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Characterization of the polyphenolic fraction of pomegranate samples by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry detection

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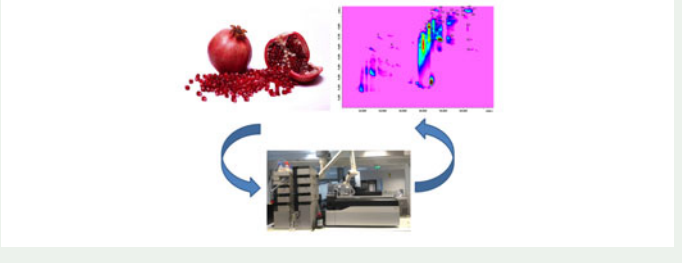
ABSTRACT

Punica granatum L., commonly known as pomegranate, is an ancient fruit widely consumed all over the world as fresh fruit or juice. In addition, it is extensively used in therapeutic formulas, cosmetics and food seasonings. The fruit is native to Afghanistan, Iran, China and the Indian sub-continent. The pomegranate market has steadily grown, presumably due to the increasing demand of health-conscious consumers for products with potential beneficial effects on human health, due to the synergistic presence of a unique and complex phytochemical composition that enclose anthocyanins, phenolic acids and hydrolysable tannins. Conventionally, for their analysis liquid chromatography is employed even though it can present some drawbacks in terms of resolving power. In this contribution, as a valuable alternative, comprehensive two-dimensional liquid chromatography with "shifted gradients" in the second dimension, was applied for the characterization of three pomegranate samples, leading to the identification of 37 different polyphenolic compounds.



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1. Introduction

Punica granatum L., commonly known as pomegranate, is an ancient fruit widely consumed all over the world as fresh fruit or juice. In addition, it is extensively used in therapeutic formulas, cosmetics and food seasonings (Viuda-Martos et al. 2010). The fruit is native to Afghanistan, Iran, China and the Indian sub-continent, but pomegranate cultivation has spread throughout the Mediterranean region, Central and South America (Viuda-Martos et al. 2010; Ismail et al. 2012). The fruit can be divided into three main anatomical parts: the arils, translucent juice-containing sacs which surround the seeds, the mesocarp, the white tissue to which the arils are attached inside the fruit, and the exocarp or pericarp, the external fibrous layer of the fruit (Teixeira da Silva et al. 2013). The pomegranate market has steadily grown, presumably due to the increasing demand of health-conscious consumers for products with potential beneficial effects on human health (Fischer et al. 2011). This increasing interest in the health-promoting properties of pomegranate is fully justified by the most recent findings, according to which the fruit can be a useful agent for the prevention and treatment of a wide range of human disorders and diseases, including infectious and cardiovascular diseases, diabetes and cancer (Viuda-Martos et al. 2010; Ismail et al. 2012; Lansky & Newman 2007; Wu & Tian 2018). Pomegranate juice is used in cosmetics and food for its well-known antioxidant activity (Lansky & Newman 2007). Pomegranate mesocarp and exocarp extracts are documented as having anti-inflammatory, antimicrobial and anti-proliferative properties (Viuda-Martos et al. 2010; Duman et al. 2009; Hamad & Al-Momene 2009; Negro et al. 2012; Orgil et al. 2014; Rafiq et al. 2016; Imperatori et al. 2018; Mastrogiovanni et al. 2018; Meselhy et al. 2018; Zhang et al. 2018). The wide range of health-promoting biological activities are related to the presence of bioactive molecules, well represented in nature, comprising anthocyanins, phenolic acids and hydrolyzable tannins (Cacciola et al. 2016; Cicero et al. 2018; Clodoveo et al. 2015; Gervasi et al. 2016). Anthocyanins represent the predominant class of phytochemicals in pomegranate juice, imparting the typical red colour. Pomegranate mesocarp and exocarp are rich sources of hydrolyzable tannins, which are multiple esters of gallic acid with glucose and products of their oxidative reactions (Li et al. 2013). The large number of possible combinations of monomers gives rise to an enormous structural diversity. Hydrolyzable tannins are divided into gallotannins and ellagitannins, since their hydrolysis yields gallic acid and ellagic acid, respectively (Arapitsas 2012). Among ellagitannins, Fischer et al. (2011) have recognized a unique group named gallagyl esters, such as the main hydrolyzable tannin of pomegranate known as punicalagin. Hydrolyzable tannins have been found in higher amounts in the juice extracted upon commercial processing of the whole fruit. HPLC coupled with PDA and ESI-MS has typically been the technique of choice for the quali- and quantitative analysis of pomegranate polyphenols (Fischer et al. 2011; Qu et al. 2012; Calani et al. 2013; Romani et al. 2012; Borges & Crozier 2012; Di Stefano et al. 2018; Russo et al 2018). However, when the polyphenols content is very complex, a single separation system often does not provide sufficient resolving power for attaining rewarding results.

The scope of this research was the use of comprehensive two-dimensional liquid chromatography coupled to photodiode array and mass spectrometry detection

Table 1. Polyphenolic compounds identified in the pomegranate samples investigated by LC × LC-PDA-MS.

No.	Trivial name	$[M-H]^-$	λmax (nm)	Juice	Peel	Pulp	1D-LC
1	Gallic Acid	169	216, 271	X	X	X	Unresolved
2	Delphinidin 3,5-O-diglucoside	627	525	X	—	X	Unresolved
3	Ellagic Acid derivative	805	282	X	—	X	Resolved
4	Galloylglucose	331	279	X	X	X	Resolved
5	Ellagitannin	643	276	X	X	X	Unresolved
6	Cyanidin 3,5-O-diglucoside	611	515	X	X	X	Unresolved
7	Vanillic acid derivative	525; 561[a]	270, 297	X	—	—	Unresolved
8	Gallocatechin	305	270	X	X	X	Unresolved
9	Phlorizin	435	280	—	X	X	Unresolved
10	Pelargonidin 3,5-O-diglucoside	595	530	X	—	—	Unresolved
11	Coumaric Acid-hexoside	325	315	X	X	X	Unresolved
12	Diosmetin glucoside	461	280	X	X	X	Unresolved
13	α-punicalagin	1083	278	X	X	X	Resolved
14	Hexahydroxydiphenic hexoside	481	266	X	X	X	Unresolved
15	Ellagic Acid hexoside	463	254, 360	X	X	X	Unresolved
16	Delphinidin-3,5-O-diglucoside	625	515	X	X	X	Unresolved
17	Cyanidin-3-glucoside	449	273, 530	X	X	X	Unresolved
18	β-punicalagin	1083	278	X	X	X	Resolved
19	Pelargonidin-3-glucoside	433	515	X	—	X	Resolved
20	Delphinidin-3-glucoside	465	525	X	X	X	Unresolved
21	Diosmetin-glucoside	462	350	X	X	X	Unresolved
22	Acetil prunin	475	280	X	—	X	Unresolved
23	Syringetin hexoside	508	280	X	X	X	Unresolved
24	Vanillic acid hexoside	329	266, 298	X	X	X	Unresolved
25	Quercetin 3-O-rhamnoside	447	263, 355	X	X	X	Unresolved
26	Ellagic Acid deoxyhexose	447	255, 362	X	X	X	Unresolved
27	Hexahydroxydiphenic hexoside II (HHDP)	481	266	X	X	X	Resolved
28	bis-HHDP-hexoside (pedunculagin I)	783	259, 380	X	X	X	Resolved
29	Ellagic Acid pentoside	433	254, 360	X	X	X	Unresolved
30	cis-dihydrokaempferol hexoside	449	365	X	X	X	Unresolved
31	trans-dihydrokaempferol hexoside	449	365	X	X	X	Resolved
32	Cyanidin-3-O-rutinoside	595	515	X	—	—	Unresolved
33	Cyanidin-3-O-pentoside	419	515	X	—	—	Unresolved
34	Galloyl-HHDP-hexoside (Corilagin)	633	259, 380	X	—	—	Resolved
35	Ellagic Acid	301	270, 380	X	—	—	Resolved
36	Quercetin hexoside	463	263, 355	—	X	X	Resolved
37	Kaempferol 3-O-glucoside	447	263, 365	X	X	X	Resolved

A comparison with peaks separated in 1D-LC is also provided.
[a]$[M + HCOOH-H]^-$; X = identified; — = not detected.

(LC × LC-PDA/MS). The system was capable to provide simultaneous separation and identification of bioactive polyphenolic constituents in pomegranate samples.

2. Results and discussion

The aim of this work was to develop an innovative novel analytical method for the characterization of pomegranate polyphenolic constituents. Despite RP-HPLC is the most frequently applied technique for the analysis of polyphenolic compounds in plant extracts, many closely or completely overlapping compounds occur and this compromises the accurate identification. Figure S1 shows the RP-HPLC profile of the poyphenolic fraction of pomegranate juice, where several pair of compounds overlap (Table 1). As a consequence, to overcome such a limitation, the LC × LC technique was employed (Cacciola et al 2017a, 2017b); in particular polyphenolic extracts from pomegranate peel, pulp and juice were characterized by RP-LC × RP-LC-PDA/ESI-MS. A

microbore cyano column (1.0 mm I.D.) was chosen for first dimension (^{1}D) separations, and operated at suboptimal conditions, in order to reduce the ^{1}D flow thus achieving better sample focusing (Leme et al 2014; Cacciola et al 2018; Wong et al. 2018). Fast 60 s gradients were run on a short C18 superficially-porous particle column, at a flow rate of 0.8 mL min^{-1}, including a re-equilibration step, obtaining narrower bandwidths and fast ^{2}D separations. The developed RP-LC × RP-LC approach provided a good separation of the bioactive components in pomegranate extracts with higher resolving power than one-dimensional methods. However, the use of correlated separation mechanisms in the two dimensions may significantly decrease the attainable peak capacity as well as the orthogonality of the two-dimensional system. To overcome such a problem a shift gradient (SG) has been applied in the second dimension (^{2}D) (Figure S2) and performed through dedicated software (LC × LC Assist Software ver 2.0; Figure S2). For the presence in samples of interest of different polarity compounds and molecular weights, a mobile phase mixture of acidified water and acetonitrile were employed. The use of formic acid in the mobile phase allowed to obtain a reduction of peak tailing of polyphenolic compounds under RP conditions and an enhancement of the ionization in ESI-MS. The chromatographic blobs were preliminarily assigned to a chemical class according to their UV/vis spectra. PDA detectors provide important information for the identification purposes: e.g. ellagic acid derivates have UV/vis spectra with a strong λ_{max} usually below 270 nm and a second less intense absorbance maximum around 360–380 nm. On the other hand, anthocyanins have a typical λ_{max} between 515 and 540 nm, which make them easily distinguishable from the other chemical classes mentioned previously. Regarding MS detection, the experiments were carried out both in the positive and in the negative ionization mode. In general, the positive ion mode was used for anthocyanins, due to the fact that these compounds are present as flavylium ions at acidic conditions and produce molecular cations $[M+H]^+$ in their ESI mass spectra (Motilva et al. 2013). For polyphenolic acids, including vanillic acid, hydroxycinnamic acid derivatives and also hydrolyzable tannins, the negative ion mode was employed for their MS characterization, as described in the literature (Arapitsas 2012). As an example, Figure S1 shows the RP-LC × RP-LC plot of detected polyphenolic compounds in pomegranate juice. The composition of the bioactive molecules identified in the three pomegranate extracts is reported in Table 1. Overall a total of 37 polyphenolic compounds belonging to different classes were detected and identified using the complementary information coming from PDA, MS and literature data. Juice sample was the richest one as 35 compound were identified in it (Figure S3, Table 1).

3. Conclusions

The development of a new LC × LC-PDA-MS method for the analysis of pomegranate polyphenols in different parts of the fruit has been carried out in this study. The LC × LC technique, due to an increased peak capacity allowed to identify 37 compounds in pomegranate samples, which were grouped in the obtained 2D plots according to their chemical class. Chromatographic analyses of the juice, peel and pulp highlighted the presence of both phenolic acid derivates and anthocyanins.

Pomegranate samples investigated in this work, have showed interesting characteristics of composition, making them potential candidates for further studies and employment in the food industry.

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Conflict of interest

The authors declare no potential conflict of interest, including any financial interest.

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