0. INTRODUCTION

Throughout the EEC the use of xenobiotic anabolic agents is prohibited in food producing animals. Subsequently the Maximum Residue Level (MRL) for residues of these anabolics in animal products is zero (non-detectable). Analytical strategies are needed for monitoring the use by checking biological samples.

1. SCOPE

This modular method of analysis describes the detection and confirmation of the presence of individual anabolic agents in samples of animal origin.
Within the field of application only semi-quantitative methods are needed and method validation procedures are based on this. However, when deuterated internal standards are available and the purity of the standards used for identification and calibration are known, the method can be considered quantitative.

2. FIELD OF APPLICATION

The method is used to perform routine screening and confirmatory analyses in bovine urine and muscle tissues for the compounds listed in Table 1. Bile can be analysed too, but supplementary validation is necessary prior to analyses. Special samples, including application sites (primary extracts and homogenates) can be analysed, provided appropriate precautions are made.

The limit of detection is below 1 µg/l or µg/kg. The limit of detection is based on the detection of the most abundant diagnostic ion with a response at the correct retention time exceeding the average noise + 3 SD. The limit of identification ranges from
0.5 - 2 µg/l of µg/kg, depending on the analyte and the matrix. The limit of identification equals the limit of detection as based on the fourth diagnostic ion.
Detailed validation studies were performed on a number of analyte- matrix combinations (references included in chapter 7). However, in principle more compounds than those listed in Table 1 can be analysed within this procedure, provided an adequate supplementary validation study is included in the study plan concerned. This plan, unequivocally identified, contains information with respect to; the identification and storage conditions of the samples, sample pre-treatment steps (e.g. additional homogenisation), the analyte or combination of compounds to be analysed for, the selected analytical steps and details with respect the analytical quality control.

3. REFERENCES

Van Ginkel, L.A., Stephany, R.W., Van Rossum, H.J., Steinbuch, H.M., Zomer, G., Van de Heeft, E. and De Jong, A.P.J.M. (1989), Multi-immunoaffinity Chroma-tography: A simple and highly selective clean-up method for multi-anabolic residue analysis of meat. J. Chromat. 489,111-120.

Zomer, G. (1987), Synthesis of deuterated anabolic compounds, RIVM Report no. 378106 001, Februari 1987.

Haagsma, N. Ellen, G. De Ruig, W.G. en Stephany R.W. (1991), Begrippen bij de bepaling van residuen in voedingsmiddelen van dierlijke oorsprong. Overleggroep Residu-Analyse (ORA), Werkgroep Kwaliteit Analyse, De Ware(n) Chemicus, 21, 82-95.

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Zomer, G. (1988), The synthesis of trideuterated 19-nortestosterone ([16,16,17? -D_3]-17ß-hydroxyestr-4-en-3-one, NT-d3]), RIVM Report no. 328608001, Februari 1988.

4. DEFINITIONS

The mass concentration is taken to mean the amount of analyte in the substance in question, regardless of the chemical form, determined according to the described method and expressed as µg/kg or litre of test sample. All other definitions are according to Haagsma et al.

5. MATERIALS

Reference to a company and/or product is for purposes of identification and information only and does not 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 suitable.

5.1. Standards

The use of BCR certified standards is recommended. It is foreseen that these internal standards will be made available through BCR. Relevant standards are listed in Table 1.

TABLE 1: Compounds included in this SOP

Analyte	Abr.	CAS #	Formula	Mol. weight
17ß-19-Nortestosterone	(ßNT)	434-22-0	$C_{18}H_{26}O_2$	274,3
17? -19-Nortestosterone	(? NT)	4409-34-1	$C_{18}H_{26}O_2$	274,3
17ß-Testosterone	(ßT)	58-22-0	$C_{19}H_{28}O_2$	288,4
17? -Testosterone*	(? T)	481-30-1	$C_{19}H_{28}O_2$	288,4
17? -Methyltestosterone	(MT)	58-18-4	$C_{20}H_{30}O_2$	302,4
Boldenone	(BOL)	846-48-0	$C_{19}H_{26}O_2$	286,4
17? -Ethynyl estradiol	(? EE2)	57-63-6	$C_{20}H_{24}O_2$	296,4
17ß-Estradiol	(ßE2)	50-28-2	$C_{18}H_{24}O_2$	272,2
Zeranol	(ZER)	26538-44-3	$C_{18}H_{26}O_5$	322,4
Taleranol*	(TAL)	42422-68-4	$C_{18}H_{26}O_5$	322,4

*not an anabolic by itself but a metabolite of the previous compound

The use of internal standards is of great importance for the quality control of analytical procedures:

- for accurate correction for the analytical recovery in quantitative analyses
- for control for false negative results in qualitative methods.

For this purpose a number of deuterated internal standards is available (Table 2). It is foreseen that these standards will get a wider distribution through CEC/BCR in the near future.

The standards used for identification and calibration are registered (ARO-MIS CARDBOX database). Minimum available data are: A data-sheet of the source preparation which includes, the chemical name, the synonym used, the CAS #, the molecular weight and a mass spectrum of the actual preparation from which the correct identity can be checked.
From these standard preparations stock solutions containing 1 mg/ml are prepared.
These solutions are registered and stored in the dark at approximately -20^{o}C (not higher than -10°C) for a maximum period of 5 years.

Working solutions are prepared by 10-fold dilution of the stock solutions. These solutions are stored in the dark at approximately 4^{o}C (range 1-10°C) for a maximum period of 6 months. Quality control includes the registration of a mass spectrum (identity) and a HPLC Diode Array chromatogram/UV-spectrum.

TABLE 2: Compounds available for use as isotope enriched internal standard

Internal standard	RIVM/ARO sample no.	Source
Diethylstilbestrol-d6	H145661	RIVM
Dienestrol-d2	H143275	RIVM
Hexestrol-d4	H143274	RIVM
Zeranol/taleranol-d4	87M1553	RIVM
17ß-Nortestosterone-d3	87M1056	RIVM
Methyltestosterone-d3	H146525	RIVM
17ß-Testosterone-d2	89M1692	MSD md-2962
17ß-Estradiol-d3	89M1691	MSD md-2325
Medroxyprogesterone-d3	H148155	RIVM

If the corresponding internal standard is not available the compound which elutes as close as possible to the compound of interest should be used as internal standard (only applicable in case of HPLC).

Notice: Isotopically labelled internal standards are in general not commercially available. However, several research institutes have synthesised suitable compounds. These primary sources are, amongst others, RIVM, Bilthoven, NL and RIKILT, Wageningen, NL.

5.2. Immunoaffinity chromatography-(IAC) materials

The compounds for which IAC is possible are the same as those for which deuterated internal standards are available (Table 2). For multi-residue methods appropriate IAC materials can be combined (MIAC).

Notice: The preparation and characterisation of IAC-materials and -columns is described in SOP ARO/172. 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 measured again. The capacity of the gel is evaluated and, if adequate, individual columns are filled.

5.3. Chemicals

All listed chemicals are of Pro Analyse quality or better, unless stated otherwise.
Compound solutions are stored at room temperature and expire 6 months after preparation.

5.3.1. Acetone (Merck, 14)
5.3.2. Ethanol (Merck, 983)
5.3.3. Hexane (Baker, 8044)
5.3.4. Methanol (Merck, 6007)
5.3.5. Thiomersal (BDH, 304162)
5.3.6. Acetonitrile (Merck, 30)
5.3.7. tert-Butylmethylether (Merck, 1845)
5.3.8. Sodium hydroxide (Merck, 6498)
5.3.9. Hydrochloric acid, 37% solution (Merck, 317)
5.3.10. Ethyl acetate (Merck, 9623)
5.3.11. Acetic acid (Merck, 63)
5.3.12. Sodium acetate (Merck, 6268)

5.3.13. Sodium chloride (BDH, 10241)

5.3.14. Potassium hydroxide (Merck, 5033)
5.3.15. Isooctane (Merck, 4718)
5.3.16. Disodium-hydrogenphosphate (Merck, 6586)
5.3.17. Potassium-dihydrogenphosphate (Merck, 4873)
5.3.18. ß-glucuronidase/sulfatase (suc d'Helix Pomatia containing 100.000 units ß-glucuronidase and 1000.000 units sulfatase per ml, Industrie Biologique, France, code IBR 213473)
5.3.19. Subtilisin A (Sigma, P-5380)
5.3.20. derivatisation reagent: N,O-bis(trimethylsilyl)trifluoracetamide (BSTFA) with 1% TMCS (Pierce 38832).
5.3.21. Heptafluorbutyric acid anhydride (HFBA), (Pierce 63163).
5.3.22. Phosphate buffer 0.02 mol/l, pH 7.4. Dissolve in 800 ml of water 2.278 g of disodium-hydrogenphosphate (5.3.16.), 0.416 g of potassium-dihydrogenphosphate (5.3.17.), 9.0 g of sodium chloride (5.3.13.) and 0.05 g of thiomersal (5.3.5.). Adjust the pH (5.5.18.) at 7.4. ± 0.1 and add water to a final volume of 1000 ml.
5.3.23. Acetate buffer, 2 mol/l, pH 5.2. Dissolve 25.2 g of acetic acid (5.3.11.) and 129.5 g of sodium acetate (5.3.12.) in 800 ml of water. Adjust the pH (5.5.18.) at 5.2 ± 0.1 and add water to a final volume of 1000 ml.
5.3.24. Tris buffer, 0.1 mol/l, pH 9.5. Dissolve 12.1 g of Tris(hydroxymethyl)-amino-methane (Merck, 8382) in 800 ml of water. Adjust the pH (5.5.18.) at 9.5 ± 0.1 and add water to a final volume of 1000 ml.
5.3.25. IAC-eluting solution. Add to 50 ml of ethanol (5.3.2.) water to a final volume of 100 ml.
5.3.26. IAC-washing solution. Add to 80 ml of ethanol (5.3.2.) water to a final volume of 100 ml.
5.3.27. Alkaline hydrolysis solution. Dissolve 5.6 g of potassium hydroxide (5.3.14.) in 100 ml of methanol (5.3.4.).
5.3.28. Acidic buffer. Mix 1.7 ml of hydrochloric acid (5.3.9) with 98.3 ml of 2 mol/l buffer (5.3.23.).
5.3.29. Methanol : water (4:1, (V/V)). Add to 80 ml of methanol (5.3.4.) water to a final volume of 100 ml.
5.3.30. Petroleum ether (Merck 1775)

5.4. Samples

Samples of urine and liver are stored in the dark at approximately -20°C, but not higher than -10°C, until analysis, or at approximately 4°C (range 1 - 10°C) if analysis is foreseen to be within 2 days.

5.5. Apparatus

For operating instructions and maintenance status files see ARO Cardbox databases.
Standard laboratory glassware and equipment is used, with in addition:

5.5.1. Glass roundbottomed flasks, 150 ml (Quickfit, FF 150/4S).
5.5.2. Glass vials 20 ml with screw caps (Packard).
5.5.3. Automatic pipettes (Gilson P20, P200, P1000 and P5000).
5.5.4. Refrigerated centrifuge (RC-3, Sorvall).
5.5.5. Bench-top centrifuge (GLC-4, Sorvall).
5.5.6. Centrifuge tubes, glass (55 mm x 11,5 mm) (Renes, RB55)
5.5.7. Electric water bath with thermostat adjustable ± 5°C (GFL) with nitrogen facility.

5.5.8. Vortex (Vortex-genie).

5.5.9. Ultrasonic water bath (Bransonic 32).
5.5.10. Glass derivatisation vials (Chromacol 2SV (A)) with screw caps (Chromacol 8SC) and septa (Chromacol 8-ST15).
5.5.11. Incubator thermostat adjustable ± 5°C (Salvis).
5.5.12. Heating module thermostat adjustable ± 5°C (Pierce no. 18790) with nitrogen facility.
5.5.13. Glass injection vials (Chrompack, 10201) with glass inserts (Chrompack, 10381).
5.5.14. Aluminium caps (Chrompack, 10210).
5.5.15. GC-MS equipment (see 6.3.3).
5.5.16. Rotavapor with water bath at 40 ± 5°C (Rotavapor).
5.5.17. Centrifuge tubes (50 ml), glass (Pyrex, Z14,588-2)
5.5.18. pH-meter, (Applikon).
5.5.19. HPLC-equipment. HPLC equipment is not specified in this SOP since the system used, including sample injection and collection facilities depend on the application. Standard equipment is suitable, provided that performance is checked and adequate.
5.5.20. Moulinette S (Moulinex).

TABLE 3: Analytes, and UV detection wavelength

Compound	UV-Wavelength (nm)
17ß-Estradiol	280
Zeranol	265
Ethinylestradiol	280
17ß-Nortestosterone	254
17? -Nortestosterone	254
Methyltestosterone	254
17ß-Testosterone	254
17? -Testosterone	254
Boldenone	254
Medroxyprogesterone	254
Chlormadinone	280
Megesterol	280

6. ANALYTICAL PROCEDURES

Before experimental work is started, the appropriate analytical strategy is decided on (Annex I lists relevant matrix-analyte combinations).
Procedures for the detection and identification of anabolic agents in biological materials are described, based on a four-step approach:
6.1. Preparation of a primary extract
6.2. Extract purification and concentration
6.3. derivatisation, detection and identification
6.4. Interpretation and calculation

Figure 1 (see 10.) shows a flowdiagram for the analysis of anabolic agents.

Additionally, the QC-samples to be analysed must be defined. Each analytical series must contain known blank and known blank spiked (positive control) samples. Each analyte tested for, must be included in the positive control. The frequency of analysing positive control samples must be at least one positive control per 10 unknown samples. Negative controls must be included at least once during each experiment. The level of spiking must be defined in the study plan.

6.1. Preparation of a primary extract

If a laboratory sample is considered suitable for (confirmatory) analysis (adequate sample size, proper storage history and representative for the study) the first step in the analytical procedure is the preparation of a primary extract, including procedures for deconjugation and if appropriate (e.g. tissue) for defatting.

6.1.1. Liquid samples

For extraction of liquid samples liquid-liquid partition is most frequently used. Alternatives like direct application to immunoaffinity (IAC) materials and HPLC column switching, however, can be suitable or preferable in particular cases.

6.1.1.1 Urine samples

From the laboratory sample a testportion of 5.0 ml is taken to which the internal standards are added, preferably the isotopically substituted analyte (e.g. in the case of nortestosterone (NT) 5 ng of trideutero-NT (NT-d3)). The pH is adjusted to 5.2 with diluted acetic acid (HAc) or 1.0 mol/l NaOH and 1.0 ml 2.0 mol/l buffer (5.3.23.) is added. To deconjugate glucuronide- and sulphate conjugates of the analytes 0.1 ml ß-glucuronidase/sulfatase (5.3.18) is added and the sample is incubated during 2 hours at 37°C (5.5.11.).

After incubation the test portions are cooled to room temperature and extracted twice with 5 ml t-butylmethylether (TBME) (5.3.7). The combined extracts are evaporated to dryness under a stream of nitrogen in a water bath at 50°C (5.5.7.).

6.1.1.2 Bile samples

The procedure for bile is the same as for urine with the exception that the test portion for analysis is a mixture of 1 ml of the laboratory sample and 4 ml of water.

6.1.2. Tissue samples

Two different procedures are considered, the first is an enzymatic procedure which uses a protease, the second a mechanical procedure. The advantage of the enzymatic procedure is the protein digestion allowing to free analytes from cells and from conjugates with proteins (non covalently protein bound residues). However, some analytes are not stable under the conditions used (pH 9.5, 55°C) and therefore sometimes a mechanical procedure has to be used. As far as data are available (based on incurred tissues) enzymatic digestion results in slightly higher values of analyte contents.

Notice : It should be noted that internal standards do not give adequate information about the extractability of incurred residues.

6.1.2.1 Enzymatic digestion

From the laboratory sample a test sample of 50-100 g is homogenised thoroughly (5.5.20.) and a test portion of 5.0 g is weighted into a 50 ml glass centrifuge tube. Internal standards are added and mixed with the test portions at least 30 minutes prior to the addition of 20 ml 0.1 mol/l Tris buffer, pH 9.5, (5.3.24.) containing 5 mg Subtilisin A (5.3.19). The mixture is shaken a few times and incubated during 2 hours at 55°C (5.5.7.), shaking at least every 15 minutes.

After the samples are cooled to room temperature and extracted twice with 20 ml TBME (5.3.7). The combined extracts are evaporated to dryness under a stream of nitrogen in a waterbath at 50°C (5.5.7.).

6.1.2.2 Mechanical extraction

From the laboratory sample a test sample of 50-100 g is homogenised thoroughly (5.5.20.) and a test portion of 5.0 g is weighted into a 50 ml glass centrifuge tube. Internal standards are added and mixed with the test portions at least 30 minutes prior to the addition of 10 ml methanol (5.3.4.). The samples are shaken thoroughly during 15 minutes and subsequently placed in an ultrasonic water bath (5.5.9.) for 15 minutes. The supernatant is removed (centrifuging 5 minutes at 1600 g) (5.5.4.) and the extraction procedure is repeated. The combined supernatants are evaporated to dryness under a stream of nitrogen in a water bath at 50°C (5.5.7.).

6.1.3. Defatting of the primary extract

The dry residue is dissolved in 4 ml methanol-water (4:1, V/V) (5.3.29.) and washed twice with 6 ml petroleum ether (5.3.30.). Subsequently the methanol/water phase is evaporated to dryness. Alternatively the methanol/water phase is concentrated to ± 0.8 ml, the volume is adjusted to 2.0 ml with water and the solution is extracted twice with 4 ml TBME (5.3.7).

6.1.4. Alkaline hydrolysis

In the case of analysis for compounds which might be present in the form of small esters (e.g. the gestagens and trenboloneacetate) an additional basic hydrolysis is applied.

The residue obtained is dissolved in 0.2 ml alkaline hydrolysis solution (5.3.27.). This mixture is incubated at 37°C (5.5.11.) for 30 minutes. The hydrolysis is ended by the addition of 1.0 ml acidic buffer (5.3.28.). This mixture is extracted twice with 6 ml TBME (5.3.7). The combined extract is evaporated to dryness under a stream of nitrogen. In some cases it is necessary to repeat the above-described procedure for defatting (6.1.3).

6.2. Extract purification and concentration

To allow detection and identification of low concentrations of analytes in extracts of biological samples adequate extract clean up is necessary.

6.2.1 Immunoaffinity chromatography (IAC)

One of the most powerful techniques is immunoaffinity chromatography (IAC), however, more accessible alternatives like high performance liquid chromatography (HPLC) are also suitable.

The procedure for extract purification by IAC depends on the characteristics of the IAC-material used. The following procedure is suitable for the polyclonal rabbit antibodies tested and used at RIVM.

The IAC materials usually have a capacity of 20 ng or more per ml of gel. For multi-residue analysis combinations of gels can be made.

The primary extract is dissolved in 0.05 ml ethanol (5.3.2.). Subsequently 5 to 10 ml water is added and the total mixture is applied to the IAC column. After sample application the column is washed with 5 ml water and eluted with 4 ml IAC-eluting solution (5.3.25.). The eluate is evaporated to dryness under a cold stream of nitrogen in a water bath at 50°C (5.5.7.) or alternatively, after evaporation of the ethanol from the mixture, extracted twice with 5 ml TBME (5.3.7.). At the same time the columns are washed with 10 ml IAC-washing solution (5.3.26.), 20 ml water and 20 ml phosphate buffer (5.3.22.). The columns are stored at 4°C (range 1-10°C)

6.2.2. High performance liquid chromatography

A variety of HPLC systems for the analysis of anabolic agents have been described. The following reversed phase system has proven to be adequate for extract purification prior to GC-MS :

pre-column	Lichrocart 4x4 lichrospher 100RP-18e 5?
analytical column	Lichrocart 125-4 superspher 100RP18 endcapped

The residue is dissolved in 0.30 ml of the HPLC eluens of which subsequently 0.25 ml is injected into the system. The fractions of interest are collected, usually starting 0.5 minutes before the retention time of the analyte and ending 1 minute later.

Sometimes it is possible or advantageous to combine different analytes in a single fraction. The eluens is removed under a cold stream of nitrogen in a water bath at 50°C (5.5.7.).

6.3. Gas chromatography-mass spectrometry

For GC-MS analysis on the system described below both the TMS- and HFB-derivatives are suitable.

6.3.1. Preparation of TMS-derivatives

The residue obtained after extract clean up is transferred to a derivatisation vial with 0.5 ml absolute ethanol. The ethanol is evaporated and 0.10 ml 1% TMCS in BSTFA (5.3.20) is added. The vial is vortexed (5.5.8) and the reaction mixture incubated during 1 hour at 60°C (5.5.11). After incubation the reaction mixture is evaporated to dryness under a stream of nitrogen at 50°C (5.5.12.) and the derivatised residue is dissolved in 0.025 ml isooctane (5.3.15).

6.3.2. Preparation of HFB-derivatives

The residue obtained after extract clean up is transferred to a derivatisation vial (5.5.10) with 0.5 ml absolute ethanol (5.3.2). The ethanol is evaporated and 0.05 ml HFBA (5.3.21) in acetone (1:4,V/V) is added. The vial is vortexed and incubated during 1 hour at 60°C (5.5.11). After incubation the reaction mixture is evaporated to dryness under a stream of nitrogen at 50°C (5.5.12) and the derivatised residue is dissolved in 0.025 ml isooctane (5.3.15).

6.3.3. GC-MS analysis

The following (or similar) conditions are used during GC-MS analysis :

GC-MS	Agilent 5973N
column	Cp SIL 5CB (Varian)
injection	1-5 µl splitless 250°C
temperature program	100°C - 280°C at 20°C/min.
temperature transfer line	280°C
acquisition:	see Table 4

The number and concentrations of GC-MS standards (standards derivatised 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 derivatisation vial for all analytes included in the experiment. Each vial includes the internal standard in an amount equal to the amount added to the samples (6.1). For confirmation of the identity similar standards are prepared without the inclusion of the internal standard. After the analysis of the standards sufficient derivatisation blanks are analysed in order to prevent contamination. Known positive samples are always preceded and followed by a derivatisation blank.

The ions monitored during GC-MS analysis are listed in Table 4. The most suitable derivative is indicated (++), the ion used during initial screening is underlined.

TABLE 4: Diagnostic ions monitored during confirmation analysis

Compound	derivative TMS	HFB	ions most suitable (++) derivative
17ß-NT	+	++	666 453 306 133
17? -NT	+	++	666 453 306 133
17ß-T	+	++	680 467 355 320
17? -T	+	++	680 467 355 320
MT	(+)	++	480 465 369 355
Bol	+	++	678 464 369 169
17ß-E2	+	++	664 451 409 356
EE2	+	++	474 459 446 353
MPA*		++	479 331 317 147
CMA*		++	540 497 462 401
MGA*		++	520 477 421 381
ZER	++	+	538 433 335 307
TAL	++	+	538 433 335 307

*after alkaline hydrolyses

6.4. Interpretation and calculation

The first step in interpreting the results is to check for

- adequate performance characteristics of the GC-MS system (MS-tuning)
- adequate sensitivity for external derivatised standards
- adequate signals for internal standards
- acceptable results for positive and negative control samples.

6.4.1. Calculation of quantitative results

Quantitative results are obtained by constructing calibration curves of the response variable versus the concentration. Quantification is only valid if

- the maximum of the signal originating from the analyte exceeds the noise + 3 sd
- the coefficient of correlation is better than 0.99
- the numerical value of the intercept does not deviate more than ± 3 SD from zero.

Calibration curves are calculated using least squares linear regression analysis.

6.4.1.1 Calculation in case of deuterated internal standards

For the compounds mentioned in 2. deuterated internal standards are available and therefore quantification is straight forward. The area of the selected ion of the standard and internal standard are calculated and the ratio is the response variable. A calibration curve is constructed by analysing different concentrations of standard. A linear curve is fitted using least squares linear regression calculation. Unknown concentrations are calculated by interpolation.

6.4.1.2 Calculation without corresponding deuterated internal standard

For the compounds not mentioned in 2 the use of deuterated analogues was not validated extensively yet or not available. For these analytes (alternative) internal standards are used to control for false negative results at the limit of detection. Quantification of the analyte content results in semi quantitative (indicative) results only.

6.4.2. Identification.

For identification according to the EC-criteria it is mandatory that at least 4 ions are monitored. Each ion monitored (response) should fulfil the criterion that the maximum exceeds the average noise + 3 sd. If this criterion is fulfilled the 3 different ratios are calculated. The same ratios are calculated for the standard analyte, preferably at the corresponding concentration. For positive identification the ratios obtained for the unknown sample should preferably within the range specified below.

Maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques.

Relative intensity (% of base peak)	**EI-GC-MS (relative)**	**CI-GC-MS, GC-MS-MS^n LC-MS, LC-MS-MS^n (relative)**
>50 %	? 10 %	? 20 %
> 20% - 50%	? 15 %	? 25 %
> 10% - 20%	? 20 %	? 30 %
? 10%	? 50 %	? 50 %

6.5. Analytical follow-up

If the experiment fulfils the quality control criteria:

- validated GC-MS detection
- good recovery of internal or external standards
- no contamination in blank control samples
- acceptable results for positive control samples

and the results of the analysis is negative, the experiment is closed and administrated as such. If one or more of the samples shows a response possibly related to any of the analytes the sample is reanalysed under strict conditions.

For confirmation it might be necessary to repeat the analysis without the addition of an isotope enriched internal standard since fragment-ions of the internal standard can interfere with the fragment-ions of the analyte.

7. VALIDATION OF THE PROCEDURE

The procedure described was tested in several co-operative and validation studies. Here only a brief overview of the most relevant studies is given.

7.1. Nortestosterone in muscle

Within the Benelux Public Health Workingroup "Hormones and anti-Hormones" a co-operative study of GC-MS methods for nortestosterone in muscle was undertaken (Reports SP/LAB/h 89[12] and SP/LAB/h 90[01]). Several Benelux reference and other research laboratories participated in this study. The results obtained with the procedure described here were in good agreement with the target values.

7.2. Repeatability and within laboratory reproducibility

Repeatability and within laboratory reproducibility were determent for 17? -Nortestosterone and taleranol by analysing 15 samples of spiked blank urine over three different days (ARO/DG 4.1992.32). At the level of 1.0 µg/kg it was possible to confirm the identity based on 4-ion confirmation.

TABLE 5: Repeatability and within laboratory reproducibility

Analyte	Concentration	repeatability	reproducibility
17? -Nortestosterone	0.5 µg/kg	11.2	25.2
17? -Nortestosterone	1.0 µg/kg	11.7	25.6
Taleranol	0.5 µg/kg	8.1	36.4
Taleranol	1.0 µg/kg	6.9	22.4

7.3 Validation of the method for the detection and identification of a range of steroids in muscle tissue

The method was extensively validated for methylboldenone, ethynyl estradiol, 17? -boldenone, methyltestosterone and 17? -nortestosterone in samples of muscle tissue (report 573005 013, Februari 2001). The limit of detection varied from 0.2 - 0.5 ? g/kg depending on the steroid/matrix combination. The repeatability ranges from 6 - 30% (n=6 at 0.5 ? g/kg), the within-laboratory reproducibility from 6-38% (n=3 at 0.5 ? g/kg).

7.4 Validation of the identification

The method is also suitable for the confirmation of the identity of the analyte, based on the criteria laid down in document SANCO 1805/2000 rev 1. The method was validated for confirmation, based on these criteria for five individual steroids in samples of muscle tissue from bovine and porcine animals, poultry and fish.
The limit of identification varied from 0.5-1.0 µg/kg. Applying the EU-criteria for confirmation of the identity of the analyte to the results as obtained for samples with a mass concentration corresponding to the limit of identification, revealed that in 50% of all cases these criteria were not fulfilled in full (less than 3 ratio's within the limits defined). Increasing the S/R of this ion to approximately 10 resulted in an increase of the number of confirmed cases to > 90%. When the criteria are not fulfilled in full, additional measurement must be made. This approach is valid in terms of the criteria mentioned.

8. RELATED DOCUMENTS

ARO AMAP 3770 or following : kwaliteitscontrole laboratorium standaarden.
ARO AMAP 3202 or following : registratie immunochemische materialen.
SOP ARO/172, Preparation of IAC-columns.
For additional general laboratory procedures see: ARO-MIS.

9. FLOWDIAGRAM

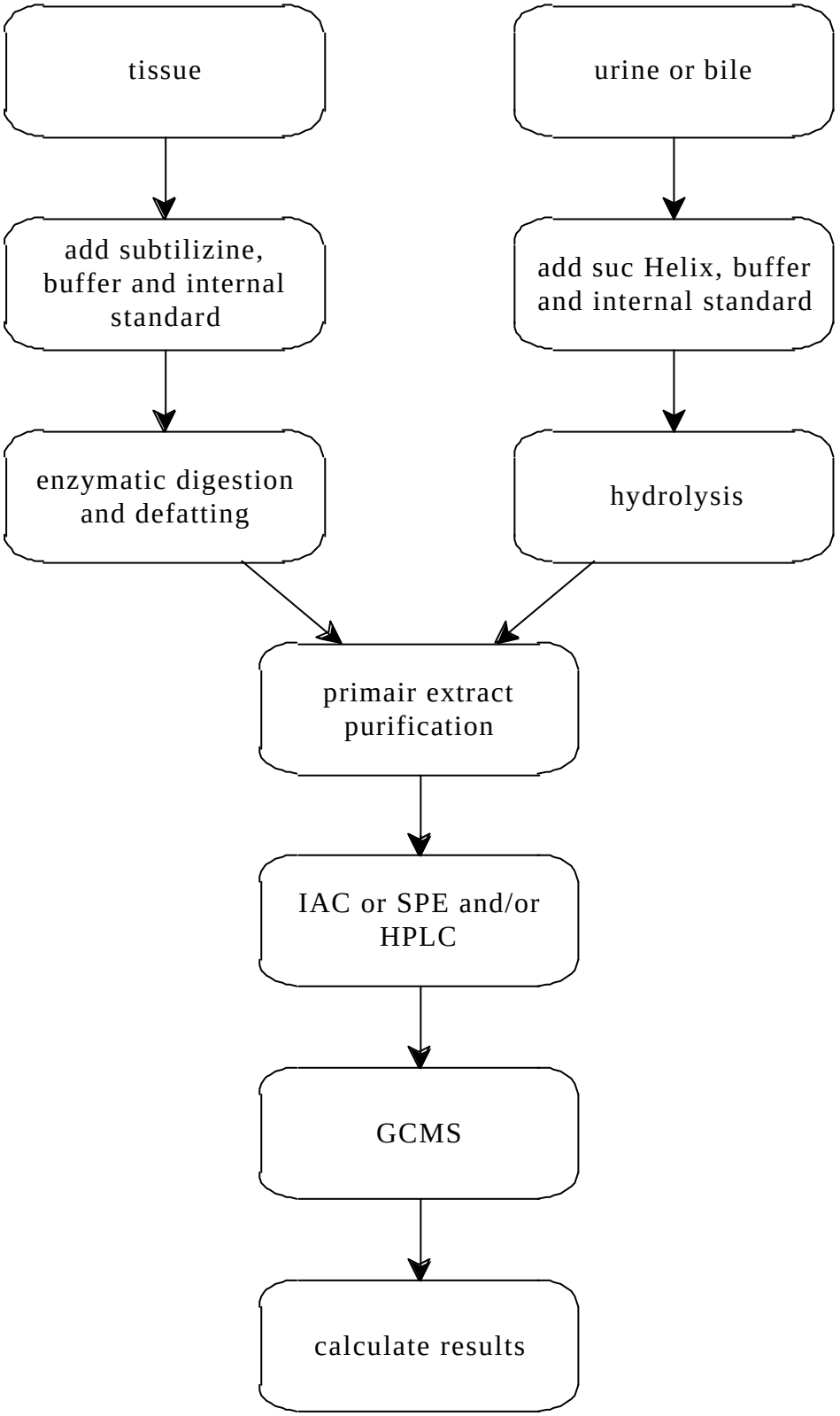

Figure 1: Flowdiagram for the analysis of anabolic agents