J Sci Food Agric 85:622–628 (2005)
DOI: 10.1002/jsfa.2020

Micellar electrokinetic capillary chromatography of methylxanthines-containing beverages: discussion of the molecular species involved

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Abstract: Micellar electrokinetic capillary chromatography (MECC) experimental conditions were applied to 12 samples of methylxanthine-containing infusions of different commercial brands of *yerba mate*, coffee, tea and cocoa as well as two cola drinks. The best resolution in this mode of automated high-performance capillary electrophoresis (HPCE) was achieved here when using 15 kV voltage in an uncoated fused-silica capillary of 45 cm length (40 cm effective length), 50 mM sodium dodecylsulfate, 90 mM pH 8.5 borate buffer and UV detection. Theobromine, caffeine and theophylline were separated, and the peak splitting due to tautomeric species was observed. Experimental conditions were controlled, keeping constant the size of the elution window in each analysis. The limit of detection was less than 1 mg l^{-1}, the limit of quantitation was 2.5 mg l^{-1} and the work range was 2.5–300 mg l^{-1}. This HPCE–MECC system has proved suitable for the analysis/quality control of xanthines in beverages for consumption. Roles of various parameters as well as distinctly charged species of each xanthine and the origin of peak splitting in this MECC system are discussed.

Keywords: automated high-performance capillary electrophoresis (HPCE); micellar electrokinetic capillary chromatography (MECC); beverages; theobromine; theophylline; caffeine; keto–enol tautomerism

INTRODUCTION

Micellar electrokinetic capillary chromatography (MECC)[1–3] is a mode of automated high-performance capillary electrophoresis (HPCE) which offers a combination of unique features of CZE and RP-HPLC such as high efficiency, rapid analysis and versatility of modifying chemical selectivity in the separation process. An optimized HPCE–MECC separation is described of the three current methylxanthines, theobromine (**I**), caffeine (**II**) and theophylline (**III**) (Fig 1), occurring in 12 samples of infusions of *yerba mate*, coffee, cocoa and tea as well as cola drinks.

The intake of these beverages results in a mild stimulant effect on the central nervous system (CNS) and some adverse effects due to the occurrence of caffeine (**II**) as the main constituent, followed by theobromine (**I**) and finally theophylline (**III**). There are many reports[4] on the action of **II** on the CNS, also producing a condition of wakefulness and increased mental activity. The influence of caffeine on sustained attention by an event-related potentials (ERP) study,[5] and the role of dopamine in the locomotor stimulant effects and tolerance to these effects of caffeine[6] have been also reported. Both **I** and **II** and other synthetic nitrogen bridgehead compounds also possess some bronchodilating properties.[7]

There have been several studies on tea and coffee. Comparative antimutagenic and anticlastogenic effects of green tea and black tea have recently been reviewed.[8] Gas chromatographic–mass spectrometric quantitation of 4-(5-methylimidazole) in roasted coffee was performed after ion-pair extraction.[9]

HPLC has been the preferred technique for evaluation of the caffeine content of various foods and drugs.[10] Caffeine and theobromine were studied in epicuticular wax of *yerba mate* (*Ilex paraguariensis* St Hil).[11] Standards of caffeine and its metabolites, caffeine in four beverages, and caffeine and acetaminophen in four pharmaceuticals were analysed by MECC[12] using pH 11.0 phosphate buffer and sodium dodecyl sulfate (SDS).

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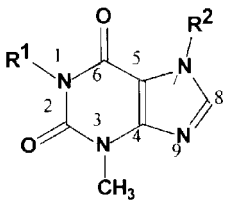
(*Received 21 March 2003; revised version received 28 June 2004; accepted 24 September 2004*)
Published online 26 November 2004



Theobromine (I): $R^1 = H$; $R^2 = CH_3$

Caffeine (II): $R^1 = CH_3$; $R^2 = CH_3$

Theophylline (III): $R^1 = CH_3$; $R^2 = H$

Figure 1. Chemical structures of compounds **I**, **II** and **III**.

We have recently reported[13] the HPCE analysis of *mate* infusions from stems and leaves of *Ilex paraguariensis*, and *yerba mate* of several brands, using MECC for comparison purposes. The remaining solids from such infusions after using sieves of different mesh have been also analysed.[13]

The aim of this work was to obtain electropherograms of theobromine (**I**), caffeine (**II**) and theophylline (**III**) in a short analysis time. However, under such experimental MECC conditions we found a splitting of the peaks. Analysis of the MECC experimental conditions showed that different degrees of ionization and a keto-enol tautomerism of each xanthine were taking place. In fact, dissociation equilibria and keto-enol tautomerism by micellar capillary electrophoresis of another family of compounds, the 4,6-diamino-*s*-triazines, have been previously reported.[14]

In this paper we describe the origin of the peak splitting due to the occurrence of the three methylxanthines in distinct ionized and keto-enol forms, and the partition between the micelle and the bulk aqueous phase, which had not been previously reported.

EXPERIMENTAL

Chemicals

Caffeine, theobromine, theophylline, quinine hydrochloride and SDS were purchased from Sigma (St Louis, MO, USA). HPLC-grade solvents and twice-distilled MilliQ® deionized water were used.

Preparation of the running buffer

Running buffer was freshly prepared prior to use. Boric acid was weighed and dissolved in deionised water, and pH was adjusted with 0.1 M NaOH. Then, the buffer was filtered through a 0.45 μm Millipore® filter, and pH was adjusted after the addition of SDS. All sample solutions were prepared with deionized water and further filtered through a 0.22 μm Millipore® filter.

Instrumental

A Bio-Rad BioFocus® 3000 Automated High-Performance Capillary Electrophoresis System (Bio-Rad Instruments Inc, Hercules, CA, USA) with a diode-array UV detector with a 45 cm length (40 cm effective length × 50 μm id, 400 μm od) uncoated fused-silica capillary and software supplied by Bio-Rad was used. The UV detector was operated at 200 nm. Prior to use for the first time, the capillary was conditioned by washing with 0.1 M NaOH for 5 min, keeping it in this solution for an hour and then washing with deionized water. Between two consecutive analyses, the capillary was sequentially rinsed with 0.1 M NaOH for 2 min, deionized water for 1 min and running buffer for 2 min, in order to improve repeatability and reproducibility of the migration time, peak shape and retention factor (k). This conditioning step is important to ensure that the capillary silica internal surface is uniformly charged. Samples were introduced by an injection at 138 mbar s using the built-in injection system at 25 °C. The same temperature was setup for the capillary.

MECC running conditions

Voltage was +15 kV, injection 138 mbar s, temperature 25 °C and UV detection $\lambda = 200$ nm. The preparation cycles were: first, 0.1 M NaOH (2 min), second, H_2O (1 min) and third, running buffer (2 min).

Preparation of samples

Infusions were prepared as follows: 100 ml of deionized water were added to the required amount of the corresponding dried material and further heated under stirring at 75 °C in a water-bath for 5 min. The prepared infusion was filtered *in vacuo* and passed through a LiChrolut RP_{18} (Merck, Darmstadt, Germany) cartridge.

Sample dilutions were carried out with diluted running buffer (1:10), pH was adjusted to 8.5 and SDS was added to achieve a 50 mM concentration.

Samples of yerba mate

Aliquots of 10.00 g of powdered material were used and worked up as above. After filtration, solutions were diluted to a 1:125 ratio.

Samples of coffee

Aliquots of 4.00 g of milled coffee and 1.00 g of soluble coffee and of soluble decaffeinated coffee were used, and worked up as above. After filtration, solutions were diluted to 1:5 and 1:10, respectively. Decaffeinated soluble coffee was diluted to 1:2.

Samples of cocoa

Aliquots of 1.00 g of powdered cocoa were used, and worked up as above. After filtration solutions were diluted to a 1:2 ratio.

Samples of tea

Aliquots of 1.00 g of tea were used, and worked up as above. After filtration solutions were diluted to a 1:2 ratio.

Samples of cola drinks

Aliquots of 100.00 ml of these carbonated beverages were previously degassed by purging with argon and further 1:2 diluted.

External standardization

Linear calibration graphs were obtained from separated standard solutions containing seven different concentrations of caffeine and theobromine: 5, 10, 20, 40, 60, 80 and 100 mg l^{-1} in diluted borate buffer (1:10), respectively. A high correlation ($r = 0.996$) was obtained. Determinations were carried out in triplicate.

Determination of elution window in this SDS–MECC system

The two extremes of the elution window were determined using methanol as the electroosmotic flow (EOF) marker, which does not interact with micelles ($P_{mw} \approx 0$), spends all of the migration time in the bulk aqueous phase, migrates at the electroosmotic mobility and elutes at t_{eo}. Quinine hydrochloride was used as micelle marker, which elutes at t_{mc} and interacts strongly with the micelles ($P_{mw} \approx \infty$). The size of the elution window was kept constant throughout these MECC analyses.

Statistical analysis

The parameters of the method (limit of detection, LOD, limit of quantitation, LOQ, and working range) were determined. A linear regression analysis was used for determining the working range. LOD was determined by triplication of the average standard deviation of 10 independent determinations of standards of caffeine and theobromine approaching the expected detection limit. The repeatability test was evaluated with standards ($n = 9$) and samples ($n = 5$) by carrying out consecutive injections. For the reproducibility evaluation, injections were done over 3 days.

Identification of the methylxanthines in the samples

Peak identification of extracts was achieved by comparing electrophoretic mobilities with those of standards and by spiking samples with the standards.

RESULTS AND DISCUSSION

A MECC separation was selected due to its hydrophobic and polar interactions, partitioning mechanism and electromigration. Accordingly, MECC is suitable for the separation of neutral, ionic and some compounds subjected to ionization, depending on the experimental conditions. Neutral solutes with different micelle–water partition coefficients (P_{mw}) can then be separated. The retention behavior of ionizable compounds, such as methylxanthines, is much more complicated than that of uncharged solutes.

Size of the elution window

In MECC, as is well known, resolution (R_s) for neutral molecules is a function of the retention factor, k, selectivity (α), efficiency (N), and the size of the elution window (t_{mc}/t_{eo}). The elution window limits peak capacity in MECC owing to the fact that these neutral ionizable xanthines are separated between the migration time of an unretained solute (eg $t_{eo} = 3.81$ using methanol) and a fully retained solute (eg $t_{mc} = 11.40$ using quinine hydrochloride). Then, the size of the elution window in all the electropherograms of this paper accounts for $11.40/3.81 = 2.99$.

The optimum k for achieving maximum R_s is the square root of the elution window: $k_{opt} = (t_{mc}/t_{eo})^{1/2}$, and here accounts for $k_{opt} = (2.99)^{1/2} = 1.73$. Better R_s is achieved as the size of the elution window is increased, but the window is large enough here to separate the methylxanthines under study.

Retention factor, selectivity, efficiency and the size of the elution window are influenced by experimental parameters such as type and concentration of the surfactant, type and concentration of the buffer, pH, temperature, ionic strength and applied field strength. These parameters are discussed below.

Voltage

Under our experimental conditions at higher voltages than 20 kV, the migration time of caffeine is too short and the R_s of the other xanthines decreases. An applied voltage of 15 kV provided good R_s of the three xanthines within 6 min, showing efficiencies typical for HPCE.

Surfactant

MECC can be viewed as a chromatographic mode with migrating charged organized micelles acting as a pseudostationary phase. The chemical nature of the latter plays an important role on R_s. Variations in the hydrophobic moiety, the ionic head group and/or the type of counterion can influence retention, selectivity, the size of elution window and efficiency in MECC. The long alkyl-chain anionic surfactant SDS was chosen in the present study, and is also the most widely used, due to its high aqueous solubility, low critical micelle concentration (CMC = 8.1 mM in pure water at 25 °C), low Kraft point (K_p 16), small UV molar absorptivity even at low wavelengths, and availability.[15,16] Above this CMC, SDS forms micelles that are in dynamic equilibrium with the monomers in the bulk aqueous solution. Other surfactants could have been also chosen instead of SDS, eg sodium cholate (SC). However, SDS micelles are stronger hydrogen-bonding donors (HBDs), whereas SC micelles are stronger hydrogen-bonding acceptors (HBAs). Therefore, it can be expected that HBA solutes, such as xanthines, would have stronger interaction with SDS micelles than with SC micelles.

Effect of surfactant concentration

The primary role of SDS concentration is to adjust k within an optimum range in order to achieve the best R_s.

At low micelle concentrations there is a linear relationship between k and SDS concentration (C_{sf}):

$$k = P_{mw}\nu(C_{sf} - CMC) \backsimeq P_{mw}(V_{mc}/V_{aq})$$

where ν is surfactant molar volume ($0.25\,l\,mol^{-1}$ for SDS),[14] P_{mw} is the partition coefficient of a solute between aqueous phase and micelles, V_{mc} is the micellar volume and V_{aq} is the aqueous phase volume.

Then, increasing SDS concentration results in increasing k for **I** and **II** because of increasing the partition into the micelles, especially in the latter (Table 1).

The SDS concentration may also modify the size of the elution window and the efficiency. However, it has little if any effect on selectivity (α), which is the ratio of k_{II} and k_I, and is nearly equal to the ratio of each P_{mw} at low SDS concentration:

$$\alpha = k_{II}/k_I \backsimeq P_{mwII}/P_{mwI}$$

The observed migration time (and therefore analysis time) is a function of k and EOF. Even when using a 60 mM SDS, $R_s = 3.00$ and $\Delta x = 0.78$ were obtained, while a 70 mM SDS gave $R_s = 3.23$ and $\Delta x = 1.05$, the time analysis being larger than 6 min in both cases (Table 1). Therefore, a concentration of 50 mM SDS was selected due to the good R_s and a shorter analysis time.

Buffer concentration

The variation of R_s and migration times of **I** and **II** with buffer concentration is shown in Table 1.

A good separation of **I** and **II** ($\Delta x = 0.67$ min) and the largest R_s value ($R_s = 2.40$) were obtained at pH 8.5 using a 90 mM borate buffer.

Table 1. Variation of resolution and migration times of theobromine and caffeine with pH, buffer borate concentration and SDS concentration[a]

pH	Borate (mM)	SDS (mM)	R_S	Migration time of theobromine (min)	Migration time of caffeine (min)
7.8	80	50	3.10	4.87	5.49
8.1	80	50	2.38	5.03	5.70
8.5	80	50	2.25	5.36	6.23
8.8	80	50	1.91	4.94	5.48
8.5	70	50	2.06	5.26	6.11
8.5	80	50	2.25	5.36	6.23
8.5	90	50	2.40	5.03	5.70
8.5	100	50	2.33	5.05	5.76
8.5	110	50	2.01	8.36	9.37
8.5	90	40	1.34	4.61	5.00
8.5	90	50	2.40	5.03	5.70
8.5	90	60	3.00	5.51	6.29
8.5	90	70	3.23	5.89	6.94

[a] Fixed voltage: 15 kV.

pH of the running buffer

The pH of the run buffer was varied, while keeping a fixed surfactant concentration of 50 mM SDS. The variation of R_s and migration times of theobromine **I** and caffeine **II** with the pH of the buffer is shown in Table 1.

Good separation of **I** and **II** ($\Delta x = 0.62$ min) and the largest R_s value ($R_s = 3.10$) were obtained at pH 7.8 using borate buffer. Likewise, good separation of **I** and **II** ($\Delta x = 0.67$ min) and $R_s = 2.38$ were obtained at pH 8.1 using borate buffer. However, the best separation ($\Delta x = 0.84$ min) was attained at pH 8.5, even with a somewhat small R_s (= 2.25; Table 1).

Order of elution and origin of the peak splitting

Retention in MECC is based on hydrophobic interaction (eg solute–micelle interactions), and hydrogen-bonding interactions (eg solute acceptor–solvent donor). Stronger hydrogen bond acceptor solutes (HBA) would have less interaction with the micelles and therefore will be less retained. Thus, more basic methylxanthines (solutes) would have a stronger interaction with the bulk aqueous media than with the micelles. The bulkier solute but with greater HBA (more basic), caffeine (**II**), elutes slightly earlier than theophilline (**III**).

In addition to the primary partitioning mechanism into micelles, secondary chemical equilibria (SCE) are occurring here in the bulk aqueous solution, eg the acid–base equilibria, and the keto–enol tautomerism.

The methylxanthines analysed here are all subjected to a keto–enol tautomerism, the keto species being usually predominant in the crystalline and/or solid state. However, in the bulk aqueous media outside the micelles, enolates are favoured. These charged forms would migrate in the bulk aqueous media under their own electrophoretic mobility.

Effect of pH

As is well known, anionic micelles migrate in the opposite direction to EOF in the uncoated capillary. Therefore, the normal polarity of the electrodes is used in this HPCE–MECC setup. The EOF velocity is stronger than the electrophoretic velocity of anionic micelles when using an uncoated capillary and pH greater than 6.

Methylxanthines (**I**, **II** and **III**) may be ionic depending on their pK_a. Theobromine (**I**; 3,7-dimethylxanthine; MW = 180.2) has a pK_{a1} of 1.0 and a pK_{a2} of 10.0 at 25 °C, accounting for H-1 and H-8, respectively. At a running pH of 8.5, only H-1 is lost, giving rise to the species **Ia**, which, due to the keto–enol tautomerism, occurs as both **Ib** and **Ic** species (Fig 2). Consequently, the peak of theobromine is split (see electropherograms).

Caffeine (**II**; 1,3,7-trimethylxanthine; 7-methyltheophylline; MW = 194.2) has a pK_a of 12.3, which refers to H-8. Therefore, at a running pH of 8.5 caffeine (**II**), which is mainly subjected to a keto–enol tautomerism, gives rise to both **IIb** and **IIc** enolate

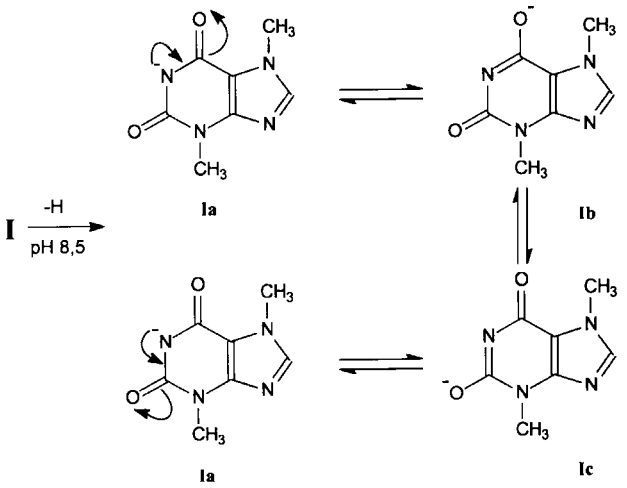


Figure 2. Theobromine species involved at a pH = 8.5 MECC running.

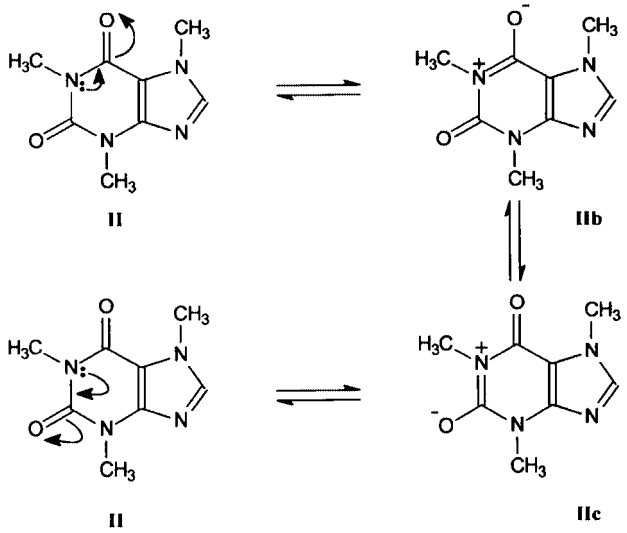


Figure 3. Caffeine species involved at a pH = 8.5 MECC running.

zwitterionic species (Fig 3), and to the subsequent splitting of the main peak (see electropherograms).

Theophylline (**III**; 1,3-dimethylxanthine; MW = 180.2) shows a pK_{a1} of 8.77, a pK_{a2} of 11.5 and a pK_{a3} of 13.5 at 25 °C. In this case ring A remains as a diketo form owing to the fact that at a running pH 8.5, H-7 of N-H of ring B (similar to an imidazol-moiety) is lost, giving rise to an electron delocalisation in the five-membered ring, in which the double bond $\Delta^{4(5)}$ takes place. This prevents the formation of enolates in ring A. The resonance (**III′**) hybrid (Fig 4) is the only ionic species at the run pH = 8.5 in the bulk aqueous phase outside the micelles. The small difference with $pK_{a1} = 8.77$ reveals a probable contribution of the neutral (**III**) species, which is located into the micelle. Actually, the electropherogram shows as expected a sharp peak due to the species (**III′**).

In fact, due to the relevance of hydrophobic interactions, the behaviour of compounds as caffeine resemble that of a neutral molecule. Therefore, for **II** the SDS concentration is certainly more important than pH.

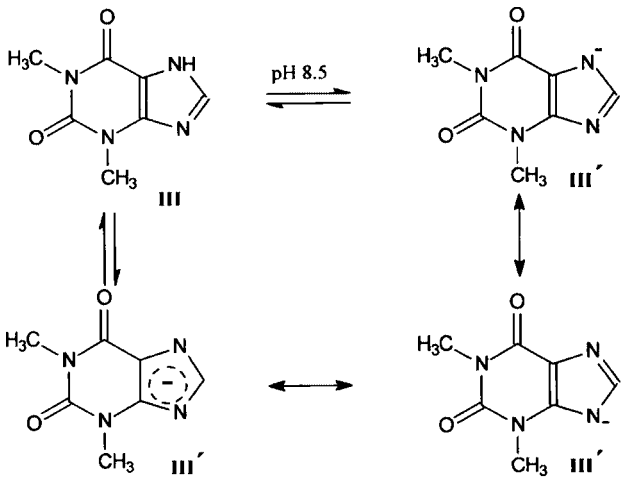


Figure 4. Theophylline species involved at a pH = 8.5 MECC running.

On the other hand, for both theobromine (**I**) and theophylline (**III**) there is first an acid–base equilibria (formation of **Ib-Ic** and **III′**, respectively), pH being thus the main determining factor on the retention compared with SDS concentration at a run pH = 8.5. However, **I** is further affected by a keto–enol tautomerism (species **Ib** and **Ic**) in the bulk aqueous phase outside the SDS micelles. Then, **I** migrates before the others (**II** and **III**) due to the electroosmotic mobility, which is faster than EOF.

Theophylline (**III**) is also affected by a keto–enol tautomerism but in ring B, thus yielding a charged species (**III′**) which is stabilized because of electron delocalization. Furthermore, a *ca* $pK_{a1} = 8.77$ indicates a partial dissociation at a run pH = 8.5, with a major amount of the non-dissociated (**III**) inside the micelle. The latter moves due to EOF toward the cathode opposite to the electroosmotic mobility. That is the reason for its slower migration rate (higher migration time than **I** and **II**), and lower sensitivity when compared with **I** and **II** and in the presence of other constituents, as in the case of beverages.

Consequently, the order of migration time of these methylxanthines is theobromine (**I**), caffeine (**II**) and theophylline (**III**).

Electropherograms

A typical electropherogram of theobromine (**I**) and caffeine (**II**) under these optimized separation conditions (90 mM sodium borate buffer, pH 8.5, containing 50 mM SDS, a 45 cm × 50 μm i.d. capillary with 40 cm to the detection window, and an applied voltage of 15 kV) is shown in Fig 5. An electropherogram of theobromine (**I**), caffeine (**II**) and theophylline (**III**) under the same conditions, but 65 mM SDS, is shown in Fig 6, where the migration times of **II** and **III** increase with the increasing SDS concentration. The entire analysis required around 6 min, with the expected LOD less than 1 $mg\,l^{-1}$ for all compounds and a linear response upto 150 $mg\,l^{-1}$ (Table 2). The repeatability was less than 0.6% RSD

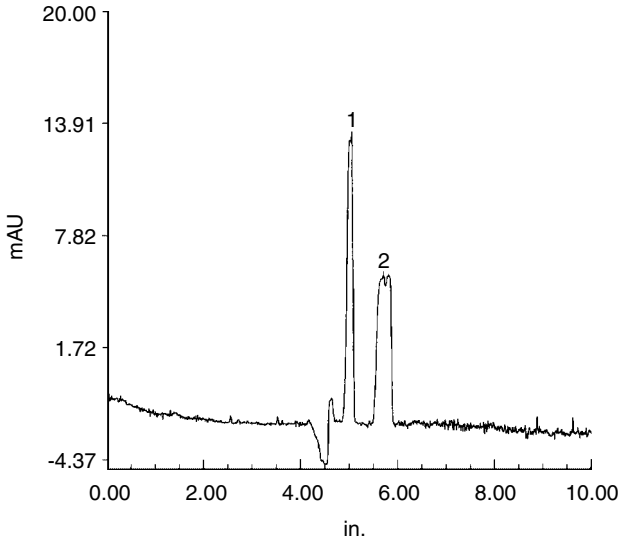


Figure 5. Electropherogram of standards using a 90 mM borate buffer, pH 8.5, and 50 mM SDS. Peaks: 1, theobromine (**I**); and 2, caffeine (**II**).

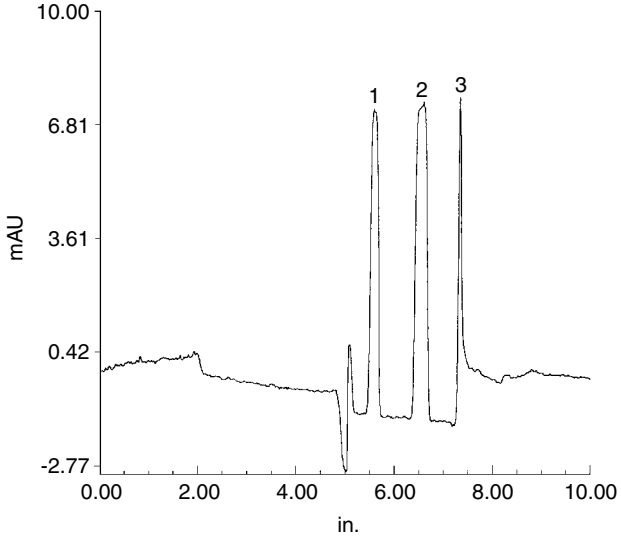


Figure 6. Electropherogram of standards using a 90 mM borate buffer, pH 8.5, and 65 mM SDS. Peaks: 1, theobromine (**I**); 2, caffeine (**II**); and 3, theophylline (**III**).

(relative standard deviation) for migration times and better than 0.8% RSD for apparent retention factors. The reproducibility of the migration time was also very good at less than 2.0% RSD.

SDS-MECC analysis of xanthines-containing beverages was carried out. Theobromine and caffeine contents ($g\,kg^{-1}$) of 12 commercial samples are shown in Table 3. No sample preparation other than filtration to prevent clogging of the capillary was necessary for analysis of non-carbonated beverages. For carbonated beverages, degassing by purging with argon was required prior to filtration. The main methylxanthine detected in cola drinks was caffeine. External calibration was used for quantitation of **I** and **II** with an excellent reproducibility (see the Experimental section).

Table 2. Parameters of the method: LOD, LOQ and working range

	Caffeine	Theobromine
LOD, $mg\,l^{-1}$	0.8	0.6
LOQ, $mg\,l^{-1}$	2.5	2.0
Working range, $mg\,l^{-1}$	2.5–300	2.0–300
Coefficient of correlation (*r*)	0.996	0.996

CONCLUSIONS

Twelve samples of methylxanthine-containing infusions of different commercial brands of *yerba mate*, coffee, tea and cocoa as well as two cola drinks were analysed. A standard mixture of theobromine, caffeine and theophylline was used for optimization of experimental HPCE–MECC conditions.

In this SDS–MECC separation the best resolution was achieved using 15 kV voltage in an uncoated fused-silica capillary of 45 cm length (40 cm effective length), 50 mM SDS, 90 mM pH 8.5 borate buffer and UV detection. Theobromine (**I**), caffeine (**II**) and theophylline (**III**) were separated, and peak splitting due to tautomeric species was observed. Migration behavior of methylxanthines in MECC

Table 3. SDS–MECC determination of caffeine and theobromine content in 12 samples of several infusions and beverages, eg *yerba mate*, chocolate, tea and coffee using an external standard method

Sample (brand)	Caffeine ($g\,kg^{-1}$)	Theobromine ($g\,kg^{-1}$)	Type	RSD (%)
Yerba Taragüí	10.70	3.90	Milled	0.90
Yerba Cruz de Malta	9.90	6.60	Milled	1.80
Yerba Nobleza Gaucha	10.70	2.00	Milled	1.70
Yerba Sol de Acuario	10.80	4.00	Organic	1.40
Yerba Cruz de Malta	3.60	1.60	Bags	1.60
Coffee Bonafide	14.40	ND	Milled	0.70
Coffee Dolca	13.20	ND	Soluble	1.30
Decaff. Coffee Nestlé	0.34	ND	Soluble decaffeinated	1.70
Chocolate Cocoa Poo	1.90	17.60	Soluble powder	2.00
Coca Cola	91.20	ND	Cola drink	0.50
Pepsi Cola	85.70	ND	Cola drink	0.40
Tea Ceylon	14.50	1.40	Dried processed leaves	1.10

has been described on the basis of the acid–base equilibrium and micelle–water partitioning equilibria for the charged and uncharged forms of solute. The acid–base equilibria occur in the aqueous phase, and there is a different partitioning contribution of (**I–III**) with the micelle. Experimental conditions were controlled, keeping the size of the elution window constant in each analysis. The limit of detection was less than $1\,mg\,l^{-1}$, the limit of quantitation was $2.5\,mg\,l^{-1}$ and the working range was $2.5–300\,mg\,l^{-1}$. This HPCE–MECC system has proved suitable for rapid and accurate quantitation of caffeine and theobromine in beverages for consumption; it is also important to take into account the peak splitting for the analysis/quality control of the xanthine-containing foods and beverages.

ACKNOWLEDGEMENTS

Thanks are due to Universidad de Buenos Aires (Argentina) and CONICET (Argentina) for financial support.

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