Analytical Letters, 39: 2687–2697, 2006

ISSN 0003-2719 print/1532-236X online
DOI: 10.1080/00032710600824805

Taylor & Francis
Taylor & Francis Group

HPLC

Reversed-Phase High-Performance Liquid Chromatography with Electrochemical Detection of Anthocyanins

Przemysław Kośmiński and Ana Maria Oliveira Brett
Departamento de Química, Faculdade de Ciências e Tecnologia,
Universidade de Coimbra, Coimbra, Portugal

Abstract: Anthocyanins, flavonoid compounds present in grapes and wines, were determined by reverse-phase high-performance liquid chromatography (RP-HPLC) with electrochemical detection (RP-HPLC-ED). The method developed consists of RP-HPLC gradient elution with voltammetric detection using a glassy carbon electrode after separation in an Inertsil ODS-3V analytical column. Good peak resolution was obtained following direct injection of a 50 μL sample of anthocyanins in a mobile phase of pH 2.20. The results show that six different anthocyanins: cyanidin-3-O-glucoside chloride (kuromanin chloride), cyanidin-3,5-di-O-glucoside chloride (cyanin chloride), malvidin-3-O-glucoside chloride (oenin chloride), malvidin-3,5-di-O-glucoside chloride (malvin chloride), delphinidin-3-O-glucoside chloride (myrtillin chloride), and peonidine-3-O-glucoside chloride, all with antioxidant properties, can be separated in a single run by direct injection of solution. The limit of detection (LOD) for these compounds was lower than 0.3 μM. The method can also be applied to the analysis of these compounds in red wines and in skins and pulp extracts of red grapes, since all these antioxidants are electroactive.

Keywords: RP-HPLC-ED, anthocyanins, antioxidant, gradient elution

Received 21 March 2006; accepted 28 April 2006

Financial support from Fundação para a Ciência Tecnologia (FCT), POCI (co-financed by the European Community Fund FEDER), ICEMS (Research Unit 103), and European Project HPRN-CT-2002-00186 are gratefully acknowledged.

Address correspondence to Ana Maria Oliveira Brett, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, 3004-535 Coimbra, Portugal. E-mail: brett@ci.uc.pt

INTRODUCTION

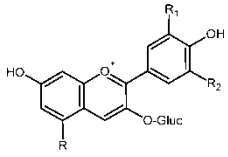
Anthocyanins, which occur in fruits and vegetables, belong to the family of flavonoids comprising the most common group of plant polyphenols. This class of molecules is responsible for the bright purple, red, and blue colors and flavor. Owing to their color and water solubility, flavonoids are a group of substances that were already well known in the fourth century A.D. as natural substances used for dying of Coptic textiles (Trojanowicz et al. 2004). The knowledge derived from identification of the chemical composition of dyes used for Coptic textiles enables the determination of the different kinds of plants from which they were extracted, finding their place of origin, reconstructing historical trade paths, and age determination, and are of great significance for conservation and restoration processes.

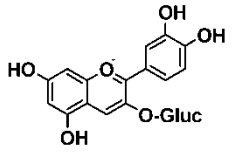
Besides the colorant features, the most important property of flavonoids is their function as antioxidant, playing an important role in human health. Anthocyanins reduce free radicals, so that eating fruits and vegetables rich in these compounds helps to protect against pathological conditions such as cancer and heart disease. Grapes contain anthocyanins in the peel, seeds, and pulp. The total amount of these compounds depends on the grape variety and on the different cultivars because anthocyanins are responsible for the color and the flavor; the biggest quantity of anthocyanins is in the peel (Kallithraka et al. 2005; Zhang et al. 2004; Castro-Gamboa et al. 2003).

Anthocyanins contain several phenolic hydroxyl groups attached to ring structures, which confer antioxidant activity. The anthocyanin structures are presented in Fig. 1. Depending on the substitutions in the structure, these compounds have different antioxidant and electrochemical properties.

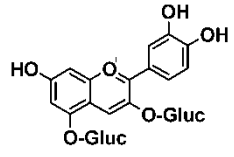
The anthocyanins, like all flavonoids, have a three ring structure. The catechol group in ring B and the resorcinol group in ring A can be oxidized electrochemically. The oxidation of one hydroxyl or two hydroxyl groups in the meta-position is more difficult. A different situation occurs when the hydroxyl groups are in the ortho- or para-position in the phenol rings (Brenna et al. 1998). The electrochemical oxidation mechanism of catechin, a representative flavonoid, was investigated using cyclic, differential and square wave voltammetry and its oxidation mechanism was proposed (Janeiro and Oliveira Brett 2004), Fig. 2. The oxidation mechanism has two steps: the oxidation of the catechol (ring B) occurs first at a low positive potential and the oxidation of the hydroxyl group of the resorcinol (ring A) is observed at a higher potential. The oxidation potential depends on the pH, such that an increase of pH was found to be associated with a decrease of the oxidation potential.

The most used methods for separation and determination of anthocyanins in grapes and wines are chromatographic methods. Anthocyanins are compounds that can absorb UV and visible light, so that HPLC with UV-visible detection has been the most used (Casteele et al. 1983; Preston and Timberlake 1981; Revilla et al. 2001; Berente et al. 2000). Nevertheless, HPLC with mass spectrometric detection (MS) (Zhang et al. 2004; Košir

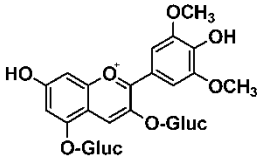
Structure	Name	R	R_1	R_2
	cyanidin-3,5-di-O-glucoside (cyanin)	Gluc	OH	H
	cyanidin-3-O-glucoside (kuromanin)	OH	OH	H
	malvidin-3-O-glucoside (oenin)	OH	OCH_3	OCH_3
	malvidin-3,5-di-O-glucoside (malvin)	Gluc	OCH_3	OCH_3
	delphinidin-3-O-glucoside (myrtillin)	OH	OH	OH
	peonidine-3-O-glucoside (peonidin)	OH	OCH_3	H



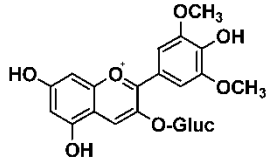
cyanidin-3,5-di-O-glucoside
(cyanin)



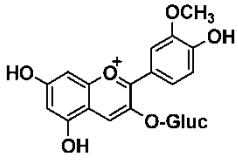
cyanidin-3-O-glucoside
(kuromanin)



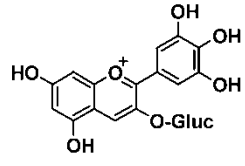
malvidin-3,5-di-O-glucoside
(malvin)



malvidin-3-O-glucoside
(oenin)



peonidine-3-0-glucoside
(peonidin)



delphinidin-3-O-glucoside
(myrtillin)

Figure 1. Structure of anthocyanins.

et al. 2004), diode array detection (DAD) (Surowiec et al. 2003), and electrochemical detection (ED) are also used (Castro-Gamboa et al. 2003; Brenna et al. 1998). The HPLC with ED is more sensitive, selective, and has a lower detection limit than that obtained by photodiode array detection (DAD).

In the majority of studies the separation of similar phenolic compounds and anthocyanins is accomplished most effectively by gradient elution (Lee and Hong 1992), and the mobile phase is usually methanol or acetonitrile. The desired pH is obtained by addition of a small amount of an inorganic acid such as trifluoroacetic acid, phosphoric acid or perchloric acid, or by adding an organic acid, usually formic or acetic acid. Depending on the pH,

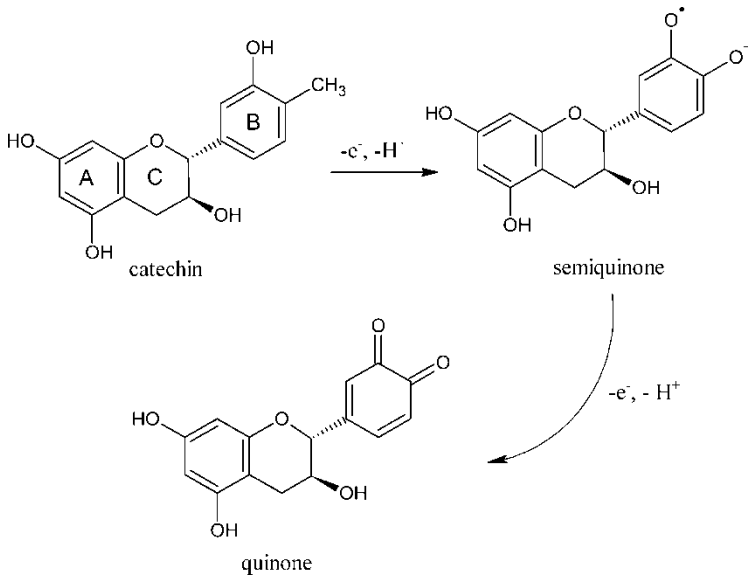


Figure 2. Mechanism of oxidation of catechin.

anthocyanins can exist in one of four structures that are flavylium cation, quinoidal base, carbinol base, and the chalcone. When the pH of the mobile phase is below 3.20 anthocyanins exist in two forms: flavylium cation and quinoidal base. The quantities of different anthocyanin structures change rapidly with pH (Casteele et al. 1983; Preston and Timberlake 1981). Almost 100% is in the flavylium cation form at pH 1.50 but at pH 2.50 it is only 67% (Košir et al. 2004). The majority of studies for the determination of anthocyanins in wines by high performance liquid chromatography are described for a mobile phase below pH 2.0.

Due to their flavonoid structure, anthocyanins can be oxidized electrochemically at potentials in the range $+0.30$ V to $+0.6$ V vs. Ag/AgCl (2 mM KCl) and these conditions are tested in this paper as appropriate operating potentials for the detection of anthocyanins after reverse phase HPLC separation with ED detection.

EXPERIMENTAL

Reagents

Acetonitrile of HPLC-gradient grade was obtained from Merck (Darmstadt, Germany). Formic acid for analysis–ACS was obtained from Panreac (Barcelona, Spain). The six anthocyanins: cyanidin-3-O-glucoside chloride (kuromanin chloride), cyanidin-3,5-di-O-glucoside chloride (cyanin

chloride), malvidin-3-O-glucoside chloride (oenin chloride), malvidin-3, 5-di-O-glucoside chloride (malvin chloride), delphinidin-3-O-glucoside chloride (myrtillin chloride), and peonidine-3-O-glucoside chloride, were prepared from Extrasynthese (Genay, France). All other chemicals were obtained from Merck (Darmstadt, Germany). Deionized water was prepared in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Stock standard solutions of 10^{-3} M anthocyanins were prepared in Milli-Q water and stored at -20 °C. These solutions were diluted to 10^{-5} M and from this concentration the standard solutions for direct injection into the HPLC system were prepared.

Equipment

Chromatographic measurements were carried out using a Waters (Milford, MA, USA) HPLC system, which consisted of a separation module 2690 with Concorde electrochemical detector from Waters Corporation, Milford, USA, and a Millenium^{32} Workstation Chromatography Manager from Waters Corporation. The separation of anthocyanins was accomplished on an Inertsil ODS-3V analytical column (5 μm particle size, 4.6 × 150 mm) from GL Sciences Inc. (Tokyo, Japan).

The electrochemical cell was a VT-03 flow cell from Antec Leyden, Zoeterwoude, Netherlands, of a confined wall-jet design, in a three-electrode configuration: a glassy carbon working electrode with 2 mm diameter, an in-situ Ag/AgCl reference electrode, and a stainless steel auxiliary electrode. The in-situ Ag/AgCl reference electrode, referred to here as ISAAC (in situ Ag/AgCl) is in direct contact with the mobile phase that contains 2 mM KCl. There is a difference of +0.19 V between the potential of the Ag/AgCl reference electrode saturated with 3 M KCl and the ISAAC reference electrode in contact with 2 mM KCl. Thus, for an application running at +0.70 V vs. Ag/AgCl with saturated KCl, the potential setting using ISAAC should be +0.51 V. All potentials are referred to the ISAAC reference electrode and, unless otherwise stated, the cell potential was set at + 0.50 V vs. ISAAC (+0.69 V vs. Ag/AgCl).

Experimental Conditions

All mobile phase solvents were degassed during 15 minutes in an ultrasound bath. For the separation two water-acetonitrile mixtures were used after adjusting to pH 2.20 with formic acid.

The mobile phase was solvent A (water-acetonitrile-formic acid 94 : 5 : 1 by volume) and a mobile phase B (water-acetonitrile 40 : 60), with 2 mM KCl, and elution was in the linear gradient mode as described in Table 1. The guard column and analytical column were kept in a Faraday cage with a

Table 1. Linear gradient elution used for separation of anthocyanins

Time/min	A (%)	B (%)
0	100	0
3	100	0
14	85	15
27	25	75
29	20	80

thermostatic oven at T = 35 °C together with the electrochemical flow cell. The flow rate was 1 mL min^{-1} and the applied potential + 0.50 V vs. ISAAC.

RESULTS AND DISCUSSION

The Inertsil ODS-3V chromatographic column is specific for use within the pH range of 2.0 to 7.5. A higher pH will dissolve the silica gel and a lower pH can strip away some of the bonded phase. As mentioned, 100% of anthocyanin is in the flavylium cation form at pH 1.50 but at pH 2.50 only 67% is in the flavylium cation form. Consequently, to keep the flavylium cation concentration higher for the separation and determination of anthocyanins, a pH of 2.20 was chosen.

Several mobile phases were tested. First, a mobile phase containing methanol as an organic modifier in 40%, 20%, and 10% concentrations, pH 3.30 was used. Afterwards the eluting condition was changed from isocratic to gradient using a flow rate of 1 mL min^{-1}. The final eluting mixture contained water, formic acid, and acetonitrile, as an organic modifier, the final pH being 2.20. The range of oxidation potentials tested was between +0.30 V and +0.55 V.

Anthocyanins contain several phenolic groups attached to the aromatic ring structures and all these groups are electroactive, so that more than one oxidation occurs. The first oxidation of the anthocyanins occurs on the catechol group at +0.30 V, the next at +0.50 V, and the last at +0.80 V (Aaby et al. 2004).

The chromatogram of a mixture of peonidin, cyanin, myrtillin, malvin, kuromanin, and oenin was first recorded at $E_{ap} = +0.30$ V and only two peaks were observed, the first very small, and the second well formed and symmetric. At this applied potential only the first oxidation peak of cyanin and peonidin appears. This potential was not sufficiently high to oxidize all compounds so that higher values of the applied potential were investigated. When a potential of +0.35 V vs. ISAAC was used it was possible to observe the oxidation of almost all compounds in the mixture except for malvidin-3-O-glucoside chloride (oenin) and delphinidin-3-O-glucoside chloride (myrtillin). Both these compounds are oxidised at a more positive

potential, the first oxidation peak of oenin and myrtillin occurs at + 0.40 V. At a potential of +0.40 V both compounds were oxidized and peaks from the oxidation of all the anthocyanins were detected. However, even at + 0.40 V vs. ISAAC the anthocyanin peaks were not very well shaped. The best oxidation potential when all peaks are well shaped was found to be at +0.55 V vs. ISAAC, Fig. 3A. A higher potential of + 0.60 V was also tested, but the time necessary for baseline stabilization was too long. Finally the oxidation potential of + 0.55 V was chosen. The baseline stabilization time is a very important parameter and before each measurement it is necessary to wait for baseline stabilization. When $E_{ap} = +0.35$ V was applied the time was 25 minutes, but for $E_{ap} = +0.55$ V more than 1 hour of stabilization time was needed.

The chromatogram presented in Fig. 3C was recorded with an applied oxidation potential of + 0.45 V, and it is possible to observe that the

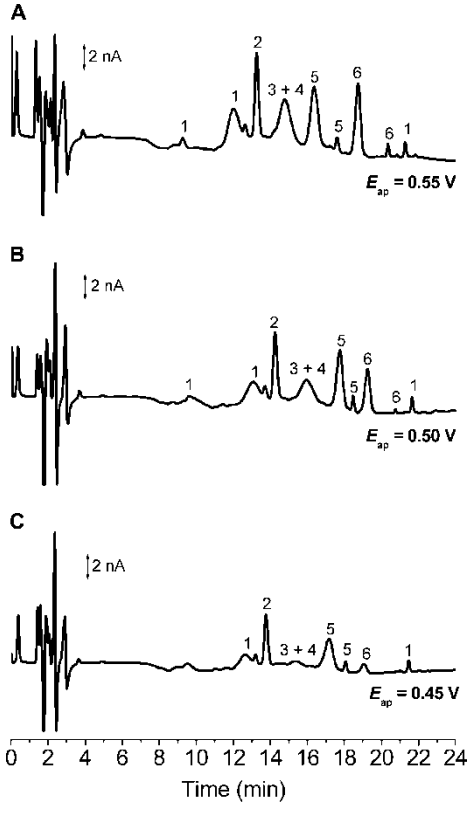


Figure 3. RP-HPLC-ED chromatogram of a mixture of 0.1 μM peonidin [2], and 1 μM cyanin [1], myrtillin [3], malvin [4], kuromanin [5], and oenin [6]. Inertsil ODS-3V column, **A** = E_{ap} = +0.55V, **B** = E_{ap} = +0.50V, and **C** = E_{ap} = +0.45V vs. ISAAC. Conditions as described in the experimental section.

chromatogram contains more peaks than the six compounds injected, the same situation is also found for $E_{ap} = +0.50$ V, Fig. 3B, and $E_{ap} = +0.55$ V, Fig. 3A. As mentioned earlier, the separation and determination of these anthocyanins is studied at pH 2.20, because of the characteristics of the chromatographic column, at which there is more than one form of the anthocyanins. The flavylium cation exists in a quantity larger than the quinoidal base, and there is also a small quantity of chalcone. This situation is very well demonstrated for oenin, for which three peaks can be distinguished. Table 2 shows mean retention times for the anthocyanins analyzed, which corresponds to the retention time for the flavylium cations, the major anthocyanin species for these conditions. Table 2 also shows that the anthocyanins with one glucose group are more slowly eluted than are the diglucoside molecules, in agreement with the literature (Casteele et al. 1983).

Hydrodynamic voltammograms, Fig. 4, were obtained from RP-HPLC-ED at different applied potentials. Except for delphinidin-3-O-glucoside chloride, the current peak increased rapidly in height with increasing applied potential. From the hydrodynamic voltammogram it is also concluded that the best oxidation potential is + 0.55 V vs. ISAAC. Nevertheless, comparing the results in Fig. 3A and 3B, and baseline stabilization time, $E_{ap} = +0.50$ V vs. ISAAC was chosen as the appropriate optimal operating potential for the detection of these anthocyanins, Fig. 3B, and a injection volume of 50 μL.

For chromatographic separation, a linear gradient was used. The conditions are described in the experimental section and Table 1.

The limit of detection was calculated from the calibration curves for all anthocyanins. The data obtained by linear regression for each anthocyanin over the concentration range in which the peak current has a linear response with concentration is presented in Table 2. The limit of detection was 10^{-7} M for the six compounds analyzed. Myrtillin had a higher limit of detection (3×10^{-7} M) and the peak obtained was not well shaped. The highest

Table 2. Linear regression analysis and limits of detection (LOD) using reversed-phase high-performance liquid chromatography with electrochemical detection (RP-HPLC-ED) for anthocyanins ($p < 0.0001$)

Sample	Retention time (min)	LOD (μM)	Linear range (μM)	Standard deviation (μV/μM)	Regression coefficient	n
Cyanin	11.85	0.1	0.1–1	1.81×10^{-3}	0.9998	5
Peonidin	13.10	0.001	0.001–0.2	1.72×10^{-2}	0.9995	9
Myrtillin	14.70	0.3	0.3–3	3.42×10^{-3}	0.9992	5
Malvin	14.85	0.1	0.1–3	2.72×10^{-3}	0.9999	6
Kuromanin	15.50	0.1	0.1–3	2.12×10^{-2}	0.9995	6
Oenin	18.70	0.1	0.1–3	1.32×10^{-2}	0.9995	6

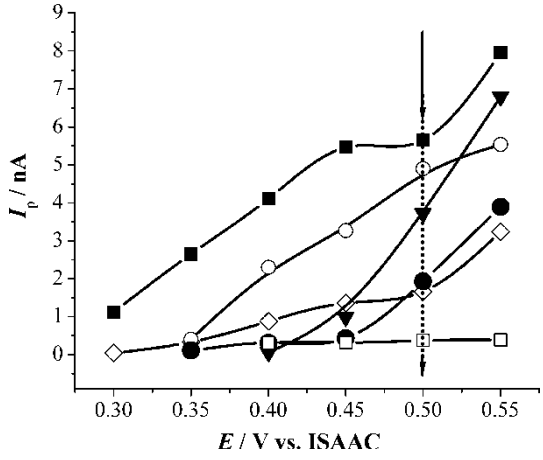


Figure 4. RP-HPLC-ED hydrodynamic voltammograms of 0.1 μM peonidin (■), and 1 μM cyanin (◇), myrtillin (□), malvin (●), kuromanin (○), and oenin (▼). The arrow indicates the optimum working potential section.

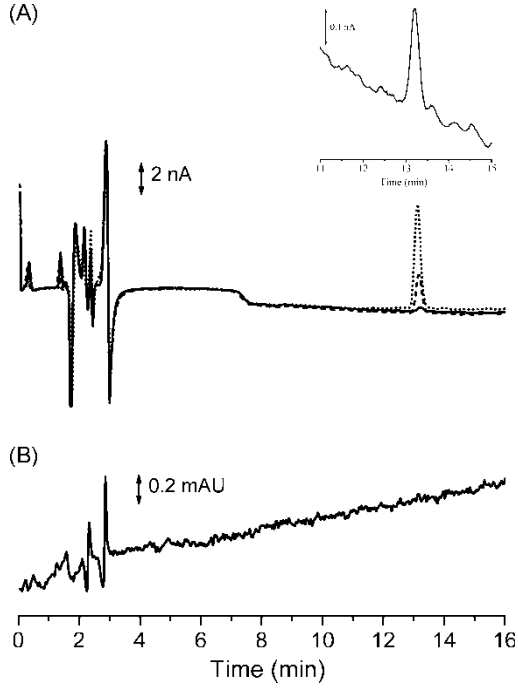


Figure 5. Chromatograms obtained from 50 μL injection of peonidin: A—RP-HPLC-ED, 3 nM (—), 30 nM (---), and 80 nM (···), $E_{ap} = +0.50$V vs. ISAAC. B—RP-HPLC-PDA, 3 nM, at $\lambda = 280$ nm. Inertsil ODS-3V column. Conditions as described in the experimental section.

sensitivity was obtained for peonidine-3-O-glucoside, and the LOD was 10^{-9} M, much lower than for other anthocyanins.

In Fig. 5 the chromatograms for peonidin with PDA and ED detector are compared from which it is clear that the electrochemical detection allowed the determination of a lower concentration. In Fig. 5A the chromatograms obtained from 50 μL injection of a peonidin for three different concentrations are presented. As is well known, a lot of organic compounds can absorb UV and visible light, making analysis in natural samples where the matrix contains several compounds very difficult, but only a few are electroactive. In this respect RP-HPLC with ED is characterized by an excellent sensitivity and selectivity, which is an advantage in comparison with the PDA detector, and RP-HPLC-ED should be of particular relevance to analyze these compounds as they are electroactive.

CONCLUSIONS

The method proposed using reversed-phase high-performance liquid chromatography with electrochemical detection (RP-HPLC-ED) for the determination and separation of anthocyanins, is more sensitive than using a PDA detector. The limit of detection depends on the electrochemical activity of each anthocyanin and also on the electrochemical detector applied potential. All peaks except those of myrtillin and malvin are well separated and characterized and have good reproducibility and correlation. Electrochemical detection has excellent sensitivity and selectivity, and should be of particular interest to analyze antioxidants that are all electroactive. The advantage of selectivity is a very important factor that should be more exploited, especially for the routine analysis of natural samples with complex matrices.

REFERENCES

Aaby, K., Hvattum, E., and Skrede, G. 2004. Analysis of flavonoids and other phenolic compounds using high-performance liquid chromatography with coulometric array detection: relationship to antioxidant activity. *J. Agr. Food Chem.*, 52 (15): 4595–4603.

Berente, B., De la Calle Garcıa, D., Reichenbacher, M., and Danzer, K. 2000. Method development for the determination of anthocyanins in red wines by high-performance liquid chromatography and classification of German red wines by means of multivariate statistical methods. *J. Chromatogr. A*, 871 (1–2): 95–103.

Brenna, O., Buratti, S., Cosio, M.S., and Mannino, S. 1998. A new HPLC method for the determination of polyphenols in wine based on the use of less aggressive eluent and a coupled revelation system. *Electroanalysis*, 10 (17): 1204–1207.

Casteele, K.V., Geiger, H., de Loose, R., and van Sumere, C.F. 1983. Separation of some anthocyanidins, anthocyanins, proanthocyanidins, and related substances by reversed-phase high-performance liquid chromatography. *J. Chromatogr.*, 259: 291–300.

Castro-Gamboa, I., Cardoso, C.L., Silva, D.H.S., Cavalheiro, A.J., Furlan, M., and Bolzani, S.V. 2003. HPLC-EICD: An useful tool for the pursuit of novel analytical strategies for the detection of antioxidant secondary metabolities. *J. Braz. Chem. Soc.*, 14 (5): 771–776.

Janeiro, P. and Oliveira Brett, A.M. 2004. Catechin electrochemical oxidation mechanisms. *Anal. Chim. Acta*, 518 (1–2): 109–115.

Kallithraka, S., Abdel-Azeem Mohdaly, A., Makris, D.P., and Kefalas, P. 2005. Determination of major anthocyanin pigments in Hellenic native grape varieties (Vitis vinifera sp.): association with antiradical activity. *J. Food Compos. Anal.*, 18 (5): 375–386.

Košir, I.J., Lapornik, B., Andrenšek, S., Wondra, A.G., Vrhovšek, U., and Kidrič, J. 2004. Identification of anthocyanins in wines by liquid chromatography, liquid chromatography-mass spectrometry and nuclear magnetic resonance. *Anal. Chim. Acta*, 513 (1): 277–282.

Lee, H.S. and Hong, V. 1992. Chromatographic analysis of anthocyanins. *J. Chromatogr.*, 624 (1–2): 221–234.

Preston, N.W. and Timberlake, C.F. 1981. Separation of anthocyanin chalcones by high-performance liquid chromatography. *J. Chromatogr.*, 214 (2): 222–228.

Revilla, E., Garcıa-Beneytez, E., Cabello, F., Martın-Ortega, G., and Ryan, J.M. 2001. Value of high-performance liquid chromatographic analysis of anthocyanins in the differentiations of red grape cultivars and red wines made from them. *J. Chromatogr. A*, 915 (1–2): 53–60.

Surowiec, I., Orska-Gawryś, J., Biesaga, M., Trojanowicz, M., Hutta, M., Halko, R., and Urbaniak-Walczak, K. 2003. Identification of natural dyestuff in archeological coptic textiles by HPLC with fluorescence detection. *Anal. Lett.*, 36 (6): 1211–1229.

Trojanowicz, M., Orska-Gawryś, J., Surowiec, I., Szostek, B., Urbaniak-Walczak, K., Kahl, J., Rejniak, H., and Wróbel, M. 2004. Chromatographic investigation of dyes extracted from Coptic textiles from collection of National Museum in Warsaw. *Stud. Conserve.*, 49 (2): 115–130.

Zhang, Z., Kou, X., Fugal, K., and Mclaughlin, J. 2004. Comparison of HPLC for determination of anthocyanins in bilberry extracts. *J. Agr. Food Chem.*, 52 (4): 688–691.