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Rapid analysis of caffeine in "smart drugs" and "energy drinks" by microemulsion electrokinetic chromatography (MEEKC)

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ABSTRACT

A novel method based on microemulsion electrokinetic chromatography (MEEKC) with diode array detection (DAD) for rapid determination of caffeine in commercial and clandestine stimulants, known as "energy drinks" and "smart drugs", is described. Separations were carried out in 50 cm × 50 μm (ID) uncoated fused silica capillaries. The optimized buffer electrolyte was composed of 8.85 mM sodium tetraborate pH 9.5, SDS 3.3% (w/v), *n*-hexane 1.5% (v/v) and 1-butanol 6.6% (v/v). Separations were performed at a voltage of 20 kV. Sample injection conditions were 0.5 psi, 3 s. Diprofilline was used as internal standard. The determination of the analytes was based on the UV signal recorded at 275 nm, corresponding to the maximum wavelength of absorbance of caffeine, whereas peak identification and purity check was performed on the basis of the acquisition of UV radiation between 200 and 400 nm wavelengths. Under the described conditions, the separation of the compounds was achieved in 6 min without any interference from the matrix. Linearity was assessed within a caffeine concentration range from 5 to 100 μg/mL. The intra-day and inter-day precision values were below 0.37% for migration times and below 9.86% for peak areas. The present MEEKC method was successfully applied to the direct determination of caffeine in smart drugs and energy drinks.

1. Introduction

Caffeine is the most ancient and widely consumed psychoactive drug, being naturally present in coffee and cacao beans, kola nuts, guarana berries, tea leaves etc. which are used worldwide in many cultures. The main effects of caffeine include physical endurance, reduction of fatigue and enhancement of mental alertness [1]. Because of its positive activity on the cardio-respiratory system and on the brain function, from year 1984 to year 2004 caffeine was included in the list of doping drugs, when detected in urine above 12 μg/mL. The physical and mental stimulation exerted by caffeine meets the modern trends of the young generations towards the use of "legal" stimulants, instead of the traditional but illegal cocaine and amphetamines. Also, it is noteworthy that caffeine availability has expanded since this compound is present as an additive in "energy drinks" and dietary supplements, often perceived as "safe", but not free from relevant adverse effects. Quite recently, different preparations containing caffeine (capsules, strips, powders) have become available through the Internet and in the so called "smart shops" as "legal", easily available stimulant drugs (smart drugs).

In recent years, the use of alcohol in combination with caffeine-containing drinks or drugs has become fairly popular, for the ability of caffeine to offset the sedating effects of alcohol and to enhance alertness [2–4].

On the other hand, evidence of clinical syndromes of caffeine dependence and overdosing have been reported [5] as well as numerous caffeine-related intoxications and even deaths [6–9].

Current methods for caffeine analysis are based on: gas chromatography–mass spectrometry (GC–MS) [10,11] and HPLC–MS [12,13]. Unfortunately, these techniques are available only in specialized laboratories, but rarely in the laboratories of clinical chemistry and clinical toxicology, causing a clear underestimation of the phenomenon of caffeine abuse in the population.

Because a higher versatility and easiness to switch between different analytical conditions, capillary electrophoresis (CE) may look preferable to the above mentioned techniques for the analysis

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of caffeine, which at present is only rarely requested in laboratories of clinical and forensic toxicology.

In recent years, indeed, CE methods for the determination of caffeine have been reported [14,15]. For the neutral characteristics of the molecule of caffeine precluding any charge-to-mass ratio based separations, usually micellar electrokinetic chromatography (MEKC) methods were proposed. In MEKC analytes are separated on the basis of their partitioning between an aqueous separation buffer and the hydrophobic core of charged micelles, which acts as a pseudo stationary hydrophobic phase [16,17].

More recently, microemulsion electrokinetic chromatography (MEEKC) has been introduced as an attracting alternative to MEKC for the separation of neutral as well as charged molecules. The MEEKC separation buffer is a microemulsion in which an organic water immiscible solvent forms the core of the microdroplets, which are stabilized by a charged surfactant located at their surface, which confers to them a net electric charge and consequently an electrophoretic mobility. According to this scheme, the separation of neutral compounds in MEEKC is based on the analyte partitioning between the moving charged "oil" droplets and the aqueous buffer phase. Particularly, the oil-in-water (o/w) microemulsions are similar to micelles for their ability of solubilizing hydrophobic compounds, but display a much larger capacity due to a larger droplet size [18]. Moreover, in comparison to MEKC, MEEKC, because of a higher complexity of the buffer, is more flexible and can be more finely tuned to optimize separations.

To date, the most common applications of MEEKC are in the pharmaceutical field [19,20], but, to the best of our knowledge, only two method were reported applying this separation mode for the determination of caffeine (and catechins) in green tea [21] or for the detection of caffeine as adulterant in illicit preparations of heroin and amphetamine [22].

The present work was aimed at the development and validation of a rapid and simple MEEKC method for the quantitative analysis of caffeine in commercial beverages and in "smart drugs" preparations.

2. Materials and methods

2.1. Chemicals and reagents

Ultrapure deoxycholic acid, sodium tetraborate and pure caffeine (Sigma Reference Standard) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Diprofilline [used as the internal standard (IS)] was obtained from a pharmaceutical product named Katasma™ (Bruschettini s.r.l., Genova, Italy). Stock solutions of caffeine and diprofilline were prepared in 50/50 methanol/water at individual concentrations of 1 mg/mL and stored at +4 °C until use.

Sodium dodecyl sulphate (SDS), *n*-hexane and 1-butanol were obtained from Merck (Darmstadt, Germany). Buffer electrolytes were prepared by proper dilution of the stock solutions of sodium tetraborate 100 mM and SDS 100 mg/mL, each one obtained by dissolution of the respective powders in deionised water.

The deionised water used throughout the study was obtained from an Aqua MAX-Ultra 370 Series water purification system (Young Lin Instrument, Anyang, Korea).

2.2. Sample preparation

Six different energy drink cans, namely Imola® (Getranke GmbH Traisental, Austria), Semtex® (Pinelli spol s.r.o., Czech Republic), Burn® (Coca-Cola Company, USA), Red Bull® (Red Bull GmbH, Austria), Shock® (Al.Namura spol s.r.o., Czech Republic), Mixxed Up® (LIDL Stiftung &Co. KG, Germany) were collected from Italian supermarkets. Samples of "smart drugs", namely Minikikke®, Koru®, Finalkat®, Happy caps XXX®, Happy caps 4U®, were purchased in different Italian Smart Shops, in the frame of a research project (Smart Search) in collaboration with the National Early Warning System.

All the samples were stored in their original cans or packages at room temperature until analysis.

2.3. Capillary electrophoresis

The present study was performed by using a P/ACE MDQ automated capillary electropherograph (Beckman Coulter, Fullerton, CA, USA) equipped with a diode array detector. The software "32 Karat" Version 5.0 (Beckman Coulter) controlled hardware operation, data acquisition and data reporting.

The electrophoretic analysis was performed in an uncoated fused-silica capillary (50 μm i.d., 50 cm total length) from Composite Metal Services (The Chase, Hallow, UK), with an effective length of 40 cm. Separations were carried out by applying a constant voltage of 20 kV at a capillary temperature of 25 °C. The optimized buffer electrolyte was composed of 8.85 mM sodium tetraborate pH 9.5, SDS 3.3% (w/v), *n*-hexane 1.5% (v/v) and 1-butanol 6.6% (v/v). Under these conditions, the generated current was about 60 μA. In order to obtain reproducible separations, the fresh buffer was prepared at the beginning of each day and degassed by sonication for 15 min before use.

Before each run, the capillary was rinsed sequentially with NaOH 1 M, water and buffer electrolyte, for 5 min each.

Hydrodynamic injections were carried out by applying 0.5 psi for 3 s at the inlet of the capillary. Detection was performed by monitoring the wavelengths corresponding to the maxima of absorbance of caffeine: 200 nm and 275 nm. However, for peak identification and peak purity check the UV spectrum in the range of 200–400 nm was also recorded.

Before CE analysis, all beverages were centrifuged at 10,000 rpm in a benchtop centrifuge for 5 min to remove particulate material and then diluted 1:2 with buffer containing 100 μg/mL diprofilline (IS). An aliquot of each smart drug in powder form was weighed and diluted in methanol to a final concentration of 20 mg/mL. The obtained solutions were then sonicated for 15 min and centrifuged at 3500 rpm for 10 min. The surnatants were diluted in buffer solution containing the IS (to a final concentration of 50 μg/mL). Each solution was sonicated for 10 min before injection to avoid outgassing.

Quantification was carried out on the basis of peak areas detected at 275 nm by using the internal standard method (IS: diprofilline). Standard curves were prepared by spiking buffer solutions with caffeine to obtain concentrations of 5, 10, 20, 40, 75 and 100 g/mL. which were diluted with the IS solution and injected.

3. Results and discussion

On the basis of existing literature [15], for the separation of caffeine, plain capillary zone electrophoresis (CZE) with a basic background electrolyte (15 mM sodium tetraborate at pH 9.5–11.0) was initially tested. However it was soon clear that at these pH values, caffeine poorly ionized and consequently migrated close to the EOF, not resolved from the neutral compounds present in the samples.

Thus, in order to solve this problem, the introduction of a new separation mechanism, in addition to electrophoresis, looked necessary. To this aim, MEEKC looked attractive, because of its ability to deal with charged and neutral compounds as it is reported by a number of recent papers [19]. In MEEKC the background electrolyte is composed of a dispersion of two immiscible liquids, consisting either in "oil" finely dispersed in an aqueous buffer (o/w microemulsion) or water dispersed in "oil" (w/o microemulsion). In this system, the resulting droplets are formed in the presence of an ionic surfactant coating their surface which reduces the surface tension, thus allowing the formation of a stable emulsion. The MEEKC buffer is further stabilized by the addition of a short-chain alcohol, such as butanol or octanol. By application of voltage across the capillary, the oil droplets bearing on the surface the charged surfactant molecules migrate toward the electrode with opposite polarity. In the present case, the droplets covered with SDS and hence negatively charged move towards the anode, i.e. in the direction opposite to the electroosmotic flow (EOF). However, at high EOF values, such as in the present case (because of the basic pH of the buffer electrolyte), the negative droplets are swept to the cathode (i.e. towards the detector) by the prevailing velocity of the EOF, which exceed their own electrophoretic velocity directed backwards in the capillary.

In o/w MEEKC, as in the present system, neutral solutes are separated on the basis of their solubility in the "oil" phase (log P) with the more water-insoluble solutes migrating last.

In this fairly complex separation system, specific conditions to be optimized include the choice and concentration of the "oil" phase, of surfactant and co-surfactant. Moreover, it should be stressed that the buffer pH plays an important role in any

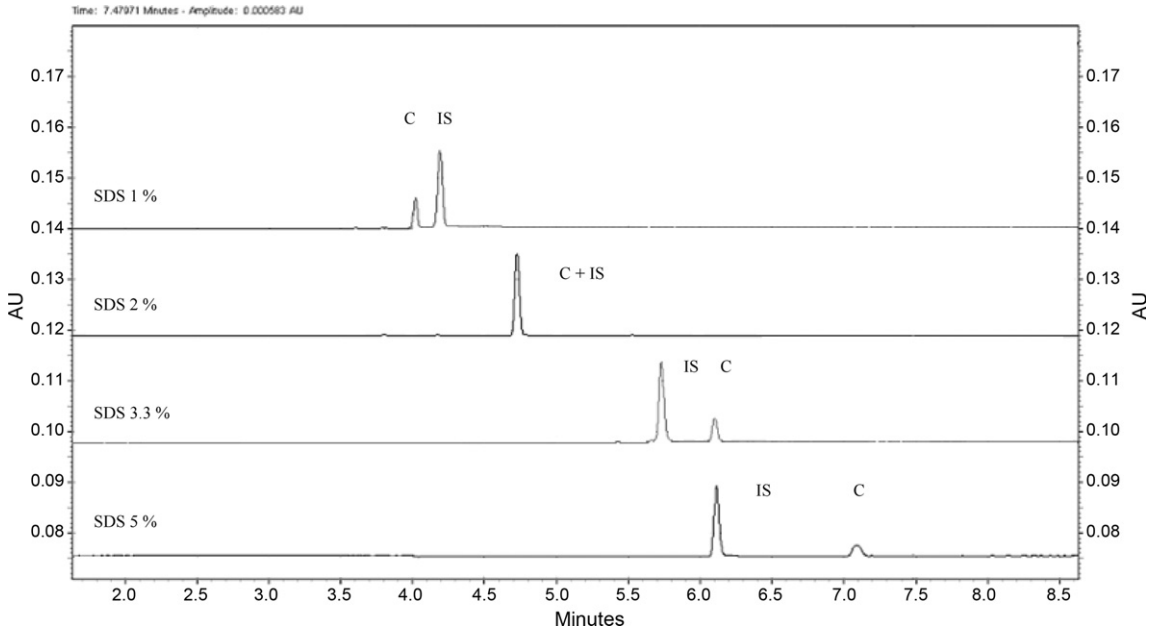

Fig. 1. Influence of % SDS on the separation of caffeine (C) and IS.

electrophoretic process, since it affects both the degree of ionization of the analytes and of the capillary walls, and hence the EOF magnitude.

In the present work, the effect of the buffer pH was investigated within the range 7.5–10.5 at a fixed buffer concentration (15 mM). The best results in terms of resolution and peak shape was obtained by using borate buffer at pH 9.5; as expected, the increase of pH produced shorter analysis times as a result of the increased EOF, but a worse separation of caffeine and IS; at pH values lower than 9.5, the separation was also unsatisfactory, because of a poor ionization of the analytes (data not shown). For the known influence of the buffer ionic strength on the EOF, by increasing the borate concentration in the buffer (from 10 up to 30 mM) a neat increase of the migration time for both caffeine and IS was observed (originated by a decrease of the EOF), with co-migration of the analytes. On this basis, 15 mM borate pH 9.5 was finally chosen as optimal separation buffer.

As it is well known, in MEEKC the formation of a stable microemulsion is affected by the ratio between the surfactant and the cosurfactant; on the other hand, the amount of surfactant in the in the microemulsion buffer strongly affects the separation. SDS is by far the most common anionic surfactant used in MEECK separations. However, other surfactants have been used, among which bile salts (e.g. deoxycholic acid) have a prominent role [17]. In our preliminary experiments, both SDS or deoxycholic acid were tested when added to a buffer solution consisting of 15 mM sodium tetraborate pH 9.5, containing *n*-hexane 1.5% (v/v) and *n*-butanol 6.6% (v/v). As expected, the increase of surfactant concentrations from 1 to 5% (w/v) and, consequently, of the ionic strength led to reduced EOF and increased analysis times. As depicted in Fig. 1, the increase of the concentration of SDS was paralleled by the increase of the migration times of caffeine and internal standard. With the separation buffer containing 1% SDS, caffeine migrated immediately before the IS at about 4 min; with 2% SDS, caffeine and IS co-migrated in the same peak, while above 2% SDS an inversion of the migration order was observed with IS migrating before caffeine. Because of the elevated concentration of SDS, high current was observed with 5% SDS, leading to broadening of the caffeine peak, because of excessive joule heating. An SDS concentration of 3.3% in the separation buffer was eventually chosen as the best compromise between the analysis time and the separation efficiency.

Since it is widely reported that, differently from other conditions, the nature of the "oil phase" plays only a minor role in the separation selectivity, hexane, producing a stable microemulsion was the only "oil phase" tested. Experiments were performed with MEEKC buffers with increasing amounts of hexane, ranging from 0 to 3%. An interference on the caffeine peak was found when analyzing real samples using buffers with less than 1.5% of hexane. Thus hexane concentration of 1.5% was chosen, also with the aim of minimizing organic solvent use as much as possible.

In agreement with the literature, a percentage of 6.6% *n*-butanol was chosen to stabilize the formed microemulsion. Under the above described optimized conditions, the microemulsion proved to be physically stable for at least one day, above which it was necessary a further 15 min sonication step.

The analytical method was fully validated in terms of linearity, limit of detection and limit of quantification, within-day and between-day variability and accuracy.

The limit of detection (LOD), calculated as the lowest caffeine concentration giving a signal-to-noise ratio (S/N) ≥ 3 was 2 μg/mL. The lower limit of quantification (LLOQ), defined as the lowest concentration of caffeine that can be determined with an accuracy and precision below 20%, was 5 μg/mL ($n = 10$).

Under the optimized conditions, the linearity was assessed in the range 5–100 μg/mL by injecting aqueous solutions containing known concentrations of caffeine. Three replicates of six calibration points were analyzed for 7 non consecutive days.

The resulting final equation was

$$y = (0.0266 \pm 0.001)x + (0.024 \pm 0.015) \text{ with an average } R^2 = 0.9995.$$

The precision and accuracy of the assay were determined on six different injections of spiked samples with caffeine concentrations of 10, 40 and 75 μg/mL on the same day and in five different days.

As summarized in Table 1, the intra-day precision CV's for relative peak areas and relative migration times were $\leq$8.89% and $\leq$0.37%, respectively; the intra-day precision CV's for relative peak

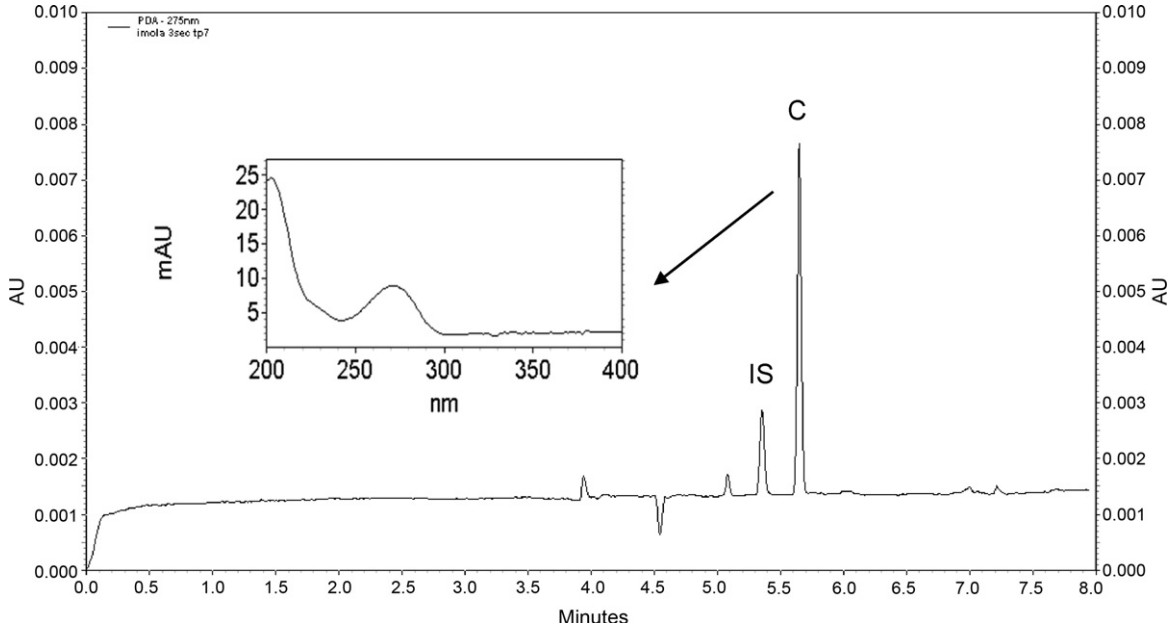

Fig. 2. Electropherogram of an energy drink (Imola®). Insert: UV spectrum of caffeine in the range 200–400 nm. Conditions: 8.85 mM sodium tetraborate pH 9.5, SDS 3.3% (w/v), *n*-hexane 1.5% (v/v) and 1-butanol 6.6% (v/v), capillary, 50 cm full lengh, 40 cm effective length, 50 um; hydrodynamic injection at 0.5 psi, 3 s; 20 kV; detection by UV absorbance at 275 nm. Peak identification: (1) IS, diprofilline; (2) C, caffeine.

areas and relative migration times were ≤4.23% and ≤0.95%, respectively.

Average analytical accuracy was ≤101.59% for peak areas (Table 1).

The method was successfully applied to the analysis of a wide range of samples. Notwithstanding the great variability of the analyzed products, including tablets, sublingual strips, capsule and powder, soft drinks, coffee, decaf coffee, green tea etc., no interferences on the caffeine and I.S. peaks were observed in the electropherograms. It is worth nothing that neither theophylline nor taurine interfered with the determination of caffeine.

Eight different types of energy drinks and five different types of smart drugs were analyzed for caffeine, without any sample preparation but dilution (liquid samples) and/or solubilization in methanol (solid samples). The results are reported in Table 2. Figs. 2 and 3 showed the electropherograms of real samples. The energy drinks showed a range in caffeine content from 3 to 144 mg per can (note: the average content of a coffee cup is about 80 mg). These results are comparable with those described in literature for similar products [23]. Great variability was also found among the different preparations of smart drugs, the content of caffeine of which ranged from 23 to 343 mg per dose.

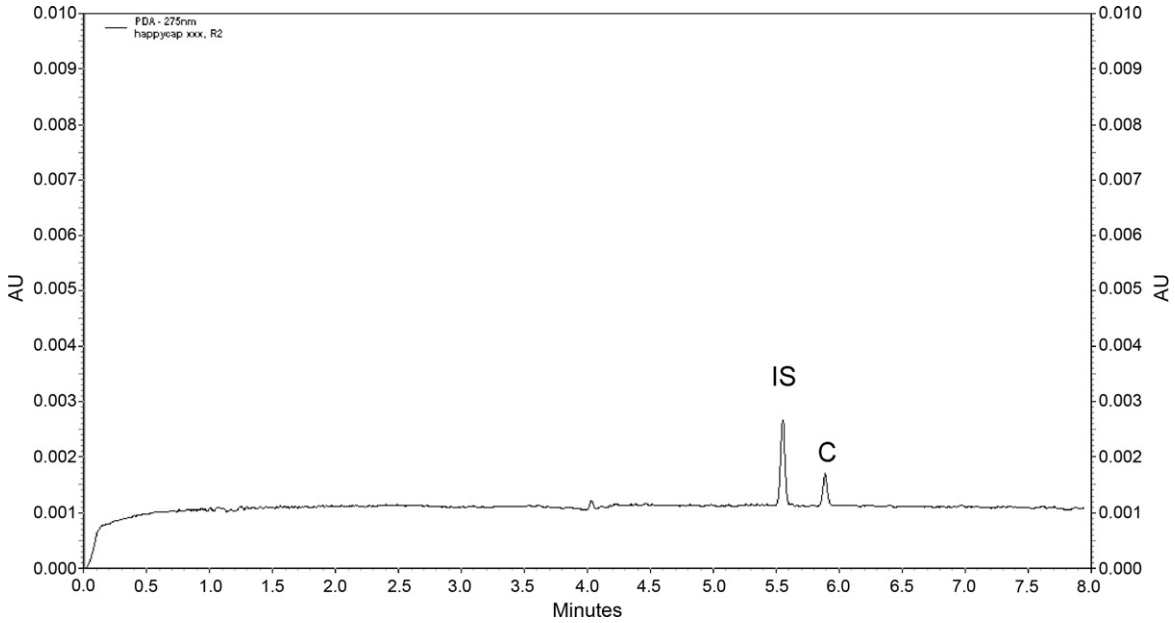

Fig. 3. Electropherogram of a commercial tablets (Happy caps XXX®). Conditions: 8.85 mM sodium tetraborate pH 9.5, SDS 3.3% (w/v), *n*-hexane 1.5% (v/v) and 1-butanol 6.6% (v/v), capillary, 50 cm full lengh, 40 cm effective length, 50 um; hydrodynamic injection at 0.5 psi, 3 s; 20 kV; detection by UV absorbance at 275 nm. Peak identification: (1) IS, diprofilline; (2) C, caffeine.

Table 1
Validation figures for precision and accuracy calculated as peak area ratios (analyte/internal standard) of standards in water.

	Intraday relative area										Interday relative area ($n=5$)	
	Day 1		Day 2		Day 3		Day 4		Day 5			
	Precision CV %	Accuracy %	Precision CV %	Accuracy %	Precision CV %	Accuracy %	Precision CV %	Accuracy %	Precision CV %	Accuracy %	Precision CV %	Accuracy %
10 μg/mL	8.89	104.41	6.06	97.34	6.35	108.28	7.38	97.49	7.39	98.14	2.84	101.14
40 μg/mL	1.98	102.57	6.59	99.64	2.26	106.13	3.30	98.31	3.50	96.62	4.23	100.65
75 μg/mL	3.63	98.62	2.00	103.57	2.44	103.3	3.79	98.51	4.54	103.94	1.93	101.59

Table 2
Real samples analyzed and relative amount of caffeine.

Drinks	mL	Caffeine mg
Decaffeinate coffee	50	3
Coffee	50	76
Green tea	200	16
Burn	250	90
Red Bull	250	80
Semtex	250	78
Imola	250	66
Shock	500	144
Mixxed Up	250	62
Smart drugs	**Drug form**	**Caffeine mg**
Happy Caps XXX	Capsule	343
Happy Caps 4U	Capsule	259
Minikikke	Tablet	29
Final Cut	Powder	264
Koru Strip	Sublingual strip	23

4. Conclusions

The MEEKC-DAD method herein described offers a fast and non-expensive tool for a rapid determination of caffeine in commercial products and clandestine preparations of stimulant drugs.

The availability of a low cost and easy to use method looks particularly important for monitoring the presence of caffeine in the fairly obscure market of the ‘‘smart drugs’’.

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