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Quantification of theobromine and caffeine in saliva, plasma and urine via liquid chromatography–tandem mass spectrometry: A single analytical protocol applicable to cocoa intervention studies

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ABSTRACT

Targeted analyses of clinically relevant metabolites in human biofluids often require extensive sample preparation (e.g., desalting, protein removal and/or preconcentration) prior to quantitation. In this report, a single ultra-centrifugation based sample pretreatment combined with a designed liquid chromatography–tandem mass spectrometry (LC–MS/MS) protocol provides selective quantification of 3,7-dimethylxanthine (theobromine) and 1,3,7-trimethylxanthine (caffeine) in human saliva, plasma and urine samples. The optimized chromatography permitted elution of both analytes within 1.3 min of the applied gradient. Positive-mode electrospray ionization and a triple quadruple MS/MS instrument operated in multiple reaction mode were used for detection. $^{13}C_3$ isotopically labeled caffeine was included as an internal standard to improve accuracy and precision. Implementing a 20-fold dilution of the isolated low MW biofluid fraction prior to injection effectively minimized the deleterious contributions of all three matrices to quantitation. The assay was linear over a 160-fold concentration range from 2.5 to 400 μmol L^{-1} for both theobromine (average R^2 0.9968) and caffeine (average R^2 0.9997) respectively. Analyte peak area variations for 2.5 μmol L^{-1} caffeine and theobromine in saliva, plasma and urine ranged from 5 and 10% (intra-day, $N = 10$) to 9 and 13% (inter-day, $N = 25$) respectively. The intra- and inter-day precision of theobromine and caffeine elution times were 3 and <1% for all biofluids and concentrations tested. Recoveries for caffeine and theobromine ranged from 114 to 118% and 99 to 105% at concentration levels of 10 and 300 μmol L^{-1}. This validated protocol also permitted the relative saliva, plasma and urine distribution of both theobromine and caffeine to be quantified following a cocoa intervention.

1. Introduction

Robust, rapid and validated analytical techniques are essential for further elucidating the potential therapeutic benefits of cocoa consumption. Several examples of this emerging area of health research can be found in literature describing the potential holistic benefits of consuming this food product [1–9]. Such studies have ranged from a reduction in the likelihood of preeclampsia [10] to the inhibition of atherosclerotic plaque progression [11]. This work however, is preliminary and further research is needed to elucidate the underlying mechanism of these effects and to identify the active cocoa components responsible for the observed health benefits [12–14]. To this end, the contribution of cocoa's methylxanthine fraction has garnered much research attention. 3,7-Dimethylxanthine (theobromine) and 1,3,7-trimethylxanthine (caffeine) respectfully represent the major and minor physiologically active methylxanthine components of the food product. Each has been implicated in the observed beneficial health impacts of nutritional cocoa intervention. From an analytical perspective, these previous studies illustrate the importance of accurately measuring the levels of both of these metabolites in human saliva, plasma and urine. A single platform capable of quantitating these small molecules in multiple biofluids matrices would be attractive to health researchers investigating the effects of cocoa intervention on disease manifestation.

Directed metabolite analyses within complex biological matrices presents significant analytical challenges. Conventional approaches often include distinct off-line sample pretreatments including preconcentration, de-salting and protein removal. Although these procedures isolate and enhance the response of the target analyte, they are often time consuming, difficult to automate, reduce precision and incur sample loss. Several different pre-analytical protocols have been employed to minimize the deleterious effects of complex biofluid matrices, with liquid–liquid extraction (LLE) [15–24] and solid-phase extraction (SPE) [25–30] being the most common for these analytes. Once isolated from

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the matrix, numerous analytical techniques have been used for multiplex methylxanthine analyses including radioimmunoassay (RIA) [31], thin-layer chromatography (TLC) [26], capillary gas chromatography (GC) [32] and micellar electrokinetic electrophoresis (MEKC) [33,34]. However liquid chromatography (LC) with spectrophotometric detection [15–20,22,25,27,35–41] remains the most widely applied technique, despite the possibility of spectral interferences from co-eluting endogenous compounds. This limitation can be avoided by coupling LC with mass spectrometric detection (LC–MS), although there are relatively fewer reports using this methodology for methylxanthine analyses [28,29]. The selectivity and sensitivity of this technique may be further enhanced by employing compound specific mass transitions with tandem mass spectrometry (MS/MS) [10,42]. The LC–MS/MS analysis of eleven different methylxanthine and methylurate urine metabolites communicated by Schneider et al. demonstrates this benefit as their extractionless sample preparation and total LC run time of 60 min, including analyses in both positive and negative electrospray ionization (ESI) modes, permitted baseline resolution and measurements of all isomeric species [43].

In this report, a rapid LC–MS/MS procedure has been developed and validated for the quantification of μmol L^{-1} levels of theobromine and caffeine in human saliva, plasma and urine. Fast separations (3 min total run time) coupled with a single ultracentrifugation based sample preparation for all three matrices provided interference-free quantitation of both metabolites in all biofluids. This universal protocol circumvents the multiple conventional pre-analytical treatments typically required for their targeted measurement and provides a single platform for which cocoa intervention studies may be performed.

2. Methods and materials

2.1. Chemicals

1,3,7-Trimethylxantine (caffeine), 3,7-dimethylxantine (theobromine), 1,7-dimethylxanthine (paraxanthine), and 1,3-dimethylxanthine (theophylline) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Double-distilled water and HPLC-grade acetonitrile (Sigma–Aldrich) were used with analytical-grade formic acid (Fisher-Scientific, Fair Lawn, NJ, USA) for mobile phase preparations. A 624 μmol L^{-1} stock solution of $^{13}C_3$ caffeine from Isotec (99 atom% ^{13}C, Miamisburg, OH, USA) was used as an internal standard (IS) to improve quantitation. Individual 10 mmol L^{-1} standard solutions of caffeine, theobromine, paraxanthine, and theophylline were prepared in distilled water and serially diluted to create all standard solutions used in the study. 41 g Hershey's Special Dark chocolate bars (The Hershey Company, Hershey, PA, USA) were used in the cocoa intervention studies.

2.2. Biological fluid sample preparations

All biological fluids used in the study were obtained from healthy volunteers, who were free of dietary restrictions. Plasma was isolated from whole blood via 7 min of centrifugation at 1800 × *g* and 5 °C. Untreated saliva, plasma and urine samples were stored at −80 °C and processed using the protocol outlined below upon thawing at room temperature. All samples were initially inoculated with the $^{13}C_3$ caffeine internal standard and centrifuged at 10,000 × *g* for 5 min to remove any particulate matter from the fluid. 500 μL of the supernatant was then transferred to a 10 kDa molecular weight cut-off (MWCO) filter (Millipore Corporation, Billerica, MA, USA) and centrifuged for 10 min at 10,000 × *g*. The low MW filtrate was then diluted and used for LC–MS/MS analysis.

2.3. LC–MS/MS protocol

An Acquity Ultra-Performance LC system with a diode-array UV spectrophotometer was coupled to a Waters Micromass® Quattro Premier triple quadruple instrument (Waters, Milford, MA, USA) and used throughout the study. The ESI source of the mass spectrometer was operated in positive ion mode, with a capillary voltage of 3 kV. The MS parameters and optimization protocol outlined by Mensch et al. for ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) analyses was implemented in this study [44]. Briefly, the source and desolvation temperatures were set to 120 and 350 °C respectively. Flow rates for the cone and desolvation gases, as well as source and desolvation temperatures were maintained at 100 and 800 L h^{-1} and 120 and 350 °C respectively. Multipliers were set at 650 V, with argon used as the collision gas at a regulated flow rate of 0.35 mL min^{-1}.

Multiple reaction monitoring (MRM) for each of the target compounds required both the cone voltage and collision energy to be respectively optimized for the metabolites precursor and product ions. 5 μmol L^{-1} of the test compounds were infused into the mass spectrometer via a syringe pump running 0.1% (v/v) formic acid in doubly distilled water at a constant flow rate of 20 μL min^{-1}. The derived optimal conditions were incorporated into the final MRM method for sample acquisition.

0.7 μL of all standards and samples were injected into a LC C18 bridged-ethyl hybrid (BEH) column (1.7 μm particles × 2.1 mm × 50 mm, Waters) maintained at a thermostatted 30 °C. The metabolites were separated using a gradient elution with mobile phases of 0.1% (v/v) formic acid in double-distilled water (phase A) and acetonitrile (phase B) respectively. A constant flow rate of 0.6 mL min^{-1} was employed throughout, with phase B initially maintained at 2% from 0 to 0.5 min. The sample was eluted with increasing the % of solvent B to 10, 13, 14 and 50% at 0.5, 0.7, 1.25 and 1.5 min respectively. The column was then equilibrated to 2% phase B over 0.1 min where it was held for an additional 1.5 min to recondition the column and eliminate any potential sample carry-over prior to the next injection. All compounds were detected by the in-line photodiode array UV detector (λ_{abs}: 280 nm) and mass spectrometer using their individually optimizing MRM methods. For all analyses the MS dwell time was 0.1 s and the spectrometer paused between mass transitions 0.005 s. All data acquisition and processing was performed using MassLynx™ 4.1 and QuanLynx™ software (Waters) with retention times (RT) and peak areas (PA) given in minutes and arbitrary units (au) respectively. All figure schematics presented were constructed using Igor Pro 5.0 (Wavemetrics Inc., Lake Oswego, OR, USA).

3. Results and discussion

3.1. Methylxanthine ESI-MS/MS behavior

Accurate assessment of theobromine biofluid levels via LC–MS/MS requires either chromatographic or mass spectrometric separation from both paraxanthine and theophylline. These isomeric alkaloid metabolites represent the primary *N*-demethylated metabolism products of caffeine and cannot be distinguished based on their precursor masses alone. Thus to facilitate accurate theobromine quantitation, compound specific multiple reaction monitoring (MRM) was employed with ESI-MS/MS. This protocol required two sample infusion experiments to optimize the cone voltage and collision energy for precursor and product ions of each compound respectively.

When infused into the mass spectrometer, operated in positive-ion mode, each of the three methylxanthine metabolites produced stable $[M+H]^+$ ions. Mass spectra of each compound were obtained

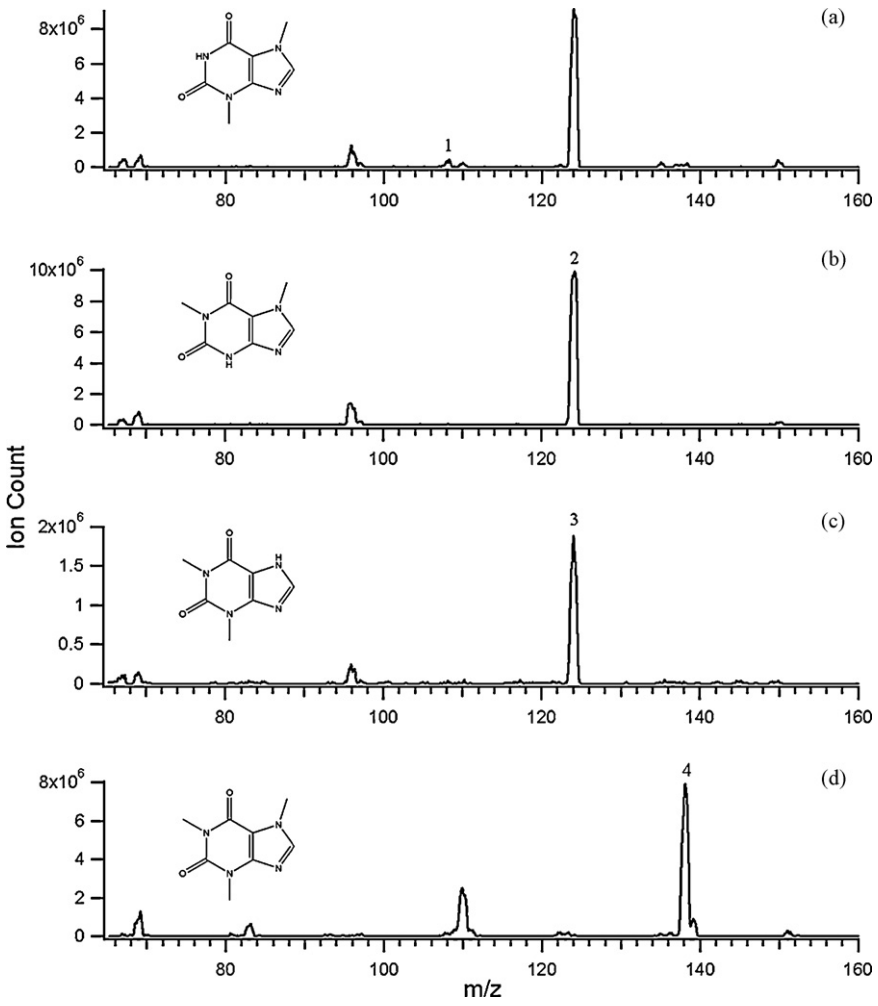

Fig. 1. Product ion scans of theobromine (a), paraxanthine (b), theophylline (c), and caffeine (d). Analyte peak numbering corresponds to the product ion implemented for quantitation; 1, theobromine; 2, paraxanthine; 3, theophylline; and 4, caffeine.

over a range of cone voltages (10–50 V) and evaluated in terms of ion intensity and stability. When combined with the fixed MS parameters outlined in Section 2.3 cone voltages of 40, 25 and 45 V produced the optimal paraxanthine, theobromine and theophylline precursor ion signals respectively. For the second injection experiment, the precursor ions for each compound were transmitted into the collision chamber using their updated MRM files. Product ion spectra were then obtained as the collision energy was incrementally varied between 10 and 50 eV. Given the separation requirement of these species, the respective fragmentation patterns were monitored and evaluated both in terms of ion intensities and the production of unique, methylxanthine specific product ions. The latter of which could be exploited as a potential MS/MS methylxanthine separation mechanism.

Under the collision energy range tested, the spectrometer failed to distinguish paraxanthine and theophylline since their respective fragmentation patterns (181.1 > 124.0, 96.0 and 69.0) were identical (Fig. 1). For both compounds a collision energy of 20 eV was implemented in their MRM files to produce the highest ion signal for the most prevalent MS/MS transition (181.1 > 124.0). The spectrometers inability to achieve a separation necessitates full chromatographic resolution of the pair to be achieved prior to MS/MS detection, if the developed protocol were to provide quantitative information on paraxanthine and theophylline. Analogous direct infusion experiments employing theobromine however showed that an optimal collision energy of 22 eV produced a mass transition (181.1 > 108.0) that was absent in the spectra of paraxanthine and theophylline. Thus, based on the respective MRM files the spectrometer dimension of instrumentation provides sufficient separation of theobromine from its isomeric methylxanthine species. The relative additional mass afforded to caffeine and its $^{13}C_3$ labeled analogue permitted the use of their most abundant MS/MS transitions. Cone voltages of 30 and 40 V combined with a collision energy of 20 eV produced stable transitions of 195.1 > 137.9 and 197.9 > 139.9 for caffeine and $^{13}C_3$ caffeine respectively. Similar MS/MS transitions for these metabolites have been previously reported [43] and all were employed in subsequent optimization and validation studies.

3.2. Minimizing the effects of sample matrix on LC–MS/MS quantitation

Prior to optimizing the LC separation, a single extractionless sample pretreatment was sought to minimize the use of organic solvents and facilitate batch processing of unique sample types. A well-designed protocol would provide complete analyte recovery and reduce matrix impact on both the derived analytes MS/MS signal and the system instrumentation. Since saliva, plasma and urine specimens contain varying levels of interfering matrix species, proteins and salt each of their deleterious contributions to assay precision and accuracy must be minimized. For instance, sample proteins may precipitate, denature and adsorb onto the packing material of the LC column. This occurrence could potentially

induce chromatographic shifts, lead to increased back pressure, and decrease column efficiency and capacity.

Several techniques for protein removal and biological sample clean-up have been reported for theobromine and caffeine quantitation, with LLE [10,15–24,42] and SPE [25–30] being the most widely used. Co-extraction of interfering species and incomplete analyte isolation due to the varying heterogeneity of the metabolite class [16] may occur with these techniques. In addition, the multiple-steps typically involved in the extractions may introduce pre-analytical errors and sample loss into the assay. Protein precipitation [45–47] has also been applied to methylxanthine biofluid analyses despite the possibility of co-precipitating the low-abundance target analyte. Micro-extraction techniques based on stir-bar sorption (SBSE) [40] and a carbowax/divinylbenzene-coated fiber [32] have also been communicated. Surprisingly though, there are relatively fewer examples of sample pretreatments via size exclusion chromatography [35,36] or ultra-centrifugation [38]. Both mass based sample fractionations avoid the use of multiple extraction steps and organic solvents, while permitting the selective isolation of the targeted metabolites from endogenous biopolymers and other high molecular weight species.

The protocol developed in this communication combines the use of sample dilution [24,39,43,48] with commercially available ultra-centrifugation devices to reduce the respective impacts of saliva, plasma and urine matrices on methylxanthine quantitation. Initially each biofluid was centrifuged to eliminate solid particulate matter from the sample. This step has been shown to be of particular importance for saliva analyses [47]. Subsequent filtration of the supernatant with a 10 kDa MWCO filter further fractionates the sample and isolates the targeted theobromine and caffeine analytes. A simple sample dilution of the methylxanthine containing low MW filtrate was then implemented as the final sample pretreatment prior to injection.

The efficacy of developed sample clean-up, in terms of analyte ion signal variations, was evaluated by determining the matrix factor (MF) [49] for duplicate analyses of spiked saliva ($N = 12$), plasma ($N = 8$) and urine ($N = 8$) samples. All methylxanthine standards were spiked after the final dilution step to evaluate the respective matrix effects on the ESI process. The factors obtained represent the ratio of the internal standard corrected analyte peak area within the spiked biological matrix to that of a neat standard solution. A factor of 1.0 shows the matrix has no effect on the generated ESI signals. Ratios of >1.0 and <1.0 indicate ESI signal enhancement and suppression respectively. For all three matrices, increased low MW filtrate dilution (ranging from 2- to 20-fold) minimized the significant effects experienced by theobromine and caffeine ion signals at 1, 5 and 15 μmol L^{-1} levels respectively (data not shown). At the highest dilution tested, the average matrix factor for all theobromine concentrations tested ranged from 1.2 in saliva to 1.0 in plasma and urine respectively. Identical factors were observed for equivalent analyses of caffeine spiked samples. The approximate 23% ESI signal enhancement observed for these analytes in saliva was likely caused by unknown co-eluting species unique to saliva, as the same enhancement was not observed for the diluted plasma and urine. Since further reducing the sample volume in the final dilution step did not improve ion signals for either analyte, a 20-fold dilution of the isolated low MW filtrate was implemented for all matrices in all subsequent analyses. Schneider et al. employed a similar dilution methodology for urine methylxanthines in which samples were diluted 4- or 20-fold prior to centrifugation and injected into an LC–ESI-MS/MS system [43]. However, by introducing a protein removal step before sample dilution both column integrity and interference-free quantitation may be further enhanced with the current protocol. It should be noted that although 500 μL of the biofluids supernatant was employed for the ultra-centrifugation step, the protocol is amendable to smaller sample volumes as only 0.7 μL of the 20-fold diluted filtrate was injected for quantitation. Additionally, commercially available ultra-centrifuge tubes and microcentrifugation devices permit the processing of multiple batches of specimens within 15 min. This may facilitate the pretreatment of relatively large plasma, saliva and/or urine samples studies as the procedure effectively combines the benefits of several distinct sample clean-up steps (i.e., centrifugation, ultra-centrifugation and dilution) into a single protocol that is applicable to reducing the matrix effects of three diverse biofluid specimens. The reproducibility and recovery of all analytes using this methodology and the optimized LC–MS/MS procedure is detailed within.

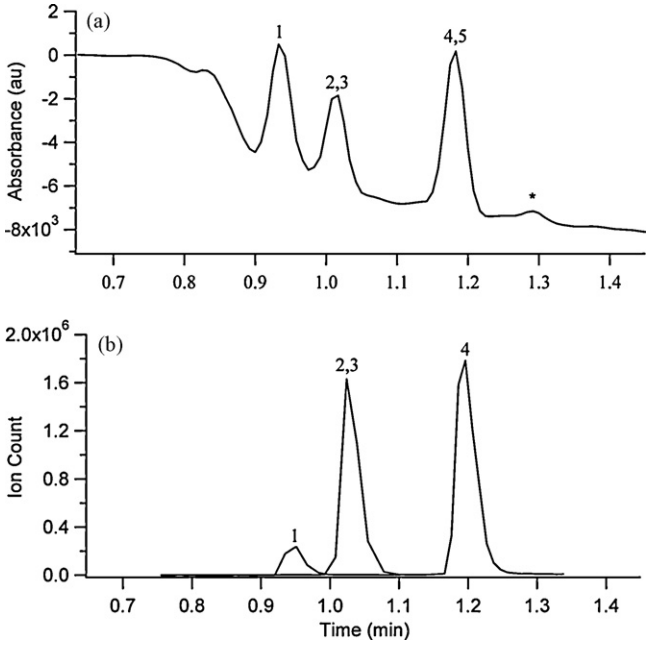

Fig. 2. Chromatograms comparing (a) UV absorbance (λ: 280 nm) and (b) positive ESI-MS/MS detector responses for caffeine, paraxanthine, theobromine and theophylline for a spiked urine sample. The overlaid extracted MRM chromatograms were obtained using the MS/MS transitions: 181.0 > 108.0, theobromine; 181.0 > 124.0, paraxanthine and theophylline; 195.1 > 137.9, caffeine. The injected 2.8 μmol L^{-1} internal standard $^{13}C_3$-caffeine (197.9 > 139.9) co-eluted with caffeine and was omitted from (b) for clarity. Analyte peak numbering corresponds to 400 μmol L^{-1}: 1, theobromine; 2, paraxanthine; 3, theophylline; and 4, caffeine. The notations: 5 and * represent $^{13}C_3$-caffeine and an unidentified metabolite respectively.

3.3. LC separation and MS/MS sensitivity

The initial chromatographic conditions employed in this study were based on the recommendations of Mensch et al. [44]. The mobile phase gradient was further optimized to provide the highest resolution of paraxanthine, theobromine, and theophylline in the shortest total analysis time. The final program permitted the elution of all methylxanthines within the first 1.3 min of the gradient with detection by both UV absorbance (λ_{abs}: 280 nm) and their respective MRM methods. The representative chromatograms presented in Fig. 2 illustrate the separations achieved and contrast the two detection modalities. Theobromine (RT, 0.9 min) and caffeine (RT, 1.2 min) are baseline resolved from a co-eluting paraxanthine and theophylline peak (RT, 1.0 min) contained within the spiked urine sample. The $^{13}C_3$-caffeine internal standard co-eluted with native caffeine and was omitted from Fig. 2b for simplicity. As expected, UV detection demonstrated reduced sensitivity and selectivity compared to the tandem MS/MS detector. The sloping

baseline absorbance (Fig. 2a) mirrors the mobile phase gradient employed and prevents the detection ($S/N \approx 3$) of methylxanthine concentration levels below 400 μmol L^{-1}. This sensitivity limitation was true for all three biofluids tested. Combined with the high background signal and potential interference from co-eluting endogenous metabolites [48] the benefits of employing compound specific MS/MS transitions for targeted methylxanthine analyses can be seen in the increased selectivity and sensitivity of the assay (Fig. 2b).

Further manipulation of the mobile phase gradient and optimization of the flow rate, sample injection volume and column temperature failed to resolve paraxanthine and theophylline. A similar co-elution of these isomers has been observed with other LC-based separation protocols [15,24,39], although methods capable of profiling ten or more caffeine metabolites, have achieved baseline resolution of the methylxanthine pair [16,20,25,43,47]. These separations typically require longer total analysis time and are associated with comprehensive caffeine metabolite quantitation. Achieving baseline resolution of all methylxanthine and methylurate species associated with caffeine metabolism is essential for such studies [15,16,18,22,39] as well as correlating the observed relative metabolite levels with xenobiotic-metabolising enzymatic activity and physiological conditions [15,16,18,19,22,41]. It should be noted that such research pursuits were beyond the scope of this current work and thus further optimization of the chromatographic conditions was not attempted.

A single, rapid, sensitive and selective assay that is applicable to multiple human biofluids was desired for the targeted quantification of the physiologically active methylxanthine cocoa components. Since the optimized LC and MS/MS conditions employed could not differentiate paraxanthine and theophylline, only qualitative information regarding their relative biofluid levels can be obtained. However, the optimized chromatography combined with the selective mass transitions of both theobromine and caffeine permits their desired respective quantitation. The sensitivity of the individual MRM transition was enhanced by scheduling the MS detection window to be ±0.15 min around the respective retention time. Under these conditions, an optimal dwell time of 0.1 s for all analytes permitted good peak shape and sensitivity. Fouling of the ESI interface and MS system from biofluid salts and contaminants was also reduced by diverting the LC effluent to waste both before and after data collection. Column back pressure remained constant through-out all experiments at approximately 8000 psi.

3.4. LC–MS/MS method validation for theobromine and caffeine

Complex matrices of pooled unprocessed saliva ($N = 12$), plasma ($N = 8$) and urine ($N = 8$) samples were created to challenge the validation of the optimized assay. Each biofluid pool was then prepared using the protocol outlined in Section 2.2. The isolated low MW filtrate was diluted 20-fold with distilled water prior to injection as described. Each respective validation parameter was assessed through spiking the appropriate concentration of caffeine, theobromine and $^{13}C_3$-caffeine (IS) into the pooled sample. A blank sample (no analyte spike) for each biofluid was subtracted from the spiked samples to account for the presence of any methylxanthines in the sample pool while allowing the biofluid matrix to influence quantitation. The theobromine and caffeine PA used in the validation were normalized to the IS PA to account for any observed variations in ion signal and sample loss. Table 1 summarizes the method validation parameters for all biofluids tested. Theobromine and caffeine concentration levels quoted represent the concentrations of standards in the unprocessed biofluid sample.

3.4.1. Linearity

The dynamic range of the method was determined by performing duplicate measurements of seven different concentrations over the 160-fold concentration range of 2.5–400 μmol L^{-1}. Linear regression of these measurements revealed good linearity for both theobromine and caffeine in all three matrices with the average correlation coefficients (R^2) of 0.9968 and 0.9997 respectively. This dynamic range closely reflects the linearity achieved in a previous LC–MS/MS technique for urine theobromine and caffeine quantitation (0.05–25 μmol L^{-1} within the injected sample) [43]. It should also be noted that the simplicity and low volume requirements of the current sample preparation protocol allow for a greater final dilution and repeat analyses of concentrated caffeine or theobromine samples beyond the dynamic range (e.g., >400 μmol L^{-1} in the sample).

3.4.2. Intra- and Inter-day reproducibility

Intra-day PA precision of theobromine and caffeine was determined by quantifying 10 individually pooled biofluid samples containing spiked concentration levels of 2.5, 50 or 150 μmol L^{-1} for both metabolites respectively. Inter-day PA precision was obtained using the same protocol with 25 individual pooled samples analyzed over five different days. Average intra-day PA variance was 10 and 5% for theobromine and caffeine respectively at the lowest concentration level tested. The inter-day PA variance slightly increased to 13 and 9% for theobromine and caffeine respectively at the same concentration level. In terms of RT, both the intra- and inter-day precision of theobromine (RT, 0.9 min) and caffeine (RT, 1.2 min) at all three levels tested were 3 and <1% respectively. However, a slight (~5%) chromatographic shift in RT was observed for replicate analyses of biofluid samples spiked with >400 μmol L^{-1} analytes. This was likely caused by column fouling from the increased analyte spike. Flushing with 100% mobile phase B for 1.5 min and re-equilibrating the column at the initial gradient conditions for an addition 1.5 min restored the chromatography and eliminated all significant analyte carry-over.

3.4.3. Lower limit of detection and functional sensitivity

The LC–MS/MS optimization outlined permitted the lower limit of detection (LOD) for theobromine and caffeine to be 1.25 μmol L^{-1}. At this concentration level the theobromine and caffeine PA CV ($N = 15$) showed moderate inter-day variability ranging from 6% for caffeine and upwards to 23% for theobromine. However, it was noted that at the lower limit of the linear dynamic range the accuracy and precision of the assay was improved (Table 1). From this experience, a concentration level of 2.5 μmol L^{-1} in the unprocessed sample should be considered the functional sensitivity of the protocol.

3.4.4. Spike and recovery

The recovery of different theobromine and caffeine concentration levels was assessed by spiking the appropriate standard volumes into aliquots of unprocessed saliva, plasma and urine respectively. The analytes were spiked at four different concentrations covering the linear range of the assay. Table 1 shows that despite the wide variability in initial matrix compositions adequate recoveries for both methylxanthines were achieved in all biofluids tested. As expected, improved recoveries were observed at higher analyte spike concentrations. Note that spiking 10 μmol L^{-1} analyte into an unprocessed sample translates to an injection of 0.5 μmol L^{-1} on-column. This concentration is approaching the lower limit of the assays dynamic range and increased variability in the recovery is expected. The effects of both ion signal variations and relative recoveries for all experiments were factored into quantitation through the use of the $^{13}C_3$-labeled caffeine internal standard.

Table 1
Validation parameters for the analysis of methylxanthine metabolites in human biofluid samples.

Validation parameter	Saliva		Plasma		Urine	
	Theobromine	Caffeine	Theobromine	Caffeine	Theobromine	Caffeine
Intra-day precision[a] ($N = 10$)						
Peak area (CV)						
2.5 μmol L^{-1}	8.5	5.2	14.6	3.5	5.8	7.0
50 μmol L^{-1}	3.4	3.6	5.6	3.1	4.1	3.4
150 μmol L^{-1}	5.0	4.4	6.3	4.0	4.5	3.0
Inter-day precision[a] ($N = 25$)						
Peak area (CV)						
2.5 μmol L^{-1}	11.0	7.3	17.0	9.1	11.2	11.9
50 μmol L^{-1}	7.0	5.7	10.2	5.7	9.5	8.2
150 μmol L^{-1}	6.4	6.7	7.1	5.1	8.5	11.7
Spike and recovery[a] ($N = 2$) (%)						
10 μmol L^{-1}	114	118	117	109	73	80
20 μmol L^{-1}	111	109	109	95	91	92
100 μmol L^{-1}	95	100	94	95	111	112
300 μmol L^{-1}	105	100	96	100	98	99

[a] $^{13}C_3$ caffeine was used as the internal standard in all assays to normalize analyte peak area variations.

This validation demonstrates that the developed protocol effectively mitigates the interfering matrix effects typically observed with targeted theobromine and caffeine biofluid analyses via LC–MS/MS. The ability to selectively quantitate μmol L^{-1} concentrations of these species via their respective MRM transitions, with a single sample pretreatment methodology presents the possibility of employing this platform for multiple fluid-type evaluations. Such combined methylxanthine studies have previously been conducted by LC–UV for plasma-and-urine [24,38,41], saliva-and-urine [22] and serum-and-urine [36] quantifications respectively. Alternatively, gradient capillary LC with frit fast atom bombardment (FAB) MS [28] and TLC–UV [26] have also been employed for plasma-and-urine and saliva-and-urine measurements respectively. While LC–UV [20,29,47,50] and RIA [31] have shown methylxanthine saliva-and-serum and saliva-and-plasma concentrations to be similar following an oral dosage. However, determinations of plasma-and-saliva-and-urine levels in a single study are relatively less common, with a MEKC–UV technique being reported by Thormann et al. [33]. Their study analyzed multiple substituted purines in all three matrices with the open tubular MEKC format and pseudo-stationary phase employed permitting the direct injection of saliva and serum samples. Only urine purine analyses required extraction prior injection with this technique [33]. Such minimized pretreatments are difficult to implement with LC separations due to column fouling from protein and matrix adsorption. The current validated protocol effectively employs both ultra-centrifugation and dilution to mitigate these deleterious matrix effects and enable the accurate and precise evaluation of theobromine and caffeine levels in three challenging biofluid matrices. To the best of our knowledge this is the first report of an LC–MS/MS platform capable of their targeted analysis in saliva, plasma and urine.

3.5. Biofluid methylxanthine distribution following cocoa consumption

For researchers studying the potential beneficial effects of cocoa consumption, the ultimate attractiveness of this validated protocol would lie in its ability to selectively quantify the biofluid levels of theobromine and caffeine pre- and post-intervention. To demonstrate this trait, five volunteers were asked to consume two commercially available chocolate bars each. The product manufacturer stated that each bar contained 188 and 26 mg of theobromine and caffeine respectively. Samples of the saliva, plasma and urine were collected immediately prior to consumption and 90 min post-ingestion. Each specimen was then split into two identical aliquots and prepared using the protocol outlined in Section 2.2. As implemented for the method validation (Section 3.3) all isolated low MW filtrates were diluted 20-fold with distilled water prior to injection. The precision and accuracy of the validated protocol was investigated through duplicate measurements of the identical split sample portions.

Table 2
Theobromine and caffeine concentration levels for the saliva, plasma and urine of volunteers undergoing a cocoa intervention.

Subject	Saliva		Plasma		Urine	
	Pre-dose	Post-dose	Pre-dose	Post-dose	Pre-dose	Post-dose
Theobromine concentration (μmol L^{-1})[a]						
1	<2.5	31.1 ± 0.1	<2.5	52.3 ± 5.3	<2.5	180.0 ± 2.4
2	<2.5	39.3 ± 1.1	<2.5	67.5 ± 7.9	9.8 ± 0.4	277.5 ± 9.2
3	<2.5	27.2 ± 0.2	<2.5	60.1 ± 1.9	12.8 ± 0.9	131.9 ± 3.8
4	<2.5	28.6 ± 1.7	7.1 ± 0.8	60.0 ± 8.1	94.9 ± 4.1	318.1 ± 13.2
5	<2.5	33.2 ± 1.9	<2.5	43.2 ± 2.1	10.9 ± 0.2	449.4 ± 39.4
Caffeine concentration (μmol L^{-1})[a]						
1	<2.5	4.6 ± 0.6	<2.5	5.6 ± 0.1	<2.5	5.7 ± 1.0
2	<2.5	3.4 ± 0.2	<2.5	4.6 ± 0.5	<2.5	4.2 ± 0.3
3	3.1 ± 0.3	3.6 ± 0.5	4.0 ± 0.3	8.4 ± 1.0	4.5 ± 0.1	6.6 ± 0.4
4	12.5 ± 2.6	12.4 ± 2.2	21.4 ± 0.1	25.3 ± 0.1	23.4 ± 0.8	24.7 ± 0.9
5	12.2 ± 1.3	12.5 ± 1.9	15.2 ± 1.9	14.7 ± 2.6	13.2 ± 0.1	18.4 ± 0.5

Samples were collected prior to chocolate bar ingestion and 90 min post consumption. The lower limit of quantification for all biofluids was 2.5 μmol L^{-1}.
[a] $^{13}C_3$ caffeine was used as the internal standard in all assays to normalize analyte peak area variations.

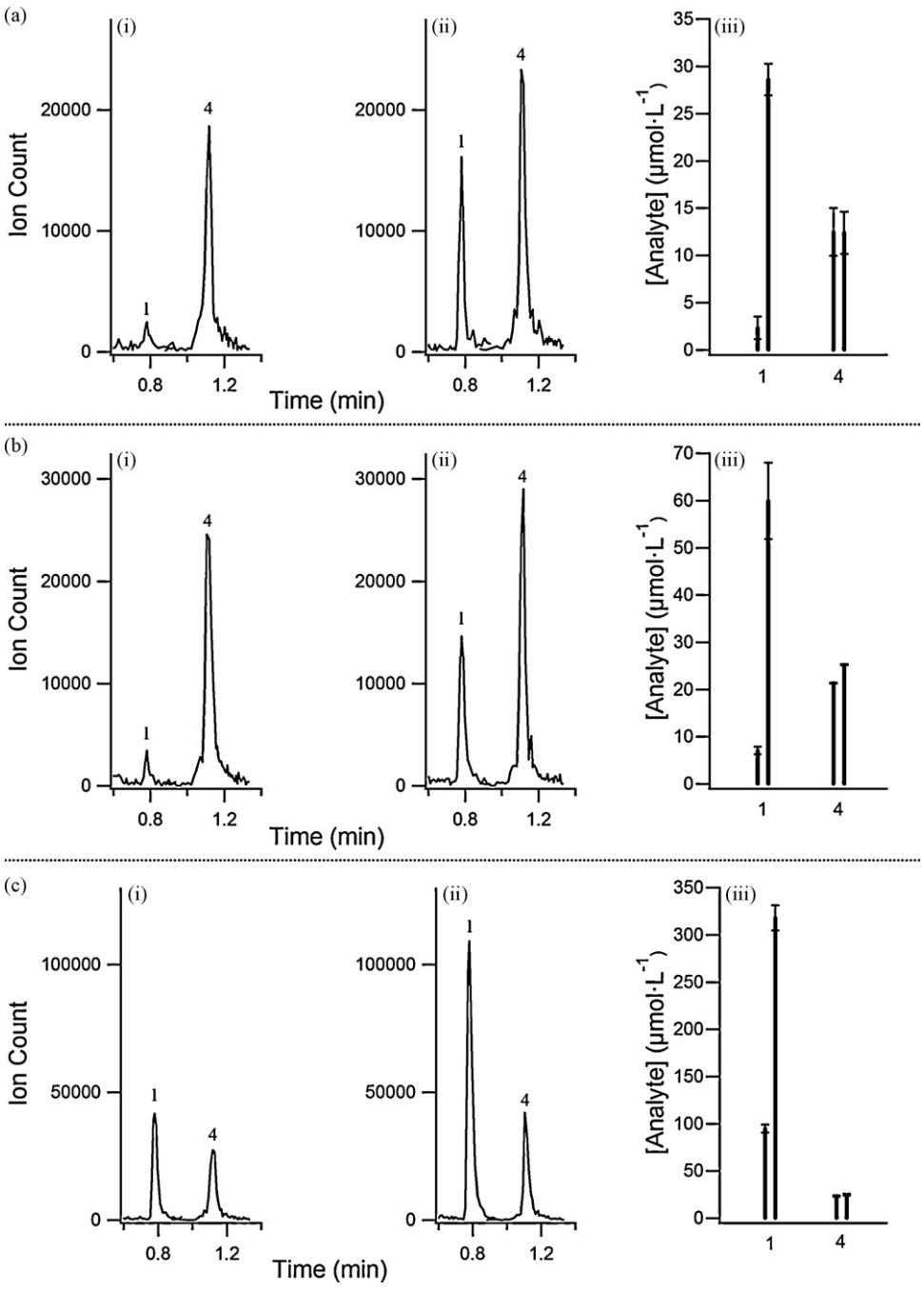

Fig. 3. Extracted MRM chromatograms illustrating the respective levels of both theobromine and caffeine in the (a) saliva, (b) plasma, and (c) urine of a volunteer. Samples were collected prior to chocolate bar ingestion (i) and 90 min post consumption (ii). Each chromatogram was obtained using the MS/MS transitions detailed in Fig. 2. Analyte peak number corresponds to: 1, theobromine; and 4, caffeine. The $^{13}C_3$-caffeine internal standard trace was omitted for clarity. The derived biofluid methylxanthine levels (iii) represent the average metabolite concentrations obtained from duplicate measurements of two identical sample aliquots. For all biofluids, the post-dose levels of each methylxanthine are represented by the right-most plots within their respective graphs (iii).

Fig. 3 illustrates the quantitative assessment of both theobromine and caffeine pre- and post-cocoa consumption. The implemented sample pre-treatment effectively mitigated the deleterious matrix effects typically observed in challenging biofluid separations, as evidenced by the observed consistency in elution of times across the diverse matrices (Fig. 3). In addition, this single protocol permitted the accurate and precise evaluation of these cocoa-derived components within each of the three biofluid sample types. Parts (iii) of Fig. 3 graphically demonstrate this ability by presenting the intervention induced changes in both theobromine and caffeine levels across the evaluated matrices. Table 2 summarizes these relative concentrations for all volunteers and sample collection points, with the biofluid levels quoted representing the average concentrations derived from the duplicate analyses of the two split-sample fractions. The quantified concentrations covered the assays 160-fold linear range and the 2.5 μmol L^{-1} LOQ failed to offer significant impedance to the dietary intervention trial. In addition, the average concentration CV of the theobromine and caffeine levels listed in Table 2 ranged from 4.1 (saliva) to 9.3% (plasma) and 3.1 (urine) to 14.7% (saliva) respectively.

From this data it is clear that the oral consumption of 376 mg of cocoa-derived theobromine produced significantly elevated lev-

els in all three sample types. Sampling 90 min post-dose revealed the highest levels in urine, signaling that the majority of the theobromine had undergone renal filtration. The expected theobromine distribution amongst the respective sample types (e.g., concentrations in urine > plasma > saliva respectively) was also observed at this sampling time. It is highly probable that this concentration increase is a direct response to the cocoa consumption. In the 90 min prior to obtaining the post-dose sample, all study volunteers were forbidden from consuming any food or drink products containing either of the target analytes. However, individuals were free of dietary restrictions prior to enrollment and the metabolism of residual caffeine levels (e.g., Volunteers 3, 4 and 5) may have contributed to elevated theobromine levels.

Interestingly, in-spite of its known extensive metabolism upon consumption [15,16,18,22,39] the ingestion 50 mg of cocoa-derived caffeine also produced detectable levels in previously naïve subjects (e.g., Volunteers 1 and 2). Overall these concentration perturbations did not show the same marked elevations as theobromine (Table 2). In part, this may be explained by the approximate 3.8-fold reduction in the chocolate bars relative caffeine content. The significant enzymatic transformations into various methylxanthine and methylurate products upon ingestion [15,16,18,22,39] likely also contributed to this observed difference. It should be noted again that a mechanistic study of the relative rates of methylxanthine metabolism was not the intent of this communication. The goal was to develop a single, robust, LC–MS/MS platform that is universally applicable to the quantification of these physiologically active cocoa components in saliva, plasma and urine. The precision and accuracy of this communicated study makes the methodology an attractive alternative to previous LC-based bioanalytical approaches for monitoring these metabolites following a high-dose administration, as only a single platform with an estimated 20 min total analysis time (from sample collection to chromatogram integration) is required for such studies.

4. Conclusion

The unique sample preparation technique detailed within permits adequate reduction of the matrix effects typically seen in targeted theobromine and caffeine biofluid analyses by LC–ESI-MS/MS. The ability to simultaneously batch process saliva, plasma and urine samples with a single protocol reduces the pre-analytical treatment typically required for the measurement of these metabolites. Unbiased quantitation of both metabolites following a nutritional intervention was provided by the optimized LC and MRM procedures with a total run time of 3 min. Further work on increasing the sensitivity and improving the analyte recovery of this protocol at lower theobromine and caffeine concentrations is on-going.

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