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Journal of Chromatography A, 880 (2000) 69–83

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

β_2-Agonist extraction procedures for chromatographic analysis

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Abstract

Normally, different procedures were necessary to prepare sample matrices for chromatographic determination of β_2-agonists. The present review includes sampling, pre-treatment and extraction/purification for urine, plasma, liver, meat, feeds, hair and milk powder, as previous steps for chromatographic analysis of β_2-agonists. Six methodologies were especially revised for extraction/purification namely, liquid–liquid extraction, solid-phase extraction (SPE), matrix solid-phase dispersion, immunoaffinity chromatography, dialysis and supercritical fluid extraction. SPE was discussed in detail and five mechanisms were described: adsorption, apolar, polar, ion-exchange and mixed phase. A brief conclusion in this field was also outlined.

Keywords: Reviews; Extraction methods; β-Agonists

Contents

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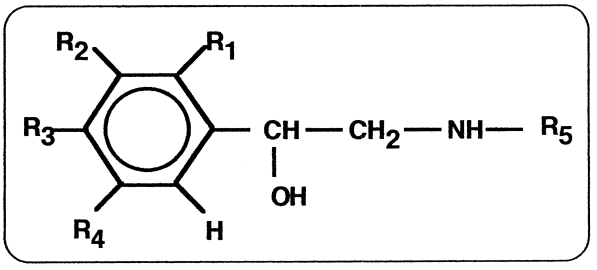
0021-9673/00/$ – see front matter
PII: S0021-9673(00)00240-5

1. Introduction

Food quality has always been a permanent concern for most people. It can be considered from various aspects, although, for the sake of a system, it usually fits into four major groups: purely nutritional, chemical residues, physical contaminants and those resulting from microbiological contamination, involving bacteria, fungi, viruses and parasites.

Control of chemical residues, particularly substances used to promote animal growth, has been the cause of some transnational concern, as can be seen from the residue control plans which are compulsory in all European Union (EU) countries [1], as well as from the so-called 'hormone war' between Europe and the US, which began in 1988 with a ban on their use within the EU [2].

It was in 1988 that the use of β_2-adrenergic agonists (Fig. 1), previously authorised for human and veterinary therapeutics, mainly as anti-asth-



	R1	R2	R3	R4	R5
Mabuterol	H	Cl	NH_2	CF_3	$C(CH_3)_3$
Mapenterol	H	Cl	NH_2	CF_3	$C(CH_3)_2CH_2CH_3$
Clenproperol	H	Cl	NH_2	Cl	$CH(CH_3)_2$
Terbutaline	H	OH	H	OH	$C(CH_3)_3$
Clenbuterol	H	Cl	NH_2	Cl	$C(CH_3)_3$
Salbutamol	H	CH_2OH	OH	H	$C(CH_3)_3$
Clenpenterol	H	Cl	NH_2	Cl	$C(CH_3)_2CH_2CH_3$
Bromobuterol	H	Br	NH_2	Br	$C(CH_3)_3$
NA 1141	H	Cl	NH_2	Cl	$C(CH_3)_2CH_2OH$
Tulobuterol	Cl	H	H	H	$C(CH_3)_3$
Cimaterol	H	CN	NH_2	H	$CH(CH_3)_2$
Cimbuterol	H	CN	NH_2	H	$C(CH_3)_3$
Orciprenaline	H	OH	H	OH	$CH(CH_3)_2$
Ractopamine	H	H	OH	H	$CH(CH_3) - CH_2 - PhOH$
Fenoterol	H	OH	H	OH	$CH(CH_3) - (CH_2)_2 - PhOH$

Fig. 1. General structure of β_2-agonists.

matics, bronchodilators and tocolithics, becames widespread as 'repartitioning agents'. The beneficial effects on growth and carcass composition of β-agonists in meat-producing animals have been extensively proved by a marked expansion of the muscular mass, together with a decrease in fat accumulation [3–6]. However, these substances have never been authorised for such purposes, and were classified in the list of substances which were formally banned from zootechnical applications [7].

The use of highly sensitive analytical methodologies has become indispensable to achieve an efficient control of these substances. All current strategies, however, agree in one point: there is no ideal method [8–10].

Nevertheless, the search for the best possible method has been a constant concern of the various research groups. A perfectly defined strategy can be observed: on one hand, the development of multi-residue methodologies capable of determining the highest possible number of substances, particularly, in this case, of β_2-adrenergic agonists [11]; on the other hand, the utilisation of fast determination methods, followed by the identification of the compounds and their subsequent confirmation, always using spectrometric methods [12]. However, the complexity of the composition of the matrices and the small amount of xenobiotic which is normally present often change the process leading to the evaluation of residues, in general, and of β-agonists, in particular, in a rather difficult procedure. Steps of sampling, pre-treatment and, above all, extraction/purification, proved to be of vital importance for β-agonist residue analysis, regardless of the chromatographic method used.

The present paper reviews the aspects mentioned above, adding comments taken from the author's own experience on the subject whenever possible.

2. Sampling

Experience shows that sampling may be an important source of errors in chemical analysis, particularly where foodstuffs are concerned; Lichon [13] even suggests that sampling should ideally be done by the analyst himself.

In the case of tissue samples, the choice of the various functional parts of an organ can have a considerable influence in the amount of the analyte determined. For example, if bovine liver shows no discrepancy in the clenbuterol contents in the various lobes, a marked difference in the clenbuterol concentrations can be observed in the medulla and the cortex of the kidney: the medulla always presents more clenbuterol than the cortex [14].

Some precautions can also be taken in hair sampling for clenbuterol. The choice of black hair, instead of white hair, is mandatory, due to the special binding of the drug to the melanin of black hair [15–17]. Clenbuterol is fixed by hair in the hypodermis. Thus, if hair sampling is made by razor blade instead of scissors, a more recent drug intake could be detected.

Microbiological contamination of animal carcass favours protein, water and increasing pH, as result of β-agonist action [18]; thus, tissue sampling after animal slaughter was advised to avoid this. Also, transportation of biological samples from the slaughterhouse to the laboratory must be done immediately after sampling at a temperature of about 4–6°C.

However, regardless of the sample, it should always be representative and large enough to enable an adequate analysis, its repetition and the respective confirmation tests. Samples should also be marked in such a way that no problems may arise at any time with regard to their identification, both at the time of sampling and at later stages of the analytical procedure [19].

3. Pre-treatment

Homogenisation is a step of vital importance in sample pre-treatment, particularly as regards the accuracy of the results. Solid matrices, for example feeds and tissues, must be thoroughly mixed, with size reduction of their particles kept to a minimum, in order to enable the later penetration of the enzymes and/or solvents [13,20]. However, since sample homogenisation is done before or after storage (−30°C), this can influence the final contents, depending on the matrix. For example, kidney homogenisation, before or after freezing, does not appear to influence the final contents of clenbuterol.

On the other hand, in the case of liver, a considerable loss of clenbuterol contents can be observed when homogenisation is done before storage and the result is compared with non-homogenised liver samples. This fact may be connected with the activation of some enzymatic processes triggered in the liver by homogenisation. Liver and muscle storage, without previous homogenisation, at low temperatures (−30°C), have shown that there was no evidence for clenbuterol losses for at least 5 months.

As regards the retina, when one wants to repeat the determinations of clenbuterol contents, it should be subjected to homogenisation before storage, due to the small amount of sample. Thus, just cutting small portions of the retina does not guarantee that the results can be reproduced. It is advisable to remove the whole retina and duly homogenise it with a buffer solution. The eventual division of the volume in amounts which will suffice to replicate the analyses is also advisable. This procedure leads to a much smaller variation of clenbuterol results for the retina with previous homogenisation, when compared with the same number of retina determinations omitting that previous step [14].

Comparing various homogenisation techniques, mixer, stomacher®, ultra-turrax and ultrasonification, is not enough to make a clear choice, since they do not appear to have a significant influence on the final result. However, taking into consideration our experience in this field, we do make a distinction between the ultrasound bath and probe, due to the power difference between them. Our advice is to use the bath, 80 W, to homogenise liquid samples and solutions; while the probe, 375 W, is indispensable for destruction of cell membranes of solid samples, as in the case of liver, for instance. On the other hand, the use of the stomacher® in the homogenisation process should be preferred when homogenising solid samples with solvents, due to the utilisation of disposable bags with corresponding decrease in the risk of inter-sample contamination.

Centrifugation or filtration, in order to eliminate the substances in suspension, and eventual dilution are the main pre-treatment steps of the liquid samples, such as blood, bile and urine, particularly when the latter are taken from adult ruminants [20–23].

The enzymatic hydrolysis of conjugated esters, particularly in the form of glucuronide and sulphate, is also compulsory in β-agonist multi-residue analysis. This hydrolysis is indispensable to isolate the free parent compound from the biological matrices in order to obtain an accurate extraction procedure. This step, which is essential for β_2-adrenergic agonists similar to salbutamol must take place at acid pH (4.8±0.2) with *Helix pomatia* (a preparation containing β-glucuronidase and arylsulfatase) for periods between 45 min and 20 h and temperatures between 37 and 60°C [11,24–28]. For clenbuterol and related substances, this enzymatic deconjugation could be omitted because the hydroxy and alkylhydroxy groups responsible for sulpho or glucuronidation are not present at the benzenic rings of the compounds (Fig. 1).

Protein elimination from samples — muscle, liver or kidney, for example — is also advised, because it contributes to release the analytes from the cells. The eventual bond of β_2-adrenergic agonists to proteins is broken and a greater and better recovery of analytes is obtained, particularly when compared with mechanical homogenisation [20,29]. This proteinaceous denaturation can be done enzymatically [29–32], by acidic precipitation [33–35] or by both processes together [29,32].

A further step can be used on sample pre-treatment: addition of an internal standard, preferably a deutered β_2-adrenergic agonist, which can minimise certain problems, such as:

(i) losses during the analytical procedure;
(ii) variability of the injection volume, in the case of the chromatographic methods;
(iii) efficiency of derivatisation, whenever necessary;
(iv) sensitivity of the determination system; and
(v) control of results.

4. Extraction/purification

This step of the analysis of β_2-adrenergic agonists can be undertaken by six different procedures (liquid–liquid extraction (LLE), solid-phase extraction (SPE), matrix solid-phase dispersion (MSPD), immunoaffinity chromatography (IAC), dialysis and supercritical fluid extraction (SFE)) either by themselves or with one another.

4.1. Liquid–liquid extraction

LLE is the earliest classic extraction/purification procedure and is based on the mixture, either manual or mechanic, of the solution containing the sample with an immiscible solvent. After a complete separation phases, the elected analyte is collected in the other phase without the main interferences.

There are various studies which use this procedure. Probably, the best example is the extraction of clenbuterol and related compounds from urines through organic solvents, provided they are previously adjusted at pH>10 [36–38]. However, significant losses could be observed when salbutamol and similar substances were extracted by this mechanism, they present significant losses. To reduce this losses, the process must be done with an 'ionic pair' which adapts itself to the sample pH and the saline concentration to the pK_a of the referred β_2-adrenergic agonists. DEHP [di(2-ethylhexyl) hydrogenphosphate] being the most commonly used substance for this purpose [39–43].

The application of this mechanism to liver samples and other bovine tissues is also described by Girault and Fourtillan [44], as well as by Leyssens et al. [45]. The latter undertook the simultaneous extraction of tulobuterol, clenbuterol, salbutamol, mabuterol, fenoterol and terbutaline with a mixture of *tert*.-butanol–ethyl acetate (30:70), after a previous digestion with subtilisin and alkalinisation at pH≈9.8.

Clenbuterol, salbutamol, mabuterol and terbutaline were also extracted from liver, previously digested with a protease and alkalinised at pH≈11, with a mixture of ethyl acetate–isopropanol (60:40) [26,30].

4.2. Solid-phase extraction

SPE can be defined as a process in which retention takes place on a solid sorbent and elution is done through a liquid which crosses the said sorbent. Today this undoubtedly is one of the most popular techniques used for sample extraction/purification, particularly when one must determine small analyte concentrations in complex matrices, as in the present case [46]. The use of SPE membranes instead of the traditional columns enables the use of greater solvent fluxes. This procedure decreases obstruction risks, due to the larger available area in a much smaller volume, as compared with the traditional column. The retention and elution are thus faster, and the latter is even possible with a smaller amount of solvent than in the classic SPE. This methodology produces a more concentrated extract, with obvious advantages [47–49]. The automation of SPE, for which various equipment is already on the market, adds to the previously mentioned advantages a decrease in analysis times, thus freeing the analyst for other tasks, particularly when a large number of sample analyses is necessary [50].

In the analysis of β_2-adrenergic agonist residues, SPE can be the only method in the extraction/purification step, or can be used together with other procedures, such as LLE, IAC, or even SPE itself, although using different mechanisms. Five types of mechanism are described in the extraction/purification of this type of residues: adsorption, apolar, polar, ionic exchange and mixed phase.

4.2.1. Adsorption

The most commonly used sorbent in SPE, which best characterises the interaction by adsorption, is diatomaceous earth. As a rule, this sorbent is not submitted to previous activation by any solvent. The sample being transferred in an aqueous solution which is completely adsorbed by the sorbent. It is only afterwards that the eluent, usually composed by isolated or mixed organic solvents, is added [51].

Clenbuterol and urine are the pair which has been submitted to most tests. The extraction/purification of clenbuterol from urine is preceded by an alkalinisation step up to pH≈10 [52–54], 11 [55,56], 12 [57] or 13 [58]. After being alkalinised, the urine is added to the diatomaceous earth column, and a period of time is allowed for its fixation/absorption, which can be 10 [55–57] or 15 min [52–54]. Clenbuterol elution is done with 3×20 ml of hexane [52–56], 3×20 ml of toluene–dichloromethane (3:1) [57] or 2×20 ml of dichloromethane [58].

Sangiorgi and Curatolo [59] applied the abovementioned strategy to the extraction of bromobuterol from bovine urine, although they submitted the extract to another, later SPE procedure with CN columns before undertaking the determination.

Van Ginkel et al. [60] were the only consulted

authors who undertook an acidification of urine at pH 5.2±0.1 followed by enzymatic hydrolysis with β-glucuronidase and arylsulfatase for 18 h at 37°C. Sample was then added to the diatomaceous earth column and was given 15 min for its fixation/absorption. Elution was attained with 60 ml of ethyl acetate, and the sample was subsequently subjected to a second extraction/purification step through an immunoaffinity column.

On the other hand, Byrem et al. [61] undertook the purification of cimaterol from urine by alkalinising it to pH≈9.5 and liquid–liquid extraction with ethyl acetate. Cimaterol is re-extracted from the ethyl acetate with 0.1 *M* hydrochloric acid, and this extract was alkalinised at pH≥11 and placed in the diatomaceous earth column. Five minutes are allowed for its absorption/fixation, and the elution is subsequently done with 30 ml of chloroform.

However, the diatomaceous earth sorbent was not suitable for use in a β-agonist multi-residue extraction from urine [62]. The presence of hydroxyl groups, besides β-hydroxylamine (Fig. 1) was liable to a strong adsorption in this sorbent [63]. The terbutylic group related to the secondary amine (R5, Fig. 1) seems to play an important role in this type of extraction mechanism, namely on the choice of the elution solvent. When the referred *N*-group is an isopropyl or an isopentyl, a strong or light decrease in β-agonist recoveries were observed with *n*-hexane as sorbent elution [62]. The use of a toluene–dichloromethane mixture (3:1, v/v) to improve elution performance of cimaterol (*N*-isopropyl group, R5, Fig. 1) also confirm previous sentence [57].

The β_2-adrenergic agonists can also be extracted/purified from other biological liquids, such as blood, plasma, serum and bile, using the same procedure which has already been described for urine [54,57,61].

As regards solid matrices, such as milk powders, pre-mixers, feeds, liver, meat and feces, a previous extraction with solvents is necessary before using the diatomaceous earth column. For example, Degroodt et al. [53] and Tantillo et al. [54] have extracted clenbuterol from meat and liver with TRIS buffer at pH≈8, calcium chloride and subtilisin A at 55°C/1 h, following which they adjusted the pH at 10. Batjoens et al. [64] have extracted clenbuterol from feces by using the same procedure that Courtheyn and co-workers [57,65] have used for clenbuterol and cimaterol in feeds, that is to say, with 0.5 *M* hydrochloric acid saturated in ethyl acetate. The extract being then alkalinised at pH≈12. An identical procedure was also followed by de Wasch et al. [66] for extracting clenbuterol, tulobuterol, bromobuterol and mabuterol from liver. Clenbuterol, cimaterol, mabuterol, salbutamol and terbutaline were extracted from milk powders, pre-mixers and feeds by Van Ginkel et al. [60] in water under the action of ultrasound for 30 min; before SPE procedure the pH of the extract being adjusted at 9.8±0.2. The following steps are similar to that already mentioned for urine by various authors, with the exception of de Wasch et al. [66] who have awaited the fixation of the β_2-adrenergic agonists in the column for 45 min, before doing their elution with 3×20 ml of toluene.

In a comparative study undertaken by Leyssens and co-workers [67,68] for the determination of clenbuterol in feeds, it was concluded that the adsorption mechanism showed the best results in the extraction/purification step. The extract was alkalinised at pH≈12 and introduced into the column, about 20 min being allowed for its complete fixation, and the elution was done with toluene. The same paper considers that, in the case of salbutamol, the best results are also obtained with an extraction/purification mechanism based on adsorption. The extract, however, was alkalinised at pH≈9.8, and the elution was done with ethyl acetate, but a supplementary SPE process with cationic-exchange sorbent was required.

4.2.2. Apolar

The apolar interactions are due to the so-called Van der Waals forces, and take place between the carbon–hydrogen links of the analyte and those of the functional sorbent group [51]. The reversed-phase columns need a previous step for activation their sorbent through a solvation process. Methanol was always used as first solvent, usually followed by water [29,43,69–75], by water and sodium phosphate buffer, 0.1 *M* [76,77] or 0.4 *M* [25,78], with pH 7.2 [25] 7.3 [77] or 7.6 [76,78], just phosphate buffer, pH 7.6 [79], by bicarbonate buffer, pH 8.6 [80], by borate buffer, pH 9.5 [81], or just sodium hydroxide 1 m*M* [82].

Urine, the most commonly studied matrix, is then added to the columns, either directly [82] or following a number of previous treatments. The most common one is the adjustment of the urine pH to values from 7.2 to 7.6 [25,77,79], 8 [72,75], 9 [69] or even 10–11 [74].

However, the extraction/purification of β_2-adrenergic agonists can be done from other matrices, such as milk powders, feeds, serum, plasma, liver, muscle or hair. If plasma and serum, after previous sorbent solvation, can be directly placed [70,73] or diluted in water [43] or 0.1 *M* phosphate buffer, pH 7.6 [76], in the column, the other matrices require a previous preparation.

Howells et al. [29] took 10 g of homogenised liver in 30 ml of TRIS 1 *M* buffer, pH≈10.5, added 10 mg of protease and submitted the whole mixture to a magnetic agitation for 1 h at 55°C. Enzyme deactivation, with boiling water for 15 min, is followed by cooling and acidification with 3.5 ml of concentrated hydrochloric acid. After centrifugation, the supernatant is decanted and its pH adjusted at 9.1 with 10 *M* sodium hydroxide, following which the extract can be added to the SPE column.

Degroodt et al. [71] start from 25 g of ground liver and then extract the β_2-adrenergic agonists with two aliquots of 50 ml of hydrochloric acid 0.01 *M* in an ultrasound bath for two periods of 15 min. The extract is taken to pH≈9 with 5 *M* sodium hydroxide and, following a centrifugation, the supernatant is passed through the column.

Hair, after being thoroughly washed, dried and ground, is digested with 0.1 *M* hydrochloric acid at 56°C for 12 h. After centrifugation, the supernatant is neutralised with 1 *M* sodium hydroxide, adjusted at pH 8.6 with bicarbonate buffer and passed through the column [80].

After the sample or its extract is fixed in the sorbent, an intermediate washing step follows in order to eliminate polar interferents. The most commonly used solvents in this step are just water [43,69,72,76,78,79], water and a mixture of methanol–water (35:65) [81], (25:75) [75] or (10:90) [73,80], water, a mixture of methanol–water (25:75) and 0.05 *M* sodium hydroxide [69,71], water and acetonitrile [29,70], 1 *M* phosphate buffer, pH≈8.6, and acetonitrile [83], 0.1 *M* phosphate buffer, pH≈ 7.3, and water [77], 1 m*M* sodium hydroxide and methanol–water (15:85) [82], or even a plain mixture of methanol–water (50:50) [74].

Methanol is the most commonly solvent used for the elution step. Thus, about 70% of consulted studies refer methanol as the only eluent, whereas in the remainder one can find mixtures of methanol with acetonitrile (85:15) [76,78], with 0.25 *M* ammonium phosphate buffer (75:25) [73], with 1 *M* ammonium acetate buffer (99:1) [81], with acetic acid (95:5) [69], or with formic acid (99:1) [75].

Some authors, before undertaking the determination step, also submit the eluate to subsequent purification procedures. SPE with CN columns [83], liquid–liquid extraction [72] or immunoaffinity column [69] were the most frequent procedures used for this purpose. Nevertheless, Elliot et al. [25] have also utilised this apolar mechanism after SPE ractopamine extraction with mixed-phase sorbent.

The β_2-adrenergic agonists mentioned earlier, with an extraction/purification basically done by apolar mechanisms, are clenbuterol [47,69,71,74, 75,78,79,81,82] salbutamol [29,43,69,70,72,73,76, 77,82,83], cimaterol [71,82], ractopamine [25,29], terbutaline [76] and fenoterol [76].

Finally, it should be mentioned that, although the great majority of the authors utilise C_{18}, four of the consulted studies used different sorbents, namely C_2 [75,81] and C_8 [62,77]. However, the presence of a larger number of carbons on the *N*-group, such as isopentyl (R5, Fig. 1), seems to improve retention and recovery of the corresponding β-agonists, while the sorbent is more apolar ($C_{18}>C_8>C_2$) [62].

4.2.3. Polar

The polar interactions take place between the reactive groups of the analyte which are capable of presenting dipolar moments and the functional groups of the sorbent with similar characteristics. Besides the dipole–dipole connections, both natural or induced, polar interactions include hydrogen bonding and a variety of other interactions capable of giving a polar behaviour to the reactive groups, both of the analyte and of the sorbent [51].

Contradicting what happened regarding the previous mechanism, few authors have utilised this interaction in the extraction/purification of β_2-adrenergic agonists. Liver being the most common matrix with this type of sorbents. Besides Collins et al. [31], who

have combined diatomaceous earth and silica columns to extract/purify clenbuterol, Leyssens et al. [45] and Schmitz et al. [84] also undertook the extraction/purification of salbutamol and clenbuterol from liver samples by SPE, using mainly polar interaction mechanisms.

Although previously activating this type of sorbent is not an usual procedure, McCarthy et al. [85] undertook its conditioning. Thus, after the passage of methanol, water and 0.1 *M* *di*potassium hydrogenphosphate, pH≈9.2, the plasma was introduced into the silica column so that salbutamol and terbutaline would be retained. Possible interferents were eliminated by water and the sorbent was then dried by centrifugation at 1000 *g* for 5 min. The elution of the β_2-adrenergic agonists was done with methanol.

This kind of interaction was studied in urine with three different sorbents: native silica (Si), diol (2OH) and amine (NH_2) [62]. The introduction of a previous step of LLE to use this mechanism [51,86] in the SPE of urines or other aqueous matrices is a limiting condition for the utilisation of these column types. However, Si sorbent seems to be recommendable for the use of this type of interaction, particularly for more polar β-agonists, like salbutamol and terbutaline.

4.2.4. Ionic exchange

The interactions by ionic exchange take place between a load-bearing analyte (either positive or negative) and an opposed-charge sorbent. This kind of interaction can be divided into two different classes: cationic and anionic [51].

SPE by ion-exchange mechanisms, using a weak cationic-exchange sorbent, was used as a previous step of extraction/purification before IAC. The column was successively activated with ethanol, water, 0.1 *M* phosphate buffer, pH≈6, and water, and, then, sample extract, adjusted to pH≈6, was passed. The washing step was done with water and ethanol and the elution was made by ethanol with 2% of ammonia [34].

Extraction/purification of salbutamol from feeds, in a comparative study undertaken by Leyssens and co-workers [67,68], was adequately done by strong cationic exchange (SCX). The procedure have utilised a previous step in which a diatomaceous earth was used. The SPE adsorption extract was evaporated to dryness and the residue was recovered with methanol–water–0.1 *M* acetic acid (45:45:10). This solution was then passed through the sorbent, which had previously been activated with the above-mentioned mixture of solvents. Washing was successively done with water, methanol and *tert*.-butyl methyl ether, and the elution with diethylamine–ethyl acetate (50:50).

Van Vyncht et al. [87] also utilised SPE with SCX for the extraction/purification of clenbuterol, salbutamol, cimaterol, mabuterol and terbutaline from liver and urine, following the principles described above. Whaites and Murby [88] have utilised cationic exchange to extract clenbuterol from bovine urine. The urine was acidified to pH≈2.2 and passed through the column, without any previous activation of the sorbent. The washing step was made by water and sodium hydroxide and analyte elution was obtained with ethyl acetate–methanol (90:10).

Vanoosthuyze et al. [89] undertook the extraction/purification of β_2-adrenergic agonists clenbuterol, mabuterol, mapenterol, clenproperol, clenpenterol, bromobuterol, cimaterol, salbutamol and terbutaline from bovine urine with a cation-exchange mechanism using extraction membranes instead of the traditional columns. The urine, filtered and adjusted to pH≈1, was passed through the membrane, which had previously been activated with methanol and 0.01 *M* hydrochloric acid. Washing was undertaken with the acid followed by methanol. Elution was obtained by a mixture of methanol–ammonia (97:3).

Knowing that the ideal pH values where about 99% of the molecules are charged can be defined as related to pK_a ($pH \geq pK_a + 2$ for negative molecules and $pH \leq pK_a - 2$ for positives) [51,86], it is possible to propose a theoretical explanation for this kind of interaction. Phenol group pK_a values are around 9 for the first and about 11 for the second (Fig. 1). The pK_a of the amine groups shows, as a rule, values below 1 for the primary amine bonded to the benzenic ring and values around 10 for the secondary amine. Thus, β-agonists without phenylamine group, like salbutamol and terbutaline, are completely protonated at $pH < 7$ and presented better recovery results in SCX sorbents when compared with SAX columns [62]. On the other hand, the poor performance obtained with the anionic interactions may

be related to the pH under which the samples need to be passed through the sorbent. That value, according to the described above, should be a minimum of 12, or even 13 for β-agonists with two phenol groups, in order to ensure that almost all molecules were negatively charged. The latter pH values were normally avoided because they could induce the hydrolysis of the bonded silica sorbents [46].

4.2.5. Mixed phase

Mixed-phase sorbents are an association of apolar and ionic mechanisms [90,91]. The interactions of these sorbent types are a mixture of those previously mentioned in Sections 4.2.2 and 4.2.4.

The use of mixed-phase columns allows the extraction/purification of the majority of β_2-adrenergic agonists. In order to give an idea of the potential of these sorbents, it should be mentioned that Montrade et al. [11] used them to extract/purify 13 compounds of the group of β_2-adrenergic agonists simultaneously: tulobuterol, mabuterol, clenbuterol, terbutaline, salbutamol, cimaterol, fenoterol, ractopamine, mapenterol, clenpenterol, cinbuterol, orciprenaline and hydroxymethylclenbuterol (NA 1141). If we add to these isoxsuprine [24], we are led to conclude that, in theory, all β_2-adrenergic agonists can be extracted/purified with this type of sorbent.

Exemplifying what was mentioned for the apolar sorbents, a previous solvation is also needed for this type of column. By consulting various studies, we noted that activation can be done with methanol, water and 0.1 *M* phosphate buffer, pH≈6 [11,26,30,92,93], with methanol and phosphate buffer only [24,27,45] or just with methanol [94].

Collins et al. [30], after a previous liquid–liquid extraction of a liver extract, evaporated the organic phase and placed the residue in 0.1 *M* phosphate buffer, pH≈6, which was passed through the column (C_8+SCX); clenbuterol, salbutamol, mabuterol and terbutaline being the β_2-adrenergic agonists studied. Haasnoot et al. [15] have adjusted the extract of bovine hair at pH≈6 before passing it through a mixed-phase column.

After the sample was passed, the column was washed with acetic acid and methanol [11,24–27,45,92,93] with acetic acid, methanol and acetone–chloroform (50:50) [15], with water, 0.1 *M* buffer acetate, pH≈4.0, and methanol [94], or just with methanol and water [30]. Elution was obtained with ethyl acetate–ammonia chloride (98.95:1.05) [25], with ethyl acetate–ammonia at 32% in proportions of 97:3 [11,26,92,93] or 98:2 [24,27], with a mixture of dichloromethane–isopropanol (80:20) containing 2% of ammonia [45,94] or with methanol–ammonia (98:2) [30].

It should be added that Solans et al. [95] utilised a method for the control of forbidden substances during the 1992 Barcelona Olympic Games in which over 100 compounds, including β_2-adrenergic agonists clenbuterol, orciprenaline, salbutamol and terbutaline, were checked in the athletes' urine. The extraction was undertaken with SPE in mixed phase (C_{18}+SCX) after enzymatic hydrolysis of the urines and pH adjustment to values between 8 and 9. The sorbents were activated with methanol and water, and the washing step, following the vehiculation of the sample, was done with water, pH≈4 acetate buffer and methanol. Elution was attained with isopropanol–chloroform (80:20) containing 2% of ammonia.

For the simultaneous interaction of hydrophobic and ionic exchange mechanisms a pH between 6 and 9 for sample passage seems to be essential [11,15,24–27,45,92–95]. Thus, in a first step, β-agonists are retained by apolar interactions and, subsequently, by cationic retention, after acidic washing when the protonation of the above referred substances takes place [62].

Finally, as regards SPE, a comparative study of the various types of sorbents, adsorption, apolar, polar, ionic exchange and mixed phase, was made by our group using bovine urine as matrix. The obtained results are summarised in Tables 1 and 2 and show clearly that the mixed-phase sorbent present the best results in multi-residue extraction [62].

4.3. Matrix solid-phase dispersion

MSPD is a process that presented a high potential for isolating compound residues from tissue samples. This procedure involves a solid-phase dispersion of animal tissues in a sorbent, normally C_{18}. The sample is mixed with a mortar and pestle to produce a semi-dry homogeneous material. This is added to a syringe barrel-column containing a filter paper disk

Table 1
Average (n=5) of SPE β-agonist recoveries

β-Agonist	Recovery (%)				
	Adsorption	C_8	Si	SCX	C_8+SCX
Mabuterol	58.1	38.4	13.4	28.4	66.9
Mapenterol	45.0	27.3	25.3	18.7	61.9
Clenproperol	20.3	36.5	14.3	28.1	58.8
Terbutaline	2.9	60.7	18.9	56.8	25.0
Clenbuterol	53.8	49.0	12.7	26.9	60.8
Salbutamol	4.8	58.6	17.6	47.6	17.7
Clenpenterol	40.1	21.7	22.1	23.4	58.6
Bromobuterol	42.1	12.0	13.9	14.2	44.6
NA 1141	3.7	36.1	20.8	23.7	21.6

and about 0.5 g of C_{18} in its lower end. The contents are compacted with a syringe piston to obtain a packed column with a unique chromatographic character. This process results in the rupture of the cell membranes releasing intracellular residues [96].

Thus, clenbuterol was extracted from bovine liver, after its homogenisation with C_{18} (1:4). A washing step was made with hexane and water, and clenbuterol elution was done by methanol [97,98]. The same group have utilised the same technique for the extraction of salbutamol from bovine liver. However, due to the higher polarity of salbutamol, the procedure is almost identical to that already described for clenbuterol. The exception is column washing in which the amount of water is halved. This procedure allows to avoid the losses of about 25% which occur when the washing is done with an equal volume to that used for clenbuterol. This process was also successfully tested for clenbuterol, mabuterol, terbutaline and cimaterol in a multi-residue strategy [99].

Table 2
Inter-assay RSD of SPE β-agonist recoveries (n=5)

β-Agonist	RSD (%)				
	Adsorption	C_8	Si	SCX	C_8+SCX
Mabuterol	22.3	10.2	13.2	9.8	11.9
Mapenterol	22.0	16.7	16.1	12.5	11.9
Clenproperol	21.0	4.5	12.5	7.5	16.8
Terbutaline	25.9	13.9	16.9	17.9	16.3
Clenbuterol	20.1	5.9	28.2	11.9	9.4
Salbutamol	24.7	17.8	18.0	16.0	12.4
Clenpenterol	20.4	12.2	19.9	8.4	12.0
Bromobuterol	20.6	7.8	16.2	17.1	10.9
NA 1141	26.8	13.5	26.7	10.0	11.4

Collins et al. [100] have successfully tested a variant of the classic MSPD, simultaneously using two interaction mechanisms, apolar and cationic exchange, for the extraction of clenbuterol and salbutamol from liver. The matrix was mixed in a mortar with C_{18} and phenylsulphonic acid, in the proportion of 1:4:2. The mixture was packed in a column and successively washed with 50 m*M* acetic acid, 50 m*M* phosphate buffer, pH≈6, and methanol. Elution was obtained with methanol containing 8% of ammonia, and substance recovery reached between 86% for clenbuterol and 70% for salbutamol.

Horne et al. [101] advise the utilisation of MSPD in the extraction/purification of clenbuterol from bovine liver with C_{18} but endcapped, whenever the free silanol groups are protected, since the extraction recoveries are higher, as compared with those of the unprotected groups.

Finally, stressing the importance of the various mentioned processes, le Boulaire et al. [102] have combined MSPD with LLE and SPE in order to obtain a multi-residue extraction procedure for β-agonists and steroids from livers of Gallinaceae. Obtained recoveries from the three joined procedures were about 60% for clenbuterol and for steroids (methyltestosterone, nandrolone, ethinylestradiol and zeranol), and 40% for salbutamol, which could be considered satisfactory results in residue analysis.

4.4. Immunoaffinity chromatography

Immunoaffinity chromatography is a procedure which consists in the extraction/purification of drugs or other compounds, through specific antibodies which were previously prepared, isolated and eventually linked to a gel, such as sepharose [103]. The sample is passed in an aqueous solution through the column, and the molecules of interest are retained in the antibodies, while the remaining components of the matrix are eliminated. The non-linked components which remain in the column are removed by washing with phosphate buffer, which does not interfere with the immunochemical interaction established between the antibody and the compound of interest. Elution of the analyte is usually done with an acidified alcohol. The regenerated column can be used for another extraction, up to the maximum limit of 200 times [21], although, after 20 utilisations [34],

safety may be affected, particularly if no previous purification step was introduced earlier.

Various authors have utilised this extraction/purification procedure, applied to various matrices and β_2-adrenergic agonists:

- Godfrey et al. [104] have extracted clenbuterol from bovine hair samples which had previously been enzymatically digested with papain in an alkaline medium;
- Ong et al. [105] have utilised IAC for the extraction of salbutamol from human plasma, applying it directly into the columns after just adding an anticoagulant;
- Pickett and Sauer [106] have extracted clenbuterol from bovine urine;
- Haasnoot et al. [21] have used IAC coupled in series to HPLC in order to determine clenbuterol in urine, injecting it directly in the system without any previous treatment, apart from filtration and dilution;
- Lawrence and Ménard [34] have utilised IAC in a process of extraction/purification of clenbuterol from liver and muscle, after an acidic deproteinisation and SPE with weak cationic-exchange sorbent.

The multi-residue assay of β_2-adrenergic agonists was tested by IAC in urines [35,107], in feeds [35,108], in chicken muscle [108], in liver [35] and in bovine bile [23]. The substances tested were clenbuterol, salbutamol, mabuterol, mapenterol, clenpenterol, terbutaline, tulobuterol and cimaterol.

4.5. Dialysis

Dialysis is a procedure which is based on the separation between compounds of high- and low-molecular mass, through a semipermeable membrane, by a simple molecular exclusion process. In theory, a static dialysis, in an infinite time interval with equal volumes of sample and solvent, will cause 50% of the substance of interest to cross the dialysis membrane to the side of the solvent, balancing their concentrations in either side of the membrane [109]. This efficiency can be modified by changing the ratio between the sample or solvent volumes, or the solvents themselves from either side of the membrane, temperature, agitation, time, or even using electrodialysis [110].

The principle of diphasic dialysis, thus called due to the use of two types of solvents, consists in the introduction of the organic solvent in the previously hydrated dialysis membrane, which is then dipped into the aqueous substrate containing the analyte. Agitation, temperature and time are optimised, as is the organic solvent, which must be immiscible with the aqueous phase containing the analyte to be extracted/purified. This analyte must be more soluble in an organic medium than in an aqueous medium, and one may change its physico-chemical properties, if necessary, in order to make it so. The pH, in the case of the β_2-adrenergic agonists, is a factor which substantially changes the said properties, modifying the organic/aqueous exchange coefficient of these drugs. Thus, a pH value which is identical or slightly higher than the pK_a makes them much more soluble in an organic medium and enables the use of this technique for the extraction of β_2-adrenergic agonists from various matrices.

Dominguez et al. [110] have extracted/purified clenbuterol from urine by diphasic dialysis with ethyl acetate, utilising an agitation of 120 rpm during 5 h at 37°C, pH≈12. González Gigosos and co-workers [111,112] have applied this technique for bovine liver: homogenisation being attained with a barium buffer, pH≈13.8, utilising *tert*.-butylmethyl ether as a solvent for the extraction of clenbuterol and a temperature of 37°C during 4 h, with an agitation of 150 rpm in an incubator shaker.

Clenbuterol was already subjected to extraction by diphasic dialysis from bovine hair [113] and retina [114], and it was noted that, at least in the case of the said β-agonist, the dialysis proved to be a good extraction/purification procedure, since it is relatively economical, requires a lower number of manipulations, which decreases the losses during the analytical process, and enables to obtain extracts without interferents from rather complex matrices.

4.6. Supercritical fluid extraction

SFE is considered by many authors as an important alternative to the use of organic solvents in sample extraction/purification [115]. Carbon dioxide is the most commonly used substance as a supercritical fluid, due to its excellent properties, such as low toxicity, low cost, good chemical stability, and to the

fact that it is easy to manipulate and easily attains critical parameters (temperature, 37°C; pressure, 73 atm (1 atm=101 325 Pa)) [116].

The change of temperature and pressure to values approaching the critical point enables a significant change in the solubilisation power of carbon dioxide, which, even if it was identical to that of the organic solvents, always presents in the critical point a similar viscosity to that of the gases. This means a greater and, more importantly, a faster diffusion and penetration in the solid samples, with added advantages in extractive recovery [115,117].

Jiminez-Carmona et al. [118] have presented a SFE study on the extraction of clenbuterol from spiked samples of feeds, powdered milk and lyophilised liver. Clenbuterol extraction, using carbon dioxide,– was obtained after the formation of a less polar form with camphorsulphonic acid, which was thus more soluble in CO_2.

O'Keeffe et al. [116] have extracted clenbuterol from bovine liver utilising methanol as an adjuvant. They concluded that methanol only brought some added advantage if its amount was not more than 1.5 ml, which led them to recommend only SFE with simple CO_2, with a temperature increase from 40 to 100°C. The extraction recovery is not significantly changed, less components are co-extracted from the matrix and, therefore, there are less interferents for the determination step.

Applying SFE to the extraction/purification of β_2-adrenergic agonists presents some advantages, as compared to the above-described processes. The extraction process is quick and safe for both the operator and the environment, since the use of toxic solvents is avoided [115,119]. However, the high cost of the equipment and the fact that it can only be applied to solid substances does restrict its current use. A previous lyophilisation step is, then, required in order to give an effective extraction procedure. This demand increases both the analysis times and the equipment costs [117].

5. Conclusion

The extraction/purification step in a β_2-adrenergic agonist residues determination process is, in actual fact and as previously demonstrated, of the greatest importance, regardless of the chromatographic method utilised.

Liquid–liquid extraction, due to the high amount of solvents it utilises, presents some inconvenience. The most important are, apart from the high-priced solvent, the normally lengthy operating time, the deterioration of laboratory hygiene and work safety conditions, due to the manipulation of organic solvents, and environmental contamination.

SPE is, undoubtedly, the first choice for a multiresidue β_2-adrenergic agonist extraction procedure, preferably with mixed-phase sorbents. Matrix solid-phase dispersion, which is really an SPE adaptation, can be recommended for tissue samples, mainly liver.

Immunoaffinity chromatography, although it is a good extraction/purification procedure, has a considerable restriction. The possibility of column contamination is great, which limits subsequent utilisation. Besides this fact, IAC can only be utilised with aqueous solutions. Furthermore, the relation between IAC column costs and the (im)possibility of single use has never been able to confirm the extensive use of this technique, despite its excellent specificity, particularly when applied to a single analyte.

Diphasic dialysis presents an excellent potential for a single β-agonist extraction/purification, independent of matrix type. Adjustment of dialysis conditions, mainly pH and solvent choice, are the most important steps, as was described above for clenbuterol.

Finally, the applicability of SFE in the extraction of β-agonist residues should be evaluated. Besides its cost, the referred technique has not yet given sufficiently credible steps in this field.

Acknowledgements

The author gratefully acknowledges the expert advice of Professor Maria Irene Noronha da Silveira, Vice-Rector of Coimbra University and Director of the Laboratory of Food Analysis, Nutrition and Hydrology of the Faculty of Pharmacy, whose suggestions and comments have been of invaluable help in the critical revision of the manuscript.

References

[1] Commission of the European Union, Off. J. Eur. Community L125 (1996) 10.
[2] Commission of the European Union, Off. J. Eur. Community L70 (1998) 16.
[3] G. Asato, P. Baker, R. Bass, T. Bentley, S. Chari, R. Dalrymple, D. France, P. Gingher, B. Lences, J. Pascavage, J. Pensack, C. Ricks, Agric. Biol. Chem. 48 (1984) 2883.
[4] H.J. Mersmann, Am. J. Physiol. 252 (1987) E85.
[5] J.P. Hanrahn (Ed.), Beta-agonists and Their Effects On Animal Growth and Carcass Quality, Elsevier, London, 1987.
[6] J. Inkster, F. Hovell, D. Kyle, D. Brown, G. Lobley, Br. J. Nutr. 62 (1989) 285.
[7] Commission of the European Union, Off. J. Eur. Community L125 (1996) 3.
[8] H. Holmsberg, J.R. Andersen, in: H.A. Kuiper, J.R. Andersen (Eds.), In Vitro Toxicological Studies and Real Time Analysis of Residues in Food-FLAIR Concerted Action No. 8: Final Report, Wageningen, 1995, p. 107.
[9] M. Petz, in: N. Haagsma, A. Ruiter, P.B. Czedik-Eysenberg (Eds.), Proceedings of EuroResidue Conference, Noordwijkerhout, 1990, p. 53.
[10] C. van Peteghem, In Vitro Toxicological Studies and Real Time Analysis of Residues in Food — FLAIR Concerted Action No. 8, in: H.A. Kuiper, L.A.P. Hoogenboom (Eds.), Proceedings of the Workshops, Ghent, 22–24 May 1992 and Thessaloniki, 30–31 October 1992, Wageningen, 1992, p. 41.
[11] M.P. Montrade, B. le Bizec, F. Monteau, B. Siliart, F. André, Anal. Chim. Acta 275 (1993) 253.
[12] R.J. Heitzman, Residues in Food-Producing Animals and their Products: Reference Materials and Methods, Commission of the European Communities, Report 14126 EN, Luxembourg, 1992.
[13] M.J. Lichon, J. Chromatogr. 624 (1992) 3.
[14] Th. Gude, I. Schmädicke, S. Rahn, L. Benesch-girke, A. Hashem, A. Preiss, B. Jülicher, in: N. Haagsma, A. Ruiter (Eds.), Proceedings of EuroResidue III Conference, Veldhoven, 1996, p. 157.
[15] W. Haasnoot, P. Stouten, R. Schilt, D. Hooijerink, Analyst 123 (1998) 2707.
[16] I. Dürsch, H.H.D. Meyer, H. Karg, J. Anim. Sci. 73 (1995) 2050.
[17] M.J. Sauer, S.P.L. Anderson, Analyst 119 (1994) 2553.
[18] P.D. Warris, S.C. Kestin, S.N. Brown, Anim. Prod. 48 (1989) 385.
[19] Commission of the European Union, Off. J. Eur. Community L118 (1996) 64.
[20] D. Boyd, M. O'Keeffe, M.R. Smyth, Analyst 121 (1996) 1R.
[21] W. Haasnoot, M.E. Ploum, R.I.A. Paulussen, R. Schilt, F.A. Huf, J. Chromatogr. 519 (1990) 323.
[22] E.A. Hogendoorn, P. van Zoonen, A. Polettini, G.M. Bouland, M. Montagna, Anal. Chem. 70 (1998) 1362.
[23] R. Sayers, P. Kwasowski, M. O'Connor, in: N. Haagsma, A. Ruiter (Eds.), Proceedings of EuroResidue III Conference, Veldhoven, 1996, p. 848.
[24] M.C. Dumasia, E. Houghton, J. Chromatogr. 564 (1991) 503.
[25] C.T. Elliot, C.S. Thompson, C.J.M. Arts, S.R.H. Crooks, M.J. van Baak, E.R. Verheij, G.A. Baxter, Analyst 123 (1998) 1103.
[26] M.P. Montrade, S. Riverain, B. le Bizec, F. André, In Vitro Toxicological Studies and Real Time Analysis of Residues in Food — FLAIR Concerted Action No. 8, in: H.A. Kuiper, L.A.P. Hoogenboom (Eds.), Proceedings of the Workshops Ghent, 22–24 May 1992 and Thessaloniki, 30–31 October 1992, Wageningen, 1992, p. 143.
[27] G. van Vyncht, P. Gaspar, Ph. Degand, E. Depauw, G. Maghuin-Rogister, In Vitro Toxicological Studies and Real Time Analysis of Residues in Food — FLAIR Concerted Action No. 8, in: H.A. Kuiper, J.R. Andersen (Eds.), Proceedings of the Workshops, Liège, 30 April–1 May 1993 and Nantes, 22–23 October 1992, Wageningen, 1994, p. 104.
[28] Ph. Delahaut, M. Dubois, Y. Colemonts, A. Boenke, G. Maghuin-Rogister, in: N. Haagsma, A. Ruiter, P.B. Czedik-Eisenberg (Eds.), Proceedings of Euro Residue II Conference, Veldhoven, 1993, p. 257.
[29] L. Howells, M. Sauer, R. Sayer, D. Clark, Anal. Chim. Acta 275 (1993) 275.
[30] S. Collins, M. O'Keeffe, M. Smyth, Analyst 119 (1994) 2671.
[31] S. Collins, M. O'Keeffe, M.R. Smyth, In Vitro Toxicological Studies and Real Time Analysis of Residues in Food — FLAIR Concerted Action No. 8, in: H.A. Kuiper, J.R. Andersen (Eds.), Proceedings of the Workshops, Liège, 30 April–1 May 1993 and Nantes, 22–23 October 1992, Wageningen, 1994, p. 97.
[32] R. Jackman, S.D. Bucknall, L. Thorne, A.L. Mackenzie, in: N. Haagsma, A. Ruiter, P.B. Czedik-Eisenberg (Eds.), Proceedings of EuroResidue II Conference, Veldhoven, 1993, p. 395.
[33] M.H.A. Botterblom, M.G.P. Feenstra, E.B.H.W. Erdtsieck-Ernste, J. Chromatogr. 613 (1993) 121.
[34] J.F. Lawrence, C. Ménard, J. Chromatogr. B 696 (1997) 291.
[35] R. Schilt, W. Haasnoot, M.A. Jonker, H. Hooijerink, R.J.A. Paulussen, in: N. Haagsma, A. Ruiter, P.B. Czedik-Eisenberg (Eds.), Proceedings of EuroResidue Conference, Noordwijkerhout, 1990, p. 320.
[36] L. Debrauwer, G. Bories, Anal. Chim. Acta 275 (1993) 231.
[37] J. Girault, P. Gobin, J.B. Fourtillan, Biom. Environ. Mass Spectrom. 19 (1990) 80.
[38] A. Polettini, M.C. Ricossa, A. Groppi, M. Montagna, J. Chromatogr. 564 (1991) 529.
[39] P.M. Brandts, R.A.A. Maes, J.G. Leferink, C.L. de Ligny, G.H.E. Nieuwdorp, Anal. Chim. Acta 135 (1982) 85.
[40] M.J. Hutchings, J.D. Paull, D.J. Morgan, J. Chromatogr. 277 (1983) 423.
[41] J.G. Leferink, I. Wagemaker-Engels, R.A.A. Maes, H. Lamont, R. Pauwels, M. van der Straeten, J. Chromatogr. 143 (1977) 299.
[42] L.G. Miller, D.J. Greenblatt, J. Chromatogr. 381 (1986) 205.
[43] Y.K. Tan, S.J. Soldin, J. Chromatogr. 311 (1984) 311.
[44] J. Girault, J.B. Fourtillan, J. Chromatogr. 518 (1990) 41.

[45] L. Leyssens, C. Driessen, A. Jacobs, J. Czech, J. Raus, J. Chromatogr. 564 (1991) 515.
[46] M.C. Castilho, F. Ramos, M.I.N. Silveira, Quimica 59 (1995) 38.
[47] C.N. Carducci, S.E. Lucangioli, G.C. Fernández-Otero, J. Chromatogr. A 730 (1996) 313.
[48] J. Horack, R.E. Majors, LC·GC Int. 6 (1993) 208.
[49] K. Vanoosthuyse, C. van Peteghem, in: N. Haagsma, A. Ruiter (Eds.), Proceedings of EuroResidue III Conference, Veldhoven, 1996, p. 958.
[50] R.E. Majors, LC·GC Int. 6 (1993) 346.
[51] F. Ramos, M.C. Castilho, M.I.N. Silveira, Quimica 62 (1996) 26.
[52] P.A. Biondi, L. Guidotti, M. Montana, F. Manca, G. Brambilla, C. Lucarelli, J. Chromatogr. Sci. 29 (1991) 190.
[53] J.M. Degroodt, B.W. de Bukanski, H. Beernaert, D. Courtheyn, Z. Lebensm. Unters. Forsch. 189 (1989) 128.
[54] G. Tantillo, R. Augelli, M.P. Todaro, Ind. Aliment. XXXI (1992) 1039.
[55] C. Eddins, J. Hamann, K. Johnson, J. Chromatogr. Sci. 23 (1985) 308.
[56] A. Papillon, B. Turck, G. Briantais, Rev. Aliment. Anim. 423 (1984) 63.
[57] D. Courtheyn, C. Desaever, R. Verhe, J. Chromatogr. 564 (1991) 537.
[58] G.F. Brambilla, S. Castelli, A. Riberzani, M. Montana, F. Manca, Ind. Aliment. XXIX (1990) 674.
[59] E. Sangiorgi, M. Curatolo, J. Chromatogr. B 693 (1997) 468.
[60] L.A. van Ginkel, R.W. Stephany, H.J. van Rossum, J. Assoc. Off. Anal. Chem. 75 (1992) 554.
[61] T.M. Byrem, T.F. Robinson, Y.R. Boisclair, A.W. Bell, W.S. Schwark, D.H. Beermann, J. Anim. Sci. 70 (1992) 3812.
[62] F. Ramos, M.C. Bañobre, M.C. Castilho, M.I.N. Silveira, J. Liq. Chromatogr. Rel. Technol. 22 (1999) 2307.
[63] F. André, M.-P. Montrade, B. le Bizec, in: G. Enne, H.A. Kuiper, A. Valentini (Eds.), Residues of Veterinary Drugs and Mycotoxins in Animal Products, Wageningen Pers, Wageningen, 1996, p. 139.
[64] P. Batjoens, D. Courtheyn, H.F. de Brabander, J. Vercammen, K. de Wasch, M. Logghe, J. Chromatogr. A 750 (1996) 133.
[65] D. Courtheyn, R. Moermans, R. Schilt, A. Boenke, Food Addit. Contam. 13 (1996) 493.
[66] K. de Wash, H. de Brabander, D. Courtheyn, Analyst 123 (1998) 2701.
[67] L. Leyssens, D. Courtheyn, R. Schilt, Y. Berthoz, R. Moermans, A. Boenke, Food Addit. Contam. 13 (1996) 795.
[68] L. Leyssens, J.P. Noben, D. Courtheyn, A. Boenke, Food Addit. Contam. 13 (1996) 883.
[69] R. Angeletti, M.P. Oriundi, R. Piro, R. Bagnati, Anal. Chim. Acta 275 (1993) 215.
[70] P.V. Colthup, F.A.A. Dallas, D.A. Saynor, P.F. Carey, L.F. Skidmore, L.E. Martin, K. Wilson, J. Chromatogr. 345 (1985) 111.
[71] J.M. Degroodt, B.W. de Bukanski, J. de Gtoof, H. Beernaert, Z. Lebensm. Unters. Forsch. 192 (1991) 430.
[72] J. de Groof, J.M. Degroodt, B.W. de Bukanski, H. Beernaert, Z. Lebensm. Unters. Forsch. 193 (1991) 126.
[73] T. Emm, L.J. Lesko, J. Leslie, M.B. Perkal, J. Chromatogr. 427 (1988) 188.
[74] M.E. Ploum, W. Haasnoot, R.J.A. Paulussen, G.D. Bruchem, A.R.M. Hamers, R. Schilt, F.A. Huf, J. Chromatogr. 564 (1991) 413.
[75] J. Schmid, A. Bücheler, Biomed. Environ. Mass Spectrom. 17 (1988) 415.
[76] J.G. Leferink, J. Dankers, R.A.A. Maes, J. Chromatogr. 229 (1982) 217.
[77] Y.K. Tan, S.J. Soldin, J. Chromatogr. B 422 (1987) 187.
[78] J.A. van Rhijn, W.A. Traag, H.H. Heskamp, J. Chromatogr. 619 (1993) 243.
[79] J.A. van Rhijn, J. Kanis, W.A. Traag, H.H. Heskamp, In Vitro Toxicological Studies and Real Time Analysis of Residues in Food — FLAIR Concerted Action No. 8, in: H.A. Kuiper, J.R. Andersen (Eds.), Proceedings of the Workshops, Liège, 30 April–1 May 1993 and Nantes, 22–23 October 1993, Wageningen, 1994, p. 111.
[80] Y. Gaillard, A. Balland, F. Doucet, G. Pépin, J. Chromatogr. B 703 (1997) 85.
[81] H. Hooijerink, R. Schilt, W. Haasnoot, D. Courtheyn, J. Pharm. Biomed. Anal. 9 (1991) 485.
[82] H.H.D. Meyer, L.M. Rinke, I. Dürsch, J. Chromatogr. 564 (1991) 551.
[83] G. Fedrizzi, A. Riberzani, Ind. Aliment. XXXI (1992) 903.
[84] P. Schmitz, G. Pelzer, J. Degraeve, G. Degand, P. Gaspar, G. Maghuin-Rogister, in: N. Haagsma, A. Ruiter, P.B. Czedik-Eysenberg (Eds.), Proceedings of EuroResidue Conference, Noordwijkerhout, 1990, p. 326.
[85] P. T McCarthy, S. Atawal, A.P. Sykes, J.G. Ayres, Biomed. Chromatogr. 7 (1993) 25.
[86] M.C. Castilho, F. Ramos, M.I.N. Silveira, Rev. Port. Farm. XLVII (1997) 155.
[87] G. van Vyncht, S. Preece, P. Gaspar, E. Depauw, G. Maghuin-Rogister, in: G. Sontag, W. Pfannhauser (Eds.), Proceedings of EuroFood VIII, Vienna, 1995, p. 93.
[88] L.X. Whaites, E.J. Murby, J. Chromatogr. B 728 (1999) 67.
[89] K.E.I. Vanoosthuyze, C.J.M. Arts, C.H. van Peteghem, J. Agric. Food Chem. 45 (1997) 3129.
[90] X. Chen, J. Wijsbeek, J. van Veen, J.P. Franke, R.A. de Zeeuw, J. Chromatogr. 529 (1990) 161.
[91] X. Chen, J. Wijsbeek, J.P. Franke, R.A. de Zeeuw, J. Forensic Sci. 37 (1992) 61.
[92] F. Ramos, C. Santos, A. Silva, M.I.N. Silveira, J. Chromatogr. B 716 (1998) 366.
[93] F. Ramos, M.C. Castilho, M.I.N. Silveira, J. AOAC Int. 81 (1998) 544.
[94] C. Gabiola, in: N. Haagsma, A. Ruiter, P.B. Czedik-Eysenberg (Eds.), Proceedings of EuroResidue II Conference, Veldhoven, 1993, p. 289.
[95] A. Solans, M. Carnicero, R. de la Torre, J. Segura, J. Anal. Toxicol. 19 (1995) 104.
[96] S.A. Barker, A.R. Long, C.R. Short, J. Chromatogr. 475 (1989) 353.
[97] D. Boyd, M. O'Keeffe, M.R. Smyth, In Vitro Toxicological Studies and Real Time Analysis of Residues in Food — FLAIR Concerted Action No. 8, in: H.A. Kuiper, L.A.P. Hoogenboom (Eds.), Proceedings of the Workshops, Ghent, 22–24 May 1992 and Thessaloniki, 30–31 October 1992, Wageningen, 1992, p. 133.

[98] D. Boyd, P. Shearan, J.P. Hopkins, M. O'Keefffe, M.R. Smyth, Anal. Chim. Acta 275 (1993) 221.
[99] D. Boyd, M. O'Keeffe, M.R. Smyth, Analyst 119 (1994) 1467.
[100] S. Collins, M. O'Keeffe, R. Calverley, M.R. Smyth, in: N. Haagsma, A. Ruiter (Eds.), Proceedings of EuroResidue III Conference, Veldhoven, 1996, p. 340.
[101] E. Horne, M. O'Keeffe, C. Desbrow, A. Howells, Analyst 123 (1998) 2517.
[102] S. le Boulaire, B. le Bizec, F. Monteau, M.P. Montrade, F. André, in: N. Haagsma, A. Ruiter (Eds.), Proceedings of EuroResidue III Conference, Veldhoven, 1996, p. 278.
[103] L.A. van Ginkel, J. Chromatogr. 564 (1991) 363.
[104] M.A.J. Godfrey, S.P.L. Anderson, B. Jülicher, P. Kwasowski, M.J. Sauer, in: N. Haagsma, A. Ruiter (Eds.), Proceedings of EuroResidue III Conference, Veldhoven, 1996, p. 421.
[105] H. Ong, A. Adam, S. Perreault, S. Marleau, M. Bellemare, P. du Souich, N. Beaulieu, J. Chromatogr. 497 (1989) 213.
[106] R.J.H. Pickett, M.J. Sauer, Anal. Chim. Acta 275 (1993) 269.
[107] J. Cai, J. Henion, J. Chromatogr. B 691 (1997) 357.
[108] A. Malucelli, A.F. Rizzo, H.H.D. Meyer, T. Hirvi, in: N. Haagsma, A. Ruiter (Eds.), Proceedings of EuroResidue III Conference, Veldhoven, 1996, p. 675.
[109] D.T. Plummer, in: An Introduction to Practical Biochemistry, McGraw-Hill, London, 1987.
[110] L. Dominguez, J.L. Blanco, M.A. Moreno, S.D. Diaz, J. Prieta, J.M. Cámara, J. Bayo, G. Suárez, J. AOAC Int. 75 (1992) 854.
[111] P. González Gigosos, C.A.F. Sampayo, C.F. Abuin, B.V. Belda, E.Q. Fernández, A.C. Sáez, Chromatographia 43 (1996) 271.
[112] P. González Gigosos, C.A. Fente, C. Franco, B. Vázquez, E. Quinto, A. Cepeda, J. Chromatogr. B 693 (1997) 321.
[113] C.A. Fente, B.I. Vázquez, C. Franco, A. Cepeda, P.G. Gigosos, J. Chromatogr. B 726 (1999) 133.
[114] F. Ramos, A. Matos, A. Oliveira, M.I.N. Silveira, Chromatographia 50 (1999) 118.
[115] D.E. Games, M.P.L. Games, H. Hernandez, P.J. Jackson, D. Thomas, J.C. Young, In Vitro Toxicological Studies and Real Time Analysis of Residues in Food — FLAIR Concerted Action No. 8, in: H.A. Kuiper, L.A.P. Hoogenboom (Eds.), Proceedings of the Workshops, Ghent, 22–24 May 1992 and Thessaloniki, 30–31 October 1992, Wageningen, 1992, p. 149.
[116] M.J. O'Keeffe, M. O'Keeffe, J.D. Glennon, A. Lightfield, R.J. Maxwell, Analyst 123 (1998) 2711.
[117] G. van Vyncht, P. Gaspar, L. D'Urzo, E. Depauw, G. Maghuin-Rogister, in: H.A. Kuiper, J.R. Andersen (Eds.), In Vitro Toxicological Studies and Real Time Analysis of Residues in Food — FLAIR Concerted Action No. 8: Final Report, Wageningen, 1995, p. 95.
[118] M.N. Jiminez-Carmona, M.T. Tena, M.D. Luque de Castro, J. Chromatogr. A 711 (1995) 269.
[119] J.W. King, J. AOAC Int. 81 (1998) 9.