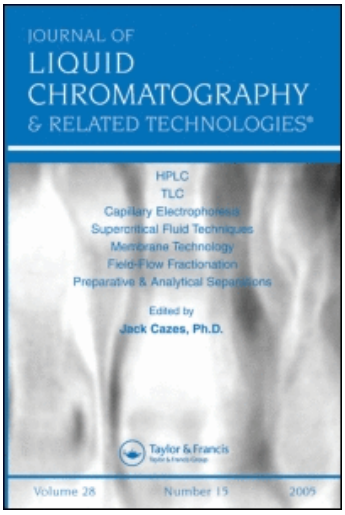

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[a] Faculdade de Farmácia da Universidade de Coimbra, Coimbra, Portugal [b] Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Campus de Lugo, Universidad de Santiago de Compostela, Lugo, Spain

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OPTIMIZATION OF DIPHASIC DIALYSIS PROCEDURE FOR CLENBUTEROL RESIDUES EXTRACTION IN BOVINE RETINA AND HAIR

Fernando Ramos,[1,*] Pilar González,[2] Anabela Oliveira,[1] Alexandra Almeida,[1] Cristina Fente,[2] Carlos Franco,[2] Alberto Cepeda,[2] and Maria Irene Noronha da Silveira[1]

[1]Laboratório de Bromatologia, Nutrição e Hidrologia, Faculdade de Farmácia da Universidade de Coimbra, 3000-295 Coimbra, Portugal
[2]Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Campus de Lugo, Universidad de Santiago de Compostela, E27002 Lugo, Spain

ABSTRACT

Development of a simple, rapid, and accurate methodology for clenbuterol residue extraction in bovine hair and retina by diphasic dialysis was evaluated. After bovine hair and retina samples digestion, diphasic dyalisis was used as an extraction procedure using four organic solvents (dichloromethane, n-hexane, ethyl acetate, and diethyl ether) and five different buffers (acetate, phosphate, borate, carbonate, and citrate). The organic extract was evaporated to dryness under a nitrogen stream and the

*Address correspondence to Fernando Ramos. E-mail: fjramos@ci.uc.pt

residue was derivatized with butylboronic acid. The results of GC-MS determination show that acetate buffer [sodium acetate 0.2M: acetic acid (95:5) pH ≈ 5.8] was the best choice for buffer dialysis, when dichloromethane and diethyl ether were elected as extraction organic diphasic dialysis solvents for hair and retina, respectively. Discussion of validation data show that diphasic dialysis could be a good, rapid, accurate, and economic extraction procedure to determine clenbuterol in bovine hair and retina samples.

Key Words: Clenbuterol; Diphasic dialysis; Retina; Hair; GC-MS

INTRODUCTION

β_2-Adrenergic agonists have been used for quite some time, both in human and veterinary medicine, mainly as bronchodilators and tocolithics. However, various studies have been performed since 1984 on the anabolic effects of clenbuterol (4-amino-3,5-dichloro-α-[(*tert*-butylamino)methyl] benzylalcohol), Figure 1, in species of high economic interest, such as chickens, pigs, sheep, and cows (1–4).

The potential of pigmented tissues in control programmes of unauthorised residues in zootechny has been enhanced by various studies, which proved the special fixation which occurs between melanin and clenbuterol. The importance of the accumulation of β_2 adrenergic agonists in the eye and hair of animals, including human hair, is a well-known fact (5–12). Thus, it is no longer considered as an oddity to find a greater concentration of clenbuterol in black hair than in blank hair (13), or a greater amount of the referred drug in the retina than in any other eye tissue (8,9).

However, although various procedures have been used for the extraction/purification of β_2-adrenergic agonists (14) and also for clenbuterol, in particular from these matrices, either through liquid–liquid extraction (12,15,16), or through immunoaffinity chromatography (12,17,18), or through solid-phase extraction (18–20), a study of the best conditions of diphasic dialysis, applied to

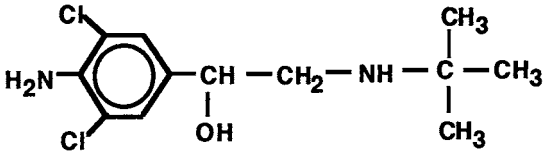

Figure 1. Molecular structure of clenbuterol.

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both hair and retina, was carried out. This option was due to the excellent selectivity and efficiency of this procedure in the separation of analytes of low molecular weight, such as clenbuterol, between an aqueous medium and an organic solvent, without any need for an additional purification procedure at a later step, as was already demonstrated for urine (21) and for liver samples (22,23).

EXPERIMENTAL

Reagents and Materials

Clenbuterol D3 (internal standard) was kindly offered by Doctor Jan Rud Andersen (Danish Meat Institute, Roskilde, Denmark) and clenbuterol hydrochloride was supplied by Interchim (Montluçon, France). Butylboronic acid (BBA) and ethyl acetate over molecular sieve were bought from Fluka (Buchs, Switzerland). All other reagents were purchased from Merck (Lisbon, Portugal), except purified water, which was obtained through a Milli-Q Plus system from Millippore (Bedford, MA, USA).

Retina and hair digestion was performed in a Memmert oven (Schwabach, Germany) and visking dialysis tubing 20/32, with a molecular exclusion size of 12000–14000 Da (Reagente 5, Oporto, Portugal), and an incubator shaker model G25 New Brunswick Scientific (Edison, NJ, USA) were used for diphasic dialysis.

The determination of clenbuterol was undertaken by gas chromatography in a Hewlett Packard (HP) apparatus, composed of an HP5890 series II gas chromatograph, HP6890 autosampler, HP5972 MSD detector, HP Vectra VL2 4/50 computer, and HP Deskjet 520 printer (Soquímica, Lisbon, Portugal).

The gases utilized were nitrogen N45 and helium N55 supplied by Sofager (Coimbra, Portugal).

A Mettler AE200 balance (Zurich, Switzerland), a CD 7400-WPA pHmeter (Cambridge, U.K.), a Stuart Scientific hemolysis tube evaporation system (Reagente 5, Oporto, Portugal), and a vortex type mixer (Retsch, Haan, Germany) were also utilized.

Samples

The hair needed for the studies was obtained from a two-month-old calf which was fed with 0.5 mg Kg^{-1} of clenbuterol-doped feed for a month. The average daily amount of ingested clenbuterol was calculated as 1.5 mg of clenbuterol per day (20). The eyes were obtained in the Figueira da Foz Municipal Abattoir, although some samples came from the Lugo Faculty of Veterinary Medicine.

Sample Preparation

Hair

The collection of black hair was performed ten days after the first ingestion of clenbuterol, with ten days' interval between collections. The hair was cut with a razor blade and the sample was washed three times with an aqueous solution of Tween 80 at 10%. The sample was then passed three times through purified water and dried at 40°C, being subsequently cut with scissors in small pieces of 1–2 mm size and stored in a refrigerator until the time appointed for clenbuterol determination.

Eyes

The collection of bovine eyes for analysis was done with surgical gloves and a scalpel. The whole eyeball was removed and both eyes were removed from the head of the slaughtered animal, transported at 4 ± 2°C, and frozen at -20°C as soon as they arrived at the laboratory.

Digestion

An amount of about one gram was used as a sample aliquot. In the case of hair, that aliquot was only weighed, but in the case of the eye, the retina had to be isolated from the remaining components. Thus, the whole semifrozen eye was cut in half. Then, crystalline and vitreous and aqueous humors were eliminated. The retina of both halves was separated from the remaining parts of the eye, cut with a scalpel blade, and weighed.

The sample was placed into a 20 mL screw capped centrifuge tube, and 3 mL of NaOH 1M and 50 μL of a methanol solution of clenbuterol D3 were added, in a concentration of 1.0 μg mL^{-1}. Digestion was carried out at 80°C with regular agitation, during one hour for hair samples or two hours for retina samples.

Diphasic Dialysis

After cooling at room temperature, the digest was transferred with 20 mL of buffer for a 250 mL erlenmeyer, where 20 cm of dialysis membrane, previously hydrated with purified water, had been placed, containing 25 mL of organic solvent, and with its extremities tied up with fishing thread. The pH of the digest was verified, and adjusted as necessary, to values of 11.9 ± 0.1 with NaOH 1M.

Diphasic dialysis was performed during 4 h at 37°C and 150 rpm in the incubator shaker (21–23). Afterwards, membrane contents were transferred to a

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separation funnel, and, after phase separation, the organic layer was put into a centrifuge tube.

Derivatization

The organic solvent was evaporated to dryness at 55°C under a nitrogen stream, and the residue was transferred to a derivatization vial with 2 × 200 μL of methanol and 30 seconds of vortex agitation, following each methanol addition. The methanol was also evaporated under the same conditions, and the dry residue was dissolved with 50 μL of a BBA ethyl acetate solution in a concentration of 5 mg mL^{-1}. The derivatization then took place for 1 hour at 55°C, after vial encapsulation and a 30-s homogeneisation period on the vortex (24).

Chromatography

An aliquot of 2 μL was injected in the GC-MS system in splitless mode, 1 min, using helium as carrier gas under a pressure of 10 psi at the head of the column (HP1 of 15 m × 0.25 mm d.i. × 0.25 μm of film thickness, in the case of hair, and Permabond OV1-DF of 25 m × 0.32 mm d.i. × 0.25 mm of film thickness, in the case of retina). Oven temperatures were programmed as follows: 120°C (0.1 min) $\longrightarrow$ 15°C min^{-1} $\longrightarrow$ 245°C (0.0 min) $\longrightarrow$ 30°C min^{-1} $\longrightarrow$ 245°C (5.0 min).

The temperatures of the injector and the detector were 260°C and 280°C, respectively, and determination was obtained in the electron impact (EI) mode with selective ion monitoring (SIM). Clenbuterol-BBA was evaluated by m/z ions 342, 327, 245, and 243, and m/z ions 246 and 345 were measured for the corresponding tri-deutered derivative. Analytical data were obtained from the area ratios of m/z 243 and m/z 246, respectively, for clenbuterol and for clenbuterolD3.

Figures 2 and 3 show chromatograms of hair and retina samples obtained through the above-mentioned methodology in optimised conditions, as described for each case at results and discussion section.

Validation

Once the extraction procedure conditions were optimised for each of the matrices, a validation study was undertaken. Linearity was evaluated for a month with five different standard concentrations, between 0.4 and 2.0 ng of injected clenbuterol; *r* values were found spanning between minima of 0.972 and 0.985 and maxima of 0.991 and 0.999, for hair and retina, respectively. Table 1 shows intra- and inter-CVs, recovery percentages, and detection limits; it will be noted that they fit within the reference values proposed by various authors (25–29).

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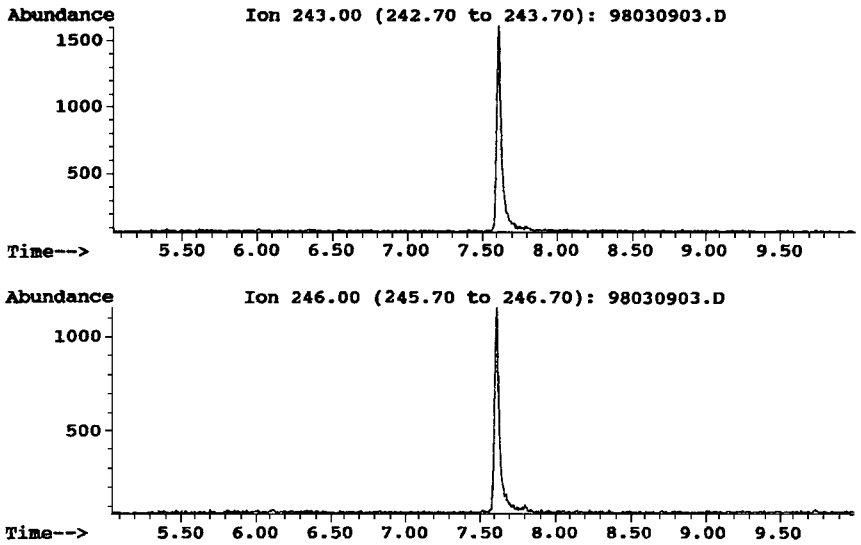

Figure 2. Ion chromatograms at m/z 243 and 246 for a sample of hair with clenbuterol at 18 ng g^{-1}.

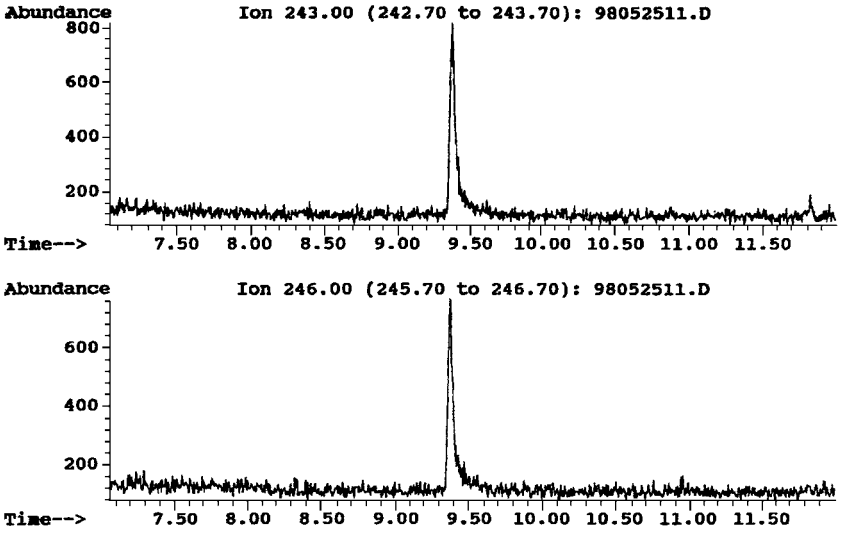

Figure 3. Ion chromatograms at m/z 243 and 246 for a retina sample with clenbuterol at 13 ng g^{-1}.

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Table 1. Validation Data ($n = 5$)

Criteria	Hair	Retina
Detection limit	5 ng g^{-1}	2.5 ng g^{-1}
Recovery		
60 ng/g	85.2%	96.1%
80 ng/g	84.4%	86.1%
100 ng/g	90.5%	88.1%
Repeatability (CV)	7.3%	9.0%
Reproducibility (CV)	15.5%	22.9%

RESULTS AND DISCUSSION

Clenbuterol residues in hair could only be detected after thirty days of drug administration. Thus, hair was pulled off the animal hide in order to verify if an earlier determination could be done, owing to that the fixation of clenbuterol in hair structure took place during the formation of the hair at the hypodermis. Unfortunately, and despite the fact that such a case is theoretically feasible, all it could get with this procedure was the animal's lack of collaboration, with no additional advantage. So, it was advised to collect this matrix with a razor blade, in order to keep to a minimum the time between clenbuterol fixation in the hair and the possibility of its determination.

Generally, better results are obtained in clenbuterol extraction at acid pH than at alkaline medium. However, an alkaline pH is mandatory during diphasic dialysis, as was explained further on. So, it was decided to undertake the digestion in an alkaline medium. In any case, although it needs two hours to be completed in the case of the retina, it proved to be markedly faster, as compared with the attempt made in an acid medium, which had not yet been homogeneously terminated after four hours.

Closing the dialysis membrane with fishing thread was due to the fact that the sisal thread initially used contain some dyed substances in its composition, and these were extracted by some organic solvents employed, thus increasing the contamination of the extract.

Some dialysis parameters, like pH, solvents, temperature, time, and agitation speed, were optimised with aliquots of clenbuterol-free sample spiked with 60 ng g^{-1} of the referred drug. The results obtained by Fente and co-workers (30) were confirmed, except in the case of the buffer in which the digest was recovered.

Therefore, the need for a pH value close to the pKa_2 of clenbuterol, 9.7, so as to enable the maximum clenbuterol transfer from the aqueous to the organic layer, led to the demand for a buffer to contain the strongly alkaline digest, during the dialysis procedure. However, it was noted that, during diphasic dialysis, there

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always was a pH decrease in aqueous layer, which was much greater when hair was the analysis matrix. In our opinion, such a fact may be due to hair sulfhydryl radicals, which means that only an initial pH with an approximate value of 12 can make it possible to reach the end of diphasic dialysis period with a pH $\approx$ 9.7.

Five different buffers of 0.2 M concentration were tested in the diphasic dialysis procedure: acetate, phosphate, borate, carbonate, and citrate. Dichloromethane was used as organic extraction solvent for these studies. The choice of acetate buffer over the remainder, both for hair and retina, was due to a better peak resolution and, therefore, greater sensitivity, as shown in Figures 4 and 5, which show the two buffers which presented the best recovery results in the case of the retina.

In the study leading to the best organic solvent choice to be used within the dialysis membrane, dichloromethane, n-hexane, ethyl acetate, and diethyl ether were considered. The digest was kept in acetate buffer during these experiences.

The dichloromethane choice, confirming the option of Fente and co-workers (30) when the analysis matrix was hair, was due to the fact that it led to the best extraction performances and also originated cleaner extracts.

In the case of the retina, Figures 6 and 7 make it plain that diethyl ether is the best organic solvent for the diphasic dialysis procedure; n-hexane was the second best choice among all those tested.

The study for the diphasic dialysis optimum temperature was undertaken for values between 30°C and 40°C; it was noted that extraction performance improved with temperature, although a clenbuterol extraction with acceptable recovery data,

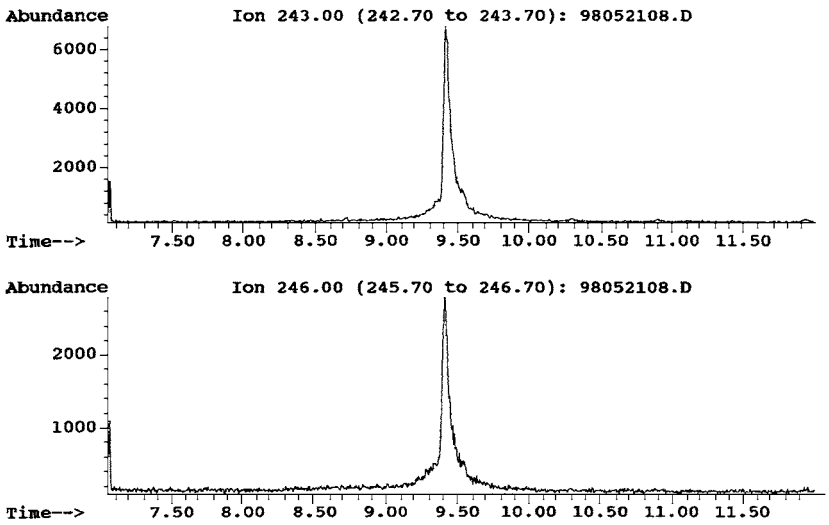

Figure 4. Ion chromatograms at m/z 243 and 246 obtained after diphasic dialysis procedure with acetate buffer.

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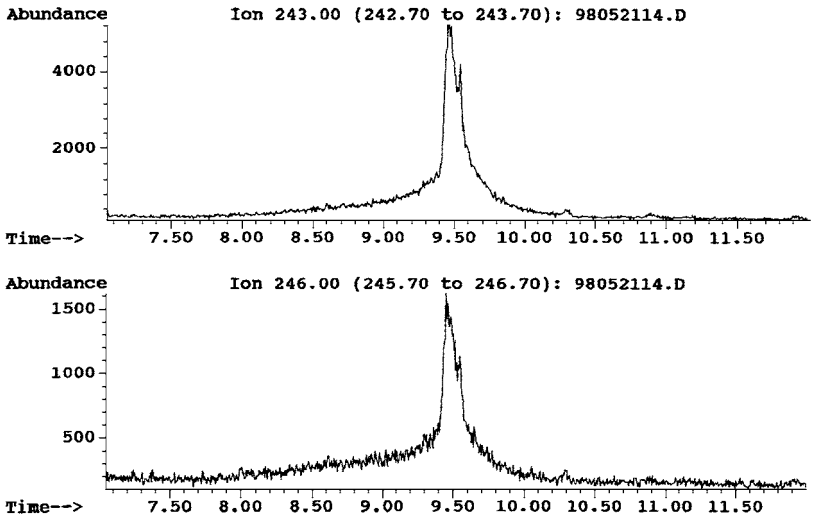

Figure 5. Ion chromatograms at m/z 243 and 246 obtained after diphasic dialysis procedure with phosphate buffer.

41 ± 8.9% ($n = 3$), was not obtained until 35°C was reached. The best results were obtained at 37°C. Besides this value, an increased evaporation of the organic solvent was observed and, even at 38°C, it is no longer found after 4 h of extraction.

Likewise, it can be stated that, the longer the dialysis took, the greater was the performance obtained in the extraction procedure, which reached a maximum after 4 h. However, it was noted that after a 5-h period, although a better recovery of clenbuterol was probably done, it was not possible to quantify this result, due to the larger amounts of dirt which were being extracted and led to the appearance of a fair amount of interferent peaks at the final chromatogram.

The agitation speed was also optimised: 150 rpm being selected as optimum stirring rate. Lesser speeds would give poorer clenbuterol recovery data, and speeds over 150 rpm invariably led to the evaporation of the organic solvent in the dialysis membrane, causing poorer extraction rates, or even invalid ones, as was the case with 250 rpm, which, after 4 h, led to the complete evaporation of dichloromethane, the solvent utilised in this test.

On the other hand, it should be noted that the extraction by diphasic dialysis in optimised conditions for hair cause the evaporation of about 25% of dichloromethane. However, this evaporation was indispensable in order to enable an efficient transfer of clenbuterol from the alkaline aqueous solution to the dichloromethane within the membrane.

Various experiments were undertaken in Erlenmeyer flasks stoppered with carded cotton wool wrapped in aluminium foil, which caused a decrease in the recovery of the extraction process of about 60%. In our opinion, this may be due

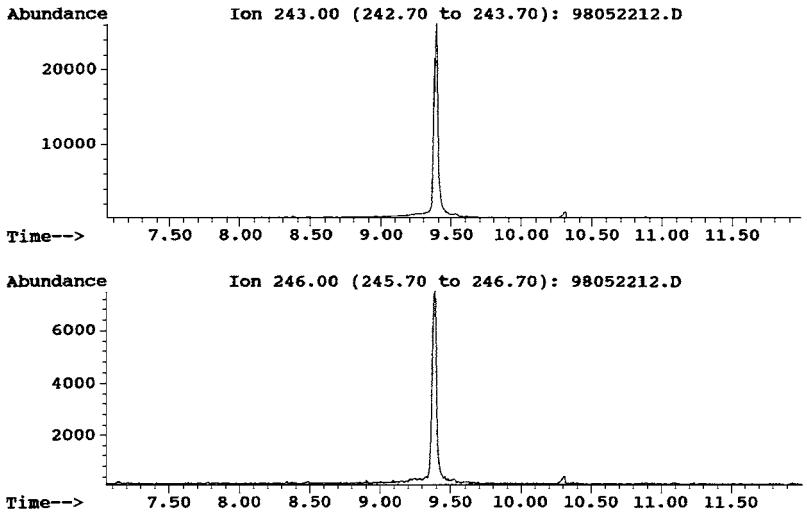

Figure 6. Ion chromatograms at m/z 243 and 246 obtained after diphasic dialysis procedure with diethyl ether as organic solvent.

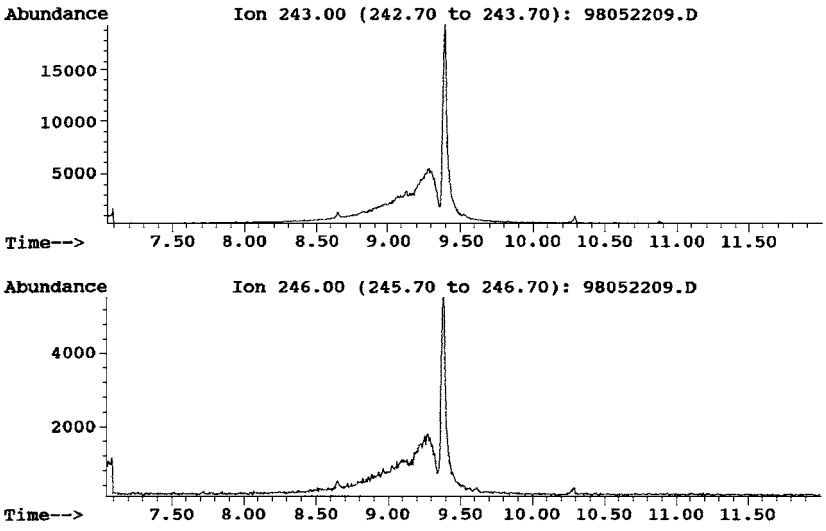

Figure 7. Ion chromatograms at m/z 243 and 246 obtained after diphasic dialysis procedure with n-hexane as organic solvent.

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to a dichloromethane critical vapor pressure around the dialysis membrane, which prevented the transfer of clenbuterol, because the extraction of this was nearly total when the step was done in open Erlenmeyers. This behaviour leads us to theorize that a vacuum system connected to the incubator shaker, so as to control the extraction atmosphere by eliminating any vapours of the organic solvent, would improve the efficiency of diphasic dialysis.

The use of diphasic dialysis as an extraction procedure of clenbuterol proved to be an efficient methodology, because, although the overall analysis time is slightly longer than those of the previously-described methods, both for retina (15,19) and and hair (12,17,18,20), it does substantially reduce the number of manipulations by the operator, thus decreasing the number of losses during analysis and providing additional freedom for undertaking other tasks. On the other hand, considering the complexity of hair and retina, the extraction/purification process used prove to be relatively easy and with good detection limits, and therefore, adequate to those purposes.

Finally, the developed methodology is a very good aid to veterinary inspection activity, particularly in live animals, since it can be used to determine clenbuterol in hair, at least 120 days after stopping drug administration.

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