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Determination of chloramphenicol residues in shrimps by liquid chromatography–mass spectrometry

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Abstract

A liquid chromatographic method with mass spectrometric detection and identification (LC–MS) is presented for the determination of chloramphenicol (CAP) in shrimp tissues. Homogenized shrimp samples were extracted with phosphate buffer (pH 7.0). Clean-up was carried out on a C_{18} SPE cartridge. Chloramphenicol was determined by LC–MS-ESI in negative mode. The column used was a Symmetry Shield with a mixture of acetonitrile–water (25:75) as mobile phase. Shrimp samples were fortified at CAP levels between 0.2 and 50 ng g^{-1} with 5D-CAP as internal standard. At these levels, accuracies lay between 101 and 110% and between-day reproducibilities were lower than 7.1%, expressed as the variation coefficient of the mean. Limit of decision (CCα) was 0.02 ng g^{-1}. Limit of quantitation (LOQ) was 0.2 ng g^{-1}.

Keywords: Shrimp; Chloramphenicol

1. Introduction

Chloramphenicol is a broad spectrum antibiotic, often used in veterinary practice, including treatment of aquaculture species. World shrimp farming has grown considerably in the last decade and chloramphenicol is also often used prophylactically in these farming practices. This could result in the occurrence of chloramphenicol residues in commercial shrimps.

Adverse reactions and side effects in humans have been extensively demonstrated [1]; therefore, to protect the consumer, the European Community banned the use of chloramphenicol in food producing animals and established a zero tolerance level for this compound in edible tissues [2].

In order to effectively monitor the occurrence of residues of chloramphenicol, specific and sensitive analytical methods are required. For screening purposes, immunological methods are claimed to be very sensitive, but positive results need further confirmation [3].

Till now, antibiotic residues were often analysed by chromatographic methods using liquid chromatography which requires UV–diode array detection as confirmation technique to avoid false positive results produced by the possible influence of interfering compounds [4–6].

Some authors use gas chromatography with electron capture detection or tandem MS–MS for chloramphenicol residue analysis in tissues, milk and

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eggs. However, gas chromatography involves derivatization of the sample extract, which makes the analytical methods based on this technique more tedious although they are very sensitive [7–9]. Some authors [10–12] have developed analytical methods based on LC–MS, avoiding the need to derivatise the sample extracts. These workers used particle beam or thermospray interfaces and therefore their detection limits were rather high (2 ng g^{-1} or higher). Atmospheric pressure ionization (API) interfaces as used in the proposed method allow to reach much lower detection limits.

The Commission Decision 2002/657/EC [13], the revised criteria Decision 93/256/CE [14], defines performance criteria for the analytical methods to be used for veterinary drug residue analysis and establishes that analytical methods with MS confirmation must be used for the determination of residues of banned substances.

In this work a rapid analytical method for the detection and confirmation of chloramphenicol in shrimps with LC–MS detection was developed, using negative ESI as ionization mode.

2. Experimental

2.1. Materials

Water was purified by demineralization (MilliQ, Millipore).

Chloramphenicol standard (purity ≥99%) was purchased from Fluka (Buchs, Switzerland).

5D-Chloramphenicol was obtained from the Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV, Berlin, Germany).

Ethylacetate, acetonitrile and methanol (HPLC grade) and acetic acid (for analysis) were from Merck (Darmstadt, Germany). Sep-Pak C_{18} cartridges (Waters, Milford, USA) and Millex AP 20 filters, 28 mm and 2 μm (Millipore, Milford, USA) were used. For sample extraction 0.05 *M* phosphate buffer pH 7.0 was used. Mobile phase for HPLC was acetonitrile–water (25:75, v/v), which was filtered through a 0.45 μm Millipore membrane filter. Preparation of the sample extracts was performed in an ultrasonic bath (Selecta, Madrid, Spain), a Macrotonic centrifuge (Selecta), a Moulinex homogenizer (Moulinex, Bilbao, Spain) and a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA).

2.2. LC–MS equipment and conditions

The HPLC equipment consisted of an Alliance pump and autosampler (Waters) and a ZMD2000 mass spectrometric detector (Waters). The autosampler temperature was set at 10 °C. The separation was performed on a Symmetry Shield RP8 5 μ column, 150×3.9 mm I.D. (Waters) and a Phenomenex C_8, 4 mm (L)×3 mm (I.D.) guard column. The column oven temperature was set at 40 °C. The mobile phase was pumped at 1 ml min^{-1}. The injection volume was 25 μl. The analysis run time was 7.5 min (isocratic elution), followed by a washing step of 10 min with acetonitrile (to avoid possible cross-contamination) and a conditioning step of 10 min with mobile phase after every sample injection.

Operation of the mass spectrometer, fitted with an ESI probe, was in negative mode, and the source parameters of the tune page were: capillary voltage 3 V, cone voltage 30, 35 or 45 V (depending on the mass), extractor voltage 3 V, block temperature 100 °C, desolvation temperature 400 °C and nitrogen flow of 750 l h^{-1}. The MS method was a single ion recording (SIR) of five masses (*m*/*z* 321, *m*/*z* 323, *m*/*z* 257 and *m*/*z* 152 for CAP, and 157 for D5-CAP) with a different cone voltage for each ion: 30 V for *m*/*z* 321 and 323, 35 V for *m*/*z* 257, and 45 V for *m*/*z* 152 and *m*/*z* 157.

2.3. Standard solutions

A CAP standard stock solution of 1.0 mg ml^{-1} was prepared by dissolving 100 mg of CAP in 100 ml of methanol and this solution was diluted 50 times in methanol obtaining the intermediate standard solution of 20 μg ml^{-1}. Different working standard solutions were prepared with concentrations in the range of 100–1000 ng ml^{-1} by diluting 0.5–5.0 ml of the intermediate standard solution each time in 100 ml of the mobile phase. Internal standard solution of 5D-chloramphenicol was prepared by dissolving the ampoule with 100 μg CAP in 25 ml of

methanol, which was adequately diluted till a working solution of 0.04 ng μl^{-1}. Standard solutions were stored at $-4\,^{\circ}C$.

2.4. Sample preparation

Frozen shrimps were held at room temperature until they felt limber. Then, in case of entire shrimps, heads, chitinous shell and body appendages were removed and the clean shrimps were homogenized in the Moulinex apparatus.

For spiking studies, homogenized shrimps were used. Shrimp samples were spiked at 0.2, 0.5, 1.0, 5.0 and 50 ng g^{-1} levels using CAP working solutions, at least 15 min before the beginning of the described extraction procedure. All samples were spiked with 150 μl of the internal standard working solution achieving a concentration of 0.6 ng g^{-1} of 5D-chloramphenicol.

2.5. Sample extraction and clean-up

Ten grams of blended shrimps were weighed into a centrifuge tube of 100 ml, and 150 μl of the internal standard working solution of 5D-chloramphenicol and 40 ml of phosphate buffer were added. The tubes were introduced in an ultrasonic bath for about 15 min, centrifuged till separation of the sediment and the supernatant was filtered through a Millex filter.

After conditioning a C_{18} cartridge with 5 ml of methanol and 5 ml of water, an aliquot of 30 ml of the filtered supernatant was gently pressed through the cartridge by means of a disposable syringe. The cartridge was then washed with 5 ml of water and 5 ml of a mixture of 5% acetonitrile in water. Chloramphenicol was eluted from the cartridge with 10 ml of 30% of acetonitrile in water in a 20-ml reagent tube, to which 2 ml of ethylacetate were added. The mixture was shaken and after separation the upper layer was transferred to a 10-ml tube and the extraction with ethylacetate was repeated twice. The combined organic phases were evaporated till dryness in a water bath at $50\,^{\circ}C$ under a gentle stream of nitrogen. The dry residue was dissolved in 300 μl of the HPLC mobile phase and 25 μl of this final extract were injected in the chromatographic system as described in Section 2.2.

2.6. Regression curve

For quantitation, an external regression curve was used with CAP concentrations corresponding to 0.2, 0.5, 1.0 and 5.0 ng g^{-1} of CAP in sample and containing a fixed amount of 5D-CAP. To four vials, 2, 5, 10 and 50 ng of CAP were, respectively, transferred. Six ng of 5D-CAP were added to each vial together with mobile phase up to a total volume of 300 μl. Twenty-five μl of each vial were injected at least in triplicate.

The regression curve was prepared relating the ratio CAP area (m/z 152)/5D-CAP area (m/z 157) with CAP concentration in ng g^{-1}, at a fixed internal standard amount.

2.7. Detection and confirmation

In each chromatographic run five ions were monitored, which allowed to perform detection, quantitation and confirmatory analysis.

The presence of m/z 152 was checked for screening analysis. The m/z 152/157 relative response was used for quantitation and the amount of CAP was calculated with the aid of the obtained external regression curve of spiked samples.

For confirmatory purposes, the presence of all diagnostic ions of CAP was checked and three m/z ratios were measured: 323/321, 257/321 and 152/321, considering the area of m/z 321 as base peak. At least two ratios had to fulfil the criteria of Commission Decision 2002/657/EC of which one had to be 152/321. The ratio 152/321 was important as m/z 152 was used for the quantitation.

2.8. Validation parameters

To validate the proposed method, five series of different shrimp samples were analysed and their results evaluated. Each series consisted of a reagent blank, a blank shrimp control sample and five spiked samples at the 0.2, 0.5, 1.0, 5.0 and 50 ng g^{-1} level.

Repeatibility (within-day) and within-laboratory reproducibility (between-day) were calculated and expressed as C.V. of the mean. External curve linearity in the work range (0.2 to 5 ng g^{-1}), was studied: slopes, intercepts and correlation coefficients.

Limit of decision (CCα) and limit of quantitation (LOQ) were calculated following criteria of the Commission Decision 2002/657/EC. Confirmation criteria have been presented in Section 2.7.

3. Results and discussion

3.1. Optimization of the method

3.1.1. Extraction and clean-up

Generally, organic solvents are used as extractant for quantitative procedures for chloramphenicol analysis, predominantly ethylacetate [4–6]. The extracts are then evaporated in a rotary evaporator, followed by liquid–liquid or solid-phase extraction for sample clean-up and concentration. We propose an extraction of the sample with phosphate buffer followed by C_{18} solid-phase (see Section 2.5) clean-up instead of the previously described [6] ethylacetate extraction followed by Silica cartridge clean-up. This extraction mode is less time-consuming and less tedious because it avoids pH adjustment and the organic solvent evaporation step in a rotary evaporator.

3.1.2. LC optimization

Almost all the samples (reagent blank and also blank samples) presented a peak in the m/z 152 channel at a retention time near CAP. Different columns were tested to achieve the best separation of this interference peak from CAP. In the described chromatographic conditions the Symmetry Shield column yielded a separation of more than 1 min. The column oven temperature was increased to 40 °C to achieve lower run times and system pressure.

Typical chromatograms and spectra of blank and spiked shrimp samples are presented in Fig. 1.

3.1.3. MS parameter optimization

To optimize MS-parameters, a CAP working solution of 1 ng μl^{-1} was infused. A ''T piece'' was used to introduce mobile phase in the system at 1 ml min^{-1} flow to reproduce the same conditions as during analysis. APCI (mobile phase water–acetonitrile, 75:25) and ESI probe (mobile phase 0.1% formic acid in water–acetonitrile, 75:25), both in negative mode, were compared. Results were similar: good sensitivity, same diagnostic ions and chromatograms without interfering peaks. ESI probe was selected as ionization technique, due to its ruggedness, easy handling and maintenance. The influence of formic acid on sensitivity was also studied and, surprisingly, the results were similar using mobile phase with or without 0.1% formic acid.

Although the flow-rate was 1 ml min^{-1}, the electrospray was formed perfectly well with a nitrogen flow of 750 l min^{-1} and a desolvation temperature of 400 °C.

Sensitivity was very high and the addition of any modifier, such as acetic or formic acid to the mobile phase to ionize the CAP molecules was not necessary.

3.1.4. Diagnostic ions selection

The CAP spectra at different cone voltages were studied to select characteristic CAP fragments. Preliminarily, ions m/z 321, 257, 249, 194 and 152 were selected and the cone voltage was optimized for each fragment to obtain the maximum signal. Standards and positive sample injections were studied, comparing sensitivities and interferences in each channel. The CAP spectrum obtained in the described conditions is shown in Fig. 2.

Following Commission Decision 2002/657/EC, four diagnostic ions are required for confirmation. The best results were obtained with 321, 257 and 152. The fourth selected ion was m/z 323, an isotopic fragment, because of the low response of m/z 249 and m/z 194 in the described ionization conditions.

For quantification purposes, m/z 152 (CAP) and m/z 157 (5D-CAP) were chosen. At the beginning, many samples were analysed monitoring channels 321 and 326, but very often the latter one presented peaks due to matrix interferences.

The reproducibility of the three ratios used for confirmatory purposes (m/z 323/321, m/z 257/321

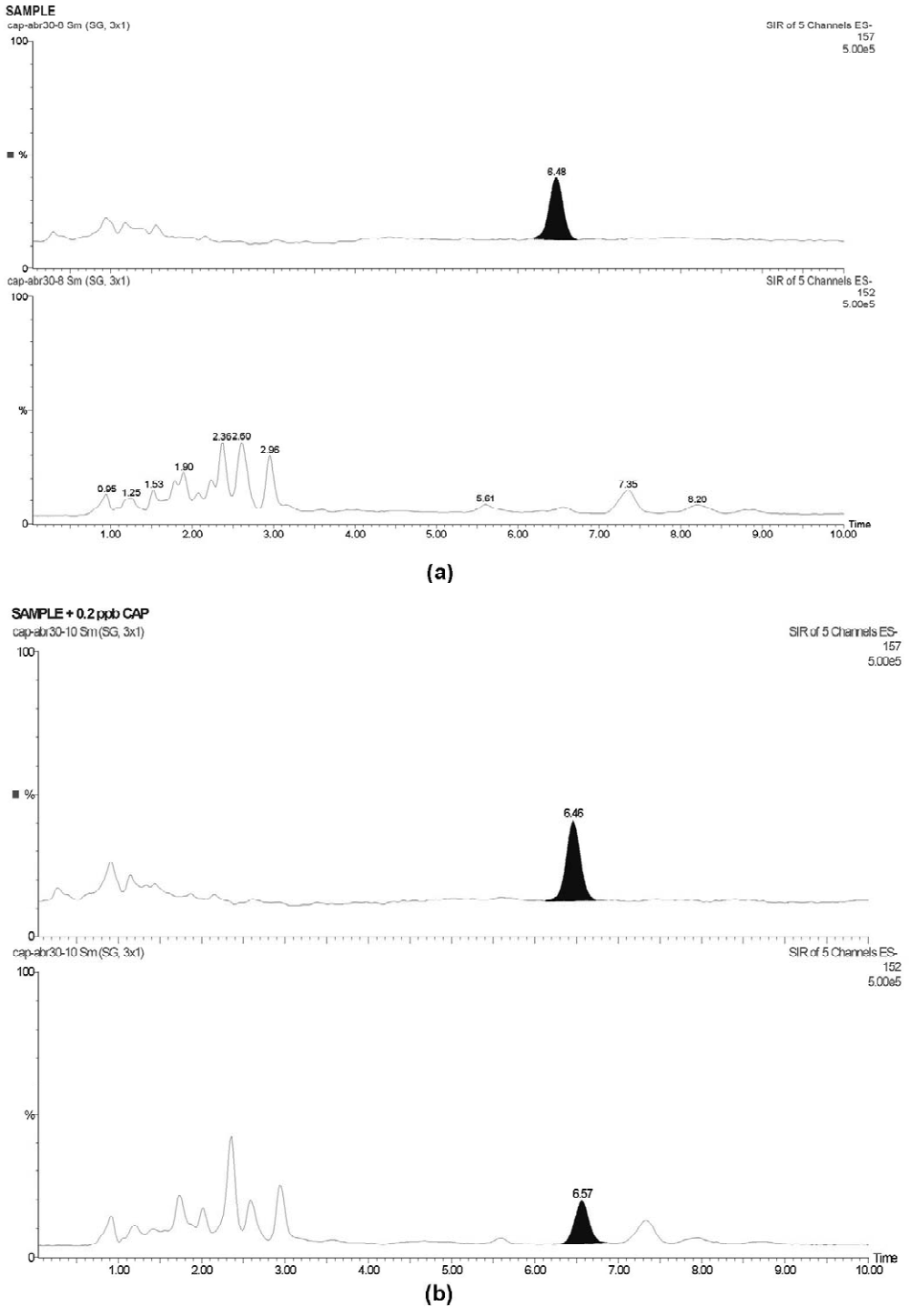
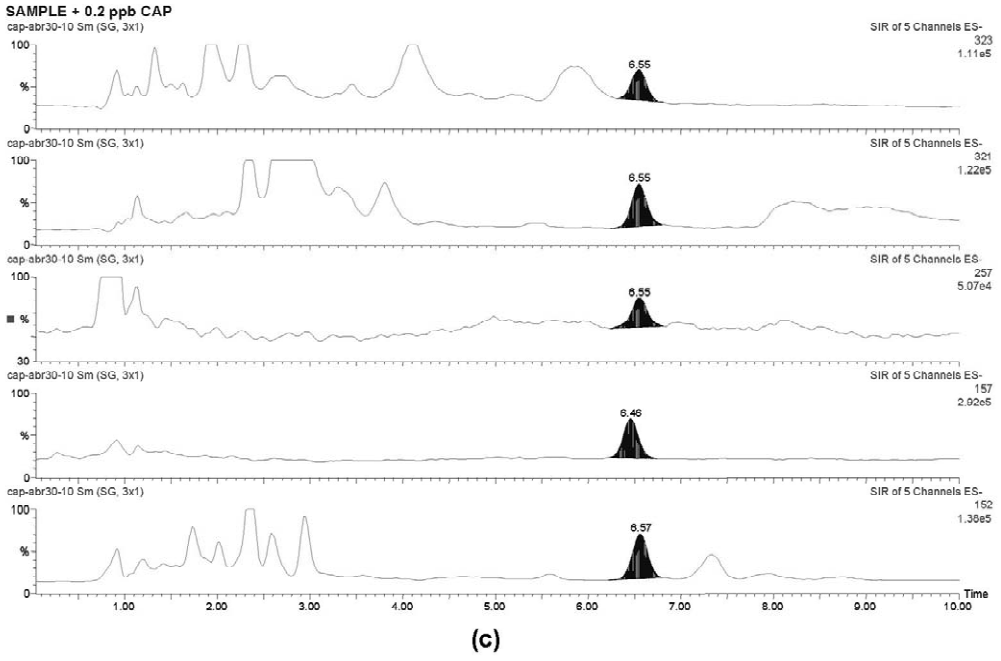


Fig. 1. (a) Chromatogram of blank CAP sample with internal standard (0.6 ng g^{-1}). (b) Sample spiked with 0.2 ng g^{-1} CAP and with internal standard (0.6 ng g^{-1}). (c) Sample spiked at the 0.2 g g^{-1} level: chromatograms of the typical fragments.



(c)

Fig. 1. (*continued*)

and m/z 152/321) was studied. All of them presented a good variation coefficient over 20 different injection days ($n=50$): mean 0.659 with C.V. 5.75%, mean 0.200 with C.V. 12.22% and mean 0.930 with C.V. 11.39%, respectively.

3.2. Validation results

3.2.1. Linearity

External calibration curves obtained for CAP/5D-CAP ($n=8$) in the 0.2 to 5 ng g^{-1} range were linear

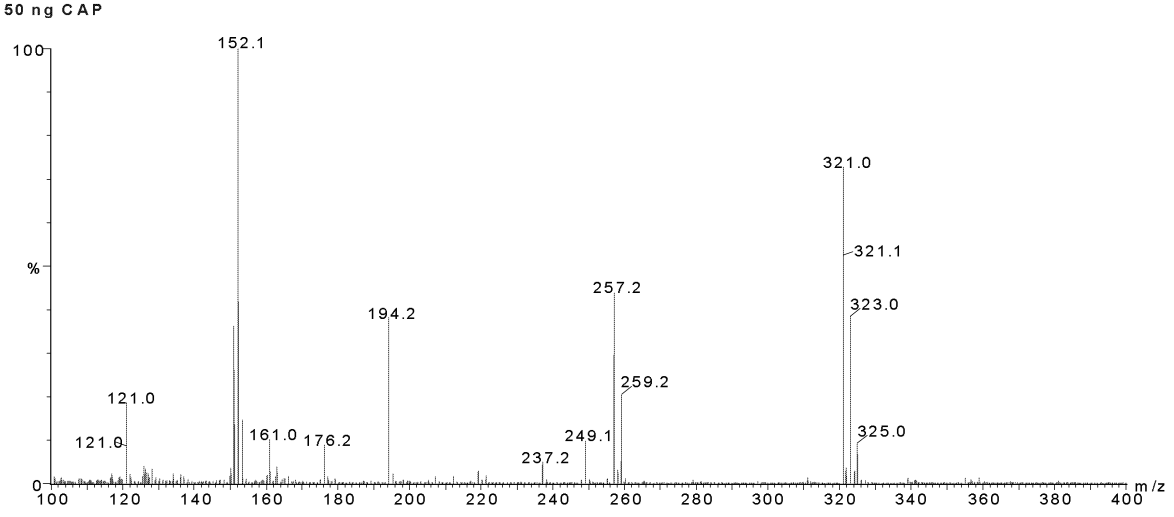


Fig. 2. Typical spectrum of chloramphenicol in LC–MS, obtained in ESI negative mode (CV: 40 V).

Table 1
Accuracy and in-house reproducibility in spiked samples

Spiked level (ng g^{-1})	Mean found concentration $n \geq 4$	SD	C.V. %	Accuracy %
0.2	0.2	0.02	7.1	110
0.5	0.5	0.03	5.5	108
1.0	1.1	0.03	3.0	106
5.0	5.0	0.17	3.4	101
50.0	50.5	0.50	1.0	101

with correlation coefficients $R^2 > 0.998$ and $R > 0.999$. The mean slope was 2.404 with a 3.58% variation coefficient and the mean intercept was 0.056.

3.2.2. Precision and accuracy in spiked samples

Reproducibility (between-day) was calculated and expressed as C.V. of the mean calculated CAP concentration, as presented in Table 1.

Accuracy, expressed as mean measured CAP concentration/nominal CAP concentration, lay between 101 and 110% and as can be seen also at the lowest tested level, C.V. of the results are lower than 7%.

3.2.3. Precision in real positive samples

Precision in real positive samples was also studied. Four different positive shrimp samples were analysed ($n = 5$). The results are shown in Table 2. The C.V. of the mean concentration of each sample was lower than 10%.

3.2.4. Analytical limits

The limit of decision (CCα) was 0.02 ng g^{-1} (mean of the responses obtained in 30 blank samples plus 2.33 SD).

The limit of quantification (LOQ), defined as the lowest studied concentration with acceptable precision and accuracy, was 0.2 ng g^{-1}.

Table 2
Repeatibility in four real samples (positives)

Sample	Mean found concentration (ng g^{-1}) ($n = 5$)	SD	C.V.%
A	0.5	0.03	5.6
B	4.4	0.24	5.3
C	4.8	0.39	8.2
D	2.2	0.08	4.9

The less abundant fragment of all the diagnostic ions (m/z 321, m/z 323, m/z 257 and m/z 152) is m/z 257, although its intensity was high enough to allow the confirmation of all spiked samples at the 0.2 ng g^{-1} level.

4. Conclusions

The proposed method allows the detection, quantitation and confirmation of CAP in shrimps with high accuracy and sensitivity. Decision and quantitation limits are rather low, although a single quadrupole instrument was used.

This work demonstrates the possibility of routine work at a high flow with an ESI probe, which increases the applicability of this technique.

A remarkable characteristic of this method is the high effectivity of CAP ionization with an ESI probe without the presence of any modifier in the mobile phase.

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