

Flavonoids in Lemon and Grapefruit IntegroPectin

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Abstract: Following the analysis of terpenes present in new lemon and grapefruit “IntegroPectin” pectins obtained via the hydrodynamic cavitation of industrial lemon and grapefruit processing waste, the HPLC-MS analysis of the flavonoid compounds reveals the presence of eriocitrin, naringin, hesperidin and kaempferol typical of the respective citrus fruits. The pectic fibers rich in rhamnogalacturonan-I “hairy” regions act as chemical sponges adsorbing and concentrating at their outer surface highly bioactive citrus flavonoids and terpenes. These findings, together with the unique molecular structure of these new whole citrus pectins, provide preliminary insight into the broad-scope and powerful biological activity of these biomaterials. Numerous new biomedical applications beyond prevention and treatment of microbial infections and neurodegenerative disease are anticipated.

Keywords: IntegroPectin, naringin, eriocitrin, citrus, flavonoids, pectin, circular economy

1. Introduction

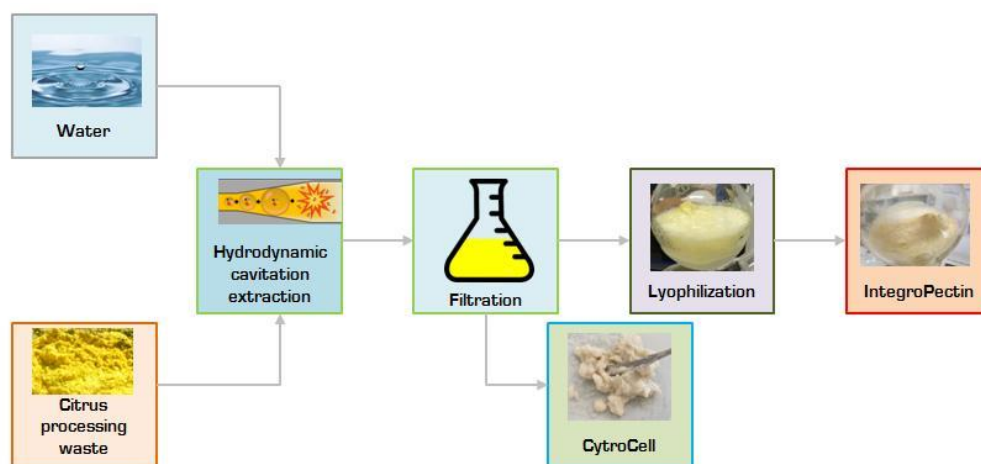
Communicating the discovery of the high antioxidant activity, and lack of cytotoxicity on human pulmonary cells, of lemon pectin obtained via hydrodynamic cavitation (HC) of citrus processing industrial waste [1], we named this new citrus pectin “IntegroPectin”. Reporting shortly afterwards its antibacterial activity, largely superior to that of commercial citrus pectin, we ascribed it to the “combined antibacterial action of lemon oil..., and of the water-soluble lemon flavonoids found in abundant amount at the surface of lyophilized IntegroPectin” [2].

Subsequent investigation of the terpenes adsorbed at the surface of these new whole lemon (and grapefruit) pectins, actually showed the presence of relatively high amount of highly bioactive α -terpineol and terpinen-4-ol, likely originating from limonene and linalool decomposition, during the HC-based extraction, catalyzed by residual citric acid in the citrus peel by-product of the industrial production of the juice [3]. Further reporting the broad-scope bactericidal activity of grapefruit IntegroPectin against Gram-positive and Gram-negative bacteria and suggesting also the bacterial inactivation mechanism [4], we noted that analysis of the flavonoids and terpenes present in the new IntegroPectin was necessary *en route* to develop new applications of these new pectic polymers. Indeed, along with the aforementioned antibacterial activity, lemon IntegroPectin was recently shown to exert exceptionally high *in vitro* neuro- and mitoprotective activity towards human neuronal cells [5].

Having been obtained after hydrolysis with mineral acid in hot water followed by precipitation with isopropyl alcohol and by extensive washing [6], commercial citrus pectin does not contain either citrus terpenes or citrus flavonoids. Furthermore, the polymer structure is extensively degraded, chiefly because of hydrolytic loss of the

“hairy” rhamnogalacturonan-I chains bound to the main homogalacturonan chain of the heterogeneous polysaccharide [6].

Citrus flavonoids contained in large amount in citrus processing waste are the target of specific extraction procedures [8], as they are increasingly added to several new nutraceutical products and food supplements. It is now well known, indeed, that the broad-scope biological activity of these flavonoids, including anti-inflammatory, antiproliferation, anti-angiogenesis and cell cycle regulation effects are due not only to their free radical scavenging activity, but also to their ability to act as modulators of several key molecular events implicated in cell survival and apoptosis [9].



Scheme 1. Biorefinery scheme for citrus processing waste based on hydrodynamic cavitation.

It is also of relevance to this account the growing evidence, recently emphasized by Valtchev and co-workers reviewing citrus peel flavonoid anti-cancer properties [9], of synergy between these bioactive molecules, with citrus peel whole extracts showing higher activity than the fractionated extracts or isolated single compounds. Finally, citrus peel extracts obtained from freeze-dried peel, due to enhanced rupture of the cell vacuoles and release of phenolic compounds accumulating therein, have >50% higher concentrations of total phenolic and total flavonoid compounds when compared to extracts obtained from the fresh peel [10].

Now, we report the qualitative and quantitative analysis of the main flavonoids and phenolic compounds accumulated at the surface of lemon and grapefruit IntegroPectin obtained after freeze-drying the aqueous solution obtained after the the HC-based extraction of lemon and grapefruit citrus processing waste using water as the only solvent (Scheme 1).

2. Experimental Section

A 600 mg sample of each (lemon and grapefruit) IntegroPectin obtained as described elsewhere [1,3] was weighted and added to a 100 mL flask. A 50 mL aliquot of an EtOH/H₂O mixture (v/v, 4:1) was added to each flask. The extraction was carried out under sonication (150 W) for 15 min using a Transsonic 460 H ultrasonic bath (Elma Hans Schmidbauer, Singen, Germany) operating at 35 kHz ultrasonic frequency. The bath temperature during sonication reached 40°C. After sonication, the content of each flask transferred into in centrifuge tubes underwent centrifugation at 10,000 rpm for 10 min in an Allegra X-22 R (Beckman Coulter, Brea, CA, USA) centrifuge, after which a small sample of the supernatant of both samples was transferred to glass vials for analysis.

The high performance liquid chromatography-mass spectrometry (HPLC-MS) analyses were conducted using a Alliance e2695 (Waters, Milford, MA, USA) HPLC system equipped with autosampler, degasser and column heater coupled with a Q-ToF Premier (Waters, Milford, MA, USA) quadrupole time of flight mass spectrometer. The compounds were separated by a Thermo Scientific (Thermo Fisher Scientific, Waltham, MA, USA) BetaBasic HPLC C18 column (50 × 2.1 mm I.D., particle size 1.8 μm) kept at 20 °C injecting each time a 5 μL volume sample.

All samples were injected in duplicate using a thermostated autosampler kept at 4 °C. The HPLC analyses were carried out using a mixture of 0.1 wt% aqueous formic acid and 0.1 wt% formic acid methanolic solution at 0.25 mL/min flow rate.

Elution started with 95% aqueous formic acid and 5% methanol formic acid, isocratic for one minute. In the subsequent 14 min the solvent becomes 100% MeOH, remaining isocratic for the subsequent 5 min (from min 15 to min 20). After 30 s, the eluting solvent mixture is reverted to 95% aqueous formic acid and 5% methanolic formic acid, and kept as such for another 30 s. The whole run lasted 21 min. Every sample was injected twice. The concentration values reported in Table 1 are the average of the two values measured.

Quantification of quercetin, resveratrol, caffeic acid, gallic acid, eriocitrin, naringin, and hesperetin was carried out using commercial samples of the phenolic compounds obtained from Sigma Aldrich (Gallarate, Italy) as standards. For the remaining compounds, we used the calibration curve of quercetin for the detection of flavonoids, and that of resveratrol for the assessment of other biophenols.

The MS experiments were performed on Q-ToF Premier using dynamic range enhancement (DRE) as acquisition mode that avoids MCP saturation keeping a fairly good sensitivity. This allows to correctly quantify very abundant as well as trace level compounds, providing results suitable for statistical analysis. Atmospheric pressure chemical ionisation (APCI) in negative mode was used under the following conditions: capillary, 2.0 kV; extraction cone, 2.0 V; ion guide, 2.0 V; source temperature 80 °C, cone gas, N₂, flow 35 L h⁻¹; desolvation gas, N₂, flow 300 L h⁻¹.

The following compounds were researched in both lemon and grapefruit IntegroPectin samples: quercetin, naringin, sinapinic acid, rutin, quercetin-3-glucuronide, naringenin, hesperidin, hesperetin, eriodictyol, eriocitrin, diosmin, caftaric acid, petunidin 3-O-(6''-acetyl)glucoside, cyanidin 3-O-glucoside, malvidin 3-O-pentoside, peonidin 3-O-hexoside isomer, resveratrol, resveratrol dimer, resveratrol trimer, resveratrol tetramer, resveratrol hexoside, kaempferol, kaempferol 7-O-glucuronide, quercetin-3-O-(6''-O-malonyl)-glucoside, quercetin-3'-O-glucuronide, kaempferol-3-O-glucoside, quercetin-3-O-galactoside, myricetin-3-O-hexoside, myricetin, epigallocatechin-3-O-coumarate, epicatechin-3-O-vanillate, epigallocatechin, procyanidin B3, catechin, procyanidin B2, procyanidin C2, isovanillic acid, gallo catechin, prodelphinidin dimer B type, catechin-7-O-glucoside, caffeic acid methyl ester, 3-caffeoyl-4-*p*-coumaroylquinic acid, ferulic acid, ellagic acid, *p*-coumaric acid, gallic acid ethyl ester, vanillic acid, caffeic acid, *p*-hydroxybenzoic acid, and gallic acid.

3. Results and discussion

Table 1 lists the flavonoid and other phenolic compounds found using the highly sensitive HPLC-MS technique employed. In agreement with the literature, the two main flavonoids found are eriocitrin, for lemon IntegroPectin, and naringin for grapefruit IntegroPectin. Particularly abundant in the lemon peel (mesocarp, epicarp, and pulp vesicles) but also significantly present in the juice (but absent in the seed), eriocitrin (eriodictyol 7-O-rutinoside) is a powerful antioxidant with an activity level equal to that of α -tocopherol, which is even enhanced in the presence of citric acid [11]. Supported by clinical studies in pre-diabetic patients showing benefits in glycemic control, reduced systemic inflammation and oxidative stress, up to reversing the prediabetic condition in 24% of the patients [12], nutraceutical products whose active ingredient is lemon-derived

eriocitrin are regularly commercialized for reducing blood glucose levels by administering to the subject a composition comprising 100-200 mg eriocitrin once per day [13].

Table 1. Flavonoids in lemon and grapefruit IntegroPectin identified by HPLC-MS.

Biophenol	Lemon IntegroPectin (mg/g)	Grapefruit IntegroPectin (mg/g)
Naringenin	-	0.34
Naringin	0.11	73.66
Hesperidin	0.60	0.60
Eriocitrin	3.35	-
Kaempferol	0.26	-
Kaempferol-7-O- glucuronide	0.26	0.26
<i>p</i> -Coumaric acid	0.28	
Gallic acid	0.56	0.31

Along with naringin, hesperidin (hesperetin 7-*O*-beta-rutinoside) is also present in significant amount at the surface of lemon IntegroPectin. Administered with eriocitrin, this dietary lemon flavonoid suppresses the oxidative stress in diabetic mice [14], and it is nowadays the most studied citrus flavonoid due to its broad-spectrum bioactivity in the prevention of cancer [15], neurodegenerative [16] and cardiovascular [17] diseases.

Flavanone glycoside naringin, (4',5,7-trihydroxyflavone-7-rhamnoglucoside), by far the predominant flavanone found in grapefruit [18], was found to be exceptionally concentrated in grapefruit IntegroPectin approaching 74 mg/g. For comparison, the highest yield values for naringin extracted from fresh or dry grapefruit albedo so far reported are 10.5 mg/g (for fresh albedo) and 43.28 mg/g [19].

Besides showing anti-cancer, cholesterol-lowering, anti-apoptotic, anti-atherogenic, anti-inflammatory and antioxidant activity, naringin is an antimicrobial agent inhibiting growth of bacteria such as *Actinomyces naeslundii*, *Actinomyces viscosus*, *Aggregatibacter actinomycetemcomitans*, *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus* and *Lactobacillus casei* as well as of *Candida albicans* fungal species typical of the dental plaque [20]. Remarkably, naringenin (the aglycone of naringin) was lately found by scholars in South Korea to exert powerful antimicrobial activity against Methicillin-resistant *Staphylococcus aureus* and its mutant strain Δagr by inhibiting the microbial fatty acid synthesis [21], in full agreement with the bactericidal activity of grapefruit IntegroPectin against *S. aureus* lately discovered [4].

Kaempferol 7-*O*-glucuronide, namely kaempferol (3,4',5,7-tetrahydroxyflavone) with a β -*D*-glucosiduronic acid residue attached at the 7-position, was found in similar amount (0.26 mg/g) in both citrus IntegroPectins studied. The latter is a tetrahydroxyflavone likely formed during cavitation via reaction of kaempferol in the presence of residual glucose and citric acid abundant in citrus processing waste, which was recently reported to have strong antimicrobial activity [22]. Kaempferol, whose beneficial effects in reducing the risk of chronic diseases including cancer, was found in lemon IntegroPectin only, in the same amount of its aforementioned *O*-glucuronide. Molecular mechanism investigations have shown that the flavanol controls (modulates) a number of intracellular signaling cascades that regulate cellular signal transduction pathways linked to apoptosis, angiogenesis, inflammation, metastasis [23] and allergic airway inflammation and associated asthma symptoms [24]. The main problem limiting nutraceutical applications of kaempferol has been its poor bioavailability due to poor solubility. As argued for other citrus flavonoids, such as hesperidin, naringin and the

respective aglycones, the hydrodynamic cavitation-based extractions process and the complexation with pectin could substantially relieve the bioavailability issue [25], thus further opening the way to effective nutraceutical applications.

4. Conclusions

The analysis of the main flavonoids present at the surface of the newly obtained citrus pectins, namely lemon and grapefruit IntegroPectin, complements the analysis of the adsorbed volatile compounds unveiling the presence of highly bioactive flavonoids. Eriocitrin predominates in lemon IntegroPectin, followed by significant amounts of hesperidin, kaempferol, and kaempferol-7-O- glucuronide. In the case of grapefruit IntegroPectin, naringin is by far predominant, with minor amounts of hesperidin and naringenin.

Along with the unique molecular structure of these new pectins, with low degree of esterification and high amount of RG-I "hairy region" (repeating disaccharide units [\rightarrow 2)- α -L-Rhap-(1 \rightarrow 4)- α -D-GalpA-(1 \rightarrow) and neutral sugar side-chains mainly consisting of arabinose and galactose with different linkages) [4,5], these results shed light on the exceptionally high bactericidal activity of grapefruit IntegroPectin [4], and the neuro- and mitoprotective action of lemon IntegroPectin [5].

In the former case, the IntegroPectin polymer chains rich in RG-I regions act as carriers of adsorbed naringin, α -terpineol and linalool [3], which are powerful antibacterial agents particularly against Gram-positive bacteria, permeating the hydrophobic *S. aureus* cell membrane [26], with subsequent likely hydrolysis of naringin into highly antimicrobial naringenin [20].

The neuro- and mitoprotective action of lemon IntegroPectin might be due to the combined neuroprotective, antioxidant and anti-inflammatory action of eriocitrin in synergy with hesperidin and with α -terpineol and terpinen-4-ol neuroprotective species abundant in the newly obtained citrus pectin [3], again in combination with the RG-I enriched structure of the lemon IntegroPectin favoring interaction with the cancer cells studied [5] (which include human pulmonary cells [1]).

The aforementioned RG-I side-chains, indeed, play a central role in enhancing the bioactivity of citrus pectin, including anti-cancer, immunomodulatory, anti-hyperlipidemic, and anti-hyperglycemic activity [27], with the key ability of said chains to inhibit the pro-metastatic protein galectin-3 [28] by binding to its carbohydrate recognition domain. Furthermore, RG-I enriched citrus pectin forms gels both adding Ca^{2+} cations and under acid conditions via a gelation mechanism, different from that of commercial pectin, in which the neutral side-chains improve network formation [29].

Numerous new biomedical applications beyond the treatment of microbial infections and neurodegenerative disease can be anticipated in light of the broad scope bioactivity of the citrus flavonoids and terpenes identified in whole citrus pectins studied in synergy with that of pectin, recently called "an universal medicine" [30], especially considering the unique molecular structure of citrus pectin obtained via hydrodynamic [4,5,7] or acoustic [31] cavitation-based extraction. The results of further ongoing investigations will be reported in due course.

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This research received no external funding. The authors declare no conflict of interest.

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