IDENTIFICATION OF PEPTIDES POTENTIALLY RESPONSIBLE FOR *IN VIVO* HYPOLIPIDEMIC ACTIVITY OF A HYDROLYSATE FROM OLIVE SEEDS

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ABSTRACT

Previous studies demonstrated that peptides produced by the hydrolysis of olive seed proteins using Alcalase enzyme showed *in vitro* multifunctional lipid-lowering capability. This work presents a deeper insight in the hypolipidemic effect of olive seed peptides. The capability of olive seed peptides to inhibit endogenous cholesterol biosynthesis through the inhibition of HMG-CoA reductase enzyme was evaluated observing a 38 ± 7% of inhibition in comparison with a blank. Two *in vivo* assays using different peptides concentrations were designed to evaluate the hypolipidemic effect of olive seed peptides in male and female mice. A low concentration of hydrolysate (200 mg/kg/day) reduced total cholesterol in male mice in a 20% after 11 weeks treatment compared to the mice feeding the hypercholesterolemic diet. A higher hydrolysate concentration (400 mg/kg/day) showed a greater reduction in total cholesterol (25%). Hypocholesterolemic effect was attributed to the capacity of the hydrolysate to increase HDL cholesterol and to decrease LDL cholesterol. The analysis of the olive seed hydrolysate by RP-HPLC-MS/MS enabled the identification of peptides that could be responsible for this hypolipidemic effect.

Keywords: Hypolipidemic activity, peptides, *in vivo* assay, olive seed, mass spectrometry.

1. Introduction

Cardiovascular diseases are a serious worldwide health problem. Indeed, above 31 % of all world deaths are due to these diseases.[1] Main risk factor in the development of cardiovascular diseases is hyperlipidemia, characterized by high serum triglycerides and cholesterol levels.

There are different strategies to treat hyperlipidemia. Most of these strategies are based on the disruption of micellar cholesterol transport and the inhibition of enzymes involved in the absorption and biosynthesis of cholesterol and triglycerides. In fact, cholesterol is solubilised in micelles for its transportation and absorption and, thus, the displacement of cholesterol in micelles has been a target to decrease cholesterol absorption.[2] Moreover, the inhibition of the pancreatic cholesterol esterase enzyme, that hydrolysates dietary cholesterol esters into free cholesterol, also results in the reduction of the bioavailability of dietary cholesterol esters.[3] Additionally, oxidation of endogenous cholesterol produces bile acids in the liver and the sequestering of bile acids has been an additional strategy to decrease plasma cholesterol level.[4] Regarding triglycerides, their absorption can be ameliorated by the inhibition of pancreatic lipase enzyme that is responsible for their hydrolysis into monoglycerides and free fatty acids.[5] Furthermore, biosynthesis of endogenous cholesterol consists of different cascade reactions and a proposed mechanism to disrupt cholesterol biosynthesis is based on the inhibition of the 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA, EC 1.1.1.34) reductase enzyme that catalyses the rate-limiting step in this process.[6]

Different synthetic drugs based on previous strategies (inhibitors of cholesterol endogenous synthesis, inhibitors of membrane proteins that promote the intestinal absorption of cholesterol, bile acid sequestrants, etc.) have been developed for the treatment of hyperlipidemia. However, these drugs can cause diverse side effects

65 including digestive disturbances, nausea, increase of hepatic transaminases and creatine
66 kinase, muscle weakness, headache, *etc*.[7,8] On the other hand, there is an increasing
67 interest in searching for alternative natural compounds to treat and/or prevent
68 hyperlipidemia. Different authors have demonstrated that peptides from soybean, lupin,
69 rice bran, potato, seafood, and fish[9-10] could exert *in vitro* hypolipidemic activity. An
70 additional source of lipid-lowering peptides are some food by-products. Moreover, the
71 exploitation of food by-products contributes to the sustainability of food processing. As
72 example, our research group demonstrated, in a previous work, that peptides from olive
73 seeds proteins presented *in vitro* multifunctional lipid-lowering capacity. Indeed, olive
74 seed peptides could decrease exogenous lipid absorption by the reduction of the micellar
75 cholesterol solubility and the inhibition of cholesterol esterase and pancreatic lipase
76 enzymes.[11]

77 Despite *in vitro* experiments are useful as a preliminary evaluation of potential
78 bioactivity, physiological processes and components bioavailability can make that *in vitro*
79 activity was not correlated with results at *in vivo* level. The main aim of this work was to
80 further investigate the hypolipidemic capacity of the olive seed hydrolysate by studying
81 its capacity to inhibit the biosynthesis of cholesterol, its cytotoxicity, and its effect at
82 physiological level. Moreover, peptides potentially responsible of this effect were
83 identified by HPLC-MS/MS.

84

2. Materials and methods

2.1 Chemicals

Water was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). Acetone, hydrochloric acid (HCl), acetonitrile (ACN), and acetic acid (AA) were obtained from Scharlau (Barcelona, Spain). Tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), and di-sodium tetraborate were from Merck (Darmstadt, Germany). DL-dithiothreitol (DTT), albumin from bovine serum (BSA), β-mercaptoethanol, o-phthaldialdehyde (OPA), L-glutathione (GSH), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Dulbecco's modified eagle's medium (DMEM), antibiotics (penicillin, streptomycin, and amphotericin), fetal bovine serum, phosphate buffered (PB), and the HMGCoA-reductase (HMGR) assay kit were purchased in Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent (Coomassie Blue G-250) was acquired at Bio-Rad (Hercules, CA, USA). All the chemicals were analytical grade, except for ACN and AA, which were MS grade. Ezetimibe was obtained from Merck Sharp & Dohme (Kenilworth, NJ, USA) and Alcalase 2.4 L FG (EC 3.4.21.62) was kindly donated by Novozymes Spain S.A. (Madrid, Spain). All cell lines (HeLa and HK-2) were from the American Type Culture Collection (Rockwell, MD, USA).

2.2 Production of hydrolysate

Raw olives of ‘Manzanilla’ variety were kindly donated by the olive company FAROLIVA S.L. (Murcia, Spain). Olive seeds were extracted from olive stones by manual pressing. Olive seeds were next ground in a domestic mill and defatted with hexane for 30 min (four times). Defatted seeds were dried at room temperature. Seed

proteins were extracted and digested following the method described by Prados et al.[11] Briefly, 0.03 g of deffated olive seed were mixed with 5 mL of 0.1 M Tris-HCl buffer containing 0.5% SDS and 0.5% DTT (pH 7.5). For that purpose, a high intensity focus ultrasonic (HIFU) probe (model VCX130, Sonic Vibra-Cell, Hartford, CT, USA) was employed at a 30% of amplitude for 5 min. After centrifugation of the extract at 6000g for 10 min, supernatant containing proteins was collected. Proteins were next precipitated with cold acetone for 24 h followed by centrifucation and drying. Finally, proteins were dissolved in a 5 mM borate buffer (pH 8.5) and hydrolyzed with Alcalase at 50 ºC for 4 h. Protein concentration was estimated by Bradford assay[12] and content of peptides was determined using OPA assay.[11]

2.3 HMGR in vitro assay

HMGR inhibition assay was carried out following the method used by Marques et al.[13] HMGR assay kit included the assay buffer, pravastatin, β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), HMGCoA, and HMGR enzyme. Solutions containing 181 µL of the assay buffer (diluted 5 times), 1 µL of inhibitor (pravastatin/sample), 4 µL of NADPH, 12 µL of HMGCoA, and 2 µL of HMGR enzyme were prepared. After mixing the sample thoroughly, the absorbance of the NADPH was measured every 10 s up to 10 min at a wavelength of 340 nm. The activity (A) of HMGR, expressed as µmol of oxidized NADPH/min/mg protein, was calculated using the following equation:

$$\mathrm{A} = \frac{(\mathrm{Abs}_{340}/\mathrm{min}_{\mathrm{sample}} - \mathrm{Abs}_{340}/\mathrm{min}_{\mathrm{blank}}) \cdot \mathrm{TV}}{12.44 \cdot \mathrm{V} \cdot 0.6 \cdot \mathrm{LP}}$$

where TV is the total volume (mL), V is the volume of enzyme (mL), 0.6 is the enzyme concentration in mg protein/mL, LP is the light path (cm), 12.44 is the product of the molar absorption coefficient of NADPH at 340 nm (6.22 mM^{-1} cm^{-1}) by two (number of NADPH molecules consumed in the reaction), Abs_{340}/min_{sample} is the absorbance observed when adding the hydrolysate, and Abs_{340}/min_{blank} is the absorbance corresponding to the blank. Each sample was measured by triplicate. Two different concentrations of hydrolysate were measured, 3.1 mg/mL and 26.7 mg/mL. The percentage of inhibition of HMGR was calculated as:

$$\%\ \text{inhibition of HMGR} = \frac{A_{máx} - A_{sample}}{A_{máx}} \times 100$$

where $A_{máx}$ is the activity of the enzyme without inhibitor and A_{sample} is the activity of the enzyme when adding sample or pravastatin.

2.4 Cytotoxicity of the olive seed hydrolysate

The MTT assay was carried out following the method described by Prados et al.[11] Different concentrations of the olive seed hydrolysate were used, and two different cell lines were employed: a cancer cell line (HeLa, human cervical cancer cells) and a healthy cell line (HK-2, human renal proximal tubule cells). Cell bioavailability was expressed related to the control (hydrolysis buffer).

2.5 Animals

Six-month-old C57BL/6 mice (20-30 g weight) from Envigo (Huntingdom, UK) were housed at the Animal Research Centre of the University in groups of two or three animal

153 per cage with stable room conditions as room temperature (20-24 ºC), 55 ± 10% of
154 humidity and a 12:12 h dark/light cycle. All animals were fed *ad libitum*. Two kinds of
155 diets were used to feed the mice, a standard laboratory animals' diet (Teklad ref. 2014)
156 and a high cholesterol diet (HCD) (Teklad ref. TD.88051), both from Envigo. The animals
157 were supplemented by oral gavage and euthanized at the end of the studies by Oxygen
158 Dioxide and terminal heart puncture. Blood samples were collected to determine plasma
159 lipid profile.

160 The *in vivo* assays with animals were carried out implementing the European
161 Directive 63/2010/EU and the Spanish regulations (RD 53/2013) on animal
162 experimentation. These assays were favourably assessed by the Ethics Committee of the
163 University of Alcalá (CEI: CEI-UAH-AN-2015006) and authorized by the Spanish
164 Competent Authority (PROEX 223/18).

165

166 *2.6 In vivo experiment design*

167 Two sets of *in vivo* assays were designed using two different doses of the olive
168 seed hydrolysate. In all cases, the animals were supplemented once per day, at 10:00 in
169 the morning. In the first study, male and female mice were divided in the following four
170 groups. Group 1 was fed with a standard diet and supplemented with a low concentration
171 of hydrolysate (200 mg/kg/day) while group 2 was supplemented with the same dose but
172 fed with HCD. Group 3 was fed with HCD and supplemented with 5 mg/kg/day of
173 ezetimibe (positive control) while Group 4 was fed with HCD but not supplemented
174 (negative control). Every group was constituted by 9-11 mice with at least 4 female mice
175 per group. Mice blood was collected after 5 and 11 weeks.

176 The second experiment was carried out only with male mice. Three groups were
177 made up: groups 3 and 4 were identical to the previous experiment and a new group
178 (group 5) fed with HCD and supplemented with 400 mg/kg/day of hydrolysate. Every
179 group was constituted by 5-6 mice. In this case, blood was collected after 4 and 8 weeks.

180

181 *2.7 Analyses of mice plasma*

182 Blood samples were collected using Z/Serum separator tubes (Aquisel, Barcelona,
183 Spain) and let 15-30 min at room temperature until blood coagulation. After
184 centrifugation at 1600g for 10 min, serum was collected and storage at -80 ºC until
185 analysis. Serum levels (total cholesterol (TC), total triglycerides (TG), low-density
186 lipoproteins (LDL-C), and high-density lipoproteins (HDL-C)) were determined using
187 routine laboratory methods with a Cobas C311 Autoanalyzer (Roche, Basel,
188 Switzerland). Two indices, atherogenic index (AI) and coronary risk index (CRI), were
189 calculated using the following equations[14]:

190 $$\mathrm{AI} = \frac{\mathrm{LDL} - \mathrm{C}}{\mathrm{HDL} - \mathrm{C}}$$

191 $$\mathrm{CRI} = \frac{\mathrm{TC}}{\mathrm{HDL} - \mathrm{C}}$$

192

193 *2.8 Identification of peptides by HPLC-MS/MS*

194 MS/MS detection was carried out with an electrospray quadrupole-time-of-flight
195 (ESI-Q-TOF) series 6530 mass spectrometer coupled to a liquid chromatograph (model
196 1100), both from Agilent Technologies (Pittsburgh, PA, USA). Analytical separation was
197 carried out in an Ascentis Express Peptide ES-C18 column (100 mm x 2.1 mm, 2.7 µm

198 particle size) with a guard column (5 mm x 2.1 mm, 2.7 μm particle size), both from
199 Supelco (Bellefonte, PA, USA). Mobile phases were: 0.3% (v/v) AA in water (phase A)
200 and 0.3% (v/v) AA in ACN (phase B). Other chromatographic conditions were: injection
201 volume, 15 μL; flow rate, 0.3 mL/min; column temperature, 25 ºC. The elution gradient
202 was: 5% B for 10 min, 5–65% B in 35 min, 65-95% B in 2 min, and 95% B for 3 min. A
203 reversed gradient from 95 to 5% B in 5 min was used to return to the initial eluting
204 conditions.

205 Mass spectrometer was operated in the full-scan mode from 100 to 1500 m/z and
206 in the positive ion mode. ESI conditions were: fragmentator voltage, 200 V; nebulizer
207 pressure, 50 psig; capillary voltage, 3500 V; gas temperature, 350 ºC; drying gas flow, 12
208 L/min; and skimmer voltage, 60 V. The Jet Stream sheath gas temperature was 400 ºC
209 and the flow was 12 L/min. MS/MS was carried out using *Auto* mode with the following
210 conditions: 5 precursors per cycle and a collision energy of 4 V per each 100 Da. Two
211 Agilent compounds (HP0921 and purine) yielding ions at 922.0098 m/z and 121.0509
212 m/z, respectively, were simultaneously introduced and used as internal standards
213 throughout the analysis.

214 PEAKS Studio Version 7 software from Bioinformatics Solutions Inc. (Waterloo,
215 Canada)[15] was used for the analysis of MS/MS spectra and the *novo* sequencing of
216 peptides. Two independent samples of the olive seed hydrolysate were injected by
217 triplicate. Only peptides appearing in the three injections of the two independent samples
218 with average local confidence (ALC, expected percentage of correct amino acids in the
219 peptide sequence) equal or higher than 90 % were selected. Since it is not possible to
220 differentiate between I and L amino acids due to their equal masses, only isoforms with
221 L were presented, although peptide sequences containing I amino acid instead of L are

222 also possible. Isoelectric point and solubility in water were determined using Innovagen's
223 peptide property calculator.

224

225 *2.9 Statistical analysis*

226 Statistical analysis was performed using Statgraphics Software Plus 5.1 (Statpoint
227 Technologies, Inc., Warranton, VA, USA). Data comparison was carried out by the
228 analysis of variance (ANOVA) or the test-t. Duncan's Multiple Range test was used to
229 determine statistically significant differences (p-value < 0.05). Results were expressed as
230 mean ± standard deviation.

231

3. Results and discussion

3.1 In vitro evaluation of hypocholesterolemic activity

The capacity to reduce the bioavailability of dietary cholesterol and triglycerides of a hydrolysate obtained from olive seed proteins has been explored in a previous work observing a multifunctional effect.[11] Indeed, results demonstrated that the olive seed hydrolysate showed capacity to reduce cholesterol micellar solubility and to inhibit the activity of cholesterol esterase and pancreatic lipase enzymes although it did not show capacity to bind bile acids. Biosynthesis of cholesterol is an additional source of cholesterol that contributes to hypercholesterolemia as much as exogenous cholesterol. Biosynthesis of cholesterol consists of different steps being the transformation of HMG-CoA to mevalonate, the limiting one. This reaction is catalysed by the enzyme HMGR in presence of the cofactor NADPH. Therefore, HMGR is an important target against biosynthesis of cholesterol. *In vitro* HMGR inhibitory activity of the olive seed hydrolysate was determined at two different concentrations and results were compared with that of pravastatin, a drug usually employed for the treatment of hypercholesterolemia (see Figure 1). Olive seed hydrolysate reduced the activity of HMGR in 16.8 % when the peptide concentration was 3.1 mg/mL. This inhibition increased to a 40 % when the peptide concentration was 26.7 mg/mL. This activity is similar to the observed for hydrolysates obtained from amaranth[16] and snakehead fish skin collagen.[17]

3.2 Cytotoxicity of the olive seed hydrolysate

Safety of the olive seed hydrolysate was determined by the MTT assay using two different human cell lines, a cancer cell line (HeLa cells) and a healthy cell line (HK-2 cells). Results shown in Figure 2 demonstrated cytotoxic effects in HeLa cells at concentrations higher than 3 mg/mL and a high cell viability in healthy HK-2 cells at any concentration.

3.3 In vivo assay using a low olive seed hydrolysate concentration

Olive seed hydrolysate has showed hypolipidemic activity in *in vitro* assays. Nevertheless, *in vitro* results do not always correlate with *in vivo* capacity since it depends on the bioavailability and temporal and spatial distribution of peptides in the body.[18] Therefore, a first *in vivo* assay was designed to evaluate the physiological effect of the olive seed hydrolysate.

A preliminary assay was conducted using male and female mice in order to establish the effect of following a commercial hypercholesterolemic diet in serum lipids. The experiment took 11 weeks and serum TC was determined at week 4, 5, 8, and 11 (Figure 3). Both male and female serum TC increased up to weeks 4-5 and, afterwards, it kept up to week 11.

From these results, a new set of *in vivo* assays were designed to evaluate the effect of the administration of the olive seed hydrolysate. Four different groups were established: Group 1 was fed with a standard diet and daily treated with 200 mg/kg/day of the olive seed hydrolysate. This dose was selected based on previous studies found in bibliography that used doses ranging from 100 to 2500 mg/kg/day.[19-22] Mice fed with normal diet and daily treated with the olive seed hydrolysate did not show any significant

278 variation in their serum lipids after 5 weeks (data not shown). Moreover, Group 2 was fed
279 with HCD and daily treated with 200 mg/kg/day of the olive seed hydrolysate, Group 3
280 was fed with HCD and daily treated with 5 mg/kg/day of hypocholesterolemic drug
281 ezetimibe, and Group 4 was fed just with HCD. Table 1 shows lipid serum parameters
282 obtained when using male mice. As expected from results in Figure 3, male mice fed with
283 HCD showed a huge increase in TC level after 4 weeks but this level kept up to week 11.
284 The administration of the olive seed hydrolysate reduced this serum TC in male mice in
285 a 17% and a 20% after 5 and 11 weeks of administration, respectively. This result was
286 compared with the obtained for Group 3 (supplemented with ezetimibe) observing that
287 ezetimibe decreased the TC in a 11%, after 11 weeks.

288 A similar experiment was conducted with female mice (see Table 2). The
289 administration of 200 mg/kg/day of the olive seed hydrolysate for 11 weeks in female
290 mice resulted in an increase in serum TC. Moreover, female mice neither showed a
291 significant reduction in serum lipids when they were treated with ezetimibe for 11 weeks.
292 The difference between male and female behaviour could be explained by the different
293 hormonal cycles in the female organism.[23]

294 In addition to TC, serum HDL-C and LDL-C were also determined (see Table 1
295 and 2). HDL-C kept in serum male mice feeding with just HCD after 5 weeks and doubled
296 when the olive seed hydrolysate was administrated for the same period. However, HDL-
297 C levels increased, in the same way, in the groups 2, 3, and 4 after 11 weeks. LDL-C in
298 mice from group 4 increased more than LDL-C in mice from group 2, after 5 weeks. The
299 same effect was appreciated in mice from groups 2 and 4, after 11 weeks. LDL-C levels
300 in mice treated with ezetimibe did not show significant differences with mice from group
301 2. Therefore, the hypocholesterolemic effect of the olive seed hydrolysate, observed after

5 and 11 weeks treatment, in male mice seems to be mainly due to the decrease in LDL-C. This effect was similar in the ezetimibe group.

Female mice showed an increase in HDL-C when they were treated with the olive seed hydrolysate while no significant variation in HDL-C was observed when they were treated with ezetimibe (see Table 2). This fact could explain the increase in TC observed for female mice treated with the hydrolysate. Female mice LDL-C kept after treatment with hydrolysate while it decreased when they were treated with ezetimibe. This result showed that, for female mice, the hypocholesterolemic effect of the hydrolysate was based on the increase of the HDL-C.

Two different indexes, the atherogenic index (AI) and the coronary risk index (CRI), were calculated from lipid serum levels in male and female mice and results are shown in Table 1 and 2. Both, AI and CRI, are strong biomarkers to predict the risk of atherosclerosis and coronary heart diseases and they are more useful than independent serum parameters.[24] Male mice feeding just with HCD resulted in a 215% and a 123% increase in AI and CRI, respectively, after 5 weeks treatment. After 11 weeks, these indexes showed a slight decrease due to the increase of HDL-C level, but they kept significantly higher than the rest of groups. Indeed, AI and CRI indexes in mice from groups supplemented with ezetimibe or hydrolysate were significantly lower than the indexes in the group fed only with HCD. The administration of the olive seed hydrolysate kept AI and CRI indexes in male mice during the whole experiment confirming the hypocholesterolemic effect, at physiological level, of the olive seed hydrolysate.

Moreover, no significant variation in TG level was observed in male mice fed with HCD regardless they were treated with hydrolysate or ezetimibe. Similar results were observed for female mice except when they were treated with ezetimibe that resulted in a significant increase in TG level.

Taking into account the hypolipidemic effects observed in mice when they were treated with 200 mg/kg/day of the olive seed hydrolysate, a second *in vivo* assay with a higher dose was designed in order to find out whether it can produce a more significant effect.

3.4 In vivo assay using a high olive seed hydrolysate concentration

A new *in vivo* assay was carried out using a dose of 400 mg/kg/day of the olive seed hydrolysate and serum lipids were determined after 4 and 8 weeks of treatment. Figure 4 shows TC levels in the group fed with HCD and treated with ezetimibe (Group 3), in the group fed with just HCD (Group 4), and in the group fed with HCD and treated with the new concentration of the olive seed hydrolysate (Group 5). As expected, TC increased in three groups after 4 weeks treatment not observing any significant difference in serum TC between them. However, after 8 weeks' treatment, serum TC in mice treated with the olive seed hydrolysate (Group 5) decreased in a 25% while TC in mice within groups 4 and 3 kept at same levels than at week 4. This behaviour has also been observed in Figure 3 and in Table 1. The effect of ezetimibe treatment was observed after treatment for 11 weeks. The effect observed by the administration of 400 mg/kg/day of the olive seed hydrolysate at week 8 (25% reduction in TC) was more significant than the decrease in TC observed when using the dose of 200 mg/kg/day of the olive seed hydrolysate (20% reduction in TC at week 11).

Like in the first assay, HDL-C, LDL-C, TG and two indexes were also determined at 8 weeks' treatment (see Table 3). Serum HDL-C increased in the three groups after 8 weeks. These increased was higher in mice supplemented with ezetimibe than in mice supplemented with hydrolysate. Furthermore, LDL-C levels decreased in a 44% and 26%

in groups 5 and 3, respectively, compared with negative control (group 4). Additionally, both indexes calculated, AI and CRI, were kept at initial values when mice were fed with HCD and supplemented with hydrolysate. The two indexes were significantly lower in mice supplemented with hydrolysate than in mice feeding just HCD. In the case of CRI, after 8 weeks, the index was even slightly lower than at the beginning of the treatment. The mice treated with ezetimibe showed the same AI value than those treated with hydrolysate. However, there was no significant difference in CRI indexes of groups 3 and 4. Furthermore, Figure 5 shows the variation of mice weight during the assay in groups 4 (HCD), 3 (HCD + ezetimibe), and 5 (HCD + hydrolysate). Animal weight increased in the same way in the three groups. At the end of the assay, all mice increased their body weight above 50%. This fact discards a secondary satiating effect of the olive seed hydrolysate.

In vivo hypolipidemic effect of the olive seed hydrolysate was similar to the effect observed when purified peptides fractions were employed.[25] Indeed, Rho et al.[26] demonstrated a reduction up to 25% in TC after treatment with a concentrated black soybean hydrolysate fraction for 4 weeks. Nagaoka et al.[2] observed that the administration of a tryptic hydrolysate of the standard protein β-lactoglobulin produced a 17% reduction in TC in 48 h fasted rats. Moreover, Lapphanichayakool, Sutheerawattananonda, and Limpeanchob[26] evaluated the hypocholesterolemic activity of sericin-derived peptides isolated from silk cocoons observing around a 28% TC reduction when a dose of 200 mg/kg/day was administrated for 4 weeks. Another example was the work carried out by Zhong et al.[19] that studied the *in vivo* hypocholesterolemic effect of a fraction from a soybean protein hydrolysate. They observed a 24% TC decrease when feeding mice with 500 mg/kg/day and no effect when using 100 mg/kg/day doses.

375 In order to estimate the equivalence dose to obtain a similar effect in humans, it
376 is necessary to consider that the metabolism of a high organism is slower than that of a
377 mouse and it is necessary to apply a conversion factor. In the case of a mouse, the
378 conversion factor from animal dose to human dose is 0.081[28]. Then, the human dose
379 necessary to observe a similar effect, considering a 60 kg person, ranges from 0.97-1.9 g.

380

381 *3.5 Identification of peptides potentially responsible for the hypolipidemic effect of the*
382 *olive seed hydrolysate*

383 In order to identify which peptides could be responsible for the observed
384 hypolipidemic effect, the olive seed hydrolysate was analysed by RP-HPLC-MS/MS.
385 Figure 6 shows the EIC (Extracted Ion Chromatogram) and the mass spectra of abundant
386 peptides, FLPH and WNVN. Moreover, Table 4 shows the sequence of the identified
387 peptides along with their molecular masses, retention time, mass accuracy, isoelectric
388 point, and abundance (based on the areas obtained from extracted chromatograms). Only
389 peptides appearing in the three injections of the two independent samples and with ALC
390 equal or higher than 90 % were selected. Thirty-three different peptides with Mw lower
391 than 1 kDa were identified. Identified peptides appeared in the first half of the TIC
392 (between 1-24 min). Peptides had between 4-7 amino acids and showed isoelectric points
393 ranging from 0 to 10. Almost half of peptides showed good solubility in water. Peptides
394 contained a 56% of hydrophobic amino acids (alanine (A), leucine (L), phenylalanine (F),
395 proline (P), methionine (M), valine (V), and tryptophan (W)), 10% of acidic amino acids
396 (glutamic (E) and aspartic (D) acids) and 8% of basic amino acid lysine (K). Most
397 abundant peptides were ADLY, FLPH, KLPLL, and TLVY. Two of these sequences,
398 FDGEVK and VPLSPT, were previously observed, according to BIOPEP-UWM
399 database, and showed antioxidant bioactivities while FAVV, KALM, KGAL, SSPL, and

TLVY are part of antibacterial peptides.[29] Some peptides sequences were also observed in previous works of our research group[11,30]. Despite olive seed proteins are not sequenced in proteomic databases (there is no genomic data for the vast majority of olive proteins) yet, it has been possible the comparison of sequenced peptides with proteins identified in a previous work by homology with other plant species[31]. Indeed, two peptides, identified in this work, were within sequences of olive seed proteins: peptide ADIY (the sequence has been observed in protein Triticin OS=Triticum aestivum PE=2 SV=1 (tr|B2CGM5|B2CGM5_WHEAT)) and peptide VYIE (the sequence has been observed in 11S globulin seed storage protein 2 OS=Sesamum indicum PE=2 SV=1 (sp|Q9XHP0|11S2_SESIN))[31]. Among identified peptides, it is interesting to highlight peptide FDGEVK since it has the same two C-terminal amino acids that a hypocholesterolemic peptide identified in the β-lactoglobulin tryptic hydrolysate.[2]

In conclusion a hydrolysate obtained from olive seed proteins using Alcalase enzyme has demonstrated *in vivo* capacity to reduce serum cholesterol in mice at two different concentration levels (200 and 400 mg/kg/day). This effect was attributed to both, the increase of HDL-C and the decrease of LDL-C in serum. The treatment with the olive seed hydrolysate also kept initial atherogenic and coronary risk indexes. According to *in vitro* studies, this effect could be attributed to the capability showed by the olive seed hydrolysate to reduce the micellar cholesterol solubility and to inhibit important enzymes (pancreatic lipase, cholesterol esterase, and HMGR) involved in the biosynthesis and absorption of cholesterol. Analysis of mice weight also enabled to discard a possible satiating effect of the hydrolysate. Thirty-three different peptides were identified in the olive seed hydrolysate. All peptides showed a high content of hydrophobic amino acids being peptides ADLY, FLPH, KLPLL, and TLVY the most abundant. These peptides could be responsible for the observed hypolipidemic effect.

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518

519 **Figure captions**

520 **Figure 1.** Capacity of the olive seed hydrolysate to inhibit the enzyme HMGR at two
521 different concentrations (3.1 and 26.7 mg/mL) in comparison with the positive control
522 pravastatin. Means with different letters are significantly different ($p < 0.05$).

523 **Figure 2.** Cell viability of human cell lines HK-2 and HeLa after treatment with the olive
524 seed hydrolysate at different concentrations. Results are expressed as percentage related
525 to the control value. Means with different letters are significantly different ($p < 0.05$).

526 **Figure 3.** Variation of total cholesterol (TC) in serum male and female mice fed with
527 HCD for 11 weeks. Values are expressed as mean ± standard deviation. Means with
528 different letters are significantly different ($p < 0.05$).

529 **Figure 4.** Variation of total cholesterol (TC) in serum male mice fed with HCD (Group
530 3), with HCD and ezetimibe (Group 4), and with HCD and the olive seed hydrolysate
531 (Group 5) after 4 and 8 weeks. Means with different letters are significantly different (p
532 < 0.05).

533 **Figure 5.** Variation of the body weight observed in male mice within Groups 3, 4, and 5
534 during the assay that used 400 mg/kg/day. Means with different letters are significantly
535 different ($p < 0.05$).

536 **Figure 6.** Extracted Ion Chromatograms at 512 m/z (corresponding to peptide FLPH) and
537 531 m/z (corresponding to peptide WNVN) and fragmentation spectra of these peptides.

Table 1. Serum plasma levels in male mice fed with HCD (Group 4) and supplemented with 200 mg/kg/day of hydrolysate (Group 2) or ezetimibe (Group 3). Means with different letters are significantly different ($p < 0.05$).

	WEEK 0	WEEK 5		WEEK 11		
		GROUP 4 (HCD)	GROUP 2 (HCD + Hydrolysate)	GROUP 4 (HCD)	GROUP 2 (HCD + Hydrolysate)	GROUP 3 (HCD + Ezetimibe)
TC	138 ± 17^{a}	274 ± 7^{c}	227 ± 9^{b}	263 ± 9^{c}	209 ± 10^{b}	234 ± 27^{b}
HDL-C	110 ± 6$^{a'}$	97 ± 2$^{a'}$	191 ± 10$^{b'}$	188 ± 9$^{b'}$	177 ± 7$^{b'}$	185 ± 21$^{b'}$
LDL-C	22 ± 5$^{a''}$	61 ± 4$^{cd''}$	48 ± 2$^{bc''}$	66 ± 5$^{d''}$	48 ± 18$^{bc''}$	40 ± 11$^{b''}$
TG	158 ± 36$^{a'''}$	145 ± 14$^{a'''}$	131 ± 26$^{a'''}$	182 ± 35$^{a'''}$	142 ± 45$^{a'''}$	169 ± 31$^{a'''}$
AI	0.20 ± 0.03$^{a''''}$	0.63 ± 0.05$^{c''''}$	0.25 ± 0.00$^{a''''}$	0.35 ± 0.01$^{b''''}$	0.27 ± 0.09$^{a''''}$	0.23 ± 0.03$^{a''''}$
CRI	1.26 ± 0.09$^{a'''''}$	2.82 ± 0.13$^{b'''''}$	1.18 ± 0.02$^{a'''''}$	1.39 ± 0.05$^{b'''''}$	1.18 ± 0.02$^{a'''''}$	1.25 ± 0.06$^{a'''''}$

Table 2. Serum plasma levels in female mice fed with HCD (Group 4) and supplemented with 200 mg/kg/day of hydrolysate (Group 2) or ezetimibe (Group 3). Means with different letters are significantly different ($p < 0.05$).

	WEEK 0	WEEK 11		
		GROUP 4 (HCD)	GROUP 2 (HCD + Hydrolysate)	GROUP 3 (HCD + Ezetimibe)
TC	93 ± 6^{a}	122 ± 26^{b}	160 ± 24^{c}	118 ± 11^{ab}
HDL-C	75 ± 4$^{a'}$	108 ± 24$^{b'}$	137 ± 29$^{c'}$	98 ± 9$^{b'}$
LDL-C	19 ± 3$^{b''}$	19 ± 4$^{b''}$	24 ± 7$^{b''}$	12 ± 4$^{a''}$
TG	131 ± 35$^{a'''}$	182 ± 53$^{a'''b'''}$	164 ± 13$^{a'''b'''}$	315 ± 42$^{b'''}$
AI	0.25 ± 0.03$^{b''''}$	0.18 ± 0.01$^{a''''b''''}$	0.17 ± 0.02$^{a''''}$	0.12 ± 0.04$^{b''''}$
CRI	1.25 ± 0.05$^{c'''''}$	1.12 ± 0.06$^{b'''''}$	1.05 ± 0.28$^{b'''''}$	1.20 ± 0.06$^{a'''''}$

le mice fed with HCD (Group 4) and supplemented with 400 mg/kg/day of hydrolysate (Group 5) or ezetimibe

ers are significantly different ($p < 0.05$).

		WEEK 8	
	GROUP 4 (HCD)	**GROUP 5 (HCD + Hydrolysate)**	**GROUP 3 (HCD + Ezetimibe)**
	259 ± 61^{c}	194 ± 31^{b}	271 ± 23^{c}
	271 ± 20$^{c'}$	172 ± 36$^{b'}$	213 ± 20$^{c'}$
	62 ± 17$^{c''}$	35 ± 6$^{b''}$	46 ± 11$^{b''}$
	65 ± 36$^{a'''}$	163 ± 75$^{a'''}$	141 ± 27$^{a'''}$
	31 ± 0.04$^{b''''}$	0.21 ± 0.02$^{a''''}$	0.24 ± 0.02$^{a''''}$
	28 ± 0.07$^{b'''''}$	1.14 ± 0.04$^{a'''''}$	1.28 ± 0.04$^{b'''''}$

Table 4. Peptide sequence, average local confidence (ALC), retention time, molecular mass, mass accuracy (ppm), isoelectric point, water solubility, and abundance of peptides sequenced in the olive seed hydrolysate.

PEPTIDE	ALC (%)	RT	MASS	PPM	ISOELECTRIC POINT	SOLUBILITY IN WATER	ABUNDANCE ($X10^6$)
ADLY	92	9.63	480.222	-3.8	0.74	Good	115.3 ± 7.9
AVFDD	95	13.73	565.2383	-5.1	0.73	Good	47.8 ± 4.4
FAVV	94	21.32	434.2529	-5.2	0.00	Poor	9.8 ± 0.7
FDGEVK	96	4.46	693.3333	-6.4	3.93	Good	75.1 ± 7.3
FLPH	93	9.69	512.2747	-5.1	0.10	Poor	306.2 ± 8.9
HTLY	92	5.02	532.2645	-5.4	0.10	Poor	36.5 ± 2.8
KALM	96	3.24	461.2672	-4	9.91	Good	15.9 ± 0.9
KFVEGDE	90	8.89	822.3759	-7.7	3.69	Good	7.3 ± 0.5
KGAL	94	1.56	387.2481	-2.9	9.91	Good	20.5 ± 1.9
KLGNF	93	16.03	577.3224	-7.6	9.91	Good	33.3 ± 0.8
KLPL	97	18.9	469.3264	-5.9	9.91	Good	86.3 ± 4.0
KLPLL	96	23.78	582.4105	-7	9.91	Poor	193.4 ± 10.8
KVSSPL	93	12.37	629.3748	-7.7	9.91	Good	22.1 ± 2.7
LAFK	93	9.13	477.2951	-7.8	10.12	Good	3.4 ± 1.3
LLGL	94	23.36	414.2842	-7.5	3.63	Poor	4.2 ± 0.6
LPVNTL	92	22.04	655.3904	-7.2	3.63	Poor	13.8 ± 2.1
NDVFK	94	6.06	621.3122	-6	6.22	Good	14.7 ± 2.1
NFVVLK	93	21.11	718.4377	-7.7	9.70	Poor	53.0 ± 7.5
SSPL	92	5.18	402.2114	-3.2	3.37	Poor	15.1 ± 1.2
SSPLL	96	20.18	515.2955	-6.1	3.37	Poor	20.4 ± 2.9
STLF	94	21.42	466.2427	-6.6	3.43	Poor	28.2 ± 4.5
SVLY	94	17.6	480.2584	-5.3	3.39	Poor	96.3 ± 8.6
TLVY	95	17.97	494.274	-5.3	3.35	Poor	177.7 ± 15.1
VDLE	96	7.51	474.2325	-3.9	0.71	Good	11.3 ± 0.7
VFDGE	93	7.28	565.2383	-5.1	0.71	Good	20.1 ± 3.4
VPLSPT	96	15.35	612.3483	-6.5	3.66	Poor	94.3 ± 7.1
VVLK	99	2.55	457.3264	-2.5	10.10	Good	11.5 ± 0.5
VVLLT	92	20.89	543.3632	-7	3.66	Poor	6.6 ± 0.4
VVVPH	90	7.3	549.3275	-6.9	7.78	Poor	12.1 ± 1.2
VYLE	92	15.37	522.2689	-4.2	1.00	Good	12.1 ± 1.9
WDPN	91	5.92	530.2125	-3.2	0.78	Good	30.7 ± 3.4
WNVN	91	13.91	531.2441	-6	3.59	Poor	75.5 ± 7.2
YTSSPL	94	18.27	666.3224	-7.3	3.34	Poor	27.8 ± 4.2

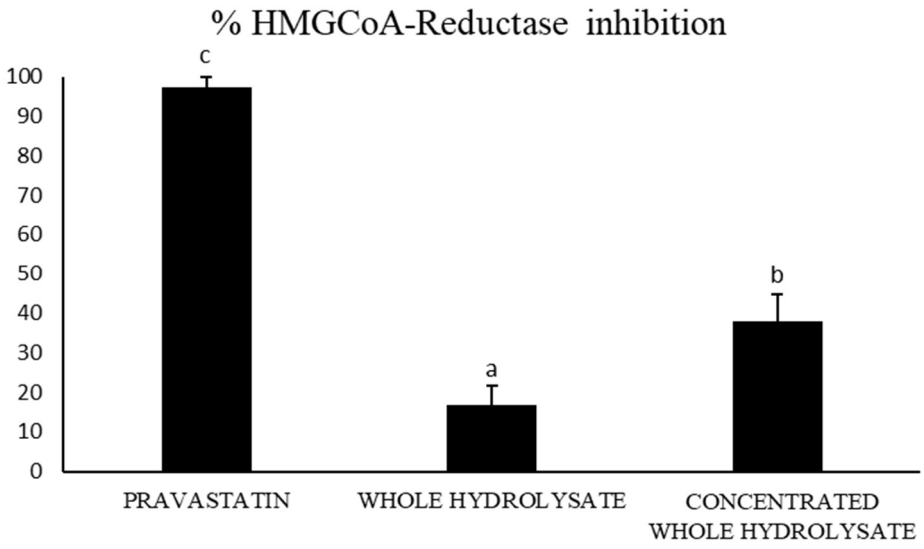

% HMGCoA-Reductase inhibition
c
a
b
100
90
80
70
60
50
40
30
20
10
0
PRAVASTATIN
WHOLE HYDROLYSATE
CONCENTRATED WHOLE HYDROLYSATE

Fig. 1

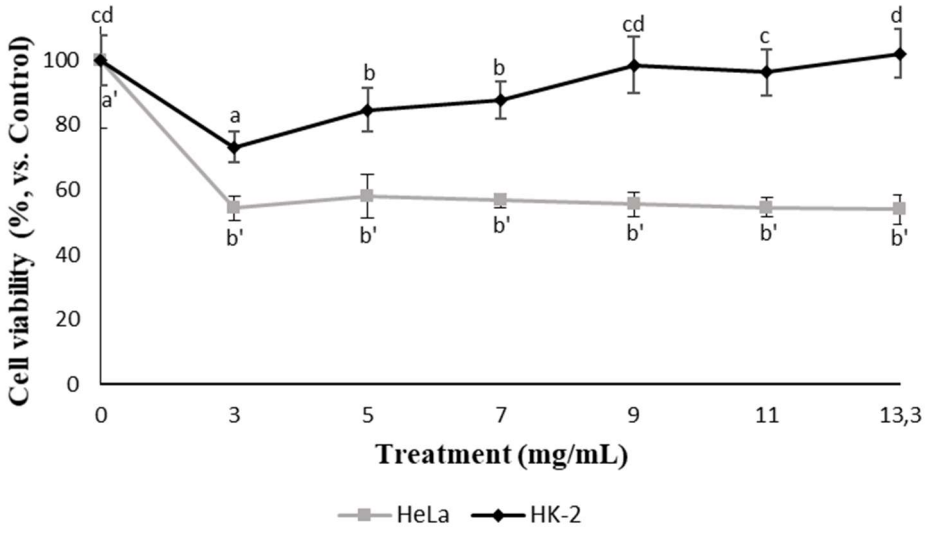

Cell viability (%, vs. Control)
100
80
60
40
20
0
cd
a'
a
b
b
cd
c
d
b'
b'
b'
b'
b'
b'
0
3
5
7
9
11
13,3
Treatment (mg/mL)
HeLa
HK-2

Fig. 2

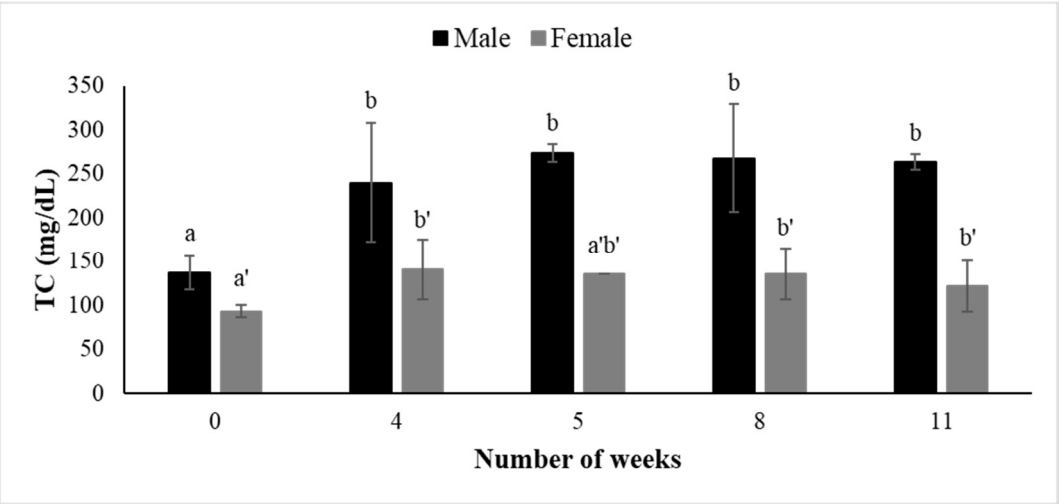

Male
Female
TC (mg/dL)
350
300
250
200
150
100
50
0
a
a'
b
b'
b
a'b'
b
b'
b
b'
0
4
5
8
11
Number of weeks

Fig. 3

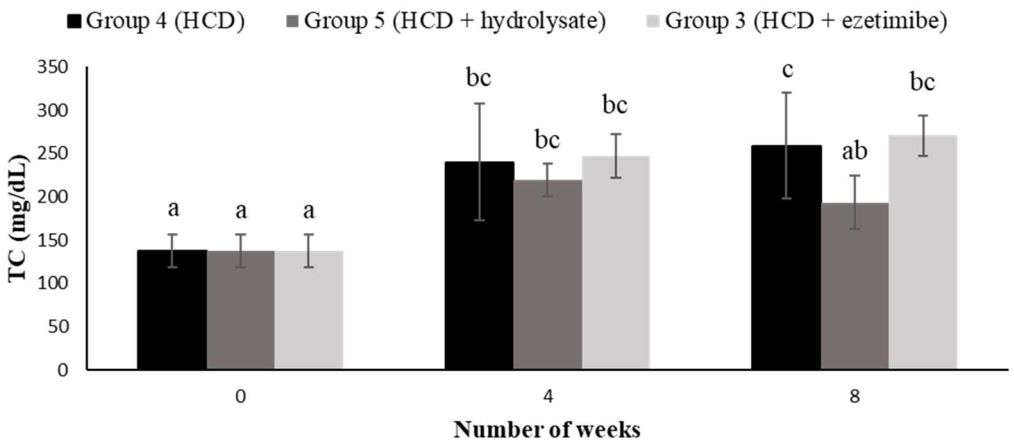
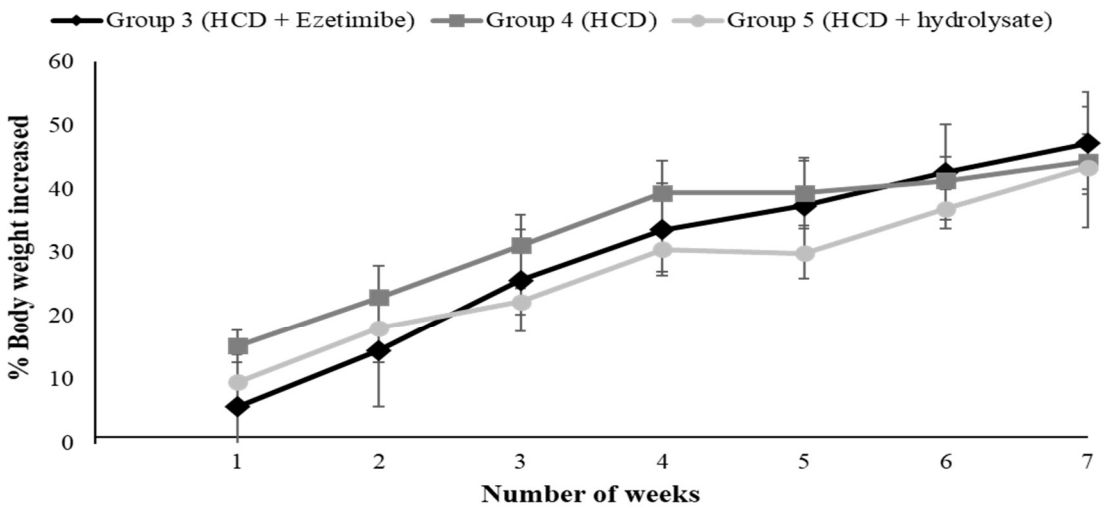

Group 4 (HCD)
Group 5 (HCD + hydrolysate)
Group 3 (HCD + ezetimibe)
TC (mg/dL)
350
300
250
200
150
100
50
0
a
a
a
bc
bc
bc
c
ab
bc
0
4
8
Number of weeks

Fig. 4



Group 3	a	a'	a"b"	ab"'	b""	a""	a"""
Group 4	b	b'	b"	b"'	b""	a""	a"""
Group 5	ab	a'b'	a"	a"'	a""	a""	a""

Fig. 5

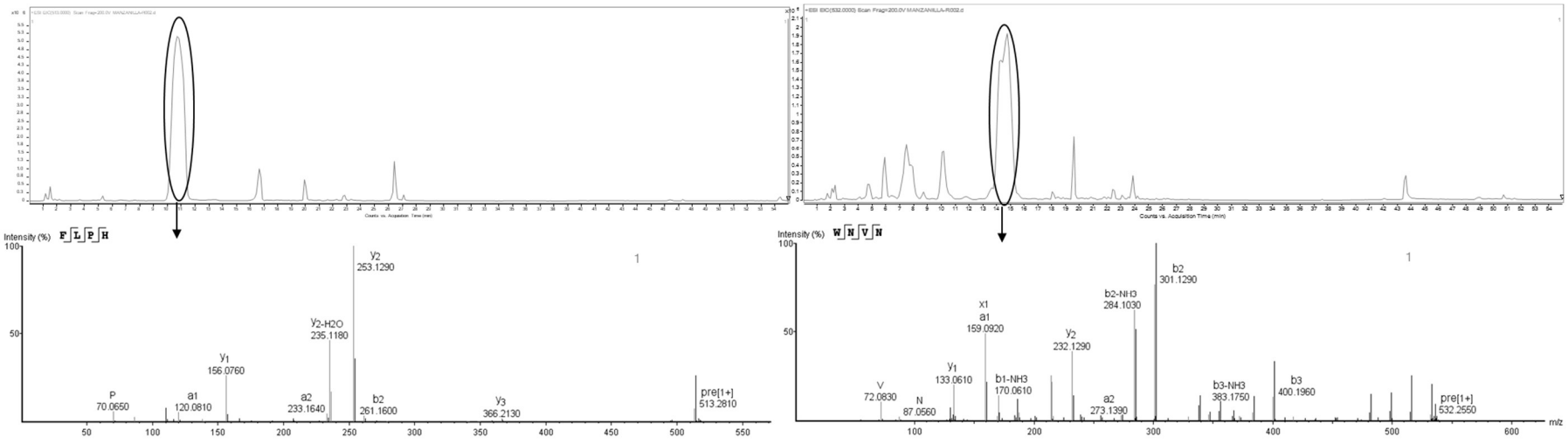

Intensity (%)
F L P H
y2
253.1290
y2-H2O
235.1180
y1
156.0760
P
70.0650
a1
120.0810
a2
233.1640
b2
261.1600
y3
366.2130
pre[1+]
513.2810
Intensity (%)
W N V N
b2
301.1290
b2-NH3
284.1030
x1
a1
159.0920
y2
232.1290
y1
133.0610
b1-NH3
170.0610
V
72.0830
N
87.0560
a2
273.1390
b3-NH3
383.1750
b3
400.1960
pre[1+]
532.2650
m/z

Fig. 6

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