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# Rapid screening of anthocyanins in berry samples by surfactant-mediated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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**Surfactant-mediated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been used for the identification of flavonoids from three berry extracts: lowbush blueberry (*Vaccinium angustifolium*), lingonberry (*Vaccinium vitis-idaea*), and blackberry (*Rubus armeniacus*). The addition of the surfactant led to suppression of matrix ions from both α-cyano-4-hydroxycinnamic acid (CHCA) and 2′,4′,6′-trihydroxyacetophenone (THAP). This is the first case of this method being successfully employed with a matrix other than CHCA. It was observed that CHCA led to a great deal of fragmentation of the sugar moiety from glycosides, whereas THAP produced more intact glycoside molecules, and thus leads to better characterization of the flavonoids in a berry sample. The flavonoids were characterized and quantified by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) with UV detection. Although MALDI-TOF-MS did not lead to the identification of as many flavonoids, it did enable us to identify many anthocyanin glycosides. Quantification was achieved and demonstrated that use of the THAP matrix can enable quantification of the intact glycosides with relative standard deviation (RSD) values of less than 10% with surfactant addition. These results are comparable with LC results. MALDI-TOF-MS with THAP matrix thus provided a rapid method for the qualitative screening of these compounds. It took only a few minutes, greatly reducing the analysis time from that in traditional LC/MS methods. Copyright © 2007 John Wiley & Sons, Ltd.**

Matrix-assisted laser desorption/ionization (MALDI)[1,2] is an excellent ionization method for the analysis of proteins, oligonucleotides, and synthetic polymers, especially when coupled to a time-of-flight (TOF) mass analyzer.[1–7] The theoretical unlimited mass range of the TOF analyzer makes possible the determination of masses not possible by gas chromatography/mass spectrometry (GC/MS) or even electrospray ionization (ESI). MALDI provides sensitivity, high-throughput capabilities, and easily interpreted mass spectra consist predominantly of singly charged protonated species.[8–10] Recently, there has been a growing interest in the ability of MALDI to analyze small molecules. This has been difficult because the small organic acids typically used as matrices for MALDI tend to fragment under most instrumental conditions and the decomposition reactions of the associated fragments tend to complicate mass spectra, making it particularly difficult to analyze compounds of molecular mass less than 1000.[11–13]

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The surfactant cetyltrimethylammonium bromide (CTAB) was used as a α-cyano-4-hydroxycinnamic acid (CHCA) matrix-ion suppressor by Guo *et al*.[14] This additive suppressed the formation of CHCA matrix ions, while still allowing for adequate resolution of several analyte classes, including peptides and cyclodextrins. This method has been further explored in other studies.[15–17] Su *et al*. demonstrated that this technique can be used to screen drug molecules in clandestine tablets. Recently, our group has shown that a larger variety of quaternary ammonium surfactants can be used to induce matrix-ion suppression. This has led to the successful analysis of phenolic acids and flavonoids.[17] For these particular analytes, CTAB was found to be a viable surfactant choice and it was shown that the matrix/surfactant ratio can be reduced to 10000:1 or lower. It was demonstrated that these surfactant-containing samples yielded greater reproducibility than those without surfactant. Higher resolution values were also obtained for multiple analytes, including a phenolic acid. Due to the specificity of this method of ion suppression, we have referred to this as 'surfactant-mediated' MALDI.

Flavonoids are a large class of biologically active non-nutrients in plants, and these can be further divided into the following categories: flavonols, flavones, catechins, proanthocyanidins, anthocyanidins, and isoflavonoids.[18] In human health, flavonoids are known to be powerful antioxidants.[19] They also have antiallergenic, antiinflammatory and antiviral properties.[19,20] Several studies have shown that they decrease the risk of coronary heart disease, stroke, and stomach and lung cancer.[21–23] Both flavonol and anthocyanidin glycosides have been found in a multitude of fruit juices, wines, and berries which include blueberries, raspberries, partridgeberries, and strawberries.[24–27] These compounds are often responsible for the blue or reddish colour of the berries. Several studies have focused on methods to extract these compounds, and they have then been analyzed by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS).[26,27] Typically, ESI is used as the ionization method, but some studies have explored the used of MALDI-time-of-flight (TOF)-MS as an alternative.[28,29] Wang and Sporns demonstrated that 2,4,6-trihydroxyacetophenone (THAP) is a suitable matrix for anthocyanin glycosides.[28] Qualitative screening of fruit juices and berry samples was successful, and the linear response of anthocyanins indicated that quantitation was possible.[28]

The current study carries forward the use of MALDI-TOF-MS with the focus on the application of surfactant-mediated MALDI to aid in the rapid analysis of flavonoids from a variety of berry extracts. We investigated both CHCA and THAP as potential matrices for the flavonoids, with the assumption that the CTAB would cause suppression of the matrix ions, and in turn lead to mass spectra with minimal noise. We also illustrate separation of flavonoids via LC with ESI-MS to aid in peak identification. We demonstrate this method as a complementary rapid-screening technique that can qualitatively identify flavonoids in just minutes, whereas LC methods require longer run times to adequately separate the flavonoids from berry extracts.

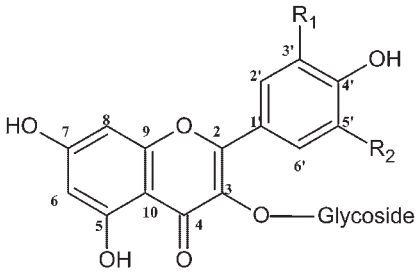
## EXPERIMENTAL

### Chemicals

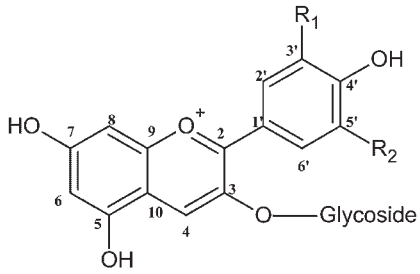
α-Cyano-4-hydroxycinnamic acid (CHCA), quercetin (302.24 g/mol) and rutin (610.52 g/mol), (quercetin 3-rutinoside) were purchased from Sigma (St. Louis, MO, USA). Cetyltrimethylammonium bromide (CTAB) was obtained from Aldrich (Mississauga, ON, Canada). Deionized water and methanol were HPLC grade purchased from Fisher Scientific (Fair Lawn, NJ, USA). Cyanidin (287.25 g/mol), cyanidin 3-glucoside (cyanidin 3-O-β-D-glucopyranoside) (449.39 g/mol), delphinidin (303.25 g/mol), malvidin (331.22 g/mol), and malvidin 3-galactoside (malvidin-3-O- β-D-glucopyranoside) (493.44 g/mol) were all chloride salts and purchased from Fluka (Seelze, Germany). Petunidin chloride (aglycone molar mass of 317.27 g/mol) was purchased from Extrasynthese (Genay, France). All chemicals were used without further purification. For structures of flavonols and anthocyanins, see Fig. 1.

### Sample preparation

CHCA stock solution was prepared fresh daily at a concentration of 10 mg/mL in a solution that had a 4:1 volumetric ratio methanol to water. THAP was prepared daily at 20 mg/mL in 50:50 methanol/water. We chose four analytes as standards due to their availability; quercetin, rutin (quercetin 3-rutinoside), petunidin, and cyanidin 3-glucoside. A four-component mixture was prepared that contained the following concentrations of each: 246, 125, 252 and 24 μmol/L, respectively. Various dilutions were made of this standard to prepare calibration curves for high-performance liquid chromatography (HPLC) and MALDI quantification. All standards were stored at −20°C. For MALDI analysis, all samples were prepared by the dried-droplet preparation method in plastic centrifuge vials, which included being mixed with a matrix, vortexed for 30 s, and centrifuged for 30 s. Aliquots of 0.5 μL were then spotted onto a 96 × 2 well MALDI plate with a hydrophobic coating




| Flavonol | Abbrev. | $R_1$ | $R_2$ |
|---|---|---|---|
| Kaempferol | K | H | H |
| Quercetin | Q | OH | H |
| Myricetin | M | OH | OH |
| Isorhamnetin | I | $OCH_3$ | OH |




| Anthocyanin | Abbrev. | $R_1$ | $R_2$ |
|---|---|---|---|
| Cyanidin | Cy | OH | H |
| Peonidin | Pe | $OCH_3$ | H |
| Delphinidin | De | OH | OH |
| Petunidin | Pt | OH | $OCH_3$ |
| Malvidin | Mv | $OCH_3$ | $OCH_3$ |

**Figure 1.** Structures of flavonols and anthocyanins. See Table 1 for glycosidic units.





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(Applied Biosystems, Framington, MA, USA). Samples were left to crystallize in a desiccator before being loaded into the MALDI-MS instrument.

### MALDI-TOF-MS instrumentation

The MALDI-TOF mass spectrometer was a Voyager DE™-PRO purchased from Applied Biosystems. The instrument was equipped with a video camera and the sample image was displayed on a monitor, which enabled the laser to be focused on a given spot and controlled manually. The positive ion reflectron mode was used. The instrument was equipped with a pulsed nitrogen laser (337 nm, 3 ns pulse duration, 3 Hz frequency) and a delayed extraction source. An accelerating voltage of 20 kV and a grid voltage setting of 69% were used. The guide wire was adjusted to 0.004%. The laser fluence was set to 2800 arbitrary units (unless otherwise stated) and an extraction delay time of 145 ns was used. The acquisition mass range was *m/z* 100–1000 unless otherwise shown and all spectra were obtained by averaging 25 laser shots. Mass spectra were analyzed using Voyager Data Explorer™ v.4 software. All resolution values were calculated at 50% of the maximum peak height.

### LC/UV-ESI-MS

An Agilent 1100 Series LC/MSD Trap SL ion trap mass spectrometer (Palo Alto, CA, USA) was used. All chromatograms were processed using ChemStation for LC 3D software (Rev.A.10.02; Agilent). The ESI mass spectra were analyzed using Bruker© LC/MSD Trap Control 5.2 (Bruker Daltonics, Bremen, Germany). The parameters for the ion trap were as follows: nebulizer pressure, 60.0 psi; drying gas flow rate, 11.0 L/min; drying temperature, 350°C; target mass, *m/z* 500; scan range, *m/z* 150–900; capillary voltage, 3500 V. An Agilent diode-array detector (G1315B) was used for quantitative experiments. UV wavelength detection for flavonols and anthocyanins was at 360 and 520 nm, respectively. A 25 μL aliquot injected using the autosampler was separated on a Symmetry® C-18 RP column (150 × 3.9 mm i.d.; Waters, Mississauga, ON, Canada), protected by a Symmetry® C-18 guard column (W31921). A binary solvent system was employed, following the work of Wang *et al*.[29] Solvent A was 5% aqueous formic acid (v/v) and solvent B was 100% methanol (HPLC grade). The flow rate was maintained at 0.8 mL/min. The gradient elution profile was as follows: 0 min, 14% B; 1–10 min, 14–17% B; 10–35 min, 17–23% B; 35–60 min, 23–47% B; 60–80 min, 47–60% B; 80–85 min, 60–14% B.

### Extraction method

Three berry samples were chosen for analysis; a lowbush blueberry (*Vaccinium angustifolium*), a lingonberry (*Vaccinium vitis-idaea*), and a blackberry (*Rubus armeniacus*). Samples (30 g, frozen) were ground in a coffee grinder to a paste, and then 30 mL of 40:40:20:0.1 $CH_3CN$/MeOH/$H_2O$/formic acid were added. Samples were stirred for 10 min with a magnetic stirring bar before the solid residue was removed by suction filtration using Whatman No. 4 filter paper. The residue was rinsed with extra solvent to make a final volume of 50 mL. The extract was dried by rotary evaporation, and then re-dissolved in 50 mL of water. A 5 mL aliquot of the extract was loaded onto a C-18 Sep Pak cartridge (Supelclean™ ENVI™ -18 solid-phase extraction (SPE) tubes; Supelco, Bellefonte, PA, USA) that had been pre-rinsed with 5 mL of water and 2 mL of methanol. Once loaded, the cartridge was rinsed with 5 mL of water to remove non-flavonoid components. The anthocyanins and flavonols were eluted with 10 mL of methanol containing 0.1% formic acid. Samples were stored at −20°C and later thawed 1 h prior to analysis. For LC analysis the extracted samples were dried and re-dissolved in 86% solvent A and 14% solvent B.

## RESULTS AND DISCUSSION

### Identification and quantification of flavonoids by LC-UV/ESI-MS

To identify the flavonoids in berry extracts, LC/ESI-MS was employed. Table 1 lists the various anthocyanins and flavonol glycosides identified in the three chosen berry samples (for structures, see Fig. 1). Twenty-eight compounds were identified, based on the chromatographic behaviour of the standards, the elution order as found in the literature,[24,26] and the resulting ESI mass spectra. ESI-MS was used to determine the major ions, and fragment ions also aided in the peak labeling. Some general chromatographic trends can be observed from this data. First, the general order of elution of anthocyanins under these conditions was delphinidin, cyanidin, petunidin, malvidin, peonidin; for the flavonols only quercetin and myricetin were identified, with quercetin generally eluting first. More specifically, the order of elution for a specific aglycone group was dependent on the attached glycoside; the order being galactoside < glucoside < arabinoside < rutinoside. Acetylated sugars were also identified, with 3-acetylglucoside being the dominant species.

The individual chromatograms for anthocyanins are shown in Fig. 2, with specific detection set at 520 nm, which was also used to quantify the anthocyanins as listed in Table 1. Although flavonols were not measured by MALDI, Fig. 2(d) shows their chromatogram at 360 nm from blackberry extract. The blueberry contained the largest variety of both anthocyanins and flavonols. In comparison, the lingonberry and blackberry contained mostly cyanidin glycosides. The blackberry was the only species to contain cyanidin 3-dioxalylglucoside.

The flavonoid concentrations (Table 1) were determined by calibration curves using peak area UV data, using cyanidin 3-glucoside and quercetin 3-rutinoside as standards. For anthocyanins, amounts were converted into cyanidin equivalents, and the flavonols were determined using rutin equivalents.[24] The blueberry had a fairly even distribution of anthocyanins, most ranging between 20 and 50 μg/mL in the extract. The flavonols present included myricetin galactoside, myricetin glucoside and quercetin rutinoside at 171.3 ± 0.5, 221.1 ± 0.6 and 83.4 ± 0.1 μg/mL, respectively. The lingonberry may contain a much smaller variety of anthocyanin glycosides, but it contains a very large amount of cyanidin 3-galactoside (171.3 ± 0.3 μg/mL). The blackberry also had a smaller distribution of flavonoids, but a high abundance of cyanidin 3-glucoside. The results of our





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**Table 1.** Summary of chromatographic peaks and mass spectrometry information obtained by LC/ESI-MS of berry samples and quantification by UV detection

| | | Structure | *m/z* values | | | | |
|---|---|---|---|---|---|---|---|
| Peak # | Retention time (min) | Anthocyanins | $M^+$ | Major fragment ion | Blueberry (μg/mL)* | Lingonberry (μg/mL)* | Blackberry (μg/mL)* |
| 1 | 12.0 | De 3-galactoside | 465 | 303 | 46.4 ± 0.1 | | |
| 2 | 14.5 | De 3-glucoside | 465 | 303 | 39.5 ± 0.2 | | |
| 3 | 15.6 | Cy 3-galactoside | 449 | 287 | 36.1 ± 0.2 | 171.3 ± 0.3 | |
| 4 | 17.4 | De 3-arabinoside | 435 | 303 | 31.8 ± 0.2 | | |
| 5 | 19.0 | Cy 3-glucoside | 449 | 287 | 28.6 ± 0.1 | 18.4 ± 0.5 | 222.2 ± 0.6 |
| 6 | 21.6 | Cy 3-arabinoside | 419 | 287 | 44.6 ± 0.3 | 29.1 ± 0.6 | |
| 7 | 24.0 | Cy 3-rutinoside | 595 | 449, 287 | | | 24.4 ± 0.6 |
| 8 | 25.5 | Pt 3-galactoside | 479 | 317 | 45.8 ± 0.2 | | |
| 9 | 29.1 | Pt 3-arabinoside | 449 | 317 | 15.5 ± 0.1 | | |
| 10 | 32.7 | Mv 3-galactoside | 493 | 331 | 42.5 ± 0.1 | | |
| 11 | 37.3 | Mv 3-glucoside | 493 | 331 | 50.3 ± 0.1 | | |
| 12 | 40.7 | Pe 3-galactoside | 463 | 301 | 24.9 ± 0.1 | | |
| 13 | 43.3 | Pe 3-glucoside | 463 | 301 | 1.8 ± 0.1 | | |
| 14 | 47.2 | Cy 3-dioxalylglucoside | 593 | 287 | | | 23.2 ± 0.6 |
| 15 | 47.3 | De 3-acetylglucoside | 507 | 303 | 13.8 ± 0.1 | | |
| 16 | 50.5 | Cy 3-acetylglucoside | 491 | 287 | 13.5 ± 0.1 | | |
| 17 | 51.4 | Mv 3-acetylglucoside | 535 | 331 | 16.0 ± 0.1 | | 10.2 ± 0.6 |
| 18 | 52.5 | Pt 3-acetylglucoside | 521 | 317 | 9.0 ± 0.1 | | |
| 19 | 54.7 | Pe 3-acetylglucoside | 505 | 301 | 3.1 ± 0.1 | | |
| 20 | 55.4 | Cy 3-malonylglucoside | 535 | 387 | 29.6 ± 0.1 | | |
| | | Flavonols | $[M–H]^{\cdot-}$ | Major fragment ion | | | |
| 21 | 44.7 | Q 3-galactoside | 463 | 301 | | | 9.5 ± 0.5 |
| 22 | 45.1 | M 3-galactoside | 479 | 319 | 171.3 ± 0.5 | | |
| 23 | 45.6 | Q 3-glucoside | 463 | 301 | | 64.5 ± 0.5 | 1.3 ± 0.4 |
| 24 | 46.1 | M 3-glucoside | 479 | 319 | 221.1 ± 0.6 | | |
| 25 | 47.5 | Q 3-rutinoside | 609 | 301 | 83.4 ± 0.1 | | 3.1 ± 0.1 |
| 26 | 52.1 | Q 3-glucosylxyloside | 595 | 433, 301 | | 65.6 ± 0.5 | |
| 27 | 52.3 | Q 3-acetylrhamnoside | 489 | 301 | 41.4 ± 0.1 | 39.1 ± 0.5 | 12.2 ± 0.2 |
| 28 | 61.2 | Q | 301 | | <LOQ | <LOQ | |

* Based on 30 g of berry and extract dissolved in 50 mL of water.

analysis are comparable with other studies, which have reported that cyanidin glucoside accounted for 80% or more of the total anthocyanidins in blackberry species.[30,31]

## MALDI-MS versus surfactant-mediated MALDI-MS of flavonoid standards

Figure 3 shows the mass spectrum obtained when MALDI is tested as a method for the analysis of petunidin, cyanidin 3-glucoside, quercetin and rutin flavonoids, with the use of either CHCA or THAP matrix. In each case, 10 μL of matrix were mixed with 10 μL of the standard stock solution. In the case of CHCA addition (Fig. 3(a)), the masses observed correspond to the aglycones at *m/z* 287 (cyanidin, $[M^+]$) and 317 (petunidin, $[M^+]$), with protonated molecules at *m/z* 303 (quercetin) and 611 (rutin). The intact cyanidin 3-glucoside molecule $[M]^+$ was identified at *m/z* 449, but in lower abundance. The detector was saturated when a laser power of 2800 (arbitrary units) was employed. Decreasing the power to 2400 was found to be sufficient for the CHCA matrix. As expected, many CHCA matrix ions are observed, including the protonated and sodiated molecules at *m/z* 190 and 212. (Note that for all MALDI spectra, the ions with glycosides have been labeled such that glucoside = glu, galactoside = gal, arabinoside = arab, and rutinoside = rut.)

When THAP was used as the matrix (Fig. 3(b)), similar aglycone analyte ions were obtained; however, the $[M]^+$ ions of the glycosated anthocyanins are clearly observed. In addition, sodiated molecules were often observed at *m/z* 471 and 633 for cyanidin 3-glucoside and rutin, respectively. The presence of sodiated molecules, in addition to the $[M]^+$ or $[M+H]^+$ ions, aids in identification when considering samples with multiple analytes. The cyanidin and petunidin aglycones were also observed, but at much lower signal intensity than when CHCA was used. From our data, we believe that CHCA is more energetic than THAP, making it a 'hotter' matrix, and thus leads to more glycoside cleavage. However, this can complicate the analysis, as the *m/z* values for certain flavonols $[M+H]^+$ and aglycones $[M]^+$ are the same. For example, protonated kaempferol and the aglycone of cyanidin 3-glucoside will both yield ions of *m/z* 287. Therefore, THAP matrix has a distinct advantage in that it can more easily distinguish between the glycosides and aglycones. However, for the remainder of this work we will compare results obtained with CHCA alongside those with THAP because of the common usage of CHCA.

Figure 3 also shows the addition of a surfactant, cetyltrimethylammonium bromide (CTAB), to the mixture of standards with each matrix. As seen, the presence of the surfactant leads to suppression of the matrix ions and, to a





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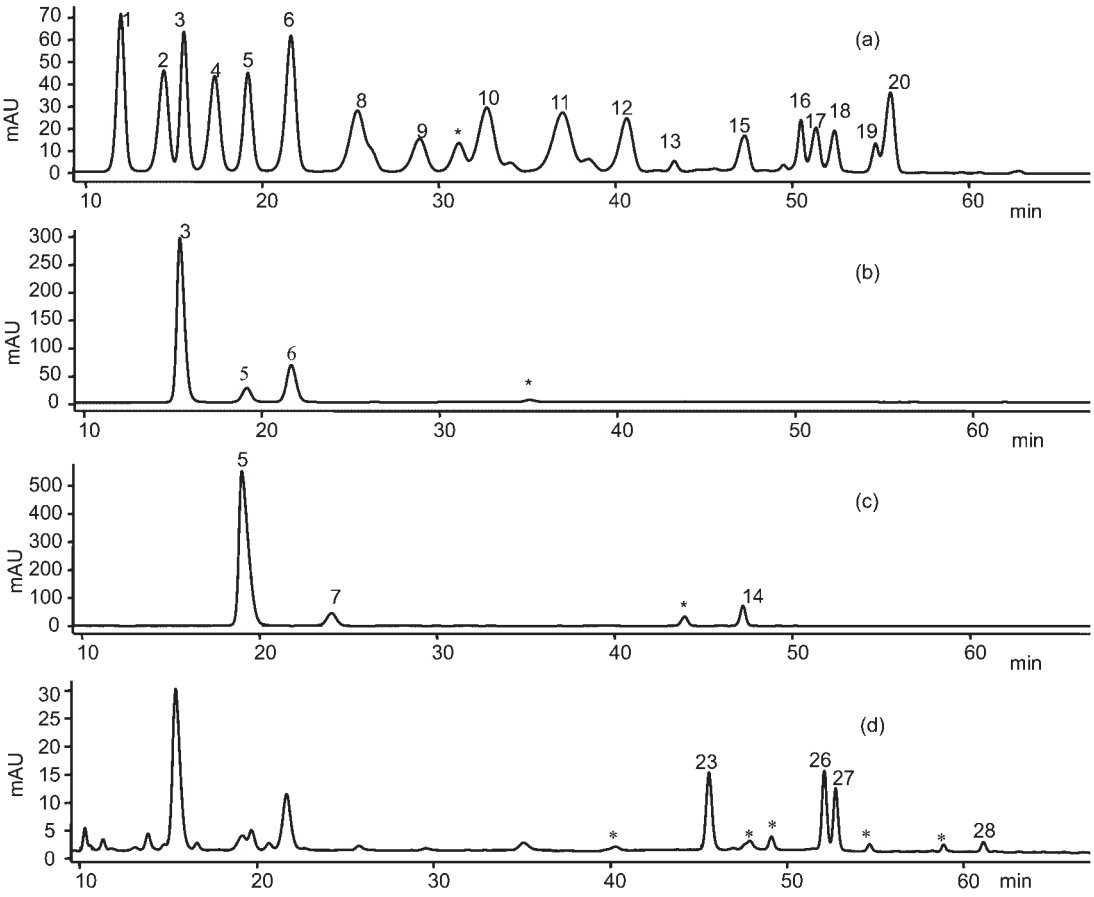


**Figure 2.** UV chromatographic profile of anthocyanins (520 nm) in extracts from (a) blueberry, (b) lingonberry, (c) blackberry, and (d) UV detection (360 nm) of blackberry flavonols.

lesser extent, the analyte ions, as observed in our previous study.[17] In both mass spectra the analyte ions are still readily observed. The use of the surfactant seems to improve the analysis in terms of the standard deviation. For example, without the surfactant the resolution (n = 5) for cyanidin 3-glucoside, petunidin, quercetin and rutin was 3432 (±19.0%), 3018 (±18.6%), 3370 (±17.1%) and 4352 (±19.9%), respectively, when THAP matrix was used. In contrast, when

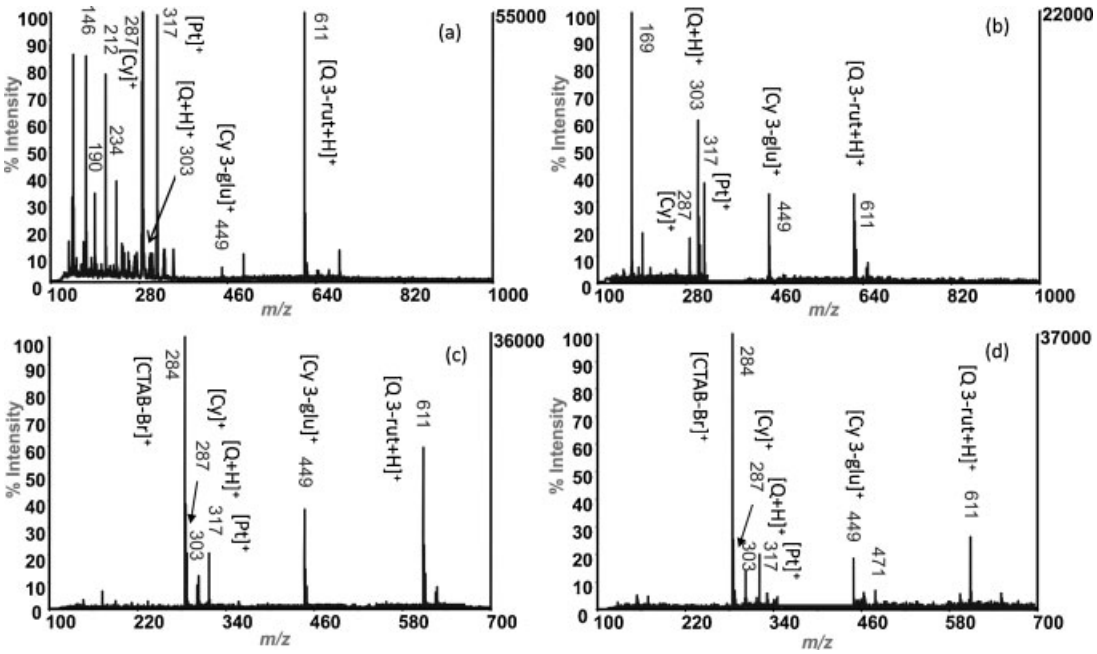


**Figure 3.** Positive ion MALDI-TOF mass spectra of flavonoid standards obtained with the addition of (a) CHCA, (b) THAP, (c) CHCA/CTAB, and (d) THAP/CTAB. See Fig. 1 and Table 1 for abbreviations.





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the surfactant was used the resolution was 3779 (±5.9%), 3570 (±6.0%), 4290 (±5.3%) and 5485 (±6.2%). This demonstrates that, for each ion, the resolution was improved, and this increase in resolution ranged from 10.1 to 27.3%. To the best of our knowledge, this is the first report of this surfactant being used to improve the performance of the THAP matrix. Since the surfactant does not contain conjugated moieties, it clearly does not allow for absorption in the wavelength of the $N_2$ laser (337 nm). Thus, the surfactant itself is not behaving as a matrix. Mixing the CTAB and the standards mixture together, without matrix, resulted in a lack of any ionization from the mixture.

## Analysis of berry extracts by MALDI-MS and surfactant-mediated MALDI-MS

Useful MALDI mass spectra were obtained when berry extracts were analyzed by MALDI (10 μL of the matrix with 10 μL of original extract). A summary of these results is presented in Table 2. When CHCA matrix was used for the blueberry extract, we obtained a mass spectrum with ions due to only the aglycones of anthocyanins and flavonols. No flavonoid glycosides were observed. Using MALDI alone we cannot confirm whether the ion at *m/z* 287 is due to kaempferol or cyanidin, but, considering other reports[25,29] regarding the composition of various blueberry species and our ESI-MS data, we believe that it is probably due to cyanidin. For the lingonberry extract, ions for cyanidin, cyanidin 3-arabinoside and cyanidin 3-glucoside (or cyanidin 3-galactoside) were observed. Note that cyanidin 3-glucoside or cyanidin 3-galactoside can yield an ion at *m/z* 449, but unfortunately MALDI cannot differentiate between isomers. As an example of our data, Fig. 4(a) displays analysis of the blackberry extract using the CHCA as a matrix, revealing only the presence of the cyanidin.

When THAP was used as a matrix for blueberries, all the aglycone ions observed in the CHCA matrix spectra were present but, in addition, many intact glycosides observed by LC/ESI-MS were clearly visible, including cyanidin 3-arabinoside, cyanidin 3-glucoside, peonidin 3-glucoside, delphinidin 3-glucoside, petunidin 3-glucoside, malvidin 3-glucoside and malvidin 3-acetylglucoside. This demonstrates that the use of THAP provides improved structural information on sugar-containing flavonoids. In the analysis of lingonberries, it is again demonstrated that the use of THAP gives better resolution of the intact glycosides, and the aglycone of cyanidin observed with CHCA was also visible (Table 2). The blackberry analysis yielded results similar to those with the blueberries, with the glycoside ion being observed, as illustrated in Fig. 4(b).

The effect of CTAB addition to each sample was then monitored. CTAB was added to each sample so that the matrix/CTAB ratio was 10000:1. The results are shown in Table 2. For example, in the blueberry extract, the flavonoid ions were slightly suppressed, but they remained very well resolved in comparison with the matrix ions. All the same ions were observed as when CHCA was used alone. The presence of the ion at *m/z* 284 $[CTAB\text{-}Br]^+$ make it difficult to observe the cyanidin aglycone, but careful observation did indeed reveal its presence. In the case of the lingonberry, all ions were still observed and well resolved, except for the quercetin 3-glucosylxyloside. However, in the blackberry analysis (Fig. 4(c)), the cyanidin aglycone was still observed, and in this case the glycoside was also observed.

When CTAB was added to the THAP matrix at the same ratio, the blueberry analysis led to identification of the same glycosides; however, the aglycones were all suppressed except for malvidin. In the lingonberry analysis, the same ions were found, with a dominant ion at *m/z* 449, and the blackberry result (shown in Fig. 4(d)) demonstrated the appearance of glycosides that had not been detected in any of the other MALDI experiments (malvidin 3-acetylglucoside, cyanidin 3-dioxalylglucoside, quercetin 3-glucosylxyloside). Figure 5 illustrates the results of the surfactant addition to THAP in the analysis of lingonberry and blueberry extracts.

In comparison with the MALDI-TOF-MS analysis, LC/ESI-MS was able to identify more flavonoids in each sample. For example, delphinidin 3-acetylglucoside was present in the blueberry extract, but was not identified in the

**Table 2.** Flavonoids detected in berries using MALDI-TOF-MS with CHCA or THAP matrix. (C = CHCA matrix, C/C = CHCA matrix + CTAB addition at a 10000:1 ratio, T = THAP matrix, T/C = THAP matrix + CTAB addition at a 10000:1 ratio)

| | | Blueberry | | | | Lingonberry | | | | Blackberry | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *m/z* | Compound | C | C/C | T | T/C | C | C/C | T | T/C | C | C/C | T | T/C |
| 287 | cyanidin | ✓ | ✓ | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 301 | peonidin | ✓ | ✓ | ✓ | | | | | | | | | |
| 303 | delphinidin | ✓ | ✓ | ✓ | | | | | | | | | |
| 317 | petunidin | ✓ | ✓ | ✓ | | | | | | | | | |
| 331 | malvidin | ✓ | ✓ | ✓ | ✓ | | | | | | | | |
| 419 | cyanidin 3-arabinoside | | | ✓ | * | * | * | ✓ | ✓ | | | | |
| 449 | cyanidin 3-glucoside (galactoside) | | | ✓ | ✓ | * | ✓ | ✓ | ✓ | | ✓ | | ✓ |
| 463 | peonidin 3-glucoside (galactoside) | | | ✓ | ✓ | | | | | | | | |
| 465 | delphinidin 3-glucoside (galactoside) | | | ✓ | * | | | | | | | | |
| 479 | petunidin 3-glucoside | | | ✓ | ✓ | | | | | | | | |
| 493 | malvidin 3-glucoside (galactoside) | | | ✓ | ✓ | | | | | | | | |
| 535 | malvidin 3-acetylglucoside/cyanidin 3-malonylglucoside | | | ✓ | ✓ | | | | | | | | ✓ |
| 593 | cyanidin 3-dioxalylglucoside | | | | | | | | | | | | ✓ |
| 595 | cyanidin 3-rutinoside | | | | | * | | | | | | | ✓ |

* Denotes a minor ion; less than 2% relative intensity of largest ion signal intensity.





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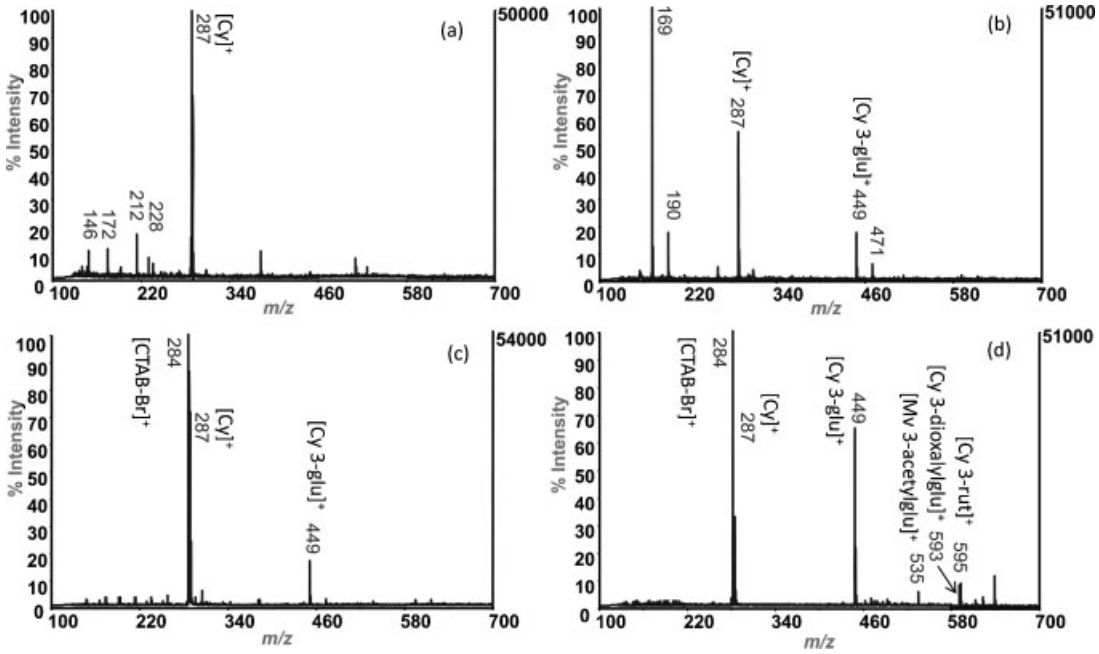


**Figure 4.** Positive ion MALDI-TOF mass spectra of blackberry extract obtained with the addition of (a) CHCA, (b) THAP, (c) CHCA/CTAB, and (d) THAP/CTAB. See Fig. 1 and Table 1 for abbreviations.

MALDI-MS spectra. One reason for this is that UV-VIS detection gives almost no background interference, as opposed to the ubiquitous background noise of MALDI. Another problem with MALDI-TOF-MS of the complex samples may stem from the analyte-analyte ion suppression.[32] Essentially, species that are present in much larger quantity than others, or species that have a higher proton affinity than others, may abstract protons more easily from a matrix. This can lead to an observed suppression of ions from the species with a lower concentration. MALDI will not always qualitatively identify as many species as LC/MS, but it can rapidly screen for major constituents and illustrate the main species from a sample.

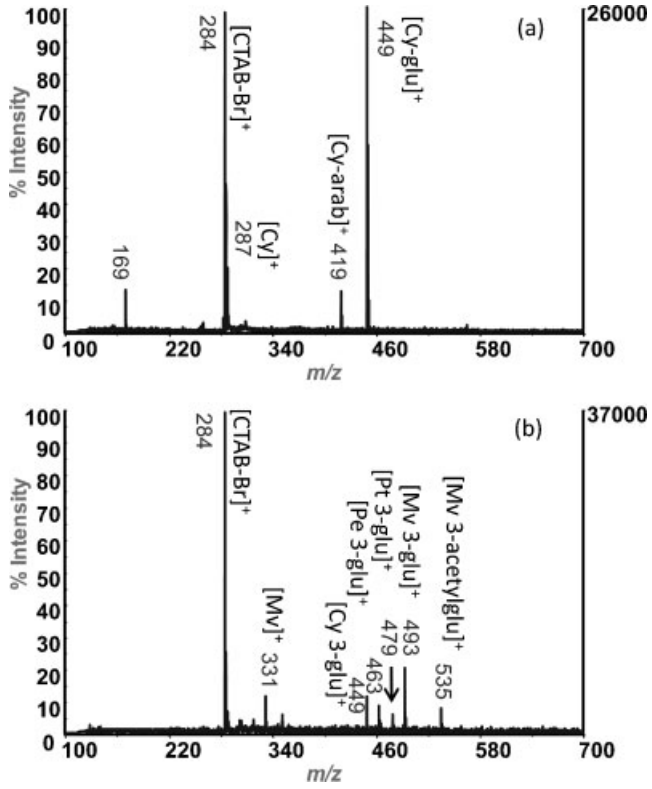


**Figure 5.** Positive ion MALDI-TOF mass spectra obtained when THAP/CTAB was used for the analysis of (a) lingonberry extract and (b) blueberry extract. See Fig. 1 and Table 1 for abbreviations.

In our MALDI-TOF-MS experiments, the anthocyanins were more easily detected than the flavonols. Thus, Table 2 contains information only on the former. Changing the solvent system to a more acidic medium, such as one containing some trifluoroacetic acid, might improve this analysis but we recognized that, even then, many of the flavonols and anthocyanins would generate ions of the same *m/z* value. Thus, this is an inherent limitation of the method.

## Quantification by surfactant-mediated MALDI-TOF-MS

Although a very powerful qualitative analytical tool, MALDI-TOF-MS has not yet become as widely used for small molecule quantification. Wang and Sporns[28] demonstrated that in MALDI-TOF-MS analysis, anthocyanins ionize in a proportional manner. We designed an experiment to compare the results of quantification of the flavonoids in berry extracts by normal MALDI-TOF-MS and the surfactant-mediated approach. Mass spectral calibration was achieved using cyanidin 3-glucoside standard for both matrices, with and without surfactant. Figure 6 shows the calibration curves for THAP matrix, where Fig. 6(a) shows the calibration curve by THAP only, yielding a correlation coefficient of 0.981 and an average relative standard deviation (RSD) value of 32%, with a range from 24–46%. With the addition of CTAB surfactant, as shown in Fig. 6(b),





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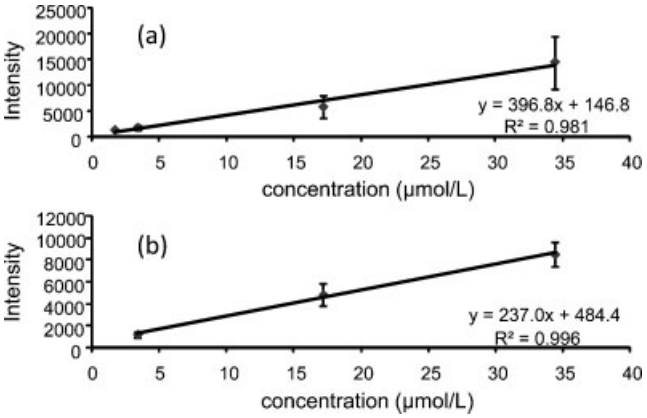


**Figure 6.** MALDI-TOF-MS calibration curves of cyanidin 3-glucoside standard by analysis with (a) THAP and (b) THAP/CTAB.

the correlation coefficient increases to 0.996 and the average RSD was 18% (range of 13–21%).

Each standard curve was prepared by analyzing four different concentration levels of a standard. However, in each case the most dilute level (2 μmol/L) could not be detected by surfactant-mediated MALDI. As the ion suppression also partially suppresses the analyte ions, these ions could not be distinguished from the background noise. We found that the use of CHCA led to calibration curves with better correlation coefficients (data not shown), if the aglycone ion was monitored, and lower standard deviations were observed. However, in this study we sought to quantify the intact glycosides, and thus solely THAP was used.

Using the calibration curves from the THAP/CTAB work, quantification of anthocyanins in berry extracts was undertaken. Based on the LC quantitation results, analysis of the blueberry extract was a problem since it was very complex and not all ions were observed. Analyte-analyte suppression would hinder quantification and, as multiple species have the same *m*/*z* value, this makes it difficult to determine which component is giving a particular signal. The MALDI-TOF-MS quantitation results are shown in Table 3. Using THAP alone, there was a large discrepancy in the results for cyanidin 3-glucoside against the LC/ESI-MS results; 14.8% in blackberries and 12.5% in lingonberries. Both values have a RSD value greater than 30%. Cyanidin 3-arabinoside was also determined, and its discrepancy was over 90%. However, adding CTAB into the matrix greatly improved the discrepancy against the LC result and reduced the RSD. For cyanidin 3-glucoside in both berries, the discrepancy decreased by about 10% in each berry to 5.5% and 1.8% and the RSD dropped to less than 10%. In addition, malvidin acetylglucoside, cyanidin 3-dioxalylglucoside and cyanidin 3-rutinoside could be quantified with results differing from those in the LC/ESI-MS analysis by 11.3 to 18.9%, and RSD values ranging from 10.0 to 16.7%. These results show that quantitation is markedly improved using CTAB and reproducibility is excellent compared with traditional analyses, where experiments often have RSD values of 30% or greater.

This work shows that surfactant-mediated MALDI-TOF-MS is a viable approach for the fast screening of flavonoids in berries. Although LC/ESI-MS provides more qualitative and quantitative information, the long run times are a significant drawback compared with the speed of a MALDI-TOF-MS analysis. Thus, this is an example of MALDI being an excellent tool for rapid screening and it provides a complementary analysis to LC.

## CONCLUSIONS

It has been shown that the addition of the surfactant CTAB to common matrices for the analysis of flavonoids improves the MALDI-TOF-MS data by decreasing matrix-ion signals and providing more reproducible signals that can be used for quantitative purposes. CHCA led to more fragmentation of the sugar moiety than THAP. This method was successfully applied to the analysis of an anthocyanin standard and extracts from multiple berry samples. Surfactant-mediated MALDI-TOF-MS can be a rapid screening technique for these flavonoids, and reduces analysis time compared with LC/ESI-MS, to just a few minutes. Work is now being pursued to further the applications of screening small biomolecules using this method.

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**Table 3.** Results from quantitation by MALDI-TOF-MS analysis of anthocyanins in blackberry and lingonberry extract. Results shown are averaged (n = 5). %Disc. = Percentage of discrepancy from results obtained by LC-MS with UV detection. Note: THAP indicates that only THAP matrix was used; THAP/CTAB indicates THAP was used with added surfactant at a 10000:1 mole ratio

| | | THAP | | | THAP/CTAB | | |
|---|---|---|---|---|---|---|---|
| Berry | Anthocyanin | Amount (μg/mL) | %RSD | %Disc. | Amount (μg/mL) | %RSD | %Disc. |
| Blackberry | cyanidin 3-glucoside | 189.2 | 38.5 | 14.8 | 209.9 | 8.3 | 5.5 |
| | malvidin 3-acetylglucoside | n/d | — | — | 13.4 | 16.7 | 11.3 |
| | cyanidin 3-dioxalylglucoside | n/d | — | — | 28.7 | 10.0 | 13.7 |
| | cyanidin 3-rutinoside | n/d | — | — | 29.0 | 14.0 | 18.9 |
| Lingonberry | cyanidin 3-glucoside | 213.4 | 47.3 | 12.5 | 191.3 | 6.1 | 1.8 |
| | cyanidin 3-arabinoside | 56.01 | 18.5 | 92.5 | 34.3 | 8.9 | 17.8 |





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