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Journal of Chromatography B, 770 (2002) 297–301

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Influence of storage conditions on the stability of monomeric anthocyanins studied by reversed-phase high-performance liquid chromatography

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Abstract

The effect of light, storage time and temperature on the decomposition rate of monomeric anthocyanin pigments extracted from skins of grape (*Vitis vinifera* var. Red globe) was determined by reversed-phase high-performance liquid chromatography (RP-HPLC). The impact of various storage conditions on the pigment stability was assessed by stepwise regression analysis. RP-HPLC separated well the five anthocyanins identified and proved the presence of other unidentified pigments at lower concentrations. Stepwise regression analysis confirmed that the overall decomposition rate of monomeric anthocyanins, peonidin-3-glucoside and malvidin-3-glucoside significantly depended on the time and temperature of storage, the effect of storage time being the most important. The presence or absence of light exerted a negligible impact on the decomposition rate.

Keywords: Decomposition rate; Anthocyanins

1. Introduction

The commercial value and the buyer's acceptance of any food products markedly depend on the quality and amount of pigments present. Because of the legislative actions resulting in the continuous withdrawal of artificial dyes approved before, the interest in the development of food colorants from natural sources enormously increased [1,2]. Because of the bright attractive color, high water solubility and possible health benefits [3] anthocyanins are considered as potential replacement for synthetic dyes. Anthocyanins can act as antioxidants by donating hydrogen to highly reactive radicals preventing further radical formation [4]. Unfortunately, monomeric anthocyanins are not stable, oxygen, elevated temperature, metal ions and enzymes may enhance their decomposition rate [5,6] and promote polymerization. The polymeric forms have generally a brownish shade [7]. Anthocyanins can be extracted with organic solvents from solid matrices [8] and preconcentrated by solid-phase extraction [9] before analysis. The total pigment concentration can be easily measured by spectrophotometric techniques after extraction, by the direct determination of the absorption of transparent liquids or by measuring surface reflectance. The reliability and reproducibility of spectrophotometric techniques is high, they are

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00055-7

generally easy to carry out, and can be employed in routine control laboratories [10]. However, spectrophotometry, even multiwavelength spectrophotometry is unsuitable for the separation of pigment and for the quantitative determination of fractions. The exact knowledge of anthocyanin composition is important because it may facilitate the detection of adulteration [11] and can enhance the reliability of the authenticity test [12].

Liquid chromatography has been frequently used for the analysis of anthocyanins. Thus, thin-layer chromatography has been recently used for the separation of anthocyanins in callus cultures of different plant species [13], and in the flowers of some *Delphinium* species [14]. The high separation power and low detection limit made high-performance liquid chromatography (HPLC) a method of preference for the analysis of pigments even in complicated accompanying matrices [15]. The considerable commercial value of food products containing anthocyanins encouraged the development of a considerable number of HPLC methods suitable for their separation [16]. The application of HPLC for the measurement of anthocyanins in fruits [17] and fruit juices [18] have been reported. Because of the highly different retention capacity of anthocyanin pigments, gradient elution has been proposed for their effective analysis [19].

The objectives of the recent study were the separation of anthocyanin pigments extracted from grape skins by reversed-phase (RP) HPLC and the assessment of the effect of light, time and temperature of storage on the overall decomposition rate of monomeric anthocyanins (peonidin-3-glucoside and malvidin-3-glucoside) using stepwise regression analysis.

2. Experimental

All solvents were analytical or HPLC grade and were purchased from Merck (Darmstadt, Germany). Solid-phase extraction (SPE) cartridges and anthocyanin standards were purchased from Isolute (Mid-Glamorgan, UK) and Extrasynthése (Genay, France), respectively. Analytical and pre columns were also purchased from Merck.

2.1. Extraction of monomeric anthocyanin pigments

Pigments were extracted form the skins of the grape *Vitis vinifera* var. Red globe. Skins of 128 g were mixed with 100 ml of acetone in an Ultra Turrax homogeniser and were centrifuged at 20 000 *g* for 10 min. The solid residue was re-extracted with 100 ml of water–acetone (30:70, v/v) until a clear solution was obtained. The combined supernatants were shaken in a separatory funnel with chloroform in the ratio 1:2 (v/v). After overnight storing of the mixture at 1 °C the aqueous phase was separated and the residues of acetone were removed at 35 °C. The aqueous extract was brought to 100 ml with distilled water containing 0.01% HCl.

2.2. Purification and preconcentration of anthocyanins

ENV+ cartridges of 3 ml volume containing 0.1 g of sorbent were conditioned with 2 ml of methanol followed by 2 ml of 0.01% (v/v) aqueous HCl. An aliquot of 0.5 ml of aqueous extract was passed through the cartridge, and sugars and organic acids were removed with 1 ml of 0.01% of aqueous HCl. Pigments were eluted with 2 ml of methanol containing 0.01% HCl. This procedure was repeated to obtain 8 ml of eluate than methanol was evaporated at 35 °C and anthocyanins were dissolved in 10 ml of distilled water containing the same concentration of HCl.

2.3. RP-HPLC separation

Separation of anthocyanins was carried out on a HPLC system consisting of an L-7450 diode array detector, an L-6200 Intelligent pump (Hitachi, Tokyo, Japan) and a Valco injector (Houston, TX, USA) with 20-μl injector loop. The analytical column was a Li-Chrocart endcapped RP-18 (250×4 mm I.D.; particle size 5 μm) column coupled with a LiChrosphere 100 RP18 precolumn (4×4 mm I.D.; particle size 5 μm). Separation was performed with gradient elution. The eluents were A: 20 ml of formic acid (98-100%) was diluted to 1 l by distilled water, B: acetonitrile–water–formic acid (80:18:2,

v/v). The binary gradient was: from 5 to 30% B in 40 min, from 30 to 50% B in 20 min, and from 50 to 80% B in 10 min. Flow-rate was 1 ml/min, column temperature was set to 30 °C. To avoid the possible decomposition of anthocyanins each analytical step was performed under subdued light. Detection wavelength was between 250 and 600 nm, and calibration curve was measured at 520 nm. Each determination was run in triplicate and the relative standard deviation (RSD) of the retention time and peak areas was calculated. Identification of anthocyanins was obtained by using authentical standards, and by comparing the elution order and UV and visible spectra with those of found in the literature. Quantification was carried out by using the external standard method. Peonidin-3-glucoside and malvidin-3-glucoside were dissolved at five different concentrations between 250 and 50 ng/ml in distilled water containing 0.01% HCl and linear correlations were calculated between the peak areas and the concentration of anthocyanins in the samples.

2.4. Storage experiment and statistical analysis

Storage experiments were carried with the extract of pigments redissolved in distilled water containing 0.01% HCl. Samples were stored in the air at 24, 32 and 40 °C in light using a lamp of 1.5 W and in the dark and analysed after 1, 3, 6, 8, and 14 days of storage as described above.

The simultaneous effect of storage time and temperature, and light was assessed by using stepwise regression analysis [20]. The original pigment concentrations were taken as 100% and the relative percentage of pigments remained intact were calculated. These values were the dependent variables. The independent variables were the storage time (days), temperature and the presence (marked by 1) or absence (marked by 0) of light. The number of accepted variables was not limited, the acceptance level for the individual independent variables was set to a 95% significance level. The impact of the storage conditions on the anthocyanins was compared by calculating the "*t*" probe between the corresponding coefficients of regression.

Software for stepwise regression analysis was purchased from Compudrug (Budapest, Hungary).

3. Results and discussion

The average yield of skin was 80 g/kg of grape, and the total amount of monomeric anthocyanins was 446±29 mg/100 g of skin. This finding indicates that skins of grape can be used for the production of natural food colorants and represent a valuable source of anthocyanins. The chromatogram of original extract of monomeric anthocyanins is shown in Fig. 1. The pigments identified are well separated indicating that the RP-HPLC method is suitable for the analysis of monomeric anthocyanins in the extract of grape skin. The chromatogram contains also unidentified pigment peaks but their concentration is relatively low. The chromatogram of extract stored for 8 days at 32 °C in light is shown in Fig. 2. The quantity of pigments is considerably reduced (compare absorbance values), however, the ratio of identified pigments is fairly similar to that of the original extract. This finding suggests that the differences among the decomposition rate of pigments is negligible. It can be also established that the number and relative amount of unidentified pigment fractions is higher indicating the formation of new pigments during storage. The RSDs of retention times and peak areas were 0.7–1.3% and 1.4–2.1%, respectively. These data prove the good stability and reproducibility of the HPLC system and the reliability of results. The calibration curves were different

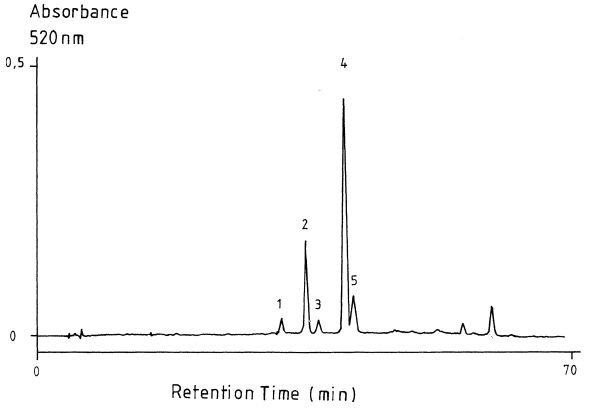

Fig. 1. RP-HPLC separation of the original extract of monomeric anthocyanins. For details see Experimental. Peak identification: 1=Delphinidin-3-glucoside; 2=cyanidin-3-glucoside; 3=petunidin-3-glucoside; 4=peonidin-3-glucoside; 5=malvinidin-3-glucoside.

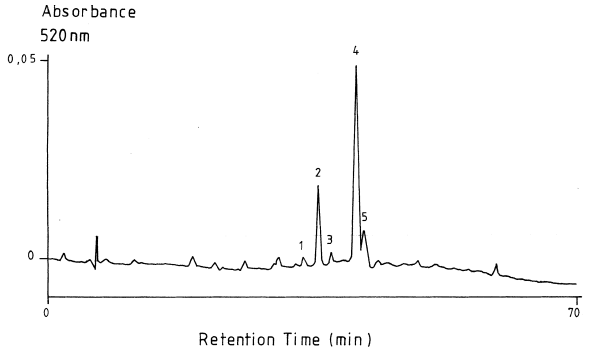

Fig. 2. RP-HPLC separation of the extract of monomeric anthocyanins after 8 days of storage at 32 °C in light. For details see Experimental. Peak identification as in Fig. 1.

for peonidin-3-glucoside (Eq. (1)) and malvidin-3-glucoside (Eq. (2)):

$$\text{Concentration (ng/ml)} = -(18.0 \pm 16.0) + (1.39 \pm 0.05) \cdot 10^{-5} \cdot \text{peak area} \quad r_{\text{calc.}} = 0.9970 \qquad (1)$$

$$\text{Concentration (ng/ml)} = -(4.33 \pm 12.8) + (1.94 \pm 0.13) \cdot 10^{-5} \cdot \text{peak area} \quad r_{\text{calc.}} = 0.9912 \qquad (2)$$

The intercept values do not differ significantly from zero indicating that the method of quantitation does not contain systematic error. The slope values are significantly different suggesting that the specific absorption of these anthocyanins is distinct at the wavelength of detection.

The amount of anthocyanins found at various sampling times are compiled in Table 1. The data clearly show that the concentrations of both peonidin-3-glucoside and malvidin-3-glucoside decreases with increasing length of storage and the decomposition rate is higher at elevated temperatures. These findings emphasize again the importance of adequate storage conditions for products containing anthocyanins as natural ingredients or as added colorants.

The results of stepwise regression analysis are compiled in Table 2. In the traditional multiple linear regression analysis the presence of independent variables that exert no significant influence on the dependent variable lessens the significant level of the independent variables that significantly influence the dependent variable. Stepwise regression analysis overcomes this difficulty by eliminating automatically from the selected equation the insignificant independent variable increasing in this manner the information power of calculation. Calculations proved that both the storage time and temperature influence significantly the decomposition rate of

Table 1
Effect of storage conditions on the stability of peonidin-3-glucoside and malvidin-3-glucoside

Storage time (days)	Peonidin-3-glucoside			Malvidin-3-glucoside		
	24 °C	32 °C	40 °C	24 °C	32 °C	40 °C
Storage in dark						
1	99.9	83.3	76.5	97.7	91.1	81.0
3	72.9	70.0	58.1	85.0	70.4	58.9
6	65.4	47.4	14.2	62.4	58.5	20.8
8	38.7	35.2	4.9	46.0	42.2	6.6
14	27.9	13.7	0.0	35.3	21.7	0.0
Storage in light						
1	96.1	82.4	69.2	89.2	85.9	68.6
3	59.8	56.7	53.1	76.1	69.2	59.1
6	54.3	21.4	13.4	55.2	41.7	4.8
8	34.9	7.8	0.1	38.0	16.9	1.3
14	21.1	0.2	0.0	33.5	5.7	0.0

Numbers are relative percentages the original pigment concentrations taken as 100%.

Table 2
Parameters of linear correlations between the relative percentages (Rel.%) of anthocyanins and the storage time (days) and temperature (T)

Rel.% $= a + b_1 \cdot \text{days} + b_2 T$		
Parameter	Peonidin-3-glucoside	Malvidin-3-glucoside
a	134.9	145.9
b_1	−5.62	−5.47
s_{b1}	0.55	0.53
b_2	−1.76	−1.98
s_{b2}	0.38	0.36
b_1' (%)	68.76	65.51
b_2' (%)	31.24	34.49
r^2 (%)	82.54	83.47
$F_{calc.}$	63.82	68.18

Results of stepwise regression analysis ($n = 30$; $F_{99.9\%} = 5.94$).

anthocyanins (compare calculated F values with tabulated ones). The change of storage time and temperature explains more than 80% of the change of the decomposition rate (see r^2 values) suggesting that the decomposition rate of these anthocyanins can be predicted at other temperatures and storage times. The concentration of pigments decreases linearly with the length of storage and the decomposition is more rapid at elevated temperatures. The normalized slope values (b_1' and b_2') indicate that the effect of storage time is considerably higher than that of temperature. The calculated ''t'' values indicated that the sensitivity of peonidin-3-glucoside and malvidin-3-glucoside towards storage time and temperature did not differ significantly, therefore, the loss of these pigments under storage conditions occur parallel. Interestingly, the presence or absence of light did not exert a significant impact on the amount of pigments indicating that this storage condition is of secondary importance.

4. Conclusions

It can be concluded from the results that the decomposition of anthocyanin monomers extracted from grape skin can be successfully followed by RP-HPLC–diode array detection and visible spectrophotometry. Stepwise regression analysis facilitate the elucidation of the effect of storage conditions on the decomposition rate.

Acknowledgements

This work was supported by the Portuguese–Hungarian cooperation project ''Development of new methods and their application for the assessment of the effect of environmental conditions on the stability of color pigments in foods''.

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