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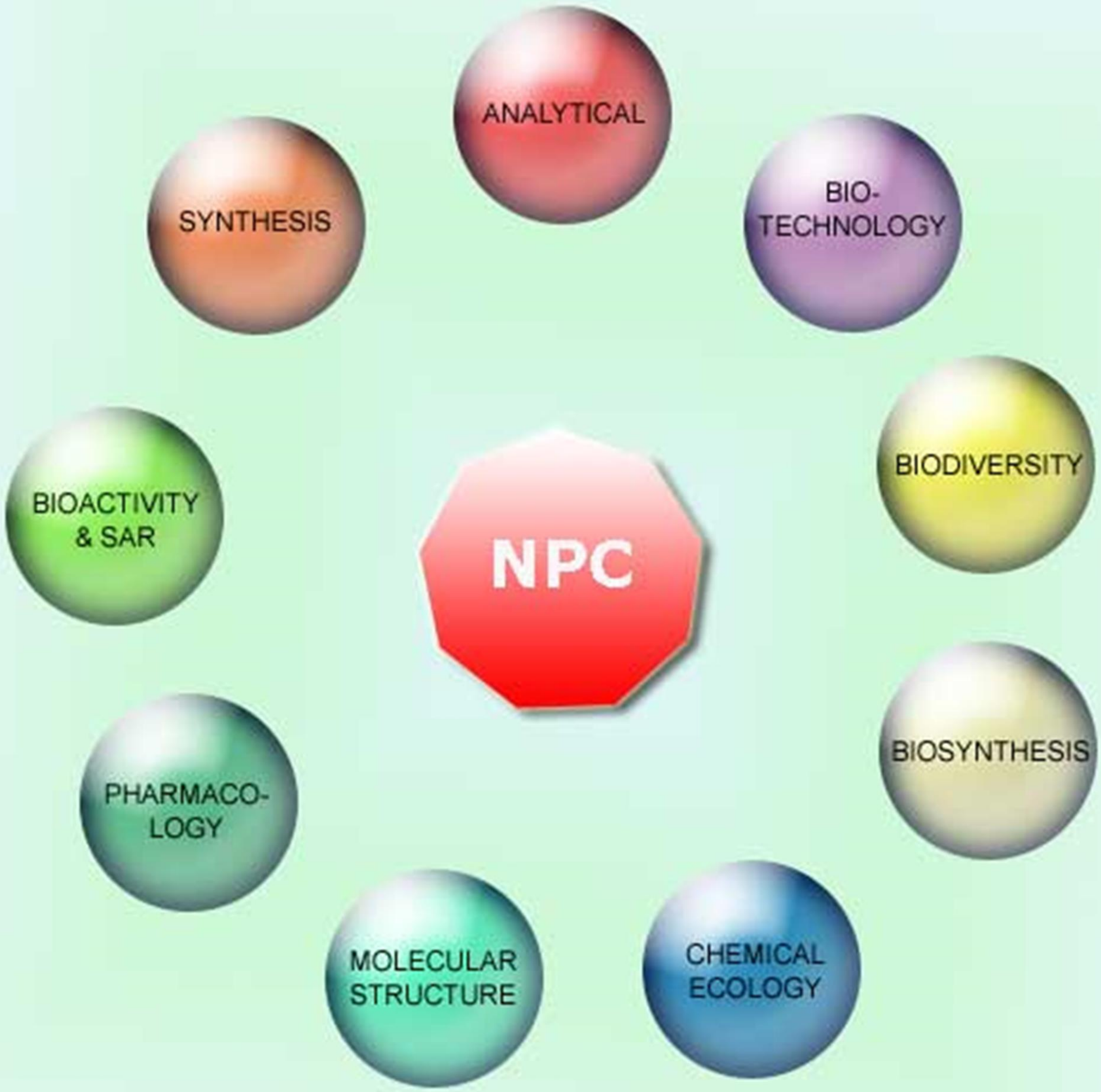

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Polyphenols and Volatile Compounds in Commercial Chokeberry (*Aronia melanocarpa*) Products

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Aronia melanocarpa (Michx.) Elliott commercial products (dried fruit, juice and compote) were analyzed for their polyphenol content by chromatographic and spectrophotometric analyses in order to ascertain the fate of this group of compounds when fresh fruit is processed and sold in different forms on the market. Different classes of polyphenols were investigated: hydroxycinnamic derivatives ranged from 0.65 mg/g to 4.30 mg/g, flavonoids from 0.36 mg/g to 1.12 mg/g, and anthocyanins from 0.65 to 7.08 mg/g sample. 4-*O*-Caffeoyl-quinic acid was tentatively identified for the first time in Aronia. In order to characterize better chokeberry juice, a GC profile of aroma compounds was obtained. The aroma juice compounds belong mainly to the chemical classes of alcohols (48.9%) and ketones (30.28%). The most abundant compound is 3-penthen-2-one (23.6%).

Keywords: SPME-GC-MS, VOCs, HPLC-DAD, Spectrophotometric analyses, Chokeberry juice, Chokeberry fruits, Chokeberry compote.

While most berries contain a large number of flavonoids and phenolic acids, black and red berries contain anthocyanins, compounds considered to have a positive effect on a large number of chronic diseases (such as diabetes, cardiovascular disease and obesity) [1,2], as well as protecting against cancer [3]. Chokeberry, *Aronia melanocarpa* (Michx.) Elliott, has been intensively studied in recent years due to its high polyphenol content. Fresh chokeberry fruits, among the richest berries for their anthocyanin content [4-6], have a higher anthocyanin and flavonoid content and antioxidant capacity than cranberries, blueberries and lingonberries [7]. Prevention of gastric damage induced by ethanol in rats is inhibited by the red pigment fraction of the black chokeberry [8].

A. melanocarpa, a shrub of the *Rosaceae* family, originally from the eastern parts of North America and Canada, is now cultivated in Eastern European countries, Germany and Slovenia where its characteristics have recently been reviewed [9]. The polyphenol composition of fresh fruit has been extensively studied [10-12]. Since consumption of the fresh fruit is limited, the shelf-life may be increased through processing techniques. In order to assess the presence of polyphenols, dried Aronia powders obtained from different drying methods were analyzed [13] and the stability of anthocyanins in berry juices was assessed; in this regard, chokeberry juice exhibited the highest stability [14]. The exploitation of phenolics from industrial Aronia by-products was also taken into account [15,16].

The aim of this research was to investigate the polyphenol composition in commercial products in order to ascertain the fate of this group of compounds when fresh fruit is processed and sold in different forms on the market. We took three commercial chokeberry products into account: dried fruit, juice and compote, which were analyzed for their total content of polyphenols, anthocyanins, flavonols and hydroxycinnamic derivatives. Unfortunately, since commercial products were analyzed, it was not possible to compare the results achieved with those of fresh fruit. In order to characterize more effectively chokeberry juice, a GC profile of aroma compounds was obtained and compared with that of fresh berries [17].

Commercial *A. melanocarpa* products (dried fruit, juice and compote) were analyzed for their polyphenol content by chromatographic and spectrophotometric analyses. The mass spectra were obtained in both positive and negative modes by HPLC-TOF analysis. In particular, the two pairs of isobaric compounds: quercetin 3-*O*-rutinoside and quercetin 3-*O*-rhamnosylgalactoside (compounds **6**) and quercetin 3-*O*-galactoside and quercetin 3- *O*-glucoside (compounds **7** and **8**) were identified based on the order of exit and the mass spectra according to Slimestad *et al*. [11]. Compound **3** was identified as 4-*O*-caffeoyl-quinic acid based on its typical retention order compared with its isomers [21] and according to the relative intensities of diagnostic fragments in negative ionization mode [22]; the main ion fragment was at *m/z* 353 (quasimolecular ion), while minor ion fragments were detected at *m/z* 191 (quinic acid moiety) and *m/z* 173 (‘dehydrated’ quinic acid moiety) with an intensity of 15% and 25% respectively. The ion at *m/z* 173 is characteristic of a substituted isomer in position 4 [22]. To our knowledge this is the first time that the presence of this compound has been reported in Aronia.

Table 1 reports the quali-quantitative data of the samples analyzed. In the case of the juice (density = 1.078 g/mL), the data are reported as mg/g. Chlorogenic and neochlorogenic acids are by far the most prevalent compounds in dried fruit, juice and compote, while all the flavonoids are present in about the same amount, as already observed in fresh fruit and juice by Oszmiański and Wojdylo [10]. 4-*O*-Caffeoyl-quinic acid exhibits a peculiar behavior: its amount is practically the same when changing from dried fruit to juice, thus indicating its high water solubility.

Table 1: Quali-quantitative composition of dried chokeberry fruit, juice and compote. Data are expressed in mg/g per sample. Standard deviation in brackets.

Compounds	Dried fruit	Juice	Compote
Hydroxycinnamic derivatives			
(**1**) Neo-chlorogenic acid	1.82(0.034)	0.47 (0.009)	0.26 (0.003)
(**2**) Chlorogenic acid	2.33 (0.043)	0.51 (0.011)	0.37 (0.006)
(**3**) 4-*O*-Caffeoyl-quinic acid	0.17 (0.003)	0.13 (0.004)	0.03 (0.3*10^{-3})
Flavonoids			
(**4**) Quercetin 3- *O*-arabinoglucoside	0.13 (0.002)	0.22 (0.004)	0.02 (0.2*10^{-3})
(**5**)	0.15 (0.003)	0.02 (0.3*10^{-3})	0.023 (0.3*10^{-3})
(**6**) Quercetin 3- *O*-rutinoside + quercetin 3- *O*-rhamnosylgalactoside	0.23 (0.003)	0.04 (0.4*10^{-3})	0.03 (0.2*10^{-3})
(**7**) Quercetin 3- *O*-galactoside	0.31 (0.003)	0.05 (0.4*10^{-3})	0.06 (0.5*10^{-3})
(**8**) Quercetin 3- *O*-glucoside	0.22 (0.004)	0.03 (0.3*10^{-3})	0.03 (0.3*10^{-3})
(**9**)	0.06 (0.8*10^{-3})	0.01 (0.1*10^{-3})	0.01 (0.1*10^{-3})

Table 2 reports the total HPLC content of hydroxycinnamic derivatives and flavonols, as well as the anthocyanin spectrophotometric content and total polyphenols according to the Folin-Ciocalteu method. Hydroxycinnamic acid derivatives and the flavonoid content decrease when changing from dried fruits to compote. Blueberry jam showed a loss of chlorogenic acid and total anthocyanins, with no change in the total flavonol content [23]. For home-made jams from five different berries (strawberries, raspberries, blackcurrants, bilberries and lingonberries) a considerable loss of flavonols was observed during processing [24]. In the case of red raspberries, while the loss of anthocyanins in the industrial processing of fresh fruit to obtain jam was between 17 and 41% depending on the fruit variety, the flavonol loss was much lower, ranging between 7 and 8% [25].

Table 2: Hydroxycinnamic derivatives and flavonoids determined by HPLC analyses (mg/100 g), anthocyanins determined by spectrophotometric analysis (mg/100 g), total phenolics (Folin-Ciocalteu method) expressed as mg gallic acid / 10 0g sample. Data are the mean of three determinations.

Compounds	Dried fruits	Juice	Compote
Hydroxycinnamic derivatives (HPLC)	4.3	1.1	0.7
Flavonoids (HPLC)	1.1	0.2	0.12
Anthocyanins (spectrophotometric analyses, 550 nm)	7.1	0.6	0.8
Total phenolics (Folin Ciocalteu)	30.9	8.7	7.7

As regards the anthocyanins, which are the compounds generally most investigated in fresh berries, their content changes depending on the cultivar considered [12]: in a survey of the pertinent literature, their content is found between 252 and 460 mg/100 g fresh weight [5,11,12,26], changing from 1041 mg/100 g [27] to 6408 mg/kg [28], dry weight. Our dried commercial fruit maintained 7.08 mg/g (Table 2) of anthocyanins; since the water content of dried fruit is about 17.5%, its content is in the same magnitude as that of fresh fruit, thus indicating that the anthocyanin content is not reduced in the industrial dehydration process. Plums, on the contrary, showed flavonol and anthocyanin degradation after the drying process at different temperatures [29]. A lower anthocyanin content is observed in the juice, since these compounds are mainly found in the external layers of the berry skin and are absent in the flesh tissues, being, however, water soluble pigments [4]. The anthocyanin juice content was similar to that of laboratory-made chokeberry juice, i.e. 62 mg/100 mL [14], which is very close to our datum (0.70 mg/mL, with a density of 1.078 g/mL). The anthocyanin content of compote is similar to that of juice; in this case, however the decrease should be ascribed to the thermal process required to obtain compote [30]. In the case of unprocessed and thermally processed strawberry purées, a 30% decrease of pelargonidin-3-glucoside was observed [30]. Blueberry jam also underwent a total loss of anthocyanins compared with fresh fruit [23]. With regard to the total polyphenols (Folin Ciocalteu method), the content in dried fruit is much higher than that reported for fresh fruit [5,12], while the juice and compote showed a lower content. However, with respect to dried fruit, the loss of anthocyanins is about 90% for the juice and compote, whereas the loss of total polyphenols is 72% and 76% in the case of juice and compote, respectively. Table 3 illustrates the volatile constituents identified, together with *I* Kovats indices modified according to van den Dool [31,32].

Table 3: Volatiles of chokeberry juice tentatively identified by GC-MS and calculated retention index. RT (Retention time), **I** value (**I**: Non-isothermal Kovats retention indices from temperature-programming, using the definition of Van den Dool and Kratz [34] and % of total area are reported. The **I** values were calculated from the retention time using *n*-alkanes as retention calibration standards according to the Kovats convention (1958).

no.	Compound	RT	I (DB-WAX)	% of total Area
1	Ethyl acetate	5.85		0.09
2	Ethanol	6.46	937	5.7
3	2-Pentanone	7.28	992	3.8
4	3-Penten-2-one. -(E)	7.87	1032	0.9
5	3-Buten-2-ol, 2-methyl-	8.01	1041	0.1
6	Undecane	8.48	1100	0.04
7	Hexanal	8.96	1145	0.2
8	2-Butanol, 3-methyl-	9.39	1184	0.4
9	3-Penten-2-one. -(Z)	9.81	1223	23.6
10	1-Penten-3-ol	10.01	1171	0.08
11	3-Penten-2-ol	10.29	1184	0.08
12	Dodecane	10.63	1200	0.08
13	Eucalyptol	11.17	1225	0.2
14	2-Hexenal, (E)-	11.30	1243	0.2
15	Styrene	11.94	1285	0.3
16	Tridecane	12.17	1300	0.1
17	2-Heptanol	12.44	1318	0.04
18	unk 2-Penten-1-ol	12.54	1325	0.1
19	1-Hexanol	13.02	1358	18.2
20	3-Hexen-1-ol, (E)-	13.21	1372	0.3
21	3-Hexen-1-ol, (Z)-	13.55	1395	3.8
22	2-Hexen-1-ol, (E)-	13.80	1413	11.1
23	2-Hexen-1-ol, (Z)-	13.94	1423	0.1
24	Acetic acid	14.43	1458	0.4
25	cis-Linaloloxide	14.53	1466	0.2
26	2-Pentanone, 4-hydroxy-	14.88	1491	1.7
27	1-Hexanol, 2-ethyl-	14.97	1497	0.3
28	Citronellal	15.04	1502	0.08
29	1,6-Octadien-3-ol, 3,7-dimethyl-	15.63	1547	0.3
30	Benzaldehyde	15.98	1574	2.4
31	Isophorone	17.006	1656	0.3
32	Butanoic acid, 2-methyl-	17.28	1678	0.9
33	alpha.-Terpineol	17.82	1726	0.3
34	Hexanoic acid	19.10	1854	1.1
35	Ethanol, 2-(2-butoxyethoxy)-, acetate	19.16	1861	0.1
36	2H-Pyran-2-one, tetrahydro-6-methyl-	19.26	1872	0.6
37	2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	19.29	1876	0.3
38	Benzyl alcohol	19.65	1917	16.7
39	Dihydro-3-methylene-5-methyl-2-furanone	19.65	1917	
40	Phenylethyl Alcohol	20.01	1962	0.1
41	2-Hexenoic acid, (E)-	20.17	1982	0.4
42	Benzoic acid, 4-ethoxy-, ethyl ester	22.19		0.2
43	Phenol, 3,4,5-trimethyl-	23.98		0.5
44	Benzoic acid	24.72		3.0

While in the case of two different raspberry cultivars, very slight differences were observed in the aroma composition of fresh fruit and juice [33], in this case, with respect to the volatile constituents determined in fresh berries [17] a lower number of compounds were identified. This occurrence can be ascribed to the different procedures via which the juice was obtained however. In the case of raspberries, it was obtained by pressing frozen fruit without any stabilization processes or the use of enzymes and technological coadjutants. It has been shown in the case of pomegranate juice that different processing methods can change the aromatic composition of commercial juice compared with fresh-squeezed juice [34]. In the case of fresh chokeberry fruit of two different cultivars [17], 3.9-Epoxy-*p*-menth-1-ene is the most abundant compound, but it was not found in our samples. The most abundant compounds in our juice were 3-penthen-2-one (23.6%), 1-hexanol (18.2%) and 2-hexen-1-ol (11.1%). The juice aroma compounds belong to these chemical classes: alcohols (48.9%), ketones (30.3%), hydrocarbons (0.2%), acids (5.8 %), aldehydes (2.9 %), terpenes (0.6%), esters (0.3%), and others (1.3 %).

Therefore, also considering peaks of 39 and 40 (16.7 %) that were not resolved and contain both an alcohol and a ketone, it was found that the main juice aroma compounds are alcohols and ketones. In the case of crushed dried fruit, few peaks were recorded; however, 3-penthen-2-one was the most abundant compound, as already observed in juice. Both benzaldehyde and acetic acid were found in juice, while 1-ethoxy-2-propanol was not detected in either juice or fresh fruit [17]. On the basis of polyphenol subclasses and volatile compounds, the commercial Aronia products can be regarded as a new source of biologically active compounds that may enhance agricultural products of marginal areas.

Experimental

Samples: Three commercial chokeberry samples (juice, compote and dried fruits) from a Croatian market were analyzed.

Extraction
Chokeberry juice: Fifty mL of 70% ethanol was added to 50 mL of juice and adjusted to pH 2 with formic acid.
Chokeberry compote: Forty mg of compote was extracted with 50 mL of 70% ethanol, adjusted to pH 2 with formic acid overnight and then filtered to eliminate residues.
Chokeberry dried fruit: Dried fruits (2.5 g) were extracted with 10 mL of 70% ethanol, adjusted to pH 2 with formic acid overnight, and then filtered to eliminate residues.

Standards and solvents: Authentic standards of quercetin 3-*O*-glucoside, chlorogenic acid, neo-chlorogenic, gallic acid and kuromanin were purchased from Extrasynthèse S.A. (Lyon, France). All solvents used were of HPLC grade purity.

HPLC/DAD analysis: Analyses of flavonols and hydroxycinnamic acids were carried out using an HP 1100L liquid chromatograph equipped with a DAD detector and managed by an HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). Compounds were separated using a 150 × 4.6 mm i.d, 5 µm LUNA C18 column (Phenomenex, USA). UV/Vis spectra were recorded in the 190-600 nm range and the chromatograms were acquired at 250, 280, 330, 350 and 520 nm. The samples were analyzed by gradient elution at a flow rate of 0.6 mL/min. The mobile phase was a multi-step linear solvent gradient system, starting from 95% H_2O (adjusted to pH 3.2 by HCOOH) up to 100% CH_3CN in 53 min.

HPLC-TOF analysis: The HPLC system was interfaced with an Agilent TOF MS equipped with an ESI source (Agilent Corp, Santa Clara, CA, USA). The TOF/MS analysis worked using full-scan mode and the mass range was set to *m/z* 100–1500 in both positive and negative modes. The conditions of the ESI source were as follows: drying gas, high purity nitrogen (N_2); drying gas temperature, 350°C; drying gas flow-rate, 6 L/min; nebulizer, 20 psi; capillary voltage, 4000 V (negative) 4000 V (positive); fragmentation, 80-150 V, and skimmer, 60 V. The acquisition and data analysis were controlled using Agilent LC-MS TOF Software (Agilent, USA).

Identification and quantification of individual compounds: The identity of polyphenols was ascertained using data from HPLC-DAD and HPLC-TOF analyses, by comparison with bibliographic data and combination of retention times, UV/Vis and mass spectra with those of authentic standards. The quantification of individual polyphenolic compounds was performed directly by HPLC-DAD using a five-point regression curve ($r^2 \geq 0.998$) in the range of 0-30 µg on the basis of authentic standards. In particular, flavonols like the quercetin derivatives were determined at 350 nm using quecetin 3-*O*-glucoside as a reference compound, while the hydroxycinnamic acid derivatives were determined at 330 nm using chlorogenic acid as the reference compound. In all cases, actual concentrations of the derivatives were calculated after applying corrections for differences in molecular weight. Each sample was analyzed in triplicate, so as to express the analytical results as an average with its standard deviation.

Spectrophotometric analyses: The 3 chokeberry extract samples were analyzed using an Agilent 8453, (Agilent Technologies, Palo Alto, CA, USA) spectrophotometer. The absorbance at 550 nm of the hydroethanolic sample solutions was evaluated using a 1 cm pathway quartz cell. The total amount of anthocyanins is expressed as kuromanin through the calibration curve of kuromanin.

Total phenolic content: The total phenolic content was determined using the Folin-Ciocalteu method described by Singleton *et al*. [18], and slightly modified according to Dewanto *et al*. [19]. The total phenolics are expressed as gallic acid equivalents (GAE, mg gallic acid/g sample) through the calibration curve of gallic acid. The calibration curve ranged from 20 to 500 µg/mL (R^2 = 0.9969).

SPME-GC-MS conditions and VOCs characterization: The VOCs profile of Aronia samples was determined by SPME (Solid-Phase Micro Extraction)-GC-MS. A tentative compound identification was performed by comparing the mass spectra of each peak with those reported in mass spectral databases after Dynamic Background Compensation by Clear View software, (ALMSCO, UK). Chokeberry juice (0.5 mL) was placed in a 2 mL vial and 0.2 g NaCl added. One g of crushed chokeberry dried fruit was placed in a 2 mL vial. SPME conditions: VOCs profile was determined by absorption of VOCs at 40°C (for 10 min) on a trivalent Carboxen PDMS DVB 1 cm fiber, followed by desorption at 280°C and GC/MS analysis. An Agilent 7890a GC equipped with a 5975C MSD was used. The analyte separation was achieved with an Agilent DB WAX 50 m column, 0.20 µm id, 0.40 µm df. The chromatographic conditions were: initial temperature 40°C, then 10°C min^{-1} up to 260°C, and held for 6.6 min.

Determination of juice density: The chokeberry juice density was measured with a standard pycnometer (ISO 1183-1:2004) [20]:

$$\rho = \rho_{H_2O}\,\frac{m_x - m_o}{m_{H_2O} - m_o}$$

ρ = chokeberry juice density
ρ_{H_2O} = water density
m_x = pycnometer + chokeberry juice weight
m_0 = empty pycnometer weight
m_{H_2O} = pycnometer + water weight

Evaluation of moisture content: The moisture content of the commercial fruits was obtained by keeping the sample in a static oven at 60° for 5 days.

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