

# Development of a methodology utilizing gas chromatography ion-trap tandem mass spectrometry for the determination of low levels of caffeine in surface marine and freshwater samples

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Received: 7 March 2008 / Revised: 28 April 2008 / Accepted: 5 May 2008 / Published online: 6 June 2008  
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**Abstract** A methodology for monitoring low level of caffeine in aqueous samples via gas chromatography coupled with an ion-trap tandem mass spectrometry detection system (IT-MS/MS) was developed. Four IT-MS/MS operating parameters, including the collision-induced dissociation (CID) voltage, the excitation time (ET), the isolation time (IT) and the maximum ionization time (MIT) were optimized in order to maximize the sensitivity of the IT-MS/MS technique towards the analyte and its isotope-labeled standard. After optimization, a limit of detection of  $500 \text{ fg } \mu\text{l}^{-1}$  with  $S/N=3$  was achieved. Taking into account blank values and the matrix background, a method detection limit of  $1.0\text{--}2.0 \text{ ng l}^{-1}$  was derived and applied to all of the samples analyzed in the study. Various mass spectrometric conditions have been applied to caffeine and its trimethyl- $^{13}\text{C}$ -labeled standard to elucidate fragmentation pathways for new and commonly occurring product ions observed in the collision-induced dissociation (CID) spectra produced by the ion trap. Ion structures and fragmentation pathway mechanisms have been proposed and compared with previously published data. An isotope dilution method using  $^{13}\text{C}$ -labeled caffeine as a surrogate internal standard was employed to determine and correct the recovery of native caffeine in water samples. The developed methodology has been applied for the determination of caffeine in surface marine and freshwater samples collected on the west coast of Vancouver Island in British Columbia, Canada. The results obtained for the marine water samples indicated a wide

variation in the level of caffeine, ranging from  $4.5$  to  $149 \text{ ng l}^{-1}$ , depending on the location of the sampling site within the inlet. The concentrations of caffeine in samples from lakes associated with various residential densities ranged from ND to  $6.5$ ,  $1.8$  to  $10.4$  and  $6.1$  to  $21.7 \text{ ng l}^{-1}$  for low, moderate and high residential densities, respectively.

**Keywords** Caffeine · Surface water · Ion trap GC-MSMS

## Introduction

Caffeine is one of the main ingredients in a variety of beverages, food products, and over-the-counter medicines, including anticold, antipyretic and analgesic drugs [1]. Considering its uptake from beverages, foods and medicines, caffeine is probably the most widely consumed drug in the world [2]. Caffeine has been added to the United States Environmental Protection Agency list of chemicals with high production volumes [3]. The global average consumption of caffeine is between  $80$  and  $400 \text{ mg}$  per person per day [4]. In humans, dietary caffeine is metabolized by the hepatic cytochrome P450 1A2 pathway, although  $0.5\text{--}10\%$  is not metabolized and this caffeine is excreted in urine [5]. Because of its extensive use in food, beverages and medicines, caffeine has been detected in surface water, groundwater and wastewater effluents worldwide. Caffeine has emerged as an important chemical tracer for tracking input of human fecal waste into source water [6–10], and so it is necessary to develop an accurate, reliable and real-time monitoring method for it. Due to the wide range of concentrations of caffeine in source water, a few different analytical methodologies have been used to analyze this anthropogenic input indicator. Buerge et al. [6] and Peeler et al. [10] employed a triple-quadrupole mass

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spectrometer in MS/MS (tQ-MS/MS) and selective ion monitoring (SIM) modes using  $^{13}\text{C}$ -labeled caffeine as an internal standard to analyze their wastewater and natural water samples. The method detection limits (MDL) achieved were 2 and 10  $\text{ng l}^{-1}$  for the GC-tQ-MS/MS and GC-SIM techniques, respectively, with fortified quality control samples prepared in DI water showing recoveries in the range of 81–127%. The mean extraction efficiency of  $^{13}\text{C}$ -caffeine from natural waters was somewhat lower than in DI water (84%) and showed a larger range (34–113%). The method detection limits achieved in other studies have varied depending on the methodology employed: 40  $\mu\text{g l}^{-1}$  for high-performance liquid chromatography (HPLC) with photodiode array detection [11]; 40  $\text{ng l}^{-1}$  and 5  $\mu\text{g l}^{-1}$  for GC-SIM and HPLC systems, respectively [12]. Siegener and Chen [7] used a GC-SIM analytical methodology with a surrogate internal standard to assess the caffeine levels in seawater samples. The MDL achieved was 5  $\text{ng l}^{-1}$ , but the recoveries of the surrogate standard were very low. For terphenyl- $\text{d}^{14}$  it was on average 25–51%, and for  $^{13}\text{C}$ -(trimethyl)-caffeine it was in the range of 27–57%. Liquid chromatography coupled with a mass spectrometry detection system provided satisfactory sensitivity with MDLs of 5–10  $\text{ng l}^{-1}$  [13] and 10–25  $\text{ng l}^{-1}$  [14], although the recoveries for caffeine in the fortified quality control samples of surface or ground waters were around 48–68%. The analytical methodology based on gas chromatography with an ion-trap tandem mass spectrometry detection system developed by Verenitch et al. [15] achieved a MDL of 20  $\text{ng l}^{-1}$  without the use of an internal standard to verify the recovery of the target analyte.

The aim of the present study was to establish an analytical methodology utilizing gas chromatography with ion-trap tandem mass spectrometry for the determination of low levels of caffeine in surface marine and freshwater samples. Particular attention was paid to optimizing a set of instrumental parameters and employing the method of isotope dilution to achieve the highest sensitivity and accuracy when determining native caffeine in water samples of various origins. Four IT-MS/MS parameters, namely the collision-induced dissociation voltage, the excitation time, the isolation time and the maximum ionization time, were thoroughly optimized. The fragmentation pathways of caffeine and its  $^{13}\text{C}$ -labeled analog under the conditions of low-energy collisionally induced dissociation using ion-trap tandem mass spectrometry were also investigated. Product ions and the fragmentation pathways of caffeine and its trimethyl- $^{13}\text{C}$ -labeled analog under CID conditions were identified and compared with previously published data. The established method was then used to determine caffeine in surface water samples collected at a marine research station and from a few different watershed ecosystems on the west coast of Vancouver Island, British Columbia, Canada.

## Experimental

### Materials and sample preparation

Caffeine was purchased from Sigma-Aldrich Canada (Oakville, Canada); trimethyl- $^{13}\text{C}$ -labeled caffeine, used as surrogate internal standard (SIS), was from Cambridge Isotope Laboratories (Andover, MA, USA). Anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) purchased from Fisher and used to remove moisture from the sample extract was baked at 400 °C for 4 h prior to use. All solvents (methanol [MeOH], methyl tert-butyl ether [MTBE], acetone, hexane and dichloromethane [DCM]) were HPLC-grade and were purchased from Fisher Scientific (Ottawa, Canada). All glassware, including amber sampling bottles, were rinsed with DCM, acetone, MeOH and as a last step, double-distilled deionized water and baked at 300 °C overnight. The solid-phase extraction (SPE) cartridges used in the sample preparation procedure were Oasis HLB 6 ml, 0.2 g from Waters Co. (Milford, MA, USA).

Standard (STD) stock solutions of native and  $^{13}\text{C}$ -labeled caffeine were prepared in methanol. Calibration solutions of different concentrations (10, 20, 40, 60, 80, 100, 200, 500 and 1000  $\text{pg } \mu\text{l}^{-1}$ ) of caffeine with a constant, 200  $\text{pg } \mu\text{l}^{-1}$ , concentration of the surrogate internal standard in each of them were prepared in DCM. A standard solution of caffeine at a concentration of 100  $\text{pg } \mu\text{l}^{-1}$  in DCM was used to optimize the IT-MS/MS parameters.

One-liter grab samples were collected by hand at the water's surface at different locations on the west coast of Vancouver Island, British Columbia, Canada. The locations of sample sites were characteristic of specific human activities in that area, and included a marine research station, river samples up- and downstream from a source of sewage contamination, a sewage treatment plant (STP), lakes of various sizes from 53 to 189,000  $\text{dam}^3$  with different densities of residential development around them. All samples were collected and placed in plastic bottles and immediately transported to the laboratory where they were frozen and kept at -20 °C until the extraction procedure was performed. Samples were usually processed within a 30-day period after their collection.

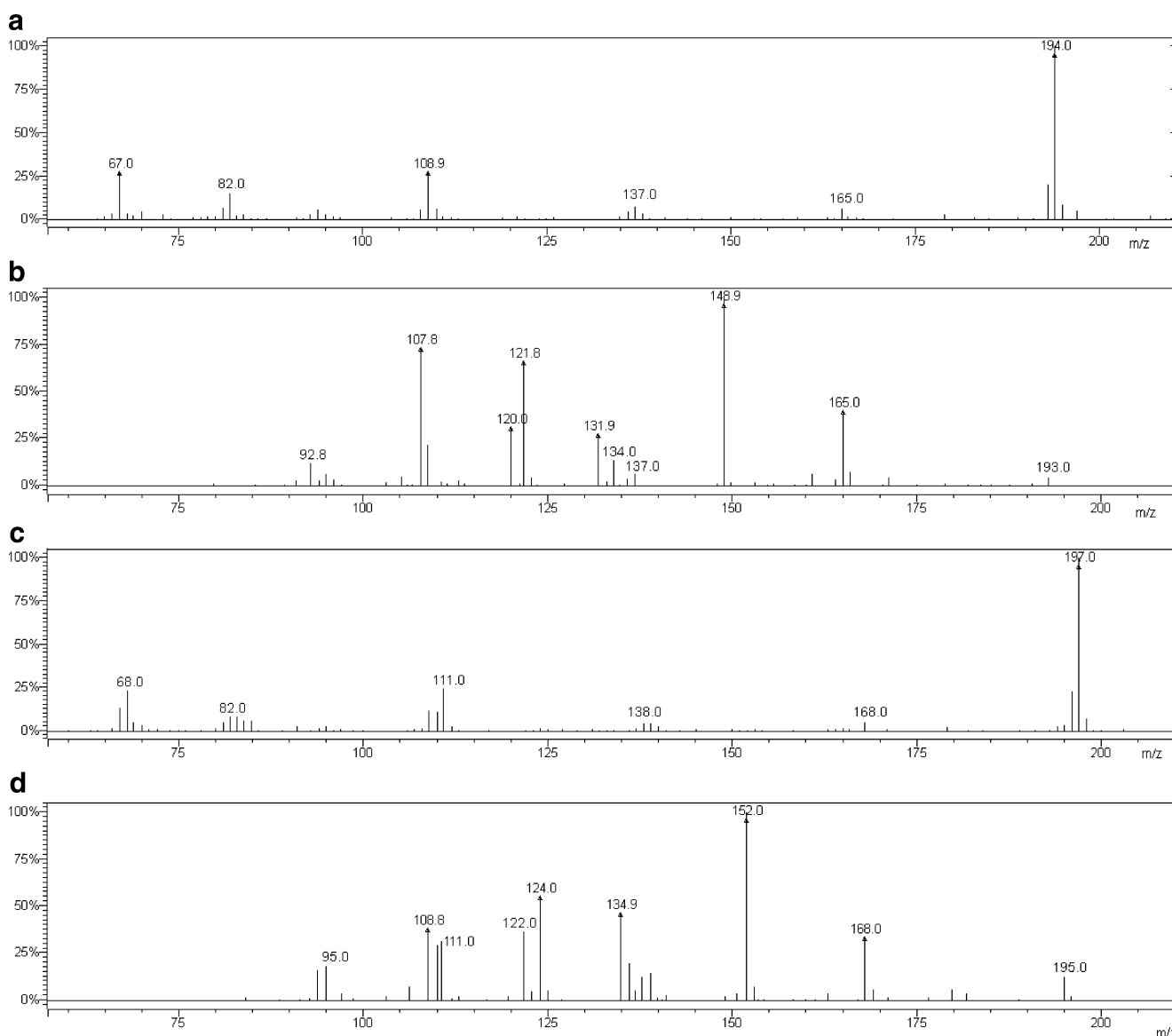
The procedure for caffeine extraction from water samples was an adaptation of a method that is well covered in the literature. Briefly, 10  $\mu\text{l}$  of a 2000  $\text{pg } \mu\text{l}^{-1}$  solution of surrogate internal standard of  $^{13}\text{C}$ -labeled caffeine in methanol was added to 1 l of each sample so that its theoretical concentration in the final extract of 100  $\mu\text{l}$  was 200  $\text{pg } \mu\text{l}^{-1}$ . The sample was then filtered through 0.45  $\mu\text{m}$  GF/C filters which had been pre-washed with hexane and DCM, oven-dried and ashed for 1 h at 500 °C. To the fortified samples of 1 l of distilled de-ionized water, 10, 20,

30, 50 and 100  $\mu\text{l}$  of 200  $\text{pg } \mu\text{l}^{-1}$  of the standard solution of caffeine in methanol was added. This corresponds to concentrations of caffeine of 2.0, 4.0, 6.0, 10.0 and 20.0  $\text{ng } \text{l}^{-1}$ . When necessary, the pH of the sample filtrate was adjusted with 1 M NaOH to pH 7.5. The SPE cartridge (Oasis HLB) was conditioned with 3 ml of MTBE, and then 3 ml of MeOH followed by 6 ml of deionized water, before the sample was passed through the cartridge at approximately 10  $\text{ml } \text{min}^{-1}$ . All sample bottles were rinsed with 10 ml of pH 7.5 deionized water three times, and the rinses were combined and passed through the SPE cartridge. After the extraction was completed, the cartridges were washed with 2 ml of 25% MeOH/water solution to remove the polar co-extractives and further dried under full vacuum for 5 min. The elution of caffeine was performed using 1 ml of MeOH

followed by 6 ml of MeOH/MTBE (1:9, v/v). The extract was dried to dryness and redissolved in 1 ml of DCM. The solution was treated with baked sodium sulfate to remove moisture and transferred to a Kuderna-Danish evaporative concentrator using 0.5  $\text{ml} \times 3$  of DCM. The volume of the final extract was reduced to 100  $\mu\text{l}$  using a gentle stream of nitrogen. The sample was then analyzed on GC-IT-MS/MS by injecting 2  $\mu\text{l}$  of the sample extract.

#### Instrumentation and IT-MS/MS conditions

Analyses were conducted on Varian (Palo Alto, CA, USA) CP 3800 gas chromatograph equipped with a 30  $\text{m} \times 0.25$  mm ID  $\times 0.25$   $\mu\text{m}$  film thickness CP-SIL 8CB-MS capillary column. The gas chromatograph was directly



**Fig. 1** Total ion counts mass spectra of caffeine (**a, b**) and its  $^{13}\text{C}$ -labeled analog (**c, d**) obtained with an ion-trap mass analyzer in EI mode (**a, c**) followed by a full mass scan and MS/MS mode performed at the optimized CID amplitude (**b, d**)

interfaced to a Varian Saturn 2200 ion-trap mass spectrometer. All injections (2  $\mu\text{l}$ ) were made using a Varian CP-8200 autosampler. The flow of He through the GC column was constant and set to 1  $\text{ml min}^{-1}$ . The programmable temperature of the vaporization injector was maintained at 250  $^{\circ}\text{C}$ , the transfer line at 300  $^{\circ}\text{C}$ , the ion-trap at 220  $^{\circ}\text{C}$  and the manifold at 80  $^{\circ}\text{C}$ . The injector was operated in splitless mode for 0.5 min, and then turned to the split mode at a ratio of 100:1. The column temperature program was as follows: initial temperature 70  $^{\circ}\text{C}$ , maintained for 1.0 min, then ramped at 25  $^{\circ}\text{C min}^{-1}$  to 180  $^{\circ}\text{C}$  and at 5  $^{\circ}\text{C min}^{-1}$  to 240  $^{\circ}\text{C}$ , where it was held for 3 min. Total run time was 20.4 min.

The first step of the IT-MS/MS optimization procedure was the selection of an appropriate precursor ion for caffeine. The precursor ion selected from the full-scan mass spectrum of the analyte for the sequential application of the MS/MS conditions was the most abundant ion. For caffeine it was its molecular ion,  $m/z$ 194. In order to improve the IT-MS/MS specificity (i.e., to decrease the potential interference from organic matter ions), the isolation width of the precursor ions for the surrogate internal standard and native caffeine with  $m/z$ 197 and 194, respectively, was reduced to 1  $m/z$ . After the application of the second ionization step (collision-induced dissociation voltage) to the selected precursor ion, the MS/MS spectrum was obtained. Figure 1a and b present the mass spectra of native caffeine obtained in different modes: electron impact (EI) ionization mode followed by a full mass scan (a) and MS/MS mode (b). As expected, the isotope-labeled caffeine gave a mass spectrum similar to that of its native analog, both in EI ionization full mass scan mode and in MS/MS mode (Fig. 1c,d, respectively). A mass segment range of  $m/z$ 80 to 200 was employed at the stage of optimizing the MS/MS conditions. Five major product ions,  $m/z$  108, 122, 149, 165 and 193, were selected on the basis of their high abundances as the characteristic ions for the selected MS/MS conditions. Besides the common MS parameters (such as the axial modulation voltage: 4.0 V; electron multiplier voltage: 1950 V), some MS/MS parameters were set at their default values [16]. Those were the filament/multiplier delay: 10 min; peak threshold: 0; mass defect: 0  $\text{mmu}/100$  u; background mass: 45  $m/z$ ; RF dump value: 650  $m/z$ ; filament current: 80  $\mu\text{A}$ ; AGC target: 2000; prescan ionization time: 1500  $\mu\text{s}$ ; scan time: 0.50  $\text{s scan}^{-1}$ ; multiplier offset:  $\pm 300$  V. Some ion preparation mode (IPM) parameters that were set at their default values or adjusted were kept constant during the process of IT-MS/MS optimization and during further analyses of water samples: isolation windows: 1  $m/z$ ; low/high offset: 6/2 DAC steps; ionization storage level: 48  $m/z$ ; ejection amplitude: 20 V; modulation rate: 3000  $\mu\text{s step}^{-1}$ ; modulation range: two steps; number of frequencies: 1; wave-

form type: resonant; CID frequency offset: 0 Hz, electron energy: 70 eV. The excitation storage level (ESL) was calculated based on the molecular weight of the analyte and a  $q_z$  value equal to its default setting of 0.400.

Under MS/MS conditions, product ions are formed from the precursor ions by collision-induced dissociation. The amount of energy that is converted to internal energy in the precursor ion exposed to the CID voltage and collisions with He atoms depends on the number of collisions (excitation time), the relative energy of the collisions (CID), and the rate that the internal energy is removed by the collisional deactivation. CID in the ion trap is always in competition with ion ejection. If the CID amplitude selected is too large, the precursor ion will be ejected to the trap electrodes before it can collide with background helium atoms. If the CID voltage is too small, the energy of the precursor ion will not exceed the internal energy threshold required to break the chemical bonds and form product ions. ET is the duration that the resonance excitation voltage is applied at constant radiofrequency (RF) to the endcap electrodes of the ion trap mass analyzer to cause the formation of product ions from an isolated precursor ion via CID with damping gas molecules. IT refers to the duration of the ion isolation waveform voltage which is applied to the endcap electrodes of the ion trap during the isolation of the precursor ions in the ion trap. A shorter IT is used when the parent ion is considered unstable. A longer IT may be desirable when stability is not an issue and high mass cleanup is required (i.e., high background/column bleed). MIT is reserved for the main scan as an upper limit for the ionization time allowed to achieve the target number of ions during the ionization mode. The effect of each parameter in general has been described in detail elsewhere [17–19].

Therefore, to achieve the highest sensitivity and accuracy in determinations of caffeine in water samples, the authors conducted a detailed optimization of a set of instrumental IT-MS/MS parameters: CID, ET, IT and MIT. Four series of injections of a standard solution of caffeine at a concentration of 100  $\text{pg } \mu\text{l}^{-1}$  were made in order to investigate the signal intensities of the product ions of caffeine by varying each one of the four instrumental parameters cited above. Initially the operating parameters chosen for optimization were set at the default values; they were then optimized one-by-one in the following order: CID, ET, IT, MIT. For each series of injections, the instrumental parameter under investigation was gradually increased and the peak areas of the product ions with  $m/z$  108, 122, 149, 165 and 193 were integrated. Three replicate injections were performed and averaged for each data point during this process of optimization in order to evaluate the deviation caused by instrumental fluctuations.

Except for isolation time, all of the parameters had a significant effect on the sum of area counts of the ions at

$m/z$  108, 122, 149, 165 and 193 produced under MS/MS conditions. Based on the results obtained in the optimization experiments, the optimum conditions for the determination of caffeine by IT-MS/MS were as follows: CID=1.0 V; ET=50 ms; IT=5 ms and MIT=40,000  $\mu$ s.

These optimum conditions for IT-MS/MS were applied to check the sensitivity and linearity of the ion-trap tandem mass spectrometry detector. For this purpose a series of standard solutions of caffeine with concentrations of between 10 and 1000  $\text{pg } \mu\text{l}^{-1}$  were analyzed. Due to the low background signal, high signal-to-noise ( $S/N$ ) ratios were obtained, even for the standard solution with the lowest caffeine concentration,  $S/N_{\text{ave}}=112$ . The limit of detection (LOD) determined using the formula  $LOD = 3 \times SDV$  was 500  $\text{fg } \mu\text{l}^{-1}$ , where  $SDV$  is the standard deviation determined from seven replicate analyses of the standard solution of caffeine at a concentration of 5  $\text{pg } \mu\text{l}^{-1}$ .

The linearity of the response of the ion-trap mass spectrometry detector in MS/MS mode for caffeine was studied by constructing a seven-point calibration curve using standard solutions covering concentrations from 10 to 1000  $\text{pg } \mu\text{l}^{-1}$ . In these solutions, the concentration of  $^{13}\text{C}$ -labeled caffeine used as the internal standard was kept constant at 200  $\text{pg } \mu\text{l}^{-1}$ . We performed the injections by gradually increasing the concentration of caffeine. The range of concentrations used was sufficiently representative of levels of caffeine in surface water. A good linearity with a correlation coefficient  $r^2=0.986$  was achieved,  $\frac{\text{Peak Size}}{\text{PS IS}} = \frac{\text{Amount}}{\text{Amt.IS}} \times 1.2908 - 0.0524$ . Here *Peak Size* is the area counts of the analyte; *PS IS* is the peak size of the internal standard; *Amount* is the amount of the analyte in  $\text{pg}$ ; *Amt.IS* is the amount of the internal standard in  $\text{pg}$ .

#### Fragmentation and interpretation of MS/MS spectra

Ion-trap tandem mass spectrometry and multistep  $\text{MS}^n$  (where  $n > 2$ ) experiments have been gaining popularity, not only for the analysis of trace levels of a variety of organic analytes [15, 20–24], but also as an essential technique for the structural analysis of a wide range of environmentally and biologically relevant compounds [25–27]. Such systems provide a considerable increase in informing and detecting power in areas such as fundamental studies of ion structure, as well as for the detection and quantification of molecules in complex matrices. The technique of collision spectroscopy (or tandem mass spectroscopy, MS/MS,  $\text{MS}^2$ ) in the presence of a collision gas in a suitable region(s) of the spectrometer increases both the number of ions and the abundance of naturally occurring decomposition products.

The energy deposition mechanism that operates in collisional experiments has been the target of many studies. These have indicated that, aside from a relatively small

quantity of ions that experience electronic excitation, most of the collisionally induced decompositions originate from vibrationally excited precursors. Quite a high internal energy distribution is observed; such a distribution allows the activation of different decomposition channels with different activation energies, which can lead to a unique fragmentation pathway for a selected precursor ion.

A few papers [28, 29] have been devoted to the elucidation of fragmentation patterns of caffeine in tandem mass spectrometry. Liguori et al. [28] have developed a method based on gas chromatography/triple quadrupole mass spectrometry (tQ-MS/MS) for the identification and characterization of caffeine and its metabolites in human urine. They used mass-analyzed ion kinetic energy (MIKE) and high-energy collision-induced dissociation spectra to elucidate the fragmentation pathways of the analytes of interest. Recently Williams et al. [29] studied the rapid desorption electrospray ionization (DESI) of caffeine as well as other drugs and their fragmentation using triple quadrupole and hybrid quadrupole time-of-flight mass spectrometry (Q-ToF). The electrospray ionization (ESI) employed by LC-MS/MS instruments [13, 14, 30] for caffeine is characterized as a soft ionization technique which primarily yields protonated or deprotonated species with little or no fragmentation occurring in the source. The most abundant product ions produced under these conditions are  $m/z$  138  $[\text{M}-\text{H}_3\text{C}-\text{N}-\text{CO}+\text{H}]^+$  and  $m/z$  110  $[\text{M}-\text{CO}-\text{N}(\text{CH}_3)-\text{CO}+\text{H}]^+$ . The ion activation mechanisms employed by various techniques presented in the literature were different to those used in the combination of EI and CID processes used by the ion-trap tandem mass spectrometry (IT-MS/MS) instrument employed in this study. The differences between the fragmentation products of caffeine obtained in a multisection instrument (triple quadrupole mass spectrometer or tQ-MS/MS “in space”) and in IT-MS/MS (MS/MS “in time”) may be explained by the fact that the parent ions that are collisionally activated via resonant excitation in an ion trap are excited between collisions; as such, the ions undergo stepwise activation coupled with collisional cooling by the He buffer gas. The resonant excitation potential or CID is applied here at an amplitude of 1.0 V for 50 ms, which should be compared to the 10–50  $\mu$ s residence times of the ions in the central quadrupole of a triple quadrupole mass spectrometer [30, 31]. For tQ-MS/MS instruments, the preselected ions undergo a variety of interactions with the target gas, leading to the formation of a family of excited species with quite a large energy distribution, which enhances the feasibility of accessing decomposition channels with large differences in activation energies, such as the loss of a H. atom due to the cleavage of a C–H bond (product ion with  $m/z$  193) and the fragmentation driven by the retro-Diels–Alder mechanism [32] that results in the loss of methyl isocyanate

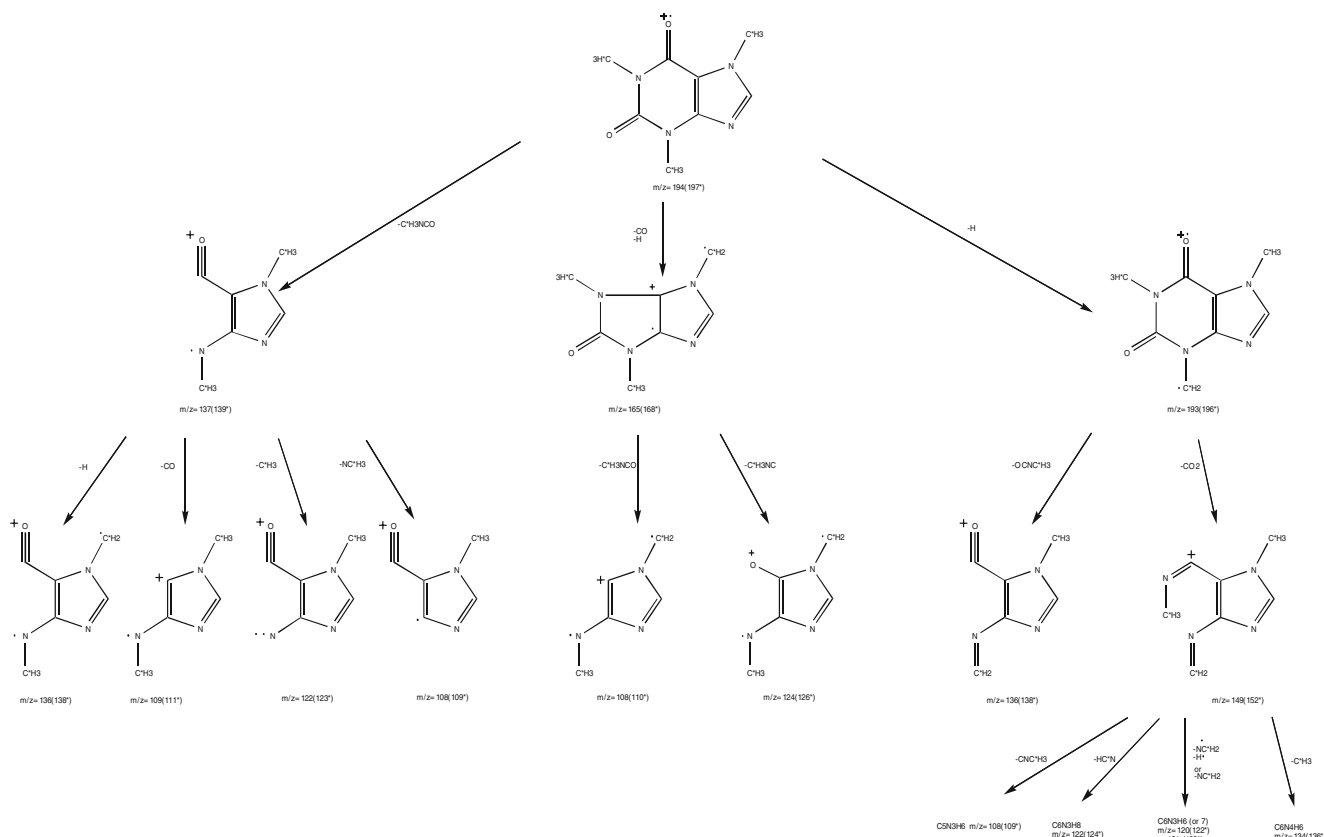
**Table 1** Relative intensities (in parentheses) of molecular and product ions obtained for caffeine and its  $^{13}\text{C}$ -labeled analog under MS<sup>2</sup> conditions by different mass analyzers

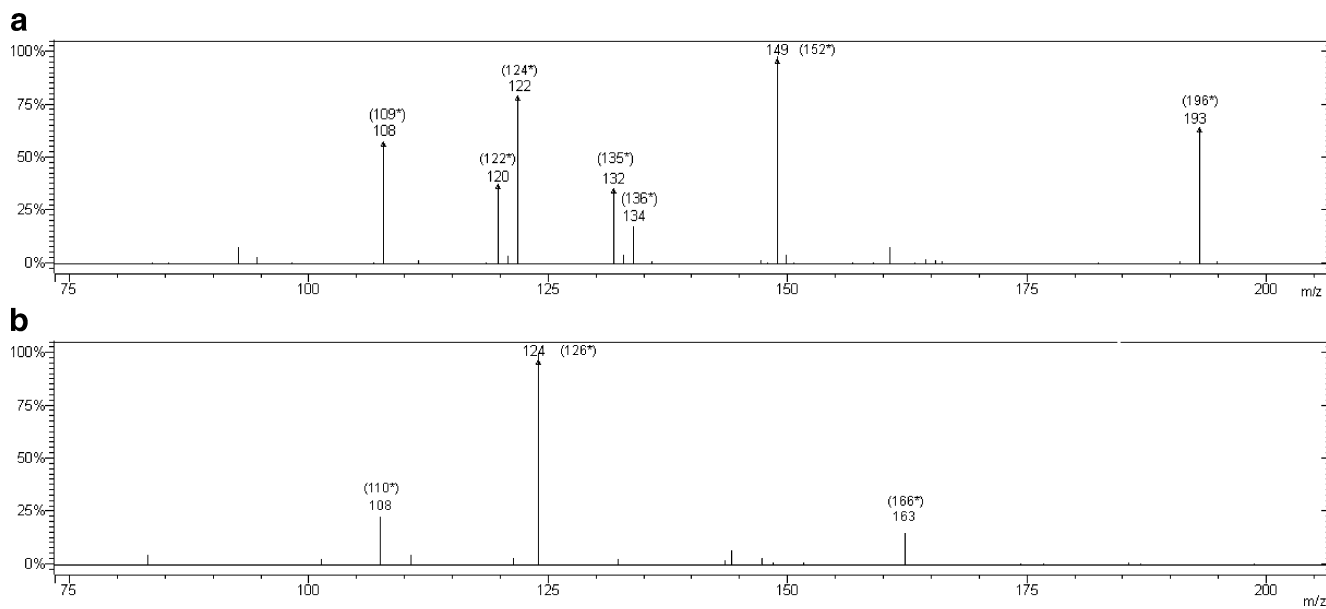
Analyte	Molecular ion, $m/z$	Product ions, $m/z$									Technique	Source
Caffeine	194(0)	193 (0.6)	165 (35.1)	149 (100)	137 (10.6)	132 (45.8)	122 (69.6)	120 (52.8)	109 (27.7)	108 (67.4)	GC-IT- MS/MS	authors
$^{13}\text{C}$ -Caffeine	197(0)	196 (1.5)	168 (49.8)	152 (100)	139 (7.3)	135 (27.7)	124 (68.8)	122 (31.3)	111 (30.3)	109 (30.1)	GC-IT- MS/MS	authors
Caffeine	195(0)				138 (100)		123 (14)		110 (52)		LC- MS/MS	Thevis
Caffeine	194(0)	193 (100)	165 (2.6)		137 (12.5)				109 (6.0)		GC-tQ- MS/MS	Liguori

[ $\text{CH}_3\text{N}=\text{C}=\text{O}$ ] (product ions with  $m/z$  137) followed by the elimination of a molecule of carbon monoxide [ $\text{C}=\text{O}$ ] (ion species with  $m/z$  109) [28].

The energy deposition mechanism in collision experiments performed using IT-MS/MS with a supplementary radio-frequency (RF) voltage is considered a step-by-step one, in which low activation energy processes are favored [33]. As a result, in the CID fragmentation spectrum of caffeine produced under IT-MS/MS conditions, a number of new ion species were formed (Table 1) along with the product ions that were also seen for triple quadrupole tQ-MS/MS [28] and

LC-MS/MS [30]. Although present at different relative abundances, the product ions of native caffeine at  $m/z$  193, 165, 137 and 109 were identified in the MS/MS spectra from the tQ-MS/MS and IT-MS/MS (Table 1) ionization techniques. The product ions at  $m/z$  149, 132, 120 and 108 were only observed in the spectra produced by the IT-MS/MS instrument. The relative abundances of the product ions obtained for caffeine and its  $^{13}\text{C}$ -labeled analog in the mass range between  $m/z$  100 and 200 under the optimized IT-MS/MS conditions are presented in Table 1 and compared with results in the literature.

**Scheme 1** Fragmentation pathways of caffeine in an ion-trap under the optimized MS/MS conditions

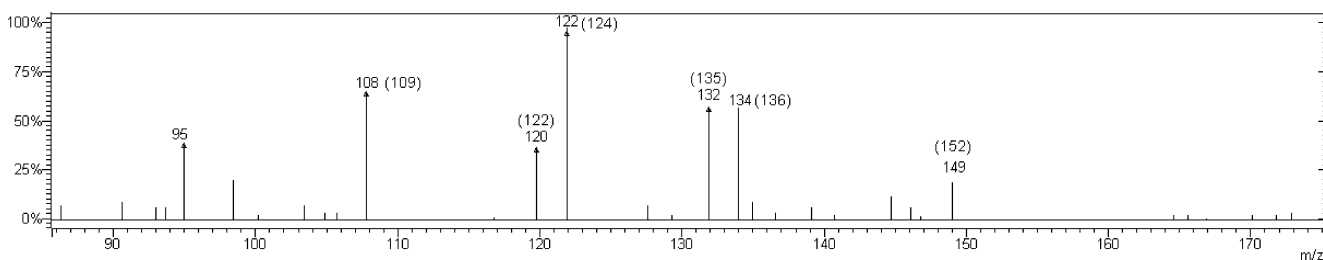


**Fig. 2** Ion-trap MS<sup>3</sup> mass spectra of caffeine, with the molecular ion used as the precursor ion at different sequential breakdowns: **a**  $m/z$  194(197) > 193(196) > product ions, with CID at 0.4 V for both MS<sup>2</sup> and MS<sup>3</sup>; **b**  $m/z$  194(197) > 165(168) > product ions, with CID at 2.2 V for MS<sup>2</sup> and 0.6 V for MS<sup>3</sup>

The strategy used for the elucidation of the fragment ions of caffeine in an ion-trap mass spectrometer under collision-induced dissociation conditions was based on the spectral information from multistep mass spectral data (MS<sup>n</sup>) obtained for both caffeine and its <sup>13</sup>C-labeled analog. Their molecular ions were used as the first precursors. Different CID amplitudes, depending on the desired product ions, were applied to initiate the second ionization step (MS<sup>2</sup>) that produced the first CID mass spectra. Product ions of various  $m/z$  values were produced and selectively isolated for further dissociation (MS<sup>3</sup>) at different CID voltages. The spectral data generated by the ion-trap analyzer were used to link compositions via genealogical relationships; see Scheme 1.

Third-generation ions were obtained using MS<sup>3</sup> experiments for the molecular ion of caffeine  $m/z$  194 (197) and selected product ions at  $m/z$  193 (196), 178 (181), 165 (168), and 149 (152) obtained under the specific MS<sup>2</sup> conditions. The ions in brackets are the corresponding species produced when analyzing the <sup>13</sup>C-labeled caffeine

analog. The isolation window in all these experiments was  $m/z=1$ . The CID amplitudes chosen to perform these experiments were selected based on the principle of maximizing the relative abundance of the investigated product ion produced under MS<sup>2</sup> conditions and its moderate subsequent fragmentation under MS<sup>3</sup> conditions. In order to identify the origin of the fragment ion with  $m/z$  149 (152) obtained under MS<sup>2</sup> conditions (Fig. 1b(d)), MS<sup>3</sup> spectra were generated for three different product ions at  $m/z$  193, 178 and 165. As it turned out, the species with  $m/z$  149 was only generated when MS<sup>3</sup> conditions were applied to the MS<sup>2</sup>-produced product ion  $m/z=193$ , not  $m/z$  178 or 165. The conditions used for these experiments were the following: CID=0.4 V applied to the molecular ion  $m/z$  194 (197) (MS<sup>2</sup> spectrum), with CID=0.4 V applied to the product ion  $m/z$  193 (196) (MS<sup>3</sup> spectrum), Fig. 2a, and CID=2.2 V applied to  $m/z$  194 (197) (MS<sup>2</sup> spectrum), with CID=0.6 V applied to the product ion  $m/z$  165 (168) (MS<sup>3</sup> spectrum), Fig. 2b. These experiments demonstrate the power of multistep mass spectral data, MS<sup>n</sup>, for identifying



**Fig. 3** MS<sup>3</sup> mass spectrum of caffeine obtained under the following dissociation conditions: CID=1.0 V applied to the molecular ion followed by isolation and CID fragmentation of the product ion with  $m/z$  149 at a CID amplitude of 0.6 V

**Table 2** IT-MS/MS data with proposed assignments for caffeine and its isotope-labeled standard

Analyte	Molec. ion, $m/z$	Product ions, $m/z$							
		-H	-O	-H, -O	-CO, -H	-H, -CO <sub>2</sub>	-C*H <sub>3</sub> NCO	-C*H <sub>3</sub> NCO, -H	-H, -CO <sub>2</sub> , -C*H <sub>3</sub>
Caffeine	194[M <sup>+</sup> ]	[M <sup>+</sup> ]-1 193	[M <sup>+</sup> ]-16 178	[M <sup>+</sup> ]-1, -16 177	[M <sup>+</sup> ]-28, -1 165	[M <sup>+</sup> ]-1, -44 149	[M <sup>+</sup> ]-57 137	[M <sup>+</sup> ]-57, -1 136	[M <sup>+</sup> ]-1, -44, -15 134
<sup>13</sup> C-caffeine	197[M <sup>+</sup> ]	196	181	180	168	152	139	138	136

the origins of ions produced under MS<sup>*n*-1</sup> conditions. In Fig. 2a and b, the  $m/z$  values of the corresponding product ions obtained under the same conditions for <sup>13</sup>C-labeled caffeine are indicated without showing their relative abundances on the graph. The use of isotope-labeled caffeine exposed to the same fragmentation conditions provided a valuable source of information that enabled the structure of a product ion of the native analyte to be identified. Without the spectral information from multistep mass spectral data obtained for trimethyl <sup>13</sup>C-labeled caffeine in parallel with its native compound, different assignments may have been proposed for some fragments.

The product ion at  $m/z$  149 (152) undergoes further fragmentation, forming ions at  $m/z$  108 (109), 120 (122), 122 (124), 132 (135) and 134 (136), see Scheme 1. Selective fragmentation of the ion at  $m/z$  149 (152) was studied under MS<sup>3</sup> conditions by applying a CID voltage of 1.0 V to the molecular ion of caffeine at  $m/z$  194 (197) and isolating and further fragmenting the product ion at  $m/z$  149 (152) at a CID amplitude of 0.6 V (MS<sup>3</sup> scheme 194(197) > 149(152) > product ions), see Fig. 3. The product ions generated under these conditions helped to elucidate the fragmentation pathway from  $m/z$  194 (197) to  $m/z$  149 (152) and further down; see Scheme 1 and Table 2.

The presence of the ions at  $m/z$  137 (139), 136 (138), 122 (124), 109 (111) and 108 (109) in the IT-MS/MS spectra of caffeine (Fig. 1b,d) can be explained by the retro Diels–Alder mechanism, which results in a loss of methyl isocyanate [CH<sub>3</sub>N=C=O] (-57u, product ions with  $m/z$  137), followed by the elimination of a molecule of carbon monoxide [C=O] (-28u, ion species with  $m/z$  109), or the expulsion of a hydrogen atom from one of the methyl groups ( $m/z$  136). The product ions with  $m/z$  137 can also lead to the formation of ions with  $m/z$  122, 108 via the cleavage of one of the N–CH<sub>3</sub> bonds (-15u,  $m/z$  122) or the loss of a NCH<sub>3</sub> group (-29u,  $m/z$  108), Scheme 1. This has been well studied using techniques such as tQ-MS/MS [28, 29], LC-MS/MS [30] and a direct mass spectrometry method [32].

The formation of  $m/z$  165 (168) ions (Fig. 1b,d) under the conditions of resonant excitation of the collisionally induced dissociation of the molecular ion of caffeine  $m/z$

194 (197) in an ion trap (MS<sup>2</sup>) can be rationalized as shown in Scheme 2. This fragmentation involves the loss of CO with the subsequent expulsion of a hydrogen atom from one of the methyl groups. The first step of this pathway involves  $\alpha$ -cleavage next to the carbonyl group, with the charge stabilized on the carbonyl O atom. Although CO could arguably be lost at this point, the generation of a potentially more stable ion in a ring seems more appealing. To accomplish this, the double bond nearest the carbonyl group donates one electron to form a new N–C single bond, and the five-membered ring closes. The charge remains on the carbonyl O. Finally, heterolytic cleavage of the C–C bond between the ring and the carbonyl group moves the electron pair onto the carbonyl C atom, expelling a neutral molecule of CO and forming the ion at  $m/z$  166 (169) with the subsequent loss of an H atom, producing the ion residue at  $m/z$  165 (168). MS<sup>3</sup> experiments conducted on the ions at  $m/z$  194 (at CID=2.2 V) followed by  $m/z$  165 (at CID=0.6 V) (Fig. 2b) demonstrated that the fragment at  $m/z$  165 subsequently expels methyl isocyanate (CH<sub>3</sub>NCO, -57u) or methyl isocyanide (CH<sub>3</sub>NC, -41u), generating the ions at  $m/z$  108 (110) and 124 (126), respectively; see Scheme 1. It is known that the removal of the residue CH<sub>3</sub>NC is involved in the latter based on the MS<sup>3</sup> analysis of the trimethyl <sup>13</sup>C-labeled caffeine. The shift of the ion at  $m/z$  124 to  $m/z$  126 in the MS<sup>3</sup> spectrum when analyzing the molecular ion of the labeled caffeine  $m/z$  197 along with the subsequent isolation and CID fragmentation of the ion at  $m/z$  168 (Fig. 2b) indicates the loss of one of the labeled methyl groups and the presence of the remaining two on the final product ion.

As demonstrated above, a combination of single and multistep MS<sup>*n*</sup> (where  $n=1-3$ ) fragmentations of caffeine and its <sup>13</sup>C-labeled analog provided sufficient information to construct the genealogical relationship (Scheme 1) between the precursors and their product ions. To elucidate some of the fragment ions for which several compositions were possible, MS<sup>2</sup> and MS<sup>3</sup> dissociation of an isotope-labeled analog was performed in order to confirm the structures assigned to the ions of the native analyte obtained using the ion-trap in MS<sup>2</sup> mode.



Product ions, $m/z$								
-CO, -H, -C*H <sub>3</sub> NC	-H, -CO <sub>2</sub> , -HC*N	-C*H <sub>3</sub> NCO, -C*H <sub>3</sub>	-H, -CO <sub>2</sub> , -NC*H <sub>3</sub>	-C*H <sub>3</sub> NCO, -CO	-CO, -H, -C*H <sub>3</sub> NCO	-C*H <sub>3</sub> NCO, -NC*H <sub>3</sub>	-H, -CO <sub>2</sub> , -CNC*H <sub>3</sub>	
[M <sup>+</sup> ] -28, -1, -41	[M <sup>+</sup> ] -1, -44, -27	[M <sup>+</sup> ] -57, -15	[M <sup>+</sup> ] -1, -44, -29	[M <sup>+</sup> ] -57, -28	[M <sup>+</sup> ] -28, -1, -57	[M <sup>+</sup> ] -57, -29	[M <sup>+</sup> ] -1, -44, -41	
124	122	122	120	109	108	108	108	
126	124	123	122	111	110	109	110	

## Monitoring of caffeine

### QA/QC in monitoring

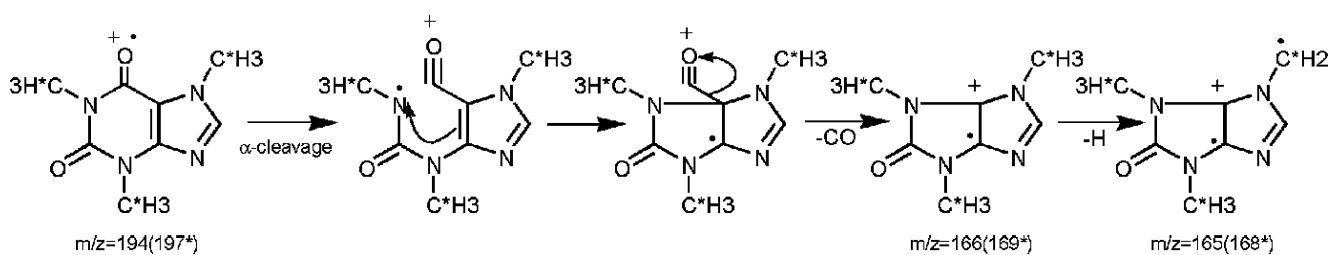
The developed method based on gas chromatography with IT-MS/MS detection was routinely applied by the authors to the analysis of caffeine in various sources of marine and freshwater. The identification of the analytes, caffeine and its <sup>13</sup>C-labeled standard was based on the chromatographic retention time and the MS/MS spectrum of each compound obtained individually using standard solutions containing just one analyte. The molecular ion of the analyte was selected as the precursor ion for the sequential application of the MS/MS conditions. Five of the most abundant product ions were selected to identify each compound. One of the criteria for analyte identification under MS/MS conditions was that the ratios of the five chosen product ions had to be within  $\pm 20\%$  of those established from the analysis of the corresponding authentic standards; see Table 3.

To ensure that the instrumental performance was maximized for the GC-IT-MS/MS system, a number of tests were performed on a routine basis. The tests were designed to assess the GC column performance, the retention time windows, the greatest sensitivity, multipoint calibration and linearity, instrument detection limits, sample carryover, and continuous calibration verification. The IT-MS/MS detector was also optimized for resolution, transmission and mass calibration using perfluorotributylamine (PFTBA).

The target compounds were identified as native caffeine or its <sup>13</sup>C-labeled surrogate internal standard only when the

chromatographic peaks that were obtained satisfied all of the following criteria. 1) The retention time of the specific analyte must be within a  $\pm 0.2$  min window of that obtained during the analysis of the authentic compounds in the calibration standards. All native and surrogate ion peak maxima must be coincident within three seconds. 2) The threshold for the MS/MS spectral match of a specific compound in a sample with its analog in a standard mixture was set to 700 out of a maximum of 1000 (a value of 1000 represents a perfect match). Only ions with intensities that were higher than 40% of the reference spectrum base peak were considered when calculating the spectral match. 3) All five product ions of each analyte (see Table 3) must be present, and must be detected at their exact  $m/z$ . 4) The signal-to-noise ratio for each compound must be  $>3$  for a sample extract, and  $>10$  for a calibration standard. 5) The ratios between the integrated signals of the product ions chosen to identify native caffeine and its isotope-labeled standard must be within  $\pm 20\%$  of the values outlined in Table 3.

Each batch of samples consisted of nine samples plus three QA/QC samples: a sample duplicate, a replicate sample spiked with a known amount of native caffeine, 20 ng l<sup>-1</sup> (200 pg  $\mu$ l<sup>-1</sup> in the final sample extract) and a procedural blank. The replicate sample spiked with a known amount of caffeine was used as a laboratory reference material (LRM). <sup>13</sup>C-labeled caffeine spiked into each sample at a concentration in the final extract of 200 pg  $\mu$ l<sup>-1</sup> was used to determine the recovery efficiency. It was added to each sample before filtration. Corrections for recoveries of the native caffeine were made against the <sup>13</sup>C-labeled surrogate internal standards. The recovery of SIS in



**Scheme 2** Representation of the fragmentation resulting in the formation of the product ions with  $m/z$  165(168)

**Table 3** Some characteristics of the product ions selected for identification

Analyte	Precursor ion, <i>m/z</i>	MS/MS product ions used for identification, <i>m/z</i>	Ratios of the product ions
Caffeine	194	108/122/149/165/193	74.8/68.4/100.0/42.6/4.6
<sup>13</sup> C-Caffeine	197	109/124/152/168/195	38.2/54.6/100.0/33.4/12.4

a sample was determined against a “standard check” containing <sup>13</sup>C-labeled caffeine at a concentration of 200 pg μl<sup>-1</sup>. The high recoveries of SIS in all of the samples analyzed (Table 4) indicate the high efficiencies of both the sample preparation procedure and the analytical determinations of the analytes of interest.

In order for the data to be acceptable, the following QA/QC criteria had to be met. The reproducibility of duplicate analysis had to be between 60 and 130%, and the % recovery of the surrogate internal standard had to be between 50 and 130%.

Concentrations of caffeine in the distilled deionized (DDI) water samples used as blanks were in the range of 0.6–1.4 ng l<sup>-1</sup>, which translated into a method detection limit (MDL) of 1.0–2.0 ng l<sup>-1</sup> (MDL=mean<sub>blank</sub>+3SD<sub>blank</sub>). Fortified samples of DDI water spiked with known amounts of caffeine covering the range 2.0–20.0 ng l<sup>-1</sup> showed acceptable recoveries of 80.2–103.4%.

#### Tracking anthropogenic input

Over a hundred samples from marine waters and freshwaters were analyzed by the authors using the optimized GC-IT-MS/MS methodology. The level of caffeine found in a blank sample was subtracted from the results obtained for surface water samples. All results were grouped into five categories, showing good correlation with the anthropogenic burden associated with domestic wastewater contamination. Group one included 76 marine water samples collected around Bamfield research station. The results for four freshwater samples taken up- and downstream of STP represented group two. Groups three, four and five contain the results obtained on freshwater samples collected from the lakes with various densities of residential developments (high, moderate and low, respectively); see Table 4. The

results obtained for the marine water samples indicated a wide variation in the level of caffeine, ranging from 4.5 to 149.0 ng l<sup>-1</sup>, depending on the location of the sampling site in the marine inlet studied. The concentrations of caffeine in the samples from the lakes with a high density of residential development around them were 6.1–21.7 ng l<sup>-1</sup>. The lakes with a moderate density of residential development showed lower levels of caffeine in their surface waters, between 1.8 and 10.4 ng l<sup>-1</sup>. The samples collected from the lakes with a low residential density or no residential development around them were characterized by low concentrations of caffeine. The levels of caffeine in this group of samples ranged from not detectable (ND) to 6.5 ng l<sup>-1</sup>. Caffeine was present in all surface water samples taken near sewage treatment plants (STP). In comparison to previously published data on the range of concentrations of caffeine in seawater [7, 34] and in streams in Canada [13], the United States [35] and Germany [14], the levels of this compound detected in our study were in good agreement with [7, 13, 34], while they were two orders of magnitude lower than those found by Ternes et al. [14] and Kolpin et al. [35]. The recoveries of surrogate internal standard spiked into the marine water samples were in the range of 76.0–104.1%. Freshwater samples spiked with <sup>13</sup>C-caffeine gave 61.0–91.6%, 73.4–106.7% and 92.3–116.4% recoveries for the groups with high, moderate and low densities of residential development around the lakes, respectively. The river samples collected up- and downstream of STPs gave recoveries for SIS of 66.0–67.0% and 74.4–97.75%, respectively.

#### Conclusion

Freshwater resources are being placed under increasing stress by the environmental loading of pathogens arising

**Table 4** The concentrations of caffeine found in sea and freshwater samples collected from sites with different human impact levels

Sample type	Number of samples analyzed	Concentration range of caffeine found, ng l <sup>-1</sup>	Recovery of SIS, %
Seawater	76	4.5–149.0	76.0–104.1
River 100 up/s STP	2	2.1–3.2	66.0–67.0
River 400 down/s STP	2	7.2–15.4	74.4–97.7
Lake of high human impact	13	6.1–21.7	61.0–91.6
Lake of moderate human impact	10	1.8–10.4	92.3–116.4
Lake of low human impact	10	ND–6.5	73.4–106.7

ND=not detected, where MDL=2 ng l<sup>-1</sup>.

from urban development, human population growth, agricultural practices and overuse of aquifers. Additionally, changes in climate have also had a significant impact on the viability, survivability and dynamics of pathogens in aquatic environments. The direct monitoring and detection of pathogens in the aquatic environment is a great challenge because of their phylogenetic diversity and because they are only present in low numbers. A freshwater management approach has been developed by the regulatory authorities to protect the source water quality that involves monitoring for fecal indicator bacteria. A key requirement to implement best management practices to reduce the loading of pathogens responsible for waterborne enteric illness is to identify fecal pollution sources and subsequently eliminate and/or abate them. The microbiological population-dependent and species-dependent phenotypic and species-dependent genotypic methods that have been developed to track the origins of sources of fecal contamination have some practical limitations (spatiotemporal variability, instability of the phenotypic and genotypic markers under different environmental stresses, and the size of the reference library), and these need to be resolved before they can be reliably applied in the field.

Caffeine has recently been used as an important chemical tracer for monitoring the input of human fecal waste into source water. When used as a marker, the level of caffeine was found to correlate significantly with fecal indicator bacteria and other aquatic nutrients. It also possesses important properties such as stability, source-specificity and long-term persistence in aquatic environments. One of the challenges when monitoring caffeine in aquatic environments is that it is only present in trace quantities. The analytical method presented in this study demonstrated that trace levels of caffeine can be detected and monitored in different aquatic systems (surface water and seawater) using gas chromatography (GC) coupled with IT-MS/MS detection. After optimizing the GC-IT-MS/MS parameters, a limit of detection of  $500 \text{ fg } \mu\text{L}^{-1}$  with  $S/N=3$  was achieved. An isotope dilution method using  $^{13}\text{C}$ -labeled caffeine as a surrogate internal standard was employed to determine and correct the recovery of native caffeine in water samples. The collision-induced fragmentation pathways of caffeine in an ion-trap tandem mass spectrometer were investigated. New product ions not previously reported in the literature have been identified in the CID fragmentation of caffeine. Various mass spectrometric conditions have been employed to elucidate fragmentation pathways for the product ions observed in the CID spectra. Ion structures and fragmentation pathway mechanisms have been presented and compared with the literature.

Surface marine and freshwater samples collected in various watersheds were analyzed for caffeine as an indicator of domestic wastewater contamination. Caffeine

concentrations in lakes and rivers as well as in seawater correlated with the anthropogenic burden introduced by domestic wastewater or human activities in the surrounding area. The results presented by the authors are in good agreement with literature data.

Further studies are required to evaluate the efficacy of caffeine as a robust surrogate for human fecal waste. The issues, such as the biological and chemical persistency of caffeine in various environments [36] and the correlation of caffeine concentration with the prevalence of human-specific enteric pathogens as well as with nutrients and other characteristic parameters of aquatic ecosystems should be addressed.

**Acknowledgements** This research was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada Industry Research Chair Grant awarded to Dr. A. Mazumder, the Capital Region District (CRD) Environmental Services Department, the CRD Water Services Department and the Ministry of Environment. Our particular thanks are extended to Kevin Rieberger and Deb Epps for organizing sample collection, and to Austin Tokarek for sample processing.

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