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Original Research Article

Determination of theobromine and caffeine in some Malaysian beverages by liquid chromatography-time-of-flight mass spectrometry

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Abstract

Purpose: *To determine the concentration of theobromine (TB) and caffeine (CF) in tea and other beverages using liquid chromatography-time of flight-mass spectrometry (LC-TOF-MS).*
Methods: *The extract of caffeine and theobromine from tea and other beverages was filtered by 0.45 µm nylon micro-syringe and then injected into a LC-ToF-MS system. Theobromine and caffeine were separated using Thermo Scientific C18-column (length 250 mm, width 2.1 mm and diameter 5 µm). Acetonitrile-methanol (ACN – MeOH, 3:1 v/v) was used as mobile phase B, while mobile phase A was 0.1 % FA in DIW. The volume injected was 30 µL at a rate of 0.3 mL/min.*
Results: *Good linearity was obtained in the range of 0.3 – 400 and 0.2 – 200 mg/L for theobromine and caffeine, respectively (regression coefficient (R^2) > 0.970). The limits of detection were 0.15 and 0.05 µg/mL for theobromine and caffeine, respectively. The highest concentrations of caffeine and theobromine determined in tea samples were 159.1 and 255.8 mg/L, respectively.*
Conclusion: *Theobromine and caffeine have been successfully analysed in tea, coffee and soft drinks. LC-TOF-MS is an accurate and promising instrument for the determination of the studied compounds in beverages.*

Keywords: *Theobromine, Caffeine, Tea, Coffee, LC-TOF/MS*

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INTRODUCTION

Methylxanthines, theobromine and caffeine are used as analgesics, diet aids, and cold/flu remedies in numerous popular carbonated drinks. They are also the main compounds found in tea, coffee, sodas, chocolate, and various energy drinks. More than 120,000 tons of caffeine is consumed worldwide annually [1,2]. Malaysia–Regular Beverage Consumption revealed that coffee and tea are the most consumed drinks by Malaysian people [3].

The present study is focused on the analysis of

TB and CF in coffee and tea; given that both drinks are preferred in restaurants, coffee shops, households, and markets. To the best of our knowledge, this study is the first to evaluate the contents of compounds in coffee and tea in Malaysia. The analysis of theobromine and caffeine in foods, biological fluids, environmental samples, plants, and water, was provided with different instrumental methods, such as high-performance liquid chromatography (HPLC) [4], gas chromatography–mass spectrometry-flame ionization detection (GC–MS-FID) [5], Fourier transform-infrared spectrophotometry (FT-IR) [6], near-infrared spectroscopy [7], UV–Vis spectrophotometry [8], FT-Raman spectrometry [9], and capillary electrophoresis (CE) [10]. Of these techniques, HPLC which offers advantages, such as simplicity and selectivity coupled with a UV–Vis detector, was deemed the preferred analysis system for the analysis of theobromine and caffeine [8,11,12]. However, the co-elution of compounds is a common phenomenon when a UV–vis detector is used with HPLC [13]. This limitation, and other challenges, can be resolved by coupling with a highly specific, sensitive, and accurate ToF-MS analyser that is considered a suitable and confirmative technology to measure trace levels of TB and CF.

ToF-MS is a highly accurate and specific detector. With this detector, data is given as a four-decimal-point *m/z* that responds, almost exclusively, to electrospray ionization as an ionization source [14]. The separation and extraction of TB and CF from tea, coffee, soft drinks, and other beverages has been reported in several previous studies with different extraction methods [15,16].

The aim of this study is to develop a simple and accurate LC-ToF-MS method, using an extremely limited reagent for the analysis of theobromine and caffeine in beverages. Direct injection, without any further sample preparation, was provided using LC-ToF-MS which is an accurate technique to extract caffeine and theobromine from the total ion chromatogram using a 20 mD mass window.

EXPERIMENTAL

Chemicals

Pure standards (≥ 99 %) of CF and TB, CAS no. 58-08-2 and CAS no. 83-67-0, respectively, were purchased from Sigma-Aldrich (USA). HPLC-grade organic solvents (acetonitrile (ACN) and methanol (MeOH)) were purchased from Merck (Germany). Formic acid as additive was obtained from Merck (Germany). Deionized water was collected from the Faculty of Sciences and Technology, UKM, Malaysia.

Standard solution preparation

Stock solutions 1 mg/mL for theobromine and caffeine were prepared by dissolving in MeOH, 0.01 g of compound was dissolved in 10 mL of MeOH, and stored at 20 °C for further preparation. Further solutions were prepared by diluting the stock solution (1 mg/L) with deionized water. All solutions were injected in triplicate and analysed using LC-ToF-MS. Calibration curves were built by plotting peak area against concentration.

Sample collection and preparation

All the samples were collected from different local markets in Malaysia and are as presented in Table 1.

Tea samples were extracted in hot water. A 1.0 g sample of tea was placed in 50 mL of distilled water at 95 - 100 ^{o}C for 5 min as per normal preparation for a daily consumer. Tea extracts were filtered through a 0.45 μm Nylon micro-syringe filter. A 5 mL sample of coffee was filtered and then injected directly into the LC-MS. Dissolved gases were excluded from beverages using an ultrasonic water bath for 10 min, prior to sample analysis. Thereafter, the samples were filtered using a 0.45 μm Nylon micro-syringe filter to obtain a transparent sample ready for analysis.

LC-ToF-MS instrumentation and conditions

Separation of theobromine and caffeine was performed using a liquid chromatography instrument (Dionex Ultimate 3000/LC 09115047 (USA)) equipped with a vacuum degasser, a quaternary pump, and an auto-sampler. Sample aliquots of 30 μL were injected into 5 μm, 2.1 mm × 250 mm Thermo Scientific C18 column. Electrospray ionisation (ESI) was utilized as an ionization source.

TB and CF were analysed in positive ion (PI) mode. The elution of compounds was achieved with ACN – MeOH (3:1, v/v) as mobile phase B and 0.1 % FA in DIW as mobile phase A at 0.3 mL/ min.

The gradient elution was as follows: 5 % B (0 min) → 60 % B (linear increased in 3 min) → 97 % B (linear increased in 3 min) → 97 % B (hold 5 min) → 5 % B (linear decreased in 0.1 min) → 5 % B (hold 5 min).

Table 1: Tea and beverages samples description

Name brand	Name manufacturer	Batch no.	Expire date
Al-Kbous tea	Al-Kbous Industrial Trading and Investment Co. W.L.L. Amman, Jordan.	6-251361-100104	12/10/2019
Lipton tea	PT Unilever Indonesia Tbk Kawasan Industri, Bekasi, Indonesia.	8-888086-021001	13/12/2019
BOH tea	BOH Plantations Sdn. Bhd. Kuala Lumpur, Malaysia.	9-556015-010080	30/11/2019
Sari-Wangi tea	Sari Wangi Teh Asli, Indonesia.	8-999999-195649	24/06/2018
Al-Wazah tea	Finlays Colombo Ltd. Welisara, Sri Lanka.	4-791002-019454	30/10/2019
BOH green tea	BOH Plantations Sdn. Bhd. Kuala Lumpur, Malaysia.	9-556015-010226	08/01/ 2020
The Cap Masjid	TEH Wangi Ros. Sdn. Bhd. Kuala Lumpur, Malaysia.	9-555195-100000	31/12/2010
Ahmad tea	Ahmad Tea Ltd. Hampshire, England.	0-54881-00616-3	16/01/2010
Mahmood tea	Mahmood Tea Int. (PVT) Ltd. Colombo-02, Sri Lanka.	4-796000-550725	30/11/2019
White coffee	ETIKA Sdn. Bhd. Selangor, Malaysia.	9-556404-116423	30/11/2019
Nescafe	Nestle products Sdn. Bhd. Selangor, Malaysia.	9-556001-054005	30/11/2018
Milo	Nestle products Sdn. Bhd. Selangor, Malaysia.	9-556001-081322	19/06/2018
Red Bull	T.C. Pharmaceuticals Industries Co., Ltd. Prachin Buri, Thailand.	8-888307-882572	07/05/2019
Lipton iced tea	ETIKA Sdn. Bhd. Selangor, Malaysia.	9-556404-049035	15/11/2018
Pepsi	ETIKA Sdn. Bhd. Selangor, Malaysia.	9-556404-001033	18-10-2018

Theobromine was separated at 5.43 min, whereas caffeine was separated at 6.51 min. The LC-ToF-MS (Bruker, Germany) was operated in single reaction mode, using the positive electrospray ionization (ESI+) mode. The source conditions were as follows: drying gas temperature, 190 ^{o}C, drying gas flow rate, 8.0 L/min, set endplate offset, − 500 V, set collision cell RF, 250 Vpp, MS capillary voltages, 4000 (PI), set capillary, 4000 V, and nebulizer pressure, 4.0 bar. A mixture of sodium hydroxide and formic acid was used as the lock mass at *m/z* 90.9766 – 974.8132.

Quantification and confirmation method

TB and CF were identified and quantified using LC-ToF-MS instrument based on exact retention times and accurate mass value (*m/z*) of the protonated molecular ions $[M + H]^+$. Theobromine and caffeine were extracted at 0.02 Da. Theobromine and caffeine were identified based on two factors; the accurate mass value (*m/z*) and the retention time of the molecular ion $[M + H]^+$.

Evaluation of selectivity

Selectivity was evaluated by comparing the chromatograms of three different samples; real sample, spiked real sample, and deionized water. The results revealed that no interfering peaks were present at the TB and CF retention times.

Determination of linearity and sensitivity

The linearity ranged between 0.3 and 400 mg/L for theobromine and 0.2 and 200 mg/L for caffeine.

Calibration curves were generated for each compound by plotting the peak area against the concentration of each compound using the linear regression model. For the analytical chromatography instruments, LOQ was the lowest concentration corresponding to S/N ratio ≥ 10.

Determination of precision and accuracy

Precision and accuracy of the LC-ToF-MS method were evaluated at three different concentrations (1, 50, and 200 μg/mL) and (5, 25 and 100 μg/mL) for theobromine and caffeine, respectively. Inter-day precision was evaluated by performing three replicates of three different concentrations. Intra-day precision was assessed within three consecutive days. The precision was presented in terms of relative standard deviation percentage (RSD %). The accuracy was expressed as the ratio of mean observed

concentration to nominal concentration, as a percentage.

Statistical analysis

Each assay was repeated at least three times. Means were analysed statistically by analysis of variance (ANOVA) followed by Duncan's test, using SPSS software, Version 19. The results were considered significantly different at $p < 0.05$.

RESULTS

The linearity of the LC-ToF-MS method of theobromine and caffeine was assessed by analysing seven concentrations (0.3, 1.0, 10.0, 50.0, 100.0, 200.0, and 400.0 mg/L) and (0.2, 1.0, 5.0, 25.0, 50.0, 100.0, and 200.0 mg/L) for theobromine and caffeine, respectively. Calibration curves were obtained by the plotting peak area of theobromine and caffeine separately against a working standard concentration. Calibration curves were fitted to least-square linear regression. The results showed good linearity for caffeine and theobromine; in which (R^2) was 0.979 and 0.999, respectively.

The precision of the LC-ToF-MS method was assessed by analysis of three samples replicates within the same day (inter-day) and for three different days (intra-day) (see Table 2). The precision was determined by analysing three different concentrations for theobromine (1, 50 and 200 μg/mL) and caffeine (5, 25 and 100 μg/mL), respectively, on three different days. The results were in accordance with standard method validation [17]. RSD % values ranged from 3.11 to 13.22 % for intra-day precision and 2.52 to 11.43 % for inter-day precision. Accuracy was determined by adding known amounts of TB and CF as a mixture to the solvent (1, 50, and 200 μg/mL) and (5, 25, and 100 μg/mL), respectively. The accuracy results were good, ranging from 99 to 108 % for intra-day and 94 to 110 % for inter-day (as shown in Table 2).

Figure 1 shows that no peaks interfered at the retention times of TB (5.43 min) and CF (6.51 min). Chromatogram **A** refers to the standard solution of a mixture of TB and CF. The tea sample was spiked with the mixture of these two compounds and then injected into LC-ToF/MS as shown in chromatogram **B**.

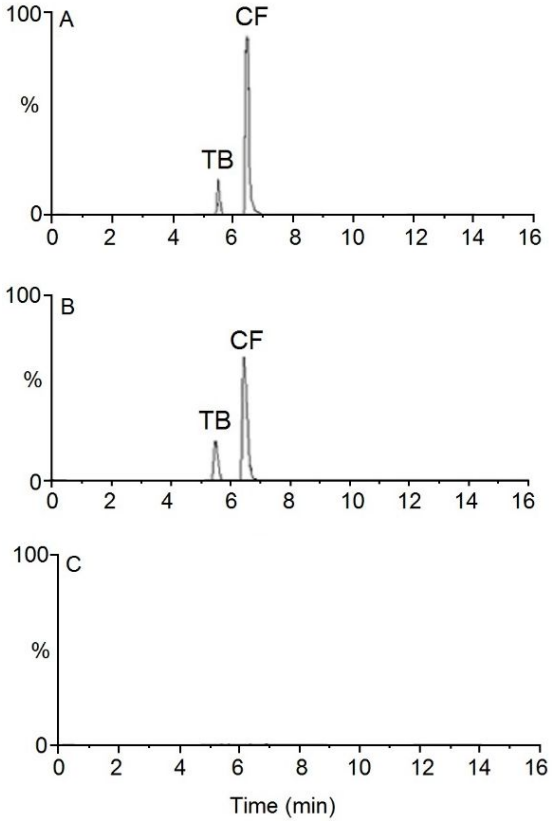

Figure 1: Representative chromatograms of caffeine and theobromine as a mixture: (A) standard mixture of caffeine and theobromine (50 μg/mL), (B) tea sample spiked with caffeine and theobromine (50 μg/mL), and (C) blank solvent

Table 2: Intra-day and inter-day precision and accuracy data for the analysis of caffeine and theobromine in real samples

Compound	Nominal concentration (mg/L)	Intra-day (n=4)		Inter-day (n=6)	
		Precision (RSD %)	Accuracy (%)	Precision (RSD %)	Accuracy (%)
Caffeine	5.0	11.37	106	10.11	94
	25.0	8.31	99	6.49	104
	100.0	3.11	102	2.55	98
Theobromine	1.0	13.22	99	11.43	98
	50.0	5.11	102	4.95	94
	200.0	4.72	108	2.52	110

No peaks appeared TB and CF in deionized water (blank solvent); this method was therefore selective for TB and CF; as shown in chromatogram **C**.

Environmental samples are commonly too polluted with organic and inorganic contaminants and are therefore called complex matrices. To prevent the alteration or interference of other compounds to the target compounds, a narrow window was used in the ToF-MS analyser to reconstruct the chromatographic traces; consequently the selectivity of the instrument was increased. Extracted ion chromatograms (EICs) from the total ion chromatogram (TIC) were provided at 0.02 Da for theobromine and caffeine (as shown in Figure 2).

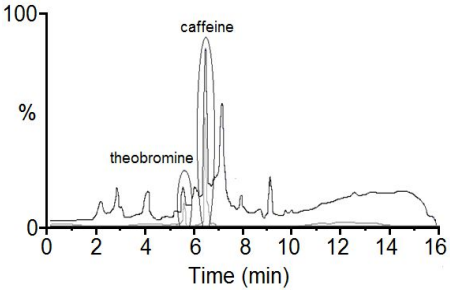

Figure 2: Extracted ion chromatogram (gray color) for TB and CF from the total ion chromatogram (black color) using LC-ToF/MS at 20 mD

Caffeine and theobromine were analysed in PI mode; therefore the resulting products are protonated molecular ions $[M + H]^+$. The product ion spectra and fragmentation patterns for CF and TB are shown in Figure 3.

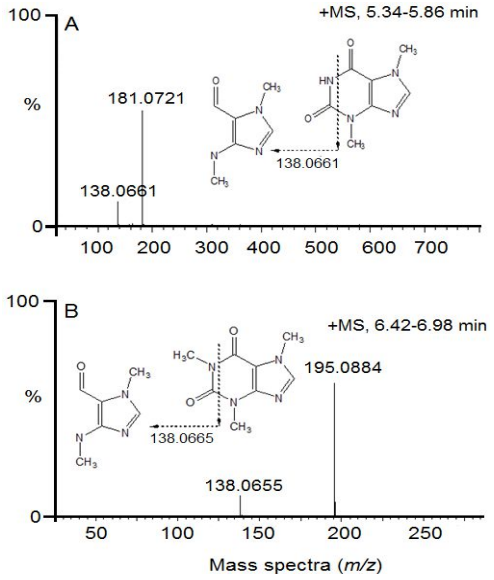

Figure 3: Mass spectra of theobromine A and caffeine B

All 15 samples were analysed using LC-ToF-MS. Retention times were 5.43 and 6.51 min for TB and CF, respectively (as shown in Figure 2). The concentrations of theobromine and caffeine in samples are presented in Table 3.

Table 3: Caffeine and theobromine concentration (mg/L) in real samples

No.	Sample	Caffeine	Theobromine
		Mean ±SD	Mean ±SD
1	Tea Al-Kbous	143.4±2.7	186±6.61
2	Lipton tea	133.9±1.8	110.6±5.2
3	BOH Tea	138.3±4.5	126.7±5.0
4	Sari wangi tea	140.6±0.6	110.2±3.7
5	Al-Wazah tea	159.1±1.5	255.8±12.4
6	BOH green Tea	107.7±2.7	102.3±5.9
7	Teh cap masjid	133.4±2.7	119.8±1.8
8	Ahmad tea	125.3±2.1	140.3±3.9
9	Mahmoud tea	117.5±3.2	104.2±10.7
10	White coffee beverage	82.7±2.9	<LOQ
11	Nescafe original beverage	83.1±2.6	16.2±0.7
12	Milo beverage	30.4±2.5	222.2±5.3
13	Red bull beverage	136.1±1.7	Nd*
14	Lipton ice tea beverage	67.2±5.1	20.4±3.4
15	Pepsi beverage	102.7±1.7	Nd*

* not detected

DISCUSSION

The investigation of the LC-ToF-MS method's selectivity was discussed after the comparison of the peak chromatograms; which resulted in standard solutions, tea sample spiked with standards and deionized water without standards. The regression coefficients were 0.999 and 0.979 for TB and CF, respectively. It was observed that limit of quantification (LOQ) values of 0.15 and 0.05 μg/mL for theobromine and caffeine, respectively, were more acceptable than in previous studies [18,19].

The precision and accuracy of the LC-ToF-MS method was assessed for theobromine and caffeine. Three replicates were performed for three different concentrations and then all samples were injected into the LC-ToF-MS instrument. The results showed that RSD % was

≤ 13.22; which is acceptable in this range of concentration.

It is well-known that analysis of complex matrices is a challenge; because the signals of target compounds could be impacted by organic and inorganic components that are both present in the real sample, together with analytes. The selectivity of the LC-ToF-MS method was enhanced by selecting a narrow window (0.02 Da) for analysis of theobromine and caffeine in actual samples. Improvement of S/N ratio, from twofold to fourfold, was observed after reducing the daltonic value from 0.5 to 0.02 Da [20]. The mass value (*m/z*) for the protonated molecular ions $[M + H]^+$ was used for quantitation analysis. The errors obtained for mass values were between 0.6 and 1.2 ppm.

All data related to accurate mass were calculated using Bruker Daltonics Data Analysis software; which provided the experimental and theoretical mass value (*m/z*) and elemental composition for molecular ions $[M + H]^+$. From the results, the mass errors were less than 0.5 ppm for both CF and TB; which are more acceptable than in previous study [21].

The most intense peaks observed, at *m/z* 195.0884 for CF and 181.0721 for TB, represented protonated molecular ion $[M + H]^+$. A small daughter peak appeared at *m/z* 138.066 after the fragmentation of CF and TB. This small, intense peak refers to the loss of C_2H_3NO from CF and CNHO from TB to form $C_6H_7N_3O$; as reported previously by Choi *et al*. They investigated the formation of metabolite of *m/z* 138.12 from theobromine and caffeine after losing C_2H_3NO and CNHO moieties, respectively [22].

The highest concentration of caffeine was present in Al-Kbous tea, with the concentration reaching 143 mg/L. Meanwhile, in Pepsi, the caffeine concentration was 103 mg/L; which is almost identical to that of original Cane from the market. For beverages, caffeine was highly detected in Red Bull at 136.1 mg/L. The mean caffeine content of carbonated soft drinks was lower than that of energy drinks. This finding is in line with the same result reported by Fatima *et al* [1]. However, the mean concentration of CF in 9 tea samples was 133 mg/L. The highest concentrations of TB were detected in Al-Wazah tea and Milo at 256 and 222 mg/L, respectively. All of these beverages are a major source of contamination of the water samples by theobromine and caffeine; as shown in previous studies [23,24].

CONCLUSION

The findings of the present study indicate that LC-ToF-MS method is accurate and sensitive enough to detect theobromine and caffeine in tea and other beverages. This study is the first to report on the analysis of theobromine and caffeine in Malaysian tea and beverages. Thus, ToF-MS is a powerful analyser that can be used to confirm the presence of theobromine and caffeine in tea and other beverages.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work

Contribution of the authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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