

Analytical Methods

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1 **Determination of cocaine, its metabolites and pyrolytic products by**
2 **LC-MS using chemometric approach**

3
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16

17

1 Abstract

2 A method to assay cocaine (COC), its metabolites benzoylecgonine (BZE),
3 ecgonine (ECG), ecgonine methyl ester (EME), benzoynorecgonine (BNE), pyrolytic
4 products anhydroecgonine (AEC) and anhydroecgonine methyl ester (AEME) and
5 adulterant levamisole (LEV) was developed and validated by liquid chromatography-
6 mass spectrometry (LC-MS) using a chemometric approach including a two-level
7 factorial design in the screening step and face-centered central composite design
8 (FCCCD) to achieve the optimization. The method was carried out on positive electro
9 spray ionization (ESI⁺) with a flow of 1 mL.min⁻¹ in isocratic mode consisting of 53%
10 methanol and 47% ammonium acetate 10 mmol.L⁻¹ pH 6.3. The chromatographic
11 separation was obtained with a Phenomenex Luna C18(2) column (250 mm x 4.6 mm,
12 particle size 5 µm), with the temperature set at 31 °C. Validation parameters such as
13 specificity, linearity, precision and accuracy were evaluated. The method was linear
14 over the concentration range of 1-100 ng.mL⁻¹ for COC, AEME, EME, LEV, BZE and
15 ECG and 5-100 ng.mL⁻¹ for AEC and BNE. The method was successfully applied to
16 identify and quantify the analytes.

17

18

19 Key words:

20 Cocaine;

21 Metabolites;

22 Pyrolytic products;

23 LC-MS;

24 Chemometric approach.

1 1. Introduction

2 According to the World Drug Report 2010 (UNODC) the estimated number of
3 cocaine users in 2010 ranged from 13.3 to 19.7 million of the global population aged
4 from 15 to 64 years.¹ The high consumption of illicit drugs severely harms users, their
5 families and society, and it is one of the major concerns of policies to reduce drug
6 related damage.² In Brazil the federal government implemented an integrated plan to
7 combat trafficking and drug consumption, investing in health treatment programs for
8 people addicted to cocaine and other illicit drugs.^{3,4,5} In Europe, the United States and
9 more recently in Brazil efforts are focused on uncovering the trafficking routes by
10 studying drug components such as adulterants, some typical alkaloids and residual
11 solvents.^{6,7,8,9,10}

12 In view of this, it is necessary to have the tools to perform a satisfactory
13 toxicological analysis to identify and quantify cocaine and its main metabolites,
14 degradation products and some adulterants.

15 Liquid chromatography- mass spectrometry (LC-MS) is a versatile technique to
16 determine illicit drugs such as cocaine and its metabolites due to high sensitivity and
17 selectivity. Unlike gas chromatography – mass spectrometry (GC-MS), it does not
18 require derivatization of some non-volatile compounds such as benzoylecgonine and
19 anhydroecgonine, the major metabolites of cocaine hydrochloride and *crack*,
20 respectively,^{11,12} further, it allows analysis without thermal degradation of the product
21 which interferes in the analysis.¹³

22 Toxicological analysis usually involves the evaluation of many factors and
23 different compounds in a same method, hindering method development. Some statistical

1 tools are important and can help the development and optimization to obtain a reliable
2 method. The chemometric approach including experimental design and response surface
3 methodology is very useful, but still little implemented in toxicological analysis, only a
4 few works apply this technique.^{14,15} When it is necessary to optimize more than one
5 response at a time a chemometric technique is helpful. Two-level factorial design is
6 important in the development stage of work to select which factors are significant and at
7 which level.¹⁶ Central composite design is a statistical technique used to obtain the
8 optimum conditions of the method.^{17,18} The best approach is to first screen for
9 significant factors followed by full optimization using a central composite design.¹⁶

10 Hence this paper aimed at developing and validating a simple LC-MS method,
11 using experimental design as an optimization tool to identify and quantify cocaine, its
12 pyrolysis product and its main metabolites (Figure 1).

13 **2. Experimental**

14 **2.1. Solvents and Chemicals**

15 Cocaine hydrochloride (COC) 98.2%, ecgonine (ECG) 99.5%, ecgonine methyl
16 ester (EME) 99.4%, benzoylecgonine (BNE) 98.9%, benzoylecgonine (BZE)
17 98.5% were kindly donated by the National Institute of Criminalistics (Brasília, DF,
18 Brazil). Levamisole hydrochloride (LEV) 100.1% was a secondary standard.
19 Anhydroecgonine (AEC) 95.9% and anhydroecgonine methyl ester (AEME) 99.3%
20 were synthesized in-house. LC-grade methanol (MeOH) was obtained from Tedia
21 (Fairfield, OH, USA). Acetic acid, ammonium hydroxide and ammonium acetate were
22 from Merck (Frankfurt, Germany). Ultrapure water was obtained using a Milli-Q Plus
23 system of Millipore (Bedford, MA, USA).

1 **2.2. Instrumentation**

2 An Agilent 1260 infinity LC system equipped with a G1311B quaternary pump,
3 a G1329B auto sampler, a G1314F UV/VIS detector, a G1316A thermostatizer coupled
4 to an Agilent 6120B series mass detector and a Chemstation (v. B.04.03) software were
5 used. They were all from Agilent Technologies (Palo Alto, CA, USA).

6 **2.3. Additional softwares:**

7 Minitab[®] 16.0 (State College, PA, USA) was used to analyze the experimental
8 designs. The statistical tests used for the validation analysis were performed using
9 Microsoft Excel 2010 (Redmond, WA, USA).

10 **2.4. Method Development**

11 Four reversed-phase columns with different stationary phases and column sizes
12 were tested for appropriate running time and resolution of the eight compounds
13 described above. Waters Xterra C18 MS (150 mm x 3.9 mm, 5.0 μ m), Agilent C8 300
14 SB (250 mm x 4.6 mm, 5.0 μ m) and Trinity P1 (100 mm x 3.0 mm, 3.0 μ m) columns
15 were tested and their performance was inadequate. The best results were achieved with
16 a Phenomenex Luna C18(2) (250 mm x 4.6 mm, 5 μ m) column which was then used to
17 perform the experimental design and validation.

18 To assess which factors really affect the response, a two-level factorial design
19 was performed aiming to spend less work time, reagents and samples. Fractional
20 factorial design is a kind of factorial design widely used in screening experiments.
21 Although faster and more practical than intuitive process development, a general

1 factorial design with a large number of factors requires numerous runs. Information on
2 main effects and two-order interactions may be assessed only in a fraction of the full
3 factorial design, without loss of credibility, the remaining fractions are related to higher
4 interactions. As an example, a general factorial design with 6 factors requires 64 runs,
5 only 15 correspond to two-factor interaction, the remainder are associated with three-
6 factor and higher interactions.¹⁷

7 One quarter fractional factorial design of five factors was applied (Table 1). The
8 notation is 2^{k-2} , where 2 is the number of levels (low level, -1; high level +1) for each
9 factor, k-2 means the number of factors applied on a one quarter fractional factorial.
10 This trial 2^{5-2} requires only 8 runs instead of 32 required in a full factorial design, which
11 confirmed the improvement in the development of these experiments. After this
12 screening, the factors that showed to be significant were selected and subsequently
13 applied in a face-centered central composite design (FCCCD). This design locates the
14 axial points on the centers of the faces of the cube, $\alpha = 1$, where α is the axial distance,
15 that supplies rotatability to provide good predictability throughout the region of
16 interest.^{17,18} The representation of a face-centered cube is shown in Figure 2. This kind
17 of central composite design is frequently used because it requires only three levels of
18 each factor making experiments easier.

19 The responses analyzed in this design were resolution between peaks (Rs) and
20 the retention factor (k') of each compound. The responses were modulated using
21 polynomial models. For an experimental design with three factors, a low-order
22 polynomial model is usually employed:

23
$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1.0)$$

1 In this first order response surface model, y represents the estimated response;
2 b_0, b_1, b_2, b_3 are a set of unknown parameters where b_0 is the average experimental
3 response, b_1 to b_3 are the main effects on the factors (x_1, x_2, x_3), and b_{12} to b_{23} are the
4 estimated effects with interactions. After model assessment, a higher order (quadratic or
5 cubic) polynomial model and/or mathematical transformation in the response is
6 sometimes necessary.¹⁹

7 When there are more than three design variables, sometimes the aim of each
8 response is contrasting, so it is necessary to use some tools to find a global optimum
9 response. There are several optimization approaches of multiple responses. One of
10 them, Derringer's Desirability, is a very useful method that utilizes desirability
11 functions.

12 **2.5. Liquid chromatography – mass spectrometry**

13 A satisfactory chromatographic condition was achieved using a Phenomenex
14 Luna C18 (2) column (250 mm x 4.6 mm, 5 μm) (Torrance, CA, USA) in an isocratic
15 condition consisting of 53% methanol and 47% 10 mmol.L⁻¹ ammonium acetate pH 6.3
16 adjusted with acetic acid. The flow rate was 1.0 mL.min⁻¹. The injection volume was 10
17 μL and the column temperature was set at 31 °C.

18 The mass spectrometer was operated with an electrospray ionization source in
19 positive mode (ESI⁺). The optimal parameters for the analysis were: gas temperature,
20 350 °C; drying gas flow 13.0 L.min⁻¹ (nitrogen); nebulizer gas pressure 40 p.s.i.
21 (nitrogen); capillary voltage at 3000 V and fragmentor at 100 V. The parameters of each
22 compound are detailed in Table 2.

1 2.6. Preparation of standard solutions

2 Stock standard solutions of each compound ($20 \mu\text{g}\cdot\text{mL}^{-1}$) were prepared in
3 methanol and stored at $-10 \text{ }^\circ\text{C}$. Working solutions were prepared by diluting stock
4 solution in methanol just before the analysis.

5 2.7. Validation

6 The validation was performed according to the Q2(R1) International Conference
7 on Harmonization (ICH) guideline and United States Pharmacopeia (USP 36).^{20,21}
8 Specificity, linearity, precision, accuracy, limit of detection and quantification were
9 assayed.

10 2.7.1. Linearity and range

11 Linearity was evaluated by constructing three calibration curves each one with 7
12 concentration levels on three different days. The range set for the analysis covers all
13 concentrations required for the proposed work. The concentration range used for COC,
14 AEME, EME, LEV, BZE and ECG were $1\text{-}100 \text{ ng}\cdot\text{mL}^{-1}$, and for AEC and BNE were 5-
15 $100 \text{ ng}\cdot\text{mL}^{-1}$. The analytical graphs were derived by plotting the peaks areas against the
16 analyte concentrations. The linearity was evaluated by the determination of correlation
17 coefficient significance (least square regression analysis) and residual analysis.

18 2.7.2. Specificity

19 Specificity is an important parameter which allows unequivocally measuring the
20 analytes when certain components may be expected to be present.²¹ Cocaine samples
21 usually contain different cutting agents. In this way it is important to determine the

1 influence of these compounds in order to avoid erroneous evaluations. Caffeine,
2 lidocaine, lactose, mannitol and phenacetin were evaluated.

3 Specificity was performed by analysis of samples at 50 ng.mL^{-1} spiked with
4 interfering compounds at $200 \text{ }\mu\text{g.mL}^{-1}$. The retention time of these compounds and their
5 peak areas were evaluated. The relative standard deviation (RSD) of peak area
6 measurements was used to express the results. Furthermore, carry-over was evaluated
7 by injecting methanol after the highest level of the analytical curve.

8 2.7.3. Accuracy and precision

9 Precision was determined by repeatability and intermediate precision. The
10 relative standard deviation (RSD) of peak area measurements was used to express
11 precision. To perform the repeatability study, six replicate experiments on an average
12 concentration (50 ng.mL^{-1}) were carried out on the same day. Intermediate precision
13 was measured by comparing the results of the assay on different days and between two
14 different analysts. Accuracy was inferred after analyzing the results of linearity,
15 specificity and precision as proposed by ICH.²¹

16 2.7.4. Determination of limit of detection (LOD) and limit of quantification (LOQ)

17 LOD and LOQ were calculated from the slope and the standard deviation of the
18 intercept of the mean of three calibration curves, determined by a linear regression
19 model. The factors 3.3 and 10 for the detection and quantitation limits, respectively,
20 were multiplied by the ratio from the standard deviation of the intercept and the slope,
21 according to the equations:

$$LOD = \frac{3.3\sigma}{s} \qquad LOQ = \frac{10\sigma}{s}$$

The LOQ was also evaluated in an experimental assay.

3. Results and discussion

3.1. Column selection

The first step to perform a chromatographic analysis with eight compounds was to select the column. Although a mass detector was used, a complete separation of the peaks is desirable because some fragments are common to more than one compound. A short column like Waters Xterra C18 MS (150mm x 3.9mm, 5.0 μ m) did not achieve a satisfactory resolution. Trinity P1 (100 mm x 3.0mm, 3.0 μ m) with mixed groups (octadecylsilane and cationic/anionic groups) attached to the stationary phase has greatly increased the running time of more non-polar compounds such as cocaine. The Agilent C8 300SB (250mm x 4.6mm, 5.0 μ m) column obtained an inadequate retention factor for the eight compounds. Therefore, a Phenomenex Luna C18(2) column (250 mm x 4.6 mm, 5 μ m) was tested and showed a satisfactory resolution and total running time.

3.2. Design of experiments

The five factors tested in this screening using 2^{5-2} design were: buffer concentration (low: 5 mmol.L⁻¹; high: 15 mmol.L⁻¹), pH (low: 4.5; high: 6.5), methanol proportion (low: 35; high: 65), flow rate (low: 0.6 mL.min⁻¹; high: 1.0 mL.min⁻¹) and oven temperature (low: 25 °C; high: 35 °C) (Table 1). The pH range was chosen due to pKa values of the compounds. All molecules are 2 pH units above or below their pKa

1 values avoiding incomplete ionization that could interfere in compound determination.
2 Retention factor and resolution were evaluated. Buffer concentration was tested in the
3 5-15 mmol.L⁻¹ range and showed no significant effect on retention factor for all
4 compounds. A buffer concentration of 10 mmol.L⁻¹ was chosen because it is a usual
5 work concentration without possible buffer interference. Resolution was not
6 significantly affected by the flow rate. Moreover, flow rates below 1.0 mL.min⁻¹ greatly
7 increased running time, thus flow rate was set at that value. Based on this, the next step
8 was evaluating the factors that showed to be significant for the resolution and retention
9 factor for the majority of the 8 compounds. The main effect plots are shown in Figure 3.

10 An FCCCD was applied using pH, MeOH proportion and oven temperature to
11 evaluate retention factor and resolution. AEC and ECG as well as their esters showed no
12 significant differences when conditions were changed. This occurred due to the high
13 polarity of these compounds and the small molecular size, resulting in a low retention in
14 the stationary phase. Based on the results of the FCCCD it can be concluded that the k'
15 of COC decreased in the higher proportion of MeOH and in the lower pH and the k' of
16 BZE increased in the lower proportion of MeOH and in the lower pH. The k' of BNE
17 increased when the proportion of MeOH decreased and the oven temperature increased.
18 The pH did not significantly affect k' for BNE. LEV was greatly affected by the tested
19 factors, in a lower proportion of MeOH and higher pH the k' increased significantly. It
20 was not possible to achieve a great separation between AEC, ECG and EME due to the
21 structural similarity and low retention power. Nevertheless, this did not cause any
22 problems for the quantification of these compounds, since it can sort them by molecular
23 ions. The resolution between AEC and AEME increased in high pH and high MeOH
24 proportions. The best resolution for AEME and BZE was found in lower pH, higher
25 temperature and lower MeOH proportion, while between BZE and BNE higher MeOH

1 proportion and higher pH were better. Resolutions among BNE, LEV and COC were
2 not as high as they could be because this would substantially increase the run time, so a
3 resolution value target for these compounds was set as 5.

4 *Derringer's Desirability* was used to improve the parameters in order to achieve
5 an optimum response. The Derringer desirability function is defined as a geometric
6 mean, weighted, or otherwise, of the individual desirability functions.¹⁸ In this
7 procedure it is necessary to convert each response into an individual desirability
8 function d_i ranging $0 \leq d_i \leq 1$, when the response y_i is at its target, $d_i = 1$, and if the
9 response is outside an acceptable region $d_i = 0$.¹⁷ Furthermore, the factors and responses
10 can be normalized by weight and importance and the function possesses a goal that can
11 be adjusted to minimize, maximize or target the factors and responses.²² The target
12 parameters were chosen to provide a more appropriate analysis. The results are shown
13 in Table 3. The comparison among the predicted values and the observed values are also
14 shown. The global optimum parameters after the optimization were: MeOH proportion
15 53%, oven temperature 31.0 °C, ammonium acetate 10 mmol.L⁻¹, pH 6.3 and flow rate
16 1.0 mL.min⁻¹. Representative chromatograms are shown in Figure 4. The optimized
17 conditions were tested and the response values presented no significant differences
18 compared to predicted values. The composite desirability value was 0.917378 which
19 shows that the responses are on target, the values of each response are shown in Table 3.

20 **3.3. Method Validation**

21 No interferences were detected in the retention time and concentration of target
22 analytes. The chromatographic run was completed in 8 minutes, only phenacetin,
23 caffeine and lidocaine are detected using the method described above at three times the

1 normal chromatographic run. The other compounds probably do not have good
2 retention or ionization under the proposed conditions. The similarity index of the target
3 compounds without and within the contaminants is shown in Table 4.

4 Linearity was evaluated in the concentration range of 1-100 ng.mL⁻¹ for COC,
5 AEME, EME, BZE, LEV; and of 5-100 ng.mL⁻¹ for AEC, ECG and BNE. All
6 correlation coefficients (r) were higher than 0.999 (Table 5). The residual coefficient
7 was also evaluated and the results were acceptable. Due to the high polarity and small
8 molecular size, AEC and ECG did not exhibit good retention in reversed phase
9 columns, such as C18 and C8, and thus quantification can be a challenge in complex
10 matrices. However, analytical measurements can be carried out. The lower levels of the
11 analytical curves were the experimental LOQ. The calculated LOD and experimental
12 LOQ were shown in Table 5.

13 Repeatability, inter-assay and inter-analysts precisions were established with the
14 concentration of 50 ng.mL⁻¹. The results are given in Table 5. The values demonstrated
15 a good precision. Accuracy is also shown in Table 5 and the values demonstrated that
16 the method was accurate within the validated range.

17 **4. Conclusions**

18 The chemometric approach including factorial design in the screening step and
19 face-centered central composite design was a very important and helpful tool to achieve
20 method optimization for the analysis of cocaine and its major metabolites as well as its
21 two main pyrolytic products. The method was successfully validated and demonstrated
22 to be adequate to identify and quantify cocaine hydrochloride, ecgonine, ecgonine
23 methyl ester, benzoylecgonine, benzoylecgonine, anhydroecgonine and

1 anhydroecgonine methyl ester in the presence of levamisole hydrochloride, a
2 contaminant commonly found in street cocaine.

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4 **Acknowledgments**

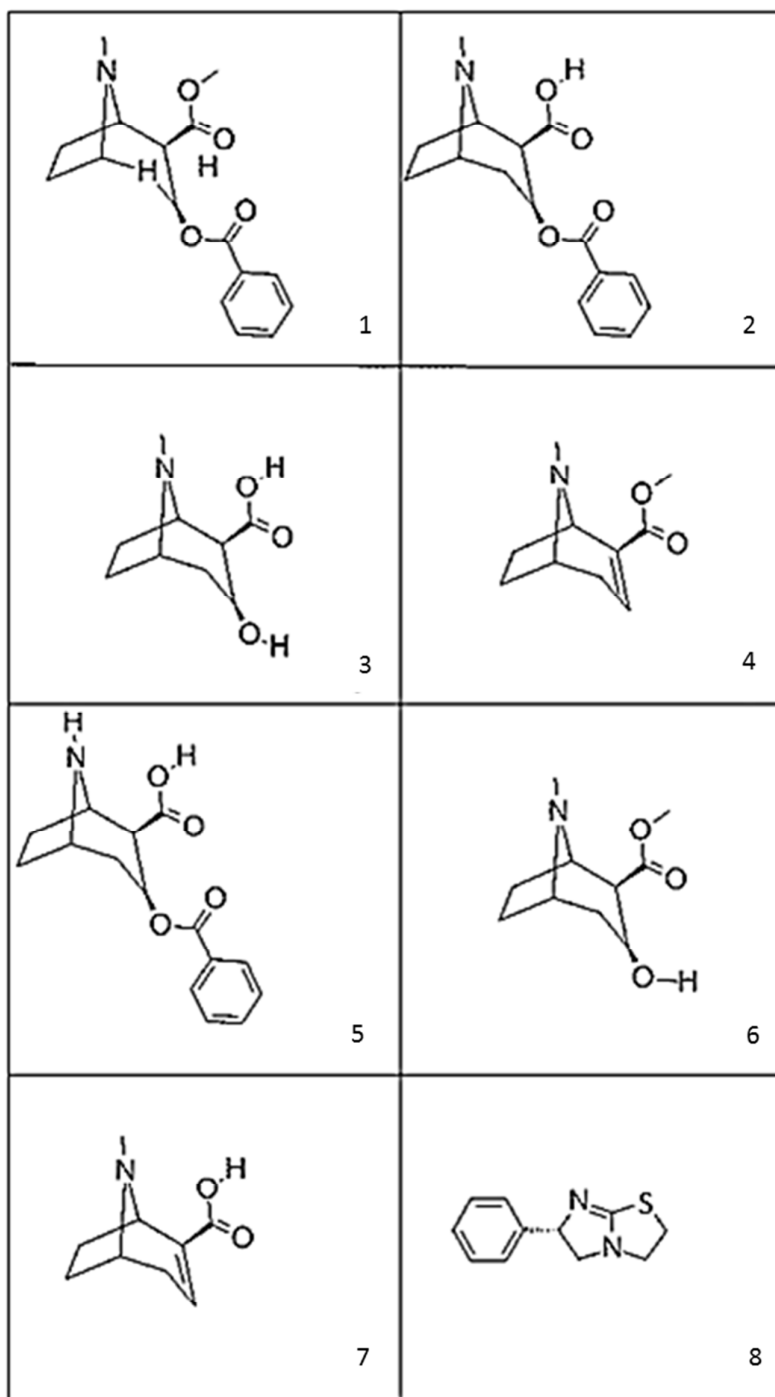
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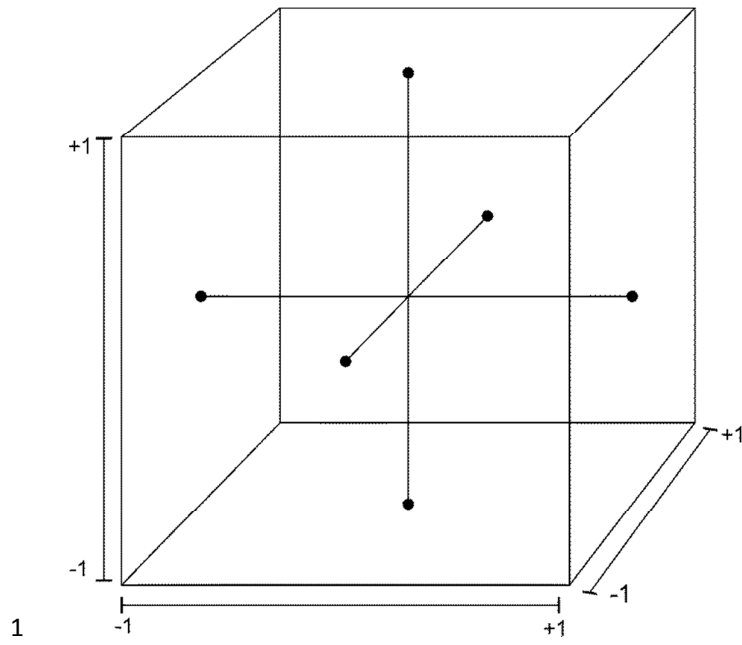
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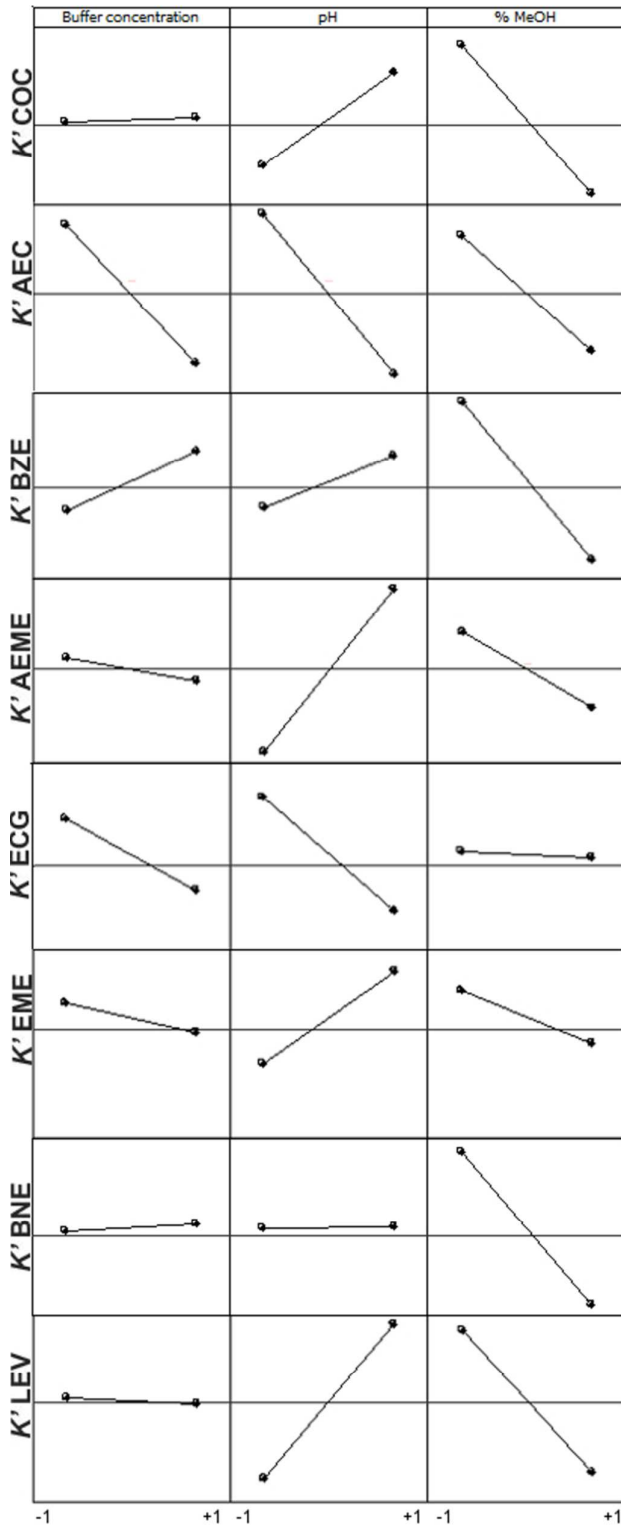
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2 **Figure 1:** Chemical structures of cocaine, its metabolites and pyrolysis products

3 1-COC; 2- BZE; 3- ECG; 4- AEME ; 5 – BNE; 6 – ECG; 7- AEC; 8- LEV



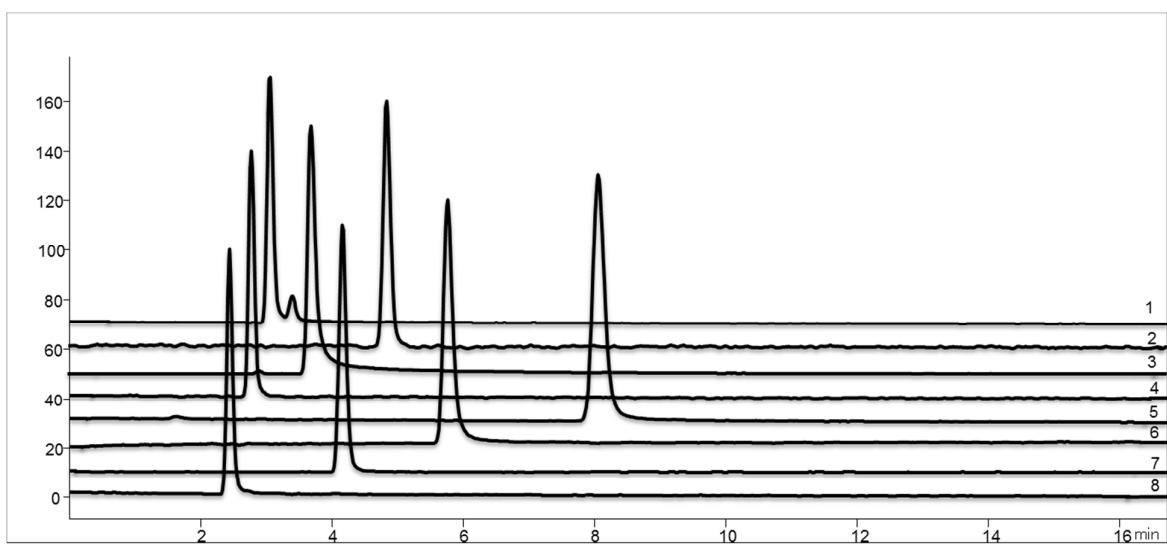
2 **Figure 2:** Graphic representation of FCDCC cube for $k = 3$



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2 **Figure 3:** Main effects plot for the 3 most significant factors of the k' of the 8 target

3 compounds



1 **Figure 4:** Representative chromatogram of EME (1), BNE (2), AEME (3), ECG (4),

2 COC(5), LEV (6), BZE (7), AEC (8).

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2 **Table 1:** Levels of one quarter fractional factorial design with 5 factors

Run Order	Factors and levels ^a				
	Buffer conc.	pH	% MeOH	Flow rate	Oven temperature
1	0	0	0	0	0
2	+1	+1	+1	+1	+1
3	-1	-1	+1	+1	-1
4	+1	-1	+1	-1	+1
5	-1	+1	-1	-1	+1
6	-1	-1	-1	+1	+1
7	-1	+1	+1	-1	-1
8	+1	+1	-1	+1	-1
9	+1	-1	-1	-1	-1

3 ^a(-1) = low levels; (+1) = high levels; (0) = nominal levels.

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1 **Table 2:** Fragmentation patterns of target compounds

Compound	Retention time (min.)	Quantifying ion (m/z)	Qualifying ions (m/z)	
COC	6.3	304.1	182	82
BZE	3.6	290.1	168	150
AEC	2.5	168.1	122	91
EME	2.5	200.1	182	150
AEME	3.2	182.1	122	118
ECG	2.5	186.1	168	150
BNE	3.8	276.2	154	136
LEV	4.9	205.2	178	118

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1 **Table 3:** Derringer desirability predicted values

Parameters	Goal	Importance	Desirability	Responses	
				Predicted	Experimental
K' COC	Maximize	1	1.0000	2.787	2.830
K' AEC	Maximize	1	0.732	0.235	0.250
K' BZE	Maximize	1	0.999	0.994	1.031
K' AEME	Maximize	1	0.924	0.672	0.642
K' ECG	Maximize	1	0.777	0.266	0.255
K' EME	Maximize	1	0.793	0.279	0.265
K' BNE	Maximize	1	1.000	1.131	1.159
K' LEV	Maximize	1	1.000	1.764	1.764
Rs 1-2	Maximize	1	1.000	3.935	3.740
Rs 2-3	Maximize	1	1.000	3.196	3.600
Rs 4-5	Target	1	0.961	5.325	4.410
Rs 5-6	Target	1	0.882	7.138	7.320

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1 **Table 4:** Analysis of specificity for method validation

	COC	BZE	AEC	AEME	EME	ECG	BNE	LEV
Control	130120.9	89482.9	81782.0	158871.0	170262.5	55565.0	12633.2	227482.4
Specificity	132771.8	87715,9	82019.3	155181.6	169549.5	54026.9	12630.1	230517.6
Diference	-2650.9	1767.0	-237.2	3689.4	713.1	1538.2	3.1	-3035.3
%	98.0	102.0	99.7	102.3	100.4	102.8	100.0	98.7

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1 **Table 5:** Accuracy and precision for method validation

Compound	Repeatability ^a			Inter-assay ^a	Inter-analysts ^a	Accuracy		LOQ ^b	LOD ^b	Correlation coefficient (r)
	Day 1	Day 2	Day 3			Assay (%)	%RSD			
COC	1.2	1.4	2.0	0.5	0.7	99.7	0.5	1.0	0.7	0.9999
BZE	1.9	1.8	1.9	0.9	0.4	99.3	0.9	1.0	0.6	0.9998
AEC	0.7	1.4	1.5	2.0	1.3	105.8	2.0	5.0	2.0	0.9997
AEME	0.9	1.6	1.5	1.0	0.4	99.9	1.0	1.0	0.7	0.9995
EME	1.5	1.9	1.9	0.3	0.3	100.3	0.3	1.0	0.7	0.9995
ECG	1.7	2.5	1.6	1.8	2.4	101.9	0.8	5.0	3.9	0.9999
BNE	0.7	0.9	1.1	1.7	2.0	99.9	1.7	5.0	3.4	0.9999
LEV	0.9	1.2	1.6	1.2	0.8	99.6	1.2	1.0	0.4	0.9998

2 ^aRSD = Relative Standard Deviation3 ^b(ng.mL⁻¹)

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