Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 1095–1100
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Capillary electrophoresis for caffeine and pyroglutamate determination in coffees
Study of the in vivo effect on learning and locomotor activity in mice

N. Maeso, C. del Castillo, L. Cornejo, M. García-Acicollar, L.F. Alguacil, C. Barbas *

Facultad de Farmacia, Universidad San Pablo-CEU, Urbanización Montepríncipe, Ctra. Boadilla del Monte, km 5, 3-28668 Madrid, Spain

Received 24 November 2005; received in revised form 29 January 2006; accepted 30 January 2006
Available online 20 March 2006

Abstract

In a preliminary study pyroglutamate showed to be over 10 times increased in some lyophilised coffees with respect to brewed or filtered coffees, and probably that increase is related to some stage of the industrial process. Pyroglutamate is known to have a number of remarkable cognitive enhancing effects, which could be also related to the properties of coffee traditionally associated to caffeine. Pyroglutamate improves memory and learning and has anti-anxiety effects in rats.

Therefore, a method has been developed and validated for the simultaneous determination of caffeine and pyroglutamate in coffee by capillary electrophoresis. Separation conditions employed MECK conditions with 50 mM borate buffer at pH 9.5 with 130 mM SDS. The applied potential was 10 kV and detection was performed at 200 nm.

Afterwards, 10 soluble coffees from the market were measured and caffeine and pyroglutamate levels were compared. Those coffees with higher pyroglutamate with or without caffeine were preliminarily tested for sedative/stimulant properties and cognition enhancing effects in mice. The most relevant finding was a partial reversal of scopolamine-induced amnesia in the passive avoidance paradigm after oral administration of one coffee.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Pyroglutamic acid; Caffeine; Brain; Coffee; Capillary electrophoresis

1. Introduction

The acid content in a coffee, an important indication of its quality, is also greatly dependent upon the degree of roasting, the type of roaster and method of infusion, but it is still unknown which acids are imperative to recreate the acidity experienced in coffee. The perceived acidity of coffee results from the proton donation of acids to receptors on the human tongue.

Knowing the problems related to short chain organic acid analysis by GC and HPLC, our research group has proved that CE is a good choice for the investigation of low molecular weight organic acids in complex aqueous samples with high efficiency and minimum sample pre-treatment [2–4,6–8,10,16,17]. Therefore, a capillary electrophoresis method was developed that permitted the direct measurement of 17 short chain organic acids in coffee extracts [9]. When it was applied to commercial coffees, important differences were observed in some of these compounds, in particular pyroglutamic acid content, which had never been previously described. Pyroglutamate was greatly increased, around 10 times in the lyophilised coffee versus grain coffee, and probably that increase is related to some stage of the industrial process.

Pyroglutamate, also known as 2-oxo-pyrrolidone carboxylic acid or PCA and 5-oxoproline, is an intermediate of the gamma-glutamyl cycle of glutathione synthesis and degradation. On the other hand, it is known that thermal conversion of L-glutamate to PGA occurs. In that sense L-glutamate could be spontaneously converted to PGA during coffee treatment. Pyroglutamate is known to have a number of remarkable cognitive enhancing effects, in animals, which could be attributed to increased

* Corresponding author. Tel.: +34 91 3724711; fax: +34 91 3510475.
E-mail address: cbarbas@ceu.es (C. Barbas).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.01.062

release of acetylcholine in the brain cortex [1], protection against glutamate-induced neurotoxicity [20] or improved cerebral perfusion [15]. Furthermore, in one double-blind study with aged human subjects, verbal memory was improved by pyroglutamate compared to placebo [11]. These antecedents raise the possibility that the pyroglutamate content of the coffees could provide them with some central activities previously associated with caffeine, another natural component of the coffees that reduces fatigue, improves attention and enhances cognition by modulating dopamine and adenosine central functions [13].

In the manufacture of instant coffee, extraction follows the roasting and grinding operations. The soluble solids and volatile compounds that provide aroma and flavour are extracted from the coffee beans using water. Water heated to about 175 °C (347 °F) under pressurized conditions (to maintain the water as liquid) is used to extract all of the necessary solubles from the coffee beans. Manufacturers use both batch and continuous extractors. Following extraction, evaporation or freeze-concentration is used to increase the solubles concentration of the extract. The concentrated extracts are then dried in either spray dryers or freeze dryers. Therefore, different treatments could produce a different pyroglutamate content. Additional operations associated with processing green coffee beans include decaffeination, the process of extracting caffeine from green coffee beans prior to roasting. Different factors, such as blend composition, brewing extraction rates, the grind or the water temperature, can slightly alter the level of caffeine in decaffeinated coffee so that it is above or below the typical caffeine level. Nevertheless, the simultaneous determination of both caffeine and pyroglutamate, has, to our knowledge, not been described.

The aim of this study was to develop and validate a simple and reliable CE method to determine simultaneously caffeine and pyroglutamate in coffee, then to measure, by applying this method, the content of these compounds in usual brands of instant coffees bought in the local supermarket and finally, to assess the effects of instant coffees with different amounts of caffeine and pyroglutamate on locomotor activity and learning/memory in mice.

2. Materials and methods

2.1. Instrumentation

The separation was performed on a capillary electrophoresis P/ACE 5010 (Beckman) with UV detection at 200 nm. The injection was by pressure (0.035 bar) for 5 s. The separation was carried out with an uncoated fused-silica capillary (57 cm × 50 μm i.d.) and was operated at 10 kV potential. Temperature was maintained at 25 °C. The background electrolyte was prepared with 0.05 M H_3BO_3 and 130 mM SDS, as anionic surfactant to decrease electroosmotic flow, and pH adjusted by adding NaOH to 9.5. The current generated in such conditions was 25 μA.

The capillary was flushed between runs with 0.1 M NaOH for 3 min, H_2O for 3 min and the background electrolyte for 5 min.

Several parameters were studied during the optimization of the method, such as nature, pH and concentration of the BGE, SDS concentration and applied potential were also tested.

2.2. Chemicals

Standards were obtained from Sigma (St. Louis, MO, U.S.A.). Boric acid was from Merck (Darmstadt, Germany), sodium hydroxide and SDS from Panreac (Madrid, Spain) and organic solvents from Scharlau (Barcelona, Spain).

2.3. Samples

Samples of coffee with different origins and treatment were obtained from different brands available in the market. The characteristics were only known through their labels, which contained scarce information and are listed in Table 1. Although our work was focused on instant coffees, a standard grain coffee, brewed in the laboratory was included as a control.

For the analysis, samples were prepared in triplicate with 1 g of instant coffee in 100 mL volumetric flasks with purified water. Then, samples were diluted 1:4 (v/v) with purified water, and filtered through 0.45 μm nylon filters prior to analysis.

Table 1
Characteristics of coffee samples assayed

Sample identification	Characteristics described in the label	Caffeine (mg/g coffee)*	Pyroglutamate (mg/g coffee)*
1	Instant coffee, lyophilised	45.9 ± 2.4 A	4.3 ± 0.52 b
2	Instant coffee, lyophilised, Colombia	36.6 ± 0.80 B	6.5 ± 0.18 c
3	Instant coffee, lyophilised, decaffeinated	3.8 ± 0.33 C	10.12 ± 0.36 e
4	Instant coffee, decaffeinated	4.7 ± 0.42 C	6.1 ± 0.28 c
5	Grain coffee, brewed in the laboratory	19.8 ± 0.32 F	0.09 ± 0.01 a
6	Instant coffee	41.8 ± 1.0 E	8.6 ± 0.29 de
7	Instant coffee, decaffeinated	4.59 ± 0.28 C	7.0 ± 0.05 c
8	Instant coffee, decaffeinated	3.5 ± 0.67 C	7.51 ± 0.15 cd
9	Instant coffee, decaffeinated	3.6 ± 0.31 C	8.7 ± 0.44 de
10	Instant coffee, lyophilised, Colombia	30.8 ± 2.2 D	9.8 ± 0.93 e
11	Instant coffee, decaffeinated	3.94 ± 0.33 C	8.67 ± 0.18 de

Mean ± S.D. Different letters show statistical difference.
* $p < 0.05$.

2.4. Validation

Individual stock solutions of caffeine (5 mM) and pyroglutamic acid (50 mM) in purified water were prepared and stored at −4 °C. On the day of the analysis they were mixed and diluted to the final working concentrations of 0.5 mM for caffeine and 0.125 mM for pyroglutamate.

Linearity of response for standards was tested by assaying in triplicate using six levels of concentrations, ranging from 12.5 to 625 μM for pyroglutamic acid and from 4 to 200 μM for caffeine, covering in this way all the expected values as much in classic as in decaffeinated coffees.

Recovery was estimated by comparing the values obtained in the linearity test for spiked samples with the corresponding standards linearity, taking into account the coffee concentrations which had been previously quantified.

Within-day precision was tested both to check the constancy of instrumental response to a given analyte and the repetitiveness of concentrations. For this purpose, the assay was performed with six solutions of standards and six of samples, in the medium concentration of the calibration curve for both compounds. The same experiment was done on a different day with buffer and samples freshly prepared for intermediate precision.

Limits of detection were calculated following IUPAC recommendations $[(a + 3S_B)/b]$, where a is the intercept, b the slope and S_B is the standard deviation of the blank, for chromatographic methods [12] by extrapolating to zero concentration the standard deviation of the last three points of linearity and interpolating this value in the corresponding equation.

3. Pharmacological studies

3.1. Animals

Male OF1 Swiss albino mice weighing 25–30 (Charles River, Lyon, France) were used. The animals were housed in a controlled environment (22 ± 1 °C, 55 ± 10% humidity, 12-h light:12-h dark cycle) and were fed ad libitum with standard rodent diet (RMM, Harlan Interfauna Ibérica, Madrid, Spain). All experiments were conducted between 09:00 and 17:00 h in a noise-free environment. The experimental protocols were approved by the Institutional Ethical Committee.

3.2. Locomotor activity

Animals received 10 ml/kg, p.o., of either tap water (controls) or coffee solutions prepared by solving 1 g of each instant coffee in 10 mL of tap water (dose = 1 g/kg). Immediately after oral administration, mice were isolated in plexiglass cages (25 cm × 25 cm × 14 cm) and left to acclimatize to the environment for 5 min. Motility counts (arbitrary units) were then recorded for 60 min by means of a magnetic device (Panlab, Barcelona, Spain) placed at the bottom of the cages.

3.3. Passive avoidance

The effects of coffees on learning/memory were assessed by means of a step-through passive avoidance procedure, in which animals learn to avoid an electrical discharge by suppressing their natural preference for dark environments. This test is widely used in testing the effects of memory active drugs [18,19].

Animals received orally either tap water or coffee solutions (1 g/kg) as explained above. One hour afterwards, mice received an i.p. injection either of saline (controls) or scopolamine (1 mg/kg), a drug commonly used to induce learning deficits. Thirty minutes after the i.p. treatment, a task acquisition trial was conducted by means of a two-compartment apparatus (Ugo Basile, Italy). For this purpose the animals were placed in a white, illuminated chamber connected to a dark compartment by a guillotine door. As soon as the animals entered the dark compartment, the door closed and mice received a mild electric footshock (0.5 mA, 2 s) by means of the electrified grid floor. The latency for this response was recorded and those animals showing latencies longer than 60 s (cut-off) were not used further. Twenty-four hours afterwards a retention trial was conducted exactly as the acquisition trial, and positive learning was defined by step-through latency higher than 60 s.

3.4. Statistics

Statistical differences between experimental groups regarding motility counts were determined using ANOVA followed by Newman Keuls post hoc test. In the passive avoidance study, learning frequencies were compared with the Fisher's exact test. Comparisons with $p < 0.05$ were considered to be significant.

4. Results and discussion

The simultaneous separation of caffeine and pyroglutamate in coffee was optimised by testing first the suitability of the already existing methods for pyroglutamate and caffeine individually [5,9]. Caffeine did not pass the detector in reversed polarity; therefore, efforts were headed towards the optimization of separation conditions in normal polarity where both compounds were eluted, with micelles formed with SDS and pH around 7.0. Final conditions, described in the previous section, were selected after testing different potentials, buffer concentrations and pH, by injecting different types of coffee and considering mainly the resolution of caffeine from other peaks in the profile, which was the main issue. Fig. 1 shows the electropherograms corresponding to the standards and a coffee sample. Selectivity was proved for caffeine by injecting a decaffeinated coffee and looking at a minimum peak appearing with the same spectrum and migration time as the large peak in normal coffee. For pyroglutamate selectivity was proved by quantifying the response obtained with the previous method with reverse polarity.

4.1. Validation

Once both compounds were identified by migration time as compared with the standards and by spiking, selectivity was proved as described above. All the calculi were performed with peak areas.

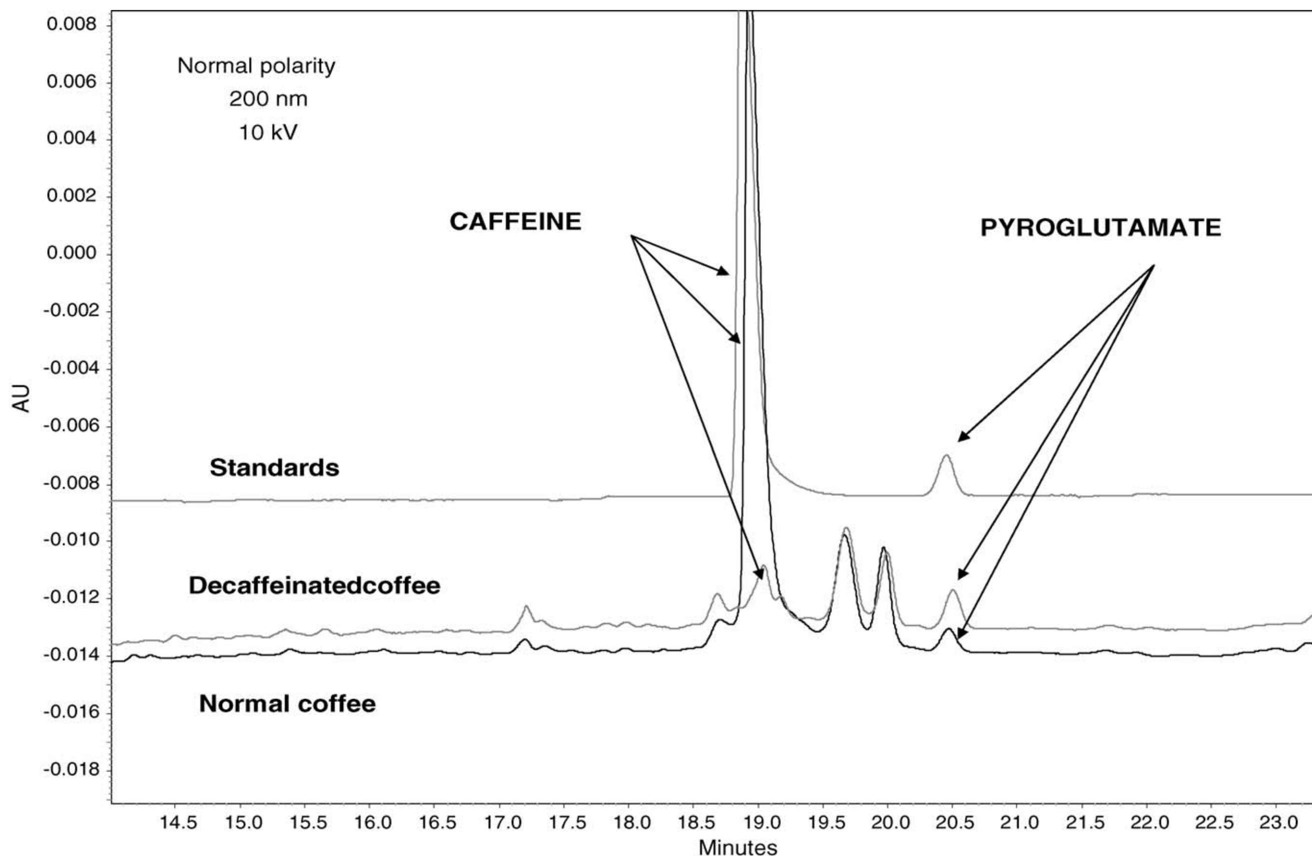

Fig. 1. Electropherograms for caffeine and pyroglutamate standards (upper); decaffeinated and normal coffees (lower); conditions: UV detection at 200 nm, voltage 10 kV, capillary untreated 57 cm and 50 μm i.d, 130 mM SDS and 0.05 M borate buffer at pH 9.5.

Linearity was determined by a series of three replicates of six levels of standards whose concentrations span a wide range to include all possible samples. As can be seen in Table 2, the linear regression equation applied to the results gave an intercept not significantly different from zero. The slopes were different from zero and correlation coefficients were over 0.99 in both cases. Samples linearity was also adequate for the same reasons, but this experiment was mainly developed to study the recoveries. All the recoveries include 100% and R.S.D.s presented very adequate values. Intra-assay precision of the method for samples gave R.S.D. ranging 4.2–6.4% considering both standards and samples. On two different days, R.S.D. values were very similar. Limits of detection were lower than the expected values in samples. Therefore, the method can be considered suitable for the intended purpose.

4.2. Coffee analysis

Once the method was optimised and validated the different coffee samples were measured and results are listed in Table 1. When an ANOVA test was applied, no statistically significant difference was found ($p < 0.05$) in caffeine content among the decaffeinated coffees (samples 3, 4, 7–9 and 11); meanwhile, normal coffees showed statistically significant differences. These results could be related with different origin and/or industrial processes.

Regarding pyroglutamate, higher values were found both in normal (samples 6 and 10) and decaffeinated coffees (samples 3, 9 and 11). Correlations are difficult to establish, as the information on the labels is quite scanty. The thermal lability of nutrients has been known for several decades, especially as it relates to food processing. In that sense, L-glutamate can be spontaneously converted to PGA. It has been shown that virtually no free amino acids are still present in coffee beans after roasting for 5 min at 220 °C [14].

4.3. Animal experimentation

After the quantification, four instant coffees were selected for in vivo experimentation: coffee 1 because it had the highest caffeine in the group with a proportionally low pyroglutamate content, coffees 3 and 11 due to their high pyroglutamate with low caffeine content and finally coffee 4 with similar content in both compounds. Coffee 1 was the most potent in producing locomotor stimulation and the only one to achieve statistical differences when compared to the control group. Coffees 3 and 4 also tended to increase locomotor activity but the effect was not significant, and coffee 11 was devoid of any apparent action on this test (Fig. 2). Concerning passive avoidance studies, the administration of coffee 1 produced in all the animals a large increase of latency, always exceeding the cut-off time during the acquisition trial. This effect disabled our procedure to measure

Table 2
Main validation parameters for the analytical method for the determination and quantification of caffeine and pyroglutamic acid in coffee

	Coffee	
	Caffeine (12.5–626.18[a])	Pyroglutamic acid (4.29–214.30[a])
Linearity		
Standards		
Intercept ± L.C.	1157 ± 10555	341 ± 1109
Slope ± L.C.	500 ± 30	90.3 ± 8.2
Accuracy		
Samples		
r	0.995	0.994
Recovery (%)	98.9	103.1
R.S.D (%)	4.5	4.8
Instrumental precision		
Standards		
n	6	6
Mean RF (UA/μmol/L)	499.98	90.77
R.S.D. (%)	2.8	1.8
Standards method precision		
Intra-assay		
n	6	6
Mean RF (UA/μmol/L)	460.75	83.6
R.S.D. (%)	6.4	5.5
Intermediate		
n	11	12
Mean (μmol/L)	471.49	81.44
R.S.D. (%)	5.79	6.48
Samples method precision		
Intra-assay		
n	6	6
Mean (μmol/L)	46.6	134.9
R.S.D. (%)	5.1	4.2
Intermediate		
n	12	12
Mean (μmol/L)	45.73	137.8
R.S.D. (%)	5.4	5.1
LOD (μmol/L)	0.23	0.69
LOC (μmol/L)	0.76	2.30

L.C.—limit of confidence.
[a] Range (μmol/L).

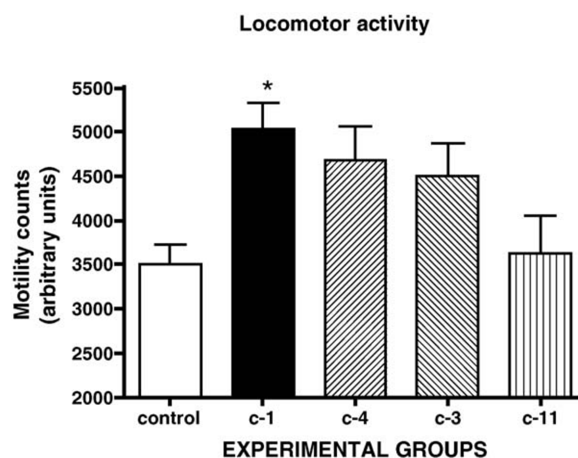

Fig. 2. Locomotor activity exhibited by mice orally treated with tap water (control) or coffee solutions (1 g/kg). Bars are means and S.E. from at least nine determinations. $^{*}p < 0.05$ vs. control.

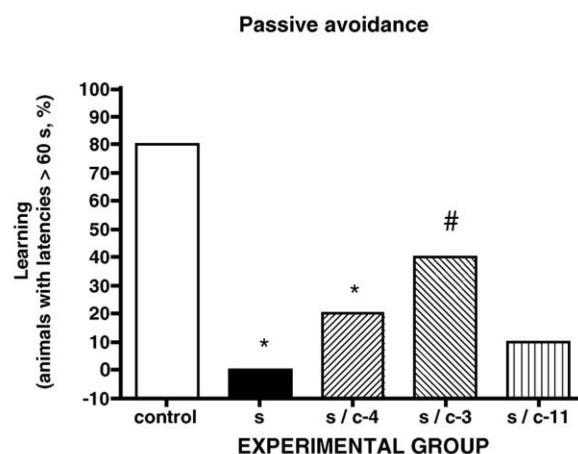

Fig. 3. Learning assessed by the passive avoidance test in mice treated with saline i.p./tap water p.o. (control), scopolamine/tap water (s) and scopolamine/coffee (s/c). $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. scopolamine.

learning in this case, and was apparently provoked by a marked increase of exploratory behaviour in the dim illuminated chamber. The rest of the coffees did not exhibit this action and partially prevented the marked amnesia provoked by scopolamine injection, coffee 3 being the only one to produce a significant increase of the number of mice that learned the task (Fig. 3).

The combined results of the biological evaluation of the coffees showed a marked stimulating effect of coffee 1, as shown by the significant increase of locomotor activity and the marked potentiation of exploratory behaviour during passive avoidance acquisition trials. This effect was expected due to the high caffeine content of the sample. The low caffeine content of coffees 3 and 4 could account for their residual tendencies to increase locomotor activity; however, these effects were not significant and both these coffees and coffee 11 lacked any prominent effect on exploratory behaviour, since all the animals treated showed normal latencies below 60 s in acquisition trials. Therefore, the psychomotor stimulant effects of coffees 3, 4 and 11, if any, was only mild at the dose tested, and hardly explain the tendency of these coffees to enhance cognition in passive avoidance experiments. Rather, the pyroglutamate content of these coffees may account for their tendency to reverse scopolamine amnesia, since coffee 3 was the most active and also exhibited the higher content of pyroglutamate. Anyway, these results must be considered preliminary and require further confirmation by assaying different doses, additional tests of learning/memory and appropriate pharmacological experiments to clarify the mechanisms of these actions.

References

[1] T. Antonelli, V. Carla, L. Lambertini, F. Moroni, C. Bianchi, Pharmacol. Res. Commun. 16 (1984) 189–197.
[2] B. Baena, D. Garcia-Martinez, C. Barbas, J. Chromatogr. A 1051 (2004) 199–205.

[3] C. Barbas, N. Adeva, R. Aguilar, M. Rosillo, T. Rubio, M. Castro, Clin. Chem. 44 (1998) 1340–1342.
[4] C. Barbas, A. García, L. Miguel, C. Simó, J. Chromatogr. B 780 (2002) 73–82.
[5] C. Chen, C. Liang, J. Lai, Y. Tsai, J. Tsay, J. Lin, J. Agric. Food Chem. 51 (2003) 7495–7503.
[6] V. Galli, A. García, L. Saavedra, C. Barbas, Electrophoresis 24 (2003) 1951–1981.
[7] V. Galli, N. Olmo, C. Barbas, J. Chromatogr. A 894 (2000) 135–144.
[8] V. Galli, N. Olmo, C. Barbas, J. Chromatogr. A 949 (2002) 367–372.
[9] V. Galli, C. Barbas, J. Chromatogr. A 1032 (2004) 299–304.
[10] A. Garcia, C. Barbas, R. Aguilar, M. Castro, Clin. Chem. 44 (1998) 1905–1911.
[11] S. Grioli, C. Lomeo, M. Quattropani, G. Spignoli, C. Villardita, Fundam. Clin. Pharmacol. 4 (1990) 169–173.
[12] G.L. Long, J.D. Winefordner, Anal. Chem. 55 (1983) 712–724.
[13] M. Lorist, M. Tops, Brain Cogn. 53 (2003) 82–94.
[14] H.G. Maier, 12th International Scientific Colloquium on Coffee, ASIC, Paris, 1987, pp. 229–237.
[15] S. Mirzoian, M. Zalinian, M. Balasanian, A. Topchian, Eksp. Klin. Farmakol. 57 (1994) 22–24.
[16] L. Saavedra, C. Barbas, Electrophoresis 24 (2003) 2235–2243.
[17] L. Saavedra, A. Garcia, C. Barbas, J. Chromatogr. A 881 (2000) 395–401.
[18] A. Sahgal, Behavioural Neuroscience, IRL Press, Oxford, 1993, pp. 49–56.
[19] H.G. Vogel, W.H. Vogel, Drug Discovery and Evaluation, Springer, Berlin, 1997.
[20] X.-Q. Xiao, G.-Q. Liu, Zhongguo Yaoli Xuebao 20 (1999) 733–736.