TITLE : Multi residue analysis IAC/GC-MS;
ß-agonists

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1. SCOPE

ß-agonists are illegally used for growth promoting in e.g. calves, cattle and pigs. This method of analysis describes the detection and confirmation of the presence of individual ß-agonists in samples of animal origin.

2. FIELD OF APPLICATION

The method described is suitable to perform routine screening and confirmatory analyses in urine and liver of calves, cattle and pigs for the presence of Salbutamol and Clenbuterol. The method however was designed to allow for the detection and identification of t-butyl- or isopropyl-ß-agonists by combining antibodies raised

Figure 1: Molecular structure of Clenbuterol

against Clenbuterol (Figure 1) and Cimaterol (Figure 2). Therefore the method is in principal also suitable for the detection and confirmation e.g. Mabuterol, Terbutaline and other components mentioned in Table 1. For these compounds the suitability was demonstrated but not validated with respect to limit of detection, limit of determination (identification) and within- and between-assay variability.

The limit of detection, defined as three times the standard deviation of the blank determination, measured in µg/kg or litre of sample is well below 1. The limit of identification according to the EC criteria for reference methods is within the range 0,5 - 1,5 µg/l or kg (ppb) but depends on the ionization technique and equipment used.
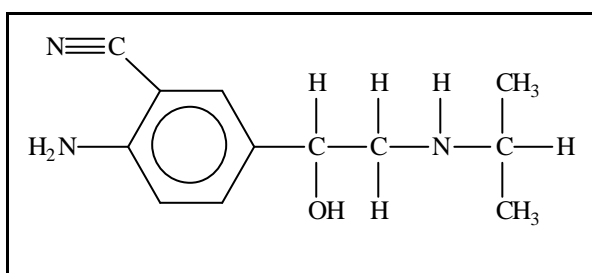
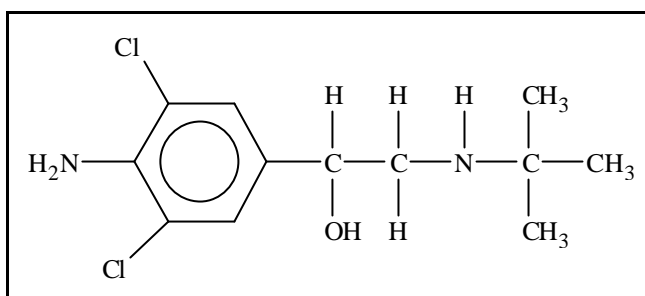## 3. REFERENCES

Figure 2: Molecular structure of Cimaterol

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4. DEFINITIONS

ß-agonists are compounds that stimulate the ß-adrenergic receptor. ß-agonists content is taken to mean the amount of ß-agonists in the substance in question determined according to the described method and expressed as µg ß-agonists per kg or litre of test sample. All other definitions are according to Haagsma et al.

5. PRINCIPLE

The method comprises 4 stages :
1. Preparation of a primary extract
2. Extract purification and concentration using immunoaffinity chromatography
3. Derivatization, detection and identification
4. Calculation and evaluation of results

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6. MATERIALS

Reference to a company and/or product is for purposes of identification and information only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and Environment (RIVM) to the exclusion of others which might also be suitable.

6.1. Chemicals and reagents
All chemicals, including standards and solutions, are of defined quality. Pure chemicals are of "Pro Analyse" quality or better, standards are checked for identity (GC-MS and/or FTIR) and purity (HPLC and/or TLC) and the shelf-life and storage conditions of all prepared reagents and solutions are defined.

6.1.1. *Reference compounds.*
In Table 1 the most important ß-agonists used are summarized.

TABLE 1: BASIC DATA SELECTED ß-AGONISTS.

Compound	CAS number	mol formula	mol. weight
Clenbuterol	37148-27-9	$C_{12}H_{18}Cl_2N_2O$	277.18
Salbutamol	18559-94-9	$C_{13}H_{21}NO_3$	239.31
Terbutaline	23031-25-6	$C_{12}H_{19}NO_3$	225.29
Cimaterol	54239-37-1	$C_{12}H_{17}N_3O$	219.29
Mabuterol	56341-08-3	$C_{13}H_{18}ClF_3N_2O$	310.75
Mapenterol	95656-68-1	$C_{14}H_{20}ClF_3N_2O$	324.75
Cimbuterol	54239-39-3	$C_{13}H_{19}N_3O$	233.29
Bromobuterol	41937-02-4	$C_{12}H_{18}Br_2N_2O$	366.09
Clenproperol	-	$C_{11}H_{16}Cl_2N_2O$	263.09

It is preferred to use isotope labelled internal standards, e.g. Clenbuterol-d6, Salbutamol-d6, Mabuterol-d9, Cimbuterol-d9, Mapenterol-d11 and Cimaterol-d7.
Note: Isotopically labelled internal standards are in general not commercially available. However, several institutes have synthesized suitable compounds. These primary sources are, amongst others, RIVM - Bilthoven - NL and RIKILT- Wageningen - NL.

6.1.2. *IAC-materials*
The immunogens used were prepared as described earlier by coupling diazonium-clenbuterol and diazonium-Cimaterol respectively to bovine serum albumin (BSA, Sigma) (Yamamoto et al., 1982). The respective antisera used to prepare the IAC-matrices were obtained by immunizing rabbits four times over a period of 5 months, 2 mg of immunogen each time. The isolation of the antiserum immuno-globulin G (IgG) fraction and coupling of this fraction to the activated matrix were performed as described previously (Ginkel et al., 1989). The final columns have a capacity > 100 ng for the compounds mentioned (6.1.1).

6.1.3. Ethanol (Merck, art. no. 983).
6.1.4. Methanol (Merck, art. no. 6007).
6.1.5. Toluene (Merck, art. no. 8327).

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6.1.6.   Thiomersal (BDH, art. no. 304162).

6.1.7.   Sodium hydroxide (Merck, art. no. 6498).

6.1.7.1. Sodium hydroxide solution 1 mol/l.
Dissolve 40 g sodium hydroxide (6.1.7.) in 1000 ml water.

6.1.8.   Hydrochloric acid (Merck, art. no. 317).

6.1.8.1. Hydrochloric acid solution 1 mol/l.
Mix 96 g hydrochloric acid (6.1.8.) with water and add water to a volume of 1000 ml.

6.1.8.2. Hydrochloric acid solution 0.1 mol/l.
Mix 9.6 g hydrochloric acid (6.1.8.) with water and add water to a volume of 1000 ml.

6.1.8.3. Hydrochloric acid solution 0.01 mol/l.
Mix 100 ml hydrochloric acid solution of 0.1 mol/l (6.1.8.2.) with 900 ml of water.

6.1.9.   Ethyl acetate (Merck, art. no. 9623).

6.1.9.1. Ethyl acetate stored above molecular sieve pellets 3Å (BDH, art. no. 54001).

6.1.10. Acetic acid (Merck, art. no. 63).

6.1.11. Sodium acetate (Merck, art. no. 6268).

6.1.12. Sodium chloride (Analar BDH art. no. 10241).

6.1.13. Extrelut^R (Merck, art. no. 11737; refills art. no. 11738)

6.1.14. Acetate buffer 0.1 mol/l, pH 4.0.
Mix 4.92 g acetic acid (6.1.10) with 800 ml water and add 2.45 g sodium acetate (6.1.11) and dissolve. Adjust the pH at $4.0 \pm 0,1$ and add water to a volume of 1000 ml.

6.1.15. IAC-eluting buffer.
Mix 400 ml of ethanol (6.1.3.) with 75 ml water and 25 ml acetate buffer (6.1.14).

6.1.16. Disodium hydrogen phosphate (Merck, art. no. 6586).

6.1.17. Potassium dihydrogen phosphate (Merck, art. no. 4873).

6.1.18. Phosphate buffer, 0.02 mol/l, pH= 7.4 ± 0.1.
Dissolve in 800 ml water 2,278 g disodium hydrogen phosphate (6.1.16.), 0.416 g potassium dihydrogen phosphate (6.1.17.), 9.0 g sodium chloride (6.1.12.) and 0.05 g thiomersal (6.1.6.). Adjust the pH at 7.4 ± 0.1 and add water to a volume of 1000 ml.

6.1.19. Stock solutions of standards containing 1 g/l and 0.1 g/l in ethanol or methanol, stored at -20°C in the dark.

6.1.20. Working solutions of standards containing 0.01 g/l, 0.001 g/l and 0.0001 g/l in ethanol, stored at +4°C for a maximum period of 6 months.
Smallest volume pipetted out of the stock solution is 0.1 ml.

6.1.21. Derivatization reagent: N,O-bis-(trimethylsilyl) trifluoracetamide (BSTFA) with 1% TMCS (Pierce, art. no. 38832).

6.1.22. Beta-glucuronidase/sulfatase (suc'Helix Pomatia, containing 100.000 units ß-glucuroni-dase and 1.000.000 units sulfatase per ml, Industrie Biologique, France, code IBR 213473)

6.1.23. Acetate buffer 2 mol/l, pH 5.2. Mix 25.2 g acetic acid (6.1.10.) with 800 ml water and add 129.5 g sodium acetate (6.1.11.) and dissolve. Adjust the pH at a value of 5.2 ± 0.1 and add water to a volume of 1000 ml.

6.1.24. Methyl Boronic Acid (MBA) (Aldrich, art. no. 16.533-6).

6.1.25. MBA-derivatization solution.
Dissolve 2 g MBA in 1000 ml of ethyl acetate (6.1.9.1.).

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6.2.   Apparatus

6.2.1. Glass roundbottomed flasks, 150 ml (Quickfit, FF 150/4S).

6.2.2. Centrifuge (Heraeus, Varifuge 3.0R).

6.2.3. Automatic pipettes (Gilson P20, P200, P1000 and P5000).

6.2.4. Centrifuge tubes, polypropylene (29 x 103 mm) (Dupont).

6.2.5. Centrifuge (RC-5B, Sorvall) with SS34 rotor.

6.2.6. Centrifuge tubes, glass (100 mm x 15 mm) (Renes).

6.2.7. Electric water bath with thermostat adjustable to $50 \pm 5^{o}C$ (GFL) with nitrogen facility.

6.2.8. Vortex (Vortex-genie).

6.2.9. Ultrasonic water bath (Bransonic 32).

6.2.10. Glass derivatization vials (Chromacol 2SV [A]) with screw caps (Chromacol 8SC) and septa (Chromacol 8-ST15).

6.2.11. Incubator $60^{o}C$ (Salvis).

6.2.12. Heating module for derivatization vials (Pierce 18790) with nitrogen facility.

6.2.13. Glass injection vials (Chrompack, 10201) with glass inserts (Chrompack, 10381).

6.2.14. Aluminium caps (Chrompack, 10210).

6.2.15. GC-MS equipment, electron impact and chemical ionization.
(Hewlett Packard, type 6890).

6.2.15.1. Automatic injector (Hewlett Packard, type 7673A).

6.2.15.2. Computer (Hewlett Packard, Vectra 486-66u)

6.2.15.3. Printer (Hewlett Packard, Laserjet type 4).

6.2.15.4. Methane reagent gas (Hoek-Loos 4.5)

6.2.15.5. GC-column, fused silica permabond SE-52, 25 m x 0,25 mm ID, film thickness 0,25 mm (Machery-Nagel, art. no. 723054).

6.2.16. Rotavapor with water bath at 40° C (Büchi, type Rotavapor R).

6.2.17. Glass centrifuge tubes (50 ml), (Corex).

6.2.18. pH-meter, (Applikon).

6.2.19. Rotating apparatus (Heidolph, RAEX2).

6.3.   Samples
Samples of urine and liver are stored in the dark at $-20^{o}C$ or at $+4^{o}C$ if analysis is foreseen to be within 2 days. Samples of animal feed are stored at $+4^{o}C$.

7.   ANALYTICAL PROCEDURE

Warning : Results of residue analysis for anabolizing agents of which the use in meat production is prohibited can have serious implications. To prevent false negative results internal standards and control samples are analyzed. However, to prevent false positive results much effort must be put into avoiding contamination during the analytical procedure of unknown samples with standard compounds or other unknown samples. Special care must be taken during pipetting, with evaporating devices and injection systems. The described procedures for regenerating IAC-columns must be followed strictly. As a rule therefore positive results are always confirmed by repeated analysis.

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For the extraction of liver several procedures, based on e.g. Subtilisin digestion (Sigma, P-5380) or ultrasonic extraction, are possible. Studies at RIVM revealed no significant difference when samples obtained from treated animals were analyzed with either of these two extraction procedures. However, ultrasonic extraction is easier applied and results in less interfering compounds. As a rule therefore ultrasonic extraction in dilute acid is the preferred technique (Courtheyn et al.) .

7.1.   Extraction procedures.

7.1.1. *Liver*
Accurately weigh 3.0 g of liver into a centrifuge tube (6.2.4) and add the internal standard(s) and 15 ml 0.01 mol/l HCl. Place the tube in an ultrasonic water bath and extract during 30 minutes. In the meanwhile shake the tube a few times by placing on a vortex (6.2.8.). Subsequently shake for 30 minutes (6.2.19.). Centrifuge the tube for 30 minutes (18,000 rpm) (6.2.5.) and decantate the supernatant into a clean tube (6.2.17.).

7.1.2. *Urine.*
Accurately pipette 10 ml of urine into a glass centrifuge tube (6.2.17) and add the internal standard(s). Adjust the pH at a value of 5.2 ± 0.2 with 1 mol/l NaOH or 1 mol/l HCl.

7.2.   Enzymatic Hydrolysis
Samples of urine and primary extracts of liver are enzymatically hydrolyzed. For analysis of Clenbuterol the hydrolysis step can be omitted. For the other ß-agonists, especially for Salbutamol, data available about hydrolysis are insufficient for definitive conclusions. For hydrolysis adjust the pH to a value of 5.2 ± 0.2 with 1 mol/l NaOH. To the aqueous samples 2 ml acetate buffer (6.1.23.) and 0.1 ml Helix Pomatia (6.1.22.) are added. All samples are incubated overnight at 37°C. After incubation the samples are cooled to room temperature and if necessary centrifuged (10 minutes 3000 rpm, 6.2.2.).

7.3.   Extrelut^{R} extraction
Prior to extraction the pH is adjusted to 9.8 ± 0.2. The volume of the aqueous extract is adjusted to 20 ml with water and is applied to an Extrelut^{R} extraction cartridge (6.1.13.). After equilibration during 15 minutes the cartridge is extracted with 60 ml ethylacetate (6.1.9.), the extract is evaporated on a rotavapor (6.2.16) at a temperature of 40°C.

7.4.   Sample clean-up; immunoaffinity chromatography
The IAC-columns are washed with at least 10 ml of water. The dry residue is dissolved in 0.2 ml ethanol. Subsequently 50 ml water is added and the residue is further dissol-ved by placing the tube in an ultrasonic water bath for at least 1 minute. The aqueous extract is applied to the IAC column and flushed through with a flow of 2 ml/minute. Subsequently the column is washed with 5 ml of water after which the ß-agonists are eluted with 5 ml IAC-eluting buffer (6.1.15.). The eluate is evaporated to dryness in a water bath at 50°C under a cold stream of nitrogen (6.2.7.).
The IAC columns are regenerated by washing it with 10 ml IAC eluting buffer (6.1.15.), 25 ml of water and 25 ml PBS (6.1.18.) and stored at 4°C under a few ml of PBS.

Note : The preparation and characterization of IAC-materials is described in SOP ARO/172: Preparing of IAC-columns. It is not included in this SOP since it concerns a series of specialized laboratory procedures not directly related to the analytical procedure. In short, the IgG-fraction is isolated from the (rabbit)serum by affinity-chromatography on protein A Sepharose[R]. The protein content is measured with e.g. the method of Lowry. The IgG-fraction can be coupled to one of a number of activated matrices. To estimate the percentage IgG coupled the protein content after coupling can be measured again. The capacity of the gel is evaluated and, if adequate, individual columns are filled.

7.5. Derivatization.

7.5.1. *TMS-derivatization.*
The dry residue is dissolved in 0.05 ml of derivatization reagent (6.1.21.). The mixture is incubated during 1 hour at 60° (6.2.11.). The reagent is evaporated (6.2.12.) and the dry derivatized residue is dissolved in 0.05 ml of toluene (6.1.5.). The toluene solution is transferred to an injection vial (6.2.13.) and subjected to GC-MSD analysis.

GC-MSD settings (example) :

injection volume	2 μl.
injection temperature	260°C
injection mode	splitless
starting temperature	70°C
gradient	25°C/min
first final temperature	200°C/min (6 minutes)
gradient	25°C/min
second final temperature	300°C/min (2 minutes)
ions monitored (screening)	m/z = 86 (t-butyl-ß-agonists)
	m/z = 72 (isopropyl-ß-agonists)
	m/z = 79 - 92 -95 and 111 (internal standards)
	m/z = 100 (pentyl-ß-agonists)

The number and concentrations of GC-MS standards (standards derivatized without additional analytical manipulations) depends on the application. For quantitative analysis within the low ppb range a minimum of five standards is prepared over the range of 1 - 50 ng per derivatization vial for all analytes included in the experiment. Each vial includes the internal standards in an amount equal to the amount added to the samples (7.1). For confirmation of the identity similar standards are prepared without the inclusion of the internal standard. After the analysis of the standards sufficient derivatization blanks are analyzed in order to prevent contamination. Known positive samples are always preceded and followed by a derivatization blank.

7.5.2. *MBA-derivatization.*

For purposes of confirmation of the identity (4-ions detection) additional studies may be necessary. An alternative derivatization is the preparation of MBA-derivatives. Especially when chemical ionization technique is not available.

The dry residue is dissolved in 0.025 ml of ethyl acetate (6.1.9.1) and 0.025 ml of MBA-derivatization solution (6.1.25.) is added. The mixture is incubated for 20 minutes at room temperature and transferred to an injection vial (6.2.13.) and subjected to GC-MSD analysis. The GC-MS settings are the same as in the case of TMS-derivatives.

7.6. <u>Calculation</u>

For quantification the most intense ions are used. For TMS-derivatives after EI-fragmentation fragment-ions [A] (see 8. confirmation) are used. The procedure used for calculating results (quantification) depends on the internal or external standards used. For some ß-agonists isotopically labelled standards are available and linear calibration curves can be fitted with the ID-ratio as independent and the concentration of standard (e.g. ng/injection vial) as dependent variable. This procedure yields linear calibration curves which give an intercept deviating not significantly from zero. Curvefitting is allowed with standard linear least squares procedures. An example of a typical calibration curve is shown in Figure 3.

ß-agonists for which no internal standard is available can be quantified using Clenbuterol-d6 as internal standard. However, these results can only be regarded as indicative.

For purposes of confirmation by the simultaneous detection of at least four ions response ratios have to be calculated for both standards and unknown compounds.

Figure 3: Typical ID-calibration curve for clenbuterol

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8.   CONFIRMATION

8.1.   EI-ionisation of TMS-derivatives.

For purposes of confirmation additional (fragment)-ions have to be monitored. TMS-derivatives of ß-agonists fragment according to the scheme shown in Figure 4. The corresponding masses are summarized in Table 2.

Table 2: Masses of diagnostic ions of TMS-derivatives after EI-fragmentation

ß-agonist	N	M_o	M_d	[A]	[B]	[C]	[D]	[E]	[F]
Terbutaline	3	225	441	86	356	336	426	370	280
Salbutamol	3	239	455	86	369	350	440	384	294
Clenbuterol	1	276	348	86	262	243	333	277	187
Cimaterol	1	219	291	72	219	186	276	234	
Cimaterol	2	219	363	72	291	258	348		
Mabuterol	1	310	382	86	296	277	367	311	221
Mapenterol	1	324	396	100	296	291	381	311	221
Cimbuterol	1	233	305	86	219	200	290	234	
Bromobuterol	1	364	436	86	352	333	425	367	
Clenproperol	1	262	334	72	262	229	319	277	

M_0 = molecular weight (based on lowest natural isotopes)
N   = number of TMS-groups
M_d = molecular weight of derivative (based on lowest natural isotopes)

Figure 4 Fragmentation pattern of ß-agonists

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8.2. EI-ionisation of MBA-derivatives.

Identification of MBA-derivatives based on the fragment-ions listed in Table 3:

Table 3: Masses of diagnostic ions of MBA-derivatives after EI-fragmentation

ß-agonist	M_o	N	M_d	diagnostic(fragment)ions
Clenbuterol	276	1	300	243 285 300
Clenbuterol-d6	282	1	306	246 288 306
Salbutamol	239	2	287	230 272 287
Salbutamol-d6	245	2	293	233 275 293
Mabuterol	310	1	334	277 319 334
Mapenterol	324	1	348	277 291 319 348
Cimaterol	219	1	243	186 228 243
Cimbuterol	233	1	257	200 242 257
Bromobuterol	364	1	388	333 375 390
Clenproperol	262	1	286	229 231 271 286

M_0 = molecular weight (based on lowest natural isotopes)

N = number of MBA-groups

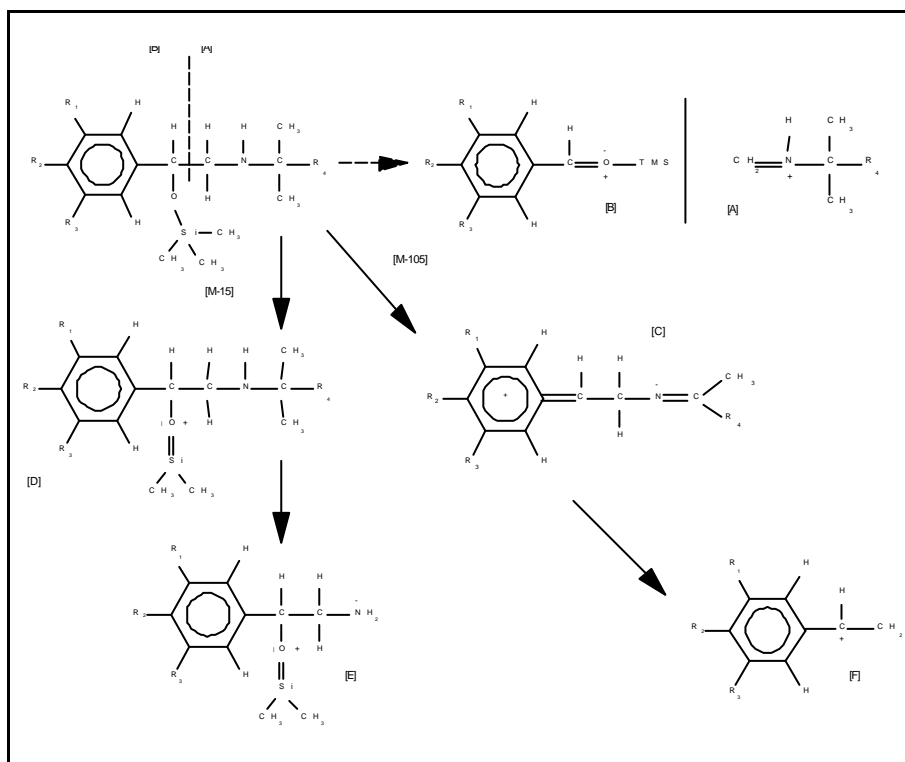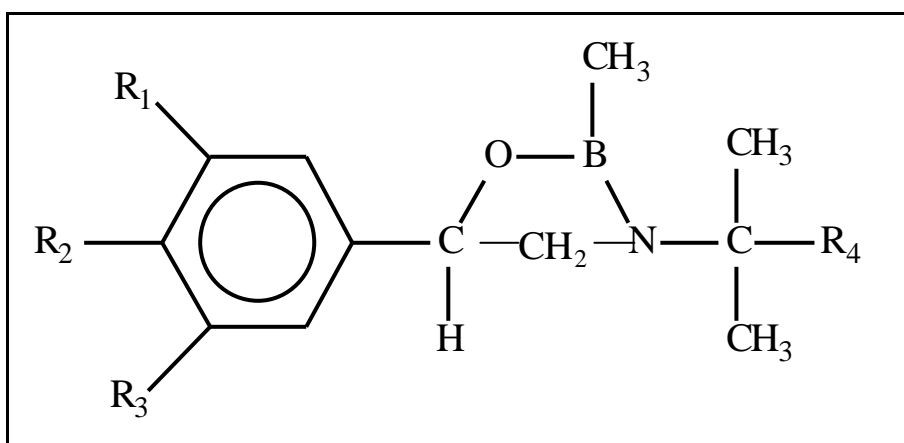M_d = molecular weight of derivative (based on lowest natural isotopes)

Figure 5 : Chemical structure of ß-agonists-MBA-derivatives.

Loss of a methyl group produces an ion of [M-15], together with the loss of $OBCH_3$ a loss of [M-57] is observed. With Mapenterol the loss of a ethyl group [M-29] and $OBCH_3$ produces [M-71]. With Terbutaline no derivatives were formed with the procedure used.

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8.3. Positive chemical ionization.

If it is not possible to fulfil the criteria mentioned, the extract is reinjected after positive chemical ionization. Table 4 summarizes the diagnostic ions observed after PCI-ionization of TMS-derivatives.

Table 4: Masses of diagnostic ions of TMS-derivatives after PCI-fragmentation

ß-agonist	M_o	N	M_d	diagnostic-(fragment)-ions
Clenbuterol	276	1	348	86 259 333 349
Terbutaline	225	3	441	86 356 426 442
Salbutamol	239	3	455	86 366 440 456
Mabuterol	310	1	382	86 293 363/367 383
Mapenterol	324	1	396	100 307 377/381 397
Cimaterol	219	1	291	72 202 276 292
Cimbuterol	233	1	305	86 216 290 306
Bromobuterol	364	1	436	86 423 437/439 349
Clenproperol	262	1	334	72 245 319 335

M_0 = molecular weight (based on lowest natural isotopes)
N = number of TMS-groups
M_d = molecular weight of derivative (based on lowest natural isotopes)

In all cases $[M+C_2H_5]^+$ and sometimes $[M+C_3H_5]^+$ was formed.

9. VALIDATION.

Initial validation studies.

The procedure has been tested within a large number of validation studies. Initial validation studies were performed within the research plans 4.1990.43 and 4.1990.44.

Repeatability and within laboratory reproducibility were determined for Clenbuterol and Salbutamol by analyzing samples of urine and liver obtained from treated animals (urine from veal calves and liver from a pig). The samples were analyzed on three different occasions, each time in duplicate.

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Salbutamol in liver.

individual values (ppb)	within assay variability	between assay variability
3.6 3.3	$s^2 = 0.016$	$s^2 = 0.065$
3.4 3.3	$s = 0.13$	$s = 0.25$
3.7 3.7	%RSD = 3.7	%RSD = 7.1

Clenbuterol in liver.

individual values (ppb)	within assay variability	between assay variability
13.8 11.2	$s^2 = 1.31$	$s^2 = 2.63$
12.9 12.6	$s = 1.14$	$s = 1.62$
15.1 14.1	%RSD = 8.5	%RSD = 12.1

Salbutamol in urine.

individual values (ppb)	within assay variability	between assay variability
2.0 2.0	$s^2 = 0.007$	$s^2 = 0.020$
1.8 2.0	$s = 0.08$	$s = 0.14$
2.1 2.1	%RSD = 4.0	%RSD = 7.0

Clenbuterol in urine.

individual values (ppb)	within assay variability	between assay variability
4.4 4.3	$s^2 = 0.035$	$s^2 = 0.122$
4.2 4.6	$s = 0.19$	$s = 0.35$
4.7 4.9	%RSD = 4.2	%RSD = 7.8

More recent studies are described in RIVM report 389201001, describing a number of validation studies on urine and liver (RIVM=lab 4). From these studies it can be concluded that the within laboratory reproducibility for the procedure is <10% for all analytes and matrices tested.

10. METABOLISM AND CONJUGATION.

This procedure does include a special step for deconjugation of ß-agonists which might be present in the form of glucuronide- or sulphate-esters. For Clenbuterol all data available led to the conclusion that no conjugates are formed. For Salbutamol the presence of sulphate-esters is described. Studies in our laboratory show that in cattle Salbutamol is for over 95% conjugated. The use of prolonged incubations and relative large amounts of enzymes is needed. Based on these results it was concluded that in routine it is necessary to perform a deconjugation step during multi residue sample preparation. In case of a specific analysis

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(e.g. confirmation) for Clenbuterol the hydrolysis can be omitted. For other ß-agonists no data are available at the moment.

11. CRITERIA FOR ACCEPTANCE/REJECTION OF RESULTS.

In order to evaluate GC-MS results the following criteria must be fulfilled :
- the GC-MS tuning report must be adequate
- the external standards must give adequate responses (0.1 ng Clenbuterol injected should give a response at $m/z = 86 > 10$ * the average noise (SIM mode, 2 ions monitored)
- response maximum of a signal of unknown compound must exceed the average noise $\pm$ 3sd.

11.1 Quantification

Linear calibration curves are constructed with the ratio [peak area $m/z(86)$]/[peak area m/z (ion of internal standard)] as dependent variable and the concentration as independent variable. For quantification the calibration curve must have a coefficient of correlation > 0.99 and the numerical value of the intercept should not differ more than 3SD from zero.

11.2 Identification

For positive identification at least four ions must be monitored. The response-ratios are calculated : r_1, r_2 and r_3. For EI the values must be within $\pm$ 10% of the corresponding value for standards (for PCI this ratio should be $\pm$ 20%). Discussion within the EC (RIVM/ARO letter 2531\93) have resulted in an acceptable alternative. Eventhough the objective is to fulfil the original criteria the alternative set can be used if necessary :

ratio fragment-ion of basepeak	variability
> 20%	< 15%
> 10%	< 20%
> 5%	< 50%
> 1%	< 100%