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Rapid characterization of anthocyanins in red raspberry fruit by high-performance liquid chromatography coupled to single quadrupole mass spectrometry

William Mullen[a], Michael E.J. Lean[b], Alan Crozier[a,*]

[a]*Plant Products and Human Nutrition Group, Graham Kerr Building, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK*

[b]*University of Glasgow Department of Human Nutrition, Queen Elizabeth Building, Royal Infirmary, Glasgow G31 2ER, UK*

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Abstract

Anthocyanins from red raspberries were extracted from the fruit by homogenizing in acidified methanol. The methanolic extract was centrifuged and the supernatant analyzed by reversed-phase HPLC. The eluent was monitored at 371 and 520 nm before being introduced into a single quadrupole mass spectrometer through an atmospheric pressure chemical ionization probe operating in positive ion mode. This method allowed the identification of eight anthocyanins. In the absence of readily available reference compounds, approaches that can be taken to analyse anthocyanins by HPLC with absorbance and mass spectrometric detection are discussed.

Keywords: Fruits; Anthocyanins

1. Introduction

Anthocyanins are best known as the compounds responsible for the red color of many fruits. A number of chromatographic techniques, including thin layer chromatography (TLC) [1] and high-performance liquid chromatography (HPLC) [2], have been employed to investigate the distribution and anthocyanin content of raspberries. However, it is the choice of detector that has received most attention. Hong and Wrolstad [3] used HPLC coupled to photo diode array detection (DAD) to analyse anthocyanins in a range of fruits and berries. The diode array absorbance spectra can be used to distinguish not only between different anthocyanins, but also whether they are glycosylated at the 3- or 3,5-positions. Furthermore, information regarding the presence of acylation by hydroxylated aromatic organic acids can also be obtained from absorbance spectra. However, DAD is unable to distinguish between compounds with close retention times and similar absorbance spectra [4]. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has also been used to identify anthocyanins [5]. This too, has limitations, as it cannot distinguish between diverse structures such as the anthocyanin delphinidin-3-glucoside and the flavonol, quercetin-3-glucoside, which have the same molecular mass (M_r=464). However, this problem can be overcome

*Corresponding author. Fax: +44-141-330-5971.
E-mail address: a.crozier@bio.gla.ac.uk (A. Crozier).

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by fractionating and purification of samples prior to analysis by HPLC [6]. Following the use of solid-phase extraction techniques, Giutsi et al. [7] were able to identify glycosylated and acylated anthocyanin derivatives by HPLC with DAD coupled to a triple quadrupole mass spectrometer. The anthocyanins were selectively fractionated and thereby separated from potential interfering compounds such as flavonols.

The aim of this investigation was to assess whether the anthocyanins present in unpurified extracts of Glen Ample raspberries could be rapidly characterized by HPLC with a dual wavelength absorbance detector coupled to a single quadrupole mass spectrometer.

2. Experimental

2.1. Extraction of plant material

Red raspberries (*Rubes idaeus* L. var. Glen Ample) were supplied by the Scottish Soft Fruit Growers (Blairgowrie, UK). Forty grams of fruit were macerated in a glass homogeniser. The resultant juice was decanted and remaining seeds and tissue were further extracted with methanol containing 0.1% HCl. The material was combined with the initial extract and centrifuged at 2000 *g* for 30 min. The supernatant was removed and stored as 2-ml aliquots in Eppendorf tubes at −80 °C. The contents of individual tubes were thawed as required and centrifuged at 15,800 *g* for 20 min prior to analysis by gradient elution reversed-phase HPLC.

2.2. High-performance liquid chromatography

Use was made of a Shimadzu (Kyoto, Japan) LC10ADvp series automated liquid chromatograph comprising an SCL-10Avp system controller, two LC-10ATvp pumps, an SIL-10ADvp auto-injector with a sample cooler and a CTO-10Avp column oven operating at 40 °C. Data were collected and processed via a Gateway 2000 G6-400 PC running Shimadzu QP8000 software. Anthocyanins in 5-μl aliquots of raspberry extract were separated using a 250×4.6 mm (I.D.) 5 μm Novapac C_{18} column fitted with a 20×4.6 mm (I.D.) Novapac C_{18} guard column (Waters Associates, Milford, MA) eluted at 0.8 ml min^{-1} with a 30-min gradient of 8–18% acetonitrile in water containing 1% formic acid. Column eluate was passed to a Shimadzu LC10AVvp UV–Vis detector operating at 371 and 520 nm (maximum wavelength separation possible on this detector) before being directed to a Shimadzu QP8000 quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface probe at a voltage of 4 kV, gas flow 2.5 l min^{-1} and a temperature of 400 °C. The mass spectrometer was operated in positive ion mode, scanning from 250 to 800 u.

2.3. Reference compounds

Quercetin-3-glucoside was purchased from Extrasynthese, Genay, France. Cyanidin-3-glucoside and delphinidin-3-glucoside were obtained from Polyphenols Labs, Sandnes, Norway. All other chemicals were supplied by Sigma–Aldrich, Dorset, UK. HPLC solvents were purchased from Rathburn, Walkerburn, UK.

3. Results and discussion

Gradient reversed-phase HPLC with absorbance detection and mass spectra analysis was used to rapidly identify the main anthocyanins in Glen Ample raspberries. An $A_{520\ nm}$ HPLC trace is illustrated in Fig. 1B, mass spectral fragmentation patterns are shown in Fig. 2 and identifications are summarized in Table 1. With the exception of cyanidin-3-glucoside, anthocyanin reference compounds were unavailable so identifications were assisted by the findings of earlier studies [1,2,8,9] and mass spectral fragmentation patterns reported by Giutsi et al. [7].

Peak 1 (t_R 15.1 min) had a molecular ion (M^+) at m/z 611 and a fragment ion at m/z 287 (M−324, loss of sophorosyl unit), which corresponds with cyanidin. On the basis of this evidence, it is concluded that peak 1 is cyanidin-3-sophoroside (**I**, Fig. 3) which is a major anthocyanin in raspberries [2].

Peak 2 (t_R 16.5 min) was also a major raspberry anthocyanin and was identified as cyanidin-3-(2^G-glucosylrutinoside) (**II**) having a mass spectrum with

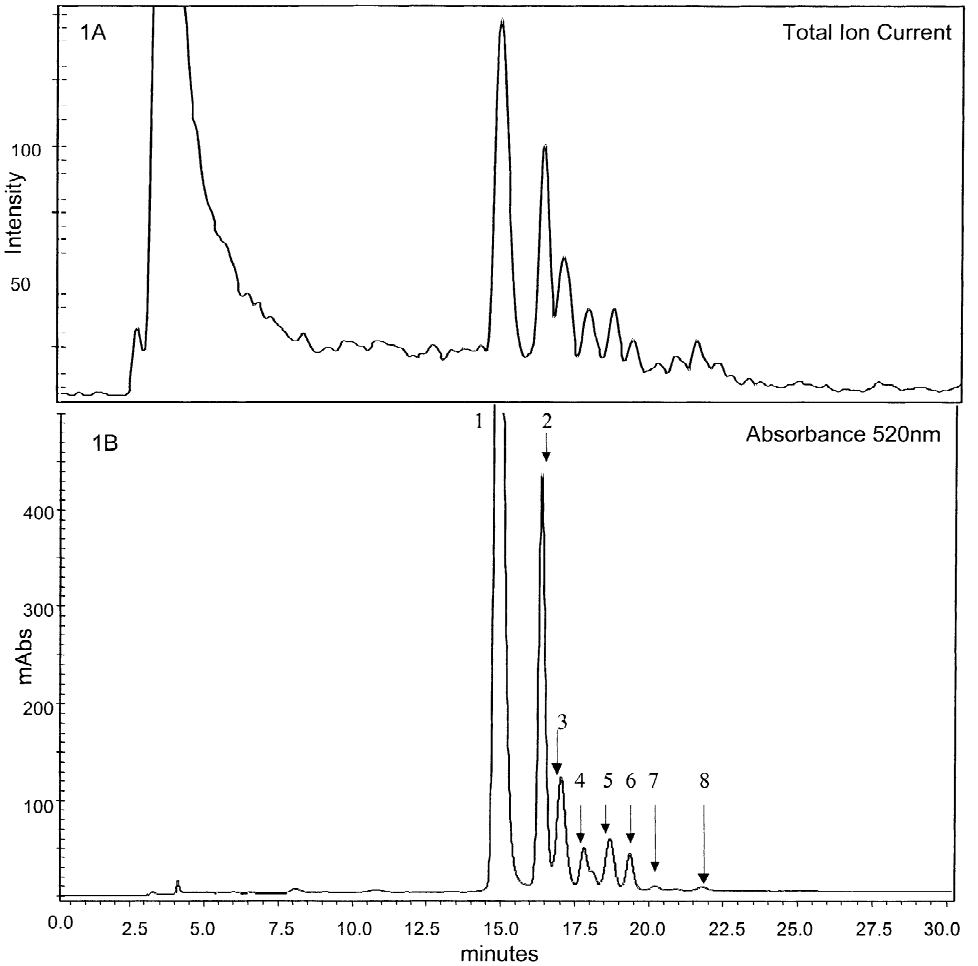

Fig. 1. Analysis of a raspberry extract by gradient reversed-phase HPLC using absorbance and mass spectrometric detection. A 5-μl aliquot of a raspberry extract was analysed using a 250×4.6 mm (I.D.) 5 μm Novapac C_{18} column fitted with a 20×4.6 mm (I.D.) Novapac C_{18} guard column eluted at 0.8 ml min^{-1} with a 30-min gradient of 8–18% acetonitrile in water containing 1% formic acid. Column eluate was passed to an absorbance monitor operating at 371 and 520 nm before being directed to a quadrupole mass spectrometer equipped with an APCI interface probe at a voltage of 4 kV, gas flow 2.5 l min^{-1} and a temperature of 400 °C. The mass spectrometer was operated in positive ion mode, scanning from 250 to 800 u. Trace A is the total ion current from the mass spectrometer. Trace B was obtained with an absorbance monitor operating at 520 nm.

an M^+ at m/z 757 and a cyanidin base peak at m/z 287 which involves a loss of M−470 corresponding to cleavage of one rhamnosyl and two glucosyl units.

Peak 3 (t_R 17.1 min) was cyanidin-3-glucoside (**III**). It co-chromatographed with an authentic standard and the mass spectrum obtained comprised an M^+ at m/z 449 and an m/z 287 cyanidin base peak formed by loss of a single glucosyl unit (M−162).

Peak 4 (t_R 17.7 min) had an M^+ at m/z 595 and a fragment ion at m/z 271 (pelargonidin, M−324, cleavage of sophorosyl group) and is therefore pelargonidin-3-sophoroside (**IV**).

Peak 5 (t_R 18.7 min) also yielded an m/z 595 M^+ that yielded a major fragment ion at m/z 287 which equates with cyanidin and a loss of M−308 corresponding to cleavage of a rutinosyl unit. Peak 5 is thus identified as cyanidin-3-rutinoside (**V**).

Peak 6 (t_R 19.3 min) produced a mass spectrum comprising M^+ at m/z 741 and loss of M−370 to yield an m/z 271 ion. This equates with cleavage of

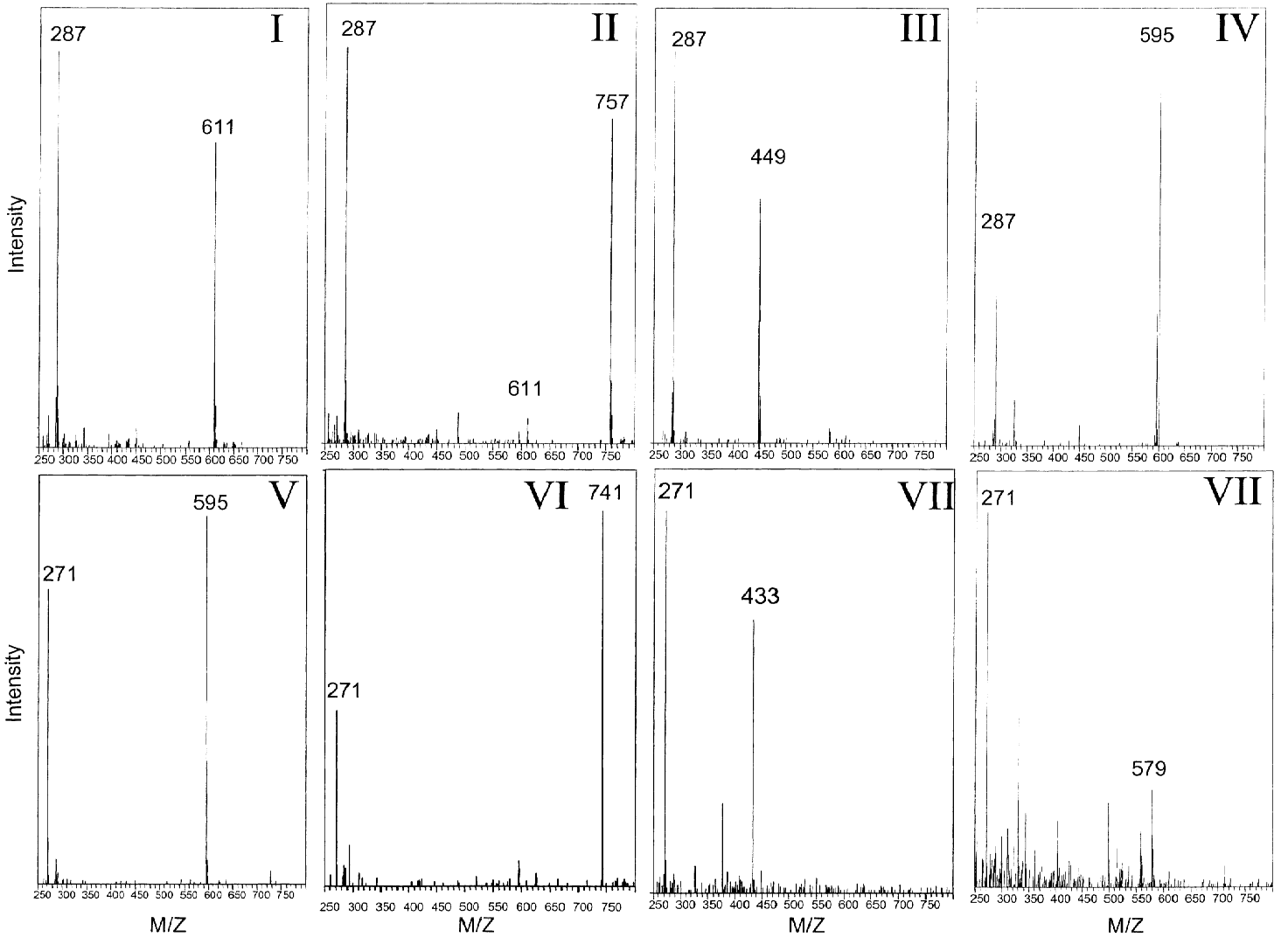

Fig. 2. Positive ion APCI mass spectra of anthocyanins detected in red raspberries; (**I**) cyanidin-3-sophoroside, (**II**) cyanidin-3-(2^G-glucosylrutinoside), (**III**) cyanidin-3-glucoside, (**IV**) pelargonidin-3-sophoroside, (**V**) cyanidin-3-rutinoside, (**VI**) pelargonidin-3-(2^G-glucosylrutinoside), (**VII**) pelargonidin-3-glucoside, (**VIII**) pelargonidin-3-rutinoside.

Table 1
Summary of the properties of compounds detected in extracts of Ample raspberries following analysis by HPLC with absorbance and MS detection. Peak numbers and retention times refer to numbers given in Fig. 1

Peak	t_R (min)	Compound	M^+ (m/z)	Fragment ions (m/z)
1	15.7	Cyanidin-3-sophoroside	611^+	287[Cyan](M^+ − Soph)
2	17.1	Cyanidin-3-(2^G-glucosylrutinoside)	757^+	611(M^+ − Rham), 287[Cyan](M^+ − Rham − Glc − Glc)
3	17.5	Cyanidin-3-glucoside	449^+	287[Cyan](M^+ − Glc)
4	18.4	Pelargonidin-3-sophoroside	595^+	271[Pel](M^+ − Soph)
5	19.3	Cyanidin-3-rutinoside	595^+	287[Cyan](M^- − Rham − Glc)
6	20.0	Pelargonidin-3-(2^G-glucosylrutinoside)	741^+	595(M^+ − Rham), 271[Pel](M^- − Rham − Glc − Glc)
7	20.7	Pelargonidin-3-glucoside	433^+	271[Pel](M^+ − Gluc)
8	22.1	Pelargonidin-3-rutinoside	579^+	271[Pel](M^+ − Rham − Glc)

Cyan, cyanidin; Pel, pelargonidin; Glc, glucosyl; Sop, sophorosyl; Rham, rhamnosyl; $M^{+/-}$, molecular ion.

one rhamnosyl and two glucosyl units from M^+ producing a fragment ion corresponding to pelargonidin. Peak 6 is, therefore, identified as pelargonidin-3-(2^G-glucosylrutinoside) (**VI**).

Peak 7 (t_R 20.2 min) was a minor anthocyanin with an M^+ at m/z 433 and a base peak at m/z 271 (pelargonidin) (M − 162, loss a glucosyl group) and was identified as pelargonidin-3-glucoside (**VII**).

Peak 8 (t_R 21.7 min), another minor anthocyanin, had an M^+ at 579 m/z and a base peak at m/z 271 (pelargonidin) (M − 308, loss of a rutinosyl unit) and is, therefore, identified as pelargonidin-3-rutinoside (**VIII**).

These identifications above are in keeping with previously published information on red raspberry anthocyanins [2,8,9]. All eight anthocyanins had previously been detected in raspberries, though not in a single variety, in an elegant TLC-based study by Ref. [1].

The elution profile of the anthocyanins provides substantial information on the nature and identity of the compounds under study. Under the chromatographic conditions used in the present study, the anthocyanin reversed-phase HPLC elution profile is consistent with that reported by Hong and Wrolstad [3]. Delphinidin elutes first followed by cyanidin, pelargonidin, petunidin and malvidin. In general as the degree of glycosylation of the conjugate increases, the anthocyanins are less well retained and have shorter retention times [3]. However, when the glycoside moiety contains rutinose, the hydrophilic methyl group increases the retention time, hence cyanidin-3-(2^G-glucosylrutinoside) elutes after cyanidin-3-sophoroside and likewise the equivalent pelargonidin derivatives (Fig. 1A). A much more marked increase in retention time is observed with anthocyanins that have been acylated. For instance, reported gradient reversed-phase HPLC retention times for delphinidin-3-glucoside and delphinidin-3-*O*-(6-*O*-*p*-coumaroyl)glucoside were 13.5 and 51.5 min, respectively [10].

The detection method of choice for anthocyanins has been an absorbance monitor since this offers the advantage that in the region of maximal absorbance for anthocyanins, 500–520 nm, very few other compounds absorb strongly and, as a consequence, extremely clean HPLC traces are obtained without sample purification. This is evident in the $A_{520\ nm}$ HPLC trace obtained with a crude raspberry extract illustrated in Fig. 1A. Examination of the mass spectrometric total ion current trace obtained with the same sample (Fig. 1A) reveals a very large early eluting multicomponent impurity peak that was not present in the $A_{520\ nm}$ trace (Fig. 1B). These, and other impurities can adversely affect the signal-to-noise ratio of the mass spectrometer, reducing sensitivity and adding a degree of uncertainty to the identification of a compound by its mass spectral characteristics. In order to prevent this in the present investigation, HPLC mobile phase conditions were employed that allowed the bulk of the impurities to elute before the anthocyanins. To minimize mass spectrometer contamination, this portion of the chromatogram was diverted to waste.

Good chromatographic separations and retention time information can play an important role in the interpretation of mass spectra data obtained with complex mixtures. As seen in Table 1 both cyanidin-

3-rutinoside and pelargonidin-3-sophoroside have an M^+ at m/z 595 and they elute within 1 min of each other. These compounds cannot be distinguished solely by molecular ion information, which is what is generated by MALDI-MS [5] and selected ion monitoring (SIM) and as a consequence, with these types of MS, accurate chromatographic information and/or fragmentation data are required to reveal the identity of the anthocyanin conjugate.

The need for further information becomes more evident when an examination of common sugars and acylating groups associated with anthocyanins is undertaken [7]. For instance, the mass spectrum of cyanidin-3-(2^G-glucosylrutinoside) contains two prominent ions, the M^+ at m/z 757 and the aglycone base peak at m/z 287 (Table 1). This mass spectrum could be interpreted as being that of cyanidin-3-sophoroside acetylated with *p*-coumaric acid. In theory DAD spectra would distinguish between these compounds as the acylated aromatic acid has a characteristic peak at 310 nm [3]. However, in a complex mixture with multiple co-eluting compounds, an increase in absorbance at 310 nm could easily arise from a co-eluting component, thereby adding a degree of uncertainty to the analysis.

Sole reliance on HPLC with DAD has already

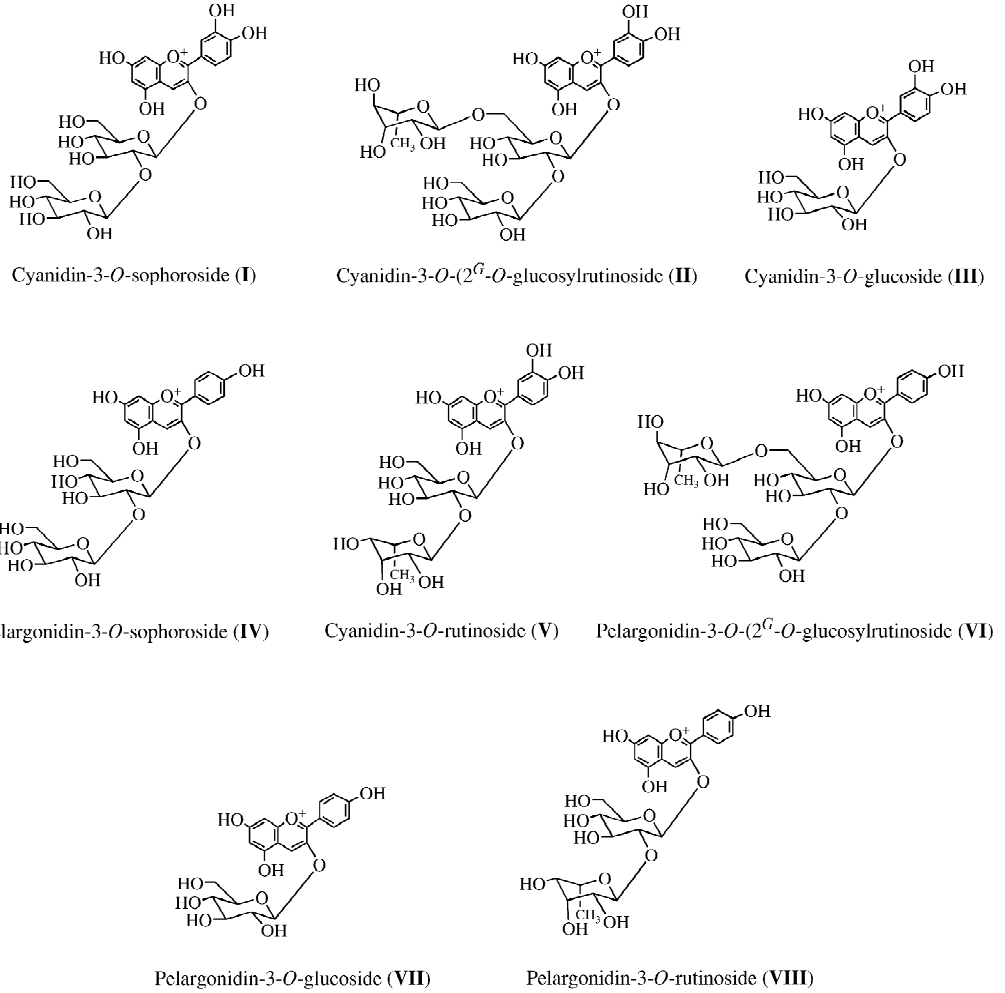

Fig. 3. Structures of anthocyanins detected in red raspberries.

been shown to have caused two misidentifications of alleged anthocyanins, as discussed by Cao and Prior [11]. In both cases the investigators, Paganga and Rice-Evans [12] and Lapidot et al. [13], claimed to have identified anthocyanins solely on the basis of absorbance spectra obtained by HPLC–DAD. However, on closer inspection, the spectrum obtained from a urine sample by Lapidot et al. [13] has a 431 nm peak as well as a peak at 520 nm. This spectrum is not that of an authentic anthocyanin and at best could be a metabolite. The peak detected in plasma by Paganga and Rice-Evans [12] is reported by Cao and Prior [11] to have too broad a 280 nm peak, ranging from 250 to 350 nm. Further doubt must be cast on this study as the identification was based on a single analysis of a single plasma sample from a single volunteer whose dietary intake of flavonoids was not controlled.

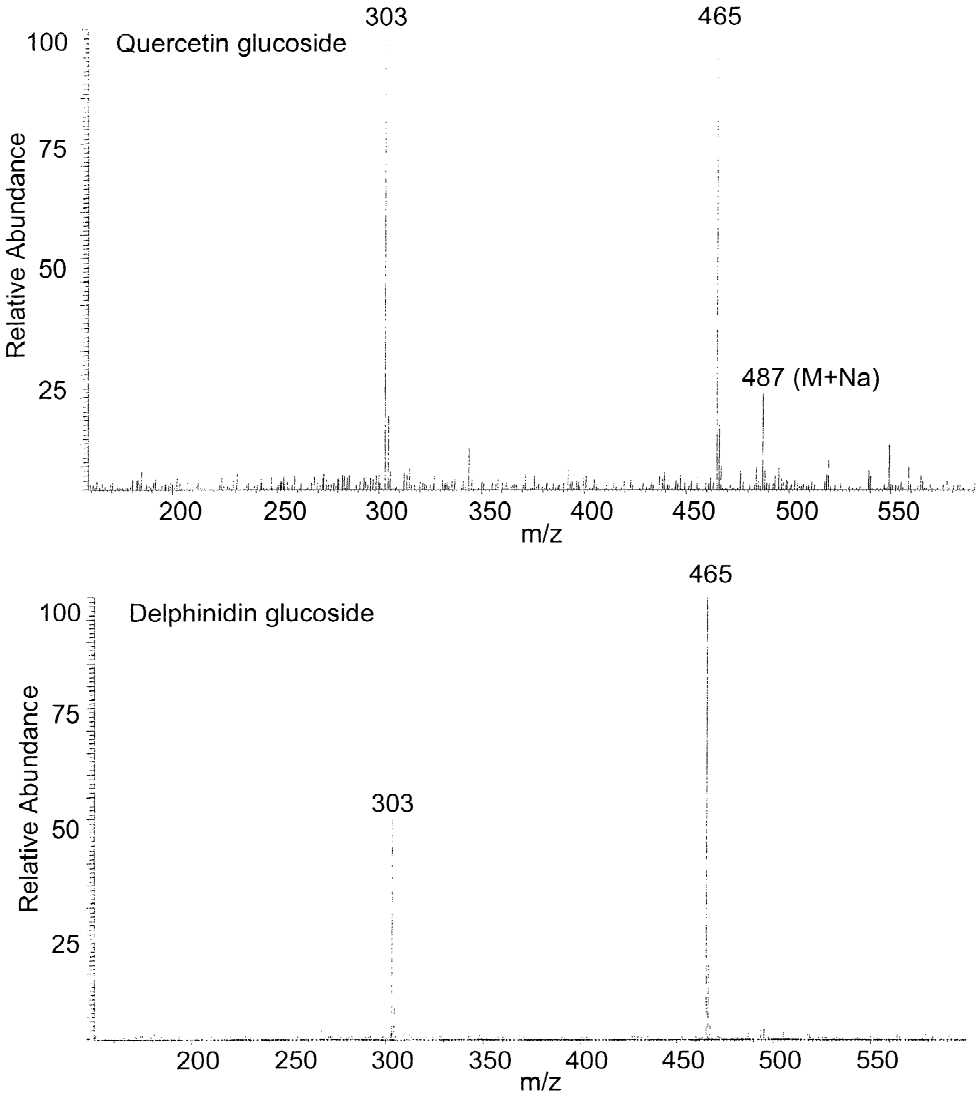

Fig. 4. Positive ion APCI mass spectra of quercetin-3-glucoside and delphinidin-3-glucoside.

Crude extracts can also contain compounds that have the same fragmentation patterns as anthocyanins. This is the case with delphinidin-3-glucoside and the equivalent flavonol conjugate quercetin-3-glucoside. After positive ionisation the anthocyanin produces an M^+ at m/z 465 and an aglycone ion at m/z 303 and the fragmentation of the flavonol is similar (Fig. 4). This potential problem is readily resolved by monitoring absorbance at 371 and 520 nm as this distinguishes between flavonols, which absorb only at the lower wavelength and anthocyanins which absorb at 520 nm and also, albeit less intensely, at 371 nm.

4. Conclusions

With state-of-the-art technology and minimal sample preparation Giusti et al. [4] were able to positively identify anthocyanins from various sources. Barritt and Torre [1] were able to achieve similar results with much simpler, but time-consuming, TLC-based procedures. However, the results obtained by Paganga and Rice-Evans [12], using gradient reversed-phase HPLC coupled to DAD, have been questioned, because of the subjective interpretation of an absorbance spectrum [11] allied to poorly controlled experimental procedures.

When analysing complex mixtures it is important to be aware of the limitations of the analytical system. The key information provided by HPLC with absorbance detection is retention time data. As well as determining the retention time of reference standards it is vital that they also be used routinely for co-chromatography to confirm the identity of peaks in extracts with greater surety.

The use of HPLC with mass spectrometric detection can reduce the reliance on retention time data. However, on its own, it does not necessarily provide positive identification of all compounds. For instance, MALDI time-of-flight and SIM-MS cannot distinguish between flavonol conjugates and anthocyanins with the same molecular mass. Therefore, either separation of these flavonoid groups must be performed before analysis or absorbance spectra must be acquired. However, there are many anthocyanins with the same molecular mass [4] in which case MS fragmentation data must be obtained. If this is not possible, acid hydrolysis could be performed to enable the released anthocyanidin to be identified.

The system in the present study can monitor absorbance at two wavelengths, so by selecting the appropriate wavelengths identification of flavonols and anthocyanins can be achieved in one chromatographic analysis. Furthermore, the mass spectrometric data contains information on the fragmentation of the compounds of interest allowing identification of the conjugate and the aglycone moiety. The method, thus, fits the criteria for the characterization of anthocyanins in raspberry extracts without the need for sample preparation.

Acknowledgements

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