JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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# Anthocyanin Characterization Utilizing Liquid Chromatography Combined with Advanced Mass Spectrometric Detection

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Supporting Information

**ABSTRACT:** Anthocyanins are naturally occurring polyphenolic plant pigments. To analyze the anthocyanin content of samples, rapid and reliable methods for separation and detection are needed. In this work an LC−DAD−ESI-MS/MS instrument was used to develop a new tandem MS data acquisition strategy for anthocyanin characterization which was subsequently compared to more conventional measurements. It has been shown that the newly developed strategy, multiple reaction monitoring-initiated anthocyanin characterization (MIAC), can successfully be used in anthocyanin analysis and has various advantages compared to some more traditional measurements, such as enhanced selectivity, better signal-to-noise ratio and simplified data evaluation. Furthermore, the number of relevant MS/MS data increased significantly with the MIAC method compared with a more common information dependent MS experiment strategy.

**KEYWORDS:** anthocyanins, multiple reaction monitoring mass spectrometry, red onion, strawberry, sour cherry

## INTRODUCTION

Anthocyanins are a subgroup of the flavonoids, a class of plant secondary metabolites, which have been the subject of several studies due to their importance as quality indicators in food,[1−3] their medical properties[4−11] and the potential use as food colorants.[12] To analyze the anthocyanin content of samples, rapid and reliable methods for separation and detection are needed. Liquid chromatography (LC) is the main technique for separation of anthocyanins combined with ultraviolet−visible (UV−vis) diode array detection (DAD).[13−15] The obtained spectra can give information on the nature of the aglycon, glycosylation and possibility of acylation.[13] Over the past few years the use of mass spectrometry (MS) detectors in anthocyanin analysis has increased tremendously due to the development of atmospheric-pressure ionization sources such as electrospray (ESI). MS detection can provide information about both the molecular mass and molecular structure, which facilitates the identification of analytes. The latter is especially true for tandem mass spectrometry (MS/MS) where fragmentation gives additional structural information.[16,17] Improved detection sensitivity and selectivity can furthermore be obtained if the data are recorded in multiple reaction monitoring (MRM) mode.[18,19]

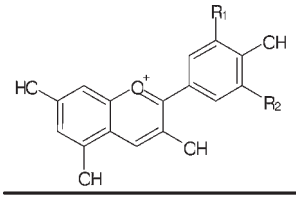
In 2005 Unwin et al.[20] introduced multiple reaction monitoring-initiated detection and sequencing (MIDAS) as a data acquisition strategy for the identification of protein phosphorylation sites. MIDAS is based on an information dependent acquisition (IDA) approach. This information dependent strategy uses multiple reaction monitoring (MRM) as a survey scan followed by product ion scans (EPI). After calculating possible MRM transitions of the potential phosphopeptides, MRM survey scans are used to trigger dependent scans according to the specified IDA criteria.

The basic structure of all anthocyanidins is the flavylium ion (2-phenylbenzopyrilium), yielding different anthocyanidins by varying methoxyl and hydroxyl substitution.[21] An overview of the six most common anthocyanidins is given in Figure 1. Anthocyanins are substituted glycosides of anthocyanidins, and aside from the structure of their aglycon, anthocyanins vary in the type, number and position of their substituted sugars as well as possible acylation, leading to more than 500 naturally occurring anthocyanins which have been identified so far.[22] The systematic structure of anthocyanins makes it possible to calculate the $m/z$ of theoretically imaginable anthocyanins. With these calculated anthocyanin $m/z$ values as precursors in MRM transitions and knowledge of common fragments,[17] a MRM-EPI method which is adapted to anthocyanin analysis can be developed. This new approach for anthocyanin characterization will have the potential to provide high sensitivity and selectivity and at the same time facilitate compound identity by providing product ion information. The development, testing and evaluation of such a method, called multiple reaction monitoring-initiated anthocyanin characterization (MIAC), is the aim of this work. The MIAC workflow is subsequently compared with more traditional IDA-MS acquisition strategies.

## MATERIALS AND METHODS

**Chemicals.** Fresh red onions (*Allium cepa L.*) as well as strawberries (*Fragaria* × *ananassa*) were purchased from a local supermarket in Uppsala, Sweden. Sour cherries (*Prunus cerasus*) were harvested from a local garden and stored in a freezer at about −18 °C until extraction. Methanol in HPLC grade was obtained from Sigma-Aldrich Chemie GmbH (Steimheim, Germany), 99.7% ethanol from Altia Corporation (Rajamäki, Finland). Formic acid in pro analysi quality was purchased from Merck KgaA (Darmstadt, Germany). All water used in the experiments was purified using a Milli-Q Plus lab water system from Millipore S.A. (Molsheim, France).

Received: December 16, 2010
Accepted: February 26, 2011
Revised: February 22, 2011
Published: March 11, 2011



| Name | $R_1$ | $R_2$ | M |
|---|---|---|---|
| Pelargonidin (pg) | H | H | 271 |
| Cyanidin (cy) | OH | H | 287 |
| Peonidin (pn) | $OCH_3$ | H | 301 |
| Delphinidin (dp) | OH | OH | 303 |
| Petunidin (pt) | $OCH_3$ | OH | 317 |
| Malvidin (mv) | $OCH_3$ | $OCH_3$ | 331 |

**Figure 1.** Structures and molecular mass (M) of the six most common anthocyanidins.

**Sample Preparation.** *Red Onion.* The edible part of a fresh red onion bulb (156 g) was cut into pieces of about 5 × 5 × 3 mm using an onion chopper. An 8 g portion of the homogenized sample was then placed in the 100 mL cell of the batch minireactor (Autoclave Engineers, Erie, PA) together with 80 mL of extraction solvent consisting of a water/ethanol/formic acid mixture (94/5/1, v/v/v). This batch reactor is a closed inert system equipped with a heating device, a stirrer, an inlet for nitrogen gas to increase pressure and an inlet and outlet for the extraction cell. After heating the filled extraction cell to 110 °C within 8 min, the extraction was continued for an additional 10 min at this temperature. During the extraction, the sample was stirred at 220 rpm. No external pressure was applied to the cell. After extraction, the extract was filtered through a Büchner funnel. The extract was aliquoted into Eppendorf tubes (1.5 mL), which were stored in the freezer at −18 °C until measurement. For measurement, thawed sample was transferred to amber screw cap vials which were kept at 4 °C by the thermostat of the autosampler.

Diluted red onion samples were prepared by mixing 10 μL of thawed sample with 990 μL of water to obtain a 1:100 sample and then diluting 100 μL of the 1:100 sample with 900 μL of water to obtain a 1:1000 sample. A sample concentrated by a factor of 21 was prepared by concentrating 6 mL of thawed sample to 80 μL of on a vacuum centrifuge, Savant ISS110 Integrated SpeedVac System (Savant Instruments Inc., Holbrook, NY) and diluting the resulting solution with 200 μL of water. Degraded red onion samples were prepared by heating one of the thawed original sample vials in an oven at 90 °C for 194 min.

*Strawberries and Sour Cherry.* 82 g of strawberries and 60 g of defrosted cherries were chopped into small pieces and homogenized. 13 g of strawberries and 13 g of cherries were then each transferred into 22 mL ASE extraction cells (Dionex Corporation, Sunnyvale, CA) and extracted with the same extraction solvent as previously used. The extraction method consisted of two 5 min extraction cycles at 110 °C after a heating period of 6 min. The applied pressure was 5 MPa. After extraction the samples were transferred into Eppendorf tubes stored in the freezer at −18 °C until measurement. For measurement, thawed sample was centrifuged and the supernatant transferred to amber screw cap vials kept at 4 °C by the thermostat of the autosampler.

**Liquid Chromatography and Mass Spectrometry: Instrumentation.** The anthocyanin separation was conducted on an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) equipped with a binary gradient pump, a degasser and a thermostated well plate autosampler. The column was a Zorbax SB-C18 column (2.1 × 100 mm, 3.5 μm, Agilent Technologies, Santa Clara, CA). Elution was performed using a flow rate of 200 μL/min with a gradient of mobile phase A (5% formic acid in aqueous solution) and mobile phase B (5% formic acid in methanol). The following gradient was used for all samples: 0−2 min, 5% B; 2−10 min, 5−20% B; 10−15 min, 20% B; 15−30 min, 20−30% B; 30−35 min, 30% B; 35−50 min, 30−45% B, 50−51 min, 45−5% B, 51−70 min, 5% B. The injection volume was 50 μL for the onion samples and 10 μL for all fruit samples. For spectrophotometric detection, an Agilent 1100 DAD (Agilent Technologies, Waldbronn, Germany) with a 6 mm flow cell was used and UV/vis spectra between 190 and 650 nm were collected for all samples while monitoring the absorption at 520 and 254 nm.

The LC−DAD system was connected online to a 3200 Q Trap mass spectrometer manufactured by AB Sciex (Concord, ON, Canada). The ion source used for the LC−MS interface was a Turbo V source in positive ESI mode. The following MS conditions were used: temperature 600 °C, curtain gas ($N_2$) 15 psi, ion source gas 1 ($N_2$) 60 psi, ion source gas 2 ($N_2$) 60 psi, ion spray voltage 5500 V and entrance potential 10 V for all measurements. For the selected ion monitoring (SIM) measurements, the declustering potential was 90 V while all other methods used 50 V. For all MS/MS and EMS (linear ion trap MS scan) methods, the collision gas parameter ($N_2$) was set to an arbitrary number, 11, which corresponds to a pressure reading of $3.9 \times 10^{-5}$ Torr. The collision energy was 30 eV for all experiments aside from the EMS where it was set to 10 eV. The monitored $m/z$ transitions for MIAC_1 to MIAC_4 methods are given in Tables S1 and S2 in the Supporting Information. In IDA mode the number of EPI experiments was three except for the general MIAC_271−301 and MIAC_303−331 methods with three aglycons and the EMS_EPI method where it was set to two.

Acquisition and processing of the DAD data was performed with ChemStation for LC 3D systems (Agilent Technologies). For the MS data, Analyst 1.4.2 (AB Sciex) was used.

## RESULTS AND DISCUSSION

**MIAC for Onion Analysis.** Red onion extracts, of different concentrations, were analyzed with LC−DAD−MS utilizing a number of different MS methods. The results from the different runs are summarized in Table 1, where boldface denotes compounds tentatively identified with help of published data,[23] and according to ref 23, at least 10 anthocyanins have been found in red onion. All of them have either cyanidin (cy) or peonidin (pn) as aglycon, and glucose (glu) as the basic sugar unit. In the case of acylation, the acid is either malonic (mlo) and/or acetic acid (act). Based on this data, the $m/z$ of likely components were calculated giving in total 16 MRM transitions (MIAC_1 method). The monitored fragments of the MRM transitions are the respective aglycons: $m/z$ 287.1 for cyanidin and 301.1 for peonidin. A total of 25 chromatographic peaks were found in the $DAD_{520nm}$ chromatogram (Figure 2a) when the initial onion extract was analyzed. However, upon analyzing a concentrated extract, 33 $DAD_{520nm}$ peaks were detected (Figure 2b). Thus to facilitate comparison of the two chromatograms, corresponding peaks are given the same number. The retention times ($t_R$) of the DAD peaks were then matched with those found in the total ion current (TIC) chromatogram with the MIAC_1 method. The dominating peaks are no **11**, **13**, **16**, **21** and **23**. Peak **11** was found to be an overlap of at least two $m/z$ signals corresponding to; cyanidin 3-glucoside (peak **11a**, $m/z$ 449, dominating signal) and cyanidin 3-(3″-malonyl)glucoside-5-glucoside (peak **11b**, $m/z$ 697, minor signal). Peak **11a** could possibly be an up-front CID fragment originating from **11b**, however the chromatographic elution profile differs slightly. Chromatographic peaks with minor signals for $m/z$ 697, which could possibly correspond to peak 1 (cyanidin 3-(malonyl)glucoside-5-glucoside) in previously published data,[23]

**Table 1. Red Onion**[a]

| peak | $t_R$ [min] DAD$_{520nm}$ | $t_R$ [min] XIC | $[M]^+$ ($m/z$) | MS/MS ($m/z$) | compound | MS method |
|---|---|---|---|---|---|---|
| 1 | 10.89 | 11.00 | 535 | 287 | cy + (glu + mlo) | MIAC_4 |
| 2a | 12.44 | 12.90 | 611 | 287, 449 | cy +2 glu | MIAC_4 |
| 2b | | 12.90 | 535 | 287 | cy + glu + mlo | MIAC_4 |
| 3 | 13.70 | 13.89 | 535 | 287 | cy + glu + mlo | MIAC_4 |
| 4 | 14.54 | 14.60 | 773 | 287, 449, 611 | cy + glu + (2 glu) | MIAC_4 |
| 5a | 15.18 | 15.35 | 611 | 287, 449 | cy +2 glu | MIAC_1 |
| 5b | | 15.36 | 697 | 287 | cy + (2 glu + mlo) | MIAC_1 |
| 6a | 16.55 | 16.61 | 611 | 287, 449 | cy +2 glu | MIAC_1 |
| 6b | | 16.77 | 773 | 287, 449, 611 | cy +3 glu | MIAC_4 |
| 6c | | 16.77 | 697 | 287 | cy + (2 glu + mlo) | MIAC_1 |
| 6d | | 16.91 | 627 | 303, 345, 465 | dp +2 glu | MIAC_2 |
| 7 | 17.05 | 17.00 | 611 | 287, 449 | cy +2 glu | MIAC_1 |
| 8a | 18.07 | 17.95 | 773 | 287, 449, 611 | cy +3 glu | MIAC_4 |
| 8b | | 17.96 | 535 | 287 | cy + (glu + mlo) | MIAC_4 |
| 8c | | 18.30 | 697 | 287, 449, 535 | cy + glu + (glu + mlo) | MIAC_4 |
| 9 | 18.64 | 18.70 | 697 | 287, 449, 535 | cy + glu + (glu + mlo) | MIAC_4 |
| 10 | 19.35 | 19.50 | 535 | 287 | cy + (glu + mlo) | MIAC_4 |
| 11a | 21.13 | **21.22** | **449** | **287** | **cyanidin 3-glucoside** | MIAC_1 |
| 11b | | **21.29** | **697** | **287, 449, 535** | **cyanidin 3-(3″-malonyl)-glucoside-5-glucoside** | MIAC_1 |
| 12 | 22.64 | 22.64 | 449 | 287, 315, 360, 405 | cy + glu? | MIAC_1 |
| 13 | 23.88 | **23.95** | **611** | **287** | **cyanidin 3-laminarbioside** | MIAC_1 |
| 14a | 25.10 | 25.10 | 611 | 287 | cy + (2 glu) | MIAC_1 |
| 14b | | 25,23 | 627 | 303, 345, 465 | quercetin +2 glu | MIAC_2 |
| 15a | 25.73 | 25.83 | 697 | 287 | cy + (2 glu+ mlo) | MIAC_1 |
| 15b | 25.98 | 26.32 | 859 | 287, 449, 697 | cy + glu + (2 glu + mlo) | MIAC_4 |
| 15c | | 25.90 | 611 | 287 | cy + (2 glu) | MIAC_1 |
| 16 | 26.82 | **26.88** | **535** | **287** | **cyanidin 3-(3″-malonylglucoside)** | MIAC_1 |
| 17a | 28.25 | 27.82 | 491 | 287 | cy + (glu + act) | MIAC_1 |
| 17b | | **28.22** | **463** | **301, 329** | **peonidin 3-glucoside** | MIAC_1 |
| 17c | | 28.59 | 535 | 287 | cy + (glu + mlo) | MIAC_1 |
| 18 | 29.64 | 29.77 | 491 | 287 | cyanidin 3-(3″-acetoyl)glucoside | MIAC_1 |
| 19 | 30.57 | 30.63 | 859 | 287, 449 697 | cy + glu + (2 glu + mlo) | MIAC_4 |
| 20 | 31.22 | 31.31 | 463 | 301 | pn + glu | MIAC_4 |
| 21 | 34.57 | 34.66 | 535 | 287, 449, 491 | cyanidin 3-(6″-malonylglucoside) | MIAC_1 |
| 22 | 36.20 | - | 287 | 137, 157, 213 | cyanidin | EMS_EPI |
| 23 | 37.51 | **37.55** | **697** | **287** | **cyanidin 3-(6″-malonyllaminarbioside)** | MIAC_1 |
| 24a | 38.45 | 38.54 | 697 | 287 | cy + (2 glu + mlo) | MIAC_1 |
| 24b | | 38.56 | 465 | 303, 345 | quercetin + glu | MIAC_2 |
| 25 | 41.87 | 41.97 | 549 | 301 | peonidin 3-(malonyl)glucoside | MIAC_1 |
| 26 | 43.05 | 43.10 | 491 | 287 | cy + (glu + act) | MIAC_1 |
| 27 | 44.01 | **44.12** | **577** | **287** | **cyanidin 3-(malonyl)(acetoyl)glucoside** | MIAC_1 |
| 28 | 45.33 | 45.5 | 711 | 301 | pn + (2 glu + mlo) | MIAC_4 |
| 29 | 46.07 | 46.20 | 463 | 301 | pn + glu | MIAC_4 |
| 30 | 46.65 | 46.75 | 653 | 287 | cy +(2 glu + act) | MIAC_1 |
| 31 | 47.7 | | | | | |
| 32 | 49.29 | | | | | |
| 33 | 51.07 | 51.14 | 551 | 303 | dp + (glu + mlo) | MIAC_2 |

[a] Peak list including retention times ($t_R$) for DAD$_{520nm}$ and matched XIC peaks as well as found precursor ions ($[M^+]$), MS/MS fragments and assigned compounds: cy = cyanidin, pn = peonidin, dp = delphinidin, glu = glucose, act = acetic acid, mlo = malonic acid. Boldface denotes compounds tentatively identified with the help of published data.[23]

were also found for peaks **5b** and **6c**. However, MS/MS of those peaks show only the 287 fragment indicating the loss of malonyl bioside. In Table 1 this is indicated by the compound label, **5b**, cy + (2 glu + mlo), and **6c**, cy + (2 glu + mlo), where the parentheses


dx.doi.org/10.1021/jf104840v |J. Agric. Food Chem. 2011, 59, 2988–2996

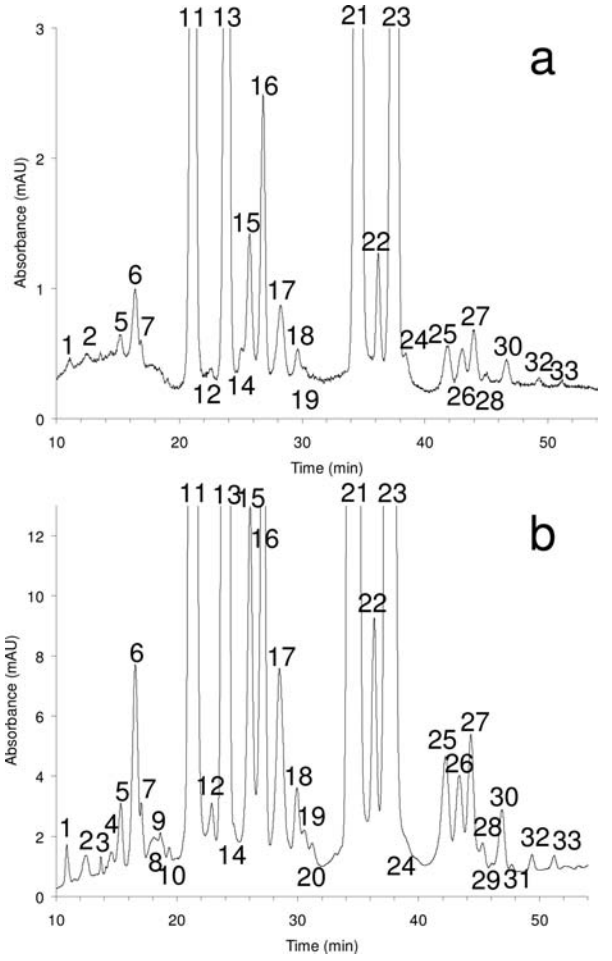



**Figure 2.** Red onion; enlarged DAD chromatogram (520 nm): (a) original sample (b) concentrated sample.

correspond to the observed loss. The $m/z$ 535 and $m/z$ 449 fragments corresponding to the loss of glucose and malonyl-glucoside respectively are not observed. The next large tentatively identified peak is peak **13**, cyanidin 3-laminarbioside, followed by **16**, cyanidin 3-(3″-malonyl)glucoside. The last two dominating peaks are **21**, cyanidin 3-(6″-malonyl)glucoside, and **23**, cyanidin 3-(6″-malonyl) laminarbioside. Further anthocyanins previously identified[23] include $DAD_{520nm}$ peak **17**, MS peak **17b**, peonidin 3-glucoside, among two unidentified compounds **17a,c**; peak **18**, cyanidin 3-(3″-acetoyl)glucoside; **25**, peonidin 3-(malonyl)glucoside; and **27**, cyanidin 3-(malonyl)(acetoyl)glucoside. Additional peaks (peak **7**, **12**, **14**, **15**, **24**, **26**, **30**) in the $DAD_{520nm}$ chromatogram were correlated to 8 MS peaks (peak **7**, **12**, **14a**, **15a**, **15c**, **24a**, **26**, **30**) utilizing the MIAC_1 method, and the results are included in Table 1. MS/MS data provide some structural information, however, exact configuration cannot be assigned based on MS data. For example, both peaks **26** and **30** belong to compounds which have cyanidin as the aglycon and a sugar moiety which is acylated with acetic acid. Considering the fact that glucose is the only hexose found in red onion, it is likely that peak **26** belongs to a cyanidin (acetoyl)glucoside. Peak **30**, $m/z$ 653, does not show any fragments where only one sugar molecule is lost. This is a sign for only one glycosidic bond.[17] Considering this and the relative position to peak **26**, it is likely that the compound is the laminarbioside equivalent to peak **26** since no other biosides were previously found in red onion.

Some of the $DAD_{520nm}$ peaks show overlapping peaks (see Table 1) with different $m/z$ in the TIC. Based on the $DAD_{520nm}$ signal, it can only be determined that there is at least one anthocyanin present in each peak, but not if all of the different peaks in the TIC belong to anthocyanins. Furthermore, some MS peaks were difficult to match with any peaks in the $DAD_{520nm}$ chromatogram from the onion extract, such as the $m/z$ 535 peaks eluted at 12.90 (**2b**) and 13.89 (**3**) min. There are two likely causes for these peaks. On the one hand the signal-to-noise ratio (S/N) for the MS is in general much better than for the DAD. This combined with the irregular background in the low $t_R$ area could be responsible for anthocyanins of low concentration not showing up in the $DAD_{520nm}$ chromatogram. On the other hand, compounds which are not anthocyanins but have similar $m/z$ values and structures, such as e.g. other flavonoids, might be present. This shows the importance of the additional selectivity provided by the DAD. For peak **3** at 13.89 min the DAD chromatogram displays a large matching peak at 254 nm, indicating the presence of another kind of flavonoid. In MIAC_1 measurements, very low signals were detected which were not visible in the $DAD_{520nm}$ chromatogram. As previously mentioned, one possible reason for this is the difference in S/N between DAD and MS. The difference in S/N between DAD and MS was investigated more thoroughly to provide a better basis for determining whether an extracted ion chromatogram (XIC) peak should be visible in the $DAD_{520nm}$ chromatogram or not. By diluting the onion extract it was concluded, based on the most intense peaks (**11**, **13**, **21**, **23** in Figure 2a), that more than ten times higher S/N were obtained with MS detection. The MS peak **3**, $m/z$ 535, found at 13.89 for undiluted sample shows S/N of 18.5. This is in the magnitude where XIC peaks for established anthocyanins are no longer visible in the $DAD_{520nm}$ chromatogram. This means that peak **3** could well be caused by anthocyanins without showing any visible $DAD_{520nm}$ signals.

Some peaks in the DAD chromatogram were not possible at all to correlate with any MS signal acquired with the MIAC_1 method. The most likely reason for this is that the $m/z$ of the respective compounds were not included in the MRM transition list (see Table S1 in the Supporting Information). Compounds that do e.g. have a different aglycon than cyanidin or peonidin would not be found. As the $DAD_{520nm}$ chromatogram, in Figure 2a, shows the presence of at least 25 anthocyanins, a further literature search was conducted and a number of additional reports dealing with anthocyanins in red onion were found. In total, 24 anthocyanins[24−28] have been reported to be present in different red onion cultivars. In previous studies, sample preparation has been reported to influence the results,[26,27] and in an attempt to ensure that the additional peaks, found in this study, are not the result of degradation during the extraction process, sample was artificially degraded and the resulting chromatograms were compared to undegraded sample. A clear decrease in peak area ($DAD_{520nm}$) for all 25 peaks was observed (15−60% after 194 min for **11**, **13**, **21**, **23**). Since none of the peaks increased during the degradation process, it can be assumed that all anthocyanins found are actually present in red onion and not a product of degradation during the extraction process. It should be pointed out that some degradation could already occur during the initial extraction[29] contributing to some uncertainty in the evaluation. However, as the main aim with this study is to evaluate a new MS strategy for anthocyanin characterization, we still believe that the proposed MIAC method is promising.

To broaden the search, three different measurements were conducted monitoring MRM transitions corresponding to ions containing: (1) the other four common anthocyanidins (see Figure 1) (MIAC_2); (2) cyanidin and peonidin, with both one and two glucose molecules acylated with one of the acids propionic acid, oxalic acid, succinic acid and malic acid (MIAC_3); (3) cyanidin and peonidin using the same acylation patterns as in MIAC_1, but including the sugar moieties with three glucose molecules instead of only one and two (MIAC_4).

Upon testing the presence of anthocyanins with petunidin, pelargonidin, malvidin and delphinidin as aglycon utilizing MS method MIAC_2, 11 major peaks were found in the TIC. However only four could be assigned to peaks in the $DAD_{520nm}$ chromatogram. The first of these four $m/z$ peaks (peak **6d**, Table 1) cannot be assigned to a specific $DAD_{520nm}$ peak, but lies in the overlap between the broad $DAD_{520nm}$ peaks **6** and **7**. Since it is relatively small, the absorption could be low enough to be covered by the neighboring peaks. Peak **6d** with $m/z$ 627 combined with the 465 and 303 MS/MS fragments suggests a delphinidin based anthocyanin with two glucose molecules attached to different glycosylation sites. The 345 fragment might be caused by fragmentation of one of the glucose molecules.[30] However, combined with the missing selectivity due to the ambiguous $DAD_{520nm}$ signal, this makes it dubious whether the compound is truly an anthocyanin. The next two TIC peaks show very intense MS signals while the absorption of the assigned peaks **14b** and **24b** in the $DAD_{520nm}$ chromatogram is very low. The assigned $m/z$ peaks are 627 for peak **14b** and 465 for peak **24b**, which suggests a delphinidin with two glucose molecules for $DAD_{520nm}$ peak **14b** and a delphinidin glucuoside for peak **24b**. The fragmentation patterns (see Table 1), however, show again the presence of the 345 ion aside from the more expected fragments in both cases. Additionally to this, an overlay of the DAD chromatograms at 254 and 520 nm shows intense peaks in the UV overlapping with peaks **14** and **24**. The low quotient of visible to UV signal suggests that the intense XIC peaks belong to compounds which are not anthocyanins. The flavonol quercetin has a mass of 302. Protonated quercetin which could be detected by the MS in positive mode would therefore have an $m/z$ of 303, just like delphinidin. Red onions are known to contain high amounts of quercetin glycosides.[31,32] It is therefore likely that the intense XIC peaks belong to a quercetin with two glucose molecules bound to different glycosylation (**14b**) sites and a quercetin glucoside (**24b**). The low absorption signal in the visible range can be explained by presence of other anthocyanins in low quantity. Another possible cause could be that the minimal absorption of the quercetin compounds at 520 nm has become noticeable due to their high concentration. The last XIC peak that was assigned, with MIAC_2, suggests that peak **33** is caused by a delphinidin (malonyl)glucoside.

Upon utilizing a method (MIAC_3), testing the presence of cyanidin and peonidin, with both one and two glucose molecules acylated with one of the acids propionic acid, oxalic acid, succinic acid and malic acid, it can be concluded that they are not likely present in red onion.

Finally, testing the presence of cyanidin and peonidin with three glucose molecules utilizing the MIAC_4 method, the onion extract was also concentrated by a factor of 21. The $DAD_{520nm}$ chromatogram shown in Figure 2b now indicates the presence of additional possible anthocyanins. In total 33 DAD peaks and 16 additional MS peaks detected with MIAC_4 are listed in Table 1. The results obtained with the MS method MIAC_4 indicate the presence of at least five anthocyanins with three glucose molecules (**4**, **6b**, **8a**, **15b**, **19**). Upon a closer examination it was found that the $DAD_{520nm}$ peak **6** consists of several components (**6a**−**d**) ($m/z$ 611, 627, 697 and 773) with slightly different elution profiles. The XIC peaks for the $m/z$ 697 and 859 found for peaks **15a**,**b** do however overlap, indicating, as previously mentioned, that the 697 ions might be fragments of the 859, an assumption supported by the MS/MS data of the 859 ion. To conclude, the analysis of red onion extract with the MIAC approach indicates that 41 anthocyanins are present, including possible isomers (excluding overlapping compound, **15a**), although the exact configuration could not be elucidated. However, MS/MS spectra of all 41 compounds were acquired.

**MIAC vs Common IDA Analysis.** Improved separation and detection selectivity as well as sensitivity is desirable to fully characterize the anthocyanin content of red onion extracts. However, the main aim with this work was to develop and evaluate a new data acquisition strategy for anthocyanins in different extracts. It has been shown in the previous sections that MIAC measurements can be successfully used as a tool in anthocyanin analysis. To evaluate the performance of MIAC in comparison to a more traditional method, the results from the MIAC measurement of red onion extract are compared to those from a more common IDA experiment based on EMS_EPI measurement. The main advantage of the MIAC compared to the EMS_EPI measurement is the increased simplicity of the data evaluation. Due to the enhanced selectivity achieved by choosing MRM for a survey scan, most background noise, as well as compounds which do not fit the transition list, are removed, giving the MIAC TIC a shape close to the $DAD_{520nm}$ chromatogram. This is a valuable visual aid in finding XIC peaks to assign to the $DAD_{520nm}$ peaks. Furthermore, the number of acquired MS/MS is greatly reduced compared to the EMS_EPI measurement and includes only relevant precursors, thereby enabling a peak search by intensity without further consideration of the precursor $m/z$ value. Since according to the IDA criteria the two largest $m/z$ traces at a given point are selected for MS/MS, many nonrelevant $m/z$ traces are selected as precursors in the EMS_EPI measurement while smaller relevant $m/z$ traces are missed due to increased signal threshold. Approximately 30% of the relevant MS/MS spectra taken in MIAC_1 were found with the EMS_EPI measurement. An additional advantage of the MIAC measurement is the superior S/N, approximately 10−40 times higher, in comparison to the EMS_EPI measurement which enables detection of smaller peaks. The main disadvantage of the MIAC measurement is that the enhanced selectivity, better S/N and simplified data evaluation come at the cost of missing analytes which were not anticipated since anthocyanins not included in the MRM transition list will not be detected. Therefore, great care has to go into the development of a fitting MIAC method. Developing a fitting method might be difficult without relying on previously collected data of the sample. Even in the case of red onion, where literature data was available to create the MIAC methods, certain relevant compounds were likely not included in the transition lists, as evident from the lack of MS data for three of the $DAD_{520nm}$ peaks (**22**, **31**, **32**). Since the EMS_EPI measurement is unbiased in regard to measuring all ions as long as they are within the scanning range, this is not a problem for these types of methods. One of the unidentified $DAD_{520nm}$ peaks, peak **22**, was tentatively identified as the free cyanidin aglycon with the data provided by the EMS_EPI measurement. Other likely anthocyanins which were not included in the transition list are

diacyl- and (dimalonyl)glucosides. One of the latter compounds has even previously been found in red onion.[24] The incorrect exclusion of masses deemed unimportant might also lead to false security in peak identity if overlapping mass peaks are not detected.

**General MIAC Analysis.** General MIAC methods were developed which do not rely on previous knowledge of the presence of anthocyanins in a particular matrix. Different MIAC methods were combined according to two different strategies. The first strategy included development of one MIAC method for each of the six common aglycons shown in Figure 1. Each of these methods combines the sugars arabinose/xylose (arb/xyl), rhamnose (rha), glucose/galactose (glu/gal), sambubiose/lathyrose (smb/lat), rutinose (rtn), sophorose/laminarbiose/gentobiose (sph/lmb/gnt) and three sugar molecule combinations thereof with the following 11 acids: acetic acid (act), propionic acid (prp), oxalic acid (oxa), malonic acid (mlo), succinic acid (suc), malic acid (mli), *p*-hydroxybenzoic acid (hyb), *p*-coumaric acid (cou), caffeic acid (caf), ferulic acid (fer) and sinapic acid (sin). After removal of isobaric transitions, each of the methods included 83 transitions resulting in a decreased dwell time for each MRM transtition, from 50 ms for MIAC_1 to 30 ms. These MIAC methods are called MIAC_271, MIAC_287, MIAC_301, MIAC_303, MIAC_317 and MIAC_331 where the number stands for the $m/z$ of the respective anthocyanidin. Since this approach requires one measurement for each aglycon, the data acquisition strategy includes a preceding SIM measurement (with an increased up-front CID potential) of the aglycons present to reduce the number of necessary runs. The second approach combines the methods MIAC_271, MIAC_287 and MIAC_301 into MIAC_287−301 and MIAC_303, MIAC_317 and MIAC_331 into MIAC_303−331. This reduces the number of measurements per sample necessary to cover every aglycon by four. Each of the two resulting methods contains 249 MRM transitions, with a dwell time for each MRM of 10 ms.

*Red Onion.* To compare the general MIAC methods with a method specifically designed for onion analysis, MIAC_271−301 was run for the red onion sample, and the majority of anthocyanins previously detected were also found with the general MIAC_271−301 method. The only exception is cyanidin 3-(malonyl)(acetoyl)glucoside, which was not detected since anthocyanins with more than one acyl group are not included in the transition lists of the general methods. Three of the minor MS peaks previously found were not detected with MIAC_271−301. This is likely due to decreased peak shape quality and S/N caused by reduction of data points per MS cycle and decrease in dwell time per $m/z$ respectively. This loss in MS measurement quality is not avoidable for the general methods due to the higher number of incorporated MRM transitions.

*Strawberry.* The most common aglycon $m/z$ in the SIM measurement for a strawberry extract is 271, which indicates the presence of pelargonidin anthocyanins. The other major peaks found had $m/z$ 287 (cyanidin) and 303 (delphinidin). Since the large 303 XIC peak cannot be assigned to any peak in the $DAD_{520nm}$ chromatogram but shows a large UV absorption, it likely belongs to a different kind of flavonoid aglycon, such as e.g. quercetin. Therefore only the general MIAC methods MIAC_271 and MIAC_287 as well as the combined method MIAC_271−301 were used on the strawberry sample. Previous studies[33,34] reported the presence of six[33] and five[34] anthocyanins in strawberry. A total of 27 peaks were found in the $DAD_{520nm}$ chromatogram (Figure 3a). For eleven of these peaks,

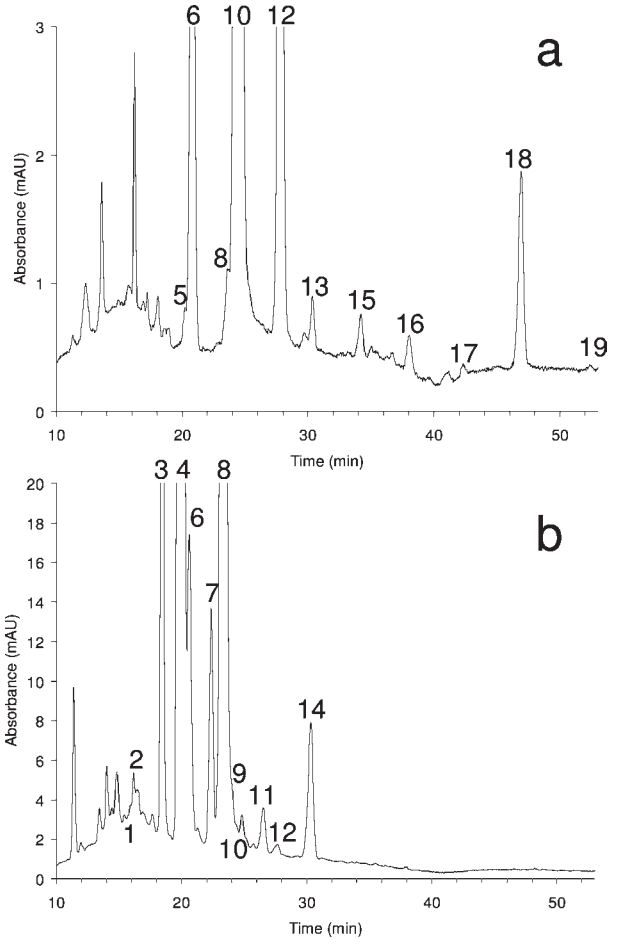



**Figure 3.** Enlarged $DAD_{520nm}$ chromatogram for (a) strawberry and (b) sour cherry.

XIC peaks could be assigned, shown in Table 2. By comparison with retention times and mass spectra from the literature,[33,34] seven of the anthocyanins could be tentatively identified. The peaks in Figure 3a,b are numbered uniformly in an attempt to make comparison between the two chromatograms easier; furthermore, only peaks that were correlated to MS data are numbered. The first two tentatively identified peaks are **6**, cyanidin 3-glucoside (peak 13), and **8**, cyanidin 3-rutinoside, followed by their pelargonidin equivalents **10**, pelargonidin 3-glucoside, and **12**, pelargonidin 3-rutinoside. The other three tentatively identified compounds were **15**, cyanidin 3-(malonyl)glucoside, **16**, pelargonidin (malonyl)glucoside, and **18**, 3-(6″-acetoyl)glucoside. The other four peaks for which XIC peaks could be assigned are peaks **5**, **13**, **17** and **19**. The assigned $m/z$ for peak **5** is 757 with cyanidin as the aglycon. While this mass allows for several different combinations of sugar moieties and esterified acids, a combination of two glucose and one rhamnose molecule seems most likely considering the structures of the identified anthocyanins. The combination of three sugar molecules would also explain the relatively low $t_R$. The next unidentified anthocyanin, peak **13**, is an acylated pelargonidin glycoside. Under consideration of the other anthocyanins present the anthocyanin is likely a pelargonidin (malonyl)glucoside isomer. Peak **17** was assigned to a XIC peak with an $m/z$ of 491. The aglycon is cyanidin. A cyanidin (acetoyl)glucoside is the most probable type of anthocyanin for this combination. The last

**Table 2. Strawberry**[a]

| peak | $t_R$ [min] DAD$_{520nm}$ | $t_R$ [min] XIC | $[M]^+$ ($m/z$) | MS/MS ($m/z$) | compound |
|---|---|---|---|---|---|
| 5 | 20.22 | 20.44 | 757 | 287 | cy + smb/lat + fer, cy + rtn + caf, cy + sph/lmb/gnt + cou, 2 glu/gal + rha, glu + gal + rha |
| 6 | 20.82 | **21.08** | **449** | **287** | **cyanidin 3-glucoside** |
| 8 | 23.61 | **23.82** | **595** | **287, 449** | **cyanidin 3-rutinoside** |
| 10 | 24.36 | **24.62** | **433** | **271** | **pelargonidin 3-glucoside** |
| 12 | 27.80 | **28.03** | **579** | **271, 433** | **pelargonidin 3-rutinoside** |
| 13 | 30.37 | 30.54 | 519 | 287 | pg + glu/gal + mlo, pg + arb/xyl + mli |
| 15 | 34.20 | **34.42** | **535** | **287** | **cyanidin 3-(malonyl)glucoside** |
| 16 | 38.04 | **38.38** | **519** | **271** | **pelargonidin 3-(malonyl)glucoside** |
| 17 | 42.33 | 42.51 | 491 | 287 | cy + arb/xyl + oxa, cy + glu/gal + act |
| 18 | 46.96 | **47.21** | **475** | **271** | **pelargonidin 3-(6″-acetoyl)glucoside** |
| 19 | 52.47 | 52.68 | 489 | 271 | pg + arb/xyl + mlo, pg + rha + oxa, pg + glu/gal + prp |

[a] Peak list for MIAC_271 and MIAC_287 measurement including retention times ($t_R$) for matched DAD$_{520nm}$ and XIC peaks as well as found precursor ions ($[M^+]$), MS/MS fragments and assigned compounds: cy = cyanidin, pg = pelargonidin, arb = arabinose, xyl = xylose, rha = rhamnose, glu = glucose, gal = galactose, smb = sambubiose, lat = lathyrose, rtn = rutinose, sph = sophorose, lmb = laminarbiose, gnt = gentobiose, act = acetic acid, prp = propionic acid, oxa = oxalic acid, mlo = malonic acid, mli = malic acid, cou = *p*-coumaric acid, caf = caffeic acid, fer = ferulic acid. Boldface denotes compounds tentatively identified with the help of published data.[33,34]

**Table 3. Cherry**[a]

| peak | $t_R$ [min] DAD$_{520nm}$ | $t_R$ [min] XIC | $[M]^+$ ($m/z$) | MS/MS ($m/z$) | compound |
|---|---|---|---|---|---|
| 1 | 16.00 | 15.25 | 595 | 287 | cy + arb/xyl + fer, cy + rha + caf, cy + glu/gal + cou, cy + rtn |
| 2 | 16.25 | 16.48 | 773 | 287 | cy + sph/lmb/gnt + caf, cy + 3 glu/gal and permutations thereof |
| 3 | 18.55 | 18.81 | 611 | 287 | **cyanidin 3-sophoroside** |
| 4 | 19.94 | 20.24 | 757 | 287, 433, 611 | **cyanidin 3-glucosylrutinose** |
| 6 | 20.73 | 21.04 | 449 | 287 | **cyanidin 3-glucoside** |
| 7 | 22.53 | 22.80 | 727 | 287, 433, 581 | cy + rha + smb/lat |
| 8 | 23.47 | 23.67 | 595 | 287, 433, 449 | **cyanidin 3-rutinoside** |
| 9 | 24.00 | 24.38 | 741 | 271, 417, 595 | pg + rha + sph/lmb/gnt |
| 10 | 24.38 | 24.75 | 433 | 271 | **pelargonidin 3-glucoside** |
| 11 | 26.77 | 27.02 | 771 | 301, 625 | pn + rha + sph/lmb/gnt |
| 12 | 27.80 | 28.10 | 579 | 271, 433, 505 | **pelargonidin 3-rutinoside** |
| 14 | 30.53 | 30.75 | 609 | 301, 463 | **peonidin 3-rutinoside** |

[a] Peak list for MIAC_271, MIAC_287 and MIAC_301 measurement including retention times ($t_R$) for matched DAD$_{520nm}$ and XIC peaks as well as found precursor ions ($[M^+]$), MS/MS fragments and assigned compounds: pg = pelargonidin, cy = cyanidin, pn = peonidin, arb = arabinose, xyl = xylose, rha = rhamnose, glu = glucose, gal = galactose, smb = sambubiose, lat = lathyrose, rtn = rutinose, sph = sophorose, lmb = laminarbiose, gnt = gentobiose, cou = *p*-coumaric acid, caf = caffeic acid, fer = ferulic acid. Boldface denotes compounds tentatively identified with help of published data.[33,35,36]

matched peak, **19**, had an $m/z$ 489 peak and pelargonidin as the aglycon. There are several probable combinations of sugar moieties and acids that could lead to this $m/z$. While the obtained signals are lower in the MIAC_271−301 measurement than the two separate MIAC_271 and MIAC_287 measurements due to decreased dwell time, all relevant signals and corresponding MS/MS data were present. This makes MIAC_271−301 the preferable data acquisition method due to the decrease in total analysis time.

*Sour Cherry.* From the SIM measurement of a sour cherry extract cyanidin was found to be the main anthocyanidin together with smaller amounts of pelargonidin and peonidin. Furthermore, XIC peaks for $m/z$ 303 (delphinidin) and 317 (malvidin) were found, however, they could not be matched with DAD$_{520nm}$ chromatogram peaks and are therefore likely not caused by anthocyanins. Petunidin peaks were not detected. Therefore the chosen MIAC methods were MIAC_271, MIAC_287, MIAC_301 and the combined method MIAC_271−301. Published studies have identified a total of 8 different anthocyanins in cherries.[33,35,36] The number of anthocyanins for each cherry sample varied between 2 and 6. While sweet cherries have cyanidin 3-rutinoside as the major anthocyanin,[33,36] the main pigment in sour cherries is cyanidin 3-glucosylrutinoside.[35,36] The measurements conducted in this study showed 27 distinguishable DAD$_{520nm}$ peaks for the cherry sample (Figure 3b). An overview of the data for matched DAD$_{520nm}$ and XIC peaks is given in Table 3. As expected, the largest DAD$_{520nm}$ peak **4** was tentatively identified as cyanidin 3-glucosylrutinose. Aside from cyanidin 3-glucosylrutinose, six other anthocyanins were tentatively identified with the help of published data:[33,35,36]

**3**, cyanidin 3-sophoroside, **6**, cyanidin 3-glucoside, **8**, cyanidin 3-rutinoside, **10**, pelargonidin 3-glucoside, **12**, pelargonidin 3-rutinoside, and **14**, peonidin 3-rutinoside. However, for five additional $DAD_{520nm}$ peaks with assigned XIC peaks, unambiguous identification of the compounds was not possible. For peaks **1** and **2**, the MS data indicates cyanidin but permits a multitude of different combinations for the glycoside and acyl moieties. Peak **7** belongs to a cyanidin glycoside as well. In this case the MS/MS fragmentation pattern indicates the presence of rhamnose at one glycosylation site and a pentose-hexose moiety like sambubiose or lathyrose. The last two anthocyanins are pelargonidin (peak **9**) and peonidin glycosides (peak **11**). As with peak **7**, the MS/MS fragmentation pattern indicates a rhamnose moiety at one glycosylation site. Considering the fragmentation pattern and the sugar moieties of other anthocyanins in the matrix, the other sugar moiety consists likely of two coupled glucose molecules, like sophorose, laminarbiose or gentobiose.

As can be seen in the chromatograms in Figure 3a,b, there are a number of additional plausible anthocyanins present, pointing out that the selectivity provided by the DAD is important if extensive characterization of anthocyanins is to be conducted. However, in our opinion we strongly believe that MIAC is a useful data acquisition strategy for anthocyanin characterization. It has various advantages such as high sensitivity and a good S/N ratio. The main restrictions of the MIAC approach lie in that appropriate MRM transitions have to be selected and that the number of MRM transitions that can be monitored with one MIAC method are generally limited; with the current instrument used in this study the upper limit is 250 transitions.

## ■ ASSOCIATED CONTENT

**Ⓢ Supporting Information.** Two tables giving detailed information about MRM transitions. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

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### Funding Sources

This study was funded by The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS, 2006-1346) and (FORMAS, 229-2009-1527).

## ■ ABBREVIATIONS USED

MIAC, multiple reaction monitoring-initiated anthocyanin characterization; LC−DAD−ESI-MS/MS, liquid chromatography diode array detection electrospray ionization tandem mass spectrometry; EMS, linear ion trap MS scan; EPI, product ion scans; IDA, information dependent acquisition; MRM, multiple reaction monitoring; SIM, selected ion monitoring; TIC, total ion current chromatogram; XIC, extracted ion chromatogram; cy, cyanidin; pn, peonidin; pt, petunidin; pg, pelargonidin; mv, malvidin; dp, delphinidin; arb, arabinose; xyl, xylose; rha, rhamnose; glu, glucose; gal, galactose; smb, sambubiose; lat, lathyrose; rtn, rutinose; sph, sophorose; lmb, laminarbiose; gnt, gentobiose; act, acetic acid; prp, propionic acid; oxa, oxalic acid; mlo, malonic acid; sus, succinic acid; mli, malic acid; hyb, *p*-hydroxybenzoic acid; cou, *p*-coumaric acid; caf, caffeic acid; fer, ferulic acid; sin, sinapic acid

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