Orange proteomic fingerprinting: From fruit to commercial juices

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Combinatorial peptide ligand library technology, coupled to mass spectrometry, has been applied to extensively map the proteome of orange pulp and peel and, *via* this fingerprinting, to detect its presence in commercial orange juices and drinks. The native and denaturing extraction protocols have captured 1109 orange proteins, as identified by LC–MS/MS. This proteomic map has been searched in an orange concentrate, from a Spanish juice manufacturer, as well as in commercial orange juices and soft drinks. The presence of numerous orange proteins in commercial juices has demonstrated the genuineness of these products, prepared by using orange fruits as original ingredients. However, the low number of identified proteins in sparkling beverages has suggested that they were prepared with scarce amounts of fruit extract, thus imparting lower quality to the final products. These findings not only increase the knowledge of the orange proteome but also present a reliable analytical method to assess quality and genuineness of commercial products.

Keywords:
Orange fruit
Orange juice
LC–MS/MS
Combinatorial peptide ligand library
Protein
Proteomics

1. Introduction

Oranges are one of the most popular fruits around the world: they are a hybrid between pomelo (*Citrus maxima*) and mandarin (*Citrus reticulata*), which has been cultivated since ancient times (Pedrosa, Schweizer, & Guerra, 2000). The fruit of *Citrus sinensis*, belonging to the family Rutaceae, is considered a sweet orange, whereas the fruit of *Citrus aurantium* is considered a bitter orange (Spiegel-Roy & Goldschmidt, 1996).

Orange trees are the most cultivated fruit trees in the world, growing in tropical and subtropical climates (United States Department of Agriculture. Foreign Agricultural Service. Citrus: World Markets, 2014). The European Union is the third largest producer of oranges worldwide, with Spain and Italy at the top of the list. The fruit of the orange tree is described as "hesperidium": a modified berry with tough, leathery rind, constituted by a peel, enriched in volatile oil glands in pits, and by an interior flesh, composed of segments called carpels made up of numerous fluid-filled vesicles. Orange fruits can be eaten fresh or processed for their juice or fragrant peel. Brazil is the largest producer of orange juice in the world, followed by the US. Often industries prepare frozen orange juice concentrate from freshly squeezed and filtered orange juice.

Citrus fruits and juice, as such, have long been valued for their wholesome nutritious and antioxidant properties and, by virtue of their abundance in vitamins, antioxidants and minerals, have many proven health benefits. Moreover, it is now well confirmed, in citrus fruits, the presence of biologically active non-nutrient compounds, such as phytochemical antioxidants (Goulas & Manganaris, 2012) and soluble or insoluble dietary fibres involved in reducing the risk for stroke (Kurl et al., 2002), chronic diseases like arthritis (Pattison et al., 2004), hypertension (Galati et al., 1994), and coronary heart diseases.

While traditional nutrition research has focused on secondary metabolites, it is important to consider also food-derived proteins and peptides. Proteins are not only the major macronutrients involved in every cellular process, but also the precursors of bioactive peptides, characterised by antihypertensive or antimicrobial functions and capability to regulate immune response (Kussmann, Panchaud, & Affolter, 2010). For example food allergy, which is an adverse reaction to food components or additives with an underlying immunological mechanism, is well studied through proteomic analysis able to detect and identify allergenic proteins (Eigenmann, 2001). As regards *C. sinensis*, a proteomic analysis of flesh at ripening time has characterised overexpressed proteins devoted to sugar metabolism, to stress response and to secondary metabolism. In particular Mucilli et al. (2009) have demonstrated

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Table 1
Proteins identified in CPLLs and control eluates of commercial Italian orange juice (Z3) and sparkling orange beverages (Z4, Z5), by consulting Uniprot_Viridiplantae database.

Sample	Accession number	Protein name	Taxonomy	Mascot score	Mr	N pep	CPLLs	Ctrl
Z3	Q8S987	Polygalacturonase-inhibiting protein	*Citrus aurantifolia*	785	36564	6	×	×
	Q6RUQ2	Glyceraldehyde 3-phosphate dehydrogenase	*Daucus carota*	506	36768	5	×	×
	D0R8T6	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	*Manihot sp. Allem 4581*	471	14536	5	×	×
	Q308A5	Fructose-bisphosphate aldolase	*Solanum tuberosum*	343	38901	5	×	
	D7NHW9	Enolase	*Poncirus trifoliata*	336	47986	8	×	×
	Q84LP5	HSP19 class II (Fragment)	*Citrus paradisi*	312	11191	4	×	×
	E4MYB4	mRNA, clone: RTFL01-47-M11	*Thellungiella halophila*	296	71410	5	×	
	Q9ZSW0	Tetra-ubiquitin	*Saccharum officinarum*	291	34202	3	×	×
	Q9M4E7	Heat shock protein 70	*Cucumis sativus*	276	71843	6	×	×
	Q40151	Hsc70	*Solanum lycopersicum*	272	71869	6	×	
	C6SV69	Putative uncharacterized protein	*Glycine max*	242	17832	3	×	
	A9QA17	Ubiquitin (Fragment)	*Catharanthus roseus*	220	15447	3	×	
	B9RQT7	Heat-shock protein, putative	*Ricinus communis*	214	17808	4	×	×
	B2D2G5	70 kDa heat shock protein	*Capparis spinosa*	212	71456	5	×	
	F6HYK6	Putative uncharacterized protein	*Vitis vinifera*	209	71814	3	×	
	A5CAF6	Phosphoglycerate kinase	*Vitis vinifera*	199	42510	3	×	
	B4UW51	Class II small heat shock protein Le-HSP17.6 (Fragment)	*Arachis hypogaea*	193	14508	4	×	
	D8RYS9	Putative uncharacterized protein	*Selaginella moellendorffii*	187	71211	4	×	
	B9GJB1	Predicted protein	*Populus trichocarpa*	167	36670	5	×	×
	D2D326	Luminal binding protein	*Gossypium hirsutum*	166	73566	4	×	
	D8TT41	Luminal binding protein Bip1	*Volvox carteri f. nagariensis*	161	72249	2	×	
	A5AS18	Putative uncharacterized protein	*Vitis vinifera*	157	21726	2	×	
	B8LM67	Putative uncharacterized protein	*Picea sitchensis*	148	17058	2	×	×
	O04428	Putative uncharacterized protein	*Citrus paradisi*	148	32737	3	×	×
	F2YRD3	Putative PDF1-interacting protein 3 (Fragment)	*Gossypium barbadense*	146	25861	2		×
	Q5UFR1	14-3-3 family protein	*Malus domestica*	146	29793	5	×	
	E9N1I2	Enolase (Fragment)	*Schiedea globosa*	139	9311	2	×	
	E3VVV8	14-3-3 protein	*Litchi chinensis*	138	29504	6		×
	A8VK65	Cytosolic glyceraldehyde 3-phosphate dehydrogenase (Fragment)	*Conradina grandiflora*	127	8508	3	×	
	B3TM07	Ribosomal protein L17	*Elaeis guineensis var. tenera*	117	20744	2	×	
	Q2PF01	Putative cytosolic factor	*Trifolium pratense*	115	67827	1	×	×
	C5X6A7	Putative uncharacterized protein Sb02g043510	*Sorghum bicolor*	113	46539	2	×	
	A5BFL3	Putative uncharacterized protein	*Vitis vinifera*	112	23370	2		×
	Q0Z864	Actin 1	*Boehmeria nivea*	105	41867	1	×	×
	Q42207	Fructose-bisphosphate aldolase (Fragment)	*Arabidopsis thaliana*	105	13708	1		×
	F6H7L5	Putative uncharacterized protein	*Vitis vinifera*	104	46874	3		×
	Q9ZWQ8	Plastid-lipid-associated protein, chloroplastic	*Citrus unshiu*	97	35252	4		×
	C0PDC7	Putative uncharacterized protein	*Zea mays*	96	101284	1		×
	D1MWZ0	GRAM domain-containing protein	*Citrullus lanatus subsp. vulgaris*	96	28613	1	×	
	P93267	Ras-related protein Rab7A	*Mesembryanthemum crystallinum*	95	23516	2		×
	B7FMJ8	Putative uncharacterized protein	*Medicago truncatula*	94	17714	1	×	
	C6T116	Putative uncharacterized protein	*Glycine max*	93	17897	2		×
	B9MUL1	Autoinhibited H + ATPase	*Populus trichocarpa*	93	105444	1	×	
	B9N0X9	Predicted protein	*Populus trichocarpa*	90	34990	1	×	
	A1ECK1	Thioredoxin	*Citrus hybrid cultivar*	89	13270	2	×	×
	B8ZV17	Phosphoenolpyruvate carboxykinase (Fragment)	*Aristida rhiniochloa*	89	56473	2	×	
	A4ZF49	Chaperone	*Agave tequilana*	87	18325	3		×
	Q5PNZ9	At1g22780	*Arabidopsis thaliana*	87	17591	4	×	
	E5FY24	Chloroplast small heat shock protein 1	*Potentilla discolor*	86	26454	3	×	×
	D7LG27	Putative uncharacterized protein	*Arabidopsis lyrata subsp. lyrata*	86	31591	2	×	
	Q3LUM6	Elongation factor 1-alpha	*Gossypium hirsutum*	85	49538	4	×	×
	A9SAK3	Predicted protein	*Physcomitrella patens subsp. patens*	85	74055	2	×	
	F2DWX8	Predicted protein	*Hordeum vulgare var. distichum*	83	21692	1		×
	A8QVI5	Putative ADP-ribosylation factor (Fragment)	*Lactuca sativa*	82	15707	1		×
	A5HE90	Hypersensitive response protein	*Triticum aestivum*	82	31519	1		×
	C0PAJ0	Putative uncharacterized protein	*Zea mays*	81	20672	2		×
	B9I496	Predicted protein	*Populus trichocarpa*	80	22694	1	×	
	A9PBU5	Putative uncharacterized protein	*Populus trichocarpa*	79	20391	2	×	
	B9SKK5	Nucleoside diphosphate kinase	*Ricinus communis*	79	16301	1	×	
	D7KXF2	Putative uncharacterized protein	*Arabidopsis lyrata subsp. lyrata*	77	17769	2	×	
	Q0D7W3	s07g0205000 protein	*Oryza sativa subsp. japonica*	77	20189	1	×	
	O81961	Heat shock protein 26 (Type I)	*Nicotiana tabacum*	76	26754	5	×	×
	Q53E30	Cytosolic class I small heat shock protein 2B (Fragment)	*Nicotiana tabacum*	76	15345	3		×
	C1KEU0	Sal k 3 pollen allergen (Fragment)	*Salsola kali*	76	83795	1	×	
	Q2XTD8	Histone H2B	*Solanum tuberosum*	76	15817	1	×	
	F4JJ94	14-3-3-like protein GF14 chi	*Arabidopsis thaliana*	75	36044	3		×
	C0SUJ6	Elongation factor 1-alpha (Fragment)	*Nelumbo nucifera*	75	39146	2	×	
	B4F976	17.4 kDa class I heat shock protein 3	*Zea mays*	75	17869	3	×	
	O49152	14-3-3 protein homolog	*Maackia amurensis*	74	29506	3		×
	A0EJ88	Glutamate decarboxylase	*Populus canescens*	74	56591	1	×	
	D2T0D8	Cytosolic class I small heat shock protein type 2 (Fragment)	*Rhododendron rubropunctatum*	73	15973	3	×	×
	C1JYE2	Phosphoglycerate kinase (Fragment)	*Henrardia persica*	71	31430	1	×	

Table 1 (*continued*)

Sample	Accession number	Protein name	Taxonomy	Mascot score	Mr	N pep	CPLLs	Ctrl
	Q75XU9	14-3-3 f-2 protein (Fragment)	*Nicotiana tabacum*	69	27533	4		×
	A6N839	UDP-glucose pyrophosphorylase	*Pinus taeda*	69	53169	3	×	
	C6SVU1	Putative uncharacterized protein	*Glycine max*	69	21811	2	×	
	Q38JC1	Temperature-induced lipocalin	*Citrus sinensis*	68	21561	2	×	
	A9YVC8	Pyrophosphate-dependent phosphofructokinase alpha subunit	*Citrus sinensis* × *Citrus trifoliata*	68	68078	1	×	
	C5YI16	Putative uncharacterized protein Sb07g005270	*Sorghum bicolor*	68	24772	1	×	
	A5BGC5	Putative uncharacterized protein	*Vitis vinifera*	68	16472	1	×	
	E0CVB4	Putative uncharacterized protein	*Vitis vinifera*	68	25128	5	×	
	B0LUL2	Cell-wall invertase	*Populus alba* × *Populus grandidentata*	68	65663	1	×	
	A5B7A4	6-phosphogluconate dehydrogenase, decarboxylating	*Vitis vinifera*	66	48484	1	×	×
	D2KU75	Thaumatin-like protein	*Citrus jambhiri*	66	27551	1	×	
	E4MXN5	mRNA, clone: RTFL01-28-H06	*Thellungiella halophila*	66	81193	3	×	
	Q9SP07	14-3-3-like protein	*Lilium longiflorum*	66	29349	3	×	
	B6TFS9	14-3-3-like protein A	*Zea mays*	66	28718	2	×	
	Q40511	Heat shock protein 70 (Fragment)	*Nicotiana tabacum*	65	63313	2		×
	Q2V995	Cytoplasmic ribosomal protein S13-like	*Solanum tuberosum*	65	17108	2		×
	D0UZK2	Terpene synthase 1	*Citrus sinensis*	65	63978	1	×	
	A5AHA8	Putative uncharacterized protein	*Vitis vinifera*	65	80247	3	×	
	C0P4Q3	Putative uncharacterized protein	*Zea mays*	64	82037	2		×
	B9S4Y1	Uro-adherence factor A, putative	*ORicinus communis*	63	174718	1	×	
	A8J1U1	Heat shock protein 90A	*Chlamydomonas reinhardtii*	63	80973	2	×	
	B6STA3	IN2-1 protein	*Zea mays*	62	26361	1		×
	C6SXW7	Putative uncharacterized protein	*Glycine max*	62	23118	1	×	
	A2Q5G2	Ras GTPase; Sigma-54 factor, interaction region	*Medicago truncatula*	61	23294	2		×
	A2ZLM3	Putative uncharacterized protein	*Oryza sativa subsp. japonica*	61	22044	3		×
	Q0JB49	Glutathione peroxidase	*Oryza sativa subsp. japonica*	61	18642	1	×	
	B6UDY2	60S ribosomal protein L17	*Zea mays*	60	19605	2	×	×
	Q5QJB8	Harpin binding protein 1	*Citrus paradisi*	59	31275	1		×
	D7TQB0	Putative uncharacterized protein	*Vitis vinifera*	59	27956	1	×	
	B9SMK4	ligopeptidase A, putative	*Ricinus communis*	59	88119	1	×	
	Q0JDA9	s04g0416400 protein (Fragment)	*Oryza sativa subsp. japonica*	58	15663	1		×
	B4FNT1	Elongation factor 1-delta 1	*Zea mays*	58	24951	1		×
	F2E8I7	Predicted protein	*Hordeum vulgare var. distichum*	58	36511	2	×	
	C0HFI5	Putative uncharacterized protein	*Zea mays*	57	57827	1		×
	D7KJI0	Putative uncharacterized protein	*Arabidopsis lyrata subsp. lyrata*	57	26722	1		×
	C0PJQ8	Putative uncharacterized protein	*Zea mays*	54	38233	1		×
	Q8GS16	Pectinesterase	*Citrus sinensis*	54	69973	1		×
	F1T197	Heat shock protein 90	*Chara braunii*	54	80700	2	×	
	C0HE21	Putative uncharacterized protein	*Zea mays*	53	16802	1		×
	Q0JGZ6	Fructokinase-1	*Oryza sativa subsp. japonica*	53	34869	2		×
	C1ECE1	Predicted protein	*Micromonas sp. (strain RCC299 / N)*	51	16934	1	×	×
	A0S5Z5	Dehydroascorbate reductase	*Sesamum indicum*	51	23731	1		×
Z4	Q84LP5	HSP19 class II (Fragment)	*Citrus paradisi*	537	11191	3	×	
	B4UW51	Class II small heat shock protein Le-HSP17.6 (Fragment)	*Arachis hypogaea*	527	14508	4	×	
	B9RQT7	Heat-shock protein, putative	*Ricinus communis*	527	17808	4	×	
	Q38HV4	Fructose-bisphosphate aldolase	*Solanum tuberosum*	353	38812	1	×	
	Q8S988	Polygalacturonase-inhibiting protetin	*Microcitrus sp. citruspark01*	320	36641	4	×	
	Q53E42	Cytosolic class I small heat shock protein 1B (Fragment)	*Nicotiana tabacum*	151	15631	3	×	
	D7NHW9	Enolase	*Poncirus trifoliata*	133	47986	3	×	
	B9N0X9	Predicted protein	*Populus trichocarpa*	108	34990	1	×	
	Q9FQ13	Cystatin-like protein	*Citrus paradisi*	105	13435	3	×	
	Q39627	Citrin	*Citrus sinensis*	90	55426	3	×	
	P34091	60S ribosomal protein L6	*Mesembryanthemum crystallinum*	88	25985	2	×	
	Q0JNS6	Calmodulin-1	*Oryza sativa subsp. japonica*	83	16878	1	×	
	E9N1I2	Enolase (Fragment)	*Schiedea globosa*	82	9311	2	×	
	A0FIJ6	Stress-related protein	*Citrus sinensis*	79	17593	1	×	
	Q6EV47	Non-specific lipid-transfer protein (Fragment)	*Citrus sinensis*	76	9793	1	×	
	A9RZ63	40S ribosomal protein S24	*Physcomitrella patens subsp. patens*	75	15499	1	×	
	E5FY24	Chloroplast small heat shock protein 1	*Potentilla discolor*	69	26454	1	×	
	A2Y3Z4	Putative uncharacterized protein	*Oryza sativa subsp. japonica*	69	22336	2	×	
	C0PCV2	40S ribosomal protein S8	*Zea mays*	68	24409	1	×	
	B7FH91	Putative uncharacterized protein	*Medicago truncatula*	67	17901	1	×	
	Q3LUL9	Elongation factor 1-alpha	*Gossypium hirsutum*	66	49750	2	×	
	Q2HUT9	Nascent polypeptide-associated complex NAC; UBA-like	*Medicago truncatula*	66	22074	2	×	
	Q84LP4	HSP19 class I (Fragment)	*Citrus paradisi*	65	6430	2	×	
	C0PTL1	Putative uncharacterized protein	*Picea sitchensis*	65	7972	1	×	
	D1MWZ0	GRAM domain-containing protein	*Citrullus lanatus subsp. vulgaris*	63	28613	1	×	
	A9PAD6	Predicted protein	*Populus trichocarpa*	62	7725	1	×	
	Q38JC1	Temperature-induced lipocalin	*Citrus sinensis*	61	21561	2	×	
	D8RNW6	Histone H2B (Fragment)	*Selaginella moellendorffii*	58	11397	1	×	

(*continued on next page*)

Table 1 (*continued*)

Sample	Accession number	Protein name	Taxonomy	Mascot score	Mr	N pep	CPLLs	Ctrl
	D2KU75	Thaumatin-like protein	*Citrus jambhiri*	58	27551	1	×	
	A5AJ83	Putative uncharacterized protein	*Vitis vinifera*	56	13751	1	×	
	C5YI16	Putative uncharacterized protein Sb07g005270	*Sorghum bicolor*	55	24772	1	×	
	C6SZ56	Superoxide dismutase [Cu-Zn]	*Glycine max*	55	15298	2	×	
	Q9FQ15	Miraculin-like protein 3	*Citrus paradisi*	51	22833	1	×	
	C5WP54	Putative uncharacterized protein Sb01g026440	*Sorghum bicolor*	51	35063	1	×	
	Q0J5J5	G-box binding factor	*Oryza sativa subsp. japonica*	50	28979	1	×	
	A9P8G8	Predicted protein	*Populus trichocarpa*	50	16760	1	×	
Z5	Q8W3U6	Polygalacturonase-inhibitor protein	*Citrus hybrid cultivar*	447	36915	5	×	
	C6SV69	Putative uncharacterized protein	*Glycine max*	418	17832	3	×	
	B9S392	Heat-shock protein, putative	*Ricinus communis*	255	17505	2	×	
	D7NHW9	Enolase	*Poncirus trifoliata*	254	47986	4	×	
	Q38HV4	Fructose-bisphosphate aldolase	*Solanum tuberosum*	252	38812	3	×	
	Q9FQ13	Cystatin-like protein	*Citrus paradisi*	203	13435	6	×	
	Q8GS16	Pectinesterase	*Citrus sinensis*	147	69973	4	×	
	B2BF98	40S ribosomal protein S6	*Glycine max*	144	28243	3	×	
	Q39627	Citrin	*Citrus sinensis*	141	55426	7	×	
	C6SWC7	Putative uncharacterized protein	*Glycine max*	139	25829	2	×	
	P04464	Calmodulin	*Triticum aestivum*	110	16893	2	×	
	C5YI16	Putative uncharacterized protein Sb07g005270	*Sorghum bicolor*	109	24772	1	×	
	Q9ZSW0	Tetra-ubiquitin	*Saccharum officinarum*	109	34202	3	×	
	Q0J5J5	G-box binding factor	*Oryza sativa subsp. japonica*	97	28979	1	×	
	B7FIB3	Putative uncharacterized protein	*Medicago truncatula*	92	29676	1	×	
	B9N0X9	Predicted protein	*Populus trichocarpa*	90	34990	1	×	
	E5FY24	Chloroplast small heat shock protein 1	*otentilla discolor*	87	26454	1	×	
	F2DWX8	Predicted protein	*Hordeum vulgare var. distichum*	81	21692	1	×	
	D7KJI0	Putative uncharacterized protein	*Arabidopsis lyrata subsp. lyrata*	78	26722	1	×	
	C0PCV2	40S ribosomal protein S8	*Zea mays*	77	24409	2	×	
	Q9FQ15	Miraculin-like protein 3	*Citrus paradisi*	75	22833	2	×	
	D7KXF2	Putative uncharacterized protein	*Arabidopsis lyrata subsp. lyrata*	75	17769	2	×	
	D8S6W2	Putative uncharacterized protein	*Selaginella moellendorffii*	75	34924	2	×	
	O82013	17.3 kDa class II heat shock protein	*Solanum peruvianum*	74	17311	2	×	
	C0KQW1	40S ribosomal protein S15-like protein	*Jatropha curcas*	74	17155	2	×	
	B3TLK8	60S ribosomal protein L24	*Elaeis guineensis var. tenera*	73	18759	1	×	
	A5BGC5	Putative uncharacterized protein	*Vitis vinifera*	72	16472	1	×	
	B4F976	17.4 kDa class I heat shock protein 3	*Zea mays*	71	17869	2	×	
	Q2PF01	Putative cytosolic factor	*Trifolium pratense*	71	67827	1	×	
	B7FH91	Putative uncharacterized protein	*Medicago truncatula*	71	17901	1	×	
	D2KU75	Thaumatin-like protein	*Citrus jambhiri*	69	27551	2	×	
	Q9M5L0	60S ribosomal protein L35	*Euphorbia esula*	67	14405	2	×	
	Q39538	Heat shock protein (Fragment)	*Citrus maxima*	67	9381	2	×	
	D2T0E3	Cytosolic class I small heat shock protein type 1 (Fragment)	*Rhododendron mariesii*	66	16320	3	×	
	D1MWZ0	GRAM domain-containing protein	*Citrullus lanatus subsp. vulgaris*	65	28613	1	×	
	Q5J0W3	Lipocalin protein	*Capsicum annuum*	64	21414	1	×	
	Q7X7E8	Peptidyl-prolyl cis-trans isomerase (Fragment)	*Triticum aestivum*	64	24864	2	×	
	A2Y0K0	Ribosomal protein L18	*Oryza sativa subsp. japonica*	64	21355	1	×	
	A9RZ63	40S ribosomal protein S24	*Physcomitrella patens subsp. patens*	63	15499	1	×	
	F4HYS8	Leucine-rich repeat/extensin 2	*Arabidopsis thaliana*	63	90545	1	×	
	A5BIA1	Putative uncharacterized protein	*Vitis vinifera*	62	20948	3	×	
	B2DD07	Chitinase	*Citrus unshiu*	61	31078	4	×	
	C1KEU0	Sal k 3 pollen allergen (Fragment)	*Salsola kali*	60	83795	1	×	
	D7LG27	Putative uncharacterized protein	*Arabidopsis lyrata subsp. lyrata*	59	31591	1	×	
	A2Y3Z4	Putative uncharacterized protein	*Oryza sativa subsp. japonica*	59	22336	1	×	
	D7TQB0	Putative uncharacterized protein	*Vitis vinifera*	59	27956	2	×	
	A5B3K6	Putative uncharacterized protein	*Vitis vinifera*	59	16124	3	×	
	O81961	Heat shock protein 26 (Type I)	*Nicotiana tabacum*	58	26754	3	×	
	O82011	17.7 kDa class I heat shock protein	*Solanum peruvianum*	57	17674	2	×	
	Q7Y045	Dehydrin	*Citrus sinensis*	57	27214	2	×	
	B2CKB7	Glyceraldehyde 3-phosphate dehydrogenase (Fragment)	*Manihot triphylla*	57	11827	3	×	
	A8WCV1	Low molecular weight heat shock protein	*Gossypium hirsutum*	57	17565	2	×	
	C1ECE1	Predicted protein	*Micromonas sp. (strain RCC299 / N*	57	16934	2	×	
	C0PK55	Putative uncharacterized protein	*Zea mays*	57	12273	1	×	
	B6U476	40S ribosomal protein S15	*Zea mays*	55	16604	1	×	
	D8SFT8	Putative uncharacterized protein	*Selaginella moellendorffii*	54	36438	2	×	
	Q0WR55	GPI-anchored protein (Fragment)	*Arabidopsis thaliana*	53	22950	1	×	
	Q0J4P2	Heat shock protein 81-1	*Oryza sativa subsp. japonica*	52	80429	1	×	
	D6BQM8	Ubiquinol-cytochrome C reductase complex 14 kDa protein	*Jatropha curcas*	52	14686	2	×	
	A9P0F0	Putative uncharacterized protein	*Picea sitchensis*	50	20043	1	×	

that protein overexpression at ripening time was specific of sweet orange varieties: while blood oranges overexpressed proteins related to anthocyanins pathway, the common oranges increased species involved in stress response. Proteomic and metabolomic profiles are also affected by temperature: after freezing stress, expression of proteins involved in regulatory

functions, in iron metabolism, in oxidative damage and in carbohydrate metabolism has been induced, suggesting an energy request from glycolysis or an activation of defence mechanisms (Perotti et al., 2015).

Considering the increasing interest in orange components and the paucity of deep proteomic investigation, the aim of the present study is to characterise and to identify the orange proteome in fruit and in juices, in order to investigate how industrial working conditions could alter and modify the protein profiles, with direct consequences for nutritional properties. The proteomic fingerprinting of orange juice could be useful to verify the genuineness of commercial products and to protect consumers from possible adulteration.

2. Material and methods

2.1. Chemicals

ProteoMiner™ (combinatorial hexapeptide ligand library beads), Laemmli buffer, 40% acrylamide/bis solution, *N*,*N*,*N*′,*N*′-tetramethy lethylenediamine (TEMED), molecular mass standards, electrophoresis apparatus for one-dimensional electrophoresis and DC protein assay were from Bio-Rad Laboratories, Inc., Hercules CA. β-Mercaptoethanol, dithiothreitol (DTT), ammonium persulphate, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfo nate (CHAPS), acetonitrile (ACN), trifluoroacetic acid (TFA), sodium dodecyl sulphate (SDS), iodoacetamide (IAA), formic acid (FA) and

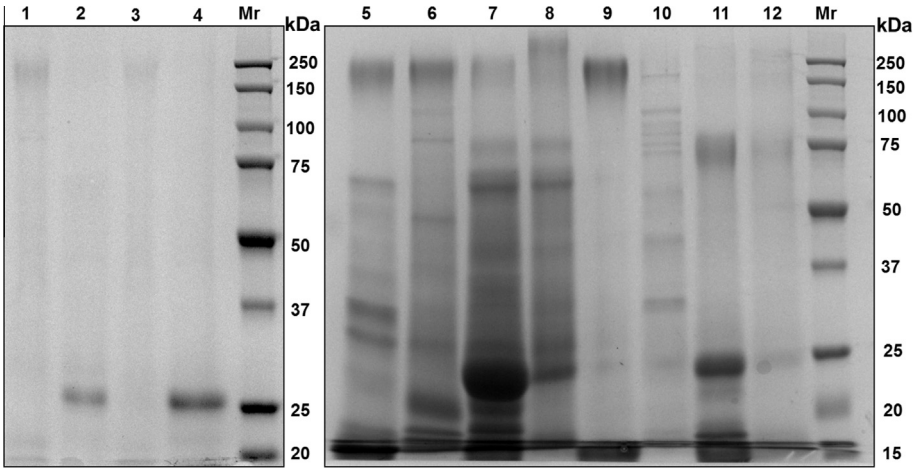

Fig. 1. Coomassie Blue SDS–PAGE gels corresponding to protein extracts from orange peel and pulp, obtained with different extraction methods. Lane 1, control native pulp; Lane 2, control native peel; Lane 3, control denatured pulp; Lane 4, control denatured peel; Lane 5, eluate pH 2.2 of native pulp; Lane 6, eluate pH 7.2 of native pulp; Lane 7, eluate pH 2.2 of native peel; Lane 8, eluate pH 7.2 of native peel; Lane 9, eluate pH 2.2 of denatured pulp; Lane 10, eluate pH 7.2 of denatured pulp; Lane 11, eluate pH 2.2 of denatured peel; Lane 12, eluate pH 7.2 of denatured peel; Mr denotes molecular mass standards.

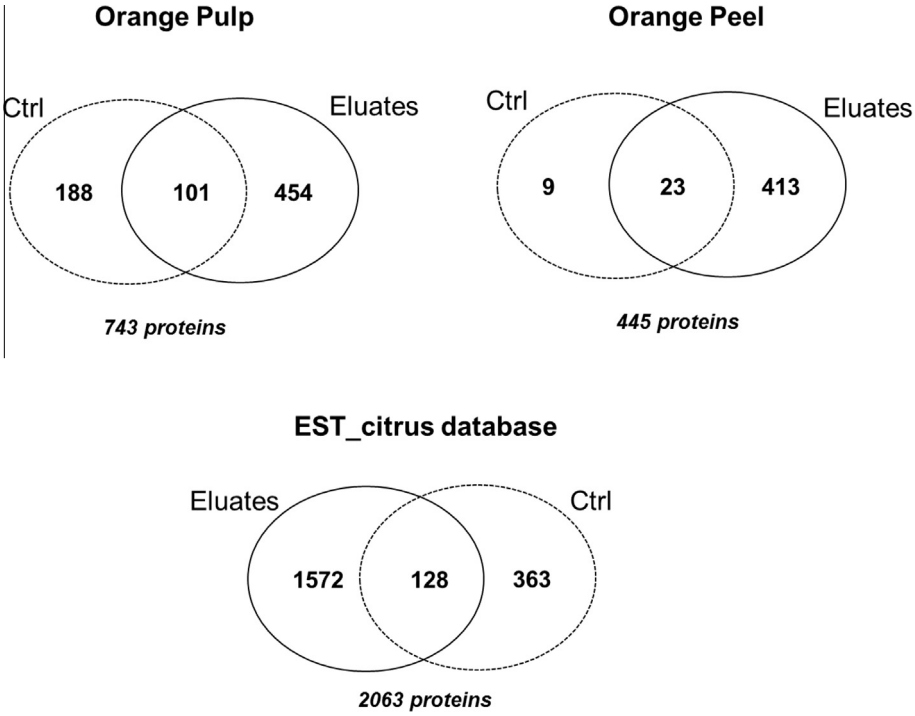

Fig. 2. Venn diagrams of total protein identifications in orange pulp and peel. In both cases, the total discoveries of untreated control were matched *vs* the CPLL capture. The lower graph gives the protein identifications obtained by using the EST_*Citrus* database.

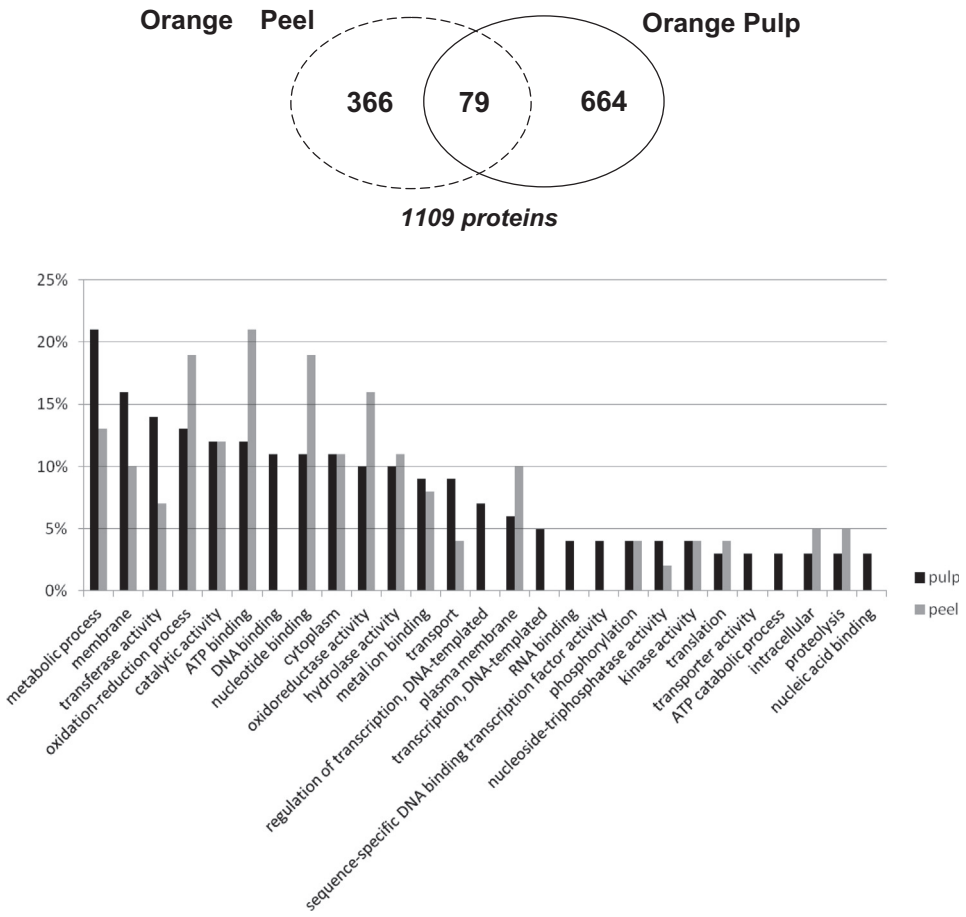

Fig. 3. Venn diagrams of total protein identifications in orange pulp *vs* orange peel and corresponding gene ontology (GO) analysis.

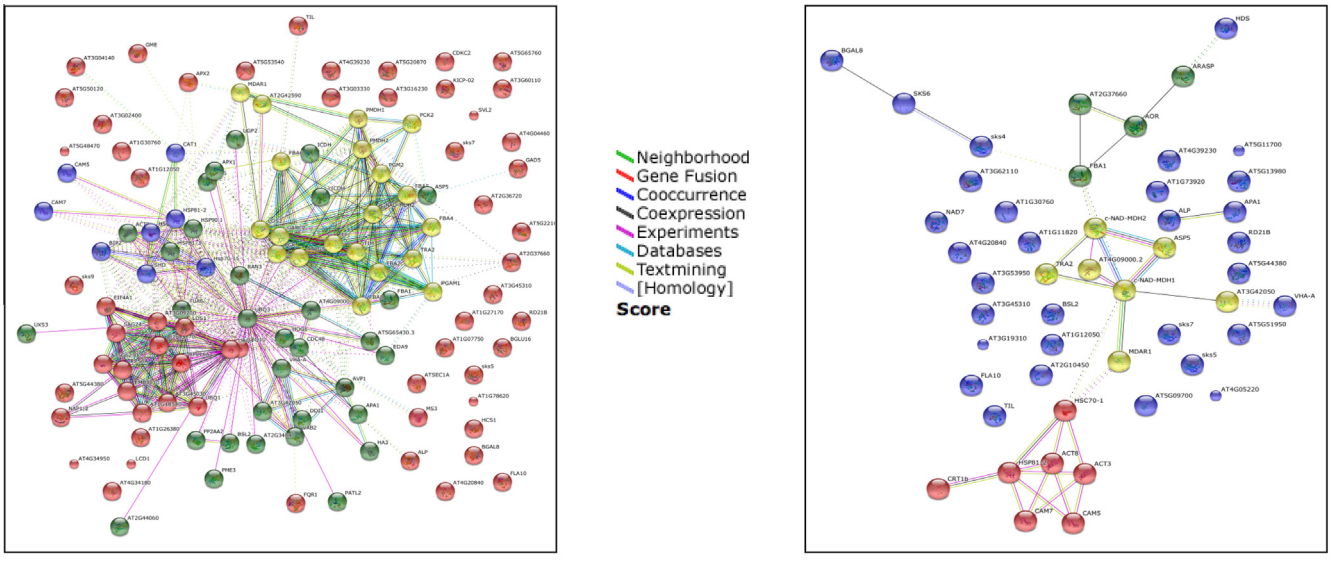

Fig. 4. Protein–protein interaction networks of orange peel and pulp, constructed by clustering K-MEANS via STRING software (p-value = 1.82e-1 for peel sample, p-value = 1.21e-5 for pulp sample). The legend describes the type of interconnections between different genes.

all other chemicals used were analytical grade products purchased from Sigma–Aldrich Corporation, St Louis, MO. Complete protease inhibitor cocktail tablets (which contained inhibitors for a broad spectrum of serine, cysteine and metalloproteases as well as calpains) and sequencing grade trypsin were from Roche Diagnostics (Basel, Switzerland).

2.2. Samples and protein extraction protocols

2.2.1. Sample origin

The oranges used in this work came from two different varieties (Navel and Sanguinelli), which were kindly donated by Fontestad company (Museros, Valencia, Spain). All oranges used in this study were collected in January 2014 in the Valencian region, Spain. Three orange juices were also analysed: the first (Z1) was kindly donated by a Spanish juice manufacturer (Zumos Valencianos del Mediterráneo S.A.); the second (Z2) was a commercial Spanish orange juice not from concentrate (Hacendado brand), and the third (Z3) was a commercial Italian orange juice 100% from concentrate (Zuegg Skipper brand). Finally, two soft drinks were also analysed: orange Fanta (Z4, whose label indicated a 12% orange juice content) and Sanpellegrino Aranciata, an Italian orangeade (Z5, whose label indicated a 15.6% juice content).

2.2.2. Protein extraction

Before orange protein extraction, the surface of orange fruits was washed with lukewarm 1% SDS solution, to eliminate bacterial and surface contamination from human hands. Next, the thin fla-vedo peel was carefully excised and reduced to very minute frag-ments *via* a treatment in a fruit blender for 10 min at maximum power. For exploring the proteomes of both peels and pulp, two extraction protocols were devised after full homogenisations of these two tissues. The native extraction buffer contained 50 mM Tris–HCl (pH 7.2), 50 mM NaCl and 2% (*m*/*v*) CHAPS, whereas the denatured buffer contained additionally 1% (*m*/*v*) SDS and 25 mM DTT dissolved in the same buffer. Protease inhibitor cocktails were added to both extraction buffers, in order to prevent protein degradation by protease action. In detail 15 g of minced peel and 75 g of pulp were mixed respectively with 40 mL and 5 mL of each extraction buffer and gently shaken for 3 h at room temperature for the native buffer and under boiling conditions for the denatured one. Finally the homogenates were centrifuged at 18,000 rpm for 10 min.

2.2.3. Combinatorial peptide ligand libraries treatment

After centrifugation, the recovered volumes of both protein extracts from orange peel and pulp were incubated with combinatorial peptide ligand libraries (CPLLs): while the native samples were ready to be incubated with CPLLs, the denatured ones were diluted 1:10 (v/v) with a buffer containing 50 mM Tris–HCl (pH 7.2), 50 mM NaCl, 25 mM DTT and protease inhibitor cocktail, in order to reduce the original 1% SDS amount to 0.1% (*m*/*v*), so as to allow an effective protein capture (Fasoli et al., 2010). Peel and pulp extracts obtained under both native and denatured conditions were divided into two aliquots prior to CPLL capture, which were titrated at pH 7.2 using 1 M NaOH and at pH 2.2 by addition of formic acid and 0.1% TFA. Three technical replicas on two different biological samples were performed.

For detection of proteinaceous material in the orange juices and soft drinks, two aliquots of 100 mL for each juice/soft drink were added to 10 mL of native and denatured extraction buffer after centrifugation (18,000 rpm for 10 min), in order to eliminate insoluble materials. One of the aliquots was adjusted to pH 7.2 and the other one was titrated to pH 2.2 as previously described. Also in this case the CPLL treatment was performed *via* gentle shaking overnight at room temperature at pH 7.2 and pH 2.2. The adsorbed proteins were then desorbed with a solution containing 4% SDS and 20 mM DTT for 15 min, under boiling conditions (Candiano et al., 2009). To determine the protein concentration, a Bio-Rad DC protein assay was performed on pulp and peel extracts, orange juices and drinks. It was a colorimetric assay based on the Lowry assay, where proteins create complexes with copper in an alkaline medium able to reduce the Folin reagent, producing a blue colour, proportional to protein concentration, with maximum absorbance at 750 nm. The absorbance reading was performed in two replicas of a triplicate for each type of sample.

2.3. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed in a Bio-Rad Mini-Protean system (Hercules, CA) according to standard protocols (Fasoli et al., 2010; Saez, Fasoli, D'Amato, Simó-Alfonso, & Righetti, 2013) using commercial Mini-PROTEAN precast gels or home-made gels composed of a 4% polyacrylamide stacking gel cast over a 12% resolving polyacrylamide gel. As positive control samples of juices and soft drinks were chosen; another positive control was obtained after protein concentration from 200 μL of juice/drink to 40 μL (since seeding a 40 μL aliquot into an SDS–PAGE gel did not enable detection of bands *via* micellar Coomassie staining).

2.4. Mass spectrometry and data analysis

SDS–PAGE bands were cut into thin slices, reduced with 10 mM DTT, alkylated with 55 mM IAA, and digested with 1 ng/μL trypsin in 25 mM ammonium bicarbonate at 37 °C overnight. The tryptic mixtures were acidified with FA up to a final concentration of 10% and 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 clean-up and pre-concentration. After clean-up, 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 Objec-tive). The peptides were eluted with a 30 min gradient from 4%buffer **A** (2% ACN and 0.1% FA in water) to 60% buffer **B** (2% water and 0.1% FA in ACN) at a constant flow rate of 300 nL/min. The liq-uid chromatography was connected to an LTQ-XL mass spectrom-eter (Thermo Scientific) equipped with a nanospray ion source. Full scan mass spectra were acquired in the mass range *m*/*z* 350 to 1800 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 by consulting the Uniprot_*Viridiplantae* data-base (30264 sequences, 184678199 residues) and the EST_*Citrus* database (604877 mRNA sequences). 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, by consulting the Mascot decoy database, was less than 1%.

2.5. Protein–protein interaction network construction and functional enrichment analysis

The STRING v.10 (Search Tool for the Retrieval of Interacting Genes/proteins) software, a database of known and predicted protein interactions, was used to find a protein–protein interaction (PPI) network both for peel and pulp orange proteins. The previous MS data were analysed again by Mascot search engine (version 2.3.01) consulting the Uniprot_*ArabidopsisThaliana* database (38489 sequences, 69767 residues) and the new identifications were analysed by STRING v.10 set on *Arabidopsis Thaliana* as organism database (Szklarczyk et al., 2011).

To investigate the cellular components of genes in PPI networks, GO (Gene Ontology) cellular component enrichment was performed for both pulp and peel samples.

3. Results

As reported in recent literature (Fasoli & Righetti, 2013), the application of two different extraction protocols and the use of two different pH values for CPLL incubation have substantially contributed to increase protein capture, as seen by SDS–PAGE profiling. Fig. 1 shows the electrophoretic profiles of the controls (untreated samples) and the eluates from CPLL captures. Considering the controls, in both native and denatured peel extracts (lanes 2 and 4) only one band of about 25 kDa was detected. Conversely the various eluates obtained after CPLL treatment (from lane 5 to lane 12) were characterised by a larger number of protein bands and by an increase of their intensity as a demonstration of the effi-ciency and potentiality of such methodology. For all orange sam-ples (peel and pulp) the eluates from CPLL incubation in native buffer appeared to be more effective and efficient in protein extrac-tion, due to the absence of interfering reagents, like SDS, able to prevent a correct interaction between proteins and ligand peptides on beads.

After MS analysis, the Venn diagrams, reported in Fig. 2, compared the proteins identified in CPLL eluates and in controls both for the peel and pulp. When considering orange pulp, the number of unique gene products, recognised after CPLL treatment, is 2.5 times greater than in the control, while in orange peel the corresponding increase of identifications in eluates is about 40 times higher than identifications in controls (Supplementary Table 1). This is a confirmation of the efficiency of CPLL for the in-depth detection of these proteomes (Fasoli, D'Amato, Kravchuk, Citterio, & Righetti, 2011), as also demonstrated by the Venn diagram surveying both orange peel and pulp by using the EST_*Citrus* database. The amino acid region of *Citrus* proteins, translated from identified mRNA sequences, encompassed more than 1500 proteins in the eluates, with 363 in the controls and 128 being in common between both eluates and controls. It has to be noticed that the non-redundant number of identified proteins using EST-database is lower, considering the overlapping and the presence of different translated amino acid regions in the same protein. For these reasons different identified mRNA sequences, belonging to the same protein accession number, would generate redundancy.

In order to describe and to compare the biological function of the total 1109 species identified both in orange peel and pulp, a gene ontology (GO) analysis was performed by using the web available software QuickGO (www.ebi.ac.uk/QuickGO) and reported in Fig. 3. On the other hand, based on confidence score calculated by STRING (confidence score > 0.4), PPI networks for orange peel (p-value = $1.82e^{-1}$) and orange pulp (p-value = $1.21e^{-5}$) were constructed and visualised in Fig. 4. For genes in PPI networks, a GO enrichment analysis based on cellular component localisation was performed for both pulp and peel samples and the top 10 significantly enriched cellular components were listed in Supplementary Table 2.

In order to understand how the proteome is preserved during the industrial processing and preparation of commercial juices, we had the possibility to analyse unpasteurised filtered orange juices and the corresponding commercial pasteurised ones (Supplementary Table 3). Fig. 5A shows the Venn diagram of identified

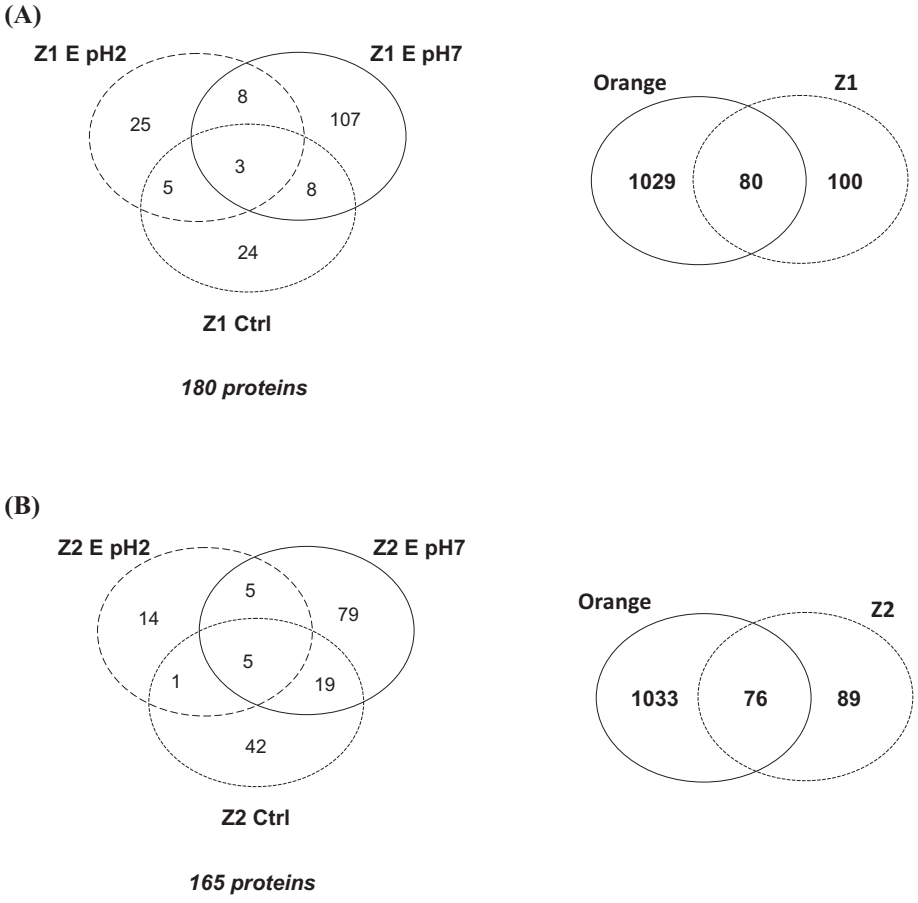

Fig. 5. Venn diagrams of (A) total protein identifications in unpasteurised filtered orange juice (Z1) and (B) in a commercial pasteurised Spanish orange juice (Z2). The first diagram gives the contributions to total discoveries of the untreated control *vs* two CPLL captures; the second diagram gives common proteins between orange extract and industrial (A) and commercial (B) juice.

proteins in a control of unpasteurised filtered juice *vs* different eluates. It was interesting to realise that only the industrial filtering passage is able to reduce 6 times the number of identified proteins as shown in the Venn diagram between orange and juice: only 180 proteins were identified in the filtered juice against the 1109 in orange fruit. Fig. 5B reports the same data for pasteurised juices. The number of unique gene products, found *via* mass spectrometry analysis, is less in pasteurised beverages than in unpasteurised ones (165 vs. 180, respectively), showing that the industrial processing further reduces the number of identified proteins.

Table 1 shows MS identifications found in one Italian commercial orange juice (Z3) and in two orange-flavoured sodas (Z4 and Z5): the number of identified proteins was dramatically reduced down to a few tens in beverages, demonstrating their low orange content with a consequently reduced nutritional value.

4. Discussion

4.1. Proteome identification of orange fruit

In order to obtain a proteomic fingerprinting of commercial orange juice, it is important to know the entire proteome of all components of the commercial product: in this case the orange fruit. In fact, as reported in recent literature (Fasoli, Colzani, Aldini, Citterio, & Righetti, 2013; Lerma-García, D'Amato, Fasoli, Simó-Alfonso, & Righetti, 2014), it is fundamental to expand the proteomic knowledge of raw materials, in order to detect them in commercial products and to test their genuineness. Recently international scientific research has focused on metabolites (Patti, Yanes, & Siuzdak, 2012) as possible nutritional biomarkers (Heinzmann et al., 2010) directly connected with human health (Baldrick, Woodside, Elborn, Young, & McKinley, 2011). Conversely we have decided to investigate the proteome for its role in biological functions of plant and for its importance in human health. Moreover we had the possibility to adopt the CPLL captures, which have detected in both peel and pulp of orange fruit more than 1000 unique gene products, thus increasing by at least 4 times for the pulp and by 40 times for the peel the number of identified proteins, in contrast with the conventional extraction protocols. Despite the high amount of identified proteins, the number of specific species for *C. sinensis* is quite low, even when using the EST-*Citrus* database too. The majority of identified proteins belongs to *Viridiplantae* species by homology matching. This aspect is quite common in food science (Esteve, D'Amato, Marina, García, & Righetti, 2013) and it is due to incomplete knowledge of the proteome of vegetables and fruits, even if their genomes are almost sequenced. We finally recognised more than 2000 unique gene products, thus obtaining an in-depth proteomic fingerprinting of the fruit. In order to complete our research we finally performed a GO analysis to compare the biological functions of proteins found both in orange peel and pulp. In orange peel the majority of proteins was connected with defensive or membrane activities, like for example oxidation–reduction processes, ATP binding and proteolysis. On the contrary in orange pulp proteins were mostly connected with metabolic activities and transcription, like for example nucleic acid binding, regulation of transcription and ATP catabolic processes. So GO has revealed specific biological functions compatible with tissutal localisation of proteins.

The interactomic maps, built up by the application of STRING v.10 software, were constructed to screen hub genes and to investigate PPIs in different orange samples. As regards orange peel, the PPI network was characterised by three hub genes with most interactions. The major hub was formed by genes (ACT8, ACT3, CAM5, CAM7) able to express proteins involved in cell motility or in the control of ion channels. Two other hub genes played a role in the energy metabolism. The results of PPI network were confirmed by enrichment analysis on cellular compartments. In fact the vast majority of genes belonged to extracellular compartments and to cell periphery, confirming the typology and function of peel as a defensive and protecting tissue. As regards orange pulp, the PPI network was characterised by a higher number of hub genes. One of them was mostly formed by genes encoded for ribosomal proteins (AT1G18540, At1G43170, UNQ1), the others were characterised by genes involved in cytoplasmic actions like glucose metabolism (PGM2, PMDH1, PGK) and vacuolar proton transport (VHAA, AVP1, APA1). Also in this case the cellular compartment enrichment analysis has reported the prevalence of intracellular locations like cytoplasm, intracellular organelle and cytosol, confirming the specific role of pulp in energy metabolism and cell life processes. Some proteins, involved in limonene synthesis, could be very interesting, like limonene synthase (A7BG59) and D-limonene synthase (Q6F5H2), both found in CPLL eluates of native extraction. In fact limonene is a lemon essential oil, which is gaining a wide interest in the food industry for its potential as a decontaminating agent (Lucera, Costa, Conte, & DelNobile, 2012). This essential oil has showed anti-yeast effects in acidic pH range, normally optimal for yeast growth (Tserennadmid et al., 2011). For this reason limonene has been proposed as an alternative to traditional artificial preservatives to prevent yeast and bacterial spoilage (Belletti, Kamdem, Tabanelli, Lanciotti, & Gardini, 2010).

4.2. Comparison of orange juice proteome during different steps of industrial processing

In order to monitor the presence of the orange proteome during industrial processes, we have investigated the proteomes of a Spanish juice (Z1), which was a filtered but unpasteurised juice, and of a commercial Spanish orange juice not from concentrate (Z2), but cor-responding to the pasteurised beverage. In both cases we have observed a strong reduction of the number of identified proteins, as reported in the Venn diagrams of Fig. 5. During the industrial processes the reduction is greater in Z2 when the pasteurisation occurs after the filtering process, while in Z1 the number of identified proteins is higher because it was subjected only to filtration. So the industrial steps, which are fundamental for the commercialisation of product, decrease the number of identified proteins and maybe change the nutritional properties of juice. The number of identified proteins decreased not only in terms of number of identifications but also in terms of protein quantity. In fact protein assay has revealed concentration values of 3.9 ± 0.02 g/kg in pulp extracts, prepared freshly in the laboratory, while it was 3.84 ± 0.25 g/L in Z1 and 3.12 ± 0.19 g/L in Z2. Hence, a reduction in protein content upon pasteurisation may result in the lower number of recognised species in juices. Moreover, in both orange juices many proteins were identified as different from orange proteins, due probably to the addition of other components during industrial processes. Finally it is interesting to underline that identification of proteins, 180 in Z1 and 165 in Z2, was possible only after CPLL treatment, which was able to extensively capture the proteome: in fact the majority of identified species were recognised in CPLL eluates, as demonstrated by the Venn diagram in Fig. 5, where proteins of different eluates were compared with untreated sample (control).

4.3. Comparison of proteome in different commercial orange juices

Finally we wanted to evaluate the proteomic fingerprinting in different commercial beverages and for this purpose we have selected one commercial Italian 100% orange juice coming from concentrate (Z3) and two soft drinks (Z4 and Z5). The Italian 100% orange juice has shown a protein concentration of 3.88 g/L, similar to Z1, and this value has confirmed the percentage declared on the label. In addition, MS analysis has identified 181 proteins,

mostly found in CPLL eluates, demonstrating the genuineness of product in accordance with the producer's label. However, only 36 proteins were identified in Z4 and 60 in Z5, suggesting the lower content of orange fruit in the commercial beverages. The low number of identifications was in agreement with the low protein amount found by Lowry assay, which detected 0.55 ± 0.09 g/L of proteins in Z4 and 0.72 ± 0.07 g/L in Z5. The CPLLs contribution to protein discovery was extremely relevant considering that all proteins in soft drinks were found only in CPLLs eluates, a final demonstration of efficiency and reliability of CPLLs to capture the entire proteome.

In the present research different types of juices were assessed during many steps of the industrial process, which used different preservation techniques, like heat treatment, acidification and synthetic chemical additives, able to inhibit growth of undesirable microorganisms. In recent years, because of greater consumer awareness, food and also fruit juices are preserved with natural additives like nitron essential oil, mainly composed of limonene (Belletti et al., 2007). The applied proteomic technology was not able to detect essential oils, but could identify D-limonene (A7BG59) only in CPLL eluates in Z1. This enzyme is involved in limonene production and also in antimicrobial activity in juice. It is interesting to realise that this potential function is preserved during the filtration step, as in Z1, but not after pasteurisation, as in Z2, where this protein was not recognised. Considering other proteins connected with antimicrobial activity, chitinase (B2DD07), a hydrolytic enzyme, involved in pathogens resistance, was identified in CPLL eluates of all analysed juices. So, despite heat treatment, a sort of intrinsic antimicrobial function was present in orange protein, preserving the juice and maintaining its sensory quality.

5. Conclusions

The results obtained in this work proved that orange proteome profiling was useful to evaluate the genuineness of commercial orange juices and drinks, detecting proteins connected not only with biological functions (Galbadon & Koonin, 2013) but also with nutritional properties (Van Ommen et al., 2010) and allergenic reactions (Crespo et al., 2006; Ferreira, Hawranek, Gruber, Wopfener, & Mari, 2004; Poltl et al., 2007). Natural orange proteins were firstly quantified *via* DC protein assay and secondly detected by analytical technologies (CPLL and MS), which seemed to be versatile and reliable also for their application to the food proteomic field. In conclusion we have proposed an analytical procedure able to assess the proteomic signature of orange and of corresponding foodstuffs, like juices and drinks, in order to confirm the natural origin of their components and to check for possible adulterations. Considering the great importance of dietary assessment for human health, we believe that our study could be a starting point to provide analytical tools able to test for food genuineness, useful for consumers and control agencies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.10.009.

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