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In-depth proteomic analysis of non-alcoholic beverages with peptide ligand libraries. I: Almond milk and orgeat syrup

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ARTICLE INFO

Article history:

Received 3 February 2011

Accepted 15 March 2011

Available online 31 March 2011

Keywords:

Peptide libraries

Hexapeptide ligands

Almond milk

Orgeat syrup

Low-abundance proteome

ABSTRACT

Combinatorial peptide ligand libraries, both commercial and home-made, have been adopted to investigate the proteome of non-alcoholic beverages, in order to assess their genuineness and detect also trace proteins, in search of potential allergens. Two such beverages have been studied: almond milk and orgeat syrup. In the first product we have been able to identify 132 unique protein species, the deepest investigation so far of the almond proteome. In the second beverage, a handful of proteins (just 14) have been detected, belonging to a bitter almond extract. In both cases, the genuineness of such products has been verified, as well as the fact that almond milk, judging on the total protein and fat content, must have been produced with 100 g ground almonds per litre of beverage, as required by authorities. On the contrary, cheap orgeat syrups produced by local supermarkets and sold as their own brands, where found not to contain any residual proteins, suggesting that they contained only synthetic aromas and no natural plant extracts. This could be the starting point for investigating the myriad of beverages that in the last decades have invaded the shelves of supermarkets the world over, whose genuineness and natural origin have never been properly assessed.

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1. Introduction

Sweet almonds (*Prunus amygdalus dulcis*), considered as stone fruits, are a very popular ingredient in the human diet, as they are used not only as slivers to grace the top of many cakes (or other delicacies like marzipan) but also to garnish platters of vegetables (e.g., steamed beans) or meats (poultry and pork). Almonds have been used for centuries in the human diet, since they have important nutritional and pharmacological properties. They provide a nutrient-dense source of vitamin E, manganese, magnesium, copper, phosphorus, fibre, riboflavin, monounsaturated fatty acids and protein (exhibiting a very high content, ca. 20% of the dry weight). Although almost 50% of

almond weight is fat, incremental intakes of 7 g per day of this tree nut reduce low-density lipoprotein (LDL) cholesterol concentration by 1%, especially within the context of diets recommended by the National Cholesterol Education Program. Habitual almond consumption does not lead to weight gain, and their inclusion in low-calorie diets appears to promote more weight loss than a comparable carbohydrate-based low-calorie diet. Also, almonds have a low glycemic index and do not adversely impact insulin sensitivity. Almonds are an excellent source of bioavailable α -tocopherol, and increasing their intake enhances the resistance of LDL against oxidation. In addition, the polyphenolic constituents of almonds have been characterised recently and found to possess antioxidant activity [1–3].

Abbreviations: CPLL, combinatorial peptide ligand library; HM-CPLL, home-made combinatorial peptide ligand libraries; PM, ProteoMiner.

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In the variegated world of non-alcoholic beverages, two drinks are quite popular in all countries facing the Mediterranean Sea, and in fact stretching from the Iberian Peninsula to East Asia: almond milk and orgeat syrup. The former has been very popular at least since the Middle Ages, when it was known in both the Islamic world and Christendom, where its vegetable composition — being a nut that is the seed of a fruit of a plant — made it suitable for consumption during Lent. Almond milk was also a staple of mediaeval kitchens because cow's milk would not keep for long without spoiling, and would usually be turned into butter or cheese immediately. Almond milk is indisputably nutritious with little to no unsaturated fat, unlike cow's milk, and no lactose, which makes it safe for people with lactose intolerance or milk allergy. A second interesting beverage also derived from almonds is orgeat syrup, which is a sweet syrup made from finely ground almonds, sugar and rose water or orange-flower water. Per se, the term “orgeat” is a misnomer, since it recalls barley (*Hordeus vulgaris*) as during the Middle Ages it was produced from barley extracts. Later on, it was made with a barley–almond blend, although today barley grains have been abandoned. It has a pronounced almond taste and is used to flavour many cocktails, perhaps the most famous being the Mai Tai. Both beverages can also contain up to 5% of extracts from bitter almonds (*Prunus amygdalus amara*), as higher levels could be toxic or even lethal due to the release of cyanide when the ground powder is mixed with water.

Curiously, there are no studies on the proteome content of these two beverages. Such studies would be of importance for at least two reasons: first of all, identification of all proteins present in such extracts will help to detect any potential allergenic macromolecule, even in traces; secondly, in order to assess whether such drinks (especially the orgeat) are indeed made with genuine extracts of almonds and not with artificial flavour only. Orgeat drinks (called horchata in Spain, rozata in Tunisia, orgeade in Suriname and orzata in Italy) are indeed the most critical ones, since in different countries they appear to be made with different ingredients, often poorly specified in the label (e.g., in Spain it is the extract of a tuber called chufa; in Mexico it is made with a rice extract; and in Italy it is made with de-acidified benzoin and aromas and oils extracted from bitter almonds, vanilla and flowers of orange trees).

In this respect, analysis of such drinks with combinatorial peptide ligand libraries (CPLs, as reviewed in, e.g., [4–6], now commercially available as ProteoMiner, PM) could be the method of choice, since such libraries have performed very well in detecting the low- to very-low-abundance proteome in plenty of biological samples (as examples see the cytoplasmic red blood cell proteome [7] and cerebrospinal fluid [8]). Recently, we have adopted this technique for exploring the hidden proteome of alcoholic beverages, namely white [9] and red [10] wines, as well as beer [11]. We could demonstrate, with this methodology, some unique features of CPLs, such as: (a) the ability to handle large sample volumes (one litre and more) in an easy and user-friendly protocol; (b) sample enrichment factors up to four orders of magnitude; (c) extremely high detection sensitivities, reaching as little as 1 µg protein/L of sample (in the case of casein detection of white wines), a sensitivity rarely obtained by present-day methodologies.

The natural extension of this work was to explore the “hidden” proteome of these two non-alcoholic beverages, as illustrated below. Some remarkable results have been obtained and will be described in the following sections.

2. Materials and methods

2.1. Chemicals, biologicals and apparatuses

ProteoMiner (combinatorial hexapeptide ligand library beads, PM), molecular mass standards, SDS-PAGE precast gels, Dc protein assay kit and electrophoresis apparatus for one-dimensional electrophoresis were from Bio-Rad Laboratories, Hercules, CA. β -mercaptoethanol, DTT, De-Streak [Bis-(2-hydroxyethyl)disulphide, $(\text{HOCH}_2\text{CH}_2)_2\text{S}_2$] and all other chemicals used all along the experimental work were current pure analytical grade products and purchased from Sigma-Aldrich, St Louis, MO. Home-made-CPLs (HM-CPLs) were produced in our laboratory and used as described in [9]. Almond milk and “orzata” were bought in local supermarkets. Most of the analyses reported here have been performed on the brand “Fabbri” (Bologna, Italy), believed to be one of the best commercial brands.

2.2. Treatment of beverages with peptide libraries

The entire content of an almond milk bottle (560 mL) was titrated to two different pH values (7.2 and 9.3) consecutively and subsequently added, for both treatments, with 100 µL of a 1:1 mixture of HM-CPLs and ProteoMiner beads. Protein adsorption was implemented via gentle shaking for 3 h at room temperature; then the beads were collected by filtration. The latter were then washed twice with proper buffers (10 mM phosphate buffer at pH 7.2 and 10 mM Tris buffer at pH 9.3) [12]. The captured proteins from each peptide library sample were then desorbed by using a solution composed of 4% SDS and 2% DTT for 15 min, under boiling conditions, (as per Candiano et al.) [13]. Desorption was carried out twice with 100 µL each and the two eluates pooled. Such extractions were done in triplicate. The bottle of orzata (560 mL) was treated as above, except that the capture was performed under very acidic conditions (pH 2.2 in formic acid, in presence of 0.1% TFA) so as to favour hydrophobic interactions. In this last case, only the HM-CPLs were used, since they were found to allow a much higher harvest of the orzata content.

2.3. SDS-polyacrylamide gel electrophoresis

Twenty microliter of each bead eluate, in presence of Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH approx. 6.8), were 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 and anodic compartments were filled with Tris-glycine buffer, pH 8.3, containing 0.1% SDS. Electrophoresis was at 100 V until the dye front reached the bottom of the gel. Staining and destaining were performed with Colloidal Coomassie Blue

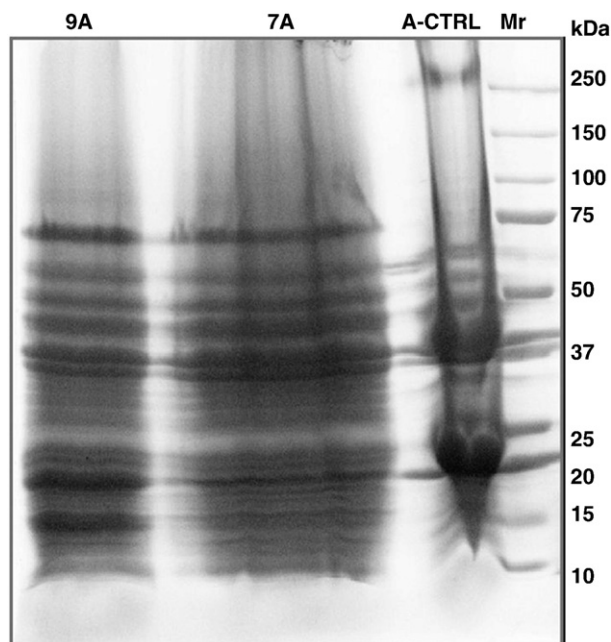


Fig. 1 – SDS-PAGE of almond milk proteome. A-CTRL = 20 μ L of almond milk before library treatment. 7A = 20 μ L of 4% SDS eluates of almond milk treated with a 1:1 mixed library of PM and HM-CPLLs at pH = 7.2. 9A = 20 μ L 4% SDS eluates of almond milk treated with a 1:1 mixed library of PM and HM-CPLLs at pH = 9.3. Mr: molecular mass markers.

and 7% acetic acid in water, respectively. The SDS-PAGE gels were scanned with a VersaDoc imaging system (Bio-Rad).

2.4. Mass spectrometry and data analysis

The sample lanes of SDS PAGE were cut in nine pieces along the migration path and proteins were reduced by 10 mM DTT, alkylated by 55 mM iodoacetamide and digested overnight with bovine trypsin. The tryptic mixtures were acidified with formic acid up to a final concentration of 1%. Ten microliter of tryptic digest for each band was injected in a nano chromatographic system, UltiMate 3000 RSLCnano System (Dionex). The peptide mixtures were loaded on a reversed-phase trap column for the cleanup and pre-concentration. After cleanup, the 2-position valve is switched to place the trap column in series with a fused silica reverse phase column (Acclaim PepMap100, C18, 3 μ m, 100 $\text{\AA}$, 75 μ m i.d. $\times$ 15 cm, Dionex). The peptides were eluted with a 30 min gradient from 4% buffer A (2% acetonitrile and 0.1% formic acid in water) to 60% buffer B (2% water and 0.1% formic acid in acetonitrile) at a constant flow rate of 300 μ L/min. The liquid chromatography 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 three most intense ions were automatically selected and fragmented in the ion trap. Target ions already selected for mass spectrometry (MS/MS) were dynamically excluded for 60 s. The MS data were

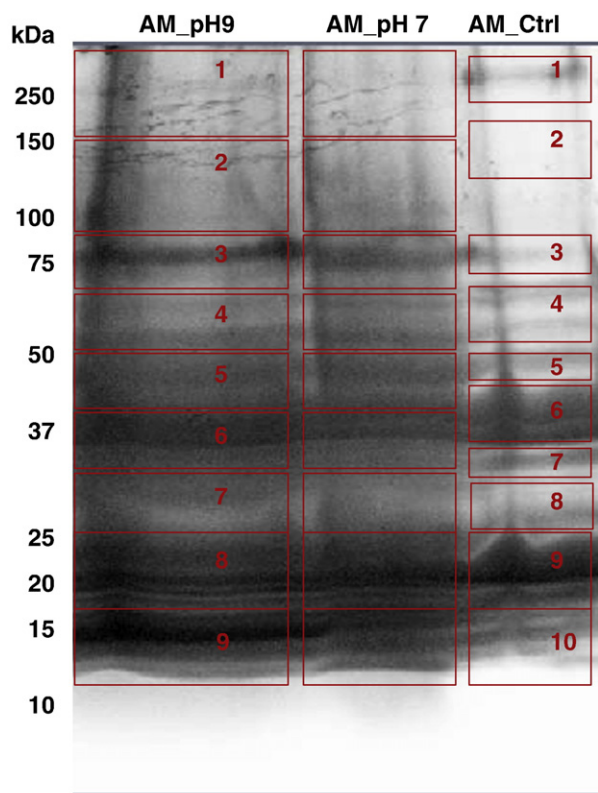


Fig. 2 – SDS-PAGE of control (AM-CTRL) and eluates from a 1:1 mixed library of PM and HM-CPLLs at pH 7.2 (AM-pH 7) and pH 9.3 (AM-pH 9) values. The left panel shows the 9–10 gel segments cut for digesting and analysing proteins, while the right panel depicts the Venn diagram of the identified protein species in the two captured proteomes and in the control, untreated sample. The total number of identifications amounts to 132 unique gene products.

TOT132

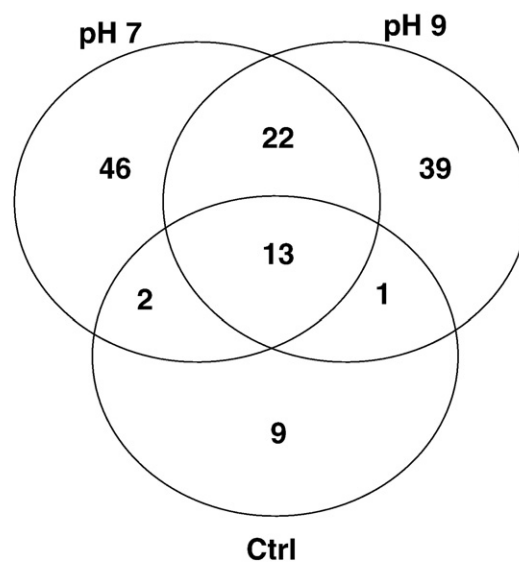


Table 1 – Proteins identified in almond milk.

Database	Accession number	Protein name	Mascot score	Mr	N peptides	pH7	pH9	ctrl
Uniprot_prunus	tr Q43607 Q43607_PRUDU	Prunin OS = <i>Prunus dulcis</i>	2839	63321	24	x	x	x
	tr E3SH28 E3SH28_PRUDU	Prunin 1 OS = <i>Prunus dulcis</i>	2489	63356	27	x	x	x
	tr E3SH29 E3SH29_PRUDU	Prunin 2 (fragment) OS = <i>Prunus dulcis</i>	722	57199	13	x	x	x
	tr Q945K2 Q945K2_PRUDU	R-oxynitrile lyase isoenzyme 1 OS = <i>Prunus dulcis</i>	432	61405	13	x	x	x
	tr Q6RFM0 Q6RFM0_PRUPE	Small heat shock protein OS = <i>Prunus persica</i>	314	17380	6	x	x	x
	tr C9EIM4 C9EIM4_9ROSA	CI small heat shock protein 2 OS = <i>Prunus salicina</i>	297	17479	5		x	
	tr Q43608 Q43608_PRUDU	Pru2 protein (fragment) OS = <i>Prunus dulcis</i>	265	57245	9		x	x
	sp P52706 MDL1_PRUPE	(R)-mandelonitrile lyase 1 OS = <i>Prunus serotina</i>	180	61447	7	x	x	x
	tr Q71SN1 Q71SN1_PRUAV	Calmodulin OS = <i>Prunus avium</i>	161	16894	1		x	
	tr B5LXC9 B5LXC9_PRUDU	Antimicrobial peptides 2-1 (fragment) OS = <i>Prunus dulcis</i>	130	17513	3	x	x	x
	tr O82435 O82435_PRUPE	(R)-(+)-mandelonitrile lyase isoform MDL5 OS = <i>Prunus serotina</i>	113	61224	3		x	
	tr A7Y7K3 A7Y7K3_PRUDU	Putative lipid transfer protein (fragment) OS = <i>Prunus dulcis</i>	86	7255	3	x	x	
	tr C9EIM3 C9EIM3_9ROSA	C I small heat shock protein 1 OS = <i>Prunus salicina</i>	72	18130	3		x	
	tr E3W0S7 E3W0S7_9ROSI	Photosystem I P700 apoprotein A2 OS = <i>Castanea mollissima</i>	69	82437	2	x	x	x
	tr C9EIM5 C9EIM5_9ROSA CII	small heat shock protein 1 OS = <i>Prunus salicina</i>	64	17572	2		x	
	tr Q6YNS1 Q6YNS1_PRUAV	Putative glycine-rich RNA-binding protein OS = <i>Prunus avium</i>	61	17374	1	x	x	
	tr Q8H2B9 Q8H2B9_PRUDU	60S acidic ribosomal protein OS = <i>Prunus dulcis</i>	58	11408	1	x	x	
	tr E3W0M6 E3W0M6_PRUPE	Ribosomal protein S11 OS = <i>Prunus persica</i>	50	15112	1	x	x	x
	tr E3W0Q0 E3W0Q0_PRUPE	Hypothetical chloroplast RF1 OS = <i>Prunus persica</i>	45	224293	2	x		x
	tr Q93WZ3 Q93WZ3_PRUPE	Thioredoxin H OS = <i>Prunus persica</i>	44	14656	1		x	
	tr Q000F7 Q000F7_PRUAV	NBS-containing resistance-like protein (fragment) OS = <i>Prunus avium</i>	42	18113	1	x	x	
	tr Q8W1W7 Q8W1W7_PRUPE	Prunasin hydrolase isoform PH B OS = <i>Prunus serotina</i>	42	61822	2		x	
	tr B6CQS7 B6CQS7_9ROSA	Putative allergen Pru du 1.06B OS = <i>Prunus dulcis</i> × <i>Prunus persica</i>	40	17413	2		x	
	tr E3W0K2 E3W0K2_PRUPE	Acetyl-CoA carboxylase carboxyltransferase beta subunit OS = <i>Prunus persica</i>	40	56468	1	x		x
	tr Q76HE4 Q76HE4_PRUMU	S-locus F-Box protein 1 OS = <i>Prunus mume</i>	40	44000	2	x	x	
	tr A4F1Y9 A4F1Y9_PRUMU	Lycopene beta-cyclase (fragment) OS = <i>Prunus mume</i>	39	38177	1	x		
	tr B6CQU8 B6CQU8_9ROSA	Profilin OS = <i>Prunus dulcis</i> × <i>Prunus persica</i>	39	14166	1	x		
	sp O64982 PRS7_PRUPE	26S protease regulatory subunit 7 OS = <i>Prunus persica</i>	38	48041	2			x
	tr A9UAM9 A9UAM9_9ROSA	Putative ethylene receptor OS = <i>Prunus salicina</i>	37	83400	2	x		
	tr Q76HE4 Q76HE4_PRUMU	S-locus F-Box protein 1 OS = <i>Prunus mume</i> GN = SFB1 PE = 2 SV = 1	36	44000	3	x	x	
	sp O82784 MDL4_PRUPE	(R)-mandelonitrile lyase 4 OS = <i>Prunus serotina</i>	36	61929	1	x		
	tr B6ZL92 B6ZL92_PRUPE	GDP-D-mannose-3~,5~-epimerase OS = <i>Prunus persica</i>	35	42956	2	x	x	
	tr B2BMQ4 B2BMQ4_PRUPE	Lipoxygenase OS = <i>Prunus persica</i>	33	106359	1		x	
	tr Q8LKR9 Q8LKR9_PRUPE	NADP-dependent sorbitol-6-phosphate dehydrogenase (fragment) OS = <i>Prunus emarginata</i>	33	28574	1			x
	tr Q9MUA2 Q9MUA2_9ROSA	Ribulose biphosphate carboxylase large chain (fragment) OS = <i>Prunus cerasifera</i>	33	44212	2	x		
	sp P54892 MP_ACLSA	Putative movement protein OS = Apple chlorotic leaf spot virus (isolate apple)	32	50594	1	x		
	tr A0MQ92 A0MQ92_9ROSA	Phenylalanine ammonia-lyase (fragment) OS = <i>Prunus cerasifera</i> × <i>Prunus munsoniana</i>	32	32393	1	x		
	tr A6XN06 A6XN06_PRUPE	Putative uncharacterized protein OS = <i>Prunus persica</i>	32	34120	1	x		
	tr Q48628 Q48628_PRUAR	Pyrophosphate-dependent phosphofructo-1-kinase (fragment) OS = <i>Prunus armeniaca</i>	32	31298	1		x	
	tr Q217H8 Q217H8_9VIRU	Coat protein OS = Asian prunus virus 2	32	43388	1	x		
	tr Q5DIW8 Q5DIW8_CRLV	Polypeptide OS = Cherry rasp leaf virus	32	252672	2			x
	sp P82353 NLTP2_PRUAR	Non-specific lipid-transfer protein 2 OS = <i>Prunus armeniaca</i> ì	31	7697	1	x		
	tr E0Y456 E0Y456_PRUAR	Oxalate oxidase 2 OS = <i>Prunus armeniaca</i>	31	24435	1		x	
	sp P25246 YR22_TORVR	Uncharacterized protein in RNA2 OS = Tomato ringspot virus (isolate raspberry)	30	22248	1		x	

(continued on next page)

Table 1 (continued)

Database	Accession number	Protein name	Mascot score	Mr	N peptides	pH7	pH9	ctrl
Uniprot_viridiplantae	tr D8V151 D8V151_PRUAR	Putative CC-NBS-containing resistance protein (fragment) OS = <i>Prunus armeniaca</i>	30	19771	1	x		
	tr Q71QD5 Q71QD5_PRUAV	Protein phosphatase 2C OS = <i>Prunus avium</i>	30	47041	1	x		
	tr C1ECS6 C1ECS6_9CHLO	Predicted protein OS = <i>Micromonas</i> sp. RCC299 (Picoplanktonic green alga)	309	100569	2	x	x	
	tr Q43608 Q43608_PRUDU	Pru2 protein (fragment) OS = <i>Prunus dulcis</i>	181	57245	10	x		
	tr B1NDJ5 B1NDJ5_9ERIC	Calmodulin OS = <i>Actinidia kolomikta</i>	135	16705	2		x	
	sp P52706 MDL1_PRUSE	(R)-mandelonitrile lyase 1 OS = <i>Prunus serotina</i>	124	61447	11		x	
	tr Q5D4W0 Q5D4W0_PANGI	Actin (fragment) OS = <i>Panax ginseng</i>	114	10856	2	x	x	
	tr A1E0V5 A1E0V5_FICAW	11S globulin isoform 1B OS = <i>Ficus awkeotsang</i>	110	58736	2	x		
	tr B6CIL7 B6CIL7_9LAMI	Cytosolic glyceraldehyde-3-phosphate dehydrogenase (fragment) OS = <i>Conradina</i> sp. Edwards 133	103	10150	2		x	
	tr C0HF32 C0HF32_MAIZE	ATP synthase subunit beta OS = <i>Zea mays</i>	94	59181	1	x		
	tr D7MG30 D7MG30_ARALY	Putative uncharacterized protein OS = <i>Arabidopsis lyrata</i> subsp. <i>lyrata</i>	91	23848	2	x	x	
	tr D0R8X7 D0R8X7_9ROSI	Glyceraldehyde-3-phosphate dehydrogenase (fragment) OS = <i>Manihot cecropiifolia</i>	90	14536	2	x	x	
	tr A5YVV3 A5YVV3_WHEAT	Glyceraldehyde-3-phosphate dehydrogenase OS = <i>Triticum aestivum</i>	89	36854	4		x	
	tr A2XIE7 A2XIE7_ORYSI	Putative uncharacterized protein OS = <i>Oryza sativa</i> subsp. <i>indica</i>	74	77957	2	x	x	
	tr Q8S5F1 Q8S5F1_ORYSA	Putative retroelement OS = <i>Oryza sativa</i>	69	120626	2	x		
	tr Q9M417 Q9M417_QESU	Heat shock protein 17a.5 (fragment) OS = <i>Quercus suber</i>	69	12629	2	x	x	
	sp Q5KTS5 GRDH_DAUCA	Glucose and ribitol dehydrogenase OS = <i>Daucus carota</i>	67	31652	2	x		
	tr A9TIZ4 A9TIZ4_PHYPA	Predicted protein (fragment) OS = <i>Physcomitrella patens</i> subsp. <i>patens</i>	67	155589	2	x	x	
	tr D7TKK0 D7TKK0_VITVI	Whole genome shotgun sequence of line PN40024, (fragment) OS = <i>Vitis vinifera</i>	66	92836	1	x		
	tr Q05JY1 Q05JY1_LOTJA	Pterocarpan reductase OS = <i>Lotus japonicus</i>	64	34204	1	x		
	tr A1Y2J9 A1Y2J9_HELAN	Phosphoglycerate kinase OS = <i>Helianthus annuus</i>	62	42334	2	x	x	
	tr B8LKD2 B8LKD2_PICSI	Putative uncharacterized protein OS = <i>Picea sitchensis</i>	62	50479	1	x	x	
	MDL3_PRUSE	(R)-mandelonitrile lyase 3 OS = <i>Prunus serotina</i>	60	62426	4	x		x
	tr B8AJF6 B8AJF6_ORYSI	Putative uncharacterized protein OS = <i>Oryza sativa</i> subsp. <i>indica</i>	59	69074	1	x		
	tr Q2PZA5 Q2PZA5_CUCME	Putative alcohol dehydrogenases OS = <i>Cucumis melo</i>	59	41728	2		x	
	tr A5AGQ2 A5AGQ2_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	58	11422	1	x		
	tr A5BLZ5 A5BLZ5_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	57	133159	2	x		
	tr B9RTX3 B9RTX3_RICCO	Short chain dehydrogenase, putative OS = <i>Ricinus communis</i>	56	32582	3		x	
	tr B9RUY3 B9RUY3_RICCO	Putative uncharacterized protein OS = <i>Ricinus communis</i>	55	52607	1		x	
	tr A2X5K7 A2X5K7_ORYSI	Putative uncharacterized protein OS = <i>Oryza sativa</i> subsp. <i>indica</i>	54	11591	1		x	
	sp Q9SJ07 Y2192_ARATH	Putative B3 domain-containing protein At2g21920 OS = <i>Arabidopsis thaliana</i>	53	32276	1		x	
	tr A5C844 A5C844_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	53	143210	1		x	
	tr Q0JQL7 Q0JQL7_ORYSJ	Histone H2B (Fragment) OS = <i>Oryza sativa</i> subsp. <i>japonica</i>	53	4392	3	x		
	tr A5YKH9 A5YKH9_9VIRI	Elongation factor 1-alpha (fragment) OS = <i>Chara australis</i>	52	47970	1		x	
	tr A5B0X5 A5B0X5_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	51	38348	1		x	
	tr A8MQL8 A8MQL8_ARATH	Uncharacterized protein At5g15230.2 OS = <i>Arabidopsis thaliana</i>	51	10088	1		x	
	tr A9S4H1 A9S4H1_PHYPA	Predicted protein OS = <i>Physcomitrella patens</i> subsp. <i>patens</i>	51	40121	2		x	
	tr Q3ZDM5 Q3ZDM5_ARAGE	Actin 8 (fragment) OS = <i>Arabidopsis gemmifera</i>	51	38737	2	x		
	tr A4S0S6 A4S0S6_OSTLU	Predicted protein OS = <i>Ostreococcus lucimarinus</i> (strain CCE9901)	50	26169	1	x		
	tr B9SP74 B9SP74_RICCO	Expressed protein, putative OS = <i>Ricinus communis</i>	50	37855	1		x	
	tr C1N636 C1N636_MICPS	Predicted protein OS = <i>Micromonas pusilla</i> CCMP1545	50	95306	1		x	
	tr Q9FKD4 Q9FKD4_ARATH	Similarity to unknown protein OS = <i>Arabidopsis thaliana</i>	50	71740	1		x	
	tr B9SWX4 B9SWX4_RICCO	Phosphatidylethanolamine binding protein, putative OS = <i>Ricinus communis</i>	49	18392	1	x		
	RR11_DIOEL	30S ribosomal protein S11, chloroplastic OS = <i>Dioscorea elephantipes</i>	48	15092	9			x
	tr D7LZD4 D7LZD4_ARALY	Putative uncharacterized protein OS = <i>Arabidopsis lyrata</i> subsp. <i>lyrata</i>	48	49037	1	x		

SwissProt_all entries	tr[B9S395]B9S395_RICCO	Heat-shock protein, putative OS = <i>Ricinus communis</i>	47	18108	2	x	
	tr[C1FET4]C1FET4_9CHLO	Predicted protein OS = <i>Micromonas</i> sp. RCC299	47	117428	1	x	
	tr[A5AWV4]A5AWV4_VITVI	Putative uncharacterized protein OS = <i>Vitis vinifera</i>	46	65763	1	x	
	tr[C1FJL8]C1FJL8_9CHLO	Vesicle coat complex AP-1/AP-2/AP-4, beta subunit OS = <i>Micromonas</i> sp. RCC299	46	113447	2	x	
	tr[C1MZX9]C1MZX9_MICPS	Predicted protein (fragment) OS = <i>Micromonas pusilla</i> CCMP1545	46	39521	1	x	
	HFA2B_ORYSJ	Heat stress transcription factor A-2b OS = <i>Oryza sativa</i> subsp. <i>japonica</i>	42	41498	11		x
	BRE1B_ARATH	E3 ubiquitin-protein ligase BRE1-like 2 OS = <i>Arabidopsis thaliana</i>	41	104472	32		x
	CALM6_ARATH	Calmodulin OS = <i>Arabidopsis thaliana</i>	143	16828	3		x
	DNAK_BARHE	Chaperone protein dnaK OS = <i>Bartonella henselae</i>	99	68333	3	x	
	ATPB_MYCMM	ATP synthase subunit beta OS = <i>Mycobacterium marinum</i> (strain ATCC BAA-535 / M)	94	52768	1	x	
	ATPB_M_ARATH	ATP synthase subunit beta-1, mitochondrial OS = <i>Arabidopsis thaliana</i>	94	59805	1	x	
	G3PC_HORVU	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (Fragment) OS = <i>Hordeum vulgare</i>	90	33443	4		x
	HS23M_ARATH	23.6 kDa heat shock protein, mitochondrial OS = <i>Arabidopsis thaliana</i>	85	23595	2	x	x
	ADHX_SPAAU	Alcohol dehydrogenase class-3 OS = <i>Sparus aurata</i>	69	40930	1		x
	ATPB_ACIC5	ATP synthase subunit beta OS = <i>Acidobacterium capsulatum</i>	67	52116	2	x	x
	GRDH_DAUCA	Glucose and ribitol dehydrogenase OS = <i>Daucus carota</i>	67	31652	2	x	x
	EBH_STAAR	Extracellular matrix-binding protein ebh OS = <i>Staphylococcus aureus</i> (strain MRSA252)	66	1156473	4	x	x
	ATPB_DESAP	ATP synthase subunit beta OS = <i>Desulforudis audaxviator</i> (strain MP104C)	65	51233	1	x	
	EPI1_CAEEL	Laminin-like protein epi-1 OS = <i>Caenorhabditis elegans</i>	65	415887	2	x	x
	HSP21_SOLPE	17.3 kDa class II heat shock protein OS = <i>Solanum peruvianum</i>	64	17311	2		x
	PGKH_SPIOL	Phosphoglycerate kinase, chloroplastic (fragment) OS = <i>Spinacia oleracea</i>	62	45658	2	x	x
	G3PC_ANTMA	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic OS = <i>Antirrhinum majus</i>	59	36776	3	x	
	RLA2_PARAR	60S acidic ribosomal protein P2 OS = <i>Parthenium argentatum</i>	58	11495	1	x	x
	TIM22_DICDI	Mitochondrial import inner membrane translocase subunit tim22 OS = <i>Dictyostelium discoideum</i>	57	19690	1	x	
	GSA_METHJ	Glutamate-1-semialdehyde 2,1-aminomutase OS = <i>Methanospirillum hungatei</i>	56	44684	1	x	x
	H42_WHEAT	Histone H4 variant TH091 OS = <i>Triticum aestivum</i>	54	11460	2		x
	G3P_DEBHA	Glyceraldehyde-3-phosphate dehydrogenase OS = <i>Debaryomyces hansenii</i>	53	35926	1		x
	H2B10_ARATH	Histone H2B.10 OS = <i>Arabidopsis thaliana</i>	53	15723	3	x	
	POLG_BCMNN	Genome polyprotein OS = Bean common mosaic necrosis virus (strain NL-3)	53	353245	2		x
	SYS_BURCE	Seryl-tRNA synthetase OS = <i>Burkholderia cepacia</i>	53	47679	2		x
	Y2192_ARATH	Putative B3 domain-containing protein At2g21920 OS = <i>Arabidopsis thaliana</i>	53	32276	1		x
	RS5_PHOPR	30 S ribosomal protein S5 OS = <i>Photobacterium profundum</i>	52	17294	1		x
	NU107_MOUSE	Nuclear pore complex protein Nup107 OS = <i>Mus musculus</i>	51	107334	1	x	
	DEFL_RICCN	Peptide deformylase-like OS = <i>Rickettsia conorii</i>	50	20922	1	x	
	EF1A_PSEMZ	Elongation factor 1-alpha (fragments) OS = <i>Pseudotsuga menziesii</i>	50	1988	1	x	
	RIMM_AZOVD	Ribosome maturation factor rimM OS = <i>Azotobacter vinelandii</i> (strain DJ / ATCC BAA-1303)	50	20377	1		x
	IFT80_MOUSE	Intraflagellar transport protein 80 homolog OS = <i>Mus musculus</i>	49	88440	2	x	
	AIFM1_RAT	Apoptosis-inducing factor 1, mitochondrial OS = <i>Rattus norvegicus</i>	48	66966	2	x	
	CLPC_CYACA	ATP-dependent Clp protease ATP-binding subunit clpA homolog OS = <i>Cyanidium caldarium</i>	47	95688	1	x	
	GPC1_HUMAN	Glypican-1 OS = <i>Homo sapiens</i>	47	62724	1	x	
	H2AZ_USTMA	Histone H2A.Z OS = <i>Ustilago maydis</i>	47	14609	2	x	
	PGK_RHOCS	Phosphoglycerate kinase OS = <i>Rhodospirillum centenum</i> (strain ATCC 51521/SW)	47	41057	1	x	
	ACCA_CAMFF	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha OS = <i>Campylobacter fetus</i> subsp	47	34672	1	x	

searched against three databases with Mascot search engine (version 2.3.01): Swiss Prot without restriction of taxonomy (523151 residues, 184678199 sequences), Uniprot_viridiplantae (30264 residues, 184678199 sequences) and Uniprot_prunes (3237 residues, 748256 sequences). The analyses of almond's milk raw data and orgeat raw data were further refined by using Proteome Discoverer software (v. 1.2.0 Thermo). The false discovery rate obtained when consulting the decoy database was around 0.05. We used tryptic cleavage, cysteine alkylation, oxidation of methionine residues and acetylation of N termini as variable modifications. Peptide mass tolerance was set to 0.5 Da, fragment mass tolerance to 0.8 Da and ion score cut-off of 20.

3. Results

Fig. 1 shows the SDS-PAGE profiling of control almond milk (A-CTRL) as compared to two eluates from the CPLL bead mixture (1:1 HM-CPLLs and PM), at pH 7.2 and at pH 9.3 (7A and 9A tracks, respectively). It can be appreciated that the untreated sample shows two major bands, centred at around 25 and 40 kDa, plus some additional bands extending from ca. 12 up to 60 kDa. Conversely the two eluates show a large spectrum of polypeptide chains, extending from 10 up to 150 kDa. Moreover, of the two eluates, the pH 7.2 capture appears to be more polydisperse, indicating the potential capture of a larger population of species.

In all three cases, the SDS-PAGE tracks were divided into 9 (the CPLL eluates) or ten (control) segments, the protein content of each gel zone digested and the peptide eluates subjected to LTQMS/MS analysis (Fig. 2, left panel). For protein identification, as the proteome of almonds is largely unknown, the following databases were searched: Uniprot_viridiplantae, Uniprot_prunus and SwissProt_all_entries. A total of 132 unique gene products could be identified (as listed in Table 1; the sequences of all peptides being deposited in the supplementary material on line), the largest description so far of the almond proteome. The Venn diagram of Fig. 2 (right panel) summarises the data obtained: the pH 7.2 capture permitted to reveal a total of 83 species, the capture at pH 9.3 a total of 75 proteins and the control barely 25 proteins. Of those, 46 were specific of the pH 7.2 capture, 39 could be detected only in the pH 9.3 eluate and 9 proteins were missed by the CPLL enrichment and could be found only in the control. It is thus confirmed that a proper CPLL treatment allows detection of 500% more species than in the control, with a minimal loss of the species present in the untreated sample (5–6%), a fact confirmed in most of our research in different proteomes [4–6], where, typically, 300 to 600 more species could be detected after CPLL treatment.

Analysis of the orgeat syrup was decidedly more difficult. Capture at three pH values (4.0, 7.2 and 9.3) did not yield any visible protein band in SDS-PAGE, even after silver staining. We thus resorted to a novel type of treatment, namely we tried to simulate reverse-phase chromatography, where protein capture occurs in general at strongly acidic pH values (pH 2.2) in presence of 0.1% TFA as an ion-pairing agent [14]. To this aim, 560 mL (the entire content of a bottle) was treated with 100 μ L of just HM-CPLL beads, since preliminary experiments indicated a much higher capture. As we suspected that the

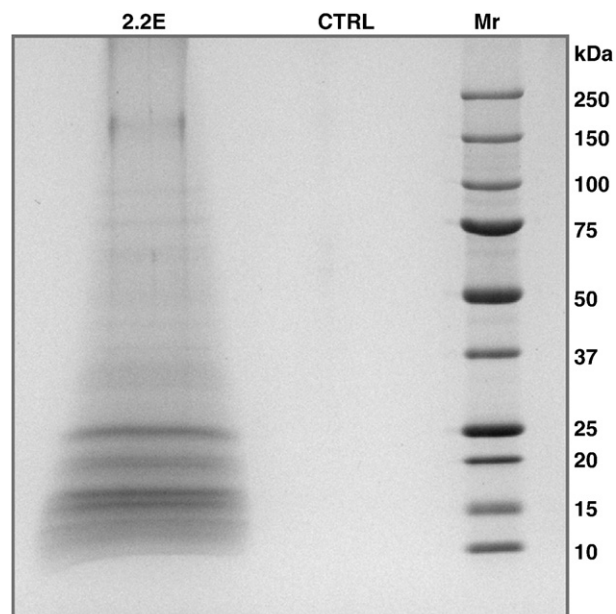


Fig. 3 – SDS-PAGE of orgeat syrup proteome. CTRL = 100 μ L of control orgeat solution before HM-CPLL treatment. 2.2E = 20 μ L 4% SDS eluate of orgeat solution treated with the HM-CPLL library at pH=2.2. No protein zones are visible in the control lane.

amount of protein harvested could have been rather minute, the 150 μ L of SDS-DTT eluate was reduced to a 20 μ L volume, which was loaded onto the SDS-PAGE gel. This permitted to analyse at once all the eluate of the CPLL beads. The results are shown in Fig. 3: ten fine polypeptide bands are visible in the 10 to 25 kDa region, with a series of very faint zones extending from 30 up to 150 kDa. Analysis of the digests of these bands permitted the identification of a reduced set of proteins (a total of 14), as listed in Table 2.

4. Discussion

The present data once more prove the power of the CPLL technique in enlarging the vision of any proteome. In the present case, when considering the control sample of almond milk, it is seen that two major proteins, centred at around 25 and 40 kDa (see Fig. 1, track A-CTRL) dominate the landscape, leaving little visibility to all minor components. These two species belong to the prunin family (prunin OS, prunin 1 and 2) and in fact represent the species best known in almonds. Conversely, in the treated sample, a much larger number of species could be identified, bringing the total number of species from a mere 25 in the control up to 132. Interestingly, of all species identified only 30 could be enucleated from the database Uniprot_prunus; the vast majority could only be identified by homology with proteins listed in the Uniprot_viridiplantae, especially those of *Oryza sativa* (rice) *Vitis vinifera*, *Triticum aestivum* (wheat), *Arabidopsis* and so on. What is even more striking is the fact that we still had a few thousand peptides sequenced which could not be attributed to any known protein in plant systems. Assuming a minimum of 3 peptides for a safe

Table 2 – Proteins identified in orgeat syrup.

Database	Accession number	Protein name	Mascot score	Mr	N peptides	pep_exp_mr	pep_ex p z	pep_calc_mr	pep_delta	pep_score	pep_expect	pep_seq	pep_var_mod
Uniprot_viridi plantae	tr[Q9AU64] Q9AU64_ELAV	7S globulin OS =	211	67008	3	702.4351	1	702.3813	0.0538	14.61	1.80E+02	TWLAGR	
		<i>Elaeis guineensis</i>				802.7357	2	802.3933	0.3424	24.09	18	KGQGEER	
		var. <i>tenera</i>				1308.6119	2	1308.547	0.0649	14.07	1.60E+02	EADSDDYPELR	
	sp[P85925] UP18_PSEMZ	Unknown protein	162	1393	1	1392.7611	3	1392.7249	0.0362	24.25	15	TNAENEFVTIKK	
		18 (fragment) OS =											
		= <i>Pseudotsuga menziesii</i>											
	sp[P52707] MDL3_PRUSE	(R)-	151	62426	3	820.9336	2	820.4079	0.5257	32.25	2.3	VLDGFR	
		mandelonitrile				842.7667	2	842.4974	0.2693	46.38	0.098	VTGINALR	
		lyase 3 OS =				1249.6263	2	1249.5939	0.0324	69.79	0.00038	FVSEDGIDNVR	
	tr[Q9SNZ2] Q9SNZ2_ELAV	<i>Prunus serotina</i>	125	42811	7								
		Glutelin OS =				648.5741	1	648.3959	0.1783	19.32	48	VVLYR	
		<i>Elaeis guineensis</i>				912.5923	2	912.5029	0.0895	46.8	0.091	LNALPEPTR	
		var. <i>tenera</i>				980.5076	2	980.4749	0.0327	24.1	12	FIQMSAER	
						996.8996	2	996.4698	0.4297	46.74	0.063	FIQMSAER	Oxidation (M)
						1254.8499	2	1254.6754	0.1745	57.58	0.0059	GLLLPSMSNAPR	
Uniprot_prun us	tr[B9G786] B9G786_ORYSJ	Putative	107	37817	1	1270.7701	2	1270.6703	0.0998	23.29	17	GLLLPSMSNAPR	Oxidation (M)
		uncharacterized				1497.323	2	1496.8926	0.4304	62.36	0.0021	ITTLNSEKLPILR	
		protein OS =				757.0731	2	756.4857	0.5873	27.72	6.6	LIKLDR	
		<i>Oryza sativa</i>				764.9138	2	764.3817	0.5322	27.42	7	AIENYR	
	tr[A2XGF3] A2XGF3_ORYSI	Putative	70	61742	3	1489.9759	2	1489.7354	0.2405	23.92	13	GGPFVFFGFTTSAR	
		uncharacterized				1617.7959	3	1617.7895	0.0064	12.25	2.10E+02	EGLMHIGFITMEPK	Oxidation (M)
		protein OS =				1768.7195	3	1768.893	-0.1735	14.84	1.00E+02	AMSATVRAGD	Oxidation (M)
	tr[E3SH28] E3SH28_PRUDU	<i>Oryza sativa</i> s	3026	63356	13								
		<i>Prunus dulcis</i>				574.3542	1	574.3074	0.0468	24.12	0.059	LSAER	
						622.5397	1	622.3439	0.1959	25.41	0.017	TSFLR	
						804.4891	1	804.413	0.0761	38.11	0.0024	ADIFSPR	
						1141.7108	2	1141.588	0.1228	57.95	1.80E-05	GNLDFVQPPR	
						1169.159	2	1168.62	0.539	53.14	3.50E-05	LKENIGNPER	
						1346.9901	2	1346.679	0.3111	75.81	1.40E-07	QETIALSSSQQR	
						1434.9359	2	1434.7103	0.2256	105.32	1.90E-10	TEENAFINTLAGR	
						2339.3106	3	2339.2386	0.072	27.91	0.011	NGLHLPYSYNA	
												PQLIYIVQGR	
						2354.457	3	2354.1224	0.3346	24.28	0.038	QQEQQQGQGR	
												PQQQQQFR	
						2451.4125	3	2451.1567	0.2558	67.99	1.10E-06	KFYLAGNPENE	
												FNQQGQSQR	
						2684.3307	3	2684.2137	0.117	56.96	8.80E-06	GVLGAVFSGCP	
												ETFEESQQSSQGR	

(continued on next page)

Table 2 (continued)

Database	Accession number	Protein name	Mascot score	Mr	N peptides	pep_exp_mr	pep_ex p z	pep_calc_mr	pep_delta	pep_score	pep_expect	pep_seq	pep_var_mod
						3039.3243	3	3039.3781	-0.0538	47.44	9.80E-05	IQAEAGQIETW NFNQEDFQCA GVAASR	
						3650.9437	3	3650.6444	0.2993	30.73	0.0046	EPDNRIQAEAGQ IETWNFNQEDFQ CAGVAASR	
	tr Q945K2 Q945K2_PRUDU	R-oxynitrile lyase isoenzyme 1 OS = <i>Prunus dulcis</i>	802	61405	8	578.5118 727.4797 820.9336 843.0888 924.4027 981.5313 1249.6263 1295.5238	1 1 2 2 2 2 2 2	578.3428 727.4592 820.4079 842.4974 924.5029 981.4767 1249.5939 1295.647	0.169 0.0205 0.5257 0.5915 -0.1002 0.0546 0.0324 -0.1232	26.87 23.46 32.25 51.48 34.44 38.83 69.79 40.36	0.027 0.038 0.0078 8.30E-05 0.0024 0.0012 8.20E-07 0.00042	YVGIK VLVLER VLDGDFR VTGINALR VGVHASVEK ITGSTFDNK FVSEDGIDNVR ITGSTFDNKGTR	
	sp P52706 MDL1_PRUSE	(R)- mandelonitrile lyase 1 OS = <i>Prunus serotina</i>	701	61447	7	578.5118 727.4797 820.9336 843.0888 981.5313 1249.6263 1295.5238	1 1 2 2 2 2 2	578.3428 727.4592 820.4079 843.4814 981.4767 1249.5939 1295.647	0.169 0.0205 0.5257 -0.3926 0.0546 0.0324 -0.1232	26.87 23.46 32.25 46.41 38.83 69.79 40.36	0.027 0.038 0.0078 0.00027 0.0012 8.20E-07 0.00042	YVGIK VLVLER VLDGDFR VTGIDALR ITGSTFDNK FVSEDGIDNVR ITGSTFDNKGTR	
	tr Q43608 Q43608_PRUDU	Pru2 protein (fragment) OS = <i>Prunus dulcis</i>	667	57245	8	446.1358 940.9721 977.8762 1025.141 1058.4591 1159.5979 1293.5218 1536.0959	1 2 2 2 2 3 3 3	446.2237 940.5342 978.5498 1024.5375 1058.4741 1159.5218 1293.7669 1535.7952	-0.0879 0.4379 -0.6736 0.6034 -0.015 0.0762 -0.2451 0.3007	3.86 63.26 1.48 4.56 35.17 2.6 0.81 5	3 2.70E-06 10 3 0.0023 3.5 7.1 3.4	SAGGR LLSATSPPR YLRLSAEK GRLMSILGY NLQGQDDNR QEEQSQSR NHLPIRLYLR CLLLLFGCLASR	Oxidation (M)
	tr Q7XJE8 Q7XJE8_PRUDU	Hnl isoenzyme 5 OS = <i>Prunus dulcis</i>	270	61168	2	1010.4713 1249.6263	2 2	1009.5192 1249.5939	0.9521 0.0324	68.5 69.79	1.30E-06 8.20E-07	HAADLLNK FVSEDGIDNVR	
	tr B5LXC9 B5LXC9_PRUDU	Antimicrobial peptides 2-1 (fragment) OS = <i>Prunus dulcis</i>	144	17513	2	764.9138 775.6389	2 2	764.3817 775.4592	0.5322 0.1797	27.42 30.19	0.022 0.007	AIENYR FQLLQK	
	sp O50048 MDL2_PRUSE	(R)- mandelonitrile lyase 2 OS = <i>Prunus serotina</i>	75	62970	1	842.7667	2	842.4974	0.2693	46.38	0.00027	VTGINALR	
	tr B9X0I1 B9X0I1_PRUMU	(R)-hydroxynitrile lyase OS = <i>Prunus mume</i>	75	62998	1	842.7667	2	842.4974	0.2693	46.38	0.00027	VTGINALR	

identification of any protein, we could have detected close to one thousand species, had the genome of almonds been decoded. This still points to the fact that more efforts should be spent by geneticists in covering to a greater extent the plant genome.

As for the orgeat syrup, the identifications of the bands captured by the HM-CPLL beads might suggest that this beverage could be made by simply diluting almond milk, since the fourteen species identified (see Table 2) can also be found in Table 1. Yet, upon verifying that with the producer, we learned that indeed such beverage is made according to the rules issued by the Italian Government (*Gazzetta Ufficiale* No. 62 of 7th March 1968, i.e. with de-acidified benzoin and aromas and oils extracted from bitter almonds, vanilla and flowers of orange trees). Thus, given the fact that benzoin is an aroma extracted from a resin exuded by the benzoin tree (*Styrax*), the source of the proteins identified in Table 2 can only be the bitter almond extract, in which, together with aromas, small amounts of proteins have been extracted as well. It is here recalled, in fact, that whereas almond milk has a rather high protein content (given by the producer as 660 mg/mL), the orzata's protein content is about two orders of magnitude less (10 mg/100 mL, as stated by the manufacturer).

Another interesting aspect of our research, whose aim was also to detect the presence of allergens, if any, is that indeed at least a major one was found, namely lipid transfer protein (LTP, see Table 1), known to be a quasi-ubiquitous allergen present in plant systems, fruits nuts, etc. [15]. Other known potential allergens to be found in this list (based on the known function of these proteins) are: calmodulin, profilin, Pru2 protein and glyceraldehyde 3 phosphate dehydrogenase. On the positive side, all of them (except the Pru2 protein) were found only among the proteins captured via CPLLs at pH 7.2 and pH 9.3, not in the control. This means that such allergens are present at quite low levels, and thus their adverse effects on sensitive patients might be mitigated.

In conclusion, thanks to the CPLL treatment, we could prove that both beverages are indeed produced according to rules and are made with vegetal extracts. Contrary to that, other cheap orzata found in local supermarkets and sampled by us at random, were found not to contain any proteinaceous material, suggesting that they are produced via use of either synthetic or natural aromas and additives. The fact that almond milk should have been produced according to rules is also confirmed by the total protein content, stated as 660 mg/100 mL. As the producer has informed us that this amount was arrived at via the Kjeldahl method (an assay that determines the total content of amino groups of the beverage and that includes also any small molecule and metabolite containing amino groups), we have repeated protein determination via two classical protein assays, namely via the Bio-Rad Dc kit (based on Lowry assay) [16] and with the bicinchoninic assay [17]. Our results (referred to both, almond milk and orzata, see Table 3) confirm such values, although they are, on the average, 20–25% lower, suggesting that the Kjeldahl over-estimated to some extent the total protein amount, no doubt due to determination of any and all amino groups present in solution, not necessarily belonging to proteinaceous material. Such average protein concentration in almond milk (around 600 to 700 mg/100 mL) should indeed be consistent with the total amount of ground almonds required in

Table 3 – Declared and experimental protein concentration in almond's milk and orgeat.

Sample	Declared average values of protein for 100 mL mg	Experimental average values of protein for 100 mL mg
Almond milk	660	540
Orgeat	10	7

the product (the official Italian rule requiring 100 g of finely ground almonds per litre of final product). In fact, considering that almond nuts are very rich in protein content (18–20% of dry weight), assuming that only one third of the proteins can be brought to solution, this would give just about the content stated in the label (0.66 g/100 mL). The total amount of fat also given in the label (2.64 g/mL) is also consistent with the total amount of ground almonds used to prepare this beverage. Thus, both beverages appear to be produced according to rules and, most importantly, to have been made with natural plant extracts, a fact which does not seem to be true, in the case of orgeat syrup, for some cheaper products directly produced by local supermarket chains.

5. Conclusions

The use of combinatorial peptide ligand libraries for detecting the trace proteome in non-alcoholic beverages might become an official test for regulatory authorities for assessing the proper preparation of such beverages and confirming the use of natural plant extracts (including leaves, roots, nuts, etc.) and might be adopted also for consumers' protection. In addition to verifying the genuineness of the products here analysed (almond milk and orgeat syrup from the Fabbri producer) our investigation has already proven that most of the other cheap orgeat syrup brands available in local supermarkets (and sold with the label of the given chain of supermarket) do not contain any genuine plant extract, as no proteins could be detected by our methodology (a method that has proven the capability of revealing as little as a few µg protein/L in white and red wines) [9,10]. Due to the fact that a myriad of such products has invaded commercial outlets, we feel it is just about time that a better survey of such products should be implemented and that the present proteomic investigation could be a good starting point.

Acknowledgments

P.G.R. gratefully acknowledges support by Fondazione Cariplo (Milano) and by PRIN-2008 (Rome). We thank Dr. Gabriel Peltre (Paris) for tips on the presence of allergens.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2011.03.016.

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