According to the CPLL proteome sheriffs, not all aperitifs are created equal!

María Jesús Lerma-García [a,1], Alfonsina D'Amato [b,1], Elisa Fasoli [b], Ernesto Francisco Simó-Alfonso [a], Pier Giorgio Righetti [b,*]

[a] Departament de Química Analítica, Facultat de Química, Universitat de València, Dr. Moliner 50, 46100 Burjassot, Spain
[b] Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, 20131 Milan, Italy

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1. Introduction

The combinatorial peptide ligand library (CPLL) is now a well-established technique able to enhance the signal of low- to very-low abundance proteins to a level at which they would be detectable and identifiable via standard analytical tools, such as an ELISA test or mass spectrometry (MS) [1–4]. In addition to the several applications reported by the scientific community (especially in the bio-medical field), our group has focused, in recent years, in the analysis of a variety of foodstuff, in order to expand the knowledge of the various proteomes and also to check for the presence of unreported allergens [5]. We have also enlarged our investigations to include the trace proteomes present in wines and beers [6], thus reporting, for instance, traces of bovine caseins (known allergens) present in wines treated with fining agents that were not detectable by conventional techniques. Another field we have covered has been the analysis of aperitifs, in order to confirm the genuineness of such products. Most of these aperitifs, indeed, are stated to be produced via secret recipes contemplating an alcoholic infusion of a mixture of herbs and roots, up to thirteen (and perhaps more). Our contention has been that, this being the case, such liqueurs should contain (at least in traces) proteins and peptides eluted from these herbal ingredients. Conversely, their absence would suggest their preparation via addition of synthetic chemicals and flavours, thus not from plant materials. The first aperitif we have analysed has been the Braulio, a very popular liqueur in Northern Italy stated to be produced from a mixture of 13 herbs, berries and roots from alpine flora [7]. Via CPLL capture we could identify >70 proteins present in alpine herbs (including a strong signal from juniper berries), thus fully confirming the genuineness of this product. Things did not go so well with another widely appreciated aperitif we studied, namely Cynar, stated to be an infusion of artichoke leaves, and thus endowed with all beneficial properties of this vegetable. Indeed no single protein/peptide could be detected in this commercial beverage, whereas, when we prepared an infusion in our lab of artichokes, our home-made Cynar-like beverage was found to contain 18 proteins from artichoke leaves, thus casting severe doubts on the genuineness of the commercial liqueur [8]. An intermediate situation was detected with another very popular aperitif in the Mediterranean area, namely Limoncello, a hydro-alcoholic infusion of lemon peels (the

Abbreviations: CPLL, combinatorial peptide ligand libraries; PM, ProteoMiner

* Corresponding author. Fax: +39 02 23993080.
E-mail address: piergiorgio.righetti@polimi.it (P.G. Righetti).

[1] These two authors have equally contributed to the experimental work and share first authorship.

Table 1
Proteins identified in the Amaro Branzi at different pH of CPLLs binding and in the control.

Database	Accession number	Protein name	Known species in recipe	Mascot score	Mr	N peptides	E1	E2	Ctrl
Uniprot_Viridiplantae	tr|Q8H985|Q8H985_CITJA	Acidic class II chitinase OS = *Citrus jambhiri*	Orange peel	676	32864	5	x	x	x
	tr|Q43752|Q43752_CITSI	Chitinase OS = *Citrus sinensis*	Orange peel	303	32459	3	x	x	
	tr|O23785|O23785_CITSI	Cu/Zn superoxide dismutase (Fragment) OS = *Citrus sinensis*		261	12777	4	x	x	
	tr|D7MRV5|D7MRV5_ARALL	Beta-xylosidase 4 OS = *Arabidopsis lyrata subsp. lyrata*		131	85306	5	x		
	tr|B9GZS2|B9GZS2_POPTR	Predicted protein OS = *Populus trichocarpa*		124	84503	3	x		
	tr|B2BDZ8|B2BDZ8_PISVE	Superoxide dismutase OS = *Pistacia vera*	*Angelica*	113	25797	1			x
	tr|A9QA17|A9QA17_CATRO	Ubiquitin (Fragment) OS = *Catharanthus roseus*	Orange peel	111	15447	8	x		
	tr|D3K376|D3K376_CITSI	Polyubiquitin (Fragment) OS = *Citrus sinensis*		111	11985	7	x		
	tr|P93135|P93135_FRAAN	Polyubiquitin OS = *Fragaria ananassa*	Orange peel	111	42656	9	x		
	tr|D7L2X7|D7L2X7_ARALL	PR4-type protein OS = *Arabidopsis lyrata subsp. lyrata*		109	16047	2	x		
	tr|F6H7L5|F6H7L5_VITVI	Putative uncharacterized protein OS = *Vitis vinifera*	Orange peel	108	46874	1			x
	tr|O04428|O04428_CITPA	Putative uncharacterized protein OS = *Citrus paradisi*	Orange peel/*Gentiana*	107	32737	2			x
	sp|P84159|GLP1_CITSI	Germin-like protein (Fragment) OS = *Citrus sinensis*	Orange peel/*Gentiana*	104	2740	2		x	x
	tr|D7L1G5|D7L1G5_ARALL	Disease resistance response OS = *Arabidopsis lyrata subsp. lyrata*		103	13649	1	x	x	
	tr|Q6DMS1|Q6DMS1_SALMI	Calmodulin OS = *Salvia miltiorrhiza*		103	16777	2		x	
	tr|B6T0C0|B6T0C0_MAIZE	Heme-binding protein 2 OS = *ZEA MAYS*	Orange peel	99	23860	1	x	x	
	tr|Q6EV47|Q6EV47_CITSI	Non-specific lipid-transfer protein (Fragment) OS = *Citrus sinensis*	Orange peel	92	9793	3	x		
	sp|P04464|CALM_WHEAT	Calmodulin OS = *Triticum aestivum*	Orange peel/*Gentiana*	91	16893	4	x		
	tr|F4K8M3|F4K8M3_ARATH	Calmodulin 1 OS = *Arabidopsis thaliana*		90	18628	3	x		
	tr|Q9ZTV2|Q9ZTV2_PHAVU	Calmodulin (Fragment) OS = *Phaseolus vulgaris*		90	7837	3	x		
	tr|C5YI16|C5YI16_SORBI	Putative uncharacterized protein Sb07g005270 OS = *Sorghum bicolor*	Orange peel/*Gentiana*	89	24772	1	x	x	x
	tr|E0Z846|E0Z846_PICSI	Thaumatin-like protein (Fragment) OS = *Picea sitchensis*		89	19265	1	x		
	tr|Q0WNZ5|Q0WNZ5_ARATH	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase-like protein OS = *Arabidopsis thaliana*		88	90993	2	x		
	tr|D5A7T4|D5A7T4_PICSI	Putative uncharacterized protein OS = *Picea sitchensis*		87	84688	3	x		
	sp|P85925|UP18_PSEMZ	Unknown protein 18 (Fragment) OS = *Pseudotsuga menziesii*	Orange peel/*Gentiana*	86	1393	2	x	x	x
	tr|F6H6V7|F6H6V7_VITVI	Putative uncharacterized protein OS = *Vitis vinifera* GN = VIT_05s0077g01760	Orange peel	80	49511	1	x		
	tr|Q9FQ13|Q9FQ13_CITPA	Cystatin-like protein OS = *Citrus paradisi*	Orange peel	80	13435	4	x	x	
	tr|A8W7L1|A8W7L1_COFAR	Pathogenesis-related thaumatin-like protein (Fragment) OS = *Coffea arabica*		79	19739	2	x		
	tr|F5ANW8|F5ANW8_MUSAC	Superoxide dismutase (Fragment) OS = *Musa acuminata*	Orange peel	79	21722	1			x
	tr|Q2HPG3|Q2HPG3_GOSHI	Osmotin-like protein I OS = *Gossypium hirsutum*		79	27438	2	x		
	tr|Q5XUG7|Q5XUG7_SOLTU	Putative thaumatin-like protein OS = *Solanum tuberosum*	*Gentiana*	79	28103	2	x		
	tr|Q7X9R0|Q7X9R0_GOSBA	Putative polyubiquitin (Fragment) OS = *Gossypium barbadense*		79	15022	6	x		
	tr|Q4LB13|Q4LB13_HORVU	Methionine synthase 1 enzyme OS = *Hordeum vulgare*		77	84853	2	x		
	tr|Q9SPU0|Q9SPU0_PETCR	Chitinase OS = *Petroselinum crispum*	*Angelica*	77	29430	1			x
	sp|P31753|RS27A_ASPOF	Ubiquitin-40S ribosomal protein S27a (Fragment) OS = *Asparagus officinalis*	orange peel	75	13810	4	x		
	tr|B9SKK5|B9SKK5_RICCO	Nucleoside diphosphate kinase OS = *Ricinus communis*	Orange peel/*Gentiana*	72	16301	1		x	
	tr|D7LT91|D7LT91_ARALL	Putative uncharacterized protein OS = *Arabidopsis lyrata subsp. Lyrata*	*Gentiana*	72	34907	2		x	
	tr|F4HWR0|F4HWR0_ARATH	Uncharacterized protein OS = *Arabidopsis thaliana*	*Gentiana*	72	34443	2		x	
	tr|D7M5B2|D7M5B2_ARALL	Putative uncharacterized protein OS = *Arabidopsis lyrata subsp. lyrata*	Orange peel	69	22828	1		x	
	tr|D8T651|D8T651_SELML	Putative uncharacterized protein OS = *Selaginella moellendorffii*		68	13986	2	x		
	tr|Q2VC78|Q2VC78_SOYBN	Thaumatin-like protein (Fragment) OS = *Glycine max*		67	14683	1	x		
	tr|B9II49|B9II49_POPTR	Predicted protein OS = *Populus trichocarpa* GN = POPTRDRAFT_777161		64	59622	1	x	x	
	tr|F4K4Q2|F4K4Q2_ARATH	Isoleucyl-tRNA synthetase OS = *Arabidopsis thaliana*		64	143927	1	x	x	
	tr|Q38JC1|Q38JC1_CITSI	Temperature-induced lipocalin OS = *Citrus sinensis*	Orange peel	64	21561	3		x	
	tr|B9RE23|B9RE23_RICCO	Aspartic proteinase nepenthesin-1, putative OS = *Ricinus communis*		62	53736	1	x		
	tr|E4MVP8|E4MVP8_THEHA	mRNA, clone: RTFL01-03-B06 OS = *Thellungiella halophila*		62	54269	1		x	

	tr|C6SWP1|C6SWP1_SOYBN	Putative uncharacterized protein OS = *Glycine max*		61	23468	1	x		
	tr|D8T7V0|D8T7V0_SELML	Putative uncharacterized protein OS = *Selaginella moellendorffii*		60	13975	1	x		
	tr|Q2ERX5|Q2ERX5_MANIN	Beta-1,3-glucanase OS = *Mangifera indica*	*Gentiana*	60	19552	1	x		
	tr|Q2HTY5|Q2HTY5_MEDTR	FAD linked oxidase, N-terminal OS = *Medicago truncatula*	Orange peel/*Gentiana*	60	61994	1		x	x
	tr|B9SAZ8|B9SAZ8_RICCO	Reticuline oxidase, putative OS = *Ricinus communis*	Orange peel	58	60401	2			x
	tr|Q0WMC3|Q0WMC3_ARATH	Putative uncharacterized protein At5g66420 (Fragment) OS = *Arabidopsis thaliana*		58	27592	1		x	
	tr|B8QWV3|B8QWV3_ZEAMP	Pathogenesis-related maize seed protein OS = *Zea mays subsp. Parviglumis*		57	18847	2	x		
	tr|B8ACG5|B8ACG5_ORYSI	Putative uncharacterized protein OS = *Oryza sativa subsp. indica*		56	28868	1			x
	tr|B9NDL0|B9NDL0_POPTR	Predicted protein OS = *Populus trichocarpa*	Orange peel	56	25026	2	x	x	
	tr|Q01JQ5|Q01JQ5_ORYSA	H0523F07.8 protein OS = *Oryza sativa*	*Gentiana*	56	20923	1		x	
	sp|P49043|VPE_CITSI	Vacuolar-processing enzyme OS = *Citrus sinensis*	Orange peel/*Gentiana*	55	54713	1			x
	tr|A9T7Y0|A9T7Y0_PHYPA	Predicted protein OS = *Physcomitrella patens subsp. patens*		53	88493	1	x		
	tr|E1ZIN7|E1ZIN7_9CHLO	Putative uncharacterized protein OS = *Chlorella variabilis*		52	21643	1	x		
	tr|F2DAJ2|F2DAJ2_HORVD	Predicted protein OS = *Hordeum vulgare var. distichum*		51	56708	1		x	
Uniprot_allentries	MRJP1_APIME	Major royal jelly protein 1 OS = *Apis mellifera*	Honey	8254	49311	34	x	x	x
	MRJP3_APIME	Major royal jelly protein 3 OS = *Apis mellifera*	Honey	5625	61966	25	x	x	x
	MAL1_APIME	Alpha-glucosidase OS = *Apis mellifera*	Honey	2822	65694	27	x	x	x
	MRJP2_APIME	Major royal jelly protein 2 OS = *Apis mellifera*	Honey	2085	51441	26	x	x	x
	MRJP5_APIME	Major royal jelly protein 5 OS = *Apis mellifera*	Honey	817	70531	16	x	x	x
	MRJP4_APIME	Major royal jelly protein 4 OS = *Apis mellifera*	Honey	589	53225	12	x	x	x
	THIO_ECOLI	Thioredoxin-1 OS = *Escherichia coli* (strain K12)		130	11913	2	x	x	
	SODM_HEVBR	Superoxide dismutase [Mn], mitochondrial OS = *Hevea brasiliensis*		127	25880	1			x
	CALM_DICDI	Calmodulin OS = *Dictyostelium discoideum*		120	17140	2	x	x	
	BXL4_ARATH	Beta-D-xylosidase 4 OS = *Arabidopsis thaliana*		112	85338	5	x		
	UBIQ_CANAL	Ubiquitin OS = *Candida albicans*		111	8552	7	x		
	GLP1_CITSI	Germin-like protein (Fragment) OS = *Citrus sinensis* PE = 1 SV = 1		110	2740	2	x	x	x
	FRYL_HUMAN	Protein furry homolog-like OS = *Homo sapiens*		109	342177	2	x	x	x
	LACB_BOVIN	Beta-lactoglobulin OS = *Bos taurus*		97	20269	2			x
	TLP_ORYSJ	Thaumatin-like protein OS = *Oryza sativa subsp. japonica*		89	18551	1	x		
	PYRD_BORA1	Dihydroorotate dehydrogenase OS = *Bordetella avium* (strain 197 N)		73	37119	1	x		
	CCD13_HUMAN	Coiled-coil domain-containing protein 13 OS = *Homo sapiens*		71	80834	2	x	x	
	EFTS_RALME	Elongation factor Ts OS = *Ralstonia metallidurans* (strain CH34/ATCC 43123/DSM 2839)		71	31043	1	x	x	x
	METE_SOLSC	5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase OS = *Solenostemon scutellarioides*		71	84822	2	x		
	GL25_ARATH	Probable germin-like protein subfamily 2 member 5 OS = *Arabidopsis thaliana*		69	22911	1		x	
	WIN_SOYBN	Wound-induced protein (Fragment) OS = *Glycine max*		68	11402	1	x		
	Y835_RICCN	UPF0192 protein RC0835 OS = *Rickettsia conorii*		68	213993	1		x	
	BXL5_ARATH	Probable beta-D-xylosidase 5 OS = *Arabidopsis thaliana*		60	86415	1	x		
	DYHC_DROME	Dynein heavy chain, cytoplasmic OS = *Drosophila melanogaster*		59	532987	1	x		
	PSMB_METTE	Proteasome subunit beta OS = *Methanosarcina thermophila*		58	23024	1	x	x	
	RL18_NITWN	50S ribosomal protein L18 OS = *Nitrobacter winogradskyi* (strain Nb-255/ATCC 25391)		58	13090	2		x	
	DCD_HUMAN	Dermcidin OS = *Homo sapiens*		57	11391	1		x	
	PRMS_MAIZE	Pathogenesis-related protein PRMS OS = *Zea mays*		56	18720	1	x		
	HUTU_BACA2	Urocanate hydratase OS = *Bacillus amyloliquefaciens* (strain FZB42)		54	61179	1			x
	YCF46_PORPU	Uncharacterized AAA domain-containing protein ycf46 OS = *Porphyra purpurea*		54	56338	1		x	
	RK18_PLAF7	Putative 50S ribosomal protein L18, apicoplast OS = *Plasmodium falciparum* (isolate 3D7)		53	24922	1		x	
	TBFG_EPTST	Thread biopolymer filament subunit gamma OS = *Eptatretus stoutii*		52	62872	1	x		
	NHA1_RHORH	High-molecular weight cobalt-containing nitrile hydratase subunit alpha OS = *Rhodococcus rhodochrous*		51	22991	2		x	x

Abbreviations: E1: eluate from CPLL beads after capture at pH 2.2; E2: eluate from CPLL beads after capture at pH 4.8; Ctrl: control.

yellow, very thin skin, called flavedo, eliminating the underlying spongy layer called albedo). In principle, Limoncello should be produced by utilizing special lemons growing in the Sorrento Region in South Italy; in reality, just about every Italian family brews homemade limoncellos. In only a single commercial product we could detect just 8 proteins (and some proteolytic fragments) together with 12 peptides, thus proving the genuineness of this product (although in our home-made infusion 264 proteins were present, but we had used a much higher amount of peels in a reduced hydro-alcoholic volume). On the contrary, cheaper Limoncellos available in supermarkets were devoid of any protein/peptide, casting doubts on their production from vegetable extracts [9].

In the present investigation, we report the proteome of yet another liqueur quite popular in the Lombardy region, namely Amaro Branzi (also called Amaro delle Orobie). This amaro (bitter) can be sipped as an aperitif, as a digestive and also as a tonic, since it is stated to be an alcoholic infusion of (among other secret ingredients) *Gentiana lutea*, *Angelica officinalis*, orange peel and honey. It figures, given the unique properties of these herbal ingredients. The *Gentiana* root has a long history of use as an herbal bitter in the treatment of digestive disorders and is an ingredient of many proprietary medicines. It is considered especially useful in states of exhaustion from chronic disease and in all cases of debility, weakness of the digestive system and lack of appetite. It has also been regarded as one of the best fortifiers of the human system, stimulating the liver, gall bladder and digestive system, and it has been thought to be an excellent tonic to combine with a purgative in order to prevent its debilitating effects [10]. *Angelica* is a genus of about 60 species of tall biennial and perennial herbs in the family Apiaceae, native to temperate and subarctic regions of the Northern Hemisphere, reaching as far north as Iceland and Lapland; its active ingredients are found in the roots and rhizomes [11]. The herb, also known by the Chinese name, Bai Zhi, and Latin name, Radix Angelicae Dahurica, is used medicinally in Traditional Chinese Medicine. According to a study, methoxy-8-(2-hydroxy-3-buthoxy-3-methylbutyloxy)-psoralen has been shown to regulate the cyclooxygenase-2 (COX-2)-dependent phase of prostaglandin D(2) generation in bone marrow-derived mast cells. In addition, this compound consistently modulated the production of leukotriene C(4), demonstrating the ability to modulate both cyclooxygenase-2 and 5-lipoxygenase activity. Furthermore, this compound also affected the degranulation reaction [12]. Its roots and seeds are often used to flavour some liqueurs, such as Chartreuse. Also orange peels are supposed to possess several health promoting properties, such as lowering the total cholesterol levels in the body, inhibiting the growth and division of cancer cells, relieving heartburns, preventing irritable bowel syndrome, curing various digestive disorders including indigestion and averting numerous respiratory problems such as bronchitis, colds, flu and asthma. Additionally, in cuisine, they are popularly used as flavouring agents to garnish and add a tangy taste to foods [13,14].

In order to prove the genuineness of this product, we have investigated the proteome not only of the liqueur, but also of two herbal ingredients, namely *G. lutea* and *A. officinalis*. As part of another project, we had obtained the proteome of orange peel (work in progress); as for honey, we had already published recently its proteome content [15].

2. Materials and methods

2.1. Chemicals and biologicals

ProteoMiner™ (combinatorial hexapeptide ligand library beads, CPLL), Laemmli buffer, 40% acrylamide/Bis solution, N,N,N′,N′-tetramethylethylenediamine (TEMED), molecular mass standards and electrophoresis apparatus for one-dimensional electrophoresis were purchased from Bio-Rad Laboratories, Hercules CA. β-mercaptoethanol, dithiothreitol (DTT), ammonium persulphate, 3-[3-cholamidopropyl dimethylammonio]-1-propanosulfonate (CHAPS), acetonitrile (ACN), trifluoroacetic acid (TFA), sodium dodecyl sulphate (SDS), formic acid (FA) and all other chemicals used all along the experimental work were current pure analytical grade products and purchased from Sigma-Aldrich, St Louis, MO. Complete protease inhibitor cocktail tablets and sequencing grade trypsin were from Roche Diagnostics, (Basel, CH). Both, *A. officinalis* and *G. lutea* roots as well as the bottles of Amaro Branzi were bought in a local herbalist shop.

2.2. Plant materials and protein extraction protocols

For preparation of root extracts, 50 g of each root (*A. officinalis* and *G. lutea*), added with 250 mL of 35% vol. ethanol solution, was homogenized in a steel-blade blender for 10 min, till pulverization of the mass. The suspensions were stirred at room temperature for 2 days to improve protein extraction in the hydro-alcoholic solution. The insoluble materials were separated by centrifugation (18,000 rpm for 10 min) and the alcoholic supernatant was diluted to a final ethanol concentration of 12.5% vol., considered optimal for capturing proteins via CPLLs. Both, *A. officinalis* and *G. lutea* extracts were divided in two aliquots, one of them maintained at its natural extraction pH (4.8), the other one being titrated at pH 2.2 by addition of formic acid and 0.1% TFA.

In order to detect proteinaceous material in the Amaro Branzi (25% ethanol content), 700 mL liqueur was 1:1 diluted with deionized water in order to reduce the ethanol content to 12.5% vol. Also in this case, the total volume was divided in two aliquots, one of them was maintained at its natural pH (4.8), and the other one was titrated to pH 2.2 (by adding formic acid and 0.1% TFA).

To all above aliquots, 100 μL of ProteoMiner (CPLL) was added and protein capture was implemented via gentle shaking overnight at room temperature; then the beads were collected by filtration. The adsorbed proteins were then desorbed by using 100 μL of 4% SDS and 20 mM DTT for 15 min, under boiling conditions [16]. As a control sample for the aperitif, since seeding a 30 μL aliquot into an SDS-PAGE gel did not enable detection of bands via micellar Coomassie staining, another control was obtained after protein precipitation of 200 μL of the Amaro (directly taken from the bottle) with MeOH/chloroform. To one volume of protein solution 4 volumes of cold MeOH were added, mixed and kept at 4 °C for 5 min. One volume of chloroform and 4 volumes of water were added, mixed and finally centrifuged at 14,000 rpm for 15 min. The supernatant was discarded, and the pellet was washed twice with cold methanol and finally centrifuged at 14,000 rpm for 5 min. The obtained pellet was dissolved in 30 μL of Laemmli buffer for SDS-PAGE (which was performed as in [16]). The results listed in Table 1 are only those entries detected in three technical replicas onto two different bottles of Amaro Branzi, considered as two biological replicates.

2.3. Mass spectrometry and data analysis

The tryptic mixtures were acidified with formic acid up to a final concentration of 10%. Eight microlitres of tryptic digest for each band was injected in a nano chromatographic system, UltiMate 3000 RSLCnano System (Thermo Scientific). The peptide mixtures were loaded on a reversed-phase trap column (Acclaim PepMap100, C18, 100 Å, 100 μm i.d. × 2 cm, Thermo Scientific) for the cleanup and pre-concentration. After cleanup, the 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 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 nL/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* 2000 Da and the five 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 30 s. The MS data were analysed separately by Mascot search engine (version 2.3.01) using Proteome Discover software (v. 1.2.0 Thermo) and consulting

Uniprot_viridiplantae database (30264 sequences, 184678199 residues) and SwissProt database without restriction of taxonomy (515203 sequences, 181334896 residues). Oxidation of methionine residues was set as a variable modification; two missed cleavages were allowed to trypsin; peptide mass tolerance was set to 1 Da, fragment mass tolerance was set to 0.8 Da, and an ion source cut-off of 20 was chosen. The false discovery rate obtained by Proteome Discoverer, consulting the Mascot decoy database, was less than 0.01.

3. Results

Fig. 1 shows the SDS-PAGE profiles of the extracts from *Angelica*'s (tracks 1–3) and *Gentiana*'s roots (tracks 4–6). It can be appreciated that the respective controls (i.e. the clear hydro-alcoholic extracts loaded as such, tracks 1 and 4) do not show any visible bands, whereas the eluates from the CPLL beads after capture at pH 2.2 (tracks 2 and 5, respectively) and at pH 4.8 (tracks 3 and 6) exhibit intense bands, especially in the Mr 10 to 70 kDa regions. There are rather severe smears, especially in the lower Mr region, but this, unfortunately, is typical of vegetable extracts, which contain tannins and polyphenols and other plant polymers that are not easily eliminated during extraction. The blue arrows indicate the centre of 10 polyacrylamide gel segments that have been excised and whose protein content has been sent to MS analysis after trypsin digestion (the total discoveries in both extracts are listed in the supplementary on-line Tables S1 and S2).

Fig. 2 displays the corresponding SDS-PAGE profiles of the proteome content of the Amaro Branzi. Here too (see track 1) if 30 μL of the amaro is directly loaded onto the gel, nothing is visible. If the amaro is preconcentrated seven-folds by precipitation with MeOH/chloroform, some faint bands at 60 kDa are visible after micellar Coomassie staining (track 2). However, only when the hidden proteome of the amaro is captured with CPLL beads at pH 2.2 and pH 4.8 (tracks 3 and 4, respectively) intense zones are visualized all along the track, from 10 up to 250 kDa. Here too all tracks are divided into 12 zones (blue arrows), whose content is digested with trypsin and sent to MS analysis. Table 1 lists all identifications combined from the different tracks, after eliminating redundancies.

The Venn diagrams in Fig. 3a and b give the contribution to the various identifications of proteins present in the Amaro Branzi as found in the control, untreated liqueur and in the eluates from the CPLL beads at the two pH values. In Fig. 3a it can be appreciated that, notwithstanding

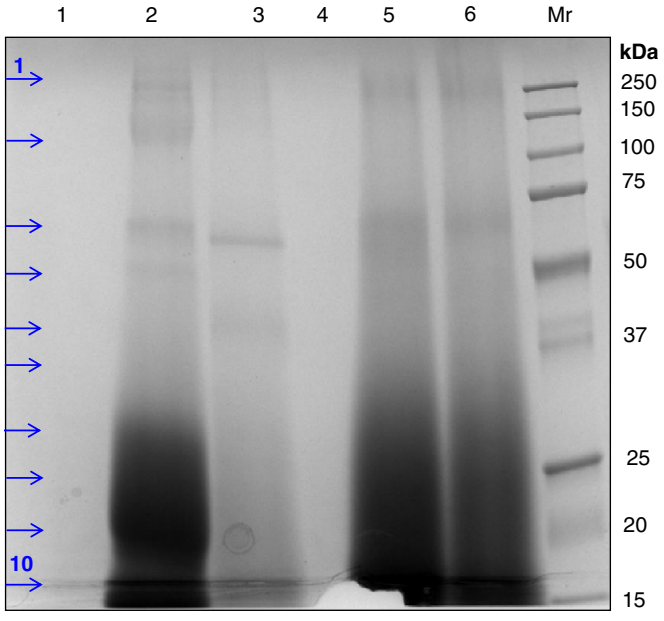

Fig. 1. SDS-PAGE analysis of the *Angelica officinalis* (tracks 1–3) and *Gentiana lutea* (tracks 4–6) roots extracts. Lanes: 1 and 4: controls; 2 and 3: eluates of *Angelica*'s root from CPLL beads after capture at pH 2.2 and pH 4.8, respectively; 5 and 6: eluates of *Gentiana*'s root from CPLL beads after capture at pH 2.2 and pH 4.8, respectively; Mr: molecular mass standards. In all cases, 30 μL of sample solution was loaded into the gel pockets. Colloidal Coomassie Blue staining. The ten blue arrows indicate the centre of each gel segment excised for band elution, trypsinization and MS analysis.

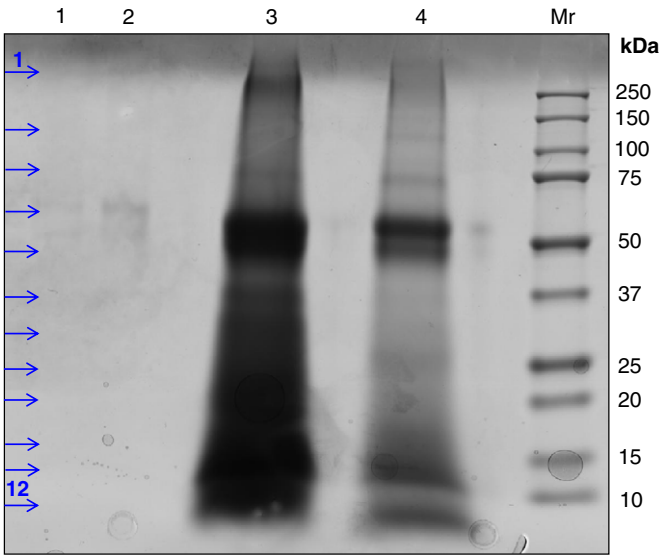

Fig. 2. SDS-PAGE analysis of the proteome content of the Amaro Branzi. Tracks 1 and 2: aperitif loaded as such or after a seven-fold concentration, respectively; 3 and 4: eluates of aperitif from CPLL beads after capture at pH 2.2 and pH 4.8, respectively; Mr: molecular mass standards. In all cases, 30 μL of sample solution was loaded into the gel pockets. Colloidal Coomassie Blue staining. The twelve blue arrows indicate the centre of each gel segment excised for band elution, trypsinization and MS analysis.

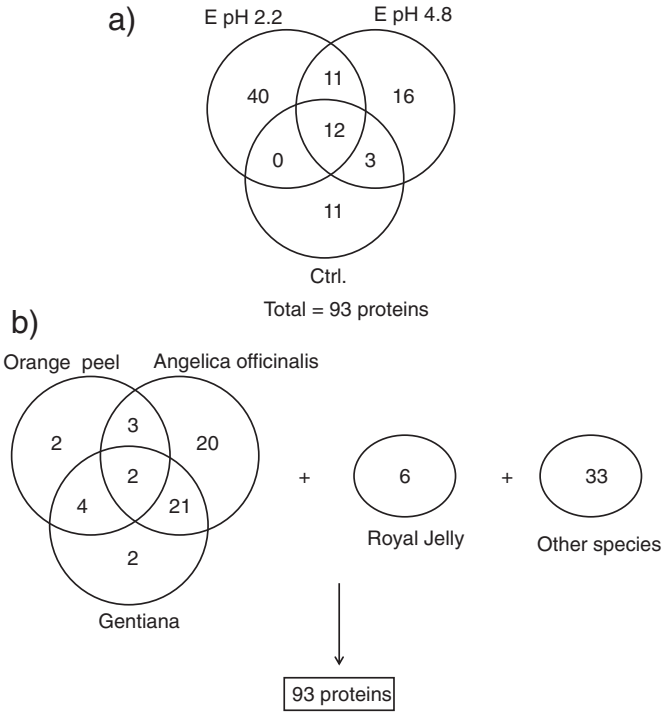

Fig. 3. Panel a: Venn diagrams of the species found upon the various captures in the Amaro Branzi. Ctrl: control sample, seven-fold concentrated; E pH 2.2: eluate from CPLL beads after capture at pH 2.2; E pH 4.8: eluate from CPLL beads after capture at pH 4.8. Panel b: Venn diagram of the contribution of the various ingredients to the total proteome (93 proteins) detected in the aperitif.

the fact that the control had been concentrated seven folds, only 26 species are detected. On the contrary, the combined discoveries of the two CPLL eluates have increased substantially the total proteome detected, up to 93 unique gene products. Here too, as already reported in many other investigations [3–5], the increment in the visibility of the "hidden" proteome has been by a factor of almost four-folds. It is of interest also to see what is the contribution of the various ingredients to the total proteome as here detected. This is highlighted in Fig. 3b: 20 species are found to be specific of the *A. officinalis*, vs. only two specific for orange peel and *G. lutea*, the others being in common between two species at a time. Interestingly, 21 proteins are shared between the two root extracts (*Angelica* and *Gentiana*) suggesting a non-negligible similarity between their respective proteomes; conversely, barely 2 species are in common among the three proteomes. This does not account for all discoveries: six additional proteins have been detected, belonging to the honey proteome, according to the manufacture declaration about its presence as an ingredient. Another 33 proteins are found not to belong to the four official ingredients and these species likely should represent the other "secret" ingredients in the amaro's recipe.

4. Discussion

The title first:

"We hold these truths to be self-evident, that all men are created equal…"

This is the opening sentence sculptured in the United States Declaration of Independence, as formulated in 1776 (13 years before the French Revolution, which claimed "Liberté, Égalité, Fraternité!") by Benjamin Franklin, Thomas Jefferson, John Adams, Robert Livingston, and Roger Sherman. This is what humanity wishes and strives at achieving, although in real life this seems to be a quite difficult goal to reach, no matter how hard we try. In terms of food industry and commercial preparations, we all hope that all products would be "created equal", i.e. by honest and truthful protocols, avoiding frauds, but this too seems to be a rather difficult proposition. In every country there is a fraud repression agency, which should exert surveillance on foodstuff placed on the market, for customer protection. Fortunately legislation, in the EC and elsewhere, is becoming more and more restricted by imposing, for instance, that labels should state the country of origin of products and where and when they were handled for commercialization. Yet repression of frauds is not always so easy, as exemplified by those existing in the production of extra virgin olive oil, where adulteration is part of the daily life and very frequent [26].

The path most frequently trodden today for quality control and fraud repression by inspecting agencies is to analyse metabolites via MS or NMR [17–20]; within the last few years, also stable isotope analysis has gained increasing importance in authenticity control of food and food ingredients [21]. When analysing non-alcoholic and alcoholic beverages our approach has been different: for those stated to be obtained from vegetable and plant extracts, we reasoned that such liquids should contain, even if in trace amounts, proteins and peptides therefrom. Our assumption has been verified in practice: thus, when analysing orgeat syrups present on supermarkets shelves, we found only one brand containing a number of almond proteins, other cheaper products being devoid of proteinaceous material, indicating the absence of plant extracts [22]; the same applied to a Cola drink [23] and to ginger ales [24]. In the present case of aperitifs, as stated in the introduction, the results have also been clear-cut: Braulio and some brands of limoncellos have been found to be genuine, whereas Cynar, stated to be an artichoke infusion, seemed to be devoid of any protein or peptide from this vegetable. The Amaro Branzi here investigated appears to be perhaps the best brand on the market, since we could verify (see Table 1) all ingredients officially stated by the producer and reported on the label. In addition, the number of proteins identified has been the largest so far in any beverage, amounting to no less than 93 species. This has been made possible by the information on the aperitif preparation kindly provided by the producer. The starting solution is 35% alcohol to which all plant and herbal ingredients are added. The infusion is allowed to continue for two weeks at room temperature, after which the liquid (which, due to the contribution of the various ingredients, has seen its alcoholic content reduced to 25%) is filtered and transferred to oak barrels to age for one year. At the end of this period, after a second filtration process, the amaro is added with honey and bottled. Knowing that, when we prepared our *Angelica*'s and *Gentiana*'s root extracts, we did that in a 35% alcohol solvent, so as to mimic the amaro's manipulations. This is why our proteome discoveries in both extracts (see Supplementary Tables S1 and S2) barely reach 100 species, hardly an in depth investigation. Notwithstanding that, the digestive has been found to contain fewer protein species than our two herbal extracts. This is no doubt due to the fact that, upon the 1-year ageing in barrels, a variety of proteins present in the original infusion must have flocculated and precipitated, due to aggregation of large Mr species with tannins and other plant polymers present in the infusion. In fact, after the oak barrel ageing, the amaro preparation had to be filtered to remove the substantial precipitate.

Although our data fully confirm the genuineness of this product, they do not allow us to infer the relative proportions of the various ingredients, since we do not know the efficiency of capture of all proteins present in solution. Although in general such efficiency has been evaluated to be on average around 60–70%, it could be as low as 20% and as high as 90% [25]. A clear example can be appreciated when inspecting the mascot score of the various entries in Table 1: according to these data, by far the honey proteins should represent the most abundant ingredient, due to their very high score, and the orange peels the lowest one, due to the paucity of species detected (11, vs. 29 for *G. lutea* and 46 for *A. officinalis*, accounting also for shared species, see Fig. 3b). Yet we know, from previous work [15], that CPLLs have a very high affinity for honey proteins (indeed all of them belonging to the royal jelly proteome), so that they might have been harvested from a solution with an efficiency close to 90%. Also the fact that the *Gentiana* proteins are present in substantially lower amounts than those of *Angelica* cannot give us any clue on their respective initial ratio in the infusion. It might simply mean that the *Gentiana* species originally present in the infusion have flocculated more extensively, during ageing in oak barrels, than those of *Angelica*. Our data, in addition, confirm the presence of other vegetable and herbal species in this aperitif, since no less than 33 proteins in our list are found to belong to "other species", although such ingredients could not be identified due to lack of genomic information for the vast majority of plants in the vegetable kingdom.

In conclusion, our strategy of assaying for the genuineness of beverages present in the market, and stated to be of plant origin, by searching for traces of proteins and/or peptides therein, seems to be working quite efficiently provided, though, one adopts the CPLL methodology, since quite often such species might be present in minute amounts or even in traces.

Supplementary data to this article can be found online.

Conflict of interest

The authors declare no conflict of interest.

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