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TRENDS IN FOOD SCIENCE & TECHNOLOGY

Review

Separation of functional macromolecules and micromolecules: From ultrafiltration to the border of nanofiltration

Charis M. Galanakis*

Department of Research & Innovation, Galanakis Laboratories, Skalidi 34, GR-73131, Chania, Greece (Tel.: +30 28210 93056; fax: +30 28210 88981; e-mail: cgalanakis@chemlab.gr)

The recovery of functional compounds from underutilized bioresources is today accomplished in five distinct stages, whereas ultrafiltration has been utilized for the separation and the clarification of macromolecules from smaller molecules or the opposite. The current article highlights the outcomes of an integral study including research articles, which cover the separation mechanisms dominating during UF (from 100 to 1 kDa) of different feed solutions and extracts, under similar processing conditions. Target macromolecules concern dietary fibers (i.e. pectin, β-glucan), proteins and polymeric anthocyanins, while assayed micromolecules were sugars, cations, monomeric anthocyanins and different phenolic classes such as o-diphenols, hyxrodycinnamic acid derivatives and flavonols.

Introduction

As it is well known, functional compounds and the so-called "nutraceuticals" are today used as additives in foodstuff due to their ability to provide advanced technological properties and health claims, respectively, to the final product (Galanakis, 2013; Galanakis, Markouli, & Gekas, 2013b; Ramaa, Shirode, Mundada, & Kadam, 2006). Indeed, epidemiological studies have shown that health benefits (i.e. reduced risk of coronary heart disease and stroke, diabetes, obesity and cancer) may be attributed to the consumption of both macro- and micro-nutrients (Elleuch *et al*., 2011; Schieber, Stintzing, & Carle, 2001). For instance, macromolecules like soluble dietary fiber is known for its ability to lower blood lipid level and at the same time possesses advanced gelling properties that can replace fat in foods, stabilize emulsions and improve the shelf-life of the product (Elleuch *et al*., 2011; Galanakis, 2011; Galanakis, Tornberg, & Gekas, 2010c; Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006). Proteins have also been used as fat replacement in meat and milk products, flavor enhancers in confectionary, food and beverage stabilizers (Kristinsson & Rasco, 2000; Pogaku, Seng, Boonbeng, & Kallu, 2007; Prakash, 1996). On the other hand, natural antioxidants have been connected to both nutritional (reduction of oxidative stress, prevention of cancer, arteriosclerosis, ageing processes) and functional (preservative of vegetable oils and emulsions) properties. Antioxidants include typically smaller compounds like polyphenols, carotenoids, tocopherols and ascorbic acid (Boskou, 2006; Kiokias & Oreopoulou, 2006; Moure *et al*., 2001).

Undervalued bioresources and natural products are considered as target substrates for the recovery of the above compounds. The later process follows typically the principles of analytical chemistry, whereas it has recently been proposed to accomplish with the so-called "5-Stages Universal Recovery Processing" including: (i) macroscopic pre-treatment, (ii) separation of macro- and micromolecules, (iii) extraction, (iv) isolation-purification and finally (v) product formation or encapsulation (Galanakis, 2012, 2014). Ultrafiltration (UF), nanofiltration (NF) and other membrane technologies are among the key physicochemical and non-destructive techniques applied in the second, third and fourth step of the above downstream processing. Particularly, researchers target to concentrate macromolecules and release smaller molecules in the permeate stream, respectively.

This procedure seems to be simple in theory since membranes are able to separate compounds via sieving mechanism, based on their molecular weight (MW). However,

* Corresponding author.

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this is not the case in practice, as the asymmetric manufacture of membrane pores does not always reflect a narrow range of molecular weight cut-off (MWCO). The effect of the latest parameter attenuates when the solubility of the solutes and the hydrophobicity of the membrane surface are incorporated (Gekas, Trägårdh, & Hallström, 1993; Pinelo, Jonsson, & Meyer, 2009). Thereby, MWCO is not an absolute barrier for the separation of macro- and micro-molecules. Besides, larger and smaller functional molecules exist in clusters inside bioresource matrixes, i.e. phenols bind either dietary fibers of plant materials (Bravo, Abia, & Saura-Calixto, 1994) or dietary proteins (non-covalently) (Rawel, Meidtner, & Kroll, 2005). Subsequently, smaller molecules (i.e. antioxidants) recaptured in the concentrate stream due to the structural characteristics of the macromolecules or the reverse. This is the main reason why natural extracts containing compounds that are not so antioxidant (i.e. oligosaccharides or proteins), appear to have advanced antiradical and reducing properties. The above aspect could be desirable depending on the product that the food technologist is willing to develop. However, in terms of food separation, the simultaneous recovery of macro- and micro-molecules in one stream is a problem leading to additional purification stages, yield loss and finally increased cost.

Nowadays, researchers focus more and more on membrane applications and separation of proteins, dietary fiber, polyphenols, anthocyanins, tannins, flavonoids, saccharides and sugars in fruit juices, solutions, agricultural wastewaters and beverages (Cassano, Conidi, Giorno, & Drioli, 2013; Díaz-Reinoso, Moure, Domínguez, & Parajó, 2009; García-Martín *et al.*, 2010; He, Girgih, Malomo, Ju, &

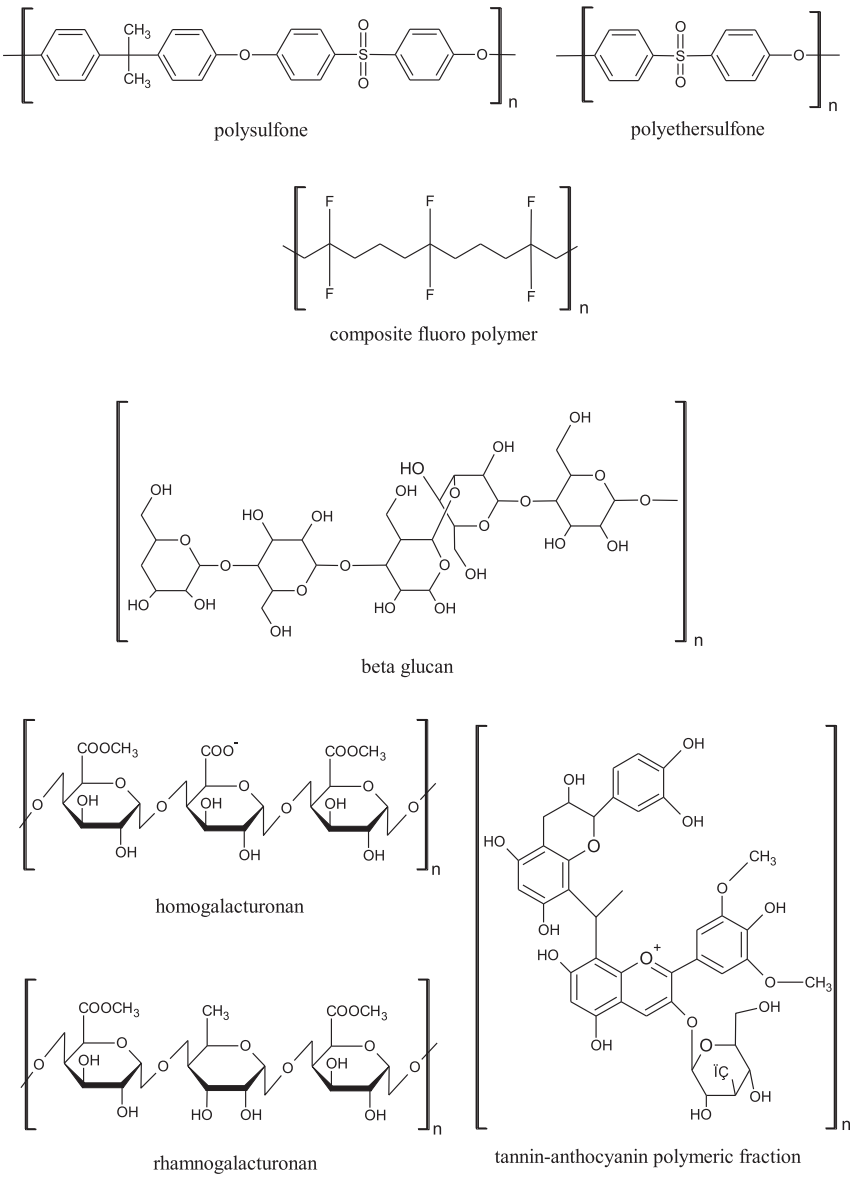


Fig. 1. Chemical structural units of polymeric membrane materials and polymeric functional compounds.

Aluko, 2013; Kuhn *et al*., 2010; Li, Bi, Lin, Bian, & Wang, 2013). Following this trend, the present review provides an overview of 5 research studies (Galanakis, Chasiotis, & Gekas, in press-b; 2013b; Galanakis, Kotanides, Dianellou, & Gekas, in press-a; Galanakis, Tornberg, & Gekas, 2010b; Pastors, Galanakis, & Gekas, 2011), dealing with the separation mechanisms of the above compounds conducted with UF under similar processing conditions. Studies were conducted in the whole range of MWCO, i.e. from 100 up to 1 kDa (border of NF). Experiments were accomplished using two cross-flow UF systems (DSS Labstak M20 and M10) and 7 membrane materials (GR40PP-100 kDa, GR51PP-50 kDa, GR60PP-25 kDa, GR70PP-20 kDa, GR81PP-10 kDa, GR95PP-10 kDa, and ETNA01PP-1 kDa) of the same manufacturer (Alfa Laval Nakskov), while several mediums derived from food wastes and products have been assayed.

Structural characteristics of the assayed membranes

Among the tested membranes, GR40PP, GR51PP, GR60PP and GR70PP membranes were made of polysulphone, whereas GR81PP and GR95PP were made of polyethersulphone. ETNA01PP was consisted of a fluoro polymer material. Fig. 1 illustrates the structural characteristics of these polymers. Specifically, polysulphone is composed of sequential aromatic and aliphatic units, which are responsible for the hydrophobic profile of the polymer by repulsing water and hydrophilic compounds. These units are connected with oxygen (aryl-O-alkyl) and sulphur dioxide (aryl-SO_2-alkyl) molecules, which provide an occasional hydrophilic character to the membrane surface due to the formation of hydrogen bonds. Polysulphone is susceptible to concentration polarization and fouling caused by deposition of polymers (Mérian & Goddard, 2012; Saha, Balakrishnan, & Ulbricht, 2009), but it is not so susceptible to fouling caused by solutes adsorption on membrane surface. On the other hand, polyethersulphone is a similar, but less hydrophobic polymer since more sulphur dioxide molecules are present compared to polysulphone. For instance, the O-atoms of SO_2-unites are able to bind water by providing their two pairs of unshared electrons. Polyethersulphone exhibits protein and polysaccharides repellency (Ma, Su, Sun, Wang, & Jiang, 2007; Peng, Su, Shi, Chen, & Jiang, 2011) and is also susceptible to concentration polarization caused by whey proteins. The latest phenomenon occurs when membrane pores are more open and feed solution undergoes higher fluxes (Atra, Vatai, Bekassy-Molnar, & Balint, 2005). Composite fluoro polymer is a material that contains an aliphatic chain with multiple carbon–fluorine bonds. It is less susceptible to hydrogen bonds and van de Waals forces that make it more hydrophobic and less susceptible to fouling compared to the other two membranes. Besides, a modeling based on ovine whey UF has revealed that mass transfer is controlled by concentration polarization in the case of composite fluoro polymer and resistance to fouling in the case of polysulphone and polyethersuphone membranes (Macedo, Duarte, & Pinho, 2011).

Structural characteristics of the assayed macromolecules

Table 1 shows the structural characteristics of the tested macromolecules in spite of their MW and charge. Among the soluble dietary fiber, β-glucan moieties were derived from an oat mill waste (Patsioura *et al*., 2011) and its MW was expected to be up to 122 kDa. These polymers are linear homo-polysaccharides composed of continuant (1,4)-linked β-D-glucose segments. The latest are separated by single (1,3) linkages (Fig. 1). β-Glucan is water soluble due to the presence of the β-(1,3)-linked β-glycosyl residue, which prevents alignment of glucose segments and increases the corresponding solubility (Lazaridou &

Table 1. Structural characteristics of the assayed macromolecules.

Group of macromolecules	Source	Compound	Molecular weight (kDa)	Charge
Soluble dietary fibers	Oat	β-glucan	122	Neutral
	Grape & olive	Homogalacturonan	70–250	Negative depending on methylation degree
	Grape & olive	Rhamnogalacturonan	70–250	Negative depending on methylation degree
	Olive	Arabinan	8–10	Neutral
	Grape	Arabinogalactan	n.a.*	Neutral
Proteins	Cheese whey	Immunoglobulin	150–1000	Positive
	Cheese whey	Bovine serum albumin	66	Positive
	Cheese whey	α-Lactalbumin	14	Weakly negative
	Cheese whey	β-Lactoglobuin	18	Positive
	Oat	Globulin	20–35	Positive
	Oat	Albumin	14–17	Weakly positive
	Oat	Prolamin	17–34	Positive
Polymeric anthocyanins	Grape & wine	Pigments of tannins & anthocyanins	n.a.*	Weakly positive

* "n.a." for "not available".

Biliaderis, 2007). Moreover, the large size of the molecule restricts its permeation through small pores, whereas the surrounding hydroxyl groups form hydrogen bonds with the water and hydrophilic membranes.

Pectin from different sources (i.e. olive mill waste and winery sludge) were studied, too, whereas the most dominant types were homo- and rhamno-galacturonans. The first polymer consists of a backbone of α-1,4-linked galacturonic acid residues (McNeil, Darvill, Fry, & Albersheim, 1984). As it can be seen in Fig. 1, homogalacturonan molecules are surrounded by numerous hydroxyl groups that provide the ability to form hydrogen bonds, whereas these molecules are negatively charged due to the demethylation of carboxylic groups. On the other hand, rhamnogalacturonan backbone contains less carboxylic groups compared to homogalacturonan structure since it is composed of repeated α-L-rhamnose-(1 → 4)-α-D-galacturonic acid units.

Proteins comprise a group of macromolecules consisting of amino acids polymeric chains (polypeptides). The latest units provide an amphoteric nature of the molecule depending on its isoelectric point (pI). Tested proteins were recovered from two sources: oat mill waste (Patsioura *et al.*, 2011) and cheese whey (Galanakis *et al.*, in press-b). Oat is known to contain mainly globulin (20–35 kDa and pI = 5.5) and other proteins like albumin (14–17 kDa, 4.0 < pI < 7.0) and prolamin (17–34 kDa, 5.0 < pI < 9.0) (Klose & Arendt, 2012). Taking into account that the tested feed solution from oat mill waste had an acidic nature (pH = 4.5), corresponding protein molecules were expected to be positively charged. The "bulk" whey proteins such as immunoglobulin (150–1000 kDa and 5.5 < pI < 8.3) and ovine serum albumin (66 kDa and pI = 5.0 ± 0.1) are positively in the acidic (pH = 4.8 ± 0.1) whey solutions. Indeed, immunoglobulin and ovine serum albumin have been recovered by 100% using a 300 kDa-tubular ceramic membrane and pH = 4 (Almécija, Ibáñez, Guadix, & Guadix, 2007). With regard to the smaller α-lactalbumin (14 kDa and pI = 4.5 ± 0.3) and β-lactoglobulin (18 kDa and pI = 5.3 ± 0.1), charge is expected to be weakly negative and positive, respectively (Bhattacharjee, Bhattacharjee, & Datta, 2006; Lawrence, Perera, Iyer, Hickey, & Stevens, 2006; Zydney, 1998). Hydrolysis of these proteins (i.e. of β-lactoglobulin) generates smaller peptides with known biological functionality, i.e. immunomodulatory activity (Rodríguez-Carrio, Fernández, Riera, & Suárez, 2014), angiotensin-converting enzyme inhibitory activity and ferrous chelating capability (O'Loughlin, Murray, Brodkorb, FitzGerald, & Kelly, 2014a; O'Loughlin, Murray, FitzGerald, Brodkorb, & Kelly, 2014b). The selective recovery of a particular protein (i.e. α-lactalbumin) or peptide can be conducted by combining several effects, including reduced pore size and enhanced electrostatic repulsion between the charged membrane and protein species (Cowan & Ritchie, 2007).

The separation of polymeric anthocyanins from winery sludge (Galanakis *et al.*, 2013b) and wine samples (Galanakis *et al.*, in press-a) were monitored, too. These compounds are mainly comprised of malvidin 3-glucoside and respective pyruvic acid derivatives as well as pigments of anthocyanins linked either to a catechin unit or to a procyanidin dimer or to a 4-vinylphenol group (Mateus, de Pascual-Teresa, Rivas-Gonzalo, Santos-Buelga, & de Freitas, 2002; Remy, Fulcrand, Labarbe, Cheynier, & Moutounet, 2000). Polymerization of anthocyanins can start from simple dimeric acetaldehyde malvidin 3-glucoside structures (Atanasova, Fulcrand, Le Guernevé, Cheynier, & Moutounet, 2002) and riches heavier fractions, but the corresponding charge was expected to be weakly positive.

Structural characteristics of the assayed micromolecules

Table 2 shows the chemical structures of the monitored micromolecules and their characteristics in terms of their MW and the corresponding numbers of aromatic rings, hydroxyl, carboxylic and methylation groups. Aromatic rings and aliphatic chains provide a hydrophobic nature to the micromolecules and increase their volume. On the other hand, the increasing number of hydroxyl and carboxylic groups, accompanied with acidic pH for all the feed samples, is leading to intermolecular negative polarity. Subsequently, solutes attract water molecules that increase the volume of the target molecules and restrict permeation through membrane pores due to the "polarity resistance" phenomenon. For instance, small sugars like glucose, fructose and galactose have the same MW (=180) and are very polar due to the numerous hydroxyl groups (=5). Their structure includes occasionally an aromatic ring, as the open-chain monosaccharides exist in equilibrium with several cyclic isomers. Disaccharides like sucrose and lactose show similar characteristics, but they are a bit bigger (MW = 342) and contain 8 hydroxyl groups.

The chemical structure of micromolecular phenolic compounds is relatively similar to sugars, although they typically contain more aromatic rings and/or less hydroxyl groups. These aspects increase their MW and reduce molecular polarity. Particularly, phenolic compounds can be divided in two major parts: (a) non-flavonoids and (b) flavonoids. Non-flavonoids include hydroxycinnamic acid derivatives (i.e. cinnamic, p-coumaric and ferulic acid) and o-diphenols like hydroxytyrosol, gallic and protocatechuic acids. All these compounds possess low MW (148–194) due to the appearance of only one aromatic ring. Indeed, o-diphenols (i.e. gallic and caffeic acid) are generally smaller and more polar molecules than the rest hydroxycinnamic acid derivatives (Galanakis, Goulas, Tsakona, Manganaris, & Gekas, 2013a). Flavonoids include phenolic alcohols (i.e. flavan-3-ols), flavonols, flavones, anthocyanins and secoiridoids (Niaounakis & Halvadakis, 2004). Phenolic alcohols (i.e. tyrosol) and aldehydes (i.e. isovanillic acid) found in grape derivatives and olive mill wastewater have also low MW (MW = 110–228) as non-flavonoids. Flavonols, such as procyanidin B2, quercetin

Table 2. Structural characteristics of the assayed micromolecules.

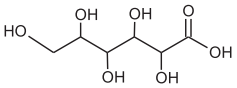
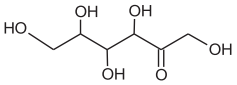
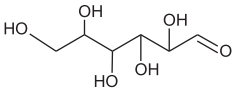
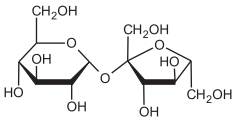
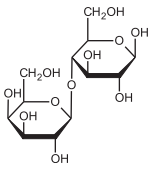
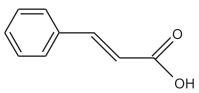
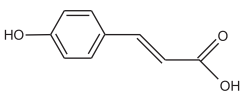
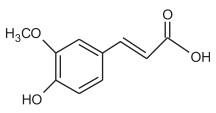
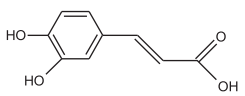
Group of micromolecules	Compound	Molecular weight (Da)	Chemical group				Molecular type
			Aromatic rings	-OH	-COOH	$-CH_3$	
Sugars	Glucose	180	0–1	5	0–1	0	
	Fructose	180	0–1	5	0	0	
	Galactose	180	0–1	5	0	0	
	Saccharose	342	2	8	0	0	
	Lactose	342	2	8	0	0	
Hydroxy cinnamic acid derivatives	Cinnamic acid	148	1	0	1	0	
	p-Coumaric acid	164	1	1	1	0	
	Ferulic acid	194	1	1	1	1	
Hydroxy cinnamic acid derivatives/ o-diphenols	Caffeic acid	180	1	2	1	0	

Table 2 (*continued*)

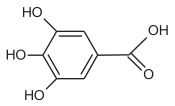
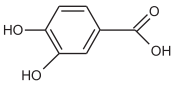
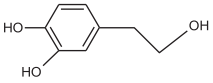
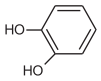
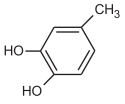
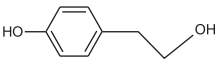
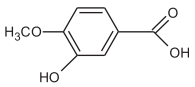
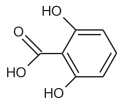
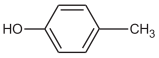
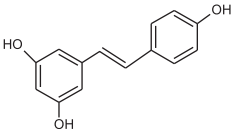
Group of micromolecules	Compound	Molecular weight (Da)	Chemical group Aromatic rings	-OH	-COOH	$-CH_3$	Molecular type
o-diphenols	Gallic acid	170	1	3	1	0	
	Protocatechuic acid	154	1	2	1	0	
	Hydroxytyrosol	154	1	3	0	0	
o-diphenols/ phenolic alcohols	Catechol	110	1	2	0	0	
	4-Methyl catechol	124	1	2	0	1	
Phenolic alcohols	Tyrosol	138	1	2	0	0	
	Isovanillic acid	152	1	1	1	1	
	γ-Resorcylic acid	154	1	2	1	0	
	Cresol	108	1	1	0	1	
	Resveratrol	228	2	3	0	0	

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Table 2 (*continued*)

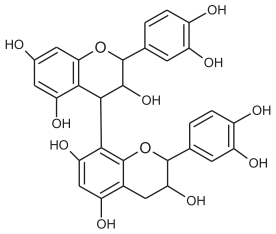
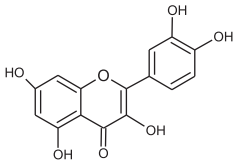
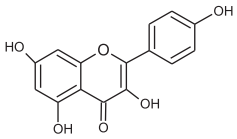
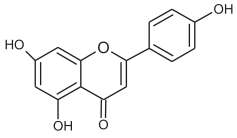
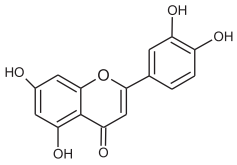
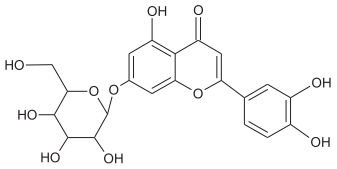
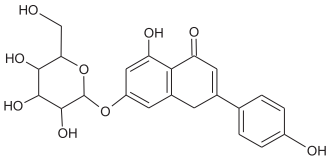
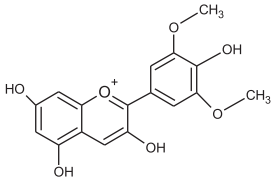
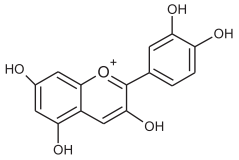
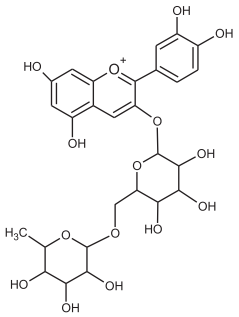
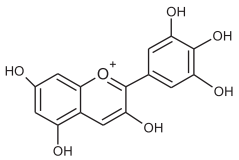
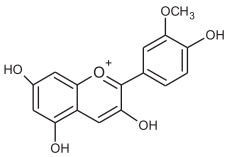
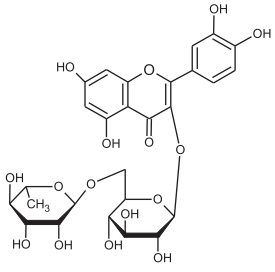
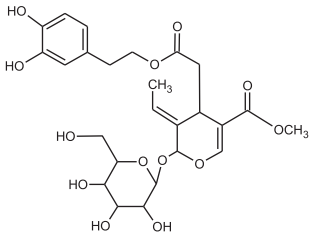
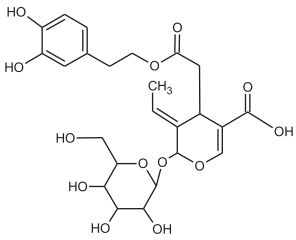
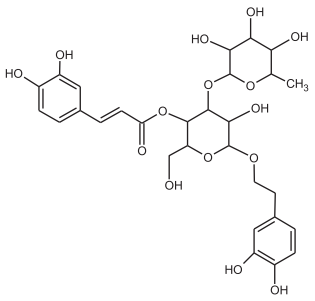
Group of micromolecules	Compound	Molecular weight (Da)	Chemical group Aromatic rings	-OH	-COOH	$-CH_3$	Molecular type
Flavonols	Procyanidin B2	579	6	10	0	0	
	Quercetin	302	3	5	0	0	
	Kaempferol	286	3	4	0	0	
Flavones	Apigenin	270	3	3	0	0	
	Luteolin	286	3	4	0	0	
	Luteolin-7-glucoside (cynaroside)	448	4	7	0	0	
	Apigenin-7-glucoside	432	4	6	0	0	

Table 2 (*continued*)

Group of micromolecules	Compound	Molecular weight (Da)	Chemical group Aromatic rings	-OH	-COOH	$-CH_3$	Molecular type
Anthocyanins	Malvidin	331	3	4	0	2	
	Cyanidin	287	3	5	0	0	
	Cyanidin-3-rutinoside	631	5	10	0	1	
	Delphinidin	303	3	6	0	0	
	Peonidin	301	3	4	0	1	
	Rutin	610	5	10	0	1	

(*continued on next page*)

Table 2 (*continued*)

Group of micromolecules	Compound	Molecular weight (Da)	Chemical group Aromatic rings	-OH	-COOH	$-CH_3$	Molecular type
Secoiridoids	Oleuropein	540	3	6	0	2	
	Demethyloleuropein	526	3	6	1	1	
	Verbascoside	625	4	8	0	1	

and kaempferol, are larger molecules (MW = 286–579) due to the existence at least of 3 aromatic rings surrounded by multiple hydroxyl groups. Flavones (i.e apigenin) have similar characteristics to flavonols. On the other hand, the structural characteristics of anthocyanins (i.e. malvidin, cyanidin-3-glucoside, rutin) varies importantly depending on their partial polymerization, as mentioned in Section 3. Other glycosylated phenolics (i.e. oleuropein, demethyl oleuropein or verbascoside), which are typically found in olive mill wastewater, have rather high MW (=526–625) similar to anthocyanins.

UF experiments with wide membrane pores (100–50 kDa)

Table 3 revises the results of the studies regarding the separation of macro- and micro-molecules using UF membranes with wide pores (100–50 kDa). At this group of comparisons, one membrane material (made of polysulphone) was applied, whereas different separations have been tested. These include: (i) β-glucan against saccharides, monovalent ions and phenols, (ii) proteins against saccharides, monovalent ions, simple sugars and phenols, (iii) pectin against simple sugars, monovalent ions, anthocyanins, total phenols and particular phenolic classes, (iv) and finally polymeric anthocyanins against monomeric anthocyanins.

In many cases, recovery of target macromolecules was successful, denoting that "sieving mechanism" governed the UF process at these high MWCO:s. On the other hand, the rather low retention percentages of many macromolecules were connected to several factors. For instance, when a standard solution of β-glucan was applied, the respective recovery was almost quantitative in the concentrate stream. When an extract (derived from oat mill waste) was applied as feed solution, β-glucan showed a lower recovery percentage (53–67%), whereas micromolecules showed a retention <11%. This result could be obtained due to the extraction procedure prior UF experiments leading to a partial breakage of polymers to lower than 122 kDa polymeric chains. Although β-glucan molecules are

Table 3. Separation of macro- and micro-molecules originated from different sources using ultrafiltration membranes of wide pores (100−50 kDa).

Substrate	Feed (target compounds)	Membrane barrier		Compounds						Reference
		MWCO[a] (kDa)	Material	Macromolecules group	C (mg/L)	R (%)	Micromolecules group	C (mg/L)	R (%)	
Standard	Solution (β-glucan)	100	PS[b]	β-glucan	200−2000	92−95	n.d.[c]			Patsioura *et al.* (2011)
Oat mill waste	Extract (β-glucan)	100	PS[b]	β-glucan	302−442	53−67	Saccharides[d]	2317−5344	4−11	Patsioura et al. (2011)
				Proteins	190−376	35−40	Total phenols	16−31	3−9	
							Monov. ions[e]	1057−1699	2−3	
Olive mill wastewater	Beverage (phenol)	100	PS[b]	n.d.[c]	–	–	Total sugars	384	<1	Galanakis *et al.* (2010b)
							Total phenols	280	<1	
							o-diphenols	57	<1	
							Hyd.-cin. acids[f]	19	<1	
							Flavonols	18	10	
							Monov. ions[e]	122	<1	
							Antirad. effic.[g]	1.7[g]	<1	
AIR[h] from olive mill wastewater	Water soluble extract (pectin)	100	PS[b]	Pectin	87	79	Total phenols	68	13	Galanakis *et al.* (2010b)
							Monov. ions[e]	1304	2	
Winery sludge	Extract (phenols & anthocyanins)	100	PS[b]	Pectin	6443	12				Galanakis *et al.* 2013b
				Pol-anthoc.[i]	172	59	Total sugars	3910	61	
							Red. sugars[j]	412	50	
							Non-red. sugars[k]	3498	62	
							Total phenols	1965	64	
							o-diphenols	560	52	
							Hyd.-cin. acids[f]	297	57	
							Flavonols	265	43	
							Total anth.[l]	249	61	
							Monom. anth.[m]	76	59	
	Diluted extract (phenols & anthocyanins)	100	PS[b]	Pectin	1670	6	Total sugars	1065	74	Galanakis *et al.* 2013b
				Pol- anthoc.[h]	172	77	Red. sugars[j]	129	58	
							Non-red. sugars[k]	935	76	
							Total phenols	476	69	
							o-diphenols	560	80	
							Hyd.-cin. acids[f]	297	99	
							Flavonols	265	68	
							Total anth.[l]	249	61	
							Monom. anth.[m]	76	77	

(*continued on next page*)

Table 3 (*continued*)

Substrate	Feed (target compounds)	Membrane barrier		Compounds						Reference
		MWCO[a] (kDa)	Material	Macromolecules group	C (mg/L)	R (%)	Micromolecules group	C (mg/L)	R (%)	
Anari cheese	Whey (protein & sugars)	100	PS[b]	Proteins	3723	76	Total sugars	49703	9	Galanakis *et al.* in press-b
							Red. sugars[j]	469	86	
							Non-red. sugars[k]	48,581	7	
							Total phenols	112	78	
Halloumi cheese	Whey (protein & sugars)	100	PS[b]	Proteins	1087	69	Total sugars	12,729	2	Galanakis *et al.* in press-b
							Total phenols	43	55	
Anari cheese	Whey (protein & sugars)	50	PS[b]	Proteins	3723	73	Total sugars	49,703	8	Galanakis *et al.* in press-b
							Total phenols	112	74	
Halloumi cheese	Whey (protein & sugars)	50	PS[b]	Proteins	1087	68	Total sugars	12729	18	Galanakis *et al.*, in press-b
							Total phenols	43	66	

[a] "MWCO" for "Molecular Weight Cut-off".
[b] "PS" for "polysulphone".
[c] "n.d." for "not determined".
[d] "Saccharides" include oligo-, di- and mono-saccharides.
[e] "Monov. ions" for "monovalent ions".
[f] Hyd.-cin. acids" for "hydroxycinnamic acid derivatives".
[g] "Antirad. effic." for "antiradical efficacy" expressed in mg DPPH/g.
[h] "AIR" for "alcohol insoluble residue".
[i] "Pol-anthoc." for "polymeric anthocyanins".
[j] "Red. sugars" for "reducing sugars".
[k] "Non-red. sugars" for "non reducing sugars".
[l] "Total anth." for "total anthocyanins".
[m] "Monom. anth." for "monomeric anthocyanins".

characterized by their bulkiness structure, they are polar and soluble in water. This means that they could come close to polysulphone surface without get absorbed on it, while they can step in membrane "gaps" (larger parts of asymmetric membrane material) and pass in the permeate stream (Patsioura *et al*., 2011). The recovery of pectin was also affected by the extraction procedure, i.e. in the case of winery sludge extract, pectinolytic enzyme has been applied during wine making and the corresponding retention was almost negligible 6–12% (Galanakis *et al*., 2013a). Besides, the application of pectinolytic enzyme preparation has been used in order to reduce viscosity of juices (grape and peach fruit) avoid fouling during membrane treatment (Bailey, Barbe, Hogan, Johnson, & Sheng, 2000; Echavarría *et al*., 2012). Pectinolytic enzyme is typically applied in membrane reactors of 10 kDa cut-off (Rodriguez-Nogales, Ortega, Perez-Mateos, & Busto, 2008), meaning that hydrolysate fragments are expected to be below this barrier. Improved fouling behavior during UF can also be obtained by changing the properties (i.e. pH) of the extract and subsequently colloidal stability of the extract (Hatziantoniou & Howell, 2002).

In the case of pectin from olive mill wastewater, pectin methyl esterase had been deactivated thermally during extraction procedure (Galanakis, Tornberg, & Gekas, 2010a; e) and subsequently retention was relatively high (87%) to the respective MW of the target macromolecule. On the other hand, since MW of oat proteins varies from 17 to 34 kDa (Table 1), the respective retention (35–40%) during UF was considered as normal. The rather high retention of whey proteins (68–76%) was expected, too, as these compounds possess a broad MW (18–1000 kDa). Cheang and Zydney (2004) reported an even higher recovery of whey α-lactalbumin using 100 and 30 kDa composite regenerated cellulose membranes in series.

Regarding the permeation of micromolecules in the permeate stream, sieving mechanism was not always as efficient as it was expected theoretically. This phenomenon was observed in two cases: (a) extracts from winery sludge and whey solutions. In the first, several classes of phenolic classes were highly retained (52–99%) despite their corresponding MW (<1 kDa). The same outcome was also obtained for sugars, i.e. retentions of 50–76%. Liu, Vorobiev, Savoire, and Lanoisellé (2011) have also reported very high retentions of phenols (87–91%) by using polyethersulphone (150 kDa) and polyvinylidene (50 kDa) fluoride membranes of another manufacturer (Nadir). Carvalho, Silva, and Pierucci (1998; Carvalho, Castro, and Silva 2008) referred that a 50 kDa-polysulphone membrane removed very efficiently tannins (71%) and pectin (93%) from pineapple juice, but sugars were retained only by 10–20%.

The high retention of micromolecules by the large membrane pores has been attributed to their polarity (Galanakis *et al*., 2013a). For instance, phenolic compounds have non-polar and polar sides within their molecules. The hydroethanolic solvent of the particular application protects these sides by providing ethanol and water molecules in each side, respectively. This fact increases their solubility and flexibility, whereas it could be the reason for preserving their antioxidant properties during storage (Galanakis, Tornberg, & Gekas, 2010d). On the other hand, this flexibility may allow them to move through membrane pores, but absorb and lock like a "key" on the narrower parts of polysulphone. The importance of matching molecular size, polar and non-polar sites with membrane's polarity and MWCO could be verified by the lower retention of phenolic compounds obtained during treatment of kiwifruit juice with a more polar, cellulose acetate 30 kDa-membrane (Cassano, Donato, Conidi, & Drioli, 2008). Interaction mechanism of polyphenols with polyethersulphone membranes and adsorptive fouling has been discussed by Susanto, Feng, and Ulbricht (2009).

In any case, the asymmetric structure of the membrane material plays again an important role by showing the reversal effect compared to macromolecules. This separation mechanism could also be followed by sugars in both cases of winery sludge extracts and whey solutions. Besides, when ethanol was very low in the solvent (beverage derived from olive mill wastewater), sugar retention was shown to be negligible (Galanakis *et al*., 2010b). Finally, the rather high retention of total phenols (66–74%) in the case of whey solutions can only be charged to their simultaneous recovery with protein, due to their high affinity (Moure *et al*., 2001).

UF experiments with intermediate membrane pores (25 – 10 kDa)

The results of macro- and micro-molecules separations using UF membranes with intermediate pores (25 –10 kDa) are revised in Table 4. Two similar membrane materials (polysulphone of 20–25 kDa and polyethersulphone of 10 kDa) were tested, whereas respective separations followed the characteristics of aforementioned assays. As it was expected, separations of macro- and micro-molecules were generally less successful compared to these obtained for membranes with wider pores (100 –50 kDa). Nevertheless, polysulphone membranes of 25 –20 kDa were very efficient in specific applications. For example, separation of pectin and phenolic compounds of the winery sludge extract was impossible since they showed moderate (52–64%) and high (65–89%) retentions, respectively. The same conclusion can be extracted by comparing pectin and sugars recovery percentages. The latest separation was also not possible in pigmented orange pulp wash, using a 10 kDa-fluoro polymer spiral membrane (Scordino, Mauro, Passerini, & Maccarone, 2007). On the other hand, separation of polymeric and monomeric anthocyanins was almost ideal (94% compared to 17%), if a more diluted winery sludge extract was assayed in UF module (Galanakis *et al*., 2013a). This outcome is in accordance

Table 4. Concentrations of macro- and micro-molecules originated from different sources and corresponding retention percentages obtained using ultrafiltration membranes (25–10 kDa).

Substrate	Feed (target compounds)	Membrane barrier		Compounds						Reference
		MWCO[a] (kDa)	Material	Macromolecules group	C (mg/L)	R (%)	Micromolecules group	C (mg/L)	R (%)	
Olive mill wastewater	Beverage (phenol)	25	PS[b]	n.d.[c]			Total sugars	384	18	Galanakis *et al.* (2010e)
							Total phenols	280	10	
							o-Diphenols	57	6	
							Hyd.-cin. acids[d]	19	32	
							Flavonols	18	37	
							Monov. ions[e]	122	26	
							Antirad. effic.[f]	1.7[f]	8	
AIR[g] from olive mill wastewater	Water soluble extract (pectin)	25	PS[b]	Pectin	87	98	Total phenols	68	40	Galanakis *et al.* (2010e)
							Monov. ions[e]	1304	10	
Winery sludge	Extract (phenols & anthocyanins)	20	PS[b]	Pectin	6443	64	Total sugars	1065	87	Galanakis *et al.*, 2013b
				Pol-anthoc.[h]	172	92	Red. sugars[i]	129	78	
							Non-red. sugars[j]	935	88	
							Total phenols	476	85	
							o-diphenols	560	87	
							Hyd.-cin. acids[d]	297	81	
							Flavonols	265	65	
							Total anth.[k]	249	89	
							Monom. anth.[l]	76	86	
	Diluted extract (phenols & anthocyanins)	20	PS[b]	Pectin	1670	52	Total sugars	1065	78	Galanakis *et al.*, 2013b
				Pol- anthoc.[h]	172	94	Red. sugars[i]	129	78	
							Non-red. sugars[j]	935	78	
							Total phenols	476	77	
							o-diphenols	560	85	
							Hyd.-cin. acids[d]	297	99	
							Flavonols	265	75	
							Total anth.[k]	249	62	
							Monom. anth.[l]	76	17	
Anari cheese	Whey (protein & sugars)	20	PS[b]	Proteins	3723	84	Total sugars	49703	21	Galanakis *et al.*, in press-b
							Total phenols	112	78	
Anari cheese	Whey (protein & sugars)	20	PS[b]	Proteins	1087	76	Total sugars	12729	34	Galanakis *et al.*, in press-b
							Total phenols	43	59	

Olive mill wastewater	Beverage (phenol)	10	PES[f]	n.d.*			Total sugars	384	32	Galanakis *et al.* (2010e)
							Total phenols	280	21	
							o-Diphenols	57	32	
							Hyd.-cin. acids[d]	19	44	
							Flavonols	18	56	
							Monov. ions[e]	122	23	
							Antirad. effic.[f]	1.7[f]	24	
AIR[g] from olive mill wastewater	Water soluble extract (pectin)	10	PES[f]	Pectin	87	98	Total phenols	68	71	Galanakis *et al.* (2010e)
							Monov. ions[e]	1304	49	

[a] "MWCO" for "Molecular Weight Cut-off".
[b] "PS" for "polysulphone".
[c] "n.d." for "not determined".
[d] "Hyd.-cin. acids" for "hydroxycinnamic acid derivatives".
[e] "Monov. ions" for "monovalent ions".
[f] "Antirad. effic." for "antiradical efficacy" expressed in mg DPPH/g.
[g] "AIR" for "alcohol insoluble residue".
[h] "Pol-anthoc." for "polymeric anthocyanins".
[i] "Red. sugars" for "reducing sugars".
[j] "Non-red. sugars" for "non reducing sugars".
[k] "Total anth." for "total anthocyanins".
[l] "Monom. anth." for "monomeric anthocyanins".

with Santamaría et al. (2002) who fractionated dimeric and trimeric proanthocyanidins from the monomeric ones in grape seeds extract (>70% and 30%, respectively) with a 20-kDa polysulphone membrane. Proteins and sugars separation in whey samples was relatively efficient (76−84% compared to 21−34%) with a similar membrane, too (Galanakis *et al*., in press-b). Separation of soy proteins from sugars has been reported to be very efficient (90% against negligible retention) using a 18 kDa polyvinylidene difluoride membrane (Kumar, Yea, & Cheryan, 2003). Polysulphone was proved to very efficient (a) in separating pectin from phenols and cations (98% compared to 40 and 10%) from a solution recaptured from olive mill wastewater. Besides, the same 25 kDa-membrane was able to preserve the antioxidants properties of the corresponding beverage by removing a part of the "heavier" hydroxycinnamic acid derivatives and flavonols (Galanakis, Tornberg, & Gekas, 2010e). The removal of hydroxycinnamic acids and flavanones from blood orange juice using an integrated membrane process (including UF, osmotic distillation and reverse osmosis) has been reported to be more difficult compared to anthocyanins and ascorbic acid (Galaverna *et al*., 2008).

On the other hand, the less hydrophobic but narrower (10 kDA) polyethersulphone membrane was not efficient for the aforementioned applications, i.e. separation of pectin with phenols or fractionation of different phenolic classes. However, the latest result was in discordance with other studies, i.e. Cassano, Conidi, and Drioli (2011) reported that a polyethersulphone membrane (10 kDa) was able to remove only 34% of total and 9% of low MW phenols from pre-filtered olive mill wastewater. A similar conclusion was obtained by Gökmen, Acar, and Kahraman (2003) who reported a negligible removal (up to 7%) of total phenols and particularly hydroxycinnamic acid derivatives from apple juice, using a 10 kDa polyethersulphone membrane. The above differences could be attributed to several factors such as the different membrane manufacturer of feed solution. Besides, the retention of macromolecular gelling compounds (i.e. pectin) is generating the formation of a second or dynamic membrane that increases the retention of lower MW solutes such as phenols and ions (Mulder, 1996). Other authors showed that a 10 kDa-polyethersulphone was efficient in whey protein recovery (70%) by removing lactose to the permeate (Baldasso, Barros, & Tessaro, 2011).

UF experiments with narrow membrane pores (2−1 kDa)

Table 5 revises the results of the studies regarding the separation of macro- and micro-molecules using UF membranes with narrow pores (2−1 kDa) in the border of NF. Two materials were assayed: polyethersulphone (2 kDa) and composite fluoro polymer (1 kDa). At this level of cut-off, separations of macro- and micro-molecules (i.e. proteins and sugars, pectin and phenolic classes or monovalent ions, polymeric and monomeric anthocyanins etc) became more difficult. An exception can be observed in case of diluted winery sludge extract, where polar hydroxycinnamic acids were highly retained (80%) by polyethersulphone in contrast to hydrolyzed pectin fragments that basically passed in the permeate stream (retention of 39%). Proteins from UF-Halloumi whey were partially recovered (47%) compared to the negligible (5%) retention of sugars, but for other feed solutions this was not the case. These results are in accordance with other studies. For instance, Chabeaud *et al*. (2009) referred that a 4 kDa-polyethersulphone did not separate fish protein hydrolysates microsolutes (recovery percentage of 73 and 56%, respectively). Later, Fernández, Zhu, FitzGerald, and Riera (2014) reported the low efficiency of β-lactoglobulin tryptic digest peptides recovery using a polyethersulphone (1 kDa) and an extremely hydrophilic membrane (2 kDa). Indeed, the selectivity of peptide fractionation is decreased at a high salt content (Fernández & Riera, 2013).

The hydrophobic but narrower composite fluoro polymer membrane was able to separate satisfactorily hydroxycinnamic acids from anthocyanins and flavonols in the diluted and concentrated winery sludge extract, respectively, since polar acids showed up to 2-fold higher recovery percentages compared to the rest phenolic classes (Galanakis *et al*., 2013a). Indeed, the efficacy of hydroxycinnamic acids separation from flavonols was recently verified in several diluted wine samples (Galanakis *et al*., in press-a), although the initial phenolic concentrations were similar to these found in the concentrated (not in the diluted) winery sludge extract. On the other hand, the same composite fluoro polymer membrane (1 kDa) has been referred to recover total and low MW phenols from pre-treated olive mill wastewater in relatively low percentages (32 and 17%, respectively) (Cassano *et al*., 2013). This discordance could be attributed to the hydroethanolic nature of winery sludge extract that increases the intermolecular polarity of the polar solutes and thus are rejected by the hydrophobic membrane.

General remarks

UF is a well established methodology with multiple advantages, restrictions and challenges driven by size and charge exclusion. When membranes with wide pores are used, UF is dominated by sieving mechanism and corresponding separation of macro- and micro-molecules is rather distinct. By applying narrower membrane pores, the molecular cut-off becomes stricter and separation is conducted in terms of components solubility, membrane hydrophobicity and polarity resistance. This fact introduces severe process restrictions due to concentration polarization of proteins and polysaccharides (i.e pectin), leading to gel formation, protein aggregation and fouling (Mohammad, Ng, Lim, & Ng, 2012; Tsagaraki & Lazarides, 2011). Nevertheless, the above fact reveals separation

Table 5. Concentrations of macro- and micro-molecules originated from different sources and corresponding retention percentages obtained using membranes in the edge of ultrafiltration (UF) with nanofiltration (2−1 kDa).

Substrate	Feed (target compounds)	Membrane barrier		Compounds						Reference
		MWCO[a] (kDa)	Material	Macromolecules group	C (mg/L)	R (%)	Micromolecules group	C (mg/L)	R (%)	
Olive mill wastewater	Beverage (phenol)	2	PES[b]	n.d.[c]			Total sugars	384	38	Galanakis *et al.* (2010e)
							Total phenols	280	25	
							o-Diphenols	57	48	
							Hyd.-cin. acids[d]	19	53	
							Flavonols	18	62	
							Monov. ions[e]	122	27	
AIR[f] from olive mill wastewater	Water soluble extract (pectin)	2	PES[b]	Pectin	87	99	Total phenols	68	81	Galanakis *et al.* (2010e)
							Monov. ions[e]	1304	55	
Winery sludge	Extract	2	PES[b]	n.p.[g]			n.p.[g]			Galanakis *et al.* 2013b
	Diluted extract	2	PES[b]	n.p.[g]			n.p.[g]			Galanakis *et al.* 2013b
Anari cheese	UF-whey (protein & sugars)	2	PES[b]	Proteins	652	35	Total sugars	38,402	13	Galanakis *et al.* in press-b
							Total phenols	27	9	
Halloumi cheese	UF-whey (protein & sugars)	2	PES[b]	Proteins	275	47	Total sugars	8212	5	Galanakis *et al.* in press-b
							Total phenols	16	31	
Winery sludge	Extract (phenols & anthocyanins)	1	ETNA[j]	Pectin	6443	47	Total sugars	1065	76	Galanakis *et al.* 2013b
				Pol-anthoc.[k]	172	56	Red. sugars[h]	129	66	
							Non-red. sugars[i]	935	77	
							Total phenols	476	74	
							o-diphenols	560	67	
							Hyd.-cin. acids[d]	297	77	
							Flavonols	265	45	
							Total anth.[l]	249	57	
							Monom. anth.[m]	76	55	
	Diluted extract (phenols & anthocyanins)	1	ETNA[j]	Pectin	1670	39	Total sugars	1065	64	Galanakis *et al.* 2013b
				Pol- anthoc.[k]	172	45	Red. sugars[h]	129	57	
							Non-red. sugars[i]	935	65	
							Total phenols	476	56	
							o-diphenols	560	64	
							Hyd.-cin. acids[d]	297	80	
							Flavonols	265	53	
							Total anth.[l]	249	44	
							Monom. anth.[m]	76	41	

(*continued on next page*)

Table 5 (*continued*)

Substrate	Feed (target compounds)	Membrane barrier		Compounds						Reference
		MWCO[a] (kDa)	Material	Macromolecules group	C (mg/L)	R (%)	Micromolecules group	C (mg/L)	R (%)	
Anari cheese	UF- whey (protein & sugars)	1	ETNA[j]	Proteins	652	24	Total sugars	38402	24	Galanakis *et al.* in press-b
							Total phenols	27	36	
	UF- whey (protein & sugars)	1	ETNA[j]	Proteins	275	42	Total sugars	8212	23	Galanakis *et al.* in press-b
							Total phenols	16	23	
Dry red wine	6-fold diluted (phenolics & anthocyanins)	1	ETNA[j]	n.d.[c]			Total phenols	1930–4413	69–90	Galanakis *et al.* in press-a
							Hyd.-cin. acids[d]	121–444	42–53	
							Flavonols	118–369	9–40	
							Total anth.[l]	75–559	21–71	
							Antirad. effic.[n]	28–63[n]	11–57	
							FRAP activity[o]	466–806[o]	66–85	
Sweet red wine	6-fold diluted (phenolics & anthocyanins)	1	ETNA[j]	n.d.[c]			Total phenols	730	65	Galanakis *et al.* in press-a
							Hyd.-cin. acids[d]	87	67	
							Flavonols	81	25	
							Total anth.[l]	19	26	
							Antirad. effic.[n]	6[n]	31	
							FRAP activity[o]	98[o]	60	
Dry white wine	6-fold diluted (phenolics & anthocyanins)	1	ETNA[j]	n.d.[c]			Total phenols	224	23	Galanakis *et al.* in press-a
							Hyd.-cin. acids[d]	19	63	
							Flavonols	30	49	
							Total anth.[l]	15	28	
							Antirad. effic.[n]	4[n]	29	
							FRAP activity[o]	95[o]	57	

[a] "MWCO" for "Molecular Weight Cut-off".
[b] "PES" for "polyethersulphone".
[c] "n.d." for "not determined".
[d] "Hyd.-cin. acids" for "hydroxycinnamic acid derivatives".
[e] "Monov. ions" for "monovalent ions".
[f] "AIR" for "alcohol insoluble residue".
[g] "n.p." for "no permeate".
[h] "Red. sugars" for "reducing sugars".
[i] "Non-red. sugars" for "non reducing sugars".
[j] "ETNA" for "composite fluoro polymer".
[k] "Pol-anthoc." for "polymeric anthocyanins".
[l] "Total anth." for "total anthocyanins".
[m] "Monom. anth." for "monomeric anthocyanins".
[n] "Antirad. effic." for "antiradical efficacy" expressed in mg DPPH/g.
[o] "FRAP activity" expressed in μg TROLOX/mL.

opportunities for particular applications. Conclusively, the following observations can be denoted:

- Macroscopic pre-treatment (i.e. thermal concentration) and extraction procedures (i.e. acid- or enzyme assisted methods) applied prior UF lead not only to the breakage of bioresource clusters, but also to the decomposition of macromolecules and the formation of oligopolymer structures. This fact affects the separation performance of UF membranes. On the other hand, if macro- and micro-molecules complexes (i.e. whey proteins with phenols) have not been separated prior UF procedure, smaller molecules are covered in the concentrate stream.
- The application of membranes (i.e. polysulphone) with a more asymmetric cut-off profile can provide a degree of flexibility to the assayed separation. For instance, macromolecules can step in "gaps" and pass through membranes pores, whereas small polar molecules can stuck in the polar membrane parts and get adsorbed on them.
- Polysulphone membranes in the range of 20–25 kDa have been found to be very efficient in the following separations: (a) polymeric from monomeric anthocyanins, (b) pectin from phenolic compounds and cations, (c) removal of "heavier" phenolic classes (autoxidated or compounds linked to macromolecules) without affecting the overall antioxidant properties of the permeate.
- The combination of less hydrophobic and narrower membrane (i.e. polyethersulphone of 10 or 2 kDa) has been shown to be efficient the other way around, i.e. by adsorbing rapidly polar micromolecules (i.e. hydroxycinnamic acids) and releasing oligosaccharide pectin fragments in the permeate stream.
- The more hydrophobic composite fluoro polymer is able to separate efficiently micromolecular classes such as hydroxycinnamic acids from flavonols.
- An assay of UF experiments from UF (100 kDa) to the border of NF (2 kDa) can be applied in order to understand the MW of compounds determined with different spectrophotometric methods. For example, it has been shown that Folin–Ciocalteau method (at 725 nm) determines total phenols with lower MW compared to these determined at 280 nm, after acidification with HCl (Galanakis *et al.*, 2010b).
- In case of NF and tight UF membranes (1–2 kDa), the electrostatic interactions between membrane surface and solutes can improve membrane selectivity, if the chemical environment is wisely selected.

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