SECOND AND THIRD DERIVATIVES OF UV SPECTRA AS A TOOL FOR IDENTIFICATION OF MAJOR ANTHOCYANINS FROM *Aronia melanocarpa* EXTRACT, SEPARATED USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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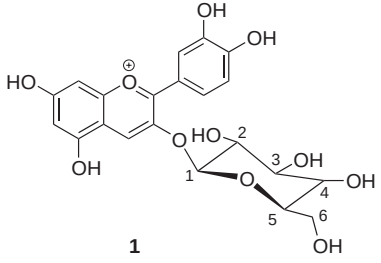
The aim of the study was to propose a method for identification of major anthocyanins in black chokeberry: cyanidin 3-*O*-β-D-galactopyranoside and cyanidin 3-*O*-α-L-arabinopyranoside, using reversed-phase high-performance liquid chromatography on line with UV spectroscopy. The similarity indexes between standard spectra and spectra of major components of black chokeberry extract were calculated. The calculations were performed for zero-order spectra and first–fifth derivatives. Only the second and third derivatives of UV spectra provided correct identification of the compounds investigated. Third derivatives of spectra gave higher values of the parameters (calculated using Student's test) indicating statistical significance between similarity indexes to the derivatives of spectra of anthocyanin standards. Third derivative UV spectroscopy may be thus recommended as a tool to identify anthocyanins.

Keywords: Anthocyanins; Glycosides; *Aronia melanocarpa*; HPLC; UV derivative spectroscopy; Photodiode-array detector; Natural products.

Hyphenated techniques in chromatography enable much more reliable identification of compounds than retention times only. Such techniques strongly support qualitative and quantitative analysis. Photodiode-array UV-VIS detectors are recently commonly used as a part of high-performance liquid chromatography (HPLC) assemblies[1]. Application of the on-line technique allows identification of the separated compounds on the basis of their UV and/or VIS spectra acquired in real time. Application of the spectroscopy on line with HPLC, however, leads to a decrease in spectra quality as compared with those obtained using the off-line technique. This disad-

vantage is caused by the fact that spectra obtained using the first technique may by affected by uncertainties of separation conditions control[2]. The above mentioned problem appears to be minor taking into consideration a possibility of facilitating the analysis. The HPLC on line with UV-VIS spectroscopy, alone or together with mass spectrometry, is recently used for, e.g., characterization of anthocyanins from various plants[3–12]. The zero-order spectra of anthocyanins were used in up-to-date experiments. Resolution of compounds in this way may be sometimes difficult in the case of close retention times and/or similarity of their zero-order spectra[3]. The latter problem may be solved using derivative spectroscopy. Derivatives of spectra provide better resolution, but a lower signal-to-noise ratio than zero-order spectra[13]. In previous paper[14] we proposed algorithm for identification of proteins using similarity index of UV spectra derivatives. We proposed statistical significance of difference between the first and second similarity index as a criterion of method reliability. In the case of proteins, the differences in chromophore composition e.g. the tryptophan to tyrosine ratio constitute the crucial cause of differences in UV spectra[15,16]. The spectra may also be affected by folding or unfolding of protein chain leading to the changes in solvent accessibility to individual aromatic residues[16]. The major anthocyanins of black chokeberry are[17]: cyanidin 3-*O*-β-D-galactopyranoside (**1**) and cyanidin 3-*O*-α-L-arabinopyranoside (**2**). Both compounds contain identical chromophores and they differ only in the sugar residue.



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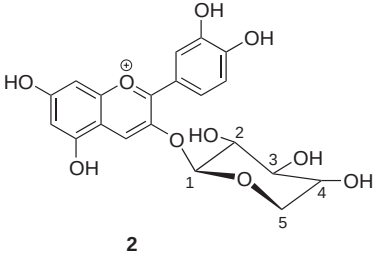

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The aim of the present study was to propose a strategy based on derivative UV spectroscopy used for identification of main anthocyanins from black chokeberry separated by RP-HPLC.

EXPERIMENTAL

Materials

Acetonitrile (ACN), trifluoroacetic acid (TFA) and methanol of HPLC grade were purchased from Baker (Deventer, Holland). All other chemicals (analytical grade) were obtained from Merck (Darmstadt, Germany). Deionised water (MilliQsystem, Millipore) was used. Samples of standards: cyanidin 3-*O*-galactoside **1** and cyanidin 3-*O*-arabinoside **2** were obtained from Department of Fruit and Vegetable Technology, Agricultural University of Wroclaw (Poland)[17]. Black chokeberry (*Aronia melanocarpa*) from field cultivation in Poland was used in the study.

Sample Preparation

Samples of fruits were extracted at room temperature with 0.1% methanolic HCl [3] overnight. Methanol was removed using a rotary evaporator and the resulting aqueous solution passed through a C-18 column (SPE). After washing with deionised water, the pigments were eluted from the column with 0.01% methanolic HCl [18,19]. Methanol was removed using a rotary evaporator and the samples were lyophilised.

Chromatography

Chromatographical analysis was carried out with a Shimadzu assembly consisting of two LC-10AD pumps, SIL-10AD autosampler, SCL-10AD controller, CTO-10AS column oven and SPD-M10AW PDA detector. Hi-Pore RP 318 column (BioRad) of 250 × 4.6 mm size was used. Class-VP 5.03 software (Shimadzu) was used for data acquisition and processing.

The samples of standards and lyophilisate of anthocyanin extract were dissolved in methanol. Their concentration was ca. 0.5 mg ml^{-1} and the injection volume varied from 5 to 20 µl. The anthocyanin separations were carried out in acetonitrile gradient. Solvents A and B consisted of acetonitrile, water and trifluoroacetic acid in the ratio 100:900:1 (v/v/v) and 900:100:0.7 (v/v/v), respectively[20]. All solutions were filtered through a nylon filter with 0.45 µm pore diameter. The separations were carried out using the flow rate 0.8 ml min^{-1} at 30 °C. The following gradient was used: 3% B at the start, 7% B after 20 min. After finishing of the gradient elution, the column was washed and equilibrated as described previously[15].

UV Spectrosopy

Spectra of anthocyanins were taken from the chromatograms. The UV spectra were recorded within the wavelength range 190–370 nm. Data acquisition time was 25 min, number of elements in the photodiode array was 512, wavelength accuracy ±1 nm, element resolution 1.2 nm/element, slit width 1.2 nm, spectrum band width 1.4 nm, noise level $\pm 0.8 \times 10^{-5}$ a.u., drift 10^{-3} a.u./h, time constant 0.64 s, sampling period 2 s.

The used program calculates only the first or second derivative of a function. Due to this fact, the following order of calculations was maintained[14]: 2nd derivative was calculated using 2nd derivative option; 3rd derivative using 2nd derivative followed by 1st derivative; 4th derivative using 2nd derivative applied twice and 5th derivative as 2nd derivative calculated twice and followed by 1st derivative. We have constructed six separate libraries containing zero-order spectra and 1st, 2nd, 3rd, 4th and 5th derivatives of anthocyanin spectra.

The spectra or derivatives of spectra were compared with the standard spectra using similarity indexes (SI) calculated using of the equation proposed previously[14]:

$$SI = \frac{\sum_i d^m A_1 / d\lambda^m(\lambda_i)\, d^m A_2 / d\lambda^m(\lambda_i)}{\sqrt{\sum_i \left[d^m A_1 / d\lambda^m(\lambda_i)\right]^2}\sqrt{\sum_i \left[d^m A_2 / d\lambda^m(\lambda_i)\right]^2}}, \quad (1)$$

where λ means wavelength; A_1 and A_2 absorbance at wavelength λ_i of standard and checked substances, respectively; $d^mA/d\lambda^m$ is m-th derivative of spectrum (m = 0 (for zero-order spectra), 1, 2, 3, 4 or 5).

The spectra and derivatives of spectra were compared with those of standards within the wavelength range 270–300 nm as recommended on the basis of the results published previously[15].

Statistical Analysis

The preliminary evaluation of method has been carried out on the basis of contingency tables constructed separately for every derivative of UV spectra of both standards as recommended by Pulido et al.[21] The statistical significance of the difference between individual similarity indexes was evaluated using Student's t-test.

RESULTS

An RP-HPLC chromatogram of anthocyanins from black chokeberry is presented in Fig. 1. The retention times of individual components of anthocyanin extract are in agreement with retention times of standards (data not shown). Peaks 1 and 2 in chromatograms of the anthocyanins from black chokeberry as well as in the chromatograms of standards do not show addi-

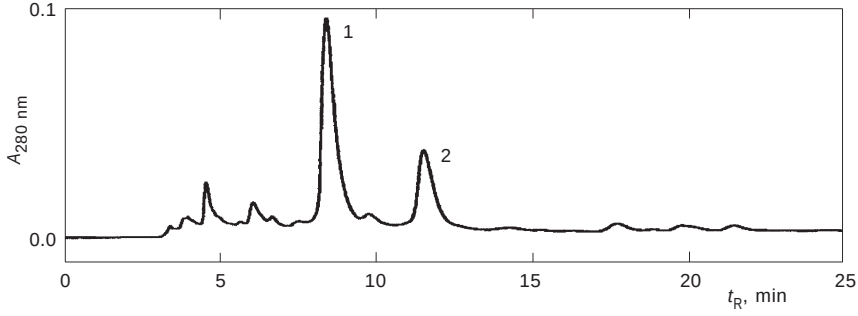

FIG. 1

RP-HPLC chromatogram of black chokeberry (*Aronia melanocarpa*) extract: 1 cyanidin 3-*O*-galactoside **1**, 2 cyanidin 3-*O*-arabinoside **2**. Experimental conditions: concentration 1 mg of black chokeberry extract per ml of methanol, injection volume 10 µl. For other details see "Experimental"

tional shoulders as compared with the peaks in the chromatograms of standards. This indicates that they do not contain substances coeluting with cyanidin 3-*O*-galactoside **1** or cyanidin 3-*O*-arabinoside **2**.

UV spectra of **1** and **2** in the wavelength range 190–370 nm and the second and third derivatives of these spectra in the range 270–300 nm are presented in Figs 2a, 2b and 2c, respectively.

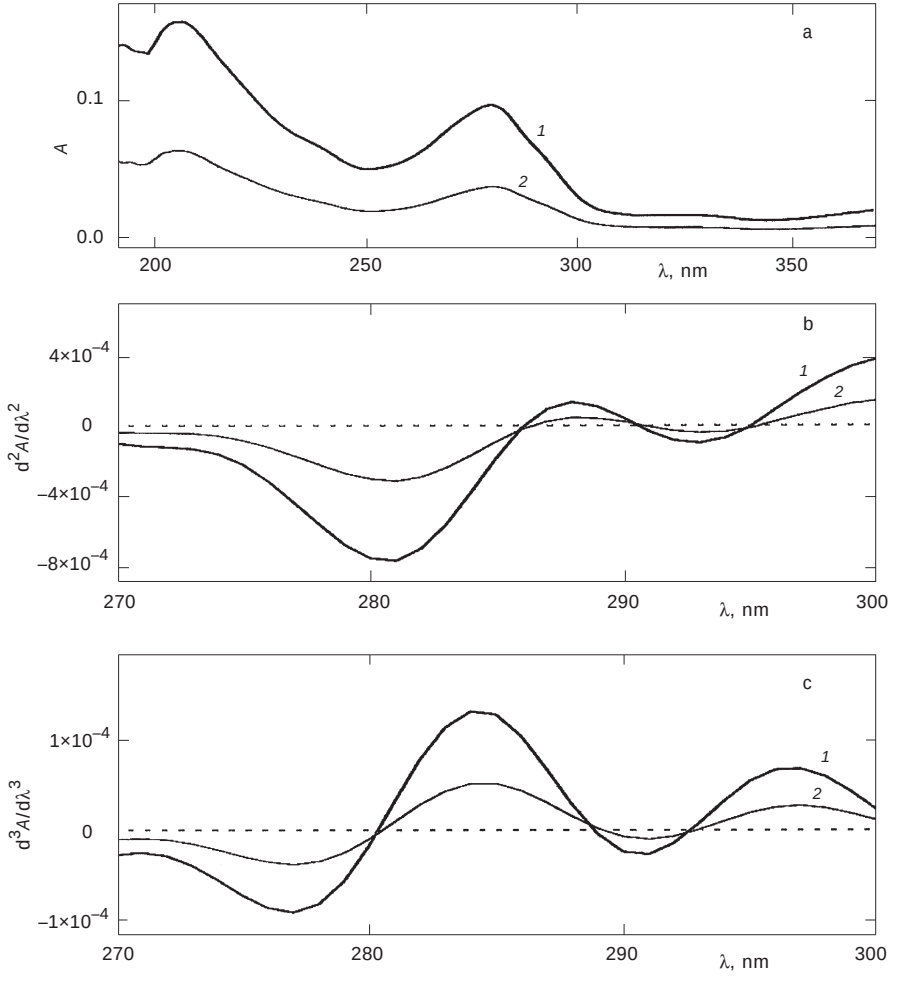

FIG. 2

UV spectra of cyanidin 3-*O*-galactoside **1** (curves *1*) and cyanidin 3-*O*-arabinoside **2** (curves *2*). Zero-order spectra (a), second derivatives (b) and third derivatives (c) of spectra in the range used for identification. Spectra of **1** and **2** were taken from peaks 1 and 2, respectively (Fig. 1). For experimental conditions see "Experimental" and legend to Fig. 1

Similarity indexes of the second and third derivatives of standard UV spectra as well as third derivatives of sample component spectra are summarised in Table I. The zero-order spectra, and their first, fourth and fifth derivatives gave incorrect identification of compounds in some cases. The statistically significant differences between individual similarity indexes are presented in Table I.

The above mentioned results, calculated according to Eq. (*1*) do not fulfil the multiplication commutative law. This phenomenon is a common problem associated with numerical calculations[22].

The detector acquired differential spectra, the spectrum at the start of run serving as a background. We have not found a detectable difference between the spectra of standard compounds obtained using the gradient starting from 100% of solvent A (data not shown) and the spectra obtained using conditions described in Experimental.

The similarity index differences between derivatives of spectra of minor components of black chokeberry extracts (see Fig. 1) and derivatives of standard spectra did not exceed 0.4 (data not shown).

TABLE I
Similarity indexes (mean ± SD) of derivatives of standard UV spectra and of sample components (*n*, number of measurements)

Parameter	Cyanidin 3-*O*-galactoside **1**		Cyanidin 3-*O*-arabinoside **2**	
	SI to standard **1**	SI to standard **2**	SI to standard **1**	SI to standard **2**
	Second derivatives			
Standard (n = 5)	0.999 ± 0.001[a]	0.999 ± 0.001	0.996 ± 0.002[a,b]	0.999 ± 0.001[b]
Sample (n = 3)	1.000 ± 0.001[c]	0.999 ± 0.001	0.996 ± 0.002[c,d]	0.999 ± 0.001[d]
	Third derivatives			
Standard (n = 5)	0.999 ± 0.001[e]	0.998 ± 0.001	0.992 ± 0.003[e,f]	0.999 ± 0.001[f]
Sample (n = 3)	0.999 ± 0.001[g]	0.997 ± 0.001	0.993 ± 0.001[g,h]	0.999 ± 0.001[h]

Pair of values different at the level: [a] 0.02 (t = 2.999), [b] 0.02 (t = 2.999), [c] 0.01 (t = 3.674), [d] 0.01 (t = 3.674), [e] 0.001 (t = 4.949), [f] 0.001 (t = 4.949), [g] 0.001 (t = 4.647), [h] 0.001 (t = 4.647).

DISCUSSION

The order of retention times of the two major components is in agreement with previous results[11]. The zero-order spectra of both major anthocyanins as well as those of their derivatives are nearly identical. The results concerning zero-order spectra are consistent with previous findings[3]. Similarity index may be defined as cosinus of angle between vectors in multidimensional space, being mathematical representation of spectra or their derivatives. Thus similarity index does not depend on vector length, i.e. on scaling or analyte concentration within the range of concentrations providing sufficient quality of spectra.

Dependence of shape of UV spectra derivatives on analyte concentration has been discussed previously[15]. Part of spectrum within the wavelength range below 270 nm strongly depends on analyte concentration[15]. Absorbance of anthocyanins within the range between 300 and 370 nm was negligible (see Fig. 2a). Thus we have used the wavelength range between 270 and 300 nm.

The ruggedness of derivatives of UV spectra, associated with change of discriminants used for the description of spectra appears at too low analyte concentration[15]. We have found that ruggedness of protein UV spectra derivatives appears, when absorbance at 220 nm is lower than ca. 0.06 [15]. The ratio of absorbance A_{280nm}/A_{220nm} for proteins and peptides is usually lower than the same ratio for anthocyanins (see Fig. 2a). For instance A_{280nm}/A_{220nm} ratio for bovine β-casein and its fragments varied within the range ca. 0.02–0.06 [23]. We have used analyte concentration (see Experimental) allowing to obtain smooth derivatives of spectra. Figures 1 and 2 show data concerning sample containing lowest amount of **1** and **2** among the samples used. The spectra used for construction of libraries were obtained using the absorbance at 280 nm within the range ca. 0.4–0.5. There was no difference between individual spectra of the same compound within A_{280} range between 0.04 and 0.5.

Sensitivity, specificity, positive predictive value and negative predictive value, calculated from the contingency tables reached 100% only for the second and third derivatives of spectra of both components, i.e. only application of these two orders of derivative allowed correct identification of all standard spectra. The resolution of zero-order spectra and their first derivatives was probably too low for identification of compounds. Fourth and fifth derivatives revealed discrete structure of spectra acquired using photodiode array detector with resolution 1.2 nm. Such resolution is typical for commercially available photodiode array detectors[1]. Fourth and fifth deriv-

atives of spectra form broken lines instead of smooth curves, especially at wavelengths close to the minima and maxima (data not shown). This phenomenon leads to partial loss of information provided by spectra.

The value of *t* parameter calculated using Student's test was higher for the third than for second derivatives of spectra. Third derivatives can thus be recommended as a tool for identification of components of black chokeberry extract.

Calculation of fourth derivatives is recommended as a procedure sufficient for interpretation of spectra obtained using "classical" spectrophotometers[13,16]. The classical spectrophotometers allow to acquire spectra with the wavelength interval 0.1 nm, i.e. sufficient to obtain smooth fourth derivatives of spectra.

The absence of influence of the gradient used on the spectra gives evidence that differences in spectral properties are not caused by the changes of the solvent composition during chromatographic elution. This finding is consistent with the results published previously[15].

In previous paper[14] we have recommended first derivatives of UV spectra of proteins as leading to highest value of parameter characterising statistical significance of difference between first and second similarity indexes (calculated using Student's test). The average first and average second similarity indexes, calculated for seven proteins, have been taken into attention. The standard deviation of average similarity indexes calculated for second and third derivatives of UV spectra was up to 0.011–0.022 and 0.136–0.139, respectively, i.e. many times higher than in present experiment (see Table I). Differences between spectra of individual proteins were caused mainly by different composition of chromophores, i.e. different tryptophan to tyrosine ratio. The changes in tertiary leading to differences in solvent accessibility of individual tryptophan and tyrosine residues during passing through detector also may affect to some extent UV spectra of proteins although the last supposition requires experimental confirmation. Similarity indexes of cyanidin 3-*O*-galactoside **1** spectra to the spectra of standard **1** and **2** did not differ significantly, although a difference occurs in the case of cyanidin 3-*O*-arabinoside **2** spectra (see Table I). This fact indicates that in the case of anthocyanins it is not sufficient to calculate values of the parameters characterizing statistical significance of difference between average first and second similarity index. Therefore, it is necessary to take into account all individual SI values as recommended by Macaud et al.[24] However, it is also necessary to take into consideration all precautions concerning the concentration of analyte[15] and the order of numerical derivative calculations[14].

The UV, as well as VIS spectra obtained on line by HPLC or off line are considered as suitable only for identification of different chromophores[25,26] or chromophore environment, e.g. to discriminate between exposed and buried aromatic amino acid residues in proteins[16]. To date, there have been no results concerning differences between UV spectra of compounds containing identical chromophore with sugar residues attached in the same position. The only difference is in composition of monosaccharide residue (pentose vs hexose).

CONCLUSION

The second and third derivatives of UV spectra may be used for identification of compounds with similar structure such as major anthocyanins from black chokeberry. The order of derivative may be chosen on the basis of correct identification of standards. The statistical significance of difference between similarity indexes calculated for derivatives of standard spectra is recommended as a second criterion for procedure choice.

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