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A rapid and sensitive LC–MS/MS method for quantification of four anthocyanins and its application in a clinical pharmacology study of a bioadhesive black raspberry gel

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ABSTRACT

Cyanidin 3-glucoside (C3GLU), cyanidin 3-rutinoside (C3RUT), cyanidin 3-sambubioside (C3SAM) and cyanidin 3-(2^G-xylosyl) rutinoside (C3XRUT) are the four constituent black raspberry anthocyanins that contribute significantly to the chemopreventive effects of freeze-dried black raspberries (FBR). A highly sensitive and specific LC–MS/MS assay was developed and validated to simultaneously quantify these four FBR anthocyanins in human saliva, plasma and oral tissue homogenates. In saliva, the lower limit of quantification (LLOQ) for these anthocyanins was 1.0 ng/mL. The within-run and between-run coefficients of variations (CVs) at the quality control concentrations (1.0, 5.0, 50 and 500 ng/mL) were all <12%, except for C3SAM and C3RUT at the LLOQ, which showed a within-run CV of 18.3% and between-run CV of 16.0%, respectively. The accuracy values ranged from 87.5 to 110%. In plasma, the LLOQ for C3GLU and C3RUT was 1.0 ng/mL and for C3SAM 5.0 ng/mL. The CVs at the above concentrations were <15%, except for C3GLU at the LLOQ, which showed the between-run CV of 16.9%. The accuracy values ranged from 90.7% to 112.7% except for C3GLU at the LLOQ, which showed 119.3%. In tissue homogenates, the LLOQ for C3GLU and C3RUT was 2.0 ng/mL, and C3SAM 5.0 ng/mL. The CVs and accuracy values at concentrations (2.0, 5.0, 50 and 500 ng/mL) were similar to those in human plasma. This assay was subsequently used in a pilot pharmacology study to evaluate the effects of topical application of a 10% (w/w) FBR bioadhesive gel to selected mucosal sites in the posterior mandibular gingiva. Measurable saliva and tissue levels of the FBR anthocyanins confirmed that gel-delivered anthocyanins are readily distributed to saliva and easily penetrate human oral mucosa.

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1. Introduction

Anthocyanins, which comprise the largest group of water soluble pigments, are widely distributed in fruits, vegetables, and flowers and are responsible for their red, purple, and blue colors [1]. More than 500 types of anthocyanins have been identified and structurally elucidated from nature [2]. Discovery of the multi-functional therapeutic effects of anthocyanins, e.g., anti-inflammatory [3], radiation-protection [3], and anti-neoplastic activities [4], through epidemiological and molecular biological studies [5–7], has triggered a surge of interest in the identification of anthocyanins and their metabolites in plants, food, animals and human excreta.

Conventionally, anthocyanins are characterized and quantified using high-performance liquid chromatography (HPLC) coupled with ultraviolet/visible (UV–vis) spectrophotometry [8]. HPLC-UV methods, however, require a long run time to achieve optimal resolution and to avoid co-elution interference substances. In addition, standard HPLC assays in general have limited sensitivity and are inadequate for quantification of anthocyanins at very low concentrations, as such would be found in biological matrices during pharmacokinetic, bioavailability and tissue penetration studies. Therefore, more sensitive analytical techniques are required for the quantification of anthocyanins in pharmacological studies.

In the last decade, HPLC coupled with tandem mass spectrometry (LC–MS/MS) has become an unprecedented technique

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for the rapid and sensitive quantification of small molecules, e.g., decitabine [9] and ansamitocin P3 [10]. The application of mass spectrometry using atmospheric pressure chemical ionization (APCI) [11], electro-spray ionization (ESI) [12], and matrix-assisted laser desorption/ionization (MALDI) [13] for detection and identification of anthocyanins in plants and foods has been reported. However, only few reports of the quantitative analyses of anthocyanins in dietary supplements and biological matrices have been published [14–17]. Capillary electrophoresis (CE) is emerging as another elegant technique for anthocyanins analysis with advantage of low sample loading and solvent consumption [18,19].

Cyanidin 3-glucoside (C3GLU), cyanidin 3-rutinoside (C3RUT), cyanidin 3-sambubioside (C3SAM) and 3-(2^G-xylosyl) rutinoside (C3XRUT) are the major anthocyanins in black raspberries (Rubus occidentalis) and in freeze-dried black raspberries that have been ground into a powder (FBR) [20]. These anthocyanins, which represent the predominant phenolic compounds in FBR, possess strong antioxidant properties and are thought to be responsible for many of FBR's chemopreventive effects [4,13,21,22]. Additionally, C3GLU and C3RUT are the major anthocyanins in several other berry fruits such as blueberries, strawberries and mulberries [20]. Recently, a liquid chromatographic electro-spray ionization tandem mass spectrometry (LC-ESI/MS-MS) method for quantitative determination of these anthocyanins was established [17] and used to quantify anthocyanins in human plasma and oral mucosal tissue explants following application of prototype FBR gels in a pilot clinical pharmacokinetic study [20]. This method, however, was not fully validated and relied upon C3GLU to serve as the reference compound for the quantification of C3RUT, C3SAM and C3XRUT. Additionally, as the run time was rather long (35 min) [20], applicability of this method in large-scale, clinical trial based pharmacokinetic studies is limited.

A phase I clinical trial in healthy volunteers who ingested 45 g of FBR daily in slurry of water for seven consecutive days indicated that less than 1% of the four anthocyanins in FBR were absorbed and excreted in urine [23]. Thus, in order to overcome the poor bioavailability of anthocyanins from systemic delivery and to increase their levels in oral mucosal tissue, a mucoadhesive gel containing 10% FBR was developed for local delivery of anthocyanins into oral mucosa [20]. The advantage of local delivery of anthocyanins after the topical application of this gel in oral mucosa has been documented in a recent report on the clinical results, which demonstrated that this gel readily distributes anthocyanins to saliva and easily penetrates human oral mucosa in health volunteers enrolled in a phase I clinical trial [24]. In the present study, we reported a highly sensitive and specific LC–MS/MS assay that is capable of simultaneously quantifying minute levels of FBR anthocyanins in human saliva, and tissue homogenates. This method was used for the aforementioned clinical trial and will be ideal for the pharmacological studies of anthocyanins in the oral cavity in future clinical trials.

2. Experimental

2.1. Reagents and chemical

FBR anthocyanins except for C3XRUT, which is not commercially available, and the internal standard malvidin-3-glucoside (M3GLU) were purchased from Polyphenols Laboratory AS (Hanaveien 4-6, N-4327, Sandens, Norway). All organic solvents were of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid (FA) was of reagent grade and was purchased from Sigma (St. Louis, MO). All chemicals and reagents were used as received. The 10% FBR mucoadhesive gels (final pH = 3.5) were prepared using current Good Manufacturing Practices (GMP) at the GMP facility within the Center for Pharmaceutical Science and Technology at the University of Kentucky (Lexington, KY). A Barnstead E-pure water purification system (Dubuque, IA) was used to obtain HPLC grade water (>18 mΩ).

2.2. Human oral mucosa and saliva samples collection

Participation of human subjects in these studies was conducted in accordance with an IRB approved protocol (The Ohio State University IRB protocol #2003C0050). None of the subjects had a diet rich in anthocyanins prior to participation in the pharmacokinetic studies. For the tissue–saliva–plasma pharmacokinetic study, the posterior mandibular gingiva of each subject was dried, then 0.5 g 10% (w/w) FBR bioadhesive gel was applied and the site was massaged for 30 s to facilitate anthocyanin uptake. Five minutes after gel application, saliva (collected for 1 min) and peripheral blood samples were obtained. The gel treated tissue overlying the third molar was then removed for surgical access.

Volumes of the saliva samples were recorded, followed by addition of 5% FA. Peripheral blood samples were centrifuged, plasma volumes recorded, followed by the addition of 5% FA. Following acidification, saliva and plasma samples were stored at −80 °C until LC–MS/MS analysis. The oral tissue samples were immediately placed in 5% FA and stored at −80 °C. Prior to analysis, tissues were washed with phosphate buffered saline (PBS), homogenized on ice [20], and appropriate aliquots were removed for protein determination using the Lowry assay [25]. To facilitate tissue preparation to constituent components, homogenized samples were subsequently treated with 2.5% trypsin and 2.0% collagenase I (Worthington Biochemical Corporation, Lakewood, NY) for 1.5 h at 37 °C, followed by acidification with FA to a final concentration of 5%. All samples were stored at −80 °C until LC–MS/MS analysis.

2.3. Calibration standards and quality controls preparation

Stock solutions (1 mg/mL) of C3GLU, C3RUT and C3SAM were prepared in acetonitrile (ACN) and stored in −80 °C. Standards solutions with concentration ranging from 2 to 10,000 ng/mL were prepared by further dilution from the stock solutions with acetonitrile. For calibration curve, 20 μL of the appropriate diluted standard solutions were spiked either into 200 μL diluted human saliva (125-fold dilution), 400 μL human plasma or 200 μL oral tissue homogenate which contains constant amount of M3GLU at a final concentration of 100 ng/mL. Quality controls (QC) were prepared at 1.0 (2.0), 5.0, 50 and 500 ng/mL. Standards prepared in diluted saliva were injected directly to LC–MS for analysis. Standards prepared in human plasma or tissue homogenate were subject to solid phase extraction prior to LC–MS analysis.

2.4. Sample preparation

Saliva samples were centrifuged at 2500 × *g* for 2 min. A 10 μL of the supernatant was taken and diluted with 990 μL 5% ACN/0.1% FA to yield an intermediate solution, from which 80 μL was taken to mix with 10 μL internal standard solution at 10 μg/mL and 10 μL 5% ACN/0.1% FA to yield the final diluted saliva samples (125-fold dilution from the original saliva). An aliquot of 10 μL of the diluted saliva sample was injected into LC–MS for analysis.

Human tissue homogenate or plasma samples were prepared by spiking 10 μL internal standard solution at 10 μg/mL to 200 μL oral tissue homogenate and subsequently extracting by the solid phase extraction method as described below. A 20 μL aliquot of the tissue homogenate or 50 μL of human plasma residue were injected into the LC–MS for analysis.

2.5. Solid phase extraction (SPE)

Anthocyanins were extracted from human tissue homogenates or plasma using Oasis 1 c.c. HLB cartridges (Waters Corporations, Milford, MA). A 200 μL aliquot of oral mucosa tissue homogenate or plasma spiked with various concentrations of anthocyanins and M3GLU were loaded onto the HLB cartridge, which was preconditioned with 1.0 mL MeOH and 1.0 mL water. After washing with 1.0 mL HPLC water, the samples were eluted with 500 μL 50% ACN/0.1% FA. The fractions were collected and dried under a mild stream of nitrogen. The residues were then reconstituted with 100 μL 5%ACN/0.1% FA. The reconstituted solutions were further purified by filtering through a 0.45 μm nylon filter (Spin-X Centrifuge Tube Filter; Corning Inc.; Corning, NY) by centrifugation at 14,000 × *g* for 2 min. The resulting solution was then analyzed by LC–MS/MS.

2.6. HPLC condition

Liquid chromatography was performed on a Shimadzu HPLC system (Shimadzu, Columbia, MD) consisted of a CBM-20A system controller, an LC-20 AD pump, a SIL-20AC auto-sampler, CTO-20A column oven, DGU-20A5 degasser and FCV-11AL valve unit. C3GLU, C3RUT, C3SAM and the internal standard M3GLU were separated from interferences in these matrices on an Aquasil C18 column (2.1 mm × 250 mm, 5 μm, Thermo Hypersil-Keystone, Bellefonte, PA) coupled with an Aquasil C18 guard column (2.1 mm × 10 mm, 2 μm, Thermo Hypersil-Keystone, Bellefonte, PA) and a drop-in guard cartridge with a uniguard holder under a gradient elution at a flow rate of 0.20 mL/min. The mobile phase A (MPA) consisted of water/0.1% FA and mobile phase B (MPB) consisted of ACN/0.1% FA. For HPLC separation of the three anthocyanins and the internal standard, the gradient was initiated at 5% B, increased to 30% B at 30 min, and then decreased to 5% B at 1 min, after which the column was equilibrated for another 4 min before next injection. For quantification of the four anthocyanins, a more rapid running program with an isocratic elution of 25% MPB for 7 min was used to increase sample throughput.

2.7. Mass spectrometry condition

Anthocyanins were monitored using a Finnigan TSQ Quantum EMR Triple Quadrupole mass spectrometer (Thermo Fisher Scientific Corporation, San Jose, CA) equipped with an electro-spray ionization (ESI) source. Xcalibur software (Home Page Version 1.4 SR1) was used for system control and data processing. The mass spectrometer was operated in the positive ESI mode with a collision gas (Argon) pressure of 1.5 mTorr, a typical electro-spray needle voltage of 4700 V, a sheath nitrogen gas flow of 25 (arbitrary unit) and a heated capillary temperature of 325 °C. Anthocyanins and the internal standard were analyzed by the multiple reaction monitor (MRM) mode using ion transitions at a proper collision energy (*E*) as follows: C3GLU *m*/*z* 449.09 > *m*/*z* 286.86 (*E* = 30%), C3RUT *m*/*z* 595.21 > *m*/*z* 286.8 (*E* = 35%), C3SAM *m*/*z* 581.20 > *m*/*z* 286.77 (*E* = 35%), C3XRUT *m*/*z* 727.00 > *m*/*z* 287.00 (*E* = 35%) and I.S. M3GLU *m*/*z* 493.17 > *m*/*z* 331.00 (*E* = 25%). The mass spectrometer was tuned to its optimal sensitivity by direct infusion of C3RUT.

2.8. Assay validation

The within-run precision values were determined in six replicates at concentrations of 1.0 (2.0), 5.0, 50 and 500 ng/mL and the between-run precision was determined across these concentrations in six different runs. The calibration curves were fitted by linear regression with no weighting factor. The mean concentration and the coefficient of variation (CVs) were calculated as the relative standard deviation (%) from the six replicates. The accuracy of the assay was determined by comparing the corresponding calculated mean concentration with the nominal concentration. The lower limit of quantification (LLOQ) was defined as the lowest concentration in the calibration curve that back-calculates with good accuracy and precision.

2.9. Recovery and matrix effect

A post-extraction spike experiment [26] was performed to evaluate the matrix effect and recovery of FBR anthocyanins in human plasma (or tissue homogenates) by HLB extraction, three separate batches of anthocyanin samples at concentrations of 5.0, 50 and 500 ng/mL were prepared as follows: (1) anthocyanins were prepared directly in 5% ACN/0.1% FA (MP); (2) anthocyanins were spiked into MP reconstituted extract of blank human plasma or tissue homogenates, and (3) MP reconstituted extract of anthocyanins in human plasma or tissue homogenates. The recovery of anthocyanins and the internal standard were calculated by the ratio of peak areas of anthocyanins of the third batch samples to their corresponding second batch samples. The matrix effect of three anthocyanins and the internal standard was evaluated by the ratio of peak areas of the second batch to that of the first batch samples.

2.10. Freeze–thaw stability

Anthocyanin plasma samples were exposed to three sequential freezing and thawing cycles prior to sample analysis. Human plasma was acidified with 5% FA in order to maintain constituent anthocyanins in their more stable flavylium cation form. Anthocyanins at concentrations of 5.0, 50 and 500 ng/mL were prepared in acidified human plasma and stored in a −80 °C freezer. At 24 h intervals, the samples were removed from the freezer and thawed at room temperature for approximately 5 min. The samples were quickly mixed and returned to the −80 °C freezer until the next cycle. All samples were stored at −80 °C until LC–MS/MS analysis.

3. Results and discussion

3.1. Mass spectrometric characterization

Chemical authenticity of the commercially available anthocyanins C3GLU, C3SAM, C3RUT and M3GLU were confirmed on a TSQ quantum triple quadruple mass spectrometer under ESI positive mode. The average full mass spectra of C3SAM, C3RUT and C3GLU showed molecular ions at *m*/*z* 581.18, 595.21 and 449.14, respectively, corresponding to their protonated molecular ions (Fig. 1). In addition to its $[MH]^+$ ion, the mass spectrum of C3SAM showed two strong ions at *m*/*z* 449.12 and 287.03 with relative intensities of 24.6% and 100% comparing to the protonated molecular ion at *m*/*z* 581.18, respectively. Similarly, a minor ion at *m*/*z* 449.12 was observed in the full mass spectra of C3RUT. The tandem mass spectra of the $[MH]^+$ ions of the three anthocyanins showed a common product ion at *m*/*z* 286.78, which likely represents cyanidin ions generated by glycosidic cleavage of their parent ions. Therefore, the ion transition channels at the optimal collision energy for C3RUT, C3SAM and C3GLU were set at *m*/*z* 595.21 > *m*/*z* 286.8 (*E* = 35%), *m*/*z* 581.20 > *m*/*z* 286.77 (*E* = 35%) and *m*/*z* 449.09 > *m*/*z* 286.86 (*E* = 30%), respectively. Similarly, the full mass spectrum (Fig. 2A) of M3GLU exhibited a base peak at *m*/*z* 493.20, corresponding to its protonated molecular ion, and the product ion spectrum (Fig. 2B) of this ion showed a single peak at *m*/*z* 330.83, corresponding to the malvidin ion generated by glycosylic cleavage of the parent ion. The ion transition channel for the internal standard M3GLU was therefore set at *m*/*z* 493.20 > *m*/*z* 330.83 (*E* = 25%, Fig. 2B). All mass and tandem mass spectra of

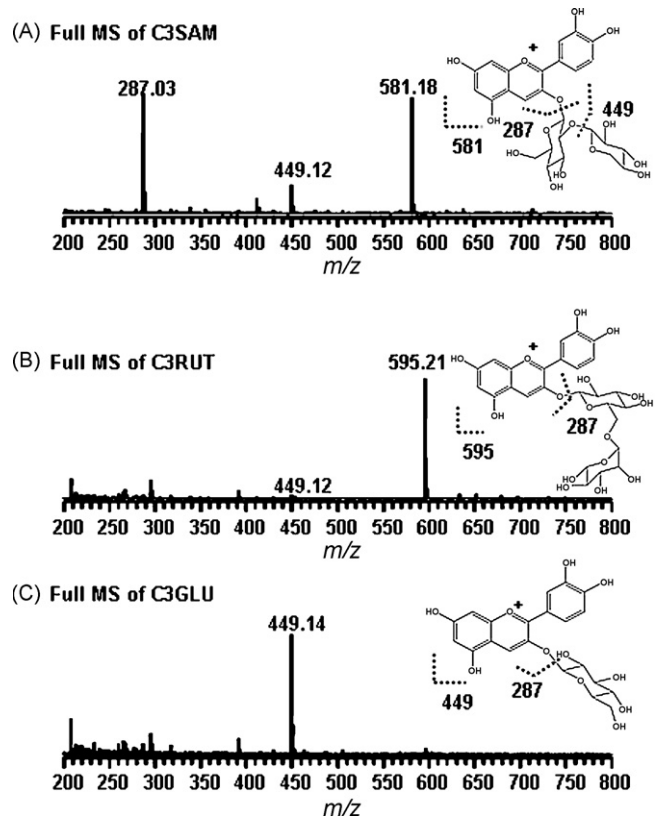

Fig. 1. The 1 min average full mass spectra of 10 μg/mL of C3SAM (A), C3RUT (B), and C3GLU (C) in 50% ACN/0.1% FA showed molecular ions at *m*/*z* 581.18 (A), 595.21 (B) and 449.14 (C), corresponding to their protonated molecular ions $[MH]^+$, respectively. The ion at *m*/*z* 449.12 corresponding to $[MH]^+$ of C3GLU was also shown in the mass spectra of C3SAM (A) and C3RUT (B). A ion at *m*/*z* 287.03 with intensities compared to that to the $[MH]^+$ ion of C3SAM was also observed in the full mass spectrum of C3SAM (A), which is the common fragment ions of C3SAM, C3RUT and C3GLU as shown in the inset chemical structures and postulated fragmentation pathways.

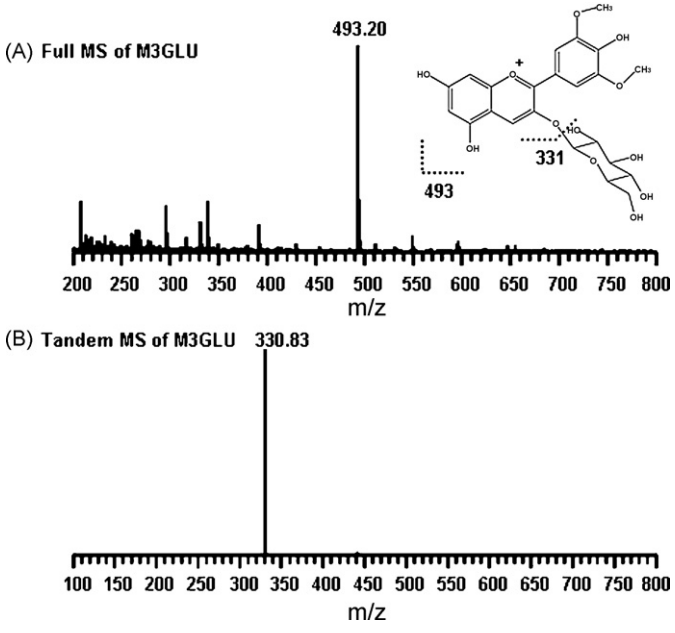

Fig. 2. The 1 min average full mass spectra of 10 μg/mL of M3GLU (A) in 50% ACN/0.1% FA showed molecular ions at *m*/*z* 493.20 (A), corresponding to its protonated molecular ion $[MH]^+$ and the product ion spectrum (B) of this ion showed a single peak at *m*/*z* 330.83, corresponding to the malvidin ion generated by glycosylic cleavage of the parent ion. Its chemical structure and postulated fragmentation pathway is inset (B).

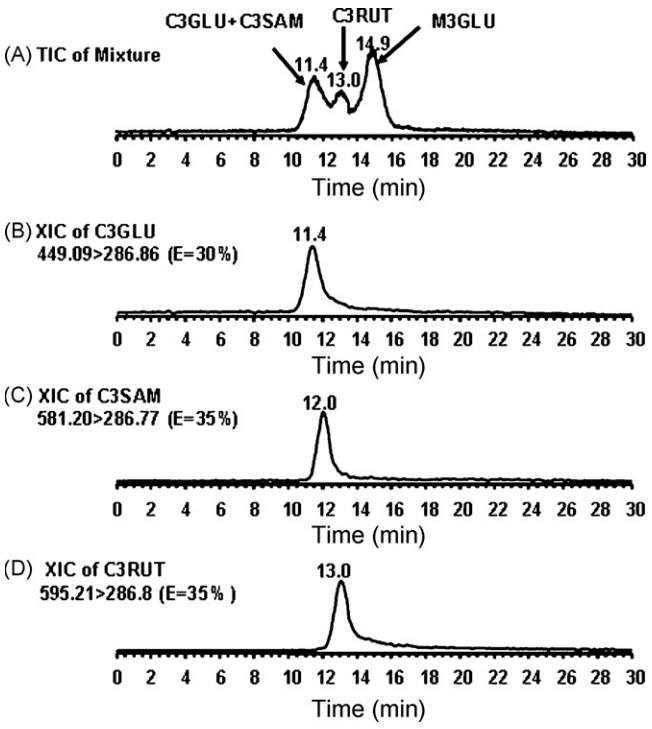

Fig. 3. The total ion chromatogram (TIC, A) of a mixture of 100 ng/mL of C3SAM, C3RUT, C3GLU, and M3GLU showed three partially resolved peaks with reaction time of 11.4 min (composite of C3GLU and C3SAM), 13.0 min (C3RUT) and 14.9 min (M3GLU). The extracted ion chromatograms (XICs) of C3GLU (B) at *m*/*z* 449.09 > *m*/*z* 286.86 (*E* = 30%), C3SAM (C) at *m*/*z* 581.20 > *m*/*z* 286.77 (*E* = 35%), C3RUT (D) at *m*/*z* 595.21 > *m*/*z* 286.8 (*E* = 35%) showed one peak at 11.4 min (B), 12.0 min (C) and 13.0 min (D), respectively, under the HPLC separation elution program detailed in Section 2.6.

the three anthocyanins and I.S. are similar to those reported [17]. The chemical structures of the three commercially available anthocyanins and the internal standard and their common fragmentation pathway as glycosylic cleavage are shown in the insets in their mass spectra (Figs. 1 and 2), respectively. C3XRUT with ion transition channel at *m*/*z* 727.00 > *m*/*z* 287.00 (*E* = 35%) was also postulated based on the protonated molecular ion and the general fragmentation patterns of anthocyanins as glycosylic cleavage.

3.2. Chromatographic separation

Presence of the ion at *m*/*z* 449.12 at different relative intensity in the mass spectra of C3RUT and C3SAM and its same *m*/*z* value as the $[MH]^+$ ion of C3GLU suggests the following two possibilities: (1) the ion could be generated from an in-source fragmentation of $[MH]^+$ ions of C3RUT and C3SAM to different extent, or (2) the ion could be the molecular ion of C3GLU formed from the spontaneous decomposition of C3RUT or C3SAM, or as an original impurity in these two anthocyanin standards. To differentiate the two possibilities, a HPLC system was developed and optimized to separate C3SAM, and C3RUT and C3GLU. As shown in Fig. 3, the three anthocyanins were separated with C3GLU eluted at 11.4 min, C3SAM at 12.0 min and C3RUT at 13.0 min. The total ion chromatogram (TIC, Fig. 4A) of C3SAM was also obtained under the same conditions. A strong peak at the retention time of C3GLU in the extracted ion chromatogram (XIC) of C3GLU (Fig. 4B) suggested that the solution of C3SAM contains detectable amounts of C3GLU. We are unable to differentiate whether the observed C3GLU is attributed to the decomposition of C3SAM or original contamination. C3RUT standard also contains C3GLU; however, at much lower extend (data not shown).

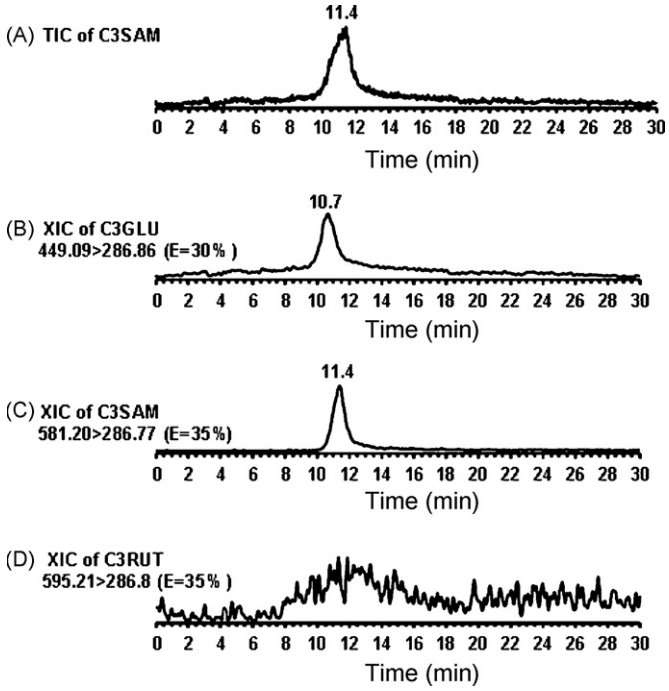

Fig. 4. The TIC (A) of 100 ng/mL C3SAM showed one broad peak at 11.4 min and the XICs of C3GLU (B) at m/z 449.09 > m/z 286.86 (E = 30%), C3SAM (C) at m/z 581.20 > m/z 286.77 (E = 35%), C3RUT (D) at m/z 595.21 > m/z 286.8 (E = 35%) showed one peak at 10.7 min (B), 11.4 min (C) and no peak (D), respectively, under a HPLC separation elution program detailed in Section 2.6.

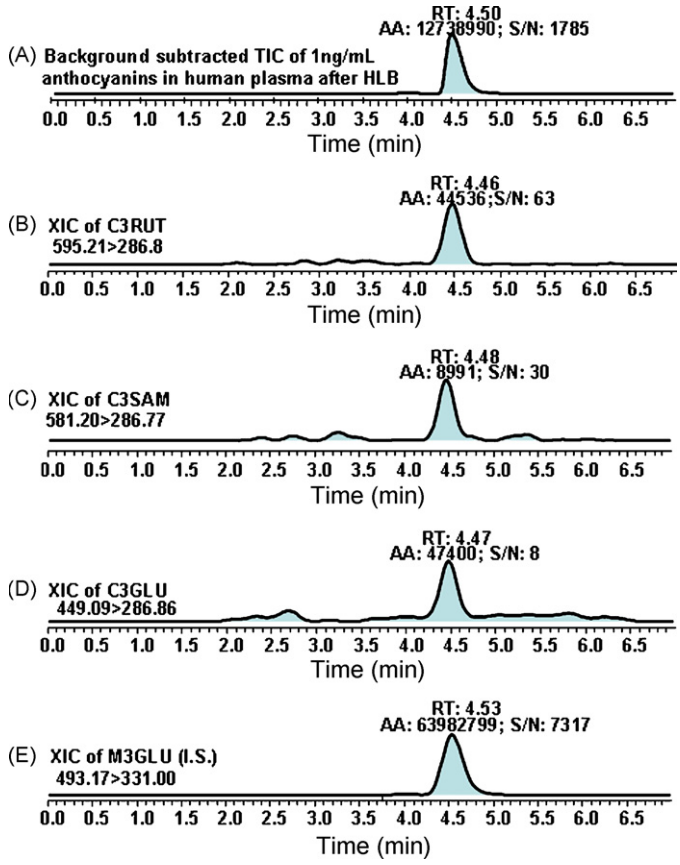

Fig. 5. The subtracted TIC (A) of MP reconstituted solution of the HLB extract of human plasma spiked with 1.0 ng/mL anthocyanins and 100 ng/mL I.S. showed one peak at 4.50 min, and the XICs of C3RUT (B) at m/z 595.21 > m/z 286.8 (E = 35%), C3SAM (C) at m/z 581.20 > m/z 286.77 (E = 35%), C3GLU (D) at m/z 449.09 > m/z 286.86 (E = 30%), and M3GLU (E) at m/z 493.20 > m/z 330.83 (E = 35%) showed one peak at around 4.50 ± 0.03 min, respectively, under a HPLC separation elution program detailed in Section 2.6.

To improve quantification efficiency and increase sample throughput, the above HPLC gradient program was modified to an isocratic elution with 25% MPB for 7 min. The first 2 min of effluent was discarded to the waste. Fig. 5 shows the background-subtracted total ion chromatogram (TIC, Fig. 5A) of 1.0 ng/mL anthocyanins in human plasma after HLB extraction and the extracted ion chromatograms (XICs, Fig. 5B–E) of three anthocyanins and the internal standard. All three anthocyanins and the internal standard were eluted around 4.5 min and the peak areas integrated at this retention time were used for quantification. All of these anthocyanins can be mass-resolved in spite of their co-elution and no cross-interference was found in our tested linear range.

3.3. Calibration curves for quantification

The presence of C3GLU in the C3SAM standard would interfere with the quantification of C3GLU, especially at the low levels. Therefore, two standard curves for quantification of the three anthocyanins were constructed to circumvent this complication. One standard curve was composed of C3GLU, which was used to calculate the C3GLU concentration; the second calibration curve was composed of a mixture of C3SAM and C3RUT, in which the peak with the transition channel at m/z 449.09 → m/z 286.86 was used to calculate the concentration of C3GLU presented in the C3SAM calibration standards. Thus, the actual concentration of C3SAM should be the nominal concentration of C3SAM subtracted with C3GLU concentration. Since C3XRUT is not commercially available, the calibration curve for C3RUT was used to quantify C3XRUT, which was eluted at the same retention time of C3RUT (4.5–4.6 min).

3.4. Assay validation

3.4.1. Human saliva

A pilot study demonstrated that the concentration of anthocyanins in human saliva samples 1 min after gel application was very high; therefore, a simplified direct dilution method for determination of these anthocyanins in saliva was adapted and validated. For all three anthocyanins, the calibration curves were linear between 1 and 1000 ng/mL with a regression coefficient (r^2) > 0.996 by injection of a 10 μL aliquot of solution. The LLOQ of C3GLU, C3SAM and C3RUT was determined to be 1.0 ng/mL. Six replicates were prepared for each of the four quality control (QC) concentrations (1.0, 5.0, 50 and 500 ng/mL) and the within-run precision and accuracy for the anthocyanins were determined. As shown in Table 1, the within-run coefficients of variation (CVs) were 11.6, 5.3, 8.9 and 2.9% for C3GLU; 18.3, 5.5, 1.8 and 10.4% for C3SAM; 7.7, 7.0, 2.5 and 10.9% for C3RUT, respectively. The accuracy values at these QC data points were in the range of 87.5–110%. The between-run (n = 6) CVs at the QC data points of 1.0, 5.0, 50 and 500 ng/mL were within 15%, except at the LLOQ of C3RUT, which shows a CV of 16.0%. The accuracy values at these QC data points ranged from 89.2 to 116.1%.

3.4.2. Human plasma and tissue homogenates

Anthocyanins were extracted from human plasma using solid phase extraction method [16]. The background-subtracted total ion chromatogram (TIC) and the extracted ion chromatograms (XICs) of the three anthocyanins and the internal standard at LLOQ of 1 ng/mL in plasma are shown in Fig. 5. The recovery at concentrations of 5.0, 50 and 500 ng/mL were determined to be about 40–50% and the matrix effect was about 70–90%. Six replicates were prepared for the four QC concentrations (1.0, 5.0, 50 and

Table 1
Validation parameters of three anthocyanins [cyanidin 3-glucoside (C3GLU), cyanidin 3-rutinoside (C3RUT), cyanidin 3-sambubioside (C3SAM)] in saliva by LC–MS/MS ($n = 6$).

Conc. (ng/mL)	Analytes	Within-day			Between-day		
		Mean	CV%	Accuracy %	Mean	CV%	Accuracy %
1	C3SAM	1.05	18.25	105.2	1.00	5.15	99.7
	C3RUT	1.05	7.68	105.0	0.92	16.01	92.0
	C3GLU	1.05	11.58	105.3	1.05	10.46	105.3
5	C3SAM	5.01	5.49	100.2	4.87	4.67	97.3
	C3RUT	4.84	7.00	96.8	4.57	9.38	91.5
	C3GLU	4.98	5.28	99.5	4.88	5.85	97.7
50	C3SAM	43.76	1.78	87.5	50.52	7.2	101.1
	C3RUT	50.2	2.45	100.4	50.16	1.85	100.3
	C3GLU	54.99	8.9	110.0	51.93	9.24	103.9
500	C3SAM	448.02	10.37	89.6	446.22	5.92	89.2
	C3RUT	523.97	10.87	104.8	507.95	1.67	101.6
	C3GLU	492.36	2.89	98.5	480.07	6.74	96.0

500 ng/mL) and the within-run precision and accuracy values are shown in Table 2. The LLOQs of C3GLU, C3SAM and C3RUT were determined to be 1.0, 5.0 and 1.0 ng/mL, respectively. For C3SAM, the calibration curve was linear between 5.0 and 1000 ng/mL with a regression coefficient (r^2) of 0.994. The within-run coefficients of variation at these QC concentrations (5.0, 50 and 500 ng/mL) were 10.8, 8.6 and 7.1%, and the accuracy values were 104.0, 88.6 and 111.1%, respectively. The between-run CVs were 1.8, 7.7 and 3.0%, and the accuracy values were 102.7, 113.0 and 100.4%, respectively. The calibration curves for the other two anthocyanins were linear from 0.2 to 1000 ng/mL with the regression coefficients >0.997. The within-run CVs at these QC concentrations (1.0, 5.0, 50 and 500 ng/mL) were below 13% and their accuracy values in the range of 90.7–106.6%, except at the LLOQ for C3GLU, which has a within-day accuracy of 119.8%. The between-run ($n = 6$) precision for these two anthocyanins ranged from 1.8 to 8.8% and the accuracy values ranged from 94.9 to 112.7%, except at the LLOQ for C3GLU, which has a between-day CV of 16.9% and accuracy of 119.3% (Table 2). In tissue homogenates, the recovery at above QC concentrations were determined to be about 30–50% and the matrix effect was about 20–40%. Similar within-day and between-day validation parameters were achieved in tissue homogenates (Table 3).

Table 2
Validation parameters of anthocyanins [cyanidin 3-glucoside (C3GLU), cyanidin 3-rutinoside (C3RUT), cyanidin 3-sambubioside (C3SAM)] in human plasma by LC–MS/MS ($n = 6$).

Conc.(ng/mL)	Analytes	Within-day			Between-day		
		Mean	CV%	Accuracy %	Mean	CV%	Accuracy %
1	C3SAM	NA	NA		NA	NA	NA
	C3RUT	0.92	13.03	92.2	1.02	5.19	102.0
	C3GLU	1.20	10.68	119.8	1.19	16.87	119.3
5	C3SAM	5.20	10.83	104.0	5.14	1.81	102.7
	C3RUT	4.54	4.19	90.7	4.70	4.76	94.1
	C3GLU	5.12	10.49	102.5	5.64	8.81	112.7
50	C3SAM	44.31	8.59	88.6	56.51	7.65	113.0
	C3RUT	47.92	6.84	95.8	47.47	4.42	94.9
	C3GLU	47.96	7.44	95.9	53.4	2.37	106.8
500	C3SAM	555.72	7.06	111.1	501.93	2.95	100.4
	C3RUT	518.69	4.51	103.7	493.36	8.20	98.7
	C3GLU	532.94	4.12	106.6	520.27	4.83	104.1

Table 3
Validation parameters of three anthocyanins [cyanidin 3-glucoside (C3GLU), cyanidin 3-rutinoside (C3RUT), cyanidin 3-sambubioside (C3SAM)] in third moral tissue homogenates by LC–MS/MS ($n = 6$).

Conc. (ng/mL)	Analytes	With in-day			Between-day		
		Mean	CV%	Accuracy %	Mean	CV%	Accuracy %
2	C3SAM	NA	NA	NA	NA	NA	NA
	C3RUT	2.09	15.5	104.43	2.20	7.6	110.17
	C3GLU	1.97	6.98	98.49	2.07	9.26	100
5	C3SAM	4.44	12.85	88.77	4.67	4.9	93.40
	C3RUT	5.64	7.38	112.80	4.49	8.31	89.73
	C3GLU	4.74	7.34	94.87	4.63	1.35	92.60
50	C3SAM	48.04	8.00	96.07	48.66	10.07	97.33
	C3RUT	57.51	2.07	115.02	48.57	5.32	97.15
	C3GLU	50.35	1.76	100.71	53.66	5.93	107.33
500	C3SAM	487.68	1.19	97.54	473.1	4.47	94.62
	C3RUT	497.51	1.65	99.50	485.01	8.71	97.00
	C3GLU	491.66	4.01	98.33	474.04	1.88	94.81

Table 4
Concentrations of the four anthocyanins [cyanidin 3-glucoside (C3GLU), cyanidin 3-rutinoside (C3RUT), cyanidin 3-sambubioside (C3SAM) and cyanidin 3-(2^G-xylosyl)rutinoside (C3XRUT)] in saliva and tissue samples.

Sample ID	C3GLU	C3SAM	C3RUT	*C3XRUT
Concentration in saliva (μg/mL)				
Saliva 001	17.61	18.98	116.00	63.26
Saliva 002	29.40	33.93	206.78	115.89
Saliva 003	17.11	25.21	146.62	86.79
Saliva 004	28.35	31.05	166.19	82.26
Saliva 005	47.40	58.94	337.19	186.99
Concentration in tissue (pmol/mg)				
Tissue 001	108.01	113.85	204.88	102.99
Tissue 002	85.33	60.91	281.89	121.45
Tissue 003	68.66	45.14	200.40	85.41
Tissue 004	139.55	35.09	298.00	76.43
Tissue 005	727.19	464.19	2345.86	960.98

* *Note*: The concentrations of C3XRUT were obtained by use of the calibration curve of C3RUT.

3.5. Freeze–thaw stability in human plasma

The stability of FBR anthocyanins after repeated freeze-thaw cycles was evaluated in 5% FA acidified human plasma. After three freeze/thaw cycles, 93, 91 and 94% of the estimated pre-freezing concentrations of C3RUT; 91, 97 and 101% of the estimated pre-freezing concentrations of C3SAM; and, 94, 92 and 96% of the estimated pre-freezing concentrations of C3GLU, each prepared at 5.0, 50 and 500 ng/mL, were found, respectively. These data indicated anthocyanins are stable in 5% FA acidified plasma over three freeze–thaw cycles.

3.6. Quantification of anthocyanins in human saliva and tissues after topical application of a 10% FBR gel

Anthocyanin levels in human saliva, plasma, and oral tissue samples obtained from human subjects after topical gel application to oral mucosa were analyzed by the validated LC–MS/MS method. Similar to our recent reported plasma level of anthocyanins [24], no anthocyanins were detected in any of the plasma samples, suggesting that local application of this gel led to localization of all of the anthocyanins in the oral matrices. The concentrations of anthocyanins in the saliva and tissue samples are listed in Table 4.

These data show that the anthocyanins in 10% FBR mucoadhesive gels are readily absorbed into human oral mucosa. Additionally, its uptake in oral tissues showed large inter-donor variability. All five participants had detectable levels of the four anthocyanins (C3GLU, C3SAM, C3RUT and C3XRUT) in their saliva and tissues. The concentrations of C3GLU were estimated between 17.6 and 47.4 μg/mL in saliva and 68.7 and 727.2 pmol/mg protein in oral tissue; C3SAM concentrations were between 19.0 and 59.0 μg/mL in saliva and 35.1 and 464.2 pmol/mg in oral tissue; C3RUT concentrations were between 116.0 and 337.2 μg/mL in saliva and 200.4 and 2345.9 pmol/mg protein in oral tissue; and C3XRUT levels, calculated by use of the calibration curve of C3RUT, were 63.3–187.0 μg/mL in saliva and 76.4–961.0 pmol/mg protein in oral tissue. Compared with their ex vivo uptake in explanted oral tissues in artificial saliva [20], the relatively higher tissue levels of these anthocyanins implicates that their in vivo penetration to oral tissues is more efficient. Notably, the detected tissue levels of these anthocyanins just reflect their normal distribution in the gel, not like that in the explant tissue studies. The discrepancy may due to the use of C3GLU as the sole calibration curve and here we constitute the calibration curve for each of these three anthocyanins for accurate quantification of these three anthocyanins, respectively. Fig. 6 shows the TIC and XICs of the four anthocyanins in a tissue sample. All four anthocyanins and the internal standard

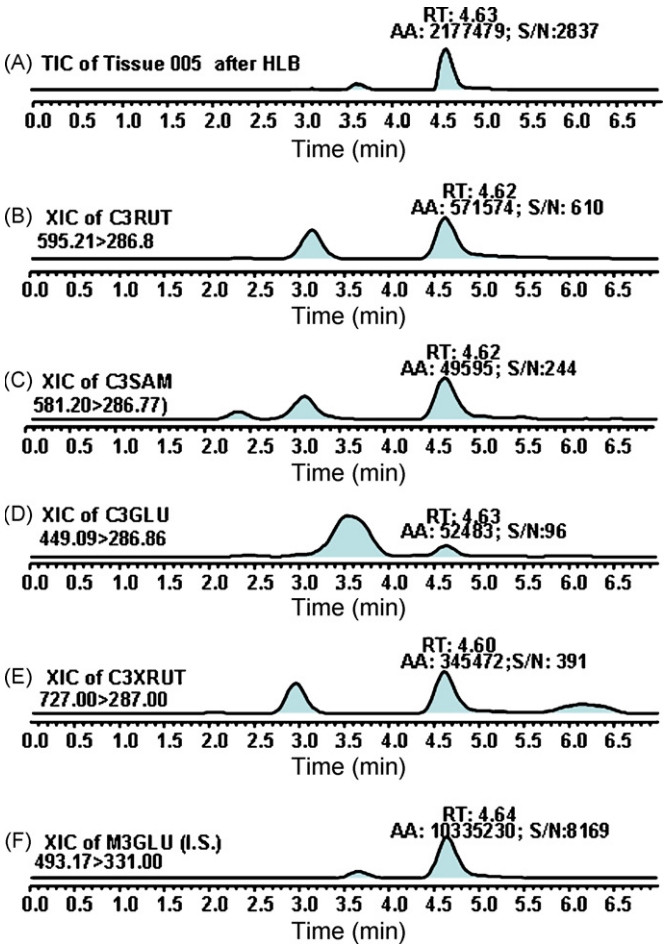

Fig. 6. The TIC (A) of MP reconstituted solution of the HLB extract of the tissue homogenate from one patient spiked with 100 ng/mL I.S. showed one peak at 4.63 min and one minor peak at 3.60 min, and the XICs of C3RUT (B) at m/z 595.21 > m/z 286.8 (E = 35%), showed two peaks at 3.20 and 4.62 min; of C3SAM (C) at m/z 581.20 > m/z 286.77 (E = 35%), showed three peaks at 2.30, 3.20 and 4.62 min; of C3GLU (D) at m/z 449.09 > m/z 286.86 (E = 30%), showed two peaks at 3.60 and 4.63 min; C3XRUT (E) at m/z 727.00 > m/z 287.00 (E = 30%), showed two peaks at 2.90 and 4.60 min; and M3GLU (E) at m/z 493.20 > m/z 330.83 (E = 35%), showed two peaks at 3.60 and 4.64 min under a HPLC separation elution program detailed in Section 2.6.

were eluted at approximately 4.6 min additionally, another apparent peak between 2.5 and 4.0 min was also observed in the XICs of C3GLU, C3RUT, C3SAM, C3XRUT, and I.S., respectively. These peaks might have originated from the tissue matrix (Fig. 6B–E) or their hydrated adduct, which possibly yields a similar mass spectrum after in-source dehydration. The concentration ranges of C3GLU, C3RUT, C3SAM and C3XRUT in saliva were significantly higher than that in tissue homogenates. The mean concentrations of FBR anthocyanins in saliva were nearly 4000-fold higher than the levels achievable in tissues. We are currently endeavoring to identify possible metabolites of anthocyanins in saliva, plasma and tissue.

4. Conclusion

A rapid and sensitive LC–MS/MS assay for simultaneously quantifying C3GLU, C3RUT, C3SAM and C3XRUT was developed and validated in human saliva, oral tissue homogenates and other biological matrices. The assay has been successfully applied to investigate the uptake of anthocyanins from a mucoadhesive gel containing 10% FBR after local application to posterior mandibular gingiva. These data showed detectable levels of anthocyanins in saliva and oral tissues and therefore confirmed the therapeu-

tic advantage obtained by topical gel application. This method, therefore, provides a useful translational tool for further characterization of the pharmacokinetics of anthocyanins and establishment of correlations with their pharmacodynamic endpoints in clinical settings.

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