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Analytical Methods

Fast chromatographic determination of caffeine in food using a capillary hexyl methacrylate monolithic column

Zeid A. Al-Othman [a], Ahmad Aqel [b], Muteb K.E. Alharbi [c], A. Yacine Badjah-Hadj-Ahmed [a,*], Abdulrahman A. Al-Warthan [a]

[a] Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia
[b] King Abdullah Institute for Nanotechnology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia
[c] King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia

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ABSTRACT

Caffeine beverages are widely consumed in the world, so new analytical techniques providing fast and reliable data are essential for a rapid and accurate evaluation of food quality. A capillary hexyl methacrylate monolithic column was designed and prepared for simple, rapid, economical and sensitive high performance liquid chromatography method for the determination of caffeine in food, using a water/acetonitrile (90:10, v/v) mobile phase with ultraviolet (UV) detection. The method was validated over the range 0.16–250 μg/mL of caffeine concentration and found to be linear ($r > 0.995$, $n = 5$) with relative standard deviation (RSD) less than 4.0%.

Calibration curves prepared for tea, coffee and cocoa extracts were linear. The limits of detection and quantification were 0.05 and 0.16 μg/mL, respectively. Recoveries of caffeine in food samples were found to be ranged from 89.0% to 116.6%, showing a good reliability of this method. Ruggedness and the case of external calibration versus standard addition for the analysis of real samples were also examined. The effect of temperature on the extraction was also investigated. This validated method was compared with the most commonly used conventional octadecylsilica packed column (C_{18}), in terms of analysis time, reagents and solvents consumption, detection and linearity limits, porosity and permeability of each column.

1. Introduction

With the exception of water, caffeine beverages like tea and coffee represent the most widely consumed in the world. Caffeine (1,3,7-trimethylxanthine) may be also considered as the most widely used drug in the world. We consume caffeine daily in coffee, tea, cocoa, chocolate, some energy or soft drinks, as well as in many painkillers and antimigraine drugs. Caffeine is also representing a mild stimulant for central nervous system, muscle, heart and circular systems of the human body. On the other hand, it induces the relaxation of bronchial muscle, acts as a diuretic and intensifies brain activity (James, 1991). It is generally associated with improvements in alertness, learning capacity and exercise performance when moderately consumed. The concentration of caffeine *in vivo* is a key mark for various disorders including heart disease, carcinogenesis, kidney malfunction and asthma (Belay, Ture, Redi, & Asfaw, 2008). However, drinking large amounts of caffeine or taking sufficiently high doses may cause many undesired symptoms and even potentially adverse effects on health, especially for infants and children, such as: agitation, chills, irritability, loss of appetite, weakness, insomnia, hypertension, gastrointestinal problem, fever, delusions, tachycardia and even death (Reissig, Strain, & Griffiths, 2009; Varnam & Sutherland, 1994). A fatal dose of caffeine has been calculated 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) (Suteerapataranon, Butsoongnern, Punturat, Jorpalit, & Thanomsilp, 2009). Therefore, it is important to monitor caffeine in beverages and food by establishing a more precise, simple, fast and cheap analytical method in order to study its physiological effects on the human body and control food quality.

Several analytical methods have been proposed for determination of caffeine in various matrices (environmental, biological, plants, food, etc.) covering a broad spectrum of instrumental techniques. Many recent publications include applications of UV–Vis spectrophotometry (Belay et al., 2008), thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) (Fenske, 2007), FT-Raman spectrometry (Armenta, Garrigues, & De La Guardia, 2005), high-performance liquid chromatography (HPLC) with different detectors and column types (Brunetto et al., 2007; Li

* Corresponding author. Tel.: +966 14677126; fax: +966 14675992.
E-mail addresses: ybadjah@ksu.edu.sa, ybadjah@hotmail.com (A. Yacine Badjah-Hadj-Ahmed).

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et al., 2010; Sun, Qiao, & Liu, 2006), gas chromatography (GC) (Shrivas & Wu, 2007), FT-IR spectroscopy (Najafi, Hamid, & Afshin, 2003), NMR spectroscopy (Talebpour, Maesum, Jalali-Heravi, & Shamsipur, 2003), mass spectrometry (Dorfner, Ferge, Yeretzian, Kettrup, & Zimmermann, 2004), and capillary electrophoresis (CE) (Meinhart et al., 2010). Among this abundant literature, it is obvious that the most popular and frequently used technique for the determination of caffeine is HPLC. Most of these methods are complicated, not very sensitive, time consuming and hence unsuitable for a quick check of caffeine concentration, moreover they require large quantities of organic solvents and reagents, so that they are expensive and not convenient for routine analysis.

Monolithic stationary phases are relatively new structures and have attracted increasing interest in all areas of chromatographic methods as an alternative to particulate columns (Smith & Jiang, 2008; Unger, Skudas, & Schulte, 2008). They consist of a single rigid piece of porous material that possesses a unique bimodal pore structure distribution with μm-sized through pores (macropores) and nm-sized mesopores. Macropores dramatically increase the column porosity, thereby considerably reducing the analysis time, while mesopores form the fine porous structure and provide the very large active surface area for high efficiency separations (Gritti & Guiochon, 2004; Minakuchi, Nakanishi, Soga, Ishizuka, & Tanaka, 1997). Due to this unique and unusual structure as well as their ease of preparation, monolithic columns offer improved chromatographic performance and favourable properties for high efficiency, fast separations, high reproducibility, low back pressure drop across the column, fast mass transfer kinetics between the mobile and stationary phases and a high binding capacity.

Kubin (1967) reported the first attempt to use a monolith material for separation; several different monolithic supports were then described in the literature since the late 1980s or early 1990s. Two types of monolithic columns have been developed for chromatography: macroporous organic polymer based monolithic columns produced by a simple moulding process (Svec & Fréchet, 1992) and silica based monolithic columns made by using the sol–gel approach (Minakuchi, Nakanishi, Soga, Ishizuka, & Tanaka, 1998). These stationary phases are basically synthesised from silica or organic monomers, such as acrylamide, styrene and methacrylate derivatives (Hjerten, Liao, & Zhang, 1989; Wang, Svec, & Fréchet, 1993). Since the 1990s, the monolithic supports became widely used in various applications such as environmental, food, pollutants, ions, and chiral analysis (Lubda, Cabrera, Nakanishi, & Lindner, 2003; McCalley, 2003; Xu, Mori, Tanaka, Ikedo, & Hu, 2004).

The present work describes the preparation of a capillary monolithic column which was applied for the quantitative determination of caffeine in different food samples. The use of a monolithic column and a miniaturised HPLC system aimed to develop a fast and sensitive analytical procedure which consumes lower amounts of solvents and needs minute samples. Furthermore, the preparation of this type of columns is more cost efficient, because it requires much smaller amount of stationary phase materials.

The analytical method was validated in terms of precision, recovery, sensitivity and ruggedness. External calibration versus standard addition experiments were carried out to evaluate the trueness and applicability of the assay for commercial real samples analyses. Comparison between the prepared monolithic column and a commercial C_{18} packed column was also performed and discussed.

2. Material and methods

2.1. Chemicals and column

Caffeine (analytical reagent grade, 98.5%) was provided by BDH (Lutterworth, UK). Tea, coffee, and cocoa samples were purchased from the local market (Riyadh, Saudi Arabia) as powder stored in vacuum packages. HPLC grade acetonitrile was purchased from BDH (Lutterworth, UK). The purified water was prepared on a Millipore system (Milli-Q Advantage Elix, Millipore S.A.S. 67120 Molsheim, France), then filtered on 0.2 μm nylon membrane filter from Whatman (Maidstone, UK). The mobile phase was always filtered using a vacuum glass filtration system through the same nylon membrane filters and degassed ultrasonically for 30 min prior to use.

Fused silica tubing (0.53 mm i.d.) was purchased from Restek (Bellefonte, USA). The chemicals used for the monolithic column preparation in this work were purchased from Aldrich (Steinheim, Germany) as follow: 3-(trimethoxysilyl)propyl methacrylate (TMSM) 98%, ethylene dimethacrylate 98% used as crosslinker, azo-bis-isobutyronitrile (AIBN) 98% as initiator and hexyl methacrylate 98% as monomer. Toluene 99.5%, ethanol 99.7%, hydrochloric acid 35.4%, sodium hydroxide 99%, orthophosphoric acid 85% and disodium phosphate 98% were acquired from BDH (Lutterworth, UK). All chemicals were used without further purification.

2.2. Preparation of hexyl methacrylate capillary monolithic column

In order to clean and activate the capillary inner surface, the fused-silica tubing (150 × 0.53 mm i.d.) was rinsed first with 1.0 M NaOH solution for 5 min and let for 10 min with the same solution, then rinsed with water and dried with an air flow for 2 min, two times for each. The column was then flushed with 1.0 M HCl for 2 min and dried with air for 5 min, after that the capillary was rinsed with toluene for 10 min then flushed with a 10% 3-(trimethoxysilyl)propyl methacrylate solution in toluene for 8 min and let with the same solution for 2 h, then rinsed with toluene for 5 min and dried with air for 5 min.

The monomer mixture was prepared as follows (wt.%): 20% hexyl methacrylate, 15% ethylene dimethacrylate as crosslinker and 1% AIBN as initiator. The porogen mixture was 64% of the total solution and prepared as follows (wt.%): 60% acetonitrile, 20% ethanol, and 20% of 5 mM phosphate buffer at pH 7.1. The monomer mixture and the porogen solvents were mixed into a homogenous solution then sonicated and purged with helium gas for 3 min. The capillary column was then filled with the reactant solution and both ends were plugged with a piece of rubber. The polymerisation was performed in a water bath at 60 °C for 20 h. After the polymerisation, the seals were removed; the prepared column was connected to an HPLC pump then washed with ACN to remove the unreacted materials and porogenic solvents. The capillary column was then connected both to injector and detector using fingertight fittings and suitable sized PEEK (polyether etherketone) sleeves.

2.3. Standard solutions

A standard stock solution with a concentration of one gram per litre was prepared using HPLC grade water, stored under refrigeration and protected from light. Working standard solutions, from 0.1 to 250 μg/mL, were freshly prepared using HPLC grade water by appropriate dilutions of the stock solution. A calibration curve was constructed each day before analysis of the samples. HPLC grade water was injected as the blank.

2.4. Sample preparation and extraction procedure

Caffeine samples (tea, coffee, and cocoa) were ground to a fine uniform powder. An accurately weighed amount of 100 mg was dispersed in 50 mL of HPLC grade water and sonicated for 30 min until complete dissolution. An adequate volume (about 20 mL) of the resulting solution was filtered through 0.45 μm membrane filter and injected without further pretreatment to the HPLC system.

In order to study the effect of temperature on the extraction process, 10 mg of tea and 100 mg of coffee and cocoa samples were added to 50 mL of water, then heated under stirring at different temperatures (25, 40, 55, 70, 85 and 100 °C); the resulting solutions were filtered and injected with the same procedure.

2.5. HPLC analysis

All analyses were performed with a Shimadzu HPLC system (Shimadzu LC-6A, Kyoto, Japan) including a pump, a Rheodyne 7125 manual injector, a UV detector and a C-R6A integrator. The detector was equipped with a 2 cm path length and 1 μL volume homemade cell and set at a 274 nm wavelength. 1 μL of each sample and standard solutions were injected in the monolithic column via the manual injector of the HPLC instrument. The manual injector was equipped with a homemade sample loop consisting of 8 cm × 0.005 inch i.d. PEEK tubing. This sample loop was filled up with each sample solution. The mobile phase consisted of an acetonitrile/water mixture (10:90, v/v). The flow rate was 41 μL min^{-1} which corresponds to a pressure of 1.14 MPa at a temperature of 25 °C. Under the above mentioned conditions the samples injection rate was 40 h^{-1}. Peak area was used for quantitative evaluations, while each sample or standard was injected five times. Standard deviations and statistical parameters were calculated using Microsoft Office Excel software 2007 package. For the comparative study, a Betasil C_{18} column, 150 mm length and 4.6 mm inner diameter packed with 3 μm size particles, was purchased from Thermo Scientific (Waltham, MA USA).

2.6. Validation

The developed method was validated in terms of linearity, limits of detection and quantification, precision and ruggedness. An additional series of experiments was carried out to compare the suitability of the external calibration and standard addition approaches for real sample analysis.

Concentrations ranging from 0.1 to 250 μg/mL of caffeine were obtained for the standard solutions. The solutions were always filtered using a 0.45 μm filter before injection and each solution was injected in five replicates. Peak areas were plotted versus the respective concentrations of caffeine, in order to study the method linearity. Limits of detection (LOD) and quantification (LOQ) were the caffeine concentrations that lead to signal-to-noise ratios of 3:1 and 10:1, respectively.

In order to study the efficiency of the extraction at the optimal point, recovery tests were performed by spiking 3.0 mL extract of 0.05 g of each sample (tea, coffee, and cocoa) with 1.0 mL caffeine standard at three levels: 1.0, 10.0 and 100.0 μg/mL.

3. Results and discussion

3.1. Preparation of the monolithic column

The general procedure for preparation of the capillary hexyl monolithic column is illustrated in section 2.2 includes four steps: (a) empty column wall modification, (b) filling the capillary with the monomer mixture, (c) *in situ* polymerisation of both monomer and difunctional crosslinker in the column, (d) connecting the column to the HPLC system.

3.1.1. Efficiency of the monolithic column

Band broadening in chromatographic columns is conveniently described by the well-known Van Deemter equation as the dependence of the height equivalent to a theoretical plate, H, on the flow rate of the mobile phase, u.

$$H = A + \frac{B}{u} + C \cdot u$$

In this equation, the A term refers to the dispersive contribution from the flow profile (eddy diffusion), B term arises from longitudinal diffusion of the solute in the mobile phase, while the C term corresponds to resistance to mass transfer in mobile and stationary phases. The H–u plot for caffeine on the column tested is shown in Fig. 1A. The Van Deemter curve shows that the lowest plate height was achieved at the optimum mobile phase flow rate 41 μL/min. Chromatogram of caffeine at the optimum flow rate is presented in Fig. 1B.

3.2. HPLC procedure

The effect of the flow rate and composition of the mobile phase on the retention time (t_R, min) of the analyte, the width at half peak ($w_{1/2}$, min) and the number of theoretical plates (N) were studied using an aqueous caffeine standard at concentration of 1.0 mg/L. The sample injection volume was 1 μL.

The selected mobile phase composition was acetonitrile/water (10:90, v/v) while the flow rate was 41 μL/min, these conditions were considered as a compromise between analyte retention time (corresponding to a sampling rate of 40 h^{-1}), separation efficiency and solvents consumption. Under these conditions, the retention time of caffeine was 1.16 min. In a recent paper, Tzanavaras and Themelis (2007) have compared eleven papers dealing with caffeine determination using different HPLC methods in terms of retention times. In the present work, the analysis time (less than 1.5 min) was shorter than most retention times previously recorded for caffeine, which were in the range 0.68–18.2 min. Moreover, all cited papers used various commercial columns with flow

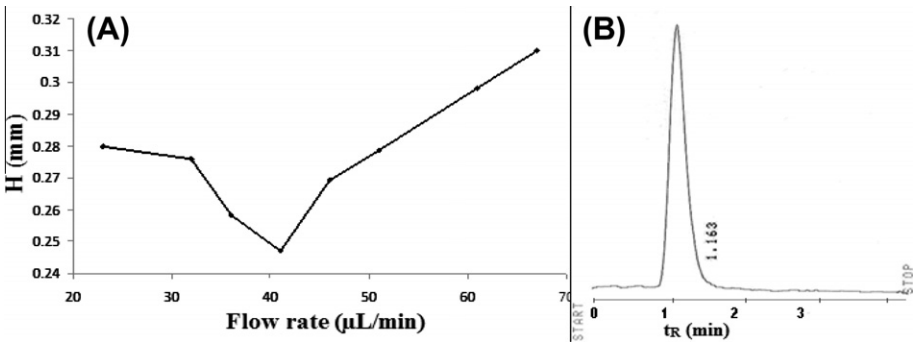

Fig. 1. (A) Van Deemter plot of the height equivalent to a theoretical plate as a function of flow rate. (B) Chromatogram of standard caffeine. Experimental conditions: monolithic capillary column, 15 cm × 530 μm i.d.; mobile phase: acetonitrile/water (10:90, v/v); 1.0 ppm standard caffeine concentration; injection volume: 1 μL; UV detection at 274 nm.

rates in the range 0.3–3.0 mL/min while our flow rate was 41 μL/min.

3.3. Validation of the method

The developed HPLC assay was validated in terms of linearity, limit of detection and quantification, precision, recovery and ruggedness. An additional series of experiments were carried out to validate the suitability of external calibration versus the standard addition approach for real sample analysis.

3.3.1. Calibration curve, linearity, LOD, LOQ and precision

Calibration curve of caffeine was prepared by diluting the standard solution in water. The developed assay was found to be linear in the range 0.16–250 μg/mL of caffeine, when peak area was used for signal evaluation. A good linearity was obtained in this concentration range ($r = 0.998$). The typical equation of calibration curve was $y = 25{,}687x + 73{,}878$, where y is the peak area and x is the concentration of standard caffeine.

The determination of both limit of detection (LOD) and limit of quantification (LOQ) of the assay were based on the signal-to-noise criteria. The respective values were found to be 0.05 μg/mL ($S/N = 3$) and 0.16 μg/mL ($S/N = 10$).

The precision of the method was evaluated through percent relative standard deviation percentage (%RSD) of five consecutive injections of different caffeine concentrations ranging from 0.16 to 250 μg/mL. The results (RSD <3.0% for all cases) confirm that good precision.

3.3.2. Ruggedness

The ruggedness of the method against small variations of the composition and the flow rate of the mobile phase was checked by calculating the retention time, width at half peak and number of theoretical plates. The experimental results are shown in Table 1. The findings verified the ruggedness of the procedure.

3.3.3. External calibration versus standard addition

In order to apply the proposed method to the analysis of caffeine in food samples, it was necessary to investigate whether caffeine could be measured accurately using either an external calibration curve or the standard addition procedure. For this reason, 0.03 g of tea, coffee and cocoa samples were prepared as described in the extraction procedure (Section 2.6) and the caffeine concentrations were determined first using a 7-point external calibration curve then using the standard addition method after spiking with aqueous caffeine standards in the range 30.0–100.0 μg/mL. The experimental results are shown graphically in Fig. 2A. The typical equations of the obtained calibration curves were as follows: tea: $y = 18{,}367x + 17{,}50{,}599$ with $r = 0.990$; coffee: $y = 17{,}749x + 961{,}614$ with $r = 0.998$; cocoa: $y = 20{,}401x + 737{,}107$ with $r = 0.999$; y being the peak area of caffeine and x the added concentration of caffeine in μg/mL.

Table 1
Ruggedness study of the proposed assay.

Chromatographic conditions	t_R[a] (min) ± % RSD	$w_{1/2}$[b] (min) ± % RSD	N[c]
Optimal conditions[d]	1.163 ± 0.33	0.111 ± 0.65	607
Effect of flow rate of the mobile phase (μL/min)			
Flow rate = 46	1.078 ± 0.23	0.108 ± 0.61	557
Flow rate = 36	1.296 ± 0.25	0.127 ± 0.80	581
Effect of mobile phase composition (H_2O/ACN, v/v)			
Acetonitrile/water = 5:95 (v/v)	1.138 ± 0.37	0.111 ± 0.66	580
Acetonitrile/water = 15:85 (v/v)	1.169 ± 0.28	0.120 ± 0.73	531

[a] Retention time (mean of three injections).
[b] Width at half peak (mean of three injections).
[c] Number of theoretical plates (mean of three injections).
[d] Flow rate = 41 μL/min of acetonitrile/water, (10:90, v/v).

3.4. Analysis of real samples

The applicability of the developed method to the analysis of food samples was demonstrated by the determination of caffeine in tea, coffee and cocoa samples after simple pretreatment. An accurate mass of tea, coffee and cocoa was extracted in 50 mL water volume and the caffeine content was determined in each sample. The curve was plotted for caffeine concentration against the mass of each sample. All curves, as shown in Fig. 2B, were forced through the origin and linearly fitted; they were linear within the tested concentration ranges. The typical equations of the obtained curves were as follows; tea: $y = 2040x + 2.694$ with $r = 0.995$; coffee: $y = 1139x + 2.568$ with $r = 0.997$; cocoa: $y = 815.1x + 4.509$ with $r = 0.993$; y being the found caffeine concentration (μg/mL) and x the mass of food sample (g). The respective values of percent relative standard deviation were <3.7% for tea,<2.4% for coffee and <4.0% for cocoa ($n = 5$). The figure confirms that the content of caffeine in tea is larger than in coffee and cocoa when the same amounts are compared. From Fig. 2B, it can be seen that the caffeine concentrations are 11.5 ± 0.16, 6.6 ± 0.18 and 5.3 ± 0.15 mg/100 mL, for 0.05 g of each sample of tea, coffee and cocoa, respectively, infused in 50 mL of water. These values are in good agreement with those found in the literature (Belay et al., 2008; Shrivas & Wu, 2007; Suteerapataranon et al., 2009).

3.4.1. Recovery study for caffeine

The trueness of the proposed procedure was evaluated by means of recovery experiments. Recovery experiments were carried out – using a 6-point calibration curve approach – after spiking the caffeine real samples (tea, coffee and cocoa) with known concentrations of caffeine at three different levels: low, medium and high (1.0, 10.0 and 100.0 μg/mL). In all cases, the recovery percentage values ranged between 89.0% and 116.6% with a percent relative standard deviation < 3.6% ($n = 3$). These values demonstrate good extraction efficiency. A summary of the recovery results is given in Table 2.

3.4.2. Temperature effect on the extraction procedure

Several previous investigations have shown that the solubility of caffeine is very much related to temperature which may affect the dissolution and diffusion rates of caffeine (Suteerapataranon et al., 2009). In order to study the effect of temperature on the caffeine extraction, 0.01 g of tea and 0.1 g of each coffee and cocoa were extracted in water under different temperatures. The influence of temperature on the extraction of caffeine from the food samples was investigated over the range from 25 to 100 °C (Fig. 3). This effect is more distinct in tea than in coffee and cocoa samples.

3.5. Comparison of packed and capillary monolithic columns

In spite of their promising characteristics, few analytical applications have been developed using monolithic columns and the transferability of analytical methods from conventional particle packed to monolithic columns is still in discussion. More investigations are needed to ensure the applicability of this type of columns.

The aim of this section was to evaluate the performance of a packed C_{18} HPLC column for the analysis of caffeine and compare the results with those obtained using the prepared capillary monolithic column. The same mobile phase and detection wavelength

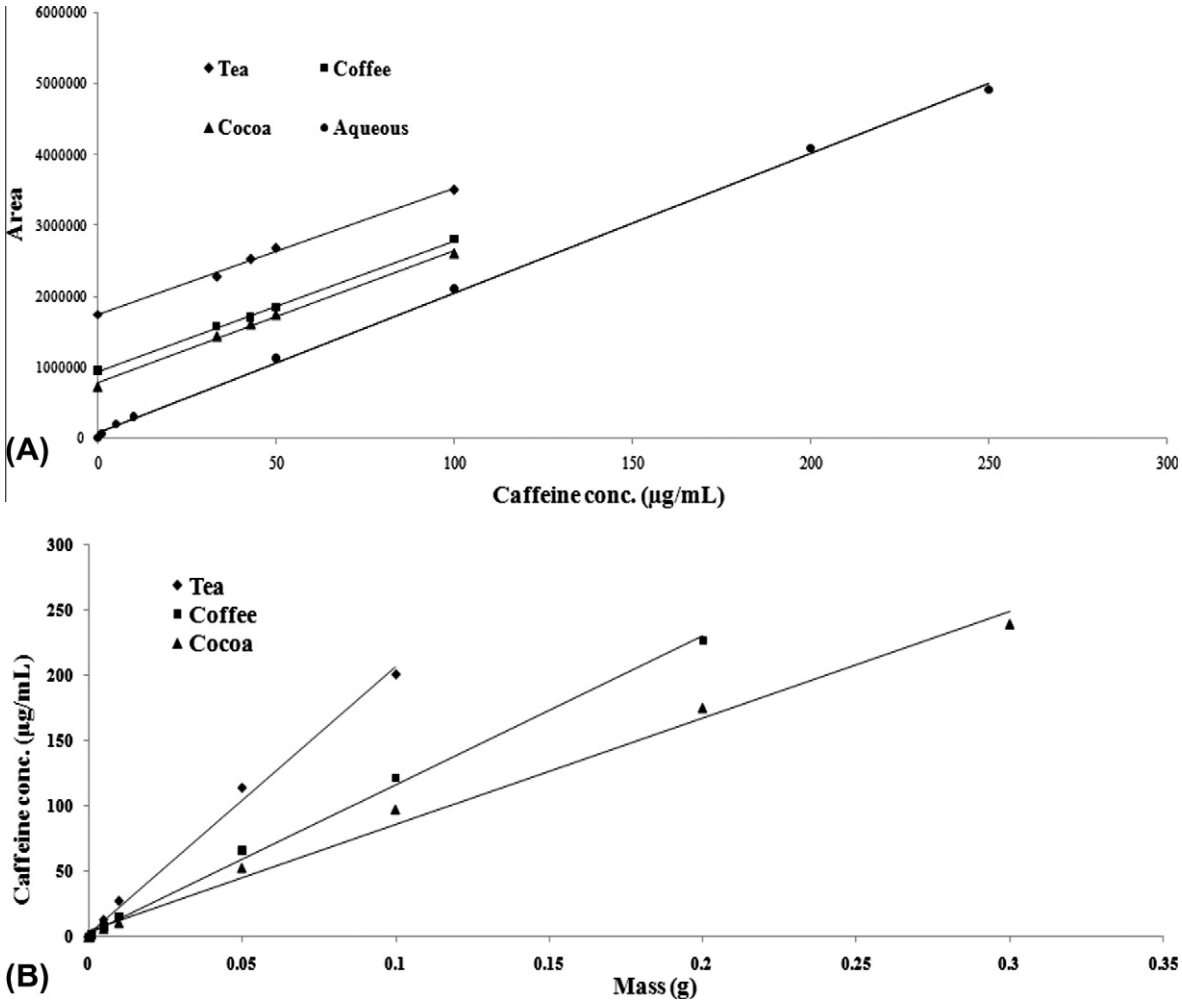

Fig. 2. (A) External calibration vs. standard addition calibration curves of aqueous, tea, coffee and cocoa caffeine samples. (B) Concentration of the extracted caffeine vs. mass of tea, coffee and cocoa samples.

were applied on conventional packed and monolithic columns, in order to enable the direct comparison of their performances and to see if a chromatographic method could be transferred from conventional to monolithic columns without further modification.

For this purpose, several experimental parameters including the porosity, pressure drop, permeability, retention time, LOD, limit of linearity (LOL) and solvents consumption of the capillary monolithic column were compared to those of a conventional (Betasil) C_{18} column. The results are presented in Table 3.

Table 2
Recovery and repeatability of the proposed analytical method at three different spiking levels.

	Caffeine added (µg/mL)	Recovery (%)	Repeatability[a] (%)
Tea	1.0	116.65	3.2
	10.0	97.78	2.1
	100.0	95.55	1.9
Coffee	1.0	105.19	2.4
	10.0	99.69	2.3
	100.0	95.61	1.6
Cocoa	1.0	102.30	3.6
	10.0	99.99	3.0
	100.0	88.98	2.2

[a] Repeatability is given as the percent relative standard deviation.

The data presented in Table 3 show explicit advantages of monolithic column compared to the conventional packed C_{18} column with 3 µm particles for the determination of caffeine. This was very clear in shortened analysis time and reduced consumption of solvents and reagents which contribute to lower the cost of analysis. The capillary monolithic column also has a significantly higher total porosity compared to the conventional one, about 80% vs. 60%, respectively. This is in good agreement with previously published papers (El Deeb & Watzig, 2006; Tanaka et al., 2002). According to these results, the monolithic column has shown to be an excellent alternative to conventional silica based columns.

4. Conclusion

A hexyl methacrylate monolithic column was prepared and successfully applied for a rapid determination of caffeine in some food samples, including tea, coffee and cocoa. The current developed method is relatively easy with simple sample preparation; it is fast, cheap and highly sensitive for the determination of caffeine content in food.

The method was linear in the 0.16 to 250 µg/mL range, with a correlation coefficient of 0.998. The LOD for caffeine measured in the sample was 0.05 µg/mL, the method showed also good recovery and precision; the ruggedness of the assay was very satisfactory. The whole procedure required approximately two hours

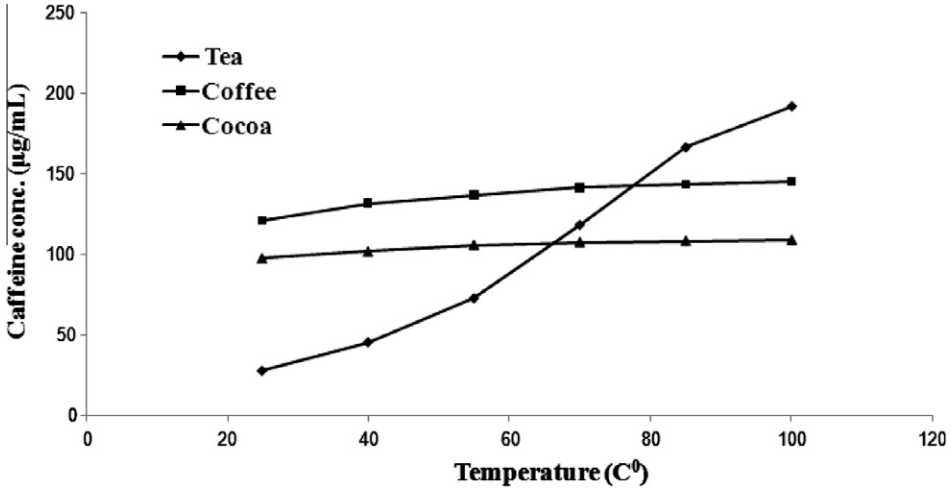

Fig. 3. Effect of temperature on caffeine extraction.

Table 3
Comparison of packed and capillary monolithic columns.

	C_{18} column	Capillary column
Column dimension	(150 × 4.6 mm) 3 µm particle size	(150 × 0.53 mm)
Caffeine analysis characteristics		
t_R (min)	15.41	1.16
Flow rate (µL/min)	500	41
Injection volume (µL)	10	1
LOD (µg/mL)	0.20	0.05
LOQ (µg/mL)	0.70	0.16
LOL (µg/mL)	150	250
Sampling rate (h^{-1})	3	40
Solvent consumption (mL/h)	30.0	2.46

(including sample preparation and analysis by HPLC) and it reduced the consumption of solvents and samples compared with the traditional methods. All these facts made the proposed method applicable to routine analysis.

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