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Multivariate optimisation of the experimental conditions for determination of three methylxanthines by reversed-phase high-performance liquid chromatography

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Abstract

Caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) are the most important naturally occurring methylxanthines. Caffeine is a constituent of coffee and other beverage and included in many medicines. Theobromine and theophylline are formed as metabolites of caffeine in humans, and are also present in tea, cocoa and chocolate products.

In order to improve the chromatographic resolution (R_s) with a good analysis time, experimental designs were applied for multivariate optimisation of the experimental conditions of an isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) method used for the simultaneous determination of caffeine, theobromine and theophylline. The optimisation process was carried out in two steps using full three-level factorial designs. The factors optimised were: flow rate and mobile phase composition. Optimal conditions for the separation of the three methylxanthines were obtained using a mixture of water/ethanol/acetic acid (75:24:1%, v/v/v) as mobile phase and a flow rate of 1.0 mL min^{-1}. The RP-HPLC/UV method was validated in terms of limit of detection (LOD), limit of quantitation (LOQ), linearity, recovery and the precision, calculated as relative standard deviation (R.S.D.). In these conditions, the LOD was 0.10 $\mu g\,L^{-1}$ for caffeine, 0.07 $\mu g\,L^{-1}$ for theobromine and 0.06 $\mu g\,L^{-1}$ for theophylline. The proposed method is fast, requires no extraction step or derivatization and was suitable for quantification of these methylxanthines in coffee, tea and human urine samples.

Keywords: Methylxanthines; Multivariate optimisation; Isocratic elution; RP-HPLC/UV

1. Introduction

The methylxanthines caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) are alkaloids naturally presents in plants used as stimulants beverages, such as black and matte tea, coffee, cocoa and soft drinks [1,2]. They also exhibit extraordinary capacity as therapeutic agents and have been utilized in many pharmaceutical formulations as analgesic, diuretics and bronchodilator. These substances are widely known by their properties as stimulants of the central nervous system and cardiac stimulants [3,4]. However, the consumption of high concentration of these compounds can cause some undesirable effect such as cardiac arrhythmia, excitement, nausea and gastric acidity.

The International Olympic Committee (IOC) has classified caffeine as a drug of abuse when it is present in human urine with concentrations higher than 12 $\mu g\,mL^{-1}$ [2,5,6]. Although theobromine and theophylline are not considered as illicit substances for humans, their administration is prohibited in racing animals, like dogs or horses [3,6].

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Several analytical methods have been proposed for determination of these methylxanthines in food [7], pharmaceutical products [8] and biological fluids [9], but the great majority of these methods need extensive sample pre-treatment or do not allow the complete separation and quantitation of different methylxanthines present in a same sample. The reversed-phase high-performance liquid chromatography (RP-HPLC) has been the technique most commonly used for determination and quantitation of these methylxanthines [10 and references cited herein], meanwhile, the chromatographic separation between the two isomeric methylxanthines, theophylline and theobromine, needs to be improved, especially in analysis of complex matrices like biological fluids.

The optimisation of the chromatographic separation was based on the following criterion: "an acceptable chromatographic resolution in the shortest possible analysis time". The minimum value of chromatographic resolution (R_s), between two peaks, that leads to a complete baseline separation is 1.0, and the maximum value, without to engage the analysis time, is 1.5. When this range is not feasible, an alternative could be to use experimental design for optimisation of factors, such as, mobile and stationary phases composition, temperature, column length, flow rate, solvent strength, that affect the quality of the separations [11].

The use of multivariate techniques for optimisation of analytical procedures is generally performed in two steps: firstly, a preliminary evaluation using factorial design, with the objective of selecting the significant variables that affect the analytical method and, afterward, estimate a function fitted among the analytical response and the significant variables, allowing calculations for determination of the optimal values for these variables in this method [12–14].

The full three-level factorial design [15,16] is a response surface methodology that has limited application in experimental design when the factor number is higher than 2, because the experiment number required for this design is calculated by expression $N = 3^k$, where N is experiment number and k is factor number. However, for two factors it has efficiency comparable with others designs as central composite, Doehlert matrix and Box–Behnken [17].

In this paper, a method for simultaneous determination of the methylxanthines: caffeine, theobromine and theophylline by RP-HPLC/UV were optimised using full three-level factorial design. In the optimisation, the response was established considering resolution among the peaks of the theobromine and theophylline and also analysis time.

2. Experimental procedure

2.1. Apparatus

A Perkin-Elmer liquid chromatograph series 200 equipped with a Rheodyne (Cotati, CA, USA) injector valve with a 20 μL sample loop and a Perkin-Elmer series 200 model UV–vis detector were used. Chromatographic separation was performed on a LiChrospher 100 RP-18 (244 mm × 4.4 mm i.d., 5 μm; Merck, Darmstadt, Germany) column linked to a LiChrospher guard column of similar characteristic (4 mm × 4 mm i.d.; Merck). The detection was performed in UV at 273 nm.

2.2. Chemicals and reagents

Methanol, ethanol acetonitrile (HPLC grade, 99.9%) and glacial acetic acid were obtained from Merck and filtered in 0.45 μm membrane. Caffeine (Carlo Erba, Milan, Italy) was purified by sublimation; theophylline was obtained by extraction from medicinal Talofiline® 100 mg (Novartis-Sandoz, Switzerland) and purified by recrystallization; theobromine (Sigma Chemical Co., St. Louis, MO) was purified by recrystallization. Purified water was obtained by distillation and filtration through E-pure Alltech system (Deerfield, IL).

2.3. Preparation of methylxanthines stock and calibration solutions

After methylxanthines purification by sublimation and recrystallization, stock solutions of caffeine, theobromine and theophylline were prepared by dissolving 40 mg of each in 200 mL of ethanol/water (50:50%, v/v) or methanol/water (50:50%, v/v) and filtered through a 0.45 μm membrane filter. They were kept refrigerated at 4 °C in the dark-glass flasks.

The standards solutions were prepared by dilution in water of the methylxanthines mixture stock solutions, in the concentration range from 1.0 to 60 μg mL^{-1} ($n = 10$ points). These solutions were stored in the dark-glass flasks at 4 °C. In these conditions, they remained stable for 60 days.

2.4. Sample preparation

All the sample solutions were filtered using Whatman 41 filter paper (twice, double filter) and diluted when necessary. Variable amounts were used to reproduce normal conditions of use.

Tea: 1.47 g of tea was placed in infusion, in about 150 mL of water (approximately one cup), at 100 °C for 3 min.
Coffee: 150 mL hot water/3.3 g coffee.
Urine: the urine samples (48 samples), obtained from volunteers people, were stored at 4 °C, defrosted before the analysis and centrifuged by 10 min.

2.5. Optimisation strategy

The optimisation was performed in two steps. Firstly, a full three-level factorial design was carried out for optimisation of the variables of mobile phase (polarity and flow rate). In the first stage, it was studied the influence of the strength (polarity) and selectivity of the mobile phase in the chromatographic resolution of the three methylxanthines. For this, methanol, ethanol and acetonitrile were used as organic

Table 1
Factors and levels used in the first full three-level factorial design

Variable	Low (−)	Medium (0)	High (+)
Polarity	Acetonitrile	Ethanol	Methanol
Flow rate (mL min^{-1})	0.6	0.8	1.0

Table 2
Factors and levels used in the second full three-level factorial design

Variable	Low (−)	Medium (0)	High (+)
% Acetic acid	2.0	6.0	10
Flow rate (mL min^{-1})	1.0	1.1	1.2

Table 3
Factors and levels used in the second full three-level factorial design

Variable	Low (−)	Medium (0)	High (+)
% Acetic acid	0.5	1.0	1.5
Flow rate (mL min^{-1})	1.0	1.1	1.2

modifiers of the mobile phase with the following composition: organic modifier/water/acetic acid (20:75:5%, v/v/v). The flow rate was changed from 0.6 to 1.2 mL min^{-1}. Maximum and minimum levels of each factor were chosen in agreement with data obtained in previous experiments and they are shown in Table 1. In all experiments, the response was established by ratio among resolution and time analysis.

In a second factorial design, for studies of optimisation of the acidity of the mobile phase, different concentrations of acetic acid have been used, and are shown in Tables 2 and 3. The experimental data were processed by using the Statistic computer program [18].

3. Results and discussion

Most of the methods described in the literature for methylxanthines analysis uses mobile phases containing only methanol–water (apparent pH 6) [19], which allows caffeine quantitation but does not separate theophylline from theobromine. Considering that methylxanthines can suffer protonation, resulting in ionic species stabilized by resonant and inductive effects, if the pH decreased below 4, the xanthines became protonated and the interaction with C18 reverse-phase columns is increased. In this way, in a previous work [10], we described a chromatographic method for separation of the methylxanthines in different samples, adding acetic acid to the mobile phase (apparent pH < 3), which allowed reasonable separation between theophylline from theobromine [10]. However, taking into account that the obtained separation is not satisfactory for analysis of the complex matrices (i.e. fluids biological), it is necessary to study the influence of the other variables that affect a complete separation (baseline resolution) of the chromatographic peaks of these compounds, applying multivariate optimisation methods, in order to improve the chromatographic resolution between the two isomeric methylxanthines, theophylline and theobromine.

3.1. Optimisation of polarity and flow rate of the mobile phase

The data of the three-level full factorial design performed (in triplicate) for optimisation of polarity and flow rate of the mobile phase are described in Table 4. In it, can be seen the responses as chromatographic resolution and also chromatographic resolution in function of the analysis time. The results of this factorial were evaluated using analysis of the variance (ANOVA). The effects and significance of the variables considering the two responses are shown in Pareto charts (Fig. 1A and B).

3.1.1. Results using chromatographic resolution as response

The results as Pareto chart (Fig. 1A) shows that the factor more significant is the polarity of the mobile phase. In Fig. 2, it can also be seen that the chromatographic resolution increases with the polarity of the mobile phase and it is independent of the used flow rate. Then, the better conditions for chromatographic resolution were achieved when methanol was used as mobile phase. However, the use of this mobile phase implies an analysis time of 13.4 min, which can constitute disadvantage for the method.

3.1.2. Results using chromatographic resolution in function of the analysis time as response

The results (Fig. 1B) considering chromatographic resolution in function of the analysis time demonstrate that the

Table 4
Design matrix and the results of the chromatographic separation

No.	Mobile phase	Flow rate (mL min^{-1})	Chromatographic resolution (theobromine and theophylline)	Chromatographic resolution/analysis time
1	+	+	2.01	0.191
2	+	0	2.23	0.134
3	+	−	1.96	0.086
4	0	+	1.00	0.180
5	0	0	1.03	0.150
6	0	−	1.05	0.116
7	−	+	0.86	0.152
8	−	0	0.92	0.133
9	−	−	0.86	0.092

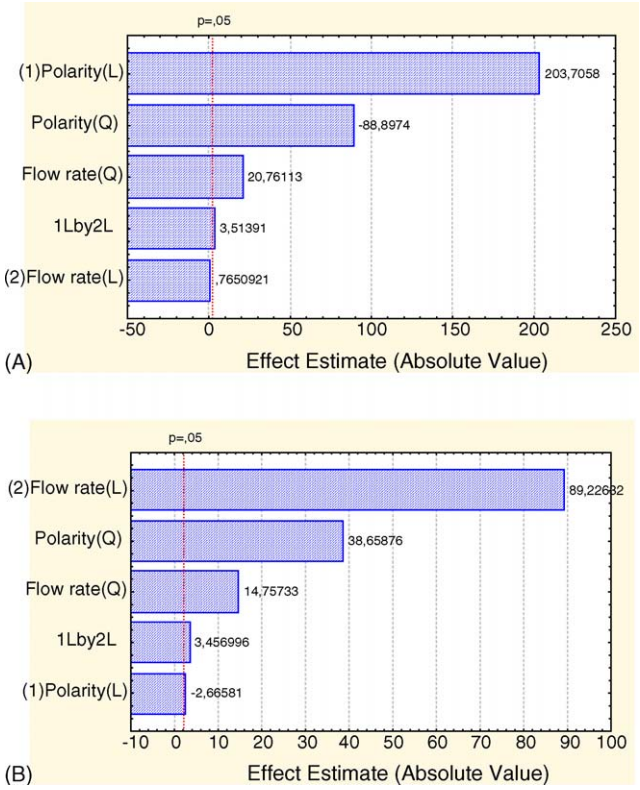

Fig. 1. Pareto chart considering chromatographic resolution: (A) as analytic response and (B) in function of the analysis time as analytic response. (L) means linear and (Q) means quadratic.

flow rate was the factor more significant. Fig. 3 shows that the better responses were achieved with the ethanol mobile phase (medium polarity) with flow rate in the maximum level.

These results are very interesting considering the smallest toxic degree and smaller cost of the ethanol when compared to the methanol. Also, the results revealed $R_S = 1.0$ in a time of analysis of 5.5 min for the ethanol mobile phase, which was advantageous considering the time of analysis of 13.4 min, when the methanol mobile phase was used. Indeed, the ethanol was recommended for the method.

3.2. Optimisation of the acidity and flow rate of the mobile phase

In a second experimental design, for studies of optimisation of the acidity of the mobile phase, different concentrations of acetic acid were used (0.5, 1.0, 1.5%). Resolution of 1.28 between the two isomeric methylxanthines (theobromine and theophylline) was obtained in the conditions flow rate optimised of 1.0 mL min^{-1} and composition of the mobile phase containing 1% of acetic acid, namely ethanol/water/acid acetic of (24:75:1%, v/v/v). The contour line graph that represents this result is shown in Fig. 4.

The relationship among the composition of the mobile phase (F), flow rate (V) and the chromatographic resolution in function of the time of analysis (R_S/t) it is represented by the equation below:

$$\frac{R_S}{t} = 0.276519 + 0.012278F - 0.033611F^2 + 0.036389V - 0.01194V^2 \quad (1)$$

Deriving this equation in function of the variables can get the great conditions for flow rate and composition of the mobile phase.

$$\frac{\partial R_S}{\partial F} = 0.012278 - 2(0.033611)F = 0 \quad (2)$$

$$\frac{\partial R_S}{\partial V} = 0.036389 - 2(0.011944)V = 0 \quad (3)$$

In this way, the optimal responses appears within the experimental domain and the corresponding values are $F = 1.09\%$ and $V = 1.2$ mL min^{-1}.

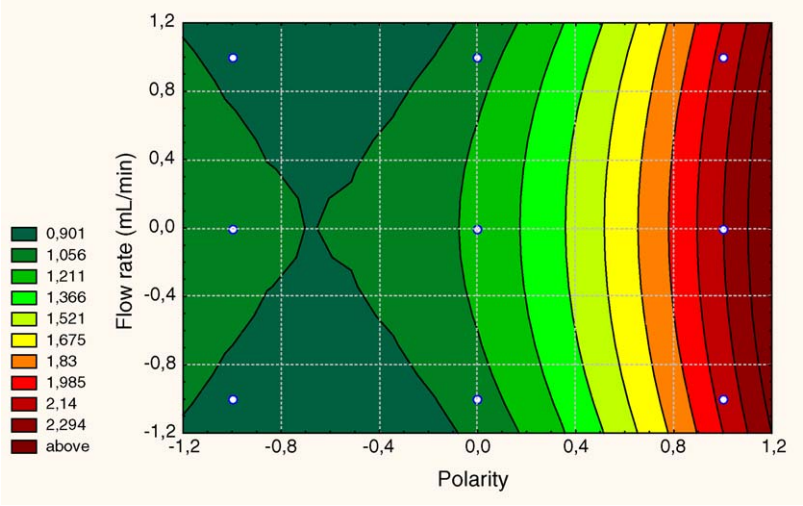

Fig. 2. Contour line graph considering chromatographic resolution as analytic response.

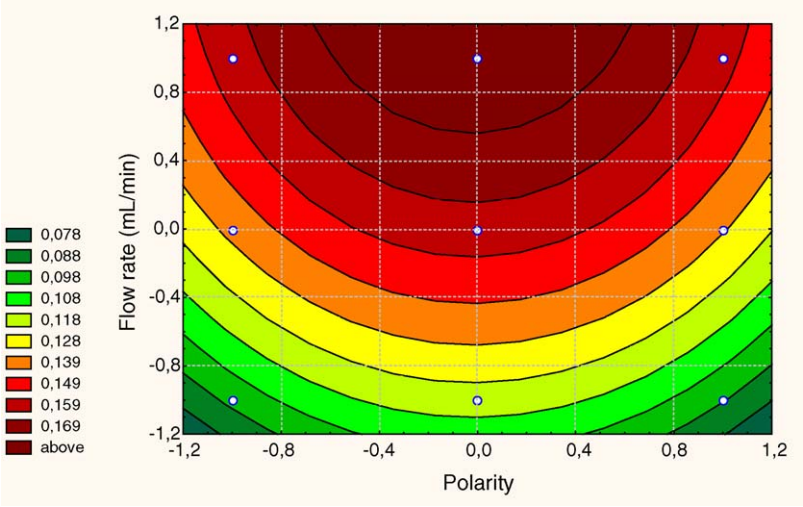

Fig. 3. Contour line graph considering chromatographic resolution in function of the analysis time as analytic response.

Nevertheless, high flow rate of the mobile phase increases a lot the pressure of the chromatography system which is not recommended and, considering that the resolution among the peaks of two isomeric methylxanthines ($R_s = 1.28$) obtained for of flow rate 1.0 mL min^{-1} and composition of the mobile phase: ethanol/water/acetic acid of (24:75:1%, v/v/v), is satisfactory, these conditions were chosen for the accomplishment of the analysis of the samples.

In this case, the objective was to achieve: the best chromatographic resolution ($1.0 \leq R_s \leq 1.5$) [11] in the shortest possible analysis time. How the response of the optimum points reached individually for two factors coincide, the application of the multiresponse analysis is not necessary [20].

The experiment performed under optimised conditions of flow rate and mobile phase gives the chromatogram of Fig. 5, in which, within 6 min, all three methylxanthines are separated in both a standard solution and a sample of human urine. This methodology, when compared to our previous work [10] has shown a better chromatographic resolution for the same time of analysis, which improves the peak identification and quantification even when applied for complex matrices like human fluids.

3.3. Method validation

The RP-HPLC method was validated in terms of limit of detection (LOD), limit of quantitation (LOQ), linearity and precision calculated as relative standard deviation (R.S.D.).

The limit of detection of the method, defined as signal equal to three times the baseline noise, were 0.07 μg mL^{-1} for theobromine, 0.06 μg mL^{-1} for theophylline and 0.10 μg mL^{-1} for caffeine and the limit of quantitation, defined as signal equal to 10 times the baseline noise, were 0.23 μg mL^{-1} for theobromine, 0.18 μg mL^{-1} for theophylline and 0.33 μg mL^{-1} for caffeine. These results are shown in Table 5.

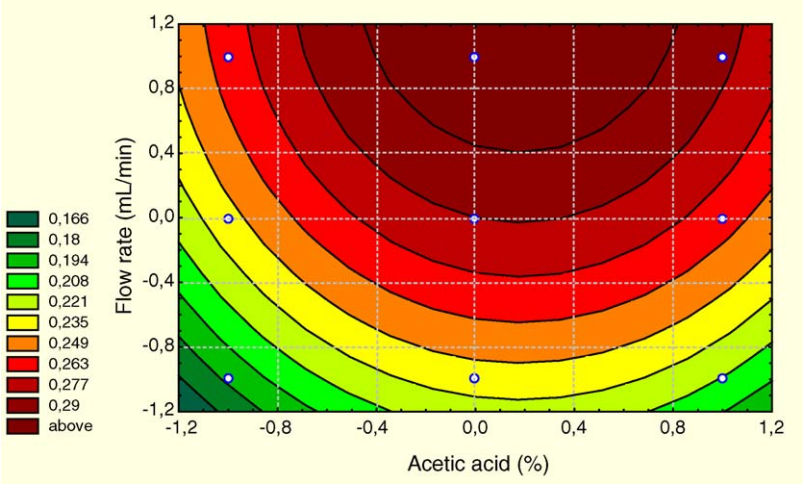

Fig. 4. Contour line graph representing the optimised conditions of the method.

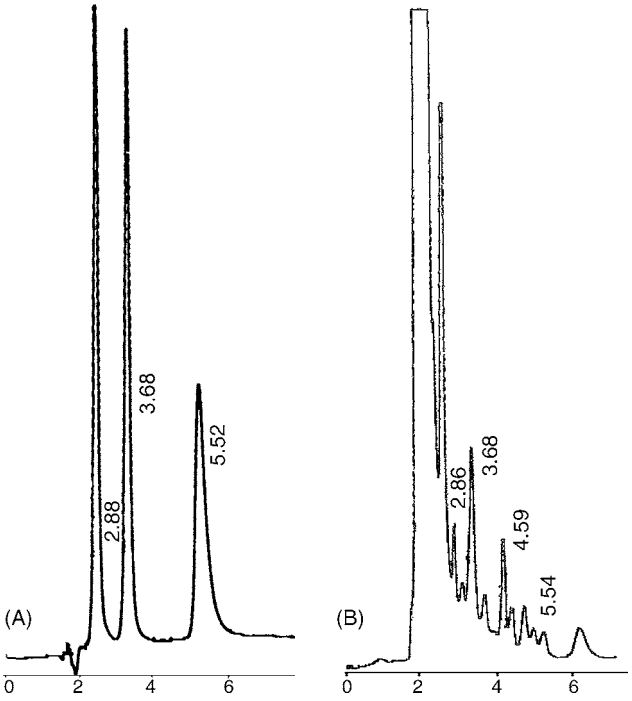

Fig. 5. Chromatograms obtained for (A) methylxanthine standards and (B) a human urine sample with ethanol/water/acetic acetic (24:75:1%, v/v/v) as a mobile phase. Numbers in the figure, 2.88, 3.68 and 5.52, represent the theobromine, theophylline and caffeine retention time, respectively.

The analytical curves were linear for the three methylxanthines in the concentration range of 1.0–60 μg mL^{-1} ($n = 10$ points). The calibration curves showed good linearity, as is shown in Table 5.

The accuracy and the matrix effects of the method were evaluated using recovery tests. Recoveries were calculated by comparison of the results with those obtained from the analysis of mixed standard solutions spiked in tea, coffee and human urine samples. In the case of coffee samples, theophylline was not added because this compound was not found in any sample analyzed. Spiked recoveries from the samples ranged from 94 to 105% for all analysts (Table 6), which indicated that matrix effects in the samples studied were minor. The recoveries also demonstrate that this method can be satisfactorily used for determination of these methylxanthines in these kinds of samples.

The relative standard deviations were determined for different matrices (tea, coffee and urine, $n = 6$ replicates) and are shown in Table 7. In the previous work [10], the LOD and R.S.D. were determined using standard solutions and in this work they were done with real samples. In this way, the obtained values herein are not comparable with those determined previously. Indeed, the results presented in Tables 5–7, confirm the feasibility of this method for the analysis of the three methylxanthines in the samples analyzed.

3.4. Analytical application

The optimised methodology was applied for determination of caffeine, theobromine and theophylline in coffee, tea and human urine samples. In the beverage, variables amounts were used to reproduce normal conditions the use. The results are described in Table 7 and are agreement with data of the literature [5,21,22].

Table 5
Performance characteristics

Methylxanthines	Calibration curves	r^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Theobromine	$Y = 0.513X - 0.2771$	0.9990	0.07	0.23
Theophylline	$Y = 0.4653X - 0.3925$	0.9987	0.06	0.18
Caffeine	$Y = 0.1849X - 0.1324$	0.9979	0.10	0.33

Note: Y: peak height; X: concentration (μg mL^{-1}); r^2: determination coefficient; LOD: limit of detection; LOQ: limit of quantification.

Table 6
Results of the addition/recovery tests

Xanthines	Tea concentration (μg mL^{-1})				Human urine concentration (μg mL^{-1})				Coffee concentration (μg mL^{-1})			
	MSC	Add	Found	%R	MSC	Add	Found	%R	MSC	Add	Found	%R
Caffeine	35.62	40.00	77.72	105	7.94	8.00	15.95	100	650.4	200.0	857.0	103
	33.42	60.00	89.98	94	7.94	8.00	16.17	103	649.8	200.00	856.6	103
	35.97	80.00	11.8.8	103	7.94	8.00	16.17	103	646.6	200.00	202.2	101
Theobromine	1.94	2.00	3.82	94	10.83	8.00	18.77	99	17.67	20.00	37.52	99
	1.45	3.00	4.43	99	10.83	8.00	19.11	103	17.45	20.00	37.27	99
	2.28	4.00	6.06	94	10.83	8.00	19.01	102	17.33	20.00	37.32	100
Theophylline	15.22	4.00	19.24	101	4.55	8.00	12.83	103	na	na	na	
	15.12	8.00	23.30	102	4.55	8.00	12.96	105	na	na	na	
	15.01	12.00	27.26	102	4.55	8.00	12.91	104	na	na	na	

Note: MSC: measured sample concentration; add: added; %R: % recovery; na: not added.

Table 7
Concentrations of methylxanthines in the samples and the respective R.S.D. (%)

Samples	Caffeine ($\mu g\,mL^{-1}$) (R.S.D.)	Theobromine ($\mu g\,mL^{-1}$) (R.S.D.)	Theophylline ($\mu g\,mL^{-1}$) (R.S.D.)
Soluble coffee[a]	771.40 (0.02)	11.50 (0.01)	<0.06
Mate tea[b]	59.70 (0.01)	13.40 (0.06)	<0.06
Sene tea	<0.10	<0.07	15.50 (0.03)
Boldo tea	<0.10	1.80 (0.03)	<0.06
Black tea	66.40 (0.03)	<0.07	<0.06
Carqueja tea	<0.10	<0.07	<0.06
Human urine	0.80–8.70 (0.02)	1.40–52.4 (0.01)	1.20–42.30 (0.01)

[a] Concentrations registered in the literature: caffeine (110–1760 $\mu g\,mL^{-1}$) [5,20]; theobromine (17–27 $\mu g\,mL^{-1}$) [5,20]; theophylline (15–47 $\mu g\,mL^{-1}$) [10].
[b] Concentrations registered in the literature: caffeine (25–433 $\mu g\,mL^{-1}$) [5,22]; theobromine (17–27 $\mu g\,mL^{-1}$) [5,22]; theophylline (9–21 $\mu g\,mL^{-1}$) [10].

4. Conclusion

The optimisation using full three-level factorial design allowed an efficient and fast chromatographic method for determination of caffeine, theobromine and theophylline.

The isocratic optimised conditions provided a high resolution of the all xanthines in less than 6 min, with LOD ranged between 0.06 and 0.10 $\mu g\,L^{-1}$ for standard solution.

The use of the ethanol as constituent of the mobile phase is advantageous, due to its low cost, its lower toxic level and shortening the time of analysis.

The method proposed was applied for analysis of teas, coffee and human urine samples. The achieved results for the samples are agreement with data of literature.

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