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Application Note

No. 85

LC×LC: sample preparation and measurement

Unravelling the polyphenolic content of berry juices using focusing-modulated comprehensive two-dimensional liquid chromatography

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LC×LC

■ Abstract

In this work a comprehensive two-dimensional liquid chromatography system, comprised of a HILIC and a C18 columns in the first and second dimension, respectively, was tuned and employed to attain the profiles of polyphenolic compounds occurring in seven commercial berry juices. A total of 104 polyphenolic compounds belonging to different chemical classes (hydroxybenzoic and cinnamic acids derivatives, flavone glycosides, flavonols, flavonol glycosides, dihydroflavonols and anthocyanin glycosides) have been characterized and quantified in the juices investigated. Despite the phenolic constituents were similar, the contents varied considerably among the analyzed berry species.

Elderberry contained the highest amount of polyphenols (917.79 mg 100 mL^{-1}), followed by chokeberry (515.73 mg 100 mL^{-1}). On the other hand, raspberry contained the lowest amount 103.56 (515.73 mg 100 mL^{-1}). The developed HILIC × RP-LC method showed a remarkable separation capability, being characterized by high values of corrected peak capacity (up to 1372) and orthogonality (A.o. up to 0.8). Such a HILIC × C18 platform based on focusing-modulation, never employed so far for berry juices, provided a great applicability to be advantageously employed for other complex food samples.

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Introduction

The ever-increasing interest in the elucidation of polyphenolic antioxidants in human health has pushed the scientific research into the determination of active polyphenolic content in a great variety of edible fruits. Among them, berries are recognized as one of the richest sources of antioxidant phytochemicals [1]. In common usage, the term berry generically refers to any small fruit usually juicy, round or semi-oblong, with a bright color and either a sweet or sour taste. Berries do have a number of different functions e.g., free-radical scavengers, peroxide decomposers and synergists. Berries are also widely recognized for beneficial health-promoting properties e.g., anti-carcinogenic, anti-inflammatory, anti-bacterial and anti-diabetics [2]. Further, beneficial aspects have been proved for prevention of neurodegenerative diseases such as Alzheimer, Parkinson, prion and motor neuron diseases, as well as specific effects in the decrease of blood pressure and improvement of plasma lipid profile and endothelial function [3]. Such functions have been ascribed to polyphenolic compounds, especially flavonols (quercetin, myricetin, kaempferol as well as their glycosides) occurring in berry fruits [4].

The polyphenolic content of such fruits can vary extensively depending on the different berry cultivars analyzed, growth conditions and methodological procedures [5].

Small berries e.g., bilberry, blackcurrant, elderberry, raspberry, blueberry, chokeberry and honeyberry are widely grown across the world and easily available in supermarkets.

Liquid chromatography coupled to mass spectrometry turned out to be the most valuable analytical tool for the analysis of polyphenolic compounds in berry fruits [6-8]. However, quantification data have been rarely reported and scattered information in literature can be found.

In the present work a novel comprehensive two-dimensional liquid chromatography system (HILIC × RP-LC) incorporating hydrophilic interaction (HILIC) and reversed phase (RP-LC) in the first (^{1}D) and the second dimension (^{2}D) respectively was tuned. The outcome of the present study can be of great aid for providing a thorough information on the polyphenolic content of berry juices, which could be used for quality assessment in the pharmaceutical and food industries, as well as for a better understanding of their potential health benefits.

Experimental

Chemicals

LC-MS grade water, methanol, acetonitrile (ACN) and acetic acid were attained from Merck Life Science (Merck KGaA, Darmstadt, Germany). Gallic acid, quercetin 3-*O*-glucoside, luteolin 7-*O*-glucoside, eriodictyol, eriodicyol 7-*O*-glucoside, quercetin, chlorogenic acid, taxifolin and cyanidin 3-*O*-glucoside chloride were obtained from Merck Life Science (Merck KGaA, Darmstadt, Germany). Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (Milan, Italy). Stock solutions of 1000 mg L^{-1} were prepared for each standard by dissolving 10 mg in 10 mL of blank matrix.

Samples

Bilberry, blackcurrant, blueberry, chokeberry, elderberry, honeyberry, raspberry juices were obtained for a commercial market. All of them were kept at -20 ˚C until their analysis. Thawing was performed at room temperature and passed through membrane filters (0.45 µm diameter) prior to injection.

Columns

Separations were conducted by using a ^{1}D HILIC column (150 × 1.0 mm I.D., 3.5 mm d.p.) and a ^{2}D Core-shell C18 column (50 × 4.6 mm I.D., 2.7 mm d.p). For peak focusing, two identical Core-shell C18 guard columns (0.5 cm × 4.6 mm I.D., 5 mm d.p.) were employed.

Instrumentation (Shimadzu)

HILIC × RP-LC analyses were performed on a Shimadzu Nexera 40 Series LC × LC (Kyoto, Japan), consisting of a CBM-20A controller, one LC-Mikros binary pump, one LC-40BX3 dual-plunger parallel-flow pump, 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 mL 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 trapping columns C18 were employed. The LC × LC instrument was hyphenated to an LCMS-8050 mass spectrometer, through an ESI source (Shimadzu, Kyoto, Japan).

^{1}D mobile phases: (A) 0.1 % formic acid in ACN, (B) 0.1 % formic acid in water (pH 3). Gradient: 0 min, 5 % B; 20 min, 5 % B; 25 min, 10 % B; 35 min, 10 % B; 70 min, 50 % B; 88 min, 80 % B. Flow rate: 10 mL 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: (1D 0-59 min), 0.01 min, 0 %B; 0.80, 50 %B; 0.81, 0 %B; (1D 60-88 min), 0.01 min, 0 %B; 0.80, 35 %B; 0.81, 0 %B. Flow rate: 3 mL min^{-1}. Modulation time: 1.00 min. Column oven: 30 ˚C. PDA conditions were in the range from 190 to 550 nm. Sampling rate was set to 40 Hz whereas the time constant was acquired at 0.025 sec.

ESI-MS conditions: mass spectral range: *m/z* 100-2000 (+/-); event time: 1.0 sec; nebulizing gas (N2) flow: 3 L min^{-1}; drying gas (N2) 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).

Validation of the quantitative method

Accurate quantitative analysis by using the HILIC × RP-LC system were developed and applied for the first time to the berry juices investigated in this work. Method performance was carried out by considering the validation of the main figures of merit reported by ANVISA [9], namely, linearity range, correlation coefficients (R^2), limit of detection (LOD), limit of quantification (LOQ), precision (intra and inter-day) and recovery.

Calibration curves were created after quintuplicate injection of six different concentration levels of a mixture of nine standards, namely gallic acid, quercetin 3-*O*-glucoside, luteolin 7-*O*-glucoside, eriodictyol, eriodicyol 7-*O*-glucoside, quercetin, chlorogenic acid, taxifolin and cyanidin 3-*O*-glucoside chloride as representative of the distinct chemical classes under evaluation. Concerning linearity, standard calibration curves for gallic acid, quercetin 3-*O*-glucoside, luteolin 7-*O*-glucoside, eriodictyol, eriodicyol 7-*O*-glucoside, quercetin and chlorogenic acid were prepared in a concentration range of 1-500 mg L^{-1}; taxifolin in a concentration range of 1-250 mg L^{-1} and cyanidin 3-*O*-glucoside chloride in a concentration range of 1-125 mg L^{-1}. LOD and LOQ values were obtained on the basis of the calibration curve parameters, with the standard deviation of the response at the lowest level of the intercept of the considered calibration curve divided by the average slope multiplied by a factor of 3.3 and 10, respectively. Precision was estimated by intra and inter-day precision; specifically, intra-day precision was expressed as the relative standard deviation (%RSD) of peak areas attained for a 50 mg L^{-1} standard solution injected five times the same day, whereas inter-day precision was determined by examining fifteen injections in the span of three consecutive days. With regards to accuracy, recovery values (%) evaluation was determined by a spiked recovery method, in which different concentrations of all standard mixtures, namely 10 ppm, 50 ppm and 125 ppm, were considered in quintuplicate analysis. Results were expressed in mg per 100 mL.

Results and discussion

An LC × LC method, based on the coupling of two independent separation systems with enhanced resolving power and peak capacity, was developed. Although different separation modes have been experienced so far, the one involving the hyphenation of HILIC and RP modes has attracted a particular attention in the last decade and sit was successfully exploited especially from Dr. Herrero and Prof. De Villiers' research groups for polyphenolic profiling of various food and natural products [10)-13)]. However, the application of such a coupling is not straightforward due to some factors which are necessary to consider and imply a proper optimization. Among all, an important issue is the solvent incompatibility; in fact, the weak solvent employed in the ^{1}D is a strong eluent solvent for the ^{2}D, thus leading to a mobile phase mismatch and poor focusing on the head of the ^{2}D column. For performing a proper method optimization, different conditions were tested independently. In the first instance, the performance of a ZIC-HILIC stationary phase, carrying zwitterionic functional groups (sulfobetaine) with a charge balance 1:1, was investigated. In terms of mobile phase composition, as organic modifiers, methanol and acetonitrile were investigated.

Comparing the results attained with the two solvent composition, acetonitrile-based mobile phases, the latter resulted in higher separation capability and with reduced backpressure values. Concerning the mobile phase additive, the use of 0.1 % formic acid at pH 3 provided the best sensitivity for all compounds in negative ionization mode. ^{1}D HILIC separations were run under suboptimal chromatographic conditions in order to decrease the amount of the eluate transferred to the ^{2}D RP-LC. The employment of a 1.0 mm I.D. in the ^{1}D, allowed to no compatibility issues arose from the mobile phases used in the two dimensions, thus achieving "peak focusing" effects on the top of the ^{2}D RP column, run with a gradient program starting with 100 % of the weaker solvent (water). The ^{2}D RP column was run with a fast gradient in order the get the highest of ^{2}D analyses per ^{1}D peak. A 4.6 mm I.D. C18 column was employed in the ^{2}D, run at 3 mL/min. Taking into consideration the different polarity of the polyphenolic compounds occurring in the samples, the HILIC × RP-LC analyses were investigated by using a segmented-in-fraction (SIF) approach. Notably, in the time frame from 0 to 59 min, the 2D %B raised up to 50 %, whereas from 60 until the end of the analysis, the 2D %B raised up to 35 %.

As an example, Fig. 1 shows the HILIC × RP-LC plots of one of the most complex berry juices samples, *viz*. elderberry.

As can be appreciated, the polyphenolic compounds were spread around the HILIC × RP-LC plots highlighting a satisfactory coverage of the separation space. Compounds eluted from the ^{1}D according to increasing polarity; on the contrary, compounds in ^{2}D eluted according to increasing hydrophobicity. In total one hundred four baseline separated polyphenolic compounds were positively detected and tentatively identified in all samples tested by combining the information obtained with PDA and MS detection and by comparison with literature data. When available, compound identification was supported by standard co-injection. Elderberry juice turned out to be the most complex being 46 different polyphenolic compounds positively identified, belonging to both phenolic acids and flavonoids (Table 1).

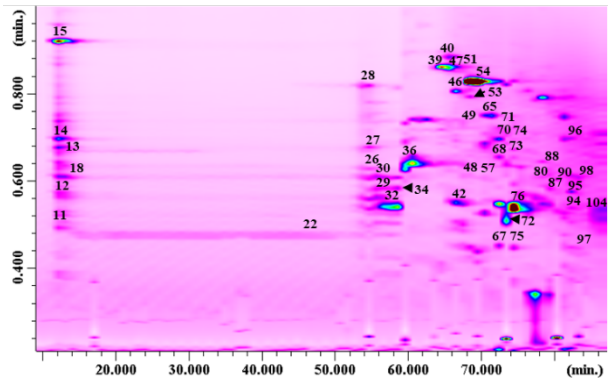

Fig. 1 HILIC × LC-PDA plots (l=280 nm) of the elderberry juice.

Table 1 List of tentatively identified compounds in the berry juices investigated.

N.	Compound	Trt	λmax	[M-H]-/ [M+H]+/[M]+	Fragment ion (m/z)	Sample	mg/100 mL
1	Vaccinoside or andromedoside	11.62	226, 312	535/-/-	581 [M-H+ HCOOH]-	BlB, BiB, RB,	n.q.
2	Dicaffeoyl tartaric acid	11.67	225, 310	-/475/-	-	HB, BlB, BC, RB	3.49; 3.39; 3.28; 3.46
3	Andromedoside or vaccinoside	11.69	228, 311	535/-/-	581 [M-H+ HCOOH]-	BlB	n.q.
4	Coumaroyl valeryl hexose	11.70	220, 270	409/-/-	-	CB	4.41
5	Coumaric acid-malonyl-hexoside	11.79	225, 311	411/-/-	163 [M-H- malonyl-hexoside group]	BiB	5.76
6	Quercetin-malonyl-hexoside	11.82	271, 310	549/-/-	505 [M-H- acetyl group]; 301 [M-H-malonyl-hexoside group]	HB, BiB	3.31; 5.21;
7	Myricetin-acetyl-hexoside 1	11.88	217, 268	521/-/-	317 [M-H-acetyl-hexoside group]	HB, BlB, BiB, RB	4.04; 5.32; 2.78; 2.19
8	Myricetin-acetyl-hexoside 2	11.92	217, 268	521/-/-	317 [M-H- acetyl-hexoside group]	HB, BiB,	2.81; 2.21
9	Acetyl eugenol	12.40	215, 294	205/-/-	-	HB, BlB, BlB, RB	n.q.
10	Kaempferol O-pentoside	12.43	217, 280	417/419/-	287 [M+H- pentosyl group]	HB, BlB, CB, BiB, RB	6.07; 4.35; 2.73; 14.06; 13.35
11	Luteolin O-hexoside	12.52	223, 300	447/-/-	285 [M-H- hexosyl group]	EB,BiB, BC,	0.81; 0.73; 0.69
12	Apigenin O-hexoside	12.57	212, 326	431/-/-	269 [M-H- hexosyl group]	EB, BlB, BiB, BC, RB	1.06; 1.22; 0.86; 0.74; 0.65
13	Laricitrin acetyl-hexoside	12.67	228, 312	535/-/-	330 [M-H- acetyl-hexoside group]	EB, HB, BlB, BiB, CB, BC, RB	1.26; 1.39; 0.98; 1.08; 1.35; 1.06; 0.93
14	Catechin O-hexoside	12.70	219, 276	-/453/-	291 [M+H- hexosyl group]	EB, HB, BlB, CB, BiB, BC, RB	15.34; 19.79; 8.60; 4.33; 13.23; 13.57; 3.03
15	Ludalbin	12.92	226, 284	307/309/-	-	EB, BC	n.q.
16	Eriodictyol	13.35	209, 265	287/-/-	-	CB, RB	< LOQ; < LOQ
17	Laricitrin O-pentoside	13.48	261, 293	463/-/-	331 [M-H- pentosyl group]	BlB, BiB,	0.75; 2.91
18	Caffeic acid	13.63	300, 325	179/-/-	134 [M-H- carboxyl group]	EB, BlB, BiB, BC, RB	4.28; 7.11; 3.31; 4.39; 3.58
19	Syringetin O-hexoside	13.73	301, 354	507/-/-	345 [M-H- hexosyl group]	BlB, CB, BiB, RB	5.06; 4.48; 2.45; 2.26
20	Coumaric acid malonyl hexoside	13.77	225, 311	411/-/-	163 [M-H- malonyl-hexoside group]	BiB	3.36
21	Quercetin	13.91	324, 370	301/303/-	-	HB, BlB, BiB, BC	2.68; 2.13; 2.74; 1.84
22	Caffeoylquinic acid (chlorogenic acid)	46.54	240, 325	353/355/-	707 [2M-H]-; 191 [M-H-caffeoyl group]-; 179 [M-H-quinic acid+ H2O]-;	EB, BlB, CB, BiB,	3.90, 4.87, 3.82, 3.76
23	Syringetin O-hexoside	54.73	300, 353	507/-/-	345 [M-H- hexosyl group]	BlB, BiB, RB	2.32; 2.41, 1.74
24	Naringenin O-hexoside	55.54	225, 285	433/435/-	271 [M-H- hexosyl group]	CB, BC, RB	0.89; 1.06; 0.82
25	Kaempferol rhamnosyl pentoside	55.59	223, 316	563/-/-	431 [M-H- pentosyl group]; 285 [M-H- rhamnosyl and pentosyl group]	CB, BiB, BC	3.35; 2.70; 2.39
26	Caffeoylquinic acid (chlorogenic acid)	55.63	242, 325	353/355/-	707 [2M-H]-; 191 [M-H-caffeoyl group]-; 179 [M-H-quinic acid+ H2O]-;	EB, HB, BlB, CB, BiB, BC	5.10; 3.46; 3.34; 3.36;3.28; 3.37
27	Hydroxybenzoyl benzoyl hexose	55.68	264, 312	403/-/-	137 [M-H-benzoyl hexoside group]	EB, CB, BiB, BC	4.85; 6.32; 4.85; 3.59
28	Myricetin	55.82	230, 373	317/319/-	-	EB, BC	4.83; 4.16
29	Caffeic acid hexoside	56.56	280, 310	341/-/-	179 [M-H- hexosyl group]	EB, BC	5.10; 3.88
30	Hydroxyferuloyl hexoside	56.61	282, 321	371/-/-	209 [M-H- hexosyl group]	EB, CB	6.02; 3.36
31	Dicaffeoylquinic acid	56.76	221, 329	515/-/-	353 [M-H- caffeoyl group]	HB, RB	12.47; 3.60
32	Caffeoylquinic acid (chlorogenic acid)	58.54	218, 325	353/355/-	707 [2M-H]-; 191 [M-H-caffeoyl group]-; 179 [M-H-quinic acid+ H2O]-;	EB, HB, BlB, CB, BiB, BC, RB	17.45; 81.86; 164.38; 117.08; 17.88; 6.02;3.51
33	Eriodictyol O-hexoside	58.69	223, 284	449/-/-	287 [M-H-hexosyl group]	CB	0.89
34	Caffeic acid hexoside	59.58	220, 310	341/-/-	179 [M-H- hexosyl group]	EB, HB, BlB, BC	4.60; 3.29; 4.65; 3.45
35	Cumaroyl-hexose hydroxyphenol	60.42	270	417/-/-	441 [M+Na]+; 307 [M-H-hydroxyphenol]; 145 [M-H-hydroxyphenol and hexosyl group]	BiB	3.56

Table 1 (continued).

N.	Compound	Trt	λmax	[M-H]-/ [M+H]+/[M]+	Fragment ion (m/z)	Sample	mg/100 mL
36	Caffeoylquinic acid	60.64	218, 325	353/355/-	707 [2M-H]-; 191 [M-H-caffeoyl group]-; 179 [M-H-quinic acid+ H2O]-;	EB, HB, BIB, CB, BiB, BC	17.60; 30.78; 77.23; 60.12; 6.10; 3.62
37	Caffeoyl hexoside	66.64	243, 325	341/-/-	179 [M-H- hexosyl group]	HB, BIB, CB, BiB, BC, RB	7.95; 18.59; 7.02; 5.68; 3.58; 3.32
38	Kaempferol O-rutinoside	66.83	264, 344	593/-/-	285 [M-H- rutinosyl group]	HB	2.57
39	Quercetin O-hexoside	66.86	256, 354	463/465/-	301 [M-H- hexosyl group]	EB, HB, BIB; CB; BiB; BC; RB	60.20; 23.70; 18.83; 40.90; 12.09; 9.16; 8.76
40	Isorhamnetin O-hexoside O-rhamnoside	66.88	264, 328	623/-/-	463 [M+H- hexosyl group]	EB, BIB,	13.46; 6.09
41	Quinic acid	67.50	-	191/-/-	-	BIB, RB	3.38; 3.58
42	Caffeoylquinic acid (chlorogenic acid)	67.55	239, 325	353/355/-	707 [2M-H]-; 191 [M-H-caffeoyl group]-; 179 [M-H-quinic acid+ H2O]-;	EB, HB, BIB, CB, BiB BC, RB	10.64; 12.30; 6.20; 128.83; 4.31; 3.48; 4.69
43	Caffeoyl hexoside	67.60		341/-/-	179 [M-H- hexosyl group]	BiB	3.51
44	Aromadendrin O-hexoside	67.65	286, 325	289/291/-	-	HB, RB	13.07; 4.51
45	Epicatechin	67.73	205, 278	289/291/-	313 [M+Na]+; 179 [M-H-dihydroxyphenyl group]	HB, CB, BiB, BC	2.94; 4.52; 3.54; 1.85
46	Dihydroquercetin (taxifolin)	67.80	268, 298	303/-/-	-	EB, HB, CB, BiB, RB	1.22; 0.59; 0.91; 0.53; 0.32
47	Quercetin O-hexoside	67.86	255, 355	463/465/-	301 [M-H- hexosyl group]	EB, HB	9.23; 3.82
48	Caffeoyl hexoside	68.61	226, 313	341/-/-	179 [M-H-hexosyl group]	EB, HB, CB, BiB, BC	3.53; 3.87; 3.38; 3.47; 3.21
49	Myricetin O-pentoside	68.73	290	449/-/-	317 [M-H-pentosyl group]	EB, BC, RB	5.70; 14.32; 2.10
50	Quercetin O-pentoside	68.80	255, 355	433/-/-	301 [M-H- pentosyl group]	RB	2.06
51	Quercetin O-glucuronide	68.86	258, 354	477/-/-	301 [M-H- glucuronyl group]	EB, BiB, RB	6.57; 9.66; 6.50
52	Caffeic acid hexoside	69.55	283, 320	341/-/-	179 [M-H- hexosyl group]	BIB, RB	3.50; 3.22
53	Myricetin O-glucoside	69.79	256, 354	479/-/-	317 [M-H- hexosyl group]	EB, HB, CB, BiB, BC, RB	7.65; 3.77; 2.64; 18.95; 18.71; 6.10
54	Quercetin O-glucosyl-xyloside	69.83	256, 355	595/-	463 [M-H-xylosyl group]; 301 [M-H- glucosyl ancd xylosyl group]	EB, HB, BIB, CB, BC	511.92; 68.15; 8.43; 28.12; 6.22
55	Quercetin O-dihexoside	70.75	256, 352	625/-	463 [M-H- hexosyl group]; 301 [M-H- 2(hexosyl group)]	CB, RB	8.25; 4.10
56	Quercetin O-hexoside	70.82	256, 354	463/465/-	301 [M-H- hexosyl group]	HB	14.13
57	Malonylglycitin	71.61	237, 285	509/-/-	-	EB, CB, BiB	0.76; 0.83; 1.27
58	Quercetin acetyl-hexoside	71.64	284, 331	507/-/-	301 [M-H- acetyl-hexoside group]	HB	3.32
59	Malvidin O-hexoside	71.69	278, 529	-/-/493	331 [M- hexosyl group]	BiB	0.80
60	Quercetin O-rutinoside	71.79	256, 355	609/-/-	301 [M-H- rutinosyl group]	HB, CB	17.80; 9.41
61	Myricetin O-rutinoside	72.63	288, 350	625/-/-	317 [M-H- rutinosyl group]	HB, BC	2.93; 2.39
62	Peonidin O-hexoside	72.66	279, 521	-/-/463	301 [M- hexosyl group]	BiB	0.80
63	Malvidin O-hexoside	72.68	278, 527	-/-/493	331 [M- hexosyl group]	BiB	0.55
64	Syringetin	72.69	266, 370	345/-/-	-	HB	5.47
65	Quercetin O-rhamnosyl-rhamnosyl-glucoside	72.75	256, 354	755/-/-	301 [M-H- glucosyl and 2(rhamnosyl) group]	EB	20.98
66	Myricetin O-rutinoside	72.79	260, 357	625/627/-	317 [M-H- rutinosyl group]	BC	5.98
67	Gallic acid O-galloylglucoside	73.45	230, 278	-/485/-	166	EB, BIB	4.75; 3.65
68	Dihydromyricetin O-rhamnoside	73.65	287, 321	465/-/-	319 [M-H- rhamnosyl group]	EB, HB, BiB,	5.08; 6.90; 2.40
69	Peonidin O-hexoside	73.66	284, 518	-/-/463	301 [M- hexosyl group]	HB, BiB	0.48; 1.42
70	Kaempferol O-rutinoside	73.69	271, 335	593/-/-	285 [M-H- rutinosyl group]	EB, CB, RB	10.39; 7.89; 2.01

Table 1 (continued).

N.	Compound	Trt	λmax	[M-H]-/ [M+H]+/[M]+	Fragment ion (m/z)	Sample	mg/100 mL
71	Myricetin O-rutinoside	73.72	260, 357	625/627/-	317 [M-H- rutinosyl group]	EB, BC	2.97; 15.28
72	Naringenin O-hexoside	74.51	221, 278	433/-/-	271 [M-H- hexosyl group]	EB, CB	2.92; 0.98
73	Taxifolin O-hexoside (Dihydroquercetin O-hexoside)	74.68	286, 320	465/-/-	-	EB, CB, BC	6.95; 16.41; 1.97
74	Quercetin O-glucosyl-rhamnosyl-glucoside	74.70	263, 344	771/-/-	609 [M-H- glucosyl group]; 301 [M-H- rhamnosyl and 2(glucosyl) group]	EB	3.31
75	Coumaroyl dihydroxybenzoyl hexose	75.45	234, 278	461/-/-	-	EB	4.48
76	Isoorientin	75.53	282, 330	-/449/-	-	EB, HB, CB	97.12; 8.70; 2.76
77	Cyanidin O-hexoside	75.60	279, 516	-/-/449	287 [M- hexosyl group]	HB	0.70
78	Quercetin O-rhamnoside	75.63	253, 353	447/449/-	301 [M-H- rhamnosyl group]	HB	13.49
79	Gallocatechin	77.52	280	305/-/-	-	BiB, BC	3.57; 3.55
80	Cyanidin O-pentoside	77.59	280, 517	-/-/419	287 [M- pentosyl group]	CB, BiB	1.04; 0.64
81	Cyanidin O-hexoside	77.60	279, 516	-/-/449	287 [M- hexosyl group]	HB	0.71
82	Cyanidin O-pentoside	76.63	279, 517	-/-/419	287 [M- pentosyl group]	EB, CB	1.22; 3.32
83	Luteolin derivate	77.67	237, 343	465/-	-	HB, BiB	1.21; 0.65
84	Quercetin O-glucoside	77.77	253, 353	463/-	301 [M-H- hexosyl group]	RB	1.76
85	Eriodictyol O- glucopyranoside	78.51	287, 320	449/-	287 [M-H- glucopyranosyl group]	HB, BiB	0.88; 0.89
86	Petunidin O-hexoside	78.64	277, 525	-/-/479	317 [M- hexosyl group]	BiB	0.84
87	Caffeoylquinic acid (chlorogenic acid)	80.58	253, 343	353/355	707 [2M-H]-; 191 [M-H- caffeoyl group]-; 179 [M-H- quinic acid+ H2O]-;	EB, BIB, RB	4.10; 3.51; 3.47;
88	Kaempferol O- rutinoside	80.61	272, 345	593/595	285 [M-H- rutinosyl group]	EB, BC	3.77; 1.92
89	Quercetin O-diglucoside	81.45	253, 353	625/-	301 [M-H- 2(hexosyl group)]	BiB, RB	4.43; 2.05
90	Cyanidin O-hexoside	81.60	279, 517	-/-/449	287 [M- hexosyl group]	EB, HB	1.13; 3.60;
91	Cyanidin coumaroyl hexoside	81.62	282, 520	-/-/595	287 [M- coumaroyl and hexosyl group]	RB	0.79
92	Cyanidin O-xyloside	81.60	280, 517	-/-/419	287 [M- pentosyl group]	HB, CB	0.66; 2.16
93	Cyanidin 3 rutinoside	81.63	278, 515	-/-/595	449 [M- rhamnosyl group]	BC	0.78
94	Quercetin O-hexoside	82.53	282, 327	-/465-/	303 [M+H- hexosyl group]	EB, BiB	5.00; 2.01
95	Cyanidin O-glucoside	82.59	277, 517	-/-/449	287 [M- hexosyl group]	EB, HB, CB, BiB, RB	1.70; 4.20; 2.77; 4.50; 0.50
96	Kaempferol O-pentoside	82.69	225, 344	417/-	285 [M-H- pentosyl group]	EB, CB	6.48; 3.70
97	Dihydromyricetin O-rhamnoside	83.45	274, 317	465/-	319 [M-H- rhamnosyl group]	EB, HB, BiB	4.22; 15.85; 4.39
98	Cyanidin 3-sambubioside	84.61	278, 517	-/-/581	287 [M- sambubiosyl group]	EB	5.13;
99	Cyanidin O-glucoside	84.59	278, 518	-/-/449	287 [M- hexosyl group]	HB, CB,BiB	8.42, 14.38, 2.27
100	Cyanidin O-pentoside	84.63	279, 517	-/-/419	287 [M- pentosyl group]	CB	1.60
101	Cyanidin O-diglucoside	85.59	278, 517	-/-/611	287 [M- 2(hexosyl group)]	RB	5.38
102	Cyanidin O-sophoroside	86.55	278, 527	-/-/611	449 [M- hexosyl group]	HB, BC	1.16; 0.73
103	Cyanidin O-hexoside	85.60	279, 516	-/-/449	287 [M- hexosyl group]	HB, CB	0.99; 8.15
104	Cyanidin 3-sambubioside 5-glucoside	87.54	278, 515	-/-/743	449 [M- hexosyl group]; 287 [M- hexosyl and sambubiosyl group]	EB	5.93

Further, aiming to evaluate the separation space coverage, the orthogonality degree was calculated Camenzuli & Schoenmakers [64]. Such a procedure, which takes into account the spread of each peak along the four imaginary lines crossing the HILIC × RP-LC space, highlighted quite satisfactory similar Ao values (Ao=0.70-0.80). Finally, considering both effective peak capacity and orthogonality values, the blackcurrent juice sample was the most efficient ($n_{c,corr}$=1372), followed by the raspberry ($n_{c,corr}$=13361).

After method optimization, a mixture of nine representative standards were shot in the HILIC × RP-LC system. Calibration curves for each standard were attained after a five-time injection of such standards at six different concentration levels (Table 3). Correlation coefficients (R^2) were higher than 0.993 for all the investigated compounds. LOD values ranged from 0.02 to 0.90 mg L^{-1}, whereas LOQ ones were lower than 1.0 mg L^{-1} with the exception of quercetin (2.71 mg L^{-1}). Instrumental intraday (n=5) and interday (n=15) precision were lower than 0.93 and 1.25, respectively with the exception of cyanidin 3-*O*-glucoside chloride where values as high as 4.34 and 5.86 were attained. In terms of accuracy, low (10 ppm), medium (50 ppm) and high (125 ppm) recovery values were determined. Most of the standards showed excellent accuracy values (recovery range 94 %-119 %), with a few exceptions e.g., except eriodictyol (84.51, 50 ppm), cyanidin 3-*O*-glucoside (86.37, 50 ppm), luteolin 7-*O*-glucoside (86.81, 50 ppm), chlorogenic acid (87.08, 10 ppm) and quercetin 3-*O*-glucoside (89.32, 125 ppm).

It is worth mentioning that such values were attained in the spiked samples, thus potential matrix effect-related issues were avoided. In terms of RSD% values for accuracy data, for all compounds values lower than 5 % were obtained.

On the basis the results achieved which highlighted a very good capability for quantitative purposes, the HILIC × RP-LC system was subsequently employed for determine the polyphenolic content of the seven berry juices investigated. Eldeberry juice was the sample with the highest content of polyphenolic compounds (917.79 mg 100 mL^{-1}), followed by chockeberry (515.73 mg 100 mL^{-1}), honeyberry (439.45 mg 100 mL^{-1}) and blueberry (405.98 mg 100 mL^{-1}). Notably, peak #54, viz. quercetin *O*-glucosyl-xyloside, turned out to be the most abundant ones in the elderberry sample (511.92 mg 100 mL^{-1}), whereas peak #32, viz. chlorogenic acid was the most abundant one in both honeyberry (81.86 mg 100 mL^{-1}) and blueberry (117.08 mg 100 mL^{-1}). Considering chemical classes, Fig. 2 shows their distribution (mg 100 mL^{-1}) in each sample analysed. In most cases, the flavonol glycosides class was the most representative one (elderberry, 796.33 mg 100 mL^{-1}, honeyberry 244.78 mg 100 mL^{-1}, bilberry, 102.54 mg 100 mL^{-1}, blackcurrant 91.91 mg 100 mL^{-1} and raspberry, 62.53 mg 100 mL^{-1}); on the other hand, in blueberry and chockeberry, the most abundant class was the hydroxycinnamic acids one with values as high as 300.13 mg 100 mL^{-1} and 331.37 mg 100 mL^{-1}, respectively.

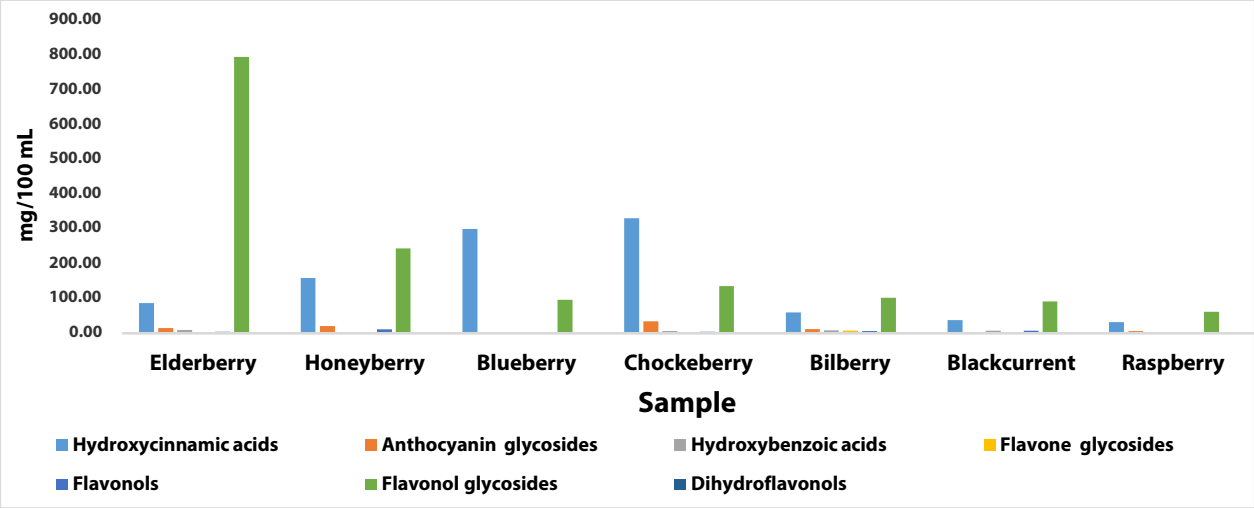

Fig. 2 Distribution of the chemical classes in each berry juice analysed by HILIC × RP-LC-PDA/ESI-MS.

Conclusions

In this work a HILIC × RP-LC-PDA-ESI-MS/MS approach for the detailed quali-quantitative profiling of the polyphenolic content of 7 commercial berry juices was developed. The method involved the combination of a HILIC column in the ^{1}D and a C18 in the ^{2}D, allowing to attain their polyphenolic profile in roughly 80 min. Excellent separation capability was achieved with values of practical peak capacity as high as 1375 and orthogonality of 0.80. 104 different polyphenolic compounds were detected and positively identified by using complementary information from PDA, MS/MS and literature data information.

The employment of a "focusing" modulation procedure with two C18 trapping columns allowed to mitigate the solvent mismatch thus providing an effective "peak focusing" at the head of the ^{2}D column. Among the berries analysed, elderberry showed the highest polyphenolic content (917.79 mg Kg^{-1}) and thus could be recommended as a primary natural source of bioactive compounds in food products. Also, the results achieved in this study could be useful for authenticity studies towards food industry. Finally, the in-depth knowledge of the polyphenolic profile in berry species might be advantageously used in clinical studies for a better estimation of potential health benefits of berries.

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First Edition: Apr. 2023

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