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Application Note

No. 83

LC×LC: sample preparation and measurement

Polyphenolic Characterization of *Rhus coriaria L.* Extracts by Comprehensive Two-Dimensional Liquid Chromatography

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LC×LC

■ Abstract

Rhus coriaria L. (Anacardiaceae), commonly known as "sumac", has been used since ancient times for many different applications; nowadays it is used mostly as a spice obtained from its grinded fruits and employed for flavoring and garnishing food predominantly in the Mediterranean and the Middle East regions. Traditionally sumac has been also used in popular medicine for the treatment of many ailments including haemorrhoids, wound healing, diarrhea, ulcer, and eye inflammation. Its drupes do contain various classes of phytochemicals namely organic acids, flavonoids, tannins and others, responsible of their powerful antioxidant capacity.

In this report, a polyphenolic characterization of six different samples of *Rhus coriaria L.* was carried out, by using comprehensive two-dimensional liquid chromatography coupled to photodiode array and mass spectrometry detection.

A total of 83 polyphenolic compounds, mainly gallic acid derivatives were positively identified. The results achieved might support the utilization of this plant as an attractive target for novel nutraceutical approaches and for drug discovery.

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Introduction

Rhus coriaria L. (*R. coriaria*), commonly known as sumac, belongs to the Anacardiaceae family. According to "The Plant List" it is one of the 131 currently accepted species names of the very large and still under evaluation Rhus genus (The Plant List (2013). Version 1.1. published on the Internet http://www.theplantlist.org/) [1] to which are usually attributed more than 200 species by most authors [2-4]. It is native to the Mediterranean and the Middle East regions, where it is a fairly common species, sumac has a wide distribution range in temperate and subtropical regions, extending from the Canary Islands, Azores and Madeira in the west to Tadzhikistan and Afghanistan in the east [5]. Since ancient times distinct parts of the plant have found several applications with significant technological value, tannins extracted from young stems as well as from leaves were utilized for tanning hides during leather preparation and in the past centuries the most extensive plantations have been indeed established for this purpose. Also, bark and fruits preparations have been extensively used in popular medicine to obtain natural remedies against different affections such as eye and urinary tract infections, ulcer, diarrhea and hepatic disorders [4, 6, 7]. Recently *R. coriaria* has also gained some interest for its ornamental features that could be of value in urban landscaping and gardening [8].

Sumac extracts have been characterized in terms of phytochemical composition: one of the earliest works was carried out in 1896 highlighting the presence of gallic acid and myricetin as a component of the leave extract [9]. Afterwards, many other components were identified in different parts of the plant [7]; recently, more than 211 phytoconstituents including isoflavonoids, tannins, terpenoids, anthocyanins and others have been determined [10].

In this report, the polyphenolic content of six samples of sumac was carried out by using comprehensive two-dimensional liquid chromatography (LC × LC): samples 1 to 4 were obtained from fruits harvested in Sicily in different seasons and subjected to specific treatments; samples 5 and 6 are commercially available processed spices.

Experimental

Samples

A total of six sumac samples were analyzed. Samples 1 to 4 were collected in the territory of Licodia Eubea Municipality (37°09'N, 14°42'E), Sicily region (Italy), at an altitude of about 600 m above sea level from wild plants growing on soils belonging to the association 'Regosols on sandy and conglomeratic rocks; the climate of this area, according to the Koppen and Geiger classification [11], is defined as 'Csa, Hot-summer Mediterranean Climate' with an average annual rainfall of 575 mm and an average annual temperature of 16.1 °C. Sample 1 consists of drupes harvested fresh in July, the most appropriate period as far as the ripening stage is concerned; Sample 2 were harvested at the same time but subsequently dried in a vacuum stove at the temperature of 40 °C. Sample 3 and 4 were collected in October (overripe stage), with the difference that also in this case Sample 4 was subjected to the same drying process previously reported.

Sample 5 and 6 were purchased as fruit dry powders on the internet (sumac spice), Sample 5 coming from the Mediterranean area without NaCl addition and Sample 6 from Iran and with the addition of NaCl as a preservative.

Sample preparation

For the extraction method optimization, different sample weights, different solvents type and volumes, pure and in mixture were tested for the polyphenol extraction. The highest yield was obtained weighting 20 g of grinded sample (fresh or dried) in 100 mL of water as solvent and using an extraction temperature of 40 °C for 1 hour. In order to produce dry extract for HPLC analysis, liquid extracts were lyophilized. The aqueous samples were frozen at -80 °C for 1 h. Drying was carried out in freeze dryer LyoQuest-55 (Telstar, Spain) at -50 °C and pressure of 0.011 mbar for 72 h. The yield of polyphenols was 13 % w/w.

Standard and Reagents

LC-MS-grade water, methanol, acetonitrile, and acetic acid were obtained from Merck Life Science (Merck KGaA, Darmstadt, Germany). Gallic acid, protocatechuic acid, isoquercetin, myricetin and cyanidin were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). Stock solutions of 1000 mg L^{-1} were prepared for each standard by dissolving 10 mg in 10 mL of methanol.

Instrumentation (Shimadzu)

LC × LC analyses were performed on a Shimadzu LC × LC instrument (Kyoto, Japan), consisting of a CBM-20A controller, one LC-Mikros binary pump for the first dimension, one LC-40BX3 dual-plunger parallel-flow pumps for the second dimension, one LC-30AD as make-up pump, a CTO-40C column oven, a SIL-40CX3 autosampler, an SPD-M40 photo diode array (PDA) detector (1.0 µL detector flow cell volume). In order to connect the two dimensions, two high speed/high pressure two-position, six-ports switching valves with micro-electric actuator (model FCV-32 AH, 1.034 bar; Shimadzu, Kyoto, Japan), equipped with two C18 guard columns (5 × 4.6 mm *I.D.*, 5 µm *dp*) were employed. A third LC pump was connected through a t-piece between the outlet of the ^{1}D and the switching valve. The LC × LC instrument was hyphenated to an LCMS-8050 mass spectrometer, through an ESI source (Shimadzu, Kyoto, Japan).

Separations were carried out on a ^{1}D HILIC column (150 × 1.0 mm *I.D.*, 3.5 µm *dp*) and a ^{2}D Core-Shell C18 column (50 × 4.6 mm *I.D.*, 2.7 µm *dp*).

Two identical C18 guard columns (5 × 4.6 mm *I.D.*, 5 µm *dp*) were used to collect and transfer the fractions from the ^{1}D into the ^{2}D.

^{1}D mobile phases: (A) 0.1 % formic acid in ACN, (B) 0.1 % formic acid in water (pH 3). Gradient: 0 min, 30 % B; 40 min, 60 % B; 50 min, 100 % B; 60 min, 100 % B; 61 min, 30 % B. Flow rate: 10 µL min^{-1}. Column oven: 30 ˚C. Injection volume: 20 µL. ^{2}D mobile phases: employed were (A) 0.1 % formic acid in water (pH 3), (B) 0.1 % formic acid in ACN. Segmented-in-fraction conditions: (^{1}D 0-12 min), 0.01 min, 10 %B; 0.89 min, 40 %B; 0.90 min, 10 %B; (^{1}D 12-17 min) 0.01 min, 0 %B; 0.89 min, 40 %B; 0.90 min, 0 %B; (^{1}D 17-51 min) 0.01 min, 0 %B; 0.89 min, 25 %B; 0.90 min, 0 %B; Flow rate: 3 mL min^{-1}. Modulation time: 1.00 min. Column oven: 30 ˚C. PDA conditions were in the range from 200 to 550 nm. Sampling rate was set to 40 Hz whereas the time constant was acquired at 0.08 sec.

ESI-MS conditions: mass spectral range: *m/z* 100-2000; event time: 1 sec; nebulizing gas (N_2) flow: 3 L min^{-1}; drying gas (N_2) flow: 10 L min^{-1}; heating gas flow (air): 10 L min^{-1}; heat block temperature: 400 ˚C; desolvation line (DL) temperature: 250 ˚C; interface temperature: 300 ˚C; interface voltage 3.50 kV; detector voltage: 1.80 kV.

The LC × LC-LCMS-8050 system and the switching valves were controlled by the Shimadzu LabSolutions software (ver. 5.93). The LC × LC data were visualized and elaborated into two and three dimensions using Chromsquare ver. 2.3 software (Shimadzu, Kyoto, Japan).

Samples were diluted 1:4 with 0.1% formic acid in MeOH:ACN solution (70:30 v/v) prior to LC × LC-PDA/ESI-MS analysis.

For the quantitative analysis of polyphenolic compounds, gallic acid, protocatechuic acid, isoquercetin, myricetin and cyanidin were employed. Standard calibration curves were prepared in a concentration range 10-500 mg L^{-1} with seven different concentration levels, run in triplicate.

Results and discussion

The polyphenolic fraction of *R. coriaria* fruits has been so far carried out by HPLC coupled with photodiode array (PDA) and/or MS detection [10),12),13)]. A comprehensive work on the phytochemical components of sumac fruit epicarp from Palestine by using HPLC-PDA-ESI-MS was reported by Abu-Reidah et al.[10)] where 211 phenolic and other phyto-constituents were described. However, in none of these works a quantification of the in-dividual polyphenolic content was reported due to the presence of overlapping peaks and matrix interferences. In this work the analysis of the polyphenolic compounds in *R. coriaria* samples was carried out by HILIC × RP-LC-PDA-ESI/MS. Prior to HILIC × RP-LC analysis, an optimization of the single separations must be carried out [14)-18)]. Normally a low mobile phase flow rate is used in the ^{1}D separation to decrease the fraction volume onto the ^{2}D and increase the ^{1}D sampling rate; as a consequence, a microcolumn is used in the ^{1}D. In this work, an easy-to-use micropump with a completely new direct-drive engineering was employed and was capable of delivering stable micro- to semi-micro flow rates [19)]. Notably when HILIC is hyphenated to RP, such coupling is not straightforward due to solvent incompatibility. To overcome such an issue a modulation procedure called "active modulation" was reported [20), 21)]. Such an approach is based on the introduction of a make-up flow of a weaker solvent (water) after the ^{1}D separation and before the entrance to the trapping column. In such a way a reduction in the solvent strength is achieved, increasing the retention of the trap columns towards the compounds separated in the ^{1}D.

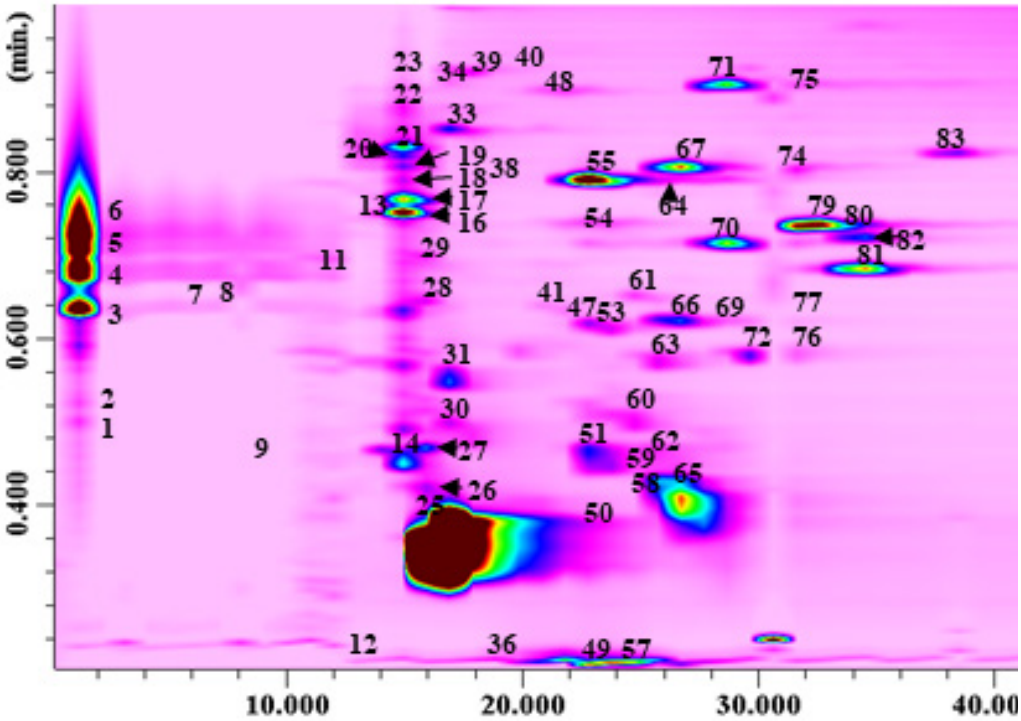

Fig. 1 HILIC × RP-LC-PDA contour plots (280 nm) of the polyphenolic profile for sample 4 (fresh and air-dried, collected in October).

Afterwards, when the valve is actuated, the retained analytes are eluted in narrow bands thanks to the ^{2}D mobile phase. Fig. 1 reports the HILIC × RP-LC-PDA-ESI/MS plots of the polyphenolic fraction of *R. coriaria* for sample 4. For MS detection a triple quadruple MS analyzer was used equipped with an electrospray interface working on both positive and negative ionization mode. The list of the compounds identified is reported in Table 1.

A total of 83 polyphenolic compounds were positively identified in the investigated samples by combining the information coming from PDA absorption (λ_{max}), mass-to-charge ratio (*m/z*) and literature data [10)-13)]. Among them, the majority were represented by gallic acid and derivates (37) and quercetin derivates (11). The rest was represented by cyanidin, luteolin, myricetin and apigenin derivates. Concerning the performance of the developed HILIC × RP-LC system, Table 1 reports the values attained for both peak capacity and orthogonality [22)].

The highest theoretical peak capacity values, resulting from the product of the peak capacity, n_c of the two single dimensions [23)], were attained for Sample 4 (3381), whereas the lowest one was attained Sample 5 (2673). The orthogonality, A_O values ranged from 0.72 to 0.90 % for Sample 6 and Sample 3, respectively. With regards to corrected peak capacity ^{2}D n_{corr} values, incorporating undersampling [24)] and A_O values [22)], the highest values were obtained for Samples 4 (1161) and 3 (1004), respectively.

In terms of quantification, a semi-quantification approach was applied, taking into account the chemical classes of the identified compounds (Fig. 2). Samples 1, 4 and 3 were the richest ones as bioactive content, accounting for roughly 2608.28 mg/100 g FW, 2489.56 mg/100 g FW and 2367.25 mg/100 g FW respectively; on the other hand, the poorest ones were represented by Sample 5 and 6, relative to commercial ones (253.28 mg/100 g FW and 338.86 mg/100 g FW). Notably, gallic acid derivatives are the most abundant ones in all samples investigated, ranging from 219.92 mg/100 g FW to 2317.46 mg/100 g FW.

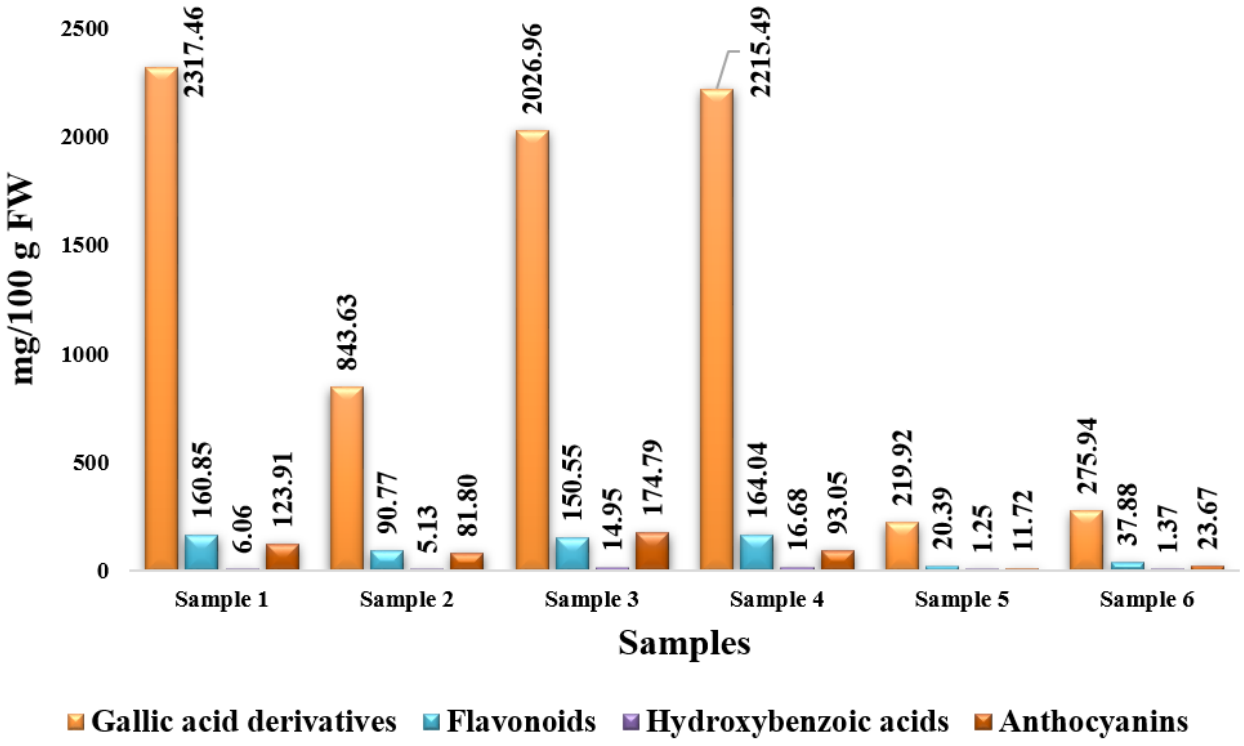

Fig. 2 Quantitative content of the six *R. coriaria* samples investigated.

Table 1 Identification of the polyphenolic compounds in *R. coriaria* extracts by using HILIC × RP-LC-PDA/MS in positive and negative ionization mode.

N.	Compound	Chemical family	Tt_r (min) RSD (%) (n=6)	$[M-H]^-$/ $[M+H]^+$	λ_{max} (nm)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
1	Tetragalloyl-hexoside	Gallic acid derivate	1.515 (0.57)	787/-	277	x	x	x	x	x	-
2	Pentagalloyl-hexoside	Gallic acid derivate	1.61 (0.47)	939/-	277	x	x	x	x	x	-
3	Hexagalloyl-hexoside	Gallic acid derivate	1.64 (0.66)	1091/-	278	x	x	x	x	x	x
4	Heptagalloyl-hexoside	Gallic acid derivate	1.70 (0.75)	1243/-	276	x	x	x	x	x	x
5	Octagalloyl-hexoside	Gallic acid derivate	1.73 (0.73)	1395/-	276	x	x	x	x	x	x
6	Nonagalloyl-hexoside	Gallic acid derivate	1.78 (0.60)	1547/-	275	x	x	x	x	x	x
7	Galloyl-valoneic acid bilactone I	Gallic acid derivate	5.64 (0.04)	621/-	279	-	-	x	x	-	-
8	Galloyl-valoneic acid bilactone II	Gallic acid derivate	7.64 (0.06)	621/-	278	-	-	x	x	-	-
9	Chrysoriol	Luteolin derivate	11.42 (0.11)	-/301	277	x	x	x	x	x	x
10	Quercetin rhamnoside I	Quercetin derivate	11.65 (0.07)	447/449	254, 352	x	x	-	-	-	-
11	Quercetin rhamnoside II	Quercetin derivate	12.50 (0.17)	447/449	254, 352	x	x	x	x	-	x
12	Malic acid	Malic acid derivate	13.19 (0.07)	133/-	237	x	x	x	x	x	x
13	Levoglucosan gallate	Gallic acid derivate	13.75 (0.06)	313/315	286	x	x	x	-	-	-
14	Protocatechuic acid hexoside	Protocatechuic acid derivate	15.47 (0.11)	315/-	258	x	x	x	x	x	x
15	Rutin	Quercetin derivate	15.75	609/-	266, 353	x	-	-	-	-	-
16	Quercetin hexoside I	Quercetin derivate	15.76 (0.09)	463/465	259, 350	x	x	x	x	x	x
17	Quercetin hexoside II	Quercetin derivate	15.77 (0.11)	463/465	259, 350	x	x	x	x	x	x
18	Quercetin hexoside III	Quercetin derivate	15.80 (0.10)	463/465	259, 350	-	x	x	x	-	-
19	Methyl digallate I	Gallic acid derivate	15.82 (0.03)	335/-	265	x	x	x	x	-	x
20	Methyl digallate II	Gallic acid derivate	15.83 (0.02)	335/-	265	-	-	x	x	-	-
21	Quercetin rhamnoside III	Quercetin derivate	15.84 (0.11)	447/449	259, 350	x	x	x	x	x	x
22	Apiin	Quercetin derivate	15.88 (0.05)	563/-	267, 332	x	x	x	x	-	-
23	Quercetin hexoside IV	Quercetin derivate	15.91 (0.09)	463/465	259, 350	x	x	x	x	-	-
24	Quercetin	Quercetin derivate	15.92 (0.06)	301/303	259, 350	x	x	-	-	-	x
25	Gallic acid	Gallic acid derivate	16.37 (0.08)	169/-	277	x	x	x	x	x	x
26	Galloyl shikimic acid I	Gallic acid derivate	16.42 (0.07)	325/-	276	x	x	x	x	-	-
27	Gallic acid O-malic acid I	Gallic acid derivate	16.48 (0.08)	285/-	276	x	x	x	x	x	x
28	Peonidin O-glucoside I	Cyanidin derivate	16.65 (0.01)	-/463	282, 515	x	x	x	x	x	-
29	Myricetin	Quercetin derivate	16.69 (0.09)	-/319	260, 359	x	x	x	x	x	x
30	Galloylshikimic acid II	Gallic acid derivate	17.52 (0.15)	325/-	273	x	-	x	x	x	-

Table 1 (continued).

N.	Compound	Chemical family	Tt_r (min) RSD (%) (n=6)	$[M-H]^-$/ $[M+H]^+$	λ_{max} (nm)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
31	Gallic acid O-malic acid II	Gallic acid derivate	17.56 (0.13)	285/-	276	x	x	x	x	x	x
32	Apigenin glucoside	Apigenin derivate	17.80	-/433	265, 344	-	-	-	-	x	-
33	Peonidin O-glucoside II	Cyanidin derivate	17.85 (0.02)	-/463	280, 515	x	x	x	x	-	x
34	Myricetin O-rhamnosylglucose	Quercetin derivate	17.92 (0.11)	-/625	262, 357	-	x	x	x	-	x
35	Myricetin O-glucuronide I	Quercetin derivate	17.97 (0.05)	493/495	262, 355	x	x	-	-	-	-
36	Quinic acid	Quinic acid derivate	18.20 (0.09)	191/-	237	x	x	x	x	x	-
37	Galloylshikimic acid III	Gallic acid derivate	18.47	325/-	274	-	-	x	-	-	-
38	Peonidin O-pentoside	Cyanidin derivate	18.81 (0.24)	-/433	273, 503	x	x	x	x	-	-
39	Myricetin O-glucuronide II	Quercetin derivate	18.93 (0.06)	493/495	261, 355	x	x	x	x	-	x
40	Quercetin rhamnoside IV	Quercetin derivate	19.94 (0.02)	447/449	262, 354	x	x	-	-	-	-
41	Di-galloyl hexoside I	Gallic acid derivate	21.70 (0.05)	483/-	275	x	x	x	x	x	x
42	Cyanidin O-hexoside I	Cyanidin derivate	21.73	-/449	279, 517	-	-	x	-	-	-
43	O-Methyl cyanidinO(2''galloyl)-galactoside	Cyanidin derivate	21.89	-/615	278, 518	-	-	x	-	-	-
44	Galloyl hexoside I	Gallic acid derivate	22.20 (0.11)	331/-	275	x	-	x	-	-	-
45	Cyanidin O-hexoside II	Cyanidin derivate	22.22	-/449	274, 516	-	-	x	-	-	-
46	Di-galloyl hexoside II	Gallic acid derivate	22.59	483/-	276	x	-	-	-	-	-
47	Di-galloyl hexoside III	Gallic acid derivate	22.70 (0.08)	483/-	276	x	x	x	x	x	x
48	O-Methyl-cyanidin O(2''galloyl)-galactoside II	Cyanidin derivate	22.90 (0.06)	-/615	278, 516	x	x	x	x	-	x
49	Galloylpyrogallol	Gallic acid derivate	23.20 (0.11)	277/-	238	x	x	-	-	-	x
50	Galloyl hexoside II	Gallic acid derivate	23.37 (0.02)	331/-	275	x	-	x	x	-	-
51	O-galloylnorbergenin I	Gallic acid derivate	23.48 (0.11)	-/467	276	x	-	x	x	-	-
52	Digalloyl hexoside malic acid I	Gallic acid derivate	23.58	599/-	276	-	x	-	-	-	-
53	Di-galloyl hexoside IV	Gallic acid derivate	23.63 (0.15)	483/-	276	x	-	x	x	x	-
54	Cyanidin O-hexoside III	Cyanidin derivate	23.74 (0.02)	-/449	279, 518	-	x	x	x	-	-
55	Tri-galloyl-hexoside I	Gallic acid derivate	23.80 (0.14)	635/-	276	x	x	x	x	-	-
56	O-Methyl-cyanidin O(2''galloyl)-galactoside III	Cyanidin derivate	23.89	-/615	278, 516	-	-	x	-	-	-
57	Galloyl hexoside III	Gallic acid derivate	24.21 (0.01)	331/-	275	x	-	x	x	x	-
58	Di-galloyl hexoside V	Gallic acid derivate	24.30 (0.01)	483/-	274	-	-	x	x	x	-

Table 1 (continued).

N.	Compound	Chemical family	Tt_r (min) RSD (%) (n=6)	$[M-H]^-$/ $[M+H]^+$	λ_{max} (nm)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
59	O-galloylnorbergenin II	Gallic acid derivate	25.44 (0.24)	-/467	277	x	-	-	x	-	-
60	Digalloyl hexoside malic acid II	Gallic acid derivate	25.52 (0.13)	599/-	277	x	x	x	x	-	-
61	Trigalloyllevoglucosan I	Gallic acid derivate	25.67 (0.10)	-/619	278	x	x	x	x	-	x
62	Digalloyl hexoside malic acid III	Gallic acid derivate	26.48 (0.12)	599/-	277	x	x	x	x	-	-
63	Digalloyl hexoside VI	Gallic acid derivate	26.60 (0.16)	483/-	274	x	x	x	x	x	x
64	Tri-galloyl-hexoside II	Gallic acid derivate	26.80 (0.08)	635/-	276	x	x	x	x	-	x
65	O-galloylnorbergenin III	Gallic acid derivate	27.43 (0.11)	-/467	277	x	x	x	x	-	x
66	O-galloylnorbergenin IV	Gallic acid derivate	27.65 (0.13)	-/467	277	x	-	x	x	-	x
67	Tri-galloyl-hexoside III	Gallic acid derivate	27.83 (0.11)	635/-	276	x	x	x	x	-	x
68	Di-O-galloyl-hexahydroxydiphenoyl-scyllo-quercitol I	Gallic acid derivate	27.95 (0.10)	-/771	278	x	-	-	-	-	x
69	Digalloyl hexoside VII	Gallic acid derivate	28.62 (0.13)	483/-	275	x	x	x	x	-	-
70	Tri-galloyl-hexoside IV	Gallic acid derivate	29.73 (0.14)	635/-	276	x	-	x	x	-	-
71	Di-O-galloyl-hexahydroxydiphenoyl-scyllo-quercitol II	Gallic acid derivate	29.91 (0.04)	-/771	278	-	x	x	x	-	-
72	O-galloylnorbergenin V	Gallic acid derivate	30.62 (0.12)	-/467	275	x	x	x	x	x	x
73	Tri-galloyl-hexoside V	Gallic acid derivate	31.80 (0.02)	635/-	276	x	-	-	-	-	-
74	Cyanidin O-(2"-galloyl) galactoside	Cyanidin derivate	31.85 (0.05)	-/601	279, 517	x	-	x	x	-	-
75	Tetra-O-galloylhexoside	Gallic acid derivate	31.89 (0.01)	787/-	277	-	-	x	x	-	-
76	O-galloylnorbergenin VI	Gallic acid derivate	32.58 (0.06)	-/467	276	-	-	x	x	-	-
77	Trigalloyllevoglucosan II	Gallic acid derivate	32.63 (0.05)	-/619	276	-	-	x	x	-	-
78	Tri-galloyl-hexoside VI	Gallic acid derivate	32.75 (0.03)	635/-	276	x	x	x	-	-	x
79	Trigalloyllevoglucosan III	Gallic acid derivate	33.73 (0.04)	-/619	276	-	-	x	x	-	-
80	Trigalloyllevoglucosan IV	Gallic acid derivate	34.74 (0.13)	-/619	276	x	-	x	-	-	-
81	Tri-galloyl-hexoside VII	Gallic acid derivate	35.68 (0.08)	635/-	276	x	x	x	x	x	x
82	Tri-galloyl-hexoside VIII	Gallic acid derivate	35.72 (0.05)	635/-	276	-	-	x	x	-	-
83	Di-O-galloyl-hexahydroxydiphenoyl-scyllo-quercitol III	Gallic acid derivate	38.83 (0.09)	-/771	278	x	x	x	x	-	-

Conclusions

In this report, the polyphenolic profile of six different fruit extracts of *R. coriaria* are reported. A total of 83 polyphenolic compounds were positively identified in the investigated samples and among them, the majority were represented by gallic acid and derivates (37). The obtained results highlight the importance of *R. coriaria* as a promising source of functional ingredients and boost its potential use in the food, nutraceutical as well as pharmaceutical industries.

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