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Identification of olive (*Olea europaea*) seed and pulp proteins by nLC-MS/MS via combinatorial peptide ligand libraries

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ABSTRACT

Different types of extraction protocols are described for identifying proteins in seed and pulp of olive (*Olea europaea*), by employing both conventional extraction methods and capture with ProteoMiner as well as with in house-made combinatorial peptide ligand libraries (HM-CPLLs) at pH 7.4 and at pH 2.2. Thanks to the use of CPLLs, able to dramatically amplify the signal of low-abundance species, a quite large number of compounds has been indeed identified: 61 in the seed (vs. only four reported in current literature) and 231 in the pulp (vs. 56 described so far), the deepest investigation up to the present of the olive proteome. In the seed, it highlights the presence of seed storage proteins, oleosins and histones. In the pulp, the allergenic thaumatin-like protein (Ole e 13) was confirmed, among the other 231, as the most abundant protein in the olive pulp. The present research has also been undertaken with the aim of identifying proteins in olive oil and ascertaining the relative contribution of seed and pulp proteins in their presence, if any, in oils.

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1. Introduction

Olive (*Olea europaea* L.) is a long-life tree from which humans have obtained wood and oil for more than 5000 years [1]. The olive is the only tree of *Oleaceae* family with edible fruit. Olive fruits have been used for several years to produce olive oil and table olives. Generally, in manufacturing olive oil the whole olive fruit is subjected to a mechanical press, thus passing both pulp and seed components to the olive oil. The consumption of olive oil has been associated with the decrease of the incidence of important illnesses like cardiovascular diseases, cancer, and Alzheimer's disease in the Mediterranean area [2].

The olive fruit has been investigated in depth to determine its composition, its nutritional and sensory values and its benefits for human health. So far, some compounds such as fatty acids, polyphenols, or sterols have been studied in

extenso [3,4]. However, despite their highly informative value, their nutritional value [5], and their suggested role in food stability [6–8] and allergenicity [9], proteins have scarcely been explored in comparison with the other olive components [10–12]. The information available about the amount of proteins able to pass from the fruit to the oil during the process of oil extraction is very scarce. In 2001, proteins were established, for the first time, as minor components in olive oils [13], demonstrating the ability of transferring a minor part of proteins from the fruit to the oil.

Since the beginning of the last decade, proteomics has rapidly become a rising science. However, plant proteomics has represented only a small percentage of the performed proteomics studies up to date, but it is growing significantly [14–17]. Until now, just one work has been focused on assessing the olive proteome [11], although only a part of it could be

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identified as a consequence of the difficulty in protein extraction. This previous study was limited by the two main handicaps of plant proteomic studies: the limited database due to the lack of sequenced genomes and the difficulty in obtaining high quality protein extracts. Biological samples contain plenty of interfering substances that hamper protein separation by gel electrophoresis [18]. Plant tissues, moreover, generate additional problems: the relatively low concentration of soluble proteins [19], the abundance of proteases [20], and the presence of different plant-specific cellular components such as polysaccharides, lipids, polyphenols and secondary metabolites that can interfere in protein separation and analysis [21]. In addition, plant tissues also present the general problems of proteome analysis, where the most abundant proteins present in the sample prevent detection and identification of the low-abundance species.

The olive fruit is an especially complex matrix due to its lipidic nature and its high amount of interfering compounds, which have hampered the extraction of proteins [22,23]. Many efforts have been focused on the development of an extraction procedure of proteins from both olive seed and pulp, but especially in the case of olive pulp the results have not been completely successful, yielding just one band when Coomassie blue staining was used [11,23]. As a consequence, new tools for the isolation of proteins from complex vegetable matrices are required. In this field, the use of combinatorial peptide ligand libraries (CPLLs), commercially available under the trade-name ProteoMiner (Bio-Rad), has demonstrated to be an excellent tool, as employed in the extraction and isolation of proteins from a variety of beverages and food-stuff [24–31]. CPLL is a protein enrichment technology used for the compression of the dynamic range of protein concentrations in difficult samples based on the treatment with libraries of hexapeptides bound to chromatographic supports. The CPLLs capture technology has provided a method for detecting low abundance proteins in complex matrices, permitting a dramatic improvement of peptides detected by mass spectrometry analysis (see [32–34] for reviews).

The main target of this work is to establish a suitable method for the isolation of proteins from olive fruit based on the application of an improved CPLL technology and for the detection of olive pulp and seed proteome. This work constitutes a first step in the study of the olive oil proteome.

2. Materials and methods

2.1. Chemicals, biologicals and apparatuses

ProteoMiner™ (combinatorial hexapeptide ligand library beads), Laemmli buffer, 40% acrylamide/Bis solution, N,N,N',N'-tetramethylethylenediamine (TEMED), molecular mass standards and electrophoresis apparatus for one-dimensional electrophoresis were from Bio-Rad Laboratories (Hercules, CA). β -mercaptoethanol, dithiothreitol (DTT), ammonium persulphate, urea, thiourea, 3-[3-cholamidopropyl dimethylammonio]-1-propanosulfonate (CHAPS), isopropanol, acetonitrile (ACN), trifluoroacetic acid (TFA), sodium dodecyl sulphate (SDS), and all other chemicals used all along the experimental work were current pure analytical grade products and were purchased from Sigma-Aldrich (St Louis, MO). Complete protease inhibitor cocktail tablets and sequencing grade trypsin were from Roche Diagnostics (Basel, Switzerland). In house-made-CPLLs (HM-CPLLs) were produced in our laboratory and used as described in [29]. Raw olives from Spanish and Italian origin were purchased in local markets.

2.2. Extraction of proteins and treatment with peptide libraries

Olive seeds were extracted from the olive stone and ground with a domestic mill. Fig. 1 shows a schematic outline of the protein extraction and capturing protocols used in the study of the olive seed. Three different extraction methodologies were employed and used as controls. In the first extraction method (CTRL 1), based on the work of Fonslow et al. [35],

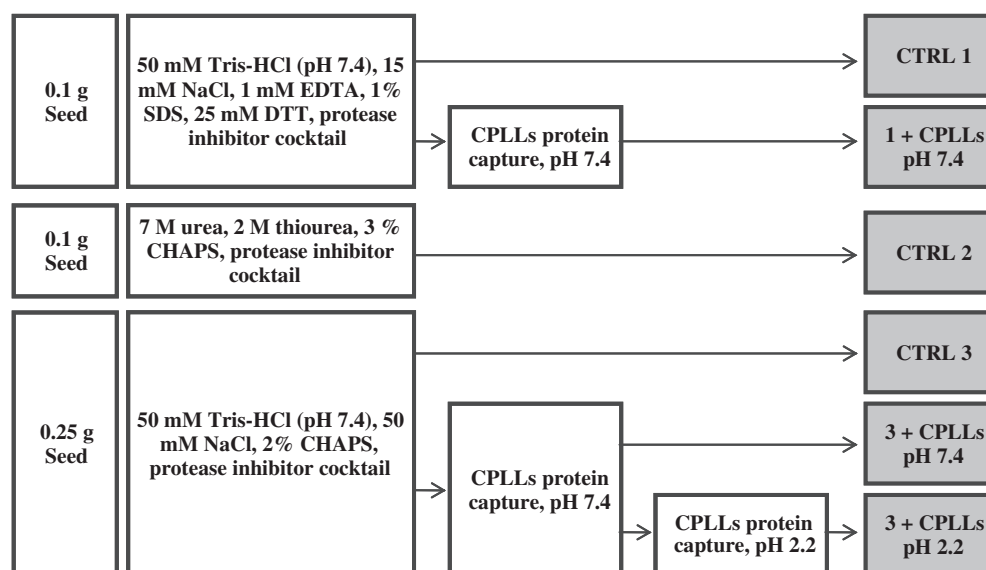


Fig. 1 – Methods designed for the extraction and capturing of proteins from the olive seed.

0.1 g of seed was mixed with 1 mL of an extracting buffer containing 50 mM Tris-HCl (pH 7.4), 15 mM NaCl, 1 mM EDTA, 25 mM DTT, 1% (m/v) SDS, and protease inhibitor cocktail. The mixture was gently shaken during 5 min and centrifuged at 9000 rpm for 5 min to recover the supernatant. In the second extraction (CTRL 2), 0.1 g of seed was mixed with 1 mL of 2-DE buffer (7 M urea, 2 M thiourea, 3% (m/v) CHAPS), and protease inhibitor cocktail. The mixture was also shaken for 5 min and centrifuged to recover the supernatant. A last extraction (CTRL 3) was performed using physiological conditions. To 0.25 g of seed were added 2.5 mL of an extraction buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2% (m/v) CHAPS, and protease inhibition cocktail. Three cycles of 10 s at 70% amplitude using an ultrasonic microprobe were carried out before centrifuging and recovering the supernatant.

We used different combinatorial libraries of hexameric peptides as capturing agents for all extracted proteins. In one of them, the terminal amino-groups are free and is commercially available as ProteoMiner. The rest of combinatorial libraries used were produced in our laboratory (HM-CPLLs), presenting diverse physicochemical properties. The supernatant obtained from the first extraction method, using SDS/DTT, was diluted 1:10 (v/v) with an extraction buffer containing 50 mM Tris-HCl (pH 7.4), 15 mM NaCl, 1 mM EDTA, 25 mM DTT, and protease inhibitor cocktail in order to reduce the amount of SDS to 0.1% (m/v) and allow an effective protein capture [35]. To the resulting solution, 50 μ L of a mixture of three HM-CPLLs and ProteoMiner were added. The capture was performed overnight at room temperature under gentle shaking. The supernatant obtained from the third extraction method, under physiological conditions, was diluted 1:2 (v/v) with the same extraction buffer and also subjected to the protein capture at pH 7.4. After this capture, the remaining solution was subjected to a second capture at pH 2.2 in the presence of 0.1% (v/v) TFA as ion-pairing reagent, by adopting a special hydrophobic library [24–27]. The capture was implemented by adding 50 μ L of such beads via gentle shaking overnight. All libraries were then recovered by filtering with Micro Bio-Spin chromatographic columns (Bio-Rad) at 4000 rpm during 5 min. The latter were then washed twice with their respective buffer to eliminate the unbound protein excess. The captured proteins from each sample were desorbed (twice, with 50 μ L each time) with Laemmli buffer (2% (m/v) SDS, 25% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% (m/v) bromophenol blue and 62.5 mM Tris-HCl, pH 6.8) for 20 min, under boiling conditions [36].

On the other hand, olive pulp protein extraction was performed by freezing 5 g of olive pulp at -20°C followed by grinding without thawing and homogenisation with 15 mL of a buffer containing 0.125 M Tris-HCl (pH 7.4), 5% (v/v) glycerol, and the protease inhibitor cocktail. Afterwards, SDS and DTT, up to concentrations of 3% (m/v) and 25 mM, respectively, were added and the resulting solution was boiled for 10 min. The remaining pellet was removed by centrifugation at 9000 rpm during 5 min. A water/methanol/chloroform precipitation [37] was next applied, obtaining an abundant white precipitate free of SDS. In order to obtain a control sample, a second precipitation with acetone at 4°C during 1 h was performed. The final precipitate was dried at room temperature

and a part of it was dissolved until saturation in Laemmli buffer by heating at 100°C .

For the protein capture with CPLL beads, after water/methanol/chloroform precipitation, the above precipitate was dissolved in 20 mL of 0.125 M Tris-HCl, pH 7.4 containing 1% (m/v) CHAPS and the protease inhibitor cocktail. A capture at pH 7.4 was performed by adding 60 μ L of beads, overnight at room temperature under gentle shaking, as described above. A capture at pH 2.2 was also performed. To the protein solution 0.1% (v/v) TFA was added and the pH was adjusted to 2.2 with formic acid (FA). Sixty μ L of HM-beads were added and the capture was performed overnight at room temperature under gentle shaking. Proteins were recovered as indicated above.

Three technical replicates of the conventional extractions and the subsequent captures with CPLLs were performed. The proteins listed in the supplementary table were only those that could be identified in at least two out of the three experiments.

2.3. SDS-polyacrylamide gel electrophoresis

A total volume of 40 μ L of each sample, in presence of Laemmli buffer (as described above), was loaded onto the gel. The SDS-PAGE gel was composed by a 4% polyacrylamide stacking gel (125 mM Tris-HCl, pH 6.8, 0.1% SDS) cast over an 8–18% resolving polyacrylamide gel (in 375 mM Tris-HCl, pH 8.8, 0.1% SDS buffer). The cathodic compartment was filled with Tris-glycine buffer, pH 8.3, containing 0.1% SDS and the anodic compartment was filled with Tris buffer, pH 8.8. Electrophoresis was at 100 V until the dye front reached the bottom of the gel and 150 V until the end of the separation. Staining and destaining were performed with Colloidal Coomassie Blue and 10% acetic acid in water, respectively. The SDS-PAGE gels were scanned with a VersaDoc imaging system (Bio-Rad Laboratories). The digitized images were further analyzed by the software Quantity One (Bio-Rad).

2.4. Mass spectrometry and data analysis

The sample lanes of SDS-PAGE were cut in pieces along the migration path and proteins were reduced by 10 mM DTT, alkylated by 55 mM iodoacetamide, and digested by 1 ng/ μ L trypsin in 25 mM ammonium bicarbonate, at 37°C overnight. The tryptic mixtures were acidified with FA up to a final concentration of 10% (v/v). Eight μ L of tryptic digest for each band were injected in a nano chromatographic system, UltiMate 3000 RSLCnano System (Dionex). The peptide mixtures were loaded on a reversed-phase trap column (Acclaim PepMap100, C18, 100 $\text{\AA}$, 100 μm i.d. $\times$ 2 cm, Dionex) for the cleanup and pre-concentration. After cleanup, the 2-position valve was switched to place the trap column in series with a fused silica reverse-phase column (picoFrit column, C18, 2.7 μm , New Objective). The peptides were eluted in a 30 min gradient from 4% phase A (2% ACN and 0.1% FA in water) to 60% phase B (2% water and 0.1% FA in ACN) at a constant flow-rate of 300 $\mu\text{L}/\text{min}$. The chromatographic system was connected to an LTQ-XL mass spectrometer (Thermo Scientific) equipped with a nano spray ion source. Full scan mass spectra were acquired in the mass range m/z 350 to m/z 1800 Da and the four

most intense ions were automatically selected and fragmented in the ion trap. Target ions already selected and for mass spectrometry (MS/MS) were dynamically excluded for 30 s. The MS data were analyzed by Mascot search engine (Version 2.3.01), using Proteome Discovery software (v. 1.2.0 Thermo) and consulting Uniprot_viridiplantae database (30264 residues, 184678199 sequences) and EST_oleaeuropea (6016 mRNA sequences). We set cysteine alkylation and oxidation of methionine residues as variable modifications; peptide mass tolerance was set to 1 Da, fragment mass tolerance to 0.8 Da and ion source cut-off of 20. The false discovery rate obtained consulting the decoy database was less than 0.05.

The Gene Ontology analysis was performed using the web available software QuickGO (www.ebi.ac.uk/QuickGO).

3. Results

Fig. 2a shows the SDS-PAGE gels corresponding to the extracts obtained from olive seeds. Lane a shows a first attempt at solubilising the seed proteins by extraction with 1% (m/v) SDS in presence of 25 mM DTT. The solubilised material was then diluted to 0.1% (m/v) SDS and capture of proteins attempted with CPLs at pH 7.4 (lane d). As shown in lane a,

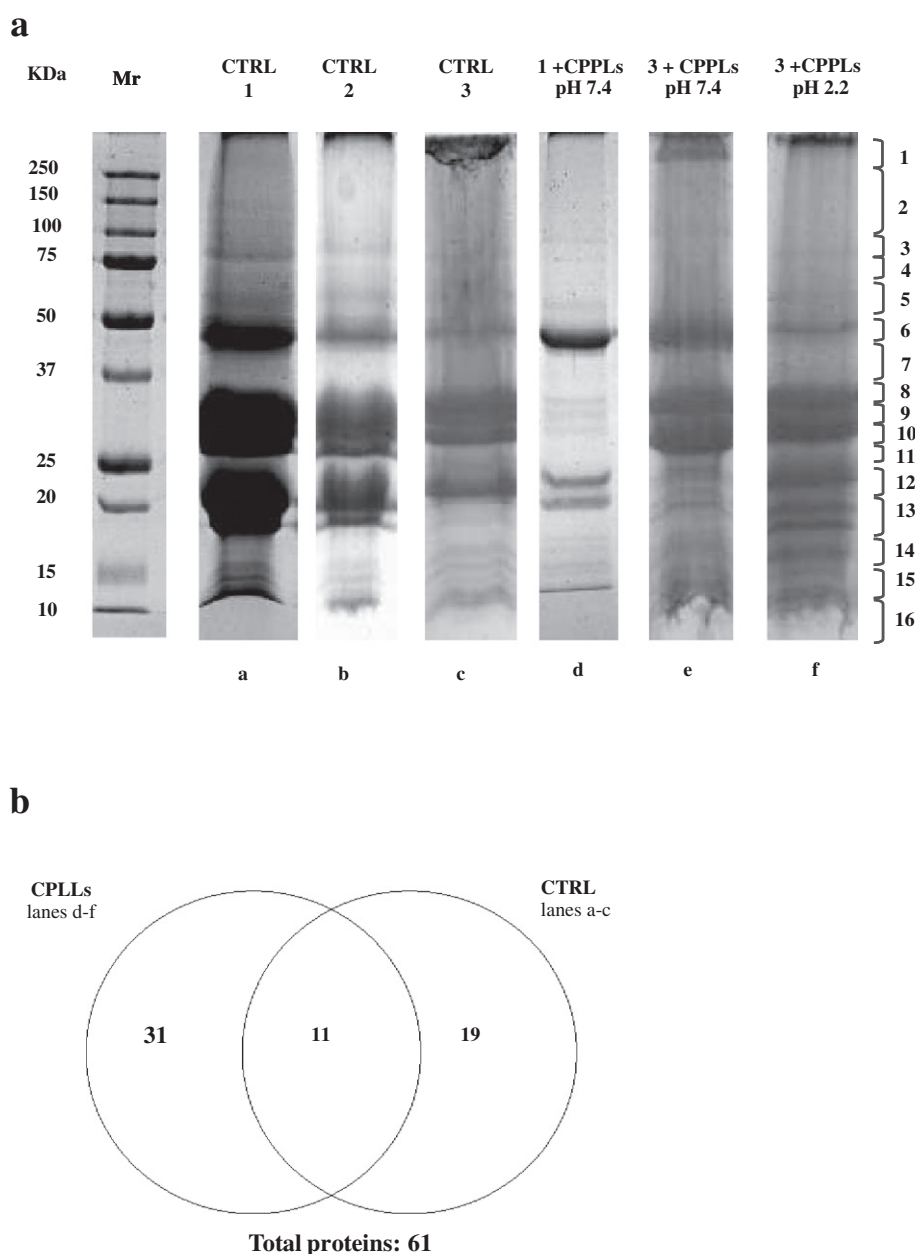


Fig. 2 – a: SDS-PAGE gels corresponding to the extracts obtained from olive seeds. Comparison of three different types of extractions: extraction with SDS-DTT (lane a); extraction with 2-DE buffer (lane b); extraction in physiological conditions (lane c) and after capture with CPLs at pH 7.4 (lanes d and e) and pH 2.2 (lane f). In all cases staining with micellar Coomassie Brilliant Blue was performed. The boxed areas represent gel segments cut out for digestion and nLC-MS/MS analyses.

b: Venn diagrams of the total number of olive seed identified species in the controls vs. those detected after CPLL treatment.

three very intense zones are visible, possibly corresponding to those proteins described as major olive seed proteins: SSPs and oleosins. However, the capture with CPLLs failed to enrich the pattern of zones (lane d), most likely due to interferences from the residual SDS. Up to sixteen gel zones were cut out, the material present in each zone digested with trypsin and the peptides analyzed by nLC-MS/MS. However, not many identifications could be made (see supplementary Table 1), thus other extraction protocols were performed. Fig. 2a also shows the patterns obtained when using: 2-DE buffer (CTRL 2, lane b), a “cytosolic” extraction (CTRL 3, lane c) and finally a capture with CPLLs at pH 7.4 of the “cytosolic” extract (lane e), showing the eluate from the CPLLs beads. It can be observed that the various patterns show strong similarities (especially in the region of the most abundant species, Mr 20–30 kDa) but also some differences. Moreover, this time the pattern exhibited by the CPLLs capture at pH 7.4 of the cytosolic fraction shows indeed an enrichment of species (compare lane e with lane c) no doubt due to the absence of SDS in the extraction buffer, known to interfere with proper CPLLs functioning. All these tracks were divided into segments, again digested with trypsin, and analyzed by nLC-MS/MS (see Table 1). The number of IDs increased to 30 unique gene products. In order to see if we could increase the number of detected species, we also adopted a capture with our in house-made CPLL beads at pH 2.2, as previously described by us (lane f). In Fig. 2a, lane f shows many more SDS bands as compared to CTRL 1 (lane a). The gel was cut into pieces and again subjected to nLC-MS/MS analyses after trypsin digestion. The total results in terms of identifications are shown in the Venn diagrams of Fig. 2b: it can be appreciated that the various captures with CPLLs permitted the additional identification of 31 unique gene products that could not be found via conventional solubilisation protocols in the absence of enrichment of low-abundance species, a task for which CPLLs are highly suited.

Having exhausted the screening of seed proteins, we next turned to analysis of pulp proteins. In this case, after solubilisation, sample clarification via centrifugation, and protein precipitation, the CPLL capture was performed with our CPLL beads at pH 2.2 and pH 7.4, since this method was found to be the one yielding the highest number of identified proteins. The SDS-PAGE pattern of the control and the material recovered from the CPLL beads at pH 2.2 is shown in Fig. 3a. While the control only shows one clear band corresponding to the one that had been already analyzed [11], several sharp bands could additionally be seen in the Mr range 10–100 kDa when the CPLL technology was employed at pH 2.2. For this gel, 13 gel zones were excised, and the resulting peptides from trypsin digestion analyzed by nLC-MS/MS. As shown in Supplementary Table 2 and Fig. 3b, a total number of 231 unique gene products could be identified. In this case, the various captures with the CPLL beads have permitted the identification of 209 unique gene products while the control barely 54 proteins. It is thus here shown that a proper CPLL treatment allows detection of 400% more species than in the control, with a minimal loss of the species present in the sample treated with CPLLs (~10%), a fact confirmed in most of the research in different proteomes [24,32,33,41], where, typically, 300 to 600 more species could be detected after CPLL treatment. In

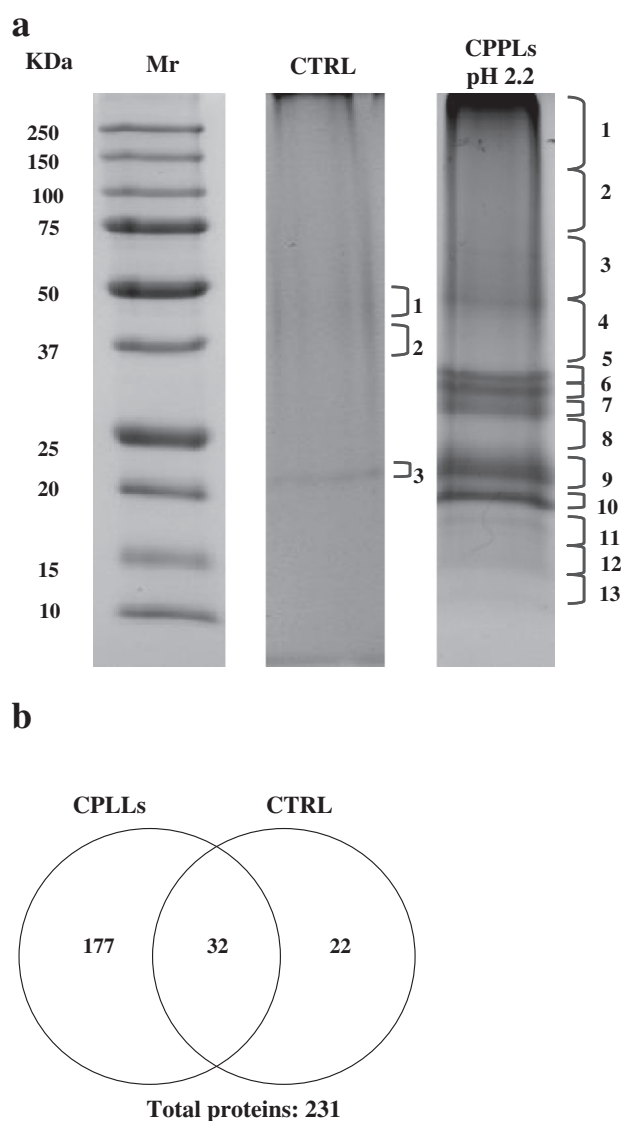


Fig. 3 – a: SDS-PAGE gel corresponding to the extracts obtained from olive pulp. Comparison of the pulp protein extract used as control and the extract treated with CPLLs at pH 2.2. Staining with micellar Coomassie Brilliant Blue was performed. The zones labelled represent gel segments cut out for digestion and protein identification via nLC-MS/MS. b: Venn diagrams of the total number of olive pulp identified species in the control vs. those detected after CPLL treatment.

addition in the Supplementary Fig. 1 four MS/MS spectra of identified proteins with only one peptide are showed.

Both in the case of seed and pulp proteins a Gene Ontology (GO) analysis was performed. As it can be observed in Fig. 4, it is possible to differentiate the two proteomes by their different functional categories. “Membrane”, “integral membrane”, “DNA binding”, and “cytosol” categories are present only in the seed proteome, while “ribosome”, “metabolic process”, “carbohydrate metabolic process”, and “chloroplast” categories are present only in the pulp proteome. Some functional categories, as ‘DNA binding’ reflect the processes performed in each tissue, like the one related with DNA storage in the seed.

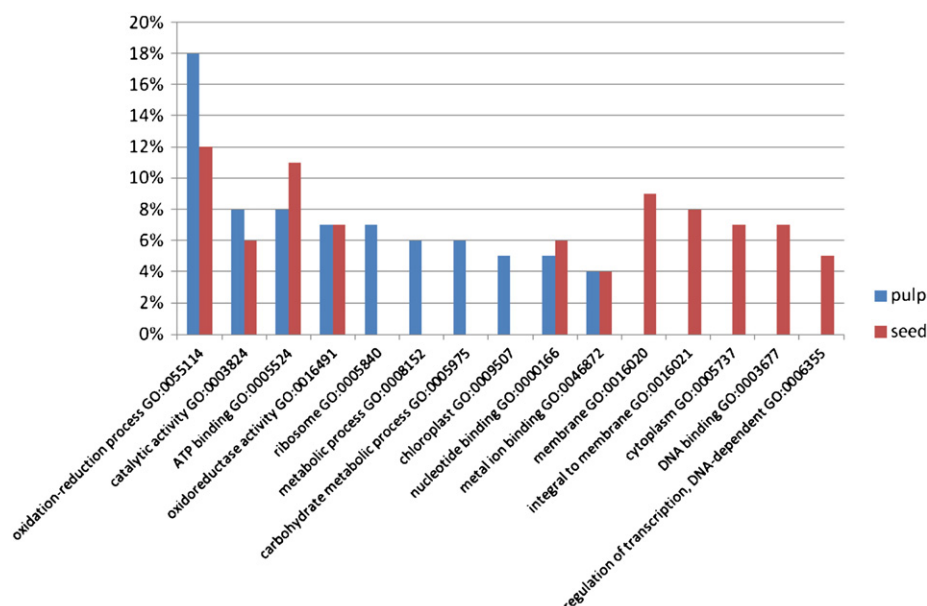


Fig. 4 – Gene Ontology (GO) functional classification of all proteins identified respectively in the pulp and in the seed (see Supplementary tables).

4. Discussion

It is noteworthy that, in previous investigations, only four seed proteins have been identified, two seed storage proteins (SSPs) called Solea I and Solea II [10,38,39] and two oleosins of 22 and 50 kDa [40]. Thus our data, identifying 61 unique gene products, represent an important increment in the exploration of seed species. As for the pulp proteins, previous work had also identified a quite high number of species (a total of 56) [11]. However, all these compounds were detected in just a single, rather broad band in the SDS-PAGE gel (see Fig. 1 in ref. [11]) centred at around 23 kDa. In our case, our extraction protocol, followed by CPLL treatment, has permitted revelation of a very high number of fine bands via SDS-PAGE profiling (see Fig. 3a) allowing thus a substantially increased identification of species (up to 231, as listed in Table 2), an increase of 400% on what could be identified previously. In both cases, seed and pulp proteins, it is here amply confirmed that the CPLL treatment permits identifying a much larger number of species than in control, untreated sample, even in such recalcitrant tissues as plant material.

Regarding protein identification, we highlight the fact that the vast majority of proteins could only be attributed by homology with other plant species, such as *Populus trichocarpa* (eleven proteins), *Zea mays* (five), *Vitis vinifera* (four), *Oryza sativa* (four) and so on. In fact, barely 1.4% of seed proteins and 4.0% of pulp proteins were attributed to the specie *Olea europaea*. This is one of the major problems when working with plant proteins, as no genomic data are available for the vast majority of them. In the seed, a high number of isoforms of histones were identified, as gleaned from Table 1 (in parenthesis the number of peptides): histone H4 (4); histone H3 (6) and histone H3.3 (6). A total of 14 histones, all with different accession numbers, have been identified, definitely an uncommon number of species to be found in any tissue. As the

seed harbours the genetic material for plant development, it is reasonable to assume that it should be very rich in DNA and thus it should contain a high level of histones. As it could be expected, because they have been established as the most abundant proteins in the seed, also different isoforms of globulin seed storage proteins were identified: globulins (2 isoforms), 11S globulins (3), and 7S globulins (1). On the other hand, just one protein has been identified as oleosin, with a molecular mass of 17.2 kDa, corresponding just to one of the two oleosins described before [40]. Conversely, in the pulp, a much higher number of proteins could be identified. Among the 231 proteins, just 9 of them were identified as proteins corresponding to the specie *O. europaea*. Of those 9, three were previously reported [11]: an acyl-carrier protein and two proteins that could be related with olive allergy, a thaumatin like protein (*Ole e 13*) and a Cu/Zn superoxide dismutase, described in the olive pollen as the allergen *Ole e 5* [9]. The non-described proteins were: an isoflavone reductase-like protein, involved in the biosynthesis of isoflavonoid phytoalexins in plants; a putative defensive protein; a beta-1,3-glucanase, with antifungal activity; an enoyl ACP reductase, involved in the fatty acid synthesis system and an important target in antibacterial drug discovery; a ribulose-1,5-bisphosphate carboxylase/oxygenase (commonly known by the shorter name RUBISCO) involved in the carbon fixation process; a putative perakine reductase, involved in the juvenile-to-adult transition in the olive tree; a chalcone isomerase, involved in the flavonoid biosynthesis; and an acyl-[acyl-carrier-protein] desaturase, that participates in the polyunsaturated fatty acid biosynthesis. Among all the identified proteins, up to 10 species could be found both in olive seed and pulp, e.g. a GluB-5 long variant, a seed storage glutelin, and two 11S globulin seed storage proteins.

The present research project has been undertaken not just for the mere identification of as many species as possible in seed and pulp tissues, but also for another intriguing reason:

recent literature had suggested that proteins could be found also in olive oil [6,12], a product of very large consumption in the Mediterranean area, associated with many health benefits [2]. The aim of said research was also to see if oils of high quality could be typed also in terms of different proteinaceous material therein and ascertain the relative contribution of seed vs. pulp proteins. In the research undertaken in our labs, we indeed could not find any proteins in the regular olive oils products sold in supermarkets (even in those labelled as extra-virgin). However, in very high quality oils, to be found only in dedicated shops, we could indeed assess the presence of very fine bands in SDS-PAGE gels, centred in the 10–30-kDa regions, whose identification is now in progress. Such species are found only in trace amounts, though, and can only be visualized via silver staining, thus their identification might not represent an easy task.

Supplementary materials related to this article can be found online at [doi:10.1016/j.jprot.2012.02.020](https://doi.org/10.1016/j.jprot.2012.02.020).

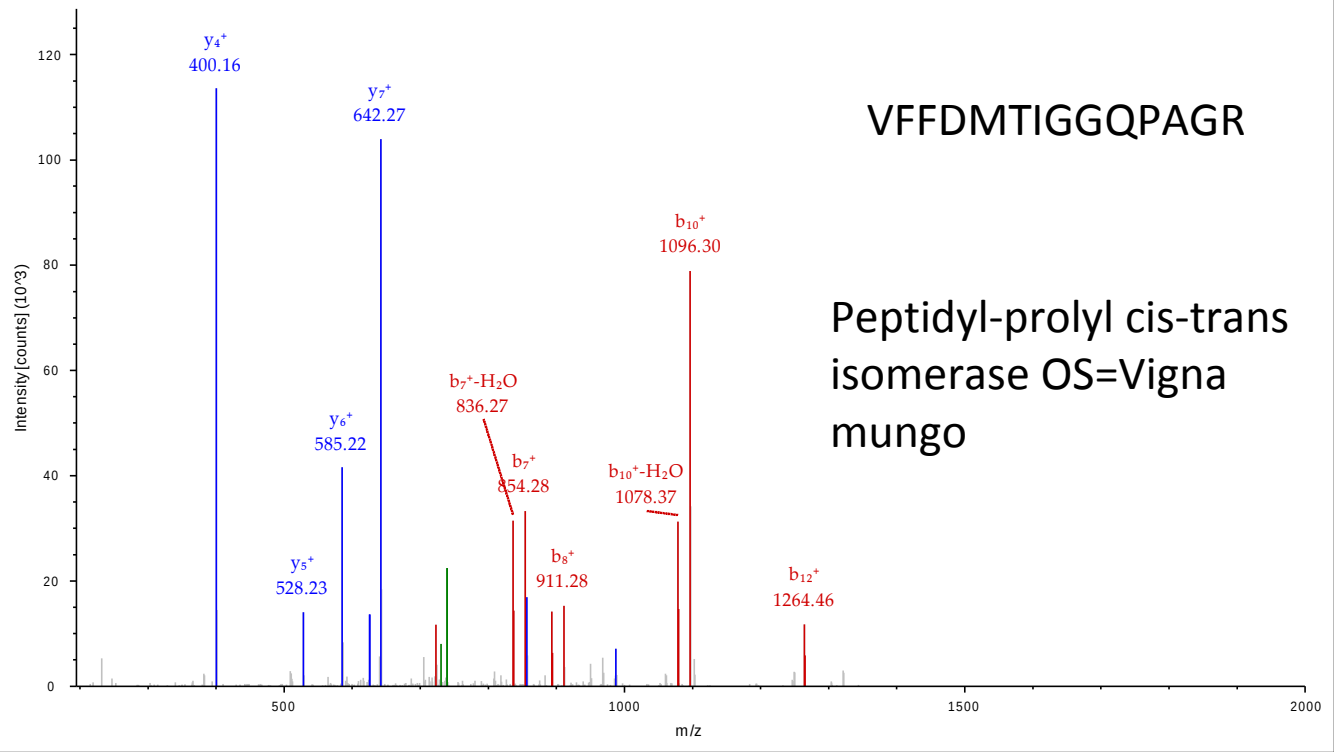
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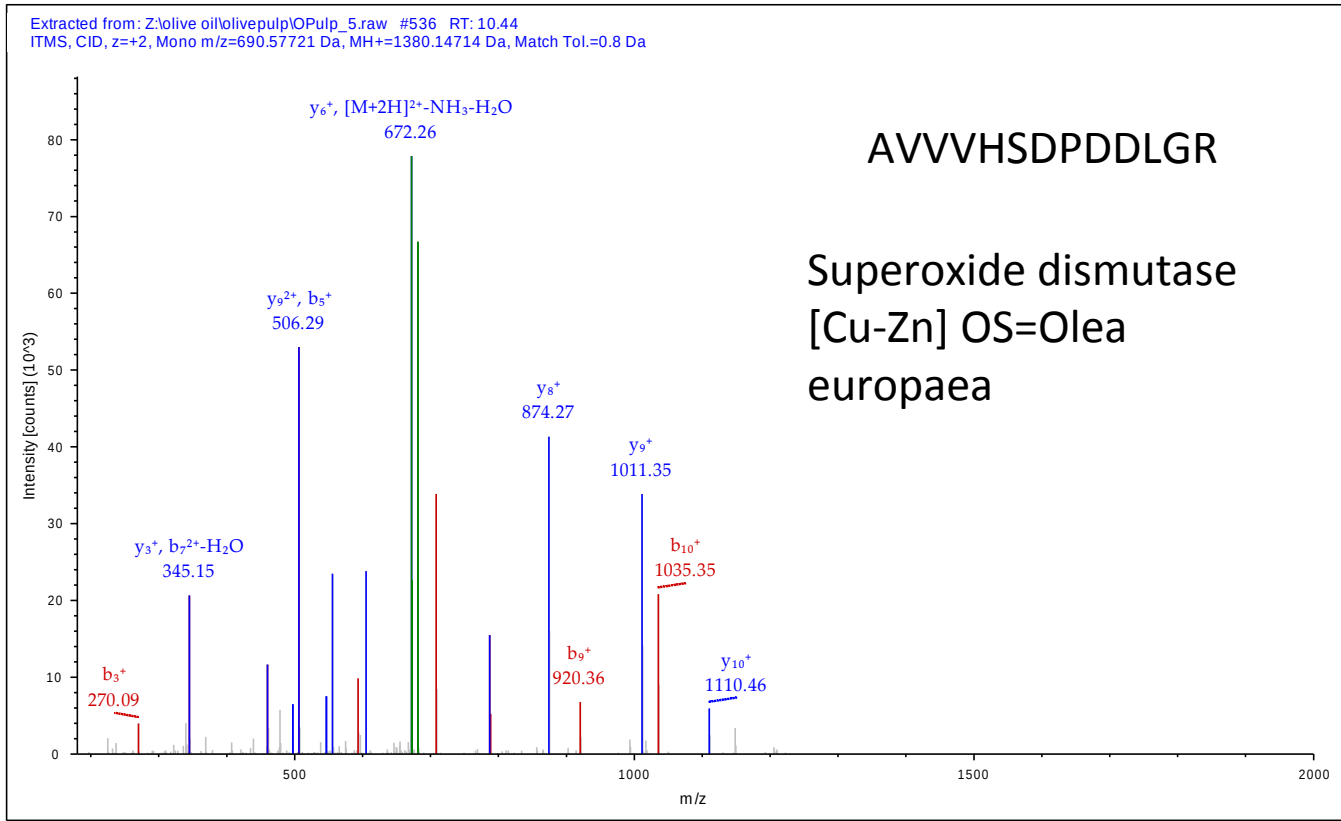
VFFDMTIGGQPAGR

Peptidyl-prolyl cis-trans
isomerase OS=Vigna
mungo

#1	b ⁺	b ²⁺	Seq.	y ⁺	y ²⁺	#2
1	100.07570	50.54149	V			14
2	247.14412	124.07570	F	1396.66788	698.83758	13
3	394.21254	197.60991	F	1249.59946	625.30337	12
4	509.23949	255.12338	D	1102.53104	551.76916	11
5	640.27999	320.64363	M	987.50409	494.25568	10
6	741.32767	371.16747	T	856.46359	428.73543	9
7	854.41174	427.70951	I	755.41591	378.21159	8
8	911.43321	456.22024	G	642.33184	321.66956	7
9	968.45468	484.73098	G	585.31037	293.15882	6
10	1096.51326	548.76027	Q	528.28890	264.64809	5
11	1193.56603	597.28665	P	400.23032	200.61880	4
12	1264.60315	632.80521	A	303.17755	152.09241	3
13	1321.62462	661.31595	G	232.14043	116.57385	2
14			R	175.11896	88.06312	1

Charge: +2, Monoisotopic m/z: 748.63965 Da (+268.04 mmu/
 +358.04 ppm), MH+: 1496.27202 Da, RT: 16.25 min,
 Identified with: Mascot (v1.16); IonScore:74, Exp Value:1.6E-
 004,

Olive Pulp

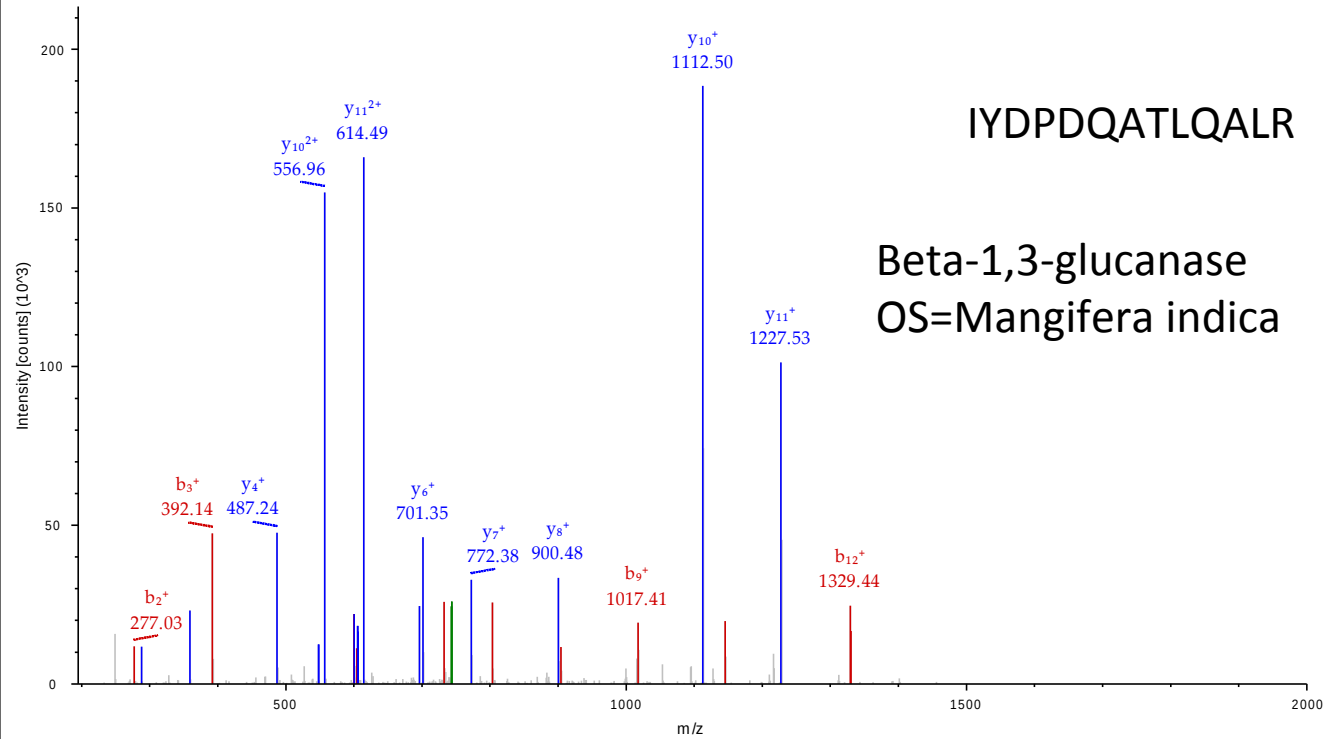


#1	b ⁺	b ²⁺	Seq.	y ⁺	y ²⁺	#2
1	72.04440	36.52584	A			13
2	171.11282	86.06005	V	1308.65432	654.83080	12
3	270.18124	135.59426	V	1209.58590	605.29659	11
4	369.24966	185.12847	V	1110.51748	555.76238	10
5	506.30857	253.65792	H	1011.44906	506.22817	9
6	593.34060	297.17394	S	874.39015	437.69871	8
7	708.36755	354.68741	D	787.35812	394.18270	7
8	805.42032	403.21380	P	672.33117	336.66922	6
9	920.44727	460.72727	D	575.27840	288.14284	5
10	1035.47422	518.24075	D	460.25145	230.62936	4
11	1148.55829	574.78278	L	345.22450	173.11589	3
12	1205.57976	603.29352	G	232.14043	116.57385	2
13			R	175.11896	88.06312	1

Charge: +2, Monoisotopic m/z: 690.57721 Da (+228.02 mmu/
+330.18 ppm), MH+: 1380.14714 Da, RT: 10.44 min,
Identified with: Mascot (v1.16); IonScore:81, Exp Value:2.8E-
005

Olive Pulp

Extracted from: Z:\olive oil\olivepulp\OPulp_4.raw #745 RT: 14.13
ITMS, CID, z=+2, Mono m/z=752.63477 Da, MH+=1504.26225 Da, Match Tol.=0.8 Da

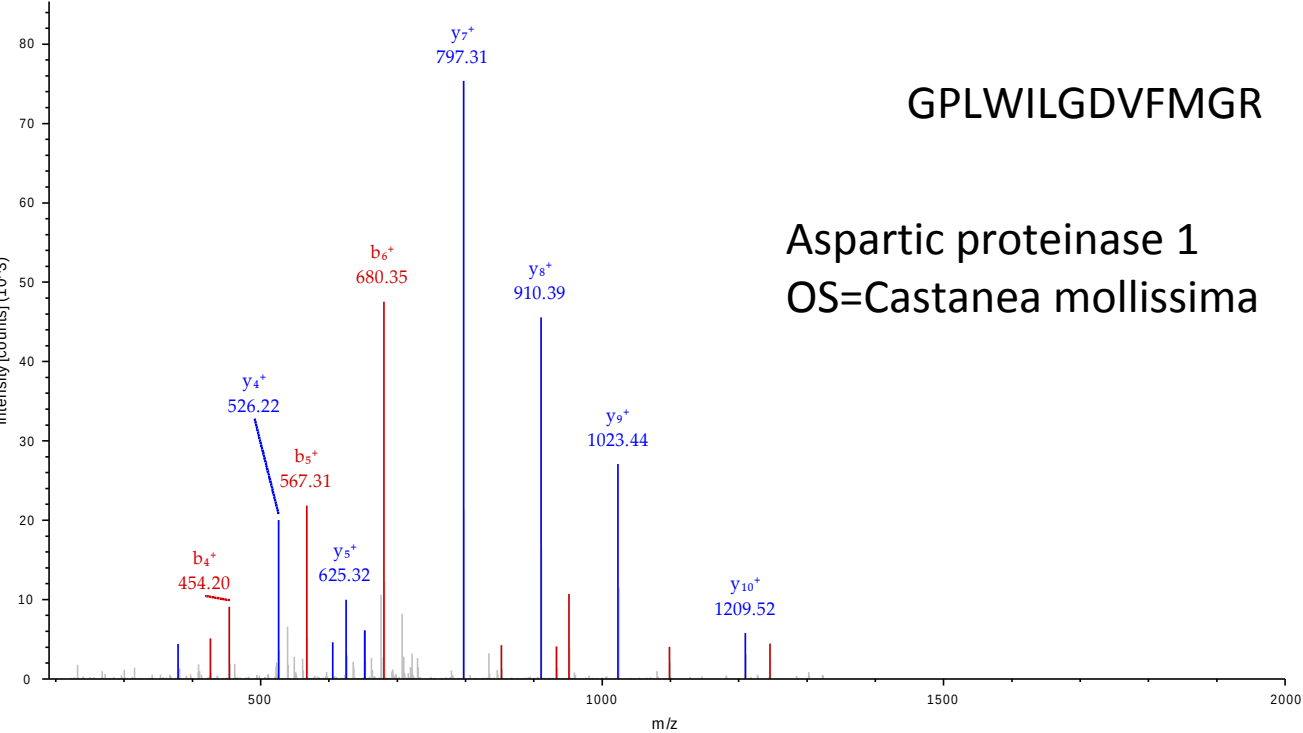


#1	b ⁺	b ²⁺	Seq.	y ⁺	y ²⁺	#2
1	114.09135	57.54931	I			13
2	277.15467	139.08097	Y	1390.69617	695.85172	12
3	392.18162	196.59445	D	1227.63285	614.32006	11
4	489.23439	245.12083	P	1112.60590	556.80659	10
5	604.26134	302.63431	D	1015.55313	508.28020	9
6	732.31992	366.66360	Q	900.52618	450.76673	8
7	803.35704	402.18216	A	772.46760	386.73744	7
8	904.40472	452.70600	T	701.43048	351.21888	6
9	1017.48879	509.24803	L	600.38280	300.69504	5
10	1145.54737	573.27732	Q	487.29873	244.15300	4
11	1216.58449	608.79588	A	359.24015	180.12371	3
12	1329.66856	665.33792	L	288.20303	144.60515	2
13			R	175.11896	88.06312	1

Charge: +2, Monoisotopic m/z: 752.63477 Da (+241.17 mmu/+320.43 ppm), MH+: 1504.26225 Da, RT: 14.13 min, Identified with: Mascot (v1.16); IonScore:91, Exp Value:3.0E-006

Olive Pulp

Extracted from: Z:\olive oil\OS_A16.raw #1125 RT: 20.73
 ITMS, CID, z=+2, Mono m/z=739.21606 Da, MH+=1477.42485 Da, Match Tol.=0.8 Da



#1	b ⁺	b ²⁺	Seq.	y ⁺	y ²⁺	#2
1	58.02875	29.51801	G			13
2	155.08152	78.04440	P	1419.74541	710.37634	12
3	268.16559	134.58643	L	1322.69264	661.84996	11
4	454.24491	227.62609	W	1209.60857	605.30792	10
5	567.32898	284.16813	I	1023.52925	512.26826	9
6	680.41305	340.71016	L	910.44518	455.72623	8
7	737.43452	369.22090	G	797.36111	399.18419	7
8	852.46147	426.73437	D	740.33964	370.67346	6
9	951.52989	476.26858	V	625.31269	313.15998	5
10	1098.59831	549.80279	F	526.24427	263.62577	4
11	1245.63372	623.32050	M-Oxidation	379.17585	190.09156	3
12	1302.65519	651.83123	G	232.14043	116.57385	2
13			R	175.11896	88.06312	1

Charge: +2, Monoisotopic m/z: 739.21606 Da
 (+329.21 mmu/+445.35 ppm), MH+: 1477.42485 Da,
 RT: 20.73 min,
 Identified with: Mascot (v1.16); IonScore:65, Exp
 Value:1.2E-003, Ions matched by search engine: 12/96
 Fragment match tolerance used for search: 0.8 Da

Olive Seed