Recovery and determination of cholesterol-lowering compounds from *Olea europaea* seeds employing pressurized liquid extraction and gas chromatography-mass spectrometry

Romy Vásquez-Villanueva [a], Merichel Plaza [a, b], María Concepción García [a, b], María Luisa Marina [a, b, *]

[a] Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.

[b] Instituto de Investigación Química Andrés M. del Río, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.

*Corresponding author: Prof. María Luisa Marina

e-mail: mluisa.marina@uah.es

Abstract

Our previous work demonstrated the presence of compounds with hypocholesterolemic capacity in olive seeds. These compounds were extracted using CO_2-expanded ethyl acetate and identified as tyrosol, hydroxytyrosol, and β-sitosterol using GC-MS. This work describes the extraction of these compounds from different olive seeds using pressurized ethyl acetate. Their solubility in ethyl acetate at temperatures ranging from 40-200 ºC was theoretically predicted by Hansen solubility parameters. The content of these compounds was estimated by GC-MS, as well as, the reduction of the micellar cholesterol solubility (RMCS) capacity of extracts enabling to establish the optimum extraction temperature at 100 ºC. A GC-MS method was developed and validated in terms of its analytical characteristics for a sensitive determination and quantification of tyrosol, hydroxytyrosol, and β-sitosterol in different olive seeds. Within varieties, Manzanilla seeds presented the highest concentration of tyrosol, hydroxytyrosol, and the lowest concentration of β-sitosterol, as well as the highest RMCS capacity.

Keywords: Cholesterol-lowering capacity, Hansen solubility parameters, olive seed, phenolic compounds, phytosterols, pressurized liquid extraction

1. Introduction

Olive stone by-product is a rich source of valuable compounds such as proteins, fatty acids, and secondary metabolites as phenolic compounds and plant sterols [1-3]. In fact, tyrosol and hydroxytyrosol, and β-sitosterol are the main phenolic compounds and phytosterol, respectively, found in several olive tissues [4, 5]. These compounds have been widely reported for their health benefits, including the reduction of high cholesterol levels in blood [6, 7]. The main limitation to obtain valuable compounds from by-products using conventional extraction methods is the high amount of toxic solvents that they usually require. As a consequence, these solvents have to be removed by evaporation after extraction, which takes long time. Additionally, these methods offer poor selectivity and reproducibility and can even degrade valuable compounds and produce undesirable species [8-10]. The use of advanced extraction techniques can show up several advantages. A recent study from our research group revealed that phenolic compounds (tyrosol and hydroxytyrosol) and β-sitosterol from olive seeds, which present very different polarities, can be extracted by using an advanced extraction technique based on the use of a CO_2-expanded liquid and ethyl acetate as bio-based solvent [11]. The extracts showed high amount of fatty acids and different proportions of tyrosol, hydroxytyrosol, and β-sitosterol, depending on the extracting conditions (pressure, temperature, and molar fraction of CO_2 in the ethyl acetate). Furthermore, these compounds seemed to be the main contributors to the *in vitro* cholesterol-lowering capacity, especially the presence of tyrosol and hydroxytyrosol [11].

These results encouraged to further study the extraction of these compounds in olive seeds using other advanced technique. Pressurized liquid extraction (PLE) has been reported as a non-conventional technique able to extract bioactive compounds from food matrices [12]. PLE processes are carried out at high temperatures and pressures keeping the solvent in liquid state [13]. These conditions enable a high diffusion and mass-transfer rates, the increase of

compounds solubility, and the decrease of solvent viscosity and surface tension. Generally, PLE process is fast and its automation improves extraction reproducibility [14-16]. PLE is considered as a "green process" since it is focused on the development of processes that reduce energy and solvent consumption avoiding the use and the generation of hazardous substances [17, 18]. Replacing the use of petroleum-based solvents for extraction purposes is recommended for obvious reasons [19-21]. Ethanol, ethyl acetate or ethyl lactate are considered bio-based solvents since they can be obtained from sugar fermentation and are sustainable alternatives to petroleum-based solvents [22]. In a previous study from our research group, Hansen solubility parameters (HSP) calculations were employed to select a suitable extracting solvent [11]. This theoretical approximation constitutes a powerful tool to estimate the interaction of solvent and bioactive compounds reducing time consumption and solvent waste and, ultimately, the economic cost [23, 24]. In that research work, ethyl acetate was selected among different bio-based solvents (ethanol, ethyl lactate, ethyl acetate and D-limonene) using HSP approximation and different proportions of CO_2 in ethyl acetate were also evaluated [11].

Hence, the novelty of the present work is the development of a methodology to extract tyrosol, hydroxytyrosol, and β-sitosterol from olive seeds using PLE and the assessment of the correlation between the presence of these compounds and the *in vitro* capacity of extracts to reduce cholesterol absorption. For that purpose, these compounds were quantitated in different olive seeds genitypes by gas chromatography coupled to mass spectrometry.

2. Materials and methods

2.1. Reagents and materials

Sodium dihydrogen phosphate was from Merck (Darmstadt, Germany). Ethyl acetate and methanol (MeOH) were provided by Scharlau Chemie (Barcelona, Spain). Cholesterol oxidase kit was purchased from BioAssay Systems (Hayward, CA, USA). Taurocholic acid, oleic acid,

phosphatidylcholine, N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA + 0.1% TMCS), and standards of tyrosol, hydroxytyrosol, β-sitosterol, and n-alkane solution C8-C40 were acquired from Sigma (St. Louis, MO, USA). Cleaned and dried sand was supplied by Labkem (Barcelona, Spain).

The olives of 'Manzanilla' (FAROLIVA S.L. company, Murcia, Spain), Picual (Baeza, Jaen, Spain), Cornicabra (Mérida, Extremadura, Spain), and Hojiblanca (Lucena, Córdoba, Spain) varieties were kindly donated.

2.2. Sample preparation

Olive seeds were extracted from olive stones using a nutcracker. Olive seeds were ground and freeze-dried (VirTis Wizard 2.0, from SP Scientific, Warminster, PA, USA). Dried olive seed samples were stored at -80 °C until use.

2.3. Estimation of Hansen solubility parameters (HSP) by HSPiP software

Hansen solubility parameters (HSP) are an extension of the Hildebrand solubility theory. Unlike Hildebrand approximation, HSP take into account some intermolecular interactions (dispersion, polar, hydrogen-bonding forces), and separate the cohesive energy density by the interaction type (Eq. 1).

$$\delta_{Total}^2 = \delta_D^2 + \delta_P^2 + \delta_H^2 \qquad \text{(Eq.1)}$$

where δ_{Total} is the total solubility parameter splitted into δ_D, δ_P, and δ_H which are dispersion, polar, and hydrogen-bond components, respectively.

Each parameter can be represented as a point in the three-dimensional (δ_D, δ_P, and δ_H) space that makes the Hansen solubility sphere. The comparison between HSP for the solvent with the HSP for the analyte, enables to define the distance (R_a) between these two points in the Hansen space as described in Eq. 2:

$$R_a = \sqrt{4\left(\delta_{Di} - \delta_{Dj}\right)^2 + \left(\delta_{Pi} - \delta_{Pj}\right)^2 + \left(\delta_{Hi} - \delta_{Hj}\right)^2} \qquad \text{(Eq. 2)}$$

where, subscripts *i* and *j* are the solute and solvent, respectively. Moreover, Hansen solubility sphere radius known as interaction radius (R_o), describes how large/small the interaction range is. The relationship between R_a and R_0 is the Relative Energy Difference (RED), calculated by Eq. 3:

$$RED = \frac{R_a}{R_o} \qquad \text{(Eq. 3)}$$

RED provides information on the affinity of the solvent for the analyte. RED = 0 means no energy difference, thus, perfect solvent; RED ≤ 1.0 indicates high affinity for the solvent; RED = 1.0 or close is a boundary condition; and higher RED numbers indicate progressively lower affinities [23] (Hansen, 2000).

Since solubility parameters of hydroxytyrosol, tyrosol, and β-sitosterol are not available in data base, HSP can be theoretically predicted from their simplified molecular line-entry systems (SMILES) C1=CC(=C(C=C1CCO)O)O, C1=CC(=CC=C1CCO)O, (CCC(CCC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C)C(C)C, respectively. Calculations are carried by Yamamoto-Molecular Break (Y-MB) tool which separates the contribution of every functional group, to estimate their properties. The software Hansen Solubility Parameters in Practice (HSPiP Version 5.0, Denmark) includes Y-MB and was used to predict the HSP values for hydroxytyrosol, tyrosol, and β-sitosterol. Finally, the temperature dependence of the hydroxytyrosol, tyrosol, and β-sitosterol was evaluated by Jayasri and Yaseen [25] equation:

$$\delta_2 = \delta_1 \left(\frac{1-T_2}{1-T_1}\right)^{0.34} \qquad \text{(Eq. 4)}$$

where T_1 is the room temperature and T_2 is the given temperature.

2.4. Pressurized liquid extraction (PLE) of olive seeds

PLE was performed on a Dionex ASE 150 (Thermo Scientific, Germering, Germany) system equipped with a solvent controller unit, an automated pressure sensor, and pressure relief during heat-up pump. Before extractions, pure ethyl acetate was sonicated during 30 min for removing the dissolved oxygen and avoiding possible oxidation reactions. All extractions were carried out in 10 mL extraction cells, containing 0.5 g of ground and freeze-dried olive seeds which were mixed homogeneously with 11 g of sand to favor uniform distribution of the sample and the extraction solvent in order to maximize the extraction yield. Extractions were achieved at seven different extraction temperatures (40, 60, 80, 100, 120, 150, and 200 °C) and the extraction time was set at 10 min. Prior to any extraction, the extraction cell was heat-up for a given time depending on the extraction temperature (i.e., 5 min when the extraction temperature was 40, 60, 80, and 100 °C; 7 min when the extraction temperature was 120 and 150 °C; and 9 min when the extraction temperature was 200 °C). The extraction procedure was as follows: (1) the extraction cell was placed inside the oven; (2) the cell was filled with ethyl acetate up to a pressure of 10 MPa; (3) heat-up time was established; (4) a static extraction was carried out when all system valves were closed; (5) the extraction cell was rinsed with ethyl acetate at 60% cell volume; (6) ethyl acetate was evacuated from the cell with N_2 for 60 s and (7) the system was depressurized. The system was cleaned to avoid carry-over between extractions. Afterwards, ethyl acetate was evaporated using a centrifugal evaporator (Concentrator Plus, Eppendorf, Hamburg, Germany), until olive oil seed extract dryness, and stored at -20 °C until use. All extractions were conducted in triplicate.

2.5. Reduction of micelle cholesterol solubility (RMCS) *in vitro* assay

Micelles were synthesized according to the method previously reported by Vásquez-Villanueva et al. [11]. Briefly, a solution consisting of 0.5 mM cholesterol, 1 mM oleic acid, and 2.4 mM phosphatidylcholine in MeOH was dried overnight at room temperature. Then, 15 mM of phosphate buffer (pH 7.4) containing 6.6 mM taurocholate salt and 132 mM NaCl were

added and the mixture was sonicated at 95% of amplitude for 1 min to form the micelles. Resulted micelles were incubated overnight in a Thermomixer Compact (Eppendorf, Hamburg, Germany) at 37 ºC. The RMCS assay was performed as follow: 150 µL of 10 mg/mL of sample was added to 50 µL of the micelle solution. The blend was sonicated for 1 min at 95% of amplitude and incubated for 2 h at 37 ºC. Afterwards, the solution was centrifuged for 10 min at 6000 xg. The supernatant was collected for the determination of the cholesterol remaining in micelles using a cholesterol kit. Remained cholesterol in micelle was calculated by interpolation in the calibration curve using cholesterol as standard.

2.6. Gas-chromatography analysis

2.6.1. Derivatization

In order to improve the peak shape and resolution of the phenolic compounds and phytosterols, the samples and standards (tyrosol, hydroxytyrosol, and β-sitosterol) were derivatized [11]. The dried olive seed extracts obtained by PLE and tyrosol, hydroxytyrosol, and β-sitosterol standards were silylated with 50 µL of BSTFA (with 1% TMCS). The mixture was sonicated during 10 min and kept for 1 h at 80 °C. Then, 1 µL of sample was injected into the GC-MS system.

2.6.2. Gas chromatography-mass spectrometry analysis

Analyses were carried out on an Agilent GC-MS system 7890B-5799B from (Agilent Technologies, Palo Alto, CA, USA). Two different GC columns were used. First column was a Zebron ZB-5HT inferno capillary column (5% -phenyl)-dimethylpolysiloxane high temperature phase, 30m x 0.25mm I.D., 0.25 µm film thickness) from Phenomenex Inc. This column was employed by our research group in a previous work [11] and was, firstly employed in this work for the optimization of the extracting conditions. In this case, chromatographic conditions were: 150 °C for 2 min, then increased to 350 °C at a rate of 5 °C/min. Then, a HP-

5 column ((5%-phenyl)-methylpolysiloxane phase, 30m x 0.32mm I.D., 0.25 µm film thickness from Agilent Technologies) was used. This column was employed for the sensitive quantification of target compounds. The optimized chromatographic conditions were: 150 ºC for 2 min and increased up to 325 ºC at a rate 5 ºC/min. Other common conditions were: the injector was heated to 270 °C in the split mode (ratio 1:20). Helium was used as carrier gas (7 psi). Mass spectrometric conditions were: energy 70 eV, working at full scan mode from m/z 50 to 700. Compounds were identified by mass spectra analysis using NIST 05 mass spectral library, data found in the literature, and linear retention indices (calculated based on n-alkanes C_2-C_{40}), and by comparing with standards, when available.

2.7. Analytical characteristics

Calibration curve was built by plotting the peak area of each analyte vs concentration following the external calibration method. For that purpose, standards of tyrosol, hydroxytyrosol, and β-sitosterol were used. Linearity was tested in a range of 5-400 mg/100 g considering, at least, five different concentrations of standard solutions, where each point was injected three times. Data was analyzed by least-squares linear regression analysis. Analytical characteristics of the method were evaluated following different international guidelines [26, 27] determining selectivity, linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, and recovery. Selectivity was determined by the injection into the GC-MS system of non-spiked olive seed extracts (n=3) and chromatographic profiles were compared to mass spectra obtained for standards of tyrosol, hydroxytyrosol, and β-sitosterol respectively. The LOD and LOQ were determined at signal-to-noise (S/N) ratios equal to 3 and 10, respectively. Precision of the optimized GC-MS method was evaluated through instrumental repeatability and intermediate precision according to the relative standard deviation (RSD %) values of peak area. The mixture of the three compounds at two different concentrations (25 mg/100 g and 80 mg/100 g) were used to calculate both, repeatability and intermediate

precision. For instrumental repeatability, ten replicate injections in the same day were injected. Intermediate precision was determined in three days by injecting, five consecutive times, three replicates at two concentration levels. The recovery was studied by spiking the olive seed extracts from four different olive varieties (Cornicabra, Hojiblanca, Picual and Manzanilla) with the standard solution at two different concentrations (low and high concentration) of tyrosol (10 and 40 µg/mL), hydroxytyrosol (10 and 40 µg/mL), and β-sitosterol (20 and 100 µg/mL). Spiking experiments were performed in triplicate and injected by triplicate. The percentage of recovery was determined as follow (Eq. 5):

$$Recovery\ (\%) = \frac{C_{spiked} - C_{initial}}{C_{added}} \times 100 \qquad \text{(Eq. 5)}$$

where, C_{spiked} is the concentration obtained after spiking the sample with a specific standard concentration, $C_{initial}$ is the sample concentration without spiking, and C_{adde} is the spiked standard concentration. Finally, the matrix effect was studied by comparing the tyrosol, hydroxytyrosol, and β-sitosterol peak areas in standard solutions with those obtained in the spiked olive seed extracts.

2.8. Statistical analysis

Statistical analyses were performed using the software Statgraphics Centurion version XVII (Statpoint Technologies, Inc., Warrenton, VA). Analysis of variance (ANOVA) enabled to evaluate the linearity of the calibration curve for every compound. Fisher's least significant difference test was used to determine statistical significant differences ($p < 0.05$) between mean values for different samples at 95% confidence level. All the analyses were carried out in triplicate. Results were expressed as mean value and standard deviation.

3. Results and discussion

3.1. PLE extraction of tyrosol, hydroxytyrosol, and β-sitosterol from olive seeds

Solvents submitted to PLE conditions present different physical properties from solvents under atmosphere conditions. One of the main changes is observed in the dielectric constant. The dielectric constant of the solvent decreases when increasing temperature and, thus, solvent polarity can be tuned by changing the temperature. Different extraction temperatures ranging from 40 to 200 ºC were studied theoretically and experimentally tested. HSP calculations were employed to predict the degree of interaction between ethyl acetate and the compounds of interest (tyrosol, hydroxytyrosol, and β-sitosterol) at the different operating temperatures. SMILE notations of tyrosol, hydroxytyrosol, and β-sitosterol were entered in HSPiP software and the Y-MB method enabled the calculation of HSPs for every compound.

Table 1 shows the solubility parameters and RED values calculated for tyrosol, hydroxytyrosol, β-sitosterol, and the bio-based solvent ethyl acetate at temperatures from 40 to 200 ºC. It is worthy to notice that HSP calculations in Table 1 were performed at 10 MPa because the experimental pressure used in PLE was 10 MPa, except the reference values (at 25 °C) that were calculated at 1 MPa. Since pressure does not exert a significant influence on HSP values when working under subcritical conditions this parameter was not studied [24]. As observed in Table 1, an increase in temperature at constant pressure led to a decrease in the total solubility parameter (δ_{total}), which is in agreement with Williams et al. [28]. For all studied compounds and ethyl acetate, the polar interactions (δ_P) remained almost constant with the increase in temperature, whilst the hydrogen-bonding (δ_H) and dispersive (δ_D) interactions showed a higher influence, with the exception of β-sitosterol that showed no significant variation in δ_H at increasing temperatures. The main differences on the solubility parameters of the studied compounds were δ_H and δ_P values, which were higher in phenolic compounds than in β-sitosterol. Additionally, at high temperatures, lower RED values were reached favoring the solubility of studied compounds in ethyl acetate. The most favorable interaction was observed for β-sitosterol, since RED values were below 1 at all temperatures. On the other hand, tyrosol

and hydroxytyrosol showed RED values higher than 1 at low temperatures, but they reached RED values near to 1 at 150-200 ºC. Consequently, the use of high temperatures with ethyl acetate could favor the extraction of tyrosol, hydroxytyrosol, and β-sitosterol.

In order to compare HSP approach with experimental extraction by PLE, Manzanilla variety was employed. Extraction by PLE was carried out by using ethyl acetate at temperatures ranging from 40 to 200 °C for 10 min at 10.3 MPa. Figure 1 shows the temperature effect on the extraction of tyrosol, hydroxytyrosol, and β-sitosterol from olive seeds (Figures 1A, 1B, and 1C, respectively). The estimation of tyrosol, hydroxytyrosol, and β-sitosterol was carried out based on peak areas, using the optimized GC-MS method (ZB-5HT capillary column) reported by Vásquez-Villanueva et al. [11]. Tyrosol extraction increased from 40 to 100 ºC, decreased from 100 to 150 ºC ($p < 0.05$) and reached the highest content at 200 ºC (Figure 1A). Hydroxytyrosol extraction increased significantly ($p < 0.05$) up to 100 ºC and, then, it decreased (Figure 1B). This behavior was in accordance with HSP prediction up to temperatures around 100 ºC. Above 100 ºC, HSP approximation did not correspond with the experimental data (see Table 1 and Figure 1). This could be due to the fact that HSP cannot predict undesirable effects that might take place at high temperature such as thermal degradation of bioactive compounds.

Indeed, some authors have suggested that phenolic compounds should be extracted at medium temperatures (not exceeding 70 °C) due to their thermolability [29, 30] while Attya et al. demonstrated that phenolic compounds present in olive oil (tyrosol, hydroxytyrosol, and oleuropein) are degraded at temperatures above 150 ºC [31]. Moreover, Tu et al. reported the thermal decomposition of hydroxytyrosol and tyrosol at high temperatures [32]. When using non-conventional extraction techniques, such as pressurized hot water, it has been also described that phenolic compounds are susceptible to degradation at temperatures above 150 °C [33].

The extraction of β-sitosterol was kept from 40 to 100 ºC ($p > 0.05$). However, it decreased ($p < 0.05$) when the temperature was higher than 100 ºC being minimum at 120-150 ºC (Figure 1C). This behavior could also be due to a thermal degradation of phytosterols. Lin et al. observed that plant sterols submitted to high temperatures were degraded leading to the formation of oxidation products [34]. Thus, accordingly to extraction yields, best extracting temperature seemed to be around 100 ºC.

On the other hand, phenolic compounds and phytosterols can improve blood lipid profile due to their capability to decrease blood cholesterol [35, 36]. Thus, in order to corroborate previous results, *in vitro* cholesterol-lowering capacity based on RMCS (%) of extracts was evaluated obtaining results plotted in Figure 2A. The extraction yields increased from 45.4% at 40 ºC to 62.3% at 200 °C ($p < 0.05$) (see Figure 2A) because the mass transfer increases at high temperatures [21]. The highest yields were obtained at 150 and 200 ºC, and there were not significant differences between both temperatures ($p > 0.05$). Taking into account previous results demonstrating the highest extraction of tyrosol, hydroxytyrosol, and β-sitosterol at 100 ºC, additional compounds are probably being extracted at higher temperatures not showing hypocholesterolemic effect or showing antagonic effect.

In vitro RMCS capacity increased with temperature until 100 ºC (Figure 2A). Above this temperature, the cholesterol-lowering capacity decreased, being minimum at 200 ºC. This fact was accompanied by a significant color change from yellowish to dark brown (see Figure 2B). This behavior can be explained taking into account that high temperatures may present negative effects on the bioactivity of some thermolabile compounds [37]. Temperatures higher than 100 ºC likely produce thermal degradation of tyrosol, hydroxytyrosol, β-sitosterol and, consequently, a decrease in cholesterol-lowering capacity. Therefore, the *in vitro* RMCS capacity showed the best results at temperatures close to 100 ºC.

3.2. GC-MS method optimization

Polar functional groups of phytosterols and phenolic compounds were derivatized in order to form less polar and more stable compounds, thus, decreasing the boiling point, increasing volatility, and improving the GC behavior of analytes. Trimethyl silylation is one of the most used derivatization strategies due to its simplicity. Among silylation reagents, BSTFA and BSTFA with 0.1% of TMCS have been widely used due to their fast and quantitative reaction with hydroxyl groups [38, 39]. In order to select the most suitable derivatizing agent, tyrosol, hydroxytyrosol, and β-sitosterol were derivatized with BSTFA and BSTFA with 0.1% of TMCS at 80 ºC. Furthermore, different reaction times were tested (30 min, 1 h, 1.5 h, and 2 h). There were not differences on peak areas at higher reaction times than 1 h (data not shown). Finally, the selected derivatization conditions were: BSTFA with 0.1% of TMCS during 1 h of reaction time at 80 ºC.

In order to improve sensitivity, a HP-5 capillary column was studied to separate tyrosol, hydroxytyrosol, and β-sitosterol. Based on optimized chromatographic separation using ZB-5HT column [11], the temperature ramp was optimized. Similarly to the ZB-5HT, column elution began at 150 ºC and reached the completely elution of all compounds at 325 ºC, at 5 ºC/min (Figure 3). Despite retention times of tyrosol, hydroxytyrosol, and β-sitosterol using HP-5 were higher than the observed for the ZB-5HT, HP-5 column offered better peak sensitivity (see Figure 3). Furthermore, the comparison of mass spectra and retention time for every compound with standards enabled their identification (Figure 4).

3.3. Analytical characteristics of the method

Different analytical characteristics of the optimized method were evaluated (selectivity, precision, linearity, sensitivity, and recovery) using olive seeds from Manzanilla variety. Results are grouped in Table 2.

Method selectivity was demonstrated by comparing chromatographic profiles and mass spectra obtained for an olive seed extract with those obtained for the tyrosol, hydroxytyrosol, and β-sitosterol standard solutions (at concentrations ranging from 10 µg/mL up to 300 µg/mL).

Linearity for tyrosol and hydroxytyrosol was demonstrated from 5 to 350 mg/100 g and for β-sitosterol from 10 to 400 mg/100 g. Method sensitivity was higher for phenolic compounds, especially, for the hydroxytyrosol. Correlation coefficients for all calibrations curves were greater than 0.995. LODs of 0.1 mg/100 g were observed for tyrosol and hydroxytyrosol and of 1.0 mg/100 g for the β-sitosterol. LOQ ranged from 0.3 mg/100 g for tyrosol and hydroxytyrosol to 3.1 mg/100 g for β-sitosterol. Precision of the developed GC-MS method was considered acceptable both within (RSD % < 2.7) and across (RSD % < 2.5) days. The recoveries of tyrosol, hydroxytyrosol, and β-sitosterol ranged from 95% to 105%. Moreover, no matrix interferences were detected.

After demonstrating the sensitivity, selectivity, accuracy, and absence of matrix interferences of the optimized method, it was applied to the determination of tyrosol, hydroxytyrosol, and β-sitosterol contents in extracts obtained from different olive seed varieties for the study of their correlation with their RMCS capacity.

3.4. Evaluation of the correlation of tyrosol, hydroxytyrosol, and β-sitosterol contents with their RMCS capacity

The optimized method using PLE with ethyl acetate as extracting solvent and the developed GC-MS method were applied to determine tyrosol, hydroxytyrosol, and β-sitosterol contents in four different varieties of olive seeds (Manzanilla, Cornicabra, Hojiblanca, and Picual). The tyrosol, hydroxytyrosol, and β-sitosterol contents expressed as mg/100 g of dried olive seeds extracts are summarized in Table 3. In addition, the cholesterol-lowering capacity was measured in all extracts and also included in Table 3. The three studied compounds were

found in the extracts from the four varieties of olive seeds. Moreover, extracts showed RMCS capacities ranging from 30 to 58%. Manzanilla was the olive variety that presented the highest tyrosol and hydroxytyrosol contents (81 ± 5 and 328 ± 10 mg/100 g dry weight, respectively) while Cornicabra showed the lowest concentration of these phenolic compounds (15 ± 1 and 11 ± 1 mg/100 g dry weight, respectively). Tyrosol and hydroxytyrosol were identified for the first time in the olive stone by Fernández-Bolaños et al., at 68.5 and 105 mg/100 g of dry weight of whole stone, respectively [4].

On the other hand, Manzanilla variety showed the lowest content (193 ± 3 mg/100 g dry weight) in β-sitosterol and Cornicabra variety, the highest (365 ± 5 mg/100 g dry weight), this could be expected since high phytosterol content is a characteristic for Cornicabra variety [40, 41].

The extracts from Manzanilla variety obtained by CO_2-expanded ethyl acetate were previously studied by Vásquez-Villanueva et al. [11]. At the best extraction conditions using CO_2-expanded ethyl acetate, Manzanilla extracts showed up to 74.5% of RMCS capacity, where, the main contributor to this capacity seemed to be the presence of tyrosol and hydroxytyrosol. The RMCS capacities of these extracts were higher than the ones reached by PLE in the present work. The main difference between both extraction techniques is the addition of CO_2 to the solvent that could contribute to the extraction selectivity. The presence of CO_2 in the solvent improves the extraction process due to the reduction in relative permittivity and the hydrogen bonding abilities of the solvent. These facts reduce solvent viscosity increasing diffusivity and reducing interfacial tension. Similarly to extracts obtained by CO_2-expanded ethyl acetate, the extracts obtained by PLE that showed the most suitable conditions to obtain the highest yields were not the same to the ones with the highest RMCS capacity. Furthermore, extracts showing RMCS capacity seemed to be positively correlated with tyrosol and hydroxytyrosol contents, confirming previous results obtained by our research group [11].

4. Conclusions

This work demonstrated that PLE with the bio-based solvent ethyl acetate enabled the extraction of tyrosol, hydroxytyrosol, and β-sitosterol from olive seeds. Hansen solubility parameters predicted a greater extraction of these compounds at higher temperatures. Experimental results corroborated this theoretical prediction up to 100 ºC. RMCS capacity of extracts also increased with extracting temperature up to 100 ºC which supported the correlation between the presence of these compounds and RMCS capability of extracts. Higher temperatures probably resulted in compounds degradation or in the extraction of other compounds with non hypocholesterolemic capacity or with antagonic effect. A GC-MS method using a HP-5 capillary column enabled a more sensitive detection than a previous method using a ZB-5HT column. Optimized GC-MS method was selective and lack of matrix interferences and demonstrated precision and accuracy. The method enabled the determination of these compounds in different olive seed varieties observing great differences among varieties. While Manzanilla seeds showed the highest tyrosol and hydroxytyrosol contents and the lowest β-sitosterol content, Cornicabra, Hojiblanca, and Picual showed similar tyrosol and hydroxytyrosol contents ranging from 11-27 mg/100 g and higher β-sitosterol content. The evaluation of the RMCS in extracts enabled to support its strong correlation with the tyrosol and hydroxytyrosol contents and the secondary contribution of β-sitosterol.

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Conflict of interest

The authors declare that they have no conflict of interest.

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523

524 **Figure Captions**

525 **Figure 1.** Peak areas of tyrosol (A), hydroxytyrosol (B), and β-sistosterol (C) of Manzanilla
526 seed extracts obtained by PLE using ethyl acetate as extracting solvent at different temperatures.
527 Peak areas were calculated from chromatograms obtained by the GC-MS method described in
528 section 2.6.2. The data are presented as the average of nine measurements from three extracts.
529 There are no statistically significant differences at the 95% confidence level ($p \geq 0.05$) for the
530 compounds showing the same letter.

531 **Figure 2.** Extraction yields (%) and capacity to reduce micellar cholesterol solubility (%,
532 RMCS) (A), and color (B) of the extracts obtained by PLE using ethyl acetate at different
533 temperatures. There are no statistically significant differences at the 95% confidence level ($p \geq$
534 0.05) for the compounds showing the same letter.

535 **Figure 3.** Comparison of chromatographic separation of olive seed extracts using two different
536 columns, ZB-5HT and HP-5 capillary columns. Chromatographic conditions used with the ZB-
537 5HT column: 150 ºC hold for 2 min and increased up to 350 ºC at 5 ºC/min. Chromatographic
538 conditions used with the HP-5 column: 150 ºC hold for 2 min and increased up to 325 ºC at 5
539 ºC/min. Peak identification: 1, tyrosol; 2: hydroxytyrosol; 3: β-sitosterol.

540 **Figure 4.** Mass spectra obtained at 70 eV using GC-MS of trimethylsylilated (TMS) tyrosol,
541 hydroxytyrosol, and β-sitosterol.

542

543 **Table 1.** Hansen solubility parameters and RED values of bioactive compounds (β-
544 sitosterol, tyrosol, and hydroxytyrosol) and the bio-based solvent ethyl acetate under subcritical
545 conditions.

Compound	Mvol (cm^3/mol)	Temperature (°C)	δ_D ($MPA^{½}$)	δ_P ($MPA^{½}$)	δ_H ($MPA^{½}$)	δ_{Total} ($MPA^{½}$)	RED
β-sitosterol	436.5	25	17.2	1.8	3.4	17.6	0.73
	NC	40	17.0	1.8	3.3	17.4	0.73
	NC	60	16.7	1.8	3.2	17.1	0.71
	NC	80	16.4	1.9	3.1	16.8	0.70
	NC	100	16.1	1.8	3.0	16.5	0.68
	NC	120	15.8	1.7	2.9	16.2	0.67
	NC	150	15.3	1.7	2.7	15.6	0.65
	NC	200	14.6	1.7	2.5	14.9	0.61
Tyrosol	122.3	25	19.3	8.1	16.8	26.8	1.53
	NC	40	19.0	8.1	16.4	26.4	1.49
	NC	60	18.7	8.0	15.9	25.8	1.46
	NC	80	18.4	7.9	15.3	25.2	1.40
	NC	100	18.0	7.9	14.8	24.6	1.36
	NC	120	17.7	7.8	14.3	24.1	1.34
	NC	150	17.2	7.7	13.5	23.2	1.28
	NC	200	16.3	7.6	12.2	21.7	1.18
Hydroxytyrosol	124.6	25	19.7	9.1	19.1	28.9	1.84
	NC	40	19.4	9.0	18.7	28.4	1.80
	NC	60	19.1	8.9	18.1	27.8	1.76
	NC	80	18.8	8.8	17.5	27.2	1.70
	NC	100	18.4	8.8	16.9	26.5	1.65
	NC	120	18.1	8.7	16.3	25.9	1.62
	NC	150	17.5	8.6	15.4	24.8	1.53
	NC	200	16.7	8.5	13.9	23.3	1.43
Ethyl Acetate	98.6	25	15.8	5.3	7.2	18.2	
	100.6	40	15.4	5.2	7.0	17.7	
	103.5	60	14.8	5.2	6.7	17.1	
	106.7	80	14.2	5.1	6.4	16.4	
	110.4	100	13.7	5.0	6.2	15.8	
	114.6	120	13.1	4.9	5.9	15.2	
	120.8	150	12.1	4.8	5.5	14.1	
	142.3	200	10.0	4.4	4.8	11.9	

546 Calculations were performed at 10 MPa, except for the reference values (at 25 °C) that were calculated at 1 MPa.

547

548 **Table 2.** Analytical characteristics of the GC-MS method developed for the analysis of

549 tyrosol, hydroxytyrosol, and β-sitosterol.

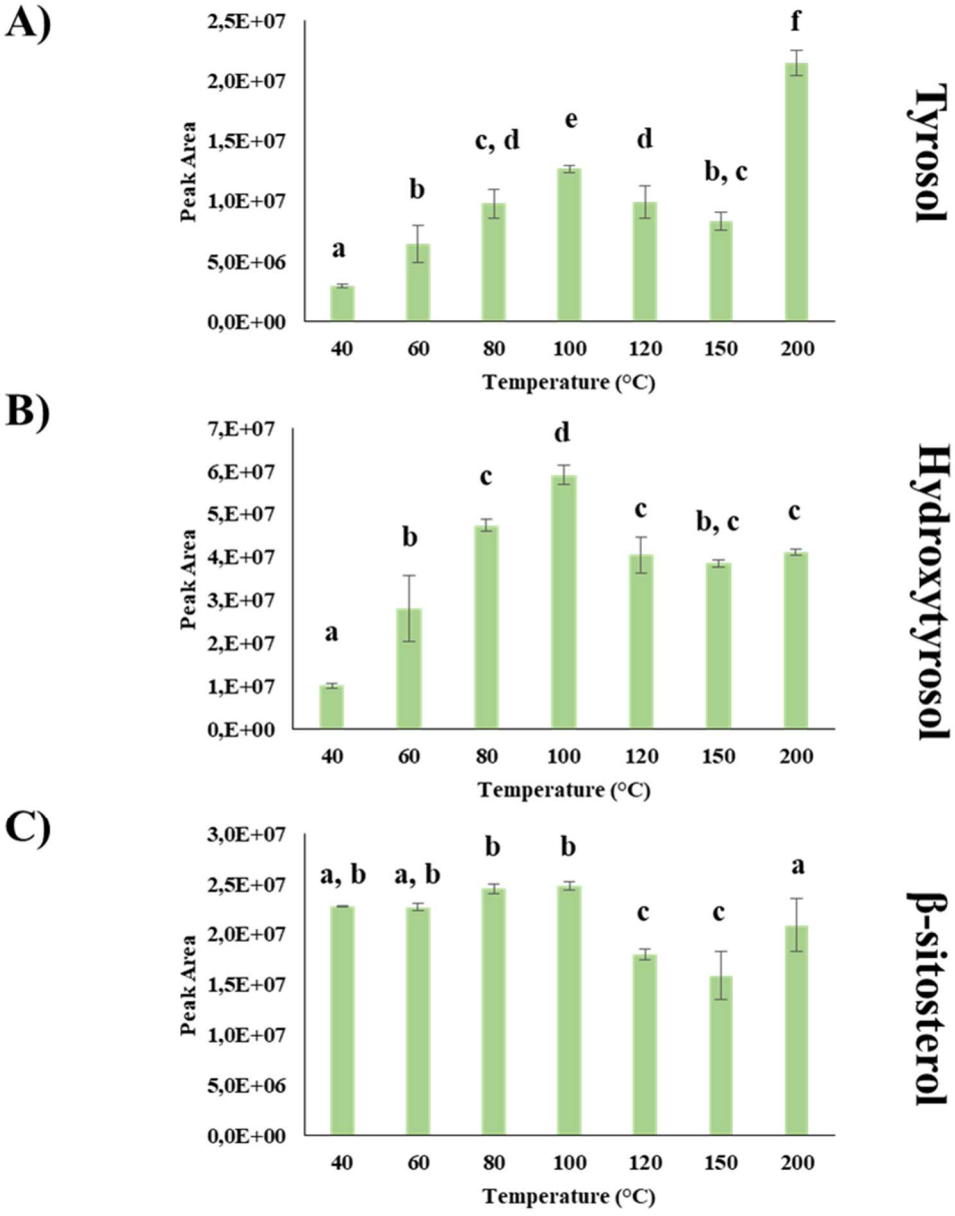
	Tyrosol	Hydroxytyrosol	β-sitosterol
Linearity			
Working concentration range (mg/100 g)	5-350	5-350	10-400
Calibration Curve			
Slope (mg/100 g) $^{-1}$	181677	224684	96880
Correlation coefficient (R^2)	0.997	0.999	0.996
Limit of detection (mg/100 g)	0.10	0.10	1.00
Limit of quantification (mg/100 g)	0.33	0.33	3.10
Precision			
Instrumental repeatability (%RSD) (n=10)			
25 mg/100 g	1.8	2.0	2.7
80 mg/ 100 g	0.90	1.2	2.1
Intermediate precision (%RSD) (n=15)			
25 mg/100 g	5.2	3.4	5.0
80 mg/ 100 g	2.0	1.5	2.5
Recovery (%)			
Low concentration			
Cornicabra	96 ± 4	97 ± 5	101 ± 5
Hojiblanca	95 ± 3	95 ± 4	97 ± 4
Picual	98 ± 3	96 ± 2	100 ± 5
Manzanilla	97 ± 2	99 ± 4	101 ± 3
High concentration			
Cornicabra	102 ± 2	105 ± 2	104 ± 3
Hojiblanca	98 ± 4	95 ± 3	102 ± 2
Picual	101 ±2	106 ± 2	98 ± 3
Manzanilla	98 ± 2	96 ± 3	103 ± 1

550

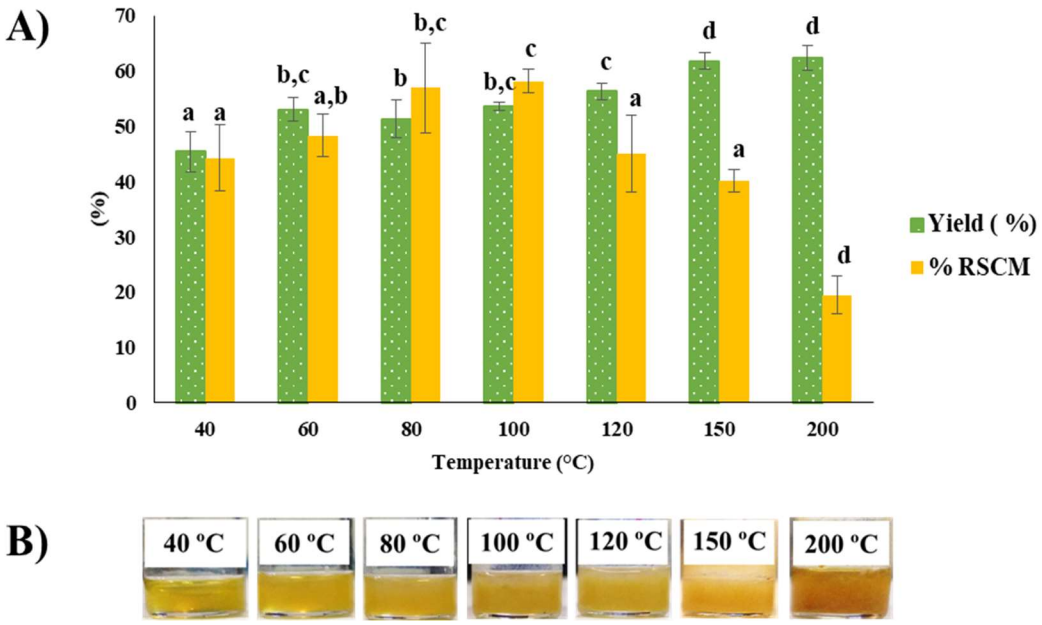
551 **Table 3.** Concentration of tyrosol, hydroxytyrosol, and β-sitosterol, and reduction of micellar
552 cholesterol solubility (RMCS) (mean ± SD, n=3) in extracts obtained by PLE with ethyl acetate
553 at 100 ºC for 10 min from four different varieties of *Olea europaea* seeds using the GC-MS
554 method described in section 3.1.

Variety	Tyrosol (mg/100 g)	Hydroxytyrosol (mg/100 g)	β-sitosterol (mg/100 g)	RMCS (%)
Manzanilla	81 ± 5	328 ± 10	193 ± 3	58 ± 2
Cornicabra	15 ± 1	11 ± 1	365 ± 5	30 ± 3
Hojiblanca	27 ± 2	13 ± 1	312 ± 7	32 ± 4
Picual	24 ± 1	12 ± 1	283 ± 3	49 ± 4

555


A)
Tyrosol
Peak Area
2,5E+07
2,0E+07
1,5E+07
1,0E+07
5,0E+06
0,0E+00
a
b
c, d
e
d
b, c
f
40
60
80
100
120
150
200
Temperature (°C)
B)
Hydroxytyrosol
Peak Area
7,E+07
6,E+07
5,E+07
4,E+07
3,E+07
2,E+07
1,E+07
0,E+00
a
b
c
d
c
b, c
c
40
60
80
100
120
150
200
Temperature (°C)
C)
β-sitosterol
Peak Area
3,0E+07
2,5E+07
2,0E+07
1,5E+07
1,0E+07
5,0E+06
0,0E+00
a, b
a, b
b
b
c
c
a
40
60
80
100
120
150
200
Temperature (°C)

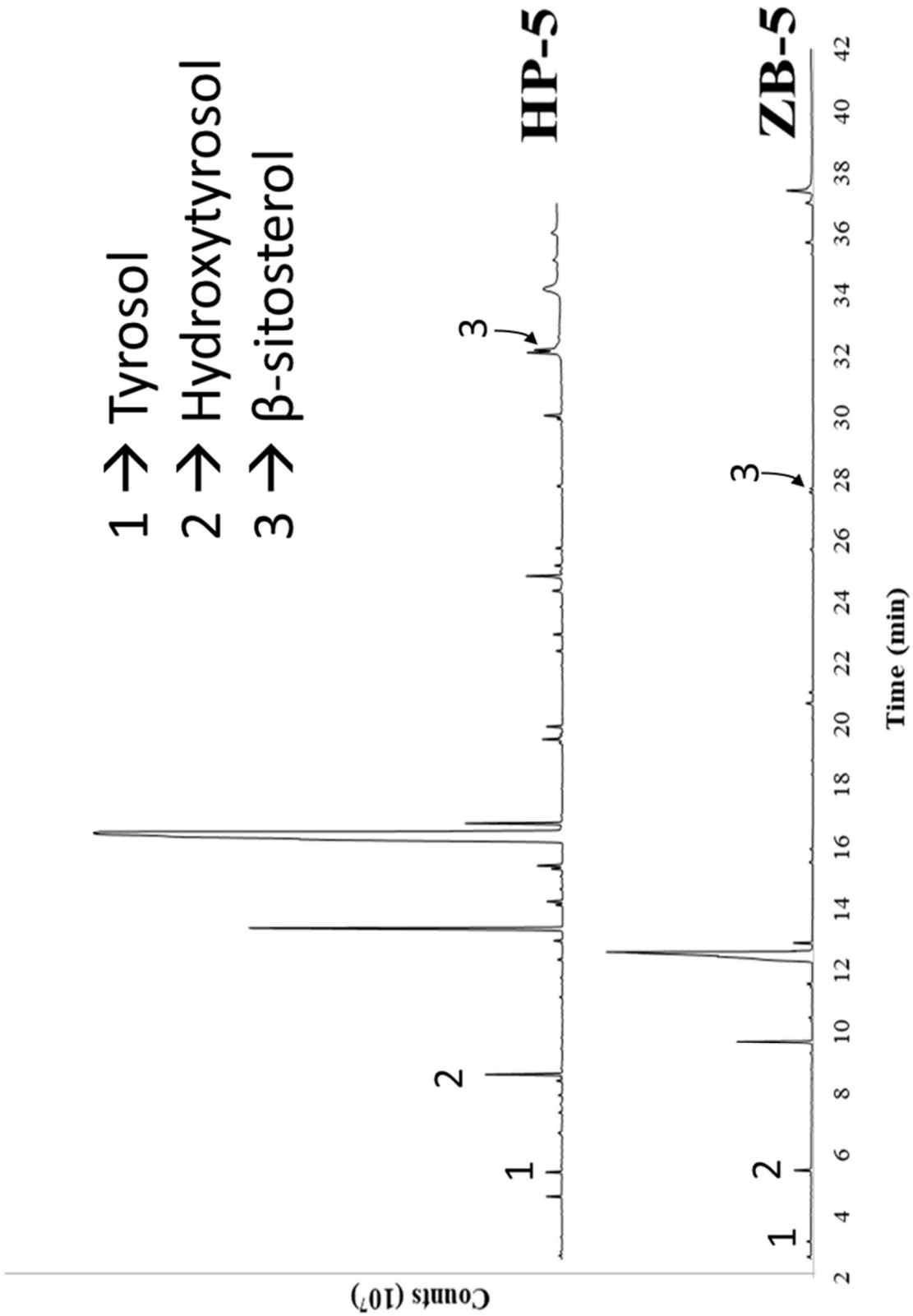
556 **Figure 1.**



557

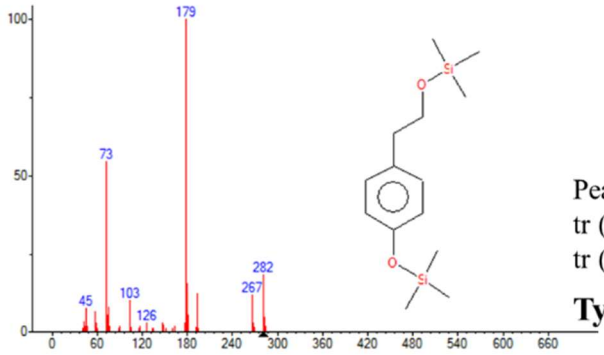
558 **Figure 2.**

559



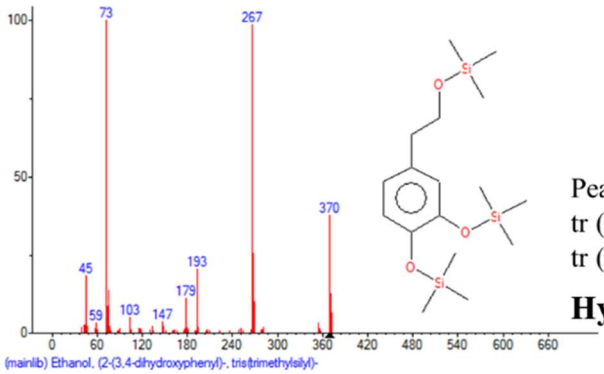
560

561 **Figure 3.**



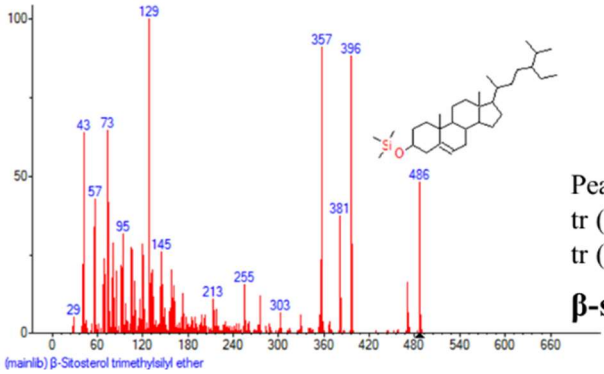
Peak 1
tr (ZB-5) = 3.17 min
tr (HP-5) = 5.47 min

Tyrosol-TMS



Peak 2
tr (ZB-5) = 5.49 min
tr (HP-5) = 8.59 min

Hydroxytyrosol-TMS



Peak 3
tr (ZB-5) = 27.53 min
tr (HP-5) = 32.20 min

β-sitosterol-TMS

Figure 4.