Analytical Methods

A rapid and simple determination of caffeine in teas, coffees and eight beverages

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ABSTRACT

Caffeine was extracted and preconcentrated by the simple, fast and green method of dispersive liquid–liquid microextraction (DLLME) and analysed by gas chromatography–nitrogen phosphorus detection (GC–NPD). The influence of main parameters affecting the extraction efficiency investigated and optimised. Under the optimal conditions, the method was successfully applied to determination of caffeine in different real samples including five types of tea (green, black, white, oolong teas and tea bag), two kinds of coffee (Nescafe coffee and coffee), and eight beverages (regular Coca Cola, Coca Cola zero, regular Pepsi, Pepsi max, Sprite, 7up, Red Bull and Hype). The limit of detection (LOD) and limit of quantification (LOQ) were 0.02 and 0.05 $\mu g\,mL^{-1}$, respectively. Linear dynamic range (LDR) was 0.05–500 $\mu g\,mL^{-1}$ and determination coefficient (R^2) was 0.9990. The relative standard deviation (RSD) was 3.2% ($n = 5$, $C = 1\,\mu g\,mL^{-1}$).

1. Introduction

Historically, the first reported consumers of caffeine were from the Galla tribe in Ethiopia at about 1000 years ago. Caffeine was isolated from coffee beans as a pure compound for the first time in 1819 by Friedlieb Ferdinand Runge (Walduogel, 2003). Caffeine with the chemical name of 1,3,7-trimethylxanthine has found an extensive applications in pharmacological preparations such as analgesics, diet aids and cold/flu remedies (Wang, Wan, Hu, & Pan, 2008). It is clinically used for the treatment of neurasthenia and the recovery of coma (Guo, Zhu, Yang, Wang, & Ye, 2011). Moreover, it can be used as an additive in many popular carbonated drinks. About 120,000 tons of caffeine is consumed world wide annually (Wang et al., 2008; Zou & Li, 2006). Pure caffeine is an odorless alkaloid with a bitter taste, which is naturally found widely in the leaves, seeds and fruits of a large number of plants such as cocoa beans, tea, coffee, cola and guarana (Zou and Lee, 2006; Armenta, Garrigues, & de la Guardia, 2005). Caffeine may be consumed daily in the form of coffee, tea, cocoa, chocolate, and energy drinks as well as many painkillers and anti-migraine drugs (Al-Othman, Agel, Alharbi, Badjah-Hadj-Ahmed, & Al-Warthan, 2012). The concentration of caffeine in vivo is a key mark for some disorders such as heart disease, carcinogenesis, kidney malfunction and asthma. The fatal dose of caffeine has been considered to be more than 10 g (about 170 mg/kg body weight). It has been also reported coma and death in cases of caffeine overdose (>200 mg/day) (Tzanavaras & Themelis, 2007).

Caffeine-containing beverages such as tea and coffee are the most widely consumed in the world. The amount of caffeine in soft drinks varies among brands and it is closely regulated by the US Food and Drugs Administration (FDA) to no more than 6 mg/oz fluid or 200 mg/L. Therefore, analysis of caffeine is required to ensure proper caffeine levels in beverages and to meet regulatory standards (Zou and Li, 2006). It is important to monitor caffeine in beverages and food by establishing a more precise, simple, fast and low cost analytical method in order to study its physiological effects on the human body's metabolism, including stimulating the central nervous system and increasing blood pressure in the short term and control food quality (Rostagno et al., 2011; Shrivas & Wu, 2007).

A variety of analytical methods have been proposed for separation and/or determination of caffeine in various matrices (environmental, biological, plants, food, etc.). Publications consisted of high performance liquid chromatography (HPLC) equipped with different types of columns and detection systems (Pura Naik, 2001; Wang, Gong, Chen, Han, & Li, 2012), gas chromatography–mass spectrometry/flame ionization detection (GC–MS/FID) (Jenkins, Liosa, Montoya, & Cone, 1996; Rahim et al., 2011), ion chromatography (IC) (Chen, Mou, Hou, & Ni, 1998), UV–Vis spectrophotometry (Belay, Ture, Redi, & Asfaw, 2008), Fourier transform-infrared spectrophotometry (FT-IR) (Paradkar & Irudayaraj, 2002), near

infrared (NIR) spectroscopy (Chen, Zhao, Zhang, & Wang, 2006; Zhang et al., 2013), FT-Raman spectrometry (Armenta et al., 2005), electrospray ionization–ion mobility spectrometry (ESI–IMS) (Jafari, Rezaei, & Javaheri, 2011), capillary electrophoresis (CE) (Meinhart et al., 2010), nuclear magnetic resonance spectrometry (NMR) (Talebpour, Maesum, Jalali-Heravi, & Shamsipur, 2003), potentiometry (Abdennabi & Sultan, 1993) and voltammetry (Guo et al., 2011). Among these techniques, gas chromatography with the advantages such as simplicity and saving considerable amounts of reagents and time (Bondati, Castelli, Latini, & Garattini, 1979), coupled with the highly specific and sensitive nitrogen–phosphorus detector (GC–NPD) is the preferred analysis system for determination of trace levels of caffeine. The NPD is a specific detector that responds almost exclusively to nitrogen and phosphorous compounds with sensitivity 10^4 times greater than that for carbon.

Although, some of the above mentioned methods such as UV–Vis, FI-IR, and NIR spectroscopy do not require stringent sample preparation process, but to resolve the analyte signal from potential interferences the application of sophisticated chemometrics techniques is necessary. This makes the method complex and difficult to implement in practical applications. However, most of the methods, due to insufficient sensitivity and matrix interferences, direct analysis of the caffeine in food and beverage samples is limited. Therefore, in order to isolate caffeine, a separation and/or a pre-concentration step prior to the analysis are necessary. Solvent extraction (Perva-Uzunalic et al., 2006), supercritical fluid extraction (SFE) (İçen & Gürü, 2010), ultrasonic assisted extraction (Zou and Li, 2006; Rostagno et al., 2011; Sereshti, Samadi, & Jalali-Heravi, 2013), soxhlet (Pura Naik, 2001), solid-phase extraction (SPE) (Jafari et al., 2011; Jenkins et al., 1996), column-chromatographic extraction (CCE) (Wang et al., 2012) and drop-to-drop solvent microextraction (DDSME) have been developed and applied for this purpose. In addition to the above mentioned pretreatment methods, an efficient extraction/preconcentration technique was introduced by Assadi et al. in 2006 named dispersive liquid–liquid microextraction (DLLME). This method provides high recovery and enrichment factor within a very short time (a few second) (Rezaee et al., 2006).

The aim of the present study was to develop a simple, fast, low cost, sensitive and efficient method for determination of caffeine in beverages. Therefore, the DLLME method was applied for extraction and preconcentration of caffeine in some popular beverages. The important parameters of DLLME were studied using one-at-a-time method. The preconcentrated caffeine was analysed by using GC equipped with a sensitive nitrogen–phosphorous detection system (NPD). To the best of our knowledge, this is the first time that DLLME coupled with GC–NPD has been introduced for determination of caffeine.

2. Experimental

2.1. Chemicals and reagents

Methanol, ethanol, acetone, sodium chloride, chloroform, carbon tetrachloride, chlorobenzene, with the purity higher than 99%, were purchased from Merck Chemicals (Darmstadt, Germany). Pure helium (99.999%) was purchased from Hiwa gas company (Tehran, Iran). Caffeine (⩾99.0%) was purchased from Fluka (St. Gallen, Switzerland). A stock standard solution of caffeine with a concentration of 2 mg mL^{-1} was prepared in double distilled water, stored under refrigeration and protected from light. The standard stock solution was stable for at least 3 weeks. Working standard solutions of 0.05–500 µg mL^{-1} were freshly prepared using double distilled water by appropriate dilutions of the stock

standard solution and used for evaluation and optimisation of methods. A calibration curve was constructed each day before analysis of the samples.

2.2. Apparatus

Centrifugation was performed by a Hermle Z 200A (Wehingen, Germany). An Eurosonic 4D (Euronda, Italy) ultrasonic water bath with a temperature control and a digital timer was used to degas the beverages. The injections into GC–NPD were carried out using a 1 µL Hamilton microsyringe model 7001 (Bonaduz, Switzerland). A 50 µL Hamilton syringe was used to inject organic solvents.

2.3. Gas chromatography

GC analyses were carried out by using an Agilent technologies gas chromatograph (Santa Clara CA, USA) equipped with a NPD detection system and a HP-5 capillary fused silica column (length, 30 m; internal diameter, 0.32 mm; film thicknesses, 0.25 µm; stationary phase, (5% phenyl)-methyl polysiloxane). The performed GC temperature program was as follows: the initial temperature was set at 100 °C and held for 1 min, then was programmed at 30 °C min^{-1} to 250 °C and held for 5 min. The injection port temperature was set at 220 °C. The detector (NPD) temperature was held at 250 °C with a hydrogen and air flow rates of 3 and 60 mL min^{-1}, respectively. The injection port was operated at a split ratio of 1:15. Other operating conditions were: carrier gas, helium (99.999%); flow rate, 1 mL min^{-1}; and inlet pressure, 6.6 psi. An enhanced ChemStation G1701 DA version D.00.01.27 was used for the data collection and processing.

2.4. Sample collection and preparation

Five types of tea (green, black, white, oolong and earl grey black tea bag), two kinds of coffee (Nescafe coffee (Nestle, Araras, Brazil) and coffee (Rio, Brazil)), and eight different beverages (regular and zero Coca Cola, regular and max Pepsi, Sprite, 7up, Red Bull and Hype) were purchased from a local market in Tehran (capital of Iran).

2.4.1. Preparation of tea samples

0.1 g of tea (dried, ground and sieved) was placed in a 100 mL beaker, and 20 mL of boiling distilled water was added to it. The solution was kept on a boiling water bath for 10 min. After that, the solution was centrifuged at 4000 rpm for 5 min to separate the solid particles from the solution. In the case of tea bag, it was placed in 250 mL of boiling distilled water for 2 min. Then, the solution was filtered on a filter paper (Whatman grade No. 44) to remove the solid particles.

2.4.2. Preparation of coffee samples

Nescafe coffee was ground to a fine powder, and then 100 mL of boiling distilled water was added to 1 g of it. In the case of coffee, 1 g of powdered coffee was added to 100 mL of distilled water and was stirred and heated to boiling. Then, the solutions were filtered on a filter paper (Whatman grade No. 44) to obtain a transparent solution.

2.4.3. Preparation of beverages

Prior to application of the procedure, beverage samples were degassed in an ultrasonic water bath for 10 min. Then, the solutions were filtered on a filter paper (Whatman grade No. 44) to obtain a transparent solution.

2.5. Procedure

1 mL of the sample solution was placed in a conical glass test tube, and 0.1 g of sodium chloride added to it. Then, 200 µL of ethanol (dispersion solvent) containing 20 µL of chloroform (extraction solvent) was injected into it by using a syringe. Accordingly, a cloudy solution (consisted of tiny droplets of chloroform dispersed in the aqueous phase) was formed. In this step, extraction of caffeine from aqueous phase into chloroform takes place. After centrifugation of the cloudy solution at 4000 rpm for 2 min, the extractant (chloroform) containing preconcentrated caffeine was sedimented at the bottom of the test tube. Finally, 0.7 µL of the sedimented organic phase was injected into GC–NPD. The performance and the robustness of the procedure were examined with lower volumes of sample solution. In this case, the procedure was examined with 0.5 mL of sample solution and 100 µL of ethanol containing 10 µL of chloroform with the salt concentration of 10%. The results confirm the possibility of performing the procedure for limited volume samples. Although the procedure may be performed directly on the undiluted samples, but the sample pre-dilution may reduce the sample consumption and also simplifies the DLLME method by reducing the matrix effect.

3. Results and discussion

In order to obtain the best performance and conditions of the extraction procedure, the effective parameters of the proposed method were investigated and optimised using the one-at-a-time method. For this purpose, an aqueous standard solution of caffeine (1 µg mL^{-1}) was prepared from stock standard solution and used as a model sample solution. The extraction efficiency of the method was evaluated as the average chromatographic peak area of caffeine from three successive injections. A literature survey based on the influence of pH on the extraction efficiency of caffeine showed that the most of investigations have been carried out at the natural pH of samples, but in some studies the results have been generated at pH of 7 (Jafari et al., 2011), and in some other researches the experiments have been carried out at pH value of 12.5 (Xiong, Chen, He, & Hu, 2010). However, in the present work in order to simplify the procedure and to avoid the risk of forming a turbid sample solution that may be produced from addition of acid, base or buffer solution, it was preferred to perform the experiments at the natural pH of samples. The natural pH values of the samples were: 6.5–7.5 for teas; 5.2–6.0 for coffee and Nescafe; and 2.5–3.5 for other beverages.

3.1. Influence of disperser solvent type and volume

The disperser solvent should be miscible with both organic (extraction solvent) and aqueous (sample) phases. Thereby, methanol, ethanol, and acetone were examined for this purpose. The tests were performed with a working solution of caffeine (1 µg mL^{-1}) according to the proposed procedure. The results indicate that ethanol and methanol represent almost the same extraction efficiency, however because a non-toxic solvent is preferred, ethanol was chosen as the extraction solvent for the subsequent experiments. Volume of disperser solvent is a crucial parameter that has an important effect on extraction efficiency. To evaluate the effect of this parameter, various volumes of ethanol including 100, 150, 200, 250 and 300 µL were examined in accordance with the procedure. The results represented in Fig. 1 show that the maximum efficiency was achieved at 200 µL. At the lower volumes, tiny droplets of the extraction solvent may not be dispersed effectively and thus cloudy state is not well formed, thus the extraction recovery decreases. However, at the higher volumes, the solubility
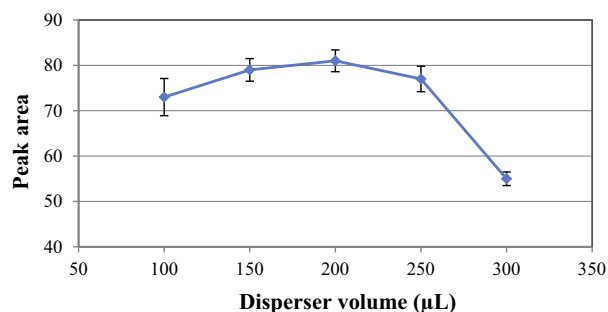

Fig. 1. Effect of disperser solvent volume on the extraction efficiency of caffeine. Extraction conditions: same as the procedure.

of caffeine in aqueous phase increases, and thus the transfer into chloroform (extraction solvent) decreases resulting in a lowered efficiency. Therefore, the volume of 200 µL was chosen for disperser solvent in further experiments.

3.2. Influence of extraction solvent type and volume

The extraction solvent plays a very important role in DLLME. The organic solvents with higher density than water were preferred because the centrifugation results in a quick phase separation. Immiscibility with water, higher extraction capacity, and good gas chromatography behaviour were the other preferred properties. Considering these characteristics, chloroform (density: 1.48 g mL^{-1}), carbon tetrachloride (density: 1.59 g mL^{-1}) and chlorobenzene (density: 1.11 g mL^{-1}) were tested. The results demonstrate that among these solvents, chloroform with higher extraction capability was the most effective at preconcentration of caffeine; therefore, it was considered as the extraction solvent in the following studies. The volume of extraction solvent is another key parameter that greatly affects the formation of cloudy state and the extraction performance (Ge & Lee, 2012). To study the effect of extraction solvent volume on the extraction efficiency, different volumes of chloroform (15, 20, 25 and 30 µL) was examined based on the proposed procedure. With the volumes lower than 15 µL, insufficient sedimented extractant at the bottom of the conical test tube was formed. This is probably attributed to the partial solubility of chloroform in water. At volumes higher than 30 µL, chloroform was not dispersed well by 200 µL ethanol in the aqueous sample solution. Fig. 2 shows that the highest chromatographic peak area was obtained when the volume of chloroform was 15 µL, but due to the low volume of the sedimented phase, the standard deviation at 15 µL is higher than the other volumes. At volumes greater than 25 µL, the dilution effect became
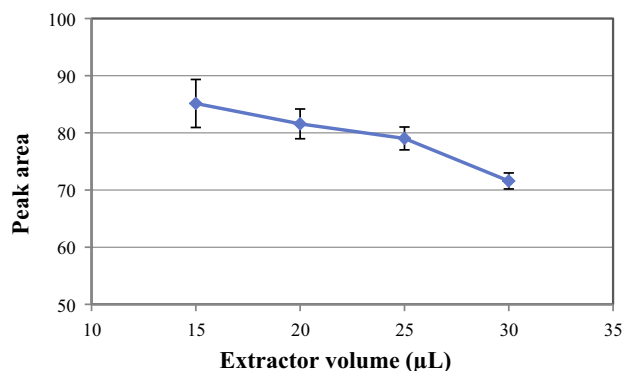

Fig. 2. Effect of extraction solvent volume on the extraction efficiency of caffeine. Extraction conditions: same as the procedure.

predominant over extraction capacity and thus the extraction efficiencies were low. Considering the above discussion, 20 μL was chosen as the optimum volume of chloroform for further experiments.

3.3. Effect of sample size

The influence of sample size on the extraction efficiency was evaluated in the range of 0.5–3.0 mL at constant experimental conditions. The results in Fig. 3 represent that the chromatographic signal increased by increasing the sample volume up to 1.0 mL. Furthur increase in sample volume up to 3.0 mL caused almost a constant peak area (plateau of the curve). Therefore, the sample volume of 1.0 mL was selected for subsequent tests.

3.4. Effect of salt addition

The addition of salt to aqueous sample solutions may have different effects on extraction (salting-out, salting-in or no effect) (Zhang & Lee, 2012). In order to study the effect of salt concentration on the performance of the proposed method, different concentrations of NaCl/H$_2$O (0, 5, 7, 10, 15 w/v%) were examined and the results were plotted in Fig. 4. By increasing the salt concentration up to 10%, the peak area was increased. Then, the peak area remained approximately constant up to 15%. At the salt concentrations greater than 10%, it is likely that the increased viscosity overcame the salting-out effect gradually, and extraction was inhibited. Considering these characteristics, further extractions were carried out at salt concentrations of 10% (w/v) NaCl.

3.5. Method evaluation

Under the optimal operating conditions (disperser solvent (ethanol), 200 μL; extraction solvent (chloroform), 20 μL; salt concentration, 10% (w/v)) the analytical figures of merit consisted of linear dynamic range (LDR), limit of detection (LOD), determination coefficient (R^2) and relative standard deviation (RSD) for determination of caffeine were obtained to evaluate the method efficiency. The calibration curve was constructed in accordance with the proposed procedure using 1 ml of different standard solutions of caffeine with 9 concentration levels in the range of 0.05–500 μg mL^{-1} and was characterised with the determination coefficient (R^2) of 0.9990. The limit of detection (LOD) based on $3S_d/m$ (where S_d and m are the standard deviation of the blank and slope of calibration graph, respectively), was equal to 0.02 μg mL^{-1}. The limit of quantification (LOQ) was calculated according to $10S_d/m$ equal to 0.05 μg mL^{-1}. The precision of the method was assessed by performing five replicates analysis of
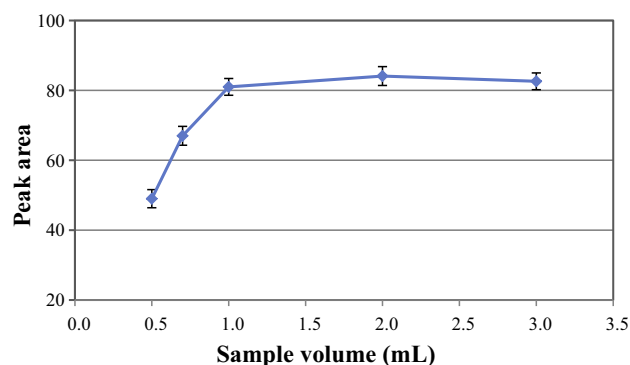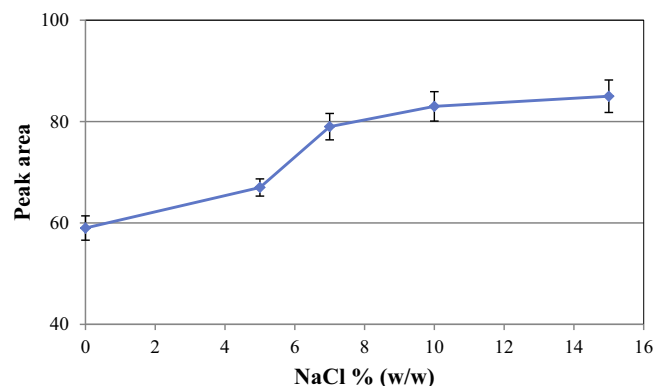
Fig. 4. Influence of salt concentration on the extraction efficiency of caffeine obtained from DLLME. Extraction conditions: same as the procedure.

standard solution of 1 μg mL^{-1}. The precision based on the relative standard deviation of the peak area was calculated equal to 3.2%. The enrichment factor (EF) was defined as the ratio of concentration of analyte between the organic extraction phase (C_{sed}) and the initial concentration of analyte (C_i) in aqueous solution as follows equation:

$$EF = \frac{C_{sed}}{C_i} \qquad (1)$$

The (C_{sed}) was calculated by direct injection of caffeine standard solutions in chloroform with concentrations in the range of 1–500 μg mL^{-1}. The enrichment factor was obtained equal to 16.

3.6. Analysis of real samples

The applicability of the proposed method for determination of caffeine in fifteen real samples was evaluated using the procedure under the optimal conditions and the results were presented in Table 1.

The relative recovery (RR) was calculated using the following equation:

$$RR = \frac{C_{found} - C_{real}}{C_{added}} \times 100 \qquad (2)$$

where C_{found}, C_{real}, and C_{added} are the concentrations of analyte after addition of known amounts of standard in the real sample, the concentration of analyte in real sample and the concentration of known amounts of the standard which was spiked to the real sample, respectively. The relative recovery values of 96.2–101.2% were obtained with three repeated measurements. The results demonstrated that the different sample matrices had no significant influence on the extraction efficiency of caffeine.

3.7. Comparison with other methods

The analytical specifications of the proposed method (DLLME–GC–NPD) were compared with those of other previously published methods for determination of caffeine (Table 2). In the present study, the extraction time is shorter than that of the other methods due to the great surface area between the extraction solvent (chloroform) and aqueous sample phase. Accordingly, the transfer of caffeine from aqueous phase to extraction solvent was intensified and resulted in an instant equilibrium. The linear dynamic range is four orders of magnitude (10^4) and thus wider than that of the other studies. Consumption of organic extraction solvent is another important characteristic that is lower than most of the mentioned methods. The limit of detection is lower than HPLC–UV (Al-Othman

Fig. 3. Effect of sample volume on the extraction efficiency of caffeine. Extraction conditions: same as the procedure.

Table 1
Determination of caffeine in various real samples by DLLME–GC–NPD method.

Sample	Caffeine ($\mu g\ mL^{-1}$)	RSD%, n = 3	Caffeine added ($\mu g\ mL^{-1}$)	Caffeine found ($\mu g\ mL^{-1}$)	Relative recovery (%)	RSD%, n = 3
Green tea	120.5	3.2	50.0	169.8	98.5	3.7
Oolong tea	145.2	4.7	50.0	193.3	96.2	4.5
White tea	165.8	4.0	50.0	214.1	96.7	3.9
Black tea	149.5	3.0	50.0	198.9	98.8	3.6
Tea bag	171.7	4.5	50.0	220.4	97.4	4.8
Coffee	267.5	2.8	50.0	318.1	101.2	3.0
Nescafe coffee	246.8	3.2	50.0	295.6	97.6	3.8
Red bull	297.9	2.9	50.0	348.2	100.5	3.4
Hype	314.7	3.3	50.0	364.4	99.4	2.8
Coca cola	94.1	4.5	25.0	118.9	99.2	4.4
Coca-zero	83.1	2.6	25.0	107.7	98.8	3.1
Pepsi	55.5	3.1	25.0	80.6	100.3	3.7
Pepsi max	51.3	2.9	25.0	76.2	99.6	2.7
7up	nd[a]	nd	25.0	24.5	98.0	4.2
Sprite	nd	nd	25.0	24.4	97.6	4.5

[a] Not detected.

Table 2
Comparison between DLLME–GC–NPD and other methods for determination of caffeine.

No.	Analysis method	Extraction method	EF[a]	LOD[b]	LOQ[c]	LDR[d]	Solventes	Extraction Time	Ref.
1	GC-NPD	DLLME	16	0.02	0.05	0.05–500	$CHCl_3$: 20 μL	Few seconds	This work
2	GC-MS (SIM)	DDSME[e]	14–16	0.004		0.05–5	$CHCl_3$: 0.5 μL	5 min	Shrivas and Wu (2007)
3	GC-MS	UAE		0.001		0.005–30	$C_4H_8O_2$: 1 mL	5 min	Zou and Li (2006)
4	HPLC	UAE	–	0.05	0.16	0.16–250	H_2O: 50 mL	30 min	Al-Othman et al. (2012)
5	HPLC	UAE	–	0.1	0.33	–	H_2O: 50 mL	30 min	Tzanavaras and Themelis (2007)
6	HPLC	UAE	–	0.004	0.014	–	CH_3OH: 25 mL	20 min	Rostagno et al. (2011)
7	FT-IR with chemometrics	–	–	5	–	–	–	–	Paradkar and Irudayaraj (2002)
8	FT-Raman	SPE	–	18	–	–	–	~2 min	Armenta et al. (2005)
9	ESI–IMS[f]	MIP-SPE	–	0.2	–	0.5–20	CH_3OH: 2 mL	~134 min	Jafari et al. (2011)
10	Voltammetry	–	–	0.02	–	0.06–19	–	–	Guo et al. (2011)

[a] Enrichment factor.
[b] Limit of detection ($\mu g\ mL^{-1}$).
[c] Limit of quantification ($\mu g\ mL^{-1}$).
[d] Linear dynamic range ($\mu g\ mL^{-1}$).
[e] Drop-to-drop solvent microextraction.
[f] Electrospray ionization–ion mobility spectrometry.

et al., 2012; Tzanavaras & Themelis, 2007), FT-IR (Paradkar & Irudayaraj, 2002), FT-Raman (Armenta et al., 2005) and ESI–IMS (Jafari et al., 2011), and is comparable with other researches.

4. Conclusion

DLLME–GC–NPD was developed for determination of caffeine in popular beverages. A very short extraction time, simple extraction process, low detection limit (0.02 $\mu g\ mL^{-1}$), good enrichment factor (16), wide linear dynamic range (10^4), a specific and sensitive detection system (GC–NPD), and requiring low sample volume (0.5 mL) are the main advantages of the proposed method. Furthermore, the method is eco-friendly because it consumes micro volume of organic solvent (20 μL chloroform), and thus can be considered as a green method.

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