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ARTICLE

Use of Accurate Mass Full Scan Mass Spectrometry for the Analysis of Anthocyanins in Berries and Berry-Fed Tissues[†]

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Anthocyanins in extracts from raspberries and blueberries were analyzed by reversed-phase HPLC coupled to a high-resolution Exactive Orbitrap mass spectrometer (HR-MS) with a resolution of 100,000, operated with an electrospray source in the positive ionization mode. As consumption of anthocyanin-rich berry extracts has been associated with improved cognitive function, brain extracts from European greenfinches (*Carduelis chloris*) that had been fed one blackberry daily for a period of 2 weeks were analyzed by both HPLC with traditional tandem MS in the selected reaction monitoring mode and HPLC-HR-MS. Cyanidin-3-*O*-glucoside was detected in the brain extracts by both methods, but because of its high level of selectivity, HR-MS was ca. 200-fold more sensitive. A further advantage of HR-MS is that unlike MS-SRM it enables both targeted and nontargeted compounds to be detected and much lower limits of detection are achieved without compromising the selectivity of the analysis.

KEYWORDS: Anthocyanins; raspberries; blueberries; birds; brain; HPLC-MS[2]; high-resolution mass spectrometry

INTRODUCTION

Anthocyanins are responsible for the red, purple, and dark blue colors of many fruits and berries. They were initially difficult to analyze by reversed-phase HPLC as they required a mobile phase containing acid at concentrations as high as 15% (*1*). However, advances in column stationary phase and support technology have resulted in optimum separations now being achieved with a mobile phase containing 1% acid. Photodiode array (PDA) detection further enhanced reversed-phase HPLC analysis of anthocyanins as differences in the $E_{440}/E_{\lambda max}$ ratio make it possible to differentiate between 3-*O*-glycosides and 3,5-*O*-diglycosides (*2*). In addition, the presence of an aromatic organic acid is characterized by the presence of a shoulder in the absorbance spectrum at 310 nm (*3*). Delphinidin, petunidin, and malvidin all have a similar absorbance spectrum, but can be distinguished from cyanidin and peonidin, which in turn differ from pelargonidin derivatives (*3*). However, identification using PDA detection relies on the availability of reference standards and cannot provide definitive structural identification of unknown peaks with close retention times and similar absorbance spectra (*4*). The use of HPLC with full scan mass spectrometry (MS) and electrospray ionization (ESI) can provide the nominal molecular weight of a compound. A simple quadrupole mass spectrometer with positive ionization can be used to identify anthocyanins and,

due to in-source fragmentation of the parent ion, can also yield information on the type of sugar substitution. At lower concentrations the fragment ion of the aglycone can be lost in the background noise and there can be difficulty in distinguishing between isobaric compounds, that is, compounds with the same nominal mass, such as cyanidin-3-*O*-rutinoside and pelargonidin-3-*O*-sophoroside (*5*). In such circumstances identification and quantification at low concentrations require a targeted analysis using selected ion monitoring (SIM) and is possible only when reference compounds are available and they can be separated chromatographically.

As mass spectrometers have developed over the years, investigators have tried to find faster and better methods to analyze anthocyanins, and to this end the use of a MALDI-TOF mass spectrometer was compared with conventional HPLC-PDA-MS for the analysis of blueberries (*6*). MALDI-TOF was quicker and provided comparable nontargeted quantitative estimates to that of PDA detection. However, as with a single-quadrupole MS, it is unable to distinguish between isomers and isobaric compounds and, furthermore, the dynamic range of MALDI-TOF is limited when compared to that of a PDA detector.

Giusti et al. (*7*) used a simple purification procedure with a C18 resin to process berry and fruit extracts prior to direct analysis of anthocyanins, without an HPLC step, using a triple-quadrupole mass spectrometer with an ESI. This was rapid, allowed MS[2] of targeted compounds, and proved to be a major advance in the characterization of anthocyanins occurring in plant extracts. It was noted that the MS[2] fragmentation of anthocyanins occurred

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only at the glycosidic bonds. Thus, in the case of a 3-*O*-diglucoside, such as cyanidin-3-*O*-sophoroside, the positively charged molecular ion ($[M + H]^+$) would yield an MS^2 ion corresponding to cyanidin, that is, m/z 611 → m/z 287. In contrast, MS^2 of diglucosides with the glucosyl groups at two loci rather than one, such as cyanidin-3,5-*O*-diglucoside, produces a monoglycosylated fragment as well as the aglycone, that is, m/z 611 → m/z 449 and m/z 287. Giusti et al. (*7*) produced an extremely useful table detailing the $[M + H]^+$ and MS^2 ions of all the common anthocyanins, anthocyanidins, and their sugar and organic acid conjugating moieties. Although this technique reveals MS^2 daughter ions produced from fragmentation of the $[M + H]^+$, and so can distinguish between isobaric anthocyanins, comprising different anthocyanidins, it cannot differentiate between an anthocyanidin conjugated with (i) a hexose sugar or acylated with caffeic acid and (ii) a rhamnose sugar and *p*-coumaric acid, as seen in purple potatoes (*8*). Furthermore, quantification still requires targeted analysis by SIM or selected reaction monitoring (SRM).

Many of these techniques work well when there is sufficient sample to allow an initial full scan investigative MS analysis followed by targeted quantitative analysis with absorbance detection or MS in the SIM or SRM mode. However, as research focuses on what effects compounds have at specific sites in vivo, the availability of a system that can carry out nontargeted identification and quantification in a single analytical step would be a major step forward. The object of this investigation was to assess whether high-resolution (HR) accurate mass MS could be such a tool for the analysis of anthocyanins in extracts of berries and the brains of birds fed blackberries.

MATERIALS AND METHODS

Berries and Chemicals. Fresh raspberries (*Rubus idaeus*) cv. Glen Ample, low bush blueberries (*Vaccinium angustifolium*), and blackberries (*Rubus ulmifolius*) were purchased at Sommerfield supermarket, Glasgow, U.K. HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Borders, U.K.). Formic acid was purchased from Riedel-DeHaen (Seelze, Germany) and acetic acid from BDH (Poole, U.K.), whereas L-(+)-ascorbic acid and cyanidin-3-*O*-glucoside was purchased from Extrasynthese (Genay, France).

Extraction of Berries. Twenty grams of berries was macerated in a glass homogenizer. The resultant juices were decanted, and the remaining seeds and tissue were further extracted with 2×15 mL of 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, made up to 50 mL with acidified methanol, and stored as 2 mL aliquots in Eppendorf tubes at −80 °C. The contents of individual tubes were thawed as required and centrifuged at 15800*g* for 20 min prior to analysis.

Feeding and Extraction of Greenfinch Brains. European greenfinches (*Carduelis chloris*) were fed either a normal greenfinch diet (Haith's, Grimsby, U.K.) comprising 10 g of seed per day (*n* = 9) or 7 g of seed plus one fresh blackberry per day (*n* = 9) for 2 weeks. The day following the last feed the birds were euthanized, blood was drawn by cardiac puncture, and the tissue was perfused in situ with 1 mL of chilled 0.15 M NaCl to remove residual blood. The brain was removed and rinsed in saline, blotted dry, and stored at −80 °C prior to extraction according to the method of Kalt et al. (*9*). Individual brains, weighing ca. 0.9 g, were homogenized in 2 mL of phosphate buffer containing 0.1% EDTA followed by extraction with 4 mL of acetone. The precipitated material was removed by centrifugation at 1500*g* for 5 min at 4 °C. The supernatant was reduced to the aqueous phase in vacuo, acidified with 100 µL of trifluoroacetic acid, and sequentially partitioned against 2 mL volumes of hexane, dichloromethane, and ethyl acetate. The aqueous layer was then freeze-dried and resuspended in 1 mL of 5% methanol in 1% aqueous formic acid.

HPLC with Full Scan HR-MS. Five microliter extracts of berries and brain extracts from blackberry-fed greenfinches were analyzed on an Accela HPLC system comprising an HPLC pump, a PDA detector, scanning from 250 to 700 nm, and an autosampler cooled to 4 °C (Thermo Fisher Scientific, San Jose, CA). Analyses were carried out at 40 °C using a 250 × 2 mm i.d. A 4 µm Synergi RP-Max column (Phenomenex, Macclesfield, Cheshire, U.K.) was eluted with a 30 min gradient of 5−50% acetonitrile in 1% aqueous formic acid at a flow rate of 200 µL/min. After passing through the flow cell of the PDA detector, the column eluate was directed to an Exactive Orbitrap trap mass spectrometer fitted with an electrospray interface (Thermo Fisher Scientific). The mass spectrometer was set up in positive ionization to scan from m/z 150 to m/z 2000 at a resolution of 100,000, at m/z 200 with a scan cycle time of 1 s. The capillary temperature was 275 °C, and the source voltage was 4 kV.

HPLC-MS². Five microliter extracts of berries and 100 µL extracts from the brain of blackberry-fed greenfinches were analyzed on a Surveyor HPLC system comprising a HPLC pump, a PDA detector, scanning from 250 to 700 nm, and an autosampler cooled to 4 °C (Thermo Fisher Scientific). Analyses were carried out at 40 °C using a 250 × 2 mm i.d., 4 µmm Synergi RP-Max column (Phenomenex) eluted with a 50 min gradient of 10−25% acetonitrile in 1% aqueous formic acid at a flow rate of 200 µL/min. After passing through the flow cell of the PDA detector, the column eluate was directed to an LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface (Thermo Fisher Scientific). Analyses utilized the positive ion mode as this provided the best limits of detection for anthocyanins. Samples were analyzed in the mass spectrometer using SRM scanning for fragments of the m/z 449 parent ion from m/z 150 to m/z 449 and for fragments from the m/z 463 parent ion between m/z 150 and m/z 463. Capillary temperature was 150 °C, sheath gas and auxiliary gas were 40 and 20 units, respectively, the source voltage was 3 kV, and SRM maximum cycle time was 200 ms. Both HPLC-MS systems were controlled by Xcalibur software (Thermo Fisher Scientific).

RESULTS

HPLC-HR-MS Analysis of Raspberries. In this study a total of 10 ions associated with 12 anthocyanins were monitored. In keeping with previous reports on anthocyanins in raspberries (*10*, *11*), the main components identified were cyandin-3-*O*-sophoroside, cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside, and cyanidin-3-*O*-glucoside, with small quantities of cyanidin-3-*O*-sambubioside, cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside, cyanidin-3-*O*-rutinoside, cyanidin-3,5-*O*-diglucoside, pelargonidin-3-*O*-glucoside, pelargonidin-3-*O*-rutinoside, pelargonidin-3-*O*-(2″-*O*-glucosyl)rutinoside, and pelargonidin-3-*O*-sambubioside and with some samples also containing detectable quantities of pelargonidin-3-*O*-sophoroside. Unlike the earlier investigations, in the present study with HR-MS, the HPLC mobile phase gradient was designed to achieve limited resolution of the raspberry anthocyanins as this enabled the strengths and weaknesses of HR-MS to be demonstrated with greater clarity. The data presented in **Figure 1** and **Table 1** show that the anthocyanins eluted from the HPLC column within a period of 3 min and that HR-MS was able to identify eight individual cyanidin- and pelargonidin-based anthocyanins. Because of their coelution from the HPLC column, HR-MS was unable to distinguish between cyanidin-3,5-*O*-diglucoside, which typically occurs as a minor component, and the main anthocyanin cyanidin-3-*O*-sophoroside and likewise between the isobaric cyanidin-3-*O*-rutinoside and pelargonidin-3-*O*-sophoroside, both of which are minor anthocyanins (**Table 1**). All four anthocyanins can be readily separated when analyzed with reversed-phase HPLC using a more typical mobile phase gradient offering an enhanced peak capacity (*10*, *11*).

Although no quantitative analysis was attempted, the main ions detected in the analysis of the raspberry extract were at m/z 611.1598, 757.2173, and 449.1076 (**Figure 1**), which, respectively, correspond with cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside, and cyanidin-3-*O*-glucoside, the known major anthocyanins in raspberries (*10*). The selectivity of the
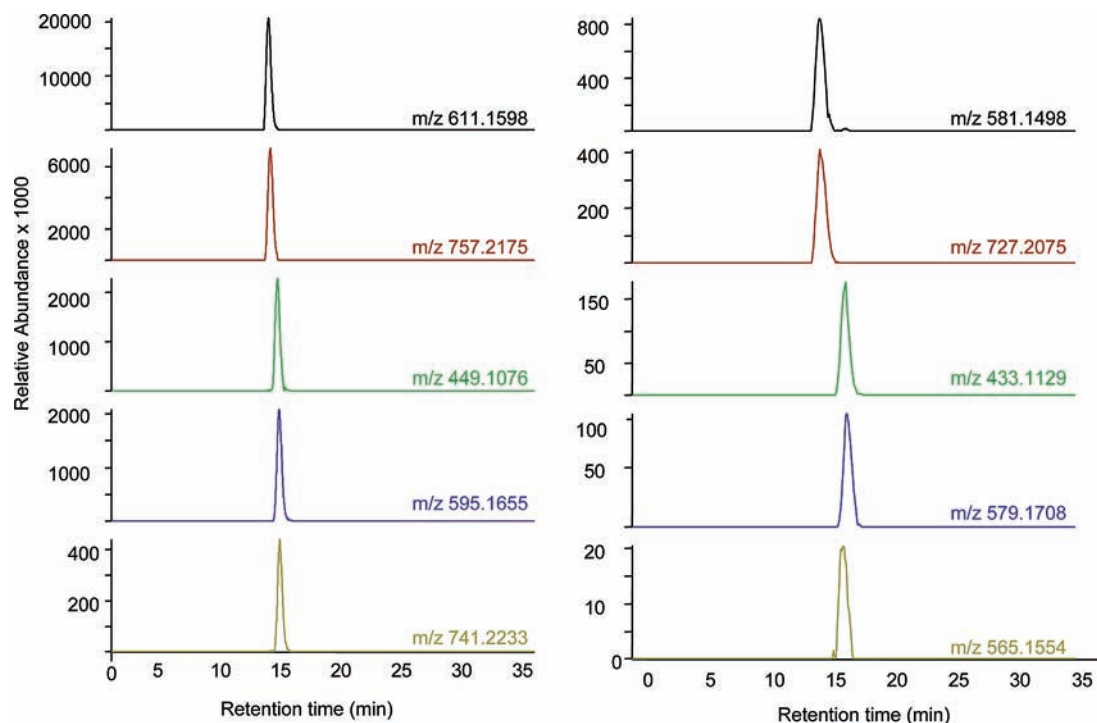
Figure 1. HPLC-HR mass chromatograms of anthocyanins in a raspberry extract. *m/z* values are ±1 mmu. *m/z* 611.1598, cyanidin-3,5-*O*-diglucoside/cyanidin-3-*O*-sophoroside; *m/z* 757.2175, cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside; *m/z* 449.1076, cyanidin-3-*O*-glucoside; *m/z* 595.1655, cyanidin-3-*O*-rutinoside/pelargonidin-3-*O*-sophoroside; *m/z* 741.2233, pelargonidin-3-*O*-(2″-*O*-glucosyl)rutinoside; *m/z* 581.1498, cyanidin-3-*O*-sambubioside; *m/z* 727.2075, cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside; *m/z* 433.1129, pelargonidin-3-*O*-glucoside; *m/z* 579.1708, pelargonidin-3-*O*-rutinoside; *m/z* 565.1554, pelargonidin-3-*O*-sambubioside.
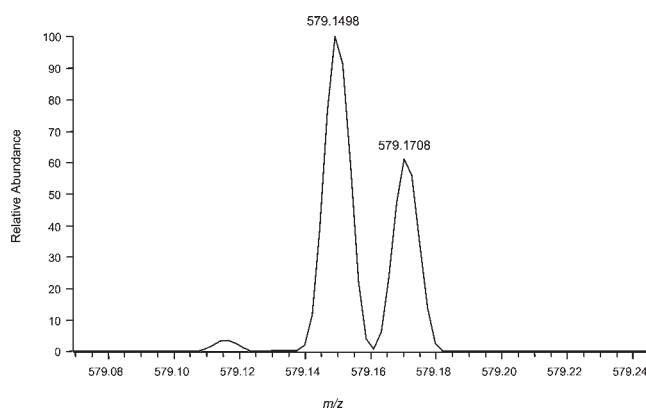
Table 1. Summary of the HPLC Retention Times and HR Mass Spectra of Anthocyanins Detected in a Raspberry Extract[a]

peak	t_R (min)	anthocyanin	estimated mass (Da)	actual mass (Da)	error (mDa)	formula
1	13.0	cyanidin-3,5-*O*-diglucoside/cyanidin-3-*O*-sophoroside*	611.1598	611.1607	−0.9	$C_{27}H_{31}O_{16}$
2	13.2	cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside	757.2175	757.2186	−1.1	$C_{33}H_{41}O_{20}$
3	13.6	cyanidin-3-*O*-sambubioside	581.1498	581.1501	−0.3	$C_{22}H_{29}O_{15}$
4	13.7	cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside	727.2075	727.2080	−0.5	$C_{32}H_{39}O_{19}$
5	13.8	cyanidin-3-*O*-glucoside	449.1076	449.1078	−0.2	$C_{21}H2_{1}O_{11}$
6	14.0	cyanidin-3-*O*-rutinoside/pelargonidin-3-*O*-sophoroside†	595.1655	595.1657	−0.2	$C_{27}H_{31}O_{15}$
7	14.0	pelargonidin-3-*O*-(2″-*O*-glucosyl)rutinoside	741.2233	741.2237	−0.4	$C_{33}H_{39}O_{19}$
8	14.7	pelargonidin-3-*O*-sambubioside	565.1554	565.1552	0.2	$C_{26}H_{29}O_{14}$
9	14.8	pelargonidin-3-*O*-glucoside	433.1129	433.1129	0.0	$C_{21}H_{21}O_{10}$
10	15.3	pelargonidin-3-*O*-rutinoside	579.1498	579.1708	−21	$C_{27}H_{31}O_{14}$

[a] For peak numbers, see **Figure 1**; t_R, retention time; Da, daltons; mDa, millidaltons; * and † indicate anthocyanins with matching exact masses.

HR-MS is such that it was possible to estimate the accurate mass of these compounds to within ±1 millidalton (mDa). As can be seen from **Table 1**, the accuracy of the system, with one exception, was 1.1 mDa for all compounds. The exception was the estimated mass for pelargonidin-3-*O*-rutinoside at *m/z* 579.1498, which is 21 mDa less than the actual mass of *m/z* 579.1708. However, by zooming in on the ion in question it was seen that two ions were in fact present, one attributable to the anthocyanin rutinoside at *m/z* 579.1708 and the second ion at *m/z* 579.1498 identified from the reported empirical formula as an (epi)catechin B-type dimer with an actual mass 579.1494 (**Figure 2**).

This analysis of raspberry anthocyanins highlights the strengths and limitations of any mass analyzer. When analyzing complex mixtures via either low-resolution HPLC or, indeed, direct infusion, HR-MS cannot distinguish between isomers and isobaric compounds that have an identical exact mass. In such circumstances, the problem can usually be overcome by a simple and rapid high-resolution HPLC step.

Figure 2. Full scan mass spectrum from *m/z* 579.08 to *m/z* 579.24 showing peaks at *m/z* 579.1498, an (epi)catechin B-type dimer, and *m/z* 579.1708, pelargonidin-3-*O*-rutinoside.

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Table 2. Summary of the HPLC and HR Mass Spectra of Anthocyanins Detected in a Low Bush Blueberry Extract[a]

peak	t_R (min)	anthocyanin	estimated mass (Da)	actual mass (Da)	error (mDa)	formula
1	12.1	delphinidin-3-*O*-glucoside/delphinidin-3-*O*-galactoside	465.1033	465.1033	0.0	$C_{21}H_{21}O_{12}$
2	13.1	delphinidin-3-*O*-arabinoside	435.0920	435.0927	−0.7	$C_{20}H_{19}O_{11}$
3	13.4	cyanidin-3-*O*-glucoside/cyanidin-3-*O*-galactoside*	449.1075	449.1084	−0.9	$C_{21}H_{21}O_{11}$
4	13.8	petunidin-3-*O*-glucoside/petunidin-3-*O*-galactoside	479.1183	479.1190	−0.7	$C_{22}H_{23}O_{12}$
5	14.2	cyanidin-3-*O*-arabinoside	419.0971	419.0978	−0.7	$C_{20}H_{19}O_{10}$
6	14.6	petunidin-3-*O*-arabinoside*	449.1075	449.1077	−0.2	$C_{21}H_{21}O_{10}$
7	14.6	peonidin-3-*O*-glucoside/peonidin-3-*O*-galactoside[†]	463.1227	463.1240	−1.3	$C_{22}H_{23}O_{11}$
8	15.0	malvidin-3-*O*-glucoside/malvidin-3-*O*-galactoside	493.1335	493.1341	−0.6	$C_{23}H_{25}O_{12}$
9	15.5	peonidin-3-*O*-arabinoside	433.1135	433.1135	0.0	$C_{21}H_{21}O_{10}$
10	15.8	malvidin-3-*O*-arabinoside[†]	463.1227	463.1240	−1.3	$C_{22}H_{23}O_{11}$
11	17.3	peonidin-3-*O*-(6′′-*O*-acetyl)glucoside	505.1339	505.1346	−0.7	$C_{24}H_{25}O_{12}$
12	17.6	malvidin-3-*O*-(6′′-*O*-acetyl)glucoside	535.1444	535.1452	−0.8	$C_{25}H_{27}O_{13}$

[a] For peak numbers, see **Figure 2**; t_R, retention time; Da, daltons; mDa, millidaltons; * and [†] indicate anthocyanins with matching exact masses.

HPLC-HR-MS Analysis of Blueberries. Low bush blueberries contain a complex array of delphinidin-, cyanidin-, petunidin-, peonidin-, and malvidin-based anthocyanins (*12*). In the current study, despite the steep mobile phase gradient, because of their structural diversity, the blueberry anthocyanins eluted from the HPLC column over a 6 min time period, which provided some degree of separation. Despite the limited chromatographic separation, HPLC-HR-MS was able to detect 12 anthocyanin peaks with extremely close correspondence between the actual mass and the detected mass values (**Table 2**). Because of their identical masses, the 3-*O*-glucoside and -galactosides could not be differentiated, but this can be achieved easily by more traditional reversed-phase HPLC methodology, in which the galactoside elutes earlier than the corresponding glucoside (*13, 14*).

Cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside both have the same mass, 449.1084 Da, as the petunidin-3-*O*-arabinoside and, likewise, at 463.1240 Da, the peonidin-3-*O*-glucoside/galactosides and malvidin-3-*O*-arabinoside. However, because they separated, even with the low-resolution HPLC step, it was possible to distinguish these nonisomeric, isobaric compounds (**Table 2**).

Analysis of Greenfinch Brain Extracts. In keeping with published data (*15*), analysis of the blackberries fed to the birds showed that the predominant anthocyanin (>95%) was cyanidin-3-*O*-glucoside. Each blackberry contained on average 13.1 μmol of cyanidin-3-*O*-glucoside, and this equates with each bird receiving a daily intake of 364 μmol/kg. The normal seed diet fed to the birds contained no detectable anthocyanins (data not shown).

The greenfinch brain extracts were analyzed by both HPLC-MS^2 in SRM mode and HPLC-HR-MS. The SRM analysis used a conventional 50 min gradient of 10−25% acetonitrile in 1% aqueous formic acid to provide a good separation of the compound(s) of interest from potential contaminants. A SRM method was set up to scan ions formed from the parent compound at m/z 449 and for the potential methylated or glucuronidated metabolites at m/z 463. Ion trap systems can scan a range of ions from the parent molecular ion downward, with no loss in sensitivity. This is a useful attribute in ensuring peak homogeneity and reducing the incidence of erroneous false-positive identifications. One hundred microliter aliquots of the 1 mL brain extracts were analyzed, and a distinctive cyanidin-3-*O*-glucoside peak was observed with a retention time of 18.5 min on the SRM trace of the m/z 449 ion fragmenting to produce a MS^2 ion at m/z 287 (**Figure 3A**). This peak also cochromatographed with a cyanidin-3-*O*-glucoside standard. The cyanidin-3-*O*-glucoside content of individual brains ranged from 12 to 148 pmol with an average of 40 ± 16 pmol (standard error, $n = 9$). No evidence was obtained for the presence of cyanidin-*O*-glucuronide

or methyl-*O*-cyanidin-*O*-glucuronide metabolites. No anthocyanins were detected in the brain extracts from control non-berry-supplemented birds (data not shown). The tissues were perfused with saline to remove any residual blood. However, as the last berry feed had occurred at least 18 h earlier, there would be little or no anthocyanins in the circulatory system present to contaminate the brain extracts.

Analysis of the brain extract by HPLC-HR-MS used the same 30 min, 5−50% acetonitrile gradient that was employed for the analysis of the raspberry and blueberry extracts. The HR-MS was operated in full scan mode from m/z 150 to m/z 2000, and the response at m/z 449.1064−449.1084 for a 5 μL aliquot of a brain from a berry-fed greenfinch revealed a clear peak with a retention time of 13.5 min (**Figure 3B**). The exact mass of this peak was 449.1079 Da, 0.4 mDa different from the theoretical value for cyanidin-3-*O*-glucoside, with which it cochromatographed. When signal-to-noise ratios of the cyanidin-3-*O*-glucoside peaks obtained with MS^2-SRM and HR-MS were compared, a value of 111 was obtained with SRM compared to 1295 for HR-MS. As the HR-MS system analyzed a 5 μL aliquot of the 1 mL green-finch brain extract, compared to 100 μL with SRM, this represents a 200-fold improvement in sensitivity.

DISCUSSION

The purpose of this investigation was to assess the value of HR accurate mass MS for the analysis of anthocyanins in berries and the potential application of this technique to the analysis of flavonoids in biological samples. The aim of many laboratories is to have methods for the rapid and accurate analysis of flavonoids and related compounds in complex mixtures, and in this context MALDI-TOF-MS and HPLC-MS^2 have both yielded substantial amounts of information with semipurified extracts (*6, 7*). However, the limitation of both systems is that it is not possible to distinguish between isomeric and isobaric compounds with the same empirical formula and, as a consequence, chromatography is still an unavoidable part of the analysis. However, as we have demonstrated, HR-MS is able to distinguish between compounds differing in mass by as little as 21 mDa. In fact, the estimated mass of all of the compounds identified in raspberry and blueberry extracts was within 1.3 mDa of their theoretical value, with most having an error of <1.0 mDa (**Tables 1** and **2**). This is substantially better than estimates of mass that can be achieved with MALDI-TOF-MS, which has a mass accuracy in the region of 10 mDa. Furthermore, the HR-MS anthocyanin peaks that were identified varied in their ion current peak heights from 2×10^3 to 4×10^7, providing a larger dynamic range than the 10^3 that is typically achieved with MALDI-TOF-MS.
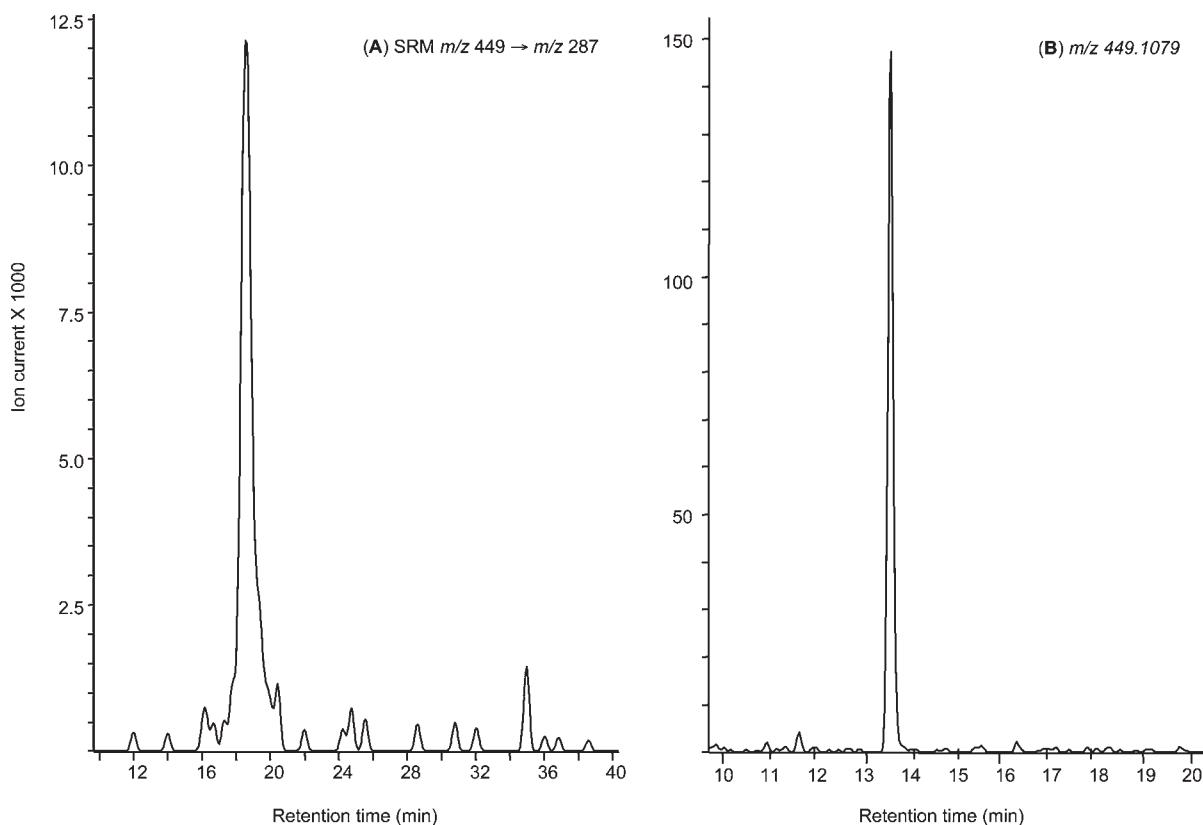
Figure 3. HPLC-MS analysis of an extract of a brain of a greenfinch fed one blackberry per day for 14 days: (**A**) HPLC-SRM trace of a *m/z* 287 fragment ion from a *m/z* 448 parent ion; (**B**) HPLC-HR-MS trace of the *m/z* 449.1074 ± 0.001 ion extracted from a full scan mass spectra.

As a result of the studies of Joseph and colleagues, there is much interest in the beneficial effects of berry anthocyanins on cognitive function (*16*), and this group and their collaborators used HPLC-MS-SRM to analyze extracts of brains from rats fed a blueberry-rich diet. Extremely low levels of anthocyanins were detected, but in such small quantities that the response was barely above that of the background noise (*17*). In the present study extracts of brains of greenfinches fed blackberries were analyzed by both HPLC-MS-SRM and HPLC-HR-MS. Both provide a convincing demonstration of the ability of anthocyanins to cross the blood−brain barrier was achieved (**Figure 3**). The concentration of anthocyanins detected in the brain of greenfinches is higher than those reported in previous studies with rats and pigs (*9, 16*). This may be a reflection of the relative dose of anthocyanin ingested or indicate that greenfinches, which eat berries as part of their normal diet, absorb blackberry anthocyanins more readily than rats and pigs absorb blueberry anthocyanins. Greenfinches consume approximately 20% of their body weight in food per day (*18*), and the consumption of one berry, as opposed to an anthocyanin-rich berry extract, is not an unnatural dietary situation.

The sensitivity of the HR-MS was ca. 200-fold better than that of SRM, so cyanidin-3-*O*-glucoside could be detected when as little as 0.5% of an extract of a greenfinch brain weighing 0.9 g (**Figure 3B**) was analyzed. Although the new generation of ion trap mass spectrometers is more sensitive than the instrument used in the current study, and likewise the latest triple-quadrupole mass spectrometers, they are both limited in requiring a priori knowledge of the analyte(s). Neither can carry out compound identification and quantification in a single analysis. HR-MS opens up the exciting possibility of quantifying anthocyanins and additional nontargeted compounds in discrete areas of the brain.

The improvement in sensitivity of mass spectrometers, especially in the case of ion trap systems, over the past 5−10 years is attributable principally to the increased number of ions that the trap can hold. More generally, there have been improvements in design to ensure maximum ion transmission into the detectors. However, there is only so much that can be done to improve the signal-to-noise ratio without a reduction in background noise. With the Oribtrap mass spectrometer, improvements in sensitivity come via an enhanced selectivity, which reduces interfering background ions. This can be achieved to such an extent with some high-resolution systems that there is no measurable background noise and, as a consequence, it is not possible to measure the signal-to-noise ratio.

Although the Orbitrap detector is a major improvement in the field of mass spectrometry, the limitations of using a mass analyzer should be appreciated. It cannot differentiate between compounds with the same empirical formula. In the analysis of plant secondary metabolites there are a large number of isomers and compounds, such as cyanidin and kaempferol and delphinidin and quercetin, that have the same empirical formula. Therefore, some form of separation, whether it be solid-phase extraction or HPLC, must be used in conjunction with mass analysis. In this context, ultraperformance liquid chromatography (UPLC) is of value as it can reduce analysis times by up to a factor of 10 over conventional HPLC (*19*). Therefore, the combination of the improved separation and high-resolution mass analysis techniques offers the promise of rapid identification and quantification in a single analysis. For more routine analysis of samples, for example, in quality control or pharmacokinetics, when known compounds are being detected and quantified, the resolution can be reduced to say 30000 to increase the number of data points acquired per peak, thereby improving quantitative accuracy. This is discussed in an authoritative paper

by Zhang et al. on quantitative mass spectrometric accuracy using an Oribitrap in full scan mode compared to a triple-quadrupole in SRM mode (*20*). In this study, analyses were carried out using an UPLC system producing peak widths of the order of 6 s. One advantage of the Oribitrap is the number of compounds measured per analysis. As it is a full scan system the number of compounds measured per analysis has no bearing on the number of data points acquired across a peak. The triple-quadrupole requires 50 ms per scan with 0.2 ms between scans. Therefore, the more compounds a triple-quadrupole mass spectrometer measures per analysis, the fewer data points are collected per HPLC peak. Zhang et al. (*20*) claim the major benefit of full scan, high-resolution mass analysis is that there is no need for compound tuning; therefore, the bioanalytical setup is simplified and information on compounds of interest, as well as nontargeted components, is readily obtained.

With resolution up to 100,000 at m/z 200 and accurate mass better than ± 2 ppm the Orbitrap mass spectrometers provide a full scan detection system that can both identify and quantify compounds in a single run. As it is a full scan system, nontargeted analytes can also be quantified. No compound tuning is necessary; therefore, the setup procedure is also greatly simplified. Despite these advances, the use of a PDA detector, as mentioned in the Introduction, should not be underestimated as in the analysis of plant secondary metabolites there are circumstances when absorbance spectra can provide useful additional structural information.

LITERATURE CITED

(1) Rommel, A.; Heatherbell, D. A.; Wrolstad, R. E. Red raspberry juice and wine − effect of processing and storage on anthocyanin pigment composition, color and appearance. *J. Food Sci.* **1990**, *55*, 1011–1017.

(2) Hong, V.; Wrolstad, R. E. Characterization of anthocyanin-containing colorants and fruit juices by HPLC photodiode array detection. *J. Agric. Food Chem.* **1990**, *38*, 698–708.

(3) Hong, V.; Wrolstad, R. E. Use of HPLC separation photodiode array detection for characterization of anthocyanins. *J. Agric. Food Chem.* **1990**, *38*, 708–715.

(4) Giusti, M. M.; Rodriguez-Saona, L. E.; Wrolstad, R. E. Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins. *J. Agric. Food Chem.* **1999**, *47*, 4631–4637.

(5) Mullen, W.; Lean, M. E. J.; Crozier, A. Rapid characterization of anthocyanins in red raspberry fruit by high-performance liquid chromatography coupled to single quadrupole mass spectrometry. *J. Chromatogr.* **2002**, *966*, 63–70.

(6) Wang, J.; Kalt, W.; Sporns, P. Comparison between HPLC and MALDI-TOF MS analysis of anthocyanins in highbush blueberries. *J. Agric. Food Chem.* **2000**, *48*, 3330–3335.

(7) Giusti, M. M.; Rodriguez-Saona, L. E.; Griffin, D.; Wrolstad, R. E. Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. *J. Agric. Food Chem.* **1999**, *47*, 4657–4664.

(8) Odake, K.; Terahara, N.; Saito, N.; Toki, K.; Honda, T. Chemical structures of two anthocyanins from purple sweet-potato, *Ipomoea batatas*. *Phytochemistry* **1992**, *31*, 2127–2130.

(9) Kalt, W.; Blumberg, J. B.; McDonald, J. E.; Vinqvist-Tymchuk, M. R.; Fillmore, S. A. E.; Graf, B. A.; O'Leary, J. M.; Milbury, P. E. Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. *J. Agric. Food Chem.* **2008**, *56*, 705–712.

(10) Mullen, W.; McGinn, J.; Lean, M. E. J.; MacLean, M. R.; Gardner, P.; Duthie, G. G.; Yokota, T.; Crozier, A. Ellagitannins, flavonoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. *J. Agric. Food Chem.* **2002**, *50*, 5191–5196.

(11) Borges, G.; Roowi, S.; Rouanet, J. M.; Duthie, G. G.; Lean, M. E. J.; Crozier, A. The bioavallability of raspberry anthocyanins and ellagitannins in rats. *Mol. Nutr. Food Res.* **2007**, *51*, 714–725.

(12) Prior, R. L.; Lazarus, S. A.; Cao, G. H.; Muccitelli, H.; Hammerstone, J. F. Identification of procyanidins and anthocyanins in blueberries and cranberries (*Vaccinium* spp.) using high-performance liquid chromatography/mass spectrometry. *J. Agric. Food Chem.* **2001**, *49*, 1270–1276.

(13) Sakata, Y.; Arisumi, K.; Miyajima, I. Studies on the flower colors in the *Camellia*. 4. Cyanidin 3-galactoside, a new anthocyanin from *Camellia japonica* subsp. rusticana (Honda) kitamura and its occurrence in the garden forms of *Camellia* of Japanese origin. *J. Jpn. Soc. Hortic. Sci.* **1986**, *55*, 82–88.

(14) Marks, S. C.; Mullen, W.; Crozier, A. Flavonoid and hydroxy-cinnamate profiles of English apple ciders. *J. Agric. Food Chem.* **2007**, *55*, 8723–8730.

(15) Cho, M. J.; Howard, L. R.; Prior, R. L.; Clark, J. R. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* **2004**, *84*, 1771–1782.

(16) Joseph, J. A.; Shukitt-Hale, B.; Denisova, N. A.; Bielinski, D.; Martin, A.; McEwen, J. J.; Bickford, P. C. Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. *J. Neurosci.* **1999**, *19*, 8114–8121.

(17) Andres-Lacueva, C.; Shukitt-Hale, B.; Galli, R. L.; Jauregui, O.; Lamuela-Raventos, R. M.; Joseph, J. A. Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. *Nutr. Neurosci.* **2005**, *8*, 111–120.

(18) Nagy, K. A.; Girard, I. A.; Brown, T. K. Energetics of free-ranging mammals, reptiles, and birds. *Annu. Rev. Nutr.* **1999**, *19*, 247–277.

(19) Cooper, K. A.; Campos-Gimenez, E.; Alvarez, D. J.; Nagy, K.; Donovan, J. L.; Williamson, G. Rapid reversed phase ultra-performance liquid chromatography analysis of the major cocoa polyphenols and inter-relationships of their concentrations in chocolate. *J. Agric. Food Chem.* **2007**, *55*, 2841–2847.

(20) Zhang, N. R.; Yu, S.; Tiller, P.; Yeh, S.; Mahan, E.; Emary, W. B. Quantitation of small molecules using high-resolution accurate mass spectrometers − a different approach for analysis of biological samples. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1085–1094.