Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 925–934
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Validation of a gas chromatography—Ion trap tandem mass spectrometry for simultaneous analyse of cocaine and its metabolites in saliva

Emmanuelle Cognard [a], Stéphane Bouchonnet [b], Christian Staub [a,*]

[a] Institute of Forensic Medicine, University of Geneva, Avenue de Champel 9, CH 1211 Geneva 4, Switzerland
[b] Laboratoire des Mécanismes Réactionnels, Ecole Polytechnique, 91128 Palaiseau Cedex, France

Received 7 November 2005; received in revised form 17 January 2006; accepted 19 January 2006
Available online 23 February 2006

Abstract

Cocaine (COC) is one of the most widely used drugs of abuse. Therefore numerous procedures are published in the literature to propose an analysis of this substance and related compounds in different matrixes. In the same way, the authors have described, in a previous work, the simultaneous analysis of COC and three of its metabolites in hair by gas chromatography–ion-trap tandem mass spectrometry (GC–MS/MS) using chemical ionization with isobutane. The present paper investigated the ability to transfer this convenient existing method for hair to another matrix, in occurrence saliva. The aim of this work was then to verify that the whole procedure (solid phase extraction (SPE) and analytical method) was also convenient to analyse simultaneously COC and three of its metabolites in this matrix. Therefore this sensitive GC–MS/MS method has been studied for the simultaneous analysis of COC, anhydroecgonine methylester (AEME), ecgonine methylester (EME) and cocaethylene (COET) in saliva samples. The method has been validated and its performances were evaluated in terms of trueness and precision using quality control (QC) samples. For quantification, the following ranges were found appropriate: 5–500 ng/ml for EME, 2–500 ng/ml for COC and COET; AEME could only be determined "semi-quantitatively" between 2 and 200 ng/ml according to our chosen acceptance criteria. Suggested dissociation pathways have also been proposed to interpret the obtained spectra.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Chemical ionization; Cocaine; GC–MS/MS; Saliva; Ion-trap; Dissociation pathways

1. Introduction

Cocaine (COC), one of the most widely used drugs of abuse, is rapidly and almost completely metabolized to benzoylecgonine (BZE) by spontaneous chemical hydrolysis as well as to ecgonine methylester (EME) and ecgonine by esterase hydrolysis [1]. When COC is smoked, a pyrolysis product, anhydroecgonine methylester (AEME), is formed. COC is also frequently consumed together with alcohol; cocaethylene (COET), an active homologue, is formed arising through transesterification following concomitant intake of COC and ethanol. Fig. 1 displays the chemical structures of COC, and its metabolites COET, EME and AEME.

The traditional media for the quantitative measurement of most psychotropic drugs are blood and urine, because many substances and their metabolites are present in these biological matrices. Nevertheless, since the two past decades, the use of saliva for drug monitoring or pharmacokinetics studies has been developed. Oral fluid presents many advantages including the non-invasive and easy technique of collection, the low possibility of sample adulteration [2] and the presence of the parent drug as the principal analyte found. However, there are several disadvantages associated with saliva sampling like the limited sample volume, the concentration of target analytes which can be considerably low, the variable nature of salivary pH and the possibility of contamination with drug residues in the oral or nasal cavity [3]. Moreover, salivary pH and stimulated conditions of collection can affect the obtained results [4,5]. That is the reason why a great number of reviews focusing on the use of saliva in forensic drugs and other chemicals detection have been published [6–10]. There are also many articles reported for drugs concentrations in saliva [3,11,12] or correlation of oral fluid levels with plasma levels [12,13].

Saliva testing for COC and its metabolites has been reported in a number of publications and with many different

* Corresponding author at: Institute of Forensic Medicine, Unit of Toxicology, Avenue de Champel 9, CH 1211 Geneva 4, Switzerland.
E-mail address: Christian.staub@hcuge.ch (C. Staub).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.01.041

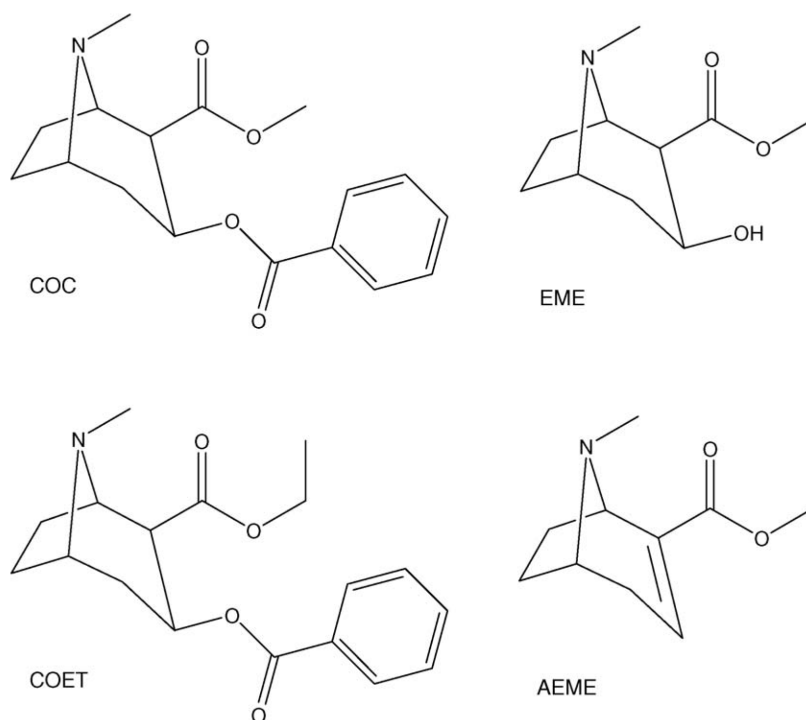

Fig. 1. Chemical structures of cocaine (COC), cocaethylene (COET), ecgonine methylester (EME) and anhydroecgonine methylester (AEME).

analytical techniques like immunoassays [14,15], spectrofluorimetry [2], liquid chromatography coupled with mass spectrometry (LC–MS) [16], gas chromatography using a nitrogen–phosphorus detector (GC–NPD) [17] or a mass spectrometer (GC–MS) [15,17,18].

In a previous work, we had developed and validated a complete procedure for the analysis of cocaine and three of its metabolites in hair by GC–CI/MS/MS using chemical ionization (CI) and ion-trap detection [19]. In hair matrix (like in saliva), the parent substance is present predominantly and only traces of metabolites are detectable. This is the main reason why ion-trap was preferred to a simple quadrupole detector such as those generally available in forensic laboratories. As a matter of fact, this spectrometric technique allows performing tandem mass spectrometry (MS/MS) at a cost much lower than triple quadrupole mass spectrometers and thus presents the advantage to permit a very selective and sensitive detection of traces. Chemical ionization stood out as the technique of choice because it carries out a "light" fragmentation (unlike electron impact) and allows to form abundant pseudo-molecular ions (MH^+) which generate characteristic fragment ions during the collision induced dissociation (CID) step of the MS/MS process. The combination of ion-trap MS/MS and CI qualities have allowed to obtained a powerful procedure for the quantitative analysis of cocaine and its metabolites in hair.

The present work consisted in adapting the analytical method mentioned above to make it suitable for analysis of COC and its metabolites in saliva, an alternative matrix. The same automated solid phase extraction (SPE) with the same analytical method (positive CI using isobutane as reagent gas with MS/MS detection) as in the previous work applied to hair were then considered. Therefore the simultaneous quantitative determination of COC and its related metabolites AEME, EME and COET in saliva by GC–MS/MS was validated. The strategy applied for the validation was based on the approach proposed by the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP) and adapted to our specific case in forensic toxicology.

The present work also suggests dissociation pathways for interpreting the CID spectra.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile solutions of COC, COET, EME, and AEME, 1000 μg/ml, were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Acetonitrile solutions of deuterated cocaine (COC-d_3) and deuterated ecgonine methylester (EME-d_3), 100 μg/ml, 98% pure, were purchased from Cambridge Isotope Laboratories Inc. too. The methyl of the amino function is deuterated in both cases. Methanol, toluene, acetic acid (100%), hydrochloric acid concentrated solution (37%), ammonium hydroxide solution (25%), sodium hydroxide, potassium hydroxide, sodium hydrogenophosphate, and potassium dihydrogenophosphate were supplied by Merck (Darmstadt, Germany). Methylene chloride and isopropyl alcohol were obtained from Fluka (Buchs, Switzerland).

2.2. Instrumentation

Automated extraction was performed on an ASPEC apparatus (Gilson Medical Electronics, Villiers-le-Bel, France) and

Table 1
Collision induced dissociation (CID) parameters and main product ions for each compound

Compounds	Precursor ions (*m/z*)	Excitation storage levels (*m/z*)	Excitation amplitudes (V)	Main product ions (*m/z*)
COC	304.1	83.6	46	182, 150
AEME	182.1	49.9	32	150, 122, 118
EME	200.1	54.9	34	182, 150, 82
COET	318.2	87.5	46	196, 150
COC-d_3	307.1	84.5	46	185, 153
EME-d_3	203.1	55.7	34	185, 153, 85

The product ion retained for quantitation is underscored.

with HCX Isolute (130 mg) cartridges, which were obtained from IST (Hengoed, UK).

Gas chromatographic analyses were performed on a Varian 3400 CX gas chromatograph (Walnut Creek, CA, USA) equipped with a Varian Saturn 2000 ion-trap detector (Walnut Creek, CA, USA). Ultra high purity helium was used as carrier gas with an inlet pressure of 0.069 Mpa (10 PSI). A DB5-MS J&W Scientifics (Folsom, CA, USA) fused silica capillary column with a 5% phenyl-95% methyl-polysiloxane stationary phase was used. The capillary column 15 m × 0.25 mm i.d. (0.25 μm film thickness) was connected to an inert retention gap of 1.5 m × 0.53 mm i.d. The column oven temperature was programmed from an initial temperature of 75 °C held during 1 min, increased to 170 °C at 15 °C/min, then increased to 210 °C at 5 °C/min, and finally to 310 °C at 30 °C/min. The injection port temperature was programmed from an initial temperature of 75 °C held during 1 min, then increased to 280 °C at 50 °C/min and held during 1.40 min. Three microlitres of sample were injected in the cool on-column mode using the Varian 8200 CX autosampler (Walnut Creek, CA, USA). The ion-trap was operated in positive chemical ionization (CI) with isobutane as reagent gas. The transfer line, manifold and trap temperatures were 290, 120 and 240 °C, respectively. Instrument control and data acquisition were carried out using the Varian Saturn Workstation version 6.3. Tandem mass spectrometry was performed in the non-resonant mode; the collision induced dissociation (CID) parameters are reported in Table 1. The following ions were retained for quantitation: COC *m/z* 182, AEME *m/z* 122, EME *m/z* 182, COET *m/z* 196, COC-d_3 *m/z* 185 and EME-d_3 *m/z* 185.

In tandem mass spectrometry, analyses are very selective. Therefore, in order to keep a supplementary source of information, as far as possible a little proportion of the precursor ion has been maintained in CID spectra so that the precursor ion intensity represented about 10% of the base peak. In the present case, it has been turned out that it was not always possible to fragment the precursor ion so that its intensity corresponded to about 10% of the base peak. The best example is protonated COET (Fig. 2C), for which the relative abundance of the precursor ion in the spectrum remained above 50% whatever the excitation voltage between 46 V (optimized value) and 100 V (maximum value available). In the same manner, the precursor ion peak of protonated COC (Fig. 2B) remained above 25%. This can be interpreted in terms of precursor ion stability. As a matter of fact, the main product ions of the CID spectra result from dissociation mechanisms that imply protonation on an ester or hydroxy group (please see Section 3.12). It is obvious that protonation on the tertiary amino function is thermodynamically favoured. The apparition of product ions from molecules protonated on the amino group implies the cleavage of at least two bonds since the nitrogen atom is involved in two rings. That is probably why the fragmentation yields of COET and COC remain low, even with high non-resonant CID voltages. Fig. 2 displays the CID spectrum obtained for each analyte under the conditions described above.

2.3. Saliva sample collection

Saliva samples were collected using Salivettes® from Sarstedt (Nümbrecht, Germany) which consist of simple cotton swab without preparation. Blank saliva was collected from different volunteers and tested for the presence of the principal substances found in forensic toxicology; it was free from them and therefore presented no traces of COC and its metabolites. Saliva was sheltered from light and stored at −20 °C until use. For analysis, after the saliva samples had been thawed at room temperature, they were shaken for homogenization. Because of the mechanistic agitation and the viscosity of this biological fluid, bubbles could be formed. Saliva volume (500 μl) was then carefully sampled in order to avoid that bubbles were sucked up.

2.4. Sample preparation

Five hundred microlitres of saliva were diluted with 1 ml of deionized water and buffered with 1 ml of phosphate buffer pH 7. Twenty-five microlitres of the internal standards (COC-d_3 and EME-d_3) solution at 1.6 μg/ml were added. After well mixing during several seconds, the saliva was transferred into a glass tube for extraction. The ASPEC system was programmed to extract the saliva samples as follows: (1) conditioning of the cartridges with 2 ml of methanol and 2 ml of deionized water, (2) loading of diluted and buffered saliva samples, (3) rinsing with 2 ml of deionized water, 1 ml of acetate buffer pH 4 and 2 ml of methanol, (4) drying with air, (5) elution of analytes with 2 ml of a (80:20:2) methylene chloride/isopropyl alcohol/ammonia hydroxide mixture [20,21]. Then the extracts were evaporated to dryness under nitrogen at room temperature before being finally dissolved in 25 μl of toluene.

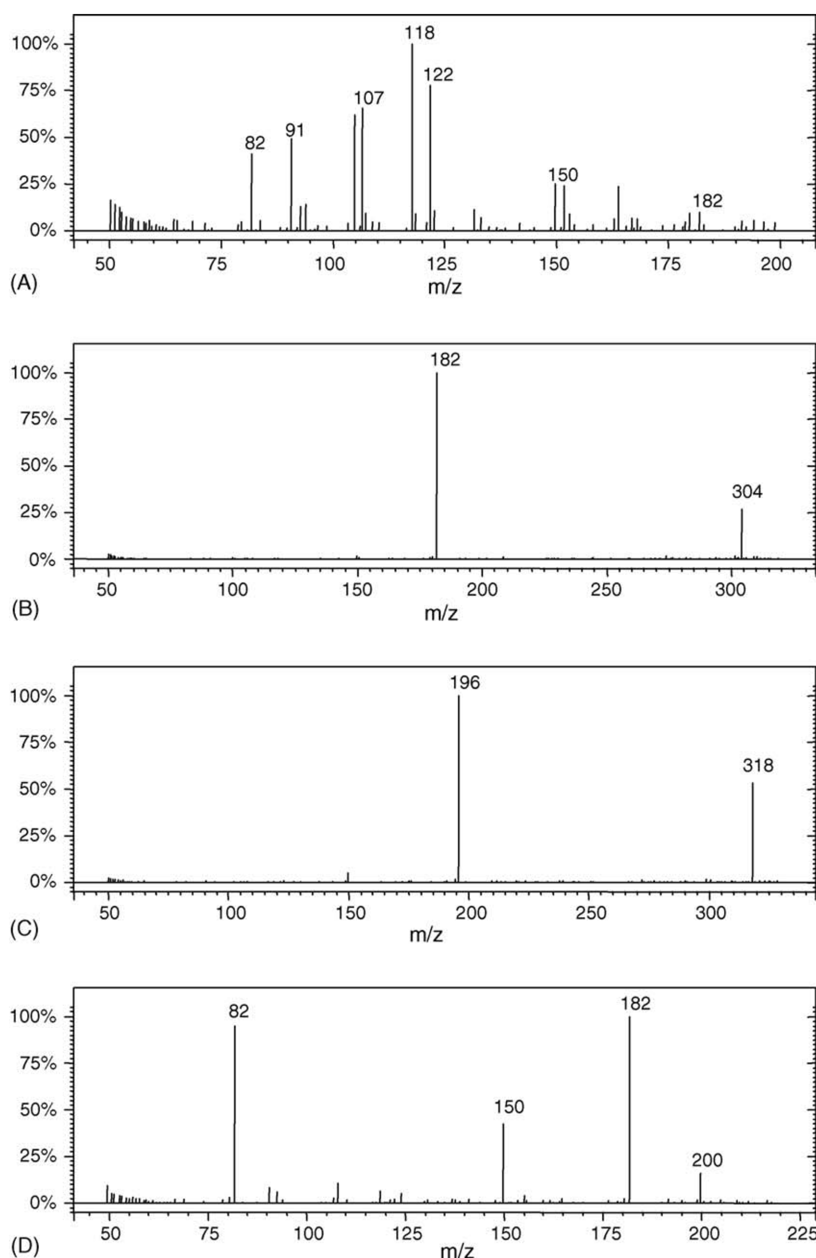

Fig. 2. Collision induced dissociation (CID) spectra of anhydroecgonine methylester AEME (A), cocaine COC (B), cocaethylene COET (C), and ecgonine methylester EME (D) obtained in the positive CI mode with an ion trap mass spectrometer.

2.5. Calibration and quality control samples

Calibration samples were independently prepared by adequately spiking blank saliva with appropriate volumes of standard COC, AEME, EME and COET solutions to reach concentrations of 2, 5, 10, 50, 200 and 500 ng/ml for each analyte.

Quality control (QC) samples were also independently obtained by spiking blank saliva with COC, AEME, EME and COET solutions to achieve concentrations of 2, 5, 10, 100 and 800 ng/ml.

In order to verify that the calibration range remains efficient for diluted samples, the 800 ng/ml QC was not used itself but was diluted twice with deionized water to yield a QC of 400 ng/ml (contained in the calibration range) which was effectively analysed.

3. Results and discussion

3.1. Saliva sampling

In spite of its viscosity, saliva has easily been handled by the robot of the automated SPE and had never formed bubbles. The SPE sampling was then not affected during the dispensing of samples. It was probably possible because saliva (500 μl) was diluted with deionized water (1 ml) and phosphate buffer (1 ml).

This dilution allowed not only to condition the samples but also to decrease the saliva viscosity.

3.2. Validation procedure

The validation process was inspired by the guidelines proposed by the SFSTP [22–25] and consisted in the determination of parameters such as trueness, repeatability, intermediate precision, limit of detection (LOD) and limit of quantitation (LOQ) for the reported GC–MS/MS analysis of COC, AEME, EME and COET in saliva. All of these typical validation characteristics are defined below in corresponding paragraphs.

The strategy applied for the validation was adapted for this specific case in forensic toxicology. The selected approach has first involved a prevalidation step. During this primary phase, the choice of the most appropriate calibration curve model, the estimation of LOD and LOQ and the study of the selectivity have been performed (data not shown). The following step consisted in the validation itself. Validation experiments were finally performed to evaluate the procedure performances on 3 days and allowed to determine trueness, precision and definitive LOD and LOQ.

Assuming that in saliva the parent drug is detectable predominantly whereas metabolites are only present as traces, a compromise has been reached between expected COC levels (very high) and expected metabolites concentrations (traces) in order to choose the best calibration range for validation. Finally, the concentrations range was selected between 2 and 500 ng/ml. Consequently, the ability to dilute samples irrelevant to the selected calibration range, was tested in order to allowed their analysis just after a simple dilution with deionized water.

To validate the whole of these criteria, two kinds of samples were prepared: calibration samples (CAL) and validation samples corresponding to QC samples used in routine analysis. These samples were independently prepared by adequately spiking blank saliva samples with appropriate volumes of COC, AEME, EME and COET standard solutions. COC-d_3 was used as the internal standard (IS) for COC and COET quantitation, whereas EME-d_3 was used as the IS for AEME and EME. Each CAL and QC sample was spiked with both IS at 80 ng/ml.

In order to establish the daily calibration curves for each substance of interest, calibrator samples (CAL) were prepared and analysed, each day, independently in triplicate ($n=3$) at six concentration levels ($k=6$: 2, 5, 10, 50, 200 and 500 ng/ml). The values of each rank were chosen inside the calibration range as following: they correspond to 100%, 40%, 10% and 2% of the upper limit (500 ng/ml) and the two lowest values were selected around the LOQ estimated during the prevalidation step. Finally 18 points were used to establish the daily calibration curves by plotting detection response (ratio analyte signal/IS signal) versus concentration.

In preliminary assays (data not shown), variance analysis indicated that the weighted linear regression was appropriate to establish the relationship between the concentration and detection response of each compound. The best weighting factor was chosen taking into account the relationship between natural variance logarithms and concentrations. For each analyte, the selected weighting factor was the inverse of the concentration raised to the λth power ($1/x^{\lambda}$), λ being the slope of the line fitted to the data on the logarithm scale round off the superior unit. Hence, the selected weighting factor was $1/x$ for each compound as described elsewhere [26].

Each day, QC samples were prepared in quadruplet ($n=4$) at five concentration levels ($k=5$: 2, 5, 10, 100, and 400 ng/ml) dispatched into the entire calibration range. The values of each rank were chosen as following: they correspond to 80%, 20%, and 2% of the upper limit of the domain (500 ng/ml) and the two lowest levels were selected around the LOQ estimated during the prevalidation step. The 400 ng/ml QC were prepared by diluting twice the 800 ng/ml samples with deionized water in order to verify if there is a risk of dilution effect.

3.3. Method selectivity

Method selectivity was first assessed by the analysis of six blank saliva samples obtained from different male or female volunteers. These blank samples have been tested by screening for the principal substances found in forensic toxicology and no traces of compounds have been detectable. Moreover, when the present method was used no interferences from the matrix have been observed in the specific time detection windows of the compounds of interest. Fig. 3 compares typical chromatograms of a cocaine abuser (real case), QC at 100 ng/ml and a blank saliva sample, and confirms the good effectiveness of the reported procedure.

3.4. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain results which are directly proportional

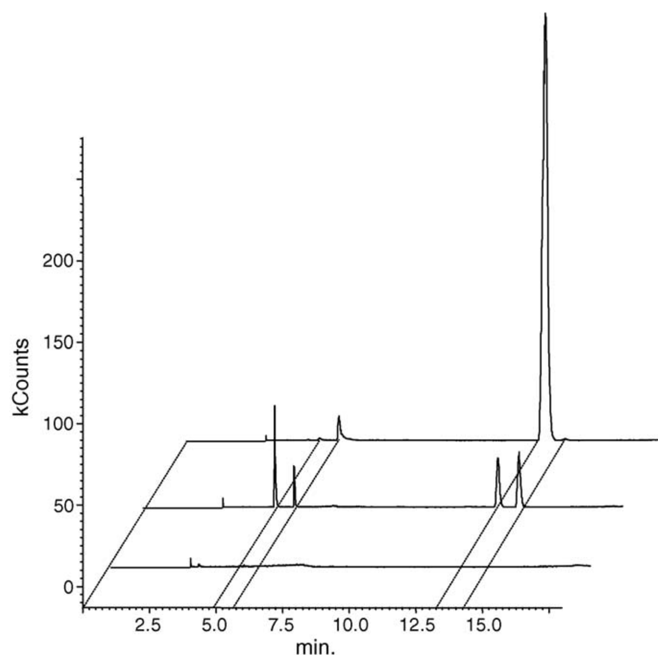

Fig. 3. Chromatograms of a cocaine abuser saliva extract (real case), a quality control (QC) at 100 ng/ml and a blank saliva.

to the concentration (or amount) of the analytes in the sample [22,23,27,28]. The linearity was calculated by fitting the back-calculated concentrations of the QC versus theoretical concentrations by applying the linear regression model based on the least square method [29]. Good linearity (slopes close to 1.00 ± 0.05) and good closeness R^2 above 0.999 for all analytes were observed.

3.5. Trueness and precision

Trueness of an analytical procedure expresses the closeness of agreement between a conventionally accepted true value (or an accepted reference value) and a mean one experimentally found [22,23,27,28]. In the present work, trueness was expressed as the mean concentration found for all QC samples at each concentration level.

The precision of a bioanalytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions [22,23,27,28]. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility (not studied here). For the present work, precision has been estimated by measuring repeatability and intermediate precision at different levels of concentration. After fitting the calibration curves for each analyte on each day, concentrations of substances of interest were computed, in all QC samples, from the analytical obtained responses by back-calculating with the linear regression model based on the least square method. Variances of repeatability and intermediate precision were computed, at each concentration rank, from the estimated concentrations and the precision was expressed by the relative standard deviation at each level. Trueness and precision results are presented in Table 2.

3.6. LOD and LOQ

The limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and trueness [22,23,27,28]. In the present work, LOQ were determined by using the validation results for repeatability and intermediate precision. The LOQ of each compound was determined as the concentration for which trueness was equal to $100 \pm 20\%$, R.S.D. was inferior or equal to 15% for repeatability and inferior or equal to 20% for intermediate precision. These criteria are those currently used in a laboratory of forensic toxicology. Hence, the LOQ were determined at 2 ng/ml for COET and COC, and at 5 ng/ml for EME.

The limit of detection (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value [22,23,27,28]. In the present work, LOD of each compound was determined by diluting calibration samples and was established as the concentration for which the signal to noise ratio (S/N) of the chromatographic peak (selected profile of the ion retained for quantitation) equals 3. Therefore the 2 ng/ml CAL samples were successively diluted and analyzed to estimate LOD. Finally LOD were determined at 0.1 ng/ml for COC and 0.5 ng/ml for AEME, EME and COET.

3.7. Ranges for quantification

Considering the chosen acceptance criteria currently applied in our laboratory (trueness $100 \pm 20\%$, repeatability $\leq 15\%$ and intermediate precision $\leq 20\%$) and the validation results, the following ranges were considered for quantification: 5–500 ng/ml for EME, 2–500 ng/ml for COC and COET (see Table 2); AEME

Table 2
Trueness, repeatability and intermediate precision for quality control (QC) samples

Compounds	Theoretical concentrations (ng/ml)	Measured concentrations (ng/ml)	Trueness (%)	Repeatability (%)	Intermediate precision (%)
COC	2	1.6	80.3	7.7	15.7
	5	4.9	99.1	5.9	6.1
	10	11.3	113.2	1.4	1.4
	100	102.1	102.1	11.0	10.5
	400	395.4	98.8	4.1	7.1
COET	2	1.7	83.8	10.9	18.4
	5	4.6	92.2	6.8	7.1
	10	9.7	96.5	6.1	6.4
	100	100.4	100.4	2.5	7.6
	400	392.8	98.2	4.8	13.7
EME	2	1.6	78.1	24.4	34.2
	5	4.5	89.4	15.2	14.1
	10	10.1	100.7	10.0	10.0
	100	96.7	96.7	1.3	13.1
	400	384.2	96.1	4.4	6.6
AEME	2	1.9	95.9	24.9	50.4
	5	4.8	95.4	27.9	45.2
	10	9.6	96.5	27.3	35.3
	100	102.5	102.5	22.4	22.8
	400	239.2	59.8	24.2	28.1

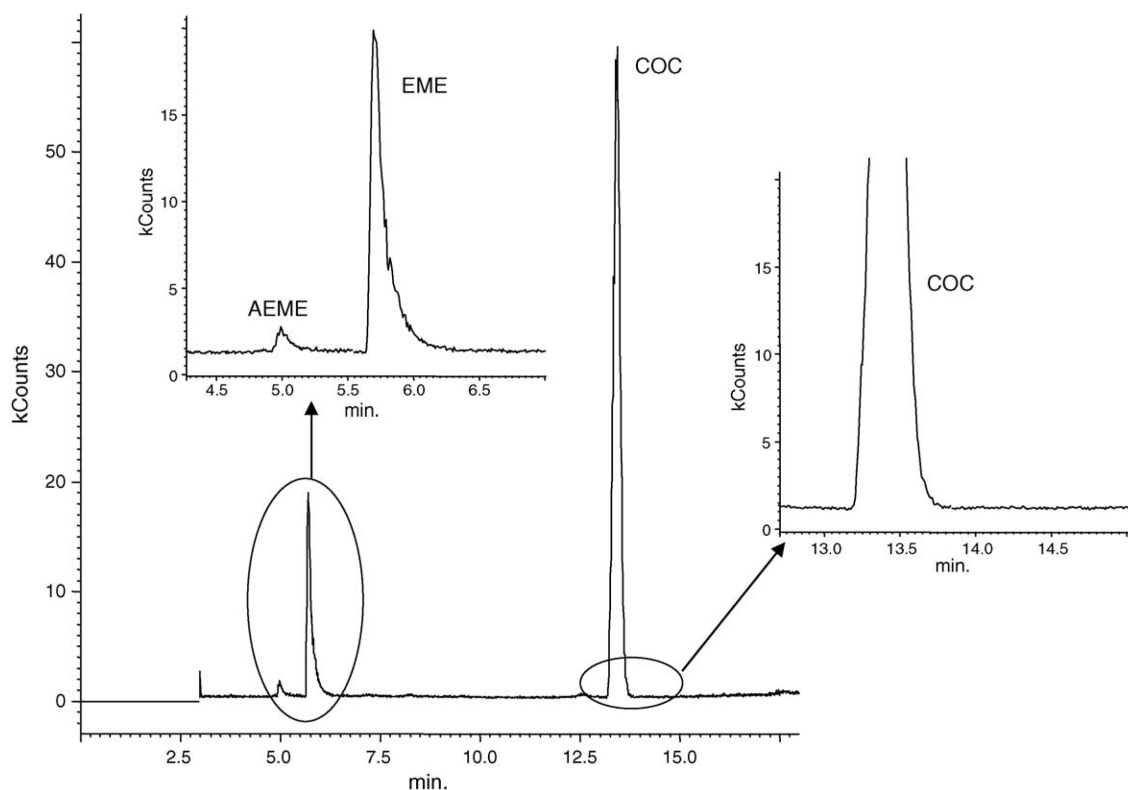

Fig. 4. Chromatograms of a cocaine abuser saliva extract showing peaks of anhydroecgonine methylester (about 4 ng/ml), ecgonine methylester (140 ng/ml) and cocaine (302 ng/ml).

could only be determined "semi-quantitatively" between 2 and 200 ng/ml according to our chosen acceptance limits.

3.8. Particular case of AEME

Concerning AEME, although its trueness values are contained in the acceptance limits of $100 \pm 20\%$ between 2 and 200 ng/ml, its repeatability and intermediate precision were higher than 15% and 25%, respectively (please see Section 3.6); the repeatability was determined between 22.4% and 27.9%, the intermediate precision values were between 22.8% and 50.4% and were irrelevant to the chosen restrictions. Therefore, the method reported is not available for the quantitation of this analyte; nevertheless it can provide a good estimation and AEME can be determined "semi-quantitatively" between 2 and 200 ng/ml.

3.9. Dilution effect

The ability to dilute samples originally above the upper limit of the calibration curve was evaluated by using the 800 ng/ml samples diluted 1:1 with deionized water to reach 400 ng/ml. The experiments allowed proving that a preliminary dilution step was possible for samples of high concentrations (outside of calibration range) without any perturbation for the quantitative results. There was no observed dilution effect. This aptitude to dilute samples was demonstrated to be valid for the four compounds of interest.

3.10. Remark

Most of the publications concerning the same purpose of the present work have been published in the 90th or before. In recent times, Campora et al. have presented a method for the quantitation of COC and two of its major metabolites (BZE and EME) in saliva by GC–MS using a quadrupole detector [30]. They obtained LOQ of 3.0 ng/ml for EME, 7.4 ng/ml for COC and 0.8 ng/ml for BZE. Even if the analysis presented by this team is very sensitive, the method described here has better LOQ for COC, the parent drug (2.0 ng/mg), and presents several other advantages over this earlier published method. First, the estimated LOD were 0.1 ng/ml for COC and 0.5 ng/ml for EME, COET and AEME whereas LOD reported by Campora et al. were 2.2 ng/ml for COC and 0.9 ng/ml for EME. Secondly, only 500 μl of saliva samples (unlike 1 ml) is needed to perform analysis. Knowing that one of the drawbacks of oral fluid consists of its small available volume, a 500 μl volume is very interesting and noticeable because it is sufficiently low to allow performing the complete procedure in duplicate and confirming the obtained results. Thirdly, the present method does not require a supplementary derivatization step which is time-consuming. Moreover BZE which needs a derivatization has been removed of the chosen metabolites for analysis because it is formed from COC by spontaneous chemical hydrolysis. Finally, the reported method was carried out using tandem mass spectrometric detection which is more selective and specific than single ion monitoring with a simple quadrupole.

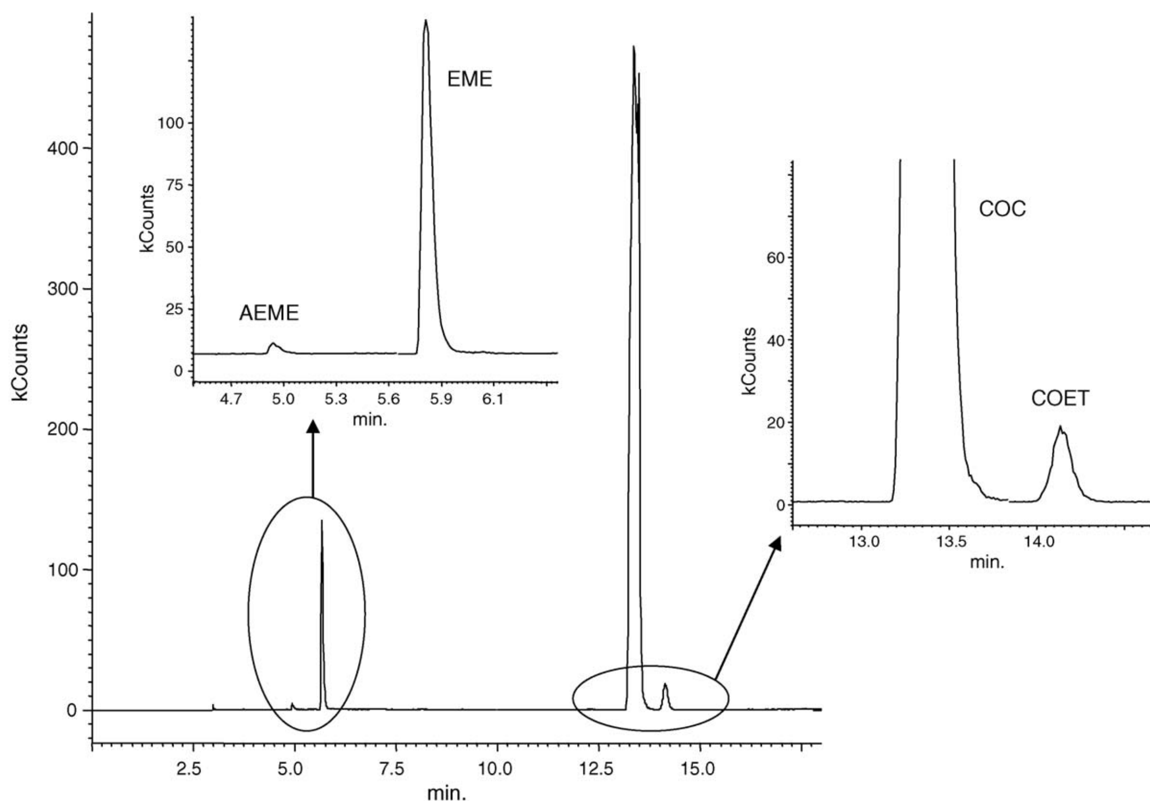

Fig. 5. Chromatograms of cocaine abuser, who had drunk concomitantly alcohol, showing peaks of anhydroecgonine methylester (about 20 ng/ml), ecgonine methylester (1500 ng/ml), cocaethylene (342 ng/ml) and cocaine (about 16,000 ng/ml).

3.11. Analysis of real cases

In order to demonstrate the applicability of the validated procedure, this latter was applied to several real human saliva samples collected from different cocaine-addicted abusers. Samples were extracted by SPE and analysed by GC–MS/MS as described above. Typical chromatograms of cocaine abuser saliva are shown in Figs. 3–5. Fig. 4 displays the chromatogram for a drug addict who has only taken cocaine whereas Fig. 5 presents the chromatogram obtained for a cocaine abuser who has taken drug concomitantly with alcohol. Fig. 3 allows comparing chromatograms obtained for a cocaine abuser (real case), a quality control at 100 ng/ml and a blank saliva sample. All the results suggested that the method is suitable for the detection of AEME and the quantification of COC, EME and COET.

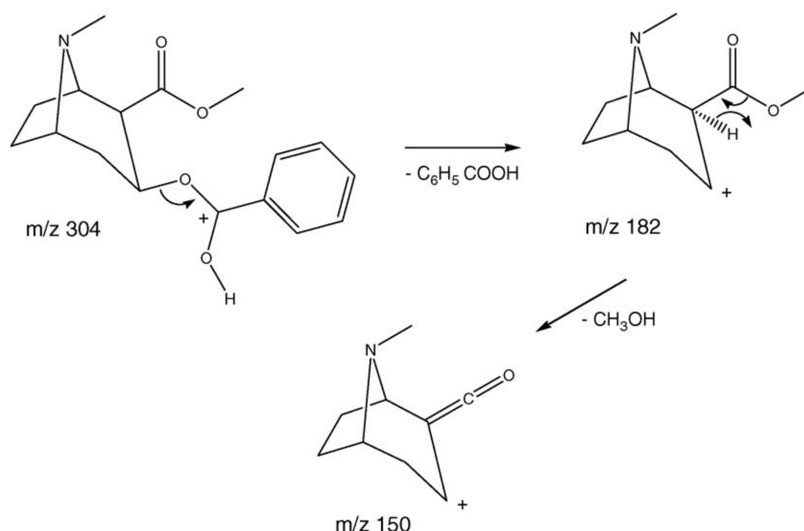

Fig. 6. Suggested dissociation pathways of protonated cocaine (COC) under collisional activation.

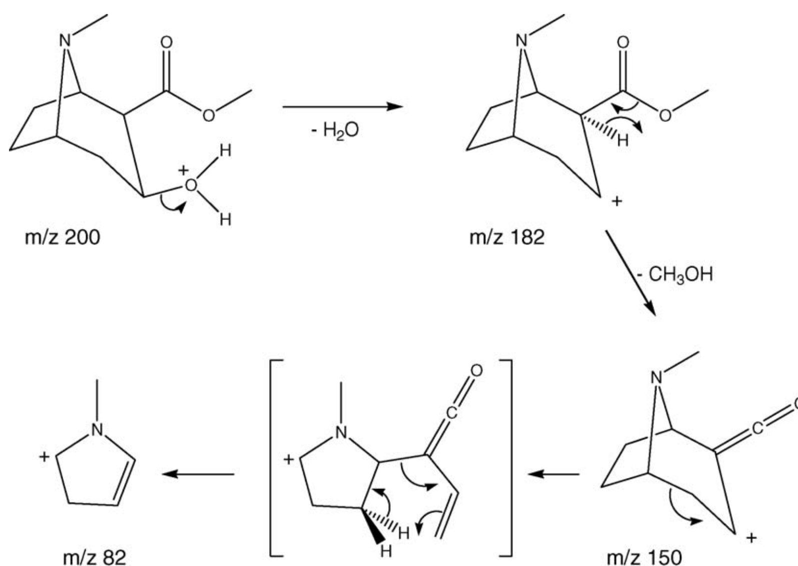

Fig. 7. Suggested dissociation pathways of protonated ecgonine methylester (EME) under collisional activation.

3.12. Dissociation pathways

Although the fragmentation of protonated COC has already been studied by LC–MS/MS with a triple quadrupole apparatus, the CID spectra obtained in this work are noticeably different from those described by Wang et al. [31]. That is why the present work also suggests dissociation pathways to interpret the CID spectra of the compounds of interest. Differences between triple quadrupole and ion trap MS/MS spectra are not surprising since the activation processes and collisions gas are not the same.

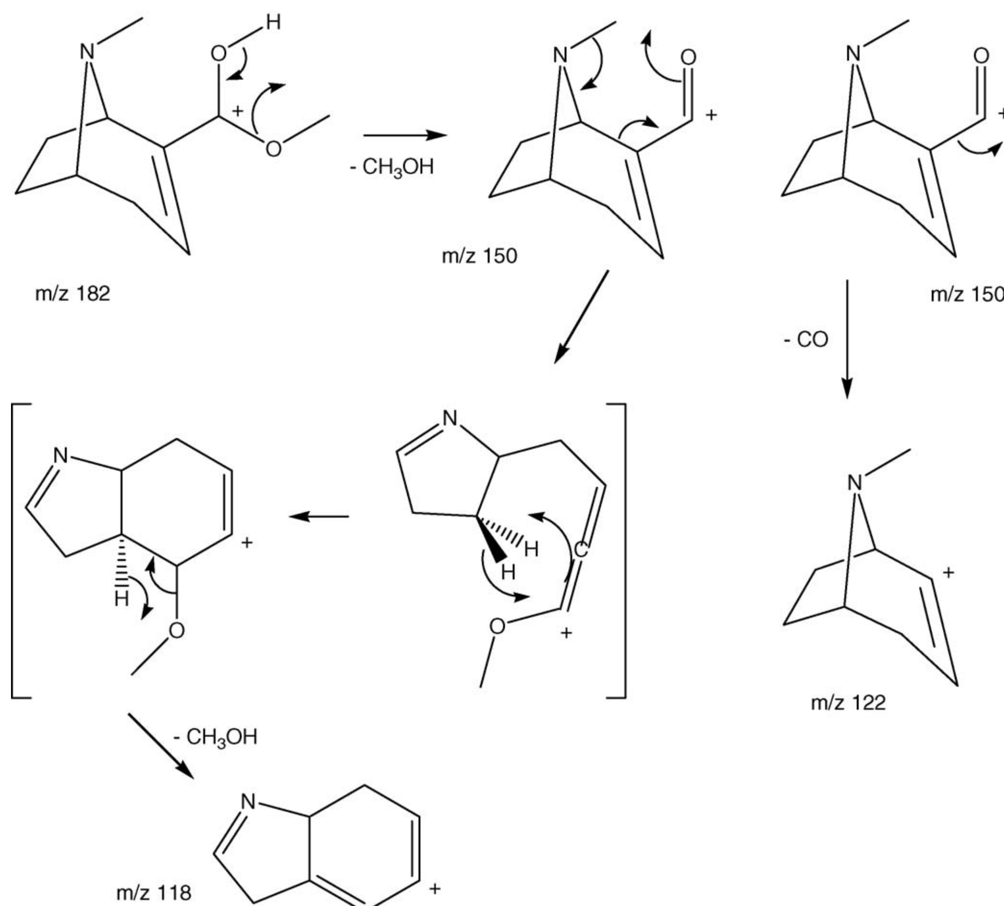

Fig. 8. Suggested dissociation pathways of protonated anhydroecgonine methylester (AEME) under collisional activation.

We have already reported such differences in a previous work devoted to LSD analysis [32]. Figs. 6–8 display the dissociation mechanisms suggested. Dissociation of protonated cocaine (Fig. 6) involves the loss of benzoic acid followed by that of methanol. The transitions *m/z* 307 → *m/z* 185 and *m/z* 307 → *m/z* 153 observed from COC-d_3 are in good agreement with the proposed mechanism. The fragmentation pathway of COET is not displayed since it is the same as that of COC, the second step involving loss of ethanol instead of methanol. Fragmentation of ecgonine methyl ester starts by water elimination to provide the *m/z* 182 ion. H_2O loss can result from direct protonation on the hydroxy group, as displayed in Fig. 7; it can also result from protonation of the ester group (more basic than the hydroxy one) followed by proton transfer to the hydroxy function. The loss of methanol from *m/z* 182 leads to *m/z* 150 that losses C_4H_4O through a six centers intermediate structure to provide *m/z* 82. The CID spectrum of EME-d_3 displays *m/z* 203, *m/z* 185, *m/z* 153 and *m/z* 85 ions. The three mass units shift on all the product ions compared with the CID spectrum of non-labelled EME shows that the methyl group of the amino function remains present in all the product ions, in good agreement with the suggested mechanisms. Dissociation of protonated anhydroecgonine methylester begins by methanol elimination after protonation on the carbonyl group. The resulting *m/z* 150 ion dissociates following two ways. The first one leads to *m/z* 122 through a classical CO elimination, the second one leads to the formation of the *m/z* 118 ion through methanol loss. The mechanism proposed in Fig. 8 to explain the formation of the *m/z* 118 ion may appear quite complex but a simpler one has not been found.

4. Conclusion

A semi automated and sensitive GC–MS/MS method for the simultaneous determination of cocaine and its metabolites has been transferred from hair to saliva matrix. It was fully validated in terms of robustness, linearity, trueness, precision and limits of quantification for COC, EME and COET. The quantification of the specific metabolite AEME was not possible according to our criteria of repeatability and intermediate precision although its trueness was totally satisfactory. Hence the method reported is only able to give a good approximation of the AEME concentrations in saliva and corresponds then to a "semi-quantitative" analysis for this compound. However the main interest in forensic toxicology is the concentration of the parent substance (COC). Concerning the metabolites, the determination of their presence is generally sufficient to confirm the consumption of cocaine. The present validated method can thus be applied for routine analysis in forensic toxicology.

References

[1] P.I. Jatlow, Clin. Chem. 33 (1987) 66B–71B.
[2] A.D. Campiglia, T. Vo-Dinh, Anal. Chim. Acta 372 (1998) 349–355.
[3] E.J. Cone, J. Oyler, W.D. Darwin, J. Anal. Toxicol. 21 (1997) 465–475.
[4] M. Navarro, S. Pichini, M. Farré, J. Ortuno, P.N. Roset, J. Segura, R. De la Torre, Clin. Chem. 47 (2001) 1788–1795.
[5] K. Kato, M. Hillsgrove, L. Weinhold, D.A. Gorelick, W.D. Darwin, E.J. Cone, J. Anal. Toxicol. 17 (1993) 338–341.
[6] O.R. Idowu, B. Caddy, J. Forensic Sci. Soc. 22 (1982) 123–135.
[7] W. Schramm, R.H. Smith, P.A. Craig, D.A. Kidwell, J. Anal. Toxicol. 16 (1992) 1–9.
[8] F.P. Smith, D.A. Kidwell, Forensic Sci. Int. 83 (1996) 179–189.
[9] N. Samyn, A. Verstraete, C. Van Haeren, P. Kintz, Forensic Sci. Rev. 11 (1999) 2–11.
[10] H.W. Peel, B.J. Perrigo, N.Z. Mikhael, J. Forensic Sci. 29 (1984) 185–189.
[11] J.C. Mucklow, M.R. Bending, G.C. Kahn, C.T. Dollery, Clin. Pharm. Therap. 24 (1978) 563–570.
[12] A.J. Jenkins, J.M. Oyler, E.J. Cone, J. Anal. Toxicol. 19 (1995) 359–374.
[13] E.J. Cone, K. Kumor, L.K. Thompson, M. Sherer, J. Anal. Toxicol. 12 (1988) 200–206.
[14] R.S. Niedbala, K. Kardos, T. Fries, A. Cannon, A. Davis, J. Anal. Toxicol. 25 (2001) 62–68.
[15] E.J. Cone, L. Presley, M. Lehrer, W. Seiter, M. Smith, K.W. Kardos, D. Fritch, S. Salamone, R.S. Niedbala, J. Anal. Toxicol. 26 (2002) 541–546.
[16] K. Clauwaert, T. Decaestecker, K. Mortier, W. Lambert, D. Deforce, C. Van Peteghem, J. Van Bocxlaer, J. Anal. Toxicol. 28 (2004) 655–659.
[17] L.K. Thompson, D. Yousefnejad, K. Kumor, M. Sherer, E.J. Cone, J. Anal. Toxicol. 11 (1987) 36–38.
[18] S.W. Toennes, S. Steinmeyer, H.J. Maurer, M.R. Moeller, G.F. Kauert, J. Anal. Toxicol. 29 (2005) 22–27.
[19] E. Cognard, S. Rudaz, S. Bouchonnet, Ch. Staub, J. Chromatogr. B 826 (2005) 17–25.
[20] C. Girod, F. De Dominicis, R. Giovannini, C. Staub, Toxicorama 11 (1999) 46–49.
[21] C. Girod, C. Staub, Forensic Sci. Int. 107 (2000) 261–271.
[22] E. Chapuzet, N. Mercier, B. Bervoas-Martin, B. Boulanger, P. Chevalier, P. Chiap, D. Grandjean, P. Hubert, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Stp Pharma Prat. 7 (1997) 169–194.
[23] P. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, B. Bervoas-Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Anal. Chim. Acta 391 (1999) 135–148.
[24] J. Caporal-Gautier, J.M. Nivet, P. Algranti, M. Guilloteau, M. Histe, M. Lallier, J.J. N'Guyen-Huu, R. Russotto, S.T.P. Pharma Pratiques 2 (1992) 205–239.
[25] B. Boulanger, P. Chiap, W. Dewé, J. Crommen, P. Hubert, J. Pharm. Biomed. Anal. 32 (2003) 753–765.
[26] S. Rudaz, S. Souverain, C. Schelling, M. Deleers, A. Klomp, A. Norris, T.L. Vu, B. Ariano, J.L. Veuthey, Anal. Chim. Acta 492 (2003) 271–282.
[27] L. Amendola, C. Colamonici, F. Rossi, F. Botrè, J. Chromatogr. B 773 (2002) 7–16.
[28] P. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, J. Pharm. Biomed. Anal. 36 (2004) 579–586.
[29] P. Chiap, A. Ceccato, B. Miralles Buraglia, B. Boulanger, P. Hubert, J. Crommen, J. Pharm. Biomed. Anal. 24 (2001) 801–814.
[30] P. Campora, A.M. Bermejo, M.J. Tabernero, P. Fernandez, J. Anal. Toxicol. 27 (2003) 270–274.
[31] P. Wang, M.G. Bartlett, J. Mass Spectrom. 33 (1998) 961–967.
[32] D. Libong, S. Bouchonnet, Int. J. Mass Spectrom. 219 (2002) 615–624.