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Rapid determination of methylxanthines in real samples by high-performance liquid chromatography using the new FastGradient® narrow-bore monolithic column

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ABSTRACT

The present study reports one of the very first analytical applications of the new narrow-bore monolithic column, FastGradient® Chromolith (50 mm × 2.0 mm i.d.). The three major methylxanthines (theobromine, theophylline and caffeine) were separated rapidly and determined simultaneously in various real samples. Based on the unique characteristics of this novel monolithic column the analytes were separated efficiently (R_s > 3.0) in less than 5 min at a low flow rate of 0.5 mL min^{-1} and using a low volume fraction of organic solvent (5% acetonitrile (ACN) in water) in the mobile phase. UV detection was carried out at 274 nm. The separation was optimized in terms of mobile phase composition, flow rate and injection volume, while the method was validated for linearity, detection and quantitation limits, within and day-to-day precision, accuracy and ruggedness. Its applicability was demonstrated by analyzing a variety of real samples including beverages, soft drinks, herbal products and pharmaceuticals. Compared to a well-established monolithic (Performance Chromolith 100 mm × 4.6 mm i.d.) and a particulate reversed phase column (Hypersil ODS 5 μm 150 mm × 4.6 mm i.d.), the narrow-bore FastGradient® column offered satisfactory performance, faster analysis time and drastic reduction in the consumption of mobile phase and organic solvents.

1. Introduction

As the regulations of international authorities become stricter in the quality control of food and pharmaceuticals the major challenge of modern analytical chemistry is to offer reliable, robust and high-throughput methods. Especially in liquid chromatography this trend is obvious in recent publications and is dictated by the critical role of this technique to quality control applications under industrial environment [1].

Currently, there are four major trends in Fast-LC applications: (i) high temperature LC [2], (ii) Ultra High Pressure LC (UHPLC) using sub-2 μm particulate columns [3], (iii) particulate columns based on fused-core technology operate at intermediate pressures between UHPLC and conventional columns [4] and (iv) highly permeable monolithic stationary phases that enable efficient separations at elevated flow rates [5]. Especially the latter technology has driven widespread attention among scientists. It is characteristic that monolithic materials have been included in the L1 packing list of the US Pharmacopoeia [6].

Very recently Merck® commercialized the new member of the Chromolith® monolithic columns family, namely the FastGradient® column [7]. This new narrow-bore monolithic column (2.0 mm i.d. × 50 mm length) provides high-performance separations at very low operating pressures. This unique feature makes the column compatible not only to UHPLC instruments but also to conventional HPLC setups offering an interesting hybrid solution. Additionally, the typical working flow rates of 0.2–1.0 mL min^{-1} are ideal for mass spectrometric detection. A literature search revealed only two published reports employing the FastGradient® column [8,9]. Fekete et al. reported that compared to other sub-2 μm UHPLC columns, the FastGradient® column provided the highest performance when the required plate number was higher than 5000 at 200 bar [8]. Spoof et al. concluded that the FastGradient® is a viable alternative column to the routine HPLC analysis of microcystins and nodularins [9].

The alkaloids theobromine (TB), theophylline (TP) and caffeine (CF) are naturally occurring substances in two of the most widely consumed plant materials worldwide, coffee and tea. Due to their bioactive properties these xanthine derivatives have attracted much attention by both the food and pharmaceutical industries.

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Table 1
Overview of recent RP-HPLC methods for the determination of methylxanthines.

Reference	Analytical column	Mobile phase	Flow rate ($mL\ min^{-1}$)	Detection	Analysis time (min)	Samples
[14]	Zorbax Eclipse XDB-C8 (150 mm × 4.6 mm i.d. × 5 μm)	ACN:water (pH = 8.0) (10:90, v/v)	0.8	UV @ 273 nm	8	Food, drinks, and herbal products
[15]	Nova-Pak C_{18} (150 mm × 3.9 mm i.d. × 4 μm)	MeOH:water (20:80, v/v)	1.4	UV @ 274 nm	10	Cocoa
[16]	Supelcosil LC-18-DB (250 mm × 4.6 mm i.d. × 5 μm)	Water:MeOH:AcH (80:19:1, v/v/v)	1.0	UV @ 275 nm	10	Cocoa and Cupuacu seeds
[17]	Supelcosil LC-18-DB (250 mm × 4.6 mm i.d. × 5 μm)	MeOH:buffer (pH = 5.8) (20:80, v/v)	1.0	UV @ 260 nm	13	Milk
[18]	LiChrospher 100 RP-18 (244 mm × 4.4 mm i.d. × 5 μm)	Water:EtOH:AcH (75:24:1, v/v/v)	1.0	UV @ 273 nm	7	Coffee, tea, and human urine
[19]	Phenomenex Luna C_{18} (150 mm × 4.6 mm i.d. × 3 μm)	Water:MeOH:AcH (75:20:5, v/v/v)	0.7	ESI-MS	10	Dietary supplements
[20]	Supelcosil LC-18-DB (250 mm × 2.1 mm i.d. × 5 μm)	MeOH:buffer (pH = 5.0) (20:80, v/v)	0.2	UV @ 280 nm	22	Human urine
[21]	Kromasil ODS C_{18} (150 mm × 4.6 mm i.d. × 5 μm)	MeOH:buffer (pH = 4.5) (30:70, v/v)	0.5	UV @ 270 nm	12	Human serum
[22]	Zorbax C_{18} (250 mm × 4.6 mm i.d. × 5 μm)	ACN:water (pH = 2.5) (10:90, v/v)	1.5	UV @ 274 nm	15	Chocolate
[23]	TSK gel ODS-80 TM (150 mm × 4.6 mm i.d. × 5 μm)	MeOH:buffer (pH = 3.5) (30:70, v/v)	1.0	UV @ 275 nm	10	Human plasma
[24]	Hypersil ODS (250 mm × 4.6 mm i.d. × 5 μm)	MeOH:water:formic Ac (48.75:200.5:0.75, v/v/v)	1.0	UV @ 280 nm	15	Chocolate
[25]	Bondesil C_{18} (150 mm × 4.0 mm i.d. × 5 μm)	Water:Me(Et)OH:AcH (75:20:5, v/v/v)	0.7	UV @ 273 nm	12	Beverages, chocolate, and human urine
[26]	RP-C_{18} (100 mm × 3.2 mm i.d. × 3 μm)	MeOH:buffer (pH = 3.5) (10:90, v/v)	0.8	Amperometric (Glassy Carbon)	16	Beverages, tea, coffee, and cocoa
This work	FastGradient® Chromolith (50 mm × 2.0 mm i.d.)	ACN:water (5:95, v/v)	0.5	UV @ 274 nm	5	Soft drinks, tea, coffee, cocoa, herbal products, and pharmaceuticals

The most important of them include stimulation of the nervous system and heart [10], increase of the metabolism rate [11], diuresis [12] and control of asthmatic manifestations [13]. However, excessive intake of such compounds has been associated to several negative side effects ranging from simple tremor and tachycardia to cancer and death.

From an analytical point of view, the majority of the methods reporting the determination of caffeine, theobromine and theophylline are based on reversed phase liquid chromatography using particulate-based columns [14–26]. A more detailed overview of these methods including columns types, chromatographic conditions and analysis time can be seen in Table 1. Most of the HPLC methods employ UV detection, while the separation cycles typically last between 7 and 22 min. In a recent study, Zambonin et al. used a narrow-bore particulate C_{18} column (250 mm × 2.1 mm i.d.) for the separation and determination of methylxanthines in urine [20]. However, using a flow rate of 0.2 mL min^{-1} and a volume fraction of 20% MeOH in the mobile phase, the separation cycle was not completed in less than 22 min.

Alternative separation-based analytical approaches for the determination of methylxanthines include capillary electrophoresis-based techniques [27,28]. Such techniques are advantageous in terms of background electrolyte consumption and usage of aqueous buffers. Although in the CZE-UV method proposed by Regan and Shakalisava the separation is completed within 2 min, several washing steps lasting 12 min are required between-injections, corresponding to an overall analysis time of 14 min [27]. Similar analysis duration (ca. 13 min) was achieved by MEKC coupled to amperometric detection and application to pharmaceuticals [28]. Amperometric detection was proven more sensitive with the LODs being in the range of 0.180–0.390 mg L^{-1} [28] versus 1.9–2.5 mg L^{-1} in CZE-UV [27]. In both CE modes the sensitivity is significantly lower compared to the proposed HPLC method. Comparable sensitivity to the present study was achieved when cation or anion exchange chromatography coupled to UV detection was employed [29]. However, in both separation mechanisms the separation cycles lasted approximately 12 min.

On the other hand, non-separation-based methods typically employ more simple and widespread instrumentation. On this basis, caffeine and theobromine can be determined simultaneously in coffees and teas by UV spectrophotometry after suitable chemometrical processing [30]. However, a more time-consuming sample preparation protocol is required involving several steps – addition of lead acetate/filtration/addition of sodium carbonate/filtration/dilution – to remove potential interfering compounds. Paper substrate room temperature phosphorescence (RTP) using KI/NaAc as heavy atom perturber [31] and anodic voltammetry using boron-doped diamond electrodes (BDD) [32] could be interesting alternatives only when a single analyte has to be determined since these approaches do not allow simultaneous analysis of the three methylxanthines in real samples.

The present study proposes a new HPLC method for the simultaneous determination of the three major methylxanthines – caffeine, theobromine and theophylline – in various real samples, using the new FastGradient® narrow-bore monolithic column. The developed analytical scheme is faster and more economical in terms of organic solvents consumption than previously reported RP-HPLC [14–26] and IC [29] methods and faster and more sensitive than CE-based separations [27,28]. Compared to non-separation protocols it offers simpler sample preparation [30] and the capability of the simultaneous determination of all analytes in a single run [31,32]. The developed assay was optimized and thoroughly validated for application to a wide variety of sample matrixes including coffee, tea, soft drinks, herbal beverages, cocoa, chocolate and pharmaceuticals.

2. Experimental

2.1. Reagents

HPLC grade acetonitrile (ACN) used in the mobile phase was provided by Merck (Darmstadt, Germany) and ultra-pure water was produced by a Millipore system. Caffeine (CF), theophylline (TP) and theobromine (TB) were all provided by Sigma (St. Louis, MO, USA). 1000 mg L^{-1} standard stock solutions of CF and TP and 250 mg L^{-1} of TB were prepared in water and stored under refrigeration and protected from light. Working solutions were prepared daily, also in water by appropriate dilutions. The standard stock solutions were found to be stable for at least 1 week.

2.2. Instrumentation

The HPLC setup comprised the following parts: a AS3000 autosampler including a column oven (Thermo Scientific); a LC9A binary pump (Shimadzu); a SPD10 UV-Vis detector (Shimadzu) equipped with a 2.4 μL flow cell and an Elite™ vacuum degasser (Alltech). Chromatographic parameters (peak areas, retention times, theoretical plates, etc.) were calculated via the Clarity® software (DataApex). The FastGradient® reversed phase monolithic column (50 mm × 2.0 mm i.d., Chromolith, Merck) was employed throughout this study. The Performance® monolithic column (100 mm × 4.6 mm i.d., Chromolith, Merck) and a Hypersil ODS particulate reversed phase column (150 mm × 4.6 mm i.d., 5 μm, MZ Analysentechnik) were also used for comparative purposes. PEEK tubing (0.18 mm i.d.) was used for all connections.

2.3. HPLC procedure

Samples and standards were injected in the monolithic column via the autosampler of the HPLC instrument (V = 5 μL in all cases). A mixture of ACN/water (5:95, v/v) was used as the mobile phase while the flow rate was set at 0.5 mL min^{-1} at ambient temperature. All analytes were detected at 274 nm. Under the above-mentioned conditions the analysis cycle was completed in 5 min. Peak area was used for signals evaluation, while each sample or standard was injected in triplicate.

2.4. Sample preparation

All real samples were purchased from the local market. It should be noted that three sub-samples were individually prepared for each sample in all cases.

Solid samples were ground to a fine powder where necessary. Accurately weighed amounts of ca. 20 mg (and ca. 100 mg for the tea samples) were dispersed in 10 mL of water and sonicated for 30 min at 80 °C. Suitable volumes of the resulting solutions were filtered through 0.45 μm nylon syringe filters (Whatman®), diluted with water (1:10) and injected without further pretreatment to the HPLC system.

Beverage samples were degassed under vacuum followed by sonication for 30 min until all air was removed. The samples were diluted 1:10 in water, filtered through 0.45 μm syringe filters and injected in the monolithic column without further preparation.

Twenty tablets of the theophylline-containing pharmaceutical formulation (Theodur® tabs, 200 mg/tab, LaviPharm, Greece) were ground to a fine powder. Accurately weighed amounts were dispersed in water under ultrasonication for 30 min. The resulting solutions were analyzed after filtration through 0.45 μm syringe filters.

It should be noted that in order to investigate and exclude the potential existence of late-eluted compounds, the first chro-

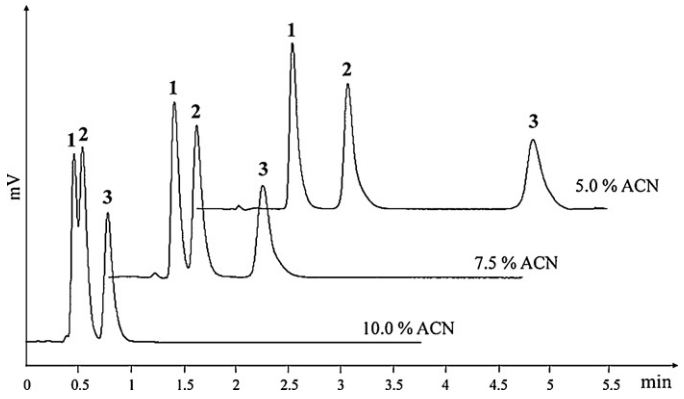

Fig. 1. Effect of the volume fraction of acetonitrile on the separation efficiency of TB, TP and CF: $Q_V = 0.5\ mL\ min^{-1}$, $V = 5\ \mu L$, $\lambda_{max} = 274\ nm$, $T = 25\ ^\circ C$.

matogram of each real sample was monitored for 60 min.

3. Results and discussion

3.1. Development of chromatographic conditions

As can be seen in Fig. 1, the volume fraction of ACN in the mobile phase has the most significant impact on the separation of the methylxanthines analytes. Using 10% ACN at a flow rate of $0.5\ mL\ min^{-1}$ all compounds were eluted in less than 1 min. However, poor resolution was achieved between TB and TP ($R_S < 0.6$) and moderate resolution between TP and CF ($R_S = 1.63$). The TB–TP resolution was improved significantly ($R_S = 1.40$) using 7.5% ACN with the separation cycle being completed in 2 min. The best compromise in terms of separation efficiency and analysis time was achieved using a mobile phase of 5% ACN in water. The resolution factor between TB and TP was 3.45 and between TP and CF 7.64, while the analysis time was quite satisfactory being 5 min.

The flow rate of the mobile phase in the range of $0.4–0.6\ mL\ min^{-1}$, as expected, did not have any practical effect on the separation efficiency of the procedure (Fig. 2). A flow rate of $0.5\ mL\ min^{-1}$ was selected for subsequent studies since it offered a satisfactory analysis time of 5 min at low mobile phase consumption.

The sample injection volume is a parameter that has to be taken into account when using narrow-bore columns, in order to avoid column overloading and therefore poor separation efficiency [33]. On this basis, the effect of the sample volume was examined in the range of 3–10 μL. No significant variation in the resolutions of the analytes was observed within the studied range. The value of 5 μL was selected for further studies.

Under the above-mentioned conditions, the performance of the FastGradient® monolithic column was compared to a Hypersil ODS particulate reversed phase column (150 mm × 4.6 mm i.d., 5 μm) and the Performance Chromolith monolithic column (100 mm × 4.6 mm i.d.). In all cases the mobile phase was ACN/water (5:95, v/v). The particulate column was operated at the "usual" flow rate of $1.0\ mL\ min^{-1}$, while the performance monolithic column was operated at $2.6\ mL\ min^{-1}$ in order to match the linear velocity of the mobile phase as it was set for the FastGradient® column [34]. Typical chromatograms of a standard mixture of the analytes ($10\ mg\ L^{-1}$ each) are shown in Fig. 3, while the performance characteristics of the columns can be seen in Table 2. The two monolithic columns showed similar performance in most cases. However, the FastGradient® column offers a 5-fold lower consumption in the mobile phase that is a critical advantage in routine applications. The particulate column has superior features in terms of peak symmetry and theoretical plates (per column meter). However, the separation cycle is completed in 30 min at a flow rate of $1.0\ mL\ min^{-1}$. This is a major drawback compared to the FastGradient® column which offers 6-fold less analysis time at half the flow rate of the particulate column.

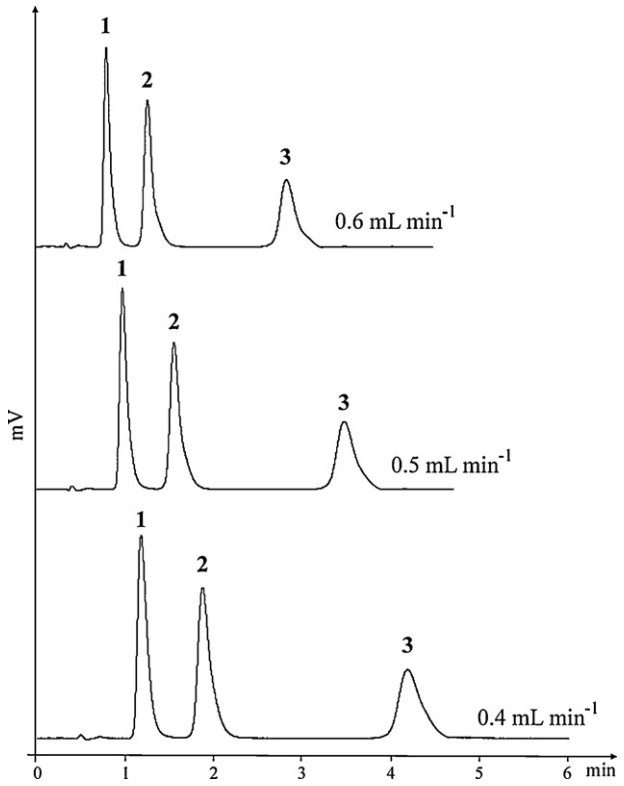

Fig. 2. Effect of the mobile phase flow rate on the separation of TB, TP and CF: $\varphi(ACN) = 5\%$, $V = 5\ \mu L$, $\lambda_{max} = 274\ nm$, $T = 25\ ^\circ C$.

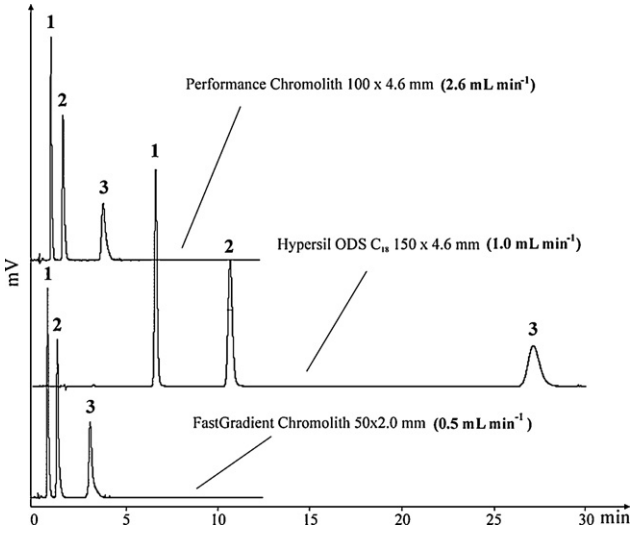

Fig. 3. Comparative chromatograms of three reversed phase columns for the separation of TB, TP and CF: for experimental details see Section 3.1.

3.2. Method validation

The developed HPLC assay method was validated in terms of linearity, limit of detection and quantitation, within and day-to-day precision, accuracy and ruggedness.

Table 2
Columns' performance for the separation of methylxanthines.

Column	Retention time, t_R (min)			Width (0.5) (min)			Symmetry/tailing			Resolution, R_s		Theoretical plates, N (per meter)		
	TB	TP	CF	TB	TP	CF	TB	TP	CF	TB–TP	TP–CF	TB	TP	CF
FastGradient® Chromolith (mm50 × 2.0 mm i.d.)[a]	0.91	1.43	3.20	0.080	0.100	0.173	1.68	1.72	1.77	3.45	7.64	14,232	22,763	37,843
Performance Chromolith (100 mm × 4.6 mm i.d.)[b]	1.29	1.92	4.08	0.063	0.097	0.227	1.63	1.58	1.44	4.75	7.88	18,390	21,780	17,950
Hypersil ODS C_{18} (150 mm × 4.6 mm i.d., 5 μm)[c]	6.68	10.72	27.19	0.150	0.253	0.693	1.18	1.18	1.27	11.82	20.53	73,247	66,133	56,814

[a] Q = 0.5 mL min^{-1}/5% ACN in water/V = 5 μL.
[b] Q = 2.6 mL min^{-1}/5% ACN in water/V = 10 μL.
[c] Q = 1.0 mL min^{-1}/5% ACN in water/V = 20 μL.

Table 3
Accuracy of the proposed HPLC method.

Sample	Caffeine		Theobromine		Theophylline	
	Added (mg L^{-1})	Recovery (%)	Added (mg L^{-1})	Recovery (%)	Added (mg L^{-1})	Recovery (%)
Cola drink 1	10.0	98.2	10.0	99.3	10.0	103.5
	20.0	98.6	20.0	102.2	20.0	96.7
	50.0	101.3	50.0	102.5	50.0	96.9
Cola drink 2	10.0	99.3	10.0	101.8	10.0	97.5
	20.0	103.5	20.0	102.0	20.0	99.2
	50.0	102.4	50.0	102.3	50.0	100.7
Instant coffee	10.0	102.5	10.0	100.6	10.0	98.3
	20.0	103.8	20.0	101.9	20.0	104.5
	50.0	103.1	50.0	97.0	50.0	98.4
Filter coffee	10.0	97.1	10.0	98.6	10.0	103.5
	20.0	96.9	20.0	103.8	20.0	97.2
	50.0	98.3	50.0	102.9	50.0	104.9
Tea	10.0	96.2	10.0	97.2	10.0	103.4
	20.0	96.8	20.0	96.3	20.0	102.7
	50.0	104.1	50.0	98.7	50.0	97.4
Cocoa	10.0	99.4	10.0	101.7	10.0	98.4
	20.0	100.7	20.0	104.3	20.0	99.4
	50.0	101.2	50.0	103.8	50.0	100.8
Chocolate	10.0	98.6	10.0	96.9	10.0	98.0
	20.0	103.5	20.0	98.0	20.0	103.2
	50.0	101.8	50.0	103.8	50.0	101.7
Theodur® tabs (200 mg TP/tab)	10.0	100.8	10.0	99.2	10.0	100.7
	20.0	98.8	20.0	101.3	20.0	101.4
	50.0	100.4	50.0	99.5	50.0	98.8

Table 4
Ruggedness study of the proposed method.

Chromatographic conditions	Recovery (%)[a] (at 10.0 mg L^{-1})			Retention time, t_R (min)			Resolution, R_s		Theoretical plates, N (per meter)		
	TB	TP	CF	TB	TP	CF	TB–TP	TP–CF	TB	TP	CF
Optimal conditions[b]	99.2	99.6	100.5	0.91	1.43	3.20	3.45	7.64	14,232	22,763	37,843
Effect of flow rate of the mobile phase											
Q = 0.48 mL min^{-1}	103.6	103.5	102.9	0.95	1.50	3.33	3.42	7.64	13,220	23,244	37,921
Q = 0.52 mL min^{-1}	97.7	97.1	97.4	0.87	1.38	3.07	3.52	7.69	14,378	24,223	37,676
Effect of mobile phase (*ACN*:*water*)											
4.8:95.2 (v/v)	101.4	101.8	98.4	0.96	1.52	3.43	3.24	7.35	11,641	20,018	34,943
5.2:94.8 (v/v)	100.1	100.0	99.7	0.85	1.33	2.93	3.21	7.34	12,607	21,080	37,157
Effect of injection volume											
V = 4.8 μL	96.4	96.1	96.8	0.91	1.45	3.21	3.43	7.63	13,309	23,189	38,079
V = 5.2 μL	104.1	103.8	103.5	0.90	1.42	3.18	3.41	7.57	14,127	22,447	37,215
Effect of detection wavelength											
λ = 270 nm	97.9	100.2	97.2	0.91	1.44	3.21	3.52	7.70	14,336	24,474	38,079
λ = 278 nm	96.1	96.7	96.6	0.91	1.44	3.23	3.41	7.70	13,809	24,082	38,396

[a] Percent recovery using the calibration curve obtained under optimal conditions (mean of three injections).
[b] Q = 0.5 mL min^{-1}, ACN:water = 5:95 (v/v), V = 5 μL, λ_{max} = 274 nm.

Table 5
Analysis of real samples.

Sample	Found by the proposed method (±SD)		
	Caffeine	Theobromine	Theophylline
Cola drink 1	123.7 (±1.4) mg L^{-1}	–	–
Cola drink 2	116.5 (±1.6) mg L^{-1}	–	–
Sprite®	–	–	–
Seven-up®	–	–	–
Instant coffee	37.9 (±0.4) mg g^{-1}	2.3 (±0.06) mg g^{-1}	–
Filter coffee	16.1 (±0.2) mg g^{-1}	1.6 (±0.07) mg g^{-1}	–
Herbal beverage	–	–	–
Tea	23.8 (±0.5) mg g^{-1}	1.8 (±0.07) mg g^{-1}	–
Cocoa	1.05 (±0.09) mg g^{-1}	18.5 (±0.4) mg g^{-1}	–
Chocolate	–	5.5 (±0.1) mg g^{-1}	–
Theodur® tabs (200 mg/tab)	–	–	203.5 (±1.2) mg/tab

3.2.1. Linearity, LOD and LOQ

Linearity was evaluated in the range of 1.0–100.0 mg L^{-1} for all analytes. Peak area was used for signals evaluation. The obtained regression equations ($n = 8$) for CF, TP and TB were respectively:

$$A = -2.244(\pm 2.739) + 26.935(\pm 0.053) \times \gamma(\mathrm{CF}) \quad [r^2 = 0.9999]$$

$$A = -4.241(\pm 6.652) + 30.845(\pm 0.130) \times \gamma(TP) \quad [r^2 = 0.9999]$$

$$A = -3.794(\pm 4.274) + 30.287(\pm 0.084) \times \gamma(TB) \quad [r^2 = 0.9999]$$

A is the peak area as integrated by the Clarity software and γ is the mass concentration of the analytes in mg L^{-1}. The validity of the regression lines was confirmed by the residuals approach. In all cases the percent residuals were distributed randomly around the "zero" line, while they ranged between −1.05 and +0.79% for CF, −2.47 and +3.50% for TP and −1.39 and +1.36% for TB.

The detection (LOD) and quantitation limits (LOQ) of the HPLC method were determined based on the signal-to-noise (S/N) criteria. The LOD values (S/N = 3) were found to be 0.150 mg L^{-1} for CF, 0.075 mg L^{-1} for TP and 0.055 mg L^{-1} for TB. In a similar manner, the LOQ values (S/N = 10) were found to be 0.510 mg L^{-1} for CF, 0.250 mg L^{-1} for TP and 0.180 mg L^{-1} for TB.

3.2.2. Precision of the method

In order to evaluate the repeatability (within-day precision) of the method mixtures of the analytes were injected at three concentration levels for eight replicates each: 10 mg L^{-1} (low level), 50 mg L^{-1} (middle level) and 100 mg L^{-1} (upper level). The calculated relative standard deviations (RSDs) were: 1.1% for CF, 1.3% for TP and 1.3% for TB at 10 mg L^{-1}, 1.0% for CF, 0.8% for TP and 1.1% for TB at 50 mg L^{-1} and 0.8% for CF, 1.0% for TP and 0.9% for TB at 100 mg L^{-1} ($n = 8$ in all cases). The RSDs of the retention times were less than 0.5% for all analytes and concentration levels.

The reproducibility (day-to-day precision) of the proposed HPLC method was validated by constructing calibration curves for the three analytes over a period of six consecutive days (0–100 mg L^{-1} CF, TP and TB, 6 calibration curves × 6 concentration levels). Fresh standards were used in all cases. The relative standard deviations of the slopes of the calibration curves were 2.1% for CF, 2.7% for TP and 2.2% for TB verifying the day-to-day precision of the assay.

3.2.3. Accuracy of the method

The accuracy of the assay was validated by spiking the real samples with known amounts of the analytes at three concentration levels, namely 10, 20 and 50 mg L^{-1}. The pretreatment of the samples prior to spiking was the same as described in Section 2.4. The experimental results are shown in detail in Table 3. In brief, the recoveries were satisfactory in all cases and ranged as follows: 98.2–103.5% for CF, 99.3–102.5 for TB and 96.9–103.5 for TP in cola drinks; 96.9–103.8% for CF, 97.0–103.8 for TB and 97.2–104.9 for TP in coffees; 96.2–104.1% for CF, 96.3–98.7 for TB and 97.4–103.4 for TP in tea; 98.6–103.5% for CF, 96.9–104.3 for TB and 98.0–103.2 for TP in cocoa and chocolate samples and 98.8–100.8% for CF, 99.2–101.3 for TB and 98.8–101.4 for TP in a pharmaceutical formulation. The slopes of the standard addition regression lines were compared statistically to the ones for the aqueous solutions mentioned in Section 3.2.1. Based on the fact that no statistical differences were observed (t-test) either approaches can be used for real samples analysis.

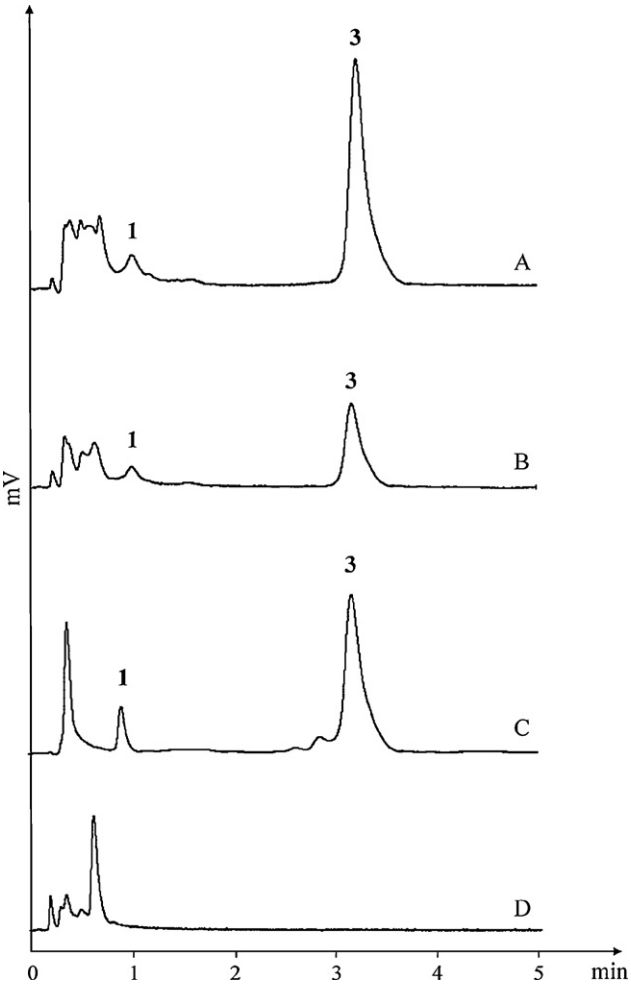

Fig. 4. Typical chromatograms from the analysis of (A) instant coffee, (B) filter coffee, (C) tea, and (D) herbal beverage samples.

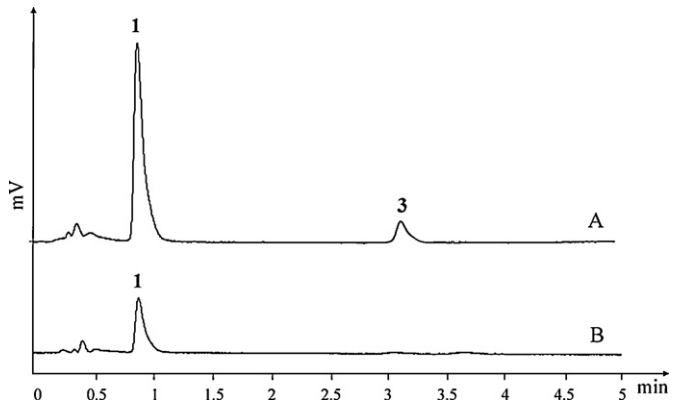

Fig. 5. Typical chromatograms from the analysis of (A) cocoa and (B) chocolate samples.

3.2.4. Ruggedness of the method

Ruggedness is an important parameter of an analytical method and provides is an indication of the ability of a method to remain unaffected by variations of critical chemical and instrumental variables. On this basis, the flow rate of the mobile phase, the volume fraction of acetonitrile and the injection volume were varied deliberately in the range of ±4% of their selected values, while the detection wavelength was varied for ±4 nm (270–278 nm). The percent recoveries of a mixture of the analytes at the 10 mg L^{-1} level were calculated in each case using calibration curves constructed under the optimal conditions. The acceptance limit was set within a range of 95.0–105%. The experimental results are shown in Table 4. The findings verified the ruggedness of the procedure since the percent recoveries were within the specified ±5% range in all cases. Table 4 also contains the effect of these variations on other important chromatographic parameters such as the retention times, the resolution factors and the numbers of theoretical plates as well.

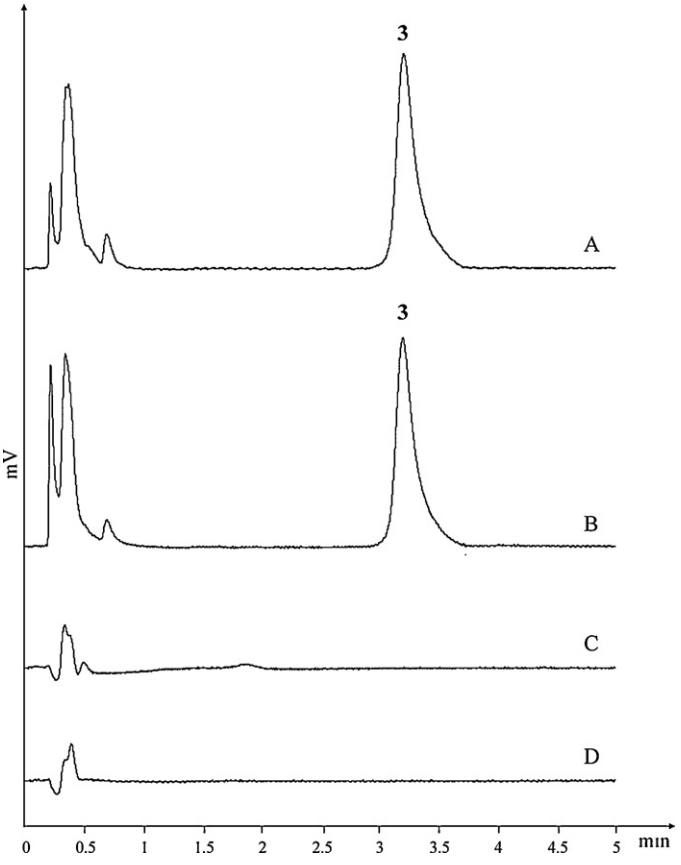

Fig. 6. Typical chromatograms from the analysis of (A) cola drink (B) diet cola drink, (C) Seven-up®, and (D) Sprite®.

3.3. Analysis of real samples

The applicability of the developed method was demonstrated by the analysis of a variety of real samples, including two cola drinks, two soft drinks (Sprite® and Seven-up®), instant and filter coffees, tea, a herbal beverage, instant cocoa and chocolate and a TP-containing pharmaceutical (Theodur® tabs, 200 mg TP/tab). The results are summarized in Table 5. Caffeine was solely detected in the cola drinks at values in good agreement with previous reported methods [14,35,36]. As expected, none of the methylxanthines was present in Sprite® and Seven-up® and in the herbal beverage as well. Both theobromine and caffeine were found to be present in the coffee, tea and instant cocoa samples, while only theobromine was determined in the chocolate sample. Finally, as expected, theophylline was present and determined in the pharmaceutical formulation (it should be noted that no interfering peaks were observed by a placebo mixture of common pharmaceutical excipients). Although some previous studies report the presence of low levels of theophylline in certain tea samples as well, this analyte was not detected by our method in the selected tea samples. Representative chromatograms of the analyzed samples can be seen in Figs. 4–6.

4. Conclusions

The present study demonstrates the advantages of the novel FastGradient® narrow-bore monolithic column for the separation and determination of the three main methylxanthines – theobromine, caffeine and theophylline – in real samples. Separation–determination of the analytes was completed in less than 5 min at a low flow rate of 0.5 mL min^{-1}. The developed method is simple, isocratic, robust and was fully validated for the analysis of a variety of samples including soft drinks, beverages, herbal products and pharmaceuticals. Compared to previous reported RP-HPLC procedures using conventional particulate-based columns the proposed method offers shorter analysis time and drastic reduction in the consumption of the mobile phase and organic solvents. The FastGradient® monolithic column has similar performance to the well-established Performance Chromolith monolithic column but with a 5-fold less consumption of mobile phase. The interesting analytical features of this new column make it ideal for process applications under industrial environment.

References

[1] A.C. Mann, LC-GC Eur. 20 (2007) 290.
[2] C. Zhu, D.M. Goodall, S.A.C. Wren, LC-GC N. Am. 23 (2005) 1.
[3] J.W. Thompson, J.S. Mellors, J.W. Eschelbach, J.W. Jorgenson, LC-GC N. Am. 24 (2006) 16.
[4] J.J. Salisbury, J. Chromatogr. Sci. 46 (2008) 883.
[5] I. Ali, V.D. Gaitonde, H.Y. Aboul-Enein, J. Chromatogr. Sci. 47 (2009) 432.
[6] US Pharmacopoeia 29 (Suppl. 1) (2006).
[7] Chromolith® FastGradient RP-18 Endcapped 50-2 mm, Merck KGaA, Darmstadt, Germany, 2008, www.merck-chemicals.com.
[8] S. Fekete, J. Fekete, K. Ganzler, J. Pharm. Biomed. Anal. 50 (2009) 703.
[9] L. Spoof, M.R. Neffling, J. Meriluoto, Toxicon (2009), in press, doi:10.1016/j.toxicon.2009.06.015.
[10] K.A. Georga, V.F. Samanidou, I.N. Papadoyannis, J. Liq. Chromatogr. Relat. Technol. 23 (2000) 1523.
[11] M.A. Ford, in: P.R. Ashurst (Ed.), Production and Packaging of Non-Carbonated Fruit Juices and Fruit Beverages, Blackie Academic and Professional, London, 1995, pp. 310–330.
[12] B. Stavric, Food Chem. Toxicol. 26 (1988) 541.
[13] I.N. Papadoyannis, V.F. Samanidou, K.A. Georga, J. Liq. Chromatogr. Relat. Technol. 19 (1996) 2559.

[14] B. Srdjenovic, V. Djordjevic-Milic, N. Grujic, R. Injac, Z. Lepojevic, J. Chromatogr. Sci. 46 (2008) 144.
[15] M.R. Brunetto, L. Gutierrez, Y. Delgado, M. Callignani, A. Zambrano, A. Gomez, G. Ramoz, C. Romero, Food Chem. 100 (2007) 459.
[16] F. Lo Coco, F. Lanuzza, G. Micali, G. Cappellano, J. Chromatogr. Sci. 45 (2007) 273.
[17] A. Aresta, F. Palmisano, C.G. Zambonin, Food Chem. 93 (2005) 177.
[18] N.M. de Aragao, M.C.C. Veloso, M.S. Bispo, S.L.C. Ferreira, J.B. de Andrade, Talanta 67 (2005) 1007.
[19] E. Marchei, M. Pellegrini, R. Pacifici, I. Palmi, S. Pichini, J. Pharm. Biomed. Anal. 37 (2005) 499.
[20] C.G. Zambonin, A. Aresta, F. Palmisano, J. Pharm. Biomed. Anal. 36 (2004) 621.
[21] Y. Fan, Y.Q. Feng, S.L. Da, Z.G. Shi, Anal. Chim. Acta 523 (2004) 251.
[22] J.B. Thomas, J.H. Yen, M.M. Schantz, B.J. Porter, K.E. Sharpless, J. Agric. Food Chem. 52 (2004) 3259.
[23] S. Emara, Biomed. Chromatogr. 18 (2004) 479.
[24] O. Tokusoglu, M. Kemal Unal, Eur. Food Res. Technol. 215 (2002) 340.
[25] M.S. Bispo, M.C.C. Veloso, H.L.C. Pinheiro, R.F.S. De Oliveira, J.O.N. Reis, J.B. De Andrade, J. Chromatogr. Sci. 40 (2002) 45.
[26] A. Meyer, T. Ngiruwonsanga, G. Henze, Fresenius J. Anal. Chem. 356 (1996) 284.
[27] F. Regan, Y. Shakalisava, Anal. Chim. Acta 540 (2005) 103.
[28] A. Wang, L. Li, F. Zang, Y. Fang, Anal. Chim. Acta 419 (2000) 235.
[29] Q.C. Chen, S.F. Mou, X.P. Hou, Z.M. Ni, Anal. Chim. Acta 371 (1998) 287.
[30] L. Lopez-Martinez, P.L. Lopez-de-Alba, R. Garcia-Campos, L.M. De Leon-Rodriguez, Anal. Chim. Acta 493 (2003) 83.
[31] D. Chuan, W. Yan-Li, S. Shao-Min, Spectrochim. Acta A 59 (2003) 1469.
[32] N. Spataru, B.V. Sarada, D.A. Tryk, A. Fujishima, Electroanalysis 14 (2002) 721.
[33] Y. Yang, C.C. Hodges, LC-GC N. Am. 23 (2005) 31.
[34] D. Guillarme, D.T.T. Nguygen, S. Rudaz, J.L. Veuthey, Eur. J. Pharm. Biopharm. 66 (2007) 475.
[35] P.D. Tzanavaras, D.G. Themelis, Anal. Chim. Acta 581 (2007) 89.
[36] R. Injac, B. Srdjenovic, M. Prijatelj, M. Boskovic, K. Karljikovic-Rajic, B. Strukelj, J. Chromatogr. Sci. 46 (2008) 137.