Universidad de Alcalá
COMISIÓN DE ESTUDIOS OFICIALES
DE POSGRADO Y DOCTORADO

ACTA DE EVALUACIÓN DE LA TESIS DOCTORAL

(FOR EVALUATION OF THE ACT DOCTORAL THESIS)

Año académico (*academic year):* 2018/19

DOCTORANDO *(candidate PHD)*: **PEREZ MIGUEZ, RAQUEL**
D.N.I. /PASAPORTE (*Id.Passport*): ******8209A**
PROGRAMA DE DOCTORADO *(Academic Committee of the Programme)*: **D415-QUÍMICA**
DPTO. COORDINADOR DEL PROGRAMA *(Department)*: **QUIMICA ANALITICA, QUIMICA FISICA E INGENIERIA QUIMICA**
TITULACIÓN DE DOCTOR EN *(Phd title)*: **DOCTOR/A POR LA UNIVERSIDAD DE ALCALÁ**

En el día de hoy 10/05/19, reunido el tribunal de evaluación, constituido por los miembros que suscriben el presente Acta, el aspirante defendió su Tesis Doctoral **con Mención Internacional** (*In today assessment met the court, consisting of the members who signed this Act, the candidate defended his doctoral thesis with mention as International Doctorate),* elaborada bajo la dirección de (*prepared under the direction of)* MARIA LUISA MARINA ALEGRE // MARIA CASTRO PUYANA.

Sobre el siguiente tema *(Title of the doctoral thesis)*: *INNOVATIVE CHIRAL AND OMICS ANALYTICAL STRATEGIES BY MICRO-SEPARATIVE TECHNIQUES AND ION MOBILITY SPECTROMETRY*

Finalizada la defensa y discusión de la tesis, el tribunal acordó otorgar la CALIFICACIÓN GLOBAL[1] de (**no apto, aprobado, notable** y **sobresaliente)** *(After the defense and defense of the thesis, the court agreed to grant the GLOBAL RATING (fail, pass, good and excellent)*: SOBRESALIENTE

Alcalá de Henares, a 10 de MAYO de 2019

Fdo. *(Signed)*: ..... Fdo. *(Signed)*: ..... Fdo. *(Signed)*: .....

FIRMA DEL ALUMNO (candidate's signature),

Fdo. *(Signed)*: .....

Con fecha 27 de mayo de 2019 la Comisión Delegada de la Comisión de Estudios Oficiales de Posgrado, a la vista de los votos emitidos de manera anónima por el tribunal que ha juzgado la tesis, resuelve:

☒ Conceder la Mención de "Cum Laude"
☐ No conceder la Mención de "Cum Laude"

La Secretaria de la Comisión Delegada

[1] La calificación podrá ser **"no apto" "aprobado" "notable"** y **"sobresaliente"**. El tribunal podrá otorgar la mención de "cum laude" si la calificación global es de sobresaliente y se emite en tal sentido el voto secreto positivo por unanimidad. (*The grade may be **"fail" "pass" "good"** or "**excellent**". The panel may confer the distinction of "cum laude" if the overall grade is "Excellent" and has been awarded unanimously as such after secret voting.).*

INCIDENCIAS / OBSERVACIONES:
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En aplicación del art. 14.7 del RD. 99/2011 y el art. 14 del Reglamento de Elaboración, Autorización y Defensa de la Tesis Doctoral, la Comisión Delegada de la Comisión de Estudios Oficiales de Posgrado y Doctorado, en sesión pública de fecha 27 de mayo, procedió al escrutinio de los votos emitidos por los miembros del tribunal de la tesis defendida por ***PEREZ MIGUEZ, RAQUEL***, el día 10 de mayo de 2019, titulada, *INNOVATIVE CHIRAL AND OMICS ANALYTICAL STRATEGIES BY MICRO-SEPARATIVE TECHNIQUES AND ION MOBILITY SPECTROMETRY* para determinar, si a la misma, se le concede la mención "cum laude", arrojando como resultado el voto favorable de todos los miembros del tribunal.

Por lo tanto, la Comisión de Estudios Oficiales de Posgrado **resuelve otorgar** a dicha tesis la

MENCIÓN "CUM LAUDE"

Alcalá de Henares, 31 de mayo de 2019
EL VICERRECTOR DE INVESTIGACIÓN Y TRANSFERENCIA
F. Javier de la Mata de la Mata

Copia por e-mail a:
Doctorando: PEREZ MIGUEZ, RAQUEL
Secretario del Tribunal: MARIA LUZ SANZ MURIAS
Directores de Tesis: MARIA LUISA MARINA ALEGRE // MARIA CASTRO PUYANA

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ESCUELA DE DOCTORADO
Servicio de Estudios Oficiales de Posgrado

DILIGENCIA DE DEPÓSITO DE TESIS.

Comprobado que el expediente académico de D./Dª RAQUEL PEREZ MIGUEZ reúne los requisitos exigidos para la presentación de la Tesis, de acuerdo a la normativa vigente, y habiendo presentado la misma en formato: ☒ soporte electrónico ☐ impreso en papel, para el depósito de la misma, en el Servicio de Estudios Oficiales de Posgrado, con el nº de páginas: 486 se procede, con fecha de hoy a registrar el depósito de la tesis.

Alcalá de Henares a 21 de MARZO de 2019

PILAR DE LA VEGA

Fdo. El Funcionario

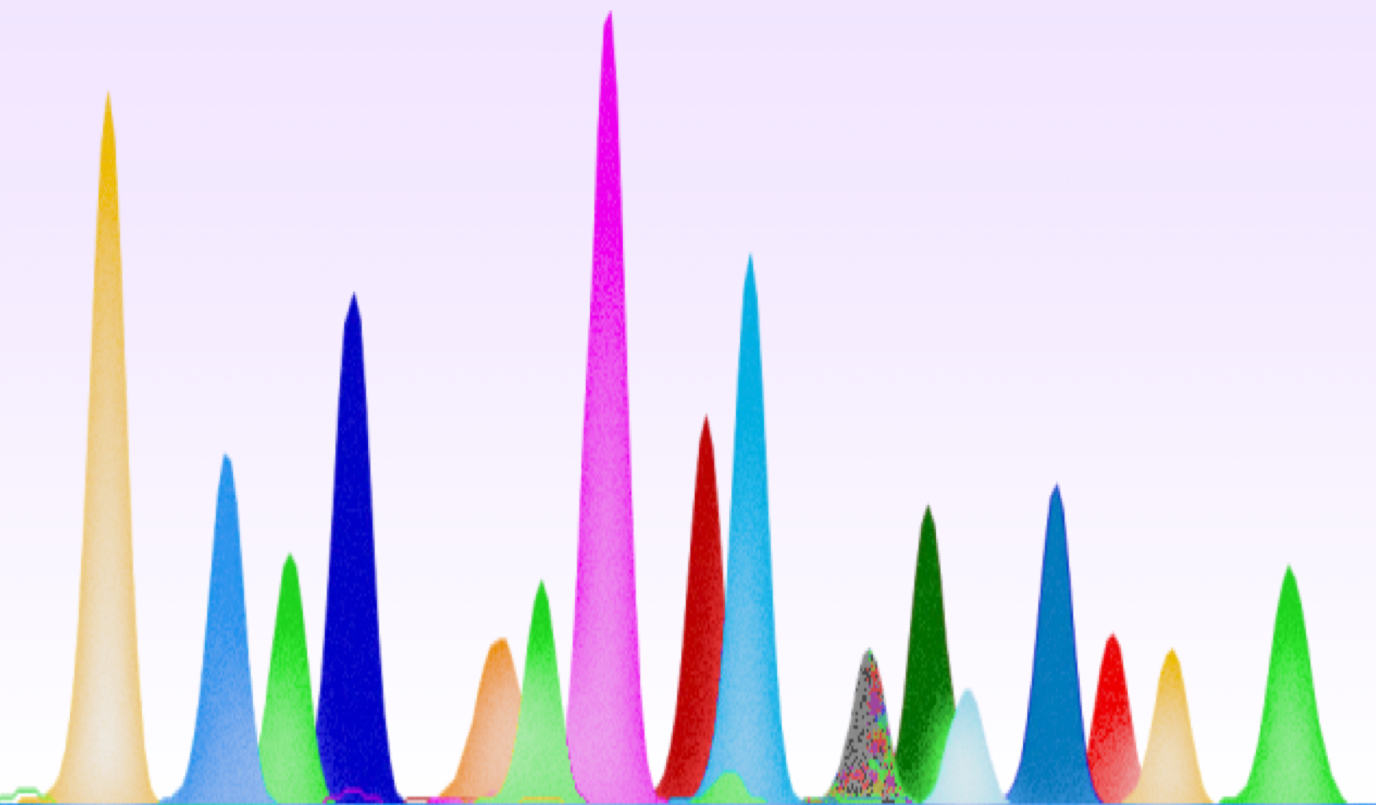
Universidad de Alcalá

DOCTORADO EN QUÍMICA

INNOVATIVE CHIRAL AND OMICS ANALYTICAL STRATEGIES BY MICRO-SEPARATIVE TECHNIQUES AND ION MOBILITY SPECTROMETRY

Raquel Pérez Míguez

March 2019



Universidad de Alcalá

Programa de Doctorado en Química

INNOVATIVE CHIRAL AND OMICS ANALYTICAL STRATEGIES BY MICRO-SEPARATIVE TECHNIQUES AND ION MOBILITY SPECTROMETRY

Tesis doctoral presentada por

RAQUEL PÉREZ MÍGUEZ

Directoras:

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Alcalá de Henares, March 2019

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ALBERTO ESCARPA MIGUEL, Coordinador del Programa de Doctorado en Química de la Universidad de Alcalá,

CERTIFICA:

Que el trabajo descrito en la presente memoria, titulado:

"**Innovative chiral and omics analytical strategies by micro-separative techniques and ion mobility spectrometry**"

ha sido realizado por **Dña. Raquel Pérez Míguez**, bajo la dirección de las **Dras. María Luisa Marina Alegre** y **María Castro Puyana**, en el Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá, a excepción del desarrollo de la metodología por TIMS-TOF para el análisis quiral de aminoácidos que ha sido desarrollada en la Vrije Universiteit de Amsterdam. Esta memoria se presenta como compendio de artículos, reuniendo los requisitos exigidos a este tipo de Tesis Doctoral, así como los requisitos científicos de originalidad y rigor metodológicos para ser defendida ante un tribunal. Esta Comisión ha tenido también en cuenta la evaluación positiva anual del doctorando, habiendo obtenido las correspondientes competencias establecidas en el Programa.

Para que así conste a los efectos del depósito de la tesis, se firma en Alcalá de Henares a 11 de Marzo de 2019

Alberto Escarpa Miguel

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MARÍA CASTRO PUYANA, Investigadora Ramón y Cajal del Departamento de Química Analítica, Química Física e Ingeniería Química de la Universidad de Alcalá,

CERTIFICAN:

Que el trabajo descrito en la presente memoria, titulado:

"Innovative chiral and omics analytical strategies by micro-separative techniques and ion mobility spectrometry",

ha sido realizado por **Dña. Raquel Pérez Míguez** y se presenta como un compendio de 10 artículos en los que el doctorando es primer autor en 9 de ellos. La contribución del doctorando en estos artículos ha sido la de realizar la parte experimental de los trabajos y la redacción de los mismos bajo supervisión.

Y para que conste y surta los efectos oportunos, firman el presente en Alcalá de Henares a 11 de marzo de 2019.

UNIVERSIDAD DE ALCALÁ · DEPARTAMENTO QUÍMICA ANALÍTICA QUÍMICA FÍSICA E INGENIERÍA QUÍMICA

María Luisa Marina Alegre **María Castro Puyana**

El trabajo de investigación recogido en esta Tesis Doctoral ha sido realizado gracias al contrato predoctoral (Formación de Personal Investigador) concedido por la Universidad de Alcalá y a la financiación concedida por el Ministerio de Economía y Competitividad (proyectos CTQ2013-48740-P y CTQ2016-76368-P), la Comunidad de Madrid y fondos europeos de los programas FSE y FEDER (proyectos S2013/ABI-3028 (AVANSECAL-CM) y S2018/BAA-4393 (AVANSECAL-II-CM)) y la Universidad de Alcalá (proyecto CCG2015/EXP-032).

"An arrow can only be shot by pulling it backward. When life is dragging you back with difficulties, it means it's going to launch you into something great. So just focus, and keep aiming."

Unknown

RESUMEN

Son muchos los campos con gran repercusión social en los que la Química Analítica posee gran relevancia a la hora de afrontar diferentes retos. En el campo de los alimentos, el desarrollo y aplicación de metodologías analíticas cada vez más rápidas, potentes, limpias y de bajo coste económico, se ha hecho imprescindible para satisfacer las exigentes demandas de los laboratorios de control, investigadores, consumidores y agencias reguladoras. La implementación de estas metodologías es crucial para obtener información relevante acerca de la composición química de los alimentos, su adulteración, contaminación, efectos de su manipulación o procesado, trazabilidad, etc., y para garantizar el cumplimiento de las normativas legales.

Esta Tesis Doctoral se centra en tres de los grandes retos actuales en el campo del análisis de los alimentos: la determinación de componentes quirales, la búsqueda de marcadores de la calidad y seguridad de los alimentos, y la identificación de compuestos bioactivos procedentes de alimentos o residuos de la industria alimentaria.

Los enantiómeros de una molécula quiral interaccionan de forma diferente con los receptores quirales presentes en el organismo, lo cual implica que pueden tener diferentes actividades biológicas. Por este motivo, la quiralidad tiene un alto impacto desde un punto de vista socioeconómico en diferentes áreas de investigación entre las que se incluye el campo de los alimentos. De hecho, conocer la composición enantiomérica de componentes quirales de los alimentos tiene una gran importancia para el control de la calidad y seguridad de los mismos. Por ello, se hace necesario desarrollar metodologías analíticas innovadoras para el análisis quiral. La Electroforesis Capilar es una de las herramientas analíticas más potentes en este campo dadas sus numerosas ventajas, tales como su elevada eficacia, el empleo de pequeños volúmenes de muestras y reactivos, o su gran versatilidad debido a la facilidad para usar un elevado número de selectores quirales. Por otra parte, la Espectrometría de Movilidad Iónica es una técnica emergente en el campo de las separaciones quirales ya que a pesar de que ha sido escasamente utilizada, ha demostrado tener un enorme potencial para la separación rápida (milisegundos) de compuestos isobáricos e isoméricos.

La búsqueda de marcadores permite proponer moléculas capaces de revelar alteraciones en sistemas biológicos. La metabolómica no dirigida es una importante técnica ómica que permite obtener el perfil o huella metabólica del metaboloma de un sistema biológico con el fin proponer metabolitos que experimentan cambios en

respuesta a enfermedades o a alteraciones genéticas o ambientales. En el campo alimentario, este tipo de estrategias metabolómicas permiten seleccionar marcadores para el control de la calidad, la seguridad y la trazabilidad de los alimentos, así como para evaluar los efectos del procesado, entre otras posibilidades. La complejidad del metaboloma de los alimentos, el cual está constituido por una gran variedad de compuestos muy diferentes desde un punto de vista químico, hace indispensable el desarrollo de métodos analíticos capaces de detectar el mayor número posible de metabolitos. Por este motivo, es necesario implementar estrategias multiplataforma capaces de aumentar el número de compuestos que se pueden analizar en un metaboloma.

En los últimos años, la búsqueda de compuestos bioactivos se ha convertido en un campo de gran interés. Ello se debe a la mejora del estilo de vida de la población que ha motivado el desarrollo de un gran número de productos con propiedades beneficiosas para la salud. Los alimentos y los residuos de la industria alimentaria constituyen importantes fuentes de compuestos bioactivos. La revalorización de los residuos alimentarios está siendo fomentada por las autoridades europeas para obtener compuestos de alto valor añadido, disminuyendo al mismo tiempo el impacto ambiental generado durante su eliminación, y aumentando los beneficios económicos derivados de su reutilización. Entre la gran variedad de moléculas que pueden tener actividad biológica, los péptidos obtenidos a partir de extractos proteicos procedentes de alimentos y residuos de la industria alimentaria constituyen un área emergente. Estos péptidos pueden encontrarse libres o encriptados en proteínas, en cuyo caso se pueden liberar mediante digestión enzimática o durante el procesado del alimento. Por tanto, el desarrollo de herramientas analíticas que permitan la separación e identificación de péptidos presenta un gran interés.

El objetivo principal de la presente Tesis Doctoral ha sido el desarrollo de metodologías analíticas quirales y ómicas sensibles y selectivas, basadas en la utilización de Técnicas Micro-Separativas y de la Espectrometría de Movilidad Iónica, todo ello con el fin de llevar a cabo la separación enantiomérica de compuestos de interés, la búsqueda de marcadores del procesado de alimentos, y la separación e identificación de péptidos bioactivos en hidrolizados proteicos procedentes de alimentos y residuos de la industria alimentaria.

El Capítulo III describe el desarrollo de metodologías analíticas basadas en Electroforesis Capilar y Espectrometría de Movilidad Iónica para la separación

enantiomérica de aminoácidos proteicos y no proteicos. Por un lado, se empleó la Cromatografía Electrocinética (EKC) con detección UV y ciclodextrinas como selectores quirales para estudiar la separación enantiomérica de diez aminoácidos no proteicos (ácido piroglutámico, norvalina, norleucina, 3,4-dihidroxifeinlalanina, selenometionina, homocisteína, ornitina, ácido 2-aminoadipico, citrulina y ácido pipecólico). Con el fin de seleccionar las mejores condiciones de separación de dichos compuestos, se investigó el poder de discriminación de un conjunto de ciclodextrinas en condiciones ácidas y básicas. Los resultados mostraron que dos de las ciclodextrinas utilizadas (α-ciclodextrina sulfatada y γ-ciclodextrina sulfatada) permitieron discriminar enantioméricamente ocho de los diez aminoácidos no proteicos estudiados con valores de resolución comprendidos entre 0.7 y 7.4, y tiempos de análisis entre 20 y 47 min. Además, fue posible llevar a cabo la separación simultánea de ácido pipecólico, citrulina y ácido 2-aminoadípico en menos de 38 min con valores elevados para la resolución enantiomérica. Para demostrar el potencial de las metodologías desarrolladas, una de ellas, basada en el empleo de formiato a pH 2.0 como medio de separación y γ-CD sulfatada como selector quiral, se aplicó a la determinación enantiomérica de citrulina en suplementos alimenticios. Con el objetivo de disminuir el tiempo de migración y mejorar la resolución enantiomérica, se llevó a cabo la optimización de diferentes variables experimentales. En las condiciones óptimas, los enantiómeros de la citrulina se separaron en 18 min con un valor de resolución de 2.7. La evaluación de las características analíticas del método desarrollado en términos de selectividad, linealidad, exactitud, precisión, LODs y LOQs, demostró el potencial del método para ser aplicado a la determinación de L-citrulina en suplementos alimenticios, al control de la impureza enantiomérica (D-citrulina), y a investigar el efecto del almacenamiento sobre la posible racemización de la citrulina.

Dada la baja resolución enantiomérica y largos tiempos de análisis obtenidos para selenometionina por EKC-UV con ciclodextrinas como selectores quirales, se desarrolló una nueva metodología basada en el empleo de la Cromatografía Electrocinética Micelar. Este método se basó en la utilización de cloroformiato de 1-(9-fluorenil) etilo (FLEC) como agente derivatizante quiral y un tensioactivo semi-volátil cargado negativamente como medio de separación. En las condiciones optimizadas, se llevó a cabo la separación enantioselectiva de la selenometionina en menos de 6 min con una resolución de 4.4. Una vez evaluadas las características analíticas del método, la metodología desarrollada se aplicó a la determinación de L-selenometionina en suplementos alimenticios.

Por último, en este Capítulo también se describe el desarrollo de una metodología innovadora basada en el empleo de Espectrometría de Movilidad

Iónica acoplada a Espectrometría de Masas para investigar la separación quiral de veintiún aminoácidos proteicos y no proteicos de forma rápida y sencilla tras su derivatización con el agente quiral FLEC. El método permitió asimismo analizar simultáneamente algunos de los aminoácidos estudiados. Los resultados preliminares obtenidos mostraron las adecuadas características analíticas del método con límites de detección del orden de nM, lo cual supone una mejora considerable con respecto a los resultados obtenidos anteriormente con esta técnica.

El Capítulo IV agrupa los resultados obtenidos en el desarrollo de una multiplataforma metabolómica no dirigida basada en el empleo de la Cromatografía de Líquidos en dos modos de separación ortogonales (fase inversa e HILIC) y dos modos de ionización (positivo y negativo), y de la Electroforesis Capilar con ionización positiva, para el análisis de los granos de café verde y granos de café sometidos a diferentes grados de tostación. La extracción sólido-líquido con metanol al 25% permitió obtener a partir de los granos de café, un gran número de compuestos en un amplio intervalo de polaridades y concentraciones. Mediante el análisis estadístico multivariante de los datos, se pudieron seleccionar los metabolitos más relevantes que ponían de manifiesto diferencias entre los granos de café verde y los granos de café sometidos a diferentes grados de tostación. Algunos de los metabolitos estadísticamente significativos, tales como ácido clorogénico y ácido neoclorogénico, ácido quínico, 1,5- ácido dicaffeoilquinic, 3-etilpiridina, colina, betaína, prolina o prolina betaína, entre otros, pudieron ser identificados de forma inequívoca y propuestos como marcadores del proceso de tostación del café.

En el Capítulo V se presenta el desarrollo de estrategias analíticas basadas en el empleo de la Cromatografía de Líquidos en fase inversa acoplada a Espectrometría de Masas de alta resolución para la búsqueda de péptidos bioactivos en hidrolizados proteicos obtenidos a partir de tres macroalgas de consumo humano (roja, verde y marrón) y de la cascarilla de café (único subproducto originado durante el proceso de tostación del café) procedente de granos de café sometidos a distintos grados de tostación. Una vez seleccionadas las mejores condiciones para la extracción de las proteínas, se llevó a cabo la hidrólisis enzimática de los extractos proteicos para liberar los péptidos encriptados en las proteínas utilizando para ello diferentes enzimas. Alcalasa y termolisina fueron las enzimas empleadas para la hidrólisis de las proteínas procedentes de las macroalgas, mientras que alcalasa, termolisina y una digestión gastrointestinal simulada fueron las condiciones evaluadas para la hidrólisis de proteínas de la

cascarilla del café. El grado de hidrólisis enzimática se evaluó mediante el ensayo OPA. Una vez obtenidos los hidrolizados proteicos, se evaluaron diferentes bioactividades antes de su análisis mediante las metodologías analíticas propuestas por RPLC-MS. Los datos obtenidos se trataron mediante la herramienta de secuenciación *de novo* del software PEAKS. Mediante la estrategia desarrollada, se identificaron treinta y siete péptidos diferentes en los hidrolizados proteicos procedentes de las macroalgas estudiadas, de los cuales cinco eran comunes en las algas roja y marrón, y cincuenta y un péptidos en la cascarilla del café (ninguno de ellos fue común para los distintos hidrolizados de cascarilla de café estudiados). Por último, se estudió la posible actividad biológica de los péptidos identificados utilizando para ello la base de datos BIOPEP. Varios de los péptidos identificados en los hidrolizados proteicos de las macroalgas formaban parte de péptidos más largos con posible bioactividad, principalmente con propiedades antibacterianas. Además, se encontró que algunos de los péptidos identificados en los hidrolizados de cascarilla de café tenían actividades biológicas como son la antibacteriana, inhibidora de la enzima convertidora de angiotensina o antioxidante.

SUMMARY

There are many areas in which analytical chemistry represents a very important tool to face different challenges. In the field of Food Science, the development and application of faster, powerful, cleaner, and cheaper analytical methodologies is imperative to satisfy the exigent demands from official laboratories, scientists, consumers, and regulatory agencies. The implementation of these methodologies is crucial to provide relevant information about chemical composition of foods, adulteration, contamination, product tampering, processing, traceability, etc., ensuring at the same time the accomplishment of legal regulations.

The determination of chiral food components, the search for markers of food quality and safety, and the identification of bioactive compounds obtained from food or food residues are three of the current trending topics in Food Analysis in which this PhD Thesis has focused considerable efforts.

Enantiomers of chiral molecules interact with the chiral environment present in the human body in a different way originating different biological activities. For this reason, chirality has a high impact from a socioeconomically point of view in different research areas among which Food Science is included. In fact, the knowledge of the enantiomeric composition of chiral food components has been shown to be relevant to control the quality and safety of food. As a consequence, the development of innovative analytical methodologies for chiral analysis is necessary. Capillary Electrophoresis is one of the most powerful tool to carry out a chiral analysis due to its numerous advantages such as high efficiency, low reagent and sample volumes required or great flexibility due to the easy use of a huge number of chiral selectors. Nowadays, Ion Mobility Spectrometry is an emerging technique in the field of chiral analysis since in spite of its scarce use, it has demonstrated an enormous potential for the rapid separation (milliseconds) of isobaric and isomeric compounds.

The search for biomarkers has become a powerful way to propose molecules that may reveal an alteration in a biological system. Untargeted metabolomics is a potent tool within omics techniques which enables the global measurement of the metabolites present in a biological system to obtain a comprehensive profile of the metabolome and to compare patterns or "fingerprints" of metabolites that may change in response to diseases, environmental or genetic alterations. In Food Science, this kind of approaches allow to point out markers enabling the control of food quality, safety, and traceability, and the evaluation of food processing, among others. Since a food metabolome comprises a large variety of compounds very

different from a chemical point of view, the development of analytical methodologies enabling the detection of as many metabolites as possible is imperative. For this reason, the implementation of multiplatform strategies allowing to increase the coverage of compounds that can be analyzed are strongly encouraged.

Bioactive compounds attract an increasing interest in the last years due to the variety of products developed with health promoting properties which have been fostered by an improvement in the lifestyle of population. Food and residues from the food industry are important sources of bioactive compounds. The revalorization of food residues has been promoted by European authorities as a means of obtaining high added-value compounds decreasing the environmental impact associated to the elimination of these residues and increasing the economic benefits derived from their reusing. Among the big variety of molecules that may have a biological activity, peptides released from protein extracts obtained from food and food residues and by-products emerge as an interesting area. Peptides can be found free or encrypted in proteins in which case they can be released by enzymatic digestion or during food processing. Therefore, the development of analytical tools for the separation and identification of peptides is of great interest.

In this context, the main objective of this PhD Thesis has been the development of sensitive and selective chiral and omics analytical methodologies based on the use of Micro-Separative Techniques and Ion Mobility Spectrometry for their application to the estereoselective separation of compounds of interest, the search for markers of food processing, and the separation and identification of bioactive peptides in protein hydrolysated obtained from food or residues from the food industry.

Chapter III deals with the development of analytical methodologies based on the use of Capillary Electrophoresis and Ion Mobility Spectrometry for the enantiomeric separation of protein and non-protein amino acids. On the one hand, Electrokinetic Chromatography (EKC) with UV detection and cyclodextrins as chiral selectors was employed to study the enantioseparation of ten non-protein amino acids (pyroglutamic acid, norvaline, norleucine, 3,4-dihydroxyphenylalanine, selenomethionine, homocysteine, ornithine, 2-aminoadipic acid, citrulline and pipecolic acid). The discrimination power of several cyclodextrins under acidic and basic conditions was investigated in order to find the most suitable conditions for the chiral separation of the compounds

analyzed. Results showed that two sulfated cyclodextrins (sulfated α-CD and sulfated γ-CD) allowed the enantiomeric discrimination of eight out of ten non-protein amino acids studied with resolutions values ranging from 0.7 to 7.4 and analysis times comprised between 20 and 47 min. Moreover, the developed methodology allowed the simultaneous separation of pipecolic acid, citrulline and 2-aminoadipic acid in a single run in less than 38 min with high enantiomeric resolutions. To demonstrate the potential of the developed methodologies, an EKC-UV method based on the use of formate buffer at pH 2.0 containing sulfated γ-CD as chiral selector was applied to the enantiomeric determination of citrulline in food supplements. Different experimental variables were modified in order to decrease the migration time and to achieve the best enantiomeric resolution. Under the optimized conditions, citrulline enantiomers were separated in 18 min with a resolution value of 2.7. The analytical characteristics of the developed method were evaluated in terms of selectivity, linearity, accuracy, precision, LODs and LOQs, showing its good performance to be applied to the determination of L-citrulline in food supplements, to assess the absence of the enantiomeric impurity (D-citrulline) in these samples, and to investigate the effect of a long storage time.

Since a poor enantiomeric resolution and a long migration time were obtained for selenomethionine by EKC with cyclodextrins as chiral selectors, a new approach based on the use of Micellar Electrokinetic Chromatography was developed. The method consisted of the use of 1-(9-fluorenyl)ethyl chloroformate (FLEC) as chiral derivatizing agent and a negatively charged semi-volatile surfactant as separation buffer. Under the optimized conditions it was possible to carry out the enantioselective separation of selenomethionine in less than 6 min with a resolution of 4.4. Once the analytical characteristics of the developed methodology were evaluated, its suitability was demonstrated by its application to the determination of L-selenomethionine in two different food supplements.

Finally, this Chapter also describes the development of an innovative methodology based on the use of Ionic Mobility Spectrometry coupled to Mass Spectrometry and FLEC as chiral derivatizing agent to investigate the rapid (less than one min) and easy chiral discrimination of twenty-one protein and non-protein amino acids. The methodology also allowed to carry out the simultaneous analysis of some amino acids in a single run. Preliminary results showed good analytical characteristics of the method with detection limits around nM, which considerably improved previous results obtained by this technique.

Chapter IV presents the results obtained in the development of a multiplatform untargeted metabolomics strategy based on the use of Liquid

Chromatography in two orthogonal separation modes (reversed phase and HILIC) and two ionization modes (positive and negative) and on the use of Capillary Electrophoresis in positive ionization, for the analysis of green coffee beans and coffee beans submitted to different roasting degrees. The use of an easy solid-liquid extraction procedure using 25% methanol, allowed to obtain a large number of compounds in a wide range of polarities and concentrations from coffee beans. By using multivariate statistical analysis, it was possible to select the most influencing metabolites that pointed out differences among green and roasted coffee beans at different degrees. Some of the statistically significant metabolites, such as chlorogenic acid and neochlorogenic acid, quinic acid, 1,5-dicaffeoylquinic acid, 3-ethylpyridine, choline, betaine, proline, or proline betaine, among others, could be unequivocally identified and proposed as markers of the coffee roasting process.

Chapter V describes the development of analytical strategies based on the use of Reversed-Phase Liquid Chromatography coupled to high resolution mass spectrometry for the search of bioactive peptides in hydrolysed protein extracts from three edible macroalgae (red, green and brown) and coffee silverskin (which is the only by-product originated during coffee roasting process) from coffee beans submitted to different roasting degrees. Once selected the most appropriate conditions for protein extraction, peptides were released from protein extracts by enzymatic digestion using different enzymes. Alcalase and thermolysin were evaluated for the protein hydrolysis of macroalgae protein extracts, while alcalase, thermolysin and simulated gastrointestinal digestion were tested in the case of coffee silversking protein extracts. OPA assay was employed to estimate the degree of enzymatic hydrolysis. Different bioactivities were evaluated for the protein hydrolysates obtained before their analysis by using the proposed RPLC-MS methodologies. Then, the data obtained were treated by the *de novo* sequencing tool from the PEAKS software. Following this strategy, thirty-seven different peptides were identified in the protein hydrolysates from the three macroalgae studied being five of them common for red and brown macroalgae and fifty-one peptides were identified in coffee silverskin being none of them common to the different coffee silverskin protein hydrolysates studied. Finally, the potential biological activity of the peptides identified was checked using BIOPEP database. Several sequenced peptides from macroalgae protein hydrolysates were found to be part of longer peptides with potential bioactivities, mainly antibacterial properties. Also, peptides identified in silverskin hydrolysates were found to have bioactivities such as antibacterial, inhibitory of angiotensin converting enzime or antioxidant.

ABBREVIATIONS, ACRONYMS, AND SYMBOLS

Ω/z	Size-to-charge ratio
μ_{ep}	Electrophoretic mobility
μ_{EOF}	Electrophoretic flow mobility
ΔK	Full width at half maximum (FWHM)
ABTS	2,2′-azino bis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin converting enzyme
Ala	Alanine
Aminoadipic	2-Aminoadipic acid
ANOVA	Analysis of variance
APA	Alkaline protein extract precipitated with acetone
APFO	Perfluorooctanoic acid
APHCl	Alkaline protein extract precipitated with HCl
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
β-ME	β-mercaptoethanol
BGE	Background electrolyte
CCS	Collisional cross section
CD	Cyclodextrin
CE	Capillary Electrophoresis
CEC	Capillary Electrochromatography
Cit	Citrulline
CMC	Critical micellar concentration
CS	Coffee silverskin
Cys	Cysteine
CZE	Capillary Zone Electrophoresis
DART	Direct analysis in real-time
DMS	High-definition differential ion mobility mass spectrometry
DOPA	3,4-Dihydroxyphenylalanine
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRC	Dark roasted coffee
DTIMS	Drift time ion mobility spectrometer
DTT	Dithiothreitol
EKC	Electrokinetic Chromatography
EOF	Electroosmotic flow
ESI	Electrospray ionization
FAIMS	High field asymmetric IMS
FITC	Fluorescein isothiocyanate
FLEC	1-(9-fluorenyl)ethyl chloroformate
FMOC	9-Fluorenylmethyloxycarbonyl
FTICR	Fourier transform ion cyclotron
FWHM	Full width at half maximum
GC	Gas Chromatography
GCB	Green coffee beans
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
Hcy	Homocysteine
HIFU	High intensity focused ultrasounds
HILIC	Hydrophilic interaction chromatography
His	Histidine
HPLC	High Performance Liquid Chromatography
HRMS	High resolution MS
ICP	Inductively coupled plasma
Ile	Isoleucine

IMS	Ion mobility spectrometry
IT	Ion trap
K0	Mobility
LC	Liquid Chromatography
Leu	Leucine
LIF	Laser induced fluorescence
LOD	Limit of detection
LOQ	Limit of quantification
LRC	Light roasted coffee
Lys	Lysine
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption ionization
MEKC	Micellar Electrokinetic Chromatography
Met	Methionine
MPP	Mass profiler professional
MRC	Medium roasted coffee
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
Norleu	Norleucine
Norval	Norvaline
NPAA	Non-protein amino acid
OPA	O-phthalaldehyde
OPLS-DA	Orthogonal partial least square discriminant analysis
Orn	Ornithine
PAA	Protein amino acid
PCA	Principal component analysis
Phe	Phenylalanine
Pipe	Pipecolic acid
PLS-DA	Partial least square discriminant analysis
Pro	Proline
Pyro	Pyroglutamic acid
Q	Quadrupole
QC	Quality control
QqQ	Triple quadrupole
QTOF	Quadrupole-time of flight
RPLC	Reversed-phase Liquid Chromatography
RSD	Relative standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SeMet	Selenomethionine
Ser	Serine
SFC	Supercritical Fluid Chromatography
TFA	Trifluoroacetic acid
Thr	Threonine
TIMS	Trapped IMS
TOF	Time of flight
Tris-HCl	Tris(hydroxymethyl) aminomethane-hydrochloride
Trp	Tryptophan
TWIMS	Travelling-wave IMS
Tyr	Tyrosine
UPLC	Ultra-High Performance Liquid Chromatography
Val	Valine
VIP	Variable importance in projection
WPA	Aqueous protein extract precipitated with acetone
WPHCl	Aqueous protein extract precipitated with HCl

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

The advances experienced by analytical techniques in the last years have opened new possibilities towards the development of sensitive and selective analytical strategies to face the upcoming challenges fostered by a society in continuous progressing. In fact, there are many areas in which analytical chemistry represents a very important tool to cope with different issues. In the field of Food Science, the development and application of faster, powerful, cleaner, and cheaper analytical methodologies is imperative to face the exigent demands from official laboratories, scientists, consumers, and regulatory agencies. Thus, these methodologies are required to be capable to provide relevant information about chemical composition of foods, adulteration, contamination, product tampering, processing, traceability, etc., while ensuring compliance with food and trade laws.

Among the current trending research topics in Food Analysis, the three approached in this PhD thesis can be cited: (i) the determination of chiral food components, (ii) the search for markers of food quality and safety, and (iii) the identification of bioactive compounds obtained from food or residues from the food industry.

Some food components such as amino acids, organic acids or phenolic compounds, among others, are examples of chiral molecules whose enantiomers have the same physicochemical properties but interact with the chiral environment present in the human body in a different way originating different effects. The different biological activities that the enantiomers of a chiral compound may have make necessary the development of enantioselective analytical methodologies enabling the determination of the enantiomeric composition of food samples as a tool for quality and safety control, detection of adulterations, evaluation of storage or processing effects, etc.

The search for biomarkers has become a powerful way to propose molecules that may reveal an alteration in a biological system. The development of omics technologies has enabled to obtain massive information from a given sample. Metabolomics, one of the main branches in the field of the omics techniques, is based on the use of advanced analytical platforms to perform the exhaustive characterization of the whole metabolome (typically molecules with molecular mass below 1500 Da) of a biological system (cell, biofluid, organism, food, etc.). Untargeted metabolomics approaches in combination with chemometric tools allow to assess food quality and safety by means of the proposal of relevant compounds as quality, safety and traceability markers. However, the comprehensive analysis of a food metabolome is not an easy task since it comprises a great variety of components very different from a chemical point of view. For this reason, new

improvements and advances in the development of metabolomics strategies are strongly encouraged.

Finally, the identification of bioactive compounds obtained from food or residues from the food industry is an increasing practice promoted by European and international authorities as a means for obtaining high value added compounds useful in the food, pharmaceutical and cosmetic industries generating economic benefits and reducing, at the same time, the impact that the elimination of residues has on the environment. In fact, bioactive molecules have the potential of improving human health and preventing some diseases. Among the big variety of molecules with a biological activity, bioactive peptides can be cited. They are specific fragments of proteins whose intake can have a positive impact on the human health. These peptides can be naturally occurring as individual entities in animal or vegetable matrices or they can be in a latent state as a part of a protein. Peptides encrypted in proteins can be released as a consequence of the proteolytic processes taking place during the gastrointestinal digestion of a protein or during food processing. However, there are a huge amount of natural sources (food and food residues) presenting high quality proteins whose potential to obtain bioactive peptides has not been investigated yet. Therefore, the development of selective and sensitive methodologies enabling the separation and identification of bioactive peptides from food or food residues would be very interesting for scientists, industry and society.

I.1. Chiral analysis

Compounds presenting the same elemental composition are called isomers. If the atoms of these isomers are bound in different position, they are referred as structural isomers. Moreover, if these isomers differ in the space atoms arrangement then they are called stereoisomers. Stereoisomers comprise enantiomers (**Figure I.1A**) and diastereoisomers (**Figure I.1B**). The term diastereoisomer is referred to two stereoisomeric molecules that are not mirror-image forms of each other. Conversely, the term enantiomer is defined as one of a pair of molecular species that are non-superimposable mirror images of each other. Enantiomer structure consists usually of an atom, which is the chiral center, surrounded by at least four different functional groups.

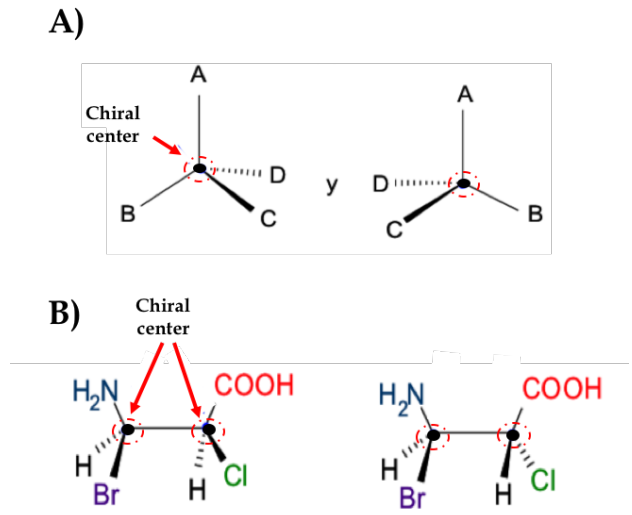


Figure I.1. Examples of enantiomers (A) and diastereoisomers (B).

According to the rules established by Cahn-Ingold-Prelog [1], the IUPAC systematic nomenclature designates the configuration of enantiomers as "R" or "S" according to a system that assigns to each functional group attached to the chiral center a priority based on its atomic number (i.e. if the priority of the remaining three substituents decreases in clockwise direction, it is labeled as R whereas if it decreases in counterclockwise direction, it is S). However, in the case of biomolecules such as amino acids or sugars, they are denoted as D- and L-enantiomers with reference to the spatial configuration of their atoms (named after Latin *Dexter* and *Laevus*, right and left). L-amino acids and D-sugars are their natural occurring forms. Other nomenclature that can also be employed for enantiomers is related to the direction in which they rotate the plane of polarized light. If the enantiomer rotates the light clockwise, it is labeled as (+) and its mirror image is labeled as (-). It should be highlighted that there is no relationship between the rotation sign and the sequence of the substituents.

I.1.1. Impact of chirality on food analysis

The analysis of chiral compounds in food has attracted the attention of several researchers [2]. Food obtained from natural products is often characterized by a definite enantiomeric composition, biological processes being mostly stereospecific [3]. This is the basis of the pursuit and the development of enantioseparative methods applied to food analysis.

Almost 30 years ago, Armstrong established different topics in which chiral analysis provides significant information in the field of Food Science [4]:

- Detection of food adulterations
- Evaluation of flavour and fragrance components
- Analysis of chiral metabolites of chiral and prochiral constituents of food and beverages
- Control and monitoring of fermentation processes and products
- 50 % less material (e.g., flavors, fragances, additives, etc.) can be used in some cases
- Differentiation of the environmental persistence of each enantiomer of a chiral compound
- Evaluation of age, storage and treatment effects
- Fingerprinting of complex mixtures

To illustrate in a clearly way the importance of the enantiomeric determination of chiral compounds in food, several examples can be considered. For instance, the presence of D-amino acids in fruit juices is an indicator that the beverage has been adulterated since amino acids are present in natural fruit juices in their L-form [5-7]. In fermented food, the presence of D-amino acids is a potent marker of the fermentation process since they are originated as a consequence of the action and autolysis of the microorganisms [8]. The enantioselective analysis of organic acids (such as lactic acid) in fermented milk products can provide information about their quality, since these compounds are naturally occurring as single enantiomers and the production of their D or L enantiomers varies depending on the microorganisms involved in the fermentation process, among other factors. For this reason, the enantiomeric purity can be used to evaluate maturation, treatment, or storage effects of fermented products [4]. The enantiomeric analysis of chiral compounds also allows the evaluation of some organoleptic properties of food such as flavour, aromas or colour that finally affect its quality. For example, the LL-diastereomer of Neotame (an artificial sweetener which presents two chiral centers) is sweet, while its DD-diastereomer is not sweet [9].

Based on all the above mentioned, the knowledge of the enantiomeric composition of a certain foodstuff is of high relevance in assessing its quality, safety and genuineness. Therefore, there is an increasing interest in the development of reliable and rapid analytical methodologies to individually analyse the enantiomers of chiral compounds in food.

I.1.2. Amino acids as markers of food quality and safety

As mentioned before, the determination of amino acids plays an important role in the field of Food Analysis since it can provide information of high interest related to the quality and safety of food samples. They are a group of organic compounds containing amine (-NH_2) and carboxyl (-COOH) functional groups, along with a side chain (R group) specific for each amino acid which determines its identity and its properties.

Only 20 amino acids have specific codons in the genetic code and are part of proteins. These amino acids (see **Table I.1**), known as protein amino acids (PAAs), regulate key metabolic pathways to improve health, survival, growth, development, and reproduction of organisms [10]. All of them, except glycine, have an asymmetric carbon and exhibit optical activity. Eight out of 20 PAAs (namely Phe, Ile, Leu, Lys, Met, Thr, Trp and Val) are known as "essential" for humans because they cannot be produced by the human body and they must be ingested from diet. In some cases, dietary supplementation with one or more of these PAAs can be used to treat some health problems. However, elevated levels of PAAs are pathogenic indicators for neurological disorders, oxidative stress, and cardiovascular diseases. Thus, it is important to establish an optimum balance of PAAs intake to guarantee body homeostasis.

In addition to PAAs, there are hundreds of other amino acids, known as non-protein amino acids (NPAAs), which are not found in proteins main chain either for the lack of a specific transfer RNA and codon triplet or because they do not arise from protein amino acids by post-translational modification. NPAAs, many of which are chiral molecules, can exist in food as metabolic intermediates, as products formed during food processing or as additives to increase some nutritional and functional properties [11]. **Table I.2** groups, as examples, the NPAAs studied in this PhD Thesis.

An important aspect to be considered in the determination of PAAs and NPAAs is their chiral nature since each enantiomer can originate different effects when interacting with chiral environments as enzymes, proteins and receptors [3]. In nature, free amino acids are generally present as their L-form, but their exposure to certain processing conditions may originate their racemization into D-enantiomers. In addition, D-forms can also be synthesized in several enzymatic pathways through the action of microorganisms or can be found in supplemented foodstuffs due to the fraudulent addition of racemic mixtures. For these reasons, the enantioselective determination of PAAs and NPAAs is especially relevant in the following areas:

Table I.1. Abbreviations, symbols and structures of protein amino acids.

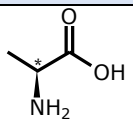
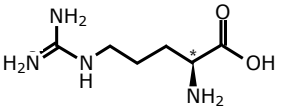
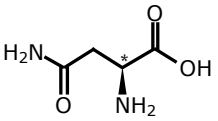
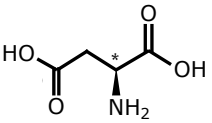
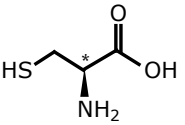
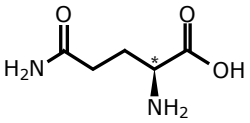
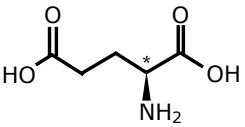
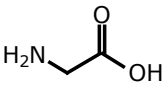
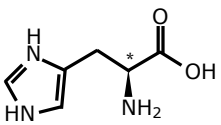
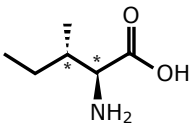
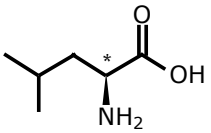
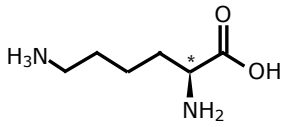
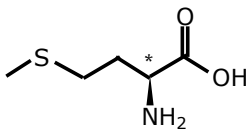
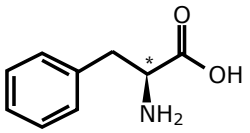
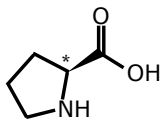
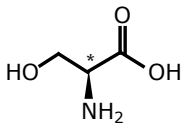
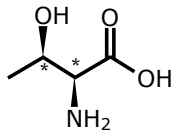
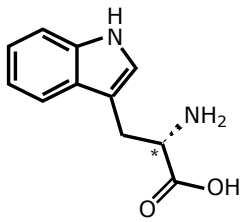
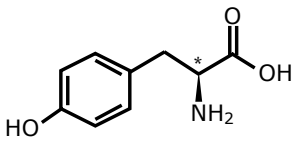
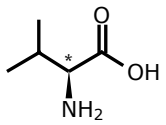
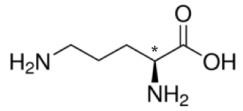
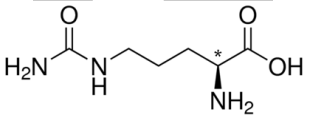
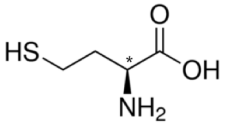
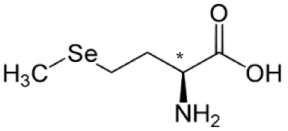
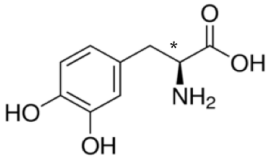
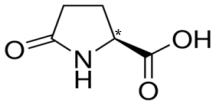
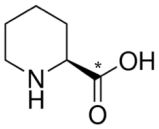
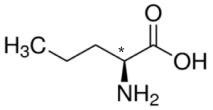
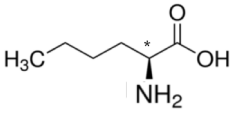
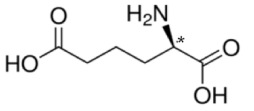
Amino acid	Abbreviation	Symbol	Structure
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic acid	Asp	D	
Cysteine	Cys	C	
Glutamine	Gln	Q	
Glutamic acid	Glu	E	
Glycine	Gly	G	
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	

Table I.1. Continued.

Amino acid	Abbreviation	Symbol	Structure
Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Serine	Ser	S	
Threonine	Thr	T	
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val	V	

* Denotes the stereogenic center.

Table I.2. Abbreviations and structures of the non-protein amino acids studied in this PhD Thesis.

Group	Amino acid	Abbreviation	Structure
Aliphatic amino acids with nitrogen in the side chain	Ornithine	Orn	
	Citrulline	Cit	
Sulfur amino acids	Homocysteine	Hcy	
Seleno aminoacids	Selenomethionine	SeMet	
Phenyl amino acids	3,4-dihydroxyphenyl alanine	DOPA	
Heterocylic amino acids	Pyroglutamic acid	Pyro	
	Pipecolic acid	Pipe	
Aliphatic monoamino-monocarboxiylic amino acids	Norvaline	Norval	
	Norleucine	Norleu	
Other	2-Aminoadipic acid	Aminoadipic	

* Denotes the stereogenic center.

i) *Detection of food adulterations and assessment of authenticity*. The addition of D-enantiomers in the food industry is forbidden since current regulations prevent their use in the elaboration of foods and dietary supplements [12, 13]. This fact implies that the presence of D-enantiomers should be controlled to avoid the fraudulent addition of racemic mixtures which have a lower cost than pure enantiomers. In fact, the enantioselective determination of different amino acids enabled to detect adulterations in fruit juices, infant formulas, and food supplements [6, 14-16].

ii) *Evaluation of fermented products and fermentation processes*. Products obtained after fermentation can have different contents of D-AAs depending on the conditions and the microorganisms involved. Thus, valuable information about maturation and microbiological contamination can be obtained throughout the chiral analysis of some PAAs and NPAAs. For example, orn is one of the AAs widely analyzed in fermented products (beer, wine and vinegar) since it has been demonstrated to be a marker of the fermentation process [6, 17, 18].

iii) *Evaluation of manufacturing processes and the effects of food treatment and storage.* Manufacturing processes, treatment and storage effects can be evaluated throughout the enantioselective analysis of AAs. Racemization of L-AAs into their D-forms can undergo during different stages of processing [19].

iv) *Evaluation of flavor and fragrance aromas*. The enantiomeric content of AAs can contribute to the taste of foods since each enantiomer can provide different organoleptic properties. For instance, D-AAs usually provide a sweet taste whereas L-AAs usually produce a bitter taste or do not give any additional flavor [20].

Undoubtedly, all these research areas are of great interest for the food industry, scientists and consumers so that there is an increasing need for developing analytical methodologies enabling the enantiomeric determination of PAAs and NPAAs.

I.1.3. Separation techniques for chiral analysis

In order to make possible the discrimination of the enantiomers of a chiral compound, different separation techniques can be employed such as Liquid Chromatography (LC), Gas Chromatography (GC), Capillary Electrophoresis (CE) or Supercritical Fluid Chromatography (SFC). In any case, the presence of a chiral

environment is mandatory. As a consequence, chiral reagents or chiral selectors are necessary, and the formation of the corresponding diastereomeric species allows in many cases to achieve the enantioseparation [21].

A chiral separation can be obtained by indirect or direct methods. In the former, the enantiomers of a chiral compound react with a chiral reagent via covalent bonds to form a pair of diastereoisomers. In this case, the enantioseparation does not require chiral conditions due to the chemical and physical differences of the diastereoisomers. The main limitation of the indirect approach is the search of an adequate chiral reagent which may have a very high cost or a very limited commercial availability. Direct methodologies are the most commonly used in chiral separations. Here, the enantiomeric separation is based on the formation of non-covalent temporary diastereomeric associates between the enantiomers and a chiral selector. The presence of the chiral selector in the separation system (either bound to an immobile support (stationary phase) or as additive in the separation medium (mobile phase)) gives rise to the formation of enantiomer-selector complexes with different stability and/or mobility.

Figure I.2 shows that the most employed analytical technique for the enantiomeric analysis of chiral molecules has been LC followed by CE, GC and SFC which is the technique used in a lesser extent. To choose the most appropriate technique, it is important to take into account the chemical structure and physicochemical properties of the enantiomers to be separated. For example, if analytes are thermally labile, SFC, LC or CE are the preferred techniques whereas GC is the technique of choice to separate volatile compounds.

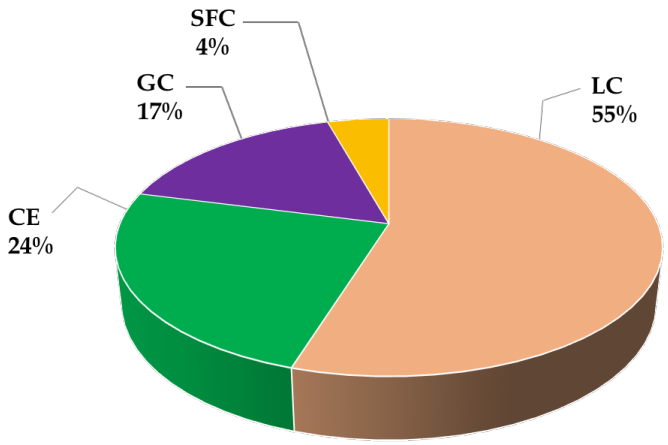


Figure I.2. Pie chart showing the contribution of different analytical techniques employed for the chiral analysis with the search "chiral or enantio*" and each of the technique in the Web of Science database on December 2018. Percentages were calculated based on the number of total publications.

An emerging technique in the field of chiral analysis is Ion Mobility Spectrometry (IMS) which, despite its scarce use, has demonstrated to be a powerful tool to separate isomeric compounds in milliseconds. In this technique, the enantioseparation is based on the enantiomers mobility in an electrical field through a neutral gas. Since the mobility is a function of the ion collision cross section (CCS) which depends on the size and shape of the ion in the gas phase, compounds with the same mass-to-charge ratio (*m/z)* ratio can be separated by IMS [22]. However, the direct separation of enantiomers by IMS is not possible since they exhibit identical CCS. Therefore, to achieve the chiral recognition of enantiomers different strategies need to be applied, such as doping the drift gas with a chiral agent to create an asymmetric environment [23, 24] or by inducing a conformational change with a complexing agent leading to different CCSs for the enantiomers [22, 25, 26].

In the following sections, CE and IMS will be briefly described since they have been the analytical techniques in which the chiral methodologies developed in this PhD Thesis were based.

I.1.4. Chiral Capillary Electrophoresis

I.1.4.1. Fundamentals of Capillary Electrophoresis

Nowadays, CE is a well-developed and powerful tool to carry out a chiral analysis [27, 28]. CE is a microseparative technique based on the differences in the electrophoretic mobilities of ions depending on their charge-to-size ratio in an electrically conductive liquid phase (background electrolyte (BGE)), within a limited-size capillary (usually with an inner diameter between 25-75 µm and a total length from 25 to 100 cm) under the influence of an electric field. **Figure I.3** depitcs the basic scheme of a CE system.

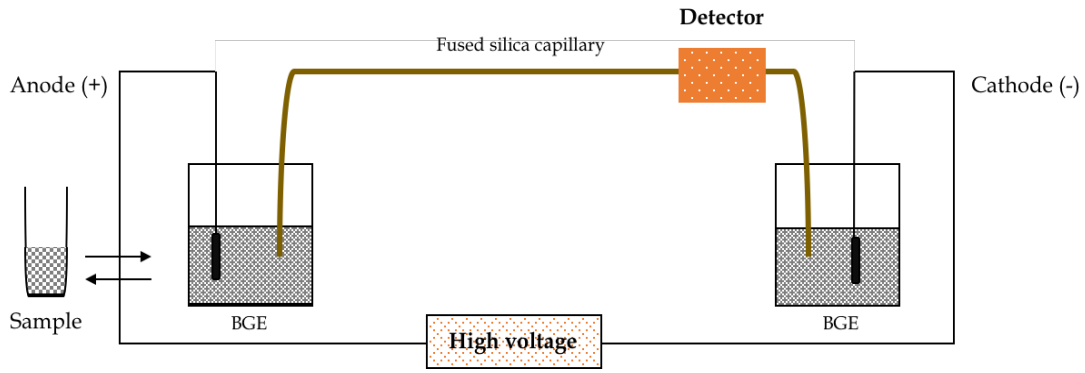


Figure I.3. Scheme of a CE system.

The mobility of an analyte in CE is determined by two factors: the mobility of the electroosmotic flow (μ_{EOF}) and its own electrophoretic mobility (μ_{ep}). The electroosmotic flow (EOF) is generated inside the silica capillary as a consequence of the ionization of silanol groups (Si-OH) present in the inner wall of the capillary which are negatively charged (Si-O$^-$) under pH conditions higher than pH ≥2-3 (See **Figure I.4**). These negative charges are neutralized by the positive ions present in the BGE. As a consequence of this organization of positive charges, when an electric field is applied, the electrolytic solution is moved towards the cathode dragging all the molecules and species. The μ_{ep} is the responsible for the movement of charged molecules towards the opposite pole to their charge. This phenomenon is directly proportional to the charge and inversely proportional to the size of the analyte. Thus, μ_{ep} will be higher for small molecules than for larger molecules, and for double charged molecules in case they have the same size. Neutral compounds will migrate together with the EOF since they do not have μ_{ep}.

Depending on the characteristics of the analytes, it is possible to work under normal or inverse polarity. When the injection takes place in the anode (positive electrode), the CE system operates under normal polarity whereas if the injection is in the cathode, the system works under the reverse polarity mode.

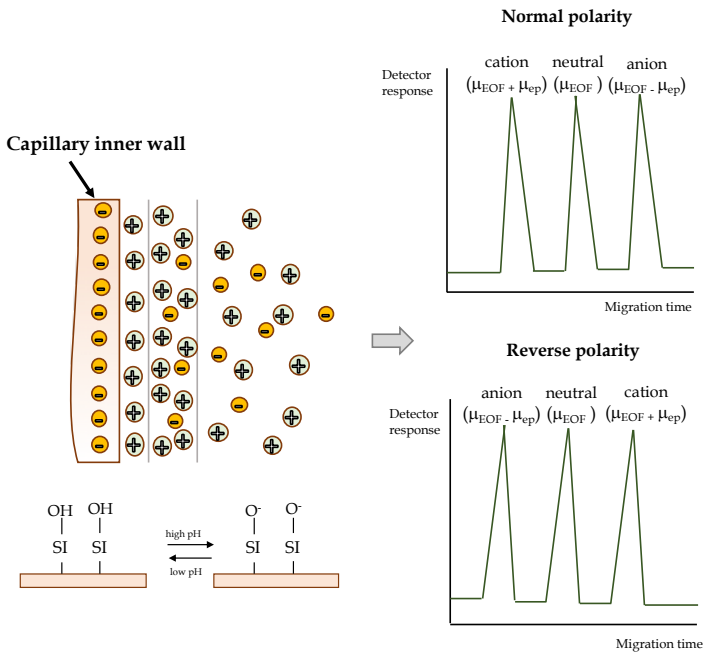


Figure I.4. Scheme of the separation inside the capillary wall and migration of the species based on their charge-to-size ratio when the CE system is working both in normal and reverse polarity.

The possibility of using different separation modes in CE has expanded the field of application of this technique allowing the analysis of a wide range of compounds presenting different characteristics and offering great versatility. Different CE modes are mostly easily accessible by changing the composition or nature of the BGE or other experimental conditions. Among all CE modes, Electrokinetic Chromatography (EKC) and Capillary Electrochromatography (CEC) are the most employed for chiral analysis. The main difference between them is that the chiral selector in EKC is added to the BGE and it acts as a chiral pseudophase whereas in CEC the chiral selector is immobilized within the capillary forming a true stationary phase.

Since EKC was the CE mode employed in this PhD Thesis to carry out the separation of enantiomers, it will be briefly described.

I.1.4.2. Electrokinetic Chromatography

EKC (introduced by Terabe in 1984 [29, 30]) is the most employed CE mode for chiral analysis. The enantiomeric separation by EKC is based on the combination of electrophoretic and chromatographic principles. Basically, the separation takes place due to the different partitioning of each enantiomer between the mobile phase and the chiral selector giving rise to the formation of enantiomer-selector complexes with different mobility. Some researchers employ the term capillary zone electrophoresis (CZE) when the enantiomeric separation of charged analytes is achieved using neutral chiral selectors whereas the term EKC is reserved to the enantioseparation of neutral analytes with charged selectors. However, in this PhD Thesis, all chiral separations using a chiral selector in solution, regardless of its charge, will be considered within the EKC mode, since the separation principle is the same for neutral and charged chiral selectors.

There is a broad range of chiral selectors that can be used in EKC such as cyclodextrins (CDs), monomeric and polymeric surfactants, antibiotics, peptides, proteins, etc. Usually, EKC has been classified according to the chiral selector employed. For instance, if a chiral surfactant is employed as chiral selector, the separation mode is Micellar Electrokinetic Chromatography (MEKC), whereas CD-EKC is the terminology employed if CDs are used as chiral selectors. Among all the chiral selectors employed in EKC, CDs and their derivatives are the most common due to their diversity, selectivity, availability, price and safety. As **Figure I.5** shows, CDs are cyclic oligosaccharides consisting of D-(+)-glucopyranose units (from 6 to 8) linked by α-(1,4) bonds. These molecules are termed as α, β, and γ-CD when they present 6, 7 or 8 D-(+)-glucopyranose units, respectively. CDs present a three-

dimensional truncated cone structure with a relatively hydrophobic inside and a hydrophilic outside owing to the presence of hydroxyl groups.

The enantiomeric separation mechanism of an analyte by EKC using CDs as chiral selectors is usually based on the partial or complete inclusion of the enantiomers into the cavity of the CD. However, external interaction might be also sufficient to achieve the enantioseparation.

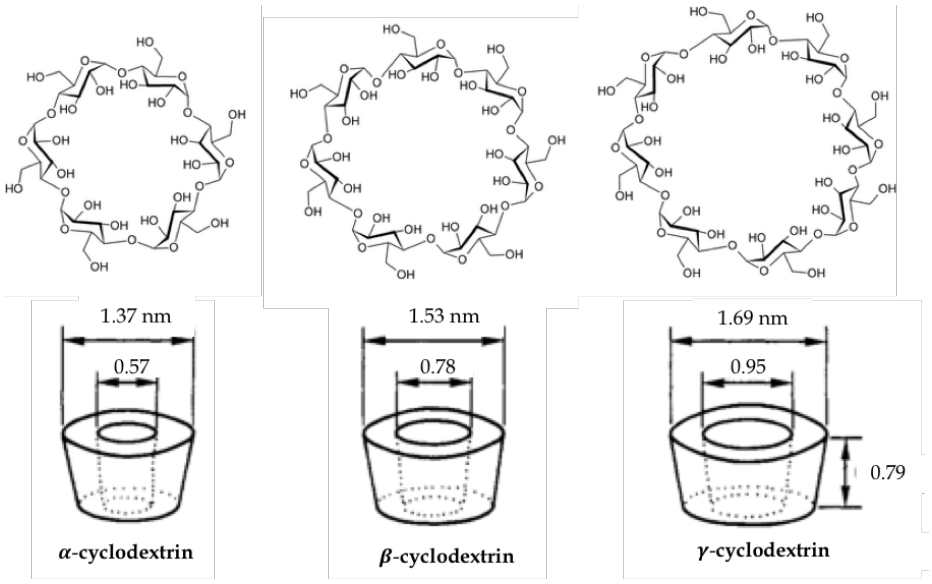


Figure I.5. Structure of native α, β, γ-CD, and their schematic side-view. Adapted and reproduced from [31] with permission from ACS Publications.

Therefore, the nature of the CD (i.e. neutral or charged), the degree of substitution and the CD concentration may affect the separation. The capability of native CDs (α, β, γ-CD) to achieve the enantiomeric resolution is limited due to their symmetry. That is why, CDs derivatives have been synthesized in order to improve the enantioresolution. In this sense, the hydroxyl groups of native CDs have been modified with different functional groups such as methyl, sulfate, acetyl and propyl, etc. In general, neutral CDs derivatives are frequently used as chiral selectors since they generate low conductivity and present low production costs. However, these CDs only allow the enantiomeric separation of charged compounds. Conversely, charged CDs, although generate higher electrical currents, offer strong interactions with opposite charged compounds and can be used to achieve the separation of both charged and neutral compounds.

The great popularity of EKC for chiral analysis is due to the enormous advantages that it presents in comparison with chromatographic techniques. Some

of these advantages include the low amount of solvents, reagents, chiral selectors and samples required, what makes it an environmentally-friendly and cost-saving technique, the high peak efficiency as a consequence of the flat EOF profile or the great feasibility for incorporating a huge number of chiral selectors in different concentrations. Nevertheless, it is not free of drawbacks. The low concentration sensitivity offered by this technique when optical detection is employed, as a consequence of the short detection path length (25-10 μm) and the low sample volumes injected, the limited repeatability of migration times and peak heights/areas sometimes obtained, and the difficulties of the coupling to Mass Spectrometry (MS) when chiral non-volatile selectors are present in the BGE, are the main limitations of this technique. To overcome these issues, some strategies can be considered. For instance, the poor sensitivity can be sorted using different strategies such as off-line and on-line sample treatments, in-capillary sample preconcentration techniques, or using alternative detectors to UV-Vis absorbance, such as MS. Regarding repeatability, a relevant consideration that might help to stabilize and improve the reproducibility of the migration times is to select an adequate rinse step before and during each CE analysis, especially for systems providing an anodic electroosmotic flow. The CE-MS coupling is usually a difficult task when using non-volatile chiral selectors since it can cause ion suppression and contamination of the ionization source and optics, leading to a decrease in sensitivity. In this sense, strategies such as the counter migration and partial filling techniques are usually employed to avoid the entry of incompatible chiral selectors to the MS system.

I.1.5. Ion Mobility Spectrometry

The motion of ions in an electric field was studied for the first time at the beginning of the 20th century by Paul Langevin [32, 33]. His research was the basis of the actual principles of IMS. However, the instrumentation took almost 70 years to be developed. In fact, it was not until 1970s-1980s when research and development programs were promoted in order to develop and improve the IMS instrumentation. As a result of these efforts, different types of reliable and powerful ion mobility devices were implemented [34]. Different types of ion mobility instruments exist such as drift time IMS (DTIMS), travelling-wave IMS (TWIMS), high field asymmetric waveform IMS (FAIMS), trapped IMS (TIMS), among others. All these IMS devices are stand-alone instruments, and sometimes they are not powerful enough to perform the analysis of complex mixtures due to their low resolving power. Therefore, hyphenated techniques are normaly used to solve this task. The hyphenation with chromatographic systems (GC, LC or CE) is usually

employed to separate components of a mixture where IMS is used as second separation technique or as detector. In addition, the hyphenation with MS is also possible. In IMS-MS, ions are separated based on the size-to-charge ratio (Ω/z) in the IMS component and according to the *m/z* in the MS component [35, 36]. This IMS-MS coupling opens the possibility to separate isobaric and isomeric compounds since they present the same *m/z* ratio but different mobility.

TIMS will be briefly described as follows as it was the IMS system employed in this PhD Thesis.

I.1.5.1. Trapped Ion Mobility Mass Spectrometry

TIMS was introduced in 2011 by Fernández-Lima et al. [37, 38]. The concept behind TIMS consists of pushing ions into the drift cell using a nitrogen gas flow while an electric field gradient is applied in the opposite direction to hold ions stationary, unlike conventional drift tube ion mobility spectrometry where the gas is stationary. Thus, the drift force is compensated by the electric field and ion packages are separated based on their size-to-charge ratio (Ω/z). This fact allows using much shorter IM drift tubes (< 5 cm), while potentially achieving a high resolving power (R > 300). In TIMS, the resolution (or resolving power) is defined as K/ΔK where K is the mobility and ΔK is the full width at half maximum (FWHM) of a compound peak in the mobilogram [39].

Figures I.6A and **I.6B** depict a schematic representation of TIMS device and its operation. As it can be seen, the device is comprised of a set of electrodes that form three regions: the entrance funnel, TIMS tunnel, and exit funnel. Basically, ions are first generated using an Electrospray Ion Source, and introduced into the entrance funnel via a glass ion transfer capillary. Then, ions are trapped in the mobility analyzer section using radially-confining RF voltages and an axial electric field that counteracts the drag force exerted from a flow of gas. The weak electric field ($E/p < 10$ V cm^{-1} Torr^{-1}) in the mobility separation section increases along the axial section while a RF applied to the electrodes confines the ions radially. Thus, the electric field compensates the gas drift force for a given Ω/z range. Ions with larger Ω/z ratios will exit the analyzing section and will not be considered in the separation since they cannot be trapped.

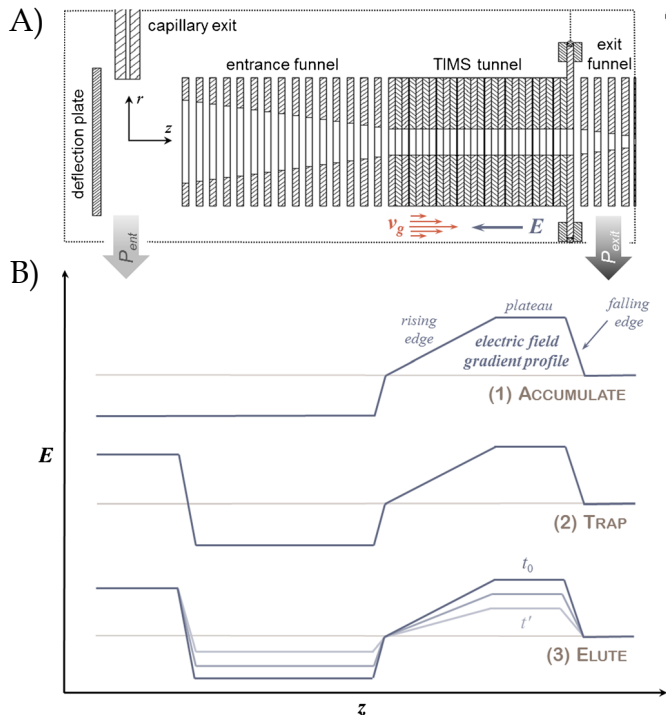


Figure I.6. Scheme of TIMS device components. Diagram of the TIMS tunnel including the orthogonal capillary ion inlet, deflection plate, entrance funnel, tunnel, and exit funnel (A) and experimental analysis sequence including plots of electric field strength, with respect to axial position (B). Reprinted from [39] with permission from Springer Nature.

After ions are injected and thermalized (fill time), the electric field in the mobility separating section is slowly decreased (ramp time), and ions elute according to their mobility (K), from high to low Ω/z values. Once ions elute from the mobility separation section, they are pushed through the exit funnel towards the mass analyser where the mass spectra are acquired [38-40].

I.1.6. State of the art of the enantiomeric separation of amino acids in food by CE and IMS

Different enantioselective methodologies were developed by EKC for the chiral separation of PAAs. In the period 2005-2015, the enantiomeric determination of PAAs, such as Pro, Ala, Arg, Glu, Asp, Tyr, Trp, Phe, Lys, Ser, and Ans was carried out enabling to obtain valuable information about fermentation processes, thermal treatments, adulterations, genetical modifications, or to assess if legal regulations are accomplished. The characteristics of these methodologies are detailed in the book chapter published in the context of this PhD Thesis and included at the end of this Chapter. Briefly, EKC with CDs as chiral selectors and

the MEKC mode were mostly employed (the use of CEC was not reported in this period). Regarding detection systems, UV, laser-induced fluorescence (LIF), and MS in a lesser extent, have been the preferred ones for PAAs detection in different food samples such as rice-brewed, vinegar, microalgae, fruit juices, maize and yeast, and beer.

After the period covered by the above-mentioned book chapter, only two works reported the enantiomeric separation of PAAs in food by CE. On the one hand, Miao et al. developed a CE methodology for the simultaneous chiral separation of 6 pairs of amino acid enantiomers (Ser, Ala, Phe, Trp, Glu, Asp) pre-capillary derivatized with 9-fluorenylmethoxycarbonyl chloride (FMOC) [41]. The use of a dual system based on a mixture of β-CD and 2-hydroxypropyl-β-cyclodextrin allowed to reach values for the enantiomeric resolution ranging from 1.06 to 9.99. The method was applied to the determination of DL-Glu and DL-Asp in several commercial rice wines. On the other hand, Nehmé et al. reported a CE method using LIF detection and β-CD as chiral selector for the simultaneous enantioselective separation of Arg, Orn, Cit, Asn, Ala, and Gln derivatized with fluorescein isothiocyanate (FITC) [42]. The developed methodology was applied to the determination of these amino acids in *Dunaliella salina* green algae grown under different conditions and the results showed a correlation between stress conditions and the production of some amino acids.

Regarding the enantiomeric separation of NPAAs, taking into account the scarce number of articles published from 2005 reporting the chiral analysis of these compounds in food, it can be said that this topic is still a quite unexplored field. The estereoselective methodologies developed were based on the use of EKC, and in a lesser extent, CEC. Neutral CDs, such as α-, β-, and γ-CD, 2-hydroxypropyl-β-CD, or succinyl-β-CD and acetylated-γ-CD have been the preferred selectors to perform chiral separations of NPAAs by EKC, although charged CDs have also been employed successfully. With respect to the detection systems, UV, LIF and MS have been the most frequently employed. In almost all the research works reported in the literature, a derivatization procedure, prior to UV and LIF detection, was needed (mainly using as labeling reagents FITC, FMOC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate or 4-fluoro-7-nitro-2,1,3-benzoxadiazole to provide chromophore or fluorophore groups enabling the determination of the analytes or improving method sensitivity. Thus, the chiral determination of NPAAs has been carried out in a broad range of complex food samples such as food supplements, infant formulas and beverages (beer, wine, vinegar, etc.) to evaluate fermented processes or to guarantee the proper use of L-enantiomers in the

elaboration of foods as established by legal regulations. The characteristics and applications of the analytical methodologies developed by CE from 2005 for the enantiomeric separation of NPAAs in food matrices are provided in the above-mentioned book chapter and in two review articles (articles 1 and 2) included at the end of this Chapter. From all this information, it can be stated that the enantiomeric separation of the NPAAs studied in this PhD Thesis (see **Table I.2**) had been very scarcely investigated in food. In fact, although some works had reported the separation of the enantiomers of Orn, Cit, Pipe, Hcy, Norleu, DOPA, and SeMet by CE-UV and CE with inductively coupled plasma-MS detection (CE-ICP-MS), only Orn, Cit and SeMet were enantiomerically determined in food samples. These NPAAs are usually employed in the formulation of some functional foods such as dietary supplements, although they can also be naturally present in other food matrices. L- and D-SeMet were determined in yeast samples using CE-UV and CE-ICP-MS [43, 44].

Despite IMS has shown to be an attractive alternative as analytical technique for chiral separations, its application to the enantioselective analysis of PAAs is scarce, and only one NPAA, namely hydroxyproline, had been enantioseparated by this technique. Up to date, all the research works published in the literature devoted to the chiral separation of amino acids by IMS are based on the use of standards as model compounds. As previously mentioned in section I.1.5, different strategies can be followed to achieve the chiral recognition of enantiomers by IMS. One of the most common strategies is by inducing a conformational change through the formation of metal-complexed forms leading to different CCSs of the studied enantiomers. Following this strategy, several works had been published using different IMS devices. For instance, FAIMS was proposed for the chiral separation of Trp, Pro, Phe, Val, Arg and Lys after forming metal-bound trimeric complexes of the form $[MII(L\text{-}Ref)_2(D/L\text{-}A)\text{-}H]^+$, where MII is a divalent metal ion, L-Ref is an amino acid in its L form acting as chiral reference compound, and A is the amino acid analyte [26]. TWIMS was also employed by Yu et al. for the separation of Trp, Gln, Tyr, Thr, His, Glu, Met, Phe, and Arg through the separation of the formed binuclear copper bound tetrameric ions [25], by Domalain et al. for the enantioseparation of Arg, Trp, Glu, Thr, His, Pro and Tyr through their cationisation with copper(II) and multimer formation using D-Pro as a chiral reference compound [22], and by Flick et al. who obtained the chiral recognition of Pro, hydroxyproline, and fluoroproline by forming complexes with alkali metal ions [45]. The other strategy usually adopted to achieve a chiral separation by IMS is creating an asymmetric environment in the drift tube by doping the drift gas with a chiral agent. In this sense, the use of (S)-(+)-

2-butanol as volatile chiral reagent has been proposed for the enantioseparation of five PAAs (Ser, Met, Thr, Phe and Trp) [23]. Recently, it has been reported in the literature the use of diastereomeric dimer ions for the chiral separation of Trp and Phe by high-definition differential ion mobility mass spectrometry (DMS). Diastereomeric proton bound complexes were formed between the enantiomers of amino acids and N-tert-butoxycarbonyl-O-benzyl-l-serine by electrospray ionization [46]. From all this information, it can be said that the PAAs and NPAAs studied in this PhD Thesis had never previously enantiomerically separated by TIMS.

I.2. Metabolomics

Omics techniques enable to obtain massive information from a system which has opened new possibilities for the study of biological systems. Metabolomics is one of the main omics techniques, and it is aimed to the exhaustive study of the whole small metabolome (molecules with a molecular mass below 1500 Da) of a particular system or organism [47]. Thus, metabolomics strategies provide the complete and unbiased analysis of the end products and intermediates of the metabolism, their dynamics, composition, interactions, and responses to changes in their environment in biological fluids, tissues, cells, or even whole organisms, under a given set of physiological conditions or under different perturbations [48, 49].

As previously reported by Dettmer et al. (2007) and Hall (2006) [50, 51], there are mainly two sorts of approaches which can be established in metabolomics: (i) targeted analysis, targeted metabolomics or metabolic profiling which is focused on the analysis of a limited group of metabolites which posses similar physicochemical properties or which are included in a specific metabolic pathway, and (ii) non-targeted analysis, untargeted metabolomics or metabolic fingerprinting which consists on the global measurement of the metabolites present in a biological system in order to obtain a comprehensive profile of the metabolome aimed to compare patterns or "fingerprints" of metabolites that change in response to diseases, environmental or genetic alterations.

Among them, untargeted metabolomics is closer to be considered a true "omic" approach since it can be simultaneously applied to the analysis of as many metabolites as possible in a biological system with the only limitation of the analytical platforms used. One of the main difficulties of metabolomics analysis arises from the heterogeneity of the broad spectrum of metabolites present in a biological system with different polarities, as well as the differences in the

abundances of these metabolites. Moreover, sample analysis, data treatment and metabolite identification are also difficult tasks in a metabolomics study.

I.2.1. Metabolomics workflow

The first step in any metabolomics study is to establish the experimental design. A detailed planning of the experiments that need to be performed to ensure that the number of samples is adequate and to reduce the technical variability to obtain the most reliable data is paramount [50].

To develop an adequate metabolomics platform, it is crucial to carefully evaluate each step involved in the metabolomic workflow. These steps are shown in **Figure I.7** and will be described in the following sections.

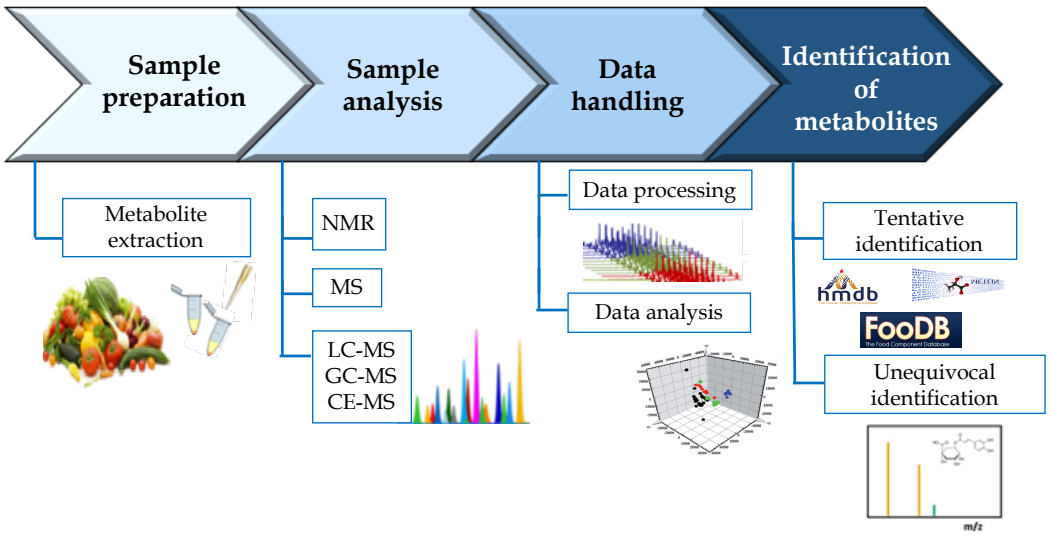


Figure I.7. Scheme of the metabolomics workflow usually followed in untargeted approaches.

I.2.1.1. Sample preparation

Sample preparation is the first step in the metabolomics workflow which is probably one of the most crucial since it will directly influence the results subsequently obtained. This procedure should involve the extraction of the metabolites into a compatible format with the analytical platform selected to perfom their analysis. Different procedures, such as extraction, derivatization or even pre-concentration of the metabolites can be included in sample preparation. All of them should be carefully selected in order to study the entire metabolome.

Obviously, the selection of the extraction solvent will depend on the sample studied. Single-phase extraction is usually recommended using solvents with

different polarities (e.g. isopropanol, water or ethanol are commonly employed to extract polar compounds whereas chloroform or ethyl acetate are usually employed to extract non polar compounds) [52] although biphasic extractions are also employed enabling to extract simultaneously polar and non-polar metabolites [53]. Moreover, the extraction procedure to be selected also depends on whether the matrix is solid or liquid. As an example, when the matrix is a liquid, simple extractions can be performed, e.g. sonication for degassing [54], centrifugation for removal solid particles [55] or even, samples can be sometimes directly injected in the system without any other treatment [56, 57]. If the sample is a solid, it is often freeze-dried or grinded to a fine powder before to carry out the extraction of metabolites with different solvents.

Ideally, the sample preparation procedure should be (i) as non-selective as possible to ensure broad coverage of the metabolome, (ii) as simple and fast as possible by minimizing the number of steps in order to avoid metabolite losses, and (iii) reproducible to ensure that small changes in metabolites are due to real sample differences [58].

To summarize, metabolite extraction should release metabolites from the sample matrix, remove interferences, make the extract compatible with the analytical technique to be employed, and concentrate metabolites which are present at trace levels, if it is possible. However, since an universal sample treatment directed to the extraction of the full metabolome of a particular sample does not exist in practice, it should be taken into account that any sample treatment may include unintended bias towards the metabolites present, and thus, some components may be lost during the sample preparation step.

I.2.1.2. Sample analysis: analytical techniques used in metabolomics

Before starting sample analysis, the metabolomics sequence must be planned. Usually, it begins with the injection of blank and quality control (QC) samples what are made by aliquoting the same volume of every sample included in the sequence. QC is also injected along the sequence (usually every 5 or 6 samples), and at the end of the sequence. The reason to do that is because the injection of QC enables to evaluate the quality of the analytical sequence, to equilibrate the analytical platform, to check the intensity of signals, to evaluate the intermediate precision and to carry out the signal correction and data normalization after the analysis [50, 59]. The samples under study are injected randomly along the sequence since it decreases the risk of bias and ensures that there is no correlation between metabolite levels and analysis order.

Despite of a metabolic study is intended to provide the holistic analysis of the metabolome of a particular sample, there is no a single analytical tool capable of extracting and detecting all the different molecules at once.

Concerning the analytical tools employed in metabolomics, vibrational spectroscopic techniques (such as infrared and Raman techniques), nuclear magnetic resonance (NMR) spectroscopy and MS-based techniques have been used to achieve fingerprinting analysis [60]. However, although most attention has been paid to the detection technique in metabolomics, the truth is that the quality of the obtained results can increase by using a proper separation technique before detection. Therefore, to obtain the most valuable information based on the study of interest, it is important to select properly the analytical platform.

NMR and MS have been the most employed detection techniques in food metabolomics. As it can be seen in **Figure I.8**, which shows the evolution of both techniques in this field, the number of publications based on the use of MS has grown exponentially from 2008 until now. This fact can be justified if it is considered that MS is more suitable by far to be coupled to a separation technique and also the development and improved affordability of high resolution MS instruments. In any case, both NMR and MS have advantages and limitations. The main characteristics of both techniques will be described below, with special attention to MS since it has been the detection technique employed in this PhD Thesis.

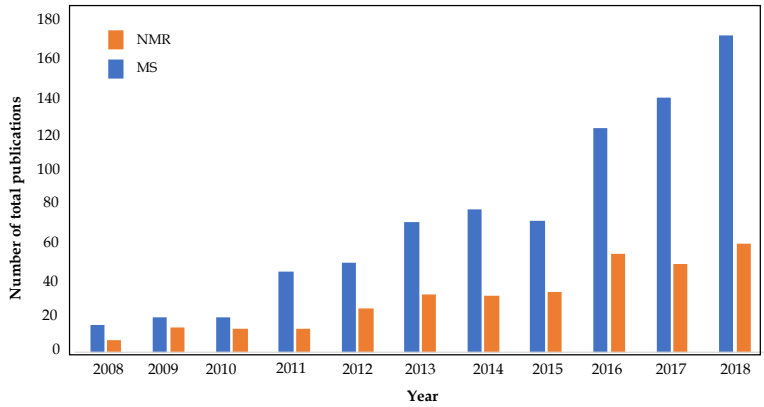


Figure I.8. Bar plot displaying the number of publications when looking up either "nuclear magnetic resonance or NMR", or "mass spectrometry" along with "metabolomics" and "food" in the Web of Science database on December 2018.

Nuclear Magnetic Resonance-based metabolomics

NMR is a non-destructive and non-invasive technique that allows the analysis of very complex samples and the determination of very different chemical

species in a single experiment. It does not require complicated sample preparation before the analysis and it can provide valuable structural and quantitative information of metabolites. The spectral NMR acquisition typically take 4-5 min which enables to achieve a huge number of analysis per day. The main limitations of NMR are its relatively low sensitivity compared to MS (typically, metabolites with concentratrion below 1 mM can not be detected [61]), and that often the signals deconvolution is a tedious process [62-64].

Mass Spectrometry-based metabolomics

Nowadays, MS is the analytical technique of choice in metabolomics since it provides a high sensitivity (up to a 1000-fold increase compared to NMR). In this technique, the metabolites of a particular sample are ionized, transferred to a gas phase into the ion source, separated based on their *m/z* in the mass analyzer, and detected.

A metabolomics analysis needs an accurate mass measurement for the determination of the elemental composition of metabolites and to carry out the identification of unknown compounds. In this regards, the use of high and ultra-high resolution MS analyzers, such as time-of-flight (TOF), orbitrap or Fourier transformed ion cyclotron resonance (FTICR), is crucial to achieve untargeted metabolomics approaches. Moreover, the possibility of using tandem MS systems (quadruple-TOF (QTOF), ion trap-TOF (IT-TOF) and TOF-TOF) in MS/MS experiments allows to obtain an additional structural information through high mass accuracy fragmentation spectra that significantly enhances the capabilities for the identification of unknown metabolites. Among them, QTOF is one of the instruments best suited for metabolomics studies and it is the one employed in this PhD Thesis so it will be briefly described as follows.

Figure I.9 shows the simplified scheme of a QTOF mass analyzer that consists of one Q and one hexapole collision cell linked to a TOF analyzer that is geometrically aligned in the orthogonal configuration with respect to the Q. In this way, this system combines the scanning capabilities of a Q and the resolving power of a TOF. It can provide high-quality, informative, simple, one-stage MS and tandem MS/MS spectra [65]. When the MS/MS mode is used, the Q analyzer acts as a mass filter to isolate the precursor ion which is subsequently fragmented in the collision cell. The resulting product ions will be analyzed by the TOF. Here, ions presenting different *m/z* are dispersed in time during their flight along a field-free drift path in a flight tube of known length where there is an absolute vacuum.

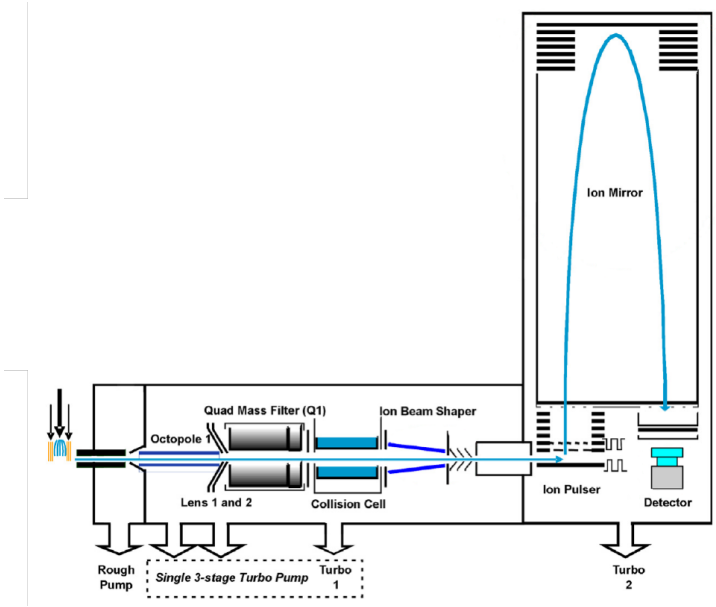


Figure I.9. Scheme of a QTOF tandem mass analyzer from Agilent Technologies catalog.

Although the direct sample injection into the MS is possible, the most common trend in MS is to use a separation technique as a system of sample introduction since this hyphenation offers the best combination of sensitivity and selectivity providing quality data for reliable metabolite identification and subsequent quantification. This kind of coupling in MS-based metabolomics can reduce ion suppression caused by coeluting compounds, isobaric interferences in the case of low-resolving mass analyzers, and sometimes isomers can be separated. In initial food metabolomics studies, GC was perhaps the separation technique of choice; however, due to the need for derivatization to increase the coverage of compounds that can be analyzed, LC is nowadays the most employed analytical technique in untargeted metabolomics. Regarding CE-MS coupling, it has been used in a lesser extent in spite of being a powerful tool for the analysis of highly polar and ionized compounds.

Since LC-MS and CE-MS have been the analytical platforms employed in this PhD Thesis for food metabolomics, they will be briefly described.

LC-MS

As mentioned before, LC-MS is nowadays the analytical platform of choice in untargeted metabolomics due to its high separation efficiency and robustness. It enables the separation of a wide range of metabolites from low to high molecular mass. Basically, the separation in LC depends on the distribution of metabolites

between a stationary phase and a mobile phase, so that the use of the appropriate column and mobile phases enables their separation depending on their physicochemical characteristics. For many years, research on LC has relied upon using smaller particle size columns. Thus, columns with sub-2 µm particles (particle size < 2 µm) were released, giving rise to the so-called Ultra-High Performance Liquid Chromatography (UPLC), improving the efficiency, the analysis times, and the detection limits. However, the use of small particle size also causes an increase in the pressure caused by the mobile phase flowing through the column. An alternative to increase the peak efficiency without causing high back-pressures in the system is the use of pellicular packings, superficially porous silica particles, so-called fused-core or core-shell particles. As it can be seen in **Figure I.10**, these particles typically consist of a 1.7 µm solid core with a 0.5 µm porous silica shell surrounding it (d_p= 2.7 µm). Due to their semi-porous surface, the analytes diffusion path is shorter than in fully porous particles which results in a faster mass transfer. It leads to more efficient peaks, without such back-pressure issues (columns with 2.7 µm pellicular particles generate less than half of the back-pressure observed with sub-2 µm particles) which makes possible the use of conventional LC systems [66].

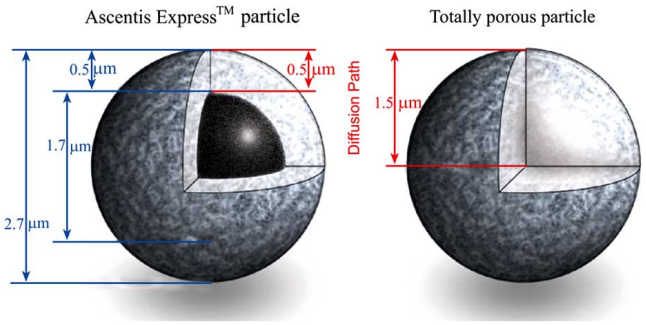


Figure I.10. Scheme of a 2.7 µm fused-core particle and a 3 µm fully porous particle. Taken and adapted from Sigma-Aldrich website.

Among the stationary phases mainly used in LC-MS, C18 columns with small particle sizes are widely used for metabolomics because of their high separation power and their good retention time repeatability and versatility. Usually, reversed phase liquid chromatography (RPLC) is employed along with aqueous-organic mobile phases containing low percentages of formic acid and with a general gradient (from 5 % to 100 % of the organic phase). In this way, it is possible to separate from medium-polar to non-polar compounds, but not highly polar and/or ionizable metabolites. An alternative, proposed by Tolstikov and Fiehn

(2002) to achieve the separation of highly polar compounds is hydrophilic interaction chromatography (HILIC) [67]. It is an orthogonal technique to RPLC which is based on the use of either silica or derivatized silica (including amino, diol, amide, polysulfoethyl aspartamide or polyhydroxyethyl aspartamide groups) as stationary phases. The separation mechanism is based on the distribution of the analyte between a polar stationary phase and a relatively hydrophobic mobile phase composed of an aqueous-organic mixture (containing from 5 to 40 % of water). The advantages of HILIC, such as having an increased analyte diffusivity in the organic-rich mobile phase [68], a lower backpressure because of the low viscosity of the mobile phase [69], and an enhanced MS signal due to the better eluent desolvation [70], make this approach an attractive alternative to RPLC. The main drawbacks associated to this technique are the higher variability in retention times, the low peak efficiency, and the long re-equilibration times needed after a gradient elution in comparison to RPLC [71, 72].

Among the different ionization sources that can be used to generate the gas-phase ions in LC-MS, electrospray (ESI) is the most frequently employed [73], both in positive and in negative modes, which enables to extend the metabolomic coverage [74]. ESI is an atmospheric pressure ionization technique which is connected to a separation technique (LC or CE) by a nebulizer (a coaxial nitrogen-containing tube) and placed at the entry of the MS. A strong electric field created at the end of the nebulizer by the application of a high voltage, gives rise to the formation of an aerosol comprised of multiple charged droplets (see **Figure I.11**). By the action of the heated inert gas flow (nitrogen), the solvent of these dropplets is evaporated so that the size of droplets decreases, and the charge density increases, resulting in a droplet instability. Then, the coulomb explosion takes place due to the electrostatic repulsion generating desorption of the ions to the gas phase which pass into the MS. The scheme described in **Figure I.11** represents ions as positively charged but the same mechanism applies in the case of negative ion mode. In any case, a reduced number of ions either protonated or deprotonated along with their adducts, and often intact molecular ions, are usually obtained by ESI. Bearing in mind that the ionization source is relevant to achieve an adequate analysis, improvements of available interfaces and ionization tools are continuously being performed. With this aim, Agilent Technologies developed the Jet Stream thermal gradient focusing technology, which is based on the use of an additional sheath gas heated at high temperature that focuses the nebulizer spray and desolvates ions more efficiently. This enables to improve the sensitivity until 5-10 times compared with normal ESI interfase [75].

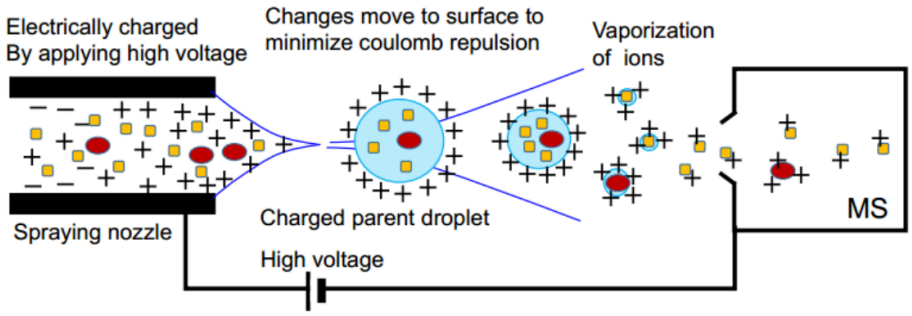


Figure I.11. ESI ionization scheme. Reprinted from [76] with permission from AIMS Press.

CE-MS

CE is a suitable tool for the rapid separation of highly polar and charged metabolites such as sugars, amino acids, organic acids or nucleotides which are usually more problematic in LC-MS. For this reason, it is considered an ortoghonal technique to LC, mainly to RPLC since CE is suitable for the determination of polar and charged compounds [77].

Among all the CE modes, CZE is the most employed in the CE-MS coupling. Here, the metabolites are separated in the capillary based on their charge-to-size ratio and subsequently detected according to their *m/z* in MS. The main difficulty of CE-MS is to establish the electrical circuit in CE needed for the separation, and to provide simultaneously an electrical potential to the spray tip to maintain a stable flow independent of droplets formation. This is generally accomplished with a co-axial sheath-liquid ESI interface (see **Figure I.12**). Achieving stable electrospray operation with a sheath liquid is often a balancing of multiple parameters such as capillary position, sheath liquid flow rate and composition, and ESI conditions.

Besides the low migration times repeatability obtained in CE-MS, which gives rise to a lower robustness if compared with LC-MS, its main limitation is its low sensitivity derived from the dilution that occurs by the sheath liquid from the co-axial sheath-liquid ESI interface. However, although there are alternative interfaces, such as the porous tip sheathless, capable to provide improvements in the sensitivity, its applicability in metabolomics has not been fully exploited up to date [50, 78, 79]. Despite the limitations of CE-MS, different reviews published have demonstrated its high possibilities in metabolomics [78, 80], although its full potential in this field is still far to be exploited.

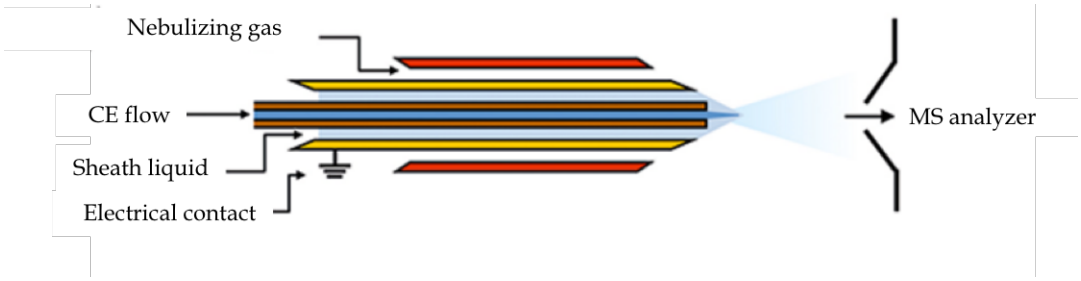


Figure I.12. Schematic representation of a sheath liquid CE-MS interface.

As it can be deduced from all the above-mentioned, there is not a single analytical technique enabling to obtain the complete insight of the metabolome, so that the way to extend the metabolite coverage is through the combination of the metabolite information obtained from different analytical platforms. Thus, by using multiple separation and detection technologies a far broader range of metabolites can be detected providing a deeper knowledge of the metabolome studied.

I.2.1.3. Data handling

The large amount of data (a high number of variables) produced in metabolomics once the samples are analyzed, has triggered the development of different tools to achieve an adequate data handling. Each step included in the data handling, i.e. data processing and data analysis will be briefly discussed.

Data processing

This step is aimed to the transformation of the raw data obtained in the metabolomics analysis to a compatible format that can be used for the subsequent steps. Data processing comprises the detection of the so-called *"features"* (a chromatographic/electrophoretic peak with a specific retention/migration time and mass or *m/z* value), deletion of noisy signals, data alignment, and normalization [81, 82]. The final dataset must be as uniform as possible to allow data comparison.

Mass Hunter and Mass Profiler Professional (MPP) softwares from Agilent Technologies have been employed in this PhD Thesis to carry out data processing. Although these softwares can be employed to carry out the processing of the data obtained from both LC-MS and CE-MS, it is necessary to adjust some scores due to the slightly different shape of the CE peaks in comparison to the LC signals and their width. Mass Hunter is used to create molecular features. By means of an algorithm, all the adducts, dimers and isotopic profile ions from a same molecule are grouped as a single compound which exact mass and retention/migration time

are provided. Then, the molecular features are exported to MPP software which is employed for the elimination of signals that do not belong to the sample, retention time and *m/z* alignment, normalization and filtering.

An step that requires special attention is the alignment. Both in LC-MS and CE-MS, retention time drift is a common issue, especially for CE-MS that presents a higher variability in migration times. The alignment is carried out by adding to the sample an internal reference compound for retention time and mass measurements corrections, or by using the most abundant peaks present in all chromatograms as reference compounds. Finally, a normalization can also be applied if it is necessary [83]. The normalization is usually achieved taking into account the signal of internal standards or the total peak area or height from each chromatogram/electropherogram. However, this procedure is not always required, only in cases with high sample variability (high %RSD) [84, 85].

Regardless of the software employed, all of them allow to obtain a data set containing all the information related to the samples, including the abundance, the mass or *m/z* values and retention time of the *features* for each analyzed sample. This data set, also referred as matrix, will be later exported to statistical tools such as SIMCA, which has been used in this PhD Thesis, where the terms "*features*" and "*samples*" are changed with "*variables*" and "*observations*", respectively.

Data analysis

Once the data have been processed and the data set has been obtained, it has to be analyzed by means of statistical tools. It should be highlighted that in some cases, a data treatment is necessary before data analysis. With this aim, different strategies such as centering, scaling and transformation or even a combination of them (which can be applied using SIMCA software) can be used to minimize the impact of variability of high-intensity peaks [86, 87]. The effectiveness of these strategies is evaluated considering those that make QC samples cluster tighter in principal component analysis (PCA) as it indicates that the intra-group samples variability is reduced. However, data treatment is only recommended when it is strictly needed since it is always desirable do not modify the data to avoid biases in the results.

Two different kinds of statistical analysis can be employed in metabolomics and the selection of one of them is based on the number of variables considered. Univariate analysis uses information related to each variable individually, whereas multivariate analysis employs information of multiple variables. In metabolomics, a large number of variables are measured so that multivariate analysis is generally

employed. However, univariate analysis is sometimes used to test the statistical significance of certain variables that are altered through multivariate analysis.

Univariate data analysis

To select the most adequate option among the broad spectrum of univariate tests (see **Figure I.13**), it is necessary to check the data distribution as well as the equality of the variance.

Normal data distribution of each variable is evaluated using Shapiro-Wilk test. p-values higher than 0.05 (for a 95 % confidence level) enable to accept such normal distribution which implies that parametric tests can be used to carry out univariate analysis. Conversely, if p-values are lower than 0.05, a normal distribution cannot be accepted and non-parametric tests must be applied. The first ones are based on the comparison of mean values whereas non-parametric tests are focused on the comparison of median values between groups.

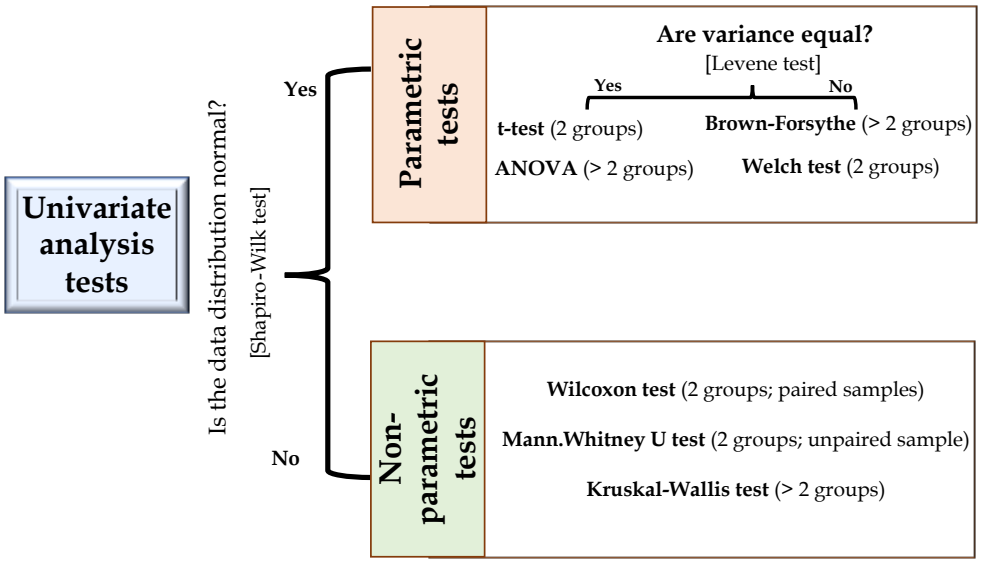


Figure I.13. Scheme displaying the different univariate tests.

As it can be seen in **Figure I.13,** parametric tests can be divided in two different groups depending on the variance equality. Equal variance for a specific variable is accepted when p-values higher than 0.05 are obtained in Levene test. In this case, univariate analysis can be achieved using a t-test (to compare two groups of samples) or an ANOVA test (when more than two groups of samples are

compared). For unequal variances, Welch and Brown-Forsythe tests are employed to compare two or more groups, respectively.

The high variability found in metabolomics makes that the use of non-parametric tests is usually the common trend. Among the different non-parametric tests (see **Figure I.13**), Mann-Whitney U test, which is the non-parametric alternative to t-test, is the one employed in this PhD Thesis.

The large number of univariate analysis tests usually carried out in metabolomics increases the risk of getting false positives or type I errors (i.e. statistically significant results that are not really different). This is usually known as multiple testing problem. To overcome it, different approaches such as Bonferroni and Benjamini-Hochberg (also called false discovery rate) corrections can be used. The first approach compensates for multiple testing by dividing the p-value threshold (usually 0.05) by the number of tests applied. This means that if 10 tests are carried out, the t-test will be 0.05/10 = 0.005 so that only those metabolites having p-values lower than 0.005 will be further considered as statistically significant. Regarding Benjamini-Hochberg correction, it is a less conservative method than Bonferroni, and presents greater power to find truly significant results. Here, all p-values are sorted from the largest to the smallest. The largest p-value remains as it is whereas the second one is multiplied by the total number of p-values (n) and divided by its rank (n-1). If the obtained value is lower than 0.05, it means that it is significant. For the third largest p-value the same process is applied but now its rank is n-2, and so on. The highest p-value which, after correction, is sorted as statistically significant, will determine the new cut-off.

Multivariate data analysis

This kind of statistical analysis is used to reduce large volumes of data into a few dimensions for sample classification. Basically, two different strategies can be applied: non-supervised and supervised methods. Multivariate analysis of all the data obtained in the different metabolomics studies developed in this PhD Thesis was performed using SIMCA.

Non-supervised methods consist of algorithms that cluster the metabolites into groups without prior knowledge of group membership and visualize the data to emphasize their similarities and differences. The most widely used non-supervised multivariate analysis in metabolomics is PCA. It shows the largest possible variance in the first principal component and each succeeding component accounts for as much of the remaining variability as possible. It provides an overview on the metabolic profile, clustering information and outliers in an easy

and quickly way. In addition, as mentioned before, PCA enables to evaluate the variability of the analytical sequence by observing how QC cluster together in the models. However, PCA just provides information on global patterns occurring in the set of samples, then to evaluate differences among the experimental groups (i.e. discrimination or classification of samples) supervised methods are required.

Partial least square discriminant analysis (PLS-DA) and orthogonal-PLS-DA (OPLS-DA) are the most frequently applied supervised methods for classification in metabolomics. In both cases, information about sample class is known a priori and it is used to build the models. PLS-DA is applied to optimize the separation between groups of samples, and it is achieved combining two data matrices: X (the dataset containing observations (samples) and variables (features)) and Y containing information about types of samples (e.g. adulterated or non-adulterated). Thus, the aim of PLS-DA is to obtain the maximum covariance between the independent variables (X) and the corresponding dependent variable (Y) by finding a linear subspace which permits the prediction of the Y variable based on a reduced number of factors (i.e. PLS components, or latent variables) [88]. On the other hand, OPLS-DA divides the systematic variation of the X matrix into two parts: one linearly-related to Y and the other unrelated, i.e. orthogonal, to Y, providing improved predictive capability [87].

One of the main disadvantages of these supervised methods is obtaining falsely significant results as a consequence of the larger number of variables compared with observations, phenomenon known as overfitting. This can take place when a model is tuned too much towards the data at hand and therefore lacks generalizability, resulting in bias of the results towards a false positive result [89]. To avoid this phenomenon, the best option is to separate training and test data sets and do not use the last one to build the model. In spite of the fact that it is the best way to overcome overfitting, it is not always possible due to the low number of samples normally used in metabolomics studies. In this case, the validation of multivariate models is usually achieved by using cross-validation strategies in combination with permutation tests. The principle of cross-validation is to leave out part of the data, build a model, and then predict the left-out samples [90]. When PLS-DA score plot shows a good class separation but the cross-validated PLS-DA model shows no class separation, it can be due to the fact that the model is overfitted and it is not valid for the intended purpose. In permutation tests, the Q^2 (predicted variance) and R^2 (explained variance) values obtained in the original model are compared to those obtained for different models constructed using randomized classification for the samples [91]. If the Q^2 and R^2 values of the original model are

the highest compared to the values obtained from all the permutated models, it indicates that the model is valid.

Regardless of the supervised method used, the aim of the statistical analysis is to find those variables (features) which differences are statistically significant among groups of samples. In this PhD Thesis, the variable importance in projection (VIP) value has been chosen to highlight the most significant candidates. The VIP value summarizes the overall contribution of each X-variable to the PLS model, summed over all components and weighted according to the Y variation accounted for each component. Thus, metabolites are classified by their VIP value, so the more they influence the projection or model, the higher VIP value they will obtain. However, according to SIMCA, variables with VIP values higher than 1.0 are considered statistically significant although the threshold of the VIP value is often stricter (e.g. 1.5).

I.2.1.4. Identification of metabolites and results interpretation

The last step of the metabolomics workflow is to identify the metabolites corresponding to the most statistically significant variables selected in the data analysis. This step is without doubt one of the most time-consuming in the workflow.

To carry out metabolite identification, it is necessary to search the masses or *m/z* values of the relevant variables in different databases. The most relevant databases employed in metabolomics are grouped in **Table I.3.** Then, the identification is carried out by looking for a match between the exact mass provided by a high-resolution MS and metabolite databases. In this point, it is important to remark that the more accurate the *m/z* provided by the MS instrument for a compound, the lower the number of possible molecular formula and the higher the chances for a correct identification. The comparison of the theoretical isotopic distribution with the experimental ones is also a useful tool to aid in metabolite identification. Combining all this information, it is possible to propose a tentative identification of the metabolites. The unequivocal identification of metabolites takes place if the MS/MS spectra and the retention/migration times from standards match those found in the samples. This is a difficult task due to the high cost or the unavailability of most standards, which makes that often just a few metabolites can be unequivocally identified. In the case of standards cannot be obtained, the tentative identification of the metabolites can be achieved by matching their MS/MS fragments to those found in databases (e.g. HMDB or Mass Bank).

Table I.3. Some of the most commonly used freely available databases in metabolomics for tentative metabolite identification.

Database	Description	Ref.
HMDB (http://www.hmdb.ca)	Database containing detailed information about small metabolites found in the human body. It is designed to contain or link three kinds of data: (i) chemical data, (ii) clinical data, and (iii) molecular biology/biochemistry data.	[92]
FooDB (http://www.foodb.ca)	FooDB is the largest and most comprehensive resource on food constituents, chemistry and biology which contains more than 100 separate data fields covering data on the compound's nomenclature, their description, information on their structure, chemical class, physico-chemical data, food source, color, aroma, taste, physiological effect, presumptive health effects, and concentrations in various foods.	[93]
KEGG (http://www.kegg.jp)	Database aimed for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies.	[94]
METLIN (http://www.metlin.scripps.edu)	Database that includes 961829 molecules ranging from lipids, steroids, plant and bacteria metabolites, small peptides, carbohydrates, exogenous drugs/metabolites, central carbon metabolites and toxicants. Over 14000 metabolites have been individually analyzed and another 200000 have *in silico* MS/MS data.	[95]
MassBank (http://www.massbank.jp)	Public repository of mass spectral data that aims to share them among scientific research community.	[96]

Once a list of known metabolites is obtained, the last step is to find the possible biological meaning of these compounds, i.e. the different metabolic pathways where they are involved in. To do that, KEGG is the common database used to obtain information related to the metabolic pathways based on the organism that is being studied (human, animals, microorganisms, or even plant metabolism).

I.2.2. State of the art of untargeted metabolomics based on LC-MS and CE-MS in food analysis

Food metabolome comprises a large variety of compounds very different from a chemical point of view (sugars, organic acids, amino acids, peptides, phenolic compounds, etc.) and that can be found in a very wide concentration range (typically millimolar to femtomolar concentrations [97]). Consequently, the comprehensive analysis of the entire food metabolite composition is a difficult task. MS-based metabolomics is the preferable tool employed for the analysis of food metabolome. As it can be seen in **Figure I.14**, the use of food metabolomics has considerably increased in the last years. The most relevant works published from 2014 to 2017 in the field of MS-based metabolomics applied to the control of food quality, safety and traceability are summarized in the review article included at the end of this Chapter. Most of the works published in this period of time devoted to the control of food quality and traceability were based on the use of untargeted strategies. With this aim, different separation techniques coupled to MS were employed being LC the most popular and CE very scarcely used. In order to update this information, a recent search (from 2017 to date) has been achieved to review the untargeted metabolomics works published dealing with food analysis and using LC-MS and CE-MS as they have been the platforms employed in this PhD Thesis (see **Table I.4**). However, as this table shows any work based on CE-MS was found in this period.

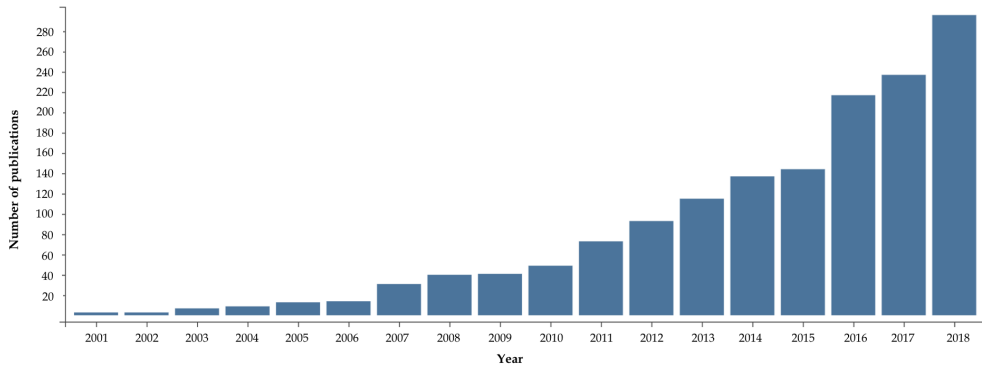


Figure I.14. Bar plot displaying the number of publications per year when looking up either "metabolomics" and "food" in the Web of Science database on December 2018.

Table I.4. Untargeted metabolomics approaches based on LC-MS in food analysis in the period from 2017 to 2019.

Food	Metabolites	MS-based technique	Application	Ref.
Indian citrus fruit/fruit juices	Didymin, rhoifolin, isorhoifolin, neohesperidin, hesperidin, naringin, narirutin, limonin glucoside, and vicenin-2.	UPLC-HRMS (QTOF)	Discrimination of authentic and adulterated Indian citrus fruit and fruit juices	[98]
Potato tubers (*Solanum tuberosum L.*) from Norkotah Russet, Atlantic, Chipeta, and Yukon Gold	Phenolic polyamines, phenolic acids, and fatty acids	LC-HRMS (TOF) GC-HRMS (TOF) ^{13}C NMR	Discrimination of native and wound periderms of four different potato cultivars	[99]
Soybean	Phosphatidylcholines and sugars	UPLC-HRMS (QTOF) DART-Orbitrap	Discrimination of genetically modified organisms and conventional soybeans	[100]
Garlic (*Allium sativum*)	Amino acids and derivatives, organosulfur compounds, saccharides and derivatives, lipids and derivatives, and others.	LC−HRMS (QTOF)	Discrimination of garlic according to its varieties and from its heat-treated products (black garlic)	[101]
Tiger nut *(Cyperus esculentus L.)*	Citric acid esters of mono-diglycerides and monoacylglycerol, biotin, phosphatidic acid and L-arginine	UPLC-HRMS	Discrimination between UHT and fresh tiger nut milks	[102]

Table I.4. Continued.

Food	Metabolites	MS-based technique	Application	Ref.
Rice bran	Amino acids, cofactors and vitamins, and secondary metabolites	UPLC-HRMS (Orbitrap) GC-MS	Search for bioactive compounds	[103]
Onion (*Allium cepa*)	Fructooligosaccharides, amino acids, S-sustitued cysteine conjugates and flavonoids.	UPLC-HRMS (QTOF)	Analysis of the metabolome of fresh onion bulbs	[104]
Meat (pork, beef, and chicken	Sulfonamides, quinolones, betablockers, tetracyclines	UPLC-MS	Search of potential risk substances to asses food safety	[105]
Garlic (*Allium sativum L.*)	Alliin, phosphatidylcholine, arginine, dehydroalanine, phosphatidylethanolamine, L-γ-Glutamyl-S-allyl-L-cysteine and choline glycerolphosphate	DART-HRMS (Orbitrap) LC-HRMS (TOF) DI-HRMS (TOF)	Discrimination of garlic according to its geographical origin	[106]
Asparagus (*Asparagus officinalis*)	Betaines, asparaptine, fatty acids	UPLC-HRMS (QTOF)	Discrimination of white asparagus according to its geographical origin	[107]
Milk samples	Organic contaminants	UPLC-HRMS (TOF)	Assessment of contamination of milk	[108]
White ginseng, taegeuk ginseng, red ginseng, and black ginseng	Ginsenosides	UPLC-HRMS (QTOF)	Evaluation of processing conditions of Ginseng products	[109]

Table I.4. Continued.

Food	Metabolites	MS-based technique	Application	Ref.
Chardonnay wines	Phenolic compounds	UPLC-HRMS (QTOF)	Discrimination of wines according to their geographical origin	[110]
Medicago truncatula	Sucrose, organic acids and tetrahydroxychalcone.	LC-HRMS	Discrimination of abiotic and biotic stress in *Medicago truncatula* root and leaf	[111]
Carrot (*Daucus carota L.*)	Fatty acids, carbohydrates, chlorogenic acids, terpenoids, arginine, citric acids, etc.	UPLC- HRMS (micrOTOF)	Evaluation of processing conditions of different carrot varieties and cultivars	[112]
Green coffee samples	O-Sinapoylglucose, 3-hydroxysuberic acid, N-acetyl-L-phenylalanine, 5-FQA trans, caffeoyl alcohol, 5-CQA cis, 5-FQA cis and palmitic acid	UPLC-HRMS (QTOF)	Discrimination of coffee according to its origin and genotypes	[113]
Green teas	Trace contaminants	UPLC-HRMS (TOF)	Assessment of contaminants in green tea	[114]
Annona cherimola Mill. (cherimoya)	Sugars, amino acids, phenolic acids and derivatives, flavonoids, phenylpropanoids, and other polar compounds	LC-HRMS (TOF)	Search of bioactive compounds present in Cherimoya	[115]

Table I.4. Continued.

Food	Metabolites	MS-based technique	Application	Ref.
Green seaweeds (*Caulerpa*, *Ulva*, and *Codium* species)	Malvidin, kaempferol, cyanidin, quercetin, apigenin, lutein, and myricetin	LC-HRMS (TOF) GC-MS	Assesment of biochemical and antiproliferative activities of green seaweeds.	[116]
Cheeses PDO GP cheeses and non-PDO grated "Grana-type" cheeses	Lipids, amino acids and oligopeptides	UPLC-HRMS (QTOF)	Discrimination of PDO Grana Padano cheeses from non-PDO Grana Padano cheeses	[117]

DART, Direct analysis in real-time; HRMS, high resolution mass spectrometry; PDO, protected designation of origin; QTOF, quadrupole-time-of-flight; TOF, time-of-flight; UPLC, ultra-high performance liquid chromatography; UHT, ultra-high-temperature processing.

The different works grouped in this table have pointed out the importance of developing untargeted metabolomics strategies for the analysis of food metabolome. A great variety of food matrices such as fruit juices, potato, soybean, garlic, onion, tiger nut, rice bran, meat, asparagus, milk, ginseng, wine, carrot, tea, cherimoya, seaweeds and cheese, has been studied by these strategies. For instance, untargeted metabolomics have been applied to the detection of contaminants in meat (pork, beef, and chicken) [105], milk [108] and green tea [114], for the search of bioactive compounds in rice bran [103] and cherimoya [115], to assess the authenticity of Indian citrus fruit and fruit juices [98], garlic [106] and Grana Padano cheese [117], or to evaluate the processing of black garlic [101], tiger nut milk [102] or ginseng products [109]. In these works, QTOF and TOF detectors have been the preferred MS analyzers. The use of these untargeted metabolomics strategies in the above mentioned works has enabled to propose different families of compounds with different polarities (lipids, phenolic compounds or amino acids, among others) as chemical markers of food quality, safety and traceability.

I.3. Separation and identification of peptides

Peptides are molecules constituted by amino acids linked by amide bonds. Commonly, peptides contain up to one hundred amino acids, more amino acids are already considered to constitute a protein. Peptides play important physiological and biochemical functions in the human body depending on their amino acid sequence, being involved in numerous biochemical processes within the nervous, immunological, and cardiovascular systems or intestine [118].

The biological activity of peptides is based on their inherent amino acid composition and sequence which commonly range from 2 to 20 amino acids [119] and they usually present molecular masses below 6000 Da [120]. Bioactive peptides have been compiled and annotated in databases that provide information on e.g. their activity *in vivo*, with the most numerous cases being attributed to antioxidant, antibacterial and antihyper/hypotensive effects. The database BIOPEP [121] collects information about the peptides and bioactive proteins discovered to date. According to this database, there are 3712 bioactive peptides and 740 bioactive proteins, which can be classified into 47 categories according to their bioactivity. As it can be seen in **Figure I.15**, the most frequent bioactive peptides show inhibitory activity of the angiotensin converting enzyme (ACE) followed by those having antioxidant and antibacterial activities. Additionally, some peptides can exert multifunctional activities (e.g. showing simultaneously ACE and antioxidant activities).

The main natural sources of bioactive peptides are food and the residues from the food industry.

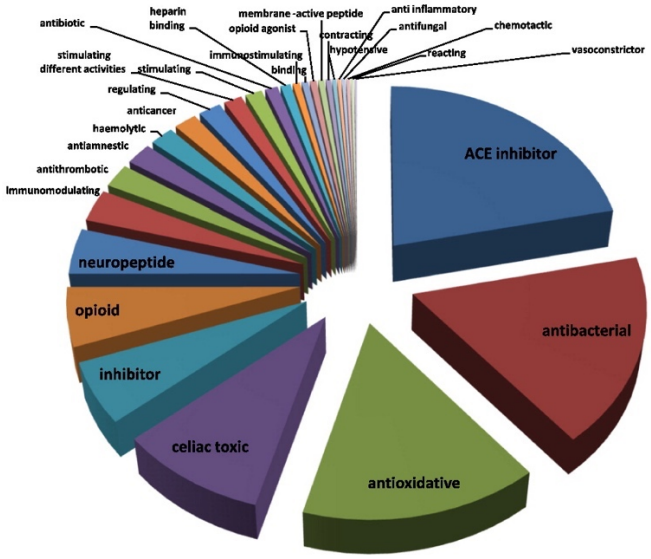


Figure I.15. Classification of known peptides based on their reported activity in the BioPep database (n = 2594). Reprinted from [122] with permission from Elsevier.

I.3.1. Peptides from food and food residues

The traditional concept of dietary proteins is based on regarding them as substances which provide the organism with essential amino acids necessary for the maintenance of life. A relatively recent interest in the identification and characterization of bioactive peptides released from dietary proteins from plant and animal sources has emerged. As **Figure I.16** shows, peptides can be natural ingredients of food (e.g. garlic or mushrooms) [123, 124] or can be released from parent food proteins by *in vivo* or *in vitro* pathways [125]. The *in vivo* pathway involves the degradation of dietary proteins by digestive enzymes (gastrointestinal tract) in the organism whereas the *in vitro* approach is due to protein processing by the action of microorganisms or enzymes derived from microorganisms or plants. It is interesting to mention peptides originated in the food processing since even though they are released from food proteins, the objective is to obtain desired products like yoghurt, cheese or other fermented products (e.g. kefir) [126-133].

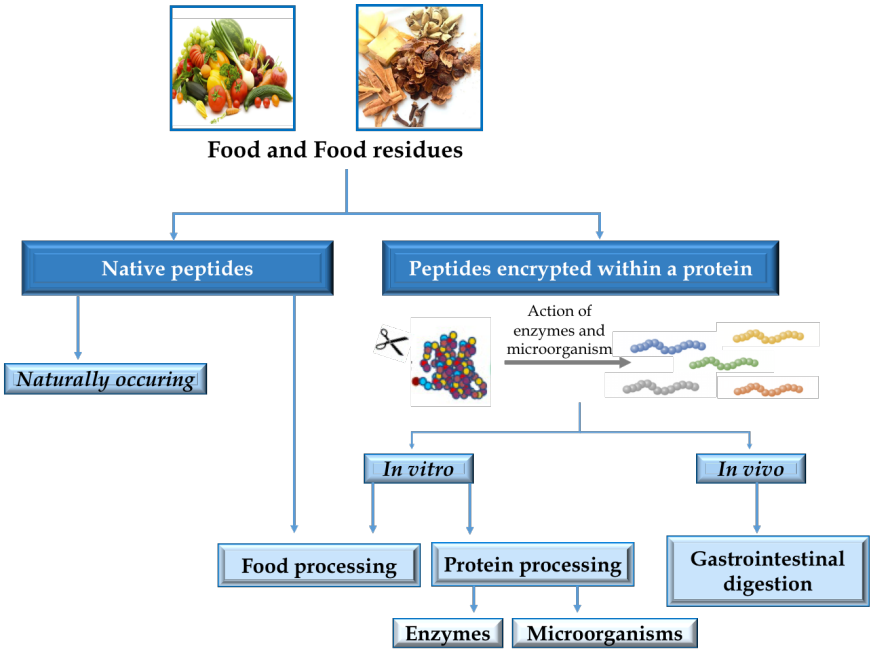


Figure I.16. Scheme summarizing the pathways to obtain peptides from food and food residues.

Numerous bioactive peptides presenting different biological functions have been found in food from animal origin like milk and egg [134]. Although milk and egg are still the main source of antihypertensive peptides, an important increase in the number of works dealing with the search of antihypertensive peptides from different foods from animal origin such as seafood (e.g. shrimps, oyster, etc.), fishes (tuna, salmon, etc.), meat (pork and chicken) or cheese, has been observed [134-136]. In addition, antioxidant peptides have been also found in several seafoods (e.g. oyster), fishes (sardine, tuna, etc.), meat (e.g. camel and bovine meat), eggs, milk and other dairy products [137-139]. Bioactive peptides of vegetable origin have been studied in a lesser extent but their relevance is increasing. Among vegetable food, maize and soybean have received special attention as a source of bioactive peptides (especially antihypertensive and antioxidant peptides); however, other sources have been also studied observing antioxidant (e.g. algae, soybean, rice, peanut, corn, sunflower, etc.) [140] or antihypertensive (e.g. wheat, maize, rice, pea, corn and apricot almond, among others) peptides [135, 140].

Although the benefits of the intake of bioactive peptides derived from food are already known, the production of bioactive peptides from sustainable and cheap sources (e.g. residues from the food industry) allows to reuse this waste and to obtain high added-value compounds resulting in the decrease of production costs of functional food and nutraceuticals. A large amount of waste is generated in the

food industry which can constitute up to 70 % of the initial product [141]. In some cases, many of these residues are used for animal feed or for fuel and compost manufacturing. However, a significant amount of these residues are disposed in landfills or incinerated, which causes the emission of pollutants constituting environmental and health problems. Thus, there is a trend in the area of peptide production from food residues oriented to obtain highly valuable material (e.g. bioactive peptides) from cheap sources, using low cost and highly efficient methods. For instance, it has been reported that meat co-products (e.g. blood, fat, skin, organs, bones, etc.) present a high content of good quality proteins and are an interesting source of bioactive peptides with different bioactivities including antioxidant, antimicrobial and antihypertensive. These peptides are generated when commercial proteases are used. Indeed, some of these meat processing co-products including blood, bones or lung have been proposed as novel sources of bioactive peptides with potential for their commercial use [142]. Moreover, waste from marine sources (e.g. skin, bones, organs, etc.) has also demonstrated to be a rich source of bioactive peptides which can be used as antioxidant, antihypertensive, antitumor, anticoagulant, and antimicrobial ingredients used in functional foods, nutraceuticals or pharmaceutical formulations [143]. Among the food residues, it is worth mentioning milk whey, that is one of the most important milk by-product which comprises the 20 % of total milk protein. It is rich in branched and essential amino acids, functional peptides, antioxidants and immunoglobulins enabling to provide benefits against a wide range of metabolic diseases. Whey milk proteins can lead to whey protein hydrolysates or peptides with important antioxidant, antihypertensive and anti-inflammatory properties [144-147].

Regarding vegetable residues, they also represent a source of important bioactive peptides although they have been much less studied than animal waste. For instance, cereals residues (e.g. wheat or rice) are one of the studied food by-products of plant origin, in terms of protein and peptide composition. Wheat bran is a by-product of the milling of wheat grain, and it contains more than 15 % high quality proteins. These proteins present a great nutritional and functional properties. Moreover, they are also an important source of amino acids and peptides with interesting bioactive properties (e.g. antioxidant, antihypertensive, antimicrobial, etc.) [148]. Oil industry derivatives such as sunflower, canola, rapeseed, palm and peanut are some vegetable waste with a high content of proteins ranging from 10 % to 54 % (dry weight) [149]. These proteins are a rich source of peptides with high antioxidant and antihypertensive properties [150]. Recently,

peptides from olive stone have also demonstrated to present lipid-lowering capacity [151]. Other seeds from fruits have shown to present diverse bioactivities. Indeed, antioxidant peptides have been reported in tomato seeds [152], Chinese cherry [153] and peach by-products [154] whereas antihypertensive peptides have been found in bitter melon seeds [155] and peach stone [156].

I.3.2. Workflow for the analysis of protein hydrolysates

The classical workflow followed in the analysis of food peptides from protein hydrolysates is depicted in **Figure I.17.** Peptides can be obtained by hydrolysis of proteins by the action of proteolytic enzymes or microorganisms.

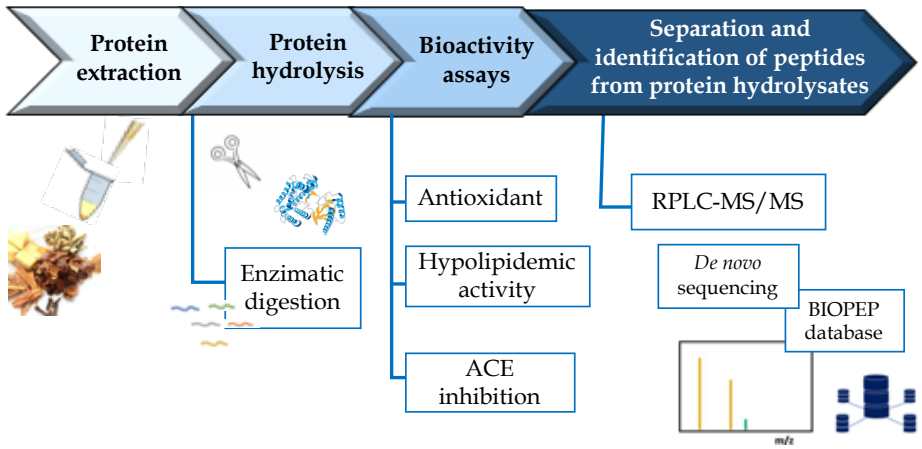


Figure I.17. Workflow usually followed for the analysis of peptides from protein hydrolysates.

Once peptides are obtained, *in vitro* bioactivity assays are needed to study if they exert some kind of bioactivity. Subsequently, peptides are separated and identified by MS with the aid of *de novo* sequencing strategy. A more detailed description of each one of these steps will be presented in the following sections.

I.3.2.1. Extraction of proteins from food and food residues

Before protein extraction, a mechanical disruption of cells (process called cell lysis) is usually required. Some of the most employed treatments to achieve cell lysis are manual homogenization, vortexing, grinding, or liquid nitrogen treatment [157]. They can be grouped into five major categories:

- mechanical homogenization
- osmotic lysis

- chemical lysis
- sonication and high-pressure treatments
- temperature treatments

Among them, chemical lysis and high-pressure procedures will be briefly described as follows as they have been the ones employed in this PhD Thesis.

Most common treatments to perform a chemical lysis include the use of antibiotics, chelating agents, surfactants, and solvents (such as acetonitrile, methanol and ethanol) capable of disintegrating cells. In some cases, the use of surfactants or strong chaotropic reagents (e.g. urea, thiourea or guanidine chloride) within the extraction buffer assures the disruption of protein interactions and the interactions established between proteins and other compounds. The most employed surfactants with this aim are: bile acid salts (charged soft surfactants, such as sodium deoxycholate, used to extract native proteins), non-ionic surfactants (e.g. Triton X series and Tween 20 which are used rather to disrupt protein-lipid interactions), zwitterionic surfactants (such as 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) that can solubilize proteins), and ionic surfactants (such as sodium dodecyl sulfate (SDS)) that cause protein denaturation. The main limitations of most traditional surfactants and chaotropic reagents are that they may interfere in the enzymatic digestion and they are not compatible with MS detection [158, 159]. Therefore, to achieve an appropriate lysis buffer for protein extraction, the presence of surfactants or chaotropic reagents, along with the effect of other variables such as buffer composition, pH, ionic strength, salt concentration, temperature, and the presence of protein reducing agents (e.g. β-mercaptoethanol (β-ME), dithiothreitol (DTT)), or even components that prevent proteolysis (e.g. protease inhibitors) [159, 160], should be carefully investigated since the selection of optimal conditions for cell lysis is crucial for further steps of the peptide workflow.

High intensity focused ultrasounds (HIFU) or pressure treatments involve the application of ultrasonic waves to the solution. It generates a cyclic sound pressure with a frequency greater than 20 kHz [161]. By the applied pressure, certain chemical reactions are accelerated, replacing traditional techniques or accelerating them. This fact is produced by the focalization of high intensity ultrasonic waves into the liquid media which creates the effect known as cavitation (a physical process by which tiny gas bubbles are produced and can be considered as microreactors inside which there are high temperatures and pressures). The use

of HIFU has demonstrated to improve extraction time and yields since it provides 100 times higher energy than the ultrasonic bath.

Once proteins have been extracted, a precipitation step is usually performed in order to separate them from interfering compounds (for example, plant tissues contain pigments, terpenes, lipids, polysaccharides or phenolic compounds, among others, that can interfere in the extraction and analysis of proteins [162, 163]), to change the environment if required in next steps, or to preconcentrate proteins. It should be indicated that removal of interfering compounds can also be performed before protein extraction. Several procedures such as precipitation at pI, precipitation at high temperatures or precipitation using various reactants/solvents (acetone, ethanol, methanol, acetonitrile) are the usual procedures to carry out protein precipitation [164].

Finally, extracted and precipitated proteins must be solubilized. Thus, the non-covalent interactions produced between proteins must be disrupted and proteins must be kept in solution for further analysis. For this purpose, the solubilization usually implies the combination of buffers containing surfactants (e.g. SDS), reducing agents (e.g. DTT or 2-mercaptoethanol) and chaotropic agents (e.g. urea or thiourea) under agitation or sonication conditions.

Once proteins are solubilized, it can be interesting to characterize them. With this aim, there are several electrophoretic techniques allowing their separation, being sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) one of the most widely used and the one employed in this PhD Thesis.

In SDS-PAGE, proteins are separated in polyacrylamide gels under denaturing conditions using SDS surfactant and β-ME or DTT. SDS anions bind to protein chains creating a complex with a large negative charge which masks the real protein charge. Thus, the net charge per unit mass is approximately constant in all separating molecules and their separation is based on their molecular masses. During the electrophoretic separation, SDS-protein complexes migrate towards the positively charged electrode (anode). Small molecules with high net charge present high electrophoretic mobility and pass through the gel faster than intermediate and larger molecules with low net charge which pass through the gel slowly or they are immobilized [165]. Gels in SDS-PAGE are characterized by the total percentage concentration (%T) of both monomers (acrylamide and cross-linker). The size range of molecules that can be separated on the gel is limited by %T value. Therefore, the higher the %T, the smaller the molecules that can be separated [166]. Once the separation is reached, the detection of proteins can be achieved using many stains like Coomassie Brilliant Blue, silver dyes, fluorescence dyes or radiolabeling, being

Coomassie Brilliant Blue or silver dyes the most common staining methods used in SDS-PAGE separation [167].

I.3.2.2. Protein hydrolysis

Different protein hydrolysis approaches can be achieved to release encrypted peptides within a parent protein. As **Figure I.16** shows, *in vivo* and *in vitro* proteolytic pathways can be followed. The former is the gastrointestinal digestion itself while *in vitro* strategy involves protein hydrolysis by the action of microorganisms, enzymes derived from microorganisms or plants, or by autolyzation (which is based on the proteolytic activity of natural ingredients in some foodstuffs) [168-170].

The most common approach employed to obtain bioactive peptides from food is the enzymatic hydrolysis of proteins. Among the different commercially available enzymes that can be employed, proteases from animal (e.g. pepsin, trypsin and chymotrypsin), microbial (alcalase, flavourzyme and neutrase) or vegetable origin (papain and bromelain) are the most used. These enzymes are frequently employed to produce peptides with high bioactivity, especially, antioxidant and antihypertensive activities [171-174]. Enzymatic digestion can be achieved using a selected enzyme or by sequential digestion with different kinds of enzymes. The effectiveness of bioactive peptides production encrypted within a parent protein will depend mainly on the protease employed as well as the hydrolysis degree obtained. Thus, the use of enzymes with low specificity is preferred in order to provide high hydrolysis degree and small peptides which present higher bioactivity, although the hydrolysis is unpredictable [175]. Moreover, conditions such as protein substrate, proteolytic enzymes employed, enzyme to substrate ratio, physicochemical conditions (pH, preheat treatment, digestion buffer, hydrolysis time, and temperature), degree of hydrolysis, post-hydrolysis modifications, etc., which affect to the final composition of hydrolysates need to be properly evaluated [176].

I.3.2.3. Bioactivity assays

Once protein hydrolysates are obtained, their bioactivity can be investigated by using one or more *in vivo* or *in vitro* assays. Since the most frequent bioactive peptides show inhibitory activity of the angiotensin converting enzyme (ACE) and antioxidant activity (see **Figure I.15**), both of them will be briefly described.

Antihypertensive activity assays

Although there are different assays to evaluate the antihypertensive activity, the most employed was proposed by Cushman and Cheung [177]. This *in vitro* assay consists of monitoring the conversion of an appropriate substrate by ACE in the presence and absence of peptide inhibitors. Thus, the hydrolysis of the tripeptide hipuryl-histidyl-leucine to hippuric acid and histidyl-leucine is performed by ACE in the presence of the compound whose activity is to be measured. Then, the hippuric acid formed can be analyzed using spectrophotometric or chromatographic techniques [178, 179]. The inhibitory capacity of a compound is measured from the IC 50 value, which is the concentration necessary to inhibit 50 % of the activity of the enzyme.

The antihypertensive activity of peptides is determined by their size and their amino acid composition. Thus, ACE inhibitory peptides usually show short sequences of amino acids ranging from 2 to 12, since larger chains may have restricted the access to the active center of the enzyme [156]. Moreover, the C terminal end of the peptides has an important influence on their activity. The existence of hydrophobic residues (Lys, Pro or Arg) or aromatic residues (Trp or Phe) in the last three positions of the C terminal end also presents a significant effect on the capacity of inhibition [154, 167]. The presence of aliphatic residues (Leu or Ile) at the N terminal group is a common trend among peptides with high antihypertensive capacity [180]. If Pro is included in the sequence, this also results in a higher antihypertensive activity and in a major resistance to gastrointestinal digestion. Unlike antioxidant peptides, a single peptide or fraction may be responsible from the antihypertensive activity of the hydrolysate.

Antioxidant activity assays

The total antioxidant capacity shown by a protein hydrolysate does not come usually from a single antioxidant peptide, but is the product of synergistic interactions established between different peptides [181].

Due to the complex nature of antioxidants, there is no official standardized method proposed to study antioxidant capacity of peptides. Thus, many *in vitro* methods such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay, oxygen radical absorbing capacity assay, ferric reducing antioxidant power, hydroxyl radical scavenging, etc. have been implemented. Most of antioxidant capacity assays can be grouped into two categories based on chemical reactions involved: (i) hydrogen atom transfer reaction based assays and (ii) single electron

transfer reaction based assays [182]. In the first group are included the capacity of inhibition of the oxidation of the fatty acids and the capacity to inhibit the formation of hydroxyl radicals, whereas the assays included into the second group are the reducing antioxidant power of the ferric ion assay and the ability to trap radicals ABTS and DPPH, although the latter can also be considered within the first group.

The most common assay to evaluate the inhibition of lipid peroxidation is to induce the oxidation of linoleic acid in presence of antioxidant compounds. The lipid hydroperoxides generated are very reactive and they produce the oxidation of Fe^{2+} to Fe^{3+}. The reaction can be monitored from the complex formed by Fe^{3+} in presence of thiocyanate [183]. The capacity to inhibit the formation of hydroxyl radicals is usually measured through the H_2O_2-$FeSO_4$-phenanthroline reaction based assay. The hydroxyl radical is generated when hydrogen peroxide reacts with Fe(II) (Fenton reaction) which results in reducing Fe(III) to Fe(II) and making the $HO^{\bullet}$ generation catalytic. The addition of antioxidant peptides would allow to inhibit the Fenton reaction and, therefore, Fe^{2+} to Fe^{3+} oxidation, observing an increase in the absorbance signal due to the complex formation between Fe^{2+} and phenanthroline [184].

The measurement of the ability to capture free radicals provides very useful information about the antioxidant capacity of a compound. There are different assays for this purpose, being the most employed the ABTS and DPPH radicals due to their rapidity, simplicity and low cost. $ABTS^{+\bullet}$ radical (green color), the oxidant, is generated by persulfate oxidation of ABTS. The action of the antioxidant compounds on this radical gives rise to the formation of the $ABTS^{+}$ cation (colorless), which can be easily monitored from the reduction of the absorbance signal observed at 734 nm wavelength (maximum absorption of the $ABTS^{+}$ radical)[185]. DPPH radical is one of the few stable and commercially available organic nitrogen radicals and has an absorption maximum at 515 nm. The reaction progress can be monitored by UV-Vis spectrophotometry [186].

I.3.2.4. Separation and identification of peptides from protein hydrolysates

The complexity of food samples makes indispensable the use of advanced analytical techniques to achieve the separation of peptides from protein hydrolysates. In this sense, LC is the technique most commonly employed [187].

Different chromatographic modes, such as RPLC, HILIC, ion exchange chromatography, size exclusion chromatography or affinity chromatography, have been employed to perform the separation of peptides [188]. RPLC with C18

stationary phases is one of the most employed approach for peptide separation and it is the one employed in this PhD Thesis. The pore size of the RPLC stationary phases used, generally ranges between 100–300 Å depending on the size of the peptides [189]. However, as mentioned in section I.2.1.2, in order to reduce analysis times and maintain column efficiency with relatively low backpressure, fused-core silica particles (*fused-core* or *core-shell*) have been developed. Usually, the mobile phases used to separate peptides with this LC mode consist of water and an organic phase (e.g. acetonitrile, methanol, ethanol or 2-propanol) with a modifier (e.g. acetic acid, formic acid and trifluoroacetic (TFA)). Among the organic solvents, acetonitrile is the most commonly used due to its volatility, low viscosity, and transparence to UV light. The elution of peptides is usually carried out under gradient conditions [190]. Regarding the modifiers, reagents are usually added to the mobile phase in order to increase the hydrophobicity of charged peptides. The most widely employed ion-pairing reagent is TFA. However, the use of TFA is not compatible with MS since it suppresses electrospray ionization causing significant signal decrease, so it is necessary to replace TFA by acetic or formic acids when MS is employed [189].

The most commonly used detection systems are UV, fluorescence and MS. Wavelengths at which peptides are detected are 210-220 nm (peptide bonds), 254 nm (absorption maximum of phenylalanine) and 280 nm (absorption maximum of tyrosine and tryptophan) [191]. Fluorescence detection is performed at a λ_{exc} of 280 nm and λ_{em} of 360 nm, which are characteristics of tryptophan [192]. Regarding MS detection, its use has increased considerably in recent decades due to its high sensitivity and the wide range of masses which can be analyzed. Nowadays, ESI and matrix-assisted laser desorption/ionization (MALDI) are the most employed techniques on the peptide research field [193]. As explained in section I.2.1.2, ESI is a soft ionization technique, achieved at atmospheric pressure that can be easily coupled to an LC system, unlike MALDI. ESI-MS system is preferred for complex samples while MALDI is often used for relatively simple peptide mixtures [194]. Most popular analyzers in this field are single Q, TOF, IT and Orbitrap [193]. Moreover, hybrid mass spectrometers such as QTOF (which has been employed in this PhD Thesis to carry out the detection of peptides from protein hydrolysates) enable to obtain additional information on the primary structure of peptides (amino acid sequence). A description of the QTOF system has already been presented in section I.2.1.2. In this MS system, peptide ion is first isolated (usually the most abundant one) and then, it is fragmented in order to obtain its MS/MS spectrum. The fragment ions produced can be separated into two classes. One class retains the

charge on the N-terminal whereas the cleavage is observed in the C-terminal. In this type of fragmentation, three different positions can occur (see **Figure I.18)** each of which is sequentially designated as types a_n, b_n, and c_n. The second class of fragment ions generated from the N-terminal retains the charge on the C-terminal, while cleavage is observed from the N-terminal, giving fragments at three different positions, x_n, y_n, and z_n (see **Figure I.18**).

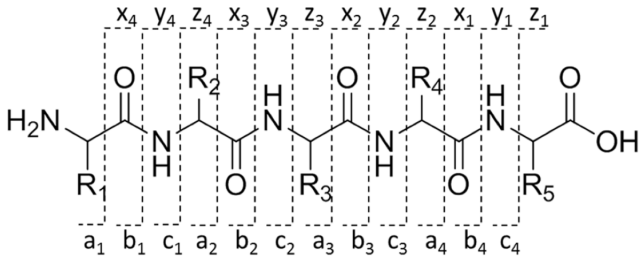


Figure I.18. Common peptide fragmentation pathway and nomenclature. Reprinted from [195] with permission from ACS Publications.

Different methods can be employed to perform MS/MS fragmentation, being the most widely employed collision-induced dissociation, in which peptide ions undergo dissociation at amide bonds generating b_n and y_n type fragment ions. However, fragments a_n, c_n, x_n o z_n types could also be produced, in a lesser extent. Sometimes, when using unspecific enzymes to carry out protein hydrolysis, peptide fragmentation information can be incomplete which results in complex MS/MS spectra analysis. Indeed, peptides obtained by enzymes, which do not cut at basic residues, do not possess charge at C- or N-terminal of peptides. Thus, the abundance of b_n and y_n ions series can be decreased and some abundant internal ions are generated. This fact complicates spectrum interpretation and peptide identification. Obtained data can be treated using databases or *de novo sequencing*, which consists of obtaining the amino acid sequence using the fragmentation spectra obtained by MS [122, 196].

I.3.3. State of the art of the analysis of peptides in food protein hydrolysates

Over the last decade, many studies have described the role of food proteins as a source of biologically active peptides and different strategies to improve their production. They are obtained mainly from vegetal and animal sources. Animal sources of bioactive peptides include milk, egg, red meat and marine animals. On the other hand, some vegetal sources of bioactive compounds and proteins are

wheat, maize, soy, rice, mushrooms, pumpkin, sorghum, and amaranth. Several works have been published in the last years concerning the obtaining and the identification of peptides from food [197, 198].

Marine sources are considered as one of the richest ones to obtain bioactive compounds. Thus, the extraction and use of marine-derived peptides have attracted much attention owing to their potential health benefits.

Table I.5 summarizes the works published in the period from 2015 to date and devoted to the identification of peptides in protein hydrolysates from marine sources. As it can be observed, the identified peptides in these matrices present different biological activities. Several fishes (lizard, tilapia, salmon, stone, giant grouper and anchovy), seafood (mussels and red king crab), algae (*Saccharina longicruris, Ulva rigida C. Agardh, Gracilariopsis lemaneformis, Tetradesmus obliquus, Palmaria palmata and Porphyra spp.*) and other marine species such as marine snail, big-belly seahorse and marine sponge have been studied. Peptides with different biological activities have been identified and in some cases quantified in these marine sources. The most employed technique in this field has been RPLC-QTOF as shown in **Table I.5.**

Regarding algae, only six works explored the potential of this natural source of peptides and were focused on the study of *Saccharina longicruris, Ulva rigida C. Agardh, Gracilariopsis lemaneformis, Tetradesmus obliquus, Palmaria palmata and Porphyra spp.* Among these works, four of them were focused on macroalgae.

The macroalga *Saccharina longicruris* proteins were extracted and hydrolysed with the enzyme trypsin in order to recover antibacterial peptides [201]. The characterization of peptides was carried out using nanoLC-MS/MS. Peptidic sequences were chemically synthesized and their bioactivity confirmed, suggesting that activity probably results from synergies among peptides [201]. *Ulva rigida* proteins were hydrolysed with pepsin plus bromelain [202]. This hydrolysate, presenting ACE-inhibitory activity, was fractionated by ultrafiltration membranes and then, the most active fraction was purified using size-exclusion chromatography RPLC, yielding two active ACE inhibitory purified peptides. Both peptides were synthesized to confirm the structure and to validate their ACE inhibitory activities [202].

Table I.5. Peptides identified in protein hydrolysates from marine sources in the period from 2015 to 2019.

Food	Peptides (bioactivity)	Technique	Ref.
Lizard fish	GMKCAF (ACE inhibitory activity)	LC-HRMS (TOF)	[199]
Tilapia mince	MILLLFR (ACE inhibitory activity)	LC-MS (IT)	[200]
Saccharina longicruris macroalga	9 peptides (antibacterial activity)	NanoLC-MS/MS	[201]
Ulva rigida macroalga	IP, AFL (ACE inhibitory activity)	LC-HRMS (QTOF)	[202]
Gracilariopsis lemaneiformis microalgae	QVEY (ACE inhibitory activity)	LC-HRMS (TOF/TOF)	[203]
Cod skin	GEIGPSGGRGKPGKDGDAGPK, GFSGLDGAKGD (matrix metalloproteinases inhibitory activity)	LC-HRMS (QTOF)	[204]
Mytilus edulis tissues	VQQELEDAEERADSAEGSLQK, RMEADIAAMQSDLDDALNGQR, AAFLLGVNSNDLLK (anticoagulant activity)	UPLC-HRMS (QTOF)	[205]
Salmo salar	21 peptides (ACE inhibitory activity)	LC-MS (Q)	[206]
Red Seaweed (*Porphyra spp*)	GGSK, ELS (α-amylase inhibitory activity)	LC-HRMS (QTOF)	[207]
Tetradesmus obliquus microalgae	WPRGYFL, GPDRPKFLGPF, WYGPDRPKFL, SDWDRF (antioxidant and ACE inhibitory activity)	Nano-LC-MS/MS	[208]

Table I.5. Continued.

Food	Peptides (bioactivity)	Technique	Ref.
Red King Crab (*Paralithodes camtschaticus)*	3 paralithocins 1−3 peptides (antimicrobial activity)	LC-MS (Q/TOF)	[209]
Marine snail (*Neptunea arthritica cumingii)*	YSQLENEFDR, YIAEDAER (antioxidant, ACE inhibitory, and antidiabetic activities)	NanoLC-HRMS (Orbitrap)	[210]
Marine Sponge (*Xestospongia testudinaria)*	KENPVLSLVNGMF, LLATIPKVG, VFSILV (cytotoxic activity)	LC-MS/MS	[211]
Big-belly seahorse *(Hippocampus abdominalis)*	GIIGPSGSP, IGTGIPGIW, QIGFIW (antioxidant, ACE inhibitory activities)	LC-HRMS (QTOF)	[212]
Stone fish (*Actinopyga lecanora*)	GVSGLHID (antioxidant activity)	LC-HRMS (QTOF)	[213]
Anchovy	TPSAGK, LE, LEE (antioxidant and antimicrobial activities)	UPLC-HRMS (QTOF)	[214]
Macroalgae *(Palmaria palmata)*	SDITRPGGNM (antioxidant activity)	Semi preparative LC-HRMS (QTOF)	[215]

Admassu et al. developed a RPLC-QTOF methodology enabling to identify bioactive peptides with α-amylase inhibitory potential from enzymatic protein hydrolysates of red seaweed (*Porphyra spp*) [207]. Enzyme-assisted extraction of proteins was carried out with subsequent enzymatic hydrolysis using different enzymes (pepsin, trypsin, alcalase and neutrase). Two novel peptides were found possessing α-amylase inhibitory activity. Moreover, Harnedy et al. developed a RPLC-QTOF methodology to identify antioxidant peptides from an enzymatically hydrolysed *Palmaria palmata* protein isolate [215]. Protein hydrolysate was obtained with the food-grade proteolytic enzyme Corolase PP and then, it was sequentially fractionated using solid-phase extraction and semi-preparative (SP) RPLC. Among the peptides selected for synthesis, SDITRPGGNM showed the highest oxygen radical absorbance capacity and ferric reducing antioxidant power activity. In this sense, proteins derived from red macroalgae have emerged as potential substrates for the generation of bioactive peptides. However, there are still numerous species and varieties of macroalgae whose potential is still unknown. For this reason, one of the objectives of these PhD Thesis was to look for other macroalgae that could constitute natural sources of peptides with potential biological activity.

I.3.4. State of the art of the analysis of peptides in protein hydrolysates from food residues

Nowadays, society depends on a number of finite resources whose processing generates many waste with significant environmental impact. For this reason, it is well recognized the importance of the efficient use of natural resources and the minimization of waste generation. In this sense, there are several food residues that contain an elevated protein content that is irretrievable lost. These proteins can be a source of bioactive peptides that could be used in the elaboration of functional foods, nutraceuticals or cosmetics. Therefore, there is an ongoing interest in the development of methodologies for the valorization of these sustainable protein sources, resulting in a minimization of waste generation.

Table I.6 groups the works published in the period from 2015 to date devoted to the identification of peptides in protein hydrolysates from food residues. Some of the animal residues explored in the last years as sources of peptides are scallop female gonads, shrimp shell discards and other wastes, waste yak milk, spotless smoothhound cartilages, tuna processing biomass, and sea cucumber.

Table I.6. Peptides identified in protein hydrolysates from food residues in the period from 2015 to 2019.

Food residue	Peptides (bioactivity)	Technique	Ref.
Peach (*Prunus persica (L.) Batsch*) stones	LYSPH, LYTPH, HLLP (ACE inhibitory activity)	LC-HRMS (QTOF)	[156]
Thornback ray skin gelatin	APGAP, GIPGAP, AVGAT (ACE inhibitory and antioxidant activities)	LC- and nanoLC-HRMS (QTOF)	[216]
Horse mackerel and small- spotted catshark	VAMPF (ACE inhibitory activity)	UPLC-HRMS (QTOF)	[217]
Bluefin leatherjacket (*Navodon septentrionalis*) heads	WEGPK, GPP, GVPLT (antioxidant activity)	LC-HRMS (QTOF)	[218]
Rice residue	RPNYTDA, TSQLLSDQ, TRTGDPFF, NFHPQ (antioxidant activity)	Nano-UPLC-HRMS (QTOF)	[219]
Olive (*Olea europaea*) seeds	22 peptides (ACE inhibitory activity)	LC-HRMS (QTOF)	[150]
Cherry (Prunus cerasus L.) seeds	36 peptides (ACE inhibitory and antioxidant activities)	LC-HRMS (QTOF)	[179]
Plum (*Prunus domestica L.*) stones	37 peptides (ACE inhibitory and antioxidant activities)	LC-HRMS (QTOF)	[178]

Table I.6. Continued.

Food residue	Peptides (bioactivity)	Technique	Ref
Oil palm (*Elaeis guineensis Jacq.*) kernel	VVGGDGDV, VPVTST, LTTLDSE (antioxidant activity)	LC-HRMS (QTOF)	[220]
Peach seeds	21 peptides (antioxidant activity)	LC-HRMS (QTOF)	[154]
Gelatin seabass (*Lates calcarifer*) skin	GLFGPR, GATGPQGPLGPR, VLGPF, QLGPLGVP (antioxidant activity)	UPLC–MS/MS	[221]
Scallop (*Patinopecten yessoensis*) female gonads	HMSY, PEASY (antioxidant activity)	LC-HRMS (QTOF)	[222]
Skate (*Raja porosa*) cartilage	FIMGPYGPAGGDY, IVAGPQ (antioxidant activity)	LC-HRMS (QTOF)	[223]
Shrimp shell discards	29 peptides (ACE inhibitory activity)	NanoLC-HRMS (Orbitrap)	[224]
Waste yak milk	RVMFKWA, KVISMI (antimicrobial activity)	LC-HRMS (TOF)	[225]
spotless smoothhound (*Mustelus griseus*) cartilages	GAERP, GEREANVM, AEVG	LC-HRMS (QTOF)	[226]
Olive stones	15 peptides (lipid- lowering activity)	LC-HRMS (QTOF)	[151]

Table I.6. Continued.

Food residue	Peptides (bioactivity)	Technique	Ref
Olive stones	10 peptides (ACE inhibitory and anti-proliferative activities)	LC-HRMS (QTOF)	[227]
Tuna processing waste biomass	YENGG, EGYPWN, YIVYPG, WGDAGGYY (antioxidant activity)	LC-HRMS (QTOF)	[228]
Cauliflower by-products (leaves and stems)	APYDPDWYYIR, SKGFTSPLF, LDDPVFRPL, LRAPPGWTGR (ACE inhibitory and antioxidant activities)	NanoLC-HRMS (Orbitrap)	[229]
Shrimp waste	SSSKAKKMP, HGEGGRSTHE, WLGHGGRPDHE, WRMDIDGDIMISEQEAHQR (ACE inhibitory activity)	LC-HRMS (QqQ)	[230]
Sea cucumber (*Stichopus japonicus*) gonad	EIYR, LF, NAPHMR (antioxidant activity)	LC-HRMS (TOF)	[231]
Tomato seeds	DGVVYY, GQVPP (ACE inhibitory and antioxidant activities)	NanoLC-HRMS (QTOF)	[232]
Johnius belengerii bone	KSA (osteogenic activity)	LC-HRMS (TOF)	[233]
Fish skin, pig skin, pig bone and bovine skin gelatins	GPA (antioxidant activity)	LC-HRMS (QqQ)	[234]

Despite vegetable proteins have been less studied as a source of bioactive peptides as mentioned before, in the last five years their relevance has increased as vegetable food residues have been increasingly explored as an interesting source of these bioactive compounds. In fact, different works (among which an important part has been achieved by our research group) have investigated in the last years the potential of different vegetable residues such as rice residues, olive seeds and stones, cherry seed, plum stones, peach stones and seeds, palm oil kernel, and cauliflower leaves and stems. These matrices have an elevated protein content which can produce peptides with different biological activities.

As it can be observed in **Table I.6**, most of the residues studied are from marine origin due to their high protein content. The most preferable technique employed for the separation of peptides is RPLC and the most preferable detector used to perform their identification is QTOF. Although the number of publications concerning this field is relatively high, there is still a huge number of residues presenting an elevated protein content whose potential has not been exploited yet. To consider an example, coffee industry generates a huge amount of residues which have never been proposed as natural sources of peptides. For this reason, one of the objectives of this PhD Thesis has been to investigate one of these residues as a source of peptides with potential biological activity.

The information included in section I.1 is extended in the following book chapter and two review articles published in the context of this PhD Thesis:

Book chapter: *Recent applications of chiral capillary electrophoresis in food analysis.*
R. Pérez-Míguez, M. Castro-Puyana, M.L. Marina.
In: A. Haynes (Ed.), Advances in Food Analysis Research, Chapter 4. Nova Science publisher, New York, 2015, pp. 89-120.

Article 1: *Capillary Electrophoresis determination of non protein amino acids as quality markers in foods.*
R. Pérez-Míguez, M.L. Marina, M. Castro-Puyana.
J. Chromatogr. A 1428 (2015) 97-114.

Article 2: *Advances in the determination of non-protein amino acids in foods and biological samples by capillary electrophoresis.*
Raquel Pérez-Míguez, Sandra Salido-Fortuna, María Castro-Puyana, María Luisa Marina.
Crit. Rev. Anal. Chem. DOI: 10.1080/10408347.2018.1546113

A part of the information described in section I.2.2 is broaden in the following review article:

Article 3: *Application of mass spectrometry-based metabolomics approaches for food safety, quality and traceability.*
M. Castro-Puyana, R. Pérez-Míguez, L. Montero, M. Herrero.
Trends Anal. Chem. 36 (2017) 102-118.

Book chapter

Recent applications of chiral capillary electrophoresis in food analysis.

R. Pérez-Míguez, M. Castro-Puyana, M. L. Marina.

in: A. Haynes (Ed.), Advances in food analysis research, Chapter 4. Nova Science publishers, New York, 2015, pp. 89-120.

Abstract

The determination of the enantiomers of chiral food components, additives or contaminants presents a high interest in Food Science since it can provide information related to food quality, authenticity and safety, also enabling to evaluate the effect of processing or manufacturing procedures and possible contaminations. Capillary Electrophoresis (CE) has demonstrated in the last years a very high potential to achieve enantiomeric separations due to its high efficiency, versatility, feasibility and the low consumption of solvents, reagents and samples which makes it an environmentally friendly analytical technique. In addition, the fact that the chiral selector in CE can be added to the electrolytic solution makes very easy the screening of different chiral selectors in order to select the most adequate for the enantiomeric separation of a given compound which is the most difficult step in the development of a chiral analytical methodology.

This chapter highlights the interesting role that chirality plays when evaluating the quality and safety of foods and points out the main practical aspects that should be considered to carry out the chiral analysis of food components, additives and pesticides by CE. The characteristics of the CE separation modes mostly employed in chiral food analysis (Electrokinetic Chromatography, EKC and Capillary Electrochromatography, CEC) are summarized together with the main detectors employed to achieve the determination of enantiomers. Moreover, a critical overview on the main applications of chiral CE methodologies developed during the last ten years including the chiral analysis of protein and non-protein amino acids, organic acids, phenolic compounds, sweeteners, and pesticides in foodstuffs is presented. Experimental conditions enabling the chiral separations are presented and some representative examples are illustrated. Finally, the main advantages and drawbacks of these procedures are discussed with the aim of showing the potential of CE to solve specific problems in the Food Science research field.

1. Introduction

Research in the field of Food Science is continuously demanding the development of robust, efficient, selective, and sensitive analytical methodologies able to provide information about chemical composition of foods and to guarantee their safety, quality and traceability. These methodologies are also crucial in order to meet the requirements from official laboratories, consumers and legal regulations [1]. The interest is focused on a big number of nutritional ingredients, additives,

adulterants or pollutants [2], many of them being chiral molecules. Food components such as amino acids, organic acids or phenolic compounds, additives such as sweeteners, or pesticides used in agriculture to control pest, are examples of chiral molecules that can exist as, at least, one pair of enantiomers (depending on the presence of one or more chiral centers). Enantiomers are non-superimposable mirror images of a molecule (they have identical atomic composition and bonds but different three-dimensional arrangement). The intrinsic properties of nature (which is chiral because mainly uses one of the two enantiomers of a chiral compound) make that the receptors of cell machinery, for example enzymes, are chiral so that they prefer to bind one of the enantiomers of a chiral compound. [3]. As a consequence, when chiral molecules present in foods are consumed, their enantiomers may interact with the chiral environment present in the human body (enzymes, proteins, receptors) in a different way originating different effects. In addition, diverse activities have been shown for the enantiomers of food components that can exhibit, for instance, different taste or different aromas. For all these reasons, the interest in chiral food analysis has increased considerably in the last years.

In 1990, Armstrong had already established different areas in which enantiomeric separations may be relevant when achieving studies on food and beverages characterization and analysis [4]. **Figure 1** shows different topics in which chiral analysis can provide a broad range of useful and significant information in Food Science; from the identification of adulterated food and beverages, to the control and monitoring of fermentation processes and products or the evaluation of age, storage and treatment effects, among others. As a result, there is a growing interest in the development of analytical methodologies enabling to increase the knowledge about the enantiomeric composition of foodstuffs in order to evaluate its effect on both quality and genuineness.

Traditionally, the analytical techniques mainly used for chiral separations have been Gas Chromatography (GC), High performance Liquid Chromatography (HPLC), and Supercritical Fluid Chromatography (SFC). Among them, HPLC is used in an important part of the publications devoted to chiral analysis due to different reasons such as (i) a larger number of laboratories possess the instrumentation, (ii) many data are available in the literature, (iii) new research work focused on instrumentation and stationary phases has been carried out, and (iv) the high interest from companies involved in the market [5]. However, even though HPLC is widely implemented, Capillary Electrophoresis (CE) has emerged

in the last decades as a powerful tool in chiral analysis. From the first enantiomeric separation by CE published 30 years ago by Gassmann et al. [6], the use of CE in chiral separations has continuously grown until be established, in the last decade, as one of the most employed and powerful techniques for analytical enantioseparations [7]. This fact is mainly due to different reasons concerning its short analysis times, high separation efficiency, versatility and feasibility to incorporate a great variety of chiral selectors to obtain high resolutions. In addition, the fact that the chiral selector in CE can be added to the electrolytic solution makes very easy the screening of different chiral selectors in order to select the most adequate to achieve the enantiomeric separation of a given compound which is the most difficult step in the development of a chiral analytical methodology. Moreover, CE has certain advantages in terms of green chemistry, mainly related to the low consumption of solvents and reagents. All these facts explain that during the last years (2005-2015), more than 700 articles (including research works and reviews) can be found in the database "ISI Web of Knowledge" using as keywords "capillary electrophoresis" and "chiral or enantio*" combined with "food", "beverages", "environmental", "agrochemical", "pharmaceutical" or "clinical". Even though pharmaceutical and clinical fields continue being the fields in which chiral CE has the highest number of applications, the number of works dealing with enantioseparations by CE in food analysis (including food and beverage samples) has been increasing in the last years [5, 8-15]. In this chapter, the fundamentals of the enantiomeric separations by CE are summarized and the main practical aspects that should be considered to carry out the chiral analysis of food components, additives and contaminants by CE are described.

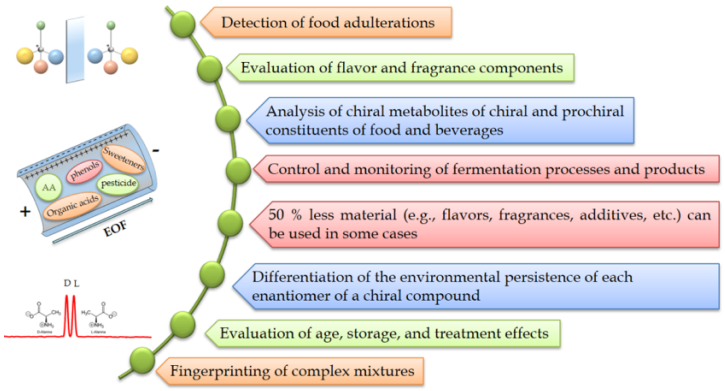


Figure 1. Areas in which enantioselective separations are relevant in Food Science. (Figure made following the table described by Armstrong et al. in [4] with permission from the American Chemical Society).

Moreover, a critical overview on the main applications of chiral CE methodologies to food and beverage analysis developed during the last ten years including the chiral analysis of protein and non-protein amino acids, organic acids, phenolic compounds, sweeteners, and pesticides is presented. Experimental conditions enabling the chiral separations are detailed and some representative examples are illustrated. Finally, the main advantages and drawbacks of these procedures are discussed with the aim of showing the potential of CE to solve specific problems in the Food Science research field.

2. Principles of chiral analysis by CE

An enantiomeric separation in CE is not based on an electrophoretic mechanism because the electrophoretic mobilities of the enantiomers of a chiral compound are equal and nonselective [3]. Indeed, the enantioselective recognition of enantiomers is due to a chromatographic mechanism based on their different interaction with a chiral selector. It implies the formation of either stable diastereoisomers by using an enantiopure chiral derivatization reagent (indirect mode in which the enantiomers are separated based on their different physicochemical properties in an achiral environment) or reversible diatereoisomeric complexes with a chiral selector (direct method where enantiomers-selector complexes are separated due to their different mobility). Although it is possible to find in the literature examples of indirect separation of chiral compounds into the food field [16], the truth is that the use of the indirect approach is much less widespread because of the practical disadvantages associated with the derivatization reactions (time-consuming) employing optically pure reagents (expensive and with limited availability). For this reason, the present chapter is focused on those chiral CE methodologies developed by employing direct separation methods. In these strategies, the presence of a chiral selector in the environment of the separation system (either bounded to an immobile support (stationary phase) or as additive in the background electrolyte (mobile phase)) gives rise to the formation of enantiomers-selector complexes with different mobility. Therefore, in the two main CE modes used in chiral analysis, *electrokinetic chromatography* (EKC) and *capillary electrochromatography* (CEC), the enantioseparation is achieved by the combination of electrophoretic and chromatographic principles. The difference between both modes is related to the way in which the chiral selector is present (immobilized or in solution). While in EKC the chiral selector acts as a chiral pseudostationary phase in the electrolytic

solution, in CEC it is immobilized inside the capillary forming a true stationary phase (like in HPLC). From these two separation modes, EKC is without doubt the most widely used in chiral CE. In 1989, it was defined by Terabe as "an analytical separation method which utilizes the experimental technique of CZE in combination with the principle of chromatography" [17]. From a mechanistic point of view, both the enantioseparation of a charged chiral analyte can been performed using a neutral chiral selector and the enantiomers of a neutral chiral analyte can be separated by a charged chiral selector [18]. For this reason, regardless of the selector charge, all chiral separations carried out by using a chiral selector in the electrolytic solution will be included within the EKC mode. **Figure 2** shows three different separation schemes that can be applied in EKC depending on the analyte and selector charge. When neutral selectors are used to perform the chiral separation, only charged analytes can be separated. On the contrary, charged selectors have a high potential not only for the separation of neutral compounds but also for analytes possessing an opposite charge to that of the chiral selector. In this case, the enantioresolution can be increased due to the large mobility difference between the analyte and the selector and to the strength of the analyte-selector interactions. From a practical point of view, at low pH and using a high concentration of negatively charged selector (or when the binding analyte-selector is strong) it is necessary to reverse the polarity (detector set at the anode) to carry out the chiral separation of basic or neutral compounds (using normal polarity they could not reach the detector) (see **Figure 2B**). On the contrary, basic or neutral compounds can be separated using a charged selector and normal polarity when alkaline pH is used as separation medium, since at high pH values, basic compounds are uncharged so that they are as neutral compounds and they are transported by the EOF to the detector (**Figure 2 C**) [7].

Before 1990, ligand-exchange compounds (LE) and cyclodextrins (CDs) were the two kinds of chiral selectors used to carry out chiral separations by CE. Nowadays, there are a great variety of compounds that can act as chiral selectors (monomeric and polymeric surfactants, antibiotics, chiral crown ether, polysaccharides, etc.), but among all of them, CDs continue being the most widely employed due to their excellent properties related to availability, diversity, universality, price and safety. Usually, EKC modes are named by adding the term of the compound used as chiral selector. Whether ionic surfactants are added at a concentration exceeding their critical micelle concentration, the acronym MEKC (*micellar electrokinetic chromatography*) is used, whereas when using CDs the mode is called CD-EKC (*cyclodextrin electrokinetic chromatography*).

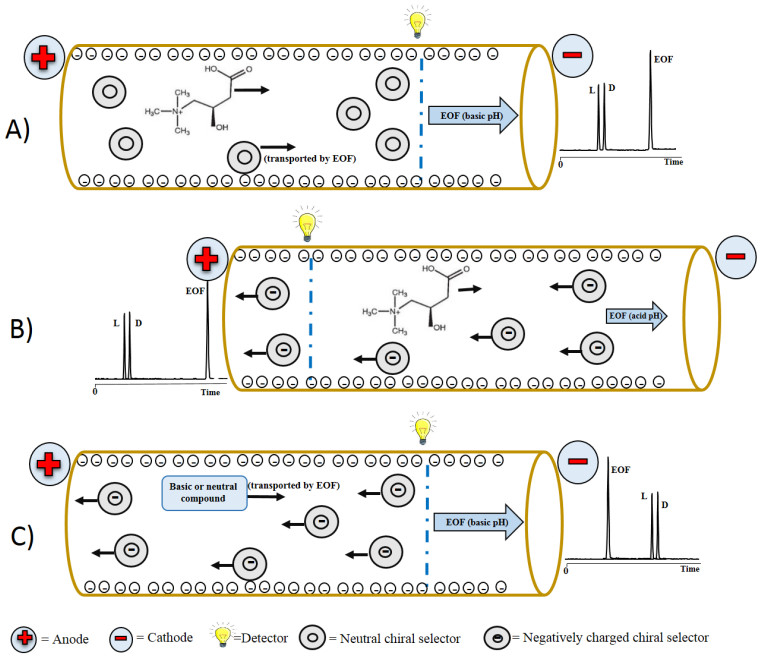


Figure 2. Some chiral separation schemes in EKC. A) A positively charged analyte with a neutral chiral selector at basic pH. B) A positively charged analyte with a negatively charged chiral selector at low pH and reversed polarity. C) A basic or neutral analyte with a negatively charged chiral selector at basic pH.

CEC mode combines the separation efficiency of CE with the high selectivity of HPLC showing a great potential for enantiomeric separations. In CEC, the capillaries are filled, packed or coated with a chiral stationary phase, the mobile phases are pushed by the EOF (which may be assisted by pressure) and an electrical field is applied to perform the separations. In this way, compounds are separated due to the combination of partitioning between the stationary and mobile phase, and if they are charged, also by their different electrophoretic mobilities. Taking into account the different filling methods of stationary phase, the columns employed to carry out chiral separations by CE can be divided into 3 groups: open tubular columns (OTCs), packed columns (PCs), and monolithic columns [19, 20, 21, 22].

OTCs are based on the use of an open capillary whose inner wall is coated or bonded with chiral stationary phases (cyclodextrins, celluloses, proteins, molecularly imprinted polymers (MIPs), or polymeric surfactants). Although chemically bonded phases are preferred to physically adsorbed phases due to their better stability and longer lifetimes, physical coating is easier to fabricate, less expensive, and can show as good stability and separation performance as covalent

coating [21]. PCs are the most used columns in chiral CEC. Here, the capillaries are packed with stationary phases (CDs, macrocyclic antibiotics, proteins, LE compounds and polysaccharides such as cellulose tris (3-chloro-4-methylphenylcarbamate), cellulose tris(4-chloro-3-methylphenylcarbamate) and amylose tris(5-chloro-2-methylphenylcarbamate)). The main drawback of these columns is that they are not commercially available and their homemade preparation is relatively complicated due to bubble formation that can occur at the end-frit what may result in current instabilities and even current breakdown. Regarding monolithic columns, they are prepared by *in situ* polymerization or solidification of monolithic stationary phases with homogeneous and porous structures. In general, these columns may be divided in monolithic silica-based columns, polymer monolithic columns, particle-fixed monolithic columns and molecular imprinted polymers (MIP) columns [7]. In the last years, molecular imprinting technique and inorganic-organic hybrid monoliths have experienced a high development being an interesting research area in separation science [23, 24, 25]. Even though CEC has evolved during the last years as separation technique for chiral separations, the lack of commercially available columns or their complicated homemade fabrication makes that this CE mode has not yet reached its full potential for practical applications.

In the following section, an in-depth description of the practical aspects directly related to the application of CE in chiral food analysis is provided.

3. Chiral Analysis of foods and beverages by CE

Different articles dealing with the application of chiral CE to food analysis have been published during the last decade (2005-2015). **Table 1** summarizes the characteristics of the enantioselective CE methodologies developed to achieve the enantiomeric determination of different chiral compounds (protein and non-protein amino acids, organic acids, phenolic compounds, sweeteners, and pesticides) in a great variety of food matrices, including fruit juices, vinegars, genetically modified organisms, microalgaes, beers, soymilk, wine, food supplements, infant formulas, cacao beans, fruits, tea, or tap water.

As shown in **Table 1**, the most employed chiral selectors to carry out the enantioselective separation of food components, additives and pesticides found in food matrices are CDs, both as unique selector and combined with anionic achiral micelles where the micelles act as carriers of the chiral compound and the neutral CD is responsible for the enantiomeric discrimination. Moreover, other selectors

such as ligand-exchange compounds [27, 28, 45, 46, 49], or to a lesser extent, macrocyclic antibiotics [48] have also demonstrated their potential for the chiral separation of the compounds of interest in the food field. In this sense, as in other scientific disciplines (such as the pharmaceutical or the environmental ones), EKC is undoubtedly the most extensively used separation mode in chiral food analysis. In fact, even though it is possible to find some applications by CEC [37, 58] showing the potential of different stationary phases to carry out the enantioseparation of standard samples by CEC, this CE mode seems to still lack of the robustness required for practical applications.

With respect to the detection systems, UV and laser-induced fluorescence (LIF) are the most popular in chiral food analysis by CE. In the case of the enantiorecognition of protein and non protein amino acids, it is necessary to include an additional analytical step (derivatization) to add a chromophore or fluorophore group into the molecule [59]. Among the different labeling agents available, fluorescein isothiocyanate (FITC), 9-fluoroenylmethylchloroformate (FMOC), dansyl chloride DNS, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), and naphthalene-2, 3-dicarboaldehyde (NDA) have been employed for the derivatization of protein and non protein amino acids. To a lesser extent, mass spectrometry (MS) and electrochemical and capacitively coupled contactless conductivity (C^4D) have also been reported as detection systems coupled to CE for enantiomerically determining chiral compounds in food samples. When EKC is hyphenated to MS to carry out chiral separations, the entry of the (nonvolatile) chiral selector into the MS ion source should be avoided because it may cause ion suppression and contamination of the ionization source decreasing the detection sensitivity [60], although in certain cases in which low concentrated chiral selectors are employed (< 5 mM), it is possible that both analyte and selector reach the MS detector without a significant detriment on the sensitivity [41, 43, 61]. Basically, the two approaches employed to avoid the entry of the chiral selector in the MS source are the partial filling technique (PFT) and the counter current migration technique (CMT) being the former the most used [60, 62]. In PFT, the CE capillary is first flushed with the buffer without the chiral selector and, before the sample is injected, a band of the separation medium containing the chiral selector is introduced into the capillary. In the case of CMT, a charged chiral selector migrates away from the detection system in opposite direction to the analytes.

Table 1 Most relevant works published in the period 2005-2015 on the chiral analysis of food samples by CE

Group	Compounds	CE Mode/ Detection	BGE	Off-line sample treatment and/or in-capillary preconcentration	Sample	Application	LOD*	Ref.
	Pro, Ala, Arg, Glu, Asp.	CD-MEKC/LIF	**20 mM β-CD** + 30 mM SDS in 100 mM borate (pH 9.7)	Filtration, FITC-derivatization	Vinegars	Determination of amino acids as markers of maduration of balsamic vinegrars or production of vinegars by microbial fermentation	< 16.6 nM	[26]
	Tyr, Trp, Phe	LE-EKC/UV	**3 mM Zn(II)/6 mM L-Arg** in 5 mM acetate, 100 mM boric acid (pH 8.20)	Centrifugation, filtration, ultrasonication, FMOC-derivatization	Rice-brewed suspensions	Relationship of the type and content of aromatic amino acids enantiomers with the brand of these fermented products	0.15 µg/mL (Trp) 0.5 µg/mL (Phe) 1.25 µg/mL (Tyr)	[27]
	17 pair of enantiomers	LE-EKC/UV	**3 mM Zn(II)/6 mM L-Arg** in 5 mM acetate, 100 mM boric acid (pH 8.0)	Centrifugation, filtration, (10-fold) dilution, DNS-derivatization	Rice vinegar	Monitoring of D,L-amino acids as markers for quality control	0.5-1.0 µg/mL	[28]
Protein amino acids	Arg, Lys, Ala, Glu, Asp	CD-MEKC/LIF	**20 mM β-CD** + 30 mM SDS in 100 mM borate (pH 9.7)	Classical extraction or PLE, FITC-derivatization	Microalgaes (*Spirulina platensis, Dunaliella salina, and Tetraselmis suecica*)	Characterization of microalgae species as well as different microalgae drying processes.	< 8.9 nM	[29]
	Arg, Pro, Ala, Asp	CD-MEKC/LIF	**10 mM β-CD** + 5 mM SDBS in 50 mM borate (pH 9.6)	FITC-derivatization	Fruit juices	Proposal of L-Asn as marker for the adulteration of pomegranate juices with apple juice	0.85-3.99 nM	[30]
	Asp, Glu, Ser, Asn, Ala, Pro, Arg.	CD-EKC/MS	**5 mM β-CD** in 100 mM acetate (pH 6.0)	Squeeze, Filtration, centrifugation, FITC-derivatization	Orange juice	Analysis of the main amino acids in fresh orange juice and detection of fraudulent addition of synthetic amino acids used to mask water dilution	16-29.4 µM	[31]

Table 1 Continued

Group	Compounds	CE Mode/ Detection	BGE	Off-line sample treatment and/or in-capillary preconcentration	Sample	Application	LOD*	Ref.
	Arg, Ser, Ala, Glu, Asp	CD-MEKC/ LIF	**20 mM β-CD** + 80 mM SDS in 100 mM borate (pH 10.0)	Acid hydrolysis, protein precipitation, centrifugation, FITC derivatization	Transgenic and nontransgenic maize	Chiral analysis of amino acids for assessing the existence (or not) of modifications in metabolic pathways linked to the amino acid profile within genetically modified organisms	160-790 nM	[32]
	Arg, Asn, Ala, Glu, Asp	CD-MEKC/LIF	**20 mM β-CD** + 30 mM SDS in 100 mM borate (pH 10.0)	Acid hydrolysis, protein precipitation, centrifugation, FITC derivatization	Transgenic and wild yeast	Analysis of amino acids profile to differenciate transgenic and wild yeast	LODs in nM range	[33]
Protein amino acids	Arg, Asn, Ala, Glu, Asp	CD-EKC/MS	**0.5 mM 3-monodeoxy-3-monoamino-β-CD** in 50 mM carbonate (pH 8.0)	Soybean: Acid hydrolysis, protein precipitation, centrifugation, FITC derivatization Vinegar: Filtration, FITC derivatization	Transgenic and wild soybean and vinegar	Analysis of amino acids profile in vinegar and soya (transgenic and wild soybean).	7.19×10^{-9}-1.09×10^{-6} M	[34]
	Asp	CD-EKC/LIF	**60 mM Hp-β-CD** +150 mM SDS in 150 mM Tris-borate (pH 9.0)	water dilution (50-fold), NDA derivatization and in-capillary preconcentration by sweeping and stacking (LVSS)	Soymilk and beer	Sensitive determination of Asp enantiomers in beer and soymilk samples	2.5×10^{-10} M (D-Asp) 2.4×10^{-10} M (L-Asp)	[35]
	Trp, Phe, Glu	CD-MEKC/UV	**35 mM β-CD** + 35 mM STDC + 12.5 % (v/v) IPA in 1.5 mM tris borate (pH 8.5)	FMOC-derivatization and ijn-capillary preconcentration by sweeping and stacking (LVSS)	Beers	Sensitive determination of Trp, Phe, and Glu enantiomers in beers	40-60 nM	[36]

Table 1 Continued

Group	Compounds	CE Mode/ Detection	BGE	Off-line sample treatment and/or in-capillary preconcentration	Sample	Application	LOD*	Ref.
	citrulline (among others 20 pairs of amino acids enantiomers)	CEC/UV	**Sepapak-2**, 0.5 M formate/H_2O/ACN (1/19/80, v/v/v) (pH 2.5)	Water solution and FMOC derivatization	Food supplements	Determination of L-citrulline and its enantiomeric impurity in food supplements	7.5×10^{-7} M	[37]
	Orn (along with 18 pairs of amino acids enantiomers)	CD-EKC/UV	**5% (m/v) HS- β -CD + 2% (m/v) Ac-γ-CD** in 50 mM phosphate (pH 2.0)	Filtration (beer was previously degassed in a ultrasonic bath) followed by AQC derivatization	Beer, wine and vinegar (fermented foods)	Analysis of Orn enantiomers in fermented foods (beer, wine, vinegar)	9×10^{-6} M	[38]
	Orn, Arg, Lys	CD-EKC/UV	**5% (m/v) HS- β -CD + 2% (m/v) Ac-γ-CD** in 50 mM phosphate (pH 2.0)	Dietary supplements: water solution, filtration, and in-capillary derivatization with AQC Wine: Filtration and in-capillary derivatization with AQC	Dietary supplements and wine	Determination of Orn, Arg and Lys enantiomers in dietary supplements and wines	6.4×10^{-6} M	[39]
Non-protein amino acids	Orn, Lys, Arg, Asp	CD-EKC/U V	**1 mM γ-CD** in 100 mM borate (pH 10.0)	Water solution, filtration, dissolution in borate buffer and accelerated derivatization with FITC using a ultrasound probe	Food supplements	Enantiomeric determination of Orn in food supplements	1.6×10^{-7} M	[40]
	Orn	CD-EKC/MS^2	**0.75 mM γ-CD** in 50 mM ammonium carbonate (pH 10.0)	Degasification (ultrasonic bath), filtration, pH adjustment (pH 10), and filtration prior to FITC derivatization	Beers	Enantiomeric determination of beers submitted to different fermentation processes	2.5×10^{-9} M	[41]
	Carnitine	CD-EKC/MS^2	**10 mM Succ-γ-CD** in 0.5 M ammonium formate (pH 2.5)	Water solution (sonication) followed by ultrafiltration (5 kDa cut-off filter) and water dissolution before FMOC derivatization	Infant formulas	Quantitative analysis of D/L-carnitine in infant formulas. Detection of D-carnitine up to 8 %.	0.1μM	[42]

Table 1 Continued

Group	Compounds	CE Mode/ Detection	BGE	Off-line sample treatment and/or in-capillary preconcentration	Sample	Application	LOD*	Ref.
Non-protein amino acids	Carnitine	CD-EKC/MS^2	**0.2 % (m/v) Succ-γ-CD** in 0.5 M formate (pH 2.5)	Drinks: Water solution, FMOC derivatization Biscuits, tablets, and capsules: Water extraction, centrifugation, dilution 1/10 in water, FMOC derivatization	Dietary food supplements (drinks, biscuits, tablets)	Identification and quantification of D/L-carnitine in dietary food supplements (drinks, biscuits, capsules and tablets). The use of racemic carnitine (not allowed by the legistation) was corroborated in one of the food sample analyzed	10 ng/mL (L-carnitine)	[43]
	S-Adenosyl-L-methionine	EKC-UV	**300 mM glycine** in 50 mM phosphate (pH 2.5)	Freezing, Liquid-liquid extraction (5 % TCA (w/v)), centrifugation and filtration	Fruits and Fruit juices	Quantification of SAM in fruits and fruit juices	0.5 µM	[44]
	Isocitric acid (citric acid)	LE-EKC/UV	**20 mM Ni(II)/ 80 mM D-quinic acid** in 30% ACN, 20 mM acetic acid (pH 5.0)	Water dilution (2-fold), centrifugation, filtration	Fruit juices	Detection of adulterations in fruit juices through the determination of the ratio of citric acid to D-isocitric acid	-	[45]
Organic acids	Malic, tartaric and isocitric acid (citric acid)	LE-EKC/UV	**100 mM D-quinic acid + 10 mM $CuSO_4$ + 1.8 mM $ScCl_3$** in 20 mM acetate (pH 5.0)	Apple, grape, pineapple juice: water dilution, centrifugation, filtration Orange and mixed fruit juice: centrifugation, SPE	Fruit juices	Detection of fruit juice adulteration through the content of α-hydroxy acids and their enantiomers.	3 mg/L (DL-malic, -isocitric) 2 mg/L (DL-tartaric,-citric)	[46]
	Lipoic acid	CD-EKC/UV	**8 mM TM-β-CD** in 100 mM phosphate (pH 7.0)	Extraction with 70 % MeOH	Dietary supplements	Determination of R/S lipoic acid in dietary supplements to control the use of racemic mixture or pure enantiomers	1.5 mg/l	[47]

Table 1 Continued

Group	Compounds	CE Mode/ Detection	BGE	Off-line sample treatment and/or in-capillary preconcentration	Sample	Application	LOD*	Ref.
Organic acids	lactic acid (α-hydroxybutyric acid, 2-hydroxycaproic acid, 2-hydroxyoctanoic acid, 2-hydroxydecanoic acid, + Asp and Glu	EKC-C^{4}D	**5 mM vancomycin** + 0.03 mM CTAB in 10 mM Tris, 4.4 mM maleic acid (pH 7.35)	ACN solution, centrifugación, drying, redissolution in buffer	Milk and yogurt	Determination of lactic acid enantiomers in milk and yogurt. The presence of D-lactate in dairy products may indicate a microbial contamination	2.8 µM (L-Lactic) 2.4 µM (D-Lactic)	[48]
	Tartaric acid	LE-EKC/C^{4}D	**7 mM $CuCl_2$** + 14 mM trans-4-hydroxy-L-proline in 100 mM ε-aminocaproic acid (pH 5.0)	Wine: water dilution, filtration Grapes: Squeeze, filtration, water dilution	Wine and grapes	Enantiomeric separation of tartaric acid as a food additive for quality control and safety of food products.	19 µM (D-Tartarate) 21 µM (L-Tartarate)	[49]
Phenolic compounds	(±)-catechin (along with other catechins and methylxanthine)	CD-MEKC/UV	**12 mM HP-β-CD** + 12 mM SDS in 50 mM Britton–Robinson (pH 2.50)	Extraction with 29 % ethanol (assisted by ultrasonication), centrifugation, filtration	*Theobroma cacao* beans	Separation of phytomarkers of *T. cacao* and proposal of (-)-catechin as marker of epimerization ocurring in *T. cacao* during manufacturing processes	0.03 µg/mL ((-)-EC/ (+)-C) 1.0 µg/mL (TB)	[50]
	(±)-catechin, (±)-gallocatechin (along with other catechins and methylxanthine)	CD-MEKC/UV	**25 mM HP-β-CD** in 25 mM borate–phosphate + 90 mM SDS (pH 2.5)	Extraction with water, filtration	Tea	Study of thermal epimerisation of C and GC to demonstrate the relevance of non-epistructured (-)-C and (-)-GC as markers of manufacturic processes involving heating	0.05-0.7 µg/mL	[51]

Table 1 Continued

Group	Compounds	CE Mode/ Detection	BGE	Off-line sample treatment and/or in-capillary preconcentration	Sample	Application	LOD*	Ref.
	(±)-gallocatechin (along with other catechins and methylxanthine)	CD-MEKC/UV	**25 mM HP-β-CD** + 90 mM SDS in 25 mM borate-phosphate (pH 2.5)	Extraction with water, filtration	Green tea	Evaluation of quality changes in commercial green tea leaves during a long-term storage. Proposal of Epigallocatechin as a marker of uncorrected storage conditions	-	[52]
Phenolic compounds	(±)-catechin, (±)-epicatechin	CD-EKC/UV	**12 mM HP-γ -CD** in 0.1 M borate (pH 8.5)	Guaraná seeds: water solution, filtration Apple juice: SPE Guaraná extracts: SPE	Apple juice, guaraná seeds and guaraná extracts	Authenticity of guaraná through the analysis of the catechin and epicatechin enantiomers	-	[53]
	Isoxanthohumol	CD-MEKC/UV	**45 mM HP-γ-CD** + 100 mM SDS in 20 mM phosphate (pH 7.0)	Filtration (Sep pack), centrifugation, concentration (rotary vacumm evaporation), ethanol solution	Beer	The racemization of IX in beer could be attributed to the production of a racemic mixture from XN during boiling and to the fact that IX enantiomers were easily interconverted.	17 ng/L	[54]
Sweeteners	Neotame	CD-EKC/UV	**30 mM TM-β-CD** in 50 mM phosphate (pH 5.5)	Extraction with methanol/water (50/50)	Mango juice, cola soft drink, orange soft drink	Enantiomeric separation of neotame in spiked beverages	0.018 nM (L,L-neotame) 0.082 nM (D,D-neotame)	[55]
Pesticides	Malathion, naled, isomalationphenthoate phenamiphos	CD-EKC/UV	**10 mM CM-β-CD** in 25 mM Tris (pH 7.0)	SPE extraction (ethylacetato-diethyle ether (50/50)) + redissolution in methanol.	Tap water	Enantiomeric determination of malathion in tap water.	200 ng/mL	[56]

Table 1 Continued

Group	Compounds	CE Mode/ Detection	BGE	Off-line sample treatment and/or in-capillary preconcentration	Sample	Application	LOD*	Ref.
	Propiconazole Tebuconazole Fenbuconazole	CD-MEKC/UV	**30 mM HP-γ-CD** + 50 mM SDS + methanol-ACN 10/5 (v/v) in 25 mM phosphate (pH 3.0)	Filtration, SPE, drying, redissolution in buffer solution and in-capillary preconcentration by sweeping	Grapes	Enantiomeric determination of triazole fungicides in grapes samples	0.09-0.1 µg/mL	[57]
Pesticides	Metalaxyl tebuconazole, diniconazole, uniconazole, hexaconazole, benalaxyl, myclobutayl, futriafol	CEC-UV	**Sepapak-4**, 90/9/1 (v/v/v) ACN/H_2O/ formate (pH 2.5)	SPE, drying, redissolution in 80/20 (v/v) ACN/H_2O dissolution	Tap water	Enantiomeric separation of metalaxyl in spiked tap water	1.4 mg/L (S-metalaxyl) 1.6 mg/L (R-metalaxyl)	[58]

ACN, acetonitrile; Ac-γ-CD, acetylated-γ-CD; Ala, alanine; AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; Arg, arginine; Asn, asparagine; Asp, aspartic acid; (+)-C, catechine; C^4D, capacitively coupled contactless conductivity; CD, cyclodextrin; CEC, capillary electrochromatography; CTAB, cetyltrimethylammonium bromide; CZE, capillary zone electrophoresis; DNS, dansyl chloride; (-)-EC, epicatechine; EKC, electrokinetic chromatography; FICT, fluorescein isothiocyanate; FMOC, 9-fluorenyl-methyloxycarbonyl chloride; GC, gallocatechin; Glu, glutamic acid; GMO, genetically modified organism; HP-β-CD, hydroxypropyl- β-cyclodextrin; HS-β-CD, highly sulfated-β-cyclodextrin; IPA, isopropanol; LE: ligand exchange; LIF, laser-induced fluorescence; LLE, liquid-liquid extraction; Lys, lysine; LVSS, Large volume sample stacking; MEKC, micellar electrokinetic chromatography; MS^2, tandem mass spectrometry; NDA; naphthalene-2,3-dicarboxaldehyde; OPs, organohosphorus pesticides; Orn, ornithine; PEO, ethylene oxide; Phe, phenylalanine; PLE, pressurized liquid extraction, Pro, proline; SDBS, sodium dodecylbenzene sulphonate; SDS, sodium dodecyl sulfate; SEPAPAK-2, cellulose tris(3-chloro-4-methylphenylcarbamate); SEPAPAK-4, cellulose tris(4-chloro-3-methylphenylcarbamate); Ser, serine; SPE, solid phase extraction; STDC, sodium taurodeoxycholate; Succ-γ-CD, Succinyl-□-cyclodextrin; TB, theobromine; Thr, threonine; TM-β-CD, trimethyl-β-cyclodextrin; Trp, tryptophan; Tyr, tyrosine; XN, xanthohumol.

A relevant challenge that chiral analysis in food samples has to face is the fact that the food samples are complex matrices which comprise a great variety of components that can be found in a very wide dynamic concentration range, from millimolar to femtomolar concentrations. For these reason, in many cases, the use of different off-line sample treatments or in-capillary pre-concentration techniques prior to chiral CE analysis are of paramount importance. As it can be seen in **Table 1**, the application of different off-line sample treatments and/or in-capillary preconcentration strategies has enabled to enhance, in some cases, the detection sensitivity up to 100/110-fold times [35, 36]. Off-line sample treatments of food matrices include dilution, filtration, ultrasonication, centrifugation, acid hydrolysis, protein precipitation, liquid extraction, solid-phase extraction or pressurized liquid extraction (Table 1). The use of these treatments sometimes allows the elimination of interferences from the sample matrix or even the preconcentration of analytes by several orders of magnitude. The selection and optimization of the sample treatment steps is a crucial point since errors during sample preparation cannot be corrected even by the best separation or detection technique so that this process cannot be underestimated.

Regarding in-capillary strategies to improve CE sensitivity by increasing the amount of sample injected into the capillary, different approaches based on electrophoretic principles have been developed during the past decades. Usually, these strategies are employed in combination with off-line sample treatments to eliminate possible matrix constituents because they also lead to the concentration of these interferences. As **Table 1** shows, strategies based on stacking and sweeping have been reported to increase the sensitivity in chiral food analysis by CE. In the former the sample is dissolved in a matrix of higher resistance (minor conductivity) than the separation buffer. In this way, when the injected analyte reachs the boundary between the sample zone and the buffer zone, it is staked. Therefore, the stacking phenomenon is based on the analyte concentration because of the difference in conductivity between the buffer and the sample. In normal sample stacking (NSS) and large volume sample stacking (LVSS) the sample is injected into the capillary by a hydrodynamic injection, whereas in field-amplified sample stacking (FASS), and anion or cation selective exhaustive injection (ASEI or CSEI), an electrokinetic injection is used [63]. On the other hand, sweeping phenomenon is based on the interactions between the sample and an additive of the buffer (usually micelles) that acts as a pseudostationary phase (component that originates the preconcentration). In this case, the key factor is that the sample matrix does not

contain the pseudostationary phase so that sample matrix may have different or the same conductivity than the buffer [63].

4. Applications

The purpose of this section is to provide the reader an in-depth overview about the usefulness of chiral CE to solve specific problems into the Food Science field through the enantioselective determination of different chiral compounds such as protein and non-protein amino acids, organic acids, phenolic compounds, sweeteners, and pesticides in a variety of food matrices. The main characteristics (CE mode and detection system, BGE, sample treatment, sample analyzed, application and LOD) of the most relevant CE methodologies applied to the analysis of these chiral compounds are summarized in **Table 1**.

4.1. Enantiomeric determination of protein and non protein amino acids

In addition to the 20 L-amino acids universally distributed as protein constituents in living organisms, there are hundreds of other amino acids of non-protein origin. These have been defined as those amino acids that are not found in the protein main chain either for lack of a specific transfer RNA and codon triplet or because they do not arise from protein amino acids by post-translational modification [64]. In nature, the free amino acids (both protein and non protein) are generally composed by their L-form whereas foodstuffs are the main source of D-amino acids. In fact, different processing conditions/treatments or technologies employed by the food industry to improve flavour, consistency or shelf life among other food characteristics, may produce the racemization of L-amino acids in their D-amino acids. In addition, D-forms can be even synthesized by microorganisms in several enzymatic pathways or can be found as a consequence of the fraudulent addition of racemic mixtures in supplemented foodstuffs where regulations establish the use of pure L-enantiomers [8, 59]. All these facts make that, in general terms, the chiral analysis of protein and non protein amino acids is very useful to obtain relevant information about food quality and food adulteration, to evaluate the effects of processing, fermentation, microbiological activity and storage, or to characterize the main L- and D-amino acids found in foods [5, 10, 65, 66].

Different CE methodologies based mainly on the use of MEKC and EKC separation modes have been developed for the chiral separation of protein and non protein amino acids in foods (see **Table 1**). CEC mode has been employed to a lesser

extent; only one work employed this mode for the chiral separation of amino acids during the period of time covered in this chapter. From the high number of references published, it is possible to infer that this is the most frequent application of chiral CE in food analysis. This fact is probably due both to the well-known structure and character of amino acids and to the large amount of information than can be obtained from their chiral analysis.

As shown in **Table 1**, neutral CDs, such as β-CD, 2-hydroxypropyl-β-CD, γ-CD or succinyl-β-CD, have been the preferred selectors to carry out the chiral separation of different protein and non protein amino acids, although other charged CDs have also been employed successfully. With respect to the detection systems, UV, LIF and MS are the most frequently employed. In both UV and LIF detection, a derivatization step is required to add a chromophore or fluorophore group into the amino acid structure. Among the reagents used for derivatization of amino acids, FITC and FMOC have been the most popular labeling reagents. Derivatization is also employed when using a MS detector in order to obtain larger molecules providing a better detection sensitivity.

The chiral analysis of *protein amino acids* by CE has been applied in the last years to obtain valuable information about fermentation processes [26, 28], thermal treatments [29], fruit juices adulterations [30], genetically modified organisms [32, 34], or just to determine the L and D-amino acids content to assess if legal regulations are accomplished [35, 36].

The formation of D-amino acids in fermented foods depends mainly on the fermentation conditions and the action and autolysis of the microorganism involved. For this reason, the chiral determination of the amino acidic profile can be used as a potent marker of these processes. For instance, the application of a MEKC-LIF methodology based on the use of β-CD as chiral selector enabled to find interesting differences in the L and D- amino acids profiles of different vinegars samples (balsamic, sherry, white wine, cava, and wine vinegar with extract of herbs). The detection and quantification of D-amino acids present in fermentation processes made possible to distinguish the type of vinegar and the degree of maturation of balsamic vinegars [26]. Along with MEKC, EKC methods based on the formation of diastereomeric ternary-mixed metal complexes between the chiral ligand (Zn (II)-L-arginine complex) and amino acids have also been employed to establish a relationship between aromatic amino acids enantiomers and the brand of fermented rice-brewed suspension (called Laozao in Chinese) [27]. Also, these methods were applied to the separation and quantification of some pairs of

enantiomers which were proposed as markers for the quality control of rice vinegar samples because their concentration depends on the time and activity of fermentation [28]. Chiral analysis of protein amino acids has also been very useful to characterize different microalgaes and to study the effect of different microalgae drying process (hot air and lyophilization) on their L-and D- amino acid contents [29]. Quantitation of five different chiral amino acids (namely Arg, Lys, Ala, Glu, and Asp) in three microalgae species (*Spirulina platensis, Dunaliella salina, and Tetraselmis suecica*) was carried out using a MEKC-LIF strategy with β-CD as chiral selector which provided a separation in less than 25 min and LODs down to 8.9 nM (see **Table 1**). Different percentages of D-amino acids were measured depending on the microalgaes species, extraction protocol (classical and pressurized liquid extraction) and drying process which demonstrated the potential of the developed methodology to differentiate among microalgaes and to investigate the effect of thermal processing on the amino acid content [29].

Fruit juice industry is one of the most important agricultural businesses in the world so that, due to its economic impact, the adulteration of fruit juices is a relevant topic which requires the development of analytical methodologies able to detect sophisticated adulteration processes. As it can be observed in **Table 1**, different chiral CE methodologies have been employed with success for the recognition of adulterated fruit juices. On the one hand, the application of a MEKC-LIF method based on the use of sodium dodecylbenzene sulphonate as surfactant, β-CD as chiral selector, and FITC as derivatization reagent, enabled to compare the amino acid profile of pomegranate and apple juices and propose L-Asn as marker for the adulteration of pomegranate juices with apple juices [30]. On the other hand, an EKC-MS methodology, in which a polymer coating protocol was used to reduce the EOF and minimize the entrance of the β-CD into the MS ion source, was developed to detect fraudulent additions of synthetic amino acids to mask water dilution of orange juices [31]. As the developed methodology enabled the determination of 2 % of the D-Asp enantiomer (derivatized with FITC), it could be used as marker for controlling possible adulterations with synthetic amino acids (containing L and D enantiomers). **Figure 3** shows the EKC-MS extracted ion electropherograms obtained from a fresh orange juice and from the same juice adulterated with 0.8 % of D/L-Asp.

The safety of the use of genetically modified organisms in food science needs to be corroborated through the equivalence between transgenic and non-transgenic foods. In this regard, the investigation of the protein amino acidic profile through

the application of enantioselective CE procedures has opened new perspectives in the study of transgenic foods since it can provide information about unexpected modifications in other metabolic pathways linked to the amino acids profile.

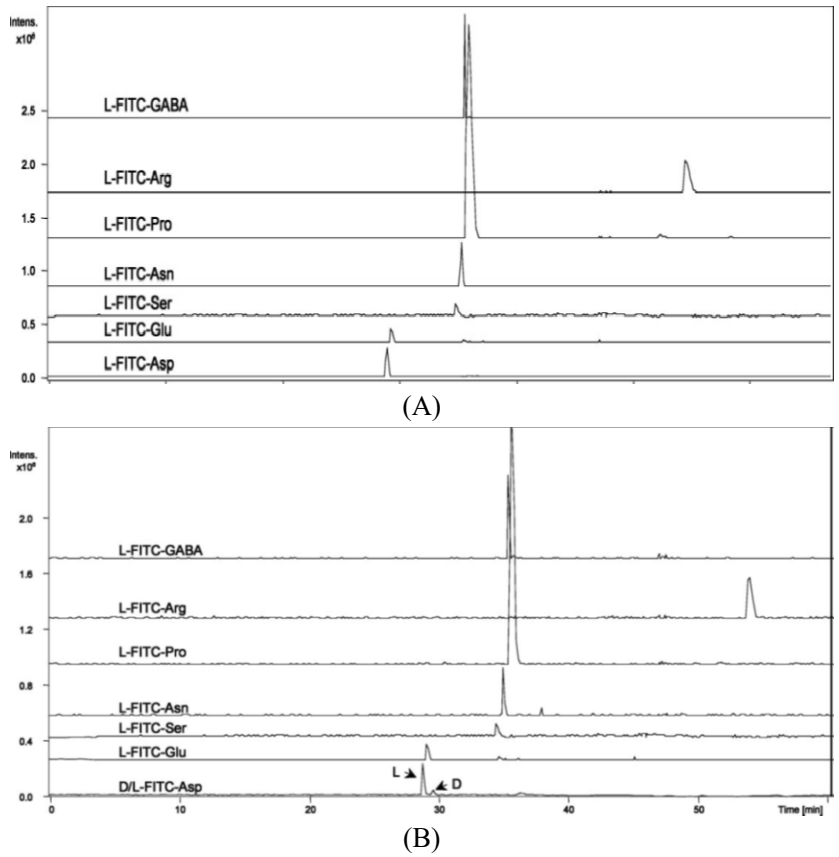


Figure 3. A) CE-MS extracted ion electropherogram (EIE) obtained from an orange juice sample derivatized with FITC. B) CE-MS EIE from an orange juice sample adulterated with 0.8 % of D/L-Asp. CE conditions: 5 mM β-CD in 100 mM ammonium acetate buffer (pH 6.0); polymer-coated capillary, 50 μm x 87 cm; voltage, -15 kV; injection, 0.5 psi, 18 s. ESI conditions: positive ion mode; sheath liquid methanol-water (1:1 v/v) with 25 % BGE without β-CD; flow rate, 3.5 mL/min; dry gas flow, 3 L/min; temperature, 350 ºC; mass scan, 150–700. Reprinted from [31], copyright (2005) with permission from John Wiley and Sons.

Two similar procedures including amino acids extraction, FITC derivatization and MEKC separation with β-CD as chiral selector and LIF detection have been applied to quantify L and D-amino acids in different lines of conventional and transgenic maize [32], and yeasts used for sparkling wine production [33]. In maize samples, very similar L and D-enantiomer composition was observed for one of the maize variety studied (Tietar) whereas significant differences were obtained

for the other maize lines (Aristis, and PR33P66) [32]. **Figure 4** depicts the D/L amino acids profile obtained from the different maize varieties studied.

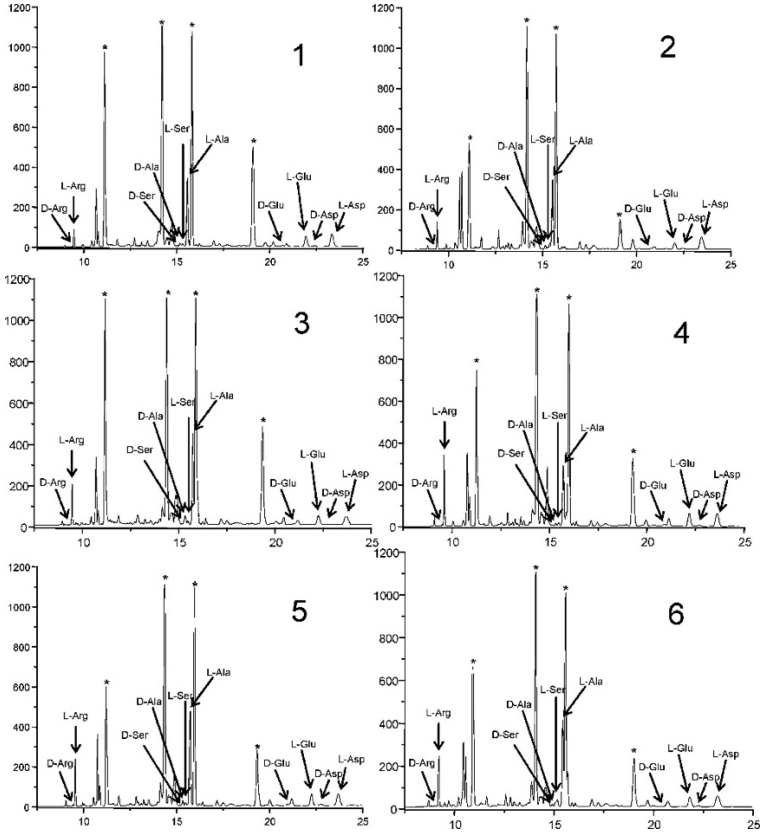


Figure 4. Chiral-MEKC-LIF electrophoregrams of free D/L-amino acids from different maize varieties: (1) Aristis; (2) Aristis-Bt; (3) Tietar; (4) Tietar-Bt; (5) PR33P66; (6) PR33P66-Bt. Peaks marked with an asterisk correspond to FITC. CE conditions: 20 mM α-CD in 100 mM sodium tetraborate, 80 mM SDS (pH 10.0); uncoated fused-silica capillary, 50 μm x 57 cm; voltage, 20 kV; LIF detection, 488 (excitation) and 520 nm (emission). Reprinted from [32], copyright (2007) with permission from American Chemical Society.

Regarding the study of yeasts, it can be concluded that a faster autolysis (which is the main source of molecules that contribute to the quality of sparkling wines made by the traditional method) takes place in the genetically modified yeast releasing a higher amount of L-amino acids to the medium in a short time [33].

Not only MEKC has been applied to distinguish conventional and transgenic varieties of foods but also EKC-MS has demonstrated to be a potent strategy in this field. Using as chiral selector a modified CD (namely 3-monodeoxy-3-monoamino-β-CD), five different D/L-amino acids (Arg, Asn, Ala, Glu, and Asp) were separated in 20 min reaching LODs in the nM range [34]. This methodology was useful for the

determination of these amino acids in wild and transgenic soybeans (different quantities of amino acids were determined in both varieties) and vinegar samples (in which only L-amino acids were detected).

Interesting methodologies are those employing an in line preconcentration technique to carry out the sensitive enantiomeric determination of amino acids in food samples. For instance, the sensitive determination of Asp enantiomers (derivatized with NDA) in beers and soymilk has been carried out using a preconcentration by stacking prior to EKC separation with LIF detection [35]. The separation was accomplished using a discontinuous system, i.e., buffer vials contained poly(ethylene oxide) (PEO, which acts as concentrating media), SDS (acting as pseudostationary phase) and HP-β-CD (acts as chiral selector) whereas the capillary was filled with SDS and HP-β-CD. The stacking mechanism is mainly based on the difference in viscosity between the sample zone and PEO whereas the sweeping is due to the interaction between Asp enantiomers and SDS. Using this system, it was possible to achieve the stacking of the derivatized amino acid without the loss of chiral resolution and to obtain a 100 and 110-fold enhancement in the sensitivity of D and L-Asp enantiomers, respectively (LODs of 2.4 and 2.5 x 10^{-10} M). Following the idea of a discontinuous system and modifying slightly the components of the buffer vial and the capillary (in this case using taurodeoxycholate and β-CD instead of SDS and HP-β-CD), it was also possible to enantioseparate Trp, Phe and Glu (derivatized with FMOC) with high sensitivity in various types of beer samples (LODs in the nM levels) [36].

Regarding the chiral analysis of *non protein amino acids* by CE, the most relevant works published during the last years have been focused on the separation of the L-enantiomer from the D-enantiomer which can have in some cases toxic properties and whose addition during the elaboration of foods is forbidden. Thus, the enantiomeric separation allows guaranteeing a good quality control of food products. Samples analyzed have been mainly food supplements, infant formulas, and fermented foods (such as wine, beer etc.) as described in detail in **Table 1.** In all the research works grouped in this table, EKC was employed as CE mode to carry out the separation of different non protein amino acid enantiomers, except in one article in which CEC was used to achieve the enaniomeric determination of citrulline in food supplements with cellulose tris (3-chloro-4-methylphenylcarbamate chiral stationary phase (CSPs) as chiral selector, FMOC as labeling reagent and formate as running buffer [37]. This methodology enabled to achieve LODs of 7.5 x 10^{-7} M for citrulline and it demonstrated to be more efficient

than nano-LC when comparing both techniques under similar experimental conditions.

Ornithine and carnitine are the non protein amino acids whose chiral separation by CE has been the most studied during the last decade. The former has received huge attention due to the beneficial properties of the L-enantiomer and the harmful effects of the D-enantiomer which may be originated during food processing or fermentation processes and can produce depletion in the urea synthesis giving toxic consequences. Carnitine has also shown to have different biological activities depending on its enantiomeric form; L-carnitine plays an important role in the long chain fatty acids metabolism while D-carnitine posseses toxical properties.

Three different EKC-UV procedures under both basic and acidic conditions have been proposed to analyze ornithine enantiomers in fermented foods (beers, wine, vinegars) and food supplements [38, 39, 40]. An EKC-UV methodology was developed using AQC as off-line derivatizing reagent, and phosphate buffer (pH 2.0) containing HS-β-CD and acetylated-γ-CD as BGE, which allowed to reach LODs of 9 x 10^{-6} M for ornithine [38]. As shown in **Figure 5,** this method made possible the chiral separation of ornithine from the enantiomers of twenty protein amino acids and GABA (**Figure 5A**), as well as their enantioseparation in fermented foods (**Figure 5B**). Using this methodology as starting point, a second procedure was developed including an in-capillary derivatization with AQC which enabled to increase CE automatization and decrease the analysis time. In this case, the ECK-UV method was successfully applied to the determination of ornithine, Arg and Lys enantiomers in wine samples (these compounds demonstrated to be responsible of wine's organoleptic properties) and dietary supplements [39].

FITC was also used as labeling reagent in the chiral determination of ornithine enantiomers in food supplements. In fact, an interesting pre-capillary derivatization with FITC using an ultrasound probe was proposed to reduce derivatization time from 16 h to 10 min. The method was employed in combination with an EKC-UV separation method based on the use of γ-CD as chiral selector and the LOD for ornithine was 1.6 x 10^{-7} M [40]. Using this fast FITC derivatization procedure, an EKC-MS^2 methodology was also developed to enhance the sensitivity and selectivity in the chiral determination of ornithine in beers submitted to different fermentation processes [41]. The method, based on the use of carbonate buffer (pH 10.0) containing γ-CD as BGE, enabled to achieve LODs of 2.5 x 10^{-9} M. The percentages for D-ornithine ranged from 1.5 % to 10 % in the analyzed samples

(the lowest value corresponding to a dietetic beer and the maximum to a double fermentation beer).

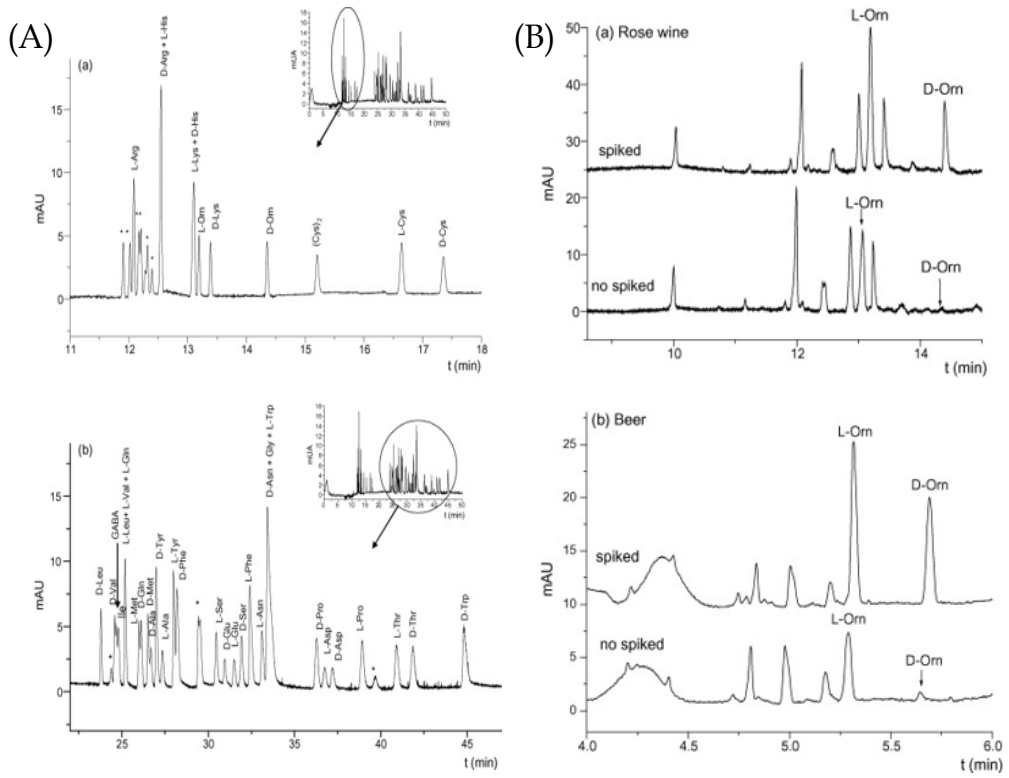


Figure 5. A) Enantiomeric separation by EKC of a mixture of the 20 protein amino acids, Orn, and GABA divided in two migration zones: (a) first-migrating zone, and (b) second-migrating zone. B) Electropherogram corresponding to different fermented foods derivatized off-line with AQC (a) a rose wine (uncoated fused-silica, 50 µm × 72.5 cm) and (b) a beer (uncoated fused-silica, 50 µm × 48.5 cm); injection by pressure, 5066.25 Pa, 5 s of sample followed of 5 s of BGE; non spiked sample and sample spiked with 5×10^{-4} M racemic Orn. CE conditions: 5 % (m/v) HS-γ-CD and 2 % (m/v) acetylated-γ-CD in 50 mM phosphate buffer (pH 2.0); uncoated fused-silica, 50 µm × 72.5 cm; voltage, -25 KV; temperature, 15 ºC; UV detection, 260 nm. (*) Unknown peaks. Reprinted from [38], copyright (2008) with permission from Elsevier.

Regarding carnitine, its chiral determination by EKC-MS^2 was carried out in infant formulas [42] and dietary supplements [43] to assess that the content of the D-enantiomer was not higher than the limits established by regulations. With this purpose, an EKC-MS^2 methodology was developed using FMOC as labeling reagent and formate containing Succ-γ-CD as running buffer. Due to the high CD concentration needed to achieve the enantiomeric separation, a partial filling technique was employed to avoid the contamination of the MS ion source. LODs

for carnitine enantiomers in the μM range were obtained and the application of the method to the analysis of supplemented infant formulas revealed amounts as high as 8 % of D-carnitine (which exceeded by far the limits established by the European Pharmacopeia) in some of the samples analyzed [42]. In order to avoid the use of the partial filling technique, the method was subsequently optimized without this step by using a low amount of Succ-γ-CD (0.2 % (m/v)) with a longer length capillary which improved the precision and sensitivity (LODs of 10 ng/mL) [43]. The application of the optimized method to the analysis of twenty two dietary food supplements demonstrated the use of racemic carnitine (not allowed by the legislation) in one of the food supplements whereas in the other samples L-carnitine was the enantiomer employed with enantiomeric impurities (D-carnitine) up to 6 %.

Other non protein amino acid whose enantiomeric separation by CE has also been reported in the last years is S-adenosyl-L-methionine. Using an EKC-UV procedure, it was possible to establish the relationship between heat treatment and S-adenosyl-L-methionine content in tomato samples which demonstrated that the content of this non protein amino acid varies depending on food processing [44].

4.2. Enantiomeric determination of organic acids

The enantiomeric separation of organic acids is also a field of huge interest in Food Science. In general, they naturally occur as single enantiomers so that the presence of racemic mixtures in foods may indicate their use as additives which must be controlled because it is not always allowed. Moreover, taking into account that different organic acid enantiomers can provide different flavor or taste to beverages and food products, their analysis is of great interest for quality control.

EKC using buffers at a pH ranging from 5.0 to 7.0 and different chiral selectors, from ligand-exchange complexes or CDs to macrocyclic antibiotics enabled the chiral determination of organic acids in foods (see **Table 1**). UV detection is, without doubt, the first choice to carry out the detection of these compounds, while C^4D seems to be a good alternative in those cases in which a chromophore group does not exist in the organic acid structure.

The development of chiral analytical methodologies for the determination of organic acids provides interesting tools to fight against fruit juice adulteration. With this aim, different strategies based on the use of ligand exchange EKC with UV detection have been proposed in the literature. Taking into account that the ratio of citric acid to D-isocitric acid is one of the most relevant parameters used to distinguish authentic and adulterated fruit juices, D- and L-isocitric acid

enantiomers were separated in different fruit juices using D-quinic acid as chiral selector and Ni(II) as central ion system (see **Figure 6**) [45]. The contents of DL-isocitric and citric acids estimated using the developed methodology were in agreement with the fruit juice industry Code of Practice. However, not only the chiral determination of DL-isocitric acid can serve to detect fruit juice adulteration; the enantiomeric determination of other α-hydroxy acids, such as DL-malic acid or DL-tartaric acid can also be used as indicators of adulteration [46].

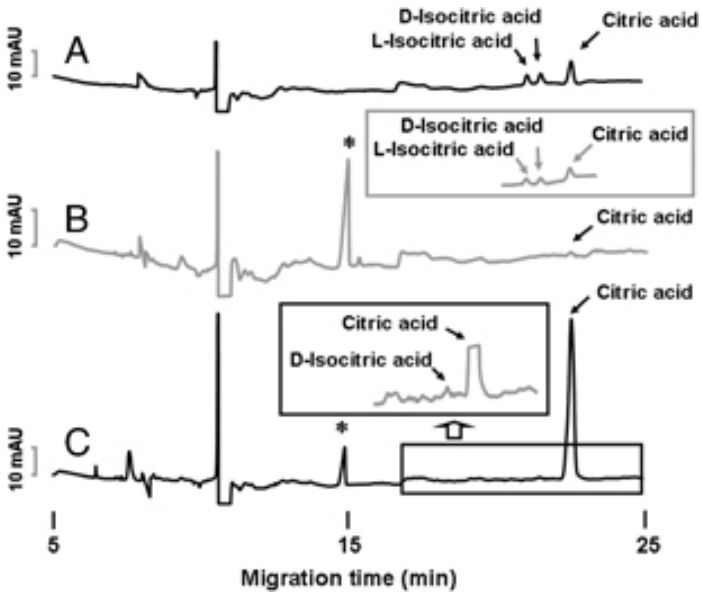


Figure 6. EKC electropherograms of (A) a standard solution (containing 400 mg DL-isocitric acid + 400 mg/L citric acid), (B) an apple juice sample, and (C) an orange juice sample (C). CE conditions: 30 % ACN, 20 mM acetic acid, 20 mM NiSO4, and 80 mM D-quinic acid (pH 5.0); FunCap-CE/Type S capillary (sulfonated capillary), 50 μm x 64.5 cm; voltage, 15 KV; temperature, 15 ºC; UV detection, 200 nm. The inset in (B) was the electropherogram obtained when 5mL of a mixture of citric acid (100 mg/L) and DL-isocitric acid (200 mg/L) was added to 5mL of an apple juice sample. The insets in (C) show part of the same data with an expanded y-axis. *represents a peak of malic acid. Reprinted from [45], copyright (2010) with permission from John Wiley and Sons.

Lipoic acid (a powerful antioxidant with multiple beneficial effects in the therapy of different diseases such as diabetes, atherosclerosis, or degenerative processes in neurons, among others) naturally occurs as the (R)-enantiomer, while synthetic lipoic acid is racemic. Since the quality control of dietary supplements should include not only the evaluation of total lipoic acid but also the quantification of each enantiomer, it is of interest to know the (R)-enantiomer content of lipoic acid (the potency of (S)-lipoic acid is not known exactly). To do that, a simple sample

treatment combined with a rapid EKC-UV methodology with TM-β-CD as chiral selector was developed to quantity both enantiomers in different dietary supplements demonstrating that in most samples analyzed the racemic mixture was present in spite of some of these samples claimed to use only the naturally occurring enantiomer ((R)-lipoic acid) [47].

As above-mentioned, C^4D has demonstrated to be a potent alternative to UV detection to determine organic acids without chromophore groups in their structure. As it can be seen in **Table 1**, this detection system coupled to EKC methodologies has been used in the determination of the enantiomers of α-hydroxy acids and two α-amino acids (Asp. Glu) [48] or the enantioseparation of tartaric acid [49]. In the first case, the determination of the lactic acid enantiomers in yogurt and milk demonstrated to be relevant since the presence of D-enantiomer acts as an indicator of natural fermentation process or microbiological contamination in dairy products [48]. In the last case, the analysis of tartaric acid enantiomers in wine and grapes by EKC-C^4D using vancomycin as chiral selector was employed to guarantee the quality and safety of both products, since the presence of D-tartaric acid could come from bacterial contamination (D-tartatic was not found in any of the samples analyzed proving that they were not contaminated) [49].

4.3. Enantiomeric determination of phenolic compounds

Phenolic compounds have demonstrated to have healthy properties associated to antioxidant, antimicrobial and antiviral activities, but the main interest they present in the food field is because they are a powerful tool for the characterization of food quality and safety. Flavonoids (group comprising flavonones, isoflavones, flavonols, flavanonoes, catechines, anthocyanidins and chalcones) are one of the largest natural occurring phenolic compounds in food and beverages, and many of them are responsible for the flavor and color of foods [8]. The chiral determination of flavonoids by CE has mainly been focused on the analysis of catechines, as it can be seen in **Table 1,** since their enantiomeric profile can be used for food characterization including the effects of processing and storage. Most of the developed methodologies described in the literature are aimed to study the epimerization of catechines to evaluate their potential as markers of manufacturing processes [50, 51], storage conditions [52] or food authenticity [53]. Thus, similar MEKC-UV methodologies using HP-γ-CD as chiral selector (see **Table 1** for specific details) have been developed to propose (-)-catechin as a phytomarker of epimerization occurring during manufacturing processes in

Theobroma cacao (since this enantiomer appears after processing) [50], (-)-catechin and (-)-gallocatechin as markers of manufacturing processes involving heating in tea (**Figure 7)** [51] and epigallocatechin as a marker of uncorrected storage conditions in green tea sample [52]. In addition, using an EKC-UV methodology with HP-γ-CD as chiral selector it was possible to investigate the authenticity of guaraná through the analysis of catechin and epicatechin enantiomers [53]. By using this methodology it was possible to demonstrate that the presence of all catechin and epicatechin enantiomers ((+)- and (-)-catechin and (+)- and (-)-epicatechin) in guaraná seeds was not due to pulping and drying by roasting. Therefore, they are natural compounds that can be used to evaluate guaraná authenticity.

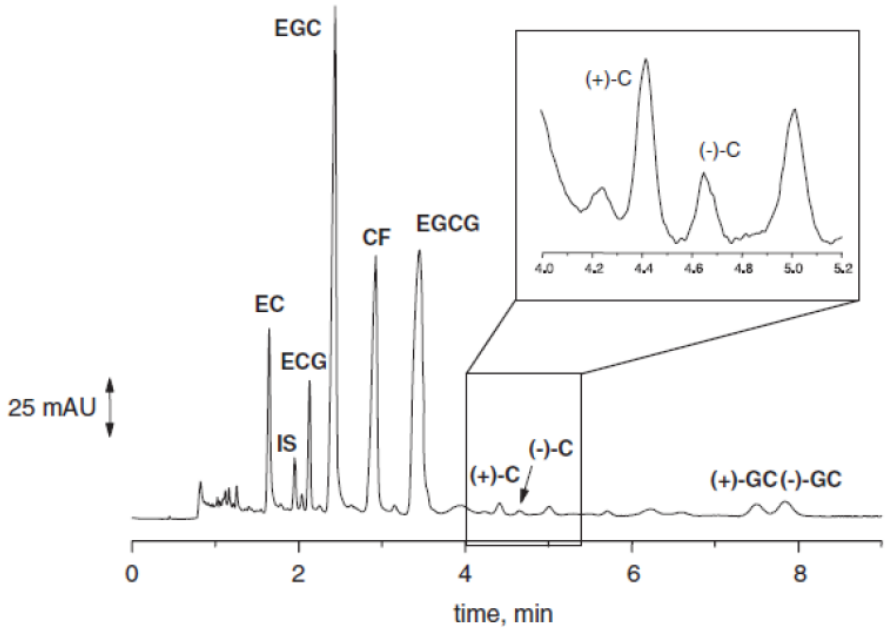


Figure 7. CD-MEKC electrophoregram of extract from Kokeicha Green tea. CE conditions; 25 mM borate-phosphate buffer (pH 2.5) containing 90 mM SDS and 25 mM HP-β-CD; uncoated fused silica capillary, 50 μm x 38.5 cm (used in the "short-end" mode (effective length 8.5 cm)); voltage, 15 kV; temperature, 25 °C; hydrodynamic injection, 3 s (pressure at 25 mbar); UV detection, 200 nm. Peak assignment: EC, epicatechin; IS, Internal standard; ECG, epicatechin gallate; EGC, epigallocatechin; CF, caffeine; EGCG, epigallocatechin gallate; (+)-C, (+)-catechin; (-)-C, (-)-catechin; (+)-GC; (+)-gallocatechin; (-)-GC; (-)-gallocatechin. Reprinted from [51], copyright (2009) with permission from John Wiley and Sons.

Other flavonoid not included in catechines group whose enantiomeric separation in foods has also been reported is isoxanthohumol. This prenylflavanone is produced from the cycling reaction of xanthohumol during brewing process of

beer samples. Both compounds have received much attention as cancer chemopreventive and/or estrogenic agents [54]. In this case, the enantiomeric analysis of isoxanthohumol in beer samples was performed using a MEKC-UV method with HP-γ-CD as chiral selector which enabled to achieve LODs of 17 ng/L. The levels of both enantiomers were almost the same showing that the isoxanthohumol was racemic in the beer samples analyzed. This result suggests that the racemic mixture is produced during boiling and the isoxanthohumol enantiomers are easily interconverted [54].

4.4. Enantiomeric determination of sweeteners

To a lesser extent, chiral CE methodologies have also been developed for the stereoselective analysis of sweeteners in food samples. Neotame is a new high intensity artificial sweetener that has two chiral centers, and hence, four diastereomers (L, L; L, D; D, D; and D, L) and its sweetness is attributed to the presence of well-oriented hydrophobic groups in the L, L-diastereomer [55]. In a racemic mixture, the L, L-diastereomer is present as the sweetener, while the D, D-diastereomer is not sweet. In any case, the presence of amino acids and organic groups in its structure makes that this compound exhibits high sweetness (nearly 10,000 times sweeter than sugar and 40 times more sweeter than aspartame) [55]. As it can be seen in **Table 1**, the chiral separation of neotame has been achieved by EKC with UV detection using heptakis-2,3,6-tri-o-methyl-β-CD as chiral selector. The main purpose of this study was to provide a better understanding of the interaction established between neotame and the chiral selector. Using molecular docking studies it was possible to show that both electrostatic and hydrophobic interactions were relevant in the stabilization of the inclusion complexes and in the elution order (a stronger interaction with the chiral selector was observed for the D, D-neotame than for L, L-neotame). Moreover, the developed EKC methodology was successfully applied to the determination of neotame in different spiked beverages, such as mango juice, cola soft drink, and orange soft drink with LODs of 0.018 and 0.082 nM for L,L- and D,D-neotame, respectively.

4.5. Enantiomeric determination of pesticides

Pesticides are active compounds used in agriculture to control pest. Approximately a 25 % of pesticides employed in the world are chiral compounds and they are introduced in the environment as racemates. However, one of the enantiomers can present the desired pesticide activity while the other can present

different activity or be inactive. For this reason, the evaluation of their real toxicity in food samples and the study of their enantioselective degradation require the use of enantioselective methodologies.

Three different examples of CE methodologies developed to perform the chiral determination of pesticides in food samples are included among the most relevant works published in the last decade (see **Table 1**). In the case of the determination of contaminants in food samples it has to be taken into account that it is imperative to have the necessary sensitivity to detect minor amounts of each enantiomer. For this reason, the analytical strategies developed included off-line sample treatments and in capillary preconcentration techniques to achieve the maximum sensitivity. For instance, to carry out the determination of malathion enantiomers in water samples spiked at μg /mL level (one of the most widely used organophosphorus pesticides), it was necessary to combine an off-line disk solid-phase extraction treatment as preconcentration step with an EKC-UV methodology consisting in the use of a Tris buffer at pH 7.0 containing CM-β-CD as BGE [56]. In this way, a LOD around 200 ng/mL was obtained for each malathion enantiomer. In addition to off-line preconcentration by SPE, it is also interesting to employ in capillary preconcentration techniques to improve the method sensitivity. A clear example which demonstrated this fact is the determination of chiral triazole fungicides (namely propiconazole, fenbuconazole and tebuconazole) by combining SPE pretreatment and sweeping-CD-MEKC procedure [57]. An enhancement factor about 100-fold in the detection sensitivity was achieved by sweeping-CD-MEKC at acidic pH compared to conventional CD-MEKC.

In addition to EKC and CD-MEKC separation modes, CEC has also been applied to analyze chiral pesticides. For instance, a polysaccharide-based chiral stationary phase known as Sepapak-4 (cellulose tris(4-chloro-3-methylphenylcarbamate)) demonstrated its potential for the chiral separation of sixteen pesticides, including herbicides, insecticides, and fungicides by CEC-UV using ACN/H_2O/phosphate at pH 2.5 as running buffer [58]. The excellent analytical characteristics of this methodology allowed its application to the separation of metalaxyl and its enantiomeric impurity in tap water samples spiked with the commercial product containing metalaxyl-M (R-enantiomer) (see **Figure 8**). The developed CEC methodology was compared with the chiral separation of the same pesticides by nano-LC under similar experimental conditions observing advantages in the use of the CEC strategy.

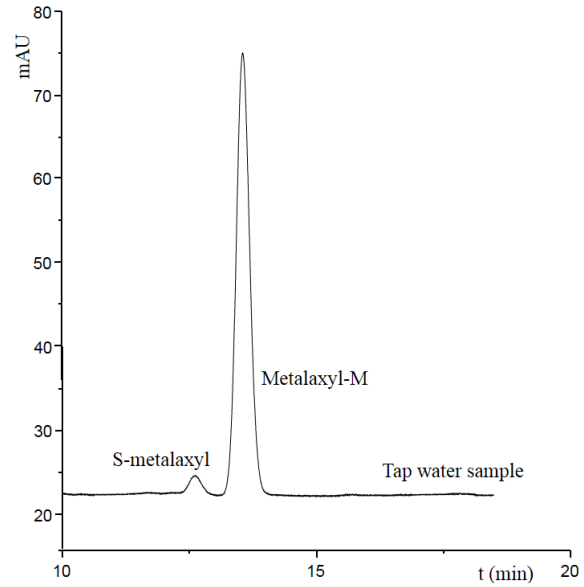


Figure 8. CEC electrochromatogram obtained for tap water spiked with the commercial product containing only metalaxyl-M at a concentration of approximately 75 mg/L (according to its label). CEC conditions: 90/9/1 (v/v/v) ACN/H2O/ formate (pH 2.5); capillary, 100 µm x 24 cm packed with Sepapak-4 and 32.5 cm total length; voltage, -10 kV with 12 bar pressure in both inlet and outlet vials; temperature, 25 ^{o}C; injection 10 bar for 0.2 min (followed by a plug of mobile phase at 10 bar x 0.2 min); UV detection, 210 nm. Figure slightly modified from [58], copyright (2012) with permission from Elsevier.

5. Conclusion

The individual determination of the enantiomers of chiral compounds in food samples has demonstrated to be an interesting tool to assess food quality, safety and traceability. The high potential of CE in chiral analysis has enabled the development of a broad range of enantiomeric methodologies that have successfully been applied to the determination of food components, additives and pesticides. Features of CE such as high efficiency, short analysis times and low consumption of solvents, reagents and samples confer this technique important advantages for chiral analysis. Moreover, the high versatility of EKC in which the chiral selector is added to the electrolytic solution has originated that this is the preferred separation mode in CE to achieve the chiral analysis of food samples although CEC using chiral stationary phases has also been employed. The most successful chiral selectors in EKC and MEKC methodologies developed have been cyclodextrins although the use of ligand exchange complexes and macrocyclic antibiotics have also been reported. The limited concentration sensitivity obtained in CE with UV detection can be overcome by using off-line sample treatment

techniques and in capillary preconcentration strategies and/or LIF or mass spectrometry detection. Bearing in mind the different applications shown in this chapter, it can be deduced that employing an adequate sample treatment sometimes combined with in capillary preconcentration strategies (stacking or sweeping) it is possible to reach LODs at nM levels.

The enantioselective analysis of different chiral compounds (protein and non-protein amino acids, organic acids, phenolic compounds, sweeteners, and pesticides) in a broad range of food matrices including fruit juices, vinegars, genetically modified organisms, microalgaes, beers, soymilk, wine, food supplements, infant formulas, cacao beans, fruits, tea, or tap water has enabled to obtain significant and useful information to evaluate food quality, safety and genuineness. In general terms, through the chiral analysis of all these food components, it is possible to propose some of the enantiomers of these compounds as potential markers to obtain valuable information about fruit juices adulterations, food authenticity, thermal treatments and fermentation processes, the effects of processing and storage, equivalence of genetically modified organisms, or to evaluate if the enantiomer content of a food ingredient fulfills the legal regulations. It is clear that all this information is of great relevance in Food Science not only from a scientific point of view but also due to the increasing concern of consumers about the quality of food. However, in spite of the work developed in the last decade and the interesting information provided in the field of food analysis, it can be said that chiral food analysis is still a quite unexplored field. Undoubtedly, CE is nowadays more than a promising technique to face the different challenges involved in the research related to the field of chiral Food Analysis.

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Article 1

Capillary Electrophoresis determination of non-protein amino acids as quality markers in foods.

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Abstract

Non-protein amino acids mainly exist in food as products formed during food processing, as metabolic intermediates or as additives to increase nutritional and functional properties of food. This fact makes their analysis and determination an attractive field in food science since they can give interesting information on the quality and safety of foods. This article presents a comprehensive review devoted to describe the latest advances in the development of (achiral and chiral) analytical methodologies by capillary electrophoresis and microchip capillary electrophoresis for the analysis of non-protein amino acids in a variety of food samples. Most relevant information related to sample treatment, experimental separation and detection conditions, preconcentration strategies and limits of detection will be provided.

1. Introduction

The determination of protein amino acids has been exploited for years in the food field because they can provide relevant information on the quality and safety of food samples [1-3]. In addition to these compounds, it is also possible to find other amino acids of non-protein origin in foods which exist as metabolic intermediates, as products formed during food processing or as additives in food to increase some nutritional and functional properties [4,5]. Non-protein amino acids have been defined as those amino acids that are not found in protein main chain either for lack of a specific transfer RNA and codon triplet or because they do not arise from protein amino acids by post-translational modification [6]. Even though non-protein amino acids have been studied to a lesser extent, they have also shown to play an important role in the quality and safety of foods. Thus, different research works have demonstrated the relevance of determining non-protein amino acids to detect food adulterations, to investigate fermentation, storage and thermal treatments, to evaluate the nutritional quality of foods or to determine their toxic effects [7- 12].

Non-protein amino acids can be chiral having one or more chiral centers providing, therefore, at least one pair of enantiomers. Obviously, each enantiomer can originate different effects when interacting with chiral environments as enzymes, proteins and receptors [3]. Although the L-enantiomers are the natural form of amino acids, their exposure to certain processing conditions may originate their racemization to D-enantiomers. Processing-induced amino acid racemization includes from heat treatments, fermentation or storage to microbiological processes [13,14]. Moreover, D-enantiomers can be found in foods as a consequence of the

fraudulent addition of racemic mixtures of non-protein amino acids in supplemented foodstuffs where regulations establish the use of pure L-enantiomers. The use of racemic mixtures originates economical benefits due to the minor cost that these mixtures have with respect to the use of pure enantiomers. In general terms, enantioselective separations may be relevant in different food areas in order to propose quality markers to assess food authenticity and to detect adulterations, to evaluate the effects of processing, fermentation, microbiological activity and storage, to analyze chiral metabolites and to investigate health-promoting compounds or evaluate flavor and fragrance aromas [2,3,15,16]. In the specific case of chiral non-protein amino acids, the analysis of their enantiomers in foods is a very useful tool not only to assess food quality and authenticity [17], but also to evaluate processing, manufacturing of food supplements and detect microbiological contaminations [12,18].

Undoubtedly, these research activities are of great interest in the area of Food Analysis due to the increasing concern of consumers about the quality of food. Therefore, taking into account that assuring product food quality, authenticity, and safety is the main demand in the food field, there is an increasing need of analytical methodologies enabling the determination of non-protein amino acids in foodstuffs. Among the advanced analytical techniques that can be used to solve some of the challenges that Food Science has to face, Capillary Electrophoresis (CE) has already demonstrated its high potential for the (achiral and chiral) determination of many compounds in foods, including amino acids, to ensure compliance with food and trade laws [1,16,19,20]. Among the CE modes used to determine non protein amino acids in food samples, CZE (based on the free mobility of analytes in the aqueous solution under an applied electric field) and MEKC (based on the combination of electrokinetic migration and the partitioning mechanism between the bulk solution and micelles) are the most employed, whereas EKC and CEC (based on the interaction of each enantiomer with a chiral selector present in the mobile phase or with a chiral stationary phase, respectively) are the most employed CE modes to achieve an enantiomeric separation.

The present article reviews the most recent advances in the analysis of non-protein amino acids in foods using capillary electrophoretic methodologies (CZE, MEKC, EKC, and CEC) and microchip electrophoresis under achiral as well as chiral conditions covering the research articles published during the period February 2007 to February 2015, following the previous article published by our research group on this topic [1]. To make easier the discussion of the literature data and demonstrate the usefulness of CE to face different challenges related to food

quality, this review has been divided in two different sections focused on the achiral and chiral determination of non-protein amino acids in foods. These sections gather the non-protein amino acids according to their structure and describe the different CE approaches (including experimental conditions, preconcentration strategies and sample treatments) developed to analyze these compounds in a great variety of food samples.

2. Determination of non-protein amino acids in foods by CE under achiral conditions

With the aim of providing an updated view on the achiral CE methodologies developed for the analysis of non-protein amino acids in the period of time reviewed in this article, **Table 1** summarizes the main characteristics of the developed methodologies. It can be observed that, as expected, most of the research articles published employed CZE as separation mode, although the use of other modes such as electrokinetic chromatography using micelles as pseudostationary phase (MEKC mode), CEC and microchip capillary electrophoresis (MCE) has also been reported. With respect to the detection systems, UV and fluorescence are the most popular despite the need to include an additional analytical step (derivatization) to add a chromophore or fluorophore group into the molecule. *o*-phthaldialdehyde (OPA), fluorescein isothiocyanate (FITC), 4-chloro-7-nitrobenzofurazan (NDB-Cl) or 9-fluoroenylmethylchloroformate (FMOC), among others, have mainly been used as reagents for the derivatization of non-protein amino acids. To a lesser extent, mass spectrometry (MS), inductively coupled plasma-MS (ICP-MS), electrochemical and capacitively coupled contactless conductivity (C^4D) have also been reported as detection systems coupled to CE for determining non-protein amino acids. It should be highlighted that a broad range of food samples, from beverages (such as tea, soy based beverages, cow milk, energy drinks, etc), to flour products, shellfish, vegetables (tomato, *brassica* or *allium* species, etc), rice, meat products, or vegetable and olive oils have been analyzed by the developed CE approaches as shown in **Table 1**. The achiral determination of non-protein amino acids in these food matrices has been carried out mainly with quality control purposes. Thus, research works focused on the detection of adulterations, the study of the effects of fermentation, storage or thermal treatments, or the evaluation of the nutritional quality of different foods samples have enabled to point out these compounds as potential markers of food quality.

A more detailed description of the different achiral CE methodologies developed for the determination of non-protein amino acids and their applications in the area of Food Analysis will be provided next.

2.1 Aliphatic monoamino-monocarboxyl amino acids

γ-Aminobutyric acid (GABA) is a non-protein amino acid whose structure contains one carboxylic group and one primary amino group attached to the gamma carbon atom. It is distributed throughout the nervous system and it is the main inhibitory neurotransmitter in the mammalian brain which helps to regulate neuron activity and keep nerve cells firing normally [45,46]. In addition, GABA has demonstrated to have potential to maintain the balance of blood pressure in individuals with hypertensive cardiovascular disease [47].

In the period of time covered by this review, different CE methodologies coupled to fluorescence o MS detection systems were developed for the determination of GABA in tea, commercial dietary supplements containing royal jelly and honey (see **Table 1**). The lack of a chromophore group in GABA makes essential to carry out a derivatization step to enhance fluorescence detection. Thus, an in-capillary labeled derivatization reaction was investigated with the aim of determining the content of GABA and alanine (Ala) in different tea samples. The in-capillary derivatization was based on the use of o-phthalaldehyde/2-mercaptoethanol (OPA/2-Me) as labeling reagent. OPA reacts rapidly with primary amines in presence of a thiol co-reactant under alkaline conditions, and 2-ME (neutral co-reactant) minimized the instability of the resulting fluorophores. Under the optimized derivatization and CZE conditions (30 mM sodium tetraborate (pH 10.0)), GABA and Ala were analyzed in only 14 min (including in-capillary derivatization and CZE separation) with LODs of 0.004 and 0.02 μM, respectively. Due to the existence of matrix interferences, a 1000-fold dilution of GABA-rich tea was needed before applying the developed methodology to the analysis of both amino acids in tea samples [21]. However, those tea samples with a low content of GABA could not be diluted.

Table 1. Characteristics of the analytical methodologies developed for the achiral determination of non protein amino acids in foods by CE.

Classification	Name (Abbr.)	CE-mode/detection Separation conditions	Separation from:	Sample treatment	Application	LOD*	Ref
Aliphatic monoamio-monocarboxyl aminoacids	γ- aminobutyric acid (GABA)	CZE-Fluorescence (λ_{ex} 200-400 nm; λ_{em} 495 nm) 30 mM sodium borate (pH 10.0); capillary, 50 µm x 65cm; 21 kV; 23ºC	Ala	Boil, filtration, and dilution with water before **in-capillary** derivatization with OPA/2-ME	Determination of GABA and Ala in tea samples	0.004 µM	[21]
		CZE-Fluorescence (λ_{ex} 200-400 nm; λ_{em} 495 nm) 50 mM sodium borate (pH 10.0); capillary, 50 µm x 65cm; 21 kV; 23ºC	Ala	Boil, filtration, and dilution with ACN:0.1 mM NaCl (2:1 v/v) before **in-capillary** derivatization with OPA/2-ME	Determination of GABA and Ala in tea samples	0.7 nM	[22]
		CZE-MS^2 1 mM formic acid (pH 1.8); capillary, 50 µm x 100 cm; 30 kV, 20ºC	16 protein amino acids	Extraction with 75 % ethanol (v/v), ultrasonication, centrifugation, addition of 1 M HCl (0.9 mL supernatant: 0.1 mL HCl) prior to CE analysis	Simultaneous determination of free amino acids in several types of royal jelly products and honey	0.84 µg/g	[23]
Aliphatic amino acids with nitrogen in the side chain	Theanine (Thea)	MEKC-UV 10 mM sodium dihydrogenphosphate + 4 mM sodium borate + 45 mM SDS + 0.5% ethanol (pH 7.0); capillary, 50 µm x 38.5 cm; 20 kV; 30 ºC	7 Catechins, 3 xanthines, gallic acid, vitamin C, and theaflavin	Different treatment for making non-fermented, partially-fermented and fully-fermented tea, drying, water extraction (under orbital shaking), and filtration before CE injection	Determination of tea fermentation through simultaneous analysis of catechins, xanthines, gallic acid, vitamin C, thea and theaflavins	-	[24]
		MEKC-LIF (λ_{ex}488 nm; λ_{em}520 nm) 20 mM Brij 35 + 10% ACN (v/v) in 20 mM sodium borate (pH 8.5);capillary, 75 µm x 40cm; 20 kV; 25 ºC	13 protein amino acids + GABA	Extraction with hot water, centrifugation, filtration and pre-capillary derivatization with NDB-Cl	Simultaneous determination of free amino acids in different tea leaves	0.1 ng/L	[25]

Table 1. Continued

Classification	Name (Abbr.)	CE-mode/detection Separation conditions	Separation from:	Sample treatment	Application	LOD*	Ref.
	Homoarginine (Har)	CZE-UV (200 nm) 18.5 mM sodium borate (pH 9.2) + 10 mM sodium sulfate; capillary, 50 µm x 42 cm; 25 kV, 30 °C	-	Extraction with ethanol/water (6:4 v/v) and centrifugation prior to CE analysis	Analysis of Har accumulation in Grass Pea dry Seeds	-	[26]
	β-N-methylamino-L-alanine (BMAA)	CZE-UV (192 nm) 5 mM sodium borate (pH 9.0); 75 µm x 50 cm; 25 kV, 25 °C	-	Sequential extraction using TCA and microwave-digested with HCL, drying, and solution in deuterated water	Determination of BMAA in 18 strains of estuarine cyanobacteria	0.5 mg/L	[27]
	Ornithine (Orn)	CZE-MS2 0.1 M formic acid (pH 2.0); capillary, 50 µm x 60 cm; 25 kV, 25 °C	5 non-protein amino acids (β-ala, GABA, allo, cit, pyro)	Extraction with methanol/chloroform (2:1 v/v), centrifugation, drying, butanol derivatization, evaporation and dilution in ACN: water (40:60 v/v).	Potential of non-protein amino acids as novel markers of adulterations of olive oils with seeds oils	0.04 ng/g (Orn, β-ala, GABA, allo) 0.19 ng/g (cit, pyro)	[7]
Heterocycle amino acids	Furosine	CZE-UV (280 nm) 50 mM sodium phosphate (pH 7.0); bubble capillary, 50 µm x 40 and 56 cm; 25 kV, 25°C	-	Acid hydrolysis, filtration, clean-up (C18 cartridge), drying and formic acid (0.2 % (v/v)) dilution prior to CE injection	Evaluation of protein damage throughout determination of furosine in commercial soy-based beverages (soy milk and cow's milk supplemented with soy isoflavones)	5.30 mg/100 g protein a	[28]
		CZE-MS2 50 mM ammonium formate (pH 2.7); capillary, 50 µm x 60 cm; 25 kV, 30°C	-	Acid hydrolysis, filtration, drying, dissolution with the running buffer and re-filtration before CE analysis	Qualitative and quantitative analysis of furosine in food products (flours, pasta, milk, and tigelle bread)	0.07 mg/L	[29]
		CZE-MS2 50 mM ammonium formate (pH 2.7); capillary, 50 µm x 60 cm; 25 KV, 30°C	-	Acid hydrolysis, filtration, drying, dissolution with the running buffer and re-filtration before CE analysis	Quantification of furosine as marker for the assessment of thermal treatment of a cereal-based model food (tigelle bread)	-	[9]

Table 1. Continued

Classification	Name (Abbr.)	CE-mode/detection Separation conditions	Separation from:	Sample treatment	Application	LOD*	Ref
	Domoic acid (DA)	pCEC-UV (242 nm) ACN: 5 mM Tris (pH 8.0) (60:40 v/v) at a flow rate of 0.05 mL/min; packed capillary column, 100 µm x 55 cm (total length of which 20 cm was packed with ODS particles); -13kV; supplementary pressure 7.2 MPa	-	Extraction with methanol/water (1:1 v/v), centrifugation, and filtration prior to CEC analysis.	Determination of DA in shellfish tissues	0.5 µg/mL (equivalent to 2 µg DA/g, ww of mussel tissue)	[11]
		CZE-EIA-EC (Ed: -0.35 V) 1 % PVP + 1 mM H_2O_2 in 10 mM BR buffer (pH 5.0); separation capillary, 50 µm x 20 cm; reaction capillary, 50 µm x 5 cm; 15 kV	-	Extraction with methanol/water (1:1 v/v), centrifugation, filtration. Then non competitive inmunoreaction between free domoic acid antigen and (HRP)-labeled antidomoic acid antibody, incubation before CE analysis	Quantitative analysis of DA in contaminated shellfish samples(mussels, oysters, clams and scallops)	0.02 ng/mL	[30]
Sulfur amino acids	Taurine (Tau)	CZE-LIF (λ_{ex} and λ_{em} unspecified) 20 mM sodium phosphate (pH 11.8); capillary, 75µ x 40 cm; 22 kV, 23 °C	-	Derivatization with FITC (Energy drink was diluted 500-fold before CE analysis)	Quantification of Tau in an energy drink and a cow´s milk	-	[31]
		MEKC-C^4D (450 kHz; 17 Vpp) 40 mM CHES + 15 mM NaOH + 50 mM SDS (pH 9.36); capillary, 50 µm x 8 cm; 5 kV	Caffeine	Sonication (to remove dissolved gases) and dilution (10-fold) with the BGE	Simultaneous determination of taurine and caffeine in energy drinks	24 mg/L	[32]
		MCE-Fluorescence (λ_{em} 545 and 605 nm) 50 mM borate, (pH 9.3); glass microchip with an orthogonal channel design (10 x 40 mm) and a channel cross section of 20 x 50 µm	-	Buffer dissolution, derivatization with NBD-Cl, and dilution (10-fold) in separation buffer prior to CE analysis	Quantitative determination of Tau energy and sports drinks	1 x 10^{-6}M	[33]

Table 1. Continued

Classification	Name (Abbr.)	CE-mode/detection Separation conditions	Separation from:	Sample treatment	Application	LOD*	Ref
		MCE-LIF (λ_{ex}635 nm; λ_{em} 495 nm) 100 mM sodium borate (pH 9.88); glass microchip with a simple cross channel design; separation channel, (60 mm x 25 µmx 70 µm (length x depth x width)) 45 mm from injection to the detector	Lys, vitamin B_3	Two-fold dilutions with 40 mM sodium borate, pH adjustment (pH 8.6), derivatization with Cy5 and dilution with sample buffer (10 mM sodium borate, pH 9.88)	Simultaneous analysis of amino acids (Tau and Lys) and vitamin B_3in functional drinks.	0.50 nM	[34]
	Homocysteine (Hcy)	CZE-LIF (635 nm) 16 mM sodium citrate (pH 7.0) + 60% v/v ACN; capillary, 75 µm x 50 cm; 22,5 kV, 25 °C	GSH, Cys, γ-GluCys, CysGly, and NAC	Extraction with PBS, trichloroacetic acid, and EDTA (added in order) centrifugation, neutralization with sodium hydroxide, and derivatization with DMDSPAB-I before CE analysis	Determination of Hcy and other thiols in cucumber and tomato samples	0.15 nM	[35]
	S-alk(en)ylcysteine-S-oxides	CZE-UV (350 nm) 20 mM sodium benzoate + 0.5 mM TTAB (pH 12.0); capillary, 50µm x 91.5 cm; - 30 kV, 25 °C	Separation between methiin and alliin.	Boiling and water extraction (microwave), water dilution, and filtration before CE analysis.	Analyze of S-alk(en)ylcysteine S-oxides in allium and brassica vegetables	-	[36]
		MECK-indirect UV (265 nm) 20 mM sodium borate + 20mM SDS + 10% (v/v) MeOH (pH 9.2); capillary, 75µm x 67 cm; 20 kV, 25 °C	Separation between Methiin, Alliin, Ethiin Isoalliin Propiin	Extraction with methanol/water (90:10 v/v) + 10 mM HCl, drying until 10-15 ml and dilution with 20 mM sodium borate (pH 9.2), filtration, and derivatization with FMOC	Simultaneous determination of *S*-alk(en)ylcysteine-*S*-oxidesin alliaceous and cruciferous vegetables (e.g. garlic, onion, leek, chive, cabbage, radish, cauliflower and broccoli)	0.2 pmol	[37]

Table 1. Continued

Classification	Name (Abbr.)	CE-mode/detection Separation conditions	Separation from:	Sample treatment	Application	LOD*	Ref
Hydroxyl amino acids	Hydroxyproline (Hyp)	MEKC-UV (214 nm) 0.05 M sodium borate (pH 9.3) + 100 mM SDS; capillary, 50 µm x 50 cm; 12 kV, 33 ºC	Pro, Gly	Acid digestion, filtration, pH adjustment (pH 10.0), solution in sodium tetraborate, filtration, derivatization with fluorescamine and evaporation until 30 µL	Determination of Hyp as a collagen content index in meat products	-	[38]
		MEKC-LIF (λ_{ex}473 nm; λ_{em} 520 nm) 25 mM sodium borate + 30 mM SDS (pH 9.7); capillary, 50 µm x 40 cm; 20 kV, 25 ºC	-	Hydrolysis, dilution (10000-fold), and **in-capillary** derivatization with NBD-F	Determination of Hyp in milk powder, liquid milk, milk drink and soymilk powder	1.6 ng/mL	[39]
Seleno aminoacids	Seleno-methionine (Se-Met) Seleno-cysteine ($SeCys_2$)	CZE-ICP-MS 20 mM sodium phosphate + 10 mM sodium borate + 0.2 mM CTAB (pH 8.6); capillary, 75µm x 80cm; -16 kV, 25 ºC	Separation between Se-Met $SeCys_2$ Se(VI) Se(IV)	Enzyme-assisted extraction (using protease and lipase), centrifugation, filtration and dilution in water prior to CE injection	Simultaneous determination of selenium species (Se(VI), Se(IV), SeCys2 and SeMet) in selenium-enriched rice	0.5 ng/L SeMet 0.9 ng/L $SeCys_2$	[40]
Betaines	Betaine	CZE-indirect UV (214 nm) 5 mM sulfanilamide (pH 2.2); capillary, 50 µm x 41.5 cm; 25 kV, 30ºC	Pro	Extraction with 80 % ethanol (v/v) and filtration before CE analysis.	Demonstrate the potential of new probes for the simultaneous determination of betaine and Pro in spinach and beetroot samples	28.3 µM	[41]
	Trigonelline	CZE-UV (195 nm) 0.1 M ammonium formate (pH 2.0); capillary, 50 µm x 66.5 cm; 25 kV, 35ºC	-	Extraction with methanol/chloroform (2:1 v/v), centrifugation, drying and dilution in ACN: water (40:60 v/v). (Different volume of extracting solvent (including wash steps) and centrifugation parameters are used in the sample treatment of seeds and oils)	Determination of trigonelline in seeds and vegetables oils as marker of adulterated olive oils	0.9 µM	[42]

Table 1. Continued

Classification	Name (Abbr.)	CE-mode/detection Separation conditions	Separation from:	Sample treatment	Application	LOD*	Ref
	trigonelline glycine betaine proline betaine carnitines	CZE-MS² 0.1 M ammonium formate (pH 2.0); capillary, 50 µm x 60 cm; 25 kV, 25 °C	-	Extraction with methanol/chloroform (2:1 v/v), centrifugation, drying, butanol derivatization, evaporation and dilution in ACN: water (40:60 v/v).	Potential of betaines as novel markers of adulterations of olive oils with seeds oils	0.050 ng/g (Carnitines, trigonelline) 0.075 ng/g (glycine and proline betaine)	[8]
	Carnitine (Carn)	CZE-C⁴D (320 kHz; 280 Vpp) 500 mM acetic acid + 0.05 % (v/v) tween-20 (pH 2.6); capillary, 50 µm x 32 cm; 20 kV	-	Dilution with 500 mM acetic acid, centrifugation and injection in the CE system	Quantification of carnitine in foodstuffs (fruit, juices, milk, yogurt, cheese, red meat and chicken meat) and food supplements	2.6 µM	[43]
Others	N-phenylpropenoyl-L-amino acids (NPA)	CZE-UV (325 nm) CE system 1: 50 mM sodium borate (pH 8.8), capillary, 70 µm x 70 cm; 30 kV, 27 °C CE system 2: 50 mM sodium borate (pH 8.8), capillary, 50 µm x 50 cm; 20 kV, 27 °C	Separation between Caff-Asp pC-Asp Caff-DOPA	Cocoa beans and shells: deffating (soxhlet), extraction with acetone/water (7:3 v/v), centrifugation, rotatory evaporation, and centrifugation prior to CE analysis Flowers, leaves, husk, pulp, and shells: same extraction protocol without deffating	Analysis of main NPA in cocoa and cocoa products	-	[44]

ACN, acetonitrile; Ala, alanine; Allo, allo-isoleucine; BR, Britton-Robinson buffer; Caff-Asp, N-[3',4'-dihydroxy-(E)-cinnamoyl]-L-aspartic acid; Caff-DOPA, N-[3',4'-dihydroxy-(E)-cinnamoyl]-3-hydroxy-L-tyrosine; C⁴D, capacitively coupled contactless conductivity; CE-EIA, capillary electrophoresis based enzyme immunoassay; CHES, 2-(N-cyclohexylamino)ethane sulfonic acid; Cit, citruline; CTAB, cetyltrimethylammonium bromide; Cy5, Sulfoindocyanine succinimidyl ester; Cys, cysteine; CysGly, cysteinylglycine; CZE, capillary zone electrophoresis; DMDSPAB-I, 1,7-dimethyl-3,5-distyryl-8-phenyl-(4'-iodoacetamido) difluoroboradiaza-s-indacene; EC, electrochemical; EDTA, ethylene diamine tetra acetic acid; EMMA, electrophoretically mediated microanalysis; FICT, fluorescein isothiocyanate; FMOC, 9-fluorenyl-methyloxycarbonyl chloride; GSH, glutathione; γ-GluCys, γ-glutamylcysteine; Gly, glycine; HRP, horseradish peroxidase; LIF, laser-induced fluorescence; Lys, lysine; 2- MCE, microchip capillary electrophoresis; ME, 2-mercaptoethanol; MEKC, micellar electrokinetic chromatography; MS², tandem mass spectrometry; NDB-Cl, 4-chloro-7-nitrobenzofurazan; ODS, octadecyl silica; OPA, o-phthaldialdehyde; PBS, phosphate buffer saline; pCEC, pressurized capillary electrochromatography; pC-Tyr, N-[4'-hydroxy-(E)-cinnamoyl]-L-tyrosine; pC-Asp, N-[4'-hydroxy-(E)-cinnamoyl]-L-aspartic acid; PDA, diode array detector; Pro, proline; PVP, polyvinylpyrolidone; Pyro, pyroglutamic acid; SDS, sodium dodecyl sulfate; Se, selenium; TCA, trichloroacetic acid; TTAB, tetradecyltrimethyl-ammonium bromide.

*LODs units expressed as in the original work. These LODs are referred to the injected solutions of standard samples except for a) in which LOD is referred to the injected solutions of food samples.

**Capillary dimension expressed as internal diameter x effective length (cm to the detector).

Then, to make possible the detection after dilution, an in-capillary sample stacking preconcentration was subsequently included in the CZE-fluorescence methodology (see **Figure 1A**) [22]. By using this strategy, both GABA and Ala were detected in jasmine green tea sample (which has a low content of GABA) as it is shown in **Figure 1B**. The use of sample stacking preconcentration allowed achieving LODs of 0.7 and 0.8 nM for GABA and Ala, respectively. This implies a relevant enhancement of the LODs, from μM up to nM level, which clearly shows that the dilution of sample solution followed by a preconcentration strategy is an interesting option to carry out the sensitive CE detection of analytes in complex matrix samples. Moreover, both methodologies could be applied with quality control purposes since they enabled to monitor the GABA content in the GABA-rich tea manufacturing process. From an analytical point of view, these methodologies are of great interest since they include, in just one analysis, from an easy sample treatment (dilution to avoid interferences and derivatization to enhance fluorescence) to an in-capillary preconcentration strategy to improve sensitivity. Undoubtedly, both methodologies provide an easy, fast, and sensitive option for determining GABA in routine analysis.

The simultaneous determination of GABA and sixteen protein amino acids could also be performed without derivatization using a CZE-MS^2 method which did not require a concentration step for sample preparation [24]. Using 1M formic acid (pH 1.8) as BGE and 50 % (v/v) methanol as sheath liquid, it was possible to carry out the identification and simultaneous determination of all the amino acids studied (see **Table 1**) in different dietary supplements containing royal jelly (tablets, capsules, powder, liquid drinks and raw materials) and honey samples. LOD for GABA was 0.84 μg/g. Since the product analyzed contained specific proportions of amino acids, the developed methodology was useful to distinguish among different royal jelly products. Moreover, taking into account that royal jelly raw material has a different composition from honey, this CE-MS^2 strategy along with the analysis of the content of trans-10-hydroxy-2-decenoic acid (the main fatty acid in royal jelly) could be employed to detect the intentionally use of honey instead of royal jelly.

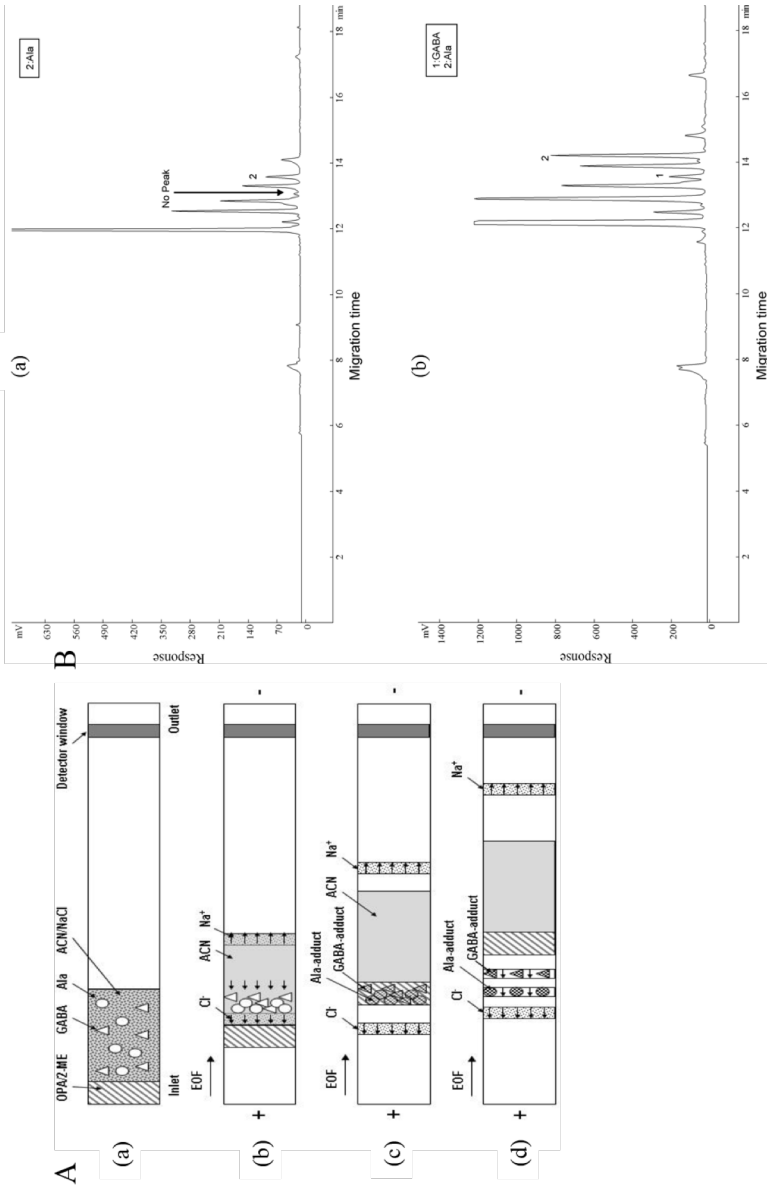

Figure 1. A) Diagram of the in-capillary labeled derivatization and stacking in ACN/salt solution: (a) working sample solution and labeling agents were introduced successively to the anodic end of the capillary column; (b) labeled derivatization occurred in the stacking process of analytes; (c) stacking of the labeled derivatives; (d) CZE began once the stacked ions left the sample zone solution. B) Electropherograms of GABA and alanine in jasmine green tea sample: (a) undiluted; (b) via stacking. CZE conditions: BGE, 50 mM sodium borate (pH 10); uncoated fused-silica capillary, 50 µm x 65 cm; voltage; 21 kV; temperature, 23 °C. Peaks: 1, GABA adduct; 2, Ala adduct. Reprinted with permission from [23]. Copyright (2010) American Chemical Society.

2.2 Aliphatic amino acids with nitrogen in the side chain

Theanine (Thea), L-homoarginine (Har), β-N-methylamino-L-alanine (BMAA), and ornithine (Orn) are non protein amino acids containing nitrogen in their side chains.

Theanine, the chief non-protein amino acid in tea (representing up to 50 % of the total amino acid content), is the main responsible of the taste of tea. It also has demonstrated to have an important role in different biological activities such as promoting relaxation, decreasing levels of serotonine and norepinephrine in brain, reducing blood pressure and enhancing anti-tumor activity [48]. A MEKC method using a BGE (pH 7.0) containing 10 mM phosphate, 4 mM sodium tetraborate, 45 mM sodium dodecyl sulfate (SDS), and 0.5 % ethanol, and UV detection (205 and 266 nm) was developed by Hsiao *et al*. [24] for the simultaneous determination of theanine, seven catechins, three xanthines, gallic acid, vitamin C and theaflavins in non-fermented, partially-fermented and fully-fermented tea samples. The successful separation of the studied compounds within 8 min enabled the application of the proposed method for the quality control of tea fermentation (in particular, the theanine content did not change significantly). Lately, Yan *et al*. [25] developed for the first time a high sensitive MEKC approach with LIF detection for the simultaneous analysis of 13 protein amino acids, theanine and GABA in five different tea leaves as shown in **Figure 2**. Brij 35 was used as surfactant and 4-chloro-7-nitrobenzofurazan (NBD-Cl) was chosen as labeling reagent to avoid time-consuming sample cleanup procedure because it does not react with other water soluble extracts in tea. This MEKC-LIF methodology enabled the detection up to 0.5 ng/mL and 0.1 ng/mL of GABA and theanine respectively, and has proved to be an efficient method for determining amino acids in tea. The two MEKC methodologies developed during the time covered by this review have a great potential to carry out an exhaustive characterization of tea samples since both enable the determination of not only theanine but also of a broad variety of compounds that can be of high interest to evaluate manufacturing processes.

L-Homoarginine (Har) and β-diaminopropionic acid (β-ODAP) are the main non protein amino acids in grass pea seeds. The impact of Har in humans and animal diets has given rise to contrasting opinions. A positive effect is considered due to its conversion into lysine and its potential to modulate β-ODAP toxicity [49, 50]. However, other studies suggested that Har modulates the biosynthesis of NO decreasing the excitation of neuronal receptors, and its presence in gene activator-repressor histones could be a cause of different cancer types [51,52].

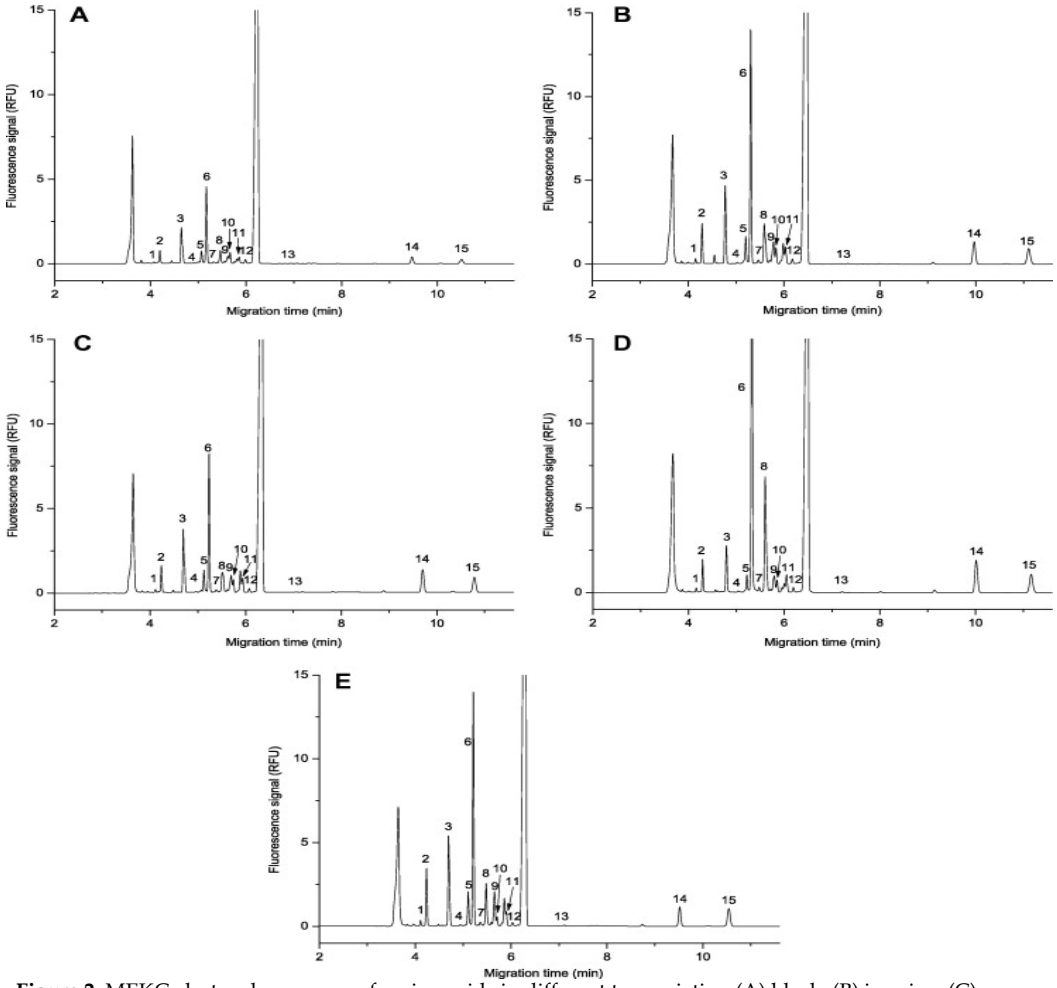

Figure 2. MEKC electropherograms of amino acids in different tea varieties. (A) black, (B) jasmine, (C) green, (D) maofeng, and (E) biluochun. Derivatization conditions: 40 mM NBD-Cl, 30 mM sodium borate (pH 8.5), reaction time of 30 min at 60 °C. Separation conditions: BGE, 20 mM sodium borate (pH 8.5) with 20 mM Brij 35 and 10 % acetonitrile (v/v); voltage, 20 kV; temperature, 25 °C. Peak 1, Lys; 2, Phe; 3, Leu; 4, Met; 5, Val; 6, Thea; 7, His; 8, GABA; 9, Thr; 10, Ala; 11, Ser; 12, Gly; 13, Cys; 14, Glu; 15, Asp. Reprinted from [26], copyright (2014) with permission from Elsevier.

The variation of the Har amount among grass pea genotypes cultivated in different Italian regions (with different soil properties and climatic conditions) in two consecutive years was investigated by using a CZE method [26]. The Har analysis was accomplished with UV detection at 200 nm using a 18.5 mM sodium borate (pH 9.2) containing 10 mM sodium sulfate as running buffer. This methodology was successfully applied to obtain information about the variation of Har in different grass pea genotypes observing that in all cases there was a trend towards increasing Har content in the second season being Har contents always

higher in one of the regions investigated (Guardia Perticara). Moreover, the variation of grain yield did not affect the Har storage.

β-N-methylamino-L-alanine (BMAA) is a neurotoxic non-protein amino acid involved in the Amyotrophic Lateral Sclerosis which has been found in strains of cyanobacteria in fresh water and marine environment [53,54]. Due to its toxicological effect its quantitation in cyanobacteria is of great interest. A CZE method based on the use of 5 mM sodium borate (pH 9.0) as BGE and UV detection at 192 nm was developed by Baptista *et al*. [27] enabling the separation of BMMA from its isomer 2,4-diaminobutyric acid (DBA). Eighteen strains of lyophilized estuarine cyanobacteria were analyzed employing the proposed method after a sequential BMMA extraction using trichloroacetic acid (TCA) and hydrochloric acid. Hydrochloric acid extraction was more effective than TCA extraction (except for one type of cyanobacteria which is called *Nostoc* sp.).

An interesting CE-MS^2 methodology was developed enabling the determination of non-protein amino acids in vegetable oils [7]. The simultaneous separation of ornithine, β-alanine, GABA, alloisoleucine, citrulline, and pyroglutamic acid, previously derivatized with butanol, was achieved in only 15 min with a 0.1 M formic acid buffer (pH 2.0). LODs between 0.04 and 0.19 ng/g were achieved when a normal stacking was used as preconcentration technique. Under these conditions, different vegetable oils (soybean, sunflower, corn and extra virgin olive oils) were analyzed in order to identify the selected non-protein amino acids. β-alanine, GABA, and pyroglutamic acid were detected in all the vegetable oils analyzed, whereas ornithine and alloisoleucine were only detected in soybean, corn and sunflower oils, and citrulline was not detected in any sample. Bearing these results in mind, and corroborating the presence of ornithine and alloisoleucine in seed oils but not in olive oils by MS^2 experiments, these two non-protein amino acids were proposed as novel markers for the detection of olive oil adulterations with sunflower, soybean and corn seed oils.

2.3 Heterocycle amino acids

Furosine and domoic acid are non protein amino acids characterized by the presence of a heterocycle group in the side chain and were analyzed by CE in the period of time covered by this review as it can be seen in **Table 1**.

Furosine is originated in the reaction between lysine and reducing carbohydrates and is one of the Maillard reaction products most widely used as markers of the nutritional quality of foods. Its presence and amount in foods is related to the Maillard reaction so that it has demonstrated to be a reliable indicator

of thermal treatment in many food products. In the last eight years furosine was analyzed by CE with the aim of evaluating protein damage in commercial soy-based beverages [28], to study the effect of milling, drying or thermal processes in different food products (flours, pasta, milk and tigelle bread) [29] or to investigate the cooking effect of different ovens on a cereal-based model food (tigelle bread) [9]. All the strategies developed with these purposes are based on simple sample treatments and fast CE analysis (around 4 min), both conditions of high interest for setting up an analytical methodology in food control laboratories.

To evaluate the protein quality of soy beverages, furosine was determined by CZE in different types of soy milk or cow's milk supplemented with soy isoflavones. A 50 mM sodium phosphate buffer (pH 7.0) was employed as BGE together with UV detection. The results were comparable with those obtained by an HPLC method. Even though HPLC proved to be slightly more sensitive than CZE (LODs of 1.30 and 5.30 mg/100 g of protein for HPLC and CZE, respectively), CZE was less time consuming (4 min versus 20 min) and caused less contamination, which showed the feasibility of the proposed CZE method [28].

The influence of different thermal processes on the quality of several food products was also investigated through the determination of furosine by CZE coupled to MS detection. Bignardi *et al.* [29] optimized different experimental conditions, such as capillary length, BGE concentration and pH, and applied voltage to establish a fast and reliable CZE-MS^2 for determining the furosine content in flours, pasta, milk and tigelle bread (treated under different milling, drying or thermal processes). Under the optimized conditions (see **Table 1**), furosine was analyzed in 4 min achieving a LOD of 0.07 mg/L. Afterwards, these authors employed the same CE-MS^2 method to evaluate the effect of different cooking treatments (two different ovens, modifying time and temperature) on tigelle bread through the determination of the furosine amount (along with the measure of maltose: maltulose ratio or colour index) [9]. The negative correlation between the furosine amount and the different cooking processes, suggested that under the cooking parameters tested furosine was transformed in other molecules.

Domoic acid (DA) is a crystalline water soluble kainoid amino acid which has neurotoxic effects. The consumption of shellfish containing high concentration of DA could be responsible of the amnesic shellfish poisoning (ASP) syndrome that produces abdominal cramps, vomits, disorientation and memory loss [55]. Despite the great evolution of CEC as separation technique, its potential in the analysis of non-protein amino acids in foods has not yet been achieved. This fact can be clearly observed in **Table 1** since the only CEC methodology proposed in the period of time

covered by this review to carry out the achiral determination of non-protein amino acids was applied to the rapid quantitation of DA in shellfish tissue extracts [11].To avoid practical problems related with bubble formation and column drying, an additional pressure was applied to the column inlet. Thus, a pressurized CEC-UV (pCEC-UV) method based on the use of a packed capillary column with octadecyl silica (ODS) particles, an isocratic separation (flow rate of 0.05 mL/min), and a supplementary pressure of 7.2 MPa, was developed to separate DA in shellfish matrices within 6 min achieving a LOD of 0.5 μg/mL. A second CE methodology based on enzyme immunoassay and electrochemical detection was also proposed to carry out the quantitative analysis of DA in shellfish samples [30]. This method, based on noncompetitive immunoreaction between DA antigen (Ag) and horseradish peroxidase (HRP)-labeled antidomoic acid antibody tracer (Ab*) in liquid phase, was able to separate the immmunocomplex (Ab*-Ag) and unbound (Ag*) in 4 min. The electrochemical detection was accomplished measuring amperometrically the enzymatic product obtained from the oxidation of *o*-aminophenol (OAP) with hydrogen peroxide. An LOD of 0.02 ng/ml was obtained enabling this approach to improve around 16 times the sensitivity reached by a commercial ELISA check kit. Although the proposed methodology provides a sensitive approach for the trace determination of DA in shellfish samples, the need to carry out a noncompetitive immune reaction between DA antigen and (HRP)-labeled antidomoic acid antibody as well as the use of an electrochemical detector can difficult its implementation in routine food laboratories.

2.4 Sulfur amino acids.

Taurine (Tau), Homocysteine (Hcy) and a group of S-alk(en)ylcysteine-S-oxides (namely, methiin, ethiin, isoallin, propiin and butiin) were the sulfur-containing non protein amino acids analyzed by CE in the reviewed period.

Taurine is a semi-essential amino acid that plays an important role in a variety of physiological functions (antioxidation, neuromodulation, etc), pharmacological properties (liver protection, low blood pressure, etc), and pathological effects (change of taurine´s levels in tissues and physiological fluids has a close relationship with different diseases such as Alzheimer, cardiovascular diseases or epilepsy, among others) [31]. Nowadays, the high consumption of energy and sport drinks makes that the normal daily uptake of taurine (one of the main components of these drinks) could be exceeded which can have undesirable effects. Therefore, reliable analytical methodologies are needed to determine taurine in foods from a quality control viewpoint. Bearing in mind this purpose,

different strategies based on CZE, MEKC and microchip capillary electrophoresis (MCE) have been proposed. For instance, a CZE method with LIF detection was developed by Zinello *et al.* [31] to determine taurine in food and clinical samples (energy drinks and cow´s milk). The use of a high incubation temperature (100 ^{o}C) enabled to reduce the reaction time between fluorescein isothiocyanate (FITC) and taurine from 6-14 h to 20 min. After optimization of different electrophoretic variables (buffer concentration, pH, and temperature), the use of 20 mM sodium phosphate at pH 11.8, a temperature of 23 ^{o}C, and a separation voltage of 22 kV allowed the analysis of taurine in less than 12 min. Different energy drinks were analyzed observing taurine contents in good agreement with the labeled ones. MEKC was also employed to determine taurine in energy drinks. In this case, the aim was to determine the caffeine and taurine contents (the major components) simultaneously in energy drinks, using a dual detection system in a short capillary (10.5 cm) employing a laboratory-home-made instrument [32]. Caffeine was detected by UV detection, whereas contactless conductivity detection (C^4D) was employed for determining taurine since it does not absorb in the UV/Vis region. Using a simple sample treatment and a 40 mM CHES buffer containing 15 mM sodium hydroxide and 50 mM SDS, taurine and caffeine analysis was accomplished in only 1 min. This method has many outstanding features including the elimination of derivatization step and the possibility to carry out a fast separation of the two main compounds in energy drinks.

Regarding MCE, it is one of the most relevant applications of micro-fluidics which offers some advantages such as miniaturization, short analysis times, and low solvent and sample consumption. From its introduction, MCE has experienced a substantial growth so that it is being considered as a new trend capable of solving a variety of problems in food analysis. In the period of time covered by this review, two different MCE methodologies were developed to determine taurine in beverages with fluorescence and LIF detection. On the one hand, using a simple sample pretreatment (just two dilution steps) and 4-chloro-7-nitro-1,2,3-benzofurazan (NBD-Cl) as labeling reagent, it was possible to quantify taurine in energy and sports drinks in only 12 s reaching a LOD of 1 x 10^{-6} M [33]. On the other hand, MCE with LIF detection was recently applied to the sensitive analysis of taurine, lysine, and B_3 vitamin derivatized with sulfoindocyanine succinimidyl ester (Cy5) in functional drinks [34]. Even though LIF has shown to be a potent detection method in sample analysis, the MCE-LIF sensitivity is sometimes not enough in sample analysis because of the low sample volume and short optical pathlength of the microchannels. For this reason, the development of on-line preconcentration

methods able to enhance the sensitivity of MCE is relevant. With this aim, an on-line pre-concentration approach combining field-amplified stacking (FASS) and reversed-field stacking was developed for the first time to achieve a sensitive analysis. The schematic mechanism of this strategy is shown in **Figure 3A**. The electrophoretic profiles obtained for the different concentration steps (see **Figure 3B**) demonstrated that both steps were crucial to achieve enhancement factors of 165-, 285- and 236-fold compared to the signal intensity without concentration. Under optimal conditions, taurine, lysine and B_3 vitamin were focused and separated within 4 min achieving LODs at the nM level. The MCE-LIF methodology was successfully applied to the analysis of these ingredients in eight different functional drinks samples with a satisfactory recovery (see **Figure 3C**) and results were in good agreement with those listed in the label of the products.

Homocysteine (Hcy) is a non-protein thiol amino acid (contains a sulfhydryl group in its structure) formed during methionine´s metabolism. Thiols play an important role in abiotic and biotic stress resistance involved in the detoxification of xenobiotics in many organisms and they have nutritional value for humans when they are present in fruits and vegetables [56,57]. To achieve thiol determination by CZE with LIF detection, a fluorescent labeling reagent is always required because these compounds do not have detectable fluorescence. Taking this into account, Zhang *et al.* [35] developed a CZE-LIF methodology to determine Hcy along with other thiol compounds (cysteine, cysteinylglycine, γ-glutamylcysteine, glutathione, and N-acetylcysteine) in cucumber and tomato samples using a new near-infrared fluorescent probe (namely 1,7-dimethyl-3,5-distyryl-8-phenyl-(4'-iodoacetamide)difluoroboradiaza-s-indacene (DMDSPAB-I)) as labeling reagent. By using the appropriate derivatization protocol (45 ^{o}C for 25 min), and the best separation conditions (16 mM sodium citrate at pH 7.0 containing 60 % (v/v) ACN), the studied thiols were analyzed within 14 min. The LOD obtained for Hcy with the proposed methodology was 0.15 nM. The authors proposed the method as a good alternative to investigate the biological function of low molecular weight thiols at trace levels.

S-Alk(en)ylcysteine-S-oxides are non-protein amino acids which appear as secondary metabolites in plants, fungi and algae and are precursors of a great variety of sensory-active and healthy compounds of *Allium* (onion, garlic, leek, etc) and *Brassica* vegetables (broccoli, cabbage, cauliflower, etc) [37].

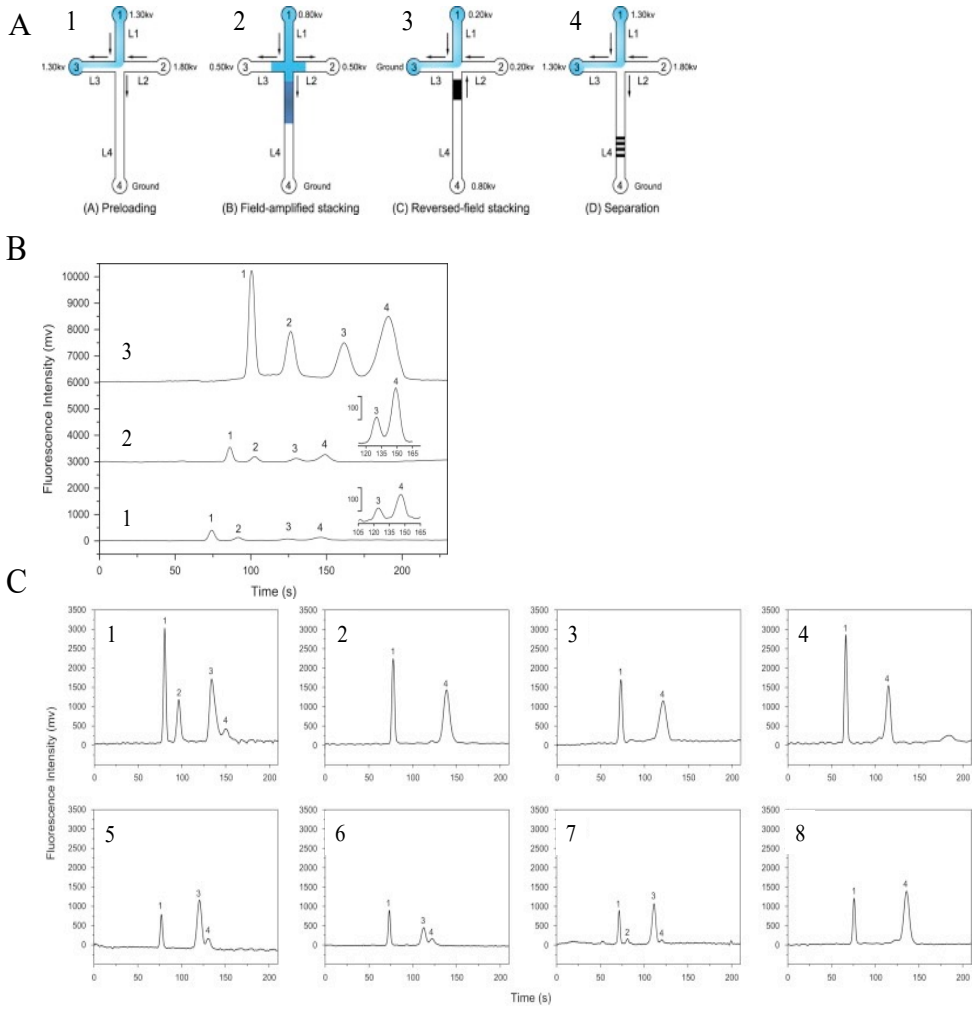

Figure 3. A) Diagram of the MCE sample loading, on line preconcentration, and separation of nutritious compounds: (1) preloading, (2) FASS, (3) reversed-field stacking, and (4) separation. The dark blue zone represents the concentrated sample by FASS before using reversed-field stacking technique, the black zone represents the area of the concentrated sample after reversed-field stacking, the light blue zone represents the sample matrix, and the clear zone represents the running buffer. B) Signal enhancement of the multiple concentration: (1) signal intensity without concentration (buffer, 100 mM borate (pH 9.88), sample diluted with the buffer, sample injection time, 2 s), (2) signal intensity with FASS (sample prepared in a 10-fold-diluted buffer, sample injection time, 2 s), (3) signal intensity with a combination of FASS and reversed-field stacking (sample injection time, 10 s; reversed-polarity time, 8 s). The concentrations of Lys, Tau, and NA in (1) were 0.6, 0.9, and 0.6 μM, respectively. The sample concentrations in (2) and (3) were 1/10 of that in (1). Peak 1, excess of Cy5; 2, Lys; 3, Tau; and 4, NA. C) MCE electropherograms of eight different Cy5-labeled functional drink samples after dilution. Dilution fold: (1), 32000; (2), 1600; (3), 2000; (4), 2000; (5), 32000; (6), 64000; (7), 320000; and F8, 2000. Reprinted from [35], copyright (2015) with permission from Elsevier.

Hideki *et al*. [36] developed a simple and rapid CZE method to analyze methiin and alliin in *Allium* and *Brassica* vegetables using indirect UV detection (at 350 nm) and 20 mM sodium benzoate containing 0.5 mM tetradecyltrimethylammonium bromide (TTAB) as BGE. The use of a sample treatment based on boiling, extraction, dilution and filtration, (without derivatization), gave rise to a total analysis time for methiin and alliin in vegetables less than 25 min per sample. An interesting advantage of this CZE methodology was the possibility to detect pyruvate which is a useful marker of unsuccessful sample preparations (the peak for pyruvate appeared instead of methiin and alliin peaks when the samples were extracted without boiling (blanching)).

Determination of the whole range of S-Alk(en)ylcysteine-S-oxides in alliaceous and cruciferous vegetables (fresh vegetables and garlic made products) was also carried out by MEKC with UV detection (previous derivatization with FMOC) [37]. Among the compounds investigated, isoalliin determination generated a special interest because it is the responsible of the pungency of onion and the undesirable decoloration of garlic [58]. The developed MEKC methodology, based on the use of 20 mM sodium borate containing 20 mM SDS and 10 % methanol as running buffer, enabled the simultaneous separation of S-Alk(en)ylcysteine-S-oxides (methiin, alliin, isoalliin, propiin, and ethiin) within 20 min and a LOD at the pmol level. Alliin was found only in garlic whereas isoalliin was the main compound in other *Allium* species (such as onion, leek, chive and shallot). On the other hand, methiin was the only compound contained in plants from the Cruciferae family (occasionally along with traces of ethiin) [37].

2.5 Hydroxyl amino acids.

Hydroxyproline (Hyp) is the only hydroxyl amino acid analyzed in food samples by CE in the period of time covered by this review. It is formed by hydroxylation of proline and it is the most abundant component in collagen so that it can be used as marker of collagen content index (collagen is often added in its natural form or as hydrolysates in some products as a protein source being its levels regulated due to collagen effects on food quality). Two MEKC methods were developed to determine Hyp content in different matrix (meat and milk products) employing UV or LIF detection. To carry out the determination of Hyp (as collagen content index) with UV detection, the MEKC method was based on the use of fluorescamine as labeling reagent and 0.05 M sodium borate containing 100 mM SDS as running buffer [38]. Under these conditions, Hyp was clearly separated not only from other amino acids (Pro and Gly) present in collagen but also from other

compounds present in meat samples. Since the results obtained using the developed MEKC-UV method were in agreement with the AOAC official colorimetric method, it can be considered as an alternative for Hyp analysis in meat products. Regarding the MEKC method with LIF detection, it was developed with the aim of providing a rapid and sensitive determination of Hyp in different dairy products for food quality assurance [39]. An in-capillary derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) and a 25 mM sodium borate buffer containing 30 mM SDS as running buffer were employed. Derivatization and separation was completed in only 7 min and the LOD for Hyp was 1.6 ng/mL, both remarkable features enabling to consider the proposed methodology as an alternative for the quality control of dairy products. In addition, these analytical features are relevant advantages compared with other chromatographic alternatives (HPLC, GC or CE), either with LIF or other types of detector, described in the literature to perform the determination of Hyp. In fact this methodology provided comparable or higher sensitivity and decreased the analysis time thanks to the use of an in-capillary derivatization which also allows a full automatization.

2.6 Seleno amino acids.

Selenium is an essential trace element for human health whose deficiency causes serious nutritional and health problems. An effective way for providing selenium is through the selenium-enriched foods intake. To carry out the nutritional and toxic evaluation of selenium compounds (Se (IV), Se (VI) and the non-protein amino acids selenocysteine ($SeCys_2$) and selenomethionine (SeMet)) in nutritional food supplements, Zhao *et al.* [40] developed a CZE-inductively coupled plasma MS (CZE-ICP-MS) methodology. As it can be seen in **Table 1**, sample treatment included an enzyme-assisted extraction approach to extract all selenium species and the CZE separation was accomplished using 20 mM sodium phosphate, 10 mM sodium borate and 0.2 mM cetyltrimethylammonium bromide (CTBA) as separation buffer (pH 8.6). LODs of 0.5 ng/L and 0.9 ng/L for SeMet and $SeCys_2$ respectively, and recoveries in the range 90-103 % were achieved. **Figure 4** shows the electropherograms obtained for a selenium-enriched rice and the same sample spiked with the selenium species studied. As **Figure 4** shows, only the non-protein amino acid SeMet was detected in selenium-enriched rice in the range of 0.136-0.143 mg Se/g dried weight.

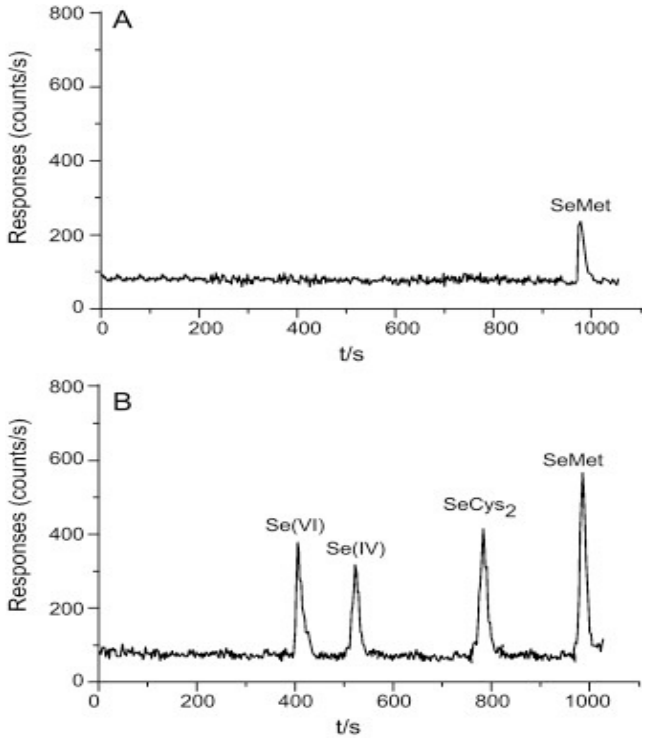

Figure 4. The electropherograms of Se(VI), Se(IV), $SeCys_2$ and SeMet under the optimal CZE-ICP-MS conditions: BGE, 20 mM sodium phosphate-10 mM sodium borate-0.2 mM CTAB (pH 8.6); uncoated fused-silica capillary, 75 μm x 80 cm; voltage; -16 kV; temperature, 25 ºC. (A) selenium-enriched rice; (B) selenium enriched rice spiked with 1.0 μg/g of Se(VI), Se(IV), $SeCys_2$ and SeMet. Reprinted from [41], copyright (2011) with permission from Elsevier.

2.7 Betaines.

Betaines are zwitterionic non protein amino acids which possess a quaternary ammonium group and a carboxylic group in their structure. They play a key role in many plants as osmoregulating compounds that help to tolerate environmental stress [60]. Betaine, trigonelline, glycine betaine, proline betaine, and carnitine have been the betaines analyzed by CE in different food samples during the time covered by this review.

The simultaneous determination of betaine and the protein amino acid proline was accomplished using a CZE methodology with indirect UV detection [41]. Among a variety of different probes (imidazole, creatinine, 4-aminopyridine, 4-aminobenzoic acid, sulfanilamide, etc), sulfanilamide demonstrated to be the most appropriate for the indirect detection of the studied compounds due to its slow mobility and good molar absorptivity. Under the optimal experimental conditions (see **Table 1**) and using 5 mM sulfanilamide, it was possible to achieve a LOD of

28.3 μM for betaine. The applicability of this methodology was demonstrated with the identification and quantification of betaine in extracts obtained from spinach and beetroot samples.

Trigonelline belongs to pyridine betaines group. It has demonstrated to have different health-promoting effects such as hypocholesterolemic, antitumor, or antimigraine, among others [60]. The determination of trigonelline in seeds and vegetables oils by CZE with UV detection (195 nm) enabled to propose this compound as a novel marker for the detection of adulterations in olive oils [42]. Under the experimental conditions detailed in **Table 1** and using an in-capillary normal stacking as sample preconcentration strategy, trigonelline was detected in both soy and sunflower seeds (and their oils) but not in olives or olives oils at least above the LOD of the developed method (LOD up to 0.9 μM) [42]. Taking into account the limitation of this CE-UV methodology to detect trigonelline in olives or olive oils, a CZE-MS^2 strategy was further carried out with the aim of detecting olive oil adulterations with seed oils through the simultaneous analysis of trigonelline and other betaines, such as proline betaine, glycine betaine (see **Figure 5A)** [8]. Following the same sample treatment described for the determination of ornithine in olive and seed oils [7] and using a derivatization with butanol to improve not only the analytical sensitivity but also the selectivity (by improving mass differentiation among analytes) the separation was achieved within 10 min using a 0.1 M formic acid (pH 2.0) as running buffer. The LOD obtained using MS detection (1 ng/g) was 20 times lower than that obtained previously with UV detection. **Figure 5B** shows the extracted ion electropherogram obtained for glycine betaine, trigonelline and total carnitines in soybean oil sample, extra virgin olive oil sample, and oil mixture of extra virgin olive oil sample with a 5 % w/w of soybean oil sample, as well as their MS^2 spectra in the oil mixture. The improved sensitivity enabled to detect low quantities of trigonelline in olive oils, so that at this low level it cannot be used as adulteration marker. By contrast, carnitines were not detected or not quantifiable in extra virgin olive oils; however, they were present in seed oils. This fact made possible to propose them as a feasible novel marker for the detection of adulteration of olive oils with seed oils [8]. From the results obtained using the developed methodology for the determination of non-protein amino acids [7] and betaines [8], both included among the variety of compounds constituting the unsaponifiable fraction of oils, the potential of ornithine, alloisoleucine and carnitines as novel markers for the detection of olive oil adulteration was demonstrated .

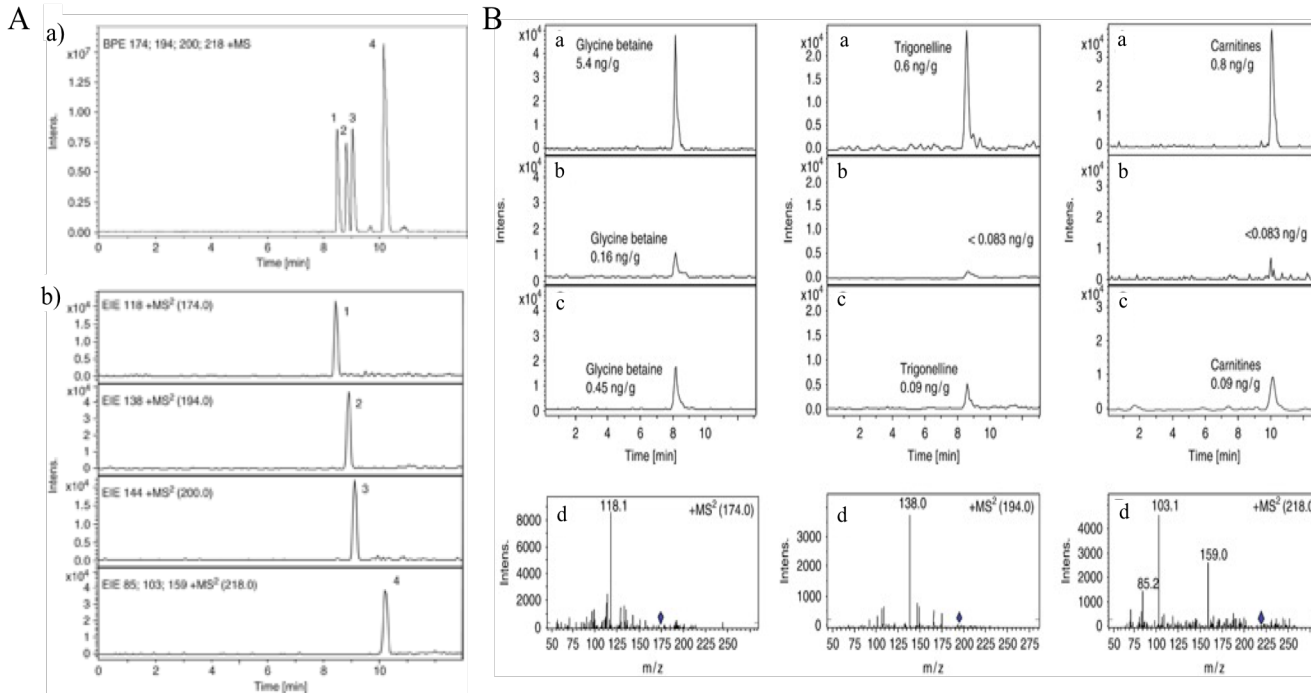

Figure 5. A) CZE-MS base-peak electropherogram (a) and simultaneous CZE-MS² extracted-ion electropherogram (EIE) (b) for standard betaines mixture (5 µg/mL each one injected at 50 mbar x 15 s (a) or 50 s (b)). B) CZE-MS² EIE for glycine betaine, trigonelline and total content of carnitines in (a) soybean oil sample, (b) extra virgin olive oil sample, (c) oil mixture of extra virgin olive oil sample with a 5 % w/w of soybean oil sample, and (d) MS² spectra for the peaks obtained in (c) of glycine betaine, trigonelline or carnitines in the oil mixtures of extra virgin olive oil sample with a 5 % w/w of soybean oil sample. CZE conditions: BGE, 0.1M formic buffer (pH 2.0); uncoated fused-silica capillary, 50 µm id x 60 cm; voltage, 25 kV; temperature, 25 °C. ESI conditions: positive ion mode (4.5 kV); sheath liquid, isopropanol/water (50/50, v/v) with 0.1 % formic acid at 3.3 µL/min; drying gas flow, 3 L/min; drying temperature, 300 °C; nebulizer pressure, 2 psi. Ion trap conditions: maximum accumulation time, 300 ms; averages, 1; scan, 50-280 m/z. MS² transitions in MRM mode with width, 4 m/z; fragmentation amplitude 1.00 V and fragmentation time, 40 ms. Reprinted from [8], copyright (2011) with permission from John Wiley and Sons.

Carnitine (Carn) is produced from lysine and methionine in low levels in human, so it is mostly introduced by diet. Due to the important role that carnitine plays in the fatty acids metabolism, its deficiency could give rise to different health problems such as hypoglycemia, hyperammonemia and hypoketosis [61,62]. A CZE method with C^4D detection was developed for the quantitative determination of carnitine in a great variety of foodstuffs (fruit, juices, milk, yogurt, cheese, chicken meat and red meat) [43]. This method consisted in the use of a 500 mM acetic acid buffer containing 0.05 % tween-20 (to prevent the wall interaction of the larger species) and a simple sample treatment. A LOD of 2.6 μM was obtained. The main advantage of the developed CZE-C^4D methodology is, undoubtedly, the elimination of the derivatization step or indirect approaches to carry out the determination of carnitine.

2.8 Other amino acids.

N-Phenylpropenoyl-L-amino acids (NPAs) are a group of non protein amino acids which are among the main contributors for the astringent taste of cocoa. For this reason, these compounds could be considered as useful quality markers concerning the cocoa´s taste [63]. In addition, NPAs have also demonstrated to have different pharmacological activities [64] Lechtenberg *et al.* [44] proposed two alternative methodologies (CZE and UPLC, both with UV detection) to determine the NPA content in cocoa and cocoa products. As it can be observed in Table 1, two different CE systems were used, but in both the experimental conditions to achieve the NPAs separation were similar (50 mM sodium borate at pH 8.8 was employed as running buffer). The CZE and UPLC methodologies developed in this work were comparable concerning the results obtained and time consumption. The former lacks in sensitivity but it just needed a simple sample treatment whereas the latter requires a SPE step in the sample cleanup but enables shorter analysis times and lower detection limits. Taking that into account, CZE was proposed for analyzing cocoa samples with high content of NPAs (mainly for the determination of N-[3', 4' -dihydroxy-(E)-cinnamoyl]-3-hydroxy-L-tyrosine (Caff-DOPA), N-[3', 4'-dihydroxy-(E)-cinnamoyl]-L-aspartic acid (Caff-Asp), and N-[4'-hydroxy-(E)-cynnamoyl]-L-aspartic acid (pC-Asp) in cocoa beans and shells).

3. Enantiomeric determination of non-protein amino acids in food by CE.

As it has been described in the introduction of this review, enantioselective separations may provide relevant information in different food areas, such as food

authenticity, detection of adulterations or evaluation of manufacturing processes among others. From the publication of the previous review devoted to describe the developed CE methods for the determination of non-protein amino acids in foods [1], the field of chiral separations has undergone a great growth. However, most of the chiral methodologies were aimed to the chiral separation of protein amino acids so it can be said that the chiral separation of non-protein amino acids in food samples is still a quite unexplored field.

Table 2 groups the main characteristics of the chiral methodologies developed to enantiomerically separate chiral non-protein amino acids by CE.

The most used CE mode has been MEKC which is based on the addition of a micellar pseudophase in which the concentration of the micellar system must be higher than its critical micelle concentration. The chiral selectors used in these methodologies were different types of cyclodextrins. CEC was also employed and compared with nano-LC. As shown in **Table 2**, the two main detection systems used were UV and MS^2 and in almost all the works a previous derivatization procedure was needed (labeling reagents employed were FMOC, FITC or AQC).

Since the L-enantiomer is usually responsible for the beneficial biological properties, the aim of these works was usually its separation from the D-enantiomer that can have in some cases toxic properties and whose addition during the elaboration of foods is forbidden. The developed methodologies enabled in these cases to guarantee a good quality control of food products. Samples analyzed were mainly food supplements, infant formulas, fermented foods (such as wine, beer etc.), etc., as described in detail in **Table 2.**

3.1 Aliphatic amino acids with nitrogen in the side chain

Ornithine (Orn) and Citrulline (Cit) enantiomers were determined in foods by CE under the experimental conditions detailed in **Table 2**.

Ornithine is a non-protein amino acid whose enantiomeric separation is receiving huge attention due to the beneficial properties of the L-enantiomers and the harmful effects of D-orn. L-Orn plays an important role in some biochemical processes such as fatty acid excess metabolism, human growth hormone synthesis, ammonia detoxification in urea cycle, synthesis of L-proline, etc. In contrast, D-enantiomer, which may occur during food processing or fermentation processes [70], can produce depletion in the urea synthesis giving toxic consequences. Humans can obtain L-orn through endogenous synthesis involving urea cycle, as well as from fermented foods (such as beer, wine, juices, cheese, etc), or functional foods (such as dietary supplements) [71].

Table 2. Characteristics of the analytical methodologies developed for the enantiomeric determination of non protein amino acids by CE.

Classification	Name (Abbr.)	CE mode/detection Separation conditions	Separation from:	Sample treatment	Application	LOD*	Ref.
Aliphatic amino acids with nitrogen in the side chain	Ornithine (Orn)	EKC-UV (260 nm) 5% (m/v) HS-β-CD + 2% (m/v) Ac-γ-CD in 50 mM phosphate (pH 2.0); capillary, 50 µm x 40 or 64 cm; -25 kV, 15 °C	20 protein amino acids, + GABA	Filtration (beer was previously degassed in a ultrasonic bath) followed by AQC derivatization	Analysis of Orn enantiomers in fermented foods (beer, wine, vinegar)	9 x 10^{-6} M	[65]
		EKC-UV (260 nm) 5% (m/v) HS-β-CD + 2% (m/v) Ac-γ-CD in 50 mM phosphate (pH 2.0); capillary, 50 µm x 64 cm; -25 kV, 15 °C;	Arg, Lys	Dietary supplements: water solution, filtration, and **in-capillary** derivatization with AQC Wine: Filtration and **in-capillary** derivatization with AQC	Determination of Orn, Arg and Lys enantiomers in dietary supplements and wines	6.4 x 10^{-6} M	[66]
		EKC- UV (240 nm) 1 mM γ-CD in 100 mM borate (pH 10.0); capillary, 50 µm x 40 cm; 20 kV, 25 °C;	Lys, Arg, Asp	Water solution, filtration, dissolution in borate buffer and accelerated derivatization with FITC using a **ultrasound probe**	Enantiomeric determination of Orn in food supplements	1.6 x 10^{-7} M	[67]
		EKC- MS^2 0.75 mM γ-CD in 50 mM ammonium carbonate (pH 10); Capillary, 50 µm x 100 cm; 25 kV, 25 °C	-	Degasification (ultrasonic bath), filtration, pH adjustment (pH 10), and filtration prior to FITC derivatization	Enantiomeric determination of beers submitted to different fermentation processes	2.5 x 10^{-9} M	[12]
	Citrulline (Cit.)	CEC-UV (210, 260 nm) 0.5 M ammonium formate/H_2O/ACN (1/19/80, v/v/v) (pH 2.5); capillary, 100 µm x 32.5 cm; -10 kV, 35 °C	-	Water solution and FMOC derivatization before CE analysis	Determination of L-citrulline and its enantiomeric impurity in food supplements	7.5 x 10^{-7} M	[17]

Table 2. Continued

Classification	Name (Abbr.)	CE mode/detection Separation conditions	Separation from:	Sample treatment	Application	LOD*	Ref.
Betaines	Carnitine (Carn)	EKC- MS^2 10 mM Succ-γ-CD in 0.5 M ammonium formate (pH 2.5) using PFT; capillary, 50 µm x 75 cm; 25 kV; 25 °C	-	Water solution (sonication) followed by ultrafiltration (5 kDa cut-off filter) and water dissolution before FMOC derivatization	Quantitative analysis of D/L-carnitine in infant formulas. Detection of D-carnitine up to 8 %.	16 ng/mL	[68]
		EKC- MS^2 0.2 % (m/v) Succ-γ-CD in 0.5 M ammonium formate (pH 2.5); capillary, 50 µm x 100 cm; 25 kV; 25 °C	-	Drinks: Water solution + FMOC derivatization Biscuits, tablets, and capsules: Water extraction, centrifugation, dilution 1/10 in water + FMOC derivatization	Identification and quantification of D/L-carnitine in dietary food supplements (drinks, biscuits, capsules and tablets). The use of racemic carnitine (not allowed by the legistation) was corroborated. in one of the food sample analyzed	10 ng/mL	[18]
Other amino acid	S-adenosyl-L-methionine (SAM)	CE-UV (260 nm) 300 mM glycine–50 mM phosphate (pH 2.5); capillary, 50 µm x 50 cm; 25 kV, 25 °C	-	Freezing, Liquid-liquid extraction (5 % TCA (w/v)), centrifugation and filtration prior to CE analysis.	Quantification of SAM in fruits and fruit juices	0.5 µM [a]	[69]

ACN, acetonitrile; Ac-γ-CD, acetylated-γ-CD; AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; Arg, arginine; Asp, asparagine; GABA, γ-aminobutiric acid; γ-CD, γ-cyclodextrin; FITC, fluorescein isothiocyanate; FMOC, 9-Fluorenylmethoxycarbonylchloride; HS-β-CD, highly sulfated-β-cyclodextrin; Lys, lysine; MS^2, tandem mass spectrometry; PFT, partial filling technique; Succ- γ-CD, succinyl-γ-cyclodextrin; TCA, trichloroacetic acid

* LODs units expressed as in the original work. These LODs are referred to the injected solutions of standard samples except for a) in which LOD is referred to the injected solutions of food samples.

**Capillary dimension expressed as internal diameter x effective length (cm to the detector).

To analyze Orn enantiomers in fermented foods, an EKC-UV method was developed by Martínez Girón et al. enabling LODs of 9 x 10^{-6} M. The method consisted of using AQC as off-line derivatizing reagent and 50 mM phosphate (pH 2.0) containing 5 % (m/v) HS-β-CD and 2 % (m/v) acetylated-γ-CD as BGE [65]. As shown in **Figure 6,** the methodology allowed the enantiomeric separation of ornithine (in less than 15 min) as well as the separation of the enantiomers of this amino acid from the enantiomers of the chiral amino acids contained in a mixture of the twenty protein amino acids and GABA in about 45 min.

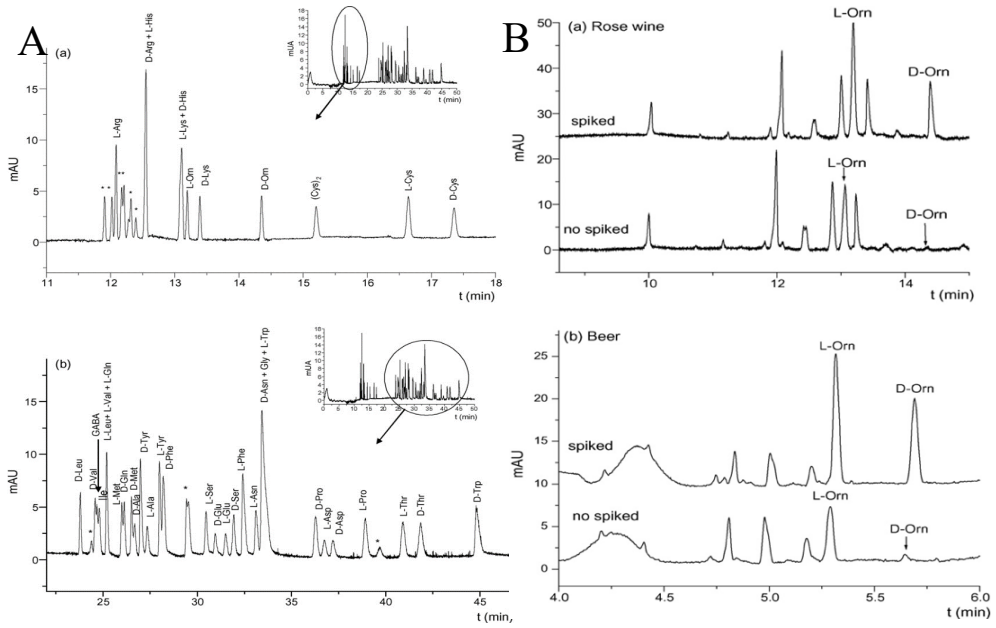

Figure 6. A) Enantiomeric separation by EKC of a mixture of the 20 protein amino acids, Orn, and GABA (upper corner) divided in two migration zones: (a) first-migrating zone, and (b) second-migrating zone. B) Electropherograms corresponding to different fermented foods derivatized off-line with AQC (a) a rose wine (uncoated fused-silica, 50 µm × 72.5 cm; injection by pressure, 5066.25 Pa for 20 s of sample followed of 5 s of BGE; non-spiked sample and sample spiked with 2.5×10^{-5} M racemic Orn) and (b) a beer (uncoated fused-silica, 50 µm × 48.5 cm; injection by pressure, 5066.25 Pa for 5 s of sample followed of 5 s of BGE; non spiked sample and sample spiked with 5×10^{-4} M racemic Orn). EKC conditions: 50 mM phosphate buffer (pH 2.0) containing 5 % (m/v) HS-γ-CD and 2 % (m/v) acetylated-γ-CD; uncoated fused-silica, 50 µm × 72.5 cm; voltage, -25 KV; temperature, 15 ^{o}C. (*) Unknown peaks. Reprinted from [66], copyright (2008) with permission from Elsevier.

To short the analysis time and to increase CE method automatization, the same authors developed a second method using in-capillary derivatization [66] with the aim of determining Orn, arginine and lysine enantiomers in wine samples (this compounds have demonstrated to be responsible of wine's organoleptic properties) and dietary supplements. The enantiomeric separation of Orn could also

be performed with FITC as labeling reagent. To do that, Domínguez-Vega et al. [78] employed an ultrasound probe which allowed to reduce derivatization time from 16 h to 10 min. The developed EKC-UV method enabled the enantiomeric analysis of this amino acid with LODs of 1.6 x 10^{-7} M, as well as the separation from the enantiomers of other protein amino acids such as lysine, arginine and asparagine (see other CE conditions in **Table 2**) in food supplements. Moreover, to enhance the sensitivity and selectivity in the enantiomeric determination of ornithine in beers, an interesting EKC-MS^2 method using the previous derivatization procedure was proposed. The method, based on the use of a 50 mM ammonium carbonate buffer at pH 10.0 containing 0.75 mM γ-CD as BGE, enabled to quantify the content of the Orn enantiomers in beers submitted to different fermentation processes with LODs of 2.5 x 10^{-9} M. The percentages for D-Orn in the analyzed samples ranged from 1.5 % to 10 %, the lowest value corresponding to a dietetic beer and the maximum to a double fermentation beer [12]. The four EKC methodologies described in the literature to carry out the chiral separation of ornithine show that this non-protein amino acid can be separated both under acid and basic conditions using different cyclodextrins as chiral selectors, depending on the characteristics of the analyzed samples or the requirements needed to perform the derivatization step. In any case, the lowest LODs for ornithine are achieved using a CE-MS^2 methodology.

Citrulline is a non-protein amino acid which has also demonstrated to have different enantiomeric behavior. L-Cit, which is naturally occurring, is precursor of protein amino acid Arginine, it is involved in urea cycle and it plays an important role in ammonia level decrease and NO cycle [72]. To carry out the enantiomeric determination of citrulline in food suplements, a CEC-UV method was developed using cellulose tris (3-chloro-4-methylphenylcarbamate chiral stationary phase (CSPs) as chiral selector, 9-fluorenylmethoxycarbonylchloride (FMOC) as labeling reagent and 0.5 M ammonium formate as running buffer. The method, which demonstrated to be more efficient in comparison with the developed nano-LC method, gave rise to LODs of 7.5 x 10^{-7} M for citrulline and made possible to achieve the enantiomeric determination of nineteen more amino acids (among twenty three amino acids analyzed) [17].

3.2 Betaines

As above-mentioned, Carn is synthesized from lysine and methionine or it can be available to human through some dietary sources. It has shown to have different biological activities depending on its enantiomeric form. L-Carn has demonstrated to play an important role in long chain fatty acids metabolism while

D-Carn posseses toxical properties [61,73]. For these reasons, the development of a method allowing to monitor the content of L/D-Carn in food samples is of great interest in order to carry out a correct quality control. With this aim, a CE-ESI-MS^2 methodology was developed using FMOC as labeling reagent (derivatization at 45 ^oC for 60 min) and 0.5 M ammonium formate containing 10 mM Succ-γ-CD as running buffer. Firstly, a partial filling technique (PFT) was employed and the method gave rise to LODs of 0.1 μM of L-Carn. The analysis of carnitine enantiomers in 14 infant formulas supplemented with this amino acid enabled the detection of amounts as high as 8 % of the toxic enantiomer which exceeded by far the limits established by the European Pharmacopeia [68]. Afterwards, in order to avoid the use of the PFT technique, the method was optimized without this step by using 0.2 % (m/v) Succ-γ-CD as chiral selector with a longer length capillary (see **table 2**) [18] improving the precision and sensitivity of the previous method (LODs of 10 ng/mL for D/L-Carn enantiomers were obtained enabling to detect enantomeric impurities up to 0.025 %). In this case, the optimized method was applied to the analysis of 22 dietary food supplements showing the use of L-Carn in 21 samples with enantiomeric impurities (D-Carn) up to 6 %. The use of racemic Carn (not allowed by the legislation) in one of the food supplements was corroborated which confirmed the need of analytical methodologies to ensure food quality [18].

3.3 Other amino acids

The only non-protein amino acid included in this group is S-Adenosyl-L-methionine (SAM), which is a chiral compound involved in many biochemical pathways and the major methyl donor in living organisms [74]. It is synthesized from methionine in the presence of ATP [75]. In addition, two diastereoisomers of this compound exist, known as (S,S)- and (R,S)-S-adenosyl-L-methionine, among which only one has demonstrated to be biologically active (S,S-). Van de Poel et al. [69] demonstrated that SAM content varies depending on food processing (relationship between heat treatment and SAM content in tomato samples is shown in **Figure 7A, 7B**).

The developed method consisted of optimizing CE-UV conditions to quantify SAM contents in different fruit tissue samples. The use of 300 mM glycine-50 mM phosphate (pH 2.5) was chosen as optimum running buffer, and the method, compared with the use of HPLC, made possible to enhance the enantiomeric separation (see **Figure 7A**) with minimum sample treatment, in half time and with

LODs 2-fold lower than those obtained by the conventional methodology (see LOD and other CE conditions in **Table 2**).

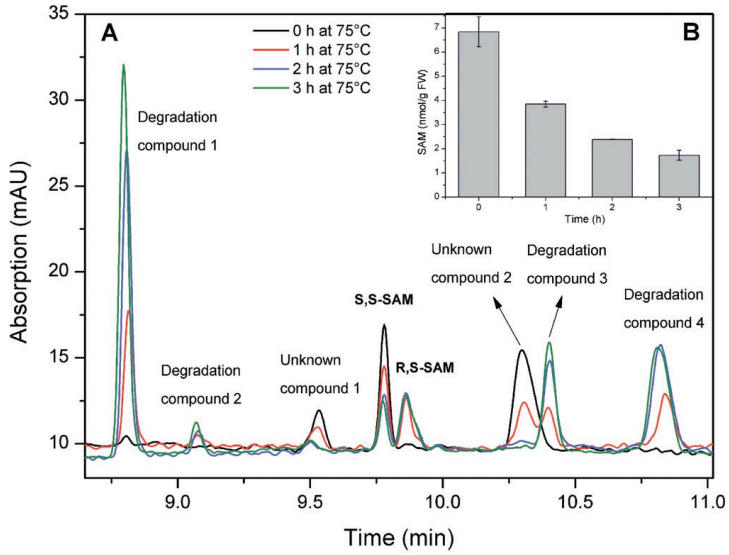

Figure 7. (A) Partial CE electropherogram showing the heat degradation of a tomato extract during several hours at 75 °C. *S,S*-SAM degrades together with two other unknown compounds (unknown compounds 1 and 2). Four degradation compounds (degradation compounds 1-4) are formed due to the heat treatment. (B) Inset showing SAM concentration (nmol/g FW) in tomato extracts during heat treatment of several hours at 75 °C. Error bars indicate the standard deviation based on two repetitions. Reprinted from [70], copyright (2010) with permission from John Wiley and Sons.

4. Concluding remarks

Non protein amino acids have shown to have a high potential as quality markers of foods. Their presence can be related to the food processing or to the existence of fraudulent practices so the development of analytical methodologies for their determination is very relevant. From the high number of research works described in the literature during the period of time covered by this review, it is quite clear that CE and MCE possess attractive capabilities to carry out the sensitive determination of non protein amino acids in a big variety of food matrices such as beverages (tea, milk, energy drinks, etc.), flour products, oil, vegetables (tomato, cucumber, spinach, etc.), food supplements, infant formulas, among others. The developed methodologies have demonstrated to be competitive in terms of sensitivity and selectivity with those involving other more conventional techniques and to present some advantages such as the low consumption of sample and solvents, the high speed of analysis and high separation efficiencies. Hence, CE techniques are nowadays not only promising techniques but real alternatives in

food analysis. In most cases, a derivatization step is required in order to introduce chromophore or fluorophore groups into the molecule of non-protein amino acids or to enhance the sensitivity obtained by different detection systems including MS. With this aim, a variety of labeling reagents has been employed such as FTIC, FMOC, NBD-Cl, DMDSPAB-1 or AQC. The latest trend in this topic involves the development of in-capillary derivatization procedures in which the labeling agent is inserted into the capillary for direct derivatization before CE separation. In this way, laborious and time consuming off-line derivatization protocols could be avoided.

The chiral separation of non-protein amino acids in food samples has received much attention in the last years. The use of a chiral selector in the separation buffer (EKC or MEKC modes) or a chiral stationary phase (CEC mode) has enabled the separation of the enantiomers of chiral non protein amino acids giving the proposed methodologies additional information of relevance in the control of the quality and safety of foods taking into account that the addition of the D-enantiomer during food production is forbidden and that racemization can occur as a consequence of some food processing or microbiological contamination.

Regarding detection systems, UV, fluorescence and MS have been the most employed detectors enabling reaching LODs at the level of 10^{-9} M. Despite the relatively poor concentration sensitivity related to the use of UV detection, this detector is the most employed in CE analysis of non-protein amino acids in foods. This fact is due to economic and availability reasons but also the development of in capillary sample preconcentration techniques based on electrophoretic principles which enable to overcome the sensitivity limitations in CE with optical detection.

Due to the high number of non-protein amino acids that exist in food samples, it can be said that their full potential as quality markers in foods remain unexplored. For this reason it can be expected than new methodologies with advances at different level (sample treatment, derivatization, preconcentration, CE separation, etc) will continue to grow in the near future.

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Article 2

Advances in the determination of non-protein amino acids in foods and biological samples by capillary electrophoresis.

R. Pérez-Míguez, S. Salido-Fortuna, M. Castro-Puyana, M. L. Marina.
Crit. Rev. Anal. Chem. DOI: 10.1080/10408347.2018.1546113.

Abstract

There are hundreds of non-protein amino acids whose importance in food and biological matrices is still unknown. Many of these compounds can be found in food as products formed during the processing, as metabolic intermediates or because they are added to increase functional and nutritional properties of food. Moreover, this kind of amino acids have also demonstrated to play relevant roles in the pharmaceutical and clinical fields since they may be used therapeutically in the treatment of some pathologies and their levels may be related with some diseases. These facts imply that the analysis of non-protein amino acids can be useful to obtain relevant information in the food and biological fields.

This article reviews the most recent advances in the development of analytical methodologies employing capillary electrophoresis for the achiral and chiral analysis of non-protein amino acids in food and biological samples. With this aim, the most relevant information concerning the separation and detection of these compounds by capillary electrophoresis is discussed and detailed experimental conditions under which their determination was achieved in food and biological samples are given covering the period of time from 2015 to 2018.

1. Introduction

Hundreds of amino acids are known, but only 20 of them are part of proteins. These 20 proteinogenic amino acids have been widely studied; however, there are others that are not found in protein main chain either for lack of a specific transfer RNA and codon triplet or because they do not arise from protein amino acids by post-translational modifications [1, 2]. Many of these non-protein amino acids (NPAAs) present an unknown origin and function, so it is difficult to attribute them a direct effect in the organism. Others have demonstrated to play relevant roles in the pharmaceutical and clinical fields since they may be used therapeutically for the treatment of some pathologies or have been related with some diseases. For instance, dihydroxyphenylalanine is used in Parkinson´s disease treatment, norleucine is related with the oxidative stress associated with Alzheimer's disease (AD), and others such as γ-aminobutyric acid (GABA) and taurine have demonstrated to act as neurotransmitters to regulate synaptic transmission and memory [3, 4, 5]. Moreover, NPAAs can provide information related to food quality and safety since some of them are present in food as products formed during processing or as additives to increase their nutritional value [6, 7]. Therefore, the determination of NPAAs constitutes an interesting tool to obtain information useful in the food, pharmaceutical and clinical fields. Consequently, it

is necessary to develop analytical methods capable to accurately determine NPAAs in real samples. Numerous works employing different techniques have been published reporting the determination of NPAAs. The most employed analytical techniques to face this challenge are High Performance Liquid Chromatography, Gas Chromatography and Capillary Electrophoresis (CE). Among these techniques, CE has emerged in the last decades as a potent separation technique thanks to its versatility, high efficiency and the low reagent and sample consumption required, among other advantages. In addition, its potential in the analysis of NPAAs has already demonstrated. The most employed CE modes to analyze NPAAs are Capillary Zone Electrophoresis (CZE) (based on the different mobility of the analyte in a conductive solution under the application of an electric field) and Micellar Electrokinetic Chromatography (MEKC) (whose separation is based on the different mobility of the analytes in a conductive solution that contains a micelle). Moreover, Electrokinetic Chromatography (EKC) and Capillary Electrochromatography (CEC) are the most employed modes to carry out the enantioseparation of chiral NPAAs using a chiral selector dissolved in the background electrolyte or a chiral stationary phase, respectively. Recently, the use of microchip electrophoresis (MCE) in the analysis of NPAAs has also become attractive. It presents some advantages over conventional systems such as the automatization, the lower sample and reagent consumption and its high efficiency [5].

The most common detection approach used in CE is the UV-Vis detector, although it requires a derivatization step because a high number of amino acids do not have sufficient UV absorption to be detected [8]. Fluorescence detection has also been widely employed for the analysis of NPAAs due to its high sensitivity; however, a derivatization procedure is also needed due to the lack of fluorescence of most amino acids. Many derivatization reagents such as 2,3-naphthalenedicarboxaldehyde (NDA) [9-14], 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [15-18], fluorescein isothiocyanate (FITC) [19], 9-fluorenylmethyl chloroformate (FMOC-Cl) [20], benzoyl chloride, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), dansyl chloride (DNS-Cl) [21] and o-phthaldialdehyde (OPA) [16] have been employed. An interesting alternative to these detection approaches is mass spectrometry (MS) that presents higher sensitivity and selectivity than other systems [5] and provides structural information being not necessary a derivatization step although sometimes it is also employed.

This article provides an overview of the most recent advances achieved in the development of analytical methodologies for determining NPAAs in foods and

biological samples by CE (including CZE, MEKC, EKC, and CEC modes). Articles published between 2015 and 2018 have been considered following the previous review of the authors on the CE determination of NPAAs in food [22]. **Tables 1** and **2** summarize the main characteristics (including CE mode, separation and detection conditions, sample treatment, LODs, and applications) of the different CE approaches developed for the analysis of NPAAs in different samples.

2. Determination of non-protein amino acids in foods by CE

The determination of NPAAs in food provides relevant information about food quality and safety. Different works have demonstrated the importance of analyzing NPAAs in food to detect adulterations [21], to evaluate nutritional quality of foods [24, 26] or to detect toxic effects [15, 19], among others. When the NPAAs of interest are chiral, their enantiomeric determination also enables to obtain valuable information on the effects of food processing or storage or on the presence of adulterations [42]. **Table 1** shows that a wide variety of food matrices were analyzed including beverages (juice, milk, beer, water or functional drinks), vegetables, fermented products or shellfish. The analysis of NPAAs was mainly achieved using CZE and MEKC, although CEC and MCE were also employed. The detection systems most frequently used were UV and LIF detectors, despite of being necessary the use of a derivatization step. Other detectors less employed were mass spectrometry (MS) and capacitively coupled contactless conductivity (C^4D).

Li et al. [21] developed a MEKC methodology enabling the simultaneous determination of hydroxyproline and hydroxylysine in different food samples (see **Table 1**). Both NPAAs are relevant components of protein collagen and they may be present in numerous food products. The developed MEKC methodology was applied to obtain amino acid profiles for authentic and fake plastron-derived functional product as a tool to detect adulterations with less expensive materials.

Table 1. Characteristics of the analytical methodologies developed for the determination of NPAAs in foods by CE.

NPAA	CE-mode/detection	Separation conditions*	Separation from:	Sample treatment	Application	LOD**	Ref
Hydroxyproline Hydroxylysine	MEKC-UV (214 nm)	20 mM sodium tetraborate and sodium phosphate + 0.1M SDS + 6% MeOH (pH 8.7); capillary, 50 µm x 50 cm; 25 kV, 25ºC	18 protein amino acids	Plastron, fish skin, pig skin, chicken tendon, calf tendon, pork, chicken and fish: dried, pulverized,dilution, alkali digestion, filtration and neutralization with HCl before derivatization with Dns-Cl Egg and milk: acid hydrolysis, filtration, lyophilization,dilution,alkali digestion, filtration and neutralization with HCl before derivatization with Dns-Cl	Potential of amino acids as markers of adulterations of plastron-derived functional foods	Hydroxyproline: 1.57 x 10^{-4} mg/mL Hydroxylysine: 6.65 x 10^{-4} mg/mL	[21]
Hydroxyproline	MEKC-UV (265 nm)	60 mM sodium borate + 30 mM SDS + 5% MeOH (pH 10.1); capillary, 50 µm x 72 cm; 25 kV, 23ºC	20 protein amino acids and Cystine	10-Fold water dilution (previously centrifuged and filtered), and derivatization with FMOC	Characterization and quantification of amino acid profile in passion fruit juices	-	[20]
Carnitine Acetyl-L-carnitine	CZE-indirect UV (200 nm)	3.0 mM melamine + 10 % MeOH (pH 2.1); capillary, 75 µm x 39 cm; 10 kV, 20ºC	-	Extraction with ACN:MeOH (4:1 v/v), centrifugation, evaporation and dilution in water	Determination of L-Carnitine and Acetyl-L-carnitine in liquid milk samples	L-carnitine: 3.0 µM Acetyl-L-carnitine: 5.0 µM	[23]
γ-Aminobutyric acid Citrulline	MEKC-LIF (λ_{ex} 442 nm; λ_{em} 485 nm)	40 mM sodium tetraborate + 60 mM SDS + 2 mM HP-β-CD (pH 9.2); capillary, 10 µm x 10 cm; -25 kV, 25ºC	17 protein amino acids, phosphoryl ethanolamine and ethanolamine	50-Fold dilution with water (previously degassed in ultrasonic bath) followed by NDA derivatization in presence of cyanide	Quantitation of 19 amino acids, phosphoryl ethanolamine and ethanolamine in beers	γ-Aminobutyric acid and Citrulline: 2.0-5.0 nM	[9]

Table 1. Continued

NPAA	CE-mode/detection	Separation conditions*	Separation from:	Sample treatment	Application	LOD**	Ref
Homoarginine β-N-Oxalyl-L-α,β-diaminopropionic acid	CZE-UV (195 nm)	25 mM sodium borate + 5 mM sodium sulfate (pH 9.2); capillary, 75 µm x 50 cm; 21 kV, 22ºC	-	Extraction with MeOH:water (60:40 v/v) (under Ultra-Turrax), centrifugation, evaporation and dilution in sample buffer (10 mM sodium borate + 5 mM sodium sulfate + 12.84 mM hippuric acid), filtration prior to the CE analysis. Extraction with ethanol:water (60:40 v/v) (under rotating shaking), centrifugation, evaporation and dilution in the sample buffer (10 mM sodium borate + 5 mM sodium sulfate + 12.84 mM hippuric acid), filtration before CE analysis.	Simultaneous analysis of β-N-Oxalyl-L-α,β-diaminopropionic acid and Homoarginine in *Lathyrus* species	-	[24]
Pyroglutamic acid Taurine	MEKC-UV (200 nm)	25 mM sodium tetraborate + 50 mM SDS (pH 9.1); capillary, 50 µm x 56 cm; 30 kV, 35ºC	Organic acids, 13 protein amino acids, Cystine and Tryptamine	Water solution, basic hydrolysis (with α-amylase), thermal sterilization, microfiltration and fermentation (tapioca starch and yeast) before CE analysis	Simultaneous determination of lactic acid and its organic impurities in fermentatively products	-	[25]
Taurine	CZE- C^4D	Commercial equipment: 20 mM CHES + 10 mM NaOH (pH 9.5); capillary, 25 µm x 11.5 cm; 10 kV, temperature, not indicated Instrument with a coaxial flow-gating interface: 20 mM CHES + 10 mM NaOH (pH 9.5); capillary, 25 µm x 18 cm; 20 kV, 25ºC	Citrate and Carbonate	Sonication (to remove dissolved gases) and 40-fold dilution for energy drink	Determinationof Taurine in energy drink	14.4 mg/L 8.2 mg/L	[26]
	MCE-LIF (λ_{ex} 635 nm; λ_{em} 495 nm)	100 mM sodium borate (pH 9.9); glass microchip with a simple cross channel design; separation channel, (60 mm x 25 µm x 70 µm (length x depth x width)), 45 mm from injection to the detector; applied potential and temperature, not indicated	Lysine and Vitamine B_3	2-Fold dilution with 40 mM sodium borate, pH adjustment (8.60), derivatization with Cy5 and dilution with 10 mM sodium borate (pH 9.88)	Analysis of amino acids (Lysine and Taurine) and vitamin B_3 in functional drinks	0.50 nM	[27]

Table 1. Continued

NPAA	CE-mode/detection	Separation conditions*	Separation from:	Sample treatment	Application	LOD**	Ref
β-N-Methylamino-L-alanine	CZE-UV (192 nm)	250 mM sodium phosphate (pH 3.0); capillary, 50 µm x 46 cm; 25 kV, 17ºC	Four β-N-Methylamino-L-alanine isomers	Acid hydrolysis, drying and dilution with HCl, clean-up, drying and re-dilution with HCl prior to CE analysis	Separation of five β-N-Methylamino-L-alanine isomers and quantification of β-N-Methylamino-L-alanine in cycad, mussel and lobster samples	0.25 µg/mL 20 mg/g[a]	[28]
	CZE-(QqQ)MS^2 ESI+ (4.0 kV); sheath liquid: MeOH:water (50:50 v/v) containing 0.1% formic acid at 1.0 µL/min. Flow and temperature of dry gas, and nebulizar gass pressure not indicated	5 M formic acid + 10 % (v/v) ACN (pH 1.55); capillary, 50 µm x 100 cm; 20 kV, 17ºC				0.8 ng/mL 16 ng/g[a]	
Domoic acid	MCE-LIF (λ_{ex} 475 nm; λ_{em} 535 nm)	5 mM sodium tetraborate (pH 9.2); glass microchipcross-channel design; separation channel, (49 mmx 30 µm x 80 µm (length x depth x width)), 27 mm from injection to the detector; 400 V/cm; temperature, not indicated	-	Extraction with MeOH:water (1:1 v/v), centrifugation, filtration and derivatization with FITC	Determination of Domoic acid in shellfish tissues	0.28 nM	[19]
	pCEC-LIF (λ_{ex} 473 nm; λ_{em} 530 nm)	5 mM phosphate:ACN (40:60 v/v) (pH 2.5) at a flow rate of 1.2 µL/min; packed capillary column, 100 µm x 55 cm (total length of which 20 cm was packed with ODS particles); 6 kV, 25ºC; supplementary pressure 7.2 MPa	-	Extraction with MeOH:water (1:1 v/v), centrifugation, filtration, clean-up, purification and derivatization with NBD-F before CEC analysis	Quantification of Domoic acid in shellfishsamples (oyster, *mytilus edulis* and *ruditapes*)	10 ng/mL[c] 15 ng/g[b]	[15]

Table 1. Continued

NPAA	CE-mode/detection	Separation conditions*	Separation from:	Sample treatment	Application	LOD**	Ref
Norvaline Norleucine 2-Aminoadipic acid Seleno-methionine 3,4-Dihydroxy-phenylalanine Pyroglutamic acid Pipecolic acid Citrulline	EKC-UV (210 nm)	10 mM sulfated-CD in 100 mM formate (pH 2.0); capillary, 50 µm x 40 cm; -20 kV,15-25ºC	-	Water solution (sonication), centrifugation, filtration, and derivatization with FMOC before CE analysis	Individual enantiomeric separation of the 8 NPAAs studied and development of a method for the quantitation of L- and D-Citrulline in food supplements	D-Citrulline: 0.21 µM L-Citrulline: 0.18 µM	[29]

ACN, acetonitrile; C^4D, capacitively coupled contactless conductivity; CHES, 2-(N-cyclohexylamino)ethane sulfonic acid; Cy5, Sulfoindocyanine succinimidyl ester; CZE, capillary zone electrophoresis; Dns-Cl, dansyl chloride; FITC, fluorescein isothiocyanate; FMOC, 9-fluorenyl-methyloxycarbonyl; HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; LIF, laser-induced fluorescence; MCE, microchip capillary electrophoresis; MEKC, micellar electrokinetic chromatography; MeOH: methanol; MS^2, tandem mass spectrometry; NBD-F, 4-Fluoro-7-nitro-2,1,3-benzoxadiazole; NDA, 2,3-naphthalenedicarboxaldehyde; ODS, octadecyl silica; pCEC, pressurized capillary electrochromatography; $(QqQ)MS^2$, triple quadrupole mass spectrometry; SDS, sodium dodecyl sulfate.
*Capillary dimensions expressed as internal diameter x effective length (cm to the detector).
**LODs units expressed as in the original work. These LODs are referred to the concentration of injected standard solutions except for a) LODs referred to the dry sample mass, b) LODs referred to the wet sample weight, and c) LOD referred to the concentration of injected real sample solutions extracts.

Table 2. Characteristics of the analytical methodologies developed for the determination of NPAAs in biological samples by CE.

NPPA	CE-mode/detection	Separation conditions*	Separation from:	Sample treatment	Application	LOD**	Ref
Homocysteine Homoarginine Ornithine Citrulline	MEKC-LIF (λ_{ex} 488 nm; λ_{em} 520 nm)	50 mM borate + 30 mM SDS + 30 % MeOH (pH 9.5); capillary, 50 µm x 50 cm; 21 kV, 21 °C	Arginine, asymmetric dimethyl-L-arginine, symmetric dimethyl-L-arginine, and Monomethyl-L-arginine	Protein precipitation with 5-sulfosalicylic, evaporation to dryness under vacuum, redisolution in BGE, and derivatization with CFSE before CE analysis.	Determination of Homoarginine, Homocysteine and Arginine metabolic derivatives in fluids from Type 2 diabetics with peptic ulcer bleeding	Ornithine: 1.70 nM Citrulline: 1.67 nM Homoarginine: 0.88 nM Homocysteine: 0.12 nM	[30]
γ-Aminobutyric acid Ornithine Citrulline Taurine	EKC-LIF (λ_{ex} 488 nm; λ_{em} 520 nm)	90 mM borate + 35 mM α-CD (pH 9.8); capillary, 50 µm x 30 cm; 21 kV	12 protein amino acids	On-line derivatization with 20 mM NBD-F/250 µM HCl in 50% MeOH	Measurement of branched chain amino acid uptake in 3T3-L1 cells	-	[18]
Ornithine Citrulline Norvaline Norleucine	CZE-LEDIF (λ_{ex} 405 nm; λ_{em} 486 nm)	1% PVP + 10 mM HEPES (pH 7.0); PVP coated capillary, 75 µm x 28 cm; -20 kV, temperature, not indicated	17 protein amino acids	Protein precipitation by heating, derivatization with NDA before CE inyection.	Separation of amino acids in human plasma	-	[14]
γ-Aminobutyric acid Ornithine Citrulline Hydroxyproline Alloisoleucine	CZE-(IT)MS^2 ESI+ (4.5 kV); sheath liquid: MeOH:water (60:40 v/v) containing 0.5% formic acid at 5.0 µL/min; dry gas: 5 L/min at 200°C; nebulizer gas pressure: 8 psi	0.8 M formic acid + 15 % MeOH (pH 1.96); capillary, 50 µm x 85 cm; 30 kV, 20°C	20 protein amino acids, Carnosine	Centrifugation and dilution prior to CE analysis	Determination of amino acids in urine samples	γ-Aminobutyric acid: 4 µM Ornithine: 4.8 µM Citrulline: 7.7 µM Hydroxyproline: 3.7 µM Alloisoleucine: 2.5 µM	[31]

Table 2. Continued

NPPA	CE-mode/detection	Separation conditions*	Separation from:	Sample treatment	Application	LOD**	Ref
Citrulline	CZE-LIF (λ_{ex} 488 nm; λ_{em} 510 nm)	20 mM carbonate (pH 10.0); 27 kV, capillary and temperature, not indicated	Arginine	Protein precipitation with ACN, derivatization with FITC and dilution before CE analysis.	Determination of plasma levels of Arginine and Citrulline in preterm and full-term neonates	-	[32]
Citrulline + Taurine	MCE-LIF (λ_{ex} 445 nm; λ_{em} 480 nm)	15 mM borate + 1.4 mM SBEC + 10 % DMSO (pH 9.2); 10 kV, capillary and temperature, not indicated	Arginine, Glutamic acid, Aspartic acid and Histamine	Fluorogenic derivatization with NDA.	Analysis of amino acid neurotransmitters in brain dialysis samples	Citrulline: 0.36 µM Taurine: 0.42 µM	[12]
Taurine	MEKC-SDED	20 mM phosphate + 20 mM SDS (pH 10.0); capillary, 25 µm x 40 cm; 12 kV, temperature, not indicated	20 protein amino acids	Extraction with water, protein precipitation wih ACN, evaporation to dryness under nitrogen stream, redisolution in BGE.	Determination of Taurine in human tear fluid	0.18 µM	[33]
Taurine γ-Aminobutyric acid	EKC-LIF (λ_{ex} 488 nm; λ_{em} 543.5 nm)	90 mM borate + 35 mM α-CD (pH 10.0); capillary, 5 µm x 6.2 cm; -23 kV, temperature, not indicated	10 protein amino acids	On-line derivatization with 20 mM NBD-F/250 µM HCl in 50 % MeOH.	Monitoring the *in vivo* dynamics of amino acids biomarkers of metabolism in adipose tissue	2.7 µM	[17]

Table 2. Continued

NPPA	CE-mode/detection	Separation conditions*	Separation from:	Sample treatment	Application	LOD**	Ref
Taurine	CZE-(TOF)MS ESI+ (4.0 kV); sheath liquid: MeOH:water (50:50 v/v) containing 0.5 µM reserpine at 10 µL/min; dry gas: flow not indicated at 300°C; nebulizer gas pressure: 10 psi	Commercial electrophoresis buffer for anion and cation analysis; capillary, 50 µm x 80 cm; applied potential and temperature, not indicated	24 metabolites including 5 protein amino acids	Centrifugation, filtration and disolution in water.	Metabolome analysis of saliva samples	-	[34]
	MEKC-LIF (λ_{ex} 488 nm; λ_{em} 515 nm)	35 mM borate + 55 mM SDS + 2.7 M urea + 1 mM BIS-TRIS propane + 23 mM NaOH (pH 9.8); capillary, 50 µm x 50 cm; 30 kV, 24°C	18 protein amino acids, Alanine-Glutamine, Cystine	Protein precipitation with MeOH. On-capillary derivatization by TDLFP: sequential injection of 1.5 mM NDA in the reaction buffer with 50 % MeOH, sample, and 10 mM NaCN in the reaction buffer.	Assessing developmental capacity of human embryos after *in vitro* fertilization	0.02 µM	[11]
	MEKC-LIF (λ_{ex} 488 nm; λ_{em} 515 nm)	73 mM SDS + 6.7 % (v/v) 1-propanol + 0.5 mM HP-β-CD + 135 mM boric acid/NaOH (pH 9.00); capillary, 50 µm x 45 cm; 30 kV, 25°C	18 protein amino acids Alanine-Glutamine, Cystine	Protein precipitation with MeOH:IPA (4:1) and dilution. On-capillary derivatization by EMMA: sequential injection of 1 mM NDA in the reaction buffer with a 12.5 % MeOH/IPA (4:1) mixture, sample, 1 mM NDA in the reaction buffer with a 12.5 % MeOH/IPA (4:1) + 2.5 mM NaCN. Reaction products were on capillary preconcentrated by sweeping.	Non-invasive targeted metabolomics of human embryos.	12 nM	[10]

Table 2. Continued

NPPA	CE-mode/detection	Separation conditions*	Separation from:	Sample treatment	Application	LOD**	Ref
Homocysteine	MEKC-UV (λ_{abs} 285 nm)	0.1 M TEA + 0.15 M formic acid + 50 µM CTAB (pH 3.9); capillary, 50 µm x 21.5 cm; -12 kV, 25°C	Cysteine, Cysteine-Glycine	Reduction with DTT before TCDI derivation.	Rapid detection of total Homocysteine and Cysteine in human plasma	0.8 µM	[35]
	MEKC-UV (λ_{abs} 285 nm)	0.1 M phosphate + 30 mM TEA + 25 M CTAB + 2.5 M SDS + 2.5% PGE-600 (pH 2); capillary, 50 µm x 23.5 cm; -17 kV, 30°C	Cysteine	Derivatized with TCDI, extraction with chloroform-ACN.	Determination of Homocysteine and Cysteine levels in human plasma and urine	0.2 µM	[36]
	CZE-LIF (λ_{ex} 473 nm; λ_{em} 510 nm)	10 mM sodium citrate (pH 7.5); capillary, 50 µm x 50 cm; 15 kV, room temperature	Glutation, Cysteine, γ-glutamylcysteine	Cells lines were resuspended in PBS and incubated with NEM. Single cells were incubated with TMPAB-o-M and mixed with the runing buffer.	Chemical cytometry of thiols in human colon cancer and breast cells	13 pM	[37]
	CZE-(QqQ)MS^2 ESI+ (4.5 kV); sheath liquid: MeOH:water (50:50 v/v) containing 5 mM acetic acid at 6.0 µL/min; dry gas: 6 L/min at 160°C; nebulizer gas pressure: 34.5 kPa	5 M acetic acid; capillary, 50 µm x 60 cm; 25 kV, 20°C	Glutamic acid, Cysteine, Methionine	Blood samples + EDTA, centrifugation. For aminothiols analysis: Mix samples with DTT, IAA and ACN, centrifugation and analysis of the supernatant.	Determination of Homocysteine as potential biomarkers of amyotrophic lateral sclerosis	35 nM	[38]
γ-Aminobutyric acid	MEKC-LIF (λ_{ex} 450 nm; λ_{em} 480 nm)	25 mM phosfate + 30 mM SDS (pH 8.3); capillary, 25 µm x 50 cm; 29 kV, 25°C	17 protein amino acids	Derivatization with NDA, cyanide, and internal standard (D- norvaline) (9:1:1:1, sample:NDA:cyanide:D-norvaline)	Measuring amino acid secretions from islets of Langerhans	3 nM	[13]
Hydroxyproline	MEKC-LIF (λ_{ex} 492 nm; λ_{em} 520 nm)	40 mM cholate + 40 mM deoxycholate + 40 mM tetraborate (pH 9.2); capillary, 10 µm x 10 cm; -25 kV, room temperature	Proline, 4 propyl dipeptides	Acid Hydrolysis, 100-fold dilution, addition of EDTA, OPA and derivatization with NBD-F prior to CE analysis.	Rapid determination of free prolyl dipeptides and Hydroxyproline in urine	70 nM	[16]

Table 2. Continued

NPPA	CE-mode/detection	Separation conditions*	Separation from:	Sample treatment	Application	LOD**	Ref
Pyroglutamic acid	CZE-UV (λ_{abs} 200 nm)	40 mM CHES/NaOH (pH 10.2); capillary, 25 µm x 23 cm; 30 kV, 25°C	Paracetamol	Protein precipitation with ACN and NH_4OH, centrifugation and analysis of the supernatant.	Quantification of Paracetamol and Pyroglutamic acid in serum	1.3 µg/mL	[39]
Betaine	CZE-(QqQ)MS^2 ESI+ (4.0 kV); sheath liquid: MeOH:H_2O (50:50 v/v) containing 5mM ammonium acetate at 6.0 µL/min; dry gas: 6 L/min at 300°C; nebulizer gas pressure: 6 psi	10 % MeOH v/v + 5 % formic acid v/v; capillary, 75 µm x 120 cm; 28 kV, 20°C, pressure assistance: 40 mbar	Choline, dimethylglycine	Protein precipitation with ACN, evaporation to dryness under vacuum, redisolution in water.	Simultaneous quantification of Choline, Betaine, and Dimethylglycine in human plasma	0.62 µM	[40]
3,4-Dihydroxy-phenylalanine	EKC-(IT)MS^2 ESI+ (-4.0 kV); sheath liquid: MeOH:water (50:50 v/v) containing 0.1% formic acid at 3.3 µL/min; dry gas: 5 L/min at 200°C; nebulizer gas pressure: 3 psi	180 mM M-β-CD + 40 mM HP-β-CD + 2 M formic acid (pH 1.2); capillary, 50 µm x 120 cm; 30 kV, 15 °C	Phenyalanine, Tyrosine, Dopamine, Norepinephrine, and Epinephrine	Precipitation of proteins with ACN (plasma/ACN, 1:2), centrifugation, dilution of the supernatant with formic acid, sonication and filtration.	Simultaneous enantioseparation of all the chiral constituents of the Phenylalanine-Tyrosine metabolic pathway	L-3,4-Dihydroxy-phenylalanine : 54 nM	[41]

α-CD, α-cyclodextrin; ACN, acetonitrile; BIS-TRIS propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; CFSE, 5-carboxyfluorescein succinimidyl ester; CHES, 2-(N-cyclohexylamino)ethane sulfonic acid; CTAB, hexadecyltrimethylammonium bromide; CZE, capillary zone electrophoresis; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, tetrasodium salt of ethylenediaminetetraacetic acid; EMMA, electrophoretically mediated microanalysis; FITC, fluorescein isothiocyanate; HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IAA, iodoacetic acid; IPA, 2-propanol; (IT)MS, ion trap; LEDIF, light emitting diode induced fluorescence; LIF, laser-induced fluorescence; M-β-CD, methyl-β-cyclodextrin; MEKC, micellar electrokinetic chromatography; MeOH, methanol; MS^2, tandem mass spectrometry; NBD-F, 4-Fluoro-7-nitro-2,1,3-benzoxadiazole; NDA, 2,3-naphthalenedicarboxaldehyde; NEM, N-ethylmaleimide; ODS, octadecyl silica; OPA, o-phthalaldehyde; PBS, phosphate buffered saline; PGE-600, polyethylene glycol 600; PVP, polyvinylpyrrolidone; (QqQ)MS^2, triple quadrupole mass spectrometry; SBEC, sulfobutylether-β-cyclodextrin; SDED, serial dual-electrode detection; SDMA, symmetric dimethyl-L-arginine; SDS, sodium dodecyl sulfate; TCDI, 1,1-thiocarbonyldiimidazole; TDLFP, transverse diffusion of laminar flow profiles; TEA, triethanolamine; TMPAB-o-M, 1,3,5,7-tetramethyl-8-phenyl-(2-maleimide)-difluoroboradiaza-s-indacene; (TOF)MS, time-of-flight mass spectrometry.

*Capillary dimensions expressed as internal diameter x effective length (cm to the detector).

**LODs units expressed as in the original work.

The MEKC-UV method based on the use of Dns-Cl as labeling reagent and 20 mM sodium tetraborate (pH 8.7) containing 0.1 M SDS and 6 % methanol as BGE allowed the simultaneous separation of 18 protein amino acids, hydroxyproline and hydroxylysine, and their by-products formed during derivatization. Also, by using principal component analysis (PCA), hydroxyproline and hydroxylysine were selected as markers to discriminate between the authentic plastron and the adulterated one since these amino acids were not present in the other low-priced materials as it can be seen in **Figure 1** [21].

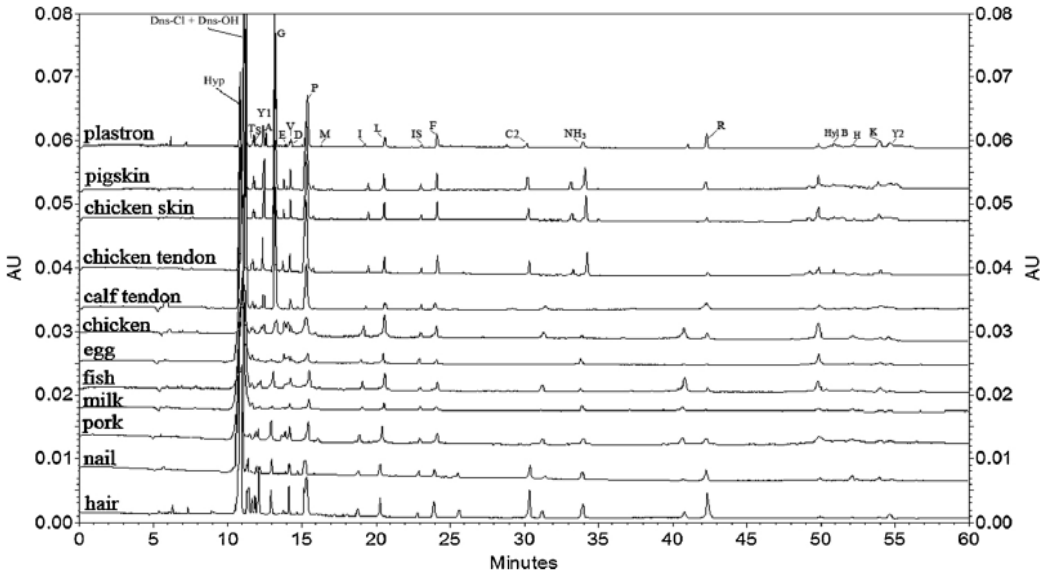

Figure 1. Comparison of electropherograms of AAs profile of plastron with other amino acid-containing materials. Experimental conditions: BGE, 20 mM borate and phosphate containing 0.1 M SDS and 6 % methanol (pH 8.74); voltage, 25 kV; temperature, 25 ºC; injection, 9 kV for 9 s at 25 ºC; UV detection at 214 nm. Reprinted from [21], copyright (2017) with permission from Elsevier.

Moreover, the determination of hydroxyproline along with the 20 protein amino acids and cysteine in passion fruit juices was also performed by Passos et al. [20]. In this case, they proposed a MEKC approach with UV detection, using a 60 mM sodium tetraborate buffer (pH 10.1) containing 30 mM SDS and 5 % methanol, and FMOC as labeling reagent. The combination of the electrophoretic approach with PCA made possible the characterization of different kinds of juices which can be used as a tool to detect adulterations on industrial juice samples.

Other group of NPAAs analyzed by CE in the last years are betaines. Betaines are a group of amino acids derivatives whose structure presents a quaternary ammonium group (positively charged) and a carboxylic group. These

compounds present osmoregulating properties in many plants to protect them from the environmental stress [43]. L-carnitine and its main ester, acetyl-L-carnitine were the betaines analyzed in the period covered by this review. They are found in different mammalian tissues, plants and microorganisms and they play a key role in fatty acid metabolism. Carnitine is produced in low levels in humans, so it may be supplied from diet [23]. Therefore, the development of analytical strategies capable of determine the content of these compounds in foods is required. Kong et al. [23] developed a new CZE method with indirect UV detection using 3.0 mM melamine and 10 % MeOH (pH 2.1) as BGE to quantify both L-carnitine and acetyl-L-carnitine in milk samples. An orthogonal experimental design (5^3) was employed to optimize the BGE pH and composition (melamine concentration and percentage of methanol). The LODs achieved for carnitine and acetyl-L-carnitine were 3.0 and 5.0 μM, respectively. As **Figure 2** shows, the methodology was applied to the analysis of milk using the indirect UV detection since under normal CZE conditions with direct UV detection, carnitine cannot be detected. Thus, 14 kinds of milks were analyzed showing carnitine contents from 43.6 to 121.5 μM and acetyl-L-carnitine contents from 17.5 to 68.5 μM.

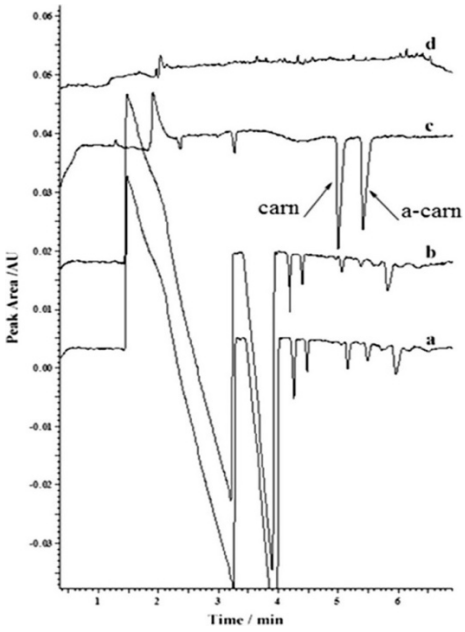

Figure 2. Electropherograms of milk sample and standard solution with established CZE indirect UV method. Sample information: **A-B)** real milk samples, **C)** standard mixture of carnitine (carn) and acetyl-carnitine (a-carn) (0.5 mmol/L), **D)** CZE with direct UV detection for sample (B) spiked with carnitine and acetyl-carnitine (4.0 mmol/L). Experimental conditions: pH 2.1, 3.0 mmol/L of melamine solution (in 10 % methanol), voltage 10 kV. Reprinted from [23], copyright (2017) with permission from Elsevier.

In the years covered by this review, the determination of GABA and citrulline by CE in food samples was reported. Besides being an important neurotransmitter in mammalians, GABA has also demonstrated to present other physiological functions as regulator of cells, hormones and blood pressure, among others [44]. Citrulline, a precursor of arginine, participates in urea and NO cycles [45]. As it can be seen in **Table 1**, a MEKC methodology with LIF detection was developed by Qingfu et al. [9] using a flow-gated CE coupled with alternate injections (electrokinetic injection -5kV for 0.3 s) in a micro-fabricated switch to perform simultaneously the determination of 17 protein amino acids, GABA, citrulline, phosphoryl ethanolamine (PEA) and ethanolamine (ETA) in beers. Using NDA as labeling reagent and 40 mM sodium tetraborate containing 60 mM SDS and 2 mM HP-β-CD as running buffer (pH 9.2) allowed a high separation efficiency for all these compounds within 90 s using a capillary length of 10 cm. The LODs obtained for amino acids, PEA and ETA with the proposed methodology were from 2.0 to 5.0 nM. The quantitative results obtained in eight different brands of beer showed that GABA, alanine and valine were the most abundant in all samples whereas citrulline, glutamine and methionine were the less abundant (indeed, the content of citrulline was lower than 40 μM in all beer brands analyzed). These differences in amino acids composition were in agreement with the differences among the characteristics of the samples (i.e. differences in flavor, raw materials, processing or enzyme activity) [9].

Sacristán et al. [24] developed a CZE methodology to analyze homoarginine and β-N-Oxalyl-L-α,β-diaminopropionic acid, that are the main NPAAs in grass pea seeds (*Lathyrus* species). *Lathyrus* species are a rich source of proteins and are cultivated for human consumption. However, a high consumption of these species may produce a disease known as "lathyrism" responsible for humans and animal's paralysis. The scientific committee of the Spanish Agency for Food safety and Nutrition recommends an occasional consumption of *Lathyrus* being the safe consumption lower than 1.5 mg/g for humans, but further research needs to be performed to ensure these safety values [46]. The developed CZE methodology employed a BGE (pH 9.2) containing 25 mM sodium borate and 5 mM sodium sulfate, and UV detection, and enabled the simultaneous determination and quantification of homoarginine and β-N-Oxalyl-L-α,β-diaminopropionic acid in *L. sativus* (grass pea) and *L.cicera* (red pea). Sample preparation was carried out by two different extraction protocols based on the use of a rotating shaker (24 h) and an Ultra-Turrax (1 min) with ethanol:water (60:40 v/v) as extraction solvent. Despite of the fact that no significant differences were found between the two extraction

protocols, the Ultra-Turrax method, which is simpler and faster, and demonstrated to provide higher yield results than the rotating shaking method, was selected to analyze all samples. Different *Lathyrus cicero* and *Lathyrus sativus* species were analyzed to evaluate the levels of homoarginine and β-N-Oxalyl-L-α,β-diaminopropionic acid showing that homoarginine contents (from 8.08 mg/g to 12.44 mg/g) were higher than the contents of β-N-Oxalyl-L-α,β-diaminopropionic acid (from 0.79 to 5.05 mg/g) in all samples. Moreover, the results obtained showed that β-N-Oxalyl-L-α,β-diaminopropionic acid levels for *L.cicera* species were lower than the recommended ones but this was not the case for *L.sativus* species, whose values exceeded those recommended [24].

Taurine is the only sulfur-containing amino acid analyzed by CE in food in the reviewed period. This NPAA can be found in mammalian tissues in high concentration levels and it presents important physiological and therapeutic functions such as bile acid conjugation, maintenance of calcium homeostasis, [47], liver protection, and treatment of low blood pressure [48]. Taurine is the most employed component in the formulation of energy and sport drinks that have gained popularity among athletes as a consequence of their energetic properties. Nevertheless, attention should be paid to the consumption of these products (especially for patients with heart disease or hypertension) since high levels of taurine intake may produce undesirable effects even in healthy people [49]. As it can be seen in **Table 1**, taurine was determined using different modes of CE such as CZE, MEKC and MCE. A CZE method with C^4D was developed for the determination of taurine in energy drinks. The results obtained with an instrument with a coaxial flow-gating interface (FGI) were compared with those obtained with an Agilent commercial equipment showing similar LODs (14.4 mg/mL and 8.2 mg/mL, respectively). Both methods were able to determine lower percentages of taurine than the declared value (4000 mg/L). The coaxial FGI presents some characteristics comparable with common commercial CE instrument such as repeatable sample injection and improved total analysis time (73 s and 225 s, respectively). As **Table 1** shows, the separation was achieved using 20 mM CHES and 10 mM NaOH (pH 9.5) as separation buffer and only a 40-fold dilution step of samples was needed to analyze them by CE [26]. Wu et al. [27] developed a methodology using MCE with LIF detection to determine taurine, lysine, and vitamin B_3 in functional drinks. The use of field-amplified sample stacking (FASS) combined with reverse-field stacking as on-line preconcentration strategy allowed improving the sensitivity and the separation efficiency in comparison with conventional MCE-LIF method. After optimizing different electrophoretic and

derivatization variables, the use of 100 mM sodium borate (pH 9.88) as running buffer and sulfoindocyanine succinimidyl ester (Cy5) as derivatization reagent enabled the quantification of lysine, taurine and vitamin B_3 (within 4 min) in eight functional drinks showing a lower concentration of taurine in one of them than the values declared in the label.

Pyroglutamic acid is an interesting cyclical NPAA that may be produced in protein biosynthesis or as an intermediate in metabolic and transport pathways [50] and it can be found as a free acid or bound at the N terminal group of proteins and peptides [51]. This NPAA is usually found in urine, plasma, bones and other tissues, and it can also be present naturally in food or can be employed in beauty or dietary formulations [52]. The only article published in the reviewed period reporting the separation of pyroglutamic acid by CE was aimed to determine lactic acid and its organic impurities in fermented products. Among these impurities, taurine and pyroglutamic acid were the NPPAs identified [25]. The MEKC methodology developed consisted of using a 25 mM sodium tetraborate buffer containing 50 mM SDS (pH 9.1) and UV detection (200 nm). Sample treatment including an enzyme-assisted extraction procedure and a fermentation process was accomplished. Thus, ten organic acids, thirteen protein amino acids, cysteine, tryptamine, taurine and pyroglutamic acid were identified and separated from lactic acid in fermentation broth of different renewable resources. It was observed a major unknown component before the lactic acid peak in some samples and it was identified as pyroglutamic acid using MS spectra followed by the standard confirmation. The methodology enabled to detect 0.3 ppm of pyroglutamic acid in presence of 718,400 ppm of lactic acid [25].

The NPAA β-N-methylamino-L-alanine is a toxin present in nature which is related to many neurodegenerative pathologies such as the amyotrophic lateral sclerosis, Alzheimer's dementia, or Parkinson's disease [53]. This NPAA presents some relevant structural isomers. Three of them, namely 2,4-diaminobutyric acid (2,4-DAB), N-2(aminoethyl)glycine (AEG) and β-amino-N-methyl-alanine have been found in food matrices (e.g. microalgae and mollusks). The major exposure pathway to β-N-methylamino-L-alanine is the dietary intake so the development of high selective methods able to separate the isomers of this NPAA is crucial [54]. However, β-N-methylamino-L-alanine analysis may be a hard task since all its isomers have the same monoisotopic mass and similar physicochemical properties making difficult their discrimination. Recently, two different methodologies based on the use of CZE with UV and MS detection were developed by Kerrin et al. [28] to carry out the separation of β-N-methylamino-L-alanine and four of its isomers in

a mussel tissue reference material. A simple sample treatment based on protein hydrolysis in acid conditions followed by Oasis-MCX cartridge cleanup procedure without any derivatization step was employed. To develop the CZE-UV methodology, the effect of different separation variables, such as the running buffer composition, buffer concentration, organic modifiers and pH, and instrumental parameters, such as temperature and voltage, were evaluated. Under the optimized conditions (see **Table 1**) β-N-methylamino-L-alanine and its isomers could be separated. However, the LOD (20 mg/g, dry mass) obtained for β-N-methylamino-L-alanine using this method was much higher than the reported content in cyanobacteria and mussels (300 μg/g and 10 μg/g, respectively). Afterwards, in order to improve the sensitivity, these authors developed a new methodology by CZE-MS. First, to select compatible CE-MS conditions, the phosphate BGE was replaced by 5 M formic acid containing 10 % acetonitrile and a custom interface was built with a straight tube enclosing the CE capillary which eliminated the plugging problems previously obtained. A 50 % aqueous MeOH containing 0.1 % formic acid was used as sheath liquid. Before the analysis by CZE-MS, a strong cation exchange solid-phase extraction (SPE) sample cleanup procedure to lower the conductivity of the extract enabling FASS was achieved with a final step of redissolution in a low conductivity solvent. This approach allowed to achieve a LOD of 16 ng/g (dry mass) for β-N-methylamino-L-alanine enabling the quantification of this NPAA in real samples (cycad leaves, lobster tail meat and lobster tomalley) [28].

Domoic acid is other neurotoxic water soluble tricarboxylic acid which is present in numerous types of shellfish and seafoods usually consumed as part of the human diet. The consumption of high levels of this NPAA may be responsible for Amnesic Shellfish Poisoning (ASP), a disease whose symptoms are cardiac arrhythmias, abdominal cramps and neurological dysfunction, among others. Therefore, analytical methods are needed to asses a safe content of this compound in food [55]. A CEC method, based on the use of a packed capillary column with octadecyl silica (ODS) particles (using a supplementary pressure) and LIF detection, was employed to analyze traces of domoic acid in shellfish samples. As it can be seen in **Table 1**, this is the only work in which CEC was applied to the analysis of NPAAs in the period of time reviewed in this article. A solid-liquid extraction followed by a clean-up procedure and a derivatization step with NBD-F was achieved before CEC analysis that was carried out in positive and negative voltage using 5 mM phosphate buffer containing 60 % acetonitrile (pH 2.5), enabling a LOD for domoic acid as low as 10 ng/mL [15]. The developed methodology was compared with a HPLC-MS/MS method showing similar LODs and RSD results,

and better recoveries in the case of CEC. MCE was also employed to determine domoic acid in shellfish tissues. The use of a 5 mM sodium tetraborate buffer (pH 9.2), FITC as derivatizing reagent, and LIF detection, allowed the determination of domoic acid within 60 s with a LOD of 2.8 x 10^{-10} M which enabled to assess the accomplishment of the official regulatory limit of 20 μg domoic acid /g wet tissue. The method constitutes a potent alternative to carry out the detection of this toxin since it presents some advantages such as simplicity, sensitivity and high separation speed [19].

Chiral analysis of NPAAs in food is of high interest to guarantee food quality, authenticity and safety. Even though the L-enantiomer is the natural form, D-enantiomers of NPAAs can be found in food due to a racemization during food processing, a microbiological processes, or by the fraudulent addition of racemic mixtures in the particular case of supplemented foodstuffs [56], for which regulations establish the use of the L-enantiomer. As a consequence of the different properties and biological activity that the enantiomers may have, their individual determination in foods present a high interest. In fact, the enantioselective determination of NPAAs has demonstrated to be relevant to detect food adulterations [42] or to evaluate manufacturing processes [57]. During the period of time covered by this review, only one work has been focused on the enantiomeric separation of NPAAs by CE in food [29]. New analytical methodologies were developed reaching the enantiomeric separation of eight NPAAs by EKC. After FMOC derivatization, the optimized separation conditions consisted of the use of a 100 mM formate buffer (pH 2.0) and an anionic cyclodextrin (sulfated-α-CD or sulfated-γ-CD depending on the amino acid). **Figure 3** shows the electropherograms corresponding to the enantiomeric separation of the NPAAs investigated under the optimized conditions. The figures of merit of the developed method were shown to be adequate for determining L-citrulline and its enantiomeric impurity in food supplements. LODs of 2.1 x 10^{-7} M and 1.8 x 10^{-7} M were achieved for D- and L-citrulline, respectively. L-citrulline was quantified in six samples (three new and three submitted to a long storage time) where D-citrulline was not detected in any case showing that storage time did not originate racemization.

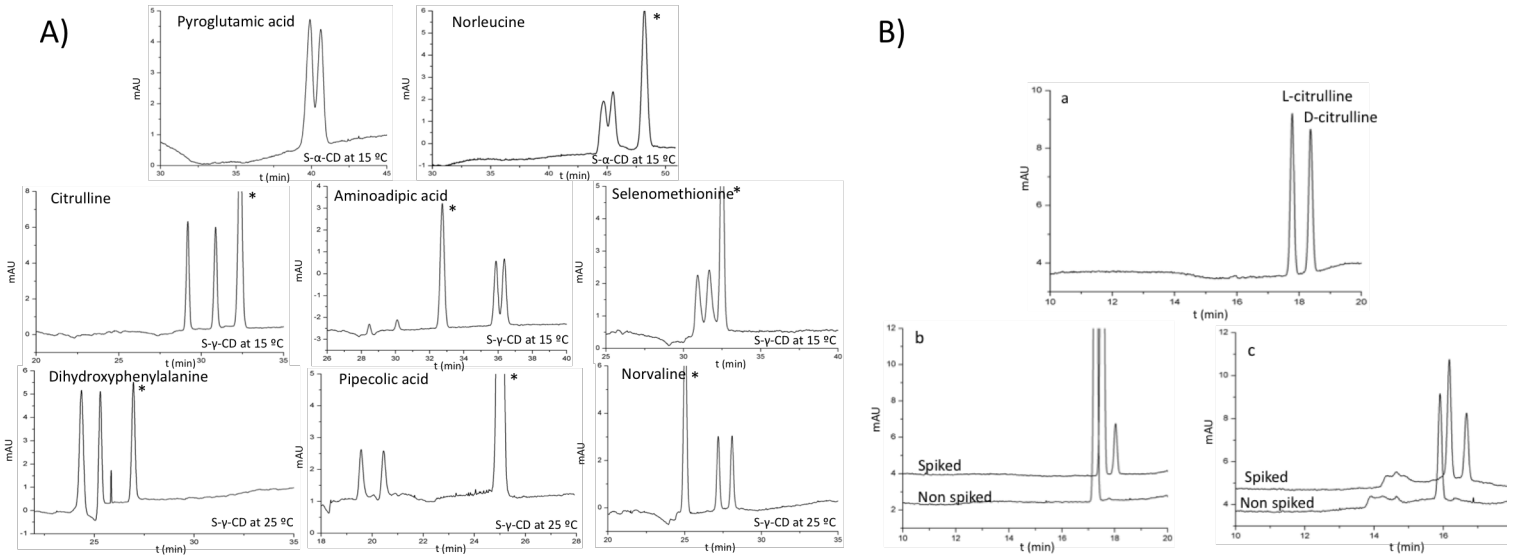

Figure 3. A) Electropherograms corresponding to the enantiomeric separation of different FMOC-NPAAs (0.2 mM) obtained under the best separation conditions. Experimental conditions: BGE, 10 mM of the corresponding CD in 100 mM formate buffer (pH 2.0); uncoated fused-silica capillary, 58.5 cm (50 cm to the detector window) x 50 µm ID; UV detection at 210 nm (except for Pyro which was detected at 200 nm); voltage, -20 kV; injection by pressure in the cathodic end, 50 mbar for 4 s. * Indicates the derivatizing reagent (FMOC). **B)** Electropherogram obtained for (a) DL-FMOC-citrulline standard (0.025 mM) and two food supplements at 0.2 mM L-Citrulline (b) FS1, spiked with D-citrulline and non spiked and (c) FS6, spiked with D-citrulline and non spiked. Experimental conditions: BGE, 10 mM sulfated γ-CD in 100 mM formate buffer (pH 3.0); uncoated fused-silica capillary, 48.5 cm (40 cm to the detector window) x 50 µm ID; UV detection at 210 nm, applied voltage, -20 kV; temperature, 25 ºC; injection by pressure in the cathodic end, 50 mbar for 15 s. Reprinted from [29], copyright (2016) with permission from Elsevier.

3. Determination of non-protein amino acids in biological samples by CE

From a biological point of view, the determination of NPAAs has a special relevance since many of them are key compounds in metabolic pathways or are related with different pathologies. In fact, several diseases related with metabolic dysfunctions originate abnormal quantities of amino acids in body fluids. Thus, the determination of NPAAs in different biological fluids can be used for the early detection of different cancer types [58, 34], as diagnostic tool to inspect vesicoureteral reflux samples [31], to detect an immature enzymatic system in preterm neonates [32], as indicator of ocular surface diseases [59], to assess the embryo viability in assisted reproduction [11, 10], as indicator of pathologies such as coronary artery disease, diabetes renal insufficiency, or Alzheimer´s disease [60], or even for clinical toxicology laboratory diagnostics [39]. These examples show the relevance of the determination of NPAAs in biological fluids and the imperative need to develop high sensitive methodologies able to detect these compounds at the low levels at which they are present in biological samples. **Table 2** summarizes the characteristics of the CE methodologies developed for the analysis of NPAAs in the period covered by this article. As it can be observed in this table, the preferred detection mode was LIF followed by MS^2 and UV. The CE approaches developed have been employed to analyze a broad range of samples: urine, plasma, serum, tear fluid, saliva, human embryos, or human colon cancer and breast cells, among others (see **Table 2**).

Some of the developed CE methodologies have been applied to the simultaneous analysis of different NPAAs, being CE coupled to LIF the approach mainly used in this kind of analysis [30, 18, 14]. For instance, Liang *et al.*, developed a MEKC-LIF methodology to achieve the simultaneous determination of homocysteine, homoarginine and five related metabolites (including ornithine and citrulline) after derivatization with 5-carboxyfluorescein succinimidyl ester (CFSE) [30]. A baseline separation was possible in 10 min using as BGE a 50 mM borate buffer at pH 9.5 containing 30 mM SDS and 30 % MeOH. LODs reached were between 0.12 and 1.70 nM which are much lower than other reported in previous works for some of the analyzed compounds by fluorescence (sensitivity was improved from 5 to 600 fold times). This methodology was fully validated using plasma and urine samples from type 2 diabetics with peptic ulcer bleeding. Also, an interesting CE-LIF method for the high-speed monitoring of branched chain amino acids uptake in 3T3-L1 cells was developed by Harstad and Bowser [18]. The interest in the measurement of these amino acids and their downstream metabolites

(where GABA, ornithine, citrulline and taurine can be included) is related to the fact that they play relevant roles in the tricarboxylic acid cycle and adipocyte lipogenesis. To carry out the analysis, analytes were sampled using microdialysis, on-line derivatized with a fluorescent reagent, separated by CE and detected by LIF using the device shown in **Figure 4A**. Under optimal conditions, the separation was obtained in less than 30 s (see **Figures 4B** and **4C)**. Other CE-LIF methodology developed within the time covered in this review has been proposed to achieve the simultaneous analysis of different NPAAs (ornithine, citrulline, norvaline and norleucine) along with 17 protein amino acids in plasma [14]. This was a polymer-based separation method in the presence of mixed micelles and the analytes were derivatized with naphthalene-2,3-dicarboxaldehyde (NDA). In spite of the fact that the analysis time was too high (180 min), the high level of resolution obtained using this methodology allowed the accurate quantitation of amino acids in plasma without the need for protein filtration. Taking in mind that the presence of free amino acids in plasma could be used for the early detection of different cancer types [59], this methodology will be useful for clinical diagnosis employing amino acids as biomarkers.

The simultaneous analysis of several NPAAs not only was performed by CE with LIF detection but also using the hyphenation of CE with MS. Thus, the targeted analysis of amino acids in urine, including 20 protein amino acids, β-alanine, the dipeptide carnosine and 5 NPAAs (GABA, ornithine, citrulline, hydroxyproline and alloisoleucine) was carried out by CE-MS^2 [31]. After optimizing the parameters related to BGE, CE-MS interface, and MS detection, the method, based on the use of 0.8 M formic acid at pH 1.96 containing 15 % MeOH and a pH stacking procedure to increase the sensitivity (a plug of 12.5 % ammonium solution was injected before the sample), enabled the separation of the 27 analyzed compounds in less than 30 min with LODs ranging from 0.63 to 29 μM. Once the method was validated according to FDA and ICH guidelines, its feasibility was demonstrated by analyzing urine samples from children with vesicoureteral reflux which proved that the CE-MS^2 method could be considered as a possible auxiliary diagnostic tool to inspect vesicoureteral reflux samples.

Even though it is possible to find in the literature different works in which CE was used for the simultaneous analysis of several NPAAs (as it has been described till now), the truth is that in most of the cases, CE analysis was focused on the determination of one or two NPAAs, normally along with other protein amino acids.

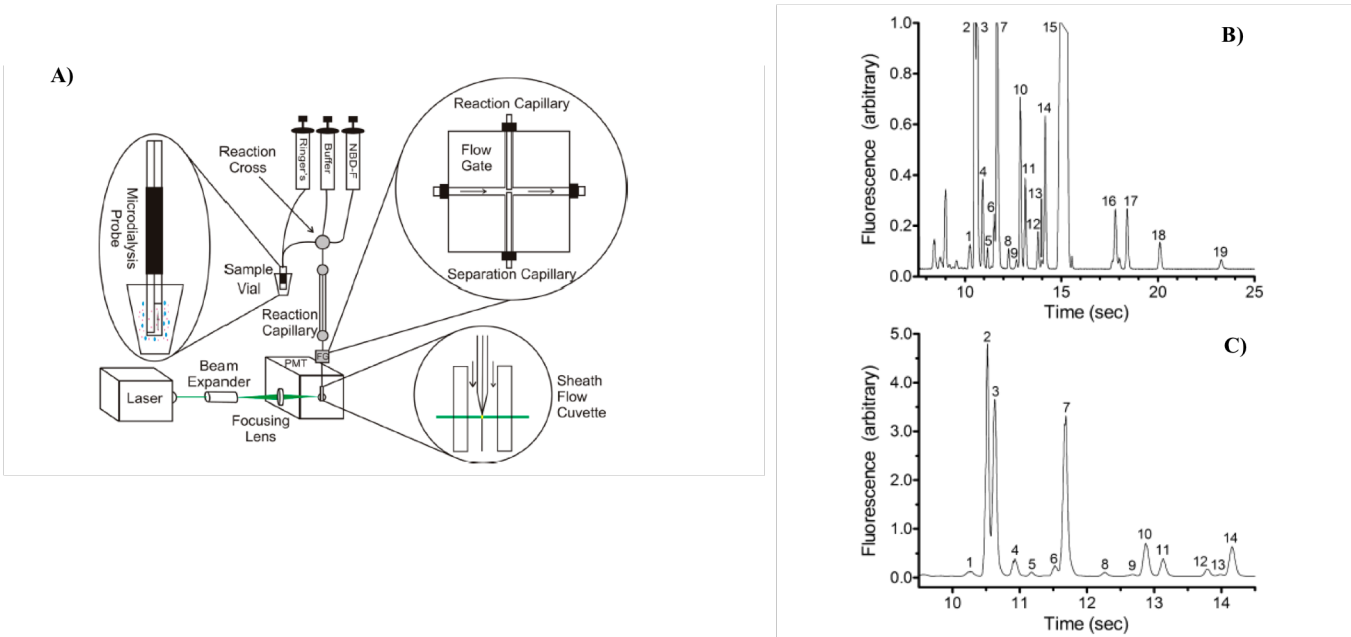

Figure 4. (a) Schematic on the online MD-CE system. (b) Full electropherogram and (c) expanded one from an online analysis of CE analysis of 3T3-L1 cells super- natant after 30 min of incubation with Ringersolution, glucose, isoleucine, leucine and valine. Experimental conditions: BGE, 90 mM borate containing 35 mM a-CD (pH 9.8); capillary, 50 mm 30 cm; voltage, 21 kV. Peaks: (1) lysine, (2) isoleucine, (3) leucine, (4) ornithine, (5) methionine, (6) phenylalanine, (7) valine, (8) ornithine, (9) GABA, (10) glutamine, (11) alanine, (12) threonine, (13) b-alanine, (14) glycine, (15) NBDOH, (16) taurine, (17) internal standard, (18) glutamate and (19) aspar- tate. Reprinted from Ref. [18] copyright (2016) with permission from ACS publications.

In this line, citrulline and arginine levels were determined by CE-LIF in blood samples from preterm newborns and mature neonates [32]. Based on the levels measured under the best separation conditions (see **Table 2**), it was possible to differentiate both groups of samples since significantly lower levels of both NPAAs were found in preterm neonates which implies an immature enzymatic system in these neonates [32]. Citrulline has also been used along with taurine and other four neuroactive amines commonly found in brain dialysate samples as model compounds to design a portable microchip electrophoresis (MCE) with LIF detection [12]. The LODs achieved ranged from 250 nM to 1.3 μM (being 0.36 and 0.42 μM for citrulline and taurine, respectively) and were adequate for the detection of the investigated analytes at physiologically relevant concentrations.

The relation of taurine levels in biological fluids with different diseases has been pointed out by different works. For instance, it was found that such levels can be related with the ocular surface disease so that it can be used as a useful indicator of this pathology [59]. For this reason, Du *et al.*, optimized a CE method with indirect amperometric detection to carry out the determination of taurine in tear fluid [33]. The methodology was based on the use of a 20 mM phosphate buffer containing 20 mM SDS (pH 10.0) as BGE and a serial dual-electrode to conduct the detection. In this indirect detection mode, bromide is oxidized to bromine which reacts quantitatively and rapidly with taurine, so that the decrease in the bromine current can provide taurine concentration. Once optimized the parameters affecting the analytical performance (bromine concentration, dual-electrode potentials, and CE separation conditions), taurine was baseline separated from other interfering amino acids within 18 min. The LOD obtained for taurine by the developed methodology (0.18 μM) was compared with those obtained by other detection modes such as pulse and direct amperometric detection or LIF, being the LOD obtained by the indirect method lower than or similar to the LOD obtained by both amperometric methods and higher than the LOD obtained by LIF detection (but it requires a derivatization step which makes the process more labor-intense) [33]. The successful application of this methodology for determining taurine in tear fluids shows the potential of this device to be applied in clinical analysis. An interesting work recently published proposes an on-line microdialysis (MD)-CE method with LIF detection to measure the *in-vivo* dynamics of amino acids (taurine, GABA, and 10 proteinogenic amino acids) biomarkers of metabolism in adipose tissue [17]. This method was applied to the monitoring of amino acids dynamics in mice adipose tissue in near real time (22 s). The LOD obtained for taurine (which represents taurine concentration out of the probe and before its derivatization with 4-fluoro-7-

nitrobenzofurazan (NBD-F)) was 2.7 μM. To demonstrate the potential of the developed strategy, *in vivo* changes were assessed after administering an insulin stimulation. In this way, it could be observed that taurine, alanine and valine levels raised within the first 5 min after insulin delivering and reaching a second baseline corresponding to higher amounts of amino acids than those present initially.

In addition to the two afore-mentioned works, in which CE strategies were employed to measure the level of taurine in biological fluids, there are other three articles in which taurine, among other compounds, is analyzed by CE. On the one hand, the use of a CE-MS platform for the metabolomics analysis of saliva samples from patients with oral squamous cell carcinoma and healthy controls enabled to propose taurine along with other 24 metabolites as oral cancer specific markers [34], and on the other hand, MEKC-LIF was employed to obtain the amino acids profiles in the culture media used in embryo cultivation after *in vitro* fertilization in order to assess the embryo viability in assisted reproduction [10, 11]. In a first attempt, Celá *et al.*, used the transverse diffusion of laminar flow profiles (TDLFP) methodology to achieve the on-line derivatization of amino acids with NDA [11]. Using a BGE composed of 35 mM borate, 55 mM SDS, 2.7 M urea, 1 mM BIS-TRIS propane and 23 mM NaOH, the derivatives of 18 protein amino acids, taurine, cysteine and the dipeptide Ala-Gln were baseline resolved in 50 min. However, due to the limitations of this methodology, the method was subsequently modified to provide better separation conditions in terms of analysis time [10]. First, the BGE was changed to avoid urea and to decrease the pH of 9.8 since both effects contribute to the dissolution of carbon dioxide giving rise to a modification of the BGE ionic strength that originated increased values for migration times due to the alteration of the micelle-analyte distribution equilibrium. The optimum BGE, based on the use of 73 mM SDS, 6.7 % 1-propanol (v/v) + 0.5 mM HP-β-CD + 135 mM boric acid/NaOH (pH 9.0), enabled the baseline resolution of the analytes in 46 min. Regarding the on-line derivatization, it was accomplished using electrophoretically mediated microanalysis (EMMA) which improved the LODs (a LOD of 12 nM was reached for taurine). Thus, the improved MEKC-LIF methodology was applied to the non-invasive analysis of human embryos in order to establish if there was a correlation between the potential of embryos to be developed and the variation in amino acids levels. Statistical analysis of the data showed that the discrimination between successfully and unsuccessfully implanted embryos was partial probably because of the small number of statistically significant samples [10].

Homocysteine is a low molecular weight aminothiol of high relevance in biological processes since higher levels in plasma or serum have been related with

different pathologies such as coronary artery disease, diabetes renal insufficiency, or Alzheimer´s disease, among others [60]. Along with homocysteine, it is also relevant to take into consideration the proteinogenic amino acid cysteine because the ratio cysteine/homocysteine reflects the bioavailability of homocysteine [61]. Ivanov *et al.*, established a CE-UV approach based on the use of 1,1'-thiocarbonyldiimidazole (TCDI) as derivatizing reagent for determining the levels of homocysteine and cysteine in plasma [35]. In this work, an electrokinetic injection with pH mediated stacking was employed, and a LOD of 0.8 μM for homocysteine was achieved. Subsequently, the authors improved the methodology introducing several modifications which enabled to achieve a LOD of 0.2 μM [36]. These modifications included the use of a liquid-liquid extraction with chloroform-ACN to purify the sample and determine homocysteine and cysteine levels in urine (the previous approach was not suitable for determining both analytes in matrices like urine in which salt levels vary considerable), a different composition of the running buffer (see **Table 2**), and an in-capillary preconcentration strategy based on the combination of field amplified sample stacking and pH mediated stacking. In this way, homocysteine and cysteine levels were determined in plasma and urine samples from healthy subjects and patients with kidney disorders (see **Figure 5**), observing a decrease in the homocysteine levels in urine from patients with kidney disorders [36]. Other detection modes different from UV were also hyphenated with CE to perform the determination of homocysteine in biological samples. Thus, LIF was used as detection mode in a high-sensitive CE method developed for chemical cytometry of homocysteine and other thiol compounds (cysteine, glutathione, and γ-glutamylcysteine) within human colon cancer (HCT-29) and breast (MCF-10A) single cells [37]. Here, 1,3,5,7-tetramethyl-8-phenyl-(2-maleimide)-difluoroboradiaza-s-indacene (TMPAB-o-M) was employed as labeling reagent in a post-column sheath flow cuvette.

Conversely, MS detection has demonstrated to be a powerful technique to carry out the analysis of homocysteine, cysteine, methionine, and glutamic acid in plasma by CE [38]. Since the levels of these compounds are altered in plasma of amytrophic lateral sclerosis (ALS) patients, their determination is relevant because they could be pointed out as potential biomarkers of this disease. Prior to analyze these compounds by CE-MS^2, the protein depletion of plasma samples was performed using DTT and cold acetone, and IAA was added to the sample to avoid the oxidation of thiol groups. After validating the CE-MS^2 methodology, it was employed to analyze plasma samples from healthy subjects and patients with ALS,

showing a significantly higher concentration of glutamic acid and cysteine in the latter group.

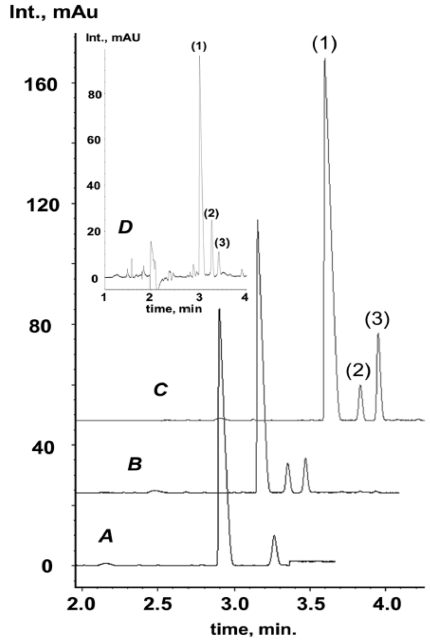

Figure 5. Electropherograms corresponding to the analysis of homocysteine and cysteine in blood plasma and urine by CE-UV. **A)** Plasma without additions, **B)** spiked with internal standard (penicillamine), **C)** spiked with internal standard, homocysteine and cysteine, **D)** urine. Experimental conditions: BGE, 0.1 M phosphate containing 30 mM TEA, 25 M CTAB, 2.5 M SDS and 2.5 % PGE-600 (pH 2); capillary, 50 µm x 23.5 cm; voltage, -17 kV; temperature, 30 ºC; injection: 2250 mbar*s. Peaks: (1) cysteine, (2) penicillamine, (3) homocysteine. Reprinted from [36] copyright (2017) with permission from Wiley-VCH.

The analysis of other NPAAs such as GABA, hydroxyproline, pyroglutamic acid and betaine by CE was also performed. To analyze the first one, Wang *et al.*, developed a MEKC-LIF method for measuring amino acid secretion from islets of Langerhans, an endocrine portion of pancreas responsible for helping to maintain glucose homeostasis, since they seem to play a key role in cell functionality [13]. Under the optimized conditions for NDA derivatization and CZE separation (25 mM phosphate buffer containing 30 mM SDS at pH 8.3), GABA and other 17 proteinogenic amino acids were analyzed in 21 min allowing the quantitation of 14 of them with LODs ranging from 0.2 to 7 nM (namely the LOD obtained for GABA was 3 nM). The developed methodology permitted to quantify the amounts of amino acids secreted from islets incubated in low or high glucose. The effect of glucose and 2,4-dinitrophenol (a pharmacological agent) in these secretions was tested observing that glucose exerted a suppression effect on GABA release

suggesting the possible ATP inactivation of glutamate decarboxylase [13]. 4-hydroxyproline and prolyl hydroxyproline in urine were proposed as biomarkers for osteoporosis and bone turnover, reason for why Zhang et al., proposed a flow-gated CE-LIF method to carry out the rapid determination of 4-hydroxyproline and prolyl dipeptides in urine samples. The developed methodology included the treatment of urine samples with OPA, which blocking primary amines, and the derivatization of the secondary amines with 4-fluoro-7-nitro-2,1,3 benzoxadiazole. Then, using a mixture of borate, cholate and deoxycholate at 40 mM each (pH 9.2) as running buffer, proline, 4-hydroxyproline and 4 propyl dipeptides were separated in just 30 s achieving LODs at the nM level [16]. An interesting investigation accomplished by Holzek et al. demonstrated the suitability of CE for clinical toxicology laboratory diagnostic [39]. High anion gap metabolic acidosis habitually complicates paracetamol poisoning and is normally attributed to lactic acidosis, renal failure or compromised hepatic function. But, it can also be due to the accumulation of pyroglutamic acid (or 5-oxoproline). Therefore, 5-oxoprolinemia could be considered to diagnose patients with acidosis after acute paracetamol overdose. Then, to determine paracetamol and pyroglutamic acid levels in serum samples from patients after ingestion of paracetamol, these authors developed a CE-UV method based on a simple sample treatment and the employ of 40 mM CHES/sodium hydroxide at pH 10.2 as BGE. By using this methodology, it was possible to carry out the quantification of pyroglutamic acid in case of paracetamol overdose. Finally, Forteschi *et al.,* designed an isotope dilution CE-MS^2 method to detect, for the first time, betaine, choline and dimethylglycine simultaneously in plasma samples, since they provide relevant information related to methyl groups flow in very relevant biological processes, particularly in folate deficiency stages [40]. Under the conditions detailed in **Table 2**, the compounds were detected in 22 min achieving LODs of 0.43, 0.62, and 0.31 μM for choline, betaine and dimethylglycine, respectively. Based on the concentration of the three analytes measured by the application of the developed CE-MS^2, it was possible to find differences between the plasma samples of healthy controls and patients with chronic kidney disease.

During the period of time covered in this review just a research work described the enantioselective determination of a chiral NPPA in a biological sample. Namely, Sánchez-Lopéz et al., developed a CE-MS^2 methodology for the simultaneous enantioseparation of all the chiral constituents of the phenylalanine-tyrosine metabolic pathway; the protein amino acids phenylalanine and tyrosine, the catecholamines dopamine, norepinephrine and epinephrine, and the NPPA 3,4-

dihydroxyphenylalanine (DOPA)) [41]. The method, consisting of the use of 180 mM methyl-β-cyclodextrin plus 40 mM 2-hydroxypropyl-β-CD in 2 M formic acid (pH 1.2) as BGE and a large volume sample stacking as in-capillary preconcentration step, enabled for the first time, the simultaneous enantiomeric separation of all the chiral compounds involved in this metabolic pathway, in 90 min with LODs from 40 to 150 nM. The usefulness of the developed method was demonstrated through the successful analysis of some of the compounds investigated in rat plasma samples (**Figure 6**).

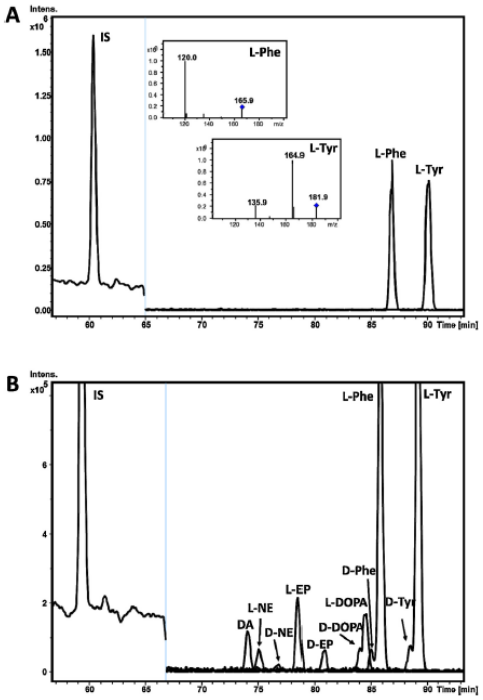

Figure 6. Extracted Ion Electropherograms obtained by CE-MS² method of **A)** a rat plasma sample (the inserts show the MS² spectra of L-Phe and L-Tyr peaks), and **B)** a spiked rat plasma sample. Experimental conditions: BGE, 2 M formic acid containing 180 mM M-β-CD and 40 mM HP-β-CD (pH 1.2); capillary, 50 µm x 120 cm; voltage, 30 kV; temperature, 15 °C; injection, 50 mbar x 250 s. Reprinted from [41] copyright (2016) with permission from Elsevier.

4. Concluding remarks

This article reviews the works dealing with the determination of non-protein amino acids in food and biological samples published from 2015 to 2018. Non-protein amino acids are related with the quality and safety of food and they have also been considered as biomarkers of some pathologies. For this reason, the determination of these compounds in real samples is relevant. During the period of

time covered by this review, the analysis of non-protein amino acids by CE in food and biological samples was mainly achieved using MEKC and CZE modes. The most employed detection systems include direct and indirect UV absorption, LIF, capacitively coupled contactless conductivity, electrochemical detection and mass spectrometry. In general, the absence of chromophore and fluorophore groups in non-protein amino acids required a derivatization step in order to enable their determination or to improve the sensitivity. Thus, many labeling reagents were employed such as NDA, NBD-F, FITC, FMOC-Cl, AQC, DNS-Cl, or OPA. CE and MCE are attractive strategies providing good sensitivity and selectivity to perform the analysis of a broad range of complex food samples such as beverages, vegetables, fermented products or shellfish and biological samples such as urine, plasma, serum, tear fluid, saliva, etc. Moreover, the chiral separation of non-protein amino acids in food and biological samples has also demonstrated to provide relevant information of these samples, but the number of publications in the last years is scarce. In fact, the enantiomeric separation of non-protein amino acids (through the addition of a chiral selector in the background electrolyte in EKC or the use of a chiral stationary phase in CEC) provides interesting information about food quality and safety or about the diagnosis or treatment of some pathologies. Since the function of numerous non-protein amino acids has not been investigated yet and their presence in real samples is still unknown, the interest of the development of analytical methodologies capable to analyze these compounds in real samples is of high interest for scientists.

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Article 3

Application of mass spectrometry-based metabolomics approaches for food safety, quality and traceability.

M. Castro-Puyana, R. Pérez-Míguez, L. Montero, M. Herrero.
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Abstract

The always more-demanding fields of food safety, quality and traceability are continuously fostering the development of robust, efficient, sensitive and cost-effective analytical methodologies. Mass spectrometry-based metabolomics is a key tool nowadays with great potential in many analytical fields and has been demonstrated to be capable of facing some important challenges related to these areas within the food science domain.

The main aim of this review is to present a critical overview of the most recent applications of MS- based metabolomics approaches for food quality, safety and traceability assessment, covering the most relevant works published from 2014 to 2017. Information about the different steps needed to develop a MS-metabolomics approach, i.e. sample treatment, analytical platform, and data processing, is also provided and discussed.

1. Introduction

Metabolomics is one of the main branches in the field of the -omics techniques, and together with genomics, transcriptomics and proteomics, is involved in the study of the food and nutrition domains through Foodomics approaches. As per definition, metabolomics includes the exhaustive study of the whole small metabolite composition of a particular system or organism, understanding by small metabolite typically those with a molecular weight below 1500 Da. In practice, this aim is difficult to achieve, due to the huge chemical variability of metabolites that is often found; this implies that a universal approach to analyze using a single method metabolites belonging to very different chemical classes (significantly different polarity) as well as present in a very wide dynamic range is not attainable. In this regard, the food metabolome is not an exception as quite diverse compounds, such as carbohydrates, lipids, proteins, amino acids, amines, steroids, phenolic compounds, carotenoids, alkaloids or volatile compounds, among others are frequently present. For this reason, the selection of more than one analytical approach, and their combination for results interpretation is often carried out.

The analytical procedures usually employed within metabolomics can be grouped in different categories. On the one hand, methods can be classified under fingerprinting approaches or under profiling methodologies. Fingerprinting is referred to the analysis of as many compounds as possible within a system, including their detection and the subsequent statistical treatment of the obtained results in order to look for sample patterns. Under this approach, the identification

and quantification of the detected metabolites may not be a necessity. In opposition, profiling refers mainly to the analysis of closely related metabolites, often belonging to the same chemical class, which are most frequently identified and quantified. Similarly, metabolomics approaches can be also classified as non-targeted or targeted analysis; whereas non-targeted approaches look for maximum coverage of metabolites that can be simultaneously identified in a particular system, targeted approaches are based on the determination and identification of a certain type of metabolites, that could either belong to the same chemical class or being involved in a particular pathway. In any case, as the complexity of the set of metabolites to be analyzed is quite high in both approaches, suitable analytical techniques are needed, as well as proper sample treatment methodologies. This latter subject is of great relevance in food analysis, as food are usually quite complex matrices full of potentially disturbing components for the analysis of metabolites. Sample treatment may be relatively simple or involve multiple steps. However, it has always to be considered that sample treatment may include unintended bias towards the metabolites present, as a universal sample treatment directed to the extraction of the full metabolome of a particular sample will not exist in practice, and thus, some components may be lost during this phase.

Concerning the analytical tools employed, most attention has been paid to the detection technique. However, it is evident that a proper separation before detection can increase the quality of the obtained results. Although gas chromatography (GC) was perhaps the separation technique of choice in the initial metabolomics studies, the need for derivatization in order to increase the coverage of compounds that can be analyzed following this approach has driven to shift the primary technique to liquid chromatography (LC). In fact, LC can be operated in several separation modes, which increases its versatility towards the separation of a variety of different metabolites. Particularly, in the last years, methods based on the use of ultra-high performance liquid chromatography (UHPLC) have gained considerable popularity thanks to the advantages that this technique can provide with, including high efficiency, good resolution, relatively short analysis times and the use of flow rates fully compatible with mass spectrometry (MS) detection.

Likewise, concerning the detection of the metabolites, nuclear magnetic resonance (NMR) was the most-used technique in the first years of metabolomics development. However, MS has gradually substituted the use of NMR. Some of the reasons behind this move include that MS is by far more suitable for coupling with a separation technique, as well as the development and improved affordability of high resolution MS instruments. In this regard, the use of high resolution

instruments, like time-of-flight (TOF) analyzers, or even hybrid instruments such as quadrupole-TOF (QTOF) or orbitrap, allows to obtain accurate mass determination, which is the key for their use in metabolomics approaches, as well as to resolve isomeric and isobaric species. Moreover, the possibility of running MS/MS experiments with some of these instruments, significantly enhances the capabilities for the identification of unknown metabolites.

As a direct consequence of the improvement on the available analytical tools, samples with higher complexity can be analyzed in which even thousands of features may be detected. Thus, the datasets generated after sample analyses in a typical metabolomics study is of extremely great complexity, including retention times, intensities, m/z, and even MS/MS spectra. Under these conditions, the manual interpretation and elaboration of all these data is impossible. For this reason, normalized procedures have been developed relying on bioinformatics tools in order to be able to subsequent statistical treatment properly extract the key information of all the huge amount of data available. Usually, data-processing involves peak detection, integration, peak alignment and normalization. After these steps, different chemometric tools can be used to statistically assess possible differences among samples. To do that, multivariate analysis is often used, although the particular statistical approach to be used will largely depend on the objectives of the study. Principal components analysis (PCA) is frequently employed at first, as it allows to group samples as a function of different variables. However, the particular statistical analyses made are usually different depending also on the topic of the study, i.e., food-health relationships, biomarker discovery, food quality, food safety or traceability, among others.

The aim of this review is to update the information provided in our previous article [1], including a critical revision of the latest research published in the field of MS-based metabolomics applied to food quality, food safety and traceability from 2014 to 2017. For the sake of clarity, each of these three topics are described and discussed in separate sections so that the basic particularities of the approaches involved in those subjects can be appropriately described.

2. MS-based metabolomics for food safety

Food safety is one of the most-important topics within food analysis; although one may tend to consider that every sold and consumed foodstuff possess proper safety, the truth is that food control is constantly required to maintain an appropriate degree of security for consumers. Food safety involves many sub-fields, including the legislation enforcement regarding the presence of selected

compounds in foods that may be present below certain limits (MRL, maximum residue limits), the detection of microbial- related spoilage, the determination of allergens, the detection of environmental contaminants as well as banned external com- pounds, or the assessment of the occurrence of natural toxins, for example. In this regard, the use of MS within metabolomics-based approaches has allowed significantly raising the level of the analytical determinations possible nowadays. In this section, the most-relevant published procedures to this aim are described and commented.

2.1. Detection of chemical contaminants: food production-related controlled substances (veterinary drug and pesticide residues), environmental pollutants and food-contact materials

Although there is a wealth of published material developing always better analytical methods for the detection of selected contaminants in foods, this section is focused to those methods that take advantage of metabolomics-based approaches to carry out those determinations, thus, targeting the detection of multiple components in just one run.

The first part of any MS-based metabolomics study for the detection of food contaminants is sample preparation. As foods may be considered as very complex matrices involving the presence of a broad array of very different components, suitable sample preparation steps are needed in order to allow a proper detection of contaminants which will surely be present in very low amounts. Some of the naturally present compounds in foods will negatively influence the analysis of the targeted compounds, and thus, different methods have been widely used to extract and/or concentrate those. Solid-liquid extraction (SLE) or liquid-liquid extraction (LLE), depending on the physical nature of the samples, using conventional solvents and solid-phase extraction (SPE) are, probably, the three sample preparation methods traditionally most employed. However, following the latest trends regarding the application of "Green Chemistry" principles, other miniaturized protocols limiting the volumes of solvents employed have been also proposed and employed in the last years. Among them, solid-phase microextraction (SPME) [2], and most notably, QuEChERS methods are highlighted [3]. Nowadays, QuEChERS involves a widely accepted methodology for the recovery of target analytes from complex matrices, which is based on an initial extraction with acetonitrile followed by a clean-up using dispersive SPE [4]. From this basic methodology, multiple modifications have been presented so far; these are mainly related to an adaptation to the nature and fat content of the sample extracted [3].

Other advanced extraction techniques, such as pressurized liquid extraction (PLE), have also been successfully employed. These environmentally green tools even allow the coupling with in-line clean-up steps using adsorbents. This strategy was followed for the extraction of pesticides from honey that were subsequently analyzed by GC-MS/MS [5]. Readers interested on gaining deeper insight on extraction methods and sample preparation for the analysis of contaminants in foods are referred to recent excellent review papers [2,6-12].

Methods directed to quantification of chemical contaminants in food are strongly influenced by current international legislation, which is generally directed to the establishment of MRLs on certain substances, and to specify the banned compounds that cannot be present at any concentration. MRLs for pesticides [13,14], veterinary drugs [15,16] and contaminants [17], are available.

The most frequent analytical approach to determine contaminants in foods relies on the use of tandem MS detection. This detection procedure allows the quantification of known compounds with great selectivity and sensitivity. Typically, triple quadrupole analyzers have been widely used to this aim, run under selected reaction monitoring (SRM), also called multiple reaction monitoring (MRM), mode. This way, each parent ion is fragmented by collision-induced dissociation (CID) and its two most-intense product ions are detected. The most-intense one is used for quantification whereas the second is employed for qualification purposes. This detection procedure allows complying with European legislation on banned and controlled substances in foods [18]. This regulation establishes the requirements that an analytical method must meet for an unequivocal identification and quantification of a controlled substance in a food sample, which means to gain, at least, four identification points. By using the mentioned approach, the legislation specifies that one identification point is gained by retention time confirmation with a commercial standard, whereas additional 1.5 identification points are gained for each ion transition successfully confirmed. As a result, and thanks to the quite fast scanning speed of modern triple quads, different remarkable applications have been developed in this field. In **Table 1**, some recent examples of this methodology for the quantification of more than 50 contaminants in foods in just one run are summarized. As it can be observed, most applications are based on the coupling of MS with a separation technique. LC and GC-based methods are widely extended, although the use of multidimensional chromatography has also been explored with success.

Table 1. Selected remarkable applications published during the period 2014-2017 dealing with the simultaneous identification and quantification of a large number of contaminants (> 50) in food samples.

Contaminants quantified	Food matrix	Sample preparation	MS-based approach	MS-based technique	Sensitivity		Ref.
					LOD	LOQ	
Pesticides (54)	Fruits and fish	QuEChERS	Targeted	UHPLC-HRMS (Orbitrap)	< 2 ng mL^{-1}		19
Pesticides (54)	Tomatoes, oranges	QuEChERS	Non-targeted	GC-EI-HRMS (Orbitrap)		10 µg kg^{-1}	20
Pesticides (55)	Bivalves (*Scrobicularia plana*)	QuEChERS	Targeted	GC-MS/MS (IT in SIM mode)		0.33-10.3 µg L^{-1}	21
Pesticides (57)	Tomato	QuEChERS	Targeted	LC-MS/MS (QqQ in MRM mode)	< 5000 µg kg^{-1}		22
Antibiotics (62)	Meat	Solvent-based extraction (ACN)	Targeted	LC-HRMS (Orbirtap)	1 µg kg^{-1}	3.3 µg kg^{-1}	23
Contaminants (68)	Food contact materials	QuEChERS (modified)	Targeted	LC-MS/MS (QqQ in MRM mode) GC-MS/MS (QqQ in MRM mode)		1.3 – 220 µg kg^{-1}	24
Pesticides (73)	Fruits, vegetables	Solvent-based extraction (ACN)	Targeted	LC-MS/MS (QqQ in MRM mode)		< 10 µg kg^{-1}	25
Contaminants (75)	Food contact materials	Soxhlet-based protocol	Non-targeted / Targeted	UPLC-HRMS (QTOF, database)	< 2 ng ml-1	< 20 ng ml-1	26

Table 1. Continued

Contaminants quantified	Food matrix	Sample preparation	MS-based approach	MS-based technique	Sensitivity		Ref.
					LOD	LOQ	
Herbicides (76) and veterinary drug residues	Shellfish	QuEChERS	Targeted	LC-MS/MS (QqQ in MRM mode) GC-MS/MS (QqQ in MRM mode)		0.25-0.50 µg kg-1 veterinary residues 2-20 µg kg-1 pesticides	27
Veterinary drug residues (76)	Meat	Solvent-based extraction (ACN)	Targeted	UHPLC-MS/MS (QqQ in SRM mode)		0.038- 74 µg kg-1	28
Pesticides and antibiotics (83)	Honey	Solvent-based extraction (ACN)	Targeted / Non-targeted	LC-HRMS (Orbirtap)	< MRLs		29
Pesticides (87)	Groundnut oil	QuEChERS	Targeted	LC-MS/MS (QqQ MRM mode)		4 - 180 µg kg-1	30
Pesticides (79) and antibiotics (13)	Honey	Solvent-based extraction (ACN) and clean-up	Targeted	UHPLC-MS/MS (QqQ in MRM mode)	0.03 to 1.51 µg kg−1	0.1 to 5 µg kg-1	31
Pesticides (103)	Chicken, fish	QuEChERS	Targeted	LC-MS/MS (QqQ in dynamic MRM mode)		1-10 µg kg-1	32
Pesticides (109)	Tomatoes	QuEChERS	Targeted	LC-MS/MS (QqQ in MRM mode)	0.5-10.8 µg kg-1	1.3-30.4 µg kg-1	33
Pesticides (113)	Rice, red pepper, mandarin	QuEChERS	Targeted	GC-MS/MS (QqQ in MRM mode)		0.1–25 µg kg-1	34
Pesticides (115)	Oranges	QuEChERS	Targeted	LC-MS/MS (QqQ in MRM mode)	1 – 11 µg kg-1	2 – 30 µg kg-1	35

Table 1. Continued

Contaminants quantified	Food matrix	Sample preparation	MS-based approach	MS-based technique	Sensitivity		Ref.
					LOD	LOQ	
Pesticides (65) and environmental contaminants (52)	Kale, salmon, pork, avocado	QuEChERS	Targeted	GC-MS/MS (QqQ in MRM mode)			36
Pesticides (120)	Fruits, cereals	QuEChERS	Targeted	GC-MS/MS (QqQ in MRM mode)	10 µg kg-1		37
Pesticides (120)	Apples, cucumbers	QuEChERS	Targeted	LC-MS/MS (QqQ in SRM mode)	1.2 – 11 µg kg-1	10 µg kg-1	38
Veterinary drugs (120)	Meat, eggs, milk	Ultrasound-assisted extraction and SPE	Targeted	LC-MS/MS (QqQ in MRM mode)	0.5–3.0 µg kg-1	1.5–10.0 µg kg-1	39
Contaminants (120)	Eggs	Solvent-based extraction (ACN) and purification	Targeted	LC-MS/MS (QqQ in MRM mode)		2.04–1316 µg kg-1 (CCβ)	40
PCBs (127), polychlorinated naphtalenes (6), PAHs (16)	Mussels, clams	PLE (100ºC, dichloromethane:hexane)	Targeted	GC-MS (quadrupole, SIM)		0.2-15 pg	41
Pesticides (105), antibiotics (49) and steroids (3)	Honey	Solvent-based extraction (ACN)	Targeted	UHPLC-HRMS (Orbitrap, in PRM mode and database)		0.009 - 6.21 µg kg-1 (CCβ)	42
Pesticides (133), PAHs (24)	Fish	QuEChERS	Targeted	GC-HRMS (QTOF)	10 µg kg-1		43

Table 1. Continued

Contaminants quantified	Food matrix	Sample preparation	MS-based approach	MS-based technique	Sensitivity		Ref.
					LOD	LOQ	
Pesticides (162)	Tea	Solvent-based extraction (ACN) and purification	Targeted	GC-MS/MS (QqQ in MRM mode)	< 10 μg kg^{-1}		44
Pesticides (164)	Apples, broccoli, oranges	Polyurethane foam disks swabbing	Targeted	DART-HRMS (Orbitrap)	10 μg kg^{-1}		45
Pesticides (167)	Honey	Solvent-based extraction (ethyl acetate)	Targeted	LC-MS/MS (QqQ in MRM mode)		10 – 100 μg kg^{-1}	46
Pesticides (172)	Wines	Solvent-based extraction (ethyl acetate)	Targeted	LC-MS/MS (QqQ in MRM mode)		10 – 50 μg kg^{-1}	47
Pesticides (177)	Soy-based products	QuEChERS	Targeted	GC-MS/MS (QqQ in MRM mode)	0.1 - 10 μg kg^{-1}	0.5-20 μg kg^{-1}	48
Pesticides (178)	Eggs	Matrix solid-phase dispersion	Targeted	LC-MS/MS (QqQ in MRM mode) GC-MS/MS (QqQ in MRM mode)		5 – 10 μg kg^{-1}	49
Pesticides (184)	Lettuce, oranges	QuEChERS	Non-targeted	LC-HRMS (Orbitrap)	10 μg kg^{-1} (SDL) for 134 compounds 50-200 μg kg^{-1} (SDL) for 39 compounds		50
Pesticides (200)	Green lettuce, orange	Ultra-Turrax homogenization with methanol and dilution	Targeted	UHPLC-MS/MS (QqQ in MRM mode)		1.0 – 5.0 μg kg^{-1}	51

Table 1. Continued

Contaminants quantified	Food matrix	Sample preparation	MS-based approach	MS-based technique	Sensitivity		Ref.
					LOD	LOQ	
Pesticides (200)	Honey	QuEChERS	Targeted	GC-MS/MS (QqQ in MRM mode)	1.00 to 3.00 ng mL^{-1}		52
Veterinary drug residues (>200)	Milk	Solvent-based extraction (ACN)	Targeted	LC-HRMS (QTOF)	< 100 ng mL^{-1} (for 72% of compounds)		53
Dioxin-like micropollutants (206)	Meat	PLE (100 °C, hexane)	Targeted	GC×GC-TOF/MS	0.050-0.100 µg kg^{-1} PCBs 65-227 ng kg^{-1} PCDD/Fs		54
Pesticides (219)	Cereals	QuEChERS	Targeted	GC-MS/MS (QqQ in MRM mode)		5 - 50 µg kg^{-1}	55
Pesticides (238)	Cabbage, cucumber	QuEChERS	Targeted	LC-MS/MS (QqQ in MRM mode)	0.02 - 6.32 µg kg^{-1}	0.06 − 21.06 µg kg^{-1}	56
Pesticides (269)	Avocado, citrus	QuEChERS with automated zirconia-based SPE	Targeted	LC-MS/MS (QqQ MRM mode)	< MRLs		57
Pesticides (317)	Vegetables, fruits	SPE	Targeted	LC-HRMS (QTOF and database)	10 µg kg^{-1} (84 %)		58
Pesticides (451)	Fruits, vegetables	QuEChERS	Non-targeted	LC-HRMS (Orbitrap)		< 5 µg kg^{-1} (85% of compounds)	59

Table 1. Continued

Contaminants quantified	Food matrix	Sample preparation	MS-based approach	MS-based technique	Sensitivity		Ref.
					LOD	LOQ	
Contaminants (492)	Milk, meat, eggs, liver, kidney, fish	Solvent-based extraction	Targeted	HPLC-HRMS (TOF-MS)	0.0005–100 ng mL^{-1}	0.003–250 ng mL^{-1}	60
Multiclass contaminants (625)	Baby foods, oranges, tomato	Solvent-based extraction (ACN)	Targeted	UHPLC-HRMS (QTOF and database)		< MRLs (excepting ca. 10% analytes)	61

ACN, acetonitrile; CCβ, detection capability; DART, direct analysis in real time; HRMS, high resolution mass spectrometry; IT, ion trap; MRL, maximum residue limit; MRM, multiple reaction monitoring; PAH, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; PCDD, polychlorinated dibenzo-p-dioxins; PLE, pressurized liquid extraction; PRM, parallel reaction monitoring; QqQ, triple quadrupole; SDL, screening detection limit; SIM, selected ion monitoring; SPE, solid phase extraction; SRM, selected reaction monitoring; TOF: time-of-flight.

Multidimensional procedures allow increasing resolving power and separation which can be beneficial for subsequent MS-based detection, considering that the targeted compounds will reach the detector more separated in time. This is the case of comprehensive two-dimensional gas chromatography (GC x GC) that has been coupled to a TOF-MS analyzer to determine dioxin-related pollutants in complex food samples [54]. Satisfactory separation of more than 200 micro-pollutants was achieved, with low limits of detection. **Figure 1** illustrates the good separation attainable using this approach.

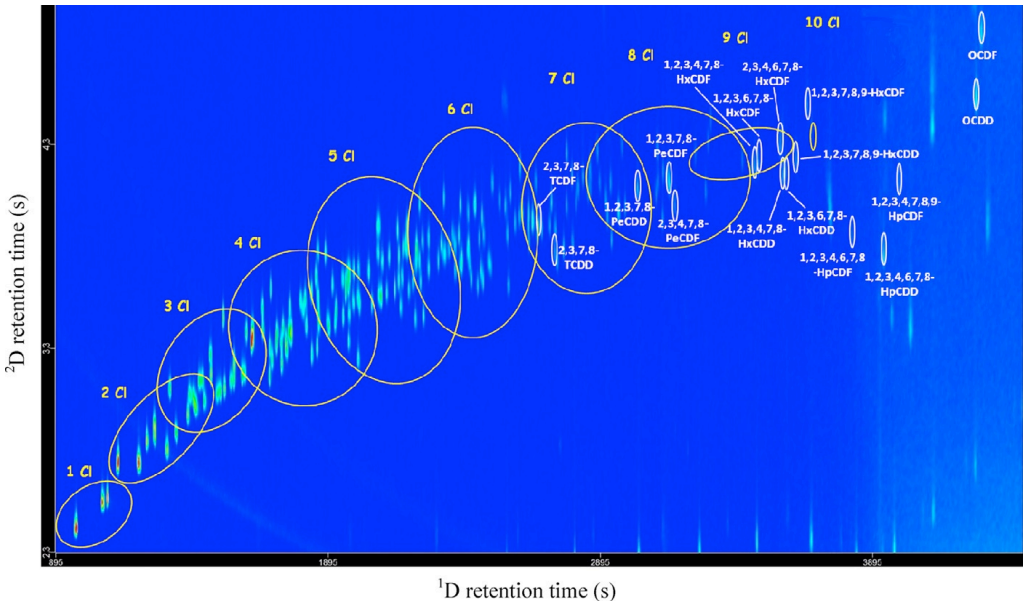

Figure 1. GC×GC-TOF/MS contour plot of the 209 PCBs and 17 PCDD/Fs with the Rtx-Dioxin2/BP-X50 column set. Adapted with permission from [54].

Although no practical application of comprehensive two-dimensional liquid chromatography (LC x LC) has been published so far for technique retains a very good potential. In fact, a first application for the quantification of pesticides in complex food samples, such as wine, has recently been presented [62]. As can be deduced from the information presented in **Table 1**, during the period covered by the present review (2014-2017), the use of triple quadrupoles in MRM mode is still the most-extended approach. Satisfactory results have been attained in a variety of applications involving the use of these approaches, using targeted approaches and reaching the quantification of a significant amount of components in relatively short analysis times with high sensitivity. Although the basic principles remain relatively constant, different modifications have pushed even forward the limits of these

procedures. This is the case, for instance, of the use of high resolution MS (HRMS) analyzers instead of the commonly employed triple quads; in fact, the use of HRMS in the field of food safety is showing an increase. For instance, thanks to the use of nano-LC and HRMS coupled through the use of ambient dielectric barrier discharge ionization (DBDI) source, extremely low detection limits, as low as 10 pg mL^{-1}, were achieved for the quantification of pesticide residues [63]. In fact, one of the possible advantages of using HRMS is the possibility of constructing databases for the sought compounds, when operating under targeted approaches. The use of these databases together with parallel reaction monitoring using a Q-Orbitrap analyzer has been shown to be effective for the appropriate screening and quantification of 157 residues of different nature in honey [42]. Similar approaches have involved an expansion on the studied compounds to more than 600 different contaminants, including pesticides, veterinary drug residues, contaminants, perfluoroalkyl substances, mycotoxins and nitrosamines [61]. In any case, each MS detection method has its highs and lows; comparative studies testing the performance of tandem MS versus HRMS to quantify polychlorinated dioxins and biphenyls in foods have concluded that although the use of GC-MS/MS allows meeting with the requirements laid by the European Commission, GC-HRMS may fit better for monitoring purposes as it was shown to produce less false positives [64].

In spite of the developed methods, the use of the above described targeted approach has important limitations, which are mainly related to the determination of unknown compounds as well as the need of reference commercial standards. For this reason, the use of similar approaches already developed in other fields for the non-targeted analysis of contaminants is increasingly proposed, taking advantage of the capabilities of HRMS modern analyzers [65]. An interesting example has recently been published in order to investigate which compounds of potential concern were present in a pizza box, as a model of food packaging material [26]. This approach involved the coupling with proper *in-vitro* assays based on aryl hydrocarbon receptor activity to limit the number of frac- tions to be studied after extraction. The most-active fractions were analyzed by using GC-QTOF-MS and UHPLC-QTOF-MS. The workflow followed in this work is shown in **Figure 2.**

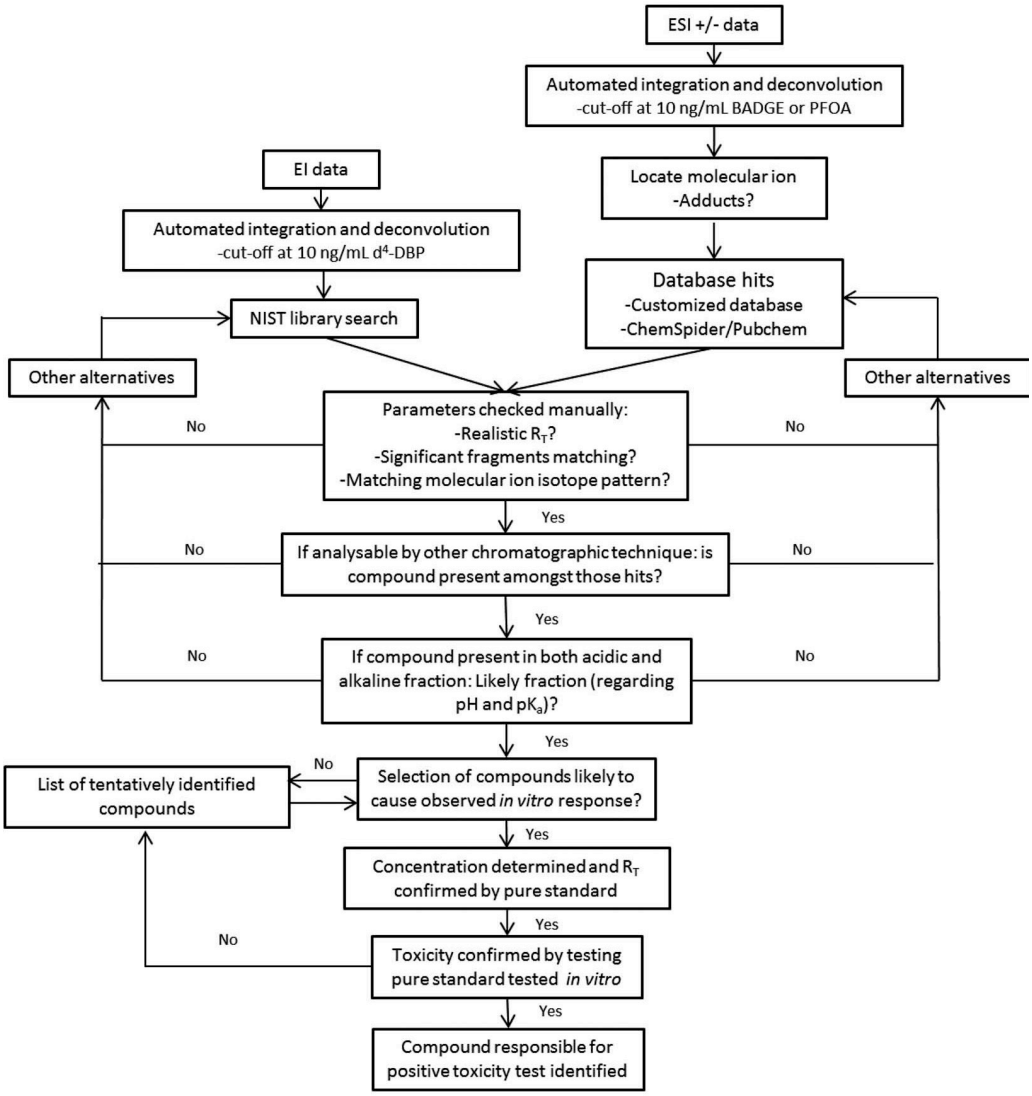

Figure 2. Workflow for the identification of compounds in fractions from pizza packaging material analyzed by GC-EI-qTOF MS and UHPLC-ESI-qTOF MS. Reproduced with permission from [26].

Seventy-five substances were tentatively identified, among which seven commercially available could be further studied but could not explain a significant proportion of the aryl hydrocarbon receptor response in the extract. Thus, it could be concluded that other very active substances still remained unidentified in the food container [26]. Using another different non-targeted approach Zomer and Mol also showed the high potential of state-of-the-art HRMS instrumentation [50]. Using a hybrid HRMS analyzer, a new fully non-targeted approach for data acquisition combining full-scan and fragmentation was developed utilizing variable data

independent acquisition for the generation of fragment ions. Quantitative validation of the methodology using a mixture of 184 pesticides in two food matrices showed that this approach was suitable for ca. 93 % of the assayed pesticide/matrix/concentration combinations studied in agreement with EU guidelines. Thus, this LC-full-scan HRMS method has been suggested as an alternative for triple quad MS-based methods. Moreover, the same data could be used to screen samples for a large number of compounds with lower probability of being present, reducing the chance for false-negatives compared to other previously used full-scan-based protocols [50].

The most interesting aspect related to the non-targeted methodology is based on the possibility of detecting substances not previously preselected, thus, increasing the chance for the proper detection of unknown and unexpected compounds. These metabolomics approaches may gain advantage of data mining tools initially developed in other fields. A proof-of-concept study, demonstrating the ability of these tools to identify unknown chlorinated chemicals in honey samples has been reported [29]. However, the use of these diverse non-targeted methodologies is still somewhat limited compared to the targeted approach, as it is clearly illustrated in **Table 1**. Further developments on this field in the near future are expected.

2.2. Detection of microbial contaminants (pathogens and toxins)

Risks of natural origin for food safety are mainly related to the presence or activity of microorganisms. Thus, foods may be contaminated directly by the presence of pathogens, which could cause an infection to the consumer, or may be indirectly contaminated by toxins produced by a particular microorganism. Contamination of food with pathogens may imply very serious consequences on health, being the most extended diarrhea, and can occur at any point of the food production chain due to inadequate hygiene conditions. On the other hand, the presence of toxin producers within or near food related products can be a potential source of contamination. This is the case, for instance, of cereal products contaminated with mycotoxins, or shellfish contaminated with microalgal toxins that are bioaccumulated in those filter-feeding animals.

For the detection and quantification of toxins in foods, similar approaches to those already described for chemical contaminants are widely employed. The methodology to quantify those components by tandem MS is very much the same; however, in this case, the natural toxin variability potentially present in a particular food product mean that less compounds have to be analyzed, and thus, advanced

metabolomics-based approaches are not required. Instead, proper sample preparation for toxins extraction and quantification by MRM using triple quads is the most common MS- based methodology applied [66,67]. Nuts [68], maize [69], shellfish [70], tomato [71], or beer [72], among others, are examples of food products assayed following this approach. However, some modifications have been also introduced to this methodology in order to increase the performance of methods as well as to allow a very sensitive detection, as some of the natural toxins that might be potentially found in foods are very toxic (even lethal) at extremely low concentrations. For instance, the use of a multiple antibody immunoaffinity column for the selective extraction of 7 toxins before HPLC-MS/MS determination has been recently reported [73]. This method allowed extending the linear range of the determination as well as to decrease the detection limits to the low mg kg^{-1} level compared to previously developed methods. Other sample preparation-oriented improvements have been directed to the implementation of inexpensive graphitized carbon for SPE of paralytic shellfish toxins, showing excellent capabilities [74].

Other sensitive gains have been attained through the analytical tool employed prior MS. The ultrasensitive detection, with detection limits as low as 0.38 fmol of saxitoxin was achieved in seafood samples thanks to a reaction involving diethylenetriamine- N,N,N′,N″,N‴-pentaacetic acid. This compound can couple with saxitoxin and simultaneously chelate with Eu^{3+} to allow metallic labeling of this toxin, that may be quantified with extremely high sensitivity using capillary electrophoresis-inductively coupled plasma-MS detection (CE-ICP-MS) [75]. Direct determination of toxins may have the further advantage of increasing throughput in food safety laboratories. As already mentioned, some direct analysis MS techniques have been employed for the quantification of chemical contaminants (see **Table 1**). In the case of toxins, some direct methods have been also presented. Indeed, domoic acid has been quantified in mussel tissues directly by MS/MS using SRM mode without any sample extraction, clean-up or separation. This has been obtained using laser ablation electrospray ionization (LAESI), reaching limits of detection of 1 mg kg^{-1} for this compounds. This LOD is not particularly low compared to other more conventional approaches based on extraction/separation and MS/ MS detection, but it has to be considered that each analysis takes around just 10 s, thus, being very attractive for routine analysis [76]. Although these recent advances have enhanced in different manners the detection of toxins in food, any of them shows a purely metabolomics-based strategy. In this regard, this subfield of analysis should benefit in the future from applications already developed for

contaminants analysis as those previously described in Section 2.1. In spite from this, some efforts have already been made, such as the development of an analytical micro HPLC-MS/MS method for the simultaneous quantification of 26 mycotoxins in maize with total run times of 9 min and reduced solvent con-sumption (below 0.3 mL) [77].

Other food safety-related methodologies are mostly focused on the detection of pathogen microorganisms that could be present in the food products posing a serious risk to consumers' health. Although different molecular techniques and proteomics-based approaches may be used to detect and identify the microorganisms present in a sample, in recent years much effort has been also focused on the determination of microbial volatile organic compounds (MVOCs) as markers of microbiological contamination [78]. To that aim, the most-extended analytical MS-based approach is based on the use of GC-MS coupled to a proper sample preparation/extraction protocol, such as SPME or headspace (HS) sampling. After the determination of a group of volatiles as wide as possible, multivariate analysis of data is necessary to correlate the presence of specific compounds with the growth of particular pathogens. This approach has been employed to predict shelf-life, evaluating potential chemical spoilage indices of Atlantic salmon stored under aerobic conditions [79], sea bass stored under air and under modified atmosphere [80], sea bream depending on the storage conditions [81,82], as well as minced meat [83] or pork [84]. Another possibility gaining interest in recent times is the determination of MVOCs by real time analysis through the application of proton-transfer-reaction-MS (PTR-MS). This technique is able to provide with fast on-line analyses that are very appropriate for determination of the real-time evolution of volatiles. Different applications have been recently published to determine MVOCs of microbial origin from selected strains [85] as well as in food products such as chicken meat [86] or milk [87,88]. To allow the continuous on-line monitoring, different set-ups have been developed, for instance, allowing the monitoring of four meat samples in parallel [86] (**Figure 3A**), or other more manually-operated set-ups for milk (**Figure 3B**) [87].

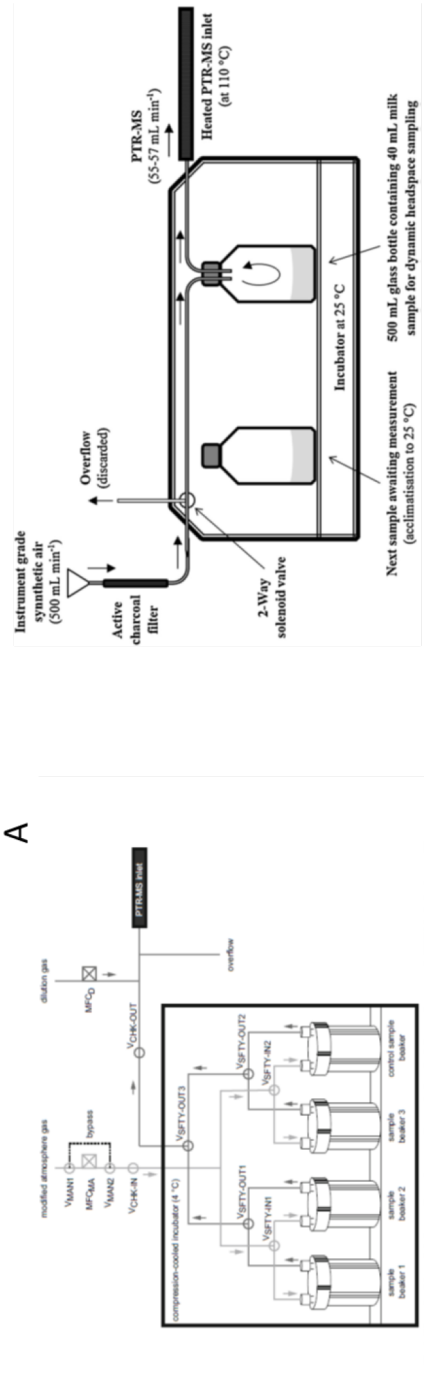

Figure 3. Schematic set-ups for continuous on-line monitoring of microbial volatile organic compounds by proton-transfer-reaction MS in A) the headspace of four meat samples in parallel (adapted with permission from [85]) and, B) in the headspace of milk samples (adapted with permission from [86]).

3. MS-based metabolomics to assess food quality

Nowadays, food quality is one of the major concerns of the food industry. Its evaluation is a complex task due to the multiple aspects that may be considered to achieve an appropriate food quality. Food composition, aroma, flavor, or nutritional properties are among the most important aspects that may be evaluated in food quality assessments. Different types of analysis are clearly needed to evaluate all these aspects. Is at this point where MS-based metabolomics approaches are gaining attention due to their demonstrated capability to establish links between relevant food aspects and food quality perception.

Table 2 summarizes the most relevant applications of MS-based metabolomics strategies for food quality published during the period of time covered by this review (2014-2017). As can be observed, these works are mainly focused on the use of this kind of platform to establish the relationship between the chemical composition and food quality, to control food authentication and adulteration, or to differentiate food samples according to their variety. To achieve these aims, non-targeted approaches have usually been employed followed by data-processing and multivariate analysis to assess possible differences among samples. An interesting strategy is the combination of non-targeted and targeted methods; its usefulness has recently been reported for the qualitative analysis of curcuminoids in turmeric [91]. This integrated strategy involves a non-targeted analysis by LC-QTOF-MS/MS and a targeted approach by LC-QTRAP-MS/MS. **Figure 4** depicts the workflow followed in this study. Ninety-six curcuminoids were fully characterized following this exclusive methodology. Anyhow, the ultimate goal of the researches developed to assess food quality is to determine relevant compounds that may be selected as quality markers. Afterwards, just a few studies have developed targeted methodologies for the routine analysis of those markers [89,90]. However, this fact is interesting from an analytical point of view, since a targeted method requires less sophisticated instrumentation, is usually simpler and the data are more easily analyzed, being, therefore, more applicable for routine analysis.

Table 2. The most remarkable MS-based metabolomics approaches devoted to food quality published during the period 2014-2017.

Food matrix	Metabolites	Sample preparation	MS-based approach	MS-based technique	Application	Ref.
Pineapple, orange, apple, clementine, pomelo, and grapefruit juices	Flavonoids and limonoid glucosides	Centrifugation and filtering	Non-targeted / Targeted	UHPLC-HRMS (QTOF)	Detection of fruit juice adulteration	88
Citrus fruits, Jaffa, Mosambi orange and Red blush grapefruit	Flavonoids and limonoid glucosides	Centrifugation and filtering	Non-targeted / Targeted	UHPLC-HRMS (QTOF) for non-targeted LC-MS/MS (QqQ in MRM mode) for targeted	Discrimination of authentic and adulterated citrus fruits/fruit juices	89
Tumeric	curcuminoids	Solvent-based extraction (using mixtures methanol:water)	Non-targeted/ Targeted	LC-HRMS (QTOF) for non-targeted LC-QTRAP-MS/MS (MRM mode) for targeted	Quality evaluation of raw turmeric from different regions	90
Grapes	Phytosterols	Solvent-based extraction (chloroform: methanol 1:1 (v/v))	Targeted	LC-HRMS (QTOF)	Discrimination of grapes according to plant sterols content	91
	Amino acids, fatty acids, acids (aromatic acids, hydroxy acids, dicar-boxylic acids, phenylpropanoic acids), flavonoid, and sugars	Solvent-based extraction (water: methanol:chloroform (1:2.5:1, (v/v/v))	Non-targeted	GC-HRMS (TOF)	Differentiation of cultivars through their metabolite profile	92
Graciano Vitis vinifera wine	Non-volatiles/ semivolatile metabolites (sugars, amino acids, higer alcohol, biogenic amines, organic acids and phenolic compounds)	Centrifugation and filtering	Non-targeted	LC-HRMS (QTOF)	Analysis of the metabolome of the Graciano Vitis vinifera wine variety	93

Table 2. Continued

Food matrix	Metabolites	Sample preparation	MS-based approach	MS-based technique	Application	Ref.
Tropical fruits (Mango, pineapple, jackfruit, baobab, tamarind)	Non-volatiles metabolites (carbohydrates, organic acids, amino acids, and fatty acids).	Solvent-based extraction (water), acid hydrolysis and derivatization with trimethylsilyl cyanide	Non-Targeted	GC-HRMS (TOF)	Comparison of non-volatile metabolites of tropical fruits	94
Soybean sprouts	Amino acids, organic acids, lipids, sugars, phytosterol, isoflavones, and soyasaponins.	Solvent-based extraction (50 % methanol for UHPLC; 50 % methanol followed by methoxylation, and derivatization with BSTFA for GC anlysis)	Non-targeted	GC-MS/MS (QqQ in MRM mode), and UHPLC-HRMS (QTOF)	Evaluation of the relationship between germination and nutritional quality	95
Infant formulas	Low-molecular-weight compounds (nicotinic acid and nicotinamide were identified)	Solvent-based extraction (water) and ultrafiltration	Non-targeted	HILIC-HRMS (QTOF)	Assessment of contamination and degradation of infant formulas	96
Saffron	Volatile metabolites	UASE-DLLME	Non-targeted	GC-MS (Q with EI)	Investigation of the effect of volatile components on the saffron´s classification	97
	Glycerophospholipids and their oxidized lipids	Solvent-based extraction (ethanol:water 70:30 v/v) with sonication	Non-targeted	UHPLC-HRMS (QTOF)	Authentication of saffron	98
	Mainly flavonols and anthocyanins	Solvent-based extraction (ethanol:borate buffer at pH 9.0, 50:50 v/v) with sonication	Non-targeted	LC-HRMS (QTOF)	Investigation of the quality and authenticity of saffron	99
Olive oil	Volatile organic compounds	SPE	Non-targeted	GC-HRMS (QTOF)	Classification of olive oils according to their quality	100
Vinegar	Amino acids, carboxylic acids, sugars, sugar alcohols, fatty acids, vitamin, peptides and aroma compounds	MCF derivatization/TMS derivatization/ or extraction with diethyl ether	Non-targeted	GC-MS (Q with EI)	Comprehensive metabolite profile of vinegar	101

Table 2. Continued

Food matrix	Metabolites	Sample preparation	MS-based approach	MS-based technique	Application	Ref.
Milk	Short-chain hydroxylated carboxylic acids, long-chain stearic and palmitic acids, free amino acids, and sugars	Solvent-based extraction (methanol:chloroform) and derivatization with pyridine	Non-targeted	GC-MS (Q with EI)	Discrimination between milk typologies and detection of milk fraud	102
Orange juice and red wine	Mainly sugars, amino acids, and organic acids	Filtering	Non-targeted	CE-HRMS (TOF)	Comprehensive anionic metabolite profile of orange juice and red wine	103
Meat	Organic acids, amino acids, sugars, sugar alcohols, phosphorylated intermediates and lipophilic compounds	Solvent-based extraction (methanol:water 80:20 (v/v)) Derivatization with MSTFA for GC analysis	Non-targeted	GC-HRMS (TOF)/HILIC-HRMS (QTOF)	Identify biomarkers of meat quality traits	104
	Amino acids, sugars, nucleotides, nucleosides, and organic acids	Solvent-based extraction (methanol:water 80:20 (v/v) followed by chloroform:water 67:33 (v/v))	Non-targeted	HILIC-HRMS (Orbitrap)	Study of colour stability of ovine meat	105
	Amino acids, organic acids, alkane hydrocarbon, and sugar alcohols,	Solvent-based extraction (chloroform:methanol:water) and derivatization with MSTFA	Non-targeted	GC-HRMS (TOF)	Detection of the adulteration of beef meat	106
Rice (Jasmine phenotype)	Volatile organic compounds	Static HS extraction	Non-targeted	GC×GC-TOF/MS	Determination of the metabolites that define the 'Jasmine' quality of rice	107
Gochujang (fermented pepper paste)	Amino acids, organic acids, fatty acids, sugars, sugar alcohols, flavonoids, capsaicinoids, capsinoids, lipids	Solvent-based extraction (80 % methanol) Derivatization with MSTFA for GC analysis	Non-targeted	GC-HRMS (TOF)/ UHPLC-IT-MS	Quality characterization	108

Table 2. Continued

Food matrix	Metabolites	Sample preparation	MS-based approach	MS-based technique	Application	Ref.
	Mainly amino acids, organic acids, and sugars	Solvent-based extraction (80 % methanol) Derivatization with MSTFA for GC analysis	Non-targeted	GC-HRMS (TOF)/ UHPLC-HRMS (QTOF)	Evaluation of the metabolite differences according to the raw material used in the production of gochujangs	109
Grean tea	Mainly catechins, amino acids, caffeine	Solvent-based extraction (hot water)	Non-targeted	UHPLC-HRMS (QTOF)	Study of the chemical composition of green tea to assess it quality	110
Peach fruit	Sugars, organic acids, and amino acids	Solvent-based extraction (methanol) and derivatization with MSTFA	Non-targeted	GC-MS (Q with EI)	Explore the chemical composition which defines fruit quality	111
Strawberry	Phenolic acids, flavonoids, flavan-3-ol derivatives, terpenes, and many types of glycosidically bound aroma and flavor precursors	Solvent-based extraction (80 % methanol)	Non-targeted	LC-HRMS (QTOF)	Separation and identification of major metabolites showing significant variation between strawberry cultivars	112
	Sugars, organic acids, and amino acids	Solvent-based extraction (methanol:water 1:1 (v/v)) and derivatization with MSTFA	Non-targeted	GC-MS (IT)	Differentiation of strawberry cultivars and assessment of the influence of agronomic conditions	113
Date palm fruit	Volatile metabolites (lipid-derived volatiles, phenylpropanoid derivatives, amino acid derived volatiles, and sugar derived volatiles)	HS-SPME	Non-targeted	GC-MS (Q with EI)	Differentiation among date varieties	114
Honey	Not described	Solvent-based extraction (methanol:water 1:1 (v/v) containing 1 % formic acid)	Non-targeted	UHPLC-HRMS (QTOF)	Discrimination of honeys according to their floral origin	115

BSTFA, bis(trimethylsilyl)trifluoroacetamide; DLLME, dispersive liquid-liquid microextraction; EI, electron ionization; HILIC, hydrophilic interaction liquid chromatography; HRMS, high resolution mass spectrometry; HS-SPME, headspace solid-phase micro-extraction; IT, ion trap, MCF, Methylchloroformate; MRM, multiple reaction monitoring; MSD, mass selective detector; MSTFA, N-Methyl-N-(trimethylsilyl) trifluoroacetamide; Q, quadrupole; QqQ, triple quadrupole; QTOF, quadrupole-time-of-flight; QTRAP, hybrid triple-quadrupole linear ion trap; SPE, solid-phase extraction; TMS, trimethyl Silyl; TOF, time-of-flight; UASE-DLLME, ultrasound-assisted solvent extraction in tandem with dispersive liquid–liquid microextraction.

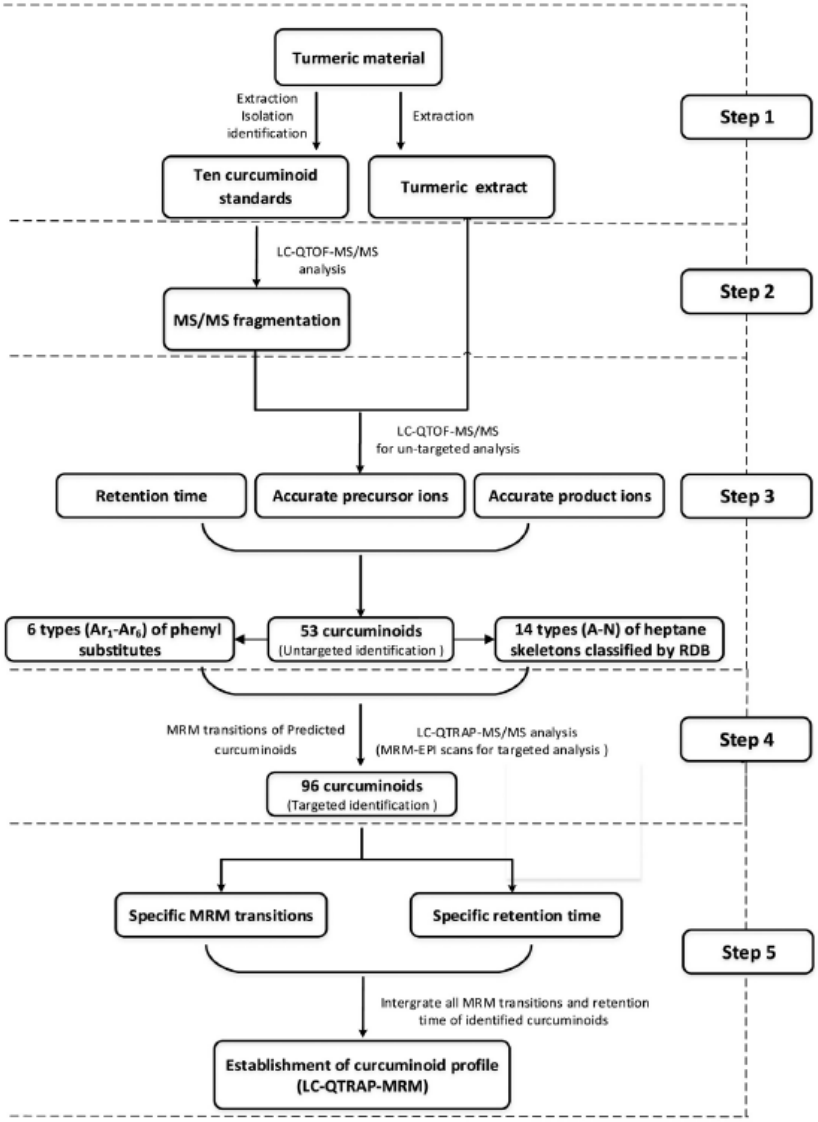

Figure 4. Workflow for establishment of curcuminoid profile in turmeric by an integrated strategy. Reproduced with permission from [100].

One of the relevant points to assess food quality by MS-based metabolomics is, again, the choice of proper sample preparation procedures. This fact will depend not only on the analytical technique employed to perform the analysis but also on the particular aim of the study. Although nowadays the use of modern mass spectrometers enables to perform analysis with high sensitivity which may simplify sample preparation, the inherent complexity of food samples makes this step a critical factor in the determination of metabolites, as previously mentioned. In any case, to prevent any substantial loss of possible relevant metabolites, minimum

sample preparation is preferable. Even though simple solvent-based extraction procedures have been the method of choice during the last years (see **Table 2**), certain GC-MS methodologies have required the use of other sample preparation techniques such as ultrasound-assisted extraction in tandem with dispersive liquid-liquid microextraction (UAE-DLLME) [98], solid-phase extraction (SPE) [101], static headspace extraction (HS) [108] or headspace solid-phase micro-extraction (HS-SPME) [114], in order to improve the extraction of volatile compounds or to achieve a preconcentration effect, thus, increasing method sensitivity and efficiency.

As can be deduced from the information shown in **Table 2**, the majority of applications of MS-based metabolomics approaches included the coupling LC-MS and/or GC-MS. Concerning LC-MS, the use of methods based on the UHPLC has increased considerably in the last years due to its capability to perform complex analysis with high efficiency and resolution in a short time. Different metabolomics studies have employed UHPLC technology for example to carry out the authentication and the evaluation of possible adulterations in fruits juices [89,90] or saffron [99], demonstrating the feasibility of these methodology to face one of the most growing problems in the global market. Another point that should be highlighted regarding LC is that although C18 columns are by far the most utilized, methods based on the use of hydrophilic interaction chromatography (HILIC) have also successfully been applied to food quality. This allows profiling highly polar and hydrophilic compounds providing complementary metabolic information to reversed-phase LC. Even though there are some drawbacks associated with HILIC (variability in retention times, low peak efficiency, and long re-equilibration times after gradient elution), this methodology has been used for the assessment of contamination and degradation of infant formulas [97] or to identify biomarkers of meat quality [104,106].

Regarding GC-MS, in spite of the need to include a derivatization step in the sample treatment to increase the range of metabolites that can be analyzed, GC-MS metabolomics approaches have been broadly used to evaluate food quality as it can be observed in **Table 2**. In these cases, GC has been hyphenated to a great variety of mass analyzers including simpler MS instruments, like quadruple (Q) working at electron ionization mode [98,102,103,111,114], or ion trap (IT) [113], as well as high resolution instruments [93,95,105,109,110], and even hybrid analyzers [96,101,107]. An interesting work based on the use of GC coupled to TOF-MS has been employed to develop a non-targeted metabolomics approach capable to establish differences between wine grape cultivars [93]. To do that, two grape cultivars were profiled and 115 metabolites were identified and quantified. Among

them, sugars and amino acids showed an opposite behavior in both cultivars. To carry out the biological interpretation of the data and to obtain an overview of the abundance of these compounds in the development of the cultivars, their behavior in the primary metabolism pathways was investigated. **Figure 5** depicts the level of each metabolite within each cultivar during the grape development stage in different pathways (tricarboxylic acid cycle, glycolysis, amino acid synthesis, and sucrose synthesis). Other interesting strategies based on GC-MS metabolomics platforms have been applied, for instance, to investigate the effect of volatile compounds for the classification of saffron based on the concentration of biomarkers [98], to classify olive oils according to their quality parameters [101], or to detect milk or meat adulteration [103,107].

Although LC-MS and GC-MS have been the preferred platforms to assess food quality, GC x GC [108] and CE methods [104] coupled to TOF analyzers have also been applied with success. The first one has allowed to establish associations between volatile metabolites and perception of rice aroma, creating a panel of biomarkers of rice flavor quality [108]. These results are valuable for breeding programs since can be used to choose pleasant rice aromas. In the latter, the feasibility of using a polymer-coated-capillary for the separation of anionic metabolites both in orange juice and wine has been demonstrated [104]. It offers a complementary coverage of the metabolome of these samples to those provide by other analytical techniques. Due to the demonstrated capabilities of both GC x GC and CE, it is expected that future developments in this field will gain advantage of those methods, since the full potential of these techniques in food metabolomics has not been reached.

4. MS-based metabolomics for food traceability

Food traceability is also a relevant topic within food analysis, whose main purpose is to provide a continuous monitoring of a food in the entire supply chain; this monitoring has been often defined as "from farm to fork". Undoubtedly, food traceability is closely related to food quality, food safety and public health. This consumers who are increasingly demanding more information about each stage of the food that they consume. In this regard, MS-based metabolomics approaches are essential since they are capable to provide the level of accuracy needed for traceability management.

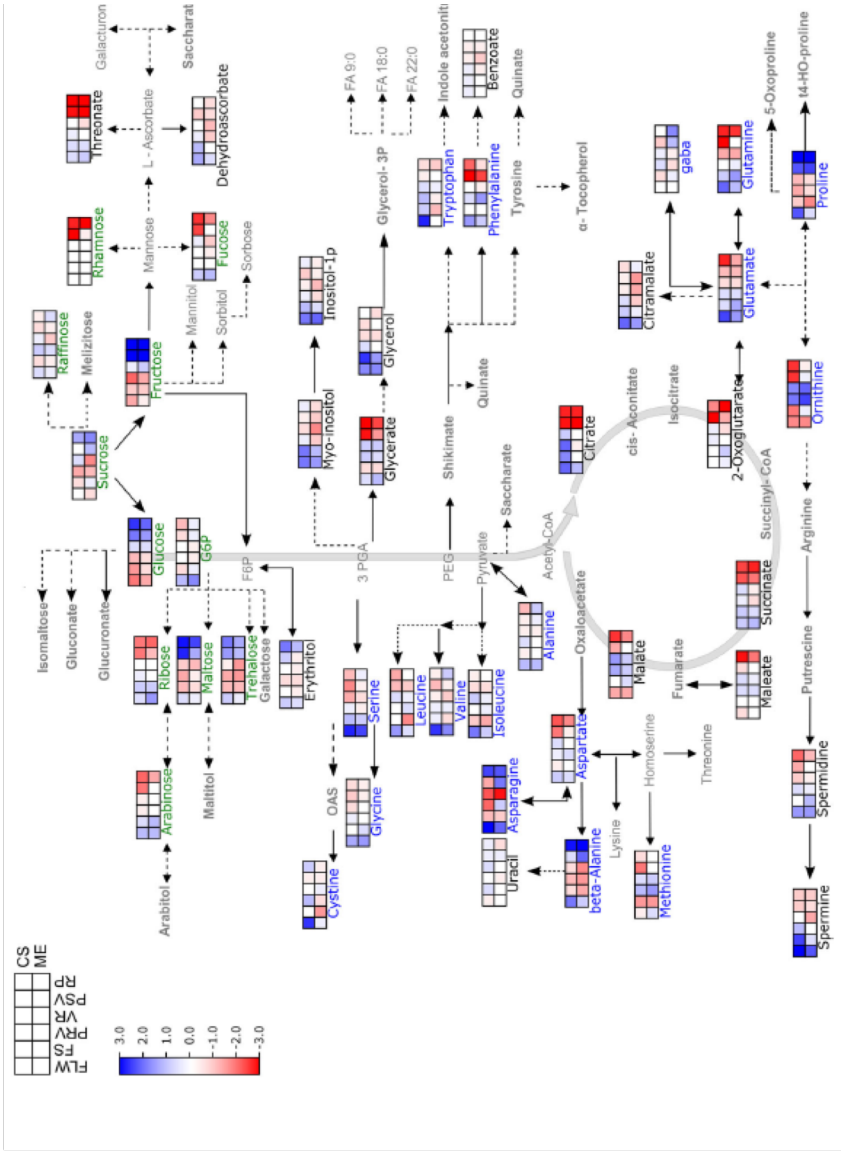

Figure 5. Scheme of the primary metabolism pathways of metabolites in Cabernet Sauvignon (CS) and Merlot (ME) cultivars during different grape development stage. Pathways are simplified version of tricarboxylic acid cycle, glycolysis, amino acid and sucrose synthesis. FLW, flowerin; FS, fruit setting; PRV, pre-veraison; VR, veraison; PSV, post-veraison; RP, ripening. Metabolite intensity is color coded. Reproduced with permission from [92].

Bearing in mind that traceability involves knowing the composition and origin of a food, it is clear that the determination of the geographical origin may be considered the starting point for food traceability. Geographical origin assessments have not only relevant implications from an economical point of view but also they are a key parameter in terms of food quality. The most common metabolomics strategies developed to discriminate food samples according to their geographical origin are non-targeted approaches based on the use of LC (mainly UHPLC) coupled with HRMS. Using the most suitable sample preparation protocols according to the features of each food sample and the appropriate multivariate data analysis, these MS-based methodologies are able to point out different metabolites as potential markers of food origin. This kind of approaches has successfully been applied for the origin assessment of extra virgin olive oil (EVOO) [116] orange [117], hazelnuts [118] or cocoa beans [119].

Other relevant branch in food traceability is focused on monitoring changes in the food metabolic profiles produced by food processing. Production steps, including for instance, heat treatments, fermentation, and storage, among others, can alter nutritional and organoleptic properties of foods, as well as lead to a substantial loss of health-promoting compounds. This fact has been demonstrated by a recent and interesting non-targeted UHPLC-QTOF-MS method developed to evaluate the phenolic profiles of three different processed tomato products and tomato paste produced by three different treatments [120]. The combination of the results obtained from the metabolomics analysis with total phenolic and lycopene content, and antioxidant capacity showed that processing affects the nutritional and health- promoting potential of tomato products. Besides, the metabolomics approach shows its high potential in traceability purposes since the treatment provides a characteristic phenolic profile.

Other non-targeted LC-HRMS platforms have also been applied with success to study the effect of storage conditions on the metabolic profile of red wine [121] or iceberg lettuce [122], as well as to compare the effects of thermal processing on *Brassica* vegetables [123]. After processing and carrying out the multivariate data analysis, the final purpose of this kind of studies is to find the relationship between the changes on the metabolite profile with a loss of food quality. **Figure 6** shows an example of the data analysis procedure followed to explore the metabolome of lettuce in order to evaluate changes related to storage time and genetics.

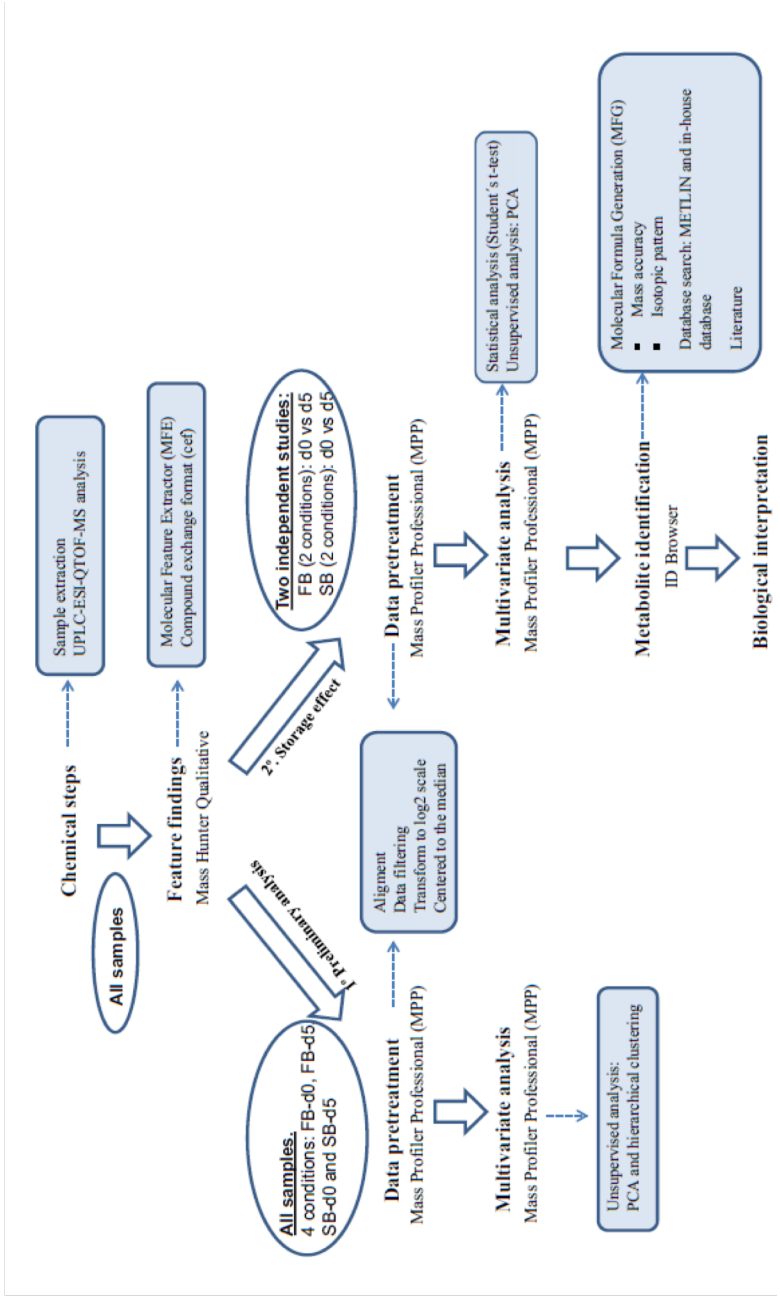

Figure 6. Data analysis workflow to explore the metabolome of lettuce in order to evaluate changes related to storage time and genetics. FB fast-browning cultivar, SB slow-browning cultivar, d0 day 0, d5 day 5. Reproduced with permission from [122].

Fermentation and ripening are also relevant process which may change the food metabolome. Two interesting examples have been described in the literature to explore the changes that occur in the metabolic profile of cocoa beans [124] and cheese [125] as a consequence of fermentation and ripening process, respectively. Bearing in mind that these two processes are critical steps in the processing of high quality cocoa beans or in the formation of specific characteristics of cheese, the results obtained in these metabolomics assays are of high value for the food industry since they shed new light into fermentation and ripening optimization.

Even though most applications developed for food traceability in the period of time covered by this review are based on the coupling of MS with LC, GC-MS methodologies have also been proposed. For instance, using headspace GC-MS non-targeted approach was possible to distinguish the effect of different process steps (including not only thermal processing but also blanching and high hydrostatic pressure) on the chemical composition of mango [126]. Once again, the results obtained clearly demonstrate the influence of these steps on the volatile profiles of processed products. GC-MS metabolomics approach has also proven to be an excellent tool to evaluate the modifications that may occur during the cooking of different types of pasta [127].

Another possibility gaining interest in recent times is the use of CE coupled to MS as analytical platform for traceability assays. For example, Sugimoto et al. developed two CE-TOF-MS methodologies for anionic and cationic metabolite analysis of dry-cured ham [128]. The results obtained enabled to establish a correlation between the metabolite profiles of twelve kinds produced in different countries and processed under different conditions and the ripening period and processing conditions. Even though CE-MS strategies are being mainly developed and applied for biological samples, nowadays, is possible to find some applications devoted to food analysis. Further progress in this field is expected in the near future.

Although non-targeted strategies have been the most-extended approach to evaluate changes in the metabolic profiles of food samples during food-processing, targeted analysis may also be very useful; this kind of approaches has been employed to evaluate the metabolic changes that take place in two starch potato genotypes in response to osmotic stress [129] or during avocado development and maturation [130].

5. Conclusion and future outlooks

As it can be deduced from the update shown in this review paper, the use of MS-based approaches for food safety, food quality and traceability is still far from reaching its maximum potential. It is quite obvious that the use of MS, particularly high resolution MS, will still be dominant in studies on the mentioned fields in the years to come. In this regard, the continuous improvement of available instruments will be translated to enhanced capabilities of the developed methods. As MS is most frequently used hyphenated to other analytical tools, the improvement on robustness of couplings and available interfaces and ionization tools, including those employed in direct analysis, will positively influence the obtainable results. This way, new to-be-controlled substances appearing in the market as well as unknown ways to perform frauds during production of valuable food products could be discovered. Specifically, within the food safety field, new multi-residue and multi-targeted methods will surely continue appearing, ready to help on the food control area. However, more interestingly, the development of novel non-targeted metabolomics-based approaches will help to gain a holistic view of the food safety issue. Those procedures are clearly more capable of discovering new safety hazards beyond the use of the regulated compounds and contaminants. But those approaches could have even more potential if accompanied by proper *in-vitro* and *in-vivo* assays, so that the perspectives may be further opened, for instance, to the discovery of markers of toxicity.

Food quality will also benefit from the extension of metabolomics MS-based approaches to other studies. Within this field, the further application and development of these methodologies could help to increase the available knowledge on which compounds present in food that may have a still concealed importance for food quality perception. This is the case, for example, of the application of this kind of procedure to reveal the whole sensory pattern of a food product, a concept already applied in flavoromics researches. Likewise, as metabolomics methods evolve in the future, new relationships between food components and particular characteristics related to food quality will be discovered.

Regarding traceability, much effort is expected to be focused on the development of new methodologies to assess food authentication and geographical origin of valuable food products. However, this field is intimately linked to food quality as some traceability aspects are related to quality. For instance, development of traceability potential will help to discover how production processes throughout the food production and commercialization chain may affect quality parameters. In this regard, the use of alternative analytical techniques to LC and GC, such as CE or

multidimensional approaches (including LC x LC and GC x GC) could offer complementary selectivity and thus, information, that would help to increase the metabolite coverage of the studied system. This enhanced coverage could positively influence the applicability of MS-based metabolomics studies in the three different mentioned fields.

In summary, it is clear that although the interest of using MS-based metabolomics approaches in food safety, quality and traceability is already high, further developments in these methodologies will have a great influence on the mentioned fields in the near future.

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CHAPTER II

OBJECTIVES

The main objective of this PhD Thesis has been the development of sensitive and selective chiral and omics analytical methodologies for their application to the estereoselective separation of compounds of interest, the search of markers of food processing, and the separation and identification of bioactive compounds from natural sources and by-products from the food industry. To achieve this goal, innovative analytical strategies were developed using Micro-Separative Techniques and Ion Mobility Spectrometry.

With this aim, the following specific objectives were proposed:

- To develop advanced chiral analytical methodologies by Electrokinetic Chromatography and Ion Mobility Spectrometry enabling the estereoselective separation of protein and non-protein amino acids and/or their determination in food samples.

- To implement a multiplatform metabolomic strategy based on two orthogonal platforms (LC-MS and CE-MS) to provide an exhaustive characterization of the metabolome of coffee beans and to apply it to the search of markers of the coffee roasting process.

- To propose new analytical methodologies based on LC-MS for the separation and identification of bioactive peptides in protein hydrolysates obtained from edible macroalgae and coffee silverskin in order to investigate their potential as new sources of bioactive peptides.

A working plan was designed to achieve these objectives, which is schematized in **Figure II.1.**

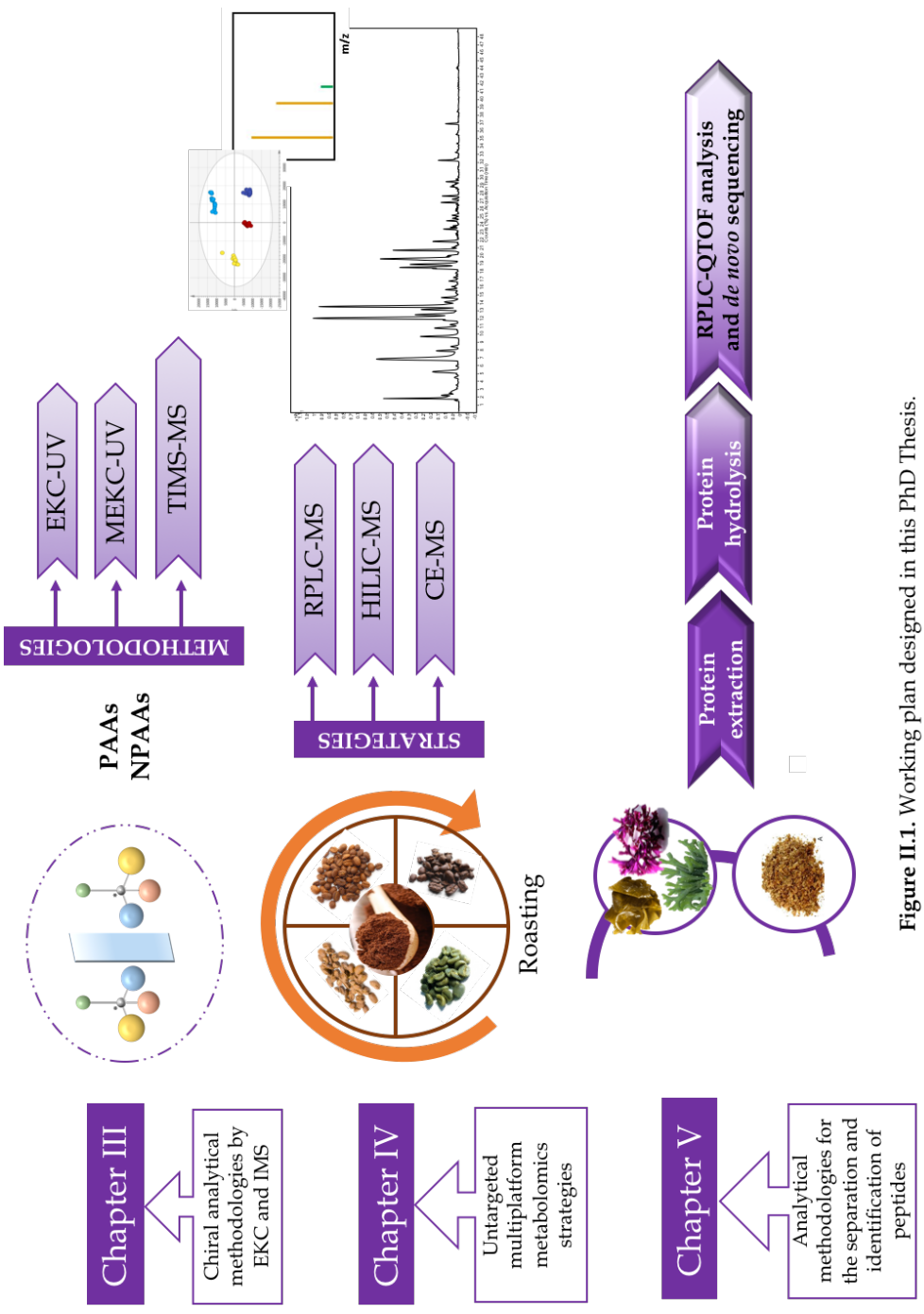

Figure II.1. Working plan designed in this PhD Thesis.

CHAPTER III

DEVELOPMENT OF ANALYTICAL METHODOLOGIES FOR THE ENANTIOMERIC SEPARATION OF PROTEIN AND NON-PROTEIN AMINO ACIDS BY ELECTROKINETIC CHROMATOGRAPHY AND ION MOBILITY SPECTROMETRY-MASS SPECTROMETRY

III.1. Preface

As mentioned in the Introduction of this PhD Thesis, the enantiomeric separation of PAAs and NPAAs is an interesting topic in different research fields due to the different biological activities that the enantiomers of a chiral AA may have. In the field of Food Analysis, the enantioseparation of AAs has demonstrated to have a high potential to obtain relevant information in different areas such as the detection of food adulterations, the assessment of food authenticity or the evaluation of food processing, among others. Bearing in mind that the addition of D-enantiomers in the elaboration of foods and dietary supplements is forbidden by regulatory agencies [12, 13], the development of analytical methodologies able to provide the enantiomeric separation of PAAs and NPAAs and the determination of enantiomeric impurities is essential.

As it can be deduced from the low number of articles reporting the chiral analysis of AAs in foods by CE and IMS (section I.1.6), it is clear that the use of both techniques in this topic is still far from reaching its maximum potential. Then, to explore the possibilities of CE and IMS as separation techniques capable of providing the chiral separation of PAAs and NPAAs, three different approaches have been investigated in this PhD Thesis. The first one is based on the use of EKC with UV detection and CDs as chiral selectors to achieve the enantiomeric separation of ten NPAAs and the determination of Cit enantiomers in food supplements. The second one is aimed to the enantioselective separation of SeMet in food supplements by MEKC-UV using a diastereomeric derivatization and a volatile surfactant. The last one is focused to investigate the possibilities of developing a new, fast and simple TIMS-MS methodology enabling the discrimination of the enantiomers of twenty-one PAAs and NPAAs.

III.2. Objectives

The objectives of this chapter were:

- To develop novel EKC-UV methodologies based on the use of CDs as chiral selectors to achieve the enantiomeric separation of ten NPAAs.

- To apply the developed EKC-UV methodologies to the determination of L-Cit in food supplements, to assess the absence of the enantiomeric impurity (D-Cit) in these samples, and to investigate the effect of a long storage time on Cit racemization.

- To develop a MEKC-UV methodology based on the use of FLEC as chiral derivatizing reagent enabling the separation of SeMet diastereomers and the determination of L-SeMet in food supplements.

- To investigate the potential of TIMS-MS using FLEC as chiral derivatizing reagent for the individual and simultaneous enantiomeric separation of twenty-one PAAs and NPAAs.

III.3. Results and discussion

III.3.1 Enantiomeric separation of NPAAs by EKC

Due to the lack of absorbance of most of the NPAAs studied, a pre-capillary derivatization step with FMOC was strictly needed to achieve their UV detection. This fact also enabled to obtain bigger molecules which can favor the selector-selectant interactions.

To carry out the enantioselective separation of the ten NPPAs studied (Pyro, Norval, Norleu, DOPA, SeMet, Hcy, Orn, Aminoadipic, Cit, and Pipe), the first step was the selection of the chiral selector. Thus, the discrimination power of seven anionic CDs under acid conditions (100 mM formate buffer at pH 2.0 and pH 4.5) and ten neutral CDs under basic conditions (100 mM borate buffer at pH 9.0) at a concentration of 10 mM was evaluated. Under basic conditions only Aminoadipic and SeMet were partially resolved, whereas none of the NPAAs analyzed were enantioseparated employing anionic CDs at pH 4.5. On the contrary, the use of formate buffer at pH 2.0 containing some sulfated CDs (α, β, or γ) or phosphate-β-CD enabled to obtain the enantiomeric resolution of eight of the ten NPPAs studied. Under these conditions, the effect of three different temperatures (15, 20 and 25 ^{o}C) was evaluated. From the results obtained, it was not possible to establish a general trend since the temperature variation gave rise to a decrease or an increase in the resolution of each NPAA studied. Basically, the use of sulfated α-CD or sulfated γ-CD as chiral selectors enabled to achieve the separation of eight of the ten NPAAs (Pyro, Norval, Norleu, DOPA, Aminoadipic, SeMet, Cit, and Pipe). Among them, the separation of Pyro and Norleu was only achieved using sulfated α-CD, whereas Pipe and Cit were only separated using sulfated γ-CD. The effect of the CDs concentration (1, 2, 5 and 10 mM) and the separation voltage (-20, -25 and -30 kV) were also evaluated with the aim of improving the chiral separation in terms of resolution and analysis time. However, it did not allow to obtain an improvement of previous results.

Under the optimal conditions obtained for each NPAA, it was possible to achieve the enantioseparation of Cit, Norval, DOPA, and Pipe with analysis times between 18 and 35 min and resolution values from 1.5 to 7.4. For other NPAAs, resolutions were lower than 1.5 (Pyro, Aminoadipic, Norleu and SeMet with resolutions of 0.9, 1.1, 0.7 and 1.2, respectively). It should be highlighted that when using sulfated γ-CD as selector, it was also possible to obtain the simultaneous separation of mixtures of some NPAAs such as Pipe, Cit, and Aminoadipic.

To demonstrate the potential of the developed method, the EKC-UV methodology based on the use of formate buffer at pH 2.0 containing sulfated γ-CD as BGE was applied to the enantiomeric determination of Cit in food supplements. Some experimental variables such as capillary length, buffer pH, temperature, and injection time were modified in order to decrease the migration time and to achieve the best enantiomeric resolution. Moreover, the analytical characteristics of the method were evaluated in terms of selectivity, linearity, accuracy, precision, LODs and LOQs for Cit enantiomers, showing its potential to determine Cit enantiomers in food supplements. In addition, the LOD obtained for D-Cit (2.1×10^{-7} M) allowed detecting up to a 0.1 % of this enantiomeric impurity. Finally, the methodology was applied to the analysis of L-Cit in six different food supplements (three of them recently acquired and other three submitted to a long storage time). L-Cit was determined in percentages between 65-97 % with respect to the labeled amounts in the samples recently acquired. The results obtained for the contents of L-Cit in samples submitted to a long storage demonstrated (by comparison with the values obtained before the expiration of these samples) that the content of L-Cit decreased with the storage time. However, this fact could not be attributed to a racemization from L to D-Cit since the D-form could not be detected in any of the analyzed samples.

The proposed EKC-UV methodology showed its suitability for routine food quality control to guarantee the accomplishment of legal regulations and the requirements of the food industry.

III.3.2. Enantiomeric separation of selenomethionine by MEKC

Taking into account the poor enantiomeric resolution (1.2) and long migration time (32 min) obtained for the separation of SeMet enantiomers by EKC with CDs as chiral selectors (section III.3.1), a new approach, based on the use of MEKC and the formation of stable SeMet diastereomers with a chiral reagent, was developed.

In this new strategy, FLEC was employed as chiral derivatizing reagent for the rapid formation of two SeMet diastereomers that could be subsequently separated in a MEKC system. Moreover, this derivatization also made SeMet detectable in a UV system.

To propose a MEKC methodology enabling the chiral separation of SeMet, a surfactant above its critical micellar concentration (CMC) was added to the separation buffer. Usually, SDS is described in the literature as the surfactant most employed for the chiral separation of AAs using chiral selectors; however, the use of perfluorooctanoic acid (APFO) as negatively charged semi-volatile pseudostationary phase is a good alternative mainly thinking in a future coupling with MS detectors. With this aim, APFO at a concentration above its CMC (25 mM) at pH 9.5 was selected for the formation of micelles. Under these conditions, the diastereomers FLEC-SeMet were negatively charged.

To achieve the most favorable experimental conditions allowing the shortest migration time and the best chiral resolution, different parameters concerning BGE and CE conditions were optimized. For instance, the influence of APFO concentration (50, 75, 100, 125 and 150 mM) was evaluated. Concentrations higher than 100 mM resulted in high current values and certain instability (>85 μA). Moreover, migration times also increased. On the contrary, concentrations lower than 100 mM gave rise to shorter migration times, but also to a loss of resolution. For this reason, 100 mM of APFO was selected for further experiments as a compromise between migration times and current values. The effect of the temperature (15, 20 and 25 ^{o}C) was also evaluated showing that higher temperature values resulted in shorter migration times, but also in a loss of enantioresolution and current instability. Therefore, 20 ^{o}C was selected as optimum temperature since it was possible to obtain enantioresolutions of 3.6 in less than 6 min. With the aim of improving the sensitivity, the effect of the injection volume was investigated by testing different injection times (4, 8, 12, 16 and 20 s). Obviously, the higher is the injection volume, the greater is the sensitivity; however, it also gives rise to a loss of resolution. Since an injection time of 20 s still enabled to reach a high resolution (2.8), it was selected for further analyses. Finally, conditions related to the derivatization procedure such as the FLEC/SeMet ratio and the reaction time, were also investigated to reach the maximum reaction yield. On the one hand, the study of the variation of FLEC concentration (1, 3, 6, 8 10 and 12 mM) keeping constant the concentration of SeMet showed that the maximum yield for the derivatization reaction was reached at 6 mM. On the other hand, when the effect of different

reaction times (2, 5, 10, 15, 20, 30 min) was studied, it was pointed out that the maximum yield of reaction was obtained at 10 min.

Under the optimized conditions, SeMet diastereomers were separated with good resolution; however, after several analyses, the use of high pH values (as it was previously indicated in the literature [235]) generated problems related to current instability resulting in the damage of the capillary. For this reason, the pH was decreased from 9.5 to 8.5 to avoid the current leakage. It allowed the SeMet separation in less than 6 min with a resolution value of 4.4.

The analytical characteristics of the developed MEKC-UV methodology were evaluated in terms of selectivity, linearity, accuracy, precision, LODs and LOQs for SeMet enantiomers. Adequate values were obtained for all these parameters and LODs of 3.7×10^{-6} M were reached both for L- and D-SeMet.

The suitability of this methodology was demonstrated by its application to the determination of L-SeMet in two different food supplements. L-SeMet was determined in a percentage of 126%, with respect to the labeled amount, for one of the sample analyzed whereas the amount of L-SeMet in the second one could not be quantified since the concentration was lower than the LOD, indicating that the real content of this supplement was lower than the labeled one. Even though the developed MEKC methodology enabled the separation of L- and D-SeMet with high resolution in a short analysis time, its main limitation was its low sensitivity for the detection of D-SeMet since the control of the enantiomeric impurities requires lower LOD values. However, the separation conditions employed in this method, based on the use of the semivolatile surfactant APFO, are fully compatible with MS systems, which open the possibility of using MS detection to enhance the sensitivity and to obtain the enantioselective determination of SeMet in more complex food matrices.

III.3.3. Enantiomeric separation of PAAs and NPAAs by TIMS-MS

As mentioned in section I.1.6, the direct separation of AAs enantiomers by TIMS is not possible; however, this technique has demonstrated its potential for the separation of isomeric compounds, so that its capacity to resolve chiral AAs previously derivatized to form their corresponding diastereomers was investigated by TIMS-MS.

For this purpose, FLEC was used as chiral derivatizing reagent (using a concentration in excess) to make possible the reaction with one or more amino groups of AAs. SeMet and Orn were selected as model compounds for the optimization of derivatization conditions. Preliminary experiments showed the

formation of a mixture of protonated and sodiated ions after ESI ionization for both compounds. This fact was attributed to the formation of sodium-adducts produced due to the nature of the buffer employed for the derivatization, which contained sodium ions. After studying the TIMS results, the extracted-ion traces of the disodiated ions $[FLEC_2\text{-Orn+2Na-H}]^+$ and $[FLEC\text{-SeMet+2Na-H}]^+$ revealed two baseline separated peaks, suggesting the separation of the respective diastereomers, whereas the protonated ions ($[FLEC_2\text{-Orn+H}]^+$, $[FLEC\text{-SeMet+H}]^+$), and ions containing one Na ($[FLEC_2\text{-Orn+Na}]^+$ and $[FLEC\text{-SeMet+Na}]^+$) showed only one peak for each AA. This seemed to indicate that the presence of Na is a requirement for the chiral recognition of AAs.

To confirm the separation of diastereomers and exclude the possibility of multiple analyte peaks due to different positions of the sodium-adduct charge, the L-enantiomer of both AAs, as well as D/L mixtures of both at different ratio were analyzed. In both cases, only one peak was observed for the L enantiomer whereas two peaks were observed for the 1:1 and 1:3 D/L mixtures. Moreover, clearly higher peak intensities were observed for 1:3 D/L mixture compared with 1:1 D/L mixture, which confirmed the diastereomeric separation.

To evaluate if Na-adducts formation is a requirement for the enantiomeric discrimination of AA enantiomers, the effect of using other alkali-cations (K^+ and Li^+) was investigated. No separation was obtained when Li^+ was used whereas a partial separation was achieved for FLEC-Orn when K^+ was tested. These results pointed out that the presence of Na^+ was effectively essential for the chiral recognition. At this point, the effect of sodium concentration used to prepare buffer derivatization was studied showing no changes either in peaks intensity or in the diastereomeric resolution when it was increased from 26 to 50 mM. Keeping the buffer concentration constant, the effect of AA concentration (0.1, 0.18, 0.35, and 0.7 mM) was also investigated. Regardless of the AA concentration employed, both the relative intensity of sodium adduct ions formed and the ratio between ions containing one and two sodium ions remained constant.

Finally, to demonstrate the potential of the methodology to achieve the diastereomeric discrimination of different AA, it was applied to the chiral separation of 17 PAAs (Ile, Leu, Val, Asn, Glu, Gln, Pro, Thr, Arg, Lys, Tyr, Ala, Met, Phe, Ser, Trp, and His) and 4 NPAAs (Orn, SeMet, Aminoadipic, and Pipe). To achieve the diastereomeric separation of each AA, different parameters, such as voltage range, voltage ramp time and accumulation times, were adjusted. Among them, the accumulation time demonstrated to have a high influence since the sensitivity is determined by the time that ions are accumulated. A total of 17 FLEC-AAs from the 21 studied were separated (Glu, Pro, Thr and Ala were not separated).

As expected, the separation was obtained in most of the cases for disodiated FLEC-AAs except for Ile, Leu and Val whose separation was observed for the singly sodiated species, and in some cases two FLEC molecules were observed for AAs containing two amino groups in their structure. The ΔK0 value determined for AAs ranged from 0.09 for Lys (only partially resolved) to 0.061 for Asn, and the resolution (K0/ΔK0) ranged from 80.3 for Tyr to 160.0 for Ser. Preliminary results of the analytical characteristics showed detection limits in the nM range and the possibility to determine enantiomeric ratios down to 2.5 %. Good reproducibility was observed for diastereomer mobilities as well as for CCS values (RSD lower than 1 %). Moreover, the feasibility of the methodology to resolve multiple AAs in a run was demonstrated by using different voltage ramps.

The developed TIMS-MS methodology enabled the rapid (less than one min) and easy chiral discrimination of AAs avoiding the use of chiral volatile reagents in the gas-phase, the formation of chiral volatile complexes and the use of specific reference compounds. The proposed method simplifies and uniforms the formation of diastereoisomers compared with other approaches previously reported by IMS [22, 23, 25, 26, 45] and it can be applied to a larger number of AAs.

The results obtained in this chapter are included in the following scientific articles:

- **Article 4:** *Enantiomeric separation of non-protein amino acids by electrokinetic chromatography.*
  R. Pérez-Míguez, M. L. Marina, M. Castro-Puyana.
  J. Chromatogr. A 1467 (2016) 409–416.

- **Article 5:** *A micellar electrokinetic chromatography approach using diastereomeric derivatization and a volatile surfactant for the enantioselective separation of selenomethionine.*
  R. Pérez-Míguez, M. L. Marina, M. Castro-Puyana.
  Electrophoresis, under first revision.

- **Article 6:** *Fast chiral discrimination of DL-amino acids by trapped ion mobility spectrometry after derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate.*
  R.Pérez-Míguez, B. Bruyneel, M. Castro-Puyana, M. L. Marina, G. W. Somsen, E. Domínguez-Vega.
  Anal. Chem. 91 (2019) 3277-3285.

Article 4

Enantiomeric separation of non-protein amino acids by electrokinetic chromatography.

R. Pérez-Míguez, M. L. Marina, M. Castro-Puyana.

J. Chromatogr. A 1467 (2016) 409-416.

Abstract

New analytical methodologies enabling the enantiomeric separation of a group of non-protein amino acids of interest in the pharmaceutical and food analysis fields were developed in this work using Electrokinetic Chromatography. The use of FMOC as derivatization reagent and the subsequent separation using acidic conditions (formate buffer at pH 2.0) and anionic cyclodextrins as chiral selectors allowed the chiral separation of eight from the ten non-protein amino acids studied. Pyroglutamic acid, norvaline, norleucine, 3,4-dihydroxyphenilalanine, 2-aminoadipic acid, and selenomethionine were enantiomericaly separated using sulfated-α-CD while sulfated-γ-CD enabled the enantiomeric separation of norvaline, 3,4-dihydroxyphenilalanine, 2-aminoadipic acid, selenomethionie, citrulline, and pipecolic acid. Moreover, the potential of the developed methodologies was demonstrated in the analysis of citrulline and its enantiomeric impurity in food supplements. For that purpose, experimental and instrumental variables were optimized, and the analytical characteristics of the proposed method were evaluated. LODs of 2.1 x 10^{-7} and 1.8 x 10^{-7} M for D- and L- citrulline, respectively, were obtained. D-Cit was not detectable in any of the six food supplement samples analyzed showing that the effect of storage time on the racemization of citrulline was negligible.

1. Introduction

Non protein amino acids are a group of compounds which are not found as protein constituents. A huge amount of non-protein amino acids (more than 800) from different origin and with diverse functions have been described. Many of them are the end products of secondary metabolism, whereas many others are intermediate of metabolic pathways [1]. Although these compounds have been studied to a lesser extent than protein amino acids, they have demonstrated to be relevant in the pharmaceutical, clinical, and food fields. In fact, some of them present relevant biological activities, for instance, DOPA is applied in the treatment of Parkinson´s disease [2] and norleucine is related to the inhibition of the oxidative stress associated with Alzheimer's disease (AD) [3]. Non-protein amino acids can also be present in foods as metabolic intermediates, as products formed during food processing or as additives in food to increase some nutritional and functional properties [4, 5], and they have demonstrated to be relevant markers of the food quality and safety. For example, the study of these compounds allowed to detect adulterations of olive oil with seed oils [6-8], to evaluate food processing

(fermentation, storage, thermal treatment) [9-12] as well as to determine the nutritional quality and toxicity of some foods [7, 8, 13].

A relevant factor that should be considered in the analysis of non-protein amino acids is their chiral behavior. In nature, they are generally composed by their L-forms, however the racemization into the D-form may be produced through different metabolic pathways, by different processing conditions employed by the food industry to improve food characteristics [14-16], synthesized in enzymatic pathways by the action of microorganisms or they can even be present in supplemented foodstuffs due to the fraudulent addition of racemic mixtures [17, 18]. In fact, although the use of racemic mixtures of non-protein amino acids is allowed in the pharmaceutical industry [19] the use of D-enantiomers in the food industry is forbidden and the regulatory agencies prevent their use in the elaboration of foods and dietary supplements [20]. All these facts originate that nowadays the chiral analysis of non-protein amino acids is of high interest in different fields.

CE has shown to be a powerful tool to carry out chiral separations since it allows short analysis times, high separation efficiency, versatility and feasibility to incorporate a great variety of chiral selectors to obtain high resolutions. EKC is the CE mode usually employed to achieve chiral separations. From the first research works dealing with the use of EKC [21-23], this technique has experienced an enormous growth being nowadays one of the best options for analytical enantioseparations [24]. Regarding non-protein amino acids, just a few research works reported the enantiomeric determination of non-protein amino acids in foods by CE. Thus, EKC-UV methodologies were developed for the chiral separation of ornithine in fermented foods (beer, vinegar and wine) and food supplements [11, 25-27], S-adenosyl-L-methionine (SAM) in fruit juices [28] whereas the coupling EKC-MS2 enabled the evaluation of fermentation processes through the enantiomeric determination of ornithine in beer samples or to identify and quantitate L-carnitine in infant formulas and dietary supplements as well as its enantiomeric impurity (D-carnitine) [29, 30]. Also, a CEC method with UV detection has been described to the determination of citrulline in food supplements [31].

The aim of this work was to develop novel EKC-UV methodologies based on the use of cyclodextrins as chiral selectors to carry out the chiral separation of ten non-protein amino acids (pyroglutamic acid, norvaline, norleucine, 3,4-dihydroxyphenilalanine (DOPA), selenomethionine, homocysteine, ornithine, 2-aminoadipic acid, citrulline and pipecolic acid) that have demonstrated to play an important role in some metabolic routes or to have some interesting properties and healthy benefits. L-citrulline and L-ornithine, which can be synthetized during the

metabolism of L-arginine [32], have been used in dietary supplements to favour the metabolism of corporal fatty excess and reduce ammonia levels [31, 27]. D-Cit has demonstrated to have different in vivo/in vitro behavior compared with L-Cit [31] while D-orn has shown toxic effects at high levels and to be an inhibitor of urea synthesis by competition with the L-enantiomer [25]. L-norvaline is also related with L-arginine metabolism since it is an inhibitor of arginase activity, reducing urea production and increasing NO production [32]. L-pyroglutamic acid and selenomethionine are also of high relevance in human health since the first has a number of remarkable cognitive enhancing effects [33] (its L-enantiomer is bioactive to improve blood circulation in the brain, whereas D-pyroglutamic remains inactive [34]) and the second is considered the major source of selenium to humans which is indispensable for human health since its deficit could cause an adverse impact on immune system. While L-selenomethionine occurs naturally, D-selenomethionine is toxic at high levels [35]. Regarding pipecolic acid, it is considered to be a neuromodulator which play a role in the central inhibitory γ-aminobutyric acid system. The presence of its D enantiomer in fluids and tissues can be due to food intake or production by intestinal bacteria. [12]. Due to the scarce number of studies devoted to the enantiomeric separation of these non-protein amino acids by CE, the development of analytical methodologies with this aim presents a high relevance.

2. Materials and methods

2.1. Reagents and samples

All reagents employed were of analytical grade. Boric acid, sodium hydroxide, ammonium hydroxide, and pentane were obtained from Sigma-Aldrich (Madrid, Spain). Formic acid, hydrochloric acid, and acetonitrile were from Scharlau (Barcelona, Spain). The chiral selectors β-CD, Heptakis (2,3,6-tri-O-methyl)-β-CD, (2-Hydroxi)propyl-β-CD (DS~3), γ-CD, carboxymethyl-β-CD were purchased from Fluka (Buchs, Switzerland), methyl-β-CD, Heptakis(2,6-di-O-methyl)-β-CD, sulfated-β-CD, succinyl-β-CD from Sigma-Aldrich (Madrid, Spain), and (2-hydroxi)propyl-γ-CD, methyl-γ-CD, acetylated-β-CD, acetylated-γ-CD, carboxyethylated-β-CD (DS~3), phosphated-β-CD, sulfated-γ-CD (DS~14), and sulfated-α-CD (DS~12) from Cyclolab (Budapest, Hungary). Water used to prepare solutions was purified through a milli-Q System from Millipore (Bedford, MA, USA).

DL-Norvaline (Norval), DL-Norleucine (Norleu), 3,4-Dihydroxy-DL-phenylalanine (DOPA), DL-Homocysteine (Hcy), DL-2-Aminoadipic acid (Aminoadipic), DL-Selenomethionine (SeMet), D-Pipecolic acid (D-pipe) and D-

Ornithine (D-Orn), were supplied from Sigma-Aldrich (Madrid, Spain), whereas D-Citruline/L-Citruline (D-Cit/L-Cit), D-Pyroglutamic acid/L-Pyroglutamic acid (D-Pyro/L-Pyro), L-Pipecolic (L-Pipe) and L-Ornithine (L-Orn) were obtained from Fluka (Buchs, Switzerland).

The derivatization reagent 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) was purchased from Sigma-Aldrich (Madrid, Spain).

The six different food supplements analyzed were acquired in different Madrid´s markets (Spain).

2.2. CE conditions

CE experiments were carried out with an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) equipped with a DAD working at 210 nm with a bandwidth of 4 nm. The instrument was controlled by HP3DCE ChemStation from Agilent Technologies. Separations were performed using uncoated fused-silica capillaries of 50 μm ID (362.8 μm OD) with a total length of 58.5 cm (50 cm effective length) or with a total length of 48.5 cm (40 cm effective length) purchased from Polymicro Technologies (Phoenix, AZ, USA). The samples were injected by applying a pressure of 50 mbar from 4 to 20 s, and the electrophoretic separation was achieved using reverse-polarity mode (voltage of -20 kV) and working temperature from 15 to 25 ^{o}C.

Before its first use, new capillaries were rinsed (applying 1 bar) with 1M sodium hydroxide for 30 min, followed by 5 min with Milli-Q water and conditioned with buffer solution for 60 min. At the beginning of each day the capillary was pre-washed (applying 1 bar) with 0.1 M sodium hydroxide during 10 min, Milli-Q water for 5 min, and buffer for 40 min, and BGE during 10 min. Between injections, the capillary was conditioned with 0.1 M hydrochloric acid (2 min), Milli-Q water (1 min) and BGE (5 min).

2.3. Preparation of solutions and samples

The borate buffer solution (200 mM, pH 9.0) required to dissolve the non-protein amino acids before the derivatization step was prepared by dissolving the appropriate amount of boric acid in Milli-Q water. The separation buffer solution was prepared by diluting the appropriate volume of formic acid with Milli-Q water and adjusting the pH to the desired value with hydrochloric acid before completing the volume with water to get the desired buffer concentration (100 mM). The BGE was obtained by dissolving the suitable amount of the appropriate chiral selector (CDs) in the separation buffer.

Stock standard solutions of each non-protein amino acid were prepared dissolving the appropriate amount in borate buffer (200 mM, pH 9.0). These solutions were stored at 4 ºC until the derivatization step with FMOC.

To prepare sample solutions of the six dietary supplements analyzed, the content of four capsules was weighed, powdered and mixed homogeneously. Taking into account the labeled amount of Cit in each supplement, an appropriate amount of the powdered obtained was dissolved in Milli-Q water to obtain a standard solution of 50 mM. Dissolution was performed by ultrasonication for 10 min followed by centrifugation (15 min, 4000g at 25 ºC) and filtration. Before derivatization, these sample solutions were diluted in borate buffer (200 mM, pH 9.0) to obtain the appropriate concentration.

All solvents and samples were filtered prior use through 0.45 μm pore size disposable nylon filters from Scharlau (Barcelona, Spain). A pH meter (Metrohn 744, Herisau, Switzerland) was used for the pH adjustment of buffer solutions.

2.4. Derivatization

Derivatization of non-protein amino acids (except pyro which was not derivatized) was carried out following a methodology previously described in the literature with slightly modifications [36, 37]. The desired concentration of FMOC chloride in ACN was freshly prepared each day taking into account that an excess of at least three times of FMOC was necessary to obtain a complete derivatization of the non-protein amino acids.

Briefly, 300 μL of a 10 mM non-protein standard amino acid or sample solutions (diluted with 200 mM borate buffer at pH 9.0) were mixed with 300 μL of FMOC chloride dissolved in ACN. The solution was kept at room temperature for 2 min to complete reaction, and the resulting solution was extracted with 0.6 mL pentane to remove FMOC excess. Then, this solution was diluted ten times with Milli-Q water before injection in the CE system.

2.5. Data treatment

Chiral resolution values (calculated from the migration times of enantiomers and their peak widths at half height) and migration times were obtained using the Chemstation software from Agilent Technologies. Experimental data analysis, calculation of different parameters, and composition of graphs with different electropherograms were carried out using Excel Microsoft, Statgraphics Centurion XVI, and Origins 8.0 software.

3. Results and discussion

3.1. Development of enantioselective analytical methodologies for the separation of non-protein amino acids.

In order to develop chiral analytical methodologies for the separation of the enantiomers of the non-protein amino acids investigated, a pre-capillary derivatization step with FMOC chloride was performed since the lack of absorbance of most amino acids makes essential their derivatization when UV detection is employed. FMOC chloride was chosen because it is a fast labeling reagent producing stable amino acids derivatives using simple reaction conditions [38]. Besides to allow the UV detection, FMOC derivatization enables to obtain large molecules which can favor in some cases the interaction with the chiral selectors. Regarding chiral selectors, cyclodextrins were chosen because they have demonstrated to be one of the best options in the field of chiral separations by EKC [39, 40].

3.1.1 Effect of the cyclodextrin nature and the separation buffer (nature and pH)

The buffers employed were 100 mM borate at pH 9.0 and 100 mM formate at two different pH values, pH 2.0 and pH 4.5. Under basic conditions, the discrimination power of ten different neutral cyclodextrins (β-CD, Methyl-β-CD, Heptakis(2,6-di-O-methyl)-β-CD, Heptakis(2,3,6-di-O-methyl)-β-CD, (2-Hydroxi) propyl-β-CD, (2-Hydroxi) propyl-γ-CD, Methyl-γ-CD, Acetylated-β-CD, Acetylated-γ-CD, γ-CD) at a concentration of 10 mM was investigated. These experiments were carried out using a separation voltage of 30 kV, and a working temperature of 25 ^{o}C. From the ten amino acids analyzed, only Aminoadipic and SeMet were partially resolved (Rs values of 0.2 and 0.9, respectively) using Heptakis(2,6-di-O-methyl)-β-CD as chiral selector.

On the other hand, when acidic conditions were employed, the potential of different anionic cyclodextrins was evaluated. In this case, the selectors tested were sulfated-α-CD, sulfated-β-CD, sulfated-γ-CD, phosphated-β-CD, carboxymethylated-β-CD, carboxyethylated-β-CD, and succinyl-β-CD when pH 4.5 was employed, and the three sulfated CD along with the phosphated-β-CD when pH 2.0 was used. All these experiments were performed at a fixed concentration of cyclodextrin (10 mM), using a separation voltage of -20 kV, and a working temperature of 25 ^{o}C. None of the cyclodextrins employed in 100 mM formate buffer at pH 4.5 allowed the enantiomeric discrimination of the non-protein amino acids studied. However, at pH 2.0, the use of the above-mentioned four anionic

cyclodextrins enabled the resolution of eight of the ten non-protein amino acids studied (Pyro, Norval, Norleu, DOPA, Aminoadipic, SeMet, Cit and Pipe) (see **Table 1**). Consequently, 100 mM formate buffer at pH 2.0 was chosen as running buffer for further experiments and the effect of the temperature was investigated for the cyclodextrins giving rise to the best enantiomeric resolutions (sulfated-α-CD, sulfated-β-CD, sulfated-γ-CD or phosphated-β-CD).

3.1.2 Effect of the temperature

In order to evaluate the influence of the temperature on the enantiomeric separation of the non-protein amino acids, the values of the chiral resolution obtained with the four cyclodextrins tested (sulfated-α-CD, sulfated-β-CD, sulfated-γ-CD, and phosphate-β-CD) at 15 ºC, 20 ºC, and 25 ºC were compared. **Table 1** shows both the migration times for each enantiomer and the chiral resolution for each non-protein amino acid analyzed at different temperatures. As it can be observed, when phosphate-β-CD was used as chiral selector, the variation of temperature did not affect significantly either migration time or chiral resolution of those amino acids which could be enantioseparated. In general, both migration times and chiral resolutions are slightly lower when increasing the value of temperature. For this reason, 15 ºC seemed to be the most appropriate working temperature when phosphated-β-CD was used, since it allowed a high number of enantiomeric separations (Norval, DOPA, SeMet and cit). A different behavior regarding the effect of the variation of the temperature could be observed in the case of the use of sulfated cyclodextrins as chiral selectors. In fact, when using sulfated-β-CD, the resolution of Norval was higher when increasing the temperature from 15 to 20 ºC while the value of resolution obtained for Pipe was almost constant for the three temperatures tested. An important factor to take into consideration is that the migration times obtained with this cyclodextrin were too high, in most of cases more than 60 min (**Table 1**).

Both sulfated-α-CD and sulfated-γ-CD showed a high discrimination power for the non-protein amino acids studied. For these two cyclodextrins, the influence of the temperature was also different. The results obtained with sulfated-α-CD were better at 15 ºC whereas those obtained with sulfated-γ-CD were not affected by the temperature. This fact can be clearly observed in **Table 1**.

Table 1. Migration time and chiral resolution for NPAA studied by EKC using four different CDs at different temperatures.

		15 °C			20 °C			25 °C		
		t_1	t_2	R_s	t_1	t_2	R_s	t_1	t_2	R_s
Phosphate β-CD	Pyro*	21.3	-	-	19.6	-	-	22.6	-	-
	Norval	17.1	17.2	1.0	16.1	16.2	0.7	16.3	16.4	0.7
	Norleu	16.9	-	-	15.9	-	-	15.5	-	-
	DOPA	15.5	15.7	1.0	14.8	15.0	1.0	16.1	16.3	1.0
	aminoadipic	18.4	-	-	16.9	-	-	16.8	-	-
	SeMet	16.2	16.4	0.6	15.2	15.3	0.5	14.9	15.0	0.6
	Hcy	18.1	-	-	17.0	-	-	17.5	-	-
	Orn	18.4	-	-	17.0	-	-	16.5	-	-
	Cit	17.7	18.1	2.6	16.6	16.8	0.9	16.3	-	-
	Pipe	>40			>40			>40		
Sulfated β-CD	Pyro*	40.9	-	-	38.8	-	-	36.2	-	-
	Norval	49.2	50.0	0.9	54.5	58.5	3.4	>60		
	Norleu	50.6	-	-	>60			52.3		
	DOPA	>60			>60			44.9	-	-
	aminoadipic	>60			>60			51.4	-	-
	SeMet	>60			>60			50.0	-	-
	Hcy	>60			>60			51.3	-	-
	Orn	53.4	-	-	52.5	-	-	51.7	-	-
	Cit	50.3	-	-	50.5	-	-	49.0	-	-
	Pipe	25.0	25.8	1.4	25.4	26.2	1.5	26.1	27.0	1.5

Table 1. (Continued)

		15 °C			20 °C			25 °C		
		t_1	t_2	R_s	t_1	t_2	R_s	t_1	t_2	R_s
Sulfated α-CD	Pyro*	32.4	32.8	0.9	37.5	38.1	0.9	40.2	41.0	0.9
	Norval	47.3	48.4	1.6	>60			>60		
	Norleu	45.6	46.4	0.7	33.7	34.1	0.5	31.8	32.2	0.5
	DOPA	49.6	52.7	3.5	>60			>60		
	aminoadipic	47.2	48.0	1.2	49.1	-	-	57.3	58.2	0.8
	SeMet	44.7	45.3	0.6	33.8	-	-	30.4	-	-
	Hcy	50.9	-	-	>60			>60		
	Orn	49.3	-	-	>60			>60	-	-
	Cit	52.0	-	-	>60			>60		
	Pipe	41.4	-	-	>60			>60		
Sulfated γ-CD	Pyro*	30.1	-	-	26.7	-	-	26.5	-	-
	Norval	34.6	35.9	2.6	30.5	31.5	2.4	27.3	28.2	2.6
	Norleu	35.3	-	-	31.5	-	-	27.9	-	-
	DOPA	30.4	32.9	6.9	30.2	-	-	24.5	27.1	7.4
	aminoadipic	35.8	36.3	1.1	32.1	32.5	1.0	28.9	29.2	0.9
	SeMet	30.9	31.7	1.2	28.1	-	-	25.1	25.6	1.1
	Hcy	32.9	-	-	28.5	-	-	25.0	-	-
	Orn	31.9	-	-	27.9	-	-	24.5	-	-
	Cit	29.2	30.9	5.1	28.0	28.3	4.3	24.2	25.3	4.2
	Pipe	24.0	25.0	2.0	21.4	22.4	2.0	19.5	20.4	2.9

Experimental conditions: BGE, 10 mM of CD in 100 mM formate buffer (pH 2.0); uncoated fused-silica capillary, 58.5 cm (50 cm to the detector window) × 50 µm ID; UV detection at 210 nm; applied voltage, −20 kV; injection by pressure, 50 mbar for 4 s of sample. *Pyroglutamic acid without derivatization (UV detection at 200 nm). t_1, time of the first-migrating enantiomer; t_2, time of the second-migrating enantiomer.

A decrease of the temperature to the lowest value (15 ºC) allowed an increment of the number of non-protein amino acids which can be enantiomerically resolved as well as a decrease in the migration times when sulfated-α-CD was employed. From the ten compounds studied, six of them (Pyro, Norval, Norleu, DOPA, Aminoadipic, and SeMet) were separated with resolution values from 0.6 to 3.5. When sulfated-γ-CD was used, the best results were obtained using both 15 and 25 ºC, since at 20 ºC an overlapping between the peaks corresponding to FMOC and some enantiomers was observed. In this case, an increase in the temperature gave rise to a decrease on the migration times, and slightly variations in the resolution values. Using both 15 or 25 ºC, it was possible to achieve the chiral separation of Norval, DOPA, Aminoadipic, SeMet, Cit, and Pipe with resolution values from 0.9 to 7.4 (see **Table 1**). Although it is well known that the enantiomeric resolution of chiral compounds can be modified with the temperature, it is not possible to establish a general behavior when this parameter varies since a decrease or an increase in the resolution can be found for different analytes. These differences can be explained by the effect that the temperature may have on the buffer viscosity, peak efficiency or selector-selectand interactions [41, 42].

From the results obtained, it can be concluded that eight out of ten non-protein amino acids investigated could be separated employing sulfated-α-CD or sulfated-γ-CD. However, the chiral separation of Pyro and Norleu was only achieved when sulfated-α-CD was used, whereas Pipe and Cit were only enantioseparated employing sulfated-γ-CD. For those amino acids for which the chiral separation could be reached by using both cylodextrins, the best results concerning chiral resolution as well as migration times, were obtained using sulfated γ-CD.

The variation of the concentration of both cyclodextrins (sulfated-α-CD and sulfated-γ-CD) from 1 to 10 mM (1, 2, 5, and 10 mM) and of the separation voltage (-20, -25, -30 kV) did not enable to improve the results obtained so a CD concentration of 10 mM and -20 kV were selected. **Figure 1** shows the electropherograms obtained for the eight FMOC-non-protein amino acids enantioseparated under the conditions giving rise to the highest chiral resolution.

It can be observed in **Table 1** that the simultaneous separation of mixtures of some non-protein amino acids were also possible as shown in **Figure 2** corresponding to the separation of Pipe, Cit, and Aminoadipic using sulfated γ-CD as chiral selector, as an example.

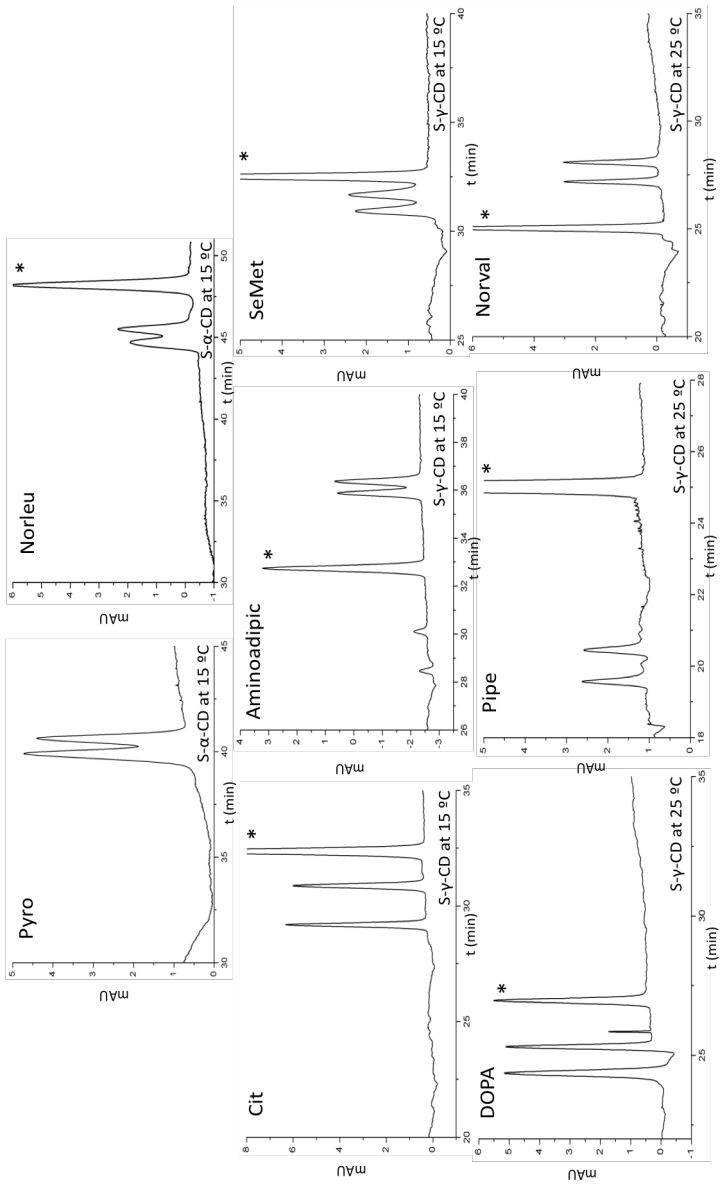

Figure 1. Electropherograms corresponding to the chiral separation of different FMOC-non-protein amino acids (0.2 mM) obtained under the best separation conditions. Pyro; using sulfated α-CD (S-α-CD) at 15 ºC, Norval; using sulfated γ-CD (S-γ-CD) at 25 ºC, Norleu; using sulfated α-CD (S-α-CD) at 15 ºC, DOPA; using sulfated γ-CD (S-γ-CD) at 25ºC, Aminoadipic; using sulfated γ-CD (S-γ-CD) at 15 ºC, SeMet; using sulfated γ-CD (S-γ-CD) at 15 ºC, Cit; using sulfated γ-CD (S-γ-CD) at 15 ºC, and Pipe; using sulfated γ-CD (S-γ-CD) at 25ºC. Experimental conditions: BGE, 10 mM of the corresponding CD in 100 mM formate buffer (pH 2.0); uncoated fused-silica capillary, 58.5cm (50 cm to the detector window) x 50 µm ID; UV detection at 210 nm (except for Pyro which was detected at 200 nm); applied voltage, -20 kV; injection by pressure in the cathodic end, 50 mbar for 4 s. * Indicates the derivatizing reagent (FMOC).

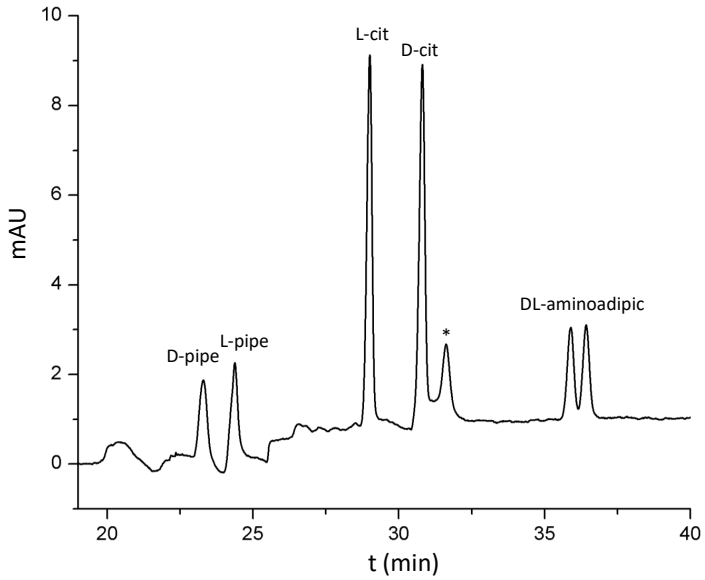

Figure 2. Electropherogram corresponding to the chiral analysis of a mixture of DL-pipe, DL-cit and DL-aminoadipic (0.2 mM each non-protein amino acid). Experimental conditions: BGE, 10 mM sulfated γ-CD in 100 mM formate buffer (pH 2.0); uncoated fused-silica capillary, 58.5 cm (50 cm to the detector window) x 50 µm ID; UV detection at 210 nm, applied voltage, -20 kV; temperature, 15 ºC; injection by pressure in the cathodic end, 50 mbar for 4 s. * Indicates the derivatizing reagent (FMOC).

3.2 Application of the developed methodologies to the analysis of food supplements.

The developed EKC methodology using a 100 mM formate buffer at pH 2.0 and 10 mM sulfated γ-CD as chiral selector was applied to the enantiomeric determination of Cit in food supplements. This non-protein amino acid is added to food supplements due to its capability to remove the lactic acid produced during strong training. While L-Cit is involved in the urea cycle, it is the precursor of arginine, reduces ammonia levels, and has a relevant role in NO cycle [43], D-Cit has shown a different behavior both in vitro and in vivo [44-46].

3.2.1 Optimization of the enantioseparation of Citrulline

In order to decrease the migration time for Cit enantiomers, the capillary length was shortened up to 40 cm. However, using this capillary and a working temperature of 15 ºC, an overlapping between the second-migrating enantiomer (D-Cit) and FMOC was observed. To avoid that, both the buffer pH (from pH 2.0 to pH 3.0) and the temperature (from 15 ºC to 25 ºC) were modified. Under these conditions, the migration time obtained for Cit was around 18 min with Rs > 2.5, and the separation between FMOC and D-Cit was higher than 4 min (Rs 10.3) which

avoided any possible overlapping between their peaks. Finally, to obtain the best sensitivity without a loss in resolution, the influence of the variation of the enantiomeric resolution as a function of the injection time (4, 10, 15 and 20 s) using a fixed pressure of 50 mbar was investigated. The results obtained demonstrated that an injection of 50 mbar during 15 s gave rise to the highest signal with enough resolution (Rs 2.7).

3.2.2 Analytical characteristics of the developed EKC methodology for Citrulline determination

Under the optimized conditions and prior to the enantiomeric determination of Cit in food supplements, the analytical characteristics of the developed EKC methodology (linearity, precision, accuracy, limits of detection (LOD) and limits of quantification (LOQ)) were evaluated in order to demonstrate the method suitability for routine quality control.

The linearity of the method was established from five calibration levels ranging from 0.2 to 2.5 x 10^{-4} M of L-Cit (i.e ranging from 10 to 125% of a nominal concentration (0.2 mM) of L-Cit), and from 0.1 to 2.0 x 10^{-5} M of D-Cit (i.e from 0.5 to 10 % of a nominal concentration (0.2 mM) of D-cit). As it can be seen in **Table 2**, satisfactory results were obtained in terms of linearity with a correlation coefficient higher than 0.997 for both enantiomers, confidence intervals for the slopes did not include the zero value, and confidence intervals for the intercept included the zero value (in both cases for a 95 % confidence level). In addition, an ANOVA test enabled to confirm that experimental data fit properly to a linear model (p-values > 0.05 for L and D-cit). A comparison between the confidence intervals for the slopes obtained by the external standard and the standard additions calibration method (four known amounts of DL-Cit were added to a food supplement containing a constant concentration of L-Cit) showed that there were no statistically significant differences between the slopes of each calibration straight line (for a 95 % confidence level). Therefore, there are not matrix interferences and the external calibration method can be used to quantify the content of Cit in food supplements. Moreover, taking into account that the response relative factor (RRF, factor which enables to study if the response of a minor component is equivalent to those for the major component and it is calculated dividing the slopes of the calibration lines, slopeminor component/slopemajor component) was between 0.8 and 1.2 such as the European Pharmacopoeia establishes [47], the response for D-Cit can be considered equal to that of L-Cit which implies that the percentage of D-Cit can be determined from the ratio between the areas of L and D-Cit.

Precision was evaluated through instrumental and methodology repeatability as well as intermediate precision. Instrumental repeatability was determined from six repeated injections of a standard solution of DL-Cit at two concentration levels (12.5 x 10^{-6} M and 1.5 x 10^{-4} M). RSD values were 1.5 % for migration times and lower than 2.9 % for corrected peak areas. Regarding method repeatability, it was evaluated with three replicates of a standard solution of DL-Cit at two concentration levels (12.5 x10^{-6} M and 1.5 x 10^{-4} M) injected in triplicate on the same day. RSD values obtained in this case, were lower than 1.7 % and 5.8 % for migration times and corrected peak areas, respectively. Finally, intermediate precision was assessed injecting (in triplicate) three replicates of a standard solution of DL-Cit at two concentration levels (12.5 x10^{-6} M and 1.5 x 10^{-4} M) during three consecutive days. RSD values for migration times were lower than 4.7 % whereas for peak areas were lower than 5.6 % (see **Table 2**).

Accuracy of the method was evaluated as the recovery obtained for Cit enantiomers when spiking (prior to derivatization) the six food supplements with known concentration of L-Cit standard solutions (5 and 100 % of a nominal concentration of L-Cit of 0.1 mM) or D-Cit (5 % of a nominal concentration of 0.1 mM L-Cit). **Table 2** shows that the mean recoveries obtained for the six food supplements analyzed ranged from 85 to 111 %.

LODs and LOQs were calculated as the minimum concentration yielding an S/N ratio of 3 and 10 times. LODs were 2.1 x 10^{-7} M for D-Cit and and 1.8 x 10^{-7} M for L-Cit and LOQs were 7.1 x 10^{-7} M and 6.1 x 10^{-7} M for D- and L-Cit, respectively. According to the LOD calculated for D-Cit and the nominal concentration injected for L-Cit (0.2 mM), it was possible to determine the relative limit of detection (RLOD) which allows to measure the minimum enantiomeric impurity that can be detected as a function of the amount of the main compound analyzed (calculated as (LOD for the minor enantiomer)/concentration of the major enantiomer injected) x 100). The RLOD calculated was about 0.1 %, which implies that using the developed EKC methodology is possible to carry out the detection of impurities up to a 0.1 %. This LOD is similar to that reported previously for D-Cit using CEC [31].

Table 2: Analytical characteristics of the developed EKC method for the determination of cit in food supplements.

		L-Cit	D-Cit
Linearity			
Linear range [a]		0.2-2.5 x 10^{-4} M	0.1-2.0 x 10^{-5} M
Slope ± t·s_b		213.9 ± 12.4	183.4 ± 25.7
Intercept ± t·s_a		0.26 ± 1.87	-0.06 ± 0.31
R^2		0.9995	0.9972
p-value of ANOVA [b]		0.209	0.075
Matrix Interferences [c]	*Sample*	*Confidence interval (Slope ± t·s_b)*	
	Standard	213.9 ± 12.4	183.4 ± 25.7
	Food supplement	191.9 ± 10.2	186.5 ± 11.6
Precision	*Concentration level*	RSD (%)	RSD (%)
Instrumental Repeatability [d] (n = 3)	12.5 x 10^{-6} M	t, 1.5; Ac, 2.5	t, 1.5; Ac, 2.9
	1.5 x 10^{-4} M	t, 1.5; Ac, 1.9	t, 1.5; Ac, 1.9
Method Repeatability [e] (n = 9)	12.5 x 10^{-6} M	t, 0.9; Ac, 4.3	t, 0.8; Ac, 4.0
	1.5 x 10^{-4} M	t, 1.7; Ac, 5.6	t, 1.5; Ac, 5.8
Intermediate precision [f] (n = 9)	12.5 x 10^{-6} M	t, 4.2; Ac, 4.2	t, 4.7; Ac, 4.6
	1.5 x 10^{-4} M	t, 2.6; Ac, 5.4	t, 2.7; Ac, 5.6
Accuracy [g]	*% Mean Recovery*		*% Mean Recovery*
FS1 / FS2	97 ± 8 / 97 ± 11		93 ± 3 / 85 ± 1
FS3 / FS4	104 ± 7 / 99 ± 10		95 ± 4 / 92 ± 4
FS5 / FS6	100 ± 2 / 106 ± 3		108 ± 1 / 111 ± 1
LOD [h]	1.8 x 10^{-7} M		2.1 x 10^{-7} M
LOQ [i]	6.1 x 10^{-7} M		7.1 x 10^{-7} M

[a] Five standard solutions at different concentration levels were injected in triplicate for 3 consecutive days.
[b] p-value for ANOVA to confirm that experimental data fit properly to linear models.
[c] Comparison of slopes corresponding to the standard addition and the external standard calibration methods.
[d] Instrumental repeatability was determined from six consecutive injections of citrulline standard solutions at two concentration levels.
[e] Method repeatability was calculated by using the value obtained for three replicates of citrulline standards solutions at two concentration levels injected in triplicate on the same day.
[f] Intermediate precision was calculated by using the mean value obtained each day for three replicates (injected in triplicate during three consecutive days) of citrulline standard solutions at two concentration levels.
[g] Accuracy was evaluated as the mean recovery obtained when six different samples solutions were spiked with known concentrations of L-Cit standard (5 % and 100 % of a nominal concentration of 0.1 mM L-Cit) or D-Cit (5 % of a nominal concentration of 0.1 mM L-Cit).
[h] LOD calculated as the concentration yielding an S/N ratio of 3 [i] LOQ calculated as the concentration yielding an S/N ratio of 10

3.2.3 Analysis of food supplements.

Once demonstrated the suitability of the developed EKC method for the enantioselective determination of Cit, it was applied to the analysis of six different food supplements, three of them recently acquired (FS1, FS2, and FS3) and other three submitted to a long storage time (FS4, FS5, and FS6). The results obtained for the content of L-Cit in samples FS1, FS2 and FS3 were 478.2 ± 5, 647.8 ± 2, and 1457.7 ± 36 mg L-Cit per capsule, respectively, what corresponded to percentages of 96, 65 and 97 % with respect to the labeled content of L-Cit. D-Cit was not detected in any of these supplements so its concentration was below the LOD of the method. In the case of FS4, FS5 and FS6 samples, the contents for L-Cit were 488.4 ± 7, 395.8 ± 2, and 72.5 ± 3 mg L-Cit per capsule, respectively. These values corresponded to percentages of 65, 79 and 8 % of the labeled amounts demonstrating that the content of L-Cit decreased with the storage time since these percentages determined before the expiration of the samples were 112, 98 and 11 % [31]. However, the decrease of L-Cit content could not be attributed to a racemization from L to D-cit since the D enantiomer could not be detected in any of these samples and therefore its concentration was below 0.1 %.

Figure 3 shows the electropherograms corresponding to DL-Cit standard and to two of the food supplements analyzed (non-spiked and spiked with D-Cit). As it can be observed D-cit was not detected in any of the sample analyzed.

As it has been mentioned in the introduction, the analysis of Cit in food supplements was described previously by using a CEC methodology based on the use of cellulose tris(3-chloro-4-methylphenylcarbamate) as chiral stationary phase. Taking into account that EKC is undoubtedly a more versatile technique due to the easy use of chiral selectors in the separation buffer and considering that the EKC methodology developed in this work can be successfully applied to the quantitative determination of L-Cit and the analysis of its enantiomeric purity, it can be considered an interesting alternative for the routine quality control of Cit in food supplements.

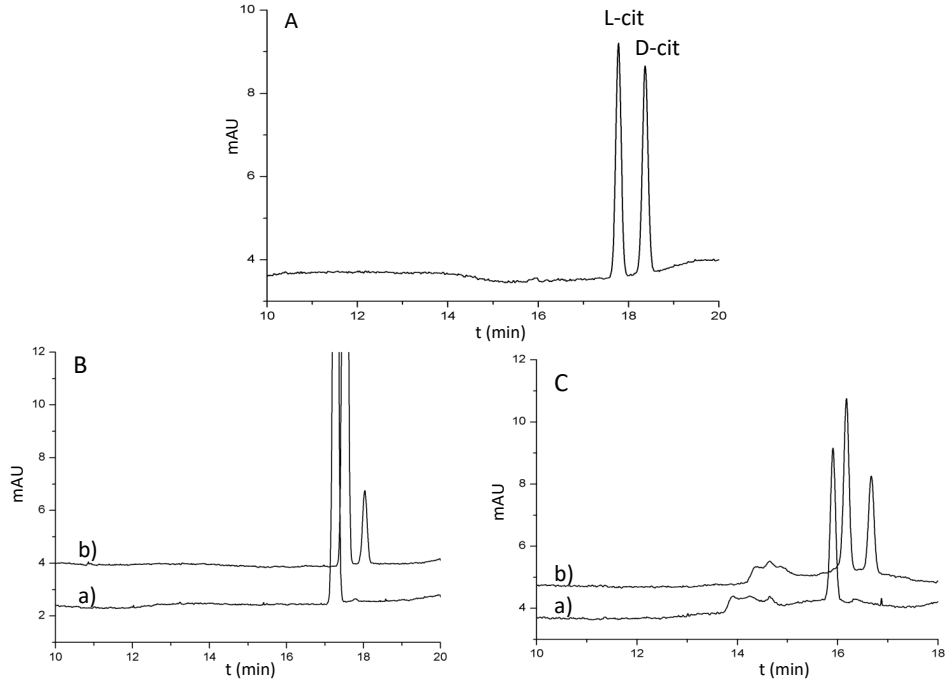

Figure 3. Electropherogram obtained for (A) DL-FMOC-cit standard (0.025 mM) and two different food supplements at 0.2 mM L-Cit (B) FS1 non-spiked (a) and spiked with D-cit (b) (C) FS6 non-spiked (a) and spiked with D-cit (b). Experimental conditions: BGE, 10 mM sulfated γ-CD in 100 mM formate buffer (pH 3.0); uncoated fused-silica capillary, 48.5 cm (40 cm to the detector window) x 50 μm ID; UV detection at 210 nm, applied voltage, -20 kV; temperature, 25 ºC; injection by pressure in the cathodic end, 50 mbar for 15 s.

4. Conclusions

Different EKC methodologies based on the use of sulfated-α-cyclodextrin or sulfated-γ-cyclodextrin as chiral selectors were developed to carry out the enantiomeric separation of a group of non-protein amino acids. The use of a 100 mM formate buffer at pH 2.0 as running buffer and pre-capillary derivatization with FMOC enabled the enantiomeric separation of eight from the ten non-protein amino acids studied (pyro, norleu, norval, DOPA, aminoadipic, SeMet, pipe, and cit) and the simultaneous separation of some of them in a run. Moreover, an optimized methodology using sulfated-γ-cyclodextrin as chiral selector was applied to the enantiomeric analysis of Cit in food supplements. The LOD obtained for D-Cit was 2.1 x 10^{-7} M which represents the possibility of detecting up to a 0.1 % of this enantiomer showing the high sensitivity of this methodology. L-Cit was determined in six food supplements where D-Cit was not detectable in any case. Since three of the food supplements were submitted to a long storage time, the results showed that no racemization occurred due to the effects of storage time.

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Article 5

A micellar electrokinetic chromatography approach using diastereomeric derivatization and a volatile surfactant for the enantioselective separation of selenomethionine.

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Electrophoresis, under first revision.

Abstract

A MEKC methodology with UV detection was developed for the enantioselective separation of selenomethionine. The use of FLEC ((+)-1-(9-fluorenyl)ethyl chloroformate) as chiral derivatization reagent to form selenomethionine diastereomers enabled their subsequent separation using ammonium perfluorooctanoate (APFO) as a volatile pseudostationary phase. The effect of APFO concentration and pH, temperature, injection volume and derivatization conditions (time and FLEC/selenomethionine ratio) was evaluated in order to select the best separation conditions. A chiral resolution of 4.4 for DL-selenomethionine was achieved in less than 6 min using 100 mM APFO at pH 8.5 as electrophoretic buffer. Satisfactory results were obtained in terms of linearity, precision (RSD from 3.4 to 5.1% for migration times and from 1.8 to 4.6% for corrected peak areas), and LODs (3.7×10^{-6} M for D and L forms). The method was successfully applied to the analysis of L-selenomethionine and its enantiomeric impurity in food supplements.

1. Introduction

Selenium is a trace element which plays a relevant role in several enzymatic processes and biological functions [1-3]. Depending on its concentration and chemical speciation, it can be beneficial or harmful to humans [4]. In fact, it is believed that at low concentration it is beneficial, whereas at higher concentrations it becomes toxic (causing problems such as dermatitis, fatigue and hair loss [5], being the range among deficiency, essentiality, and toxicity, very narrow [6]. Selenium deficiency is associated with serious nutritional and health problems such as hypertension, infertility, arthritis and ageing, and different types of cancer, among others [5, 7-10].

Among the selenium forms employed for food supplementation, selenomethionine (SeMet), as other seleno-amino acids, has demonstrated to be more bioavailable and less toxic than inorganic species [11-13]. For this reason, it is one of the main selenium specie employed in the formulation of dietary supplements [14].

Since SeMet is a chiral compound (it contains an asymmetric carbon in its structure), it must be taken into account that the bioavailability of its two enantiomers can be different because of the stereoselectivity of organisms [15, 16]. In fact, studies performed in mice and rats indicate a different uptake of L and D enantiomers but a similar bioavailability [15, 17, 18] while in humans, the use of D-selenomethionine seems to be poorer compared to L-selenomethionine [19, 20]. In

addition, the European Food Safety Authority (EFSA) only recognize L-SeMet as a source of selenium to be added to food supplements for nutritional purposes [21], being forbidden the use of the D-enantiomer in the elaboration of foods and dietary supplements [22]. As a consequence, the ratio of D- and L-SeMet content in food supplements is an important quality parameter [23], so that the development of efficient and simple analytical methodologies to discriminate SeMet enantiomers has become an interesting area of research.

The enantioselective separation of SeMet has been carried out by HPLC [24-30] and GC [31-33]. Some of these methods were applied to the enantiomeric determination of SeMet in different food (food supplements, yeast, breast milk and infant formulas) [24-26, 32, 34]. CE, one of the most powerful separation techniques in the field of chiral separations due to its enormous advantages such as high separation efficiency, low cost and rapid analysis times, among others [35, 36], has also been employed for the chiral separation of SeMet [5, 13, 37-40]. Some of these works were performed using the direct mode in which reversible diastereomeric complexes formed between the SeMet enantiomers and a chiral selector are separated due to their different mobility. Using this principle, the enantioselective determination of SeMet was achieved within 18-30 min with resolution values between 0.7 and 1.5 when vancomycin alone or combined with cyclodextrins was used as chiral selector [13, 37], or in longer analysis times (70-80 min) with high resolution (up to 8.0) in the case of using a mixture of cyclodextrin and taurodeoxycholic acid as selectors [38]. The use of a negatively charged cyclodextrin (sulfated-γ-cyclodextrin) under acidic conditions also allowed the enantiomeric separation of DL-SeMet in less than 32 min with a resolution of 1.2 [40]. However, none of these methods have been applied to the analysis of this non protein amino acid in real samples. An alternative approach to obtain the chiral separation of SeMet by CE is the use of an indirect mode in which stable diastereoisomers formed by the interaction between the enantiomer with an enantiopure chiral derivatization agent are separated based on their different physicochemical properties in an achiral environment. For instance, Day *et al.*, achieved the chiral separation of DL-SeMet by CE with UV detection and inductively coupled plasma mass spectrometry (ICP-MS) after chiral derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) [5]. A baseline resolution of the D- and L-forms was obtained in 14 min with LODs of 250 ppb using CE-UV, and 50 ppb using CE-ICP-MS. This methodology was applied to the analysis of selenized yeast digested with proteinase K. Also, Duan *et al.*, reported the separation of DL-SeMet in selenized yeast samples within 10 min with baseline resolution, employing a phenylalanine derivative (l-N-(2-hydroxy-propyl)-phenylalanine) and its chelate with Cu(II) as

chiral selector by MEKC-UV [39]. By using two different preconcentration techniques (off-line solid phase extraction and on-line large volume sample stacking), it was possible to reach LODs of 0.44 and 0.60 ng/mL for L-SeMet and D-SeMet, respectively. In order to improve the enantiomeric resolution obtained for SeMet in a short analysis time and to develop adequate methodologies enabling the fast enantioselective analysis of SeMet in complex samples such as food supplements, in the present work, a MEKC methodology was developed based on the use of a semi-volatile negatively charged surfactant above its critical micellar concentration. Diastereomers were formed by derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC). Experimental conditions were optimized, and the analytical characteristics were assessed. The applicability of the developed methodology was demonstrated by the determination of D,L-selenomethionine in food supplements.

2. Materials and methods

2.1 Reagents and samples

All reagents employed were of analytical grade and water used to prepare solutions was deionized and purified with a Milli-Q System from Millipore (Bedford, MA, USA). Methanol and ACN were purchased from Fisher Chemical (Leicestershire, UK). Formic and hydrochloric acids were from Scharlau (Barcelona, Spain). Sulfated-γ-cyclodextrin (DS~14) was from Cyclolab (Budapest, Hungary). DL-Selenomethionine (DL-SeMet), L-Selenomethionine (L-SeMet), pentane, boric acid, sodium hydroxide, ammonium hydroxide (28 %, w/v), sodium tetraborate, perfluorooctanoic acid (APFO 96 %) and the derivatization reagents 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) and (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) were supplied by Sigma-Aldrich (Madrid, Spain). Two different food supplements containing L-selenomethionine were acquired in different Madrid´s markets (Spain).

2.2 CE conditions

Electrophoretic experiments were carried out in an HP^{3D}CE system (Agilent Technologies, Palo Alto, USA) equipped with a DAD working at 210 nm with a bandwidth of 4 nm and controlled by the HP^{3D}CE ChemStation software. Analyses were performed on uncoated fused-silica capillaries of 50 μm ID (362.8 μm OD) with a total length of 58.5 cm (50 cm effective length) provided by Polymicro Technologies (Phoenix, USA). New capillaries were rinsed with 1M sodium hydroxide for 30 min, Milli-Q water for 5 min, and buffer solution for 60 min. Before

each analysis, the capillary was flushed with BGE for 5 min. At the beginning of each working day, the capillary was rinsed with BGE for 30 min, and at the end of the day it was flushed with 0.1 M NaOH for 5 min and Milli-Q water for 10 min.

2.3 Preparation of solutions and samples

Stock standard solutions of SeMet were prepared dissolving the appropriate amount in the derivatization buffer. When FMOC-Cl was employed as labeling agent, SeMet was dissolved in 200 mM borate buffer at pH 9.0 whereas a 26 mM borate buffer at pH 9.2 was required when the derivatization was performed using FLEC as derivatizing agent. These two buffer solutions were prepared dissolving the appropriate amount of boric acid or sodium tetraborate respectively in Milli-Q water.

For the CD-EKC method, the separation buffer was prepared by diluting formic acid with Milli-Q water to obtain a concentration of 100 mM and adjusting the pH to 2.0 with ammonium hydroxide (28 %, w/v). The BGE was obtained by dissolving a certain amount of sulfated-γ-cyclodextrin in the separation buffer to get a concentration of 10 mM.

For the MEKC approach, the separation buffer was prepared by diluting the appropriate volume of APFO with Milli-Q water to achieve the desired buffer concentration (100 mM) and adjusting the pH to the chosen value with ammonium hydroxide (28 %, w/v)

To prepare sample solutions of dietary supplements, the content of ten or twenty capsules (depending on the labeled amount) was weighed, powdered and mixed homogeneously. Then, bearing in mind the labeled amount of L-SeMet in each supplement, an appropriate amount of the powder previously obtained was dissolved in 26 mM borate buffer (pH 9.2) to obtain a standard solution of 0.2 mM. Dissolution was performed by ultrasonication for 10 min followed by centrifugation (15 min, 4000*g* at 25 ºC) and filtration. Before derivatization, these sample solutions were diluted in borate buffer (26 mM, pH 9.2) to obtain the appropriate concentration. Stock and sample solutions were stored at 4 ºC until the derivatization step.

All solvents and samples were filtered prior use through 0.45 μm pore size disposable nylon filters from Scharlau (Barcelona, Spain).

2.4 Derivatization

Derivatization of SeMet with FMOC-Cl was carried out following a methodology previously described by our research group [40]. A FMOC-Cl solution

in ACN was freshly prepared each working day taking into account that an excess of at least three times of FMOC-Cl was necessary to obtain a complete derivatization of SeMet. The following derivatization protocol was carried out: 300 µL of a 10 mM SeMet standard solution were mixed with 300 µL of FMOC-Cl solution. The mixture was kept at room temperature for 2 min to complete reaction, and the resulting solution was extracted with 0.6 mL pentane to remove FMOC excess. Before injection in the CE, the solution was diluted ten times with Milli-Q water.

Derivatization of SeMet with FLEC was performed using a methodology described by Prior, et al. [41] with slight modifications. The desired concentration of FLEC in methanol was freshly prepared each day. The derivatization was as follows: 50 µL of SeMet standard solution or sample solutions were mixed with 50 µL of FLEC solution, (SeMet/FLEC concentration ratio of 1:6). The mixture was kept at room temperature for 10 min to complete reaction. Then, it was diluted ten times with Milli-Q water before injection in the CE system.

2.5 Data treatment

Enantiomeric resolution values (calculated from the migration times of enantiomers and their peak widths at half height) and migration times were obtained using the Chemstation software. Experimental data analysis, calculation of different parameters, and composition of graphs with different electropherograms were carried out using Excel Microsoft, Statgraphics Centurion XVI, and Origin 8.0 softwares.

3. Results and discussion

As previously stated, our research group demonstrated the potential of EKC to achieve the enantiomeric separation of SeMet using 10 mM sulfated-γ-cyclodextrin and pre-capillary derivatization with FMOC-Cl in 32 min with a resolution of 1.2 [40]. With the aim of developing a fast methodology to carry out the quality control of food supplements containing SeMet, the effect of different experimental conditions was investigated in order to improve the results previously achieved in terms of enantiomeric resolution and migration time. Different values for the buffer pH (2.0 and 3.0), the temperature (20 and 25 ^{o}C), and the CD concentration (8, 10 and 12 mM) were tested. Results obtained were not satisfactory since the enantiomeric resolution and migration time did not improve and also an overlapping of L-SeMet with FMOC-Cl peaks took place using the lowest cyclodextrin concentration tested. CD concentrations higher than 12 mM were also tested but they gave rise to current instability. These results demonstrated that this

strategy was not adequate and, therefore, a new approach was developed using MEKC and the formation of stable diastereomers.

3.1. Development of a MEKC method for the enantioselective separation of SeMet based on the formation of stable diastereomers

An alternative strategy to achieve the chiral separation of SeMet by CE is to carry out its derivatization with a chiral labeling agent and then to achieve the separation of the formed diastereomers under achiral conditions. Different works have shown the feasibility of FLEC as chiral labeling agent of amino acids [42, 43]. Even though the potential of using FLEC as chiral labeling reagent to form diastereomers and their subsequent separation with APFO as separation buffer for the enantioselective determination of protein amino acids in cerebrospinal fluid has been demonstrated by Prior et al [41], it has never been tried for the chiral separation of non-protein amino acids or applied in food analysis. Following this idea, in this work, (+)-FLEC was chosen, which reacts covalently and fast with the amino group of the amino acid SeMet resulting in two diastereomers (**Figure S1**). Besides to form covalently a pair of diastereoisomers which can be separated in a non-chiral environment, the derivatization is also essential to perform the UV detection due to the lack of absorbance of SeMet.

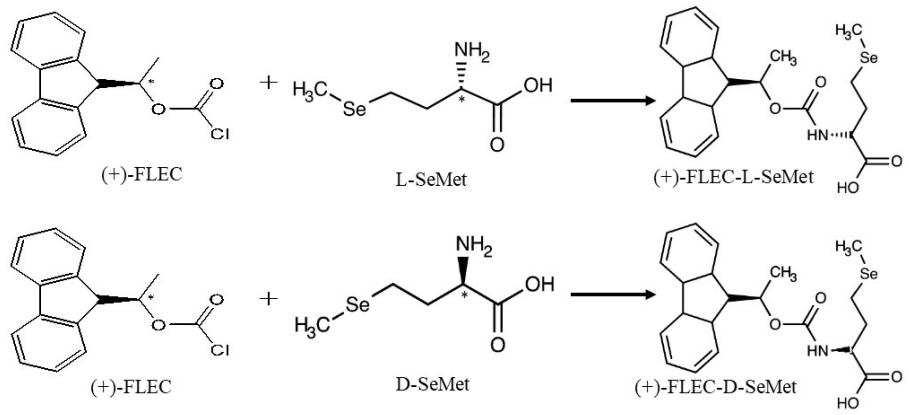

Figure S1. Scheme of the derivatization reaction of D- and L-SeMet with FLEC

For an appropriate CE separation of the formed diastereoisomers, the addition of a surfactant above its critical micellar concentration (CMC) is usually required to enable the separation. Although SDS is the most common surfactant employed to develop MEKC methodologies, the suitability of the use of APFO as

alternative negatively charged semi-volatile pseudostationary phase has been already demonstrated in different works [41, 44]. APFO (pK_a = 2.8; CMC = 25 mM) is fully deprotonated at basic pH, ensuring an effective micelle formation at concentrations higher than 25 mM [45, 46]. It should be keept in mind that to achieve an appropriate separation using an anionic pseudostationary phase, the pH of the BGE should be 7 or higher to induce a strong EOF [47]. Under these conditions, the diastereoisomer complex FLEC-SeMet will be negatively charged.

In order to establish optimal conditions for the chiral separation of SeMet, the influence of several experimental parameters, such as APFO concentration, working temperature, injection volume, and derivatization conditions (time and ratio between FLEC and SeMet), on the chiral resolution was investigated. First, the effect of the APFO concentration was evaluated using a standard sample obtained by the derivatization of 10 mM DL-SeMet with 12 mM FLEC at room temperature for 10 min, and using as preliminary separation conditions 15 ºC, 30 kV and a hydrodynamic injection of 50 mbar x 4 s. APFO concentration was varied between 50 and 150 mM (steps of 25 mM), keeping constant the pH at 9.5. A total separation with a resolution value of 4.0 in less than 7.0 min, and a high current stability, was obtained using a concentration of 100 mM APFO. Lower concentrations gave rise to shorter migration times but also a loss of resolution whereas surfactant concentrations higher than 125 mM slightly improved the enantiomeric resolution, but also increased the migration times and current instability (>85 µA). As a consequence, 100 mM APFO was chosen for further experiments.

The effect of the temperature (15, 20 and 25 ºC) was also studied. As **Figure 1** shows, an increase of the temperature originated an increase in the current intensity inside the capillary, resulting in shorter analysis times and a loss in enantiomeric resolution.

A temperature of 20 ºC was chosen since it allowed the chiral separation of DL-SeMet with high resolution (Rs = 3.6) in less than 6 min with relatively stable currents. Considering the good results obtained in terms of resolution and analysis time, the next step was to obtain the maximum injection volume enabling the best sensitivity without loss of resolution.

Thus, a study on the variation of the SeMet enantiomeric resolution with the injection time (4, 8, 12, 16 and 20 s) keeping constant the injection pressure at 50 mbar was performed. Improvements in the sensitivity were observed when longer injection times were employed; however, they also gave rise to a loss of resolution. Since the highest injection time value tested (20 s) still allowed to achieve a high resolution (2.8), it was selected for further experiments.

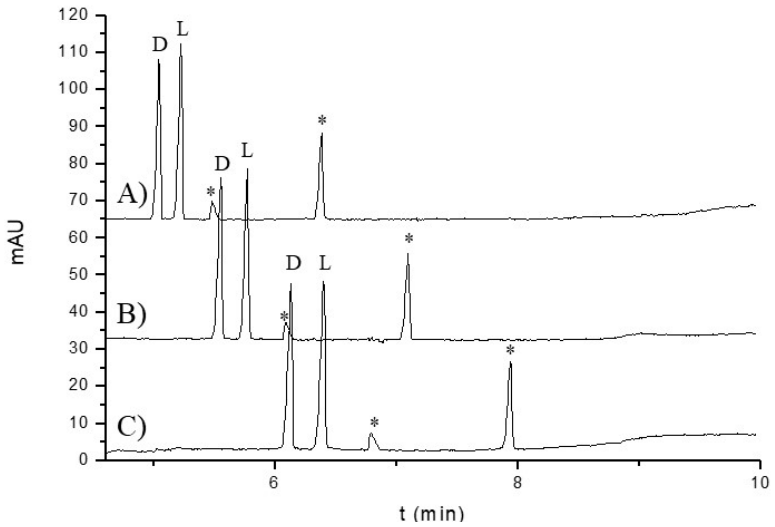

Figure 1. Electropherograms corresponding to the chiral separation of SeMet (0.5 mM) obtained at A) 25 ºC B) 20 ºC and C) 15 ºC. Experimental conditions: BGE, 100 mM of APFO buffer (pH 9.5); uncoated fused-silica capillary, 58.5 cm (50 cm to the corresponding the detector window) x 50 µm ID; UV detection at 210 nm; applied voltage, 30 kV; injection by pressure in the cathodic end, 50 mbar for 4 s. *Indicates the derivatizing reagent (FLEC).

Once optimized the MEKC methodology to achieve the chiral separation of SeMet, the derivatization procedure was also investigated in terms of the SeMet/FLEC ratio and derivatization time in order to reach the maximum derivatization efficiency. First, keeping constant the DL-SeMet concentration (1 mM) and the derivatization time (10 min), the FLEC concentration was varied from 1 to 12 mM. As it can be seen in **Figure 2A**, which shows the variation of the corrected area for L-SeMet when increasing the FLEC concentration, the maximum yield of derivatizing reaction was achieved at 6 mM FLEC since no changes were observed for L-SeMet signal when higher concentrations were used. Then, keeping constant a FLEC/SeMet ratio of 1:6, the reaction time was studied in a range from 2 to 30 min (steps of 5 min). The results demonstrated that the maximum signal for L-SeMet was obtained after 10 min of derivatization (see **Figure 2B**), so this value was selected to perform the derivatization.

In spite of the developed MEKC methodology enabled a good separation of DL-SeMet in a short time, some problems related to current instability resulted in the damage of the capillary, which could be attributed to the use of the aggressive basic BGE conditions [48].

To avoid this drawback, the pH of the BGE was decreased from 9.5 to 8.5. As it can be seen in **Figure 3**, this modification did not affect significantly the chiral separation of DL-SeMet.

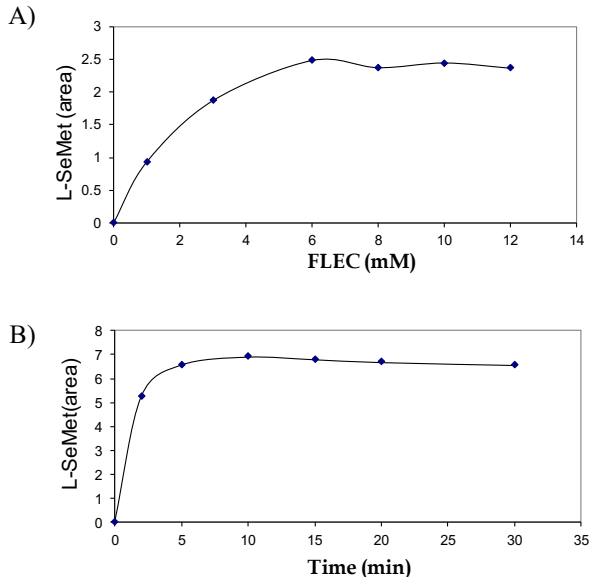

Figure 2. Variation of the corrected peak area obtained for L-SeMet as a function of A) the concentration of FLEC and B) the derivatization reaction time. Experimental conditions: BGE, 100 mM of APFO buffer (pH 9.5); uncoated fused-silica capillary, 58.5 cm (50 cm to the corresponding the detector window) x 50 μm ID; UV detection at 210 nm; applied voltage, 30 kV; injection by pressure in the cathodic end, 50 mbar for 20 s.

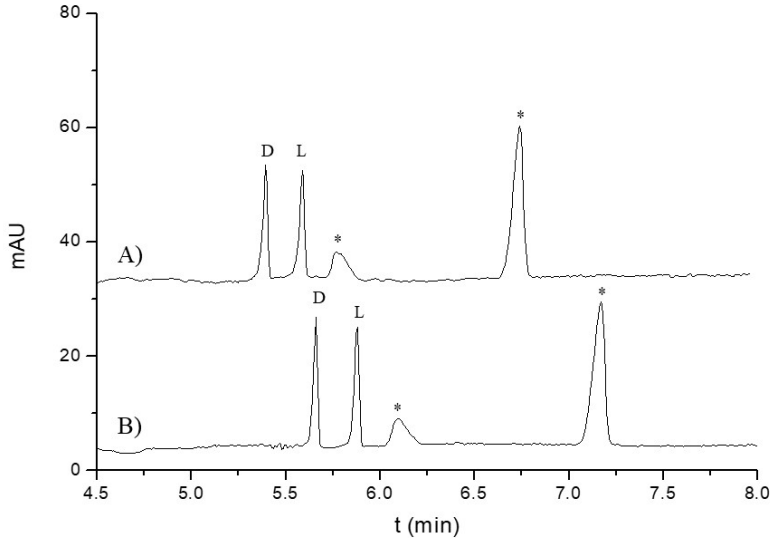

Figure 3. Electropherograms corresponding to the chiral separation of SeMet (0.05 mM) for A) buffer pH 9.5 and B) buffer pH 8.5. Experimental conditions as in Figure 2. *Indicates the derivatizing reagent (FLEC).

Under the final conditions, the MEKC approach developed in this work enabled the enantioselective separation of DL-SeMet with a resolution value of 4.4 in less than 6 min.

3.2. Application of the developed MEKC methodology to the analysis of food supplements

To demonstrate the suitability of the developed MEKC method for the quality control of food supplements containing SeMet, several analytical characteristics such as linearity, precision, accuracy, limits of detection (LOD) and limits of quantification (LOQ) were evaluated.

Seven calibration levels ranging from 0.1 to 0.7 mM M of L-SeMet and D-SeMet were injected in triplicate in 3 different days to establish the linearity of the method. As it can be seen in **Table 1**, a good correlation coefficient (higher than 0.996) was obtained for both SeMet diastereomers. In addition, confidence intervals for the intercept (at a 95 % confidence level) included the zero value, whereas the confidence intervals for the slopes (at a 95 % confidence level) did not included the zero value. An ANOVA test confirmed that experimental data properly fit to a linear model since p-values > 0.05 were obtained for L and D-SeMet.

Possible effects of matrix interferences were evaluated by establishing standard addition calibration method (four known amounts of DL-SeMet were added to a food supplement (FS1) containing a constant concentration of L-SeMet). Comparison of the slopes of this calibration curves with the slopes obtained by the external calibration method did not show statistically significant differences (for a 95% confidence level). As a consequence, there were not matrix interferences and the external calibration method could be used to determine the content of L-SeMet in food supplements. Taking into account that the response relative factor (RRF, factor which enables to study if the response of a minor component is equivalent to that for the major component and it is calculated dividing the slopes of the calibration lines, $slope_{minorcomponent}/slope_{majorcomponent}$) was between 0.8 and 1.2 such as the European Pharmacopoeia establishes [49], the response for D-SeMet can be considered equal to that of L-SeMet which implies that the percentage of D-SeMet can be determined from the ratio between the areas of L and D-SeMet. A standard solution of 0.8 mM DL-SeMet was employed to evaluate the method precision in terms of: (i) instrumental repeatability, obtained from six consecutive measurements of the standard solution in the same day, (ii) method repeatability assessed from three replicates of the standard solution injected in triplicate on the same day, and (iii) intermediate precision, determined from the measurement of

three replicates of the standard solution injected in triplicate in three consecutive days. As **Table 1** shows, acceptable precision values were obtained in all cases with RSD values not higher than 4.6 for corrected peaks areas and not higher than 5.1 for migration times. Accuracy was evaluated as the recovery obtained for L-SeMet when spiking (prior to derivatization) the food supplement FS1 (prepared at a concentration of 0.1 mM of L-SeMet taking into account the labeled amount) with four known concentrations of L-SeMet. The recovery obtained was 92 ± 3%. LODs and LOQs were calculated as the minimum concentration yielding a S/N ratio of 3 or 10, respectively. LODs for both enantiomers were 3.7×10^{-6} M and LOQs for L-SeMet and D-SeMet were 3.7×10^{-5} M and 3.1×10^{-5} M, respectively (see **Table 1**).

Table 1: Analytical characteristics of the developed MEKC methodology for the enantioselective determination of SeMet in food supplements.

	L-SeMet	**D-SeMet**
Linearity		
Linear range [a]	0.1 - 0.7 m M	0.1 - 0.7 m M
Slope ± t·s_b	4.471 ± 0.432	5.414 ± 0.380
Intercept ± t·s_a	0.156 ± 0.176	0.144 ± 0.154
r	0.996	0.998
p-value of ANOVA[b]	0.0894	0.8647
Precision	RSD (%)	RSD (%)
Instrumental Repeatability [c] (n = 3)	t, 3.5; Ac, 1.8	t, 3.4; Ac, 2.8
Method Repeatability [d] (n = 9)	t, 5.1; Ac, 4.6	t, 4.9; Ac, 3.1
Intermediate precision [e] (n = 9)	t, 4.7; Ac, 4.5	t, 4.5; Ac, 4.3
LOD [f]	3.7×10^{-6} M	3.7×10^{-6} M
LOQ [g]	3.7×10^{-5} M	3.1×10^{-5} M

[a] Seven standard solutions at different concentration levels were injected in triplicate for 3 consecutive days.
[b] p-value for ANOVA to confirm that experimental data fit properly to linear models.
[c] Instrumental repeatability was determined from six consecutive injections of SeMet standard solutions at 0.8 mM.
[d] Method repeatability was calculated by using the value obtained for three replicates of SeMet standards solutions at 0.8 mM injected in triplicate on the same day.
[e] Intermediate precision was calculated by using the mean value obtained each day for three replicates (injected in triplicate during three consecutive days) of SeMet standard solutions at 0.8 mM.
[f] LOD calculated as the concentration yielding a S/N ratio of 3
[g] LOQ calculated as the concentration yielding a S/N ratio of 10

Once demonstrated the suitability of the developed MEKC method for the enantioselective determination of SeMet, it was applied to the analysis of two different food supplements. The quantitative results obtained for the content of L-

SeMet in FS1 was 126 ± 6 μg/capsule, which corresponded to a percentage of 126% with respect to the labeled content of L-SeMet. The quantification of L-SeMet in FS2 was not possible since the content of this amino acid was lower that the LOD, indicating that its content was lower than that labeled (200 μg/capsule). As it can be seen in **Figures 4**, which show the electropherograms obtained for the two supplements analyzed, D-SeMet could not be detected in any of these samples, so its concentration was below the LOD of the method.

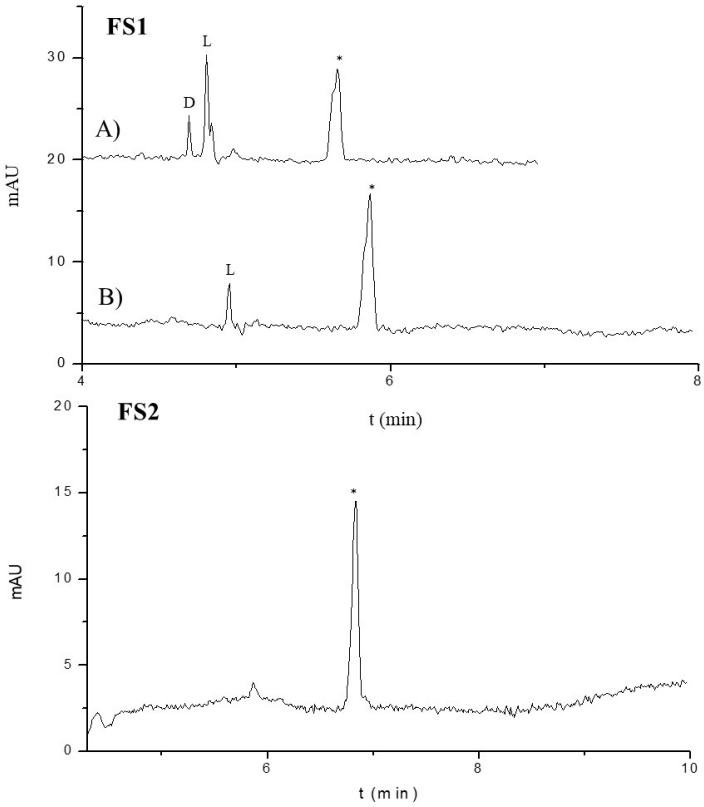

Figure 4. Electropherograms obtained for FS1 (0.1 mM L-SeMet) a) spiked (0.8 mM DL-SeMet) and b) non-spiked; and electropherogram obtained for FS2 (0.5 mM L-SeMet). Experimental conditions described in Figure 2.

4. Conclusions

In the present study, a MEKC methodology was developed based on the use of 100 mM APFO (pH 8.5) as pseudostationary phase and a fast and efficient pre-capillary derivatization with FLEC for the analysis of SeMet diastereoisomers in food supplements. The method allows a rapid separation of DL-SeMet (less than 6 min) with a resolution value of 4.4. LODs of 3.7 x 10^{-6} M were achieved for both SeMet diastereomers with satisfactory precision in terms of migration time and

peak area. Moreover, the method has been successfully applied to the quantitative determination of L-SeMet in food supplements.

The results obtained in this work highlight the potential of the developed methodology for the separation and selective detection of other neutral and/or charged non protein amino acids. In addition, the use of the semivolatile surfactant APFO, which is fully compatible with ESI-MS detection, open new possibilities for the enantioselective determination of SeMet in more complex food samples requiring more sensitive approaches.

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Article 6

Fast chiral discrimination of DL-amino acids by trapped ion mobility spectrometry after derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate.

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Abstract

A novel analytical method based on hybrid trapped ion mobility spectrometry-time of flight mass spectrometry (TIMS-TOFMS) has been developed to achieve fast enantiomeric separation of amino acids (AAs). Resolution of chiral AAs was achieved by forming diastereomers through derivatization with the chiral agent (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) without the use of reference compounds. Electrospray ionization (ESI) in positive mode yielded sodiated FLEC-AAs ions of which the diastereomers could be separated by TIMS. The effect of other alkali metal ions (such as Li and K) on the enantioselectivity was studied, but chiral discrimination was only observed for Na. TIMS conditions, including voltage ramp, ramp time and accumulation time were optimized for each AA and collision cross sections (CCSs) were determined for all diastereomers. The migration order of the DL enantiomers was found to be dependent on the structure of the AA. The resulting TIMS resolution (K0/ΔK0) for the FLEC-AA diastereomers on average was 115, requiring a mobility (K0) difference of about 0.009 cm^2/Vs to achieve 50%-valley separation. From the 21 AAs studied, enantiomer separation was achieved for 17 AAs with mobility differences ranging from 0.009 for lysine up to 0.061 cm^2/Vs for asparagine. Moreover, the presented methodology provided mutual separation of various AAs, allowing chiral analysis of multiple AAs simultaneously which may be challenging with previous enantioselective IMS approaches. It appeared possible to fully resolve all studied DL-AAs using three distinct TIMS methods, resulting in a total MS run time of about 3 min (1 min per method) and a total analysis time (including derivatization) of less than 15 min. The method demonstrated capable to determine enantiomeric ratios down to 2.5% with detection limits for the D enantiomers in the nM range. This new TIMS-based methodology opens up possibilities for easy and fast analysis of AA enantiomers.

1. Introduction

The intrinsic chiral environment of living systems makes enantiomers of a chiral compound often show mutually different chemical behaviour or biological activity. In fact, when interacting with enzymes, proteins or receptors, the enantiomeric configuration of the molecule is critical and changes may result in modulated biological function [1]. Molecular chirality clearly plays an essential role in biological, medical, pharmaceutical and food sciences, generating a strong requirement for enantioselective analytical methods.

Amino acids (AAs) are an important class of chiral molecules which constitute the building blocks of proteins and are key molecules in maintaining the

physiology of the organism [2]. Along with the 20 proteinogenic AAs, there are hundreds of AAs of non-protein origin, which also have different key functions [3, 4]. Most AAs are chiral compounds, and although L-AAs are the predominant active form in mammalian biology, the presence of D-AAs in living organisms and in the environment has been reported extensively [1]. Over the years, several analytical methods able to distinguish D and L AA enantiomers have been developed. Separation techniques, such as liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE), are the most commonly employed techniques in chiral AA analysis [5-7]. Their enantioseparation principle mostly relies on the selectivity provided by chiral stationary phases and/or selectors and separation times typically range between 5-30 min [8-11]. Mass spectrometry (MS), and in particular tandem MS, has emerged as an alternative technique for the fast chiral analysis of AAs. In this approach, enantiomers are first transformed into diastereomeric complexes using a chiral agent, and the chiral distinction is based on the different relative intensities of the fragment ions obtained for the respective AA enantiomers. However, interpretation of complex fragmentation patterns can be difficult, and as no actual separation of enantiomers takes place, enantiopure reference compounds are required [12, 13]. Ion mobility spectrometry (IMS) has demonstrated to be a powerful tool for the fast separation (milliseconds) of isobaric and isomeric compounds. In drift-tube IMS, ions are separated based on their mobility in an electric field through a neutral gas. As the mobility is a function of the ion collision cross section (CCS), which depends on the size and shape of the ion in the gas phase, compounds may be separated by IMS, even if their *m/z* ratio is the same [14]. In 2011, Fernández-Lima et al. introduced trapped ion mobility spectrometry (TIMS) [15, 16] in which ions are carried into the drift cell using a nitrogen gas flow. An electric field gradient is applied in the opposite direction in order to trap and separate ions depending on their size, charge and shape [17]. This allows use of much shorter IM drift tubes [18], while potentially achieving a high resolving power (up to 300), providing the possibility to attain fast separations of isobaric and isomeric compounds [19]. In TIMS, resolution (or resolving power) R is often defined as $K/\Delta K$ where K is the mobility and ΔK is the full width at half maximum (FWHM) of a compound peak in the mobilogram [17].

Compound enantiomers exhibit identical CCSs, making their direct separation by IMS not feasible. In order to achieve chiral separation of enantiomers by IMS, several strategies have been adopted, such as creating an asymmetric environment in the drift tube by doping the drift gas with a chiral agent [20, 12] or by inducing a conformational change with a complexing agent leading to different CCSs of the studied enantiomers [21, 14, 22]. Chiral separation of AAs by IMS has

been studied by few researchers. Prabha et al. achieved the enantioseparation of the AAs Ser, Met, Thr, Phe and Trp by doping the drift gas with a volatile chiral reagent ((S)-(+)-2-butanol) [12]. Further works on the enantioseparation of AAs by IMS employ the formation of non-covalent diastereomeric complexes of the AAs with a metal-ion containing chiral reference agent prior to IMS analysis [22, 14, 21]. Mie et al. firstly demonstrated the potential of this approach by the enantioseparation of six AAs using high-field asymmetric waveform ion mobility spectrometry (FAIMS). They formed metal-bound trimeric complexes of the form $[M(II)(L\text{-}Ref)_2(D/L\text{-}AA)\text{-}H]^+$ where M was Ni, Zn, Mg, or Cu, AA was Trp, Pro, Phe, Val, Arg and Lys, and L-Ref is the AA acting as chiral reference compound [22]. Using travelling wave ion mobility spectrometry (TWIMS), Domalain et al. described the differentiation of seven AA enantiomers through their cationisation with copper(II) and a reference AA. After screening eleven AAs, D-Phe was selected as the most suitable chiral reference compound [14]. In a similar way, Xiangying Yu et al., studied the chiral separation of Trp, Gln, Tyr, Thr, His, Glu, Met, Phe, and Arg by forming binuclear copper bound tetrameric ions with L-Trp, L-Pro, L-Tyr, L-Phe and L-His as reference [21]. The main limitation of these approaches is the need for specific reference compounds for each AA, limiting general application. Moreover, the enantiomeric discrimination was only demonstrated for individual AAs, i.e., no mixtures of AAs were analyzed.

The present work proposes a new alternative and generic approach for achieving chiral separation of AAs by employing TIMS. To this end, prior to TIMS analysis the AA enantiomers were quickly converted to diastereomers with the chiral reagent (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC). Subsequently, the diastereomers were efficiently separated based on their difference in ion mobility. Twenty-two proteogenic and non-proteogenic AAs were included in the study and parameters affecting IMS separation were evaluated. The novel enantioselective TIMS method is fast and applicable to any chiral AA, omits the need for AA-specific reference compounds, and allows to resolve multiple AAs in one run.

2. Experimental section

2.1 Chemicals

All reagents employed were of analytical grade. Sodium tetraborate, lithium carbonate, (+)-FLEC (18 mM in acetonitrile), 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl), ammonium hydroxide (28%, w/v), acetic acid and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium carbonate was obtained from Across Organics (New Jersey, US). Acetonitrile (ACN) was

supplied by Fluka (Steinheim, Germany). Water was deionized and purified with a Milli-Q purification system (Milli- pore, Belford, NJ, USA).

DL- and L- forms of tyrosine (Tyr), alanine (Ala), valine (Val), phenylalanine (Phe), tryptophan (Trp), histidine (His), arginine (Arg), lysine (Lys), isoleucine (Ile), methionine (Met), leucine (Leu), asparagine (Asn), serine (Ser), glutamic acid (Glu), proline (Pro), threonine (Thr), 2-Aminoadipic acid (Aad), and selenomethionine (SeMet), and D-glutamine (D-Gln), L-glutamine (L-Gln), D-pipecolic acid (D-Pipe), D-ornithine (D-Orn) and glycine (Gly) were purchased from Sigma-Aldrich (Madrid, Spain), whereas D-citrulline (D-Cit), L-citrulline (L-Cit), L-pipecolic acid (L-Pipe) and L-ornithine (L-Orn) were obtained from Fluka (Buchs, Switzerland).

2.2 Trapped ion mobility spectrometry

TIMS-TOFMS analysis was performed on a tims-TOF™ instrument from Bruker Daltonik (Bremen, Germany) equipped with an ESI source. A daily calibration was performed using the Agilent ESI low concentration Tune mix. Two types of calibrations were applied. In the first, a wide voltage scan allowed detection of all ions from the calibrant solution. Herewith a calibration from *m/z* 322.0212 to *m/z* 2121.9331 covering a 1/K0 range (K0 is the reduced mobility) from 0.7319 to 1.7286, allowing correction of small day-to-day variations in measured mobilities. For higher resolution IM analysis, calibration was performed over a smaller mobility ranges (1/K0, 0.7319-0.9848) using two or three *m/z* values (322.0481, 622.0290, 922.0098). In the latter case, the ramp voltage range was kept constant, but shifted to lower or higher voltages to detect all the required calibrant ions. Samples were directly injected using a syringe pump from Cole-Parmer (Vernon Hill, IL U.S.A.) with glass syringe of 1 mL from Hamilton (Bonaduz, Switzerland) at a flow rate of 180 µL/h. The TIMS was operated in positive-ion mode with an electrospray voltage of 4.5 kV. The mass range was set at *m/z* 100-3000. The ionization source conditions were: end plate offset, 500 V; nebulizer pressure, 0.5 bar; drying gas, 4 L/min at 200 °C. The TOFMS settings were: RF funnel 1, 150 Vpp; RF funnel 2, 300 Vpp; isCID energy, 0 eV; multipole R, 150 Vpp; quadrupole ion energy, 5 eV at low mass of 50 *m/z*; collision energy, 8 eV; collision RF, 600 Vpp; transfer time, 55 µs; pre-pulse storage, 1 µs.

TIMS was operated using two different modes. First, samples were analyzed using a wide voltage scan (109-160 V) to provide a general overview of the elution voltages. This mode was operated using general conditions of ion mobility such as: ramp start V, 109 V; ramp end V, 160 V; ramp time, 255 ms; accumulation time, 50 ms. Subsequently, TIMS parameters (accumulation, ramp voltages and ramp time) were adjusted to obtain maximum resolving power for a particular set of DL-AAs.

The duty cycle was 80 %. Optimum conditions are reported in the result and discussion section.

2.3 Sample preparation

Stock solutions of each AA were prepared dissolving the appropriate amount of AA in a buffer solution. Sodium tetraborate buffer solution (26 mM in Milli-Q water, pH 9.2) was employed as buffer solution to dissolve the AAs before the derivatization step. Lithium carbonate and potassium carbonate solutions (26 mM each) were prepared in Milli-Q water and adjusted with 20% acetic acid to pH 9.2. These AA solutions were stored at 4 °C until the derivatization step with FLEC.

Derivatization of AAs was performed according Prior et al. [23] 18 mM (+)-FLEC in ACN was freshly prepared each day. Briefly, 50 µL of a 90 µg/mL AA solution in 26 mM sodium tetraborate (pH 9.2) were mixed with 50 µL of 12 mM (+)-FLEC dissolved in ACN. The solution was kept at room temperature for 10 min to complete reaction, and the resulting solution was diluted 1:1 in Mili-Q water-ACN (50:50, v/v) before infusion into the ESI-TIMS-TOFMS system.

2.4 Data analysis

Resolution (R) was determined from the measured mobilograms (1/K0 vs. intensity) according $R=K0/\Delta K0$ where K0 is the reduced mobility of the respective analysed diastereomer and $\Delta K0$ its peak width at half height (FWHM). The peak mobilities (1/K0), the FWHMs and ions collision cross sections (CCS) were obtained using the Compass Data Analysis 5.0 software from Bruker Daltonik.

3. Results and discussion

3.1 Chiral resolution of FLEC-AAs by TIMS

Ions of enantiomers of an AA have identical charge, *m/z* and CCS making their direct separation by IMS not feasible. Up to date, separation of AA enantiomers by IMS has been achieved by using gas-phase ion complexing with volatile chiral agents, or by forming metal-ion non-covalent complexes with reference compounds [12, 22, 24, 25]. Since TIMS has demonstrated to be a powerful tool for the fast separation of isobaric or isomeric compounds [19], we have explored the potential of TIMS to resolve chiral AAs by first forming diastereomers in solution. For that purpose we selected the chiral agent (+)-FLEC which reacts covalently and fast with the amino group of an AA, resulting in two diastereomers per pair of enantiomers (**Figure 1A**). Initial experiments were performed using DL-SeMet and DL-Orn as model compounds. Orn contains two amine groups leading

to two FLEC molecules per AA. No singly derivatized FLEC-Orn was observed when analyzed by MS. ESI of the FLEC-AAs resulted in a mixture of protonated and sodiated ions.

The sodium ions largely originate from the buffer employed for derivatization. When analyzed by TIMS, for the protonated ions ([$FLEC_2$-Orn+H]$^+$, [FLEC-SeMet+H]$^+$) and ions containing one Na ion per molecule ([$FLEC_2$-Orn+Na]$^+$, [FLEC-SeMet+Na]$^+$) a single peak was observed for each AA (**Figure S1)**.

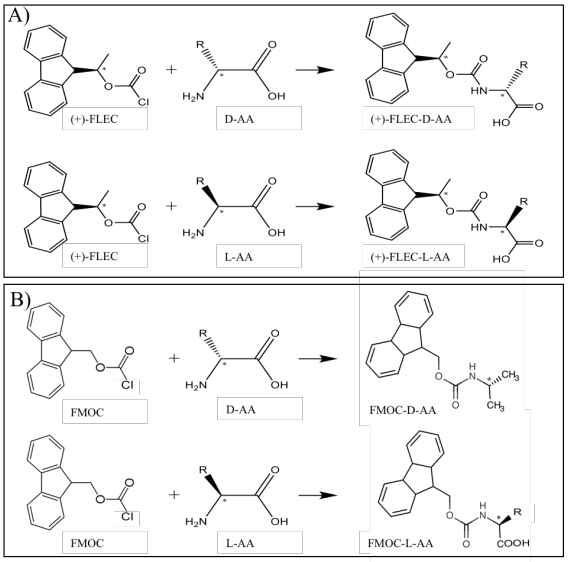

Figure 1. Scheme of the derivatization reaction of D- and L-AAs with A) FLEC and B) FMOC.

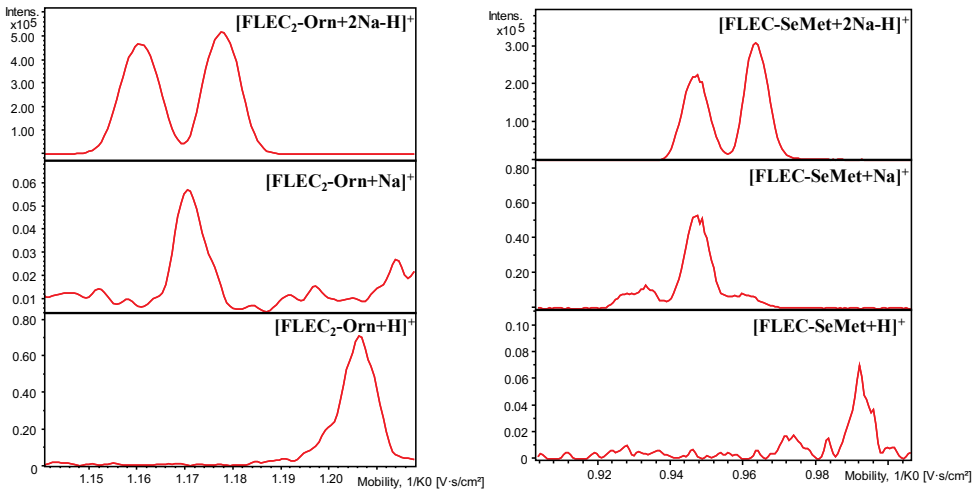

Figure S1. Mobilograms obtained during TIMS-TOFMS of FLEC-Orn and FLEC-SeMet.

On the other hand, the extracted-ion traces of the disodiated ions [FLEC2-Orn+2Na-H]+ and [FLEC-SeMet+2Na-H]+ both revealed two baseline separated peaks, suggesting separation of the respective diastereomers (**Figures 2A** and **2C**, respectively). As negative control, we also carried out the AA derivatization by FMOC-Cl. FLEC and FMOC have very similar structure, only differing a methyl group on the acyl moiety, rendering FMOC to be non-chiral (*cf.* **Figures 1A and 1B**). Consequently, FMOC derivatization of AAs results in two pair of enantiomers, and not diastereomers. When monitoring [$FMOC_2$-Orn+2Na-H]$^+$ and [FMOC-SeMet+2Na-H]$^+$ no separation is observed (**Figure 2**), indicating that TIMS indeed provides the FLEC-AA diastereomer separation.

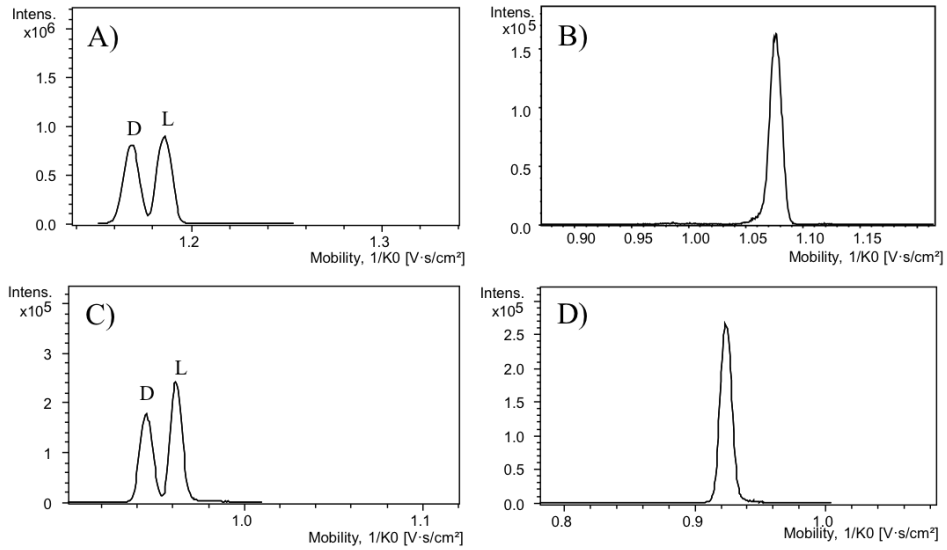

Figure 2. TIMS-TOFMS of FLEC-derivatized Orn and SeMet showing mobilograms corresponding to A) [$FLEC_2$-Orn+2Na-H]$^+$, B) [$FMOC_2$-Orn+2Na-H]$^+$, C) [FLEC-SeMet+2Na-H]$^+$, D) [FMOC-SeMet+2Na-H]$^+$.

Theoretically, multiple analyte peaks in (T)IMS may result from different positions of the adduct charge (i.e. occurrence of protomers and/or sodium-adduct isomers) [19, 26]. In order to exclude this possibility and to confirm that separation of diastereomers was achieved, L-Orn and L-SeMet, and D/L mixtures of these AAs (ratio D/L, 1:1 and 1:3). were analyzed. For both FLEC-AAs, only one peak was observed for the L enantiomer (**Figures 3A and 3B**), whereas two peaks were observed for the 1:1 and 1:3 D/L mixtures, with the former showing similar peak heights and the latter showing clearly higher peak intensities for the L-form. The analysis of the non-chiral AA Gly was also carried out after derivatization with FLEC.

This yielded one single peak in the mobilogram (**Figure S2**), again supporting the conclusion that the separation observed in TIMS indeed is caused by structural difference of the AA diastereomers and not by difference in adduct-ion position in the molecule or another reason.

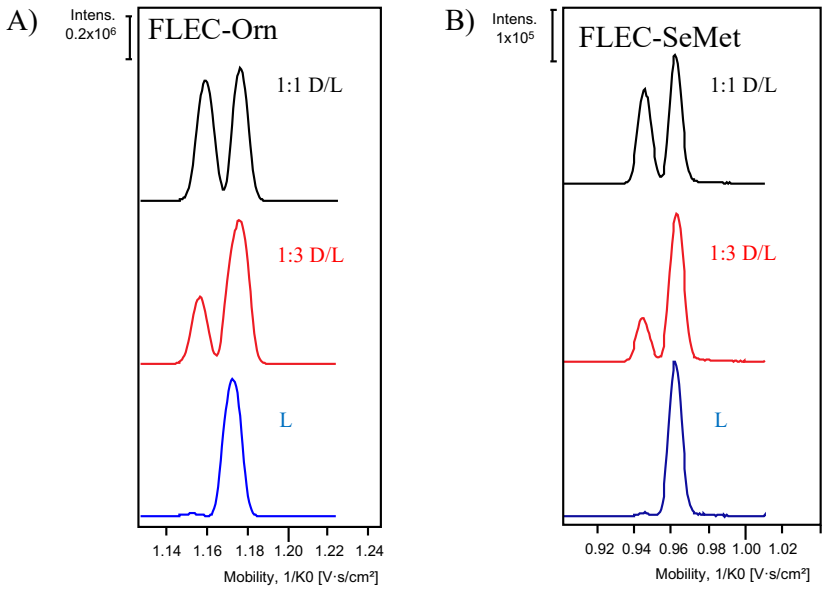

Figure 3. TIMS-TOFMS of FLEC-derivatized Orn (A) and SeMet (B) showing mobiligrams obtained for L-AA, 1:1 D/L-AA and 1:3 D/L-AA.

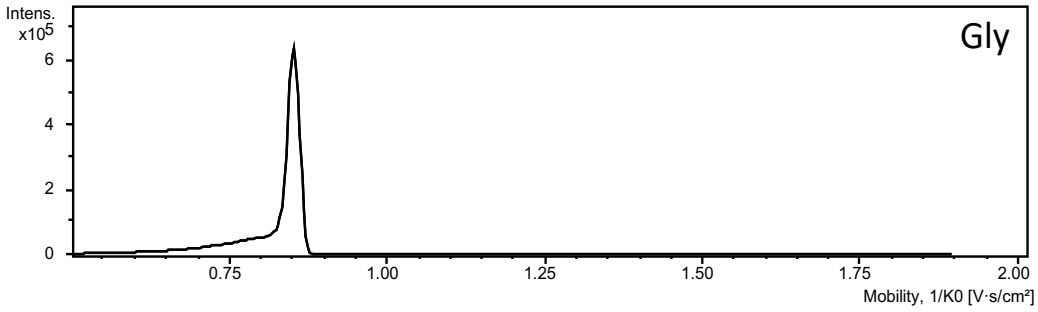

Figure S2: Mobilogram of [FLEC-Gly+H⁺] obtained during TIMS-TOFMS of FLEC-Gly

3.2 Effect of the alkali cation on the enantioseparation

Our initial results indicated that sodium-adduct formation was essential for the separation of the AA enantiomers by TIMS. This observation is in line with previous research on separation of isomeric molecules by IMS, where the formation

of alkali-cation adducts demonstrated to be a prerequisite for their efficient separation [14, 19, 21]. We also studied the effect of potassium and lithium cations on the chiral separation of AAs. FLEC derivatives of SeMet and Orn were prepared in solutions containing potassium or lithium ions, and then analyzed by TIMS. **Figure 4** shows the mobilograms obtained for the FLEC-AAs in presence of Li^+, Na^+ or K^+.

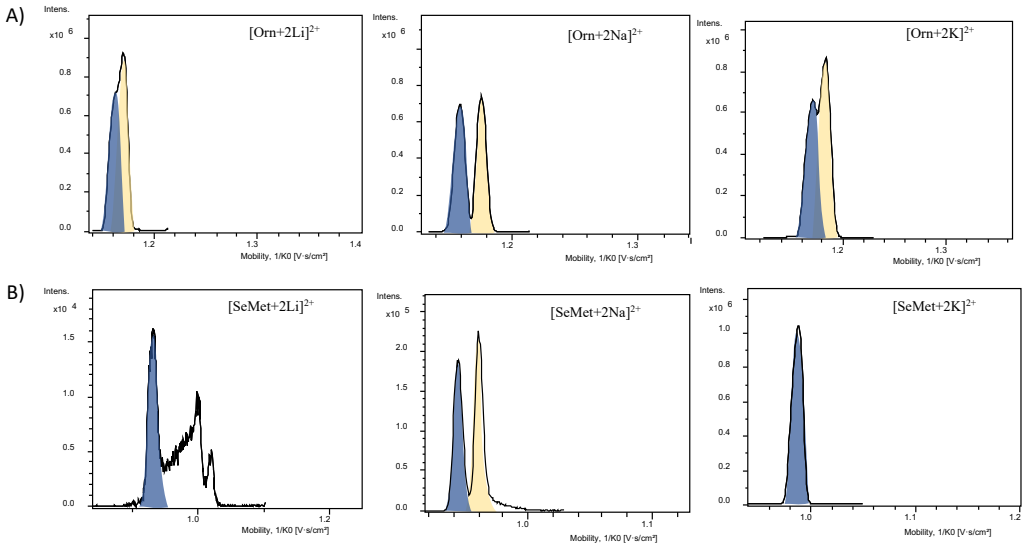

Figure 4. TIMS-TOFMS of FLEC-derivatized Orn (A) and SeMet (B) showing mobilograms obtained for in presence of lithium, potassium and sodium ions

No separation of FLEC-AA diastereomers was obtained when Li^+ was used, whereas with K^+ partial separation was observed for FLEC-Orn. **Table S1** (supporting information) lists the CCS values obtained for the different FLEC-AA adducts. For both AAs the CCS increase with the size of the metal ion. Looking at the CCS of the individual diastereomers it could be discerned that, when diastereomer separation is achieved, the first migrating diastereomer exhibits a deviation from this trend in comparison with the second diastereomer (*e.g.* CCS 240.3 for $[FLEC_2\text{-Orn+2Li-H}]^+$, and 237.7 and 241.5 for the first and second diastereomer of $[FLEC_2\text{-Orn+2Na-H}]^+$, respectively) (**Figure S3**). This most probably is the result of a conformational change of the first migrating diastereomer to a more compact form in presence of Na^+ in comparison to the second diastereomer.

As the formation of sodium adducts showed to be crucial for the separation, the amount of sodium ions in the derivatization buffer was increased (from 26 mM

to 50 mM). No change in diastereomer separation and intensity was observed, and therefore the concentration of 26 mM was maintained for further experiments (**Figure S4A**). When the AA concentration was varied (0.1-0.7 mM), the relative intensity of sodium adduct ions formed remained constant. Furthermore the ratio between ions containing one and two sodium ions was constant between experiments (**Figure S4B**).

Table S1. Observed *m/z*, collision cross section (CCS) obtained for Orn and SeMet during TIMS-TOFMS in presence of different cations.

	Li^+	***m/z***	**CCS [Å²]**
Orn	**2**	617.2803	240.3
SeMet	**1**	446.1019	193.3
	Na^+	***m/z***	**CCS [Å²]**
Orn	**1**	649.2281	237.7
	2	649.2281	241.5
SeMet	**1**	478.0497	194.9
	2	478.0498	198.3
	K^+	***m/z***	**CCS [Å²]**
Orn	**1**	681.1775	239.9
	2	681.1774	242.4
SeMet	**1**	510.0001	203.8

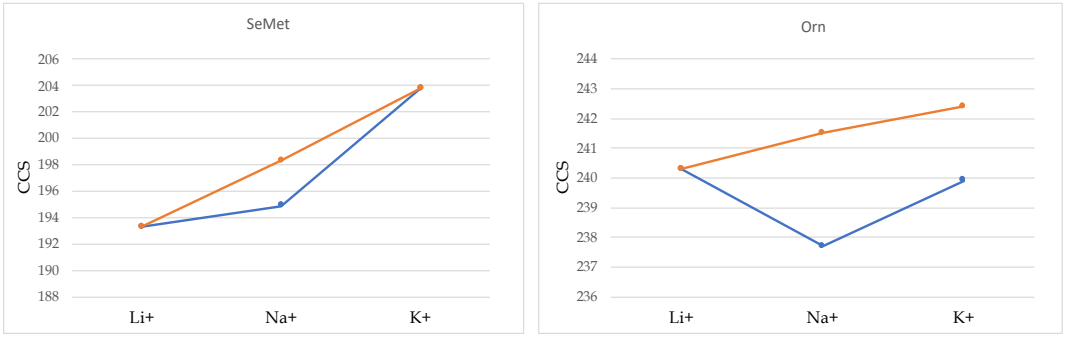

Figure S3. CCS values obtained for the two diastereomers (blue and orange lines) for FLEC-SeMet and FLEC-Orn adducts of Li, Na and K.

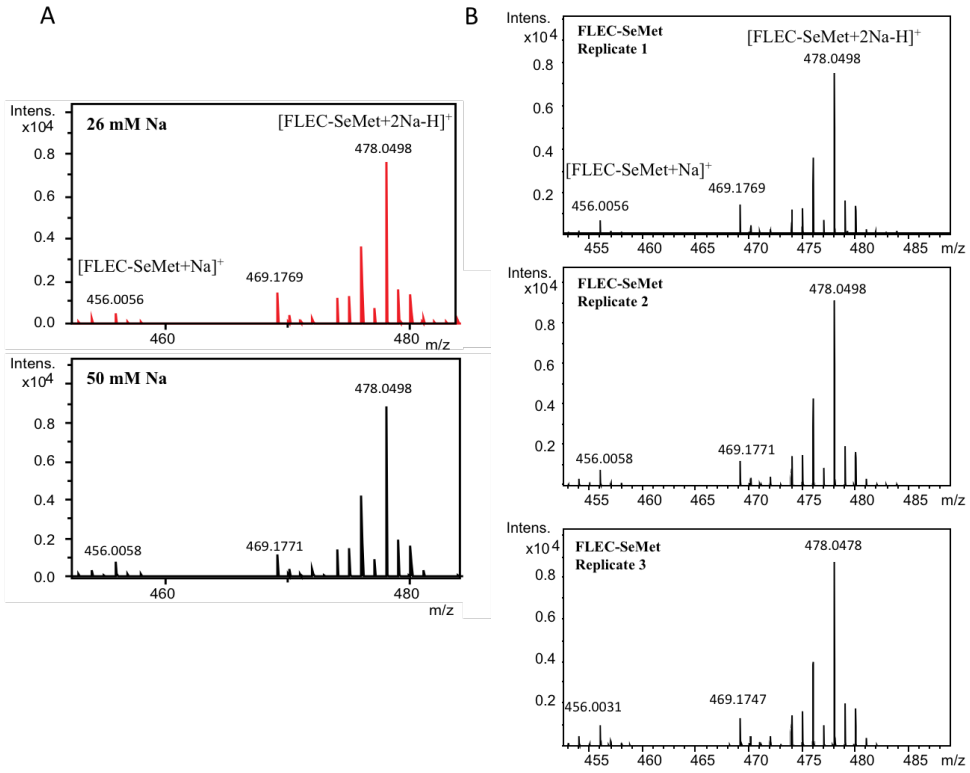

Figure S4. Mass spectra obtained for FLEC-SeMet using 26mM or 50 mM sodium tetraborate (pH 9.2) during derivatization (A). Mass spectra obtained for FLEC-SeMet in three different injections using 26 mM sodium tetraborate (pH 9.2) for derivatization.

3.3 Evaluation of TIMS conditions and applicability to multiple AAs

In order to further evaluate the potential of TIMS-TOFMS for separating FLEC-AA diastereomers, 21 AAs (17 proteinogenic and 4 non-proteinogenic AAs) were analyzed. AA solutions prepared in an enantiomeric ratio (D/L) of 1:1 and 1:3 were derivatized with FLEC and analyzed by direct infusion TIMS-TOFMS. Ions exiting the glass capillary are transferred into the TIMS cell by a nitrogen gas flow, whereas an opposite electrical field gradient is applied to trap and separate the ions. This takes place in a first TIMS analyzer. After trapping, the ions are moved to a similar second TIMS analyzer section where after trapping the electric field is decreased gradually by lowering the exit potential over time so that the ions are sequentially liberated into the time-of-flight mass spectrometer. The exit voltage range and the speed at which this voltage is changed (ramp time) define the slope of the electric field gradient which affects the resolving power of TIMS.

Initially, TIMS analysis were carried out using a wide voltage scan (109-160 V) providing the ion mobilities for all analyzed AAs. AAs carrying one FLEC per molecule were eluted at lower voltages than the AAs which contained two FLEC per molecule, such as Orn and Lys. In order to achieve optimum separation for each pair of diastereomers, the voltage range was narrowed down to a specific range and the voltage ramp time was adjusted (**Table 1**).

The TIMS sensitivity depends of the number of ions trapped in the first TIMS analyzer, which is determined by the time that ions are accumulated. Too long accumulation times can result in saturation of the trap and space-charge effects, and therefore the accumulation was studied between 10 and 100 ms. The FLEC-AA signal intensities increased with accumulation time until 50 ms, except for Ser, which showed the highest intensity at 25 ms. Further increase of the accumulation up to 100 ms did not result in higher signal intensities for most FLEC-AAs (except for His), suggesting saturation of the trap. Therefore, the accumulation time was set to 50 ms, but for His and Ser, 100 and 25 ms were selected, respectively. **Table 1** summarizes the ramp voltage, ramp time and accumulation time employed for each FLEC-AA as well as the mobility and mobility differences between the diastereomers obtained under these conditions.

From the 21 chiral AAs studied, separation of the FLEC diastereomers of 17 AAs was achieved. For Glu, Pro, Thr and Ala no enantioresolution was obtained. Diastereomer separation was obtained in general for disodiated FLEC-AAs ($[M+2Na-H]^+$), however, for Ile, Leu and Val, the diastereomer separation was observed for the singly sodiated species ($[M+Na-H]^+$). Next to AAs containing two amino groups, Tyr also appeared to be derivatized with two FLEC molecules, presumably due to a reaction of FLEC with the Tyr hydroxyl group under the conditions employed in this study. **Figure 5** shows the mobilograms obtained for the resolved AAs and **Table S1** their calculated CCS. For a large part, baseline separation of the FLEC-AA diastereomers was achieved. In the case of Arg, Lys, His, Met and Leu, the diastereomers were partially resolved. The resulting TIMS resolution ($K0/\Delta K0$) for the FLEC-AA diastereomers on average was 115, requiring a mobility difference ($\Delta K0$) of about 0.009 cm^2/Vs to achieve 50%-valley separation. Experimental $\Delta K0$ determined for the analysed diastereomers ranged from 0.09 for Lys (only partially resolved) to 0.061 for Asn. The particular migration order of the D and L enantiomers seems to be dependent on the structure of the AA (**Table 1**). Interestingly, this observation is opposite to the behavior of diastereomeric FLEC-AAs in capillary electrophoresis, where D-AAs commonly migrated first [23].

Table 1. Separation conditions, mobilities, ion mobility resolution and first migrating enantiomer for 21 FLEC-AAs analyzed by TIMS-TOFMS

AA	Observed m/z	Observed ion	Voltage range (V)	Ramp time (ms)	Accu. time (ms)	K0 diast1 (cm^2/Vs)	K0 diast2 (cm^2/Vs)	K01 - K02 (cm^2/Vs)	ΔK0 needed for 50% valley separation	R diast1	R diast2	First migrating enantiomer
Ile	390.0901	[FLEC-Ile+Na-H]$^+$	115-125	510	50	1.109	1.092	0.017	0.008	128.9	156.0	L
Leu	390.1680	[FLEC-Leu+Na-H]$^+$	109-160	255	50	1.099	1.081	0.018	0.011	91.0	108.1	L
Val	376.0901	[FLEC-Val+Na-H]$^+$	109-160	255	50	1.139	1.120	0.019	0.011	97.6	112.0	-[a]
Asn	413.1086	[FLEC-Asn+2Na-H]$^+$	109-130	510	50	1.126	1.065	0.061	0.010	111.0	118.3	L
Glu	428.1080	[FLEC-Glu+2Na-H]$^+$	109-160	255	50	1.064			0.010	94.0		-
Orn	649.2285	[$FLEC_2$-Orn+2Na-H]$^+$	146-160	510	50	0.861	0.849	0.012	0.008	116.1	94.3	D
SeMet	478.0505	[FLEC-SeMet+2Na-H]$^+$	115-130	510	50	1.056	1.037	0.019	0.008	118.4	129.7	D
Aad	442.1237	[FLEC-Aad+2Na-H]$^+$	115-135	510	50	1.057	1.037	0.020	0.007	135.1	148.2	D
Pipe	410.1338	[FLEC-Pipe+2Na-H]$^+$	115-130	510	50	1.089	1.064	0.025	0.009	114.7	118.2	D
Gln	427.1244	[FLEC-Gln+2Na-H]$^+$	105-130	510	50	1.068	1.042	0.027	0.010	104.0	115.7	L
Pro	396.1174	[FLEC-Pro+2Na-H]$^+$	109-135	510	50	1.092			0.010	91.6		-
Thr	400.1113	[FLEC-Thr+2Na-H]$^+$	109-160	255	50	1.109			0.09	100.2		-
Arg	455.1665	[FLEC-Arg+2Na-H]$^+$	120-130	510	50	1.032	1.017	0.015	0.009	107.7	127.2	D
Lys	663.2454	[$FLEC_2$-Lys+2Na-H]$^+$	145-155	510	50	0.861	0.852	0.009	0.009	116.2	85.2	D
Tyr	698.2139	[$FLEC_2$-Tyr+2Na-H]$^+$	115-170	510	50	0.818	0.803	0.016	0.009	101.8	80.3	L
Ala	370.1008	[FLEC-Ala+2Na-H]$^+$	105-160	255	50	1.153			0.009	96.3		-
Met	430.1047	[FLEC-Met+2Na-H]$^+$	110-125	510	50	1.089	1.075	0.014	0.008	114.8	153.6	D
Phe	446.1330	[FLEC-Phe+2Na-H]$^+$	115-130	510	50	1.083	1.049	0.034	0.009	115.4	131.2	L
Ser	386.0989	[FLEC-Ser+2Na-H]$^+$	105-120	510	25	1.145	1.120	0.026	0.008	124.7	160.0	L
Trp	485.1446	[FLEC-Trp+2Na-H]$^+$	109-135	510	50	1.024	0.995	0.029	0.010	97.7	99.5	L
His	672.2053	[$FLEC_2$-His+2Na-H]$^+$	140-155	510	100	0.833	0.823	0.010	0.008	120.0	82.3	D

[a]migration order was not addressed as pure enantiomers were not available.
accu. time, accumulation time.
diast, diastereomer.

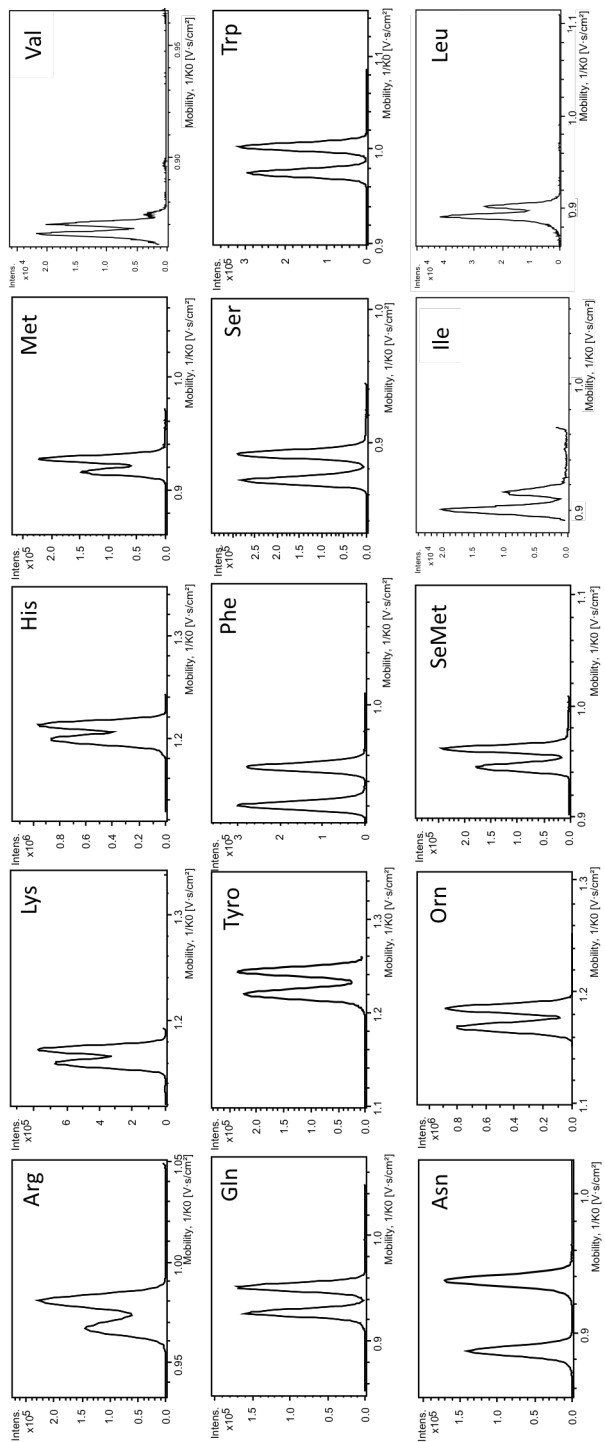

Figure 5. TIMS-TOFMS of 17 FLEC-AAs using conditions optimized for each AA.

3.4 Chiral resolution of FLEC-AAs in mixtures

The developed TIMS-TOF method allowed separation of FLEC-AA diastereomers in less than one minute. As all AAs are derivatized under the same conditions, we addressed the possibility to resolve multiple AAs in one TIMS-TOFMS run while maintaining chiral selectivity. Considering that the FLEC-AAs require specific voltage ramps to be chirally resolved, no single method was able to resolve all AAs. Therefore, three different voltage ramps were applied in order to achieve separation of most of the FLEC-AAs. A mixture of Orn, Lys, His and Tyr was analyzed using a voltage ramp from 135 to 170 V, a mixture of Asn, Ser, Pipe and Gln using 100-130 V, and a mixture of Met, Phe, SeMet and Arg using 109-135 V. For all mixtures a ramp time of 510 ms was used. The mobilogram obtained for the analysis of the mixture of Asn, Ser, Pipe and Gln (**Figure 6**) shows diastereomer separation for all four AAs. Some diastereomers comigrated, but still the individual AAs could be discerned by their specific *m/z* value.

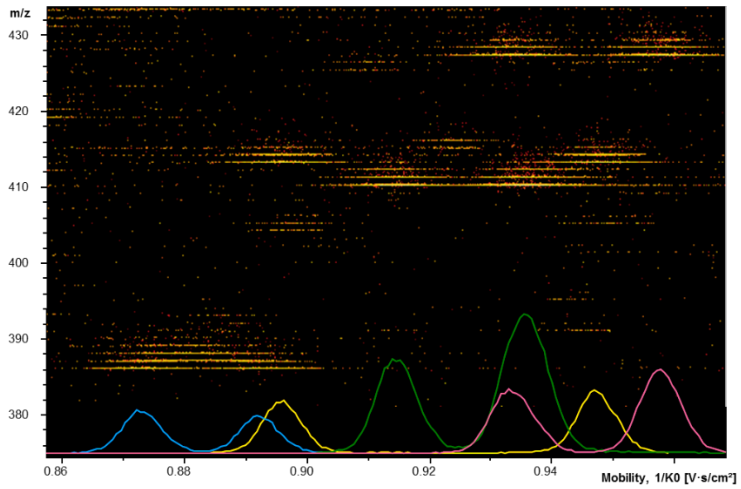

Figure 6. Heat map and extracted-ion mobilograms obtained during TIMS-TOFMS of a mixture of DL-Asn (*m/z*, 413.1086), DL-Ser (*m/z*, 386.0989), DL-Pipe (*m/z*, 410.1338) and DL-Gln (*m/z*, 427.1244) using a voltage ramp of 100 to 130 V in 510 ms.

Similar results were obtained for the other mixtures of AAs (**Figures S5A and S5B)**. This open new possibilities of analysis of AAs in mixtures which is not possible with ion mobility approaches in which a pure enantiomer of a different amino acid is required as chiral reference compound [22, 14, 21].

The TIMS-TOF method allowed detection of FLEC-AAs down to the low-nM level, demonstrating similar sensitivity to previously described analysis of FLEC-AAs by ESI-MS [23, 27]. TIMS-TOF showed able to detect an DL enantiomeric

ratio down to 2.5% D indicating the potential of the method for the determination of the enantiomeric excess in real samples (**Figure S6**).

The method showed good reproducibility with RSDs between 0.09% and 0.8% for diastereomer mobilities, between 1.3% and 9.6 % for diastereomer peak areas, and between 0.1% and 0.9% for the calculated CCS values.

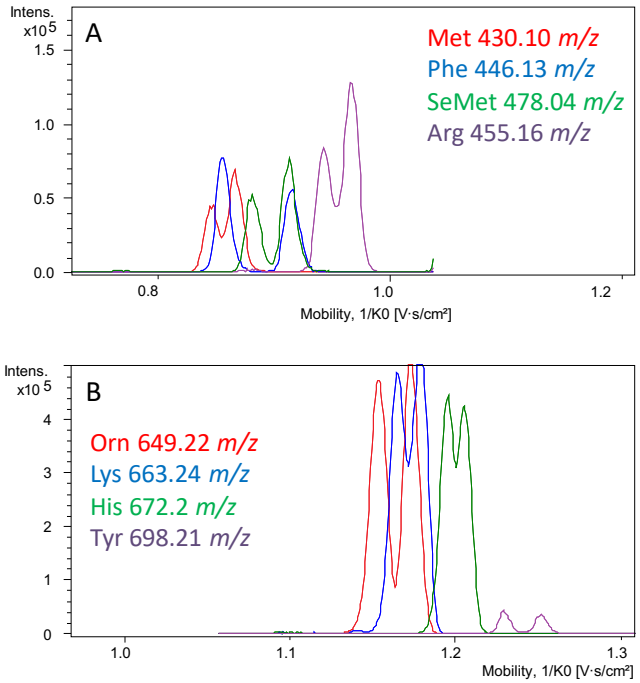

Figure S5. TIMS-TOFMS of mixtures of FLEC-AAs using a voltage ramp of (A) 109-135 V and (B) 135-170 V in 510 ms.

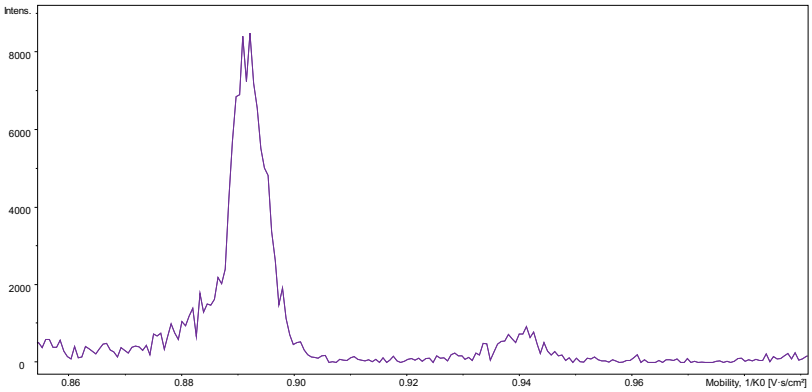

Figure S6. TIMS-TOFMS of a mixture of FLEC-L-Asn (0.225 ng/mL) and FLEC-D-Asn (0.0056 ng/mL) showing the mobilogram of $[FLEC\text{-}Asn+2Na\text{-}H]^+$.

4. Conclusions

This work presents a new and fast method for the separation of DL-AA enantiomers by TIMS after derivatization with the chiral reagent (+)-FLEC. By forming diastereomers before analysis, doping of the IMS drift gas with a chiral volatile complexing agent is not needed. When compared to earlier reported approaches, the proposed method simplifies and uniforms diastereomer formation, avoiding the use of specific reference compounds, and extending the applicability to a large number of AAs. Our study indicates that adduct formation with alkali ions is essential for achieving the separation of the diastereomeric FLEC-AAs. The new method has shown to be applicable to a variety of AAs and chiral separation was achieved for 17 from the 21 AAs studied. The analyte ions are separated on the millisecond time scale and TIMS analysis times of lower than 1 min are sufficient to attain adequate results. We also have demonstrated that the simultaneous separation of diastereomers in a mixture by TIMS is very well possible. Taking the derivatization time (only 10 min) into account, mixtures of AA enantiomers can be resolved in a total analysis time of less than 15 min. Limits of detection are in the nM range and determination of enantiomeric ratios down to 2.5% are feasible. Overall, the presented methodology provides a promising general approach for separating chiral compounds with potential applications in different fields, such as environmental or pharmaceutical analysis. Applications to more complex matrices, such as food or biological fluids, will require further evaluation of the effect of varying sodium ion concentrations in the samples.

Acknowledgements

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CHAPTER IV

DEVELOPMENT OF METABOLOMIC STRATEGIES TO EVALUATE THE COFFEE ROASTING PROCESS

IV.1. Preface

Coffee is one of the most consumed drinks in the world. The flavor, the aroma and the color of a freshly prepared cup of coffee is the last expression of a long chain of transformations linked from the seed to the cup.

Roasting process of green coffee beans is one of the most influencing steps in coffee production since it leads to the development of typical aroma and taste of a cup of coffee. It is a time-temperature dependent complex process which involves mechanical, thermal and chemical changes with the formation and/or degradation of many compounds that affect the final organoleptic characteristics of the brew coffee. For instance, during this thermal process, Maillard reactions and pyrolysis take place which gives rise to the formation of a different molecules responsible for a range of aromas [236-238]. Therefore, the understanding of the transformations occurring during roasting is of highly importance to establish a target final flavor and to achieve a coffee of high quality both for the coffee industry and for consumers. Coffee roasting is usually controlled in the food industry by physical measurements of the roasting degree at the end point of the process, consisting of measuring the color of the roasted ground beans and the weight loss during roasting. Other parameters such as texture, density and size are also considered to evaluate this procedure. However, by using these parameters, certain changes of the chemical composition of coffee beans cannot be appreciated.

In the last five years, several works were published aimed to develop analytical strategies enabling to evaluate the effects of the roasting process on the chemical composition of coffee beans and brew. Most of these works were based on the analysis of a single component or a specific class of compounds such as diterpens (cafestol and kahweol) [239, 240], chlorogenic acids [241-243], lipids [244], acrylamide, and polyphenols [245, 246]. Just a few works dealt with the use of untargeted metabolomics strategies to carry out the evaluation of the coffee roasting process [247-252]. These works were mainly focused on the discrimination of green and roasted coffee through the metabolomics analysis of volatile organic compounds using different techniques such as non-separative headspace solid phase microextraction-mass spectrometry (HS-SPME-MS) [247], resonance-enhanced multiphoton ionisation time-of-flight mass spectrometry (REMPI-TOFMS) [248], the combination of proton-transfer-reaction time-of-flight mass-spectrometry (PTR-ToF-MS) with HS-SPME-GC/MS [249], photoionization mass spectrometry with single-photon ionization (SPI) or REMPI [250], HS-SPME-GC/MS and/or GC–MS [251], and IMS–MS [252]. Only two of the published works were focused on the untargeted metabolomics analysis of the non-volatile fraction

of coffee. Kučera et al. proposed an UPLC-MS platform to evaluate changes in the chemical composition of espresso coffee during the roasting process which enabled to point out chlorogenic acids and related lactones, atractylosides and particular melanoidins, as chemical markers of the roasting process [253]. On the other hand, Rosa et al., developed an untargeted approach based on the use of ambient sonic-spray ionization (EASI) coupled to MS to monitor roasting chemical changes in the coffee bean highlighting the presence of N-alkanoyl-5-hydroxytryptamides as chemical markers for a light roast and diacylglycerols and triacylglycerols as markers of dark to a very dark roasting degree [254].

Taking into account that the evaluation of coffee roasting process has scarcely been investigated by LC-MS and it has never been studied using CE-MS, and to ensure a broad coverage of the coffee metabolome to obtain as much information as possible on the effect of coffee roasting, different metabolomics strategies based on the use of RPLC, HILIC and CE coupled to high resolution MS (QTOF) have been developed in this Chapter to provide a comprehensive characterization of the entire metabolome of *Arabica* green coffee beans submitted to different roasting degrees.

IV.2. Objectives

The objectives of this chapter were:

- To optimize an extraction protocol enabling to obtain the largest number of possible compounds from green and roasted coffee beans.

- To develop untargeted metabolomics strategies based on the use of RPLC-MS, HILIC-MS and CE-MS for the analysis of green coffee beans and coffee beans submitted to different degrees of roasting.

- To select the most influencing variables (metabolites) differentiating the experimental groups of green and roasted coffee samples using multivariate statistical analysis.

- To identify the maximum number of metabolites showing statistically significant differences among the different degrees of coffee roasting.

IV.3. Results and discussion

Arabica green coffee beans (GCB) analyzed were roasted in a pilot plant using three different degrees (light (LRC), medium (MRC), and dark (DRC) roasted coffee) under the conditions shown in **Table IV.1**. The process was controlled by the weight loss of each sample which corresponded to a 13 % in LRC, 15 % in MRC and 17 % in DRC.

Table IV.1. Temperature and time of roasting process.

Roasting conditions	LRC	MRC	DRC
T (ºC)	175	185	195
Time (min)	12.36	14.11	17.06

As discussed in the Introduction of this PhD Thesis (section I.2.1.1), the metabolite extraction protocol is a crucial step in the development of a metabolomics strategy. Therefore, in this work, water and different organic solvents (methanol and ethanol) at different percentages, and aqueous solutions at two different pH values (2.0 and 9.0) were tested as extracting solvents to carry out the metabolite extraction from coffee samples. To evaluate the extraction efficiency, all the extracts obtained were analyzed by RPLC-MS both in positive and in negative ionization modes. Among them, 25 % methanol enabled to achieve the highest number of molecular features in both ionization modes. The influence of the use of different devices (ultrasound bath and Thermomixer) and extraction times (15 or 30 min) on the extraction procedure was also investigated. No substantial differences in the number of molecular features extracted were observed among the different conditions so that the Thermomixer system was selected since it enabled the control of the extraction temperature. An extraction time of 15 min was chosen in order to short the sample preparation step. This extraction protocol was employed for RPLC-MS, HILIC-MS and CE-MS analyses.

Some chromatographic and electrophoretic conditions and MS parameters were individually optimized for the three metabolomics platforms to improve sensitivity, to avoid peak co-elution and, consequently, to reduce possible ion suppression events. Mobile phase composition, gradient elution, flow rate column temperature and injection volume are some of the variables studied in terms of chromatographic separation and peak efficiency when using RPLC and HILIC approaches. For CE, some parameters related to the CE-MS coupling (composition and flow rate of the sheath liquid), and the electrophoretic separation (BGE, temperature, voltage, and injection time) were optimized in order to achieve a good

electrophoretic separation. Regarding MS conditions, the effect of the fragmentator voltage, nozzle voltage, drying gas flow and temperature capillary voltage, sheath gas temperature, etc., was investigated in order to improve sensitivity to ensure the detection of the largest number of molecular features in coffee samples. The values tested and optimized for all the above-mentioned experimental variables for the three untargeted metabolomics strategies developed in this work are detailed in the articles included at the end of this chapter except for the HILIC platform (the corresponding article is under preparation). In this case, the chromatographic separation conditions consisted of the use of water containing 10 mM ammonium formate plus 0.2 % formic acid (solvent A) and acetonitrile containing 2 mM ammonium formate plus 0.2 % formic acid (solvent B) as mobile phase, a flow rate of 0.2 mL/min, a column temperature of 30 °C, and an injection volume of 5 µL, being the MS conditions the same as the employed for the RPLC-based metabolomics platform.

Only the untargeted analysis of cationic metabolites (positive ionization mode) was carried out using the CE-MS platform since in this case the outlet of the capillary is not immersed in the vial electrolyte. Thus, by using a fused silica capillary and normal polarity, the EOF moves from the anode to the cathode (where the MS detector is located), and the CE electrical current is stabilized. When using a fused silica capillary and reversed polarity for the analysis of anionic species, the direction of the EOF is opposed to the MS detector, and then, unstable electrical connection between the tip of the capillary and the grounded electrospray needle occurs. For this reason, the analysis of anions (negative ionization mode) by CE using fused silica capillaries requires to adopt additional approaches such as the use of coated capillaries.

To achieve the untargeted analysis of coffee samples by the three different analytical platforms developed, the experimental design included four experimental groups: GCB, LRC, MRC, and DRC. From each coffee group, five replicate samples injected in triplicate were analyzed, so that the total number of coffee samples was 60. All these samples, including QC (prepared by combining equal aliquots from each coffee extract (GCB, LRB, MRB, and DRB)), were analyzed in a randomized order.

MPP was used individually in data processing and treatment stages for each one of the three metabolomics platforms developed. In the case of CE-MS, due to the high variability in migration times, MPP was first used to carry out a time correction and data alignment, and then, a normalization step of data was performed using the intensity of the internal standard. Signal feature filtering was achieved by retaining features present in the 100 % of QC samples and excluding

those having RSD values higher than 30 % for both RPLC-MS and HILIC-MS whereas features present in the 80 % of QC samples and excluding those having RSD values higher than 35 % were considered for CE-MS due to the variability among samples.

The resulting number of features to be further statistically analyzed were 280 and 522 for RPLC-MS in positive and negative ionization modes, respectively, 53 and 40 for HILIC-MS in positive and negative ionization modes, respectively, and 39 for CE-MS in positive ionization mode. The analytical consistency of the sequences was estimated from the degree of clustering of QC samples in the PCA score plots. Usually, the CE-MS metabolomics sequence offered the less tight cluster, probably because of the higher variability and less robustness inherent to this technique. However, a good clustered was observed for QC for the three metabolomics platforms. When the PCA models were built without taking into account the QC group, the explained variable (R^2 value) obtained for the three platforms were: 93.8 % and 91.3 % for RPLC-MS (in positive and negative ionization modes, respectively), 86.7 % and 66.6 % for HILIC-MS (in positive and negative ionization modes, respectively) and 88 % for CE-MS. The variance covered by the two first principal components for the three models was quite similar: 82.4 % and 78 % for RPLC-MS (in positive and negative ionization modes, respectively), 71.2 % and 66.7 % for HILIC-MS (in positive and negative ionization modes, respectively) and 82 % for CE-MS.

Regardless of the analytical technique employed, in all cases, a clear difference of groups (GCB, LRC, MRC and DRC) was found in PCA analysis revealing metabolic differences among the four groups. Later on, the PLS-DA models for each platform, were built. All samples submitted to different roasting degree were compared with green coffee beans to get an insight into metabolic changes associated with roasting conditions. The cross-validated score plots and permutation tests were performed to assess the risk of models overfitting. In all cases, the cross-validated score plots displayed the class separation as that from original PLS-DA models and the R^2 and Q^2 values were the highest from those of permutated models, demonstrating that all models were valid.

Using VIP values, a subset of the most influential variables was selected for each model on each platform. Following this strategy, 24 and 33 variables were selected for RPLC-MS in positive and negative ionization modes, respectively, 18 and 13 variables were found for HILIC-MS in positive and negative ionization modes, respectively, and the use of CE-MS highlighted 13 variables in positive ionization mode. Using the *m/z* value of the statistically significant variables (signal

features) and looking them up in CEU mass mediator and FooDB databases, a list of statistically significant metabolites tentatively identified was provided (19 different metabolites by RPLC-MS, 16 by HILIC-MS, and 7 by CE-MS). **Figure IV. 1.A** groups the list of metabolites tentatively identified by each of these platforms highlighting those metabolites common to two platforms. For example, as shown in the Venn diagram (see **Figure IV.1.B**), one isomer of caffeoylshikimic acid was a common tentatively identified metabolite for both LC-MS-based platforms developed (RPLC-MS and HILIC-MS) while choline was common for HILIC-MS and CE-MS platforms. Limited availability and high cost of the standards made complicated in some cases the unequivocal identification of metabolites. Those metabolites unequivocally identified by each platform using standards are marked (in bold and with an asterisk) in **Figure IV.1.A.** A total of nine different metabolites were unequivocally identified by the whole multiplatform developed in this work.

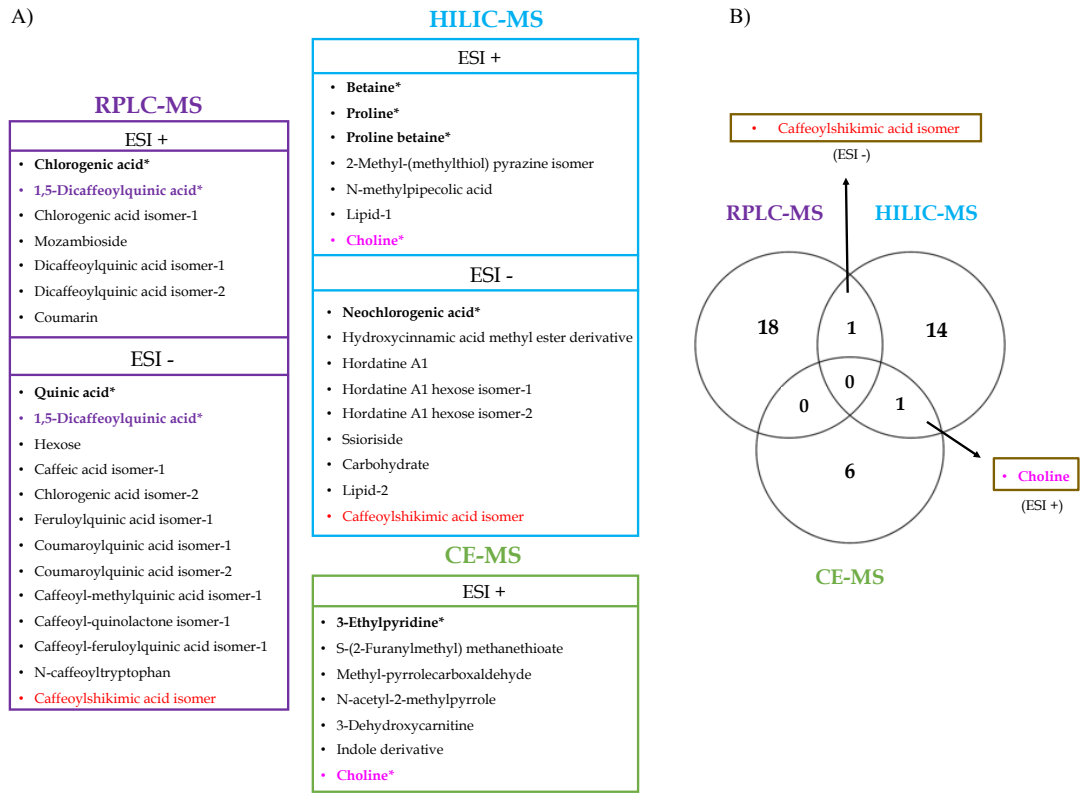

Figure IV.1. A) List of metabolites tentatively identified by each of the platforms developed. Those metabolites common to two platforms are highlighted using different colors. Metabolites unequivocally identified for each platform by the injection of standards (matched retention/migration times and MS/MS spectra fragmentation) are marked in bold and with an asterisk. B) Venn diagram displaying the total number of different metabolites tentatively identified by the untargeted multiplatform developed in this work and those common to two platforms.

These results highlight the importance of the use of different analytical techniques to provide information on the whole metabolome. They also showed that some isomers of the same compound (e.g. dicaffeoylquinic acids or coumaroylquinic acid) with the same *m/z* but different retention times were found. However, the analytical techniques employed in this PhD Thesis cannot discern between isomers, being their unequivocal identification only possible when standards are available.

As both ionization modes (positive and negative) for RPLC-MS and HILIC-MS were considered for statistical analysis and subsequent identification, they provided complementary information. For instance, for RPLC-MS, some metabolites with the same nominal mass were found in both ionization modes (one of them was unequivocally identified as 1,5-dicaffeoylquinic acid). These metabolites were identified as hydroxycinnamic acids that, in agreement with the literature, can be ionized in both ESI modes. However, after analyzing the MS/MS fragmentation of these compounds, only one of them seemed to be common for both RPLC-MS and HILIC-MS platforms (caffeoylshikimic acid isomer). Moreover, this compound showed the same trend along the roasting process by the two analytical techniques (RPLC-MS and HILIC-MS). The content of this compound seemed to be relatively stable whereas the contents of other compounds from the same family decreased. This fact highlights the consistency of the results regardless of the analytical platform used. In general, different trends could be observed for the variation of the identified metabolites with the coffee roasting process. As expected, the levels of many compounds decreased with the roasting process (e.g. chlorogenic acids) whereas other formed as products of Maillard reaction, increased (e.g. 3-ethylpyridine, methyl-pyrrolecarboxaldehyde or N-acetyl-2-methylpyrrole).

Results presented in this Chapter demonstrate the high potential of the cross untargeted multiplatform developed in this work and based on RPLC-MS, HILIC-MS and CE-MS to study a food processing such as the coffee roasting process.

The results obtained in this Chapter are included in the following scientific articles:

- **Article 7:** *A non-targeted metabolomic approach based on reversed-phase liquid chromatography-mass spectrometry to evaluate coffee roasting process.*
  R. Pérez-Míguez, E. Sánchez-López, M. Plaza, M. Castro-Puyana, M.L. Marina.
  Anal. Bioanal. Chem. 410 (2018) 7859-7870.

- **Article 8:** *Capillary electrophoresis-mass spectrometry metabolic fingerprinting of green and roasted coffee.*
  R. Pérez-Míguez, E. Sánchez-López, M. Plaza, M.L. Marina, M. Castro-Puyana.
  Submitted.

Article 7

A non-targeted metabolomic approach based on reversed-phase liquid chromatography-mass spectrometry to evaluate coffee roasting process.

R. Pérez-Míguez, E. Sánchez-López, M. Plaza, M. Castro-Puyana, M. L. Marina.

Anal. Bioanal. Chem. (2018) 410:7859–7870.

Abstract

In this work, a non-targeted metabolomics approach based on the use of reversed-phase liquid chromatography coupled to a high resolution mass spectrometer has been developed to provide the characterization of coffee beans roasted at three different levels (light, medium, and dark). In this way, it was possible to investigate how metabolites change during the roasting process in order to identify those than can be considered as relevant markers. 25 % methanol was selected as extracting solvent since it provided the highest number of molecular features. In addition, the effect of chromatographic and MS parameters was evaluated in order to obtain the most adequate separation and detection conditions. Data were analyzed using both non-supervised and supervised multivariate statistical methods to point out the most significant markers that allow groups discrimination. A total of 24 and 33 compounds in positive and negative ionization modes, respectively, demonstrated to be relevant markers, being most of them from the hydroxycinnamic acids family.

1. Introduction

Coffee beverage is one of the most consumed drinks in the world. Arabica Coffee, produced by *Coffea arabica* species, is the most consumed and exported coffee variety since it has been considered to have higher sensory properties than other species [1]. Chemical composition of coffee comprises alkaloids, phenolic compounds, carbohydrates, amino acids, proteins and lipids, and some of them are known to present beneficial properties in humans, which makes this beverage a natural source of bioactive compounds. For instance, caffeine, the main alkaloid present in coffee that grants its stimulant nature, has demonstrated, together with chlorogenic acids, to present antioxidant properties [2, 3]. Due to the fact that there are numerous steps in the production of coffee which affect the chemical composition of coffee beans [4], even modifying the organoleptic properties of the drink, it is important to evaluate how coffee chemical composition behaves under the different processes which take place from the growth of its beans until coffee is consumed.

From the different steps carried out during coffee production, the roasting process is notably one of the most important. In this process, several physical and chemical reactions happen with the formation and/or degradation of many compounds responsible for specific organoleptic properties (aroma, flavor and color) that affect the quality of coffee. For instance, total chlorogenic acids composition is reduced during roasting whereas the formation of chlorogenic acid lactones takes place [5, 6], the combination of sugars and amino acids during the

Maillard reaction results in the formation of melanoidins [7, 8]. Also, the exposition of carbohydrates to high temperatures affects their composition (they are degraded to lower molecular weight compounds such as mono and oligosaccharides) due to the conversion of part of sugar in the coffee bean into Maillard reaction and pyrolysis products during roasting, and is responsible for the bean color. Indeed, among the reactions that take place during pyrolysis, pyrazines, molecules known to affect the aroma, are formed by pyrolysis of hydroxy amino acids, while protein pyrolysis together with the degradation of trigonelline are involved in pyridine formation [9]. On the other hand, the composition of other compounds such as lipids or caffeine has demonstrated to be slightly affected by thermal processes [10]. All these changes in the chemical composition of coffee have a great impact in its quality, which makes relevant the search of markers capable of discriminating the changes occurring during the roasting process to ensure the quality and safety of the coffee not only from an industrial point of view but also for the consumers. However, despite the great interest and relevance in studying the changes occurring in coffee during roasting process, the vast majority of reported studies are based on the analysis of a single component or specific class of compounds [11-13]. In this sense, non-targeted metabolomics emerges as a promising tool to obtain the exhaustive and comprehensive analysis of the set of metabolites present in a given system, without prior knowledge on what to look for [14]. Up to date, only few metabolomic works were focused on coffee analysis. On this matter, nuclear magnetic resonance (NMR) [15, 16] and both liquid chromatography mass spectrometry (LC-MS) and gas chromatography mass spectrometry (GC-MS) have been used in the discrimination of different coffee varieties or origins [17-20], being the MS-based techniques also used to discriminate between caffeinated and decaffeinated coffee [21].-However, to the best of our knowledge, the evaluation of the coffee roasting process has only been performed employing a targeted analysis based on the use of ion mobility spectrometry–mass spectrometry (IMS-MS) [22], and using non-targeted metabolomic approaches based on NMR, ambient sonic-spray ionization-mass spectrometry (EASI-MS) and GC-MS [23- 26]. On the one hand, Wei et al. [23], using a NMR-metabolomics based approach together with a human sensory test, found the chemical substances in roasted coffee bean extracts that could distinguish and predict the different sensations of coffee taste (two degrees of roasted coffee, light and dark were studied). Despite notable advantages of NMR for metabolomic analysis, the major drawback of this technique is its low sensitivity when compared to MS. On the other hand, Santos da Rosa et al. [24] proposed an untargeted and non-volatile approach with EASI coupled to MS to monitor roasting chemical changes in the coffee bean, whereas Sgorbini et al. [25,

26] developed a non-separative headspace solid phase microextraction-mass spectrometry methodology to discriminate volatile compounds among coffee beans submitted to different roasting degrees using GC-MS. Despite LC-MS is the analytical technique most widely used in metabolomics, it has never been applied to study the metabolites changing over the roasting process in coffee. Thus, the aim of this work was to develop, for the first time, a non-targeted metabolomic strategy based on the use of Reversed-Phase Liquid Chromatography (RP-LC) coupled to high resolution MS in order to evaluate changes in the metabolic profiles of coffee samples submitted to different roasting degrees. To accomplish this task, the workflow followed in this study was: (i) the optimization of the sample preparation procedure to obtain the largest number of extracted metabolites, (ii) the optimization of the RP-LC-MS conditions to maximize number of detected peaks, (iii) the metabolic analysis, including data processing and chemometric analysis, and (iv) the identification of the molecular features which show statistical differences along different roasting degrees, i.e. markers of the roasting process.

2. Materials and methods

2.1. Reagents and samples

Acetonitrile, ethanol, methanol, and formic acid of MS grade were purchased from Fisher Scientific (Hampton, New Hampshire, USA). Ultrapure water for the chromatographic mobile phase and for preparing coffee extracts was obtained from a Milli-Q system (Millipore, Madrid, Spain). Ammonium formate, sodium borate, phosphoric acid, verapamil, niflumic acid, propranolol, terfenadine, chlorogenic acid, shikimic acid, trans-caffeic acid, paraxanthine, mannose, quinic acid, theobromine, caffeic acid were purchased from Sigma (St. Louis, MO, USA). Neochlorogenic acid, 1,3-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid were purchased from Plantachem (Pinnow, Germany).

2.2. Coffee beans

Green coffee beans (GCB) of the Arabica variety were roasted to light level at 175 ^{o}C during 12.36 min (LRC), medium level (MRC) at 185 ^{o}C during 14.11 min, and to dark level (DRC) at 195 ^{o}C during 17.06 min. The weight loss of each sample was evaluated in order to control the roasting process being 13 % in light coffee, 15 % in medium coffee and 17 % in dark coffee. All these samples were grounded and provided by "Café Fortaleza" (Vitoria, Spain).

2.3. Sample preparation

Grounded coffee samples were extracted using methanol (25 % in water) as extraction solvent (50 mg in 1.5 mL). The solid-liquid extraction procedure was performed using a Thermomixer Compact (Eppendorf AG, Hamburg, Germany) at 700 rpm during 15 min at room temperature (25 ºC). After extraction, the samples were centrifuged at 3500 rpm for 10 min at 25 ºC. Next, the supernatant fraction was injected in the RP-LC-MS system. Replicate extraction of each group of coffee samples (GCB, LRC, MRC and DRC) (n = 5) were prepared for the metabolomic sequence.

A quality control (QC) sample was prepared by combining equal aliquots from each coffee extract (GCB, LRC, MRC, and DRC). Moreover, a test sample was prepared by adding four standards (verapamil, niflumic acid, propranolol, and terfenadine) at 0.1 µg/mL to the QC sample.

2.4. RP-LC-MS conditions

A 1100 series LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a 6530 series quadrupole time-of-flight (QTOF) mass spectrometer (Agilent Technologies, Germany) equipped with a Jet Stream thermal orthogonal electrospray ionization (ESI) source was employed to perform the analyses. MS control, data acquisition and data analysis were carried out using the Agilent Mass Hunter Qualitative Analysis software (B.07.00).

Chromatographic separation was performed on a porous-shell fused-core Ascentis Express C18 analytical column (150 x 2.1 mm, particle size 2.7 µm) protected by an Ascentis Express C18 guard column (0.5 cm × 2.1 mm, 2.7 µm particle size), both from Supelco (Bellefonte, PA, USA). LC analyses were performed using mobile phases composed of water containing 0.1 % formic acid (solvent A) and acetonitrile containing 0.1 % formic acid (solvent B) eluted according to the following gradient: 5-100 % B in 45 min; 100 % B during 4 min; 100-5 % B in 2 min; and then the column was re-equilibrated for 15 min using the initial solvent composition. The mobile phase flow rate was 0.2 mL/min, the column temperature was set to 30 ºC, and the injection volume was 5 µL.

MS analyses were carried out both in positive and negative ESI modes with the mass range set at *m/z* 100-1700 (extended dynamic range) in full scan resolution mode at a scan rate of 2 scans per second (mass resolution greater than 5000 on the 118 m/z and 10000 on the 1522 m/z according to the instrument specifications). ESI parameters for the mass spectrometer were as follows: capillary voltage for positive and negative ionization modes of 3000 V with a nozzle voltage of 0 V; nebulizer

pressure at 25 psi; sheath gas of jet stream of 6.5 L/min at 300 ºC; and drying gas of 10 L/min at 300 ºC. The fragmentator voltage was set at 175 V whereas the skimmer and octapole voltages were 60 V at 750 V, respectively. For MS/MS experiments, the selected precursor ions were fragmented by applying voltages between 20 and 40 V in the collision chamber.

In order to obtain proper mass accuracy, spectra were corrected using ions *m/z* 121.0508 ($C_5H_4N_4$) and 922.0097 ($C_{18}H_{18}O_6N_3P_3F_{24}$) in ESI positive, and *m/z* 119.0363 ($C_5H_4N_4$) and 966.0007 ($C_{18}H_{18}O_6N_3P_3F_{24}$ + formate) in ESI negative. To achieve this task, a solution from Agilent Technologies containing those ions was continuously pumped into the ionization source at a 15 µL/min flow rate using a 25 mL Gastight 1000 Series Hamilton syringe (Hamilton Robotics, Bonaduz, Switzerland) on a NE-3000 pump (New Era Pump Systems Inc., Farmingdale, NY, USA).

2.5. Metabolomics sequence

The metabolomics sequence was designed as follows: blanks and QC sample were injected at the beginning of the metabolic sequence to ensure good stability and repeatability of the chromatographic system. Then, a total of 60 coffee samples (four groups of samples and five replicates for each group injected in triplicate) were randomly injected and a QC sample was injected every six coffee samples.

Moreover, a QC sample containing four known standards (see section 2.3) was injected eight times during the sequence in order to evaluate the mass accuracy (lower than 4 ppm) and retention time shifting (RSD around 0.2 %).

2.6. Data processing and multivariate analysis

Molecular Feature Extraction (MFE) tool from Mass Hunter Qualitative Analysis (B.07.00) was used to obtain the information related to the molecular features, i.e. chromatographic peaks, present in each sample. The MFE extraction algorithm selected was "small molecules (chromatographic)" using the following parameters: ions ≥ 500 counts; peak spacing tolerance = 0.0025 m/z, plus 7.0 ppm; isotope model = common organic molecular; and limited assigned change was set to 2. To identify different ion species coming from the same molecular feature, H^+, Na^+, K^+, and NH_4^+ adducts were taken into account in positive ionization, whereas that only the $HCOO^-$ adduct was considered for negative ionization.

Filtering and alignment of the extracted molecular features were performed with Agilent Mass Profiler Professional (MPP) software (B.02.00). Molecular feature filtering was carried out using a minimum absolute abundance of 10.000 counts; number of ions 2 and all charges permitted. Molecular feature alignment was

performed using a retention time window of 0.15 min, a mass tolerance of 0.02 Da and a mass window of 15 ppm. To clean data matrix from background signals, only molecular features present in 100 % of all injected QC samples with a coefficient of variation below 30 % were retained for further data analysis.

Multivariate statistical analysis was carried out using SIMCA 14.0 software (MSK Data Analytics Solutions, Umeå, Sweden) where data were centered and divided by the square root of the standard deviation as scaling factor (Pareto scaling). An unsupervised principal component analysis (PCA) was first applied to investigate clustering existing in the analyzed samples. Then, partial least squares discriminant analysis (PLS-DA) was used to discriminate samples according to their roasted degree. The quality of the models was evaluated by the goodness-of-fit parameters R^2X, R^2Y and Q^2.

2.7. Metabolites identification

Molecular features which displayed significant differences in the PLS-DA models were subjected to the identification process. Metabolite identification was performed by matching the obtained accurate mass values and the theoretical mass values (considering an error of 30 ppm in order to increase the number of possible metabolites) in the CEU Mass Mediator [27], which is a tool for searching metabolites in different databases (Kegg, Metlin, LipidMass and HMDB), and in the database FooDB (http://foodb.ca/).

In those cases, in which the standard compounds could be commercially acquired, they were analyzed under the same analytical conditions to obtain their retention time and MS/MS fragmentation in order to confirm the metabolite identity. When standards could not be acquired, experimental MS/MS spectra obtained for each molecular feature were compared to those described both in HMDB database and literature, and/or predicted MS/MS spectra obtained in CFM-ID (cfmid.wishartlab.com).

3. Results and discussion

3.1 Extraction procedure optimization

Sample treatment is probably one of the most crucial steps in metabolomics, especially in non-targeted studies where metabolites of interest are not known *a priori*. To obtain the greatest number of metabolites from the coffee samples, different solvents, such as methanol (25, 50, 70 and 90 % in water), ethanol (25, 50, 70 and 90 % in water), 100 % water, and aqueous solutions at two different pH (2.0 and 9.0) (obtained by adding to water small amounts of phosphoric acid and

sodium borate, respectively) were considered during the extraction procedure performed in a Thermomixer during 30 min at 25 ºC. All the coffee extracts obtained for every extraction solvent were analyzed by RP-LC-MS, both in positive and negative ionization modes, and the number of molecular features obtained by the MFE algorithm was employed to evaluate the extraction efficiency. 25 % methanol was selected as extracting solvent since it provided the highest number of molecular features in both ionization modes, followed by 100 % water, aqueous buffer at pH 2.0 or 9.0 (which resulted in a similar molecular feature content) and 25 % ethanol (see **Table S1**). Once selected the extracting solvent, the influence of the extraction procedure was evaluated in terms of using an ultrasound bath or a Thermomixer system. Even though the ultrasound bath enabled to obtain a slightly higher number of molecular features for both ESI modes, the use of the Thermomixer system was chosen since it enabled controlling the temperature of the extraction process, an important parameter which severely affects the sample stability. Next, the extraction time was evaluated. No substantial differences in the number of molecular features were observed when 15 or 30 min were used as extraction time, so 15 min was selected to achieve the extraction in both positive and negative ionization modes to short the sample preparation step.

Table S1. Total number of molecular features present in a QC sample obtained by MFE software using different extraction solvents.

SOLVENT	ESI +	ESI -
Methanol 25 %	1625	1523
Methanol 50 %	1555	1483
Methanol 70 %	1421	1252
Methanol 90 %	1614	1216
Ethanol 25 %	1221	1047
Ethanol 50 %	1025	975
Ethanol 70 %	994	1082
Ethanol 90 %	933	723
Water 100 %	1434	1336
Aqueous solution at pH 2.0	1329	1340
Aqueous solution at pH 9.0	1387	1305

3.2 Optimization of the RP-LC-MS metabolomics method

Optimization of the chromatographic and MS parameters is essential to improve sensitivity, to avoid peak co-elution and, consequently, to reduce possible

ion suppression events, which will negatively bias the obtained results. On the one hand, chromatographic parameters were optimized by evaluating the chromatographic peak profiles observed in the total ion chromatogram (TIC) and the based peak chromatogram (BPC). First, the addition of 0.1 % of formic acid or 10 mM ammonium formate to the water/acetonitrile mobile phase was compared in both ionization modes. It was observed that the use of formic acid provided, not only a better chromatographic separation, but also a greater number of molecular features for positive and negative modes. Along with the mobile phase composition, other parameters such as gradient elution, flow rate (ranging from 0.15 to 0.25 mL/min), column temperature (ranging from 30 to 50 ^{o}C) and injection volume (2, 5 and 10 µL) were studied in terms of chromatographic separation, peak efficiency and sensitivity to ensure the detection of the largest number of metabolites in the coffee samples. The optimal conditions were the use of water (solvent A) and acetonitrile (solvent B) both containing 0.1 % formic acid as mobile phases eluting according to the gradient described in experimental section, a flow rate of 0.2 mL/min, a column temperature of 30 ^{o}C, and an injection volume of 5 µL. On the other hand, ESI parameters were also studied: fragmentator voltage (100-200 V), nozzle voltage (0-100 V), drying gas temperature (200-350 °C) and sheath gas temperature (250-400 °C). 175 and 0 V for fragmentator and nozzle voltages, respectively, and 300 ^{o}C for both drying gas and sheath gas temperature, were the selected parameters. Using the optimized parameters, it was possible to detect 1206 and 1184 molecular features for positive and negative ESI modes, respectively. **Figure 1** shows the BPC obtained for GCB, LRC, MRC and DRC coffee samples under optimal conditions.

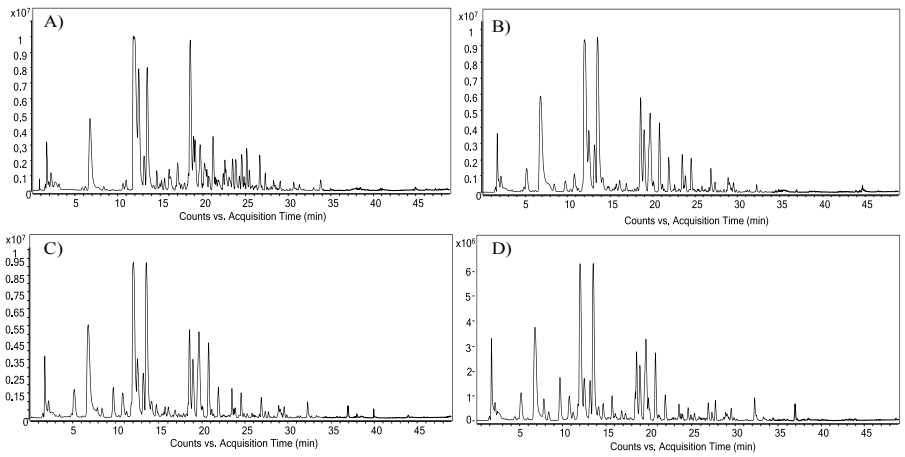

Figure 1. Base peak chromatograms (BPC) obtained in negative ionization mode for green coffee (GCB) (A); light coffee (LRC) (B); medium coffee (MRC) (C); and dark coffee (DRC) (D) under optimal separation conditions. RP-LC-MS conditions are summarized in Section 2.4.

3.3 Non-targeted analysis of RPLC-MS data by multivariate statistical analysis

Once optimized the extraction procedure and selected the most adequate RP-LC-MS parameters to carry out the metabolomic analyses, all coffee samples (a total of 60 samples) and QC samples were analyzed following the metabolomic sequence described previously (see section 2.5) and data treatment was performed according to section 2.6. A total of 10450 and 6770 features were obtained for the analyzed samples in positive and negative ionization modes, respectively. After filtering by frequency (only features present in 100 % of QC samples) and variability (features whose RSD was below 30 % in QC samples), a total of 280 molecular features were obtained in positive ionization mode whereas 580 were found for the negative ionization mode.

Multivariate data analysis, in particular, principal component analysis (PCA) has been used in most of works concerning metabolomics strategies. The goal of this statistical method is to reduce large volumes of data in order to find out the most relevant variations among groups of samples [28]. Thus, PCA analysis was first employed to evaluate the consistency of the metabolomics sequence using QC sample clustering. **Figures 2A and 2B** show the PCA score plot for all the coffee and QC samples analyzed both in positive and negative ionization modes. As it can be seen in these figures, a good clustering and high differentiation among groups of samples were obtained for both ionization modes. In addition, QC samples were also tightly clustered and were centered in the score plot, demonstrating good analytical consistency of the data during the whole metabolomics sequence. The score plots of the PCA models without the QC are shown in **Figures 2C and 2D.** The first and second components of the PCA models explained 49 % and 29 % of variance for positive ESI mode and 63 % and 20 % of variance for negative ESI mode, respectively. As the next step, supervised partial least square discriminant analysis (PLS-DA) was used to discriminate group samples. First PLS-DA models were built taking into account the four groups of coffee samples (GCB, LRC, MRC and DRC) which demonstrated that the four groups were grouped separately regardless of the ionization mode used. The quality parameters (R^2X, R^2Y, and Q^2) of both PLS-DA models are shown in **Table 1**. This table also shows the F and p-values of ANOVA test. The high values obtained for F and the low values achieved for p-values, along with the results obtained for permutation test (Q2 and R2 values are below the original values), demonstrated the quality of the model.

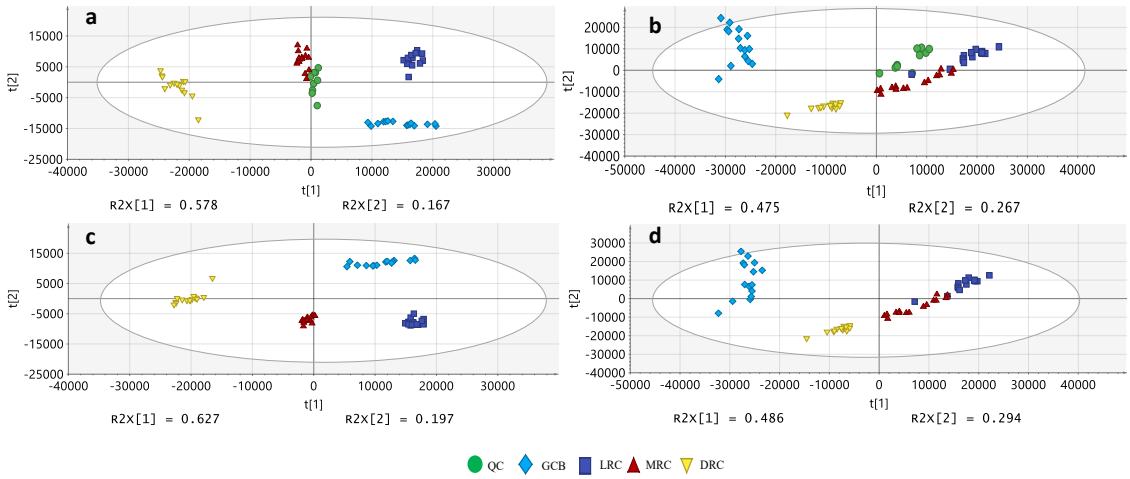

Figure 2. Principal component analysis (PCA) score plot obtained in positive (A) and negative (B) ionization modes, for the four studied coffee groups (GCB, LRC, MRC and DRC) submitted to different roasting degree with QC samples, and PCA score plot obtained in positive (C) and negative (D) ionization modes for coffee sample submitted to different roasting degree without QC samples.

Table 1. PLS-DA models for samples submitted to different roasting degree compared with green coffee.

	ESI+		ESI-	
	Quality parameters	**F (and P-values) of cross-validated ANOVA**	**Quality parameters**	**F (and P-values) of cross-validated ANOVA**
GCB, LRC, MRC, DRC	R2X = 0.931 R2Y = 0.976 Q2 = 0.951	71.4 (0)	R2X = 0.947 R2Y = 0.966 Q2 = 0.895	12.9 (1.5×10^{-30})
GCB vs LRC	R2X = 0.775 R2Y = 0.992 Q2 = 0.987	394.5 (3.4×10^{-22})	R2X = 0.819 R2Y = 0.996 Q2 = 0.984	428.0 (1.26×10^{-22})
GCB vs MRC	R2X = 0.812 R2Y = 0.989 Q2 = 0.977	234.1 (2.0×10^{-19})	R2X = 0.726 R2Y = 0.991 Q2 = 0.982	286.5 (1.7×10^{-20})
GCB vs DRC	R2X = 0.765 R2Y = 0.984 Q2 = 0.970	151.2 (3.9×10^{-17})	R2X = 0.756 R2Y = 0.992 Q2 = 0.978	202.2 (1.2×10^{-18})

Taking into account that the PLS-DA models build on four groups cannot reveal slight differences existing among the group of coffee samples, different PLS-DA models to compare the groups in a pairwise way (GCB vs LRC, GCB vs MRC, and GCB vs DRC) were built to obtain further knowledge on what metabolites are affected by the roasting process.

Figure 3 and **Table 1** show the PLS-DA models and the quality parameters obtained for each pairwise comparison.

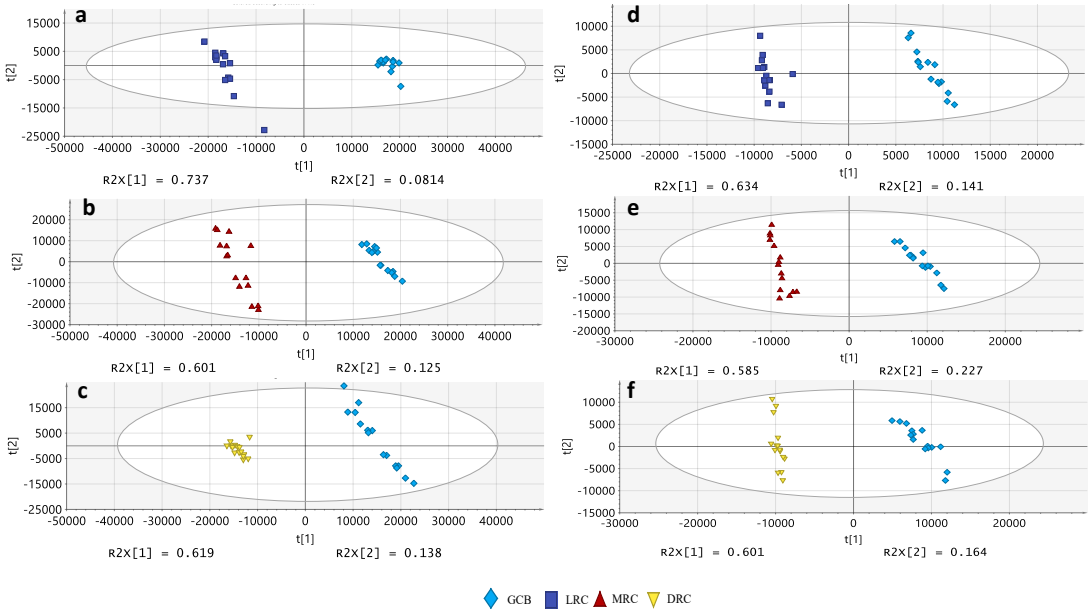

Figure 3. Partial least squares discriminant analysis (PLS-DA) score plots of LRC, MRC and DRC compared with GCB in negative ionization mode (A, B and C, respectively) and in positive ionization mode (D, E and F, respectively).

High Q2 values (> 0.970) were obtained in all models. F and p-values obtained in the cross validated ANOVA of PLS-DA models reinforced the robustness of the proposed models and demonstrated the good separation between groups (F values higher than 151.2 and p-values lower than 3.9×10^{-17}). In order to verify that group sample separation was due to real differences in metabolic profiles of analyzed samples and not just due to data overfitting, cross validation (CV) and permutation tests were performed.

As can be seen in **Figures S1 and S2,** pairwise PLS-DA models were considered as valid and robust since CV score plots showed group separation and slope obtained in the permutation tests was positive for R^2 and Q^2 values.

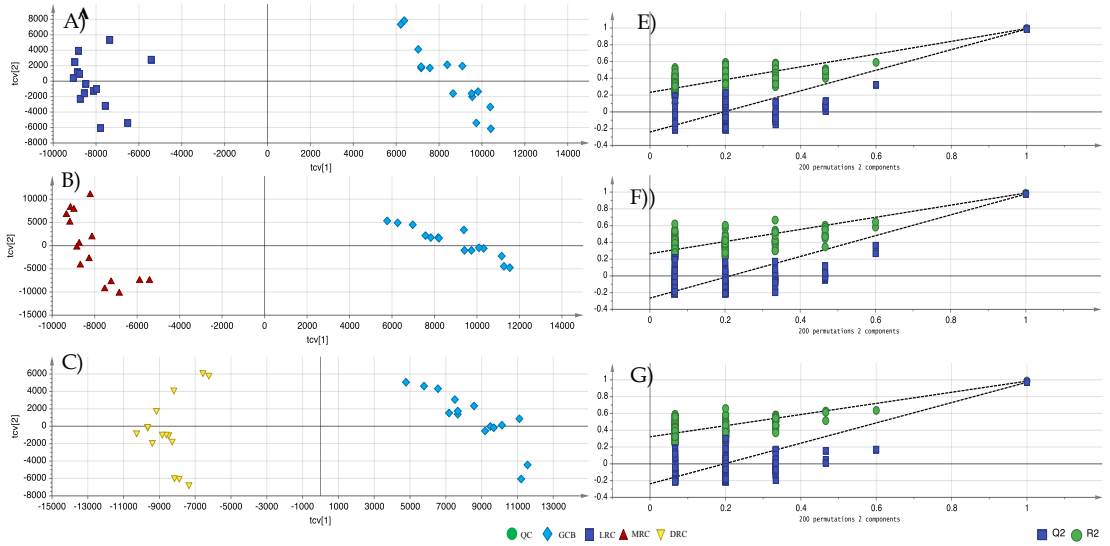

Figure S1. PLS-DA of CV score plot for (a) GCB vs LRC, (b) GCB vs MRC and (c) GCB vs DCR and permutation test for (d) GCB vs LRC, (e) GCB vs MRC and (f) GCB vs DRC in negative ionization mode.

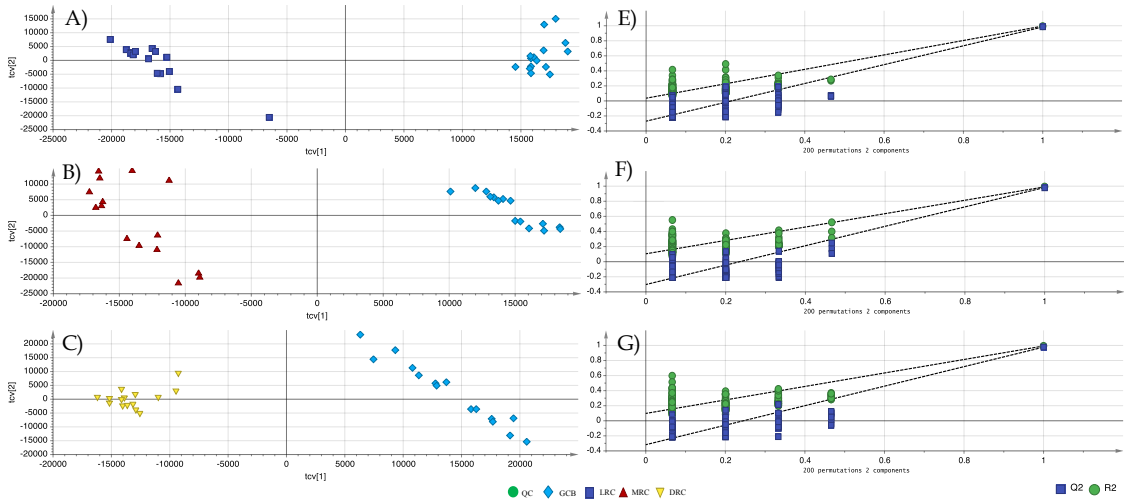

Figure S2. PLS-DA of CV score plot for (a) GCB vs LRC, (b) GCB vs MRC and (c) GCB vs DCR and permutation test for (d) GCB vs LRC, (e) GCB vs MRC and (f) GCB vs DRC in positive ionization mode.

This fact corroborated what is observed in PCA models, i.e. there were real statistical significant differences in the metabolic profiles from the studied groups and therefore it was possible to establish a set of variables responsible for these differences. The selection of these variables was performed according to the variable importance in the projection (VIP) value for each PLS-DA model. Thus a total of 25 and 34 variables with VIP values higher than 1.5 were selected as the most influencing variables in positive and negative ionization modes, respectively.

3.4 Metabolite identification

Metabolite identification usually corresponds to the most laborious step within metabolomics studies, not only for being time-consuming but also due to different factors such as the lack of standards for some compounds (or their high price) or the difficulties in the comparison of the MS/MS spectra for others. Bearing in mind that the identification is based on the accurate mass, isotopic pattern, and MS/MS pathway of each variable selected from the multivariate analysis, a useful and vital tool is the use of tandem high resolution MS to acquire both MS and MS/MS mass spectra. Using the procedure described in section 2.7, the accurate mass information was employed to propose a list of possible metabolites. Among them, only the ones corresponding with compounds whose presence was probable in food and plants were considered. Following this strategy, 24 and 33 molecular features were selected as potential markers of coffee roasting process for positive and negative ionization mode, respectively. One of these molecular features was found in both ionization modes. This fact highlights the importance of combining both ESI modes in order to maximize the number of detected metabolites.

Tables 2 (positive mode) **and 3** (negative mode) summarize the retention time, the molecular formula, the experimental m/z value, the mass error comparing with the database, the main fragments obtained in MS/MS spectra, the VIP values for the pairwise PLS-DA models, and the trend observed for all significant metabolites along the roasting process of coffee (**Figure S3** of supporting information shows the diagrams of the trends observed for all the tentatively and unequivocally compounds along the coffee roasting process). As it can be seen in both tables, 7 and 13 metabolites were identified in positive and negative ionization modes (16 were tentatively identified and 4 unequivocally identified).

Table 2. MS/MS fragmentation of the selected compounds in positive ionization mode

#	RT	Molecular formula	Tentative identification	[M-H]-	Mass error (ppm)	Main MS/MS fragments	VIP values			Roasting trend
							GCB vs LRC	GCB vs MRC	GCB vs DRC	
1	1.6		Unknown	104.1046		58.0647 44.0495 42.0338	4.7782	5.29545	2.93603	↓
2	1.7		Unknown	176.0057		118.0626 132.0802	2.56381	1.4932	1.6759	—
3	2.6		Unknown	453.1327		435.144 211.9747 140.9606	2.00091	2.0663	2.13435	↓
4	11.9	$C_{16}H_{18}O_9$	Chlorogenic acid*	355.0998	7	163. 0376 145.0236	4.30062	6.38721	8.13866	↓
5	12.0		Unknown	370.1474		147.0416 208.0939	1.4292	1.74676	2.30126	↓
6	13.4	$C_{16}H_{18}O_9$	Chlorogenic acid isomer-1	355.0990	10	163.0367 145.0261	0.3627	0.66204	2.30032	—
7	18.5		Unknown	759.2100		391.0958	1.92238	2.94479	3.25095	—
8	21.9		Unknown	303.1966		285.1718 211.1442 131.0828 119.0821	6.13668	5.15103	4.99282	↑
9	21.9		Unknown	500.2074		163.0235 239.1502	4.51142	3.74334	3.23861	↑
10	21.9		Unknown	505.2418		152.1014 180.097	1.68956	1.4904	1.4254	↑
11	22.6	$C_{26}H_{36}O_{10}$	Mozambioside	509.2402	4	147.0393 329.1696 347.1804	8.09174	8.58909	8.71963	↓

Table 2. Continued

#	RT	Molecular formula	Tentative identification	[M-H]⁻	Mass error (ppm)	Main MS/MS fragments	VIP values			Roasting trend
							GCB vs LRC	GCB vs MRC	GCB vs DRC	
12	23.5	$C_{25}H_{24}O_{12}$	Dicaffeoylquinic acid isomer-1	517.1352	2	163.0338 353.1451	3.42601	0.3839	2.42987	↓
13	23.8	$C_{25}H_{24}O_{12}$	1.5-Dicaffeoylquinic acid*	517.1372	6	163.0278 355.1305	4.62276	5.77454	3.88 E-8	↓
14	24.5	$C_{25}H_{24}O_{12}$	Dicaffeoylquinic acid isomer-2	517.1365	5	163.0338 355.1666	3.54144	0.8314	3.10321	↓
15	25.7		Unknown	513.1409		177.0479 163.0313	2.24824	1.33E-08	1.05E-08	↓
16	25.8		Unknown	421.1526		163.0353 241.1036	1.64952	1.0751	0.4846	—
17	26.4		Unknown	421.1532		163.0316 241.0982	1.2018	1.73713	1.98771	↓
18	26.9		Unknown	247.1192		171.0746 115.0453	1.8825	1.56312	1.32956	↑
19	26.9		Unknown	751.3548		Not clear MS/MS	1.60329	7.36E-10	0.9139	↑
20	27.3		Unknown	421.1531		163.0306 241.097	1.7343	2.00181	1.84203	↓
21	28.4	$C_9H_6O_2$	Coumarin	147.0442	1	65.0378 91.0531	1.0416	1.3966	1.67408	↓
22	34.9		Unknown	277.1806		137.0577	2.04446	1.95653	1.92496	↓
23	35.7		Unknown	668.4022		331.1739	1.67226	1.58306	1.40786	↑
24	39.4		Unknown	415.2130		119.0843	0.0922	0.2322	1.51976	—

* Confirmed with standard.
↑ The level of the compound increases with roasting.
↓ The level of the compound decreases with roasting.

Table 3. MS/MS fragmentation of the selected compounds in negative ionization mode

#	RT	Molecular formula	Tentative identification	$[M-H]^-$	Mass error (ppm)	Main MS/MS fragments	VIP values			Roasting trend
							GCB vs LRC	GCB vs MRC	GCB vs DRC	
1	1.8	$C_7H_{12}O_6$	Quinic acid*	191.0586	13	108.022 109.0294 127. 0384	3.37525	3.89183	5.45095	↑
2	1.8	$C_6H_{12}O_6$	Hexose	179.0585	13	134.0348 117.0325	0.890106	1.06703	1.49571	↑
3	2.1		Unknown	341.1090		119.031 101.021 113.0204	1.68073	1.90 E-8	1.52 E-8	↓
4	3.1		Unknown	371.0977		191.0533 173.0440	2.06996	2.63868	2.77613	↓
5	6.8	$C_9H_8O_4$	Caffeic acid isomer-1	179.0346	2	135.0404 134.0333	1.50793	1.23632	0.291829	—
6	6.8	$C_{16}H_{18}O_9$	Chlorogenic acid isomer-2	353.0871	2	191.0528 179.0317 135.0419	11.4834	8.53885	3.02571	—
7	10.7		Unknown	705.1616		353.081 179.0283	1.60495	0.56905	0.900738	—
8	12.3		Unknown	857.2334		503.1273 353.0793 191.0514	1.75214	2.26314	2.5821	—
9	12.5		Unknown	707.1760		353.0864 191.0548	8.16811	10.0931	12.0855	↓
10	13.2		Unknown	193.0446		134.0348 117.0325	1.61384	1.17043	0.14827	—
11	13.2	$C_{17}H_{20}O_9$	Feruloylquinic acid isomer-1	367.0978	15	193.0456 134.0325	3.6265	2.36628	0.14504	—

Table 3. Continued

#	RT	Molecular formula	Tentative identification	[M-H]⁻	Mass error (ppm)	Main MS/MS fragments	VIP values			Roasting trend
							GCB vs LRC	GCB vs MRC	GCB vs DRC	
12	13.8		Unknown	379.1584		113.0233 119.0322 191.0555	1.26436	1.63921	1.73346	↓
13	15.2		Unknown	597.1870		173.0706 132.0279 481.1830	2.31266	2.66958	2.83305	↓
14	16.0		Unknown	241.1114		197.1291 141.1019	0.56638	1.02896	1.57448	↑
15	16.1	$C_{16}H_{18}O_8$	Coumaroylquinic isomer-1	337.0835	28	191.0534 163.0377 119.0467	2.07409	2.90015	3.56737	↓
16	16.9	$C_{16}H_{18}O_8$	Coumaroylquinic isomer-2	337.0824	31#	173.0444 163.0393 119.0495	1.82156	1.4922	0.877095	—
17	17.5	$C_{16}H_{16}O_8$	Caffeoylshikimic acid isomer-1	335.0712	18	179.0340 135.0444 161.0242	1.85381	2.15126	0.69881	↓
18	19.0	$C_{17}H_{20}O_9$	Caffeoyl-methylquinic acid isomer-1	367.1021	4	191.0534 173.0425	4.75652	0.840603	5.60833	—
19	19.0		Unknown	173.0422		108.0177 109.0287	1.48052	0.70964	0.501266	—
20	20.0		Unknown	569.2246		Not clear MS/MS	1.88176	1.32728	1.46 E-8	↓
21	20.5		Unknown	559.2758		351.2166	3.40067	6.38 E-8	3.43 E-8	↓
22	20.8	$C_{16}H_{16}O_8$	Caffeoyl-quinolactone isomer-1	335.0737	10	161.0217 179.0319 135.0422	8.27339	9.37878	7.51789	↑

Table 3. Continued

#	RT	Molecular formula	Tentative identification	[M-H]⁻	Mass error (ppm)	Main MS/MS fragments	VIP values			Roasting trend
							GCB vs LRC	GCB vs MRC	GCB vs DRC	
23	22.6		Unknown	553.2254		101.0229 119.0335 113.0227	3.59581	4.48496	4.86973	↓
24	23.2		Unknown	553.2262		507.2202 179.054	1.64501	2.12151	2.3711	↓
25	23.9	$C_{25}H_{24}O_{12}$	1.5-Dicaffeoylquinic acid*	515.1185	2	353.0754 173.0411 179.0305 191.0515	4.93467	6.55407	7.39515	↓
26	24.9		Unknown	319.1887		275.1927	1.10073	1.54124	1.92031	↑
27	25.3	$C_{26}H_{26}O_{12}$	Caffeoyl-feruloylquinic acid isomer-1	529.1351	0	173.0438 367.1022 193.0499 335.0749	0.84749	1.5228	1.81611	↓
28	26.5		Unknown	437.1446		101.06 275.1117	0.837543	1.30399	1.53752	↓
29	26.9		Unknown	727.3560		643.2947 113.0234 625.2838 565.2973	5.01431	4.80522	4.03683	↑
30	27.0	$C_{20}H_{18}N_2O_5$	N-caffeoyltryptophan	365.1141	0	135.0434 161.0221 229.0602 186.0543 203.0797	1.80289	1.50784	1.06821	↑

Table 3. Continued

#	RT	Molecular formula	Tentative identification	[M-H]⁻	Mass error (ppm)	Main MS/MS fragments	VIP values			Roasting trend
							GCB vs LRC	GCB vs MRC	GCB vs DRC	
31	27.3		Unknown	437.1447		173.0441 275.1122	4.36024	5.2307	5.74657	↓
32	29.0		Unknown	565.3017		101.0596 113.0232 115.0758 463.2291	3.06446	2.88513	2.88513	—
33	29.5		Unknown	565.3020		113.0231 463.2342 101.059	2.88252	3.2899	3.5306	↑

Confirmed with standard.
Metabolite identification for this compound was performed considering an error of 31 ppm.
↑ The level of the compound increases with roasting.
↓ The level of the compound decreases with roasting.
— Indicates a different trend from the previously describe

In the positive ionization mode, the 355 *m/z* ion as $[M+H]^+$ was observed for two different compounds: **compound 4** (t_R = 11.9 min) and **compound 6** (t_R = 13.4 min). The fragment ions obtained from the MS/MS spectra of both compounds were *m/z* 163 and *m/z* 145, corresponding to the caffeoyl residue and its loss of water. **Compound 4** was unequivocally identified as chlorogenic acid based on the comparison of its retention time and MS/MS fragmentation pattern to those obtained for the commercial standard. **Compound 6** was tentatively identified as a chlorogenic acid isomer-1. Interestingly, chlorogenic acid (compound 4) levels decreased with the roasting process whereas that the isomer (compound 6) is a clear marker of DRC roasting as its level only decreased when comparing GCB vs DRC. **Compounds 12** (t_R = 23.5 min), **13** (t_R = 23.8 min), and **14** (t_R = 24.5 min) all exhibited $[M+H]^+$ ions at *m/z* 517. MS/MS fragmentation of these ions gave a fragment ion at 355 *m/z* suggesting the loss of a caffeoyl residue (163 Da). By matching the MS/MS spectra obtained to those reported in the literature [29, 30], these compounds were tentatively identified as dicaffeoylquinic acid isomer. Compound 13 is the same as compound 25 obtained in negative ionization mode which was unequivocally identified as 1,5-dicaffeoylquinic acid by comparison of its retention time and MS/MS fragmentation pattern with those of the standard. In this negative mode, the MS/MS spectrum of this compound shows fragment ions at *m/z* 353 ([M-H-caffeoyl]), *m/z* 191, ([M-H-caffeoyl-caffeoyl]), and *m/z* 179 ([M-H-caffeoyl-quinic]) which were in agreement with those fragments described previously in the literature for this compound [29, 30]. The trend of 1,5-dicaffeoylquinic acid is to decrease as roasting process increases.

As it can be seen in **Table 3**, for negative ionization mode, the $[M-H]^-$ ions at *m/z* 353 and *m/z* 515 are also highlighted as markers, corresponding to compounds 6 and 25, respectively. Standards of neochlorogenic acid (t_R = 8.0 min) and 1,3-dicaffeoylquinic acid (t_R = 19.7 min) were analyzed in order to obtain their retention times and MS/MS pattern to compare with those pointed out in the tables. However, the possibility of corresponding any of these standards with a metabolite was discarded by differences in the retention time. The MS/MS spectra of **compound 6** (t_R = 6.8 min) showed a fragment ion *m/z* 191 corresponding to [M-H-caffeoyl], and *m/z* 179 corresponding to [M-H-quinic] [29, 30]. According to that, this compound could be tentatively identified as a chlorogenic acid isomer-2 (different from compounds 4 and 6 in positive ionization mode because they had different retention times (see **Tables 2** and **3**)). The levels of this chlorogenic acid isomer-2 significantly increased from GCB to LRC but then gradually decreased with further roasting.

Compound 22 (t_R = 20.8 min) obtained in negative mode displayed the deprotonated molecular ion at *m/z* 335. Its MS/MS fragmentation showed the characteristics fragments of chlorogenic acids (i.e *m/z* 179 m/z corresponding to [M-H-caffeoyl-quinic], *m/z* 161 corresponding to [M-H-caffeoyl-quinic-H_2O], *m/z* 135 [M-H-caffeoyl-quinic-CO_2]), except by the absence of signal *m/z* 191. This is an important feature which helps to distinguish between chlorogenic acids and chlorogenic acid lactones, as reported elsewhere [31]. Moreover, the increasing trend that can be observed for this compound during roasting (**Figure S3**) suggested that this compound could be a chlorogenic acid lactone isomer-1 instead of a shikimic acid derivative which presents the same fragmentation pattern (see below).

Other hydroxycinnamic acids (coumaric acid, cinnamic acid, ferulic acid, caftaric, caffeic acid, etc.) were also tentatively identified in positive and negative ionization mode. In positive mode, **compound 21** (t_R = 28.4 min, $[M-H]^+$ = 147 had MS/MS fragment ions at *m/z* 65 and *m/z* 91 that, according to Melo et al. correspond to coumarin fragmentation pattern [32]. In negative ESI mode belonging to the hydroxycinnaminc acids, **compounds 11** (t_R = 13.2 min) **and 18** (t_R = 19.0 min) displayed roughly the same $[M-H]^-$ ion at *m/z* 367. The MS/MS spectra of these ions showed the main fragment at *m/z* 193 (compound 11) and *m/z* 191 (compound 18). According to the literature, these fragments indicated the presence of feruloyl and caffeoyl groups, respectively [31].

Therefore, there were assigned tentatively as feruloylquinic acid isomer-1 (compound 11) and caffeoyl-methylquinic acid isomer-1 (compound 18). These compounds showed a different behavior when increasing the roasting level. On the one hand, feruloylquinic acid isomer-1 had significant differences in the two first roasting levels (light and medium) when compared to GCB, however, no differences were found when comparing GCB to DRC. On the other hand, interestingly feruloylquinic acid isomer-1 has a similar trend to the previously commented chlorogenic acid isomer-2 (compound 6 in negative mode), i.e. its level increased from GCB to LRC and then decreased with further roasting.

Also in negative ionization mode, two different compounds (**15 and 16**) shared the same $[M-H]^-$ ion at *m/z* 337 and had similar fragmentation; both show fragment ions at *m/z* 163 ($[M-H\text{-quinic}]^-$) and *m/z* 119 ($[M-H\text{-quinic-}CO_2]^-$). Moreover, an ion fragment at *m/z* 191 for compound 15 corresponding to quinic acid residue and *m/z* 173 for compound 16 corresponding to [quinic acid-H_2O-H]$^-$ were also observed. These compounds, according to the fragmentation described in literature by Clifford et al. were tentatively identified as coumaroylquinic acid isomers-1 and 2 [33].

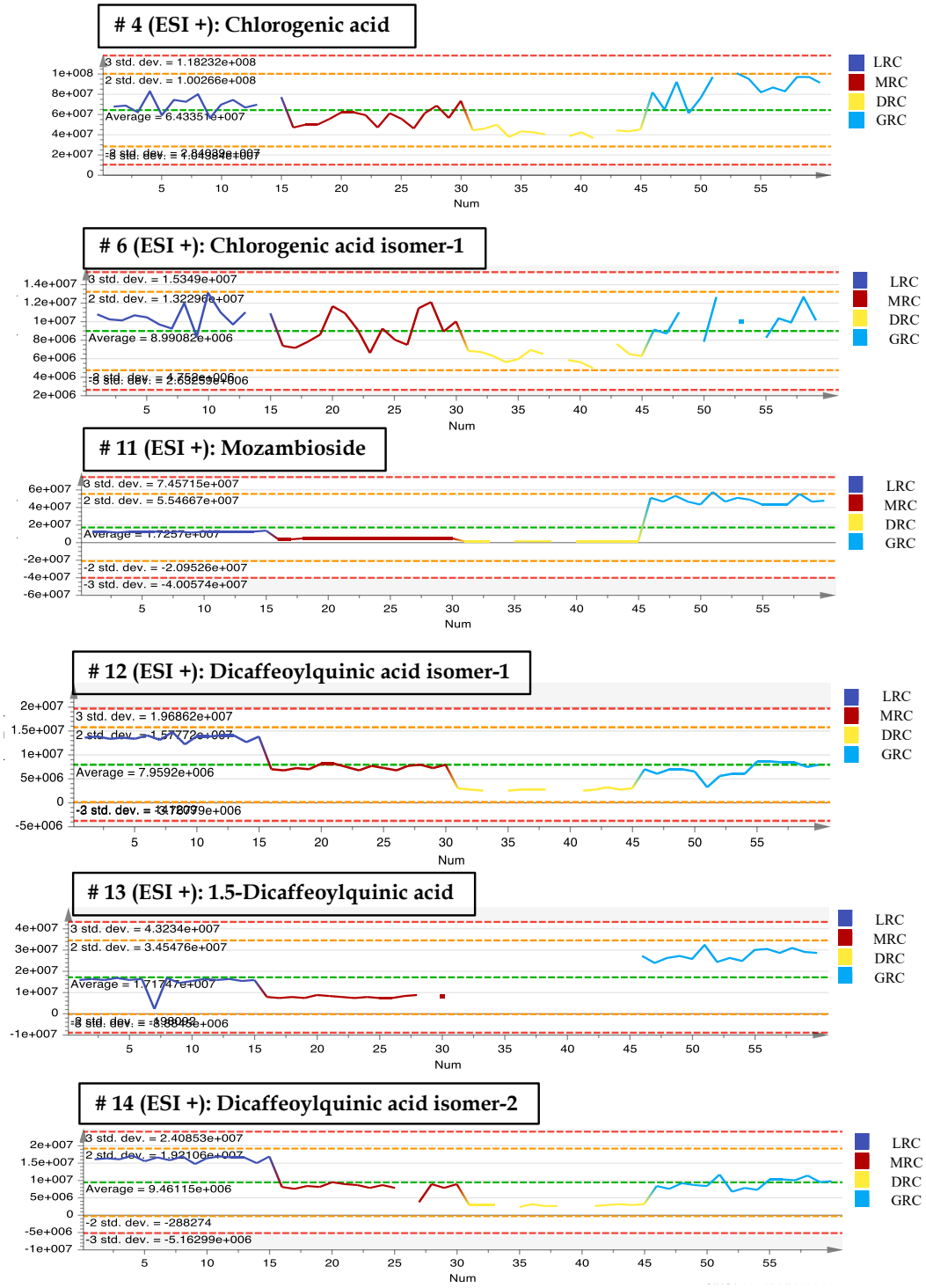

Figure S3. Diagrams of the trends observed for all the tentatively and unequivocally compounds both in positive and negative ionization mode along the coffee roasting process.

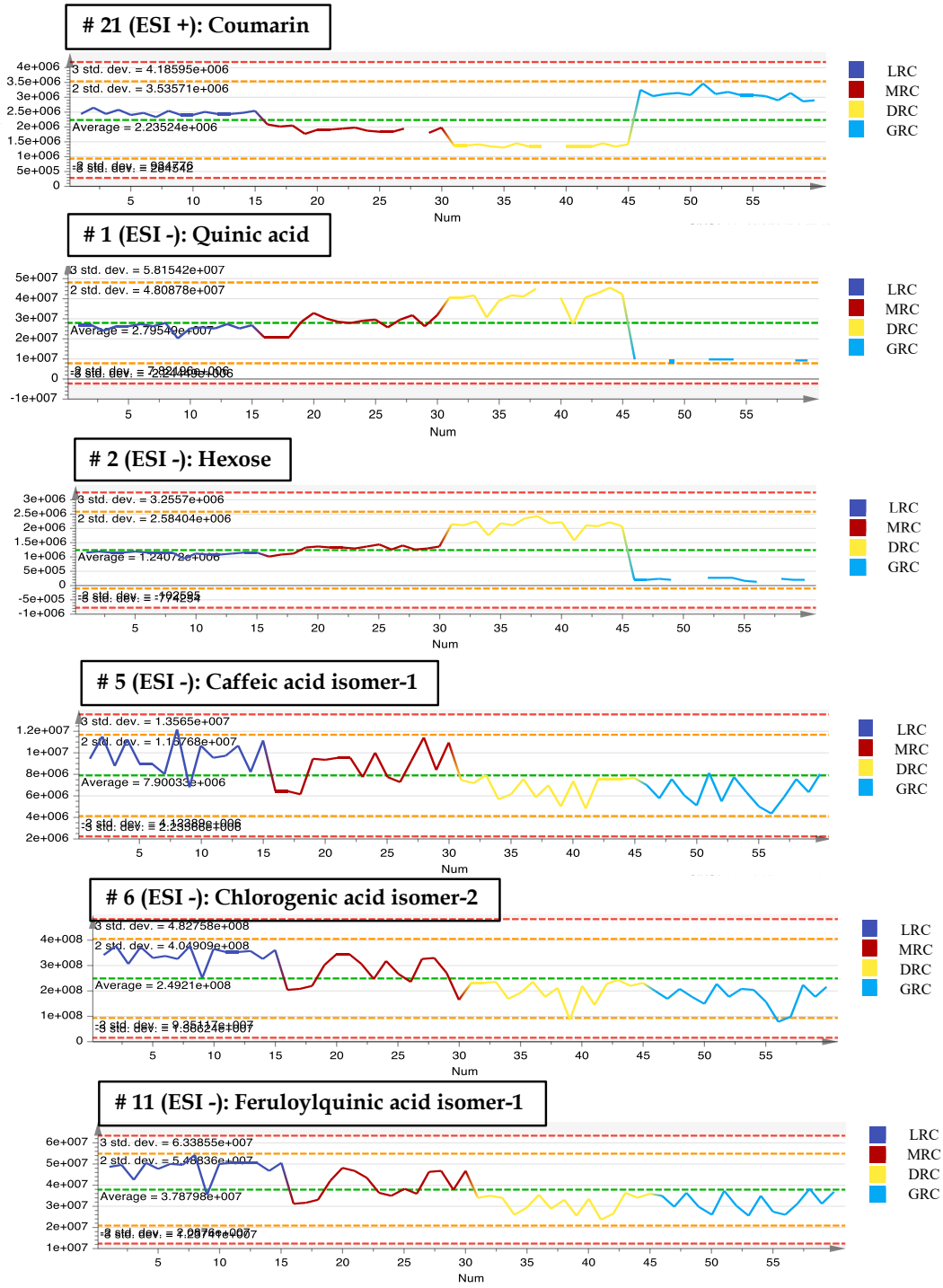

Figure S3. Continued.

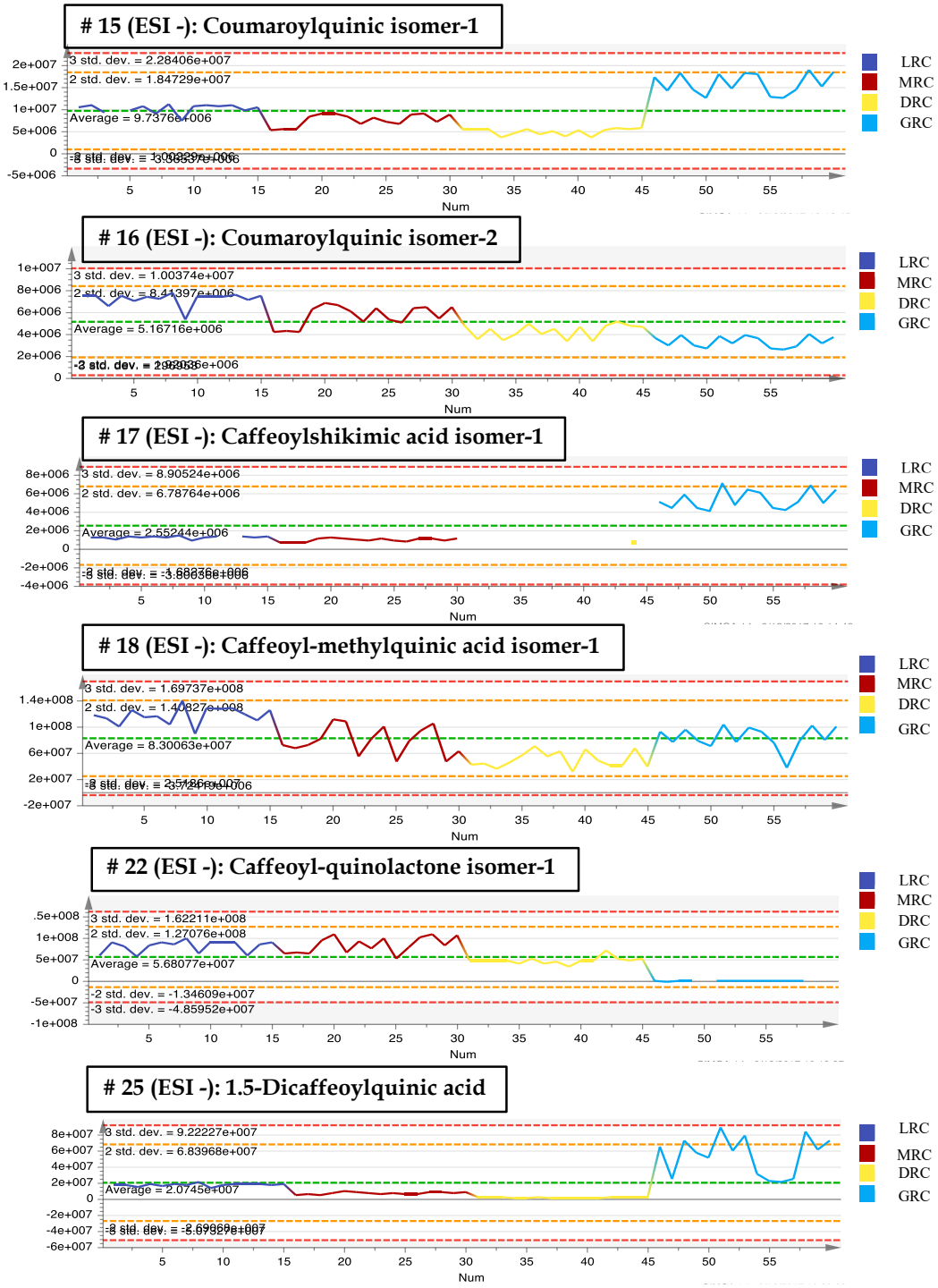

Figure S3. Continued.

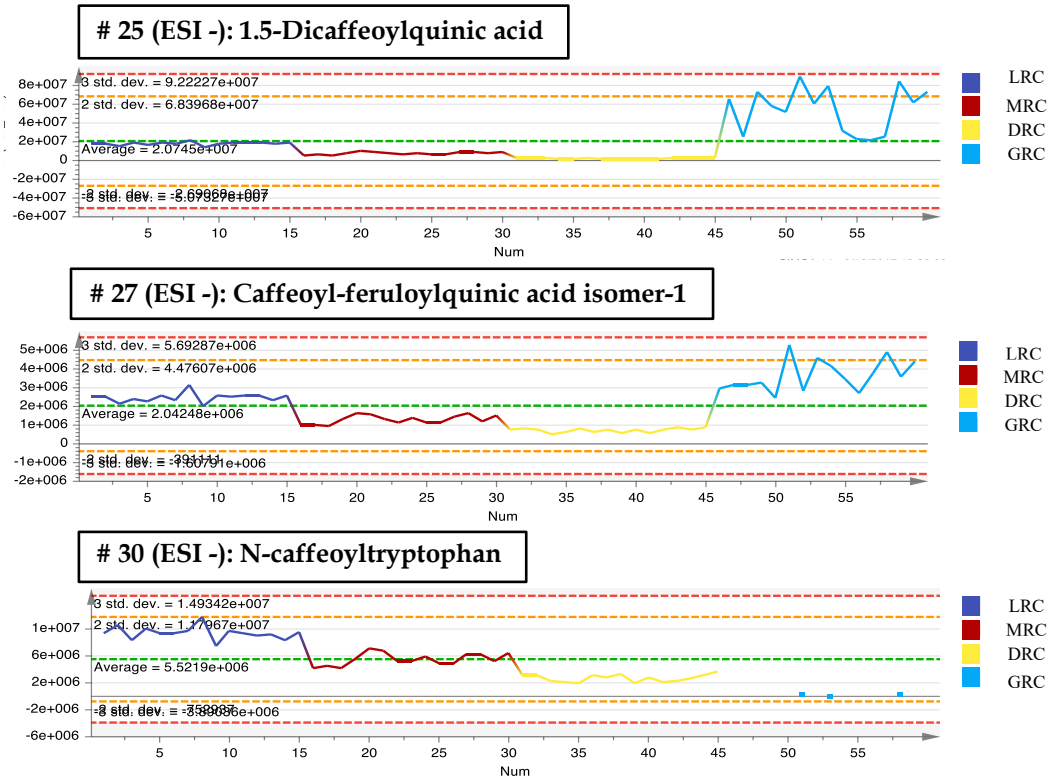

Figure S3. Continue

Under MS/MS fragmentation of **compound 17** (t_R = 17.5 min, $[M-H]^-$ = 335) in negative ionization mode, ion *m/z* 179 was obtained, which, as previously reported suggested the presence of caffeic acid, and *m/z* 161 and *m/z* 135 were formed from loss of H_2O and CO_2, respectively [29]. According to that, this compound was tentatively identified as a caffeoylshikimic acid isomer-1. Other hydroxycinnamic acids, such as **compound 5** (t_R = 6.8 min), **compound 27** (t_R = 25.3 min) and **compound 30** (t_R = 27.0 min) that displayed a $[M-H]^-$ ion at *m/z* 179, *m/z* 529 and *m/z* 365 respectively, in negative ionization mode, were shown to have differences in their levels along the roasting process. Compound 5 was tentatively identified as a caffeic acid isomer-1 since its MS/MS fragmentation pattern corresponded to the fragmentation obtained for caffeic acid and trans-caffeic acid standards (*m/z* 135 and *m/z* 134), but whose retention time differed from those of these compounds. According to the fragmentation reported by Lukáš et al. compound 27 was identified as a caffeoyl-feruloyquinic acid isomer-1 because of the presence of MS/MS fragments at *m/z* 367 ([M-H-caffeoyl]$^-$), *m/z* 193 ([M-H-caffeoyl-quinic]$^-$), and *m/z* 335 ([M-H-ferulic acidl]$^-$) characteristics of this family of compounds [31]. Finally, compound 30 gave a MS/MS fragmentation at *m/z* 135 and

m/z 161, corresponding with caffeoyl family and *m/z* 203 which corresponded to the theoretical mass of tryptophan suggesting that this compound could be tentatively identified as N-caffeoyltryptophan. Regarding the behavior of these compounds during the roasting process, it was observed, on the one hand, that caffeoyl-feruloyquinic acid isomer-1 (compound 27) and N-caffeoyltryptophan (compound 30) had an opposite trend, whereas the former decreased during the roasting process, the latter increased. On the other hand, the caffeic acid isomer-1 (compound 5) decreased during roasting disappearing in DRC.

Moreover, other compounds proposed as markers are **compounds 1 and 2** from Table 3. These compounds, with the same retention time (t_R = 1.8), gave a $[M-H]^-$ at *m/z* 191 and *m/z* 179, respectively. In this case, their MS/MS fragmentation patterns suggested that they could be quinic acid and hexose. Compound 1 was unequivocally identified as quinic acid by comparison with the standard. Paraxanthine and theobromine standards were also analyzed since their mass was in agreement with that found for compound 2. However, they showed a different retention time and MS/MS spectra than the ones experimentally obtained. For both compounds (1 and 2) their levels increased gradually with the roasting process.

The last metabolite tentatively identified in positive ionization mode (**compound 11,** t_R = 22.6 min) is of a great interest. It gave a $[M+H]^+$ ion at *m/z* 509, presented the highest VIP values, and its composition decreased with roasting. The experimental mass of compound 11 is in agreement with theoretical mass and the MS/MS fragmentation pattern of mozambioside, which has previously been reported by Roman et al., as interesting compound in coffee beans that exhibit a bitter taste [34]. In that work authors quantified this compound in Arabica coffee samples demonstrating its degradation from green to roasted coffee. In summary, the proposed RP-LC-MS methodology enables to identify markers of the roasting process that had not previously been identified using non-targeted metabolomic approaches. To obtain a more comprehensive analysis it should be necessary to apply the developed metabolomics platform to a larger number of samples.

4. Conclusions

The present study describes the development of an untargeted metabolomics strategy based on RP-LC-MS to investigate changes occurring in coffee samples submitted to three different roasting degrees (light, medium, and dark coffee). By using this method, it was possible to obtain a list of 24 and 33 compounds that have demonstrated to be potential markers of roasting process of coffee for positive and negative ionization modes, respectively. Only one of these

compounds appeared as significant in both ionization modes, highlighting the importance of using both ESI modes to carry out the metabolomics analysis. A total of 7 and 13 metabolites were identified as markers of roasting process in positive and negative modes, respectively. Most of these compounds belong to the group of hydroxycinnamic acids. In general, in most cases these metabolites decreased with the roasting process, although quinic acid, hexose, chlorogenic acid lactone and N-caffeoyltryptophan showed a different trend, i.e. they increased at high roasted degrees. Finally, an interesting compound belonging to the naphthofurans family, has demonstrated to be an important biomarker since it presents one of the highest VIP values in the list of markers and its levels decrease during roasting. Thus, the developed metabolomics strategy demonstrated not only to be a useful tool to differentiate coffee beans submitted up to three different roasting degrees but also to highlight potential markers of the roasting process.

Acknowledgements

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Article 8

Capillary electrophoresis-mass spectrometry metabolic fingerprinting of green and roasted coffee.

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Submitted.

Abstract

The aim of this work was to develop a capillary electrophoresis-mass spectrometry (CE-ESI-QToF-MS) method to carry out the metabolic fingerprinting of green and roasted coffee samples (*Arabica* variety). To evaluate changes in the metabolic profiles of coffee occurring along the roasting process, green coffee beans were submitted to different roasting degrees. The effect of different parameters concerning the electrophoretic separation (background electrolyte, temperature, voltage, and injection time), the MS detection (temperature and flow of drying gas, sheath gas of jet stream temperature, and capillary, fragmentator, nozzle, skimmer, and octapole voltages) and the sheath liquid (composition and flow rate) was studied to achieve an adequate separation and to obtain the largest number of molecular features. The analyses were carried out in positive ESI mode allowing to detect highly polar cationic metabolites present in coffee beans. Non-supervised and supervised multivariate analyses were performed showing a good discrimination among the different coffee groups. Those features having a high variable importance in the projection values on supervised analyses were selected as significant metabolites for their identification. Thus, 13 compounds were proposed as potential markers of the coffee roasting process, being 7 of them tentatively identified and 2 of them unequivocally identified. Different families of compounds such as pyridines, pyrroles, betaines, or indoles could be pointed out as markers of the coffee roasting process.

1. Introduction

Coffee production comprises different steps which affect not only its chemical composition but also its organoleptic properties [1]. Among these steps, coffee roasting is crucial for the development of coffee flavor since it involves several physical and chemical reactions that give rise to the formation and/or degradation of many components responsible for aroma, flavor or color. Some coffee components, such as lipids or caffeine, remain practically unaltered during the thermal process [2]. However, this process can lead to the formation of melanoidines due to the combination of sugars and amino acids during the Maillard reaction [3-9], pyridines because of protein hydrolysis and the degradation of trigonelline [10, 11], or even toxic compounds, such as furan derivatives, which have shown carcinogenic activity [12], among others. These changes in the chemical composition of coffee strongly affect its quality so that their evaluation has special importance to guarantee coffee quality both for the coffee industry and for consumers. In the industry, the control of roasting degree is usually carried out

through differences in beans color, dry matter loss and/or other changes in sensory characteristics. Therefore, there is a need to develop adequate analytical methodologies enabling to face the discrimination of coffee beans submitted to different roasting degrees.

Different research works have already pointed out the importance to carry out a control of the chemical composition of coffee beans along roasting process [13-15]. Most of the works focused on this topic are based on target (detection of a single component) [16, 17] or profiling (detection of a specific class or components) analyses [18-21]. Thus, compounds such as amino acids, alkylpyrazines, chlorogenic acids or chlorogenic/caffeine ratio have been reported as markers to distinguish the roasting degree of coffee beans [22-24]. However, coffee beans, as well as other foods, are a very complex matrix that presents hundreds of components so that its fingerprinting analysis (analysis of as many components as possible) during the roasting process will provide a maximum coverage of metabolites that can be simultaneously identified. In this sense, metabolomics, a well-established omics science focused on the study of the metabolome [25], is a powerful tool capable of providing an exhaustive characterization of complex samples which is becoming one of the most relevant procedures to assess food quality, safety and traceability [26, 27]. Up to date, the number of works concerning the metabolomic study of coffee is scarce. The discrimination of coffee varieties or origins [28-31] or between caffeinated and decaffeinated coffee [32] has been performed using as analytical platforms nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS). On the other hand, a few works have been focused on the evaluation of coffee roasting process using targeted metabolomic approaches based on ion mobility spectrometry-mass spectrometry (IMS-MS) [10], and employing non-targeted metabolomic approaches based on NMR, ambient sonic-spray ionization-mass spectrometry (EASI-MS) and GC-MS [3, 4, 33, 34]. Recently, our research group developed a non-targeted metabolomics strategy based on reversed-phase liquid chromatography-mass spectrometry (RPLC-MS) to provide the characterization of coffee beans roasted at three different degrees. It enabled to identify 7 and 13 metabolites as markers of roasting process in positive and negative modes, respectively [35].

Although NMR, GC-MS, and LC-MS are well established platforms for metabolomics, capillary electrophoresis (CE) coupled to MS is also a powerful analytical technique for metabolomics research due to its particular characteristics, i.e., it provides fast and efficient separations, requires low consumption of reagents and samples, and has a high versatility considering its different modes [36].

Moreover, a large number of metabolites are polar and ionic, so they can be detected by CE-MS in contrast to LC-MS or GC-MS in which medium-polar, hydrophobic and volatile compounds are detected. However, even though several works have demonstrated the high potential of CE-MS in metabolomics studies [37-39], up to now, just a few works employed this technique to perform the metabolomics analysis of food matrices [36, 40-42].

In this work, an analytical method, based on the use of CE coupled to high resolution MS equipped with a Jet Stream thermal orthogonal electrospray ionization source, was developed in order to carry out the fingerprinting of green and roasted coffee and to evaluate changes in the metabolic profiles of coffee samples (*Arabica* variety) submitted to different roasting degrees. With this aim, different parameters concerning the electrophoretic separation and MS detection were optimized in order to maximize the number of detected peaks. In addition, the metabolic analysis, including data processing and chemometric analysis using PCA and PLS-DA models was applied to discriminate coffee beans according to their roasting degree. Finally, the identification of the significant metabolites along different roasting levels was performed.

2. Materials and methods

2.1 Chemicals and coffee samples

MS-grade methanol, acetic acid, and formic acid and HPLC-grade isopropanol were purchased from Fisher Scientific (Hampton, NH, USA). Ammonium formate and ammonium acetate of MS grade were from Sigma (St. Louis, MO, USA).

Calcium acetate, 3-ethylpyridine, 2-acetylpyrrole, 1-methyl-2-pyrrolecarboxaldehyde, L-(+)-arabinose, methyl anthranilate, indole-3-butyric acid, choline, and duloxetine were purchased from Sigma (St. Louis, MO, USA). Water employed to prepare the running buffer and the coffee extracts was purified through a Milli-Q system from Millipore (Millipore, Madrid, Spain).

Arabica green coffee beans (GCB) were roasted and provided by the company "Café Fortaleza" (Vitoria, Spain). Coffee beans were roasted at three different levels: light (LRC), medium (MRC) and dark (DRC) using temperatures of 175 ^{o}C (during 12.36 min), 185 ^{o}C (for 14.11 min), and 195 ^{o}C (during 17.06 min), respectively. The roasting process was controlled in terms of the weight loss of each sample being 13 % in LRC, 15 % in MRC and 17 % in DRC.

2.2 Preparation of coffee samples

The metabolite extraction procedure from coffee samples was performed using an extraction protocol previously optimized by our research group [35]. Briefly, 5 mg of grounded coffee samples were extracted with 1.5 mL of 25 % (v/v) methanol in water. The solid-liquid extraction was carried out in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany) at 700 rpm during 15 min at 25 ºC. After centrifugation (3500 rpm, 10 min, 25 ºC) the supernatant fraction was collected and directly analyzed by CE-MS. Five replicate extractions for each group of coffee samples (GCB, LRC, MRC and DRC) were prepared and analyzed in the metabolomic sequence. Quality control samples (QC) were prepared by pooling equal aliquots of each coffee sample, which lets monitoring instrumental drifts throughout the analysis [43]. Duloxetine was used as internal standard (IS) at a final concentration of 10 µg/mL in all the analyzed samples including QCs.

2.3 CE-MS analysis

Metabolic fingerprinting of coffee samples was carried out using a 7100 CE system from Agilent Technologies (Waldbronn, Germany) coupled to a 6530 quadrupole time-of-flight mass spectrometer from Agilent Technologies (Waldbronn, Germany) equipped with a Jet Stream thermal orthogonal electrospray ionization (ESI) source. Coupling was performed via a sheath liquid interface with a CE-ESI co-axial sprayer (G1607 model from Agilent Technologies). A sheath liquid composed of methanol:water (50:50 v/v) containing 1 M acetic acid was delivered into the ESI source at a 8 µL/min flow rate by means of a NE-3000 pump (New Era Pump Systems Inc., Farmingdale, NY, USA). Sheath liquid also included two reference standards from Agilent Technologies (purine (*m/z* 121.0508) and HP921 (*m/z* 922.0097)) to allow mass accuracy monitoring. Agilent Mass Hunter Qualitative Analysis software (B.07.00) was employed for MS control and data acquisition.

Separations took place in uncoated fused-silica capillaries of 50 µm ID with a total length of 100 cm (Polymicro Technologies, Phoenix, AZ, USA) using a solution of 1 M formic acid (pH 1.8) as background electrolyte (BGE). Before first use, new capillaries were rinsed (applying 1 bar) with 1 M sodium hydroxide for 30 min, followed by 5 min with Milli-Q water and conditioned with BGE for 60 min. Between injections, the capillary was preconditioned with BGE for 5 min. Then, the samples were injected applying a pressure of 50 mbar for 80 s. Finally, the electrophoretic separation was achieved applying +30 kV at a working temperature of 20 ºC.

MS operated in positive ESI mode and data were acquired within 100-1700 *m/z* range (extended dynamic range) in full scan resolution mode at a scan rate of 2 scans per s. Capillary voltage was set to 3000 V with a nozzle voltage of 0 V, a nebulizer pressure of 10 psi, and a sheath gas of jet stream at 3 L/min and 150 ^{o}C. Drying gas was supplied at 5 L/min at 180 ^{o}C. The fragmentator voltage was set at 175 V whereas the skimmer and octapole voltages were 60 V and 750 V, respectively.

MS/MS experiments were performed to assist in the metabolite identification. The voltage employed in MS/MS analyses ranged from 20 to 40 V depending on each analyte.

2.4 Metabolomic sequence

Blank and QC samples were injected in the CE-MS system at the beginning of the metabolomic sequence to ensure a good repeatability. Then, a total of 60 coffee samples (five replicates of each group (GCB, LRC, MRC, and DRC) injected in triplicate) were randomly injected and a QC sample was injected every six coffee samples and at the end of the sequence.

2.5 Data processing and analysis

Molecular features from the raw data were obtained using the Molecular Feature Extraction (MFE) tool from Mass Hunter Qualitative Analysis (B.07.00 from Agilent Technologies) where the migration time and abundance of the molecular features were annotated. The MFE parameters were as follows: "small molecules (chromatographic)" mode; peaks with height ≥ 500 counts; peak spacing tolerance for isotope grouping was 0.0025 m/z plus 7.0 ppm; isotope model = common organic molecules; and the charge states were limited to 2. Moreover, to identify different ion species coming from the same molecular feature, H^+, Na^+, K^+, and NH_4^+ adducts were considered.

Migration time correction and alignment of molecular features were conducted using the Mass Profiler Professional (MPP) software (B.02.00 from Agilent). To carry out the alignment, a migration time window of 0.3 min, with a mass window of 30 ppm + 0.02 Da, were employed. Then, data were normalized using the intensity of the IS. To remove non-reproducible signals before performing statistical analysis, molecular features were filtered by retaining masses present in at least 80 % of all injected QC samples and with a coefficient of variation below 35 %.

Multivariate statistical analysis of the data matrix containing the filtered molecular features was performed using SIMCA 14.0 software (Umetrics, Umeå, Sweden). After log-transformation (to approximate to a normal distribution) and Pareto scaling (for reducing the relative importance of larger values) [44] both principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) models were used to investigate clustering existing in the analyzed samples and to find differences between samples according to their roasted degree. Quality of the models was assessed by the R^2 (R^2X, R^2Y) and Q^2 values. Potential biomarkers of roasting degree of coffee were found by two-class comparisons: GCB vs LRC, GCB vs MRC, and GCB vs DRC. Only features with variable importance in the projection (VIP) values of the first component of the PLS-DA models higher than 1.0 were considered as significant. Moreover, univariate statistical analysis using the Mann-Whitney U test was performed in R (http://www.R-project.org). Benjamini-Hochberg false discovery rate was employed for multiple testing correction.

2.6 Metabolite identification

Potential markers of coffee roasting process were identified by matching the experimental accurate mass values with theoretical mass values available in the CEU Mass Mediator database [45] considering an error of 30 ppm. This database, available online, allows to obtain information simultaneously from different databases such as KEGG, METLIN, HMDB and LipidMaps. In addition, the FooDB database (http://foodb.ca/) was also employed. Those metabolites that were commercially available as standards compounds were also analyzed by the developed CE-MS methodology to perform their unequivocal identification according to their migration time and MS/MS fragmentation pattern. When the standards could not be acquired, a tentative identification was carried out comparing the experimental MS/MS spectra obtained for each molecular feature with those predicted in HMDB database, CFM-ID (cfmid.wishartlab.com) and/or literature.

3. Results and discussion

3.1 Development of a CE-MS method for metabolic fingerprinting

In order to develop a CE-MS methodology enabling the fingerprinting of green and roasted coffee and to study the changes occurring in the metabolic profiles of coffee samples submitted to different roasting degrees, those parameters related to the CE-MS coupling (composition and flow rate of the sheath liquid), the

electrophoretic separation (BGE, temperature, voltage, and injection time) and the MS detection were optimized using as model a QC sample.

The effect of the composition and flow rate of the sheath liquid was investigated because they are critical variables not only to establish an adequate electric contact between the CE system and the mass spectrometer but also to obtain a good metabolite ionization. Thus, using 1 M formic acid (pH 1.8) as BGE and a methanol:water (50:50 v/v) sheath liquid as initial conditions, different additives, such as 1 M formic acid, 1 M acetic acid, 5 mM ammonium formate and 5 mM ammonium acetate were added to the sheath liquid to study their influence on the ionization. Among these additives, 1 M acetic acid was chosen for further experiments since its addition to the sheath liquid enabled to obtain the highest number of molecular features. The next step was to evaluate the influence of the organic solvent present in the sheath liquid. Mixtures of methanol or isopropanol with water at 50:50 (v/v) containing 1 M acetic acid were tested. Since the use of methanol allowed to obtain a greater number of molecular features and a higher current stability, it was selected as organic solvent. Also, different sheath liquid compositions (methanol:water 50:50, 70:30 and 80:20 (v/v)) were compared, selecting methanol:water 50:50 (v/v) due to the higher number of molecular features observed with this mixture. Finally, the flow rate was optimized using 4, 6, and 8 µL/min. It was necessary to employ the highest flow (i.e. 8 µL/min) in order to enhance the ionization which also enabled obtaining the maximum number of molecular features.

Once the best conditions for the sheath liquid were selected, the next step was to optimize the variables affecting the electrophoretic profile. The nature of the BGE (1 M formic acid or 1 M acetic acid), the working temperature (15, 20 or 25 ^{o}C), the applied voltage (20, 25 or 30 kV) and the injection time (10, 20, 80 and 120 s) were evaluated in terms of electrophoretic separation, peak efficiency, and sensitivity to ensure the detection of the largest number of molecular features in the coffee samples. The optimized conditions were 1 M formic acid (pH 1.8), a working temperature of 20 ^{o}C, a separation voltage of 30 kV, and an injection time of 80 s. Contrary to what it would be expected, the use of acetic acid in the sheath liquid and formic acid in the running buffer allowed to detect the maximum number of molecular features. The increment in molecular features was truly due to the appearance of new compounds and not to artifacts derived from the combination of both buffers.

Finally, the effect of different ESI parameters, such as fragmentator voltage (110-175 V), nozzle voltage (0-100 V), skimmer (50-60 V), octapole (160 or 750 V), capillary voltage (2000-4000 V), drying gas temperature (150-300 °C), drying gas

flow (5-10 L/min) and sheath gas of jet stream temperature (150-300 °C), was evaluated. The most adequate values to achieve the largest number of molecular features with the highest sensitivity were 175 and 0 V for fragmentator and nozzle voltages, respectively, 3000 V for capillary voltage, 60 V for skimmer, 750 V for octapole, 180 ^{o}C and 5 L/min for drying gas, and 150 ^{o}C for sheath gas temperature.

Figure 1 shows the metabolic profiles of GCB, LRC, MRC and DRC samples analyzed by the developed CE-MS methodology.

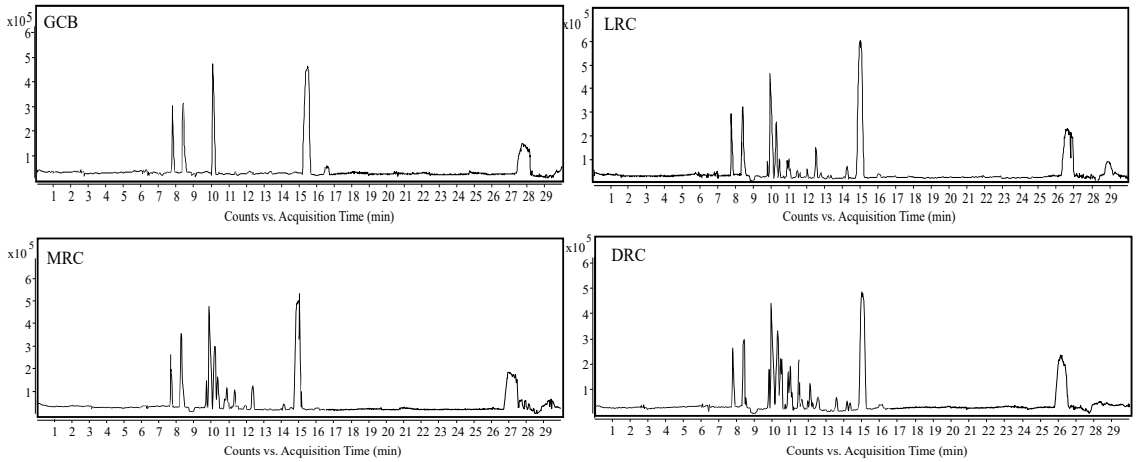

Figure 1. Base peak electropherograms obtained in positive ionization mode for green coffee (GCB), light roasted coffee (LRC), medium roasted coffee (MRC) and dark roasted coffee (DRC) under optimal separation conditions. CE-MS conditions are summarized in Section 2.4.

3.2 Metabolic fingerprinting of coffee samples by CE-MS and potential markers of roasting process

The CE-MS method developed was applied to the metabolic fingerprinting of coffee samples submitted to different roasting levels to evaluate the potential of this technique for differentiating molecules related to the roasting process. For this purpose, a total of 60 samples (five replicates of each group (GCB, LRC, MRC, and DRC) injected in triplicate) and different injections of a QC sample distributed across the metabolomics sequence (see section 2.4) were analyzed. 1275 different molecular features were found which demonstrated the potential of CE-MS for high-throughput metabolomics analysis.

It should be highlighted that data processing must be carefully performed since the algorithms used for peak fitting of some tools have been developed for LC purposes and the scores should be adjusted because of the slight different shape of the CE peaks compared to LC signals and their width [46]. In fact, the molecular

features alignment in CE-MS is normally more problematic than in LC-MS due to the higher migration time shift.

After migration time correction, alignment, normalization, and filtering (see section 2.5), the resulting dataset comprised 39 time-aligned metabolic features.

Then, logarithmic transformation and Pareto scaling were used to approximate a normal distribution and for reducing the relative importance of larger values, respectively [44]. Regarding data analysis, the data matrix was first subjected to PCA not only to evaluate the consistency of the metabolomics sequence but also to observe the variability existing in the dataset. As it can be seen in **Figure 2**, PCA clearly showed not only differences among the four groups of samples analyzed but also it demonstrated the consistence of the analytical sequence since QC samples were clustered in the center of the plot showing the low analytical variability existing between runs. Note that, high percentages of variability were explained for the first two components (66 and 15 % for the first and second component, respectively).

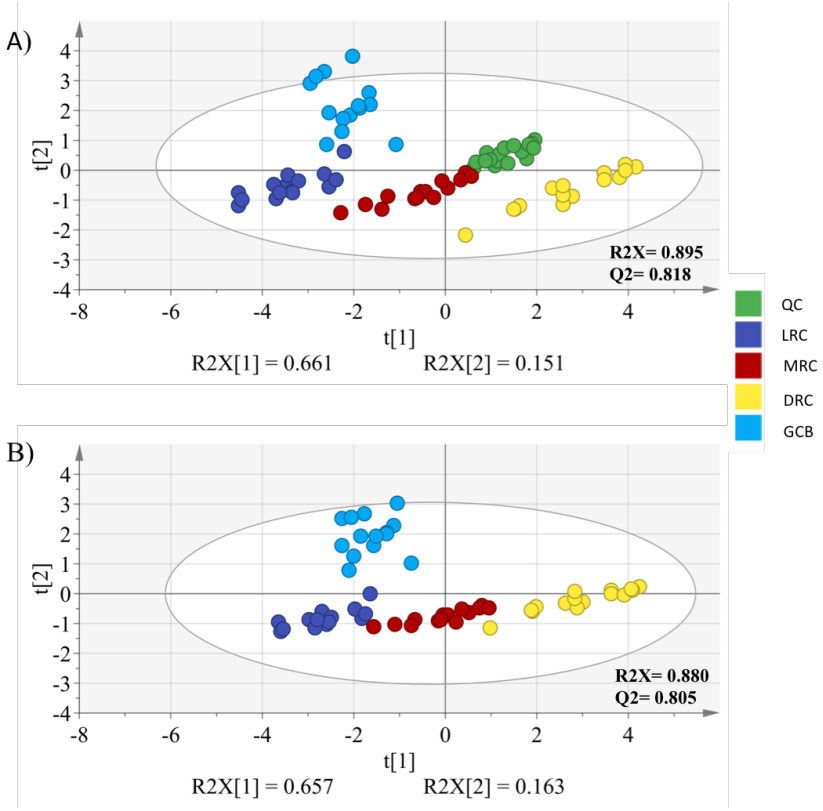

Figure 2. Score plots of the PCA models from the CE-MS data obtained from the analysis of GCB, LRC, MRC and DRC samples. (A) PCA including QC samples and (B) PCA without QC samples

To carry out the sample classification and to simplify the search of those variables that are potential markers of coffee roasting level, PLS-DA models were performed. **Figure 3** shows the PLS-DA models for different two-class comparison (GCB vs LRC, GCB vs MRC, and GCB vs DRC).

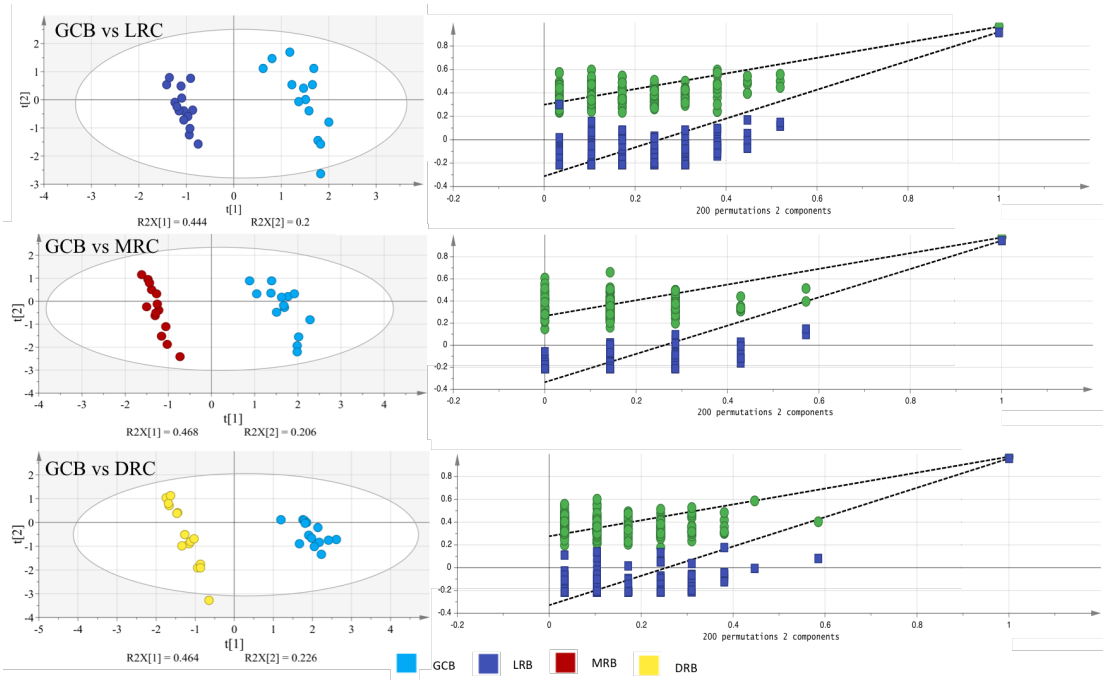

Figure 3. Score plots for the PLS-DA models of two-class comparisons and their corresponding permutation test.

In addition, **Table 1** shows R^2X, R^2Y and Q^2 quality parameters as well as F and p-values of the cross validated ANOVA for all PLS-DA models. In all cases, high Q^2 values (> 0.919) were achieved and the values obtained in the cross validated ANOVA (F values higher than 58.2 and p-values lower than 5.2×10^{-12}) demonstrated the robustness of the proposed models and a good classification existing between groups. Moreover, the results obtained in the permutation tests (based on 200 permutations), employed to validate all PLS-DA models, indicated that differences in the PLS-DA were indeed due to differences in the metabolic profile of coffee samples and not due to data overfitting (see **Figure 3**) [47].

Next, once demonstrated the differences in the metabolic profiles of the coffee samples submitted to different roasting process, the variable importance in projection (VIP) value was selected to point out potential markers since it summarizes the contribution of each variable to the PLS model. Thus, 13 variables with VIP values higher than 1.0 were chosen as potential relevant molecular

features in coffee roasting process. The extracted ion electropherograms (EIEs) of the 13 significant variables are represented in **Figure 4**.

Table 1. Quality parameters and statistical values for the PLS-DA models built for the three different pairwise groups comparisons.

	Quality parameters			Cross-validated ANOVA	
	R^2X	R^2Y	Q^2	F-value	p-value
GCB vs LRC	0.644	0.965	0.919	58.2	5.2×10^{-12}
GCB vs MRC	0.674	0.975	0.946	81.3	3.1×10^{-13}
GCB vs DRC	0.690	0.975	0.960	132.5	5.4×10^{-16}

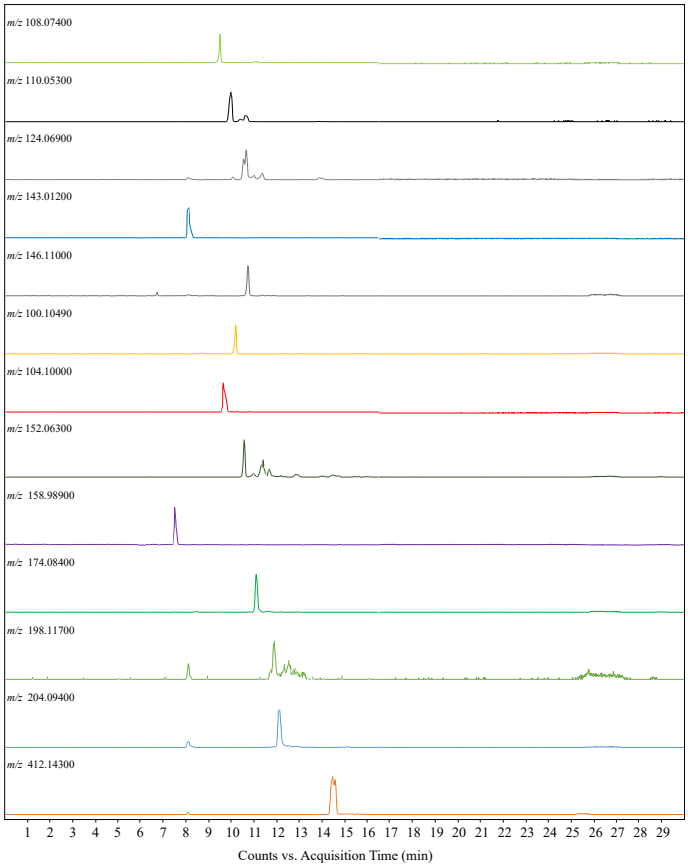

Figure 4. Extracted ion electropherograms (EIEs) for the potential markers of coffee roasting process obtained in positive ionization mode.

3.3 Metabolite identification

The identification of the 13 molecular features highlighted as markers of the coffee roasting process by the VIP values was performed using their *m/z* value, isotopic pattern, and MS/MS fragmentation pattern as it has been described in section 2.7. Using different databases, a list of possible metabolites whose presence could be probable in coffee was proposed for further interpretation. **Table 2** summarizes the migration time, the molecular formula, the experimental *m/z* value, the mass error (ppm), the main fragments obtained in MS/MS spectra, and statistical parameters, i.e. VIP values from PLS-DA models and the p-values (in brackets) from non-parametric univariate Mann-Whitney U test for each of the three pairwise comparisons. Also, the trend observed for all significant metabolites along the roasting process of coffee is included in this table. As **Table 2** shows, 7 metabolites were identified using this approach (2 of them were unequivocally identified). Interestingly, the levels of most compounds decreased with the roasting process except the compounds 3, 5, 6, 8 and 12 whose levels increased along this process.

Compound 2 (t_m = 8.4 min) exhibited a $[M+H]^+$ ion at *m/z* 143 with an intense MS/MS fragment ion at *m/z* 82. Looking to the isotopic profile of this compound hints the presence of a sulphur atom. Moreover, by comparing the MS/MS spectrum obtained to the predicted one reported in the HMDB database, this compound was tentatively identified as S-(2-furanylmethyl)methanethioate. Even though the presence of this compound in roasted coffee has been previously reported in the literature [48], as far as we know, its experimental MS/MS spectrum has not been yet reported.

From the different molecular features selected as markers of the coffee roasting process in some cases, identification could be corroborated by co-injection with commercial standards. For instance, **compound 3** (t_m = 9.9 min) with a $[M+H]^+$ ion at *m/z* 108 and **compound 4** (t_m = 10.1 min) with a $[M]^+$ ion at *m/z* 104 were unequivocally identified as 3-ethylpyridine and choline, respectively, based on the comparison of their migration times and MS/MS fragmentation patterns to those obtained for the commercial standards. The presence of both compounds in roasted coffee has been widely reported in some works [49-53]. As can be seen in the box plot (see **Figure 5),** 3-ethylpyridine (**compound 3)** is one of the compounds whose levels increased along roasting process which is in agreement with the results previously reported by Dorfner et al. [54]. This provides a way to validate our metabolomic approach herein developed. Regarding choline, Wei et al. observed that this compound slightly decreased during the roasting process [55].

Table 2. MS/MS fragmentation of potential markers of coffee roasting process found in this work

#	Migration time (t_m)	Molecular Formula	Identification	[M+H]$^+$ (m/z)	Mass error (ppm)	Main MS/MS fragments	VIP values (p-value)[a]			Roasting trend
							GCB vs LRC	GCB vs MRC	GCB vs DRC	
1	7.8	-	Unknown	158.9891	7	No fragmentation pattern	1.3 (1.7×10^{-4})	1.2 (1.8×10^{-4})	0.5 (0.0453)	↓
2	8.4	$C_6H_6O_2S$	S-(2-Furanylmethyl) methanethioate	143.0117	20	82.9977 39.0077	1.7 (5.2×10^{-6})	1.3 (1.9×10^{-6})	0.4 (0.2289)	↓
3	9.9	C_7H_9N	3-Ethylpyridine[b]	108.0737	2	65.0399 92.0575	0.9 (0.023)	1.2 (0.022)	1.4 (0.022)	↑
4	10.1	$C_5H_{14}NO$	Choline[b]	104.1061[c]	9	58.0655 60.0808 45.0564	2.3 (1.0×10^{-6})	1.8 (1.9×10^{-6})	0.9 (0.004)	↓
5	10.4	C_6H_7NO	Methyl-pyrrolecarboxaldehyde	110.0530	2	67.0417 95.0368 80.0486 41.0360	2.4 (2.0×10^{-6})	2.6 (1.9×10^{-6})	2.9 (1.7×10^{-6})	↑
6	10.6	-	Unknown	100.1049	-	44.0498 70.0649 41.0389	0.7 (0.021)	1.4 (0.022)	1.7 (0.020)	↑
7	11.0	-	Unknown	152.0631	-	53.0382 93.0546 80.0494	1.1 (0.002)	1.1 (4.4×10^{-4})	1.5 (4.9×10^{-6})	↓
8	11.1	C_6H_7NO	N-acetyl-2-methylpyrrole	124.0685	2	81.0536 109.0491 53.0401	1.4 (0.020)	1.5 (0.021)	1.7 (0.020)	↑

Table 2. Continued

#	Migration time (t_m)	Molecular Formula	Identification	$[M+H]^+$ (m/z)	Mass error (ppm)	Main MS/MS fragments	VIP values (p-value)[a] GCB vs LRC	GCB vs MRC	GCB vs DRC	Roasting trend
9	11.2	$C_7H_{15}NO_2$	3-dehydroxycarnitine	146.1101	1	43.0178 87.0428	1.4 (4.4×10^{-4})	1.9 (1.9×10^{-6})	2.3 (6.9×10^{-7})	↓
10	11.6	-	Unknown	174.0837	-	144.0793 92.0486 93.0568 65.0379 130.0620	0.9 (0.139)	0.8 (0.274)	1.2 (0.135)	↓
11	12.5	-	Unknown	198.1165	-	No fragmentation pattern	2.4 (5.0×10^{-7})	2.1 (3.6×10^{-6})	1.8 (1.7×10^{-6})	↓
12	12.7	$C_{12}H_{13}NO_2$	Indole derivative	204.0942	2	107.0731 158.0933 144.0834 186.0910	2.4 (3.6×10^{-5})	2.3 (3.7×10^{-4})	2.0 (0.612)	↑
13	15.4	-	Unknown	412.1427	-	138.0599 94.0646 192.0386 288.4826	1.5 (2.7×10^{-3})	1.6 (9.7×10^{-4})	1.6 (3.6×10^{-4})	↓

[a] p-value of Mann Whitney U (FDR) test. Cut-off value: 0.045.
[b] Identification corroborated using commercial standards.
[c] $[M]^+$
↑ The level of the compound increases with roasting; ↓ The level of the compound decreases with roasting.

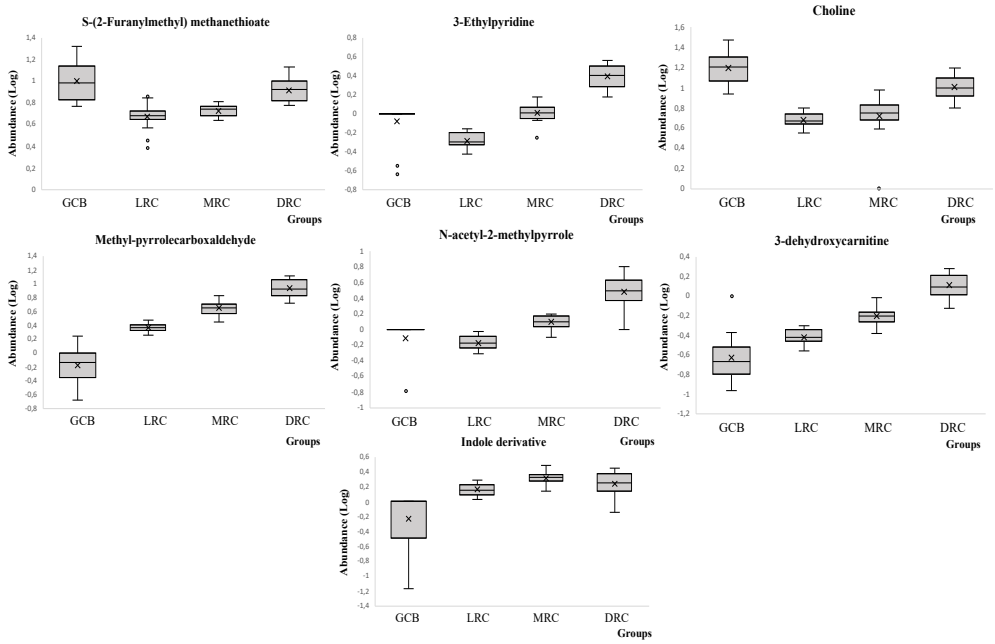

Figure 5. Box-plots for the different metabolites unequivocally and tentatively identified.

Compounds 5 (t_m = 10.4 min) and **8** (t_m = 11.1 min) displayed $[M+H]^+$ ions at *m/z* 110 and *m/z* 124, respectively. Both compounds were tentatively identified as pyrrole derivatives. Standard solution of 1-methyl-2-pyrrolecarboxaldehyde and 2-acetylpyrrole were injected to try to identify **compound 5**. Although, the migration time of these standards did not match the one obtained for **compound 5**, the fragment ions obtained from the MS/MS spectrum of 1-methyl-2-pyrrolecarboxaldehyde matched those obtained for **compound 5** (ions *m/z* 67 and 80), suggesting this compound could be a derivative of methyl-pyrrolecarboxaldehyde. On the other hand, **compound 8** was tentatively identified as N-acetyl-2-methylpyrrole since it showed fragment ions at *m/z* 80, 81 and 53, which is in agreement with previous results described in the literature [56]. Several works have reported the role of pyrrole compounds in the aroma and flavour of coffee [56-58]. An increment of levels of these two pyrrole derivatives during roasting process was observed in the box plots (**Figure 5**).

The last compound tentatively identified was **compound 12** (t_m = 12.7 min) that displayed a $[M+H]^+$ ion at *m/z* 204. A standard solution of indole-3-butyric acid (t_m = 22.5 min) was analysed in order to obtain its migration time and MS/MS pattern to compare with the ones obtained for **compound 12**. Although the migration times did not match, the MS/MS pattern shows similar fragments for both compounds at *m/z* 158, 144, and 186 suggesting that **compound 12** could be an

indole derivative. In fact, despite being very scarce the available information about the formation pathway and kinetics of indole during coffee roasting, the presence of this family compounds has been reported in coffee. Silwar and Lüllman [59] measured the formation of indole during coffee roasting and reported that it appears in considerable amounts at 170 °C and increases with roasting time. This corroborates our findings once again.

Other standard compounds such as calcium acetate (t_m = 9.1 min), methyl anthranilate (t_m = 13.4 min) and L-(+)-arabinose were analysed to know their migration times and MS/MS spectra with the aim of comparing them with those obtained for **compounds 1** and **7 (see Table 2)**. However, the possibility of matching calcium acetate or methyl anthranilate with one of our metabolites was discarded due to differences in the migration time and MS/MS fragmentation pattern. On the other hand, the ionization of L-(+)-arabinose was not possible under the given CE-MS conditions.

Thus, in the present study, several markers of the coffee roasting process have been identified. Some of these metabolites have already been proven to be related to the roasting process. This validates our approach and help us demonstrating the potential of CE-MS in the metabolomic analysis of coffee samples.

4. Conclusions

In this work, a new CE-MS method has been developed enabling the metabolic fingerprinting of coffee samples submitted to different roasting degrees. This advanced analytical methodology enables a reliable comparison of metabolic profiles in which peak alignment was successfully carried out using vendor software. The feasibility of the methodology was demonstrated by the analysis of coffee samples submitted to three different roasting degrees (light, medium, and dark coffee) in order to investigate changes occurring during this process. This approach allowed to propose 13 compounds as potential markers of the coffee roasting process. 7 of these compounds could be identified, being 2 of them unequivocally identified. Different families of compounds such as derivatives from pyridine, pyrrole, betaine, or indole have been pointed out as markers of the coffee roasting process. In summary, the developed CE-MS methodology is presented as a useful and powerful strategy to obtain information on the polar metabolome, being highly complementary to other previously used in metabolomics.

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CHAPTER V

SEPARATION AND IDENTIFICATION OF PEPTIDES IN PROTEIN HYDROLYSATES FROM EDIBLE MACROALGAE AND COFFEE SILVERSKIN

V.1. Preface

There is an increasing interest in the society on improving diet and lifestyle which encouraged the development of products with specific health promoting properties. For this reason, the search of bioactive compounds with potential beneficial health effects in order to obtain high value added compounds useful in food, pharmaceutical and cosmetic industries, has been promoted. Food and residues from the food industry are interesting and sustainable sources of bioactive compounds. The revalorization and reusing of food residues allow the generation of benefits for companies from an economical point of view and enable to reduce the environmental impact originated by the elimination of residues. In this sense, and as it has been commented in the introduction of this PhD Thesis (section I.3.1), bioactive peptides from foodstuffs are interesting compounds presenting biological activity.

As mentioned in section I.3.3 of this Thesis, marine sources are considered one of the richest to obtain bioactive compounds. Among them, macroalgae, which produce high-quality proteins at concentrations between 5-15 % in the case of brown algae (Phaeophyta), 9-26 % for green algae (Chlorophyta) and 10-47 % for red algae (Rhodophyta) (percentages referred to dry weight) [255], have emerged as potential natural sources for the generation of peptides with potential biological activity. However, there are only four published works devoted to the identification of peptides from macroalgae protein hydrolysates [201, 202, 207, 215] so it is clear that their potential as a source of bioactive peptides is still quite unexplored.

As commented in Chapter IV, coffee is one of the most consumed drinks in the world due to its excellent organoleptic properties and it is considered a natural source of bioactive compounds. Coffee industry is responsible for the generation of large amount of residues. Among them, coffee silverskin (CS), coffee husk and spent coffee grounds are generated in significant amounts and merit special attention. Although some of these residues (e.g. CS or coffee husk) have been used for the extraction of bioactive compounds such as phytochemicals, phenolics, dietary fiber, etc., in most cases they are discarded in landfills or incinerated causing environmental pollution and health risks. To date, there is no evidence about the potential of CS to produce peptides with biological activity in spite of it contains a high protein content. CS is the unique by-product generated during coffee roasting which has been used as a new potential functional ingredient for food and cosmetics [256-258]. Since CS contains from 16.2 to 19.0 % of proteins [259], it is interesting to explore the potential of its protein hydrolysates as a source of bioactive peptides.

Taking into account all the above mentioned, this chapter is focused on the development of analytical methodologies to achieve for the first time the separation

and identification of short chain peptides in protein hydrolysates from three edible macroalgae and coffee silverskin in order to explore their potential as natural sources of bioactive peptides.

V.2. Objectives

The objectives of this chapter were the following:

- To propose and evaluate extraction protocols to obtain protein extracts from edible macroalgae and coffee silverskin.
- To select the most adequate conditions to achieve the enzymatic hydrolysis of protein extracts as a means to obtain peptides with potential bioactivities.
- To evaluate different biological activities of the protein hydrolysates.
- To develop a LC-MS methodology enabling the separation and identification of potential bioactive peptides in protein hydrolysates from edible macroalgae and coffee silverskin.
- To investigate the effect of the roasting process on the peptide composition of coffee silverskin.

V.3. Results and discussion

V.3.1. Separation and identification of peptides in protein hydrolysates from edible macroalgae

This study was carried out using three different edible macroalgae, *Saccharina latissimi* (brown macroalga), *Codium spp.* (green macroalga), and *Mastocarpus stellatus* (red macroalga), whose protein contents were 6.3 ± 0.1 %, 12.4 ± 0.8 %, and 16.9 ± 0.5 % (all percentages referred to sample dry weight), respectively.

The extraction of aqueous and alkaline soluble proteins from these macroalgae was achieved following a procedure previously described in the literature for the extraction of proteins in red and green macroalgae [260] with some modifications. Then, protein precipitation was performed using chloridric acid (pH 3.5) or acetone so that four different extracts (aqueous protein extract precipitated with chloridric acid (WPHCl) or acetone (WPA), and alkaline protein extract precipitated with chloridric acid (APHCl) or acetone (APA)) were obtained. The

protein contents in the extracts were evaluated using the Bradford assay, and SDS-PAGE was employed to characterize the proteins present in the extracts. Thus, the total protein content obtained from the four extracts from each macroalga were 1.8 ± 0.5 % for *Saccharina latissima*, 2.7 ± 0.5 % for *Codium spp.*, and 4.7 ± 0.7 % for *Mastocarpus stellatus.* Electrophoretic profiles obtained by SDS-PAGE analysis showed very intense bands at molecular mass values between 75 and 250 kDa for *S. latissima*, and *Codium spp* whereas bands corresponding to molecular masses lower than 25 kDa were observed for *M. stellatus*.

In order to release the peptides encrypted in the extracted proteins, alcalase and themolysin enzymes were chosen to carry out the protein hydrolysis. Between them, alcalase was the enzyme of choice since most of the peptides obtained using thermolysin belonged to the protein sequence of this enzyme.

A RPLC-MS methodology was developed to achieve the separation and identification of peptides in the protein hydrolysates from the three edible macroalgae using *de novo* sequencing by the PEAKS software. The effect of different experimental conditions such as the gradient program (gradient time, gradient shape, and initial composition of the mobile phase), column temperature (25-50 °C), flow rate (0.2-0.4 mL/min), and injection volume (2-5 µL), was investigated in order to select those that provided the best chromatographic separation and the shortest retention times. The gradient consisted of water with 0.5 % formic acid (solvent A) and methanol with 0.5 % formic acid (solvent B) programmed as follows: 0 min, 1 % B; 0-5 min, 1 % B; 5-10 min, 1-5 % B; 10-30 min, 5-60 % B; 30-35 min, 60 % B, with 15 min of post-time at final composition. Other conditions selected were: a flow rate of 0.3 mL/min, a column temperature of 50 °C, and an injection volume of 5 µL.

As **Figure V.1** shows, thirty-seven different peptides were identified in the three macroalgae being five of them common in *Mastorcarpus stellatus* and *Saccarina Latissima.* It is worth highlighting that any of the identified peptides had previously been identified in macroalgae. The potential biological activity of these peptides was checked using BIOPEP database. Results revealed that several sequenced peptides were found to be a part of longer peptides with antibacterial bioactivity.

The results obtained remark the potential of edible macroalgae, especially red and green varieties, as a natural source of peptides with potential biological activity. However, further research is needed to attribute specific biological properties to the peptides found in this work.

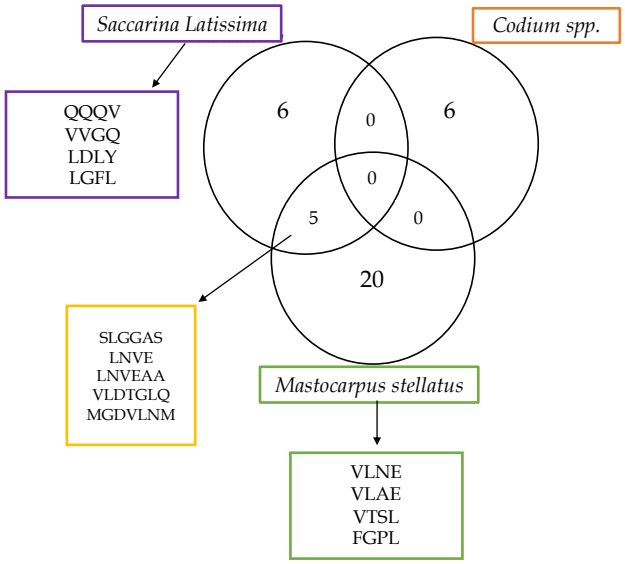

Figure V.1. Venn diagram of peptides identified in *Mastocarpus stellatus, Saccharina latissima* and *Codium spp* excluding the peptides in common with the alcalase enzyme protein sequence. The insets display those peptides found in BIOPEP database with potential biological activity.

V.3.2. Separation and identification of peptides in protein hydrolysates from coffee silverskin

The protein contents of three CS samples obtained after roasting green coffee beans at light (LCS), medium (MCS) and dark (DCS) level were 12.0 ± 0.1 %, 11.9 ± 0.1 %, and 12.0 ± 0.4 %, respectively (all percentages referred to sample dry weight). Since all CS samples had a similar protein content, MCS was chosen as model to evaluate different protein extraction procedures.

Three approaches were evaluated to perform the extraction of proteins: (i) solid-liquid extraction with acetonitrile:water (20:80 v/v) under mechanical shaking and ultrasound bath, (ii) subcritical water extraction using different temperatures (120 and 180 ºC), and (iii) HIFU extraction using 100 mM Tris-HCl buffer (pH 7.5) containing 0.5 % (v/v) SDS and 0.5 % (v/v) DTT. The protein content obtained by using each one of these protocols was estimated using Bradford assay. Percentages lower that 1 % of protein contents were obtained using the two first approaches, whereas the use of HIFU allowed to obtain a protein content of 2.6 ± 0.3 %. In order to increase the percentage of protein content extracted, the protocol based on the use of HIFU was subsequently optimized in terms of buffer composition (buffer nature, pH, the content of SDS, and DTT, and the presence of urea), HIFU conditions (time and amplitude), the extraction solvent/sample amount ratio, and the use of cleaning procedures before the extraction. The best conditions were achieved by extracting 50 mg of CS, previously defatted with

hexane, with 5 mL of 100 mM Tris-HCl buffer (pH 7.5) containing 0.25 % (v/v) SDS and 0.25 % (v/v) DTT using HIFU for 10 min at 50 % of amplitude. Under these conditions, the estimated protein content was 3.9 ± 0.5 %.

This protocol was applied to the extraction of proteins from the three different CS. Then, after precipitation with cold acetone, the protein profiles of the three CS extracts were determined by SDS-PAGE. Similar bands were observed; an intense band between 15 and 20 kD and bands with molecular masses higher than 100 kD.

Protein extracts from LCS, MCS, and DCS were submitted to enzymatic digestion using alcalase, thermolisyn and simulated gastrointestinal digestion (using pepsin and pancreatin). The hydrolysis yield, evaluated using the O-phthalaldehyde (OPA) assay, showed no significant differences among the different enzymes employed. As a consequence, the antioxidant capacity (ABTS and hydroxyl radical scavenging assays) and the capacity of peptides to reduce micellar cholesterol solubility was evaluated for all the CS protein hydrolysates. The results obtained showed that all CS protein hydrolysates exhibited antioxidant capacity in a range from 9 % to 35 %, and capacity to reduce micellar cholesterol solubility in a range from 25 to 32 %. The most antioxidant hydrolysates were obtained using thermolysin or simulated gastrointestinal digestion depending on the assay performed whereas thermolysin provided the hydrolysates with the highest cholesterol-lowering capacity.

LCS, MCS and DCS protein hydrolysates obtained by the three different enzymatic digestion procedures were subsequently analyzed by RPLC-MS. An analytical methodology was developed by studying the effect of different chromatographic conditions such as gradient program, column temperature (25, 35 and 55 °C), and injection volume (5, 10 and 15 µL) on the chromatographic resolution and analysis time. Mobile phases were composed by water with 0.3 % acetic acid (solvent A) and acetonitrile with 0.3 % acetic acid (solvent B) and the gradient consisted of 0 min, 5 % B; 0-3 min, 5 % B; 5-40 min, 5-40 % B; 40-43 min, 40-95 % B; 43-45 % 95 B with 15 min of post-time at final composition. Other parameters were: flow rate of 0.2 mL/min, a column temperature of 25 ^{o}C and an injection volume of 5 µL.

MS/MS data obtained using the developed method were analyzed using the *de novo* sequencing tool from the PEAKS software. Thus, fifty-one peptides, containing between four and twelve amino acids, were identified in the CS analyzed. None of them was common to the three different protein hydrolysates from LCS, MCS and DCS. Regarding their potential biological activity, although most of the peptides identified in LCS, MCS and DCS protein hydrolysates are not

currently included in BIOPEP database, some of them were found within longer peptide sequences with potential bioactivities such as antibacterial activity, ACE-inhibitory effect or antioxidant capacity.

From the results obtained in this work, it can be deduced that the roasting process to which coffee samples were submitted showed to have little influence on peptide composition of CS.

In summary, although the biological activity could be attributed to several factors (i.e. the presence of other bioactive compounds, the synergic effect among some peptides, etc.) and further research must be performed, this is the first time that peptide composition of CS has been studied.

The results presented in this chapter are included in the following scientific articles:

- **Article 9:** *Separation and identification of peptides in hydrolysed protein extracts from edible macroalgae by HPLC-ESI-QTOF/MS.*
  R. Pérez-Míguez, M. Plaza, M. Castro-Puyana, M.L. Marina.
  Algal Res. DOI: 10.1016/j.algal.2019.101465**.**

- **Article 10:** *High resolution liquid chromatography tandem mass spectrometry for the separation and identification of peptides in coffee silverskin protein hydrolysates.*
  R. Pérez-Míguez, M.L. Marina, M. Castro-Puyana.
  Submitted.

Article 9

Separation and identification of peptides in protein hydrolysates from edible macroalgae by HPLC-ESI-QTOF/MS.

R. Pérez-Míguez, M. Plaza, M. Castro-Puyana, M. L. Marina.

Algal Res. DOI: 10.1016/j.algal.2019.101465.

Abstract

Macroalgae contain significant amounts of high-quality proteins which, because of their structural diversity, contain a range of yet undiscovered peptides within their primary structures. In this work, an analytical methodology was developed for the separation and identification of peptides present in protein hydrolysates from three different edible macroalgae used for human consumption (*Saccharina latissima* (brown macroalga), *Codium spp.* (green macroalga), and *Mastocarpus stellatus* (red macroalga)). The extraction of aqueous and alkaline soluble proteins was carried out followed by their precipitation with HCl or acetone. The protein extracts obtained were submitted to enzymatic digestion with alcalase and subsequently analyzed by reversed-phase high-performance liquid chromatography-quadrupole-time-of flight mass spectrometry (RP-HPLC-QTOF/MS) and de novo sequencing tool to separate and identify different short chain peptides. Thirty-seven peptides were identified in the hydrolysed protein extracts from the three macroalgae, five of them being common in brown and red macroalgae. After checking against BIOPEP database, several sequenced peptides were found within longer peptides with potential antibacterial activity. Any of the identified peptides had previously been identified in macroalgae.

1. Introduction

Macroalgae are a diverse group of marine organisms which generate a wide group of functional biomolecules to survive under stress conditions [1]. They produce high-quality proteins whose concentrations can vary from 5 to 15 % in the case of brown algae (Phaeophyta), from 9 to 26 % for green algae (Chlorophyta) and from 10 to 47 % for red algae (Rhodophyta) (percentages referred to dry weight) [2]. Peptides contained in proteins from marine sources, which can be released during enzymatic hydrolysis, food processing or ripening [3], have a high interest since they could present different type of bioactivity such as anti-cancerous, anti-proliferative, anti-coagulant, antibacterial, antifungal, and anti-tumor, among others [4-9]. Although peptides contained in protein hydrolysates from macroalgae could present some type of bioactivity, their separation and identification in these macroalgae protein hydrolysates have scarcely been investigated [10-15].

One of the most relevant challenges to obtain peptides from macroalgae is related to the extraction of proteins from the matrix since it is a topic which has not been studied deeper compared to the extraction of proteins from crops [16, 17] Protein extraction from macroalgae is a difficult task due to the cross-linking between polysaccharides and proteins within the matrix, as well as the

inaccessibility of proteins within macromolecular cell wall assemblies [18]. The cross-linking between polysaccharides and proteins is especially important for brown macroalgae [19]. For instance, the extraction of proteins from the Laminaria alga *Saccharina japonica* has proved to be difficult due to the high levels of non-protein compounds (mainly viscous polysaccharides) whose presence interferes with protein extraction [20]. As a consequence, the main methods used for the extraction and precipitation of proteins are not completely useful in macroalgae.

High-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) is the analytical technique mainly employed to carry out the separation and identification of peptides [21]. Nowadays, most of the MS systems are able to detect with accuracy peptides with a length higher than 5 amino acids. However, the analysis of shorter peptides with 2 to 4 amino acids has scarcely been reported in the literature [21]. For instance, the low or high fragmentation of short peptides by tandem MS can make their detection difficult and challenging [21-23]. Thus, the development of analytical methods to carry out the separation and identification of short chain peptides presents a high interest when an in deep characterization of food is attempted.

The aim of this work was to separate and identify peptides contained in protein hydrolysates from three different edible macroalgae (*Saccharina latissima*, *Codium spp*. and *Mastocarpus stellatus*) used for human consumption. The extraction of aqueous and alkaline soluble proteins was carried out followed by protein precipitation using different approaches. Protein extracts obtained were subsequently submitted to enzymatic digestion and analyzed by reversed phase high-performance liquid chromatography coupled to a quadrupole-time-of flight mass spectrometer (RP-HPLC-QTOF/MS) and *de novo* sequencing tool.

2. Materials and methods

2.1. Chemicals and samples

All chemicals and reagents were of analytical grade. Sodium hydroxide, bovine serum albumin (BSA), and thermolysin were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid, acetone, methanol, ethanol and acetic acid were acquired in Scharlau (Barcelona, Spain). Sodium dodecyl sulfate (SDS) was purchased from Merck (Darmstadt, Germany). Alcalase 2.4 L FG was kindly donated by Novozymes Spain S.A. (Madrid, Spain). Mini-protean precast gels, Laemmli buffer, Tris/glycine/SDS running buffer, precision plus protein standards (recombinant proteins expressed by Escherichia coli with molecular weights of 10,

15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa), silver stain kit, and Bradford reagent (Coomassie Blue G-250) were acquired from Bio-Rad (Hercules, CA, USA).

For the HPLC-MS/MS analysis, MS grade methanol and formic acid from Sigma-Aldrich were employed. The ultrapure water used was obtained from a Milli-Q (Millipore, Bedford, MA, USA) instrument.

Macroalgae samples (*Saccharina latissima*, *Codium spp*. and *Mastocarpus stellatus*) consisted in dried algae kindly donated by Porto-Muíños, S.L. (La Coruña, Spain). Once the macroalgae were collected, they were washed, dried at 30-35 ºC and grinded.

2.2. Total protein content

The protein content of the macroalgae was determined by the Kjeldahl method [24]. Nitrogen data were converted into protein values employing a conversion factor of 6.25 and were expressed as g per 100 g of dried macroalga. Analyses were performed in triplicate.

2.3. Extraction of proteins

The procedure used for the extraction of water and alkaline soluble proteins from milled dried macroalgae was based on the method described by Harnedy and FitzGerald (2015) [18] with some modifications (see **Figure 1**).

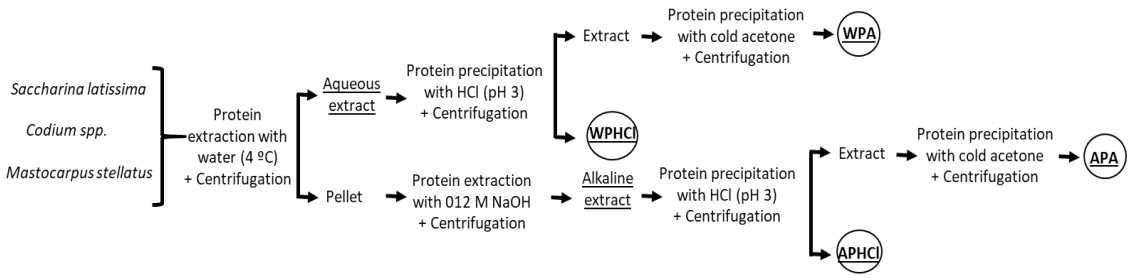

Figure 1. Protein extraction procedure employed in this work.

In brief, 0.5 g of dried milled macroalgae powder was suspended in milli-Q water (1:20 (w/v)) and stirred gently for 3 h at 4 ºC. The proteins in the aqueous extract were removed by centrifugation at 4000 x g for 15 min at 4 ºC.

For alkaline soluble protein extraction, the pellet obtained after centrifugation was resuspended in 0.12 M NaOH at a weight volume ratio of 1:15 (w/v) and stirred gently at room temperature for 1 h. Alkaline extraction was performed twice and both supernatants obtained by centrifugation at 4000 x g for

15 min at room temperature were combined.

Then, proteins from the aqueous and alkaline extracts were precipitated employing two different methods. First, the proteins were precipitated by adjusting the pH of each extract to around pH 3.5 using HCl. Aqueous protein extracts were kept for 30 min at 4 ºC while alkaline protein extracts were kept at room temperature to achieve the precipitation. The solutions were centrifuged at 4000 x g for 15 min at 4 ºC, and the pellets obtained were collected and dried by vacuum-drying. The proteins obtained were called aqueous or alkaline proteins precipitated with HCl (WPHCl and APHCl, respectively).

Secondly, the remaining proteins in both supernatants, aqueous and alkaline solutions after protein precipitation with HCl, were subjected to a second precipitation using cold acetone. The supernatants were diluted twice their volume in cold acetone and allowed then to stand for 1 h at -8 ºC. The solutions were centrifuged at 4000 x g for 15 min at 4 ºC, and the pellets obtained were over-night dried at room temperature. The proteins obtained were called aqueous or alkaline proteins precipitated with acetone (WPA and APA, respectively). The protein content for both aqueous and alkali extracts precipitated with HCl and acetone was estimated by Bradford assay [25]. WPHCl, APHCl, WPA and APA were ready to be subjected to protein digestion. Protein extraction for each algae was carried out in triplicate.

2.4 SDS-PAGE

Proteins were separated by SDS-PAGE using a Bio-Rad Mini-protean system (Hercules, CA, USA). Proteins solutions were mixed with Laemmli buffer containing 5 % (v/v) β-mercaptoethanol, followed by heating at 100 °C during 5 min and loaded into commercial Mini-PROTEAN TGX Precast Protein Gels from Bio-Rad (Hercules, CA, USA). Proteins were separated by applying 80 V for 5 min and 150 V until the separation was completed using Tris/glycine/SDS as running buffer. Molecular markers of standard proteins with molecular weights from 10 to 250 kDa were also run. After separation, proteins were treated with a fixing solution of water/MeOH/acetic acid (50/40/10 % (v/v)) by shaking for 30 min and then with a second fixing solution water/EtOH/acetic acid (85/10/5 % (v/v)) twice for 15 min each. Gels were then treated with an oxidizer solution for 5 min and washed with water followed by the addition of the silver reagent and shaking during 20 min. Afterwards, the gel was washed for 1 min with water and developer solution was added. Reaction was stopped by adding 5 % acetic acid solution.

2.5 Protein digestion

Protein extracts obtained from macroalgae were hydrolyzed using the enzyme alcalase following a procedure previously optimized by our research team for the hydrolysis of proteins from plum by-products [26]. The protein extracts were dissolved in 5 mM borate buffer (pH = 8.5) at a final concentration of 5 mg/mL with the help of an ultrasonic probe for 5-10 min and with 30 % of wave amplitude. Then, the enzyme was added at an enzyme/substrate ratio of 0.15 AU/g protein and the solution was incubated in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany) at 50 ^{o}C with agitation (700 rpm) for 4h. The digestion was stopped (100 ^{o}C for 10 min) using Thermomixer Compact and the solution was centrifuged for 10 min at 6000 g. Finally, the supernatant was collected for its analysis by HPLC-ESI-MS/MS.

2.6 Separation and identification of peptides by RP-HPLC-ESI-QTOF/MS

Peptide analysis was performed using an HPLC system 1100 from Agilent (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole-time-of flight mass spectrometer (QTOF/MS) Agilent 6530 equipped with an orthogonal electrospray ionization (ESI) source (Agilent Jet Stream, AJS). The HPLC instrument was equipped with a quaternary solvent pump, an auto-sampler, and a column heater compartment. Agilent Mass Hunter Workstation software B.07.00 from Agilent was employed for HPLC and MS control, data acquisition, and data analysis.

The separation was carried out using a porous-shell fused-core Ascentis Express C18 analytical column (150 mm x 2.1 mm, particle size 2.7 μm) with an Ascentis Express C18 guard column (0.5 cm × 2.1 mm, 2.7 μm particle size), both from Supelco (Bellefonte, Pa, USA). The column temperature was 50 ^{o}C and the flow rate 300 μL/min. Five μL of extract were injected. The mobile phases consisted of (A) water with 0.5 % formic acid and (B) methanol with 0.5 % formic acid in a gradient elution analysis programmed as follows: 0 min, 1 % (B); 0-5 min, 1 % (B); 5-10 min, 1-5 % (B); 10-30 min, 5-60 % (B); 30-35 min, 60 % (B), with 15 min of post-time.

The mass spectrometer was operated in positive ion mode and the mass range was from 100 to 1700 m/z. MS parameters were the following: capillary voltage, 3500 V; nebulizer pressure, 50 psig; drying gas flow rate, 12 L/min; gas temperature, 350 ^{o}C. The fragmentor voltage (cone voltage after capillary) was set

at 80 V. The skimmer and octapole voltage were 60 V and 750 V, respectively. Source sheath gas temperature and flow were 400 ºC and 12 L/min, respectively. MS/MS was performed employing the auto mode and the following optimized conditions; 1 precursor per cycle, dynamic exclusion after two spectra (released after 1 min), and collision energy of 5 V for every 100 Da. Internal mass calibration of the instrument was carried out using an AJS ESI source with an automated calibrant delivery system. The reference compound solution for internal mass calibration containing purine and HP-0921 (hexakis (1H,1H,3H-tetrafluoropropoxy) phosphazine) in acetonitrile-water (90:10, v/v) (4 μM and 2.5 μM, respectively, 15 μL/min) from Agilent was used, *m/z* 121.0509 and *m/z* 922.0098, respectively. The analyses were conducted in triplicate.

Tandem MS/MS spectra were obtained for the molecular ion with the highest abundance. Every sample was injected in triplicate into the MS system. In order to assure that identified peptides came from macroalgae protein, MS/MS spectra were analyzed using PEAKS Studio Version 7 (Bioinformatics Solutions Inc., Waterloo, Canada). Data analysis was performed by *de novo* sequencing tool. Only those peptides identified with an ALC (expected percentage of correct amino acids in the peptide sequence) above 85 % and with a good precursor fragmentation pattern were considered. Moreover, only those peptides appearing in at least 7 injections from 9 injections (three injections of each triplicate) were taken into account. Only isoforms with leucine (L) are presented in our results, although peptide sequences containing isoleucine (I) amino acid instead of L are also possible since it is not possible to differentiate I from L by the MS used.

3. Results and discussion

3.1. Development of an analytical methodology for the separation and identification of peptides by RP-HPLC-ESI-QTOF/MS

To achieve the separation and identification of peptides in hydrolysates from macroalgae protein extracts, an adequate analytical methodology, based on the use of HPLC-MS/MS, was developed. Taking into account that the protein contents for the three studied macroalgae (determined as described in section 2.1) were 6.3 ± 0.1 % for *Saccharina latissima*, 12.4 ± 0.8 % for *Codium spp*., and 16.9 ± 0.5 % for *Mastocarpus stellatus* (all percentages referred to sample dry weight), the macroalga *M. stellatus* was selected to perform the optimization of the chromatographic and MS parameters due to its higher protein content. Then, a protein aqueous extract was obtained and precipitated with HCl (WPHCl) following the protocol previously described (see section 2.3). The protein extract

obtained was hydrolyzed and analyzed by HPLC-MS/MS using a C18 column. To optimize the separation conditions, the effect of different parameters such as gradient program (gradient time, gradient shape, and initial composition of the mobile phase), column temperature (25-50 °C), flow rate (0.2-0.4 mL/min), and injection volume (2-5 µL), was investigated. The best resolution and shortest analysis time were achieved using a gradient elution based on water with 0.5 % formic acid (solvent A) and methanol with 0.5 % formic acid (solvent B) programmed as follows: 0 min, 1 % B; 0-5 min, 1 % B; 5-10 min, 1-5 % B; 10-30 min, 5-60 % B; 30-35 min, 60 % B, with 15 min of post-time at final composition. The other selected experimental conditions were a flow rate of 0.3 mL/min, a column temperature of 50 °C, and an injection volume of 5 µL. MS/MS parameters for peptide identification were selected taking into account those previously employed to identify peptides from different sources such as food and food by-products [26-28]. MS/MS data obtained using the developed method were analyzed using the *de novo* sequencing tool from the PEAK Software.

Figure 2 shows the Total Ion Chromatogram (TIC) corresponding to the analysis of protein hydrolysates from *M. stellatus* and the mass spectrum showing the fragmentation pattern of peak at 21.9 min and 600.3320 m/z (VGGTGPL peptide). As it can be observed, a good chromatographic profile could be obtained in an analysis time of 35 min.

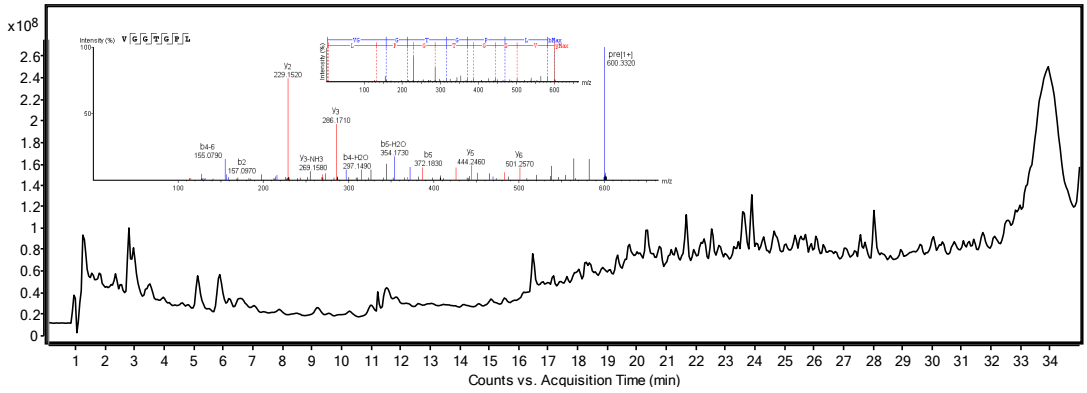

Figure 2. Total ion chromatogram from WPHCl extracts hydrolysed with alcalase from *Mastocarpus stellatus* by RP-HPCL-ESI-QTOF-MS/MS and an example of MS/MS spectrum of the peptide VGGTGPL observed at 21.9 min (molecular mass (Da): 599.3279).

3.2. Protein extraction and digestion

Protein extracts were obtained following the protocol described by Harnedy and FitzGerald (2015) [18] with some modifications. The method involved two subsequent aqueous and alkaline extractions under the conditions described in

section 2.3. Protein profiles obtained by SDS-PAGE were obtained and compared for the three algae showing electrophoretic profiles with bands at molecular mass values lower than 25 kDa for *M. stellatus* and intense bands corresponding to molecular mass between 75 and 250 kDa (see **Figure S1 from supplementary data**) for the other two macroalgae.

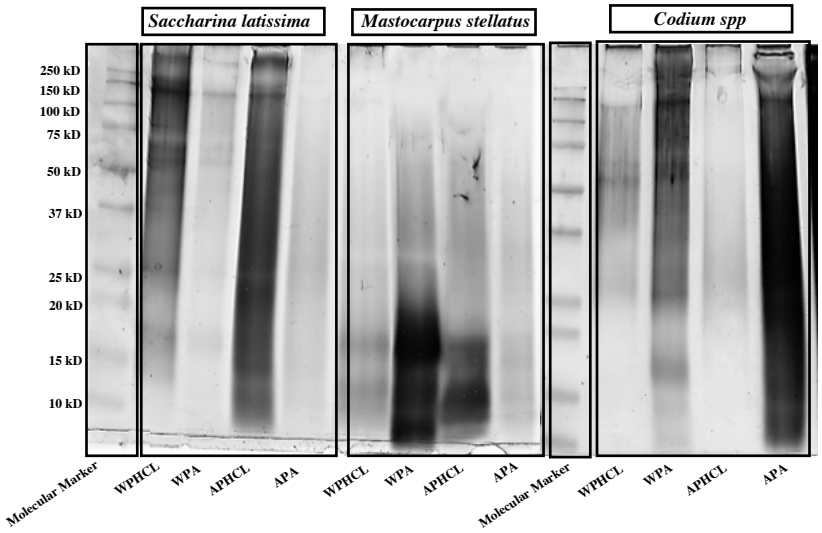

Figure S1. SDS-PAGE gels corresponding to the protein extracts obtained for WPHCL, WPA, APHCL and APA extracts for each algae.

The total protein content (expressed in %) obtained from four extracts (WPHCl, APHCl, WPA and APA) from each macroalgae, estimated by Bradford assay, was 1.8 ± 0.5 % for *Saccharina latissima*, 2.7 ± 0.5 % for *Codium spp*., and 4.7 ± 0.7 % for *Mastocarpus stellatus.* showing higher extraction yields for the red and green macroalgae since these macroalgae presented higher crude protein content before extraction. Protein extracts obtained were precipitated with HCl and digested with the enzyme alcalase. Under these conditions, 12 and 17 peptides were found in aqueous extracts (WPHCl) and 2 and 14 peptides in alkaline extracts (APHCl) from *S. latissimi* and *M. stellatus*, respectively. However, peptides were not obtained from both extracts from *Codium spp*. Moreover, a gel formation was observed in *S. latissima* after the precipitation of proteins with HCl. This fact could be explained by the high levels of non-digestible viscous polysaccharides that make especially problematic the extraction of proteins from brown macroalgae [20, 29].

Thus, based on the experience of our research group on the analysis of peptides from different sources, protein precipitation was carried out using cold acetone instead of HCl (see section 2.3) [30]. Under these conditions, 11, 6, and 11

peptides were obtained in aqueous extracts from *S. latissimi*, *Codium spp*., and *M. stellatus,* respectively, and 14 and 10 peptides in alkaline extracts of *S. latissimi* and *M. stellatus* (peptides were not found in the alkaline extract for *Codium spp)*. Bearing in mind the results obtained with HCl and acetone, a combination of both approaches was evaluated. Thus, aqueous and alkaline extracts were firstly precipitated with HCl by adjusting the pH to around 3.5 (extracts WPHCl and APHCl, respectively). Then, the supernatants obtained were submitted to a second precipitation by adding cold acetone (extracts WPA and APA, respectively) (see **Figure 1**). Following this procedure, 12, 2, 11 and 14 peptides were obtained in WPHCl, APHCL, WPA and APA extracts, respectively, from *S. latissima*; 6 peptides were detected in WPA extracts while any peptide was not found in WPHCl, APHCL and APA extracts from *Codium spp*., and 17, 14, 11 and 10 peptides were got in WPHCl, APHCL, WPA and APA extracts from the macroalga *M. stellatus,* respectively (see Tables 1, 2 and 3). Since a higher number of peptides could be obtained following this approach, it was selected to carry out the isolation of proteins from the different macroalgae.

Although two different enzymes, alcalase and thermolysin, were tested for protein digestion under the experimental conditions previously employed by our research team [26, 31], alcalase was chosen to achieve the hydrolysis since most of the peptides obtained using thermolysin belong to the protein sequence of this enzyme.

3.3. Peptide identification in protein hydrolysates

In order to carry out the tentative identification of peptides in the hydrolyzed protein extracts from *S. latissima*, *Codium spp*., and *M. stellatus*, they were analyzed by the developed HPLC-MS/MS method. Then, MS/MS data were treated by the PEAKS software to obtain *de novo* sequence.

Figures 2, **3** and **4** show the TIC chromatograms corresponding to the protein hydrolysates from WPHCl extract in *M. stellatus,* APA extract in *S. latissima* and APA extract in *Codium spp.,* respectively. These selected hydrolysates extracts presented the highest number of peptides for each macroalga. Moreover, these figures also display as an example, the mass spectrum with the fragmentation pattern of VGGTGPL, LNVE and TSFLDL peptides, respectively.

Tables 1, 2 and **3** show the different peptides identified in *M. stellatus, S. latissima* and *Codium spp*., respectively, along with their experimental molecular masses, ALC, and accuracy. Forty-nine different peptides with a number of amino acids ranging from 4 to 10 were identified.

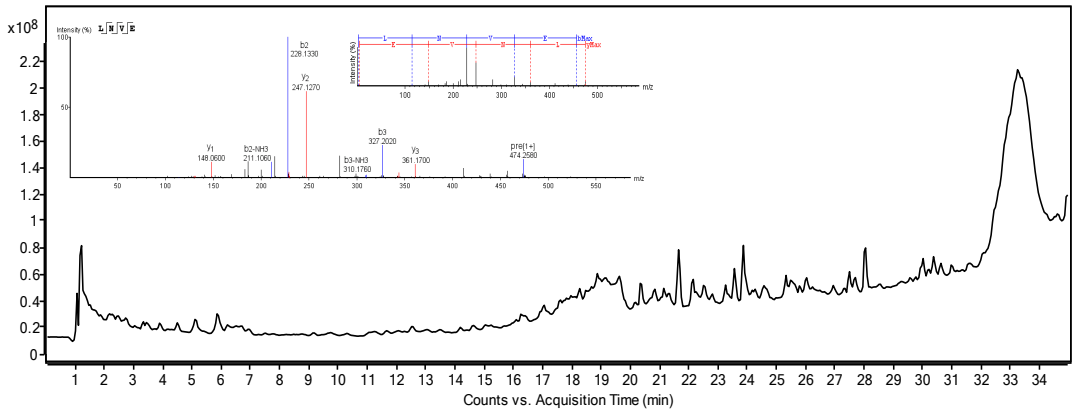

Fig. 3 Total ion chromatogram from APA extracts hydrolysed with alcalase from *Saccharina latissima* by RP-HPLC-ESI-QTOF-MS/MS and an example of MS/MS spectrum of the peptide LNVE observed at 18.8 min (molecular mass (Da): 473.2485).

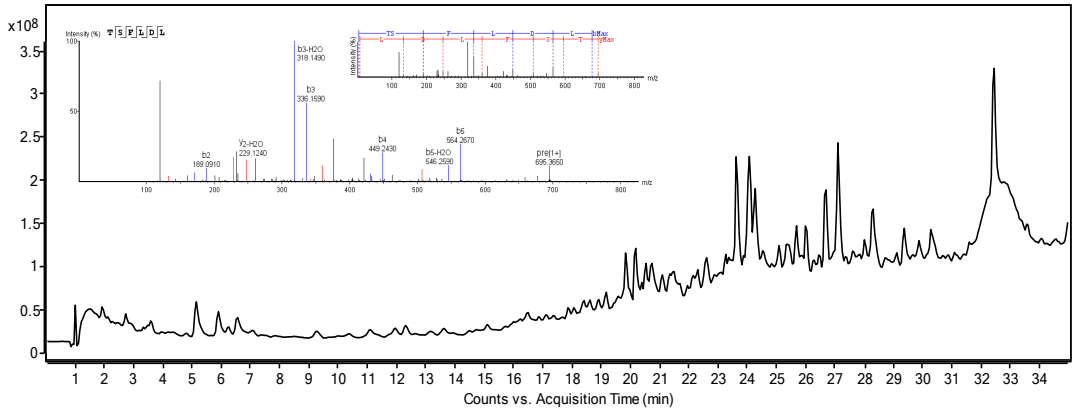

Fig. 4 Total ion chromatogram from WPA extracts hydrolysed with alcalase from *Codium spp*. by RP-HPLC-ESI-QTOF-MS/MS and an example of MS/MS spectrum of the peptide TSFLDL observed at 30.9 min (molecular mass (Da): 694.3538).

Table 1. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysates of WPHCl, WPA, APHCl and APA protein extracts from *Mastocarpus stellatus* using RP-HPLC-ESI-QTOF-MS/MS and *de novo* sequencing tool.

				WPHCl		WPA		APHCl		APA		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	LGDN	3.4	417.1859	-5 ± 2	91 ± 1	-7 ± 1	91 ± 1	-3 ± 2	91 ± 1	-	-	-
2	ATLN*	6.8	417.2224	-	-	-	-	-	-	-5 ± 3	89 ± 2	-
3	VVGGT	7.6	431.2380	-7 ± 2	89 ± 1	-	-	-	-	-	-	-
4	VVDT	9.1	432.2220	-	-	-	-	-5 ± 2	93 ± 1	-	-	-
5	SLGGAS	10.0	490.2387	-7 ± 1	90 ± 2	-7 ± 2	89 ± 2	-5 ± 2	90 ± 2	-5 ± 2	90 ± 2	-
6	AVVK	12.2	415.2794	-10 ± 1	93 ± 1	-	-	-9 ± 3	93 ± 1	-	-	-
7	VLNE	14.3	473.2485	-	-	-	-	-2 ± 2	87 ± 1	-	-	Antibacterial/ membrane-active peptide/ immunomodulating
8	LVQT	15.5	459.2693	-6 ± 1	88 ± 2	-	-	-	-	-	-	-
9	VLAE	16.5	430.2427	-	-	-	-	-3 ± 2	95 ± 1	-4 ± 2	92 ± 1	VIAE: Antibacterial
10	VTSLN	18.1	532.2857	-6 ± 1	93 ± 1	-	-	-	-	-	-	-
11	VVVE	18.1	444.2584	-7 ± 1	94 ± 2	-	-	-	-	-	-	-
12	VLVN	18.5	443.2744	-	-	-	-	-4 ± 2	88 ± 2	-	-	-
13	TLSAADAA	18.7	718.3497	-3 ± 1	92 ± 1	-	-	-	-	-	-	-
14	LNVE	18.9	473.2485	-	-	-4 ± 1	90 ± 2	-2 ± 2	90 ± 2	-3 ± 2	91 ± 2	-
15	VTSL	19.4	418.2427	-4 ± 1	91 ± 1	-4 ± 2	90 ± 2	-	-	-	-	Antibacterial
16	GTGPL	19.7	443.2380	-2 ± 2	88 ± 1	-	-	-3 ± 2	88 ± 2	-	-	-
17	ATYLGS*	19.8	610.2962	-	-	-4 ± 1	89 ± 2	-	-	-	-	-
18	APGAGVY*	20.3	633.3122	-1 ± 1	90 ± 1	-3 ± 1	89 ± 2	-2 ± 1	90 ± 2	-3 ± 2	89 ± 2	Antioxidant
19	LNVEAA	21.2	615.3228	-3 ± 1	89 ± 1	-	-	-	-	-	-	-
20	SVGAELE*	21.4	703.3388	-	-	-	-	-2 ± 2	95 ± 0	-	-	-
21	VGGTGPL	21.9	599.3279	-3 ± 1	94 ± 1	-	-	-	-	-	-	-
22	SVGAEL*	22.3	574.2962	-	-	-3 ± 1	91 ± 1	-	-	-	-	-

Table 2. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysates of WPHCl, WPA, APHCl and APA protein extracts from *Saccharina latissima* using RP-HPLC-ESI-QTOF-MS/MS and *de novo* sequencing tool.

				WPHCl		WPA		APHCl		APA		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	VAGAA*	4.5	387.2118	-	-	-	-	-	-	-2 ± 1	89 ± 1	-
2	SVGAE*	5.4	461.2122	-	-	-2 ± 1	95 ± 1	-	-	-2 ± 1	91 ± 2	-
3	QQQV	6.3	501.2547	-4 ± 2	87 ± 1	-	-	-	-	-	-	Stimulating different activities
4	ATLN*	6.8	417.2224	-5 ± 1	93 ± 1	-2 ± 1	93 ± 1	-	-	-3 ± 1	92 ± 1	-
5	SLGGAS	9.6	490.2387	-4 ± 1	93 ± 1	-1 ± 1	92 ± 2	-	-	-4 ± 1	91 ± 1	-
6	VVGQ	10.0	401.2274	-	-	-2 ± 1	87 ± 2	-	-	-	-	Antibacterial
7	DTGLQ	11.6	532.2493	-4 ± 1	88 ± 2	-1 ± 1	89 ± 2	-	-	-	-	-
8	YYGK*	13.1	529.2537	-	-	-	-	-	-	-3 ± 1	91 ± 3	
9	QQGL	15.7	444.2332	-4 ± 1	87 ± 1	-	-	-	-	-	-	-
10	LNVE	18.8	473.2485	-2 ± 1	94 ± 1	-2 ± 1	95 ± 1	-2 ± 2	92 ± 1	1 ± 1	92 ± 2	-
11	ASHPDLN*	19.7	752.3453	-	-	-1 ± 1	94 ± 2	-	-	-	-	-
12	ATYLGS*	19.8	610.2962	-1 ± 1	92 ± 2	-	-	-1 ± 1	91 ± 3	1 ± 0	91 ± 3	-
13	SHPDLN*	19.9	681.3082	-	-	1 ± 1	96 ± 1	-	-	-	-	-
14	APGAGVY*	20.3	633.3122	1 ± 1	93 ± 1	3 ± 0	94 ± 1	-	-	2 ± 1	92 ± 2	Antioxidant
15	LNVEAA	21.1	615.3228	-	-	-	-	-	-	1 ± 1	91 ± 2	-
16	SVGAELE*	21.4	703.3388	-	-	-1 ± 1	96 ± 1	-	-	-	-	-
17	VLDTGLQ	22.8	744.4017	-1 ± 0	95 ± 1	-	-	-	-	-	-	-
18	VSLY*	23.3	480.2584	-2 ± 1	97 ± 1	1 ± 1	97 ± 1	-	-	-1 ± 0	96 ± 1	-
19	VAVL*	23.5	400.2686	-3 ± 1	94 ± 1	-	-	-	-	-1 ± 1	92 ± 1	Antibacterial
20	LDLY	24.9	522.2689	-	-	-	-	-	-	-2 ± 2	93 ± 2	Antibacterial
21	MGDVLNM	27.4	778.3354	-1 ± 1	90 ± 2	-	-	-	-	-1 ± 1	89 ± 3	-
22	LGFL	28.3	448.2686	-	-	-	-	-	-	-6 ± 1	89 ± 2	Membrane -active peptide, antioxidant and ACE inhibitor

*Peptide that could belong to the alcalase enzyme protein sequence.

Table 3. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysate of WPA protein extract from *Codium spp*. using RP-HPLC-ESI-QTOF-MS/MS and *de novo* sequencing tool.

		WPA				
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	NVVDGQPVLN	24.03	1053.5454	-12 ± 3	93 ± 1	-
2	APLDVGVD	24.25	784.3967	-11 ± 2	94 ± 1	-
3	GFGDGL	25.19	564.2543	-11 ± 3	90 ± 1	-
4	LPLVF	30.75	587.3682	-14 ± 1	92 ± 1	-
5	TSFLDL	30.87	694.3538	-12 ± 2	93 ± 1	-
6	FLPLVF	33.60	734.4366	-12 ± 2	94 ± 1	-

As it can be seen in **Figure 5**, the Venn diagram showed eleven common peptides in *S. latissima* and *M. stellatus* (ATLN, SLGGAS, LNVE, ATYLGS, APGAGVY, LNVEAA, SVGAELE, VLDTGLQ, VSLY, VAVL, and MGDVLNM) and non-common peptides with *Codium spp*. (see **Figure 5a** and **Tables 1, 2** and **3**). Twelve peptides found in *S. latissima* and *M. stellatus* (VAGAA, SVGAE, ATLN, YYGK, ASHPDLN, ATYLGS, SHPDLN, APGAGVY, SVGAELE, VSLY, VAVL, SVGAEL) could belong to the alcalase enzyme sequence. Thus, thirty-seven different peptides were found in the three macroalgae being five of them common in *S. latissima* and *M. stellatus* (SLGGAS, LNVE, LNVEAA, VLDTGLQ, and MGDVLNM) (see **Figure 5b**).

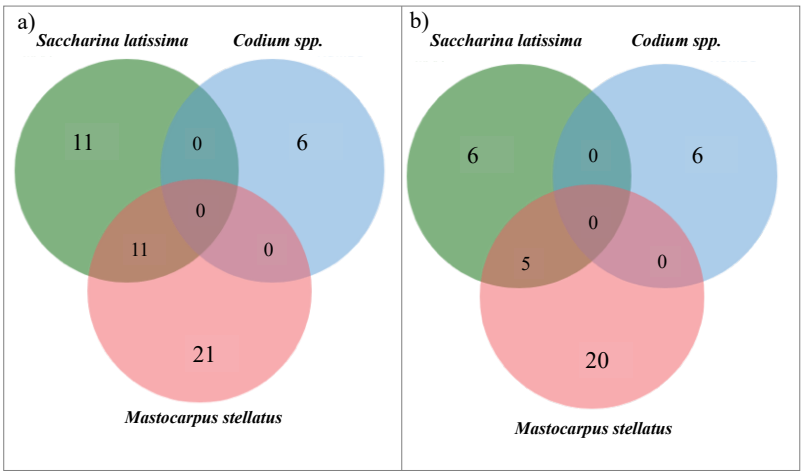

Figure 5. Venn diagram of peptides identified in *Mastocarpus stellatus, Saccharina latissima* and *Codium spp* taking into account the peptides in common with the alcalase enzyme protein sequence (a) and without taking into consideration the peptides in common with the alcalase enzyme protein sequence (b).

The data obtained demonstrated that the highest number of peptides were found in the red macroalga *M. stellatus* (25 peptides), followed by the brown (*S. latissima*) (11 peptides) and green (*Codium spp*.) (6 peptides) macroalgae. Moreover, to the best of our knowledge, this is the first time that these peptides have been found in these macroalgae. The amino acid composition of the identified peptides in these macroalgae had high percentage of hydrophobic amino acids (leucine (L)/isoleucine (I), proline (P) and valine (V)) within their sequences.

In order to know the potential bioactivity of the identified peptides found in these macroalgae, they were verified against BIOPEP database [32]. Several sequences of peptides were found within longer peptides with potential bioactivities (see Tables 1, 2 and 3). For instance, the peptides VLNE, VIAE, VTSL, VVGQ and LDLY were previously found within a longer sequence of antibacterial peptides. However, most of the peptides found in these macroalgae have not previously been reported.

4. Conclusions

An analytical methodology was developed for the first time enabling the separation and identification of short chain peptides from three edible macroalgae, *M. stellatus, S. latissima* and *Codium spp*. The extraction of aqueous and alkaline soluble proteins was achieved followed by their precipitation and enzymatic hydrolysis with alcalase enzyme. Peptide hydrolysates were analyzed by HPLC-MS/MS and *de novo* sequenced using PEAKS software. Thirty-seven peptides were identified in the three macroalgae, being five of them common in *M. stellatus* and *S. latissima*. The peptides identified in these samples were not previously found in macroalgae. After checking against BIOPEP database, several sequenced peptides were found within longer peptides with potential bioactivities mainly with antibacterial properties.

Acknowledgments

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Article 10

High resolution liquid chromatography tandem mass spectrometry for the separation and identification of peptides in coffee silverskin protein hydrolysates.

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Submitted.

Abstract

An analytical methodology was developed for the first time in this work to investigate the peptide composition of coffee silverskin (CS) protein hydrolysates. CS is the only by-product produced in the coffee roasting process and it contains a relatively high amount of proteins (16.2-19.0 %). Different extraction procedures were tested to obtain protein extracts from CS samples which were subsequently submitted to enzymatic digestion using different enzymes. Protein hydrolysates from *Arabica* CS obtained using three roasting degrees (light, medium and dark) were considered in order to evaluate the influence of this process on peptide composition. Antioxidant and hypocholesterolemic activities were investigated for these hydrolysates. A method based on the use of liquid chromatography coupled to a quadrupole-time-of flight mass spectrometer (LC-(QTOF)MS) was developed enabling the separation and identification of different short chain peptides in the CS hydrolysates using *de novo* sequencing tool. Different peptides, with a number of amino acids ranging from 4 to 12, were identified in the CS analyzed. Peptides obtained were different depending on the enzymatic hydrolysis employed. As general trend, the results obtained showed that peptide composition in CS protein hydrolysates was not significantly affected by the coffee roasting process.

1. Introduction

Coffee has an important cultural and economic impact worldwide since it is one of the most consumed beverages in the world. The production of coffee beverage comprises many stages being the roasting of green coffee beans one of the most relevant. After this process, the coffee silverskin (CS) (a thin tegument of the outer layer of the beans which represents about 4.2 % (w/w) of coffee beans) is obtained [1]. This is the only by-product produced during roasting process. The CS chemical composition includes high levels of dietary fiber (50-60 %), being mainly soluble dietary fiber (~85%), carbohydrates (glucose, xylose, galactose, mannose and arabinose), proteins (16.2-19.0 %), fat (1.56-3.28 %) and ash (7 %)[2-8]. Moreover, CS also contains other bioactive compounds such as phenolic compounds (e.g. chlorogenic acids) that together with melanoidins (that are formed as products in the Maillard reaction) are responsible of its antioxidant capacity [1]. Because of this chemical composition, CS can be considered as a natural source of bioactive compounds with beneficial properties for human health [1, 8, 9-13]. Despite the most common application of CS has been as direct fuel, for composting and soil fertilization [1, 14], nowadays, it has been proposed as a new potential functional ingredient for food. In fact, CS has been incorporated to the formulation of flakes,

breads, biscuits and snakes [9], to prepare an antioxidant beverage for body weight control [10], or used in cosmetics as active ingredient to improve skin hydration and firmness [15]. Although CS has been chemically characterized in different works [1, 4], no studies were aimed to investigate its peptide composition in spite of its relatively high content in proteins, as mentioned above.

The bioactivity of many peptides present in different foodstuff and food residues or byproducts includes antimicrobial, ACE inhibitory effect, cholesterol-lowering activity, and antioxidant properties, among others [16-18]. These bioactive capacities may be directly linked with the presence of peptides in these samples or related to peptides originated during enzymatic hydrolysis, food processing or ripening [19]. Usually, the most common bioactive peptides are formed by short amino acid chains (around 2-30 amino acids) [20, 21].

Nowadays, liquid chromatography (LC) coupled to high resolution mass spectrometry (MS) is the preferred analytical technique to carry out the accurate analysis of peptides with a chain longer than 5 amino acids [22]. The detection of shorter peptides (2-4 amino acids) has, on the contrary, some analytical limitations since, for instance, their fragmentation makes their detection complicated [22-24]. Then, the separation and identification of short peptides is a challenge.

The main objectives of this work were: i) to study the peptide composition of protein hydrolysates of CS (*Arabica* variety) and their antioxidant and hypocholesterolemic activities, and ii) to evaluate the effects of submitting coffee beans to different degrees of roasting process on peptide composition. With this aim, a high intensity focused ultrasound probe was employed to extract soluble proteins, which were subsequently precipitated and submitted to enzymatic digestion using different enzymes. A method was developed for the separation and identification of peptides in the CS protein hydrolysates using liquid chromatography coupled to a quadrupole-time-of flight mass spectrometer (LC-QTOF/MS) and *de novo* sequencing tool.

2. Materials and methods

2.1. Chemicals and samples

All chemicals and reagents used in this work were of analytical grade. Sodium hydroxide**,** thermolysin enzyme, pepsin and pancreatin digestive enzymes, bovine serum albumin (BSA), oleic acid, phosphatidylcholine, taurocholate, cholesterol, sodium chloride (NaCl), L-glutathione (GSH), β-mercaptoethanol, o-phthalaldehyde (OPA), dithiothreitol (DTT), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulphate, 1,10-phenantroline, ferrous

sulphate, hydrogen peroxide, and potassium ferricyanide were purchased from Sigma-Aldrich (Steinheim, Germany). Alcalase 2.4 L FG and Flavourzyme 1000 L were generously donated by Novozymes Spain S.A (Madrid, Spain). Hydrochloric acid, acetone, hexane, methanol, ethanol and acetic acid were acquired in Scharlau (Barcelona, Spain). Tris-(hydroxymethyl)-aminomethane (Tris), sodium dihydrogen phosphate, disodium tetraborate, and sodium dodecyl sulfate (SDS) were purchased from Merck (Darmstadt, Germany). Cholesterol oxidase kit was from BioAssay Systems (Hayward, CA, USA). Miniprotean precast gels, Laemmli buffer, Tris/glycine/SDS running buffer, precision plus protein standards (recombinant proteins expressed by Escherichia coli with molecular weights of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa), silver stain kit, and Bradford reagent (Coomassie Blue G-250) were acquired from Bio-Rad (Hercules, CA, USA).

For the HPLC-MS/MS analysis, MS grade acetonitrile (ACN) and acetic acid from Sigma-Aldrich (Steinheim, Germany) were employed. The ultrapure water used was obtained from a Milli-Q (Millipore, Bedford, MA, USA) instrument.

Different coffee silverskin samples from *Arabica* coffee variety were provided by "Café Fortaleza" (Vitoria, Spain). These samples were obtained by roasting green coffee beans at three different roasting levels: light level (LCS) using a roasting temperature of 175 ^{o}C during 12.36 min; medium level (MCS) employing 185 ^{o}C during 14.11 min; dark level (DCS) by roasting the green coffee beans at 195 ^{o}C during 17.06 min.

2.2. Total protein content

The protein content of CS samples was determined by the Kjeldahl method [25]. Analyses were performed in triplicate. Nitrogen data were converted into protein values using a conversion factor of 5.3 and were expressed as g per 100 g of dried coffee silverskin.

2.3. Protein extraction from coffee silverskin

Proteins from coffee silveskin were extracted following a procedure previously described in the literature with different modifications [26]. Briefly, 0.5 g of grounded coffee silverskin were defatted three times with 20 mL of hexane (to avoid interferences in the extraction). Then, 50 mg of proteins were extracted with 5 mL of 100 mM Tris-HCl buffer (pH 7.5) containing 0.25 % (w/v) SDS and 0.25 % (w/v) DTT using a high intensity focused ultrasound probe (HIFU) (model VCX130 from Sonics Vibra-Cell, Hartford, CT, USA) for 10 min at 50 % of amplitude. After centrifugation, the proteins were precipitated using 10 mL of cold acetone and left

at 4 ºC overnight. Precipitated proteins were centrifuged (4000 × g, 10 min, 25 ºC) and dissolved in the appropriate buffer for their digestion.

2.4. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis with a Bio-Rad Mini-protean system (Hercules, CA, USA) was performed to achieve the separation of coffee silverskin proteins. Proteins solutions were mixed with Laemmli buffer containing 5 % (v/v) β-mercaptoethanol, followed by heating at 100 °C during 5 min and loaded into commercial ready precast gels. Then, Tris/glycine/SDS was used as running buffer and protein separation was carried out by applying 80 V for 5 min and 150 V until performing the separation. Proteins standard solution with molecular weights from 10 to 100 kDa were also loaded into the gel. After separation, the gel was treated first with a fixing solution of water/MeOH/acetic acid (50/40/10 % (v/v)) by shaking for 30 min and then with water/EtOH/acetic acid (85/10/5 % (v/v)) two times (for 15 min each one). Then, an oxidizer solution was added to the gel during 5 min followed by the addition of the silver reagent during 20 min. Finally, the gel was washed with water before adding the developer solution, and the reaction was stopped adding 5 % acetic acid solution.

2.5. Protein digestion

Protein extracts obtained from CS were hydrolysed using different enzymes. CS proteins were dissolved in the corresponding digestion buffer at a final concentration of 5 mg/mL with the help of an ultrasonic probe for 5-10 min and with 30 % of wave amplitude. 5 mM borate buffer (pH = 8.5), 5 mM phosphate buffer (pH = 8.0) or acid water solution (pH 2.0) were employed as buffers for alcalase, thermolysin or simulated gastrointestinal digestion (using pepsin and pancreatin), respectively. After the addition of the appropriate amount of enzyme to the protein extract solution, it was incubated in a Thermomixer Compact (Eppendorf, Hamburg, Germany) at 50 °C (for alcalase, and thermolysin digestion) or 37 °C (for gastrointestinal digestion) with shaking at 700 rpm. The digestion was stopped by increasing the temperature to 100 °C for 10 min. After centrifugation (6000 g for 10 min, 24 ºC), the supernatant was collected for further analyses.

2.6. O-phthalaldehyde (OPA) assay

Peptide content was measured using the OPA assay following the procedure described by Wang et al. (2008) with some modifications [27]. A 40 mg/mL solution

of OPA reagent in MeOH was employed to prepare daily a mixture of 2.5 mL of disodium tetraborate (100 mM), 1.0 mL of SDS (5 % (v/v)), 1.39 mL of water, 10 µL of β-mercaptoethanol, and 100 µL of OPA solution. This mixture was employed to incubate the CS protein hydrolysates during 8 min at room temperature. Then, the absorbance was measured at 340 nm using a spectrophotometer Cary 8454 from Agilent Technologies (Santa Clara, USA). All analyses were performed in triplicate.

2.7. *In vitro* bioactivity assays

In vitro antioxidant capacity of CS protein hydrolysates was measured using two different assays evaluating the ability to scavenge free radicals: ABTS and hydroxyl radicals scavenging. Samples were prepared in triplicate and measured in duplicate. The assays were carried out following the procedures described by González-García et al [26]. The antioxidant capacity was expressed as percentage of inhibition.

In vitro hypolipidemic activity was evaluated measuring the peptides ability to reduce the absorption of dietary cholesterol (ability to reduce micellar cholesterol solubility). The assay was carried out following the procedure described by Prados et al [28]. The reduction in the micellar solubility of cholesterol was calculated using the following equation:

$$\text{Cholesterol solubility reduction (\%)} = ((C0 - CS)/C0) * 100$$

In this equation C_0 is referred to the initial concentration of cholesterol in micelles (without peptides) and Cs is referred to the concentration of cholesterol in micelles after adding peptides.

Both *in vitro* antioxidant capacity and *in vitro* hypolipidemic activity were evaluated preparing the CS protein hydrolysates in triplicate and measuring them in duplicate.

2.8. Peptide analysis by LC-(QTOF)MS

A HPLC system 1100 from Agilent (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole-time-of flight mass spectrometer (QTOF/MS) Agilent 6530 equipped with an orthogonal electrospray ionization (ESI) source (Agilent Jet Stream, AJS) was employed to carry out peptide analysis. MS control, data acquisition, and data analysis were performed using Agilent Mass Hunter Workstation software B.07.00 from Agilent Technologies. An Ascentis Express Peptide ES-C18 analytical column (100 x 2.1 mm, particle size 2.7 µm) with an Ascentis Express Peptide ES-C18 guard column (0.5 cm × 2.1 mm, 2.7 µm particle

size) from Supelco (Bellefonte, Pa, USA) were employed to carry out the chromatographic separation. The column temperature was 25 ºC and the flow rate 0.3 µL/min. Ten µL of extract were injected. The mobile phases consisted of (A) water with 0.3 % acetic acid and (B) ACN with 0.3 % acetic acid in a gradient elution analysis programmed as follows: 0 min, 5 % (B); 0-3 min, 5 % (B); 5-40 min, 5-40 % (B); 40-43 min, 95 % (B), 43-45 min, 95 % (B) with 15 min of post-time.

The MS analyses were carried out using positive ionization mode (3500 V) and masses ranged from 100 to 1700 m/z. Nebulizer pressure was set at 50 psig and the drying gas flow rate was fixed to 12 L/min and 350 ºC. The sheath gas flow was 12 L/min at 400 ºC. 80 V was chosen for the fragmentor voltage (cone voltage after capillary), whereas the skimmer and octapole voltage were 60 V and 750 V, respectively. MS/MS analyses were performed employing the auto MS/MS mode using 1 precursor per cycle, dynamic exclusion after two spectra (released after 1 min), and collision energy of 5 V for every 100 Da. Internal mass calibration in positive ionization mode was performed using a reference compound solution from Agilent Technologies containing the ions *m/z* 121.0508 ($C_5H_4N_4$) and 922.0097 ($C_{18}H_{18}O_6N_3P_3F_{24}$). This solution was continuously pumped into the ionization source at a 15 µL/min flow rate using a 25 mL Gastight 1000 Series Hamilton syringe (Hamilton Robotics, Bonaduz, Switzerland) on a NE-3000 pump (New Era Pump Systems Inc., Farmingdale, NY, USA). The analyses were conducted in triplicate.

2.9. Peptide identification by de novo sequencing

Three replicates of CS protein hydrolysates were injected in triplicate in the MS system. Blank samples containing the appropriate buffer solution instead of CS protein hydrolysates were also analyzed. MS/MS spectra were obtained for the most abundant molecular ion and analyzed using the PEAKS Studio Version 7 software (Bioinformatics Solutions Inc., Waterloo, Canada) in order to identify peptides from coffee silverskin proteins. *De novo* sequencing tool was employed to carry out data analysis. The results were refined applying a certain average local confidence (ALC). Those peptides identified with an ALC (expected percentage of correct amino acids in the peptide sequence) above 80 % and with a good precursor fragmentation pattern were considered for further interpretation. In addition, only those peptides appearing in at least 5 from 9 injections (three injections of each triplicate) from some of the analyzed CS samples were considered. Since in the MS system employed in this work was not possible to differentiate isoleucine (I) from leucine (L), only isoforms with L are presented in these results.

3. Results and discussion

3.1. Extraction of proteins

The protein contents of the three CS obtained after roasting green coffee beans at different levels were 12.0 ± 0.1 %, 11.9 ± 0.1 %, and 12.0 ± 0.4 % for light CS (LCS), medium CS (MCS), and dark CS (DCS), respectively (all percentages referred to sample dry weight). Considering that the protein content was very similar in all CS samples, MCS was chosen as model sample to evaluate different protein extraction procedures.

First, a solid-liquid extraction using acetonitrile::water (20:80 v/v) as extracting solvent was performed using shaking (5 min) and sonication (3 min). Under these conditions, the percentage of proteins extracted from the MCS sample was lower than 1 % (protein content obtained by Bradford assay). A subcritical water extraction procedure was also tested following the procedure previously described by Yusaku Narita et al., [12] with some modifications. This protocol was based on the use subcritical water as extraction solvent at two different temperatures, 120ºC and 180ºC. In both cases, the protein content of the MSC extract was lower than 1 %. Then, a method previously reported by our research group to extract proteins from olive seeds was investigated [29]. The methodology consisted of the extraction of proteins with 100 mM Tris-HCl buffer (pH 7.5) containing 0.5 % (w/v) SDS and 0.5 % (w/v) DTT using HIFU for 5 min at 30 % amplitude and followed by the protein precipitation with cold acetone overnight. Under these conditions, it was possible to obtain an extract which protein content was 2.6 ± 0.3 %. In order to increase the amount of proteins extracted from the MSC sample by this extraction procedure, an optimization of different extraction conditions, such as extracting buffer composition, HIFU probe conditions, and solvent/sample ratio was performed.

First, buffer composition was evaluated considering two buffers of different nature (100 mM Tris-HCl and 100 mM phosphate) at three different pH values (6.5, 7.5 and 8.5) keeping constant the amount of SDS and DTT as well as the HIFU conditions described above. As it can be seen in **Table 1**, the Tris-HCl buffer enabled to achieve a protein content five times higher than those obtained using phosphate buffer. In addition, the intermediate pH value allowed obtaining a protein content slightly higher than the other pH values tested. Therefore, 100 mM Tris-HCl buffer at pH 7.5 was chosen for further experiments. Protein denaturation is highly affected by SDS and DTT so that their presence in the extraction solvent could affect protein extraction.

The effect of the concentration of both denaturing agents in the extraction media was studied by varying first the amount of SDS between 0.0 and 0.5 % (w/v), and later the amount of DTT between 0.25 and 1.0 % (w/v). Bearing in mind the percentage of protein extracted under these conditions (see **Table 1**) and trying to use the lowest concentration of DTT and SDS to avoid interferences in the Bradford assay, percentages of 0.25 % (w/v) of SDS and 0.25 % (w/v) of DTT in 100 mM Tris-HCl buffer at pH 7.5 were chosen for further experiments. In this step, the possibility of adding also urea to the extraction solvent was also tested; however, it was discarded because the protein content was 1.8 ± 0.1 % when a concentration of 1 M urea was added to the Tris-HCl buffer and interferences in the Bradford assay were observed when a 4 M concentration of urea was employed.

Table 1. Evaluation of different CS protein extraction conditions using HIFU.

	Extraction conditions	**Protein extract (%) (Average ± SD)**
	Extractant composition	
Buffer nature and pH	100 mM Tris-HCl pH 6.5	2.0 ± 0.4
	100 mM Tris-HCl pH 7.5	2.6 ± 0.3
	100 mM Tris-HCl pH 8.5	2.3 ± 0.6
	100 mM PB pH 6.5	0.4 ± 0.2
	100 mM PB pH 7.5	0.5 ± 0.4
	100 mM PB pH 8.5	0.5 ± 0.7
% SDS	0 % SDS	0.8 ± 0.5
	0.25 % SDS	2.8 ± 0.4
	0.5 % SDS	2.6 ± 0.3
% DTT	**0.25 % DTT**	2.9 ± 0.3
	0.5 % DTT	2.8 ± 0.4
	1 % DTT	2.3 ± 0.5
Urea	1 M Urea	1.8 ± 0.1
	4 M Urea	Interferences Bradford assay
	HIFU conditions	
Extraction time	3 min	3.3 ± 0.3
	5 min	2.9 ± 0.3
	10 min	3.4 ± 0.4
	15 min	3.2 ± 0.1
Amplitude	20%	2.9 ± 0.2
	30%	2.9 ± 0.3
	50%	3.9 ± 0.5
	Solvent/sample ratio	
5 mL extractant	**50 mg sample extracted (1:10)**	3.9 ± 0.5
	100 mg sample extracted (1:20)	3.0 ± 0.3
	200 mg sample extracted (1:40)	3.3 ± 0.3
10 mL extractant	200 mg sample extracted (1:20)	3.3 ± 0.4
	400 mg sample extracted (1:40)	3.3 ± 0.2

The next step was to investigate the influence of the extraction time and amplitude of the HIFU probe on the percentage of proteins extracted from the MSC sample. Extraction time was tested in the range from 3 to 15 min whereas the amplitude was in the range from 20 to 50 %. As **Table 1** shows, the estimation of the protein content showed better results when 50 % amplitude during 10 min was employed. Under these optimized conditions, it was possible to extract 3.9 ± 0.5 % of proteins. The possibility to increase the amount of proteins extracted using two cycles of the extraction procedure was evaluated. However, it did not improve the value obtained using just one cycle.

Using the optimal extraction conditions, the effect of varying the ratio between extraction solvent and sample amount was also studied. Initially, 50 mg of sample were extracted with 5 mL of extracting solution (ratio 1:10). Then, the sample amount was increased up to 100 mg or 200 mg using the same volume of extracting solution (ratio 1:20 and 1:40, respectively). In both cases, a lower percentage of protein content (see **Table 1**) was observed compared to that obtained using a 1:10 ratio. An increase in the volume of the extraction solvent from 5 mL to 10 mL was evaluated keeping ratios of 1:20 and 1:40, but this increase had no effect on the protein content. Finally, trying to avoid interferences from other compounds, a cleaning procedure based on washing the MCS sample two times with methanol:water (80:20 % (v/v)) followed by washing with acetone:water (80:20 % (v/v)) was carried out before protein extraction. However, this clean up procedure did not allow to improve the protein content extracted from the MCS sample.

Once selected the best extraction conditions to obtain the highest protein content from CS samples, a SDS-PAGE analysis was carried out to evaluate if there were differences in the protein profile of the different CS samples (LCS, MCS, and DCS). **Figure S1** (see supplementary data) demonstrated that the electrophoretic profiles of the three different samples showed similar bands; an intense band between 15 and 20 kD and bands with molecular masses higher than 100 kD.

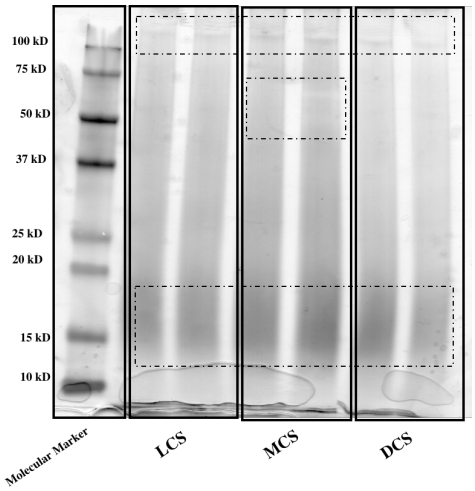

Figure S1. SDS-PAGE gels corresponding to the protein extracts obtained for LCS, MCS and DCS.

3.2. Evaluation of the bioactive capacity of CS protein hydrolysates

Peptides from the protein extracts of CS samples submitted to different roasting process were obtained by enzymatic digestion employing alcalase, themolysin and a simulated gastrointestinal digestion with pepsin and pancreatin enzymes. Then, CS protein hydrolysates were evaluated in terms of peptide content and bioactive capacity.

Figure 1A shows the hydrolysis degree obtained for LCS, MCS and DCS when alcalase, thermolysin and simulated gastrointestinal digestion were used to hydrolyse the proteins extracted from each sample. There were not significant differences in the hydrolysis degree obtained for CS samples when different enzymes were used in the protein digestion. Regarding the bioactivity of all CS protein hydrolysates, it was measured in terms of antioxidant activity (using ABTS and hydroxyl radical scavenging assays) and as the capacity of peptides to reduce micellar cholesterol solubility. In general, all protein hydrolysates exhibited antioxidant capacity with percentages ranging from 9 %, obtained for MCS submitted to simulated gastrointestinal digestion, to 35 %, obtained for DCS submitted to enzymatic digestion with thermolysin (see **Figures 1B** and **1C**). As **Figures 1A** and 1**B** show, when hydroxyl radical scavenging assays were performed, thermolysin was the enzyme yielding the highest antioxidant capacity whereas ABTS assay showed similar antioxidant capacity results for the three enzymes studied. Regarding the capacity to reduce micellar cholesterol solubility, it ranged from 25 to 32 % as it can be seen in **Figure 1D**, reaching the maximum

cholesterol-lowering activities when thermolysin was employed to hydrolyse the proteins from the three CS samples.

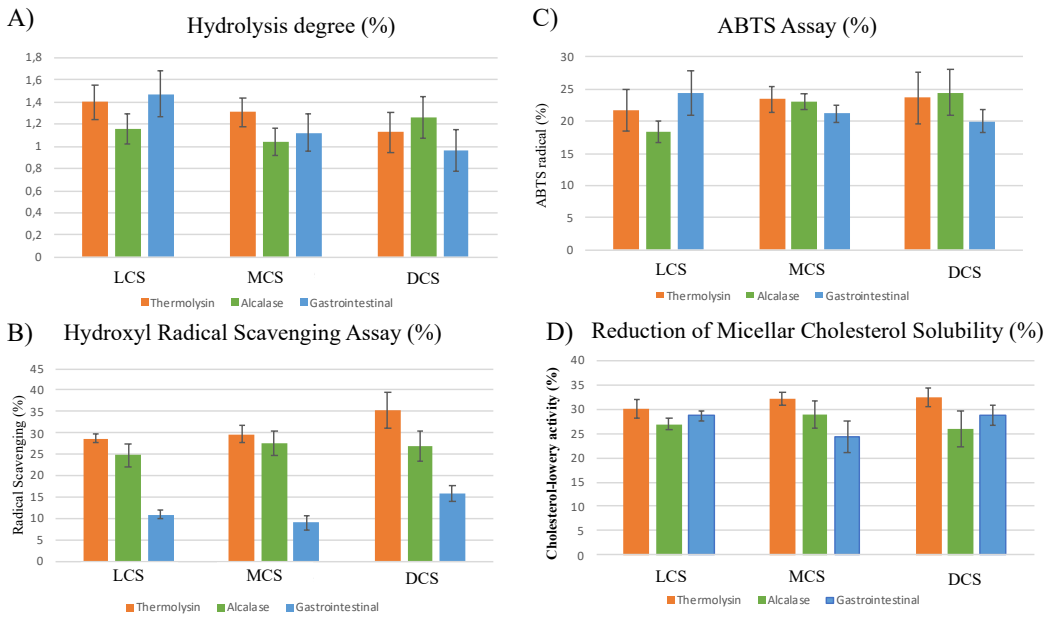

Figure 1. Hydrolysis degree (A), antioxidant capacity evaluated by two different antioxidant assays (B and C), and the capacity to reduce micellar cholesterol solubility (D) of the protein hydrolysates obtained using three different enzymatic digestions from LCS, MCS and DCS.

3.3. Peptide analysis by LC-(QTOF)MS and de novo identification

A reversed phase LC-(QTOF)MS analytical methodology was developed enabling the separation and identification of peptides in CS protein hydrolysates. To select the most appropriate chromatographic and MS/MS parameters, protein hydrolysates of LCS, MCS and DCS obtained using thermolysin were chosen as model samples. On the one hand, the effect of chromatographic parameters such as gradient program, column temperature (25, 35, 55 °C), and injection volume (5, 10 and 15 µL) was evaluated in terms of chromatographic resolution and analysis time. First, the gradient program was optimized in order to achieve a good chromatographic separation using as mobile phases water with 0.3 % acetic acid (solvent A) and acetonitrile with 0.3 % acetic acid (solvent B) at a flow rate of 0.2 mL/min, a column temperature of 25ºC and an injection volume of 5 µL. A gradient consisting of 5 % (B) at 0 min, followed by 5 % (B) at 0-3 min, 5-40 % (B) from 5 to 40 min, then 95 % (B) from 40 to 43 min and it was kept during 2 min, and finally 15 min of post-time, gave the best results. Once the gradient was optimized, the column temperature was evaluated keeping constant the flow rate and the injection

volume. The lowest value of the temperature tested (25 ^{o}C) was selected since it allowed to obtain the highest number of peptides. Then, the flow rate was varied from 0.1 mL/min to 0.3 mL/min showing that the best results were obtained for a value of 0.3 mL/min. Finally, the effect of the injection volume was also studied when injection volumes of 5, 10 and 15 µL were employed, being the injection of 10 µL which allowed to detect a higher number of peptides.

The MS/MS parameters were selected considering those previously employed by our research team [30] to carry out the identification of peptides from food by-products. Modifying the mass range from 100 to 1700 m/z instead to 1500 m/z, the collision energy was varied from 3 to 6V/100 Da, being 5V/100 Da the value that enabled to detect a high number of peptides.

Under the best LC-MS/MS conditions, protein hydrolysates from LCS, MCS, and DCS were analyzed. By using *de novo* sequencing tool from the PEAK Software, peptides in each CS hydrolysate were tentatively identified. **Figure 2** shows the total ion chromatogram corresponding to the protein hydrolysates from MCS extract obtained after thermolysin digestion and an example of the mass spectrum for LLYQ peptide present in this sample.

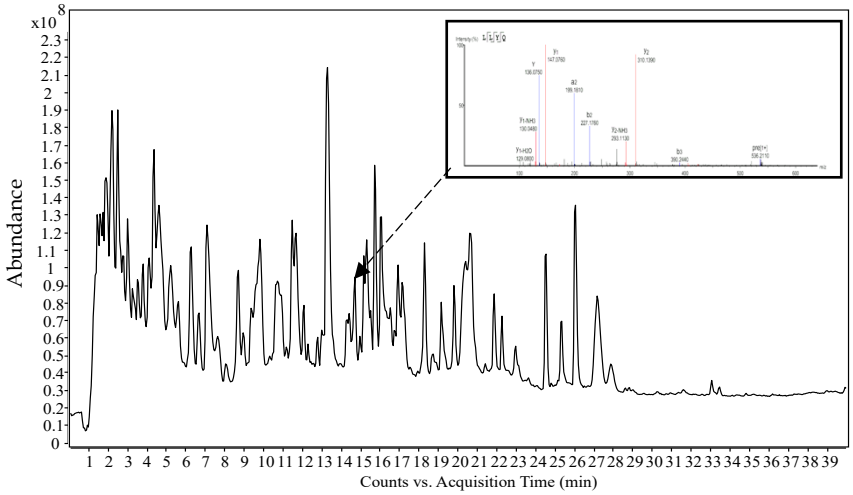

Figure 2. Total ion chromatogram from MCS protein hydrolysate using thermolysin by LC-(QTOF)MS and an example of MS/MS spectrum of the peptide LLYQ observed at 14.5 min (molecular mass: 535.3060 Da).

Tables 1, 2 and **3** summarize the peptides identified by MS/MS after the three enzymatic digestions employed (thermolysin, alcalase and simulated gastrointestinal digestion), along with their experimental molecular masses, ALC and accuracy. It should be mentioned that only isoforms with leucine (L) are presented in these results, although peptide sequences containing isoleucine (I)

instead of L are also possible (it is not possible to differentiate I from L by the MS system used). As can be seen in these tables (see also **Figure S2**), different peptides with a number of amino acids ranging from 4 to 12 were identified. After excluding those peptides that could correspond to the enzymes employed in the hydrolysis (marked in the tables), thermolysin hydrolysates were those presenting a higher number of peptides (33), followed by gastrointestinal hydrolysates (11) and alcalase hydrolysates (7). As **Figure 2S** shows, no common peptides were found for the three different hydrolysis procedures.

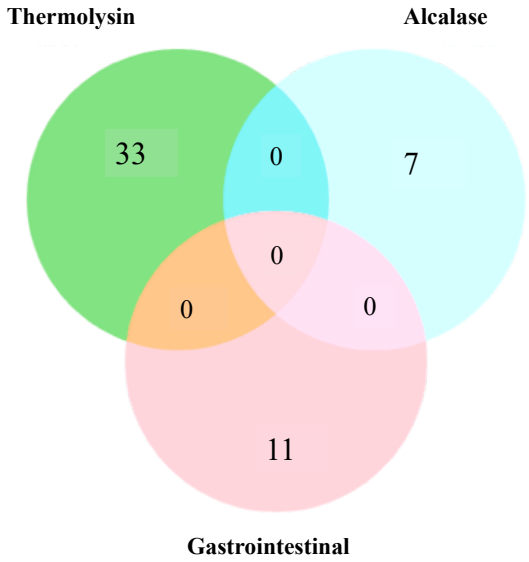

Figure S2. Venn diagrams of the total number of peptides obtained for LCS, MCS and DCS using three different enzymes.

The results obtained for the bioactive capacity of each hydrolysate showed that thermolysin was the best option, and these results were supported with peptide identification. As general trend, the results obtained showed that the peptide composition in CS protein hydrolysates was not affected by the coffee roasting process since most of the peptides identified were present in the three CS samples. For instance, using thermolysin or alcalase to hydrolyse the proteins from LCS, MCS and DCS extracts, just two and three peptides for each enzyme (LSGGLD, TTLPGS, for thermolysin, and AVPLLK, VAPLLK, ALLL for alcalase) showed differences along the roasting process. In the case of using a simulated gastrointestinal digestion, the peptides seemed to be more influenced by the roasting since five peptides identified in these CS protein hydrolysates (ALVGGTN, QVGGL, LGGLDSS, LGTVV, MMDPLA) were not present in at least one of the roasting degrees.

Table 2. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the thermolysin hydrolysates of LCS, MCS and DCS protein extracts using LC-MS/MS and *de novo* sequencing tool.

				LCS		MCS		DCS		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	LLNK[c]	1.6	486.3275	6 ± 2	93 ± 1	8 ± 1	95 ± 1	8 ± 2	96 ± 1	Antibacterial
2	AVGVK*[c]	1.71	472.3009	6 ± 4	91 ± 1	6 ± 3	91 ± 0	2 ± 4	83 ± 0	ACE-inhibitor
3	LLNAK	2.11	557.3537	4 ± 3	82 ± 1	7 ± 1	83 ± 1	8 ± 1	83 ± 1	-
4	VLGDQKN*	2.21	772.4079	3 ± 2	91 ± 0	5 ± 1	81 ± 30	6 ± 2	91 ± 0	-
5	LLSQ	3.7	459.2771	2 ± 2	94 ± 2	1 ± 1	94 ± 1	2 ± 1	95 ± 0	Haemolytic (ILSQ), Immunomodulating ((IISQ)
6	VTYD	3.75	496.2169	2 ± 2	83 ± 1	2 ± 1	83 ± 2	3 ± 2	83 ± 2	-
7	VYGDDGGQT	4.32	910.3668	2 ± 1	81 ± 1	1 ± 1	82 ± 1	2 ± 1	82 ± 1	-
8	VTDYT*	4.39	597.2646	2 ± 2	86 ± 1	1 ± 1	89 ± 1	2 ± 1	89 ± 1	-
9	LYGSTS*	5.02	626.2911	0.1 ± 3	88 ± 1	1 ± 1	87 ± 1	1 ± 1	86 ± 2	-
10	LSQGGTHYG	5.12	918.4196	2 ± 2	84 ± 1	1 ± 1	84 ± 2	1 ± 1	83 ± 2	-
11	YDAAP	5.3	535.2278	1 ± 3	82 ± 4	1 ± 1	82 ± 3	2 ± 1	82 ± 4	-
12	LYGST*	5.58	539.2591	2 ± 2	89 ± 1	1 ± 1	89 ± 1	2 ± 0	89 ± 1	-
13	YDAKTYR	5.68	915.4450	0.3 ± 3	85 ± 3	1 ± 1	85 ± 2	1 ± 1	84 ± 3	-
14	LTQY*[c]	6.96	523.2642	2 ± 2	82 ± 1	1 ± 1	82 ± 1	1 ± 2	82 ± 1	-
15	YSTY*[b]	7.31	532.2169	2 ± 2	88 ± 2	0 ± 1	85 ± 3	1 ± 2	88 ± 4	-
16	LYGSTSQE*	8.6	883.3923	2 ± 2	93 ± 2	1 ± 1	94 ± 1	2 ± 1	94 ± 1	-
17	LSGGLD[c]	8.7	561.2882	0.2 ± 2	88 ± 1	-	-	2 ± 3	87 ± 2	-
18	LSYDGNN*	9.73	781.3242	3 ± 2	91 ± 1	3 ± 1	90 ± 1	2 ± 1	91 ± 1	-
19	TTLPGS[a]	9.8	575.3036	1 ± 4	83 ± 2	-	-	1 ± 4	85 ± 2	-
20	SLGDSLSR	9.85	833.4243	2 ± 2	85 ± 2	2 ± 1	85 ± 1	1 ± 1	85 ± 1	-
21	LSGDSSLR (z=2)	9.96	833.4243	2 ± 2	87 ± 2	1 ± 1	87 ± 2	2 ± 1	86 ± 1	-
22	TYSTY*	10.14	633.2646	2 ± 3	89 ± 1	2 ± 2	88 ± 2	3 ± 2	88 ± 2	-
23	VHYSQGYNNA*	10.72	1151.4995	2 ± 3	82 ± 1	1 ± 1	83 ± 1	1 ± 1	82 ± 1	-

Table 2. Continued

ID	Peptide sequence	RT (min)	Molecular mass (Da)	LCS		MCS		DCS		Activity (BIOPEP database)
				Mass accuracy (ppm)	ALC (%)	Mass accuracy (ppm)	ALC (%)	Mass accuracy (ppm)	ALC (%)	
24	ANKNPDWE* [b] (z=2)	10.8	973.4363	2 ± 3	89 ± 2	4 ± 2	89 ± 3	1 ± 1	89 ± 1	-
25	LNTTY	11.5	610.2963	2 ± 2	80 ± 0.5	2 ± 1	80 ± 1	1 ± 1	81 ± 1	-
26	MSDPAYK [c]	11.85	810.3582	3 ± 2	86 ± 2	3 ± 1	87 ± 3	3 ± 2	84 ± 3	-
27	LDVVAHE	12.89	718.3970	3 ± 2	93 ± 1	4 ± 1	93 ± 1	3 ± 2	94 ± 1	-
28	FASY*	12.97	486.2144	2 ± 2	96 ± 1	2 ± 1	96 ± 1	3 ± 2	96 ± 0	ACE-inhibitor
29	LLSQGGTHYG	13.25	1031.5037	3 ± 2	89 ± 1	3 ± 1	88 ± 2	3 ± 1	88 ± 2	-
30	LNEAL	14.24	558.3013	3 ± 2	87 ± 1	3 ± 1	88 ± 1	3 ± 2	88 ± 1	-
31	LLYQ [a]	14.5	535.3060	2 ± 2	89 ± 2	5 ± 2	88 ± 4	4 ± 2	89 ± 2	ACE-inhibitor, Immunomodulating
32	FDAVGVK*	14.67	734.3962	3 ± 2	95 ± 0.4	3 ± 1	95 ± 0.3	3 ± 1	95 ± 0	ACE-inhibitor
33	VTYDY*	15.17	659.2802	3 ± 2	93 ± 1	4 ± 1	92 ± 1	3 ± 2	92 ± 1	-
34	LNSGLLNKA [c]	15.6	928.5380	5 ± 2	85 ± 1	4 ± 0	86 ± 1	1 ± 3	86 ± 0	-
35	LNSGLLNAK [a, c]	15.6	928.5376	6 ± 1	87 ± 1	5 ± 1	87 ± 1	1 ± 3	86 ± 4	-
36	VTYDYYKN*(z=2)	15.8	1064.4814	3 ± 3	94 ± 0.4	4 ± 2	94 ± 1	3 ± 2	94 ± 0	-
37	LWAD*	15.95	503.2380	4 ± 3	82 ± 2	5 ± 1	82 ± 2	4 ± 2	83 ± 2	-
38	AFDAVGVK*	16.58	805.4333	4 ± 3	96 ± 0.5	5 ± 1	96 ± 0	4 ± 2	95 ± 1	ACE-inhibitor
39	VGPF [a]	18.6	418.2260	6 ± 2	82 ± 1	9 ± 1	84 ± 2	6 ± 2	86 ± 2	-
40	ELLPQ	18.75	598.3326	4 ± 3	85 ± 1	6 ± 3	87 ± 4	4 ± 3	86 ± 3	-
41	FASYDAPAVDAH [a,c]	18.8	631.2829	3 ± 3	83 ± 1	6 ± 2	83 ± 1	4 ± 2	84 ± 1	-
42	LFTYD	19.25	657.3010	3 ± 2	91 ± 1	4 ± 2	91 ± 1	3 ± 2	91 ± 1	-
43	VEFY* [b]	19.9	576.8228	3 ± 3	87 ± 1	-	-	3 ± 1	88 ± 0	-
44	LAPLP [c]	19.96	509.3213	5 ± 3	92 ± 1	6 ± 2	92 ± 1	6 ± 1	88 ± 6	-
45	LSGGLDVVAHE [a,c]	20.2	1045.7616	3 ± 5	89 ± 9	3 ± 1	93 ± 1	4 ± 2	92 ± 1	-
46	LSNLDVVAHE	20.37	1095.5559	3 ± 2	90 ± 3	3 ± 1	89 ± 3	3 ± 3	90 ± 3	-
47	LFTY	20.63	542.2740	3 ± 2	90 ± 1	3 ± 2	89 ± 1	2 ± 2	89 ± 0	-
48	LVEF*	21.84	506.2740	3 ± 3	90 ± 1	4 ± 2	90 ± 1	3 ± 2	90 ± 1	-
49	FWNGSQM* [a,c]	22.2	868.3576	3 ± 5	82 ± 1	4 ± 1	82 ± 2	-	-	-
50	FGLSDLT	24.5	751.3752	2 ± 4	83 ± 0	3 ± 2	83 ± 1	2 ± 1	83 ± 1	-

Table 2. Continued

ID	Peptide sequence	RT (min)	LCS Molecular mass (Da)	MCS Mass accuracy (ppm)	MCS ALC (%)	DCS Mass accuracy (ppm)	DCS ALC (%)	LCS Mass accuracy (ppm)	LCS ALC (%)	LCS Activity (BIOPEP database)
51	LFGTL	24.8	549.3163	3 ± 3	90 ± 1	4 ± 1	90 ± 1	2 ± 2	91 ± 1	-
52	LVEFY*	25.26	669.3373	0 ± 3	89 ± 1	2 ± 1	89 ± 1	0 ± 3	90 ± 1	-
53	VLEFY [b,c]	25.3	669.3432	2 ± 3	81 ± 1	2 ± 1	81 ± 1	2 ± 2	81 ± 1	-
54	EAPWL	25.52	614.3064	4 ± 3	87 ± 1	5 ± 2	88 ± 2	6 ± 2	86 ± 4	-
55	DVLPW	28.99	628.3220	4 ± 3	84 ± 2	4 ± 3	85 ± 5	3 ± 2	84 ± 2	-

* Peptides that could belong to the alcalase enzyme protein sequence.
a Peptides that were present in < 5 injections of LCS
b Peptides that were present in < 5 injections of MCS
c Peptides that were present in < 5 injections of DCS

Table 3. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysates of LCS, MCS and DCS protein extracts using LC-MS/MS and *de novo* sequencing tool.

				LCS		MCS		DCS		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	VLVR	3.5	485.3326	0.1 ± 3	88 ± 2	0.3 ± 3	87 ± 2	4 ± 3	88 ± 2	-
2	LNVE	7.1	473.2485	0.4 ± 4	88 ± 3	0.3 ± 2	88 ± 4	5 ± 2	89 ± 3	-
3	APGAGVY*[a,b]	12.9	633.3122	3 ± 3	82 ± 2	2 ± 2	86 ± 1	4 ± 1	85 ± 2	Antioxidant
4	SVGAELE*[b,c]	14.2	703.3388	0.5 ± 2	87 ± 4	2 ± 0	89 ± 3	5 ± 1	83 ± 2	-
5	SFYYGK*[a,c]	15.6	763.3541	1 ± 2	85 ± 3	4 ± 2	88 ± 1	-	-	-
6	VVDL[a,b]	15.8	444.2584	1 ± 3	90 ± 3	2 ± 0	93 ± 2	7 ± 2	85 ± 3	-
7	VSLY*	17.7	480.2584	1 ± 3	92 ± 2	2 ± 2	93 ± 1	4 ± 3	91 ± 1	-
8	LVAL	19.1	414.2842	5 ± 3	90 ± 3	5 ± 3	90 ± 3	6 ± 2	90 ± 3	-
9	AVPLLK[b]	22.0	639.4319	0.4 ± 1	83 ± 4	1 ± 2	81 ± 2	-	-	-
10	VAPLLK[a,c]	22.1	639.4319	-	-	3 ± 2	83 ± 6	5 ± 2	82 ± 2	-
11	ALLL[a,b]	25.0	428.2999	-	-	4 ± 2	83 ± 3	6 ± 2	85 ± 6	celiac toxic (AIIL)

* Peptides that could belong to the alcalase enzyme protein sequence.
a Peptides that were present in < 5 injections of LCS
b Peptides that were present in < 5 injections of MCS
c Peptides that were present in < 5 injections of DCS

Table 4. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the gastrointestinal hydrolysate of LCS, MCS and DCS protein extracts using LC-MS/MS and *de novo* sequencing tool.

				LCS		MCS		DCS		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	WDAN[a]	3.6	505.2012	5 ± 4	81 ± 1	6 ± 1	81 ± 1	6 ± 2	82 ± 1	-
2	SSGEL*[a]	3.7	492.2265	-	-	7 ± 1	88 ± 4	7 ± 1	89 ± 3	-
3	LTGPT*[a]	4.3	488.2682	5 ± 3	86 ± 5	7 ± 1	87 ± 2	8 ± 2	86 ± 3	-
4	LLTHPN[a]	4.9	694.3833	4 ± 4	90 ± 1	9± 1	92 ± 1	8 ± 2	92 ± 1	-
5	ALVGGTN[a,b]	5.5	631.3355	-	-	9 ± 0	92 ± 2	9 ± 2	89 ± 6	-
6	TLNGV[a]	8.8	503.2786	6 ± 3	84 ± 1	7 ± 1	83 ± 2	8 ± 1	84 ± 2	Antibacterial (TIDGV)
7	TLDGV[a,b]	9.1	504.2627	5 ± 1	88 ± 1	9 ± 1	91 ± 1	7 ± 1	90 ± 1	-
8	PFAHP[a]	9.5	568.2846	5 ± 1	83 ± 2	5 ± 1	81 ± 1	6 ± 2	82 ± 1	-
9	QVGGL[a]	10.6	473.2682	-	-	8 ± 1	87 ± 2	7 ± 2	89 ± 2	-
10	LGGLDSS[a,b]	10.6	648.3151	-	-	7 ± 0	81 ± 0	-	-	-
11	NQFNHSSCST[a]	12.4	1124.4398	4 ± 4	89 ± 3	2 ± 1	87 ± 3	2 ± 2	88 ± 2	-
12	LGTVV[a,b]	14.8	488.3035	-	-	9 ± 1	84 ± 1	9 ± 1	81 ± 0	-
13	MMDPLA[a,b]	20.4	677.3035	-	-	5 ± 0	84 ± 3	6 ± 2	82 ± 1	-

* Peptides that could belong to the alcalase enzyme protein sequence.
a Peptides that were present in < 5 injections of LCS
b Peptides that were present in < 5 injections of DCS

The amino acid composition of the peptides identified in the different CS protein hydrolysates contained high percentage of leucine (L)/isoleucine (I) and valine (V) within their sequences. These amino acids are common among antioxidant peptides [31], exert radical scavenging and metal chelation capacity and allow hydrogen-transfer and lipid peroxyl radical trapping due to their high solubility in hydrophobic radical species [32]. Also, some peptides presented proline (P) in their sequences which is characteristic of antihypertensive peptides [33].

The potential bioactivity of the different peptides identified in LCS, MCS and DCS protein hydrolysates was found in the BIOPEP database [34]. Although most of them are currently not included in BIOPEP, some were found within longer peptide sequences with potential bioactivities (see **Tables 1-3**). For instance, several peptides have been previously reported to be part of longer peptides with antibacterial activity (namely, LLNK and TLNGV), ACE-inhibitory effect (peptides as AVGVK, FASY, LLYQ, FDAVGVK, and AFDAVGVK), or antioxidant capacity (APGAGVY).

4. Conclusions

The peptide composition of protein hydrolysates from *Arabica* CS obtained using three different roasting degrees (light, medium and dark) was studied for the first time in this work LC-(QTOF)MS. Proteins from CS were extracted using a Tris-HCl buffer containing SDS and DTT using a high intensity focused ultrasound probe. Subsequently, protein extracts were submitted to enzymatic hydrolysis employing different enzymes. Then, antioxidant and cholesterol-lowering capacities of the protein hydrolysates were evaluated. Despite not many differences were found among the extracts, the highest activities were obtained using thermolysin in the protein hydrolysis. Using the developed LC-(QTOF)MS method and *de novo* sequencing tool, the peptide composition of all the CS protein hydrolysates was investigated. 51 peptides, containing between 4 and 12 amino acids, were identified in the CS hydrolysates, none of them being common to the three different protein hydrolysis employed. Moreover, the roasting process to which the CS samples were submitted was shown to have little influence on their peptide composition.

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CHAPTER VI

CONCLUSIONS

From the results obtained in this PhD Thesis, the following general conclusions can be derived:

- The use of sulfated-α-CD or sulfated-γ-CD as chiral selectors enabled the enantiomeric separation of eight NPAAs (pyroglutamic acid, norleucine, norvaline, 2,3-dihydroxyphenylalanine, aminoadipic acid, selenomethionine, pipecolic acid, and citrulline) by EKC-UV with resolutions ranging from 0.7 to 7.4 and analysis times comprised between 20 and 47 min. In the case of pyroglutamic and aminoadipic acids, this was the first enantiomeric separation reported by CE. In addition, the developed EKC methodology allowed the simultaneous separation of some mixtures of enantiomers in a single run with high enantiomeric resolutions (pipecolic and aminoadipic acids, and citrulline).

- Citrulline enantiomers were separated by EKC-UV in 18 min with an enantiomeric resolution of 2.7 using a formate buffer at pH 2.0 containing sulfated-γ-CD as chiral selector. The developed methodology has demonstrated its suitability to be applied to the enantiomeric determination of citrulline in food supplements recently acquired and other submitted to a long storage time. The determination of L-citrulline in all the samples analyzed revealed that no racemization occurred due to the effect of storage time. Moreover, the content of D-citrulline was not detectable in every case even though the developed methodology enabled to detect up to a 0.1 % of this enantiomeric impurity. The high sensitivity of the methodology (LODs of 1.8×10^{-7} M and 2.1×10^{-7} M for L- and D-citrulline, respectively) allowed to propose it as a suitable tool for routine food quality control in order to guarantee the accomplishment of legal regulations forbidding the addition of D-enantiomers of amino acids to food supplements.

- An alternative MEKC-UV methodology for the enantioselective separation of selenomethionine was developed based on the use of FLEC as chiral derivatizing reagent and APFO as micellar system and separation medium. Selenomethionine diastereomers were separated in less than 6 min with a resolution of 4.4 and LODs of 3.7×10^{-6} M. The developed methodology showed its suitability for the determination of L-selenomethionine in food supplements although it was limited for the determination of D-selenomethionine since the control of the enantiomeric impurities requires lower LOD values. However, the conditions employed in this methodology are fully compatible with MS and

therefore open the possibility to enhance the sensitivity and to obtain the enantioselective determination of this NPAA in more complex food matrices.

- An innovative analytical methodology based on TIMS-TOFMS was developed enabling the enantiomeric separation of seventeen amino acids from the twenty-one studied in an easy and rapid way (less than 1 min) and with LODs in the nM range. This method was based on the formation of the amino acid diastereomers by using a previous derivatization with the chiral reagent FLEC, which simplifies and uniforms diastereomer formation, avoiding the use of specific reference compounds. The migration order of the enantiomers was found to be dependent on the structure of the amino acid. The resulting TIMS resolution ($K0/\Delta K0$) for the FLEC-amino acid diastereomers ranged between 80.3 to 160. Since the applicability of the developed methodology can be extended to a larger number of amino acids and to the simultaneous separation of their enantiomers, its potential for the analysis of complex food samples is very high.

- A multiplatform untargeted metabolomics strategy based on RPLC-MS, HILIC-MS, and CE-MS for the analysis of coffee samples was developed for the first time in this work enabling a comprehensive characterization of the metabolome of *Arabica* green coffee beans before and after submitting them to different roasting degrees.

- A different number of metabolites showing statistically significant differences among coffee bean samples submitted to different roasting degrees were obtained by each of the analytical platforms employed (fifty-seven by RPLC-MS, thirty-one by HILIC-MS and thirteen by CE-MS). Nineteen different metabolites were tentatively identified as potential markers by RPLC-MS for both ionization modes being one of them common for both ESI modes (1,5-dicaffeoylquinic acid). The use of HILIC-MS by both ionization modes allowed to identify a total of sixteen metabolites, from which one of them (caffeoylshikimic acid isomer) was common with RPLC-MS in negative ESI mode and other one (choline) was also included among the seven metabolites tentatively identified by CE-MS in positive ESI mode. From all the tentatively identified metabolites by the whole multiplatform, nine different metabolites were unequivocally identified using standards (chlorogenic acid, quinic acid, and 1,5-dicaffeoylquinic acid by RPLC-MS; proline betaine, betaine, proline,

and neochlorogenic acid by HILIC-MS; 3-ethylpyridine by CE-MS; and choline by both HILIC-MS and CE-MS).

- Most of the metabolites tentatively identified using RPLC-MS (in negative and positive mode) and HILIC-MS (in negative mode) belong to the group of hydroxycinnamic acids while betaines and amino acids were tentatively identified as potential markers of the coffee roasting process using HILIC-MS in positive mode. Regarding CE-MS, it enabled to highlight amino acid derivatives and metabolites related to the Maillard reaction as potential markers. These results reveal the relevance of combining of orthogonal analytical platforms to achieve a broad metabolomic coverage to provide a better knowledge of the changes occurring during the roasting process of green coffee beans.

- The analysis of protein hydrolysates obtained from three different edible macroalgae (using enzymatic digestion with alcalase), by the RPLC-MS methodology proposed in this work, has allowed, for the first time, the identification of thirty-seven different peptides using *de novo* sequence tool from PEAKS software. Most of these peptides were not previously found in macroalgae and five of them were common for red and brown macroalgae. Red macroalgae, *Mastocarpus stellatus*, presented the highest concentration of proteins and the highest number of peptides in its protein hydrolysates. Using BIOPEP database, some of the identified peptides were found to be a part of longer peptides with potential antibacterial properties showing that these macroalgae can constitute a potential source of bioactive peptides although more research is necessary to link the biological activity with the identified peptides.

- The study of the peptide composition of protein hydrolysates from *Arabica* coffee silverskin submitted to three different roasting degrees by the RPLC-MS methodology optimized in this work enabled to evaluate for the first time this by-product as a source of bioactive peptides. Three different enzymes (thermolysin, alcalase, and simulated gastrointestinal digestion) were used for protein digestion. Antioxidant and hypocholesterolemic activities were observed for all the protein hydrolysates being the most active those obtained using thermolysin, which pointed out the interest of coffee silverskin as a new source of peptides with potential bioactivities. Moreover, fifty-one peptides, with a number of amino acids ranging from four to twelve, were identified in

coffee silverskin being none of them common to the different coffee silverskin protein hydrolysates analyzed. In general, no significant differences were observed in the peptide composition of coffee silverskin roasted at different degrees.

CHAPTER VII

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