



# Determination of the polyphenolic content of berry juices using focusing-modulated comprehensive two-dimensional liquid chromatography coupled to mass spectrometry detection

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## Abstract

In this work, a comprehensive two-dimensional liquid chromatography system, comprised of a ZIC-HILIC and C18 columns in the first and second dimensions, respectively, was tuned and employed for attaining high resolution profiles of the polyphenolic pattern in seven commercial berry juices. The developed HILIC × RP-LC method was validated in terms of linearity range, correlation coefficients, limit of detection, limit of quantification, precision (intra- and inter-day), and recovery. A total of 104 polyphenolic compounds belonging to different chemical classes (hydroxybenzoic and cinnamic acid derivatives, flavone glycosides, flavonols, flavonol glycosides, dihydroflavonols, and anthocyanin glycosides) have been characterized and quantified in the juices investigated. Despite the constituents being similar, a notable quantitative variation among the analyzed berry species was observed. Elderberry contained the highest amount of polyphenols ( $918 \pm 1.10$  mg 100 mL<sup>-1</sup>), followed by chokeberry ( $516 \pm 0.08$  mg 100 mL<sup>-1</sup>). On the other hand, raspberry contained the lowest amount ( $104 \pm 1.21$  mg 100 mL<sup>-1</sup>). Further, total phenolic, flavonoid, and anthocyanin contents were determined spectrophotometrically, yielding consistent results. The free-radical scavenging activity (DPPH test) and reducing power of the juices, expressed as IC<sub>50</sub> (μL mL<sup>-1</sup>) and mg ASE mL<sup>-1</sup>, varied from  $2.79 \pm 0.03$  (honeyberry) to  $31.66 \pm 0.02$  (blueberry) and from  $1.71 \pm 0.01$  (blueberry) to  $8.89 \pm 0.12$  (chokeberry), respectively. Such a ZIC-HILIC × C18 platform based on focusing modulation, never employed so far for berry juices, showed a remarkable separation capability with high values of corrected peak capacity (up to 1372) and orthogonality ( $A_0$  up to 0.80), thus providing a great applicability to be advantageously employed for other complex food samples.

**Keywords** Berry juices · Polyphenols · Antioxidants · Comprehensive two-dimensional liquid chromatography · Active modulation · Principal component analysis

## 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

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are recognized as one of the richest sources of antioxidant phytochemicals [1]. From a botanical point of view, berry is a fruit deriving at maturity from the transformation of the entire ovary wall into the edible pericarp. According to this definition, some fruits traditionally considered berries fall into other classifications. Instead, in pomological nomenclature, 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 [2]. 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 [3]. Further beneficial aspects have been proved for prevention of neurodegenerative diseases such as Alzheimer, Parkinson, prion, and motor neuron, as well as specific effects in the decrease of blood pressure and improvement of plasma lipid profile and endothelial function [4]. Such functions have been ascribed to polyphenolic compounds, especially flavonols (quercetin, myricetin, kaempferol, and their glycosides) occurring in berry fruits [5].

Raspberries and blackberries are rich in cyanidin glycosides possessing high antioxidant activity whereas, on the other hand, strawberries do present higher content of pelargonidin-3-glucoside, being relatively weak antioxidants [3]. After having carried out a search in the literature, it can be highlighted that the polyphenolic content of such fruits can vary extensively depending on the different berry cultivars analyzed, growth conditions, and methodological procedures [6].

Small berries, e.g., bilberry, blackcurrant, elderberry, raspberry, blueberry, chokeberry, and honeyberry, are widely grown across the world and easily available on the market. Since some of them are often incorporated in traditional medicine, it is mandatory to evaluate their polyphenolic content. Polyphenolic compounds may be extracted by employing various organic solvents, e.g., water, ethanol, methanol, acetone, or their combinations, and evaluated by testing various in vitro spectrophotometric-based assays [7]. The polyphenolic content can be evaluated by testing various assays such as total phenolic content (TPC), total flavonoid content (TFC), and total anthocyanin content (TAC). As far as the antioxidant activity is concerned, different types of in vitro methods such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and ferric reducing-antioxidant power (FRAP) assays have been usually employed [8].

So far, liquid chromatography coupled to mass spectrometry was the most valuable analytical tool for the analysis of polyphenolic compounds in berry fruits [9–12]. However, simultaneous characterization, quantification, and determination of antioxidant potential have been rarely reported and fragmentary information can be found. In fact, most works

so far reported dealt on a single species [11, 12], on qualitative characterization [9], and quantification on selected flavonoids [10], e.g., flavonols [5].

In the present work a novel comprehensive two-dimensional liquid chromatography system (HILIC × RP-LC) incorporating hydrophilic interaction LC (HILIC) and reversed phase (RP-LC) in the first (<sup>1</sup>D) and the second dimension (<sup>2</sup>D) respectively was tuned to attain a complete polyphenolic profile of these matrices. LC × LC methods, based on the coupling of two independent separation systems with enhanced resolving power and peak capacity, have already been applied to the analysis of polyphenolic compounds in food and natural products as witnessed by several reviews devoted to this topic [13–19]. Notably, the platform investigated in this study was not straightforward due to solvent incompatibility; in fact, the weak solvent employed in the <sup>1</sup>D is a strong eluent solvent for the <sup>2</sup>D, thus leading to a mobile phase mismatch and poor focusing on the head of the <sup>2</sup>D column. To this regard, the addition of a dilution flow to decrease the strength of the <sup>1</sup>D effluent was exploited (active modulation).

In addition, total phenolic, flavonoid, and anthocyanin contents along with antioxidant activity were determined spectrophotometrically.

The outcome of the present study was to provide a thorough information on the polyphenolic content as well as antioxidant properties of berry juices coming from the market, 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.

## Materials and methods

### 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, eriodictyol 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<sup>-1</sup> were prepared for each standard by dissolving 10 mg in 10 mL of exhausted matrix.

### Samples

Bilberry (*Vaccinium myrtillus* L.), blackcurrant (*Ribes nigrum* L.), blueberry (*Vaccinium corymbosum* L.), chokeberry (*Aronia melanocarpa* (Michx.) Elliott), elderberry

(*Sambucus ebulus* L.), honeyberry (*Lonicera caerulea* L.), and raspberry (*Rubus idaeus* L.) juices were provided from the market. All of them were kept at  $-20\text{ }^{\circ}\text{C}$  until their analysis. Thawing was performed at room temperature and all samples were filtered through membrane filters ( $0.45\text{ }\mu\text{m}$  diameter) prior to chromatographic and spectrophotometric determinations.

## Columns

As  $^1\text{D}$ , a SEQuant ZIC-HILIC column ( $150\times 1.0\text{ mm}$  I.D.,  $3.5\text{ }\mu\text{m}$  dp) (Merck Life Science, Merck KGaA, Darmstadt, Germany) whereas as  $^2\text{D}$ , an Ascentis Express C18 column ( $50\times 4.6\text{ mm}$  I.D.,  $2.7\text{ }\mu\text{m}$  dp) (Merck Life Science, Merck KGaA, Darmstadt, Germany) were employed. For peak focusing, two identical Ascentis Express C18 guard columns ( $5\times 4.6\text{ mm}$  I.D.,  $5\text{ }\mu\text{m}$  dp) (Merck Life Science, Merck KGaA, Darmstadt, Germany) were used.

## Instrumentation and software

HILIC $\times$ RP-LC analyses were performed on a Shimadzu Nexera 40 Series LC $\times$ 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, and an SPD-M40 photo diode array (PDA) detector ( $1.0\text{ }\mu\text{L}$  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 C18 trapping columns, were employed. The LC $\times$ LC instrument was hyphenated to an LCMS-8050 triple quadrupole mass spectrometer, through an ESI source (Shimadzu, Kyoto, Japan). The whole set-up is illustrated in Fig. 1.

## Analytical conditions

$^1\text{D}$  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:  $10\text{ }\mu\text{L min}^{-1}$ . Column oven:  $30\text{ }^{\circ}\text{C}$ . Injection volume:  $20\text{ }\mu\text{L}$ .

$^2\text{D}$  mobile phases: employed were (A) 0.1% formic acid in water (pH 3), (B) 0.1% formic acid in ACN. Two different gradients were employed for  $^2\text{D}$  separations (segmented-in-fraction). The first one, covering the  $^1\text{D}$  analysis time 0–59 min: 0.01 min, 0%B; 0.80, 50%B; 0.81, 0%B; the second one, covering the  $^1\text{D}$  analysis time 60–88 min: 0.01 min, 0%B; 0.80, 35%B; 0.81, 0%B. Flow rate:  $3\text{ mL min}^{-1}$ . Modulation time: 1.00 min. Column oven:  $30\text{ }^{\circ}\text{C}$ . PDA parameters were performed in the wavelength range from 190 to 550 nm. Sampling rate was set to 40 Hz whereas the time constant was acquired at 0.025 s.

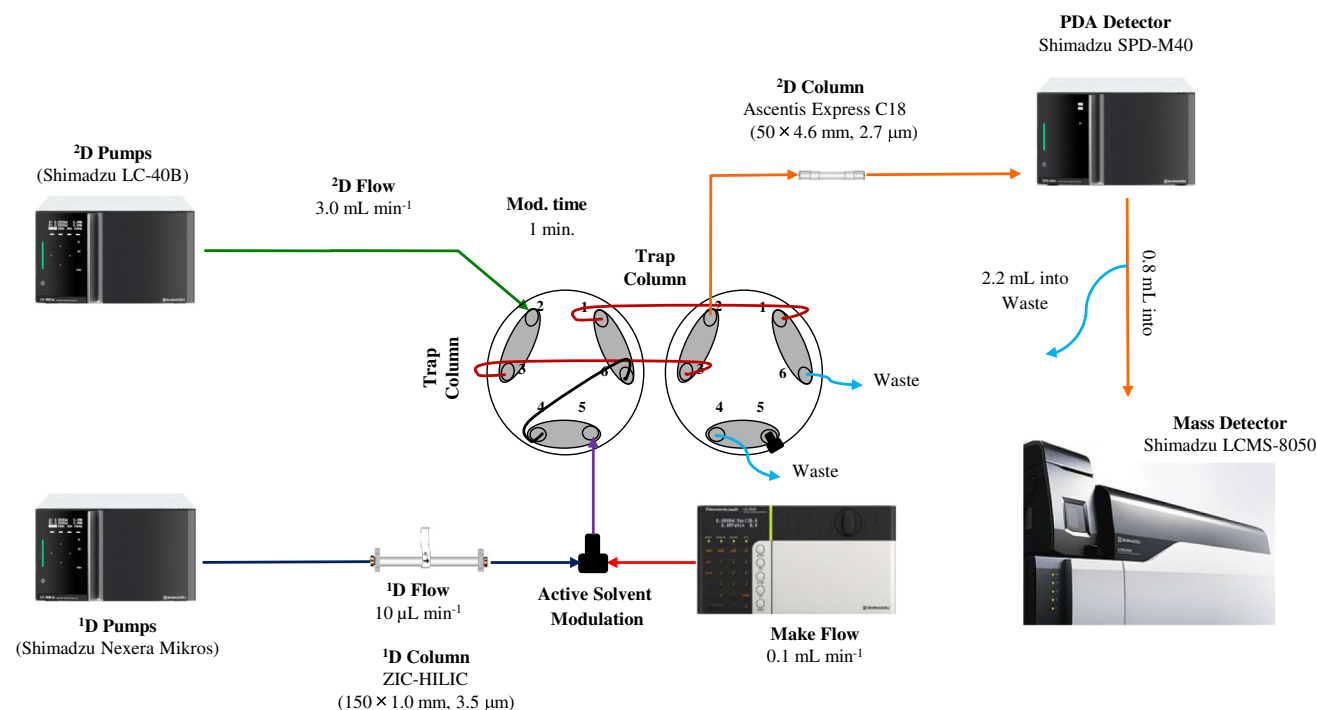


Fig. 1 Scheme of the HILIC $\times$ RP-LC instrumentation employed

ESI-MS conditions: mass spectral range:  $m/z$  100–2000 (+/-); event time: 1.0 s; 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).

### Validation of the quantitative method

Accurate quantitative analyses 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 [20], 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, eriodictyol 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, eriodictyol 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 regard 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.

### Determination of TPC

The total phenolic content of the berry juices was determined by Folin-Ciocalteu method, referring to calibration curve of gallic acid used as a standard [21]. One hundred microliters of each properly diluted sample was mixed with 0.2 mL Folin-Ciocalteu reagent, 2 mL of distilled water, and 1 mL of 15%  $Na_2CO_3$ , and the absorbance was measured at 765 nm, after 2 h incubation at room temperature, with a UV-1601 spectrophotometer (Shimadzu, Milan, Italy). The total phenolics were estimated as gallic acid equivalent (GAE) and expressed in mg GAE 100  $mL^{-1}$  juice  $\pm$  standard deviation (SD). The data were obtained from the average of three independent determinations.

### Determination of TFC

The total flavonoid content of the berry juices was determined by using the aluminum chloride ( $AlCl_3$ ) colorimetric assay, according to the protocol previously reported [21]. An aliquot of appropriately diluted sample solution (0.5 mL) was mixed with 1.5 mL MeOH, 0.1 mL of 10%  $AlCl_3$ , 0.1 mL of 1 M  $CH_3COOH$ , and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the mixture was spectrophotometrically measured at 415 nm. The amount of 10%  $AlCl_3$  was substituted by the same amount of distilled water in blank. The total flavonoids were estimated as quercetin equivalent (QE) from a calibration curve and expressed in mg QE 100  $mL^{-1}$  juice  $\pm$  SD. The data were obtained from the average of three independent determinations.

### Determination of TAC

The total anthocyanin content of the berry juices was determined according to the pH-differential method described by Lee et al. [22], using two buffer systems: potassium chloride buffer, pH 1.0 (0.025 M), and sodium acetate buffer, pH 4.5 (0.4 M).

An aliquot of appropriately diluted sample solution was transferred to a 10 mL volumetric flask and made up to 10 mL with corresponding buffer and the absorbance was measured at 510 and 700 nm. The total anthocyanins were calculated as cyanidin-3-*O*-glucoside equivalent (C3GE) and expressed in mg C3GE 100  $mL^{-1}$  juice  $\pm$  SD. The data were obtained from the average of three independent determinations.

### Antioxidant activity

#### DPPH assay

The free-radical scavenging activity of the berry juices was determined by the DPPH method [23]. Each juice was

diluted with distilled water (1:10), then serial dilutions were made to obtain five different concentrations in the range 3.125–50  $\mu\text{L mL}^{-1}$  [24]. An aliquot of 3 mL of daily prepared methanol DPPH solution (0.1 mM) was mixed with 0.5 mL of each sample and incubated at room temperature in the dark for 20 min. Then, the optical density change at 517 nm was measured with a UV-1601 spectrophotometer (Shimadzu, Milan, Italy). The results, obtained from the average of three independent experiments, are reported as mean radical scavenging activity percentage (%)  $\pm$  SD and as mean 50% inhibitory concentration ( $\text{IC}_{50}$ )  $\pm$  SD.

### Reducing power assay

The reducing power of the berry juices was evaluated by spectrophotometric detection of  $\text{Fe}^{3+}$ - $\text{Fe}^{2+}$  transformation method, according to the protocol previously reported [23]. An aliquot of each juice was diluted with distilled water (1:10), then serial dilutions were made to obtain five different concentrations in the range 3.125–50  $\mu\text{L mL}^{-1}$ . An aliquot of 1 mL of each sample was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ]. After incubation at 50 °C for 20 min, the solution was cooled rapidly, mixed with 2.5 mL of 10% trichloroacetic acid, and centrifuged at 3000 rpm for 10 min. Then, 2.5 mL of supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% fresh ferric chloride ( $\text{FeCl}_3$ ) and incubated at room temperature in the dark for 10 min; then, the absorbance was measured at 700 nm. The results, obtained from the average of three independent experiments, are expressed as mean absorbance values  $\pm$  SD and as mg ascorbic acid equivalent (ASE)  $\text{mL}^{-1} \pm$  SD.

## Results and discussion

### HILIC $\times$ RP-LC PDA-ESI-MS characterization of the polyphenolic content of berry juices

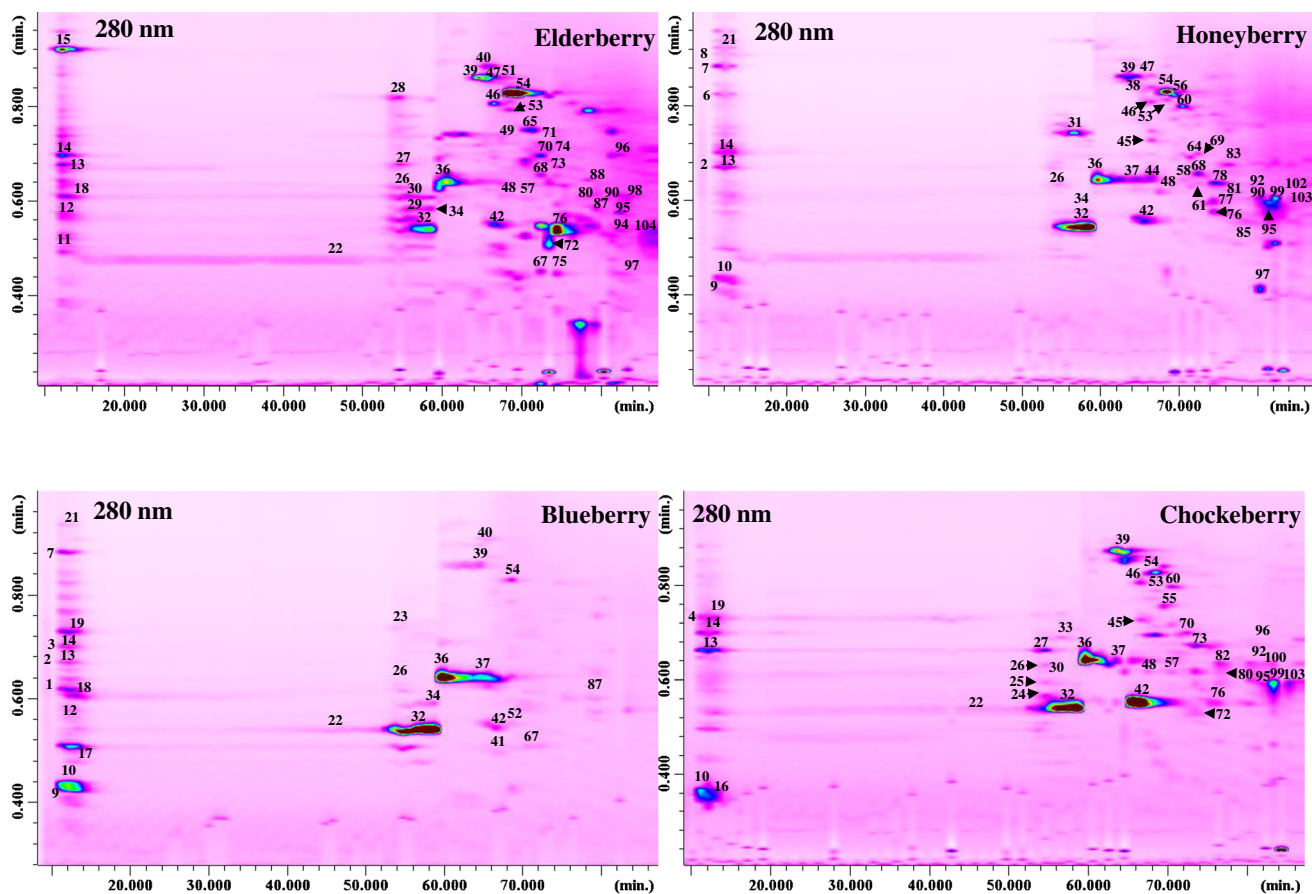
Although different separation modes have been experienced so far, the one involving the hyphenation of HILIC and RP modes has attracted a particular attention in the last decade, and it has been successfully exploited in some research groups for the polyphenolic profiling of various food and natural products [25–28]. In this work, prior to HILIC  $\times$  RP-LC analysis, a proper method optimization for both dimensions has been carried out. 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.

The employment of a 1.0 mm I.D. in the  $^1\text{D}$  allowed to reduce the amount of organic solvent (acetonitrile) from the  $^1\text{D}$  to the  $^2\text{D}$ , thus achieving effective “peak focusing” on the top of the  $^2\text{D}$  RP column; the latter was run with a gradient program starting with 100% of the weaker solvent (water). The  $^2\text{D}$  RP column was run with a fast gradient in order to get the highest of  $^2\text{D}$  analyses per  $^1\text{D}$  peak. A 4.6 mm I.D. C18 column was employed in the  $^2\text{D}$ , run at 3 mL/min. Taking into consideration the different polarity of the polyphenolic compounds occurring in the samples, the HILIC  $\times$  RP-LC analyses were investigated by using a segmented-in-fraction (SIF) approach. Notably, in the time frame from 0 to 59 min, the  $^2\text{D}$  %B raised up to 50%, whereas from 60 until the end of the analysis, the  $^2\text{D}$  %B raised up to 35%.

Figure 2 shows the HILIC  $\times$  RP-LC plots of the most complex berry juice samples, namely, elderberry, honeyberry, blueberry, and chokeberry. As can be appreciated, the polyphenolic compounds were spread around the HILIC  $\times$  RP-LC plots highlighting a satisfactory coverage of the separation space. Compounds eluted from the  $^1\text{D}$  according to increasing polarity; on the contrary, compounds in  $^2\text{D}$  eluted according to increasing hydrophobicity. In total, 104 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 (Table S1). 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. Notably, in blueberry, raspberry, and bilberry juices, two iridoid compounds (peaks no. 1 and 3) were detected. In terms of MS detection, ESI in both positive and negative ionization mode were selected made on the basis of the functional groups of the compounds identified. Specifically, for anthocyanins, the flavylium forms were detected as  $[\text{M}]^+$ , whereas the rest of the compounds was detected as  $[\text{M} + \text{H}]^+$  and/or  $[\text{M} - \text{H}]^-$  in order to achieve the best sensitivity for the OH and COOH functional groups occurring in their structures. Most peaks were detected in all samples, whereas others were uniquely present in one of a few of them. Among the identified compounds, 22 out of them have never been reported in the edible berry juices. In order to make a quantitative comparison of the separation capabilities for each sample investigated, the peak capacity ( $n_c$ ), as well as the orthogonality values, was considered (Table 1). The sample which yielded the highest





**Fig. 2** HILIC×LC-PDA plots ( $\lambda=280$  nm) of the elderberry, honeyberry, blueberry, and chokeberry. Peak numbering as in Table S1

theoretical  $n_c$  values was honeyberry ( $n_c=3790$ ), followed by raspberry ( $n_c=3746$ ). Aiming to provide more realistic values, the effective  $n_c$ , corrected for undersampling, was calculated [29, 30]. Notably, despite one of the requirements in LC×LC is not to jeopardize the resolution in the  $^1D$ , this is quite impossible to fulfil; in this context, despite a flow rate as high as 3 mL min was employed in the  $^2D$  to foster the complete elution of the compounds coming from the  $^1D$  in the  $^2D$ , a slight decrease was observed (honeyberry,  $n_c=1749$ , raspberry,  $n_c=1799$ ). Such results are in agreement with literature data [31]. Further, aiming to evaluate the separation space coverage, the orthogonality degree was calculated [32]. 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  $A_o$  values ( $A_o=0.70-0.80$ ). Finally, considering both effective peak capacity and orthogonality values, the blackcurrant juice sample was the most efficient ( $n_{c,corr}=1372$ ), followed by the raspberry ( $n_{c,corr}=1361$ ).

### HILIC×RP-LC PDA-ESI-MS semi-quantitative determination of the polyphenolic content of berry juices

In terms of quantification, nowadays at least two commercially available software are available from some

**Table 1** Peak capacity and orthogonality calculated for the HILIC×RP-LC focusing modulation set-up of the investigated samples

	Elderberry	Honeyberry	Blueberry	Chokeberry	Blackcurrant	Bilberry	Raspberry
$^1D$ peak capacity, $^1n_c$	52	63	41	48	62	55	60
$^2D$ peak capacity, $^2n_c$	53	60	64	52	59	56	63
Theoretical peak capacity, $^{2D}n_c$	2714	3790	2635	2482	3689	3068	3746
Effective peak capacity, $^{2D}n_c$	1456	1749	1635	1404	1720	1572	1799
Orthogonality $A_o$	0.80	0.76	0.70	0.79	0.80	0.72	0.76
Corrected peak capacity, $^{2D}n_{c,corr}$	1165	1322	1151	1115	1372	1126	1361

manufactures, making the methodology much easier for practical uses, and responding to the users' demands. Since peaks for each analyte are distributed into different modulation cycles, single  $^2D$  slides can be summed for calculation, considering either peak areas or volumes, thus allowing easy recognition and quantification of the target analytes in the contour plots [29–31]. After method optimization, a mixture of nine representative standards were analyzed in the HILIC  $\times$  RP-LC system. Calibration curves for each standard were attained after a five-time injection of such standards at six different concentration levels (Table 2). 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 intra-day ( $n=5$ ) and inter-day ( $n=15$ ) 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  $\times$  RP-LC system was subsequently employed to determine the polyphenolic content of the seven berry juices investigated. Elderberry juice was the sample with the highest content of polyphenolic compounds (917.79 mg 100 mL $^{-1}$ ), followed by chokeberry (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. 3A shows their distribution (mg 100 mL $^{-1}$ ) in each sample analyzed. 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 chokeberry, 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. Further,

in order to render both similarities and differences among the berry juices more evident, a multivariate analysis based on principal component analysis (PCA) was created. The results are illustrated in Fig. 3B as a biplot in the plane of the first and second PCs, accounting together for 61.01% of the total variability. The PCA separation was obtained according to the chemical classes found in the samples investigated (Fig. 3A); the latter, having similar chemical class distributions, are located closer. As can be observed in Fig. 3A and B, the chokeberry juice is characterized by a large concentration of hydroxycinnamic acids and anthocyanin glycosides accounting for 64.3 and 6.7% of the quantified compounds in such a sample. In the case of blueberry and raspberry juices, a composition consisting predominantly of hydroxycinnamic acids and flavonol glycosides was detected, accounting for 97.8% and 91.7% of the total quantified compounds in the two samples. Finally, blackcurrant juice showed a slightly different chemical class distribution profile, with the highest content of flavonols (5.3%).

### Phenolic compound estimation (TPC, TFC, and TAC)

As stated in the “Introduction” section, berries are rich sources of polyphenolic compounds [2]. First, the juices were analyzed for the estimation of TPC, TFC, and TAC, and results are illustrated in Table 3. As can be appreciated, the contents in all cases varied significantly. The determination of TPC contained in the berry juices was carried out spectrophotometrically using the Folin-Ciocalteu phenol reagent. The TPC of the juices was high in all cases, varying considerably from one juice to another. The calculated amounts of phenolics ranged from 916.74  $\pm$  2.03 mg GAE 100 mL $^{-1}$  (chokeberry) to 86.73  $\pm$  1.27 mg GAE 100 mL $^{-1}$  (blueberry), and they decreased in the following order: chokeberry > elderberry > honeyberry  $\geq$  blackcurrant > bilberry > raspberry > blueberry.

The results of the AlCl $_3$  colorimetric assay showed that the amount of TFC calculated for the juices ranged from 130.01  $\pm$  1.89 mg QE 100 mL $^{-1}$  (elderberry) to 16.77  $\pm$  0.68 mg QE 100 mL $^{-1}$  (raspberry), and it decreased in the following order: elderberry > chokeberry > honeyberry > bilberry > blackcurrant > blueberry > raspberry.

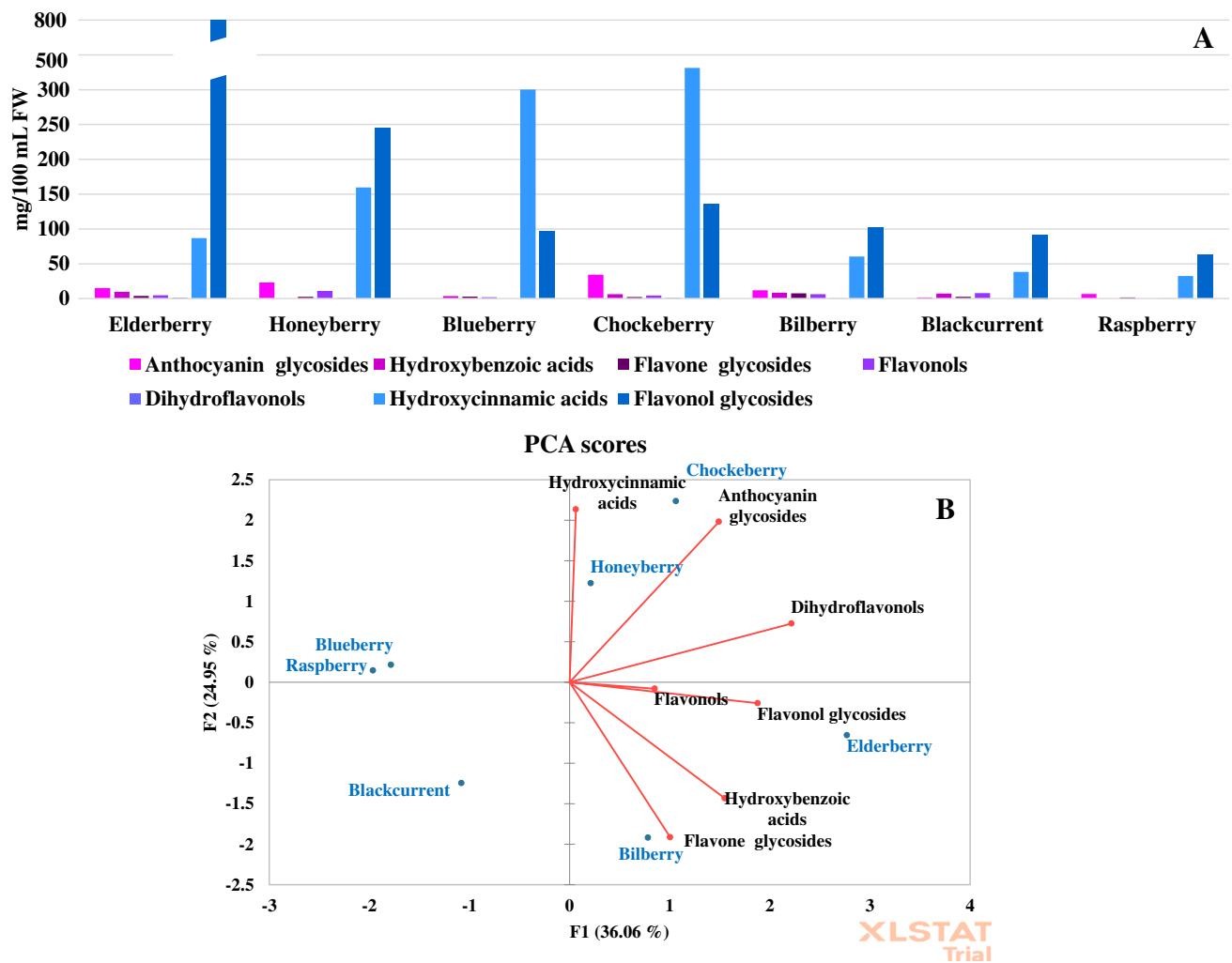
Finally, the total anthocyanins, determined by pH-differential method, ranged from 49.92  $\pm$  2.32 mg C3GE 100 mL $^{-1}$  (chokeberry) to 0.39  $\pm$  0.05 mg C3GE 100 mL $^{-1}$  (blueberry), and they decreased in the following order: chokeberry > elderberry > honeyberry > bilberry > raspberry > blackcurrant > blueberry.

The values attained with some exceptions, e.g., blueberry, are in agreement with previously published papers [9]. In fact, for blueberry, higher TPC values, viz. 1710–5230 GAE L $^{-1}$  and 7113 GAE L $^{-1}$ , were reported by Alasalvar et al. [33] and by Jayaprakasha et al. [34]; on the other hand, similar values

**Table 2** Validation parameter of HILIC×RP-LC method development for the characterization of berry juices

Compound	Phenol sub-class	[M-H] <sup>-</sup> / [M+H] <sup>+</sup>	$\lambda_{\max}$ (nm)	Linearity range (mg L <sup>-1</sup> )	R <sup>2</sup>	LOD (mg L <sup>-1</sup> )	LOQ (mg L <sup>-1</sup> )	Intra-day preci- sion RSD (%) n=5	Inter-day preci- sion RSD (%) n=15	Low recovery (%) (10 ppm)	Medium recov- ery (%) (50 ppm)	High recovery (%) (125 ppm)
Galic acid	Hydroxybenzoic acids	169/171	270	1–500	0.9972	0.12	0.80	0.72	0.85	95.90±1.47	109.14±1.07	105.32±2.02
Quercetin 3-O-glucoside	Flavonol glyco- sides	463/465	256, 354	1–500	0.9994	0.03	0.09	0.93	0.75	102.54±3.10	98.63±0.44	89.32±1.01
Luteolin 7-O-glucoside	Flavone glyco- sides	447/-	252, 350	1–500	0.9997	0.07	0.22	0.59	0.41	93.80±2.77	86.81±4.08	101.25±2.29
Eriodictyol	Flavanones	287/289	280	1–500	0.9981	0.14	0.42	0.84	0.85	94.15±2.02	84.51±2.09	115.78±1.62
Eriodictyol 7-O-glucoside	Flavanone glyco- sides	449/451	280	1–500	0.9932	0.03	0.09	0.88	0.84	106.37±3.14	96.11±1.43	110.02±1.51
Quercetin	Flavonols	301/303	324, 370	1–500	0.9992	0.90	2.71	0.69	0.47	98.59±2.10	99.36±2.40	96.32±0.63
Chlorogenic acid	Hydroxycinnamic acids	353/355	326	1–500	0.9984	0.32	0.96	0.76	0.88	87.08±4.35	112.53±3.96	115.07±3.53
Taxifolin	Dihydroflavonols	303/305	289	1–250	0.9974	0.02	0.06	0.43	1.25	102.45±3.03	94.10±2.41	118.69±1.01
Cyanidin 3-O-glucoside chloride	Anthocyanin glycosides	-/449	270, 516	1–125	0.9975	0.15	0.44	4.34	5.86	109.7±2.94	86.37±2.99	115.33±3.73





**Fig. 3** Distribution of the chemical classes in each berry juice analyzed by HILIC×RP-LC-PDA/ESI-MS: **A** quantitative contribution of the different chemical classes and **B** biplot of PC1 (36.06%) versus PC2 (24.95%) resulting of PCA

**Table 3** Spectrophotometric determination of berry juices for TPC, TFC, TAC, DPPH, and reducing power

	TPC (mg GAE 100 mL <sup>-1</sup> )	TFC (mg QE 100 mL <sup>-1</sup> )	TAC (mg C3GE 100 mL <sup>-1</sup> )	DPPH IC <sub>50</sub> (μL mL <sup>-1</sup> )	Reducing power (mg ASE mL <sup>-1</sup> )
Blueberry	86.73 ± 1.27	21.52 ± 0.36	0.39 ± 0.05	31.66 ± 0.02	1.71 ± 0.01
Raspberry	150.77 ± 1.33	16.77 ± 0.68	5.49 ± 0.62	20.49 ± 0.28	1.84 ± 0.06
Blackcurrent	393.12 ± 0.48	26.12 ± 0.40	3.74 ± 0.85	10.27 ± 0.07	3.20 ± 0.11
Bilberry	341.22 ± 1.73	32.64 ± 1.04	12.62 ± 0.26	15.32 ± 0.65	2.99 ± 0.07
Elderberry	796.11 ± 4.56	130.01 ± 1.89	33.35 ± 1.40	5.36 ± 0.09	6.25 ± 0.05
Chokeberry	916.74 ± 2.03	75.62 ± 0.50	49.92 ± 2.32	12.80 ± 1.09	8.89 ± 0.12
Honeyberry	393.17 ± 2.12	52.72 ± 0.61	30.28 ± 0.62	2.79 ± 0.03	3.38 ± 0.06

The data shown in the table are the mean ± standard deviation ( $n=3$ ); TPC, total phenolic content; TFC, total flavonoid content; TAC, total anthocyanin content; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GAE, gallic acid equivalent; QE, quercetin equivalent; C3GE, cyanidin-3-glucoside equivalent; IC<sub>50</sub>, mean 50% inhibitory concentration; ASE, ascorbic acid equivalent

were reported from other works [35, 36]. For TFC and TAC, values reported in Table 3 are lower than previously published data [9, 35, 36] despite in good correlation with other works

[37, 38]. Such variations could be ascribed to various factors, such as the plant genotype, cultivation site, time of ripening, and the technology employed for juice preparation [39, 40].

## Antioxidant activity of berry juices

### DPPH test

The results of DPPH test are shown in Fig. 4A and Table 3. All the tested juices displayed powerful free-radical scavenging activity, as also demonstrated by the calculated  $IC_{50}$  values, ranging from  $2.79 \pm 0.03 \mu\text{L mL}^{-1}$  (honeyberry) to  $31.66 \pm 0.02 \mu\text{L mL}^{-1}$  (blueberry).

Among the juices, honeyberry exhibited the best efficacy, followed by elderberry, showing an inhibition of DPPH radical of about 90% at  $6.25 \mu\text{L mL}^{-1}$  and  $12.5 \mu\text{L mL}^{-1}$ , respectively, and reaching 100% of activity at higher concentrations. The calculated  $IC_{50}$  confirmed the greatest activity of these juices ( $2.79 \pm 0.03 \mu\text{L mL}^{-1}$  and  $5.36 \pm 0.09 \mu\text{L mL}^{-1}$ , respectively). Blackcurrant juice also resulted in 100% free-radical scavenging activity at  $25 \mu\text{L mL}^{-1}$ . The  $IC_{50}$  values indicated that the activity of the juices was in the following decreasing order: honeyberry > elderberry > blackcurrant > chokeberry > bilberry > raspberry > blueberry. Compared to a recent study carried out by Ciric et al. [10], the free-radical scavenging activity of blackcurrant was found to be comparable, viz.  $10.27 \pm 0.07 \mu\text{L mL}^{-1}$  vs.  $8.56 \pm 0.02 \mu\text{L mL}^{-1}$ , whereas it was two-fold higher for raspberry  $20.49 \pm 0.28 \mu\text{L mL}^{-1}$  vs.  $10.56 \pm 0.07 \mu\text{L mL}^{-1}$ , and three-fold higher for blueberry with  $31.66 \pm 0.02 \mu\text{L mL}^{-1}$  vs.  $9.67 \pm 0.03 \mu\text{L mL}^{-1}$ . The difference in results might be due to difference in varieties, growing region, extraction solvent,

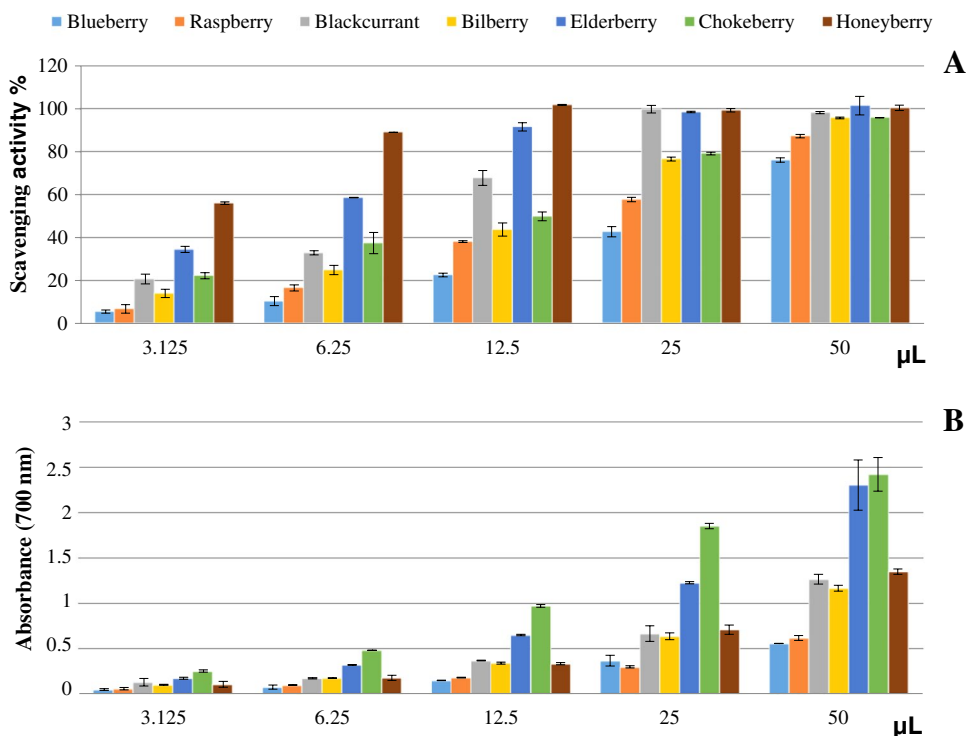
solute to solvent ratio, harvesting season, and maturation stages of berries.

Finally, linear regression analysis revealed no correlation between the total phenolic, flavonoid, and anthocyanin content of the juices and the DPPH radical scavenging activity.

### Reducing power assay

The reducing power assay was also conducted to measure the antioxidant capacity of the berries. In this assay, based on the electron transfer mechanism, the ability to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  was determined. The results of the reducing power assay showed that all the berry juices displayed activity that increased with rising concentrations (Fig. 4B and Table 3). The berries antioxidant capacity varied from  $1.71 \pm 0.01 \text{ mg ASE mL}^{-1}$  (blueberry) to  $8.89 \pm 0.12 \text{ mg ASE mL}^{-1}$  (chokeberry). Among the tested juices, chokeberry was found to possess the most powerful reducing properties, followed by elderberry, reaching an absorbance of  $2.42 \pm 0.18$  and  $2.30 \pm 0.28$ , respectively, at the highest volume tested ( $50 \mu\text{L}$ ). The calculated  $\text{mg ASE mL}^{-1}$  also confirmed the strongest reducing power of the juices ( $8.89 \pm 0.12$  and  $6.25 \pm 0.05$ , respectively). Compared to these, the other juices showed a much lower reducing power, with absorbance values at the maximum volume tested ranging from  $1.35 \pm 0.03$  (honeyberry) to  $0.55 \pm 0.01$  (blueberry). Based on the  $\text{mg ASE mL}^{-1}$ , the activity of the juices decreased in the order: chokeberry > elderberry > honeyberry > blackcurrant > bilberry > raspberry > blueberry.

**Fig. 4** Free-radical scavenging activity (DPPH test) (A) and reducing power (B) of the berry juices investigated. Values are expressed as the mean  $\pm$  SD ( $n=3$ )



A strong positive correlation was found between both the total phenolic and anthocyanin content and reducing power, as indicated by the coefficient of determination ( $R^2 = 0.9491$  and  $0.8324$ , respectively); a positive relationship was also highlighted with total flavonoids ( $R^2 = 0.5647$ ).

## 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 ZIC-HILIC column in the <sup>1</sup>D and a C18 in the <sup>2</sup>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 1372 and orthogonality of 0.80. One hundred four 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 <sup>2</sup>D column. Among the berries analyzed, elderberry showed the highest polyphenolic content ( $918 \pm 1.10$  mg 100 mL<sup>-1</sup>) 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.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00216-022-04216-y>.

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## Declarations

**Ethics approval** Not applicable.

**Conflict of interest** The authors declare no conflict of interest. Luigi Mondello is editor of Analytical and Bioanalytical Chemistry but was not involved in the peer review of this paper.

**Source of biological material** Not applicable.

**Statement on animal welfare** Not applicable.

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