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Determination of catechins and caffeine in proposed green tea standard reference materials by liquid chromatography-particle beam/electron ionization mass spectrometry (LC-PB/EIMS)

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ABSTRACT

Presented here is the quantitative analysis of green tea NIST standard reference materials (SRMs) via liquid chromatography-particle beam/electron ionization mass spectrometry (LC-PB/EIMS). Three different NIST green tea standard reference materials (SRM 3254 *Camellia sinesis* Leaves, SRM 3255 *C. sinesis* Extract and SRM 3256 Green Tea-containing Oral Dosage Form) are characterized for the content of caffeine and a series of catechin species (gallic acid, catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate (EGCG)). The absolute limits of detection for caffeine and the catechin species were determined to be on the nanogram level. A reversed-phase chromatographic separation of the green tea reference materials was carried out on a commercial C_{18} column using a gradient of water (containing 0.1% TFA) and 2:1 methanol:acetonitrile (containing 0.1%TFA) at 0.9 mL min^{-1} and an analysis time of 50 min. Quantification of caffeine and the catechin species was carried out using the standard addition and internal standard methods, with the latter providing appreciable improvements in precision and recovery.

1. Introduction

Green tea (*Camellia sinesis*) is one of the most consumed drinks worldwide, becoming a part of the daily routine of many people and a significant source of antioxidants, purportedly providing diverse health benefits [1–3]. The major class of active compounds in green tea is the polyphenols, more specifically the catechins (also known as flavan-3-ols) which make up 30% (mass fraction) of green tea leaves [4]. The most abundant catechin species in green tea include (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, (−)-gallocatechin, (−)-gallocatechin gallate and (−)-epigallocatechin gallate. Other compounds present in green tea are phenolic acids (gallic acid, chlorogenic acid and caffeic acid), flavanols (quercetin, kaempferol and myricetin) and xanthines (caffeine and theophylline) [5]. The consumption of polyphenols has acquired a great deal of attention because of their strong antioxidant properties, which have been shown to be beneficial in the prevention of cancer and cardiovascular diseases. Other reported medicinal benefits of the polyphenols include anti-inflammatory, anti-arthritic and anti-angiogenic properties [2,6,7].

Botanical supplements such as green tea, echinacea and goldenseal have become an important part of people's nutrition due to their numerous health benefits. For that reason, it is of utmost importance that the producers and manufactures of such products provide accurate content information as well as consumer safety. In 1994, the Dietary Supplement Health and Education Act (DSHEA) assigned the United State Food and Drug Administration to regulate the production of these supplements. DSHEA ensures the safety of the supplements by providing a legal definition of dietary supplements, establishing guidelines for displaying the ingredients on the labels and allowing the FDA to present good manufacturing practice (GMP) regulations [8,9]. After DSHEA, the Office of Dietary Supplements (ODS) was established within the National Institutes of Health (NIH) to promote scientific research as well as the development of standard reference materials (SRMs) for botanical supplements in order to achieve product consistency throughout the supply chain, from raw material to consumer products, in terms of chemical composition as well as the identification of potential adulterants and contaminants [10,11]. The production of these SRMs also allows the validation of new analytical methods for the characterization and quantification of the main components present in botanical supplements.

Among the various analytical methods that can be found in the literature, reversed-phase liquid chromatography (RP-LC) is the method of choice for the separation and identification of the green tea species (polyphenols) [1,12–15]. The chromatographic separations are most commonly followed by UV–vis absorbance [1,13,16] or mass spectrometry (MS) [1,3,6,17] detection, although

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electrochemical [18–20] and fluorescence [21,22] detection have also been used. However, the UV-absorbance, electrochemical and fluorescence detection methods mentioned above are not particularly analyte-specific. Therefore, the identification of the analytes requires matching their chromatographic retention times with analytical standards. On the other hand, MS has been demonstrated to be very powerful by allowing the identification/confirmation and quantification of multiple species present in a complex biological matrix. More specifically, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been reported for the identification and quantification of the catechin species present in green tea [3,23,24]. While, ESI-MS can provide molecular weight information of the polar compounds without extensive fragmentation, and in many cases the addition of MS–MS methods are necessary for the complete species-specific identification. Another important challenging aspect that needs to be considered during ESI-MS experiments is the fact that conventional RP-LC methods may not be easily interfaced to the electrospray source because of the differences between solution flow rates and matrix/mobile phase compositions [16].

In this laboratory, the particle beam mass spectrometry technique has been employed successfully for the detection and determination of an assortment of organic, organometallics, inorganic and biological compounds by the application of a glow discharge (GD) ionization source [25–29]. The ease of operation and efficient solvent removal of the PB interface allows the use of interchangeable GD or electron ionization (EI) sources to perform *comprehensive speciation*; meaning the separation and determination of elemental and molecular species in a single run. This unique analytical tool has been demonstrated the capability to affect the comprehensive speciation of organic and inorganic arsenic species for the analysis of ethanolic bladderwrack and kelp extracts as well as the chemical characterization of green tea extracts [30,31]. As well, use of the LC-PB/MS-detection method has been validated for the determination of ephedrine alkaloids present in the ephedra-containing NIST dietary supplement standard reference materials (SRMs) by a standard addition method [32].

Presented here is a RP-LC-PB/EIMS method for the chemical characterization of green tea's main constituents. More specifically, this approach is employed for the quantification of caffeine and catechin species present in three NIST standard reference materials (SRM 3254 *C. sinesis* Leaves, SRM 3255 *C. sinesis* Extract and SRM 3256 Green Tea-containing Oral Dosage Form) currently under development. Mass spectra for each of the target species were obtained using analytical standards (when available) and their class-specific signature ions identified. Calibration curves for all of the species of interest were generated and their respective detection limits determined. Two extraction procedures were employed to isolate the target species, dependent upon the physical state of the starting material. The chromatographic separation for green tea extracts was accomplished by RP-LC using a C_{18} column and monitored by UV-absorbance at 210 and 254 nm. Once the optimal separation was achieved, the column effluent was coupled to the PB/EIMS system for the quantification of caffeine and catechins by the standard addition and internal standard methods. The LC-PB/EIMS-detection method is a viable technique for the study of commercial botanical extracts, their respective active consitutents, and potential metabolites by different quantification methods.

2. Materials and methods

2.1. Particle beam electron impact mass spectrometer system

The PB-MS system used in this study was an Extrel (Pittsburgh, PA, USA) Benchmark Thermabeam LC/MS quadrupole mass spectrometer with an electron impact ionization source [27,28,33]. The particle beam serves as a "transport-type" interface for LC/MS. This allows for continuous sample introduction into the ionization source (in this case EI) in the form of dry particles by removal of the residual solvent vapors and at the same time maintaining the chromatographic integrity of the separation. The PB-MS system is equipped with a tungsten filament set at an acceleration voltage of 70 eV, the standard voltage for EI, making spectral library comparisons possible.

Data acquisition for the MS was performed under the control of the Extrel (Pittsburgh, PA, USA) Merlin Ionstation system. Total ion chromatograms (TIC) were typically acquired over the mass range of 50–500 Da in a scan time of 1.0 s. The chromatographic (temporal) trace of a specific mass can be isolated from the TIC for background correction and peak integration. The data was then exported to Sigma Plot 8.02 (Systat Software, Richmond, CA, USA), Microsoft (Redmond, WA, USA) Excel, and PowerPoint for further processing.

The Thermabeam interface (Extrel Corp., Pittsburg, PA, USA), consists of a thermoconcentric nebulizer, a desolvation chamber, and a two-stage momentum separator. The aerosol generated by the nebulizer (~86 °C tip temperature) passes through the heated desolvation chamber (~130 °C), were the wet droplets begin to dry and the solutes form particles. As the particle/gas mixture passes through a pair of 1 mm differential pumping orifices (one per stage), the low-mass solvent molecules are dispersed and pumped away as they have low momenta, while the heavier analyte-containing particles are able to pass through to the next orifice. Once the particles leave the interface there is little or no solvent vapor remaining. The resulting beam of dry analyte particles then moves into the heated (~275 °C) source block region. The optimization of the operating parameters for the EI source (electron energy and source block temperature) had been performed and described in previous work [30,31,33].

2.2. Sample preparation and delivery

A 1000 μL mL^{-1} stock solutions of catechin, epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), gallic acid (GA), proxyphylline (Sigma–Aldrich, St. Louis, MO, USA), caffeine (Aldrich Chemical Co. Inc, Milwaukee, WI, USA) and trimethyl-$^{13}C_3$ caffeine (Cambridge Isotope Laboratories Inc., Andover, MA, USA) were prepared by weighing appropriate amounts and diluting in a mixture of 95% water and 5% 2:1 methanol (MeOH):acetonitrile (ACN). Working standard solutions were prepared fresh daily to ensure minimal degradation. The green tea SRM's analyzed supplied by NIST are part of the family of SRM's under development. The suite of green tea SRMs is composed of SRM 3254 *C. sinesis* Leaves, SRM 3255 *C. sinesis* Extract and SRM 3256 Green Tea-containing Oral Dosage Form. Six boxes/packets were received for each of the green tea SRMs for analysis as well as intra- and inter-sample comparison. All test solutions were stored in light-tight vessels at 4 °C and fresh dilutions were prepared as necessary.

The samples were introduced into the PB interface via a Waters (Milford, MA, USA) Model 1525 HPLC binary system equipped with a Rheodyne (Cotati, CA, USA) Model 7125i injector and a 50 μL injection loop. A fixed flow rate of 0.9 mL min^{-1} was used throughout this work. The liquid output passed directly through a Waters Model 2487 dual wavelength absorbance detector (Milford, MA, USA) monitoring at 210 and 254 nm during the development of the chromatographic separation. Liquid chromatography separation of caffeine and the catechin compounds was accomplished using an Alltima C_{18} reversed-phase chromatography column (Alltech Associates Inc., Deerfield, IL USA, 250 mm × 4.6 mm, 5 μm) and guard column (All-Guard Holder with Alltima C_{18} Cartridge, Alltech Associates Inc., Deerfield, IL, 7.5 mm × 4.6 mm, 5 μm) operated at room

temperature. The initial composition of LC mobile phase consisted of 95% water (18.2 MΩ cm^{-1}, NANOpure Diamond, Barnstead International, Dubuque, IA, USA) containing 0.1% TFA (A) and 5% 2:1 MeOH:ACN Containing 0.1% TFA (B). A linear gradient of 5–25% B for gradient times of 0–20 min, followed by a linear gradient of 25–35% B from 20 to 40 min was used for separation of the species and quantification by the standard addition method. On the other hand, a linear gradient of 5–10% B from 0 to 5 min, followed by a linear gradient of 10–35% B from 5 to 50 min was used for the quantification of the species by the internal standard approach as resolution of the added compound (standard) was required as well.

Quantification of caffeine and the catechin species were performed using a standard addition method and the internal standard approach. For the standard addition method stock standard solutions (1.00 mg mL^{-1}) of caffeine, catechin, EC, EGC, EGCG, ECG, and GA were added in the amounts of 25 and 50 μL to aliquots of the green tea tincture and diluted to 1.0 mL. The green tea aliquots were of 50, 100 and 200 μL and diluted up to 1.0 mL, yielding 5, 10 and 20% solutions. In the case of the internal standard approach, stock standard solutions (1.00 mg mL^{-1}) of caffeine, catechin, EC, EGC, EGCG, ECG, and GA were utilized to prepare a calibration solution with final concentrations of 100 and 150 μg mL^{-1}. The internal standards proxyphylline and trimethyl-$^{13}C_3$ caffeine utilized for the quantification of the catechins and caffeine were added to the calibration solutions to achieve concentrations of 100 and 50 μg mL^{-1}, respectively. NIST SRM 3260 Bitter Orange-containing Solid Oral Dosage Form was analyzed as a quality control sample for caffeine determinations to validate the respective quantification methods.

2.3. Extraction procedures

The extraction procedures performed for the preparation of the green tea SRMs were provided by NIST. Approximately 0.2 g of SRM 3255 (*C. sinesis* Extract) material were accurately weighted, added to 15 mL polypropylene tubes, combined with the internal standard solutions containing proxyphylline and trimethyl-$^{13}C_3$ caffeine and dissolved in 2 mL of 30% MeOH solution by shaking for 1 min. After extraction, the sample was filtered to remove any suspended solids using a 0.45 μm PTFE filter (Alltech Associates Inc., Deerfield, IL, USA) for final analysis.

In the case of SRMs 3254 (*C. sinesis* Leaves) and 3256 (Green Tea-containing Oral Dosage Form), approximately 0.3 g of material and 0.1 g of diatomaceous earth (Fisher Science Education, Rochester, NY, USA) for sample dispersal were accurately weighted, combined with the internal standard solutions and placed in 50 mL polypropylene tubes. SRM 3256 was extracted in 6 mL of 30% MeOH using a rotary inversion extraction system, a laboratory built apparatus, at ~60 rpm over a period of 3 h. After extraction, the sample was centrifuged at 4000 rpm for 30 min. The supernatant was decanted and stored at 4 °C. Subsequently, 3 mL of 30% MeOH were added and the material was re-extracted in the same manner. The supernatant volumes were added together and filtered (0.45 μm PTFE filter) for final analysis. In a similar manner, SRM 3254 was extracted in 4 mL of 30% MeOH and 3 mL of 0.1% EDTA by the rotary inversion extraction system, at ~60 rpm over a period of 3 h. After extraction, the sample was centrifuged at 4000 rpm for 30 min. The supernatant was decanted and stored at 4 °C. Subsequently, 1 mL of 30% MeOH and 1 mL of 0.1% EDTA are added and the material was re-extracted in the same manner. The supernatant volumes were added together and filtered (0.45 μm PTFE filter) for final analysis.

2.4. Determination of moisture content

The moisture content of SRMs 3254, 3255 and 3256 was determined by drying in an oven at ~ 95 °C for 24 h. Conversion factors were determined based on dry-mass/received mass and used to report the quantification values on a dry-mass basis.

3. Results and discussion

3.1. Electron ionization mass spectra

The acquisition of simple and easily interpreted EI spectra via the PB interface allows spectral library comparison (when available) and demonstrates the efficiency of the interface to remove solvent residues/vapors, while also maintaining chromatographic integrity. Fig. 1a–h depicts the individual mass spectra obtained from 50 μL injections of 100 μg mL^{-1} solutions of catechin, EGC, gallic acid, caffeine, ECG, EGCG, proxyphylline and trimethyl-$^{13}C_3$ caffeine with their respective chemical structures. The spectra show the molecular ion ($M^{\bullet+}$) for each of the species with the exception of ECG and EGCG. The catechin compound spectra (catechin, EGC, ECG, and EGCG) are very similar with easy-to-interpret fragmentation patterns, as would be expected, because the family of catechin species have specific signature fragment ions as highlighted below. The absence of the molecular ion for ECG and EGCG reflects the fact that the compounds are not stable under the EI operating conditions.

The EI spectrum of catechin (Fig. 1a) shows the molecular ion at m/z = 290, with a base peak at m/z = 138 and other prominent fragments seen at m/z = 168, 153 and 124. The fragment ion at m/z = 124 represents the cleavage of the bi-phenol ring from the catechin molecular ion. While not a major peak in this spectrum, the fragment ion at m/z = 194 represents a key signature ion for the catechin family, representing the base structure. The mass spectra obtained for EC and catechin are indistinguishable, because their only structural difference is the chirality of the stereocenter (hence the spectrum is not shown here).

The mass spectrum of EGC (Fig. 1b) presents the molecular ion at m/z = 306 with a base peak at m/z = 194. The difference between catechin and EGC is simply an additional hydroxyl group on the polyphenol ring. The transition observed from the molecular ion to the fragment peak at 289 Da represents the common loss of a hydroxyl radical (M—17 Da), followed by the fragmentation of the fused ring system as the major fragments appear at m/z = 168 and 138. The mass spectra of ECG and EGCG (Fig. 1c and d) have consistent fragmentation patterns between each other, with base peaks at m/z = 170 and 194, respectively.

In addition to the antioxidant catechins, caffeine is a xanthine alkaloid and an important component in green tea extracts because of its stimulant properties. As seen in Fig. 1e, the mass spectrum of caffeine shows a base peak corresponding to the molecular ion at m/z = 194 (the fact that characteristic fragment for the catechins exists at the same mass is mere coincidence) with characteristic fragment peaks at m/z = 165, 138, and 109. Fig. 1f shows the EIMS spectrum for gallic acid, having a molecular ion at m/z = 170 and fragment peaks at m/z = 153 and 124 corresponding to the loss of hydroxyl radical and the carbonyl group, respectively; characteristic of carboxylic acids. As seen in the structure of gallic acid, the molecule is actually a pendant group common to the catechins. Lastly, Fig. 1g and h shows the spectra for the two internal standards (proxyphylline and trimethyl-$^{13}C_3$ caffeine) with molecular ions at m/z = 238 and 197, respectively. Note that proxyphylline is an excellent internal standard because of its similarity to caffeine, having an isopropanol substituent on the imidazole ring, rather than the methyl substituent in caffeine.

It should be noted that the spectra obtained for caffeine, gallic acid, catechin and epicatechin are similar to those found in the NIST mass spectral library. In the case of the other catechin species the NIST library spectra are not available due to their limited volatility and thermal instability. There are also differences between the

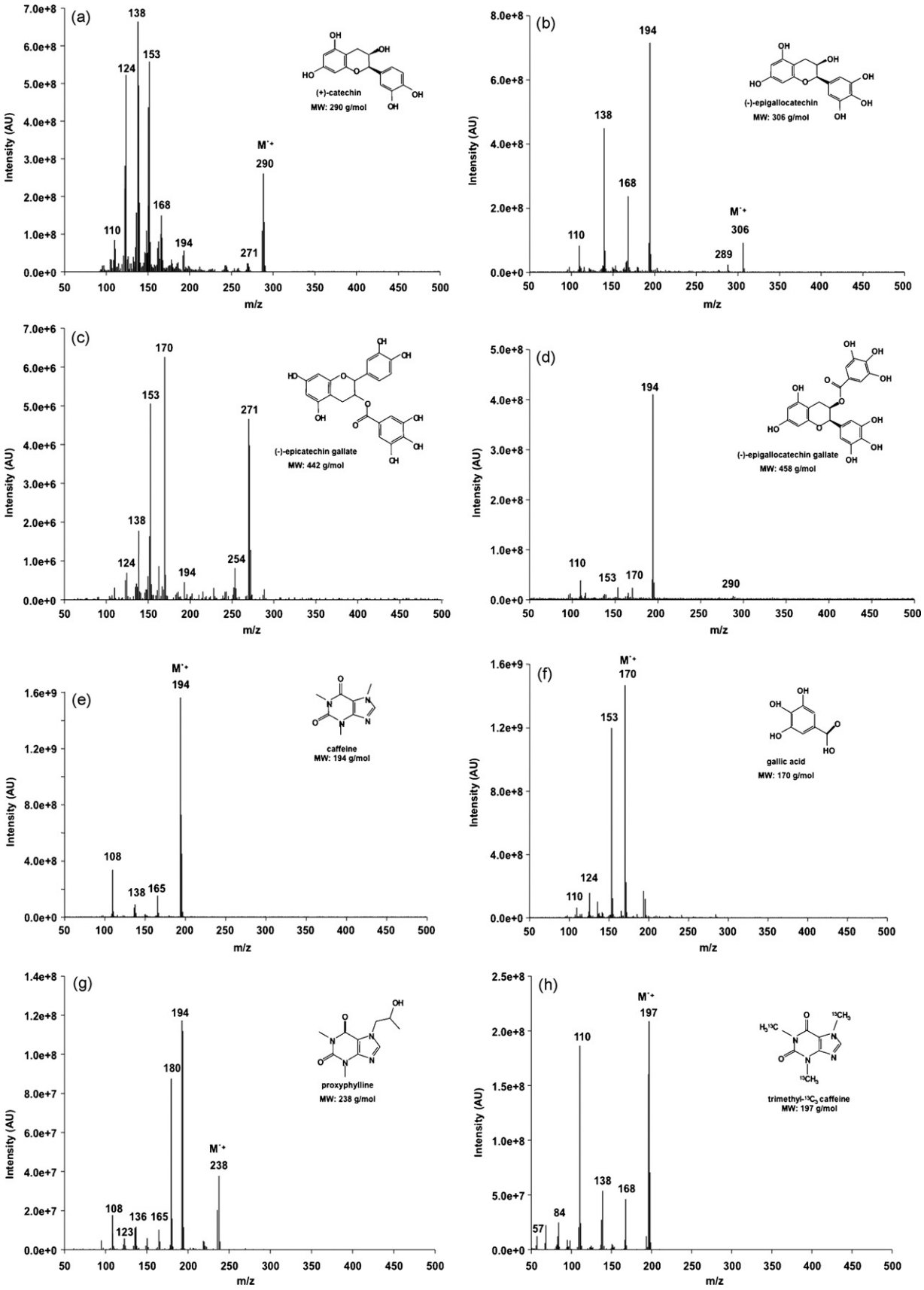

Fig. 1. LC-PB/EI mass spectra of (a) catechin, (b) EGC, (c) ECG, (d) EGCG, (e) caffeine, (f) gallic acid, (g) proxyphylline and (h) trimethyl-$^{13}C_3$ caffeine. Electron energy = 70 eV, block temperature = 275 °C, concentration = 100 μg mL^{-1}, 50 μL injection loop.

Table 1
Analytical response characteristics for green tea species via LC-PB/EIMS.

Analyte	Response function	Accuracy	Detection limit	
		(R^2)	(ng mL^{-1})	(ng)
M*$^+$ *ion				
Catechin (290 Da)	$y = 2E+07x - 5E+07$	0.9823	31	15
Epicatechin (290 Da)	$y = 8E+07x - 6E+08$	0.9470	43	2.1
EGC (306 Da)	$y = 5E+05x - 1E+06$	0.9530	74	7.4
Caffeine (194 Da)	$y = 5E+08x - 2E+09$	0.9940	3.4	0.17
Gallic acid (170 Da)	$y = 8E+08x - 2E+09$	0.9913	5.8	0.29
Base peak				
Catechin (138 Da)	$y = 1E+08x - 4E+08$	0.9940	7.5	0.38
Epicatechin (138 Da)	$y = 8E+08x - 5E+09$	0.9559	4.3	0.21
EGC (194 Da)	$y = 1E+07x - 3E+07$	0.9727	138	14
EGCG (194 Da)	$y = 1E+07x - 2E+07$	0.9911	218	11
ECG (170 Da)	$y = 1E+06x - 1E+07$	0.9856	263	26

EI spectra presented here with those of ESI-MS and APCI-MS techniques, where the molecular ion is obtained almost exclusively and collisional dissociation (MS–MS) is required for the acquisition of structural information [23,24,31,34].

3.2. Figures of merit

Table 1 presents the analytical response characteristics for caffeine and the catechin species obtained by the LC-PB/EIMS system. Response curves using the TIC and selected ion monitoring modes were generated through triplicate injections across the concentration range of 0.1–100 μg mL^{-1} (as well as an analytical blank) using 95% water and 5% 2:1 MeOH:ACN as the mobile phase composition. More specifically, for the generation of the extracted ion current (EIC) mode calibration curves for the molecular ion and base peak responses of each target species were considered. Each of the species' response functions shows good linearity with acceptable correlation coefficients (R^2 values). Overall, results show that the LC-PB/EIMS-limits of detection ($3\sigma_{blank}/m$) fall in the nanogram level for all of the species. For each of the analytes except ECG (which exhibits extensive fragmentation), the LODs are <0.4 ng and for some of the species these LODs are comparable to the LODs reported by for UV-absorbance (0.2–4 ng absolute) and ESI-MS (0.4–0.7 ng absolute) detection [1,13,14,16]. Even so, much improved LODs would result here from true selective ion monitoring (SIM) detection. In addition, the 95:5 sample introduction conditions used here (representing the most polar of elution conditions) are the least favorable in terms of nebulization efficiency, and so lower LODs would be expected in gradient mode separations. Nevertheless, the magnitude of the LODs obtained here are far below what is required for profiling botanical extracts where concentrations of the species are in the μg mL^{-1} to percent levels. In the case of metabolic studies, values on the single-ng level are quite relevant. As such, this method is likely to be an effective tool for such determinations.

3.3. Reversed-phase chromatographic separation of green tea species

3.3.1. Optimization for quantification by standard addition

Three reversed-phase liquid chromatography methods were evaluated to determine the best separation conditions for the target species in the green tea materials. During the first set of chromatographic separations, a green tea synthetic mixture containing 50 μg mL^{-1} of each of the target species was separated on the C_{18} column using the method previously published by this laboratory [31]. More specifically, a linear gradient method varying from 75:25 (0.1% TFA in water:ACN) to 55:45 over 12 min was performed and the progress of the separation monitored by UV–vis absorbance at 210 and 254 nm. The resultant chromatographic separation (Fig. 2a) demonstrates that the previously published gradient method was not able to fully-baseline resolve all of the targeted species. Gallic acid and EGC, as well as caffeine and epicatechin, co-elute at t_r = 3.75 min and ~5.0 min, respectively. The second set of chromatographic conditions attempted were provided by NIST [12], consisting of a linear gradient varying from 97:3 (0.1% phosphoric acid in water:2:1 MeOH:ACN containing 0.1% phosphoric acid) to 68:32 over 75 min at 1.0 mL min^{-1}. Fig. 2b shows the resultant chromatographic separation of the synthetic green tea mixture. As in the previous method, the green tea species do not completely separate, with EGC and catechin (t_r = 46.0 min), as well as EGCG and

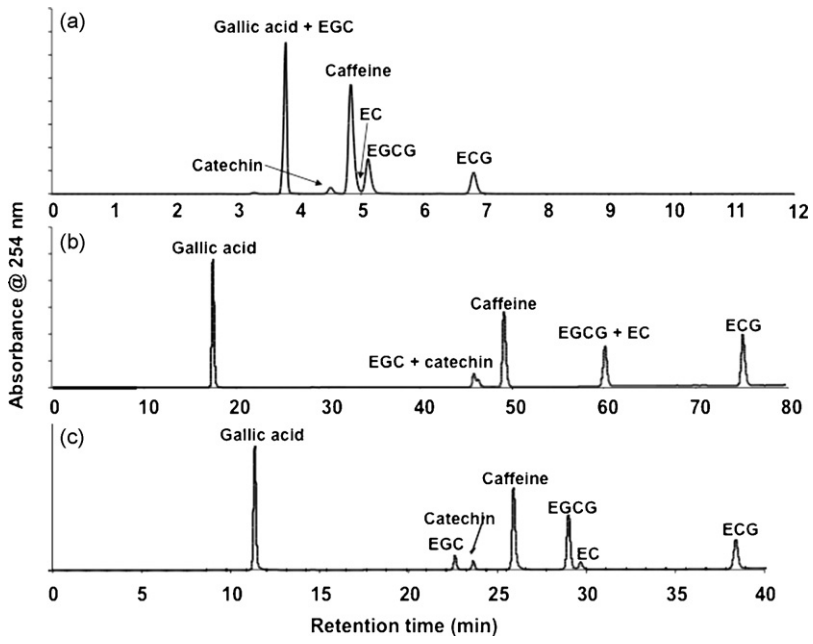

Fig. 2. Reversed-phase chromatographic separation of 50 μg mL^{-1} mixture of green tea standards using (a) published method [31], (b) NIST method and (c) optimized chromatographic conditions of 100 μg mL^{-1} mixture of green tea standards. Separation conditions presented in text.

epicatechin (t_r = 60.0 min) co-eluting during the analysis. Another drawback of this chromatographic method is the long run time of the gradient.

The third (and optimal) set of chromatographic conditions is based on modifications made to the previous method (provided by NIST) and was ultimately used for the catechin quantification via standard addition. Specifically, the phosphoric acid ion pairing agent was changed to trifluoroacetic acid (TFA) and the flow rate reduced to 0.9 mL min^{-1}. Previous work in this laboratory had demonstrated the use of TFA as a viable ion pairing agent for chromatographic separation and PB/MS of related compounds [31]. Hence, the optimized chromatographic separation conditions for the analysis of green tea SRMs includes a linear gradient varying from 95:5 (0.1% TFA in water:2:1 MeOH:ACN containing 0.1% TFA) to 75:25 over 20 min, followed by a linear gradient of 75:25 (0.1% TFA in water:2:1 MeOH:ACN containing 0.1% TFA) to 65:35 from 20 to 40 min. Fig. 2c shows the UV–vis absorbance (254 nm) chromatographic responses of a 100 μg mL^{-1} synthetic mixture of the green tea species using the optimized chromatographic conditions. In comparison to the other two chromatographic methods, the green tea species are baseline-resolved and the analysis run time is reduced in comparison to the NIST method.

3.3.2. Optimization for quantification by internal standard method

For the quantification of the species using the internal standard approach the gradient corresponding to the third set of chromatographic conditions was slightly modified to resolve the internal standard proxyphylline from caffeine. In this case, a linear gradient varying from 95:5 (0.1% TFA in water:2:1 MeOH:ACN containing 0.1% TFA) to 90:10 over 5 min, followed by a linear gradient of 90:10 (0.1% TFA in water:2:1 MeOH:ACN containing 0.1% TFA) to 65:35 from 5 to 50 min was used for the separation of the targeted species and the internal standards. Fig. 3 shows an overlay of the MS chromatographic responses of a 100 μg mL^{-1} synthetic mixture of the green tea species including the internal standards proxyphylline and trimethyl-$^{13}C_3$ caffeine at concentrations of 100 and 50 μg mL^{-1}, respectively. The MS traces shown at m/z = 197, 238 and 306 correspond to the molecular ion of trimethyl-$^{13}C_3$ caffeine, proxyphylline and EGC, respectively. Also, the MS trace at m/z = 290 correspond to the molecular ion of catechin and epicatechin. On the other hand, the MS traces shown at m/z = 138, 170 and 194 are characteristic fragment ions of the catechin species (consistent with all of the spectra), as well as 170 and 194 Da correspond to the molecular ion for gallic acid and caffeine, respectively. A proposed structure corresponding to fragment ion m/z = 194 has been published previously by this laboratory [31]. A trace at m/z = 197, representative of the trimethyl-$^{13}C_3$ caffeine reveals the same retention time as the native compound. Although the gradient conditions for the quantification by internal standard approach are about 10 min longer than the method used for standard addition, the analysis time is still less than the original NIST method.

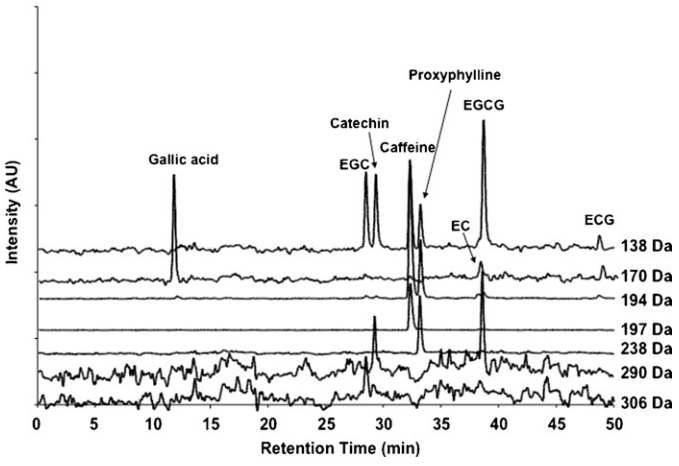

Fig. 3. Overlays of the reversed-phase chromatographic separations of 100 μg mL^{-1} mixture of green tea standards including the internal standards at various MS traces of fragment ions. Electron energy = 70 eV, block temperature = 275 °C, 50 μL injection loop.

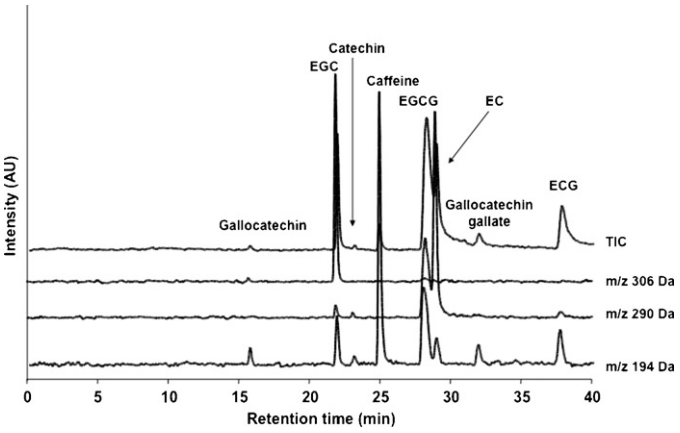

Fig. 4. LC-PB mass chromatogram of 5% SRM 3255 in TIC mode and three traces of fragment ions. Electron energy = 70 eV, block temperature = 275 °C, 50 μL injection loop.

3.4. Quantification analysis

Once suitable chromatographic conditions have been achieved, the green tea reference materials were analyzed and the targeted species quantified by standard addition and the internal standard approaches. The chromatographic separations shown in this section correspond to the chromatographic gradient used for standard addition quantification. Fig. 4 shows an overlay of the LC-PB/EIMS chromatograms of a 5% SRM 3255 solution in TIC mode and extracted traces of selected fragment ions m/z = 194, 290 and 306. As shown in Fig. 1a–f, the m/z = 138 and 194 are common fragment peaks in all the species tested. As well, m/z = 194 also corresponds to the molecular ion of caffeine. All of the target species are labeled on the chromatogram, as well as gallocatechin (t_r = 19.0 min) which is also part of the catechin family. The ability to extract mass spectral information for each of the eluting peaks allows the identification of gallocatechin, which has a molecular ion at m/z = 306 and similar fragment ions to the catechin species. This capability is simply not available in the use of ESI or APCI sources without MS/MS functionality. Fig. 5 shows the corresponding overlay of the chromatographic separation (m/z = 194) of a 5% (w/v) of SRM 3255) and 20% solutions of SRM 3254 and 3256 green tea reference materials. The mass spectra extracted from the eluted species provided consistent fragmentation patterns to the spectra acquired from the analytical standards, therefore allowing the identification of the species of interest. As in the case of SRM 3255, gallocatechin can also be observed during the analysis of SRM 3256, but is not observed in SRM 3254.

The complete analysis of active ingredients in botanical products is a very complicated process, which can be effected by a large number of factors. Perhaps the largest variable is the extraction process itself, as solvent conditions and extraction time will affect the yields of individual compounds differently. Simplistically, a different profile of caffeine and the catechins would be expected in aqueous and ethanolic extractions, thus biasing the results in comparison to other extraction protocols. In addition, the make up of the extraction media may eventually affect the

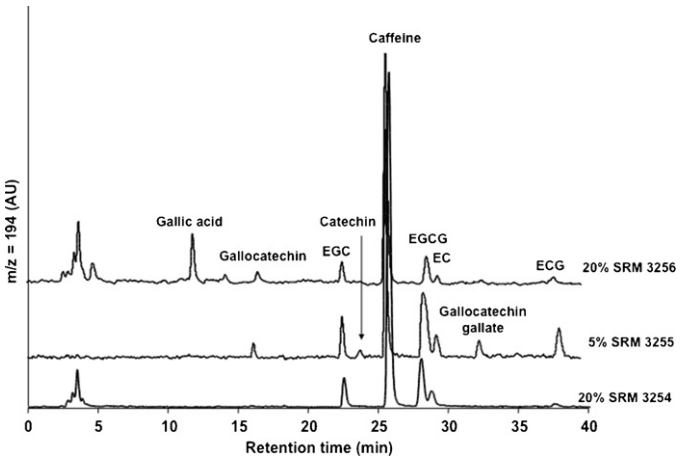

Fig. 5. Overlay of LC-PB chromatographic separation of three green tea standard reference materials at $m/z = 194$. Electron energy = 70 eV, block temperature = 275 °C, 50 μL injection loop.

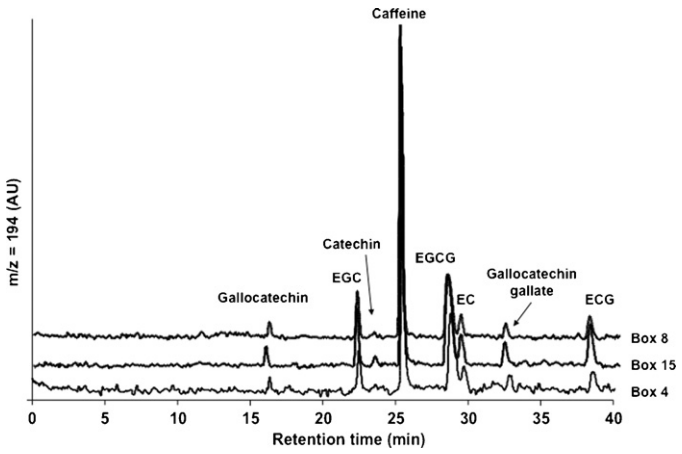

Fig. 6. LC-PB chromatographic separation overlay of three different boxes of 5% SRM 3255 at $m/z = 194$. Electron energy = 70 eV, block temperature = 275 °C, 50 μL injection loop.

performance of the chromatographic and detection steps. Both the standard addition (following extraction) and internal standard (prior to extraction) methods were evaluated in the quantification of gallic acid, EGC, EC, caffeine, EGCG, catechin and ECG in the three green tea SRMs. In order to gain a preliminary assessment of the performance of the two methods, SRM 3260 Bitter Orange-containing Solid Oral Dosage Form was used as a quality control sample. The control sample is one of the few available dietary supplement reference materials already validated by NIST, and is only certified for caffeine relative to this analyte suite. During the internal standard approach, trimethyl-$^{13}C_3$ caffeine was used as the internal standard. Recovery values of 22 and 86% were obtained for caffeine in SRM 3260 by the standard addition method and the internal standard approach, respectively. The discrepancies seen here clearly point to poor extraction efficiencies used in this procedure relative to the method(s) used in the preparation of the SRM in the certification process. Of course, the opposite could be true, resulting in greater than unit recoveries. Clearly the addition of the internal standard to be carried throughout the extraction/separation/detection processes is advantageous in these circumstances.

After analyzing the control sample, SRMs 3254, 3255 and 3256 were tested and concentration values determined for the target species. The concentration values obtained were based on duplicate chromatographic separations of the same preparations. The inter-sample reproducibility of the overall methods was evaluated by the analysis of multiple samplings (SRM boxes) of each of the materials. Of course, such a test is also a reflection of the consistency of the SRM production procedure. The reproducibility of the responses between the different boxes can be seen in Fig. 6 with the overlay of three LC-PB/EIMS chromatograms corresponding to 5% solutions of SRM 3255. Good correlation is seen for the majority of the target species in these three samples, but for example, the catechin content shows some variability.

Tables 2–4 present the results of the measurements for six different boxes of the three reference materials obtained using the standard addition and the internal standard approaches. Across these different matrices, intra- and inter-sample data analysis and comparison within each quantification method and between the two approaches can be made.

First, intra-sample variability, meaning the reproducibility of the chromatographic separation and the quantification is generally much better than 10% RSDs for all sample types and each of the ana-

Table 2
Quantification Results for SRM 3255 *Camellia sinesis* Green Tea Extract.[a].

Box	EGC (mg/g)	Gallic acid (mg/g)	Catechin (mg/g)	Caffeine (mg/g)	EGCG (mg/g)	Epicatechin (mg/g)	ECG (mg/g)
Standard addition method							
4	11.0 ± 0.8	not detected	0.9 ± 0.2	10.0 ± 3.0	10.0 ± 3.0	16.0 ± 2.0	15.3 ± 0.3
8	17.0 ± 2.0		0.88 ± 0.06	7.7 ± 0.9	15.0 ± 2.0	9.7 ± 0.5	10.4 ± 0.3
10	14.4 ± 0.3		2.0 ± 0.4	10.3 ± 0.9	20.0 ± 7.0	10.6 ± 0.3	11.9 ± 0.6
15	14.0 ± 0.5		0.91 ± 0.03	8.1 ± 0.5	45.0 ± 3.0	17.1 ± 0.9	11.3 ± 0.5
18	10.6 ± 0.6		0.85 ± 0.05	7.4 ± 0.8	8.0 ± 3.0	10.0 ± 1.0	16.4 ± 0.1
21	12.8 ± 0.9		1.1 ± 0.2	9.0 ± 2.0	22.0 ± 14.0	12.0 ± 3.0	16.2 ± 0.3
Total	13.3 ± 2.4		1.1 ± 0.4	8.8 ± 1.2	20.0 ± 13.0	12.6 ± 3.2	13.6 ± 2.7
%RSD	15		36	14	65	25	20
Internal standard approach							
4	61.4 ± 1.0	not detected	4.5 ± 0.9	32.0 ± 0.9	138.0 ± 16	53.9 ± 5.1	80.1 ± 0.4
8	67.3 ± 2.2		4.6 ± 0.9	33.2 ± 1.3	118.0 ± 11	45.3 ± 8.6	78.5 ± 2.0
10	73.5 ± 2.8		4.0 ± 0.1	36.7 ± 2.8	125.0 ± 15	49.7 ± 5.1	81.5 ± 3.3
15	63.8 ± 1.6		4.6 ± 0.6	27.8 ± 0.6	124.0 ± 11	46.5 ± 6.6	77.2 ± 0.3
18	73.5 ± 1.1		5.2 ± 0.5	27.3 ± 0.9	132.0 ± 13	42.3 ± 0.9	72 ± 11
21	67.2 ± 2.2		4.4 ± 0.6	35.7 ± 2.1	117.0 ± 7	43.8 ± 5.0	74.0 ± 5.0
Total	67.8 ± 5.0		4.6 ± 0.4	32.1 ± 3.9	126.0 ± 8	46.9 ± 4.3	77.2 ± 3.6
%RSD	7		9	12	6	9	5

[a] Concentration values are expressed on a dry-mass basis, $n = 2$ for each sample.

Table 3
Quantification results for SRM 3256 *Camellia sinesis* Green Tea-containing Oral Dosage Form.[a].

Box	EGC (mg/g)	Gallic acid (mg/g)	Catechin (mg/g)	Caffeine (mg/g)	EGCG (mg/g)	Epicatechin (mg/g)	ECG (mg/g)
Standard addition method							
4	3.6 ± 0.3	1.9 ± 0.2	not detected	9.2 ± 0.2	9.42 ± 0.01	0.91 ± 0.02	not detected
8	4.9 ± 0.2	2.3 ± 0.3		11.0 ± 0.7	9.2 ± 0.2	1.1 ± 0.2	
9	2.8 ± 0.2	1.8 ± 0.2		21.0 ± 2.0	4.8 ± 0.3	1.71 ± 0.01	
16	4.6 ± 0.2	3.1 ± 0.3		6.7 ± 0.5	3.6 ± 0.2	1.1 ± 0.1	
17	4.7 ± 0.2	3.3 ± 0.7		10.0 ± 2.0	12.0 ± 2.0	3.2 ± 0.6	
24	3.6 ± 0.1	3.0 ± 0.6		36.0 ± 3.0	10.4 ± 0.6	1.4 ± 0.3	
Total	4.0 ± 0.8	2.6 ± 0.7		15.7 ± 11.1	8.2 ± 3.3	1.6 ± 0.8	
%RSD	20	27		71	40	50	
Internal standard method							
4	7.6 ± 1.4	2.3 ± 0.3	not detected	68.3 ± 3.3	55.3 ± 7.6	7.9 ± 0.2	not detected
8	8.0 ± 1.2	1.9 ± 0.2		64.3 ± 0.5	68.3 ± 3.6	6.8 ± 0.4	
9	6.1 ± 1.2	4.4 ± 0.6		51.4 ± 3.2	55.9 ± 1.8	9.1 ± 1.1	
16	7.8 ± 0.4	2.3 ± 0.2		67.6 ± 3.3	69.5 ± 1.8	7.3 ± 0.6	
17	6.3 ± 0.6	3.0 ± 0.1		65.3 ± 4.4	74.5 ± 4.4	8.4 ± 0.4	
24	5.6 ± 0.9	3.5 ± 0.8		61.0 ± 0.8	56.2 ± 1.6	6.1 ± 0.5	
Total	6.9 ± 1.0	2.9 ± 0.9		63.0 1 6.2	63.31 8.5	7.6 ± 1.1	
%RSD	14	31		10	13	14	

[a] Concentration values are expressed on a dry-mass basis, $n = 2$ for each sample.

Table 4
Quantification results for SRM 3254 *Camellia sinesis* green tea plant material.[a].

Box	EGC (mg/g)	Gallic acid (mg/g)	Catechin (mg/g)	Caffeine (mg/g)	EGCG (mg/g)	Epicatechin (mg/g)	ECG (mg/g)
Standard addition method							
3	2.9 ± 0.3	not detected	not detected	4.44 ± 0.06	3.3 ± 0.1	1.3 ± 0.1	not quantified
4	2.1 ± 0.3			6.9 ± 0.7	5.0 ± 0.5	4.6 ± 0.5	
9	4.1 ± 0.3			4.0 ± 0.4	4.3 ± 0.3	3.6 ± 0.3	
12	2.7 ± 0.1			3.7 ± 0.3	6.8 ± 0.2	1.8 ± 0.1	
14	4.2 ± 0.2			3.8 ± 0.2	5.4 ± 0.5	1.7 ± 0.1	
20	1.01 ± 0.03			3.7 ± 0.5	2.2 ± 0.3	2.0 ± 0.2	
Total	2.8 ± 1.2			4.4 ± 1.2	4.5 ± 1.6	2.5 ± 1.3	
%RSD	43			27	36	52	
Internal standard method							
3	15.8 ± 2.4	not detected	not detected	24.3 ± 2.7	25.2 ± 3.0	2.3 ± 0.6	not quantified
4	12.9 ± 0.5			20.9 ± 1.5	20.9 ± 3.0	2.8 ± 0.1	
9	10.3 ± 1.5			24.0 ± 2.5	21.6 ± 4.0	1.3 ± 0.3	
12	13.5 ± 1.5			21.5 ± 3.3	12.4 ± 2.0	2.4 ± 0.8	
14	10.9 ± 2.2			22.8 ± 1.7	22.8 ± 1.3	1.2 ± 0.2	
20	17.6 ± 3.4			20.6 ± 3.8	24.4 ± 1.8	3.4 ± 0.7	
Total	13.5 ± 2.8			22.4 ± 1.6	21.2 ± 4.6	2.2 ± 0.9	
%RSD	21			7	22	41	

[a] Concentration values are expressed on a dry-mass basis, $n = 2$ for each sample.

lyte species. Second, the inter-sample (between the different boxes of the same material) variability is much higher with values ranging from ~5 to 70% RSDs. Overall, the variability values obtained by the internal standard approach are better than the values obtained by standard addition. Most of the quantification values obtained for caffeine and the catechins by the internal standard approach are greater than (approximately by a factor of 5×) the standard addition results. The discrepancies in concentration values obtained by the standard addition approach are more likely due to sample processing losses and/or inefficiencies in the extraction procedures. Therefore, the addition of an internal standard to the SRM material before any sample preparation procedure reduces biases from sample loss and controls for extraction procedure.

4. Conclusions

The data presented here demonstrates the capabilities of the LC-PB/EIMS as an analytical tool for the characterization of proposed green tea reference materials. The mass spectra obtained for caffeine and the catechin species demonstrate clear and easy-to-interpret fragmentation patterns. Calibration curves were generated and the analytical figures of merit extracted, illustrating linear responses and LODs down to the nanogram (injected) level. A reversed-phase HPLC method was developed for the separation of the target species in the green tea reference materials. Additional catechin species (gallocatechin and gallocatechin gallate) present in the green tea materials were identified based on their mass spectra and retention characteristics. Finally, the quantification of the target species was performed by a standard addition method and an internal standard approach, for six boxes of the three different green tea SRMs. Data analysis and comparison between the two quantification approaches revealed that the internal standard approach yielded greater concentration values (i.e. higher recoveries) as well as improved inter-sample variability.

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