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ARTICLE

Evaluation of the relationship between the peptide profile and the lipid-lowering properties of olive seeds hydrolysates as a tool for tunning hypocholesterolemic functionality

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Isabel M. Prados[a], Merichel Plaza[a,b], M. Luisa Marina[a,b] and M. Concepción García*[a,b]

Olive processing generates large amounts of stones with high protein content. Previous works demonstrated that olive seed proteins from Manzanilla variety released peptides with lipid-lowering capacity. Nevertheless, no work has demonstrated their roles in the whole hypolipidemic activity. Moreover, further studies using different olive varieties are required to purpose a solid method for the exploitation of olive seeds. Twenty different olive varieties were employed in this work. Proteins were extracted using high-intensity focused ultrasounds and digested with Alcalase. Released peptides were identified using proteomic techniques and their capabilities to reduce the absorption of dietary cholesterol (by inhibiting cholesterol esterase enzyme, binding of bile acids, and reducting micellar cholesterol solubility) or the biosynthesis of endogenous cholesterol were evaluated. Peptides with different lipid lowering capacities were obtained from all varieties although the genotype significantly affected to the hypolipidemic characteristics. Univariate and multivariate statistical analysis showed strong correlations, positive and negative, between the presence of certain peptides in the hydrolysates and their capacity to reduce exogenous cholesterol absorption and endogenous cholesterol synthesis. Therefore, the selection of the olive seed genotype can direct its lipid-lowering properties, e. g. promoting the reduction of dietary cholesterol absorption or the inhibition of cholesterol biosynthesis.

1 Introduction

Olive (*Olea europaea*) stones are a by-product from the olive industry that constitute 16 – 22% of the total olive weight.[1] This olive waste has been used to produce biomass.[2] However, olive stones are important sources of valuable compounds such as proteins that constitutes a 16-28 % of the olive seed.[3] Moreover, proteins can be precursors of peptides with bioactive properties defining a bioactive peptide as a protein fragment that has a positive impact on the functions of our body and that can condition and affect our health.[4] Exploitation of olive seeds by the recovery of bioactive peptides will enable the valorisation of this underused and sustainable material.

On the other hand, the increasing incidence of hypercholesterolemia and related diseases due to non-healthy lifestyles and high-fat diets have risen, especially when levels are moderate, the demand for foods containing smart ingredients with lipid lowering effects. Nevertheless, this rise can be caused by different reasons and, in some cases, can even be due to a genetic predisposition. Indeed, there are different mechanisms involved in the reduction of cholesterol absorption and biosynthesis being very important to characterize the type of lipid-lowering functionality of a molecule in order to adapt its use to the suitable purpose. Absorption of dietary cholesterol in humans requires its solubilisation in micelles[5] and the main mechanism to reduce the absorption of exogenous cholesterol is by the disruption of these micelles. On the other hand, bile acids are the main constituents of micelles and are released during cholesterol oxidation in the liver. Molecules with capacity to bind bile acids have a double effect; they can inhibit micelles formation and promote bile acid release by increasing cholesterol oxidation rate.[6] Other molecules can reduce the absorption of dietary cholesterol by the inhibition of pancreatic cholesterol esterase enzyme, involved in the release of free cholesterol from dietary cholesterol esters.[7] Regarding the reduction of endogenous cholesterol, molecules with this capability used to be inhibitors of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA R) enzyme, since it is the step limiting in the cholesterol biosynthesis mechanism.[8]

Different compounds from natural sources such as plants, microbes, and animals have demonstrated lipid-lowering capacity. Many of them are secondary metabolites, including phenol compounds.[9] Additionally, some peptides from milk and hempseed have shown *in vitro* and *in vivo* hypocholesterolemic capacity.[10, 11] Moreover, our research group has proposed a strategy to obtain peptides with capacity to reduce cholesterol from Manzanilla variety olive seeds.[12] Released peptides showed ability to reduce micellar cholesterol solubility and to inhibit bile acids binding and cholesterol esterase enzyme. A more recent work has also demonstrated that peptides from Manzanilla olive seeds exerted *in vitro* capacity to reduce cholesterol biosynthesis by the inhibition of HMG-CoA R enzyme.[13] These results were confirmed in two *in vivo* assays

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 Rio" (IQAR), Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares (Madrid), Spain.
† Footnotes relating to the title and/or authors should appear here.
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that showed a significant increase in blood high density lipoproteins (HDL) cholesterol (good cholesterol) when the hydrolysate obtained from the Manzanilla olive seeds was administered to mice feeding with a high cholesterol diet[13]. The study of peptides presented in the Manzanilla hydrolysate enabled the identification of up to 33 different peptides. Nevertheless, further researches are required to find out the roles of these peptides in the whole capacity showed by the Manzanilla hydrolysate to reduce the micellar cholesterol solubility, to bind bile acids or to inhibit HMG-CoA reductase and cholesterol esterase enzymes. Moreover, preliminary results obtained with the Manzanilla olive seed are promising but additional studies using other olive varieties are required to make a reliable proposal for the valorisation of olive seeds.
The aim of this work has been to evaluate the relationship between the peptide profile and the lipid-lowering properties of olive seeds hydrolysates. For that purpose, different olive seed genotypes were employed and hypocholesterolemic properties of their hydrolysates were evaluated through different mechanisms. Proteomic analysis was applied for the identification of peptides in olive seed hydrolysates and the role of peptides in the whole hypocholesterolemic capacity exerted by hydrolysates was studied using univariate and multivariate chemometric tools.
The main text of the article should appear here with headings as appropriate.

2 Materials and Methods

2.1 Chemicals and samples

All reagents were of analytical grade. Water was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). Acetone, methanol, hexane, hydrochloric acid (HCl), acetonitrile (ACN), and acetic acid (AA) were obtained from Scharlau (Barcelona, Spain). Tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), di-sodium tetraborate, and sodium dihydrogen phosphate were from Merck (Darmstadt, Germany). DL-dithiothreitol (DTT), 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA R), bovine pancreatic cholesterol esterase, p-nitrophenyl butyrate (p-NPB), sodium taurocholate, taurodeoxycholic acid, oleic acid, phosphatidylcholine, and sodium chloride (NaCl) were all from Sigma-Aldrich (Saint Louis, MO, USA). Cholesterol oxidase kit was purchased from BioAssay Systems (Hayward, CA, USA). Total bile acid kit was from Bio-Quant (San Diego, CA, USA). Alcalase 2.4 L FG, produced by fermentation of a selected strain of Bacillus licheniformis, mainly composed by Subtilisin A, with catalytic activity on serine, and with an activity of 2.4 Anson units per gram, was kindly donated by Novozymes Spain S.A. (Madrid, Spain).

Raw olives of 19 different varieties (Barnea, Bouteillan, Caballo, Cañivano Negro, Cobrancosa, Corbella, Cordobeses de Arroyo de la Luz, Cornicabra, Kalokerida, Khalkali, Lechín de Sevilla, Medjhoul, Nevado Azul, Ocal, Picual, Racimal de Jaen, Reixonenca, Sayfi, Verdiell) were kindly donated by the World Olive Germplasm Bank of IFAPA (Córdoba, Spain). Manzanilla seed variety was a gift from FAROLIVA S.L. (Murcia, Spain). All olives were collected at same maturity index (violet). Olives were manually depulped and stones extracted were stored at -20 ºC until use.

2.2 Preparation of protein hydrolysates

Olive seeds were extracted from olive stones with a nutcracker. They were next grounded in a domestic mill and defatted with hexane. The powder obtained was dried at room temperature and storage at -20 ºC until use. Protein extraction and digestion was carried out following the procedure previously optimized by our research group.[12] Briefly, 5 mL of extraction buffer (0.1 M Tris-HCl, 0.5% (w/v) SDS, and 0.5% (w/v) DTT at pH 7.5) were added to 0.03 g of olive seed powder and the extraction was carried out using a high intensity focused ultrasound (HIFU) probe (model VCX130, Sonic Vibra-Cell, Hartford, CT, USA) at 30% of wave amplitude for 5 min. After centrifugation at 4000g for 10 min, the proteins in the supernatant were collected and precipitated with cold acetone for 24 h at 4 ºC. Protein isolate was dissolved in 0.05 M borate buffer (pH 8.5) and hydrolysed with Alcalase (4 h, 50 ºC and 0.15 UA/g protein). Extraction and digestion of proteins from every variety was performed by duplicate.

2.3 Evaluation of *in vitro* hypocholesterolemic capacity

In vitro hypocholesterolemic capacity was evaluated using four different assays based on three mechanisms to reduce exogenous cholesterol (reduction of micellar cholesterol solubility, binding of bile acids, and inhibition of cholesterol esterase enzyme) and another one to inhibit cholesterol biosynthesis (inhibition of HMG-CoA R enzyme). Reduction of micellar cholesterol solubility, binding of bile acids, and inhibition of cholesterol esterase enzyme assays have been described previously.[12]

2.3.1 HMG-CoA R inhibition

The activity of HMG-CoA R in presence of hydrolysate was measured using the HMG-CoA R assay kit that included the assay buffer, pravastatin, β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), HMG-CoA, and HMG-CoA R enzyme. Solutions containing 181 µL of the assay buffer (diluted 5 times), 1 µL of inhibitor (pravastatin/hydrolysate), 4 µL of NADPH, 12 µL of HMGCoA, and 2 µL of HMGE enzyme were prepared. After mixing, the absorbance of the NADPH was measured every 10 s up to 10 min at a wavelength of 340 nm. The result was expressed as percentage of inhibition of the enzyme.[13]

2.4 Separation and identification of peptides by reversed-phase high-performance liquid chromatography coupled to mass spectrometry (RP-HPLC-MS/MS)

The analysis of peptides in the 20 olive seed hydrolysates was performed using a High Performance Liquid Chromatography (HPLC) system 1100 from Agilent (Agilent Technologies, Santa Clara, CA, USA) coupled to a high sensitive Quadrupole-Time-Of-Flight mass spectrometer (Q-TOF/MS) (Agilent 6530 series, Pittsburgh, PA, USA)

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equipped with an orthogonal electrospray ionization (ESI) source (Agilent Jet Stream, AJS). Agilent Mass Hunter Workstation software B.07.00 from Agilent was used for HPLC and MS control, data acquisition, and data analysis. Analytical separation was carried out in an Ascentis Express Peptide ES-C18 column (100 x 2.1 mm, 2.7 µm particle size) with an Ascentis Express Peptide ES-C18 guard column (5 x 2.1 mm, 2.7 µm particle size), both from Supelco (Bellefonte, PA, USA). The mobile phases consisted of water with 0.3% acetic acid (v/v) (phase A) and acetonitrile with 0.3% acetic acid (v/v) (phase B). The column temperature was 25 ºC and the flow rate was 0.3 mL/min. Injection volume was 15 µL. Elution gradient 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 reversed gradient from 95 to 5% B in 5 min was used to return to the initial eluting conditions and next injection was carried out after a 15 min post-time.

Mass spectrometry detection was carried out in the positive ion mode using a mass range from 100 to 1500 m/z. MS conditions were: fragmentator voltage (cone voltage after capillary), 200 V; nebulizer pressure, 50 psig; capillary voltage, 3500 V; gas temperature, 350 ºC; drying gas flow, 12 L/min; skimmer voltage, 60 V; and octapole voltage, 750 V. The Jet Stream sheath gas temperature and flow were 400 ºC and 12 L/min, respectively. MS/MS was carried out using the Auto mode with the following conditions: 5 precursors per cycle and a collision energy of 4 V for every 100 Da. Internal mass calibration was performed by infusing throughout the analysis a solution containing purine and HP-0921 (hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine) (injected in acetonitrile-water, 90:10 (v/v)) yielding ions at m/z 121.0509 and m/z 922.0098, respectively. All samples were injected by triplicate.

Raw data from RP-HPLC-QTOF were exported to PEAKS Studio Version 7 software from Bioinformatics Solutions Inc. (Waterloo, Canada) for *de novo* sequencing of peptides. Since it is not possible to make differences between isoleucine (I) and leucine (L) amino acids, only isoforms with L were displayed, although peptide sequences containing I amino acid instead of L are also possible. Isoelectric points and the water solubility were obtained using Innovagen's peptide property calculator. Data were also analyzed by PEAKS DB (database tool) using FASTA database that included protein sequences from *Olea Europaea* organism obtained from UniProt database. Peptides sequences were associated to a protein if the error tolerance was < 10 ppm and the mass tolerance was 0.5 Da for the fragments. Peptides with a -10lgP equal or higher to 15 confirmed the confidence between them. Only peptides appearing in all injections (six injections, three injections of each extract) were considered. Moreover, peptides with an ALC (expected percentage of correct amino acids in the peptide sequence) above 90% in, at least, four injections and above 70% in the rest of injections were taken into account.

2.5 Univariate and multivariate statistical analysis

Statistical analysis was performed using Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc., Warranton, VA, USA). Data comparison was carried out by one-way analysis of variance (ANOVA). Duncan's Multiple Range test was used to determine statistically significant differences (p-value < 0.05) between mean values from different samples at 95% confidence level. Data were presented as mean ± standard deviation of, at least, three independent experiments.

Pairwise correlations between peptides from the olive seed hydrolysates and cholesterol-lowering capacity were calculated by Pearson's correlation coefficient test using Stata software (version 12, StataCorp, Lakeway Drive, College Station, Texas, USA).

Multivariate statistical analysis was performed using SIMCA 14.0 software (MSK Data Analytics Solutions, Umetrics, Umeå, Sweden). The peak areas of peptides correlated with hypocholesterolemic capacity in the 20 hydrolysates and their hypocholesterolemic capacities were used as variables. Unsupervised multivariate principal components analysis (PCA) and hierarchical clustering (HCA) was performed. PCA was carried out without transformation and Ward distance was the criterion in HCA. Unsupervised multivariate PCA models were depicted as score and loading plots.

3 Results and discussion

Previous work using olive seeds from a Manzanilla variety has enabled to obtain a hydrolysate with high and multifunctional lipid-lowering properties.[12, 13] In order to find out whether this property is common to other olive seeds and to evaluate the role of the different peptides in the whole hypocholesterolemic capacity of hydrolysates, 20 different olive genotypes have been studied in this work. Peptides released from olive seeds genotypes were analysed to evaluate their capacity to reduce dietary cholesterol absorption and to inhibit endogenous cholesterol biosynthesis. After peptide identification, univariate and multivariate chemometric tools have been applied to find out the role of these peptides in the whole lipid-lowering capacity.

3.1 Evaluation of lipid-lowering functionalities in hydrolysates

Figure 1 shows the functionality of the 20 olive seed hydrolysates to reduce endogenous and exogenous cholesterol. All hydrolysates presented capacity to reduce the micellar cholesterol solubility that ranged from 11 to 49 %. This capacity has been related to the presence of hydrophobic and amphiphilic peptides. The varieties which displayed a significantly higher ability to reduce the micellar cholesterol solubility (44 – 49%) were Nevado Azul, Cornicabra, Cañivano Negro, Racimal de Jaén, and Picual (p ≤ 0.05), while Kalokerida, Lechín de Sevilla, and Verdiell showed lower capacity (11 – 30%). The presence of hydrophobic amino acids in the sequence of peptides has also been correlated to their ability to bind bile acids.[14] Indeed, the olive seed hydrolysates that showed the highest capacity for binding bile acids (those obtained from Verdiell, Caballo, Reixonenca, Picual, and Khalkali varieties) also exerted high capacity to reduce the micellar cholesterol solubility. On the other hand, the capacity to inhibit the cholesterol esterase enzyme was not very high in any variety (from 15 to 31 % of enzyme inhibition) and more than

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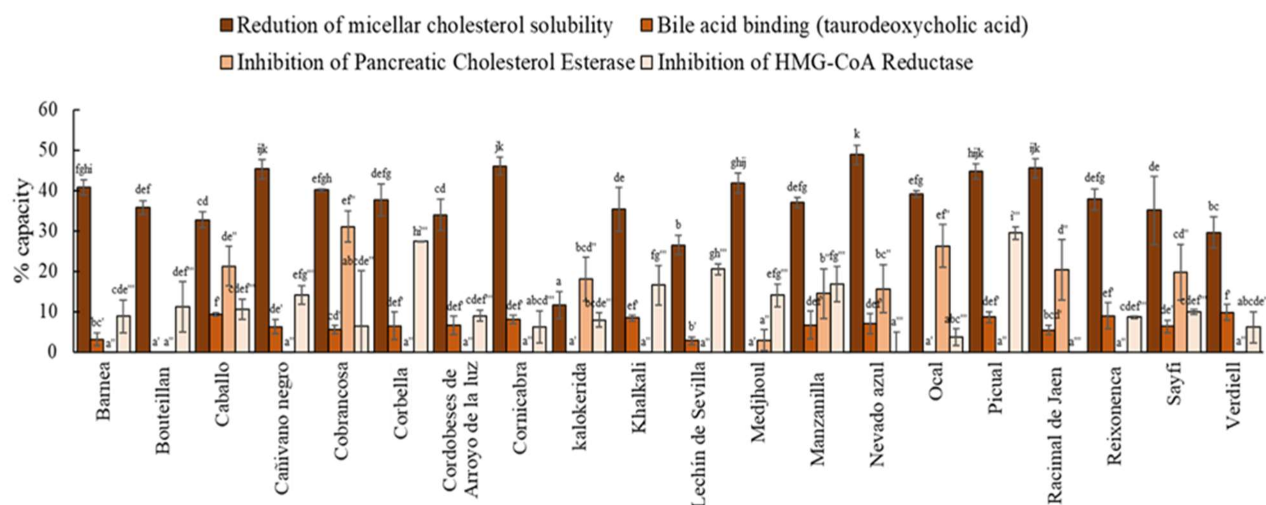

Figure 1. Capacity to reduce cholesterol levels through four different methods exerted by hydrolysates obtained from 20 olive seed varieties. Different letters represent significant differences (p ≤ 0.05).

half did not show any (Barnea, Bouteillan, Cañivano Negro, Corbella, Cordobeses de Arroyo de la Luz, Cornicabra, Khalkali, Lechín de Sevilla, Medhoul, Picual, Reixonenca, and Verdiell). All varieties, except Nevado Azul and Racimal de Jaén, had capacity to inhibit HMG-CoA R enzyme observing the highest value in the hydrolysate obtained from the Lechín de Sevilla olive seed. Hydrolysates obtained from Caballo, Cobrancosa, Manzanilla, and Sayfi seeds exerted capacity to reduce dietary cholesterol absorption by the three employed mechanisms and to inhibit endogenous cholesterol biosynthesis. Hypocholesterolemic capacity of hydrolysates obtained with alcalase from Manzanilla variety have been evaluated by different methods in previous works [12, 13] and the results obtained were similar to the obtained in this work with the Manzanilla variety.

3.2. Identification of peptides by RP-HPLC-MS/MS

Peptides present in the hydrolysates corresponding to the 20 olive varieties were identified by RP-HPLC-ESI-QTOF-MS/MS. Table S1 shows the sequence of the 103 peptides identified using the *de novo* tool along with their retention time range, experimental m/z, theoretical molecular mass, error/accuracy (ppm), ALC range, isoelectric point, water solubility, and the names of the olive varieties in which they appeared. These 103 peptides could not be identified by database searching using UNIPROT database since olive seed genome is not sequenced yet and proteins in this database refer to the *Olea Europaea* pulp or leaf. Nevertheless, the use of this database enabled the identification of 27 additional peptides, not identified by *de novo*. These peptides, that are present in the olive pulp and leaf, seem to be also in the olive seed. The sequence of these peptides along with their retention time range, experimental m/z, theoretical molecular mass, error/accuracy (ppm), isoelectric point, water solubility, proteins in which the peptides were found, and the names of the olive varieties in which they appeared are grouped in Table S2. A total of 130 different peptides were found within all hydrolysates. All peptides had between 4-12 amino acids. Every variety showed between 24 – 50 peptides within a mass range from 349-1351 Da. Almost all peptides presented the molecular ion $[M+H]^+$, except the peptides MKLADVPLCLVN and PNYQPTPR that showed the molecular ion $[M+2H]^{2+}$. Figure 2 shows the based peak chromatogram (BPC) corresponding to the hydrolysate obtained from the Verdiell variety and the tandem mass spectra of peptides WNVN (t_R = 14.4 min) and VFDGE (t_R = 6.9 min). They were the only peptides observed in all hydrolysates.

More than 50% of peptides had poor solubility in water and more than 66% presented an isoelectric point lower than 4.0. The peptides mainly contained hydrophobic (> 57%) (alanine, A (7%); leucine/isoleucine, L/I (21%); phenylalanine, F (6%); proline, P (8%); methionine, M (2%); valine, V (12%); and tryptophan, W (1%)), acidic (~10%) (glutamic, E (5%) and aspartic, D (5%) acids), and basic amino acids (8%) (lysine, K (4%); histidine, H (2%) and asparagine, R (2%)) (see Tables S1 and S2). The presence of high amounts of hydrophobic amino acids has been related to the capacity to reduce cholesterol by bile acid binding and by micellar cholesterol solubility inhibition and could justify the results previously observed.[14] Other common feature within hypocholesterolemic peptides is their amphiphilic character. Indeed, different hypocholesterolemic peptides from marine, animal, and plant sources showing a hydrophobic N-terminal and a hydrophilic C-terminal have been described.[10, 11, 15] In the case of the olive seed hydrolysates, around 33% of identified peptides showed this amphiphilic character, which could also contribute to explain their lipid-lowering capacity (Table S1 and S2).

Sequenced peptides were checked against BIOPEP database.[16] FDGEVK, VPLSPT, and VVVVPH were previously identified as antioxidant peptides in olive seed hydrolysates. Moreover, the peptides LPLL and LVVD were part of the N-terminal and C-terminal parts, respectively, of hypotensive peptides. In addition, some peptides were found within longer peptides with different bioactivities. KALM, LLDA, NLLN, and SVLY are part of peptides with antibacterial capacity. KGAL is also part of antibacterial peptides and even antioxidative and alpha-amilase inhibitor peptides. SSPL is part of antibacterial and haemolytic peptides. Moreover, EAKLA, LELL,

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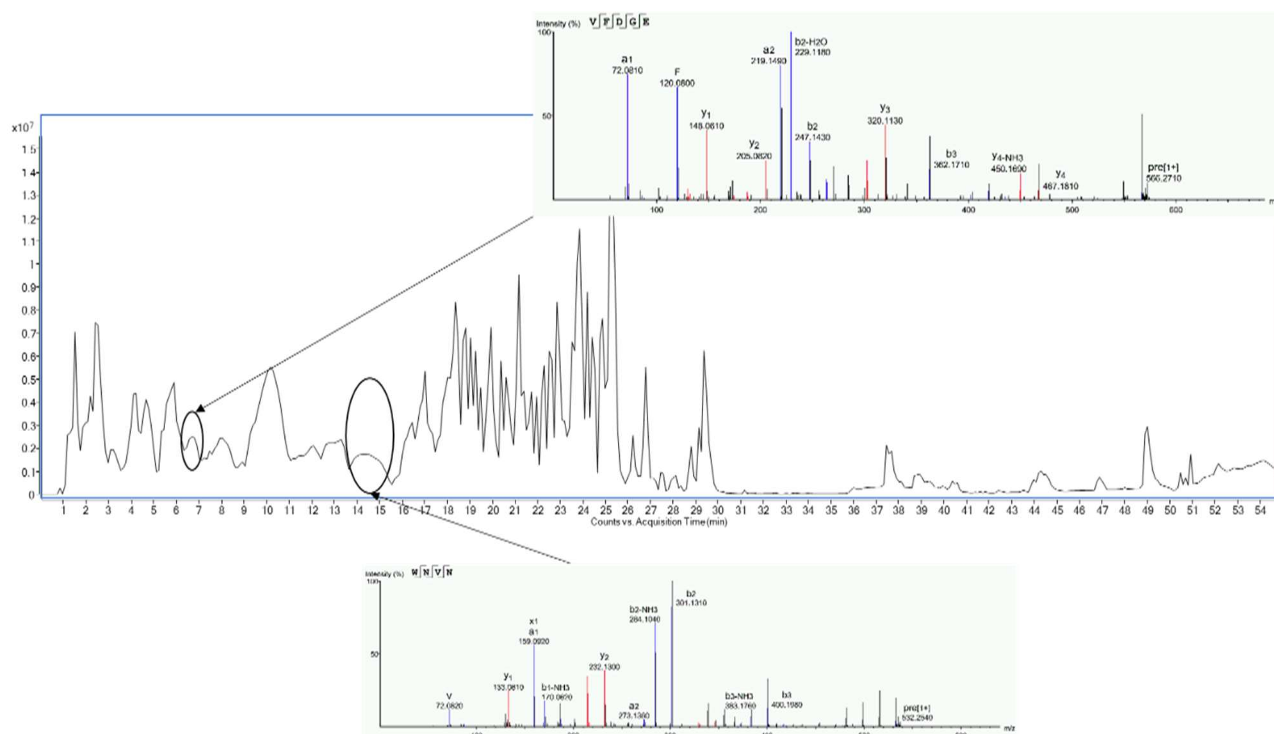

Figure 2. Base Peak chromatogram (TIC) corresponding to the separation of peptides from the olive seed hydrolysate obtained from Verdiell variety and the fragmentation spectra of peptides WNVN and NDGFE (present in all varieties with ALC ≥ 90%).

and VVLQ are part of two membrane-active peptides and a celiac toxic peptide, respectively.

Proteins in olive seed were identified in a previous work of our research group.[17] The comparison cof peptides in Table S1 with proteins identified in that work, enabled to associate 7 peptides with olive seed proteins: **ADIY** (in protein Triticin OS = Triticum aestivum PE = 2 SV = 1 (tr|B2CGM5|B2CGM5_WHEAT)), **ELLI** (in five histones H3 (tr|B9GVX4|B9GVX4_POPTR, sp|P06353|H33_HORVU, tr|Q4JKA5|Q4JKA5_RHEAU, tr|D8QUA3|D8QUA3_SELML, and tr|E9MZ24|E9MZ24_9CHLO)), **IILPQ** (in transposon protein, putative, CACTA, En/Spm sub-class OS=Oryza sativa subsp. japonica GN=LOC_Os10g04760 PE=4 SV=1 (tr|Q7XH13|Q7XH13_ORYSJ)), **ISPL** (in two predicted protein OS=Populus trichocarpa GN=POPTRDRAFT_818720 PE=3 SV=1 (tr|B9H8M5|B9H8M5_POPTR) and GN=POPTRDRAFT_830076 PE=3 SV=1 (tr|B9GS11|B9GS11_POPTR)), **TLPIL** (in 11S globulin isoform 4 OS=Sesamum indicum PE=2 SV=1 (tr|Q2XSW6|Q2XSW6_SESIN)), **VLAL** (in three histones H3 (tr|E9MZ24|E9MZ24_9CHLO, sp|P06353|H33_HORVU, and tr|B6UH77|B6UH77_MAIZE)), and **VYIE** (in 11S globulin seed storage protein 2 OS = Sesamum indicum PE = 2 SV = 1 (sp|Q9XHP0|11S2_SESIN)).[17]

Despite the identification of peptides has been important to justify hypocholesterolemic properties of hydrolysates observed in Section 3.1, further studies are needed to find out their roles in the whole bioactivity.

3.3 Evaluation of the role of peptides in the hypocholesterolemic activity of hydrolysates

In order to determine which peptides are more significant in the reduction the exogenous and endogenous cholesterol, different chemometric tools were next applied.

3.3.1 Univariate analysis

The relationship between the presence of peptides in the olive seeds hydrolysates and their capacity to reduce cholesterol micellar solubility, to bind bile acids, and to inhibit cholesterol esterase and HMG-CoA R enzymes was studied using correlation analysis. For that purpose, peptides appearing in, at least, three different varieties were considered. Thus, the correlation analysis was carried out with 78 peptides from the 130 peptides identified within varieties (see Table S1 and S2). Those peptides that showed a strong correlation (positive or negative) with, at least, one mechanism to reduce cholesterol were represented in Figure 3. Only 40 peptides were correlated (r > 0.5) with, at least, one of the capacities. Peptide NFVVLK displayed the strongest correlation with the capacity to reduce the micellar cholesterol solubility and the highest area was

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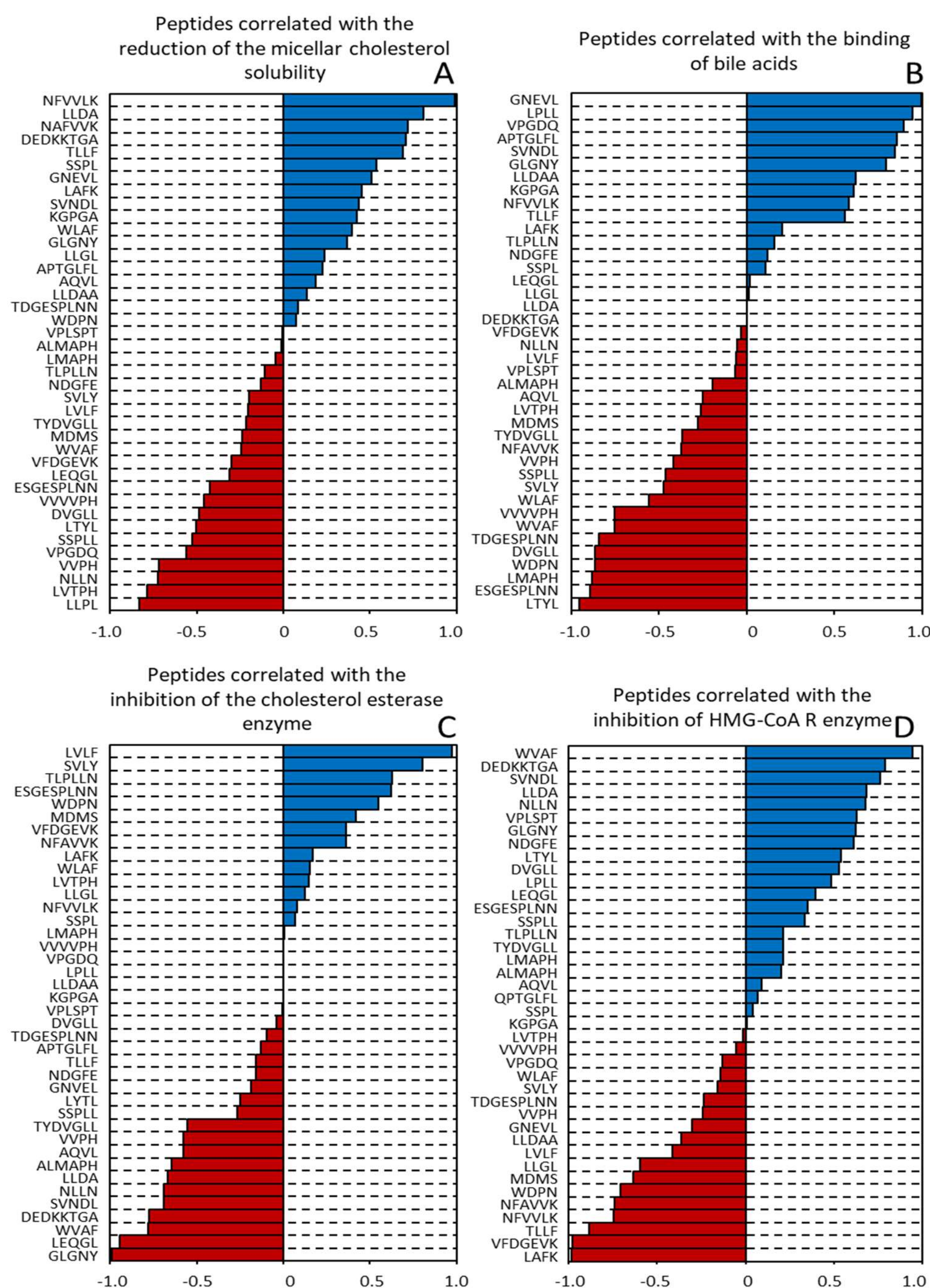

Figure 3. Pearson's correlation (r) between 40 peptides identified in olive seed hydrolysates and the capacity to (A) reduce the micellar cholesterol solubility, (B) bind bile acids, (C) inhibit the pancreatic cholesterol esterase, and (D) inhibit the HMG R. Peptides showing strong correlation presented bigger area.

observed in the Nevado Azul variety (data not shown). Indeed, this variety had demonstrated a high capacity to reduce the micellar cholesterol solubility in Section 3.1. There was not any peptide showing a strong correlation with the four measured capacities (see Figures 3 and S3). Only SSPL peptide showed positive correlation with all hypocholesterolemic capacities (see Figure S3) whereas VVPH and VVVVPH peptides had negative or zero correlation coefficients with all capacities. It was remarkable the fact that the 30% of peptides showed an opposite correlation between the capacities to reduce the endogenous and exogenous cholesterol (Figure S3). For instance, the peptides ALMAPH, LMAPH, LTYL, NLLN, SSPLL, TYDVGLL, VPLSPT, and WVAF displayed a positive correlation with the mechanism to inhibit cholesterol biosynthesis and a negative or zero correlation with the capacity to reduce dietary cholesterol absorption. The opposite behaviour was shown by LAFK, LLDAA, LLGL, and NFVVLK peptides; they presented a positive correlation with the capacity to reduce the dietary cholesterol and a negative correlation with the capacity to reduce the cholesterol biosynthesis. The best peptide for reducing exogenous cholesterol absorption was NFVVLK while WVAF exhibited the highest capacity to reduce endogenous cholesterol.

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Additionally, 73% of the 40 peptides showed simultaneously capacity to reduce micellar cholesterol solubility and to bind bile acids (see Figures 3 and S3). This seems to be related to the hydrophobic character of peptides [18] and had been observed in Section 3.1. Unlike this behaviour, most peptides (around 80%) displayed opposed correlation coefficients (correlation coefficients < - 0.5) in the capacities to inhibit the cholesterol esterase and the HMG-CoA R enzymes. Thus, those peptides with high capacity to inhibit the HMG-CoA R enzyme had low capacity to inhibit the cholesterol esterase enzyme.

3.3.2 Multivariate Analysis

Multivariate analysis was carried out using the 40 peptides that correlated with, at least, one of the hypocholesterolemic capacities. Cluster analysis (HCA) using the Ward method was able to form 4 clusters among peptides (see Figure 4). On the other hand, PCA enables to select most important variables (principal components) and to separate samples (hydrolysates from different olive varieties) according to their peptides profiles and their capacity to reduce blood cholesterol.[19] A total of 4 components explained the 77.6% of the total data variability (Figure 5A). PCA grouped the varieties in a similar way to the HCA (same colours were employed in the HCA and PCA). All groups in HCA were clearly observed in the score plot of PCA. Principal Component 1 was able to differentiate groups 1 and 2 from groups 3 and 4 while Component 2 almost separated groups 2 and 4 from groups 1 and 3. The loading plot of PCA is presented in Figure 5B in order to clarify why the hydrolysates from the different olive seed varieties have been grouped in that way. Peptides close to one capacity in the PCA loading plot are positively correlated with this capacity and vice versa. The varieties belonging to every group showed similar capacities to reduce cholesterol. For example, group 4 displayed the lowest capacity to inhibit the HMG-CoA R enzyme and the varieties pertaining to this group were opposite to this capacity in the PCA loading plot (observed by overlapping Figures 4B and 4C). Similarly, group 2 exhibited low capacity to reduce the micellar cholesterol solubility and to bind bile acids; whereas all varieties from group 1 presented high capacity to inhibit the HMG-CoA R enzyme and low or any capacity to inhibit the cholesterol esterase enzyme. Additionally, group 4, which is opposite to group 2 in the PCA score plot (Figure 5A), showed a high capacity to reduce the micellar cholesterol solubility and to bind bile acids, just in the other way around that group 4 (Figures 5A and 5B). In the same way, Ocal, Cobrancosa, and Racimal de Jaén varieties, which are part of group 4, showed the highest capacities to inhibit cholesterol esterase (observed by overlapping Figures 5A and 5B). They were even better than the rest of varieties in group 4 since they were closer to this capacity in the loading plot. However, varieties of group 1 (Lechín de Sevilla, Corbella, Picual, Khalkali, and Cañivano Negro), located at the opposite side, had the lowest capacity to inhibit cholesterol esterase. Therefore, the varieties with the highest capacities in all assays to reduce cholesterol were Manzanilla, Cobrancosa, Caballo, and Sayfi, which were placed in the middle of the PCA (Figure 5A). These results are in agreement with the observed in the Section 3.3.1.

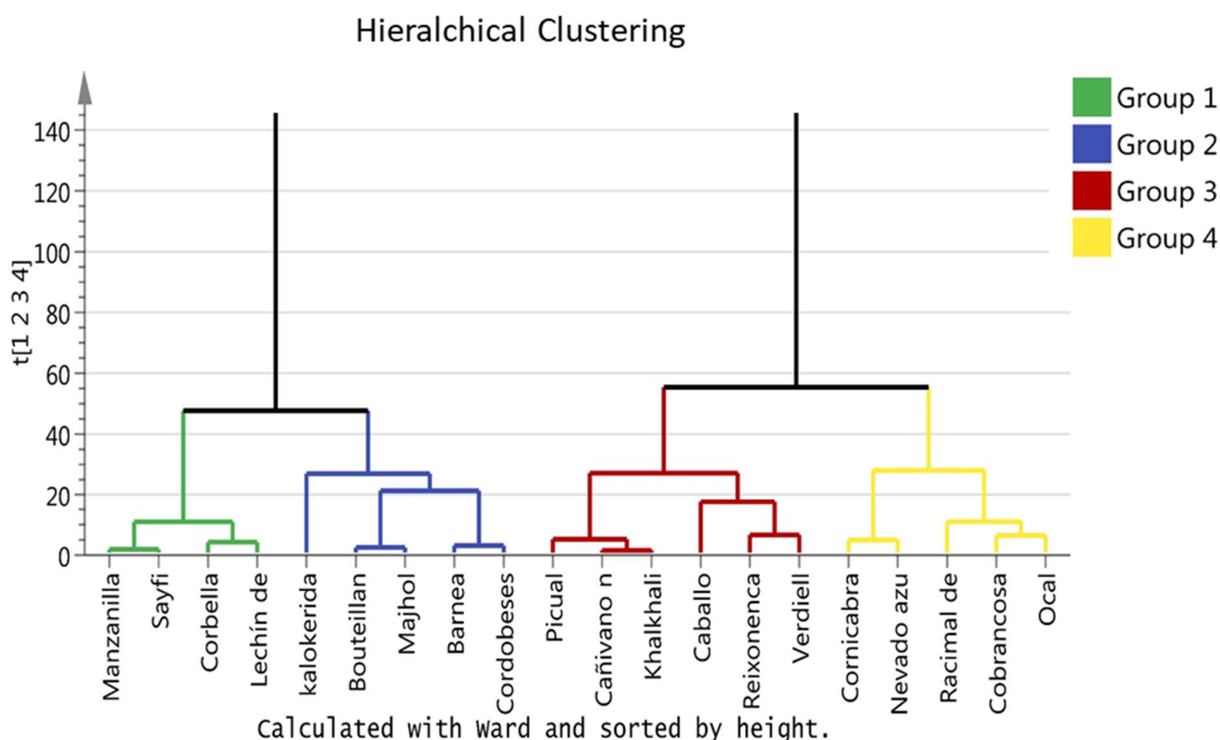

Figure 4. Dendrogram obtained by PCA using the ward method of 40 peptides correlated with hypocholesterolemic capacities to reduce the micellar cholesterol solubility, bind bile acids, inhibit the pancreatic cholesterol esterase and inhibit the HMG R from 20 varieties of olive seeds.

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According to the PCA loading plot, the capacity to inhibit the cholesterol esterase enzyme was situated in the opposite side to the capacity to inhibit HMG-CoA R enzyme (see Figure 5B). Thus, when a peptide is good to inhibit one enzyme, it will have a low or null capacity to inhibit the action of the other. An example is peptide VFDGEVK that is close to the capacity to inhibit cholesterol esterase and far from the inhibition of the HMG-CoA R. On the other hand, the capacity to reduce the micellar cholesterol solubility and to bind bile acids were close to each other in the loading plot which means that both capacities were related and peptides with high capacity for one assay, will contribute positively to the other one (Figure 5B). For instance, NFVVLK, GNEVL, and SSPL, which are located near to these capacities in the loading plot are positively correlated with both mechanisms. These conclusions confirm results withdrew from the correlation analysis (see Figure 3 and S3).

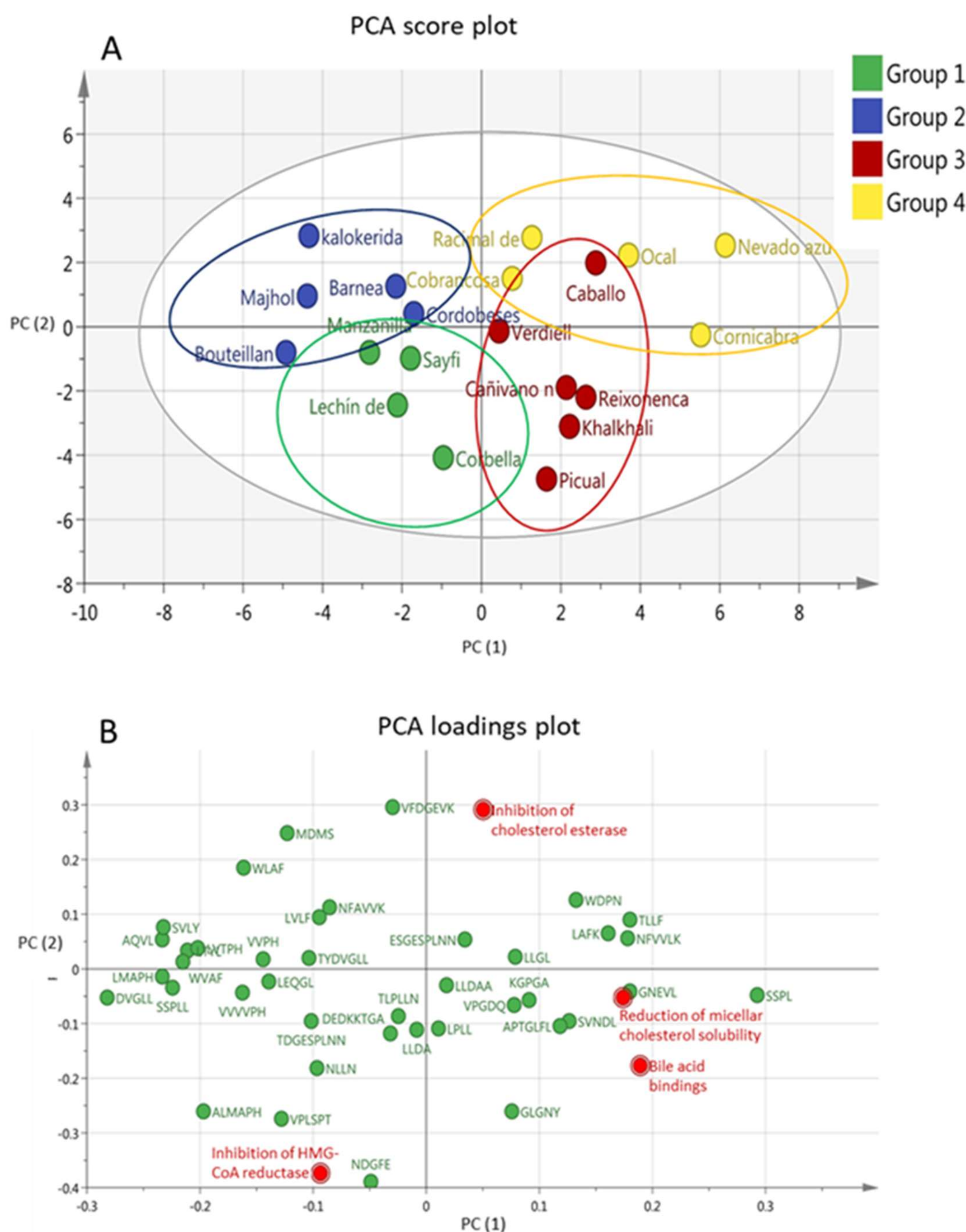

Figure 5. (A) Score plot resulting from a PCA (arbitrary groups are represented by ellipses and the colours showed the groups obtained in the HCA) of 40 peptides correlated with hypocholesterolemic capacity and their capacities to reduce the micellar cholesterol solubility, bind bile acids, inhibit the pancreatic cholesterol esterase and inhibit the HMG R from 20 varieties of olive seeds. (B) Loading plot obtained from a PCA (green colour represents peptides while red colour means hypocholesterolemic capacities).

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Conclusions

This work demonstrates that the presence of certain peptides in olive seed hydrolysates modulates their lipid-lowering properties. Forty peptides among the 130 identified within all hydrolysates showed a significant effect, positive or negative, on the capacity of hydrolysates to reduce exogenous cholesterol absorption (by the reduction of the micellar cholesterol solubility, the binding of bile acids or the inhibition of cholesterol esterase enzyme) or to inhibit cholesterol biosynthesis (by the inhibition of HMG-CoA R enzyme). Univariate analysis enabled to observe that peptide NFVVEK displayed the strongest correlation with the capacity to reduce the micellar cholesterol solubility and, in general, with the capacity of hydrolysates to reduce exogenous cholesterol absorption while the presence of peptide WVAF was related with the capacity of hydrolysates to reduce endogenous cholesterol. Multivariate analysis confirmed these results and enabled to observe a strong negative correlation between the capacity of peptides to inhibit the cholesterol esterase and the HMG-CoA R enzymes and a strong positive correlation between the capacity of hydrolysates to reduce the micellar cholesterol solubility and to bind bile acids. An opposite correlation between the capacity of hydrolysates to inhibit the absorption of dietary cholesterol and to inhibit cholesterol biosynthesis was observed in some cases. According to their peptide profile and hypocholesterolemic ability, olive varieties were grouped into 4 groups. Results show that every olive genotype can release peptides with a different lipid-lowering capacity and that their selection is a powerful tool to tune the hypocholesterolemic properties.

Conflicts of interest

There are no conflicts to declare.

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