

Article

Volatile Compounds of Lemon and Grapefruit IntegroPectin

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Abstract: The HS-SPME GC-MS analysis of the volatile compounds adsorbed at the outer surface of lemon and grapefruit pectins obtained via hydrodynamic cavitation of industrial waste streams of lemon and grapefruit peels in water only suggests important new findings *en route* to understanding the powerful and broad biological activity of these new pectic materials. In agreement with the ultralow degree of esterification of these pectins, the high amount of highly bioactive α -terpineol and terpinen-4-ol points to limonene decomposition catalyzed by residual citric acid in the citrus waste peel residue of the juice industrial production.

Keywords: IntegroPectin, lemon, grapefruit, pectin, waste citrus peel, α -terpineol, hydrodynamic cavitation, circular economy

1. Introduction

Industrially extracted from dried lemon (but also orange and lime) peel, and in far lesser amount from apple pomace, pectin is a versatile hydrocolloid in high and rapidly increasing demand from the food and pharmaceutical industries [1]. The extraction makes use of diluted mineral or oxalic acid in hot water and employs isopropyl alcohol to precipitate the polysaccharide after the extraction and extensive polymer degradation. Plentiful research has been devoted in the last decade to develop green extraction methods for pectin. Selected achievements include microwave-assisted extraction in water only [2], acoustic cavitation [3], hydro-distillation driven by concentrated solar power [4], enzymatic extraction at acidic pH [5], and controlled hydrodynamic cavitation (HC) in water only [6].

Dubbed “IntegroPectin”, citrus pectin extracted from the wet peel of the main citrus fruits residue of the juice production process using HC largely differs from conventional citrus pectin extracted from dried citrus peel using acid in hot water. Orange IntegroPectin, for instance, has a low degree of esterification (17%) and contains plentiful amounts of co-extracted citrus flavonoids and terpenes [6]. The potential antiviral activity of the main orange flavonoids hesperidin and naringin, for instance, raised interest in HC as an efficient and highly scalable flavonoid production method for the treatment of COVID-19 [7].

Lemon IntegroPectin, namely citrus pectin extracted from wet industrial waste lemon peel using again HC, has exceptional antioxidant [8] and good antibacterial [9] properties, whereas grapefruit IntegroPectin is a broad-spectrum bactericidal agent currently investigated as a natural antimicrobial likely to drive no antimicrobial resistance [10]. It is also important, in sight of medical applications of these new pectins [11,12], that lemon IntegroPectin is highly cytoprotective, preventing oxidative degradation of human pulmonary cells from oxidizing agents even at high concentrations of a strong oxidizer such as H₂O₂ [8].

Aiming to explain the high antimicrobial activity of lemon and grapefruit IntegroPectin, we ascribed the activity to the synergistic action of the chemical structure of citrus pectin extracted using cavitation, combined with the action of citrus terpenes and flavonoids adsorbed across the pectic polymer obtained upon lyophilization of the aqueous extract [10]. In this study we report the analysis of the volatile compounds in both lemon and grapefruit IntegroPectin using the headspace solid-phase microextraction (HS-SPME) technique combined with gas chromatography-mass spectrometry (GC-MS). The outcomes of this investigation provide molecular insight on the HC-assisted extraction of both terpenes and pectin from the cells comprising the waste citrus peel (exo-, meso-, and endocarp) obtained by citrus juice production lines.

2. Materials and Methods

The GC-MS analysis of the volatile compounds released in the headspace of a closed vial upon their solid phase microextraction (SPME) using a thin polymer coating fixed to the solid surface of a fiber [13] enables direct identification of the volatile compounds by comparison with the mass spectra of authentic standards. The technique, for example, has been widely used to identify volatiles in the fragrances of lemon cultivars [14]. The analytical conditions used in the present study appear in Table 1.

Table 1. Conditions for the analysis

Sample/matrix:	0.827 g freeze-dried, ground grapefruit IntegroPectin; 0.868 g freeze-dried, ground lemon IntegroPectin
SPME fiber:	50/30 μm DVB/CAR/PDMS
Sample equilibration:	24 h, room temperature
Extraction:	2 min, headspace, room temperature
Column:	TRACE TR-FAME, 100 m × 0.25 mm I.D., 0.20 μm
Oven:	50 °C (0 min), 10 °C/min to 250 °C (5 min)
Injection T:	260 °C
Detector:	DynaMax XR with off-axis dynode, discrete dynode electron multiplier and electrometer, linear range of >10 ⁷
Scan range:	full scan, m/z 50-500
Carrier gas:	He 99,9999%, 1 mL/min constant flow

2.1 Materials

Dried samples of lemon and grapefruit IntegroPectin were obtained as previously reported [10]. The divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) fiber was obtained from Supelco (Bellefonte, USA).

2.2 Headspace SPME Sampling

Lemon and grapefruit IntegroPectin samples were left in closed vials (Figure 1) at room temperature to equilibrate for 24 h before sampling. Extraction of volatile components accumulated in the headspace took place by injecting the coated fiber in the headspace and leaving the fiber to adsorb the volatiles for 2 min, after which the fiber was withdrawn from the vial and inserted in the hot (260 °C) GC injector.



Figure 1. The lemon (left) and grapefruit (right) IntegroPectin samples analyzed for the volatile content.

In order to avoid carry over effects or artifact formation, blank runs were carried out every three analyses. Prior to use and between consecutive analyses the fiber was conditioned in a GC injector at 260 °C for 3 h to remove contaminants. Nonetheless, siloxane contaminants most likely derived from the injector led to the appearance of interference siloxane peaks in the chromatogram that were readily identified. The fiber used for sampling both lemon and grapefruit pectin used a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50 µm the DVB layer and 30 µm the CAR/PDMS layer) coating suitable for analyte group flavors (volatiles and semivolatiles). In order to assess the best extraction time for this coating, several preliminary tests at increasing extraction times (2 min, 5 min and 15 min) were investigated (data not shown). The 2 min microextraction time was identified as optimal to reproduce the extraction procedure.

2.3 Gas chromatography-mass spectrometry

A ISQ LT single quadrupole mass spectrometer (Thermo Fisher Scientific, USA) next to a Trace 1310 gas chromatograph (Thermo Fisher Scientific, USA) equipped with a Trace TR-FAME, 100 m × 0.25 mm I.D., 0.20 µm (Thermo Fisher Scientific, USA) was used for the GC-MS analyses. The oven temperature program was from 50 °C (hold 0 min) at 10 °C/min to 250 °C, hold 5 min. The carrier gas was ultrapure helium (He ≥ 99,9999%, ALPHAGAZ 2, Air Liquide Italia Service, Milan, Italy) flown

at 1 mL/min rate. Injection took place in splitless mode at 260 °C. The mass analysis was carried out under conditions of electronic ionization (EI) with an ionization energy of 70 eV. The mass spectra were recorded in Full Scan in a mass range ranging from 50 to 500 m/z . The attribution of compounds corresponding to the chromatographic peaks was performed by comparing the corresponding mass spectra with those of the NIST 11.0 and Wiley 7.0 mass spectrum libraries, verifying the fragmentation through a careful study of EI spectra.

3. Results and Discussion

A total of 15 volatile compounds were identified in the case of lemon IntegroPectin (Figure 2 and Table 2).

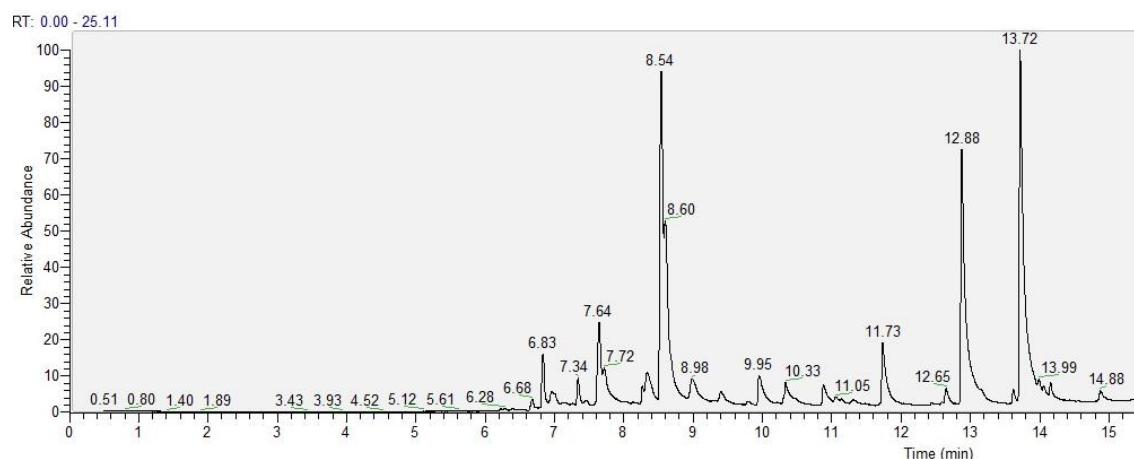


Figure 2. GC chromatogram of volatiles in lemon IntegroPectin.

Table 2. Volatile organic compounds in lemon IntegroPectin

Entry	Compound	Apex RT (min)	Area (%)
1	3-Methyl-2-buten-2-ol (prenol)	7.34	1.08
2	1-Butanol, 3-methyl acetate (isoamyl acetate)	8.27	0.73
3	<i>d</i> -Limonene	8.33	3.39
4	2-Methyl-1-butanol	8.54	17.11
5	3-Methyl-1-butanol	8.6	12.43
6	Eucalyptol	8.98	2.32
7	1-Hexanol	9.95	2.52
8	2-Hexen-1-ol	10.33	2.33
9	6-Hepten-1-ol, 2-methyl	10.88	1.52
10	α -Linalool	11.73	4.94
11	Terpinen-4-ol	12.88	21.85
12	α -Citral	13.62	0.58
13	α -Terpineol	13.72	27.42
14	Safranal	14.15	1.11
15	2,4-Cyclohexadiene-1-methanol, α,α -4-trimethyl	14.88	0.65

A highly bioactive molecule, with antioxidant, anticancer (anti-proliferative and cytostatic), anticonvulsant, antiulcer, antihypertensive, anti-inflammatory and analgesic (anti-nociceptive) properties [15], α -terpineol is the most abundant component amid volatiles found in lemon

IntegroPectin. The second most abundant terpene is terpinen-4-ol (21.85%, entry 11 in Table 1), a potent bactericidal [16] and antifungal [17] agent capable to kill *Staphylococcus aureus* strains and inhibit azole-resistant human pathogenic *Candida* species. As the main component of tea tree essential oil, terpinen-4-ol was found in a randomized trial to provide a safe and well-tolerated regimen for eradication of methicillin-resistant *S. aureus* carriage in hospitalized patients [18]. 2-Methyl-1-butanol (entry 4 in Table 2) and 3-methyl-1-butanol (entry 5) are key components imparting fragrance to grapefruit juice [19], but so far unreported in the case of lemon essential oil or juice, thereby indicating modification of certain volatile molecules during the HC-based extraction (see below). Similarly, isoamyl acetate (entry 2 in Table 2) was previously found in orange essential oil [20].

In the case of grapefruit IntegroPectin, 11 volatile compounds were identified (Figure 3 and Table 3).

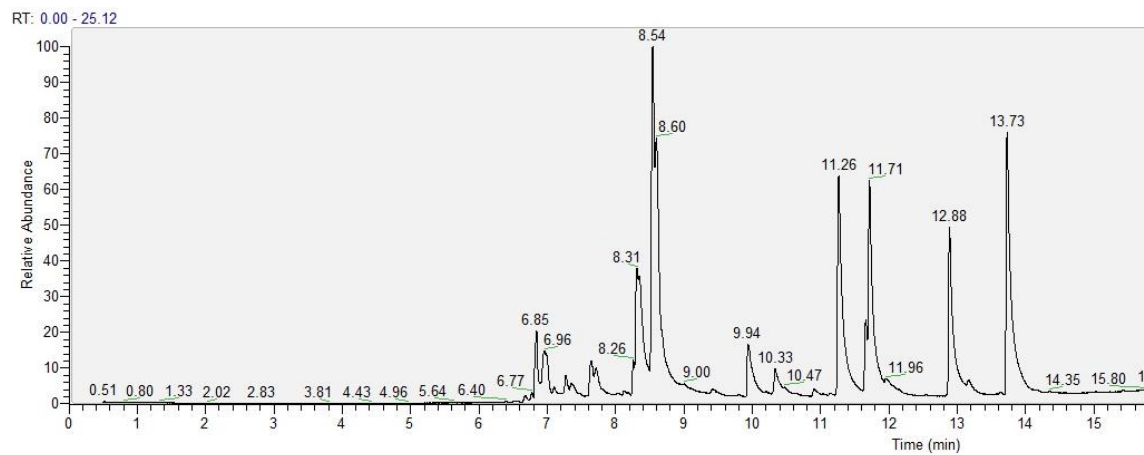


Figure 3. GC chromatogram of volatiles in grapefruit IntegroPectin.

Table 3. Volatile organic compounds in grapefruit IntegroPectin

Entry	Compound	RT (min)	%Area
1	1-Butanol, 3-methyl-acetate (isoamyl acetate)	8.26	0.67
2	d-Limonene	8.31	11.77
3	2-Methyl-1-butanol	8.54	12.91
4	3-Methyl-1-butanol	8.6	15.17
5	1-Hexanol	9.94	3.24
6	3-Hexen-1-ol	10.33	1.28
7	cis-Linalool oxide	11.26	14.4
8	trans-Linalool oxide	11.66	1.76
9	α-Linalool	11.71	12.01
10	Terpinen-4-ol	12.88	9.37
11	α-Terpineol	13.73	17.4

Besides a relatively higher amount of limonene (11.8% *vs.* 3.39%), the main difference with lemon IntegroPectin is the presence of higher amount of linalool (12% *vs.* 4.95%) as well as of substantial amounts (16%) of linalool oxide (predominantly the *cis* isomer, entry 7 in Table 3) which is absent in lemon IntegroPectin. Linalool oxide isomers are well known components of grapefruit essential oil [21].

Due to an ample and diversified spectrum of biological properties, linalool is one of the most widely studied odorant molecules [22]. Found in the essential oils (EOs) of over 200 plants including citrus, linalool exhibits antimicrobial activity against different microorganisms (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pasteurella multocida*), particularly against Gram-positive bacteria, showing activity levels comparable to that of Amoxicillin [23]. Though far less studied due to its relatively minor abundance in EOs, linalool oxide has been recently shown to have (in experiments with mice) even more powerful anticonvulsant and antinociceptive activities than linalool [24].

The low amount of *d*-limonene, by far the most abundant terpene in the peel of both lemon [25] and grapefruit [26], points to formation of α -terpineol and terpinen-4-ol from their precursors *d*-limonene and linalool in the cavitation microbubbles, in analogy to what happens in orange [27] and mandarin [28] acidic juices after storage. Following fast protonation likely taking place in the cavitation microbubbles (Figure 4), both linalool and limonene are readily converted into α -terpineol and terpinen-4-ol, with linalool being even more reactive than limonene [27].

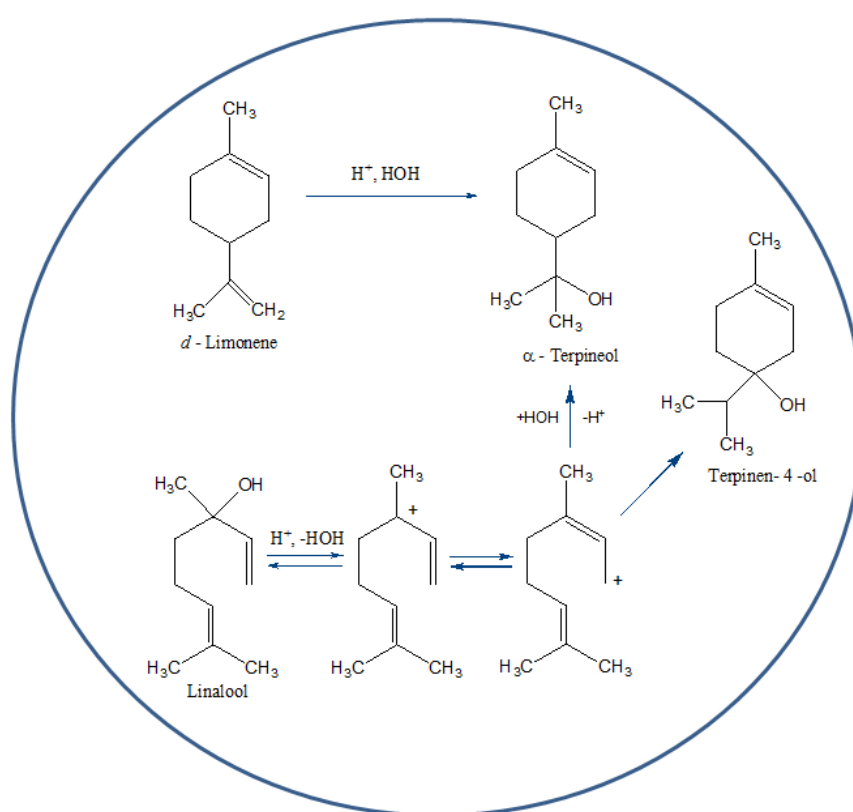


Figure 4. Molecular pathway from linalool and *d*-limonene to α -terpineol in the hydrodynamic cavitation microbubbles [Adapted from Ref.27]

The protons needed to drive the reactions displayed in Figure 4 originate from citric acid, a tricarboxylic acid particularly abundant in the lemon pulp from which it was commercially extracted until the introduction of the sugar fermentation process over *Aspergillus niger* [29]. Indeed the industrial waste citrus peel (exo-, meso-, and endocarp) used for the production of orange, lemon and grapefruit IntegroPectin obtained by citrus juice production lines, contains not only the outer skin (exocarp) and the peel (exo- and mesocarp), but also plentiful endocarp residues. Furthermore, experiments with Venturi-shaped HC reactors similar to the reactor used to manufacture the analyzed samples of IntegroPectin clearly showed that hydrophobic molecules (such as terpenes) tend to migrate to the cavity-water interface [30], increasing their concentration close to the water molecules dissolving the H_3O^+ ions produced by citric acid hydrolysis, with the extreme local pressures and temperatures created by the collapsing cavities enhance the reaction rate. This effect is

likely to provide a further contribution to the protonation of linalool and limonene and to the observed formation of terpene nanoemulsion in the HC-derived aqueous extract [6].

Based on this hypothesis, one would expect that the degree of esterification of lemon, grapefruit and orange pectin would decrease in the order lemon < grapefruit < orange, due to the action of citric acid far more abundant in the pulp of lemon (lemon juice typically has 60 g L⁻¹ citric acid concentration *vs.* 19 g L⁻¹ in grapefruit and 11 g L⁻¹ in sweet orange [31]). Indeed, this is exactly what is observed, with the ultralow DE for lemon IntegroPectin (8%), being lower than that of grapefruit (14%) [10], which is in turn lower than that of orange (17%) [6].

Catalyzed by protons, the de-esterification reaction of pectin through which the methoxyl groups in natural citrus pectin (which is an high-methoxyl pectin) are removed, is accelerated in the cavitation bubbles, as discovered in 1974 studying the acid-catalyzed hydrolysis of methyl acetate in water under acoustic cavitation conditions [32], when the reaction rate increased up to 9.6% due the “tremendous local pressure and temperature increasing the vibrating motion of the reacting molecules” [32].

The first indirect evidence of limonene conversion during the HC-assisted extraction of waste orange peel was observed measuring its concentration, when after only 10 min of hydrocavitation the concentration of limonene in the liquid phase decreased by 80% [6]. In said extraction, all the terpenes retrieved from the waste citrus peel, including those with little or no solubility in water, do not separate from the water phase as it happens in the microwave-assisted extraction of waste orange and lemon peel in water [2].

In brief, the good emulsifying and emulsion-stabilizing properties of pectin [33] are significantly enhanced under hydrodynamic cavitation conditions. As a result, the solutions obtained from the HC-assisted extraction of waste orange, lemon and grapefruit peels do not separate into a water and an oily phase even after more than 2 years of storage at room temperature, retaining their pleasant odor due to the odorant molecules stabilized against oxidation by the powerful antioxidant activity of the co-extracted citrus flavonoids [6].

Finally, further proof that acid-catalyzed molecular degradation of certain citrus terpenes occurs during the HC-based extraction is given by the presence in the lemon IntegroPectin of 2,4-cyclohexadiene-1-methanol, α,α -4-trimethyl (p-mentha-1,5-dien-8-ol, entry 15 in Table 2); as well as of 1-hexanol in the grapefruit IntgroPectin (entry 5 in Table 3). The former compound is one of the degradation products of citral (a key aldehyde mixture of neral and geranial) found in earlier studies dealing with the degradation of citral in carbonated orange juice beverages [34]; the latter is likely to be the degradation compound of 3-mercapto-1-hexanol, a sulphur volatile identified in grapefruit juice [35].

4. Conclusions

The HS-SPME-GC-MS analysis of the volatile compounds of lemon and grapefruit pectins obtained via hydrodynamic cavitation of lemon and grapefruit peel residue of juice extraction suggests important new findings *en route* to understanding the high biological activity of these new pectic materials and the hydrodynamic cavitation process applied to wet (*i.e.*, non dried) waste citrus peel.

The high amount of the highly bioactive terpenoids α -terpineol and terpinen-4-ol points to the conversion of limonene (and linalool) upon protonation and hydration, a process catalyzed by the citric acid present in the endocarp fraction of waste citrus peel and further accelerated under cavitation conditions. This hypothesis also explains the ultralow degree of esterification of lemon (DE = 8%), grapefruit (DE = 14%) and orange (DE = 17%) IntegroPectin polymers, opening the route to new medical and biotechnological applications of these citrus pectins. For example, scholars in Brazil recently discovered the bactericidal activity of α -terpineol against *S. aureus* strains, interrupting cell division and altering the “grape bunch” morphology typical of *S. aureus* colonies [36]. The findings reported in this study support similar antimicrobial action of α -terpineol in lemon

and grapefruit IntegroPectin [10]. The forthcoming analysis of the flavonoids present in these materials will shed further light on the bioactivity of these new pectic polymers.

Acknowledgments: This study is dedicated to Roberto Dal Bosco for many years of friendship with one of us (M.P.). We thank OPAC Campisi (Siracusa, Italy) for kindly providing waste lemon and grapefruit peel from which the IntegroPectin materials were isolated.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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