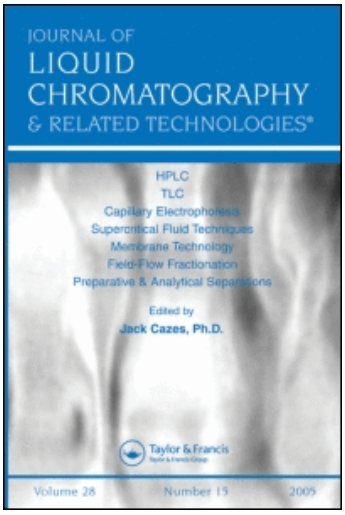

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SOLID PHASE EXTRACTION (SPE) FOR MULTI-RESIDUE ANALYSIS OF β_2-AGONISTS IN BOVINE URINE

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ABSTRACT

Solid phase extraction (SPE) for the treatment of bovine urine samples in the analysis of β_2-agonist multi-residues by gas chromatography-mass spectrometry (GC-MS) was evaluated. Mabuterol, mapenterol, clenproperol, terbutaline, clenbuterol, salbutamol, clenpenterol, bromobuterol and hidroxi-metilclenbuterol (NA1141) were tested with five different mechanisms: adsorption, reverse phase, polar, ion exchange, and mixed phase, and eleven sorbents: diatomaceous earth, octadecyl (C_{18}), octyl (C_8), native silica (Si), diol (2OH), amine (NH_2), trimethylaminopropyl (SAX), propylbenzenesulphonic (SCX), Bond Elut Certify® (C_8+SCX), Clean Screen DAU® (CSDAU) and Multimode (C_{18}+SCX+SAX).

Recoveries of the nine β_2-agonists from urine samples spiked at 20 ng mL^{-1} using metoprolol as internal standard were in ranges of 2.9% to 58.1%, of 12.0% to 60.7%, of 6.3% to 31.3%, of 3.3% to 56.8% and of 17.7% to 66.9%, respectively for adsorption, non polar, polar, ion exchange, and multiple extraction mechanisms. The coefficients of variation (C.V.) were also evaluated and discussed.

The proposed study show that mixed phase sorbents, especially those which combine hydrophobic and strong cationic exchange interactions, were recommended for a β_2-agonist multi-residue solid phase extraction procedure from bovine urine.

INTRODUCTION

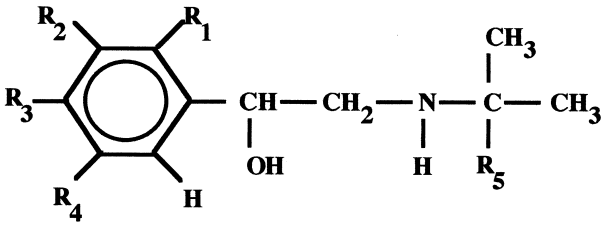
The β_2-adrenergic agonists, whose chemical structure may be observed in Figure 1, are drugs used frequently as anti-asthmatics, bronchodilators, tonicardiacs and tocolithics, both in human and veterinary medicine. However, a different use for the β_2-adrenergic agonists in the field of intensive meat production has been observed since 1984; this is displayed as a decrease of the adipose tissue in the animal's carcass and, at the same time, a considerable increase in muscle protein.[1]

The use of these drugs as growth promoters in meat-producing animals is illegal in all European Union (E.U.) and there is even a national plan for residue evaluation in every Member State which includes a compulsory checking of this class of substances.[2,3]

The solid phase extraction (SPE) is undoubtedly one of the most popular techniques utilized in the last few years in the field of sample extraction/purification, particularly when it is necessary to determine minor analyte concentrations in complex matrixes.[4,5]

The use of the various types of SPE mechanisms, adsorption,[6-11] apolar,[12-18] polar,[19] ionic exchange,[20] and multiples,[9, 21-23] in the extraction of β_2-adrenergic agonists from bovine urine may be considered in illustrating this.

The present paper is intended to contribute towards a better understanding of SPE and its mechanisms; comparing the adsorption, apolar, polar, ionic exchange, and mixed sorbent capacities, when utilized for the simultaneous extraction of mabuterol, mapenterol, clenproperol, terbutaline, clenbuterol, salbutamol, clenpenterol, bromobuterol, and hydroxymethylclenbuterol (NA1141) from bovine urine.



Mabuterol	$R_1 = H$; $R_2 = Cl$; $R_3 = NH_2$; $R_4 = CF_3$; $R_5 = CH_3$
Mapenterol	$R_1 = H$; $R_2 = Cl$; $R_3 = NH_2$; $R_4 = CF_3$; $R_5 = CH_2CH_3$
Clenproperol	$R_{1,5} = H$; $R_{2,4} = Cl$; $R_3 = NH_2$
Terbutaline	$R_{1,3} = H$; $R_{2,4} = OH$; $R_5 = CH_3$
Clenbuterol	$R_1 = H$; $R_{2,4} = Cl$; $R_3 = NH_2$; $R_5 = CH_3$
Salbutamol	$R_{1,4} = H$; $R_2 = CH_2OH$; $R_3 = OH$; $R_5 = CH_3$
Clenpenterol	$R_1 = H$; $R_{2,4} = Cl$; $R_3 = NH_2$; $R_5 = CH_2CH_3$
Bromobuterol	$R_1 = H$; $R_{2,4} = Br$; $R_3 = NH_2$; $R_5 = CH_3$
NA 1141	$R_1 = H$; $R_{2,4} = Cl$; $R_3 = NH_2$; $R_5 = CH_2OH$

Figure 1. Strucutral formula of β_2-adrenergic agonists.

EXPERIMENTAL

Reagents and Materials

The mabuterol, mapenterol, clenproperol, clenpenterol, bromobuterol, and NA1141 standards were kindly offered by Prof. François André (EVN, Nantes, France), the clenbuterol was supplied by Interchim (Montluçon, France), and the salbutamol, terbutaline, and metoprolol, internal standard, were acquired from Sigma (Madrid, Spain).

The ChemElut 1010 columns (diatomaceous earth) were purchased from Analytichem International (Harbor City, CA, USA), those of silica (Si) and silica bonded to the C_{18} (octadecyl), C_8 (octyl), 2OH (diol), NH_2 (amine), SAX

(trimethylaminopropyl), SCX (propylbenzenesulphonic) and Bond Elut Certify® (C_8 + SCX) functional groups were supplied by Varian (Harbor City, CA, USA); the CSDAU (Clean Screen DAU®, Worldwide Monitoring, EUA) and the Multimode (C_{18} + SCX + SAX) of International Sorbent Technology (UK), as well as their respective vacuum manifold, were obtained from Reagente 5 (Oporto, Portugal). All the silica and bonded silica columns were of the 300 mg / 3 mL type, with the exception of the CSDAU (500 mg / 6 mL).

The determination of the β_2-adrenergic agonists was undertaken by gas chromatography in Hewlett-Packard (HP) equipment (Soquimica, Lisbon, Portugal), comprising an HP 5890 Series II gas-liquid chromatographer, HP 6890 automatic injector, HP 5972 MSD detector, HP Vectra VL2 4/50 computer with HPG 1034G MS Chem Station software, HP Deskjet 520 printer, and SE30 fused silica capillary column (30m x 0.25 mm i.d.; 0.25 μm of film thickness) (J&W Scientific, Folsom, CA, USA).

The gases utilized were N45 nitrogen and N55 helium supplied by Arlíquido (Sogafer, Coimbra, Portugal), and all reagents were acquired from Merck (Darmstadt, Germany), except for the N,O-bis (trimethylsilil) trifluoroacetamide (BSTFA) and toluene over molecular sieve, which were purchased from Fluka (Buchs, Switzerland), and purified water, which was obtained through a Milli-Q Plus system made by Millipore (Bedford, MA, USA).

A Mettler AE200 balance (Zurich, Switzerland), a CD 7400-WPA pH meter (Cambridge, United Kingdom), a Selecta Meditronic centrifuge (Barcelona, Spain), a Stuart Scientific hemolysis tube evaporation system (Reagente 5) and a vortex-type mixer (Retsch, Haan, Germany) were also utilized.

Sample Preparation

The used lot of urine was obtained through the mixture of multiple samples of bovine urine, which had previously been centrifuged, analyzed, and considered free from β_2-adrenergic agonists.[9] Mabuterol, mapenterol, clenproperol, terbutaline, clenbuterol, salbutamol, clenpenterol, bromobuterol, and NA1141 in methanolic solution were added to the referred lot in order to obtain a β_2-agonist individual concentration of 20 ng mL^{-1} to be used as a reference matrix. The obtained lot of urine was thoroughly homogenized and divided in 5 mL aliquots which were frozen.

Before SPE procedure, urine samples were defrosted to room temperature and 100 μL of a methanolic solution of metoprolol was added in the concentration of 1 μg mL^{-1}.

Solid Phase Extraction

Adsorption Sorbent

The sample, alkalinized to pH 11.0 ± 0.5 with an ammonia solution at 32%, was added to the diatomaceous earth column, with a 15 minutes waiting period for complete fixation. The elution was undertaken with 3 x 10 mL of n-hexane[7-9].

Reversed-Phase Sorbents

The apolar columns (C_{18} and C_8) were previously activated with 2 mL of methanol and 2 mL of water. The urine was passed through the sorbent, which was subsequently washed with 2 mL of water. The β_2-adrenergic agonists were eluted, after the columns were dried, with 2 + 1 mL of methanol.[12,13]

Polar Sorbents

The sample was alkalinized to pH 11.0 ± 0.5 with an ammonia solution at 32% and submitted to a liquid/liquid extraction with 5 mL of n-hexane. After some energetic shaking and rest, for a complete separation of phases, the organic phase was added to the polar columns (Si, 2OH, NH_2), which had previously been activated with 2 mL of n-hexane. The columns were washed with 2 mL of n-hexane:ethyl acetate (50:50), and the elution of the analytes was also done with 2 + 1 mL of methanol after the columns were dried.[19]

Ion Exchange Sorbents

Strong Cationic Exchange

The activation of this type of columns was done with 2 mL of methanol and 2 mL of a 0.1 M solution of KH_2PO_4 with pH = 6.0 ± 0.1. The sample take, to which 5 mL of KH2PO_4 0.1 M, pH = 6.0 ± 0.1 were added, was passed through the sorbent, that was then washed with the 2 mL of the previously mentioned phosphate solution. After drying the columns, the elution was undertaken with 2 + 1 mL of K_2HPO_4 1 M, pH = 11.0 ± 0.1 : methanol (50:50).

Strong Anionic Exchange

The alkalinized sample at pH = 11.0 ± 0.1 with KOH 1 M and added with 5 mL of a 0.1 M buffer solution of K_2HPO_4, pH = 11.0 ± 0.1, was passed through the sorbent which had previously been activated with 2 mL of methanol and 2 mL of K_2HPO_4 0.1 M, pH = 11.0 ± 0.1. The columns were washed with 2 mL of the said phosphate buffer, and the elution was done with 2 + 1 mL of KH_2PO_4 1 M pH = 6.0 ± 0.1: methanol (50:50), after the sorbent was dried.

Table 1

Retention Times (R.T.) and Monitored Ions (m/z) of β_2-Adrenergic Agonist TMS Derivatives

Compound	No.*	R.T. (min)	Ions (m/z)
Mabuterol	1	10.9	**86**; 296
Mapenterol	2	11.8	**100**; 296
Clenproperol	3	12.8	**72**; 262
Terbutaline	4	12.9	**86**; 356
Clenbuterol	5	13.1	**86**; 262
Salbutamol	6	13.7	**86**; 369
Metoprolol (I.S.)	7	14.1	**72**; 223
Clenpenterol	8	14.3	**100; 262**
Bromobuterol	9	15.0	**86**; 350
NA 1141	10	16.2	**174**; 243

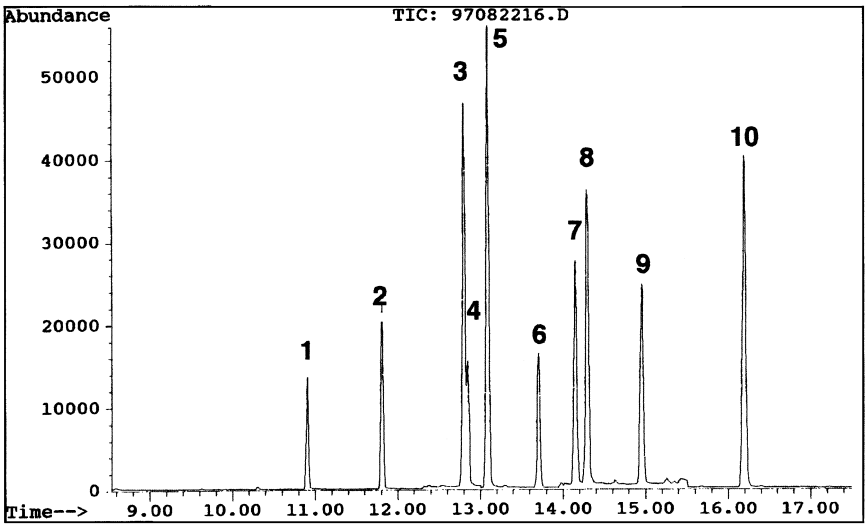
* See Figure 2.

Mixed Phase Sorbents

The conditioning of the mixed-phase columns (CSDAU, Bond Elut Certify®, and Multimode) was done with 2 mL of methanol, 2 mL of water, and 2 mL of a 0.1 M solution of KH_2PO_4 pH = 6.0 ± 0.1. To the sample, with a pH adjusted to 6.0 ± 0.1, 5 mL of KH_2PO_4 0.1 M, pH = 6.0 ± 0.1 was added and, after homogenization, it was passed through the sorbent, which was then washed with 2 mL of acetic acid 1 M and dried. It was washed a second time with 10 mL of methanol, the sorbent was again dried and the β_2-adrenergic agonists were eluted with 2 x 5 mL of ethyl acetate:ammonia at 32% (97:3).[9, 22]

Determination

The eluates resulting from the SPE were evaporated to dryness at 55°C under a nitrogen stream and the dry residues were derivatized with BSTFA for 90 minutes at 75°C. It was then left to cool off at room temperature, the derivatizer was evaporated under the previously described conditions, and the residue was recovered in 50 µL of toluene. An aliquot of toluene, 2 µL, was injected in the GC-MS system in splitless mode, 1 minute, utilizing helium as the carrier gas under a pressure of 10 psi at the head of the column, which was submitted to the following temperature gradient: 70°C (2 min.) -------› 18°C



Note: For the identification of the different peaks see table 1

Figure 2. Chromatogram of all ß$_2$-adrenergic agonist TMS derivatives studied in a spiked urine sample (20 ng ml^{-1}).

min.$^{-1}$ ----› 200°C (0 min.) ----› 5°C min.$^{-1}$ ----› 245°C (0 min.) ----› 25°C min^{-1} ----› 300°C (8 min.). The injector and detector temperatures were 260°C and 280°C respectively, and the determination was done in the electron impact (EI) mode with selective monitoring of ions (SIM).[22]

Table 1 shows the selected m/z ions, as well as the retention times of the studied compounds; Figure 2 shows a chromatogram of a sample submitted to SPE in a Multimode sorbent. The results were obtained through the ratio between the peak areas of m/z ions shown in bold on Table 1 for the β_2-adrenergic agonists and the 72 m/z ion for the metoprolol, internal standard.

RESULTS AND DISCUSSION

The optimization of the solid phase extraction following certain steps should be recommended. Thus, when conditioning the columns as necessary, the sorbent should not be left to dry until the sample sorption; this latter stage must only be done through gravity so as to ensure the maximum retention capability of the sorbent. On the other hand, drying the columns before the

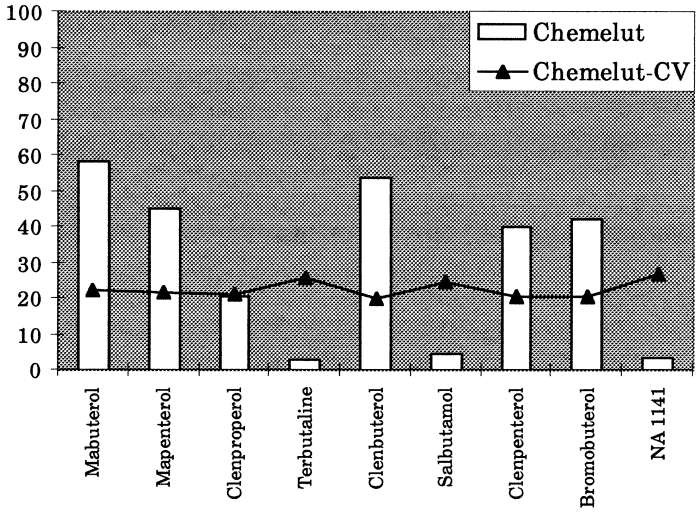


Figure 3. Adsorption sorbent results.

elution step proved to be indispensable, particularly when it is intended to elute the analytes in the smallest possible amount of solvent. However, in order to increase the precision of the obtained results, drying the columns through centrifugal force at 3000 *g* proved to be rather better than drying them for the same amount of time, 10 minutes, under strong vacuum manifold (15 inches of Hg). The elution in successive steps, two or three according to the sorbent, proved to be more efficient than if done in one step only, even when the total volume of the eluent is used.

The results can be seen in Figures 3, 4, 5, 6 and 7: The averages of the recovery percentages obtained for each β_2-adrenergic agonist being shown as bars and their respective variation coefficients (C.V.) as lines (n=5).

The diatomaceous earth sorbent, Figure 3, shows no commendable characteristics for use in a multi-residue extraction procedure of β_2-adrenergic agonists. The presence of hydroxil groups besides the β-hydroxilamine, both in the aromatic ring and in the lateral chain (Figure 1), leads to a strong adsorption in this sorbent, which is hard to eliminate with the solvent used.[24] The terbutylic group related to the secondary amine seems to play an important role in this type of extraction mechanism. The strong or light decrease in performance, when the group in question is an isopropyl or an isopentyl, respectively, seems to corroborate it.

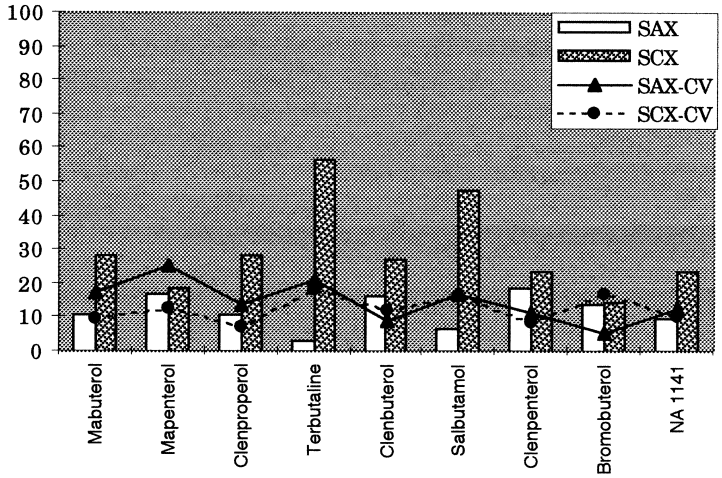


Figure 4. Ion exchange sorbent results.

Besides, Courtheyn et al.[11] had already utilized a toluene:dichloromethane mixture (3:1, v/v) in an attempt to improve the performance of this extraction mechanism for a β_2-adrenergic agonist with an N-isopropyl group, in that case, cimaterol. When the two types of ionic exchange sorbent were tested and compared, Figure 4, it is easily seen that the cationic exchange mechanism shows better results than that of anionic exchange. Considering that the pKa of the phenol groups is around 9 for salbutamol and the first terbutaline group, and about 11 for the second phenol group of the latter, and that the pKa of the amine groups of the β_2-adrenergic agonists shows, as a rule, values below 1 for the primary amine connected to the phenyl group and values around 10 for the secondary amine, an explanation could be proposed. Thus, knowing that the ideal pH values where about 99% of the molecules are charged can be defined as related to pKa, with $pH \geq pKa + 2$, the molecules are negatives or with $pH \leq pKa - 2$ when they are positives,[25,26] it is possible to define the following: salbutamol and terbutaline, the only compounds studied which possess no phenylamine group, are completely protonated when they are passed through the sorbent, and, therefore, present the best results with the SCX columns, unlike what was described by Vanoosthuyze et al.,[20] who did instead acidify to pH 1 the samples before they were passed through the columns.

On the other hand, the poor performance obtained with the SAX columns may be related to the $pH = 11.0 \pm 0.1$ under which the urines were passed through the sorbent, since such value, according to the above described, should be a minimum of 12, or even 13 for terbutaline, in order to ensure that almost all

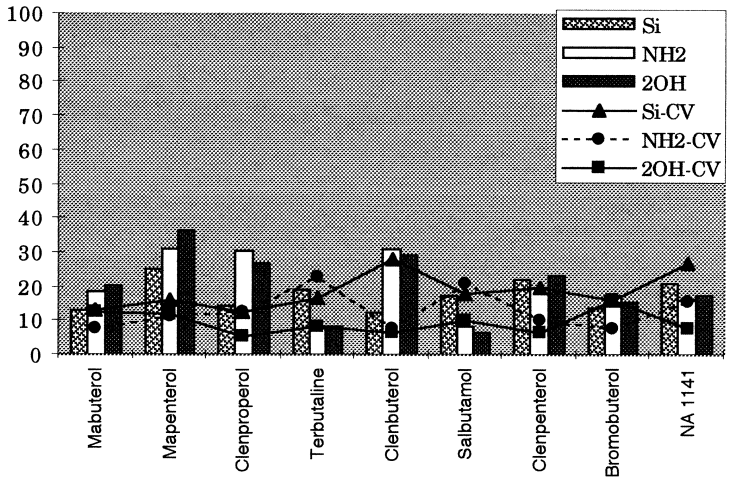


Figure 5. Polar sorbent results.

molecules of the β_2-adrenergic agonists were negatively charged. An added disadvantage of these kinds of columns is the compulsory use of eluents with a high ionic strength, which normally makes the determining stage more complex, particularly when it is necessary to evaporate the eluent, as is the case of GC-MS.

The tested polar sorbents, Figure 5, basically show the same performance, no extraction output reaching 40%. The introduction of a previous step of liquid/liquid extraction to use this mechanism[25,26] is a limiting condition for the utilization of these types of columns in the SPE of urines or other aqueous matrixes. However, the native silica sorbents, although they do not exceed the said performance, seem to be commendable among those which function mainly through this kind of mechanism, for more polar SPE of β_2-adrenergic agonists, particularly salbutamol and terbutaline, as already done by Schmitz et al.[19] and McCarthy et al.,[27] the latter utilizing plasma as matrix.

Figure 6 shows that the C_8 columns give better results than those of C_{18}, particularly in the cases of terbutaline and salbutamol, thus justifying the preference of Tan and Soldin[28] for that sorbent. However, the C_{18} columns show better results as regards the precision of the tests undertaken, as can be seen by reading the C.V., and also a slight improvement in the average extraction recoveries in the cases of mapenterol, clenpenterol, bromobuterol, and NA1141, which leads to suggest that behaviour, except for bromobuterol, may be due to a larger number of carbons in the group connected to the secondary amine, which

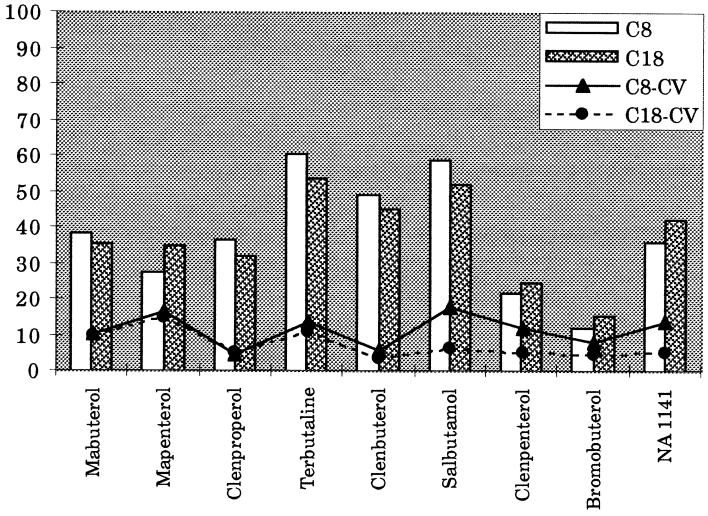


Figure 6. Apolar sorbent results.

will make retention easier in the case of a more lipophilic sorbent. The versatility of these types of columns, particularly C_{18}, has been sufficiently described, especially in applications with clenbuterol[16,17,29] and salbutamol,[13,18,30,31] both isolated and together.[14,15] However, no multi-residue application has been described with these type of columns, even though such a procedure can be undertaken as shown in Figure 6.

Figure 7 shows that the mixed-phase columns are those which present a more reasonable result as regards the extraction of the β_2-adrenergic agonists being studied, although the less polar ones do predominate, that is to say, those which are analogues to clenbuterol. The latter compounds show their best results with the Multimode columns (C_{18} + SCX + SAX) due to the greater lipophile capacity of this sorbent, since the collection of compounds of urine at pH 6.0 ± 0.1 implies that the respective analytes be retained, in a first step, by hydrophobic mechanisms, favouring C_{18} against C_8. The later acidification of these sorbents with 1M acetic acid, when promoting the protonation of the above mentioned analytes, favours their cationic retention, SCX, with no place for anionic interactions, SAX. On the other hand, the utilization of ethyl acetate as the main eluent allows a greater extraction capacity due to its lesser polarity, particularly when compared with the methanol utilized in apolar mechanisms. Thus, the presence of the anionic exchange mechanism, SAX, in the Multimode contributes little or nothing to the retention of this sorbent in the described procedure. The recovery improvement for clenbuterol analogues is solely due to the retention capabilities of C_{18} and elution of ethyl acetate.

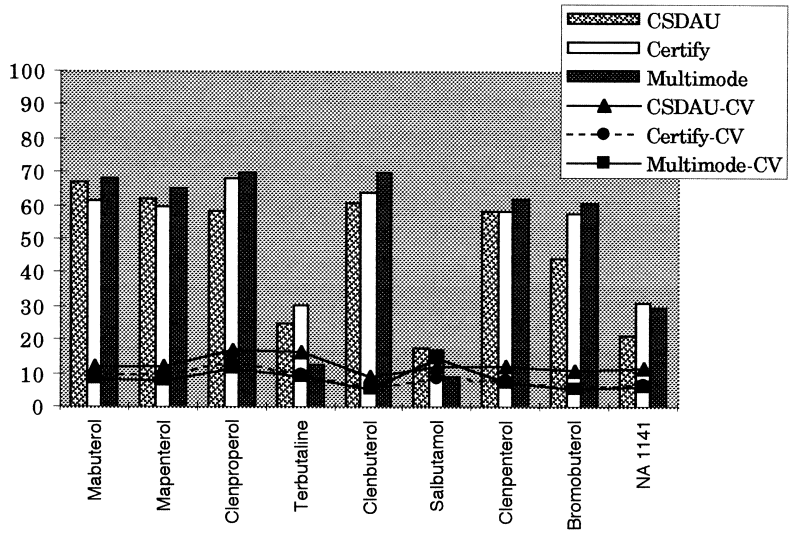


Figure 7. Mixed phase sorbent results.

Summing up, the mixed-phase sorbents (apolar and cationic exchange) are those which show better results in the simultaneous solid phase extraction of various β_2-adrenergic agonists. However, the results obtained with the Multimode column seem to suggest, in the case of clenbuterol analogues, that the lipophilia increase in the sorbent should be taken into consideration, particularly if was considered that the replacement of the anionic exchange will not adversely affect the retention capabilities of the sorbent.

ACKNOWLEDGMENTS

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