Review

Chromatographic analysis of anthocyanins

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ABSTRACT

Anthocyanins are the red, blue and purple pigments responsible for the coloration of many plants. These pigments have been the subject of many studies due to their importance as a quality indicator in foods and as an important chemotaxonomic indicator for plants. Early work with anthocyanins employed paper chromatographic methods. More recently, high-performance liquid chromatography has been widely applied to the study of these pigments. The objective of this paper is to review the chromatographic methods that have been employed in the analysis of anthocyanins with emphasis on the more recent developments in high-performance liquid chromatographic analysis of anthocyanins as applied to food quality measurement.

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## 1. INTRODUCTION

The anthocyanins are responsible for the pleasing red, blue and purple colors of most commonly grown fruit varieties and also in some citrus and tropical fruits. The different colors of the anthocyanins are obtained through extents of hydroxylation, methylation, and glycosylation. Chemically, the anthocyanins are glycosidated derivatives of the 3,5,7,3'-tetrahydroxyflavylium cation (Fig. 1). Glycosidation occurs at the 3, 5 and 7 positions. The non-glycosidated molecule (aglycone) is the anthocyanidin. Anthocyanidins rarely occur in nature and are usually found to occur as an artifact of the isolation process. The most common sugars found are monosaccharides such as glucose, galactose, arabinose and rhamnose. Di- and trisaccharides also occur. In some cases, the sugar moieties are acylated by p-coumaric, caffeic, ferulic or sinapic acids and sometimes by p-hydroxybenzoic, malonic or acetic acids. When present, these acyl substituents are usually bonded to the C-3 sugar [1].

The anthocyanins occur in plants at specific quantitative and qualitative distributions, hence they are very useful as a biochemical plant chemotaxonomic marker and as an index for quality control and quality assurance in fruit and vegetable products [2].

The objective of this article is to present a review of the chromatographic methods used to separate this class of important plant pigments.
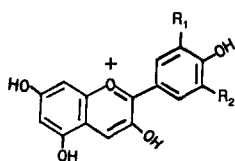
	R₁	R₂
Delphinidin	OH	OH
Cyanidin	OH	H
Petunidin	OCH₃	OH
Pelagonidin	H	H
Peonidin	OCH₃	H
Malvidin	OCH₃	OCH₃

Fig. 1. Structural formulas of the anthocyanins

2. ANALYSIS OF ANTHOCYANINS

2.1. Extraction

The anthocyanins are generally very soluble in water and can be easily extracted with polar solvents. Most workers employ acidified alcoholic solvents such as dilute (1%) HCl in methanol for extracting anthocyanins from plant materials [3,4]. Acidification is necessary to prevent oxidation, as anthocyanins are unstable at neutral and alkaline pH [5].

2.2 Sample clean-up

A number of methods have been succesfully used to clean-up crude anthocyanin extracts. They include solid-phase extraction with insoluble polyvinylpyrrolidone (PVP) [3,6–8], octadecylsilane [9–12], Sephadex G-25 [11], Sephadex LH-20 [11,12], polyamide [12,13], ion-exchange resins [4,14–18], acid alumina [19], precipitation with basic lead acetate [20,21] and solvent–solvent extraction with n-butanol [17,22]. The purification through solid-phase extraction is relatively simple. The procedure involves application of crude extracts of anthocyanins to a column or small, disposable cartridge of adsorbent and subsequent sequential elution of individual components with appropriate solvents. The anthocyanins possessing a number of unsubstituted hydroxyl groups (Fig. 1) or a sugar are strongly bound onto the adsorbents. Acidified methanol [6,7,9–12] is generally a suitable solvent for elution of anthocyanins from polymeric adsorbents.

2.3. Paper and thin-layer chromatography

Almost all of the pioneering work in the area of identification and characterization of anthocyanins has been performed with paper chromatography (PC). The literature is replete with information on solvent systems and R_F values for most of the known anthocyanins. Thin-layer chromatography (TLC) has been an attractive alternative to paper chromatography. Different stationary phases can be employed, thus opening up the possibility of many new separation mechanisms. TLC has the advantage in time over paper methods, but suffers from the disadvantage of low sample loading. Unfortunately, R_F values are not as reliable in TLC as with PC because of the differences in layer thickness

from plate to plate. In most cases, reference compounds are needed to confirm identification [23]. The paper methods are well entrenched and are unlikely to be replaced entirely by TLC methods [24].

There are a number of papers which give an excellent discussion of the uses of the various solvent systems which have been successfully used for separation of anthocyanins. The reader is referred to refs. 4, 18, 20 and 24–27 for a detailed discussion of the topic and PC and TLC.

2.4. Droplet counter-current chromatography

Counter-current chromatography (CCC) shares the same technologies as high-performance liquid chromatography (HPLC) such as pump, injector and detector except for different separation devices, columns. Although CCC could replace expensive, reversed-phase preparative-scale HPLC, it has not attracted a large number of users from industry and research involved in anthocyanin pigments. Francis and Andersen [28] described the use of droplet CCC (DCCC) for separation of the anthocyanins from black currants and raspberries using n-butanol, acetic acid and water as the solvent. These authors demonstrate that DCCC is a viable method for semi-preparative isolation of anthocyanins from berries.

2.5. Electrophoresis

The anthocyanins are ionic in nature (pH dependent) and would therefore be expected to be mobile in an electric field. Electrophoresis however, has found little use in the field of anthocyanin separations as it offers little or no advantage over PC. In acetate buffer, the anthocyanins do not migrate far. Ionization of the anthocyanins by alkali causes oxidative decomposition with air [23]. Successful results have been reported for paper electrophoresis using 0.1 M citric acid at pH 2 as an electrolyte [29]. Osawa et al. [30] obtained excellent results using cellulose acetate film. More recently, Tsuda and Fukuba [31] reported successful separation of anthocyanins by electrophoresis when using a Triton X-100/AlCl₃ containing electrolyte system.

2.6. Open column chromatography

In search of methods for larger-scale separation and quantitation of individual anthocyanins, column chromatographic methods have been developed. A number of column support materials have

been tried without success, including aluminium oxide, cellulose powders, ion exchange resins and Sephadex gel. In most cases, sample matrix components were found to interfere with the separation. Polyamide powders showed good retention but chromatographic resolution was poor. The most success has been obtained with PVP [23].

2.7. Gas chromatography

Anthocyanins exhibit limited volatility and therefore require derivatization prior to gas chromatographic (GC) analysis. The most success has been achieved with reaction of the anthocyanins with trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS). The result is a nitrogen-containing derivative which after injection into the GC system further transforms into a quinoline derivative. The quinoline derivative yields sharp peaks when chromatographed [32]. Despite the excellent results obtained by previous workers using GC, derivatization in general introduces problems of stability in the anthocyanins and thus, future development is likely to be in the direction of HPLC rather than GC [24].

2.8. High-performance liquid chromatography

HPLC is the mainstay of the separation technique in food analysis, especially for water-soluble, non-volatile, thermally labile anthocyanins. The most success for separation of both anthocyanidins and anthocyanins has been with reversed-phase HPLC (RP-HPLC). This methodology offers excellent separations along with high sensitivity and relatively short analysis time (especially when compared with PC methods). HPLC with the combination of electrochemical detection (ED) and a UV–VIS or photodiode array detection (PAD) system makes structural characterization of the pigments possible [33]. The remainder of this paper focuses on the use of HPLC for separation of the individual anthocyanins in plant products (anthocyanin profile) and its application in food analysis.

3. ANTHOCYANIN PROFILE AND ITS APPLICATIONS IN FOODS

3.1. Applications in characterization of food anthocyanins

Anthocyanin pigments in many foods have been characterized by PC [1,4,7,17,18,34–45], TLC [6,7,

9,44,47–52] and more recently HPLC [2,3,9,12,13, 53–70]. Basically similar liquid chromatographic methods have been reported in the literature by many analysts and examples of HPLC methods that can be used for analysis of each of the anthocyanins from apple, bilberry, black currant, blackberry, black cherry, blueberry, blood orange, bog whortleberry, chicory, chokeberry, cowberry, crowberry, cranberry, elderberry, grape, grape colorants, lychee, raspberry, red currant, roselle, red wine, strawberry, European cranberry (*Vaccinium oxycoccus*) and *Vaccinium japonicum* fruits are summarized in Table 1.

In most cases, the reported systems were carried out on reversed-phase chromatography on silica-based ODS (C_{18} bonded phases) columns. The average particle diameter of HPLC packings is typically between 3 and 10 μm. HPLC analysis with columns of smaller particles (*e.g.* 3 μm) permit faster separations compared with columns of larger particles. However, 5–6-μm particles generally represent a good compromise in terms of convenience, performance and column lifetime. Also, silica columns bonded with octyl (C_8) and hexyl (C_6) were used in chicory [71], red wines [56], grape [58] and in bilberry fruits [72].

Gradient elution seems ideal for separating anthocyanins which are structurally very similar. Most of the solvent systems used in analytical HPLC include binary gradient elutions with methanol or acetonitrile as organic modifiers, and occasionally isocratic elution for fruits which have a relatively simple pigment pattern such as cranberry (Table 1). Ternary gradient elution was also developed for the separation of complex mixtures of anthocyanins in *V. rotundifolia* grapes [73]. Twenty-five anthocyanins including mono- and diglucosides of acylated and non-acylated anthocyanins in varying quantities were separated from *V. rotundifolia* hybrid grapes in 80 min. Because of the high viscosity of methanol–water mixture, acetonitrile was preferred [62]. Nagel and Wulf [74] substituted acetone for methanol to obtain a similar separation. A linear gradient using 5–20% acetone in place of the methanol separated 16 different anthocyanins from Cabernet Sauvignon [74]. Drdak *et al.* [68] determined that alkylamines as mobile phase additives and butylamine (0.122 *M*) in mobile phase with gradient elution provided better resolution and less re-

tention of red wine anthocyanins. In particular, the nature of the organic modifier in the mobile phase had a dramatic influence on the separation of anthocyanin compounds. There was appreciable improvement in selectivity values for the separation of Cy-3-glurut, Cy-3-glc and Pg-3-soph (see Table 1 for abbreviations) as the organic modifier was changed from 100% methanol to 15% acetic acid in methanol [75].

The elution strength of mobile phase (water–acetonitrile–formic acid, 81:9:10) for chromatography of red fruits containing monoglycosylated anthocyanins such as bilberry, black currant, strawberry, blackberry, black cherry and morello cherry needed to be lowered to water–acetonitrile–formic acid (84:6:10) to improve the resolution of diholoside or triholoside anthocyanins from red currant, raspberry and elderberry [63].

Solvent systems for HPLC analysis of anthocyanins always include an acid to ensure that the anthocyanins are in the red flavylium cation form. Formic acid up to 10% (w/v) is most commonly used with reversed-phase columns which corresponds to pH about 1.9 [63]. Other acids such as acetic acid [57,73,75–80], phosphoric acid [9,11,54,81,82], trifluoroacetic acid [83] and perchloric acid [58,68,71] have also been used. However, extensive use of solvents more acidic than pH 2 could result in poor reproducibility and short column lifetime due to the loss of bonded phases from the surface of the silica stationary phase support. For this reason, non-silica polymer columns based on polystyrene were utilized [9,11,54,57,82]. Non-silica, polymeric columns, which are stable from pH 1 to 13, allowing their use with mobile phases at a pH close to which the anthocyanins are nearly entirely in their flavylium cation form, can produce sharp peaks.

Detection is usually carried out between 520 and 546 nm in the visible range. All the major anthocyanins have been identified based on acid hydrolysis, partial hydrolysis and subsequent determination of sugars and aglycone, spectral analysis, and co-chromatography with known available anthocyanins. Spectral acquisition was facilitated by applying on-line PAD for each peak eluting from HPLC columns [9,11,53,54]. Elucidation of structure, especially for the acylated anthocyanins, was further confirmed by fast atom bombardment mass spec-

trometry and NMR spectroscopy [12,58,71,84–87].

In an alternative method for the isolation of the anthocyanins of interest [2,58,88], a preparative HPLC system similar to that used for the analytical separation enabled isolation of pure samples of anthocyanins for use in structural studies. Hicks *et al.* [2] demonstrated that it was possible to collect multimilligram quantities of pure anthocyanins in one continuous 24-h period from blackberry and cranberry with preparative HPLC under binary gradient condition using a mobile phase consisting of 0.1 *M* phosphate buffer, pH 1.5 and acetonitrile. Sapers *et al.* [88] injected 250 μl of blackberry extract containing 15 mg of solid on Rainin Dynamax C_{18} preparative column to collect four anthocyanin peaks. Most of the semi-preparative HPLC columns are wide bore such as 10 mm [89], 16 mm [58,71] or 22 mm [2,88], and flow-rate was increased up to 14 ml/min [2].

For HPLC of anthocyanidins, isocratic conditions [3,9,63,75,78,90] are preferred because of the simplicity in commonly occurring anthocyanidins in nature and the retention of the aglycone moiety is correlated with the hydrophobicity of the molecule. RP-HPLC on a μBondapak C_{18} column (300 × 4.0 mm) with mobile phase of water–acetic acid–methanol (71:10:19) with 2 ml/min elution could resolve six anthocyanidins in 30 min [3]. Since anthocyanidins do not occur in the free form but are usually glycosylated, an acid hydrolysis step is required before analysis. As usual in RP-HPLC, the elution order is according to their polarity, delphinidin < cyanidin < petunidin < pelagonidin < peonidin < malvidin [3,63].

3.2. Applications for verification and classification of cultivars

In most of the work with anthocyanin pigmented fruits such as blueberries [43,48], raspberry [13,40,47,91], blackberry [91] and black grape [41,46], the distribution of individual anthocyanin pigments was determined by conventional techniques such as TLC and densitometry to delineate any quantitative or qualitative differences in cultivars. However, where a chemical marker is present in one plant and absent in the other, such qualitative differences make a positive identification easy. Unfortunately, at the cultivar level most of the differences found have been quantitative, and these

conventional techniques can not always be easily adapted to quantitation. Especially with PC, quantitation must be regarded as estimated and relative because of the possible preferential anthocyanin binding to the paper chromatogram during development and elution and varying stability of the anthocyanin in the solvent systems used [43]. Furthermore, anthocyanins are not always completely separated. Cyanidin-3-glucoside is virtually impossible to separate from cyanidin-3-galactoside by column or TLC [64]. Also, although traces of other anthocyanins often were present, only major anthocyanins were identified.

For chemotaxonomy, HPLC techniques offer several advantages over TLC, especially to reveal the differences in relative percentages of individual anthocyanins and make it possible to distinguish relevant cultivars [64,92]. Applications of analytical HPLC in classification of some of fruit cultivars [58,73,75,80,93–95] are included in Table 1 and Fig. 2. Sapers *et al.* [94] developed the binary gradient elution with 15% acetic acid in water (solvent A) and 15% acetic acid in methanol (solvent B) to distinguish both qualitative and quantitative differences in anthocyanins between 11 relevant blueberry cultivars [94]. A rather complex gradient elution pattern was developed for 12 cranberry cultivars. A total of 22 peaks were separated in 60 min using the two C_{18} analytical columns (Nova-Pak and μBondapak C_{18} from Waters) connected in series and eluted with ternary solvent systems of aqueous formic acid, methanol and acetonitrile mixtures [95].

Besides the above applications, HPLC analysis of anthocyanin profile has also been used in red wines [56,58] for geographical classification. Wine color, in many cases, can be related to the quality and quantity of their anthocyanins. Recently, Santos *et al.* [96] used HPLC-PAD analysis of anthocyanin profiles and HJ biplot statistical analysis to differentiate 48 young red spanish wines according to their origin. The 18 anthocyanins were resolved by gradient elution (solvent A = 4.5% formic acid in water, solvent B = acetonitrile) with initial mobile phase composition of 10% B to a final composition of 30% in the same solvent system over a period of 37.5 min at a flow-rate of 1.5 ml/min. The non-acylated/acylated anthocyanin ratio was one of the best parameters to discriminate between the relevant origins.

TABLE 1

COMPILATION OF REFERENCES ON THE SEPARATION OF ANTHOCYANINS BY RP-HPLC

Abbreviations: ac = acetyl; ara = arabinoside; caf = caffeyl; cm = coumaryl; Cy = cyanidin; Dp = delphinidin; fer = ferulyl; gal = galactoside; glc = glucoside; glcrut = glucosylrutinoside; mal = malonyl; Mv = malvidin; Pg = pelagonidin; Pn = peonidin; Pt = petunidin; rut = rutinoside; samb = sambubioside; sin = sinapyl; soph = sophoroside; xylrut = xylosylrutinoside.

Source	Anthocyanins	Stationary phase	Mobile phase	Ref.
Apple	Cy-gal,ara	μBondapak C_{18}	Water–acetic acid– methanol (71:10:19)	76
Bilberry	Dp,Cy-gal,glc Pt-gal,glc Pn-glc	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
	Dp-gal,glc,ara Cy-gal,glc,ara Pt-gal,glc,ara Pn-gal,glc,ara Mv-gal,glc,ara	LiChrospher RP-18	Water–acetonitrile– formic acid (81:9:10)	63
	Dp-gal,glc,ara Cy-gal,glc,ara Pt-gal,glc,ara Pn-gal,glc,ara Mv-gal,glc,ara	Aquapore RP-300, C_8	Binary gradient (A) 10% formic acid (B) methanol–acetonitrile– water–formic acid (22.5:22.5:45:10)	72
Black currant	Dp,Cy-glc,rut	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
	Dp,Cy-glc,rut	LiChrospher RP-18	Water–acetonitrile– formic acid (81:9:10)	63
	Cy,Dp-glc,rut	Spherisorb ODS	Binary gradient (A) 0.5% trifluoroacetic acid (B) methanol	83
Blackberry	Cy-glc,rut,soph Cly-glcrut	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
	Cy-glc,rut	LiChrospher RP-18	Water–acetonitrile–formic acid (81:9:10)	63
	Cy-glc,rut	Resolve C_{18}	Binary gradient (A) 0.1 M potassium dihydrogenphosphate (B) acetonitrile	88
Blueberry	Cy-gal,glc,ara Dp-gal,glc,ara Pt-gal,glc,ara Pg-gal,glc,ara Mv-gal,glc,ara	HS-5 C_{18}	Binary gradient (A) 10% formic acid (B) acetonitrile	93
	Cy-gal,glc,ara Dp-gal,glc,ara Pt-gal,glc,ara Pg-gal,glc,ara Mv-gal,glc,ara	μBondapak C_{18}	Binary gradient (A) 0.1 M potassium dihydrogenphosphate (B) acetonitrile	94
Bog whortle berry	Dp-gal,glc,ara Cy-gal,glc,ara Pt-gal,glc,ara Pn-gal,glc,ara Mv-gal,glc,ara	Supelcosil LC-18	Binary gradient (A) 10% formic acid (B) formic acid–water– methanol (10:40:50)	66

TABLE 1 (*continued*)

Source	Anthocyanins	Stationary phase	Mobile phase	Ref.
Cherry	Cy-glc,rut	LiChrospher RP-18	Water–acetonitrile–formic acid (81:9:10)	63
	Cy-glc,rut Cy-soph,glcrut	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
Chicory	Cy-glc-mal	Spherisorb C_6	Binary gradient (A) 0.6% perchloric acid (B) methanol	71
Chokeberry	Cy-gal,glc,ara,xyl	Spheri-5 RP-18	Binary gradient (A) 10% formic acid (B) formic acid–water–acetonitrile (10:60:30)	69
Cranberry (*V. macrocarpon*)	Cy-gal,ara Pn-gal,ara	Resolve C_{18}	Binary gradient (A) 0.1 *M* potassium dihydrogenphosphate (B) acetonitrile	2
	Cy-gal,glc,ara Pn-gal,glc,ara	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	9
	Cy-gal,ara Pn-gal,ara	μBondapak C_{18}	Water–acetonitrile–acetic acid–orthophosphoric acid (81.7:8.4:8.4:1.5)	80
	Cy-gal,ara Pn-gal,ara Cy-glc	PLRP-S	Binary gradient (A) 10% acetic acid (B) methanol–acetic acid–water (60:10:30)	57
	Cy-gal,ara Pn-gal,ara	μBondapak and Nova-Pak C_{18}	Ternary gradient (A) 4.5% formic acid (B) methanol–formic acid–acetonitrile (55:33:10) (C) methanol–formic acid–acetonitrile (55:35:10)	95
Cranberry (*V. oxycoccus*)	Cy-gal,glc,ara Pn-gal,glc,ara Dp,Pt,Mv-glc	Supelcosil LC-18	Binary gradient (A) 10% formic acid (B) formic acid–water–methanol (1:4:5)	125
Cowberry	Dp-glc Cy-gal,glc,ara	Hypersil ODS	Binary gradient (A) 10% formic acid (B) formic acid–water–methanol (10:40:50)	67
Crowberry	Dp,Pt-gal,glc Cy,Pn,Mv-gal,ara Dp,Pt-ara	μBondapak C_{18}	Water–methanol–formic acid (74:16:10)	111
Elderberry	Dp-gal,glc,rut Cy-glcrut	LiChrospher RP-18	Water–acetonitrile–formic acid (84:6:10)	63
	Cy-glc,samb Cy-sambglc Cy-diglc	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54

TABLE 1 (*continued*)

Source	Anthocyanins	Stationary phase	Mobile phase	Ref.
Grape *(V. rotundi folia)*	Cy,Pn,Dp-glc Pt,Mv-glc Mv-glc-ac Cy,Pn,Pt,Mv-glc-cm Cy,Pn,Dp,Pt,Mv-diglc Dp,Cy,Pn,Pt,Mv-glc-cm-glc	LiChrosorb RP-18	Ternary gradient (A) 15% acetic acid (B) water–acetic acid– methanol (65:15:10) (C) methanol	73
	Dp,Cy,Pt,Pn,Mv-diglc Dp-glc	HS-5 C_{18}	Binary gradient (A) 10% formic acid (B) acetone–formic acid– water (25:10:65)	65
Grape *(V. vinifera)*	Dp,Cy,Pn-glc Pt,Mv-glc Mv-glc-ac Cy,Pn,Pt,Mv-glc-cm Cy,Pn,Dp,Pt,Mv-diglc Dp,Cy,Pn,Pt,Mv-glc-cm-glc	Spherisorb C_6	Binary gradient (A) 0.6% perchloric acid (B) methanol	58
	Dp,Cy,Pt,Pn,Mv-glc Dp,Cy,Pt,Pn,Mv-diglc Dp,Cy,Pt,Pn,Mv-glc-cm Dp,Cy,Pt,Pn,Mv-glc-cm-glc	μBondapak C_{18}	Binary gradient (A) 15% acetic acid (B) water–acetic acid– methanol (65:15:20)	61
	Dp,Cy,Pt,Pn,Mv-glc Dp,Cy,Pt,Pn,Mv-glc-ac Dp,Cy,Mv,Pt,Pn-glc-cm Mv-glc-caf	LiChrosorb ODS	Binary gradient (A) 10% formic acid (B) formic acid–methanol– water (10:50:40)	13
	Cy,Pn,Dp-glc Pt,Mv-glc Cy,Dp,Pt-glc-cm Pn,Mv-glc-cm	Spherisorb ODS	Binary gradient (A) 3.5% orthophosphoric acid (B) acetonitrile	81
	Dp,Cy,Pt-glc Dp,Cy,Pt-glc-ac Pn,Mv-glc-ac Dp,Cy,Pt-glc-cm Pn,Mv-glc-cm Pn,Mv-glc-caf	μBondapak C_{18}	Binary gradient (A) 4.5% formic acid (B) acetonitrile	53
Grape skin extract (Spreda)	Dp,Cy,Pt-glc Pn,Mv-glc	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
Grape colorants (Welch)	Dp,Cy,Pt-glc Pn,Mv-glc	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
Enocyanin (Minot)	Dp,Cy,Pt-glc	PLRP-S	Binary gradient (A) 10% acetic acid (B) methanol–acetic acid–water (60:10:30)	57
Plum	Cy-glc,rut	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
Raspberry (black)	Cy-samb,glc,rut Cy-xylrut	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54

TABLE 1 (*continued*)

| Source	Anthocyanins	Stationary phase	Mobile phase	Ref.
Raspberry (red)	Cy,Pg-glc Cy,Pg-soph Cy,Pg-glcrut	Supelcosil LC-18	Binary gradient (A) 15% acetic acid (B) 15% acetic acid in methanol	75
	Dp-gal,glc,rut Cy-glcrut Cy-gal	LiChrospher RP-18	Water–acetonitrile– formic acid (84:6:10)	63
	Cy-glc,rut Cy,Pg-soph Cy,Pg-glcrut	Supelcosil LC-18	Binary gradient (A) 15% acetic acid (B) acetonitrile	77
Red currant	DP-gal,glc,rut,ara Cy-gal,glc,rut,ara Cy-glcrut Pt,Pg-gal	LiChrospher RP-18	Water–acetonitrile– formic acid (84:6:10)	63
Roselle	Cy,Dp-samb Dp,Cy-glc	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	9
Strawberry	Cy,Pg-glc Pg-rut Pg-glc-ac	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	9
	Cy-glc Pg-gal,glc,ara	LiChrospher RP-18	Water–acetonitrile– formic acid (81:9:10)	63
Red wines	Dp,Cy,Pt,Pn,Mv-glc Dp,Cy,Pt,Pn,Mv-glc-ac Dp,Cy,Pt,Pn,Mv-glc-cm	MicroPak MCH-5C_{18}	Binary gradient (A) 5% formic acid (B) methanol	126
	Dp,Cy,Pt,Pn,Mv-glc Dp,Cy,Pt,Pn,Mv-glc-ac Dp,Pn,Mv-glc-cm	CGX C_{18}	Binary gradient (A) 10% methanol–perchloric acid 0.16 M– butylamine 0.122 M (B) 90% methanol–perchloric acid 0.16 M– butylamine 0.122 M	68
	Cy,Dp,Pt-glc Pn,Mv-glc Mv-glc-cm Mv-glc-ac	LiChrosorb ODS	Binary gradient (A) 10% formic acid (B) acetone–formic acid– water (25:10:65)	74
Lychee	Cy-glc Cy-rut Mv-glc-ac	PLRP-S	Binary gradient (A) 3.5% orthophosphoric acid (B) acetonitrile	11
Blood orange	Dp,Cy,Pn-diglc Dp,Cy-glc Cy-glc-ac Cy-glc-fer Cy-glc-cmfer Cy-glc-sin Pn-glc-cm	μBondapak C_{18}	Binary gradient (A) 15% acetic acid (B) water–acetic acid– methanol (65:15:20)	79
	Dp,Cy,Pt,-diglc Pg,Pn-diglc Dp,Cy, Pg, Pt-glc Cy-glc-ac	μBondapak C_{18}	Binary gradient (A) 15% acetic acid (B) water–acetic acid– methanol (65:15:20)	78
V. japonicum	Cy,Pg-ara Cy,Pg-gal	Supelcosil C_{18}	Binary gradient (A) 10% formic acid (B) formic acid–water– methanol (1:4:5)	89
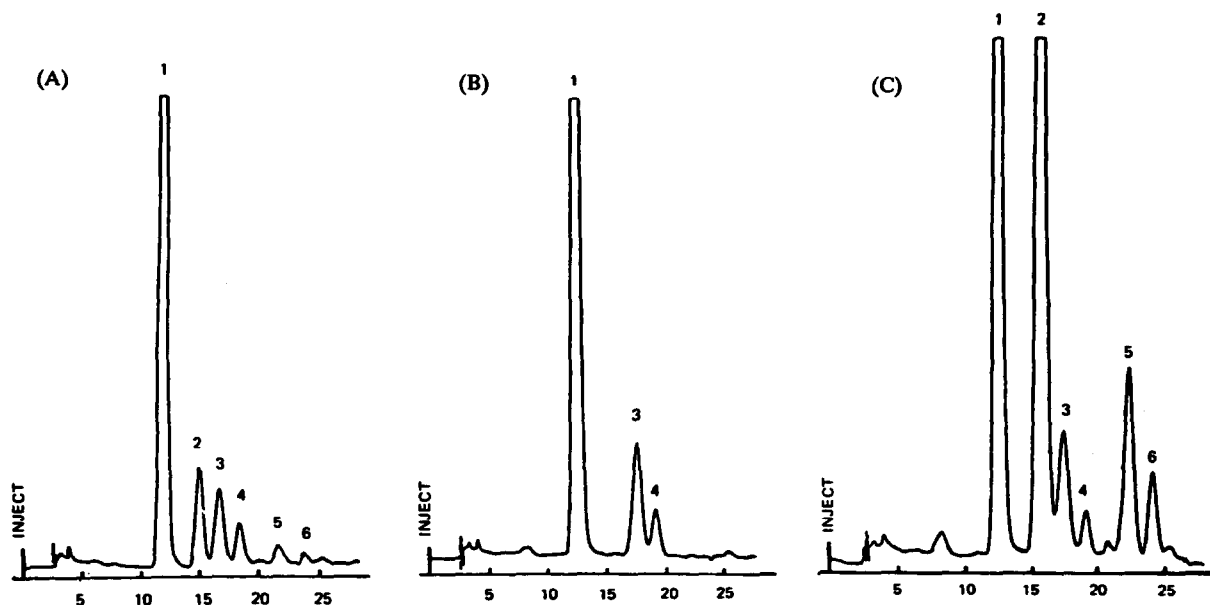
Fig. 2. HPLC of anthocyanins from red raspberries: (A) Meeker variety (Oregon); (B) Willamette variety (Oregon); (C) Marcy variety (New Zealand). Peaks (For abbreviations see Table 1): 1 = Cy-3-soph; 2 = Cy-3-glcrut; 3 = Cy-3-glc; 4 = Pg-3-soph; 5 = Cy-3-rut; 6 = Pg-3-glcrut. (Adapted with permission from ref. 75). Time scale in min.

3.3. Applications for changes in anthocyanins during ripening, processing and storage of fruit products

Very significant changes such as the accumulation of anthocyanins take place during the maturation of numerous red fruits. Quantitative determination of individual anthocyanins gave a great insight into the development of anthocyanins in the maturing red tart cherry [97], thornless blackberry fruits [88] and red grape [81]. Dekazos [97] reported the use of AG 50W-X4 (H$^+$ form), a cation-exchange resin, to purify cherry anthocyanins. Most of free sugars and organic residues were removed by washing with water, and methanol, respectively. Anthocyanin pigments were eluted by acidified methanol (0.1% to 1% HCl in methanol) and PC. There have been a few applications of HPLC in the investigation to determine the effects of ripening on the accumulation of individual anthocyanins. Sapers et al. [88] used binary gradient elution on a Resolve 5-μm C$_{18}$ column using 0.1 M potassium dihydrogenphosphate (pH 2.0) and concave (program No. 7 of the Waters solvent programmer) gradient elution from 12% to 20% acetonitrile in 25 min to determine the effect of ripening on anthocyanin patterns in thornless blackberry fruit.

Anthocyanins are very reactive and lack stability during processing and storage. The rapid degradation of the attractive red color of freshly made blackcurrant juices [83], strawberry preserves [98], cranberry cocktail [99], canned plums [100] and red raspberry juices [77] during storage has been a concern to food processors. Numerous workers have applied chromatographic analysis to estimate the losses of red anthocyanin pigments during processing and storage of fruits [76,82,101–104], and during fermentation and subsequent aging periods of red wines [74,77]. Also, applications which have been extended to evaluate many factors such as temperature, light, pH, mold, 5-hydroxymethylfurfural, furfural and sugar on anthocyanin stability [53,105–110] and to compare the influences of structural variations of the different anthocyanins on their relative rate of reactivity [90,111–115] with other component have been reported. The pH differential method was readily applicable to the studies of determination of anthocyanin pigments [90, 98,105,108–110,114] during processing and storage. Although the method is not specific for individual pigments, it provides quantitative estimations of the total concentration of pigments, which reflects the
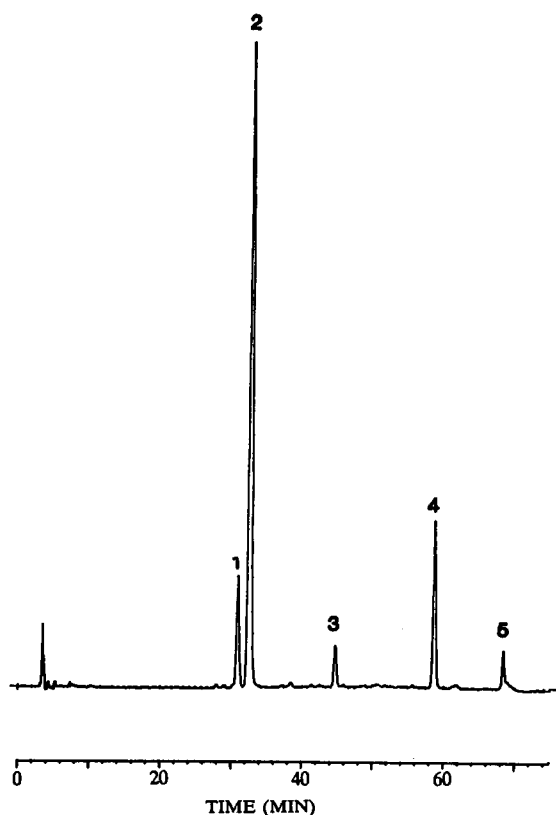
Fig. 3. HPLC of anthocyanins from lychee. Peaks: 1 = Cy-3-glc; 2 = Cy-3-rut; 3 = cyanidin; 4 = Mv-3-glc-ac; 5 = unknown. (Adapted from ref. 11).

quality of anthocyanin-containing food products.

Typical conditions for HPLC applications for anthocyanin pigment stability in manufactured fruit products [77,83] and red wines [74] are summarized in Table 1. Binary gradient HPLC analysis (Fig. 3) was applied for the quantitative determination of changes in Cy-3-glc, Cy-3-rut and Mv-3-glc-ac contents in tropical lychee fruit [11,82] during refrigerated storage conditions.

3.4. Applications for detection of adulteration of fruit juices

The distinctive anthocyanin pigment profile has been a useful tool for the verification of authenticity in fruit juice products which are rich in anthocyanin pigments, [116,117], and detecting the adulteration of red wines of V. vinifera with wines made from hybrid grapes [118]. For example, red raspberry has

been reported to have only cyanidin and pelagonidin glycosides [75]. If chromatographic analysis reveals the presence of malvidin, delphinidin, peonidin or petunidin glycosides from commercial raspberry juice samples, it can be used to confirm and pinpoint adulteration with other colored fruits.

PC of anthocyanin pigment has been used to detect adulteration of concord grape juice with other anthocyanin containing products [119,120]. This method has been applied to several dark colored fruit juices, such as blackberry, black raspberry and black cherry juices to detect adulteration with cheaper juices based on anthocyanin or anthocyanidin patterns [121]. A similar approach was used recently by Kaack [122] for fruit juice adulteration. In his procedure, semi-quantitative direct spectrophotometric determination of paper strip at 530 nm was employed to resolve the problem of visual examination from paper chromatograms, which might not see the dinstinct differences in quantity.

Analysis of anthocyanins by HPLC proved to be easier to interpret than the qualitative patterns in paper or thin layer chromatography in determination of juice adulteration. Most of the reported work has been done with cranberry juice products since cranberry has a unique pattern of anthocyanins [116] and is relatively expensive, making economic incentives for adulteration attractive [57]. Fast HPLC separation using a 5 cm long analytical column was adopted for the verification of cranberry juice cocktail [116]. Analysis was completed in about 15 min by RP-HPLC using a mobile phase of water–acetonitrile–acetic acid–orthophosphoric acid (81.7:8.4:8.4:1.5) on a Supelcosil C_{18} column (5 μm). Two major anthocyanins such as cyanidin-3-galactoside and peonidin-3-galactoside, and two minor anthocyanins of cyanidin-3-arabinoside and peonidin-3-arabinoside were well separated.

For more simple chromatographic analysis, HPLC anthocyanidin analysis of cranberry juice products, which requires acid hydrolysis of anthocyanin glycosides in sample preparation, was elected [123]. Only cyanidin (57%) and peonidin (43%) were found in the cranberry juice samples [124]. Fig. 4 presents two representative anthocyanidin chromatograms showing separations of authentic cranberry juice (Fig. 4A) and adulterated juice (Fig. 4B). Mobile phase used was water–acetic acid–methanol–acetonitrile (70:10:10:10). The presence of sub-
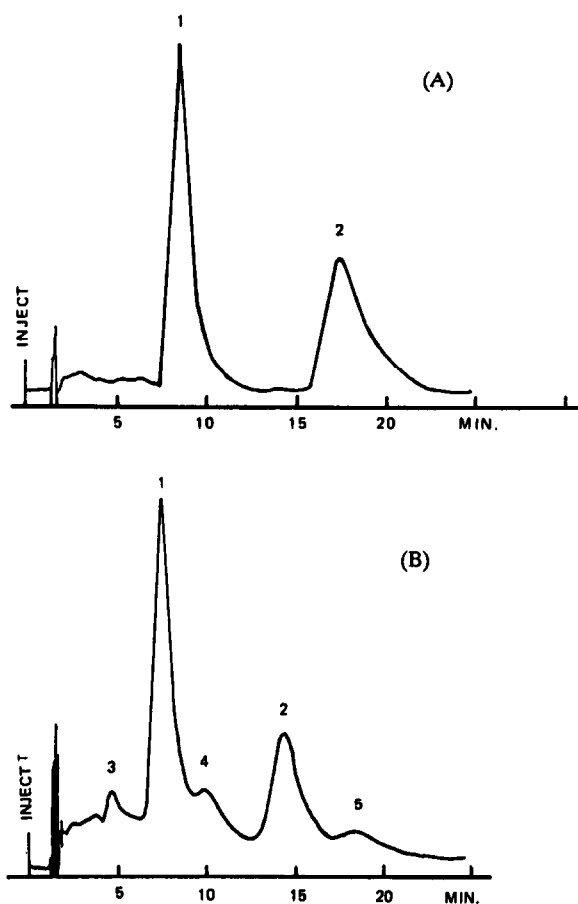
Fig. 4. HPLC of anthocyanins from cranberry juices. (A) Authentic cranberry juice; (B) adulterated commercial cranberry juice drink. Peaks: 1 = cyanidin; 2 = peonidin; 3 = delphinidin; 4 = petunidin; 5 = malvidin. (Adapted from ref. 124).

stantial quantities of delphinidin, petunidin and malvidin in the commercial samples reveals that an anthocyanin-containing colorant has been added. RP-HPLC analysis using acetic acid–water (10:90) (A) and methanol–acetic acid–water (60:10:30) (B) gradient with gradient time of t_G = 30 min was also applied to detect adulteration of cranberry juice by enocyanin, a grape skin colorant made usually as a by-product of wine making [57]. Reversed-phase separation was carried out with a C_{18} column made from polystyrene (PLRP-S, 150 × 4.6 mm from Polymer Lab.). The pH of the mobile phase was maintained at 1.3 by the addition of 3% phosphoric acid.

4. CONCLUSIONS

This article presents a brief review of analysis of anthocyanins as well as applications in food analyses. Discussion was focused on the use of HPLC for separation of individual anthocyanins. No attempt was made to give a coverage of the literature relating to anthocyanin pigments. The advent of a highly selective and sensitive mode of detection using HPLC for easy structural identification of anthocyanins where standards generally are not available will undoubtly lead to further advances in characterization of anthocyanins in fruits and berries.

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