RIVM document 389002 116

EU CRL workshop Bilthoven, The Netherlands
"Analyses of Gestagens: an analytical update"
15 - 18 October 2001

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RIJKSINSTITUUT VOOR VOLKSGEZONDHEID EN MILIEU
NATIONAL INSTITUTE OF PUBLIC HEALTH AND THE ENVIRONMENT

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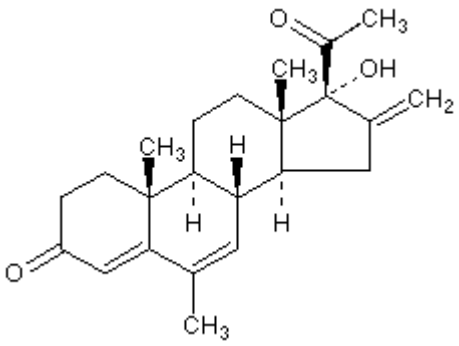


EU Community Reference Laboratory
National Institute of Public Health and the Environment
Laboratory for Residue Analysis
P.O. Box 1
3720 BA Bilthoven
The Netherlands
tel. ** 31 30 2742613
fax. ** 31 30 2744403
email: crl.aro@rivm.nl

Table of Contents

Introduction

Gestagens form a group of steroids comprising some of the oldest anabolic compounds, all related to the natural progestagenic steroid progesterone. Analytical methods for residue control mostly are based on kidney fat as sample material and to a lesser extent on samples of muscle tissue. Extensive metabolism of these compounds hampers the analyses of urine.

Methods traditionally have been based upon extensive extraction of fat with organic solvents followed by lengthy procedures for defatting the extracts obtained. Advances in Solid Phase Extraction (SPE) procedures and the increased use of Gas Chromatography Mass Spectrometry (GC-MS) significantly improved the analyses. However, in spite of these advances, analytical problems remained. One of the problems is associated with the fact that the gestagens are administered in the form of acetates, which, as a rule, do not hydrolyse upon circulation in the target animal. Therefor, analytical methods have to focus on the detection and identification of the acetylated gestagens. However, since the chromatographic characteristics of most gestagens, in the form of acetates, are very poor, a chemical hydrolysis step is used in most procedures, followed by a chemical derivatisation procedure.

Recent technological advances have been of importance on residue analysis for steroids, most particular gestagens. The use of Supercritical Fluid Extraction (SFE) and Liquid Chromatography combined with Mass Spectrometry (LC-MS) have clearly shown to be of benefit for the isolation, detection and confirmation of the identity of gestagens.

The major advantage of LC in the analyses of gestagens is the fact that derivatisation is not necessary. Subsequently, the alkaline hydrolysis step can be deleted from the procedure.

The revised performance and identification criteria, as layed down in the draft revision of Commission Decision 93/256[1], have already found wide applicability in this area.

During this workshop we will work on analytical methods for gestagens. No single methods will be demonstrated, but different modules will be combined. Traditional extraction will be used next to automated SFE and GC-MS will be used as well as LC-MS.

[1] Document SANCO 1805/2000 rev 1

Extraction						**Extract clean-up**				**Detection**
LLE (after enzymatic digestion)		Defatting		Alkaline hydrolysis		LC-fractionation		derivatisation		GC-MS LC-MS^n
SFE (for tissue after enzymatic digestion or lyophilisation)						Elution In Line trapped analytes		derivatisation		GC-MS LC-MS^n

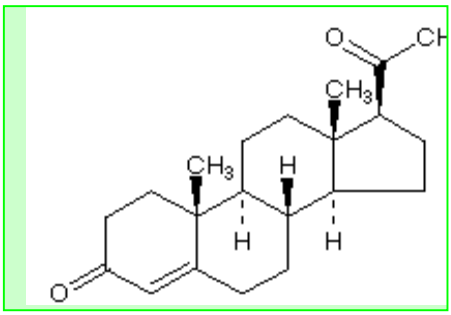
Analytical modules for gestagens

For the primary extraction, in combination with extract clean-up, there are two alternative routes. The traditional Liquid Liquid Extraction (LLE), for muscle tissue after enzymatic digestion of the tissues or Supercritical Fluid Extraction (SFE). The nature of the enzymatic digestion, in combination with LLE and alkaline hydrolysis, make extensive extract clean-up necessary. Fractionation by Liquid Chromatography (LC) is a possible approach. For detection and identification with GC-MS subsequent derivatisation is necessary. After SFE the trapped analytes can be eluted and the extract obtained is suitable for further analyses.

Overview of compounds

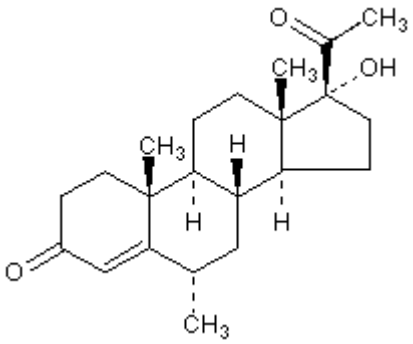
Progesterone.

Pregn-4-ene-3,20-dione: $C_{21}H_{30}O_2$; mol wt 314.47.



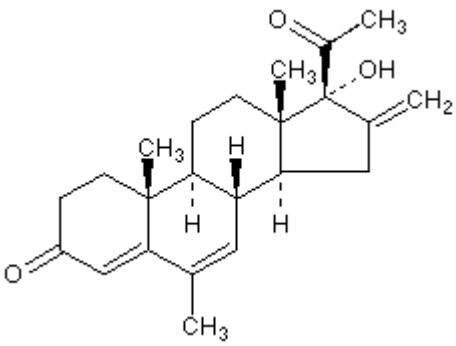
Medroxyprogesterone.

(6α)-17-Hydroxy-6-methylpregn-4-ene-3,20-dione: $C_{22}H_{32}O_3$; mol wt 344.49.
17-Acetate, $C_{24}H_{34}O_4$,



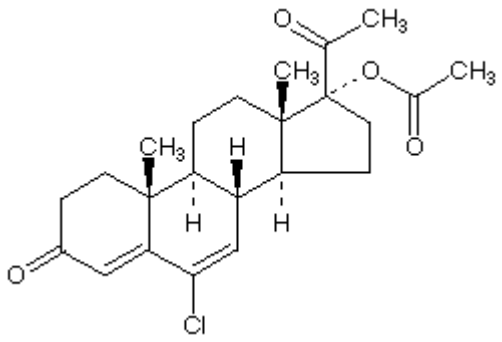
Melengestrol.

17α-Hydroxy-6-methyl-16-methylenepregna-4,6-diene-3,20-dione: $C_{23}H_{30}O_3$; mol wt 354.49.
17-Acetate, $C_{25}H_{32}O_4$.



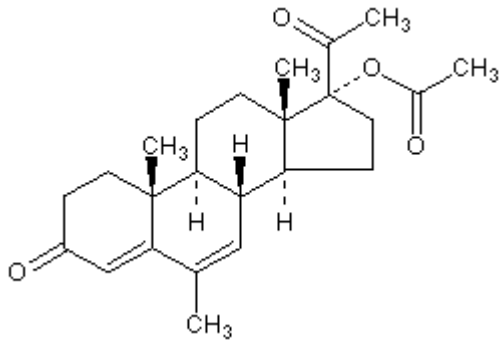
Chlormadinone Acetate.

17-(Acetyloxy)-6-chloropregna-4,6-diene-3,20-dione: 6-chloro-17-hydroxypregna-4,6-diene-3,20-dione acetate. $C_{23}H_{29}ClO_4$; mol wt 404.93.



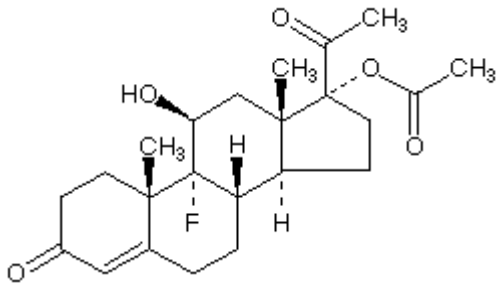
Megestrol Acetate.

17-Hydroxy-6-methylpregna-4,6-diene-3,20-dione acetate: $C_{24}H_{32}O_4$; mol wt 384.52.



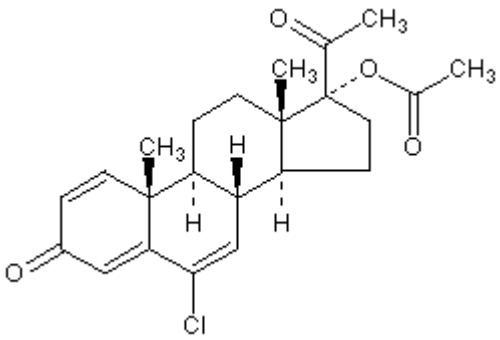
Flurogestone Acetate.

(11β)-17-(Acetyloxy)-9-fluoro-11-hydroxypregn-4-ene-3,20-dione: $C_{23}H_{31}FO_5$; mol wt 406.49.



Delmadinone Acetate.

17-(Acetyloxy)-6-chloropregna-1,4,6-triene-3,20-dione; 6-chloro-17-hydroxypregna-1,4,6-triene-3,20-dione acetate: $C_{23}H_{27}ClO_4$; mol wt 402.92.



Name compounds	Cas Reg. #	Molecular formula	Cas reg # Acetate	Molecular formula
progesterone	57-83-0	$C_{21}H_{30}O_2$		
Medroxyprogesterone	520-85-4	$C_{22}H_{32}O_3$		$C_{24}H_{34}O_4$
Melengestrol	5633-18-1	$C_{23}H_{30}O_3$		$C_{25}H_{32}0_4$
Chlormadinone	1961-77-9	$C_{21}H_{27}ClO_3$	302-22-7	$C_{23}H_{29}ClO_4$
Megestol	3562-63-8	$C_{22}H_{30}O_3$	595-33-5	$C_{24}H_{32}O_4$
Flurogestone		$C_{21}H_{29}FO_4$	2529-35-5	$C_{23}H_{31}FO_5$
Delmadinone		$C_{21}H_{25}Cl0_3$	13698-49-2	$C_{23}H_{27}Cl0_4$

Program EU-CRL-NRL Workshop " Analysis of gestagens, an analytical update "

Monday, 15 October 2001

14.00	A10.014	Registration Welcome on behalf of the CRL *Rainer W. Stephany* Introduction, Scope and Aims *Leendert A. van Ginkel*
14.30		coffeebreak, filling out of declaration forms
15.00		Presentation of NRLs on gestagen analysis
16.00		Introduction to the practical program *Saskia Sterk*
16.30		Reception on behalf of the board of directors RIVM
17.15		Departure by minibus to Hotel Heidepark, Bilthoven

Tuesday, 16 October 2001

9.00	Departure from Heidepark to RIVM by minibus
9.15	Practical program *Paul Zoontjes, Paul Schwillens, Klaas Wubs, Chris-Jan Kuijpers, Hennie van Rossum, Sylvia Linders, Hester van de Top*
10.30	Coffeebreak A10.014
12.00	Lunch
13.00	Practical program continued
16.30	Departure by minibus to Hotel Heidepark

Wednesday 17 October 2001

9.00	Departure from Heidepark by minibus
9.15	Practical program Day 2
10.30	Coffeebreak A10.014
12.00	Lunch
13.00	Practical program continued
15.00	Social program, departure to Amsterdam.

Tuesday 18 October 2001

9.00	Departure from Heidepark by minibus
9.15	Discussion on practical results
10.00	Coffeebreak
10.30	Discussion on priorities for the CRL * Update on international developments * Specific research presentations
11.30	Conclusions and recommendations
12.00	Closing of the workshop
12.15	Lunch
13.00	Departure to Schiphol by minibus

List of participants

Th. Debalis
Veterinary Laboratory of Serres
Terma Omonoias
GR-62110 SERRES
Greece

G. Kaklamanos
Veterinary Laboratory of Serres
Terma Omonoias
GR-62110 SERRES
Greece

J.M.S. Barbosa
Lab. Nacional de Investigacão Veterinaria
Estrada de Benefica 701
1500 LISBOA
Portugal

S. Berg
National Veterinary and Food Research Institute
Dept. of Chemistry
P.O.Box 45
FIN-00581 HELSINKI
Finland

M. O'Keeffe
The State Laboratory
Laboratory for Residue Analysis, Toxicology Section
Abbotstown, Castleknock
DUBLIN 15
Ireland

M. Pedersen
Institute of Food Research and Nutrition
Division of Chemical Contaminants
Mørkhøj Bygade 19
DK-2860 SOBORG
Denmark

S. A. Hewitt
Veterinary Sciences Division
Dept. Agriculture for Northern Ireland
Stoney Road, Stormont,
BELFAST, BT4 3SD
UK-NI

M. De Winter
WIP-ISP
J. Wytsmannstraat 14

B-1050 BRUSSELS
Belgium

C. Marchiafava
Istituto Superiore di Sanità
Viale Regina Elena 299
I-00161 ROMA
Italy
F. Delli Quadri
Istituto Superiore di Sanità
Viale Regina Elena 299
I-00161 ROMA
Italy

Y. Deceuninck
LABERCA
Ecole Nationale Vétérinaire de Nantes
Route de Gachet
BP 50707, F-44307
NANTES
France

F.O. van Bennekom
RIKILT/NRC
P.O.Box 230
NL- 6700 AE WAGENINGEN
The Netherlands

Th.W. Kuhn
Federal Institute for Veterinary Medicine Examinations
Dept. Chemistry, Section for Growth Promoting Agents
Robert Kochgasse 17
A-2340 MÖDLING
Austria

Sweden – not represented

P.M.Moreno
Centro Nacional de Alimentacion
Unidad de Residuos Zoosanitarios
Carretera de Majadahonda-Pozuelo, Km 2
E-28220 MAJADAHONDA (Madrid)
Spain

R. Lippold
CVUA Freiburg
Pharmakologisch Wirksame Stoffe
Bissier Str. 5
D-79114 FREIBURG
BRD

Principle of SFE

Creating a supercritical fluid (SF) is a fairly simple process. By using heat and pressure it is possible to move a substance beyond its critical point where it will become a supercritical "fluid". This is illustrated schematically in Figure 1, where the four phases of a substance (solid, liquid, gas and SF) are shown at varying temperatures and pressures. When a substance reaches the supercritical state the physical properties (density, viscosity, diffusivity) of the fluid become intermediate between those of the liquid and gas phases. The fluid's solvating powers are most like a liquid's, whereas its diffusivity and viscosity are gas-like. The properties of SFs give them the ability to dissolve non-polar solids, making them extremely useful for chemistry, especially when chemical separations and extractions are required. Supercritical fluid extraction (SFE) has been shown to be a suitable alternative to solvent extraction in a great many compounds from a wide variety of matrices.

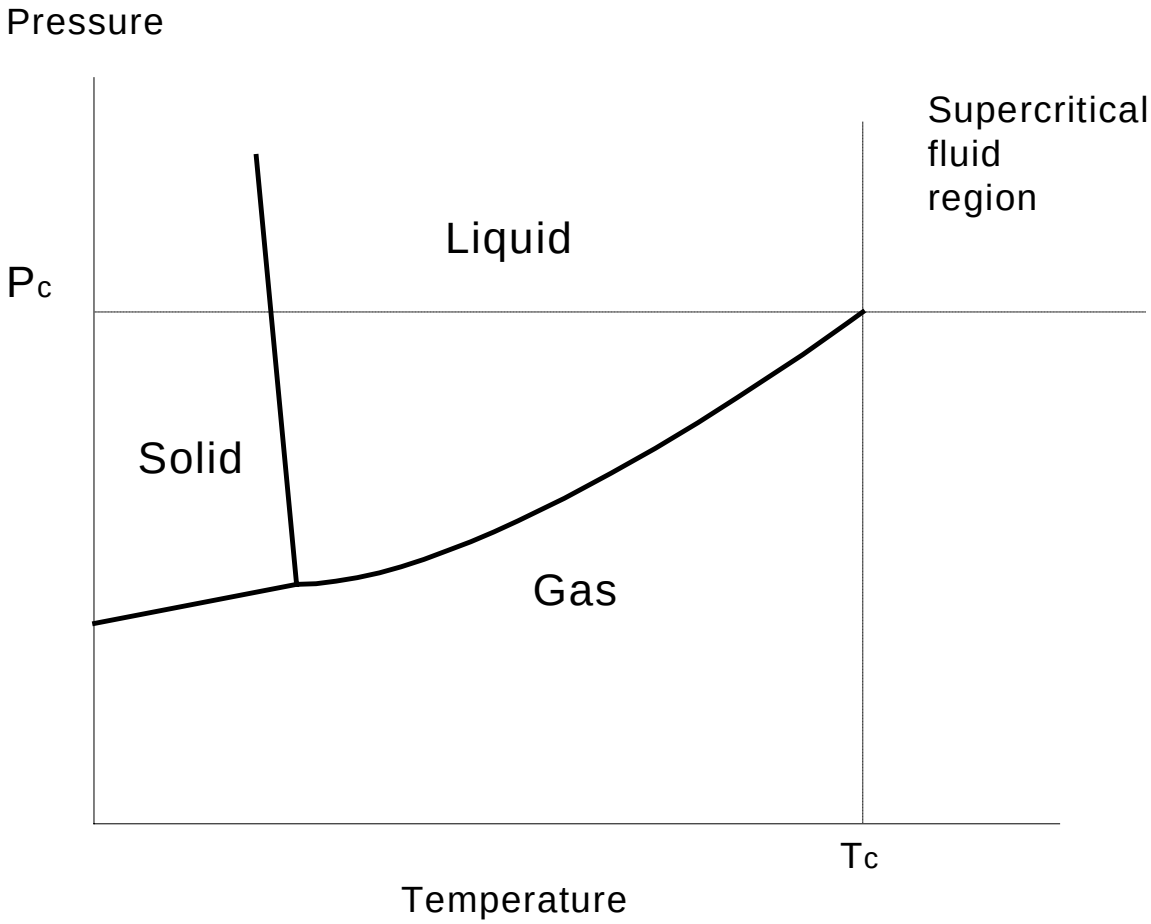


Figure 1 *Phase diagram for a liquid-gas supercritical fluid system*

Table 1 shows critical temperatures and pressures for some common solvents. Water is a poor choice for SFE because its critical temperature and pressure are among the highest of any solvent. Nitrous oxide would be a good supercritical fluid, but it is very flammable. Ammonia is a polar substance that has good solvent strength, but it is chemically reactive and corrosive, it dissolves pump seals, and it is generally considered a dangerous solvent. The hydrocarbons listed in Table 1 are flammable and are not viable for analytical SFE. Carbon dioxide (CO_2) is currently the most widely used fluid

for SFE, due primarily to its easily attainable critial parameters (31°C and 7.38 MPa), low toxicity, chemical inertness, low cost, and availability.

Table 1. *Critical values for selected solvents*

Fluid	Critical Temperature (°C)	Critical Pressure (MPa)
Ethylene	9.3	5.04
Carbon dioxide	31.1	7.38
Ethane	32.3	4.88
Nitrous oxide	36.5	7.27
Propylene	91.9	4.62
Propane	96.7	4.25
Ammonia	132.5	11.28
Hexane	234.2	3.03
Water	374.2	22.05

Fluid density and therefore solvent power change dramatically as the pressure nears the critical point. A very small change in pressure results in a large increase in solvent density.

Also, the solvent strength of a supercritical fluid can be influenced by adding small amounts of solvent modifiers. Adding a small percentage (10% maximum) of methanol, methylene chloride, or even hexane - all of which are soluble in carbon dioxide - can enhance the solvent power. Adding a modifier has some effect (though not particularly well understood) on the supercritical conditions; and the original temperature and pressure set points may require modification. However, like the use of solvent modifiers in HPLC, the use of supercritical modifiers in SFE can increase the extraction range to include more-polar analytes, such as drugs and drug metabolites in tissue and pesticides in fruit and vegetables.

The possession of unique properties intermediate between those of gases and liquids makes supercritical fluids (SFs) attractive alternatives to conventional liquid solvents for the extraction of trace analytes from complex matrices. The “gas-like” mass transport properties (i.e. low viscosities and high diffusivities) of SFs impart excellent matrix-penetrating power, thereby permitting more rapid and efficient extraction from difficult-to-access sample types when compared with liquid extractants. Densities (and, hence, solvating power) of SFs approach those of liquid solvents, and this solvating power can be easily varied by changing extraction temperature, pressure, or fluid composition. The existence of a greater number of parameters for method optimization and the ease of variation of these parameters allow a degree of selectivity “tuning” with supercritical fluid extraction (SFE) not readily available with traditional liquid-liquid extraction (LLE) or solid phase extraction (SPE) methods. The present availability of fully automated commercial

SFE instrumentation offers the potential for significant reductions in analysis time due to improvements in sample throughput.

In additon to time and selectivity advantages, SFE is also attractive from an environmental standpoint. Because CO_2 is a gas at ambient conditons, the generation of hazardous solvent waste is virtually eliminated, and post-extraction, spurred by the high costs associated with solvent purchase and disposal and regulatory measures such as the EPA's hazardous waste reduction program and the Montreal Protocol calling for production phaseout of ozone-depleting chlorofluorcarbons. With the advent of automated commercial instrumentation, analytical SFE technology is now finding routine use in many of these laboratories.

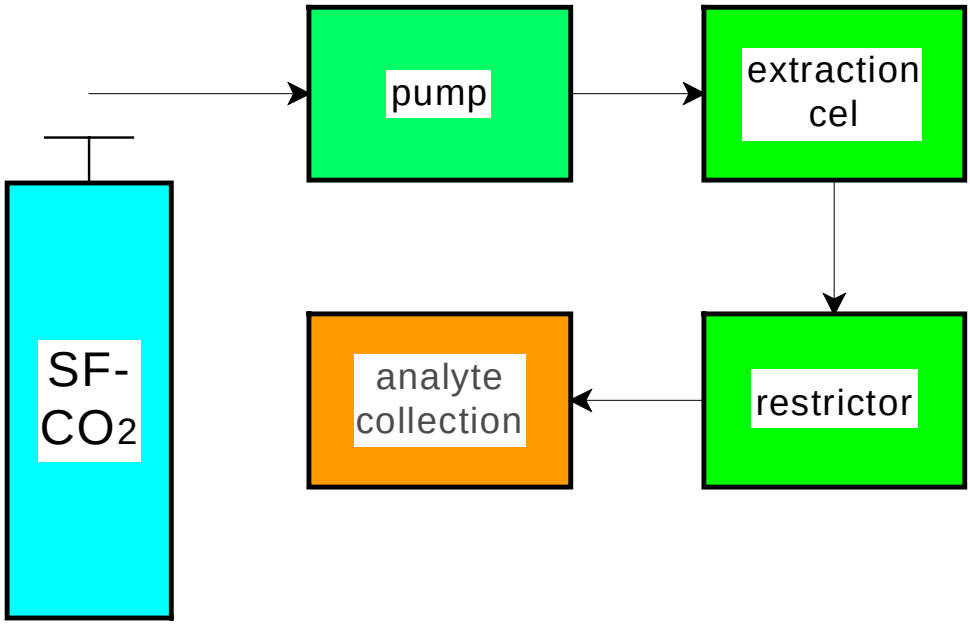


Figure 2 *Schematic representation of a SFE system*

Figure 2 is a schematic of a supercritical fluid extractor. The essential parts include a carbon dioxide source (or other SF fluid), a pump, an extraction vessel, restrictor and an analyte-collection device - normally a vessel. The quality of the carbon dioxide is very important. Generally, cryogenic-grade carbon dioxide is not pure enough for most SFE applications, and high purity carbon dioxide is required. Because the analyte is usually collected over a period of time in SFE, impurities in the supercritcal fluid - especially those with high boiling points - may also be collected and concentrated. The carbon dioxide purity is also important for certain detectors used in the analytical step. For example, when extracting trace analytes using a sensitive detection device, such as the electon-capture detector in GC, even parts-per-billion amounts of chlorinated impurities cannot be tolerated.

The pump used in SFE must generate high pressure, deliver reproducible volumes, and supply a constant flow rate. Generally, it should be able to pump liquid carbon dioxide and other fluids at the high pressures required for the supercritical state. Because the retention times of separated analytes are being measured, a constant flow rate is highly desirable. In SFE, the analyte is collected during a finite time before it is analyzed further. Therefore, the total volume of supercritical fluid passed through the extraction chamber is of great importance.

Two types of pumps have been used for SFE: reciprocating and syringe pumps. Both types meet the flow and pressure requirements of SFE. Reciprocating pumps, most

often used in HPLC, have an "infinite" reservoir and supply a continuous flow of supercritical fluid. Modifiers can be added by using doped cylinders or a second pump with proportioning valves. The major disadvantage of a reciprocating pump is that the pump head must be cooled to pump the liquid carbon dioxide. Low-cost cryogenic-grade carbon dioxide most often is used to cool the pump head.
Syringe pumps can provide pulseless flow and can be easily filled with liquid carbon dioxide. Syringe pumps do not have to be cooled because the carbon dioxide is liquefied by pressure, not temperature. However, because the pumps have limited volume, the syringe must be filled and repressurized when the pump cylinder is emptied. Also, when changing modifiers, thoroughly flushing of the pump head is necessary to prevent carryover. The pump should be able to deliver fluid at 0.5-4 ml/min flow rates. Faster flow rates enable extraction times to be reduced.

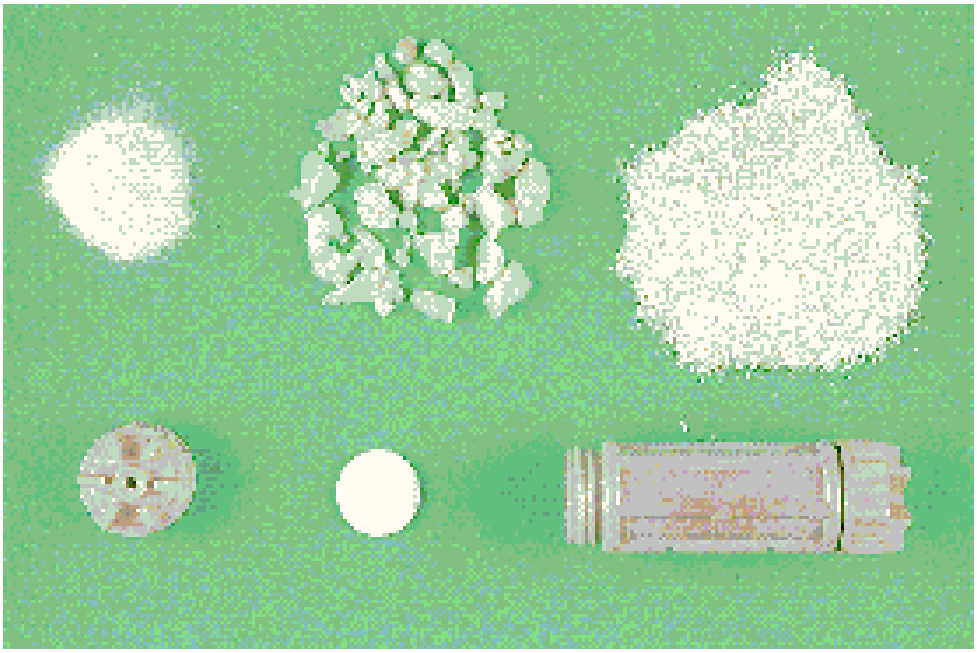
The extraction cell can be as simple as a stainless steel tube with compression endfittings or as complex as automatic sealing thimbles. The cell must withstand the pressure generated by the pump and must also be inert. Most SFE samples have masses of <10g; this sample mass represents a compromise between a sample mass requiring a large volume of supercritical fluid for quantitative extraction and the sample amount needed for a representative sample or for trace analysis. The size of most commercial analytical SFE extraction cells ranges from 100 ml to 50 ml. The amount of supercritical fluid needed to conduct a typical extraction dependents on many parameters; generally, at least three extraction-cell volumes are required. The extraction cell usually is placed in an oven to control the temperature. As discussed above, the temperture affects the supercritical fluid density. If the temperature fluctuates,the density and therefore, the solvent strength fluctuates. In addition, higher temperatures will increase the solubility of an added modifier and cause equilibria complications.
The importance of adding modifier to the supercritical fluid was discussed above. The modifier often is added using a pump or a doped carbon dioxide cylinder. Adding the modifier to the extraction cell with the sample is an easy and effective way to introduce modifier into the supercritical fluid. The procedure works not much different from adding modifier through the pump.

Automated SFE equipment

A technique for maintaining the extraction cell under pressure is required for the system to reach the supercritical state. In SFE, this pressurization often is accomplished by using a fixed or variable restrictor. Fixed restrictors are used more frequently and usually consist of a piece of capillary tubing of which the internal diameter and length can provide the appropriate back pressure. Pressure and, therefore, supercritical fluid density are changed by varying the flow rate through the restrictor. Because of the complex relationship between temperature, pressure, density, and flow rate, the restrictor must be replaced to vary the system pressure to change the density at a constant flow rate.
Thus, in SFE, several restrictors typically are used with the instrument. During method development, fixed restrictors must be changed between extractions. Fixed restrictors come in several varieties: linear, tapered, integral, and those with frits. Variable restrictors are designed to regulate pressure, independent of flow rate, by mechanically regulating the size of a small opening. Variable restrictors are more complex than fixed restrictors but do not have to be changed during method development or during any given method. They allow the "decoupling" of flow and pressure.

As the supercritical carbon dioxide passes through the restrictor, the change in pressure in the restrictor causes the pressure of the supercritical fluid to decrease, and eventually gaseous carbon dioxide may form. This step is called depressurization. The analyte is then swept into an on- or off-line collection device. In on-line collection, the analyte is directed into the analytical instrument (e.g. SFC, GC) and none can be analyzed by another technique without performing another extraction.
In off-line collection, the effluent is depressurized and the analyte is collected from the gaseous CO_2 stream. The development of efficient off-line collection methods is not always straightforward, particularly for volatile analytes, simply because a relatively low flow rate of supercritical CO_2 yields a high flow of gaseous CO_2 (e.g. 1 ml/min depressurizes to about 500 ml/min). Three general approaches for collecting analytes off-line after depressurization of the extraction fluid are trapping in a liquid solvent, thermal trapping and sorbent trapping. Linear restrictors have been used with all three collection methods, however, present forms of the commercially available variable restrictors are difficult to couple with liquid solvent trapping, and have only utilized thermal and sorbent trapping.
The simplest and most widely used method of trapping analytes involves simply depressurizing the supercritical fluid directly into a small vial containing a few milliliters of liquid solvent. This approach does not provide the potential for selectivity that sorbent trapping does. However, solvent trapping avoids the additional steps required by sorbent trapping. As is the case for sorbents, selection of the proper solvent polarity for the target analytes is important for achieving quantitative collection of the extracted analyte. While not necessary for many analytes, setting the collection solvent vial in a temperature control block (e.g. set at 5°C) helps to avoid restrictor plugging from extracted water. Further it increases the collection efficiencies for volatile analytes by avoiding the temperature fluctuations caused by the use of other restrictor heating methods such as the use of a heat gun.
Thermal trapping is inherently simple since the SFE effluent is simply depressurized into a cooled vessel. Quantitative thermal trapping has been limited to non-volatile organic compounds since the high gas flow rate causes losses of moderately volatile analytes. Non-volatile analytes may also be lost through the formation of aerosols. The two remaining approaches, trapping on a sorbent or in a liquid solvent have much better potential for quantitative recovery of high and moderate volatile analytes. Trapping on a sorbent is achieved by simply depressurizing the supercritical fluid onto the sorbent trap. Once the SFE is complete, the trapped analytes are recovered from the sorbent trap by

eluting them with a small volume (a few milliliters) of solvent. Sorbent trapping has the advantage of allowing the analyst to choose the sorbent packing that is best for the target analytes as well as providing the possibility of selective collection on the trap, followed by additional selectivity during the elution of the analytes form the sorbent.



SFE cartridge and content

For example, an SFE extract could contain three compounds with different polarities, A, B and C, of which only B is of interest. Compound A could be removed by choosing a sorbent trap with low affinity for A, but high affinity for B and C, thus causing A to be lost through the trap during the SFE step. Compound C could then be removed form the sorbent by first washing with a weak solvent, and finally, compound B could be recovered in pure form by eluting the sorbent trap with a stronger solvent. While the possibilities for selectivity using sorbent traps are attractive, it should also be recognized that sorbent trapping and subsequent elution adds additional steps (which may or may not be quantitative) to the SFE experiment.

Although a SFE instrument can be constructed with separate pieces of hardware, most users prefer to buy complete commercial units. SFE instruments range from simple manual devices to sophisticated computer-controlled units that provide automation, graphics, and other user-friendly features. As with all analytical techniques instrument design will evolve and improve further in the future.

THE USE OF AUTOMATED SFE IN ROUTINE ANALYSIS OF STEROIDS IN ANIMAL TISSUES

P.W. Zoontjes, A.A.M. Stolker and L.A. van Ginkel.

National Institute of Public Health and the Environment, Laboratory for Residue Analysis. P.O. Box 1, 3720 BA Bilthoven, The Netherlands.

Published in part previously: Proceedings Euroresidue IV (200), The Netherlands

Abstract

A method has been developed for the routine analysis of gestagens in animal tissues (e.g. bovine kidney fat) by using automated extraction with supercritical fluid (SF-CO_2). After addition of the internal standards and sample pre-treatment the analytes of interest are extracted from the matrix by unmodified supercritical CO_2 and trapped directly on alumina-sorbent placed in the extraction-vessel (in-line trapping under supercritical conditions). After extraction an alkaline hydrolysis is performed (no hydrolysis for melengestrol acetate) and the analytes are derivatised to HFB derivatives. Finally the samples are analysed by GC-MSD.

The limit of identification for megestrol, medroxyprogesterone and chloromadinone is 2 µg/kg, for melengestrol 5 µg/kg and for melengestrol acetate (without hydrolysis) 0.5 µg/kg. The with-in laboratory reproducibility ranges from 3-18% (n=3). The use of automated SFE improves the sample through put, making it possible to analyse 20 samples of kidney fat a day for gestagenic steroids.

Introduction

Different analytical methods based on LLE/SPE/HPLC and GC-MS are available to control the illegal use of anabolic steroids. The procedures, however, often are time and organic solvent consuming and the methods have to be optimised for every new steroid/matrix combination. The availability of a multi-analyte multi-matrix extraction method is essential for the development of quick and efficient methods for analyses.

Supercritical fluid extraction (SFE) has shown great potential in offering shorter extraction times with high recoveries and low consumption of organic solvents. Using a manual SFE system the obtained method validation characteristics for repeatability and with-in lab reproducibility were sometimes relatively high compared with LLE or SPE methods (1). Also the SFE procedure itself needs to be performed by an experienced technician. Using an automated SFE system both the methods characteristics and the sample throughput can be improved.

This paper describes a method for the analysis of gestagens (Melengestrol (acetate), Megestrol, medroxyprogesterone and chloromadinone) in kidney fat by the use of automated SFE and GC-MSD.

Method of analysis, SFE / GC-MSD

The sample of fat is homogenized by cutting the fat in small cubes. Two grams of sample are blended with 2 grams of Extrelut ®(Merck, Darmstadt, BRD) sorbent, the internal standard solutions (10µl of standard solution 1 ng/µl) and 0.5 ml of water are added. This mixture is transferred to an extraction vessel. After pouring the mixture in the vessel, a polypropylene frit is placed on top of the mixture and the vessel is filled with (ca. 2-3 grams) of alumina sorbent. The SFE is based on the in-line trapping technique. This means that the analytes are trapped on the alumina sorbent in the extraction vessel under supercritical conditions (2,3). The extraction conditions are mentioned in Table 1.
After extraction the alumina sorbent is poured into an empty SPE column (6 ml), and the analytes are eluted from the alumina by using 6 ml of methanol / water (65/35, (v/v)). The solvent is evaporated and 0.2 ml of the alkaline hydrolysis solution (5.6 g potassium hydroxide in 100 ml of methanol) are added. After incubation (37°C for 30 minutes) the hyrolysis is ended by the addition of 0.8 ml acidic buffer (1.7 ml of HCl in 98.3 ml of 2 mol/l acetate buffer, pH 5.2). After hydrolysis the analytes are extracted twice with 6 ml of TBME. The TBME extracts are combined and the solvent is evaporated. The obtained residue is redissolved in derivatisation reagent (HFBA:aceton = 1:4, v/v). After one hour of derivatisation at a temperature of 50°C, the reagent is evaporated and the residue is dissolved in 25 µl of iso-octane. 2 µl are injected in the GC-MS (Helwett Packard 5972A serie; Amstelveen, the Netherlands), GC-column: Chrompack CpSil 5CB 25.0 µm; film thickness 0.12µm. Table 2 shows the selected ions for the quantification of the gestagens. For confirmatory purposes the samples are reanalysed without the use of the internal standards and four diagnostic ions are monitored. The ratio's between these ions have to fulfill the EU criteria as described in Commission decision 93/256 (4).

Table 1 *SFE-conditions: for the extraction of gestagens from kidney fat*

	Static extraction	**Dynamic extraction**
Time (min)	10	20
Oven temperature in °C	50	50
Restrictor temperature in °C	110	110
Pressure in bar	300	300
Flow of CO_2 liquid (ml/min)	0	1

Table 2 *GC-MSD ions monitored for the quantification of gestagens in extracts of samples of bovine kidney fat*

	HFBA-derivative *m/z*
Medroxyprogesterone	479
Medroxyprogesterone-d3	482
Megesterol	477
Megesterol-d3	480
Melengestrol	447
Melengestrol-d3	450
Melengestrol acetate	489
Melengestrol acetate-d3	492
Chloromadinone	497
Chloromadinone-^{37}Cl	499

Method validation, SFE / GC-MSD

The detection limit is expressed as the concentration at which the maximum of the signal (of the most intensive ion) originating from the analyte shows a response with S/R =3. The limit of identification is the concentration at which four of the most intensive ions originating from the analyte show responses S/R>=3.

Repeatability and reproducibility experiments were performed by analyzing fortified samples three days in triplicate. From the analysed samples the peak areas of analyte and internal standard were measured and the ratio analyte/IS was calculated. The mean ratio of analyte/IS for every analyte was calculated within days and between days. In view of the small mass difference between analyte and internal standard the repeatability was expressed as %RSD of the mean ratio's within the days and the (within-laboratory) reproducibility was expressed as the %RSD of de mean ratio's between the days.

Results, SFE / GC-MSD

Using the above described procedure the limits of detection and the limit of identification obtained for the gestagens under investigation are respectively 1 µg/kg (melengestrol 2 µg/kg) and 2 µg/kg (melengestrol 5µg/kg). The absolute recoveries varies from 60-90% (n=9). The results obtained for repeatability and within-laboratory reproducibility are shown in Table 3.

Table 3 *Validation results of SFE/GC-MSD of gestagens from fortified (5 µg/kg) bovine kidney fat*

Analyte	Mean (n=9) in µg/kg	Repeatability in %RSD (n=9)	Within-laboratory reproducibility in %RSD (n=3)
Medroxy-progesterone	**4.6**	**5**	**9**
Chloro-madinone	**5.5**	**5**	**3**
Melengestrol	**5.4**	**7**	**18**
Megestrol	**4.4**	**8**	**18**

Figure 1 shows a GC-SIM-MS chromatogram obtained for the different gestagens by using the automated SFE technique.

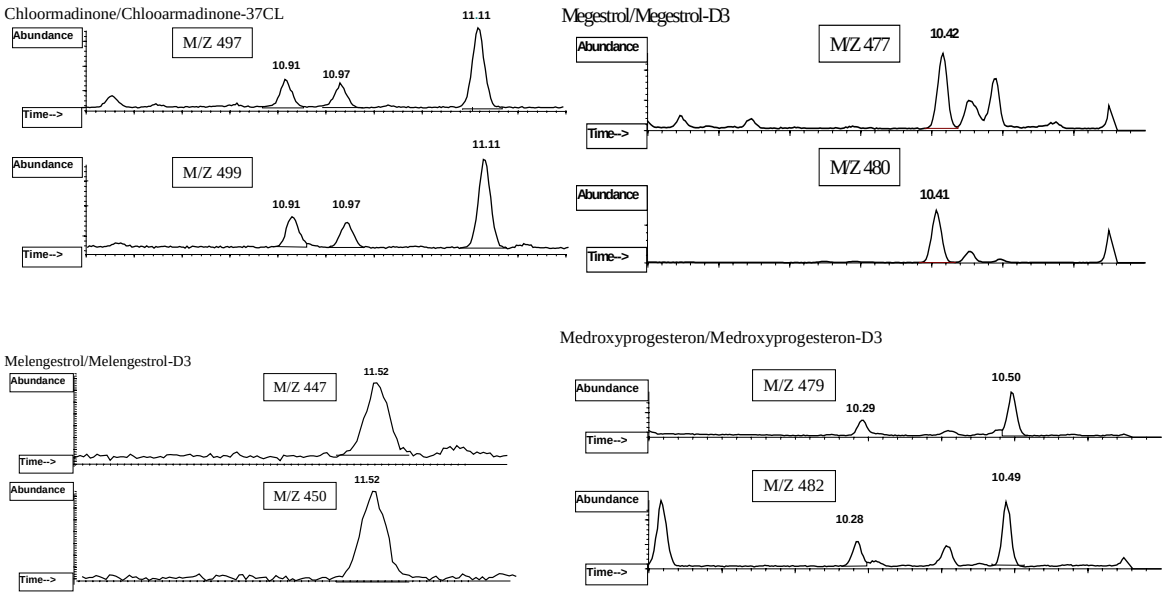


Figure 1 *GC-SIM-MS ion Chromatograms of HFB derivatives of chloromadinone(-*^{37}Cl*) megestrol(-d3), melengestrol(-d3) and medroxyprogestrone(-d3), isolated from fortified (5µg/kg) samples of bovine kidney fat by using the automated SFE technique (ISCO/Suprex SFE).*

With the method developed based on the use of automated SFE a quick and sensitive method has become available for the analysis of gestagens in bovine kidney fat. By comparing the results obtained for the automated SFE system and the manual SFE system as described (1) it can be concluded that the absolute recoveries obtained for the automated system are improved (manual system 40-60%; automated system 60-90%). Repeatability and reproducibility using the automated system are also slightly improved in comparison with the manual system. The repeatability and within-laboratory reproducibility for the automated system ranged from 5-8% and 3-18% and for the manual system from 5-14% and 7-39%.

An advantage of the manual system is the possibility to start with a large amount of sample material because of the use of an extraction vessel of 26 ml (or larger to 1 L). For the automated system only extraction vessels with a volume of 6 ml are available. With the manual system the use of in-line trapping directly on a SPE-column placed in the extraction vessel (because of the large volume of the vessel) is possible. By using the automated system the alumina sorbent is poured into the extraction vessel (loose bed) and after extraction transferred to an empty SPE column. This additional transfer step increases the risk of losses of the analyte or contamination (alumina sorbent in contant with the SFE vessel). Looking at the data such effect, however, is not observed. The average recovery for the manual system varied from 40-70% and for the automated system from 60-90%.

Depending on the purpose of the study the use of a manual or automated SFE system is selected. When there is a need for a flexible SFE system with the possiblity to use large amounts of sample material a manual SFE system is preferred (e.g. in case of quick method development). When a large number of samples has to be

analysed conform a developed SFE procedure the use of the automated system is preferred.

The limit of detection for MGA obtained with the procedure described is 2 µg/kg. Recent research on the use of this compound for growth promoting purposes shows the need for a more sensitive method. The method for gestagens was optimized for MGA by deleting the hydrolysis step. After SFE the analyte is eluted from the alumina column. An extraction step with TBME is performed and after evaporation of the TBME the residue is directly (without hydrolysis) derivatised to obtain the HFB-derivative of MGA followed by analysis with GC-MSD (monitoring *m/z* 489 and 492 for the internal standard). The limit of detection and limit of identification are respectively 0.2 and 0.5 µg/kg. The repeatability and within-laboratory reproducibility are 7 % (RSD for n=9) and 12 % (RSD for n=3). Fig. 2 shows a specific example of a four ion SFE-GC-(SIM)MS-chromatogram (used for identification) obtained for the HFB-derivative of MGA.

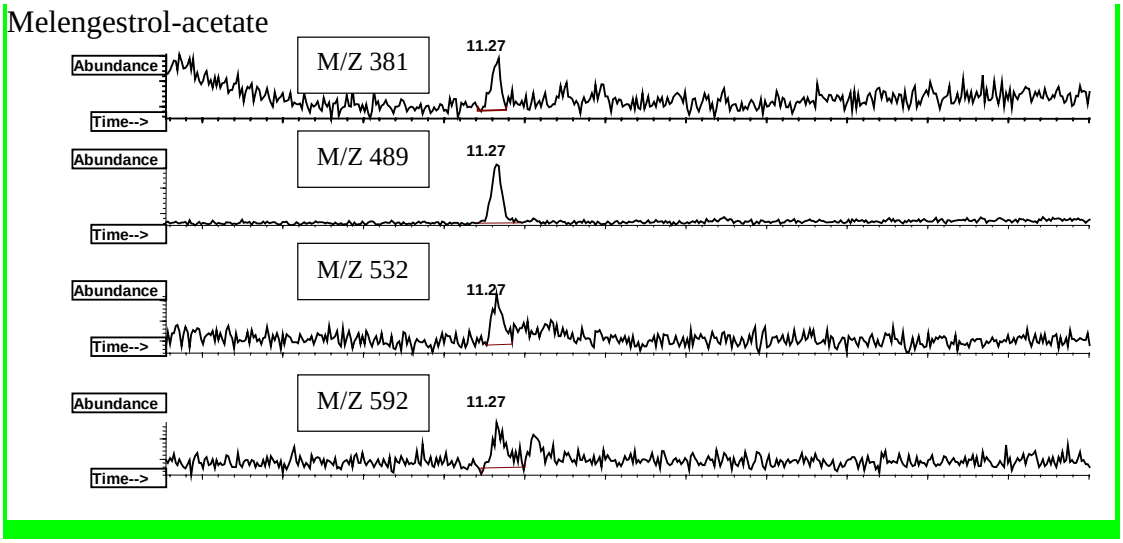


Figure 2 *GC-SIM-MS (four- ions for identification) Chromatogram of HFB derivative of melengestrol acetate isolated from a fortified (1 µg/kg) sample of bovine kidney fat by using the automated SFE technique (ISCO/Suprex SFE).*

LC-MS analysis, SFE / LC-MS

Using the conventional GC-MSD the introduction of a hydrolysis and derivatisation was necessary. MGA was the only gestagenic compounds that could be analysed without the need for hydrolysis. The other analytes are hydrolysed to the free compound to obtain the necessary sensitivity in the GC-MS analysis. After hydrolysis the compounds are transferred to their HFBA derivative before the GC-MS analysis can be performed. Introducing the LC-MS technique makes the need for hydrolysis and derivatisation superfluous.

The LC separations were performed on a Tosohaas TSK-gel Super ODS 4, 6 mm ID x 5 cm column with 2 µm particles. In comparison with conventional C18-based columns and 5 µm particles this showed high resolution for the gestagenic compounds in a short time-window (1 min). One disadvantage is the ID of 4.6 mm resulting in a flow of 1 ml/min (not useful for ESI analysis without splitting the flow).

The same type of column was not available with smaller I.D. On the used column the analytes of interest eluted separated from each other within one minute (between 5 and 6 min). Retention times obtained for the acetyl gestagenic steroids were for MA, CMA, MPA and MGA respectively 5.59, 5.71, 5.75 and 5.87 min. The APCI mode was selected because of the relatively high flow which was used 1 ml/min. This flow shows the best resolution of the compounds but for ESI this flow is too high and splitting the flow would be necessary. The use of APCI is also successfully demonstrated for corticosteroids [5].

Table 4, ions selected during LC-MSms screening

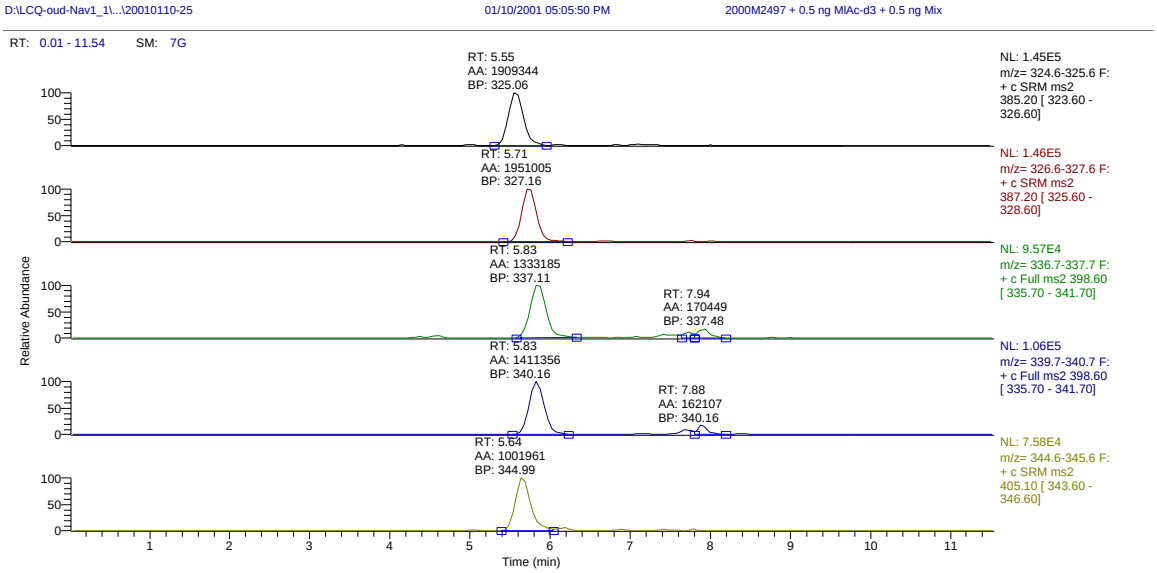
Analyte	m/z	m/z
medroxyprogesteron acetate	327	
melengestrol acetate	340	343
megestrol acetate	325	
Choormadinone acetate	345	

For the selection of the diagnostic ions in the ion-trap LCQ, first the full mass spectra of the analytes were recorded. The protonated molecules which clearly were the most intense ions, were selected in the ion trap, and application of a collision energy of 20 yielded the MS-MS spectra, as can be seen for MGA in Fig. 3. (reproduced in the annex). For CMA and MA, two ions in the MS-MS spectra were available for identification. For MPA and MGA only one ion in the MS-MS spectra was available for identification since according to the EU criteria for confirmatory purposes at least two transition ions in combination with one ion ratio are necessary the MS-MS-ion - which had an intensity was high enough for a second fragmentation - was subjected to further fragmentation to yield a third diagnostic ion in the MS3 spectrum. The diagnostic ions are mentioned in table 4. For MLA, MA, MPA and CMA the first transition ions are $[MH-60]^+$ probably these are the $[MH-CH_3COOH]^+$ ions. The second transition ions are for MLA and MA $[MH-60-58]^+$ probably the $[MH-CH_3COOH-C_3H_7O]^+$ ion. For MPA the second transition ion is $[MH-60-18]^+$ probably the $[MH-CH_3COOH-H_2O]^+$ ion and for CMA the second transition ion is $[MH-60-36]^+$ probably the $[MH-CH_3COOH-HCl]^+$ ion. For MLA and MPA are third transition ions monitored respectively $[MH-60-58-18]^+$ and $[MH-60-18-24]^+$.

During the screenings analysis the MS-MS $[M+H-C_2O_2H_5]^+$-ions were preferred to the $[M+H]^+$-ions: although the latter have a higher intensity, their selectivity is insufficient at the low (µg/kg) level. The MS-MS ions provide sufficient sensitivity to reach the required detection limits (0.5 µg/kg) and has much better selectivity. For screening purposes only one I.S. was added to the samples and only one diagonistic ion of MPA, MGA, MA and CMA and the molecular ion of the I.S. were monitored. Using GC-MS for screening all available deuterated internal standards were added to the sample. Using $LC-MS^n$ only one I.S. is added because all the analytes eluted in a time window of 1 min. and all the screening-ions have a m/z between 325-350 the use of one I.S. is sufficient. A second reason for the addition of one single I.S. is the amount of data points measured for one peak decreases when more ions in one single time-event have to be measured. By increasing the amount of ions that have to be measured, the sensitivity of the measurement will decrease (amount of integration points decreases), so for one time event, one single I.S. is used to obtain a maximum in sensitivity. If S/R ≥3 of one of the screening-ions was monitored (see Table 4) the sample was reanalysed in duplicate and to one of these samples the specific I.S. was added and to the second sample no I.S. was added. The

sample with I.S. was used for quantification based on linear regression calculation. Linear calibration curves were constructed for 0.25-2.5 µg/kg level of interest. With the data point in all instances, R^2 values ranged from 0.990-0.999 for LC-MS^2 analysis. Fig. 3 shows an example of the chromatogram obtained for the screening of a sample of fat fortified with 0.5 µg/kg of gestagenic steroids. The second sample (without I.S.) was used for confirmation.

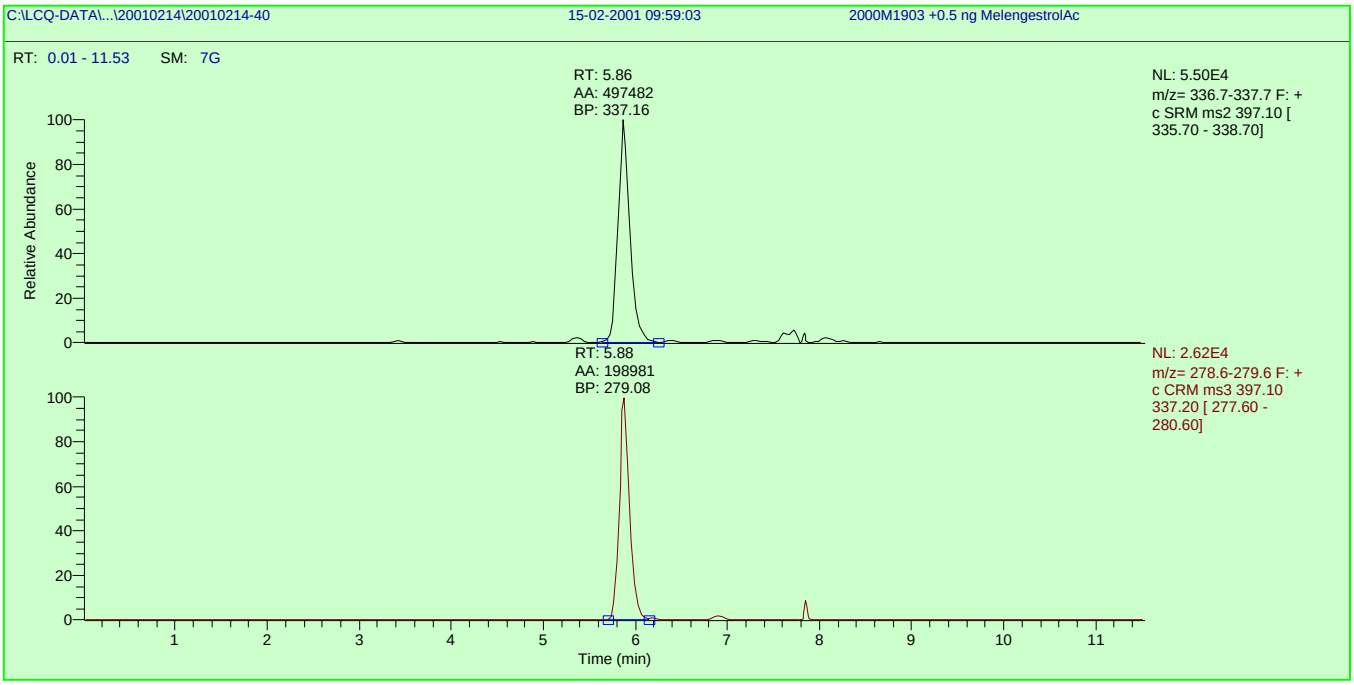
Figure 3: Screening of kidney fat for acetylated gestagens.



Chromatograms, from top to bottom;
Megestrol acetate (MS^2-ion 325, transition product of $[M+H]^+$ with m/z = 385
Medroxyprogesterone acetate (MS^2-ion 327, transition product of $[M+H]^+$ with m/z = 387
Melengestrol acetate (MS^2-ion 337, transition product of $[M+H]^+$ with m/z = 385
Melengestrol acetate-d_3 (MS^2-ion 340, transition product of $[M+H]^+$ with m/z = 385
Chlormadinon acetate (MS^2-ion 345, transition product of $[M+H]^+$ with m/z = 405

Since, according to the EU criteria for confirmatory purposes, at least two transition ions in combination with one ion ratio are necessary the MS-MS ion- which had an intensity high enough for a second fragmentation – was subjected to further fragmentation to yield a third diagnostic ion in the MS^3 spectrum. The ions selected for confirmation are mentioned in Table 4. Fig. 4 shows an example of the chromatogram obtained for the confirmation of a sample of fat fortified with 0.5 µg/kg of MGA. No signals for the specfic ions of Fig. 4 were monitored when a blank sample of fat was analysed.

Figure 4: LC-MS^n confirmation chromatogram of a sample of fat fortified with 0.5 µg/kg Melengestrol acetate upper track MS^2 ion m/z 337. Lower track MS^3 ion m/z 279.



3.3 Results method validation for SFE / LC-MSD.

The (automated)SFE- LCQ method was validated using the same procedure as was used for the (manual)SFE-GC-MS method and for the conventional LLE-GC-MS (results presented elsewhere). It is very interesting to see that no improve is obtained in reproducibility results going from SFE-GC-MS to SFE-LCQ. Because of the reduction of the necessary sample handling steps it was expected that the reproducibility (RSD%) decreases. From the results obtained it can be concluded that the SFE extraction introduced the variability in recovery. For both procedures the same extraction is used, so comparable results for the reproducibility were obtained.

Table 5 Validation results of different analytical methods for residue analysis of acetyl gestagenic steroids from fat

Sample pre-treatment	LLE	SFE Manual	SFE automated	SFE automated
Separation and detection	GC-MSD	GC-MSD	GC-MSD	LC-MSn
samples per day	8	10	25	25
limit of identification in µg/kg	2	5	5	0,5
mean within-lab reproducibility* RSD% (three days in triplo)	14-19	13-20	3-18	16-19

* at the level of identification (5µg/kg for GC-MS analysis and 0.5 µg/kg for LCQ analysis)

The most important advantage switching from conventional extraction techniques to SFE is the sample throughput is optimised. The most important advantage switching from GC-MS to LC-MS is the decrease of the identification limit. So the method available is a quick selective and sensitive method which can be used for monitoring research to the illegal use of the acetyl gestagenic steroids.

Application of the automated SFE-LCQ method

The detection capability[2] of the automated SFE-LCQ screening method is tested by the analysis of 20 different blank samples of kidney fat fortified at the level of 0.5 µg/kg. All the samples were found positive (S/R≥3 of screening ion) for all the acetyl gestagenic steroids under investigation. For MGA, MA, MPA and CMA the detection capability for the screening method is 0.5 µg/kg.
The detection capability of the confirmatory method wa tested by the analysis of 10 different blank samples of kidney fat fortified at the level of 0.5 and 1.0 µg/kg. All samples were analysed with the addition of one single analyte. No false negative results were obtained. In other words all samples fulfil the EU-criteria in respect that the ratio of the two transition ions from the sample and the standard were in the tolerance range described by the EU-criteria. Table 6 shows the results obtained for the confirmation of MGA in 5 samples of kidney fortified at 0.5 µg/kg and 5 samples of kidney fat fortified at the level of 1.0 µg/kg. From Table 5 can be concluded that there is no significant difference in the average ratio the two transitions ions for standards and fortified samples, so for sample analysis the standards can be used to calculate the ratio of transition ions.

[2] Remark: Conform the SANCO 1805/2000[2] the detection capability is defined as the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probability of ≤5%.

Table 6 Confirmation results of different samples of kidney fat fortified with 0.5 and 1.0 µg/kg MGA

	MGA in µg/kg	Ratio of the transition ions		
Standard	**0.75**	**0.33**		
Standard	**0.5**	**0.35**		
Standard	**0.38**	**0.36**		
Standard	**0.25**	**0.37**		
Standard	**0.13**	**0.44**		
			Average ratio:	**0.37**
			Tolerance:	**(0.37+30%=) 0.27** **(0.37-30% =) 0.46**
Sample 1	**0.5**	**0.31**	**Confirmed**	
Sample 2	**0.5**	**0.40**	**Confirmed**	
Sample 3	**0.5**	**0.33**	**Confirmed**	
Sample 4	**0.5**	**0.34**	**Confirmed**	
Sample 5	**0.5**	**0.35**	**Confirmed**	
Sample 6	**1.0**	**0.35**	**Confirmed**	
Sample 7	**1.0**	**0.39**	**Confirmed**	
Sample 8	**1.0**	**0.29**	**Confirmed**	
Sample 9	**1.0**	**0.39**	**Confirmed**	
Sample 10	**1.0**	**0.34**	**Confirmed**	

Conclusions

With the described method, based on automated SFE and GC-MSD, a quick and sensitive method became available for the analysis of gestagens (melengestrol, megestrol, medroxyprogesterone, chloromadinone) after hydrolysis in samples of kidney fat. With the automated system SFE detection limits of 1 µg/kg (for MGA without hydrolysis step 0.2 µg/kg) are obtained. The method shows good repeatability and reproducibility.

The use of LC-MS significantly reduces the amount of sample preparation needed. Also the formation of multiple isomers during derivatisation is avoided. Subsequently, the Limit of Detection is in the same order of magnitude as in the case of GC-MS detection. The nature of the identification criteria for MSMS, where only two ions have to be measured, allow full confirmation at lower levels than when using GC-MS. Based on these conclusions, LC-MS, in our opinion, is the preferred technique.

By using the SFE method the time consuming and laborious steps of LLE and HPLC fractionation can be omitted. A sample throughput of 20 samples a day is possible making automated SFE a technique suitable for routine applications.

References

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Approaches to "in house" method validation

The analytical modules described here can be combined into a analytical method for the (semi)-qauntitative determination of acetyl gestagens in samples of kidney fat or muscle tissue. The earlier mentioned document[3] describes in detail the procedures to be followed. Below some of the major aspects, as relevant for GC- and LC-MS methods for acetyl gestagens, are summarized below.

The methods can be considered as qualitative methods, permitting yes/no decisions (Screening) or identification without quantification (Confirmation). The use of internal standards also allow quantification. However, for residue testing quantification is optional. Validation only includes the quantitative values for the respective lower limits of the method. Therefor, full validation can be limited to the parameters listed below.

Performance Characteristics that have to be determined for qualitative methods.

	Detection Capability CCß	Decision Limit CCα	True-ness	Preci-sion	Selectivity/ Specificity	Applicability/ Ruggedness/ Stability	Limit of Identification	Limit of Quantification
S	+	-	-	-	+	+	-	-
C	+	+	-	-	+	+	-	-

Decision Limit. (CCα).

The Decision Limit is the limit from which on it can be decided that a sample is truly violative with an error probability of α. In the case of banned substances the Decision limit is the lowest concentration level, at which a method can discriminate with a statistical certainty of 1 - α whether the identified analyte is present. In the case of substances with an established MRL or MPL, this means that the Decision Limit is the concentration, above which it can be decided with a statistical certainty of 1 - α that the identified analyte content is truly above the MRL/MPL.

In the case of banned substances it can be established:

- Either by the calibration curve procedure according to ISO 11843 (here referred to as critical value of the net state variable). In this case blank material must be used, which is fortified at and above the minimum required performance level in equidistant steps. Analyse the samples. After identification, plot the signal against the added concentration. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the intercept equals the decision limit. This is applicable to quantitative assays only (α = 1%).

- Or by analysing at least 20 blank materials per matrix to be able to calculate the signal to noise ratio at the time window in which the

[3] Document SANCO 1805/2000 rev 1

analyte is expected. Three times the signal to noise ratio can be used as decision limit. This is applicable to quantitative and qualitative assays.

Detection capability (CCß)

This is the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probability of ß (Chapter 1.5). The ß-error should be less than or equal to 5%. In the case of banned substances the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1 - ß. In the case of substances with an established MRL or MPL, this means that the detection capability is the concentration at which the method is able to detect MRL/MPL concentrations with a statistical certainty of 1 - ß.

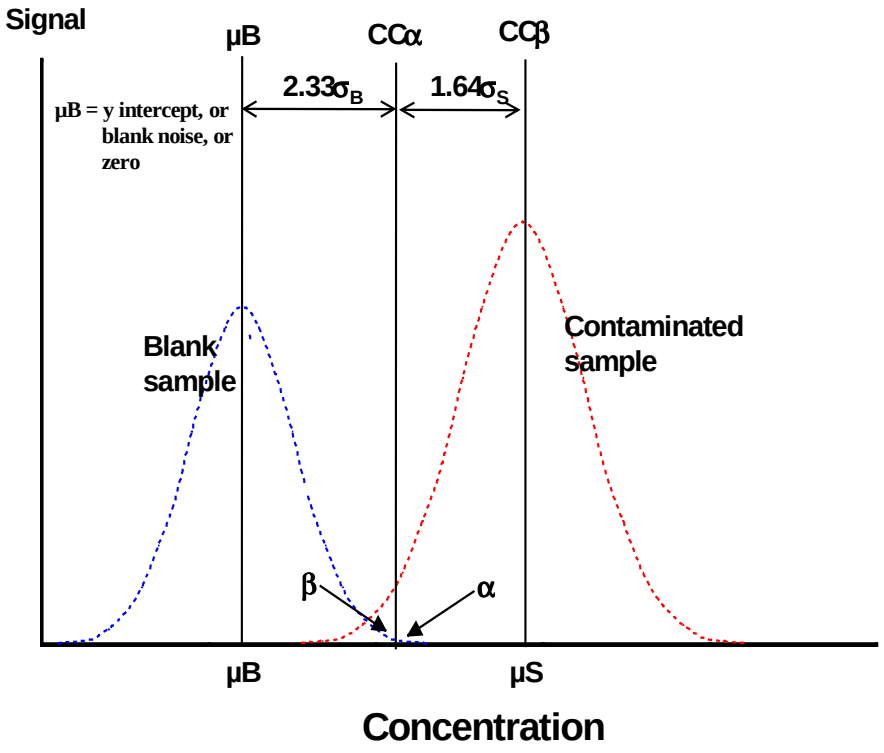
In the case of banned substances it can be established by:

- The calibration curve procedure according to ISO 11843 (here referred to as minimum detectable value of the net state variable). In this case representative blank material must be used, which is fortified at and below the minimum required performance level in equidistant steps. Analyse the samples. After identification, plot the signal against the added concentration. The corresponding concentration at the decision limit plus 1.64 times the standard deviation of the mean measured content at the decision limit equals the detection capability (ß = 5%).

- Analysing at least 20 blank materials per matrix fortified with the analyte(s) at the decision limit. Analyse the samples and identify the analytes. The value of the decision limit plus 1.64 times the standard deviation of the measured content equals the detection capability (ß = 5%).

- Where no quantitative results are available, the detection capability can be determined by the investigation of fortified blank material at and above the decision limit. In this case the concentration level, where only ≤ 5 % false negative results remain, equals the detection capability of the method. Therefore at least 20 investigations for at least one concentration level have to be carried out in order to ensure a reliable basis for this determination.

Graphical representation of the different analytical limits.



For screening purposes the detection capability (CC_β) is the most important parameter. This is the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probability of ß. A truly positive sample will be detected as such with a 95% certainty. The chances of obtaining a false negative result are < 5% (values truly at the detection capability, but giving a value below de decision limit (detection limit) (CC_α). After analysing a given set of samples (N) a sub-set (X) is obtained with a response exceeding the decision limit. With a 95% certainty the remaining (N-X) samples will all be truly negative. At this stage it is not yet known what the probability is that all positive samples are truly (confirmed) positive results. This however is known at the decision level. At this level it is known that the sample is truly positive (violative). At the level of the decision limit therefor, the method must fulfil all identification criteria applicable.
For confirmatory purposes the decision limit (CC_α) of the confirmatory method is the most important parameter. At this value confirmed positives are truly positive with an error probability < 1%.

However, for qualitative methods that are not based an a defined value for discrimination between "suspicious" samples and "truly positive" samples but on the ability of fulfilling all relevant identification criteria the use of the Limit of Identification is more appropriate. At the limit of identification the method should be able to confirm the identity of the analyte in 95% of all cases.
The table below summarizes the most practical approach to method validation for those cases where screening and confirmation are mutually dependant but performed with different analytical procedures.

Type of method	Parameter	Conclusion possible	Validation
Screening Single ion monitoring in MS	Detection capability (CC_β) No false negative results. No value known other than the LOD of th selected fragment ion as estimate for the decision limit	A truly positive sample will be detected as such with a 95% certainty	Analysing at least 20 blank materials per matrix fortified with the analyte(s) at the decision limit. The value of the decision limit plus 1.64 times the standard deviation of the measured content equals the detection capability (ß = 5%)
Screening Single ion monitoring in MS	Detection capability (CC_β) No false negative results A pre-set target value to be validated	A truly positive sample will be detected as such with a 95% certainty	Analysing at least 20 blank materials per matrix fortified with the analyte(s) at the target value. The number of false negative results must be ≤ 5% (maximum a singe observation)
Confirmation Multiple ion monitoring in MS^n or MS	Decision limit (CC_α) No false positive results	It can be decided that a sample is truly violative with an error probability of α.	Analysing at least 20 blank materials per matrix to be able to calculate the signal to noise ratio at the time window in which the analyte is expected. Three times the signal to noise ratio can be used as decision limit. This is applicable to quantitative and qualitative assays. However, in practice this is difficult to combine with the identification crietria.
Confirmation Multiple ion monitoring in MS^n or MS	Limit of identification A pre-set target value to be validated	It can be decided that a sample is truly violative with an error probability of α.	Analyse 20 spiked samples. At the limit of Identification 95% should be confirmed according to the relevant identification criteria

Analytical procedures for gestagens

Analysis of kidney fat samples for gestagens (method based on Liquid Liquid Extraction, Solid Phase Extraction, HPLC fractionation, derivatisation and GC-MS).
ARO SOP 399.
Detailed description of extraction procedures for fat, HPLC extract purification of acetylated gestagens, alkaline hydrolysis, derivatisation and GC-MS. No further details on confirmatory (identification) analysis other than listing of possible dignostic ions.

Limit of detection is 1.0 µg/kg for most compounds. For Melengestrol (acetate) the limit of detection is 2.0 µg/kg.

Analysis of muscle tissue for gestagens (method based on enzymatic digestion, HPLC fractionation, derivatisation and GC-MS).
Based on ARO SOP 113.
Detailed description of enzymatic digestion of muscle tissue, followed by LLE, defatting and HPLC extract purification of acetylated gestagens, alkaline hydrolysis and GC-MS.

Analysis of muscle tissue an kidney fat gestagens by SFE / GC-MS of SFE LC-MS (modular approach to screening for gestagens.)
Detailed description of the preparation of a primary extract of muscle tissue after lyophilisation or enzymatic digestion followed by SFE. After elution of the acetylated gestagens from an Aluminium oxide containing SPE cartridge the analytes are either analysed by GC-MS after alkaline hydrolysis and derivatisation, or, alternatively, directly by LC-MS

Evaluation of confirmatory procedures

Standard Operating Procedure (SOP)

Laboratory for Residue-Analysis (ARO)
Antonie van Leeuwenhoeklaan 9 3721 MA BILTHOVEN
Postbus 1 3720 BA BILTHOVEN
Tel. (secretariat) 31 30 2742613 Fax. 31 30 2744403

ARO doc.nr.: 16299

Title : Analysis of kidney fat sample for gestagens

SOP-no.	**: ARO/399**
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1. Introduction

Throughout the European Union, the use of anabolic agents is prohibited in food producing animals. Also the Maximum Residue Levels (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.

2. Scope

This method of analyses describes the detection and confirmation of the presence of gestagens in samples of kidney fat. Within the field of application only semi-quantitative methods are needed. However, when isotope enriched internal standards are available and the purity of the standard used for identification and calibration is known, the method can be considered quantitative.

3. Field of application

The method is used to perform routine screening and confirmation analyses of samples. The limit of detection is 1.0 µg/kg for Chloromadinone(acetate)(CM(A)), Megestrole(acetate) (MGCA), Medroxyprogesterone(acetate)(MP(A)). For Melengestrole(acetate)(ML(A). the limit of detection is 2 µg/kg. The limit of detection is based on the detection of the most abundant diagnostic ion with a response S/R ≥ 3 at the correct retention time. The limit of identification, ranges from 2.0 - 5.0 µg/kg. depending of the analyt The limit of identification equals the limit of detection as based on the diagnostic ion with the weakest intensity.

4. References

Commission decision 93/256/EEC of 14 April 1993 laying down the methods to be used for detecting residues of substances having a hormonal or a thyrostatic action.
Off. J. Europ. Comm. 118 (1993) 64-74.

Commission decision 93/257/EEC of 15 April 1993 laying down the reference laboratories for detecting residues.
Off. J. Europ. Comm. 118 (1993) 75-79.

N. Haagsma, G. Ellen, R.W. Stephany en W.G. de Ruis. Begrippen bij de bepaling van residuen in voedingsmiddelen van dierlijke oorsprong. (Ware(n) Chemicus 21 (!991) 82-95.

5. Definitions

The amount of analyt in the test sample, determined according to the described method, is expressed as µg/kg of test sample regardless of the chemical form of the analyt. All other definitions are according to Haagsma et al.

6. Principle of the Method

The method is based on the use of isotope enriched compounds as internal standards. Samples are extracted (primary extract) with an organic solvent. The primary extract is cleaned on a SPE Florisil column and subsequent liquid - liquid partition. Further purification is achieved by High Performance Liquid Chromatography. The HPLC-fraction containing the gestagens is derivatized and analysed by gas chromatography-mass spectrometry. Quantification is based on

isotope dilution linear regression lines. Additional ions have to be monitored for confirmation of the identity of the analyte.

7. Materials

7.1. Standards

Standards are checked for identity (GC-MS and/or FTIR) and purity (HPLC). All reference substances available are registered in ARO-MIS CB\PREP.

Standards : Medroxyprogesteron acetate (MPA)
Medroxyprogesteron acetate-d3 (MPA-d3)
Megestrol acetate (MA)
Megestrol acetate-d3 (MA-d3)
Melengestrol acetate (MGA))
Melengestrol acetate-d3 (MGA-d3)
Chloromadinone acetate (CMA)
Chloromadinone acetate-37Cl (CMA-^{37}Cl)

The standards used for identification and calibration are registrated in the ARO-MIS database CB\ROB. The isotope enriched internal standards are obtained through the "Bank of Reference Standards" (EC/MAT).
Stock solutions, containing 1 g/l, are prepared for all standards. in ethanol. These solutions are registered and stored in the dark at approximately -20°C (not higher than -10°C) for a period of maximum 5 years. A 10-fold dilution (0.1 g/l) of these standards is also stored under the same conditions. Working solutions are prepared by 10-fold dilution of the 0.1 g/l solution. These solutions are stored in the dark at approximately 4°C (range 1-10°C) for a maximum period of 6 months.

7.2. Chemicals

All listed chemicals are of Pro Analyse quality or better, unless stated otherwise. Solutions are stored at room temperature and expire 6 months after preparation, unless stated otherwise. Water is twice distilled.

7.2.1. Methanol (Merck, art no. 6007).
7.2.2. n-Hexane (Merck, art no. 4367).
7.2.3. Sodium sulphate anhydrous (BDH, art no. 10398)
7.2.4. Florisil column 6 ml (Baker, art no. 7213-07)
7.2.5. Ethanol (Merck, art no. 983)
7.2.6. Acetonitrile (Merck, art no. 3.)
7.2.7. Glass microfibre filters GF/F (Whatman, 1825 047)
7.2.8. TBME tert-Buthylmethyl ether (Merck, art no. 1845)
7.2.9. SPE Solvent : Acetonitrile/water 95:5 Mix 95 ml acetonitrile with 5 ml water.
7.2.10. SPE Solvent : Methanol/water 40:60. Mix 40 ml methanol with 60 ml water
7.2.11. HPLC column Lichrospher 100 endcapped RP18 (5 mm) 125 x 4 mm (Merck, art no. 50828)
7.2.12. HPLC guard column Lichrospher 100 endcapped RP18 (5 mm) 4 x 4 mm (Merck, art no. 50962)
7.2.13. HPLC Eluens pump A : methanol/water 70:30, mix 700 ml methanol with 300 ml water and filter the solution through a (Whatman GF/F) filter (7.2.7.).
7.2.14. HPLC Eluens pump B : methanol 100 % (7.2.1)

7.2.15. Heptafluorbutyric acid anhydride (HFBA), (Pierce, art no. 63163).
7.2.16. Iso-octane (Merck, art no. 4718).
7.2.17. Acetone (Merck, art no. 14).

7.2.18. Acetic acid (Merck, art no. 63).
7.2.19. Sodium acetate (Merck, art no. 6268).
7.2.20. Acetate buffer, 2 mol/l, pH 5.2. Dissolve 25.2 g acetic acid (7.1.3.8.) and 129.5 g sodium acetate (7.1.3.9.) in 800 ml water. Adjust the pH (7.1.4.20.) at 5.2 ± 0.1 and add water to a final volume of 1000 ml.
7.2.21. Hydrochloric acid, 37% solution (Merck art no. 317).
7.2.22. Acidic buffer. Mix 1.7 ml hydrochloric acid (7.1.3.11.) with 98.3 ml 2 mol/l Acetate buffer (7.2.20).
7.2.23. Potassium hydroxide (Merck, art no. 5033).
7.2.24. Alkaline hydrolysis solution. Dissolve 0.56 g potassium hydroxide (7.2.23.) in 10 ml methanol (7.2.1.). **Make this solution fresh every day.**
7.2.25. Derivatization reagent : mix 1 part of HFBA (7.2.15.) with 4 parts of acetone (7.2.17.) v/v. Make this solution just before use.

7.3 Samples

Samples of kidney fat are stored in the dark at approximately -20°C, but not higher than
-10°C, until analysis. As u rule quality control samples are included. Details on these samples are always included in the mandatory study plan.

7.4. Apparatus

For operating instructions and maintenance status see ARO-MIS database CB\INVENTAR.
Standard laboratory glassware and equipment is used, with in addition:

7.4.1. Glass centrifuge tubes 50 ml (Corex).
7.4.2. Vortex mixer.
7.4.3. Automatic pipettes (Gilson P100, P200, P1000 and P5000).
7.4.4. Refrigerated centrifuge (RC-3, Sorvall).
7.4.5. Centrifuge tubes RB100, glass (100 mm x 14/15 mm) (Renes, RB100 art no 31.00.50.).
7.4.6. Electric waterbath with thermostat adjustable ± 5°C (GFL) with nitrogen facility.
7.4.7. Incubator thermostat adjustable ± 3°C (Salvis)
7.4.8. Heating module thermostat adjustable ± 5°C (Pierce 18790) with nitrogen facility.
7.4.9. Glass derivatization vials (Chromacol 2SV (A)) with screw caps (Chromacol 02-MTV) and aluminium caps (Chromacol 11-AC5).
7.4.10. HPLC equipment.
7.4.10.1. HPLC gradient-system (2 pumps 2150 and a controller 2252) (Pharmacia).
7.4.10.2. UV detector UV 2000 (Thermo Separations Products).
7.4.10.3. Autoinjector AS3000 (Thermo Separations Products).
7.4.10.4. Fraction collector 2112 Redirac (Pharmacia).
7.4.10.5. Workstation PC1000 to intergrate chromatograms and for switching valves (Thermo Separations Products).
7.4.11. GC-MS equipment.
7.4.11.1. Gas chromatograph (Hewlett Packard, type 6890).
7.4.11.2. Automatic injector (Hewlett Packard, type 7673A).
7.4.11.3. Mass selective detector (Hewlett Packard, type 5972A).

7.4.11.4. Workstation (Hewlett Packard, MSD Chem Station).
7.4.11.5. Printer (Hewlett Packard, Laserjet 4 plus).
7.4.11.6. GC-column, HP-1 Column id: 0.20 mm, film thickness 0.11 µm, length 25 m (Hewlett Packard, 19091Z-002).
7.4.12. pH-meter, (Schott, CG 837).
7.4.13. HPLC vials (Chromacol, 1.1-STVG).

7.4.14. Ultra turrax (Janke & Kunkel 20000 rpm).
7.4.15. SPE 21 solid phase system (Baker).
7.4.16. Adaptor (Baker, 7122-00).
7.4.17. Reservoir 75 ml (Baker, 7120-03).
7.4.18. Ultrasonic waterbath (Bransonic 32).
7.4.19. Caps for tube (7.4.5.).
7.4.20. pH-meter, (Applicon)

8. Method of analysis

8.1. Preparation of a primary extract
If a laboratory sample is considered suitable for analysis (adequate sample size, proper storage history and representative for analysis) the first step in the procedure is the preparation of a primary extract, including the procedures for sample cleanup and defatting.

8.2. Preparation of a primary fat extract
8.2.1. Weigh 10 g of kidney fat (cut in small cubes) in a glass centrifuge tube (7.4.1.) and add 20 ng of a mixture of internal standards (200 µl from a mixture containing 0.1 ng/µl (7.1.) of each compound. A Series of 16 samples includes a blank sample (see 8.2.1.1.) and a spiked sample (see 8.2.1.2.).
8.2.1.1. Add internal isotope labelled standards at a level of 2.0 µg/kg (5.0 µg/kg for MLA-d3) to 10 g of blank kidney fat (= blank sample).
8.2.1.2. Add standards and internal isotope labelled standards at a level of 2.0µg/kg (5.0 µg/kg for MLA and MLA-d3) to 10 g of blank kidney fat (= ”spiked” sample).
8.2.2. Add 30 ml n-Hexane (7.2.2.) to each sample and also to the blank and spiked sample.
8.2.3. Incubate 10 minutes at 50°C.
8.2.4. Mix 30 seconds with the ultra turrax at 20000 rpm.
8.2.5. Add 1.5 g Sodium sulphate anhydrous (7.2.3.).
8.2.6. Mix by placing the tube on a Vortex (7.4.2.) for 30 seconds.
8.2.7. Centrifugate (7.4.4.) 15 minutes at 3000 rpm at 4°C. Let the tubes stand for another 15 minutes at 4°C.
8.2.8 Preparing the SPE column
8.2.8.1. Place the Florisil (7.2.4.) columns on the SPE 21 system (7.4.15).
8.2.8.2. Condition the column with 3 x 5 ml n-Hexane (7.2.2.).
(Don't let the columns run dry).
8.2.8.3. Install adaptor (7.4.16.) and reservoir (7.4.17.) on the column.
8.2.8.4. Put the funnel, filled with glasswool, on the reservoir.
8.2.8.5. Pour the supernatant (8.2.7.) in the funnel.
8.2.8.6. As the sample has passed the funnel, remove the funnel.
8.2.8.7. Draw the supernatant slowly through the column by increasing the vacuum.
8.2.8.8. As the supernatant has passed through the column, increase the vacuum, and dry the column during 10 minutes.
8.2.8.9. Place a rack with tubes (7.4.5.) under the columns.
8.2.8.10. Elute the columns with 6 ml of ethanol (7.2.5.).

8.2.9. Washing and extracting

8.2.9.1. Evaporate the ethanol to dryness under a cold stream of nitrogen in a water- bath at 50°C.

8.2.9.2. Dissolve the dry residue in 2 ml of acetonitrile/water (7.2.9.) by placing the tube in an ultrasonic waterbath (7.4.18.) for two minutes, followed by placing the tube on a Vortex (7.4.2.) during 30 seconds.

8.2.9.3. Add 6 ml of n-hexane (7.2.2.) and put a cap (7.4.19) on the tube. Mix by placing the tube on a Vortex (7.4.2.) for 30 seconds.

8.2.9.4. Centrifugate (7.4.4.) 10 minutes at 2500 rpm at 20°C.

8.2.9.5. Remove the n-hexane.

8.2.9.6. Repeat steps 8.2.9.3 - 8.2.9.5.

8.2.9.7. Evaporated to dryness under a cold stream of nitrogen in a waterbath at 50°C.

8.2.9.8. Dissolve the dry residue in 2 ml of methanol/water (7.2.10) by placing the tube in an ultrasonic waterbath (7.4.18.) for two minutes, followed by placing on a Vortex (7.4.2.) during 30 seconds.

8.2.9.9. Add 6 ml of TBME (7.2.8.) and put a cap (7.4.19) on the tube. Mix by placing the tube on a Vortex (7.4.2.) for 30 seconds.

8.2.9.10. Centrifugate (7.4.4.) 5 minutes at 2500 rpm at 20°C.

8.2.9.11. Transfer the TBME to a clean tube (7.4.5.).

8.2.9.12. Evaporate the TBME to dryness under a cold stream of nitrogen in a water- bath at 40°C.

8.2.9.13. Repeat the procedure and combine the TBME extracts (8.2.9.9 - 8.2.4.12.).

8.2.9.14. Dissolve the dry residue in 120 µl HPLC eluens (7.3.3.) by placing the tube in an ultrasonic waterbath (7.4.18.) for two minutes, followed by placing on a Vortex(7.4.2.) during 30 seconds.

8.2.9.15. Transfer the extract into a HPLC-vial (7.4.13.).

8.3 HPLC purification

The following reversed phase system has proven to be adequate for extract purification :

Guard-column	: LichroCart 4-4 (7.2.12.)
Analytical column	: LichroCart 125-4 (7.2.11.)
Flow rate	: 0.8 ml/min
Injection volume	: 100 µl

A gradient system is required in order to collect all fractions.
The variability of the retention time should be less than 0.1 minute.
A standard mixture of compounds (approximately 50 ng of each compound) is injected at least 3 times, to monitor the retention times. Before starting analysing the samples, injection of a blank sample is recommended (control for carry-over).

The gradient conditions are :
Pump A Solvent (7.2.13.)
Pump B Solvent (7.2.14.)

0.1 minute to 5.0 minutes	A = 100 %	and	B = 0 %
5.0 minutes to 12.0 minutes	A = 100 %	to	A = 58 % + B = 42 %
12.0 minutes to 14.0 minutes			A = 58 % + B = 42 %
14.0 minutes to 15.0 minutes	B = 42 %	to	A = 0 % + B = 100 %

15.0 minutes to 17.0 minutes A = 0 % + B = 100 %
17.0 minutes to 17.5 minutes B = 100 % to A = 100 % + B = 0 %

The times during which the fraction is collected are calculated as follows:
start fraction : RT from the first peak + 0.3 minute - 1.0 minute.
end fraction : RT from the last peak + 0.3 minute + 1.0 minute
(0.3 minute describes the transfer time between the detector and the collecting tube).
The eluent is removed under a cold stream of nitrogen in a water bath at 50°C.

8.4 Alkaline hydrolysis

8.4.1. Pipet directly into the tubes (7.4.5) aliquots of the standard solutions. The following amounts are used ; 0, 10, 20, 40, and 50 ng of analytes respectively. The amount of the internal standard must be identical to the amount added to the samples.
8.4.2. Dry the standards under a cold stream of nitrogen in a water bath at 50°C.
8.4.3. The dry standards and HPLC fractions are dissolved in 0.2 ml alkaline hydrolysis solution (7.2.24.). This mixture is incubated at 37°C (7.4.7.) for 30 minutes.
The hydrolysis is ended by the addition of 0.8 ml acidic buffer (7.2.22).
8.4.4. Add 6 ml TBME (7.2.8.) to the mixture. Mix by placing the tube on a Vortex (7.4.2.) for 30 seconds. Centrifugate for 5 minutes at 2500 rpm at 20°C, and transfer the TBME to a clean tube. Repeat the procedure of TBME extraction.
8.4.5. The combined supernatants (TBME) is evaporated to dryness under a stream of nitrogen in a waterbath (7.4.6.) at 40°C.

8.5 Derivatization

8.5.1. Dissolve the dry residue in 0.4 ml ethanol (7.2.5.) by placing the tube in an ultrasonic waterbath (7.4.18.) during 1 minute followed by placing on a Vortex (7.4.2.) during 130 seconds.
8.5.2. Transfer the residue to a derivatisation vial (7.4.9.).
8.5.3. The ethanol is evaporated in a heating module (7.4.8.) under nitrogen.
8.5.4. Add the 0.050 ml HFBA reaction mixture (7.2.25) and place the tube on a Vortex during 30 seconds followed by incubation of the reaction mixture during 1 hour at 60°C (7.4.7.).
8.5.5. After incubation, the reaction mixture is evaporated to dryness under a stream of nitrogen at 50°C (7.4.8.) and the derivatized residue is dissolved in 0.025 ml iso-octane (7.2.16.) by placing in an ultrasonic waterbath (7.4.18.) during 1 minute, followed by using a Vortex (7.4.2.) during 30 seconds.
8.5.6. The residue is transferred into a glass injection vial with glass insert and is closed with an aluminium cap (7.4.9.).

8.6 Gas chromatography-mass spectrometry

The following conditions are used during GC-MS analysis :

column	: HP-1 (7.4.11.6.)
injection	: 5 µl splitless
injector temperature	: 250°C
initial oven temperature	: 80°C (1 minute)
temperature programme	: 25°C/min
final temperature	: 300°C

temperature transfer line	: 280°C	
solvent delay	: 7.2 minutes	
ions monitored	: m/z 479	MP
	: m/z 482	MP-d3
	: m/z 477	MG
	: m/z 480	MG-d3
	: m/z 447	ML
	: m/z 450	ML-d3
	: m/z 497	CM
	: m/z 499	CM-^{37}Cl
dwelltime per ion	: 50 msec	

9. Interpretation and calculation

9.1 Preliminary tests

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 derivatized standards.
- adequate signal to noise ratio for internal standards (>5).

9.2 Control samples

The experiment is valid only if no signals corresponding any of the gestagens are present exceeding a level of 0.5 µg/kg within the chromatogram of the blank, the quantification of spiked compounds is between 50% and 200% of the target values.

9.3 Calculation of quantitative results

The area of the selected ion of the standard and the internal standard are calculated and their ratio is the response variable.
A calibration curve is constructed by linear regression analysis of the response variable versus the concentration.
Quantification is only valid if :
- the maximum of the signal origination from the analyt exceeds the noise + 3 SD,
- the coefficient of correlation for the calibration curve is better than 0.98,
- the numerical value of the intercept of the calibration curve does not deviate more than
± 3 SD from zero.

9.3. Confirmation (identification)

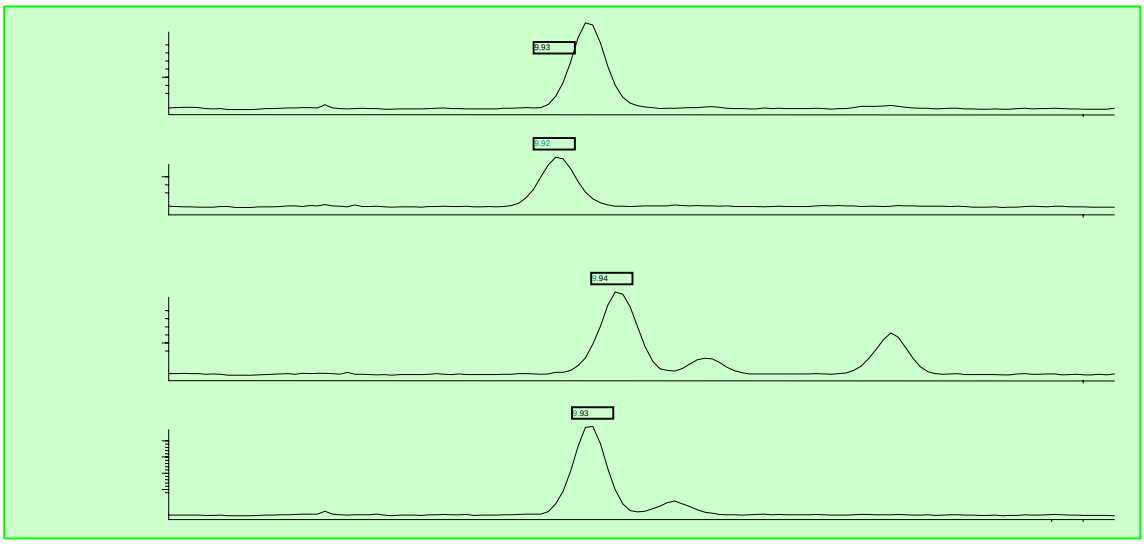
For identification according to the EC-criteria it is mandatory that at least 4 ions are monitored.

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 calcutated for the standard analyt, preferably at the corresponding concentration. For positive identification the responses obtained for the unknown sample should preferably be within ± 10 % of the average value of the standard.

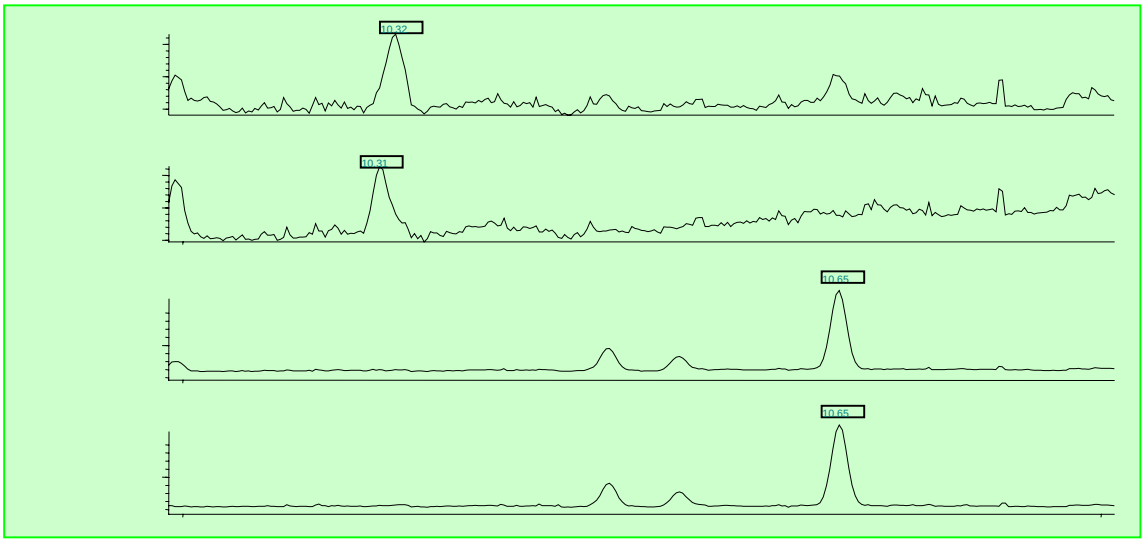
Table 1: Diagnostic ions monitored during confirmation analyses.

	HFBA ions most suitable derivative			
Medroxyprogesterone	147	317	331	479
Megestrol	381	421	477	520
Melengestrol	281	343	383	447
Chloromadinone	401	462	497	540

Typical chromatograms obtained for samples of kidney fat spiked with 5 µg/kg of each of the respective gestagens, inclusive the deuterated analogues.



From top to bottom, Medroxyprogesterone (deuterated analogue) and Megestrol (deuterated analogue)



From top to bottom, Melengestrol (deuterated analogue) and Chlormadinone (Cl^{37} analogue)

Laboratory for Residue-Analysis (ARO)
Antonie van Leeuwenhoeklaan 9 3721 MA BILTHOVEN
Postbus 1 3720 BA BILTHOVEN
Tel. (secretariat) 31 30 2742613 Fax. 31 30 2744403

ARO doc.nr.:

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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 deconjugation and if appropriate defatting.

Enzymatic digestion
From the laboratory sample a test sample of 50-100 g is homogenized thoroughly 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, containing 5 mg Subtilisin A. The mixture is shaken a few times and incubated during 2 hours at 55^{o}C, shaking at least every 15 minutes.
After the samples are cooled to room temperature and extracted twice with 20 ml TBME . The combined extracts are evaporated to dryness under a stream of nitrogen in a waterbath at 50^{o}C.

Defatting of the primary extract
The dry residue is dissolved in 4 ml methanol-water (4:1, v/v) and washed twice with 6 ml petroleum ether. 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.

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.

High performance liquid chromatography

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

pre-column	chromguard-reversed phase cartridge
analytical column	ODS-hypersil C18 (150 x 4.6 (mm x mm))
eluent	MEOH:CH_3CN:H_2O-5:40:55
flow-rate	1.5 ml/min
detection	254 nm

The residue is dissolved in 0.120 ml of the HPLC eluent of which subsequently 0.100 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 eluent is removed under a cold stream of nitrogen in a water bath at 50^{o}C.

Alkaline hydrolysis

Since gestagens will be (partly) present in the form of small esters an additional basic hydrolysis is applied. The residue obtained is dissolved in 0.2 ml alkaline hydrolysis solution. This mixture is incubated at 37^{o}C for 30 minutes. The hydrolysis is ended by the addition of 1.0 ml acidic buffer. This mixture is extracted twice with 6 ml TBME. 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.

GC-MS analysis

The following conditions are used during GC-MS analysis :

GC	HP 5890
column	Macherey-Nagel Permabond^{R} SE 52
injection	1-5 µl splitless 225^{o}C
temperature program	100^{o}C - 280^{o}C at 20^{o}C/min.
temperature transfer line	280^{o}C
MS	HP 5970
acquisition :	see Table 1

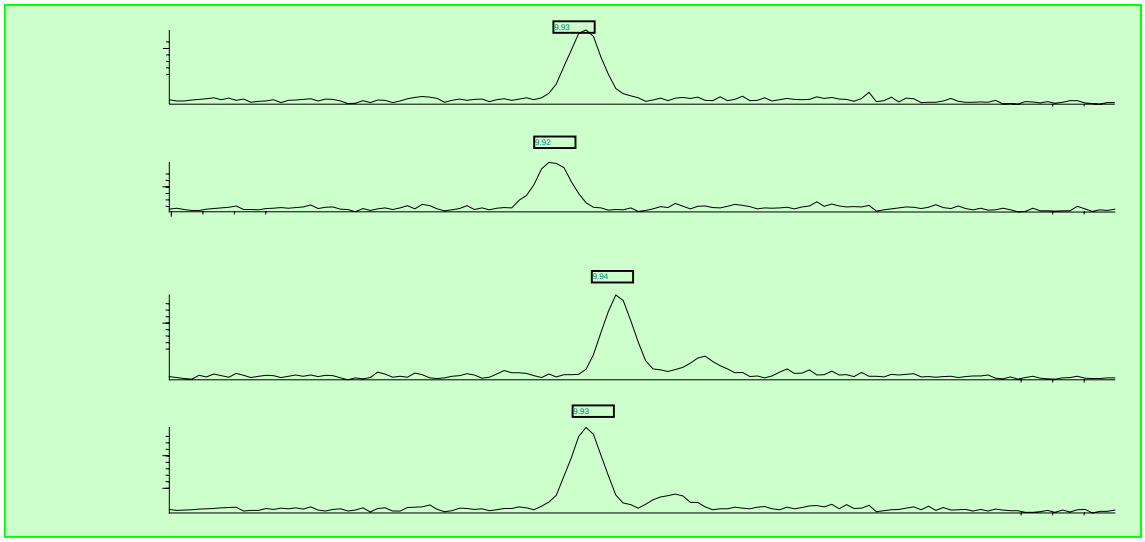
The number and concentrations of GC-MS standards (standards derivatized without additional analytical manipulations) depends on the application. For quantitative analysis within the low ppb range a minimum of five standards is prepared over the range of 1 - 50 ng per derivatization vial for all analytes included in the experiment. Each vial includes the internal standard in an amount equal to the amount added to the samples. 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.

The ions monitored during GC-MS analysis are listed in Table 1.

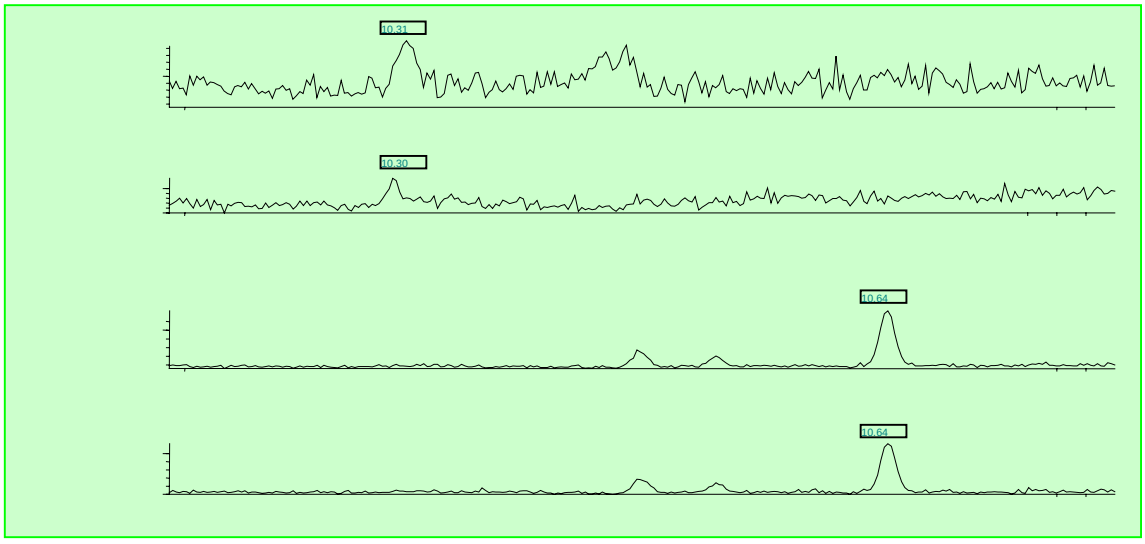
TABLE 1 : Ions monitored during screening analysis
after alkaline hydrolyses

Compound	m/z	Internal standard
Medroxyprogesterone acetate	479	482
Melengestrol acetate	447	450
Megestrol acetate	477	480
Chloromadionone acetate	497	499

Typical chromatograms obtained for samples of muscle tissue spiked with 5 µg/kg of each of the respective gestagens, inclusive the deuterated analogues.



From top to below, Medroxyprogesterone (deuterated analogue) and Megestrol (deuterated analogue)



From top to below, Melengestrol (deuterated analogue) and Chlormadinone (Cl^{37} analogue)

Laboratory for Residue-Analysis (ARO)
Antonie van Leeuwenhoeklaan 9 3721 MA BILTHOVEN
Postbus 1 3720 BA BILTHOVEN
Tel. (secretariat) 31 30 2742613 Fax. 31 30 2744403

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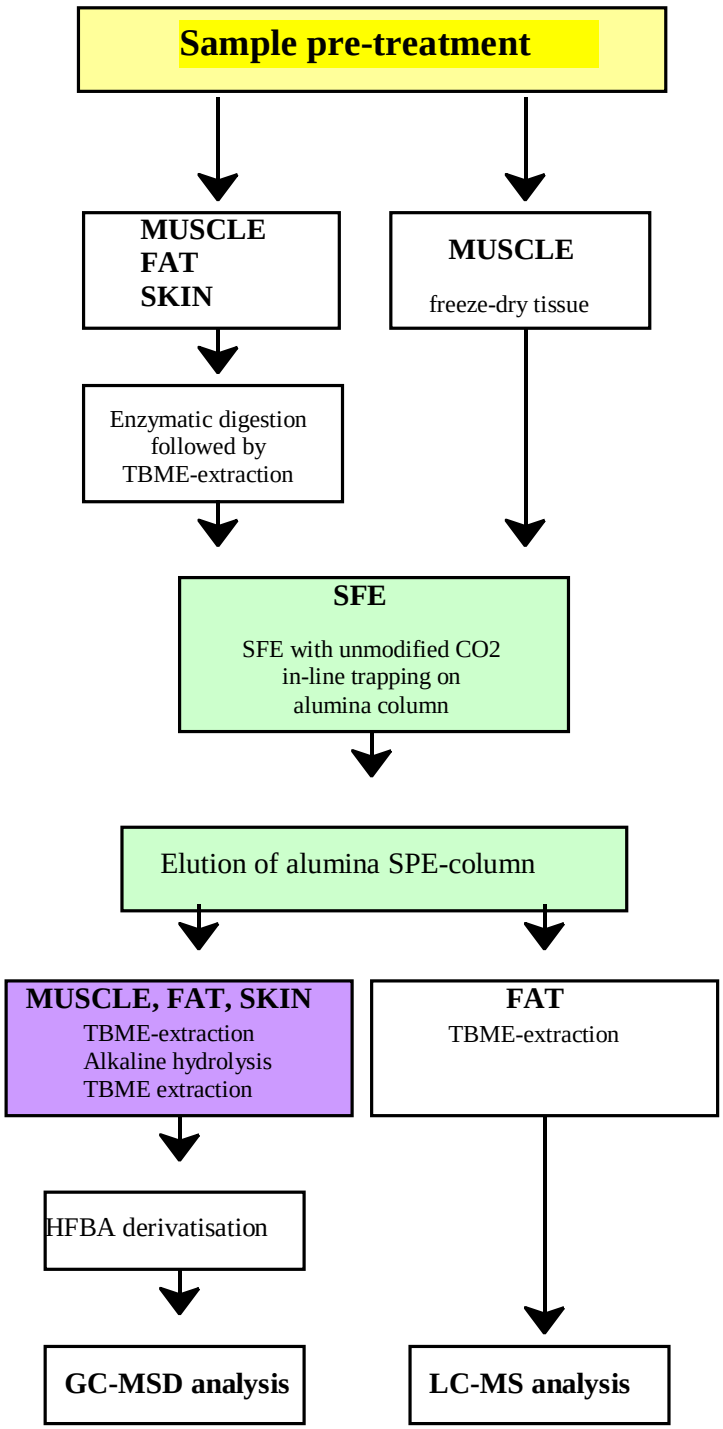

Sample pre-treatment
MUSCLE
FAT
SKIN
MUSCLE
freeze-dry tissue
Enzymatic digestion
followed by
TBME-extraction
SFE
SFE with unmodified CO2
in-line trapping on
alumina column
Elution of alumina SPE-column
MUSCLE, FAT, SKIN
TBME-extraction
Alkaline hydrolysis
TBME extraction
FAT
TBME-extraction
HFBA derivatisation
GC-MSD analysis
LC-MS analysis

Preparation of the primary extract

The preparation of the primary (SFE)-extract can be divided in three steps:

- Sample preparation for SFE
- Preparing the extraction cartridge and performing the Inline SFE extraction
- Preparing the sample for measuring

Muscle tissue:

From the laboratory sample, a test portion of 5.0 ± 0.1g is weighted into a 50 ml glass centrifuge tube.

Internal standards are added according to the relevant experimental design and (spiked) control samples are prepared (Table 1).

Allow the added standards to be fully absorbed (at least 30 min) and add 20 ml of 0.1 mol/l Tris-buffer, pH 9.5, containing 5 mg of Subtilisin A. The enzymatic digestion is performed during 2 hours at 55C, allowing non covalently protein bound residues to be freed from tissue cells.

After the enzymatic digestion the sample is cooled to room temperature and extracted twice with 20 ml of TBME. The solvent is evaporated under nitrogen and the dry residue is redissolved in 1 ml of TBME.

The TBME extract is poured on 2.3 gram of Extrelut sorbent in a beaker glass (50 ml). The solvent tube is washed with a second portion of 1 ml of TBME which too is added to the Extrelut mixture. Mix the TBME through the Extrelut with a spatula. Wait for 30 min until the TBME is evaporated and add 0.5 ml of water. Mix with a spatula.

Freeze dry Muscle:

Freeze-dry 5 grams of tissue sample conform general freeze-drying procedures for tissue (e.g. conform SOP ARO/404).

Weight 1.25 grams of the freeze-dried sample in a beaker glass (50 ml).

Internal standards are added according to the relevant experimental design and (spiked) control samples are prepared (Table 1).

Mix the sample with 2.0 gram of Extrelut in a beaker glass After at least 30 min, add 0.5 ml of water and mix with a spatula.

Fat:

Homogenise the fat sample by cutting the sample in small cubes.

Internal standards are added according to the relevant experimental design and (spiked) control samples are prepared (Table 1).

After at least 30 min, mix 1.0 grams of sample with 2.3 grams of Extrelut sorbent with a spatula. Add 0.5 ml of water and mix with a spatula.

Table 1: Adding Standards and Internal Standards

Analyte	Matrix	GCMS – ng/g	LCMS – ng/g
Medroxyprogesterone acetate	Fat	2.0	0.5
Medroxyprogesterone acetate-d3	Fat	2.0	-
Chloromadionone acetate	Fat	2.0	0.5
Chloromadionone acetate-^{37}Cl	Fat	2.0	-
Megestrol acetate	Fat	2.0	0.5
Megestrol acetate-d3	Fat	2.0	-
Melengestrol acetate	Fat	2.0	0.5
Melengestrol acetate-d3	Fat	2.0	1.0

Preparing the extraction vessel

1. Screw the unmarked end-cap fittings on the extractor vessels.
2. Poor the Extrelut mixture part by part through a funnel in the vessel and push each time with the steal tamping rod.
3. Put in a frit and push with the steal tamping rod.
4. Fill the vessel with Aluminium oxide to the top.
5. Screw the marked end-cap fittings on the top of the extractor vessels.
6. Place the vessels in the SFE –extractor with the marked-cap down.
7. Program the conditions in the extractor according the Table 2.

Table 2. *SFE conditions*

conditions SFE		Meat GCMS/LCMS	Fat GCMS/LCMS
Static	min	5	5
Dynamic	min	30	30
Oven temp	°C	60	60
Restrictor temp	°C	110	110
Flow	ml	2	1
Pressure	bar	500	300

Elution of analytes after SFE

1. Remove the extraction vessel from the SFE extraction system.
2. Place a 6 ml filtration column on the 12G SPE extractor.
3. Place the 10 ml collection tubes in the 12G SPE extractor.
4. Elute the Aluminium oxide with 2 x 3 ml Methanol/water 65:35.
5. Evaporate the collected liquid to approximately 0.5 ml.
6. Add 1 ml of water
7. Add 6 ml TBME to the mixture. Mix by placing the tube on a Vortex for 30 seconds. Centrifuge for 5 minutes at 2500 rpm at 20°C, and transfer the TBME to a clean tube. Repeat the procedure of TBME extraction.
8. The combined supernatants (TBME) are evaporated to dryness under a stream of nitrogen in a water bath at 40°C.

Extract clean up: schematic overview

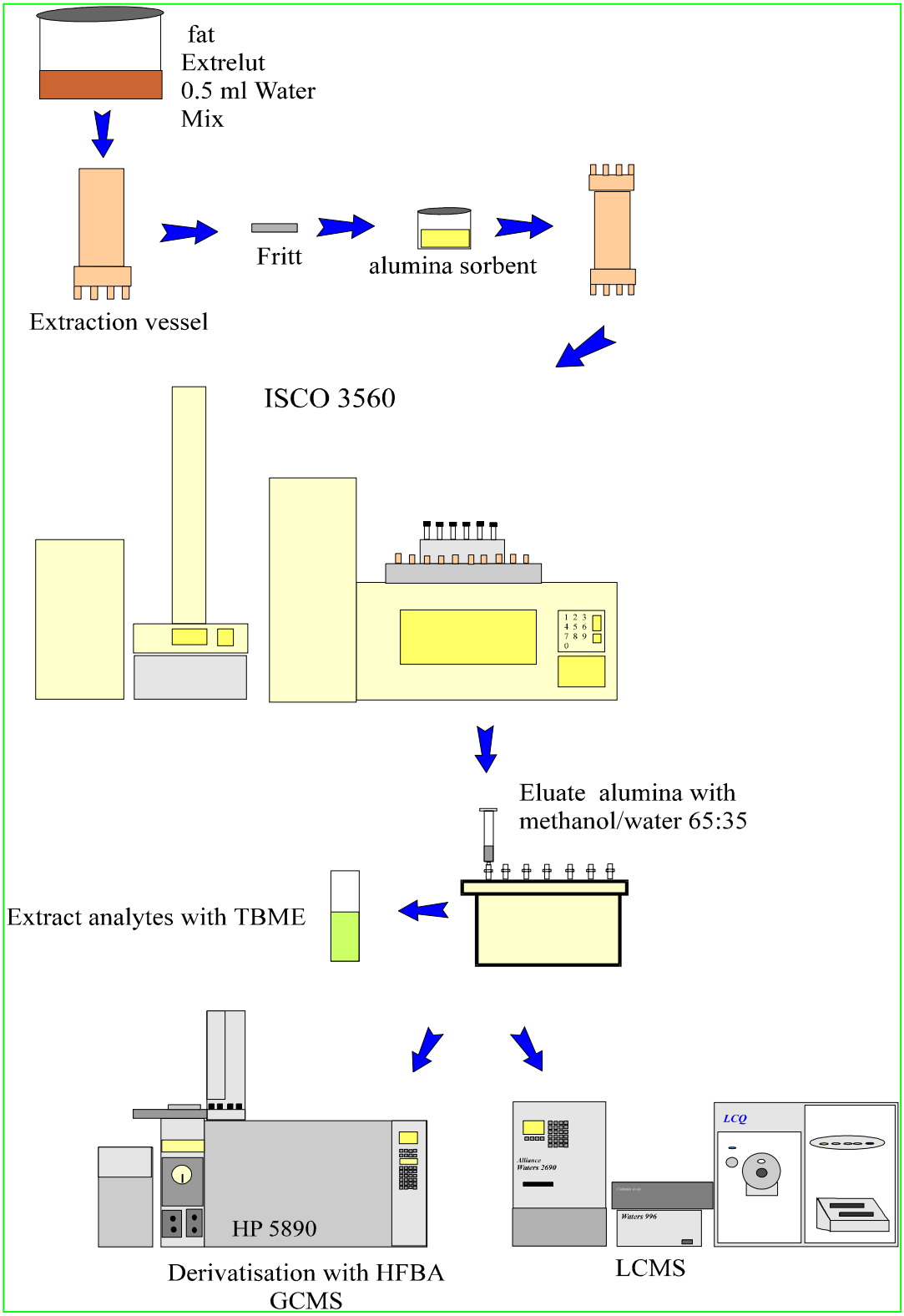


Table 3: Procedures following SFE.

Meat/Fat	**Meat**	**Fat**
	Alkaline hydrolysis	Alkaline hydrolysis
Dississolve in eluent	HFBA derivatisation	HFBA derivatisation
LC-MS	GC-MS	GC-MS

Table 4 Standard calibration curves for the different matrix analysi (absolute amounts).

No.	Fat-GCMS	Fat-LCMS
1	10 ng	4.0 ng
2	7 ng	2.0 ng
3	4ng	1.0 ng
4	2 ng	0.50 ng
5	1 ng	0.25 ng

The amount of the internal standard must be identical to the amount added to the samples.

Preparation of extracts for GC or LC-MS analysis

LC-MS Analysis

Preparation of control standards: Pipette directly into empty tubes aliquots of the standard (Table 4). The amount of the internal standard must be identical to the amount added to the samples. Evaporate the solvent under a cold stream of nitrogen in a water bath at 50°C.
Dissolve the dry extract in 120 µl of eluent. the sample is ready for LCMS analysis

LC-MS conditions

Capillary Temp (°C): 150.00
APCI Vaporizer Temp (°C): 450.00
Source Voltage (kV): 8.00
Source Current (µA): 5.00
Sheath Gas Flow (): 80.00
Aux Gas Flow (): 10.00
Capillary Voltage (V): 4.00
Tube Lens Offset (V): 30.00
Octapole RF Amplifier (Vp-p):400.00
Octapole 1 Offset (V): -4.50
Octapole 2 Offset (V): -7.50
InterOctapole Lens Voltage (V):22.00
Trap DC Offset Voltage (V): -10.00
Multiplier Voltage (V): 0.00
Maximum Ion Time (ms): 500.00
Ion Time (ms): 5.00
Data Type: Centroid
Source Type: APCI
Polarity: Positive
Zoom Micro Scans: 5
Zoom AGC Target: 10000000.00
Full Micro Scans: 1
Full AGC Target: 100000000.00
SIM Micro Scans: 1
SIM AGC Target: 20000000.00
MSn Micro Scans: 1
MSn AGC Target: 20000000.00
Injection Volume (µL): 50-100 µl,
Divert Valve: in use during run
Divert flow until (min): 3.00
Resume divert at (min): 8.00

HPLC Settings:
LC Run Time (min): 8.01
Column description: 99M0606
SuperODS 2uM (TOSOHAAS)
Inner diameter (mm): 4.600 Length (cm): 5.0
LC Pressure limits (psi): 0.0 - 3000.0
Number of solvents: 3
Solvent A = 50MeOH-50H2O
Solvent B = MeOH
Solvent C = MeOH/EtOH (50/50)

Pump program steps:

	Time (min)	Flow rate (mL/min)	%A	%B	%C
1:	0.00	0.90	100	0	0
2:	6.00	0.90	40	60	0
3:	6.01	0.90	0	0	100
4:	8.00	0.90	0	0	100
5:	8.01	0.90	100	0	0

MS Detector Settings:
Segments: 1

Duration (min) 11.50
Tune Method apcihighflo...
Scan Events 4

Segment 1 Scan Events:
1: Pos ·(385.2)->oS(323.6-326.6) CE = 20.0 % IsoW = 3.0
2: Pos ·(387.2)->oS(325.6-328.6) CE = 19.0 % IsoW = 3.0
3: Pos ·(398.6)->oS(335.7-341.7) CE = 20.0 % IsoW = 6.0
4: Pos ·(405.1)->oS(343.6-346.6) CE = 19.0 % IsoW = 3.0

Dependent Data Settings:
Reject Mass List: (none)
Parent Mass List: (none)
Default Charge State: 2
Default Isolation Width: 2.00
Collision Energy: 35.00
Min. Signal Required: 100000

GC-MS Analysis

Alkaline hydrolysis

1. Prepare control standards: Pipette directly into empty tubes aliquots of the standard (Table 4). The amount of the internal standard must be identical to the amount added to the samples.
2. Dry the standards under a cold stream of nitrogen in a water bath at 50°C. Standard solutions are treated the same as samples.
3. Evaporate the solvents from the samples solutions under a cold stream of nitrogen in a water bath at 50°C.
4. The dry standards and sample residues are dissolved in 0.2 ml alkaline hydrolysis solution. This mixture is incubated at 37°C for 30 minutes. The hydrolysis is ended by the addition of 0.8 ml acidic buffer.
5. Add 6 ml TBME to the mixture. Mix by placing the tube on a Vortex for 30 seconds. Centrifuge for 5 minutes at 2500 rpm at 20°C, and transfer the TBME to a clean tube. Repeat the procedure of TBME extraction.
6. The combined supernatants (TBME) are evaporated to dryness under a stream of nitrogen in a water bath at 40°C.

HFBA-derivatisation

Dissolve the dry residue in 0.4 ml ethanol by placing the tube in an ultrasonic waterbath during 1 minute followed by placing on a Vortex during 30 seconds. Transfer the residue to a derivatisation vial. The ethanol is evaporated in a heating module under nitrogen. Add the 0.050 ml HFBA reaction mixture and place the tube on a Vortex during 30 seconds followed by incubation of the reaction mixture during 1 hour at 60°C. After incubation, the reaction mixture is evaporated to dryness under a stream of nitrogen at 50°C and the derivatised residue is dissolved in 25µl iso-octane by placing in an ultrasonic waterbath during 1 minute, followed by using a Vortex during 30 seconds. Transfer the iso-octane to a GC-injection-vial with micro insert.

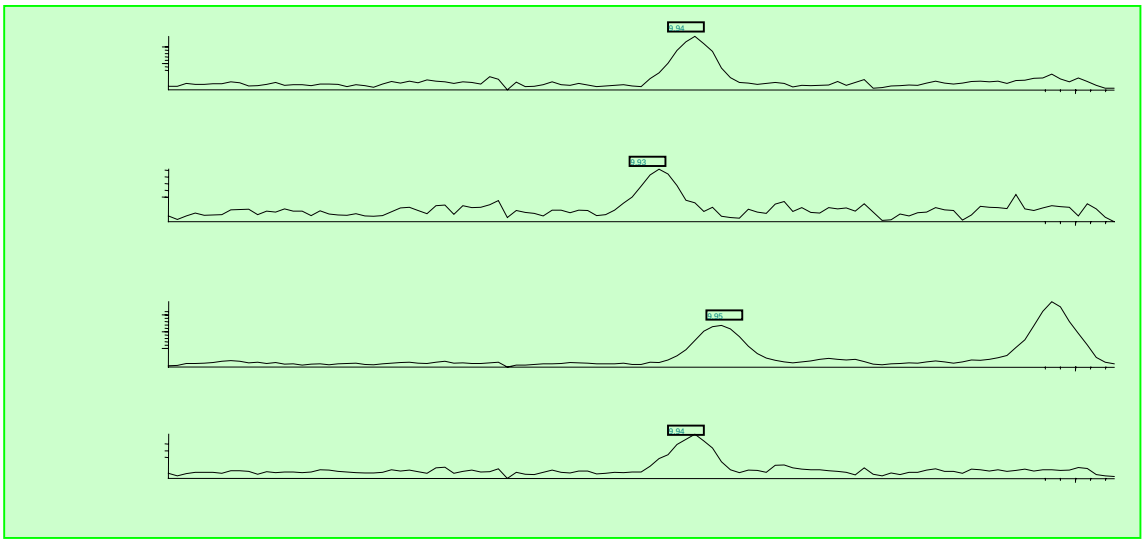
Table 5 *GC-MSD conditions*

	HFBA-derivatives
column	CP-SIL 24 CB
injection	3 µl splitless
injector temperature	250°C
initial oven temperature	80°C (1 minute)
temperature programme	25°C/min
final temp	325°C (4.2 min)
temperature transfer line	280°C
solvent delay	8.35 min
dwelltime per ion	20 msec

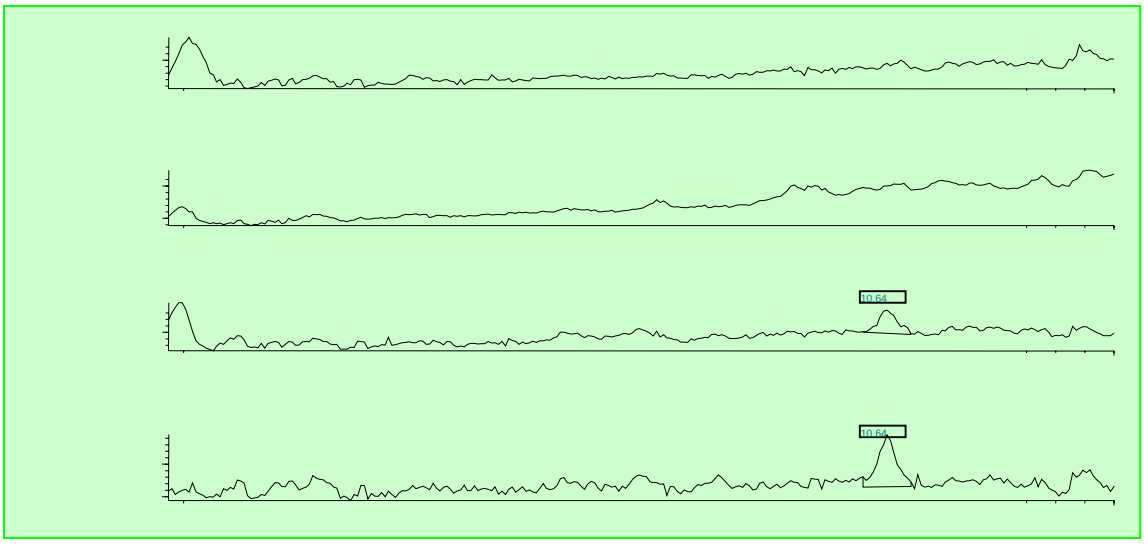
Table 6 Ions for LCMS/GCMS analysis

Fat method SFE/LC-MS	Screening	
		Internal-standard
	m/z	m/z
medroxyprogesteron acetate	327	
melengestrol acetate	340	343
megestrol acetate	325	
Choormadinone acetate	345	
Fat method SFE/GC-MS	Screening	
		int-standard
	m/z	m/z
medroxyprogesteron	479	482
melengestrol	447	450
megestrol	477	480
Choormadinone	497	499

Typical chromatograms (GC-MS analysis) obtained for samples of fat spiked with 1 µg/kg of each of the respective gestagens, inclusive the deuterated analogues.

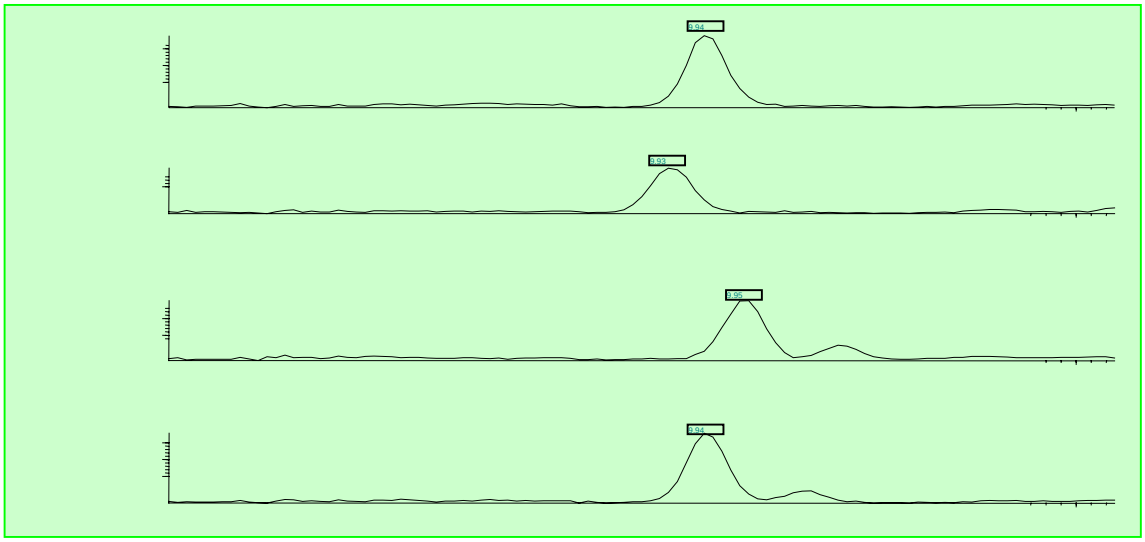


From top to below, Medroxyprogesterone (deuterated analogue) and Megestrol (deuterated analogue)

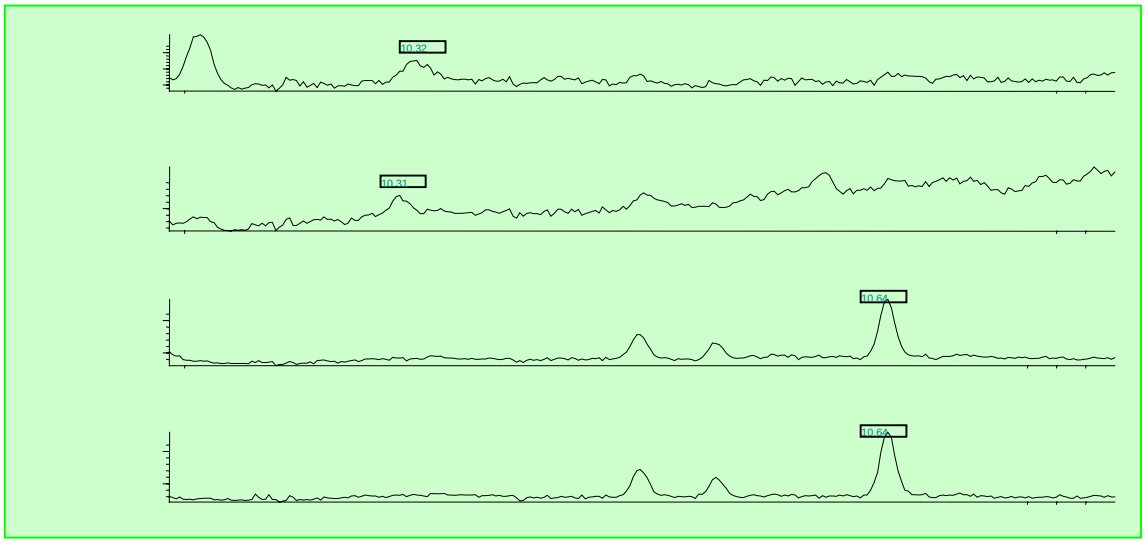


From top to below, Melengestrol (deuterated analogue) and Chlormadinone (Cl^{37} analogue)

Typical chromatograms (GC-MS analysis) obtained for samples of tissue spiked with 1 µg/kg of each of the respective gestagens, inclusive the deuterated analogues.

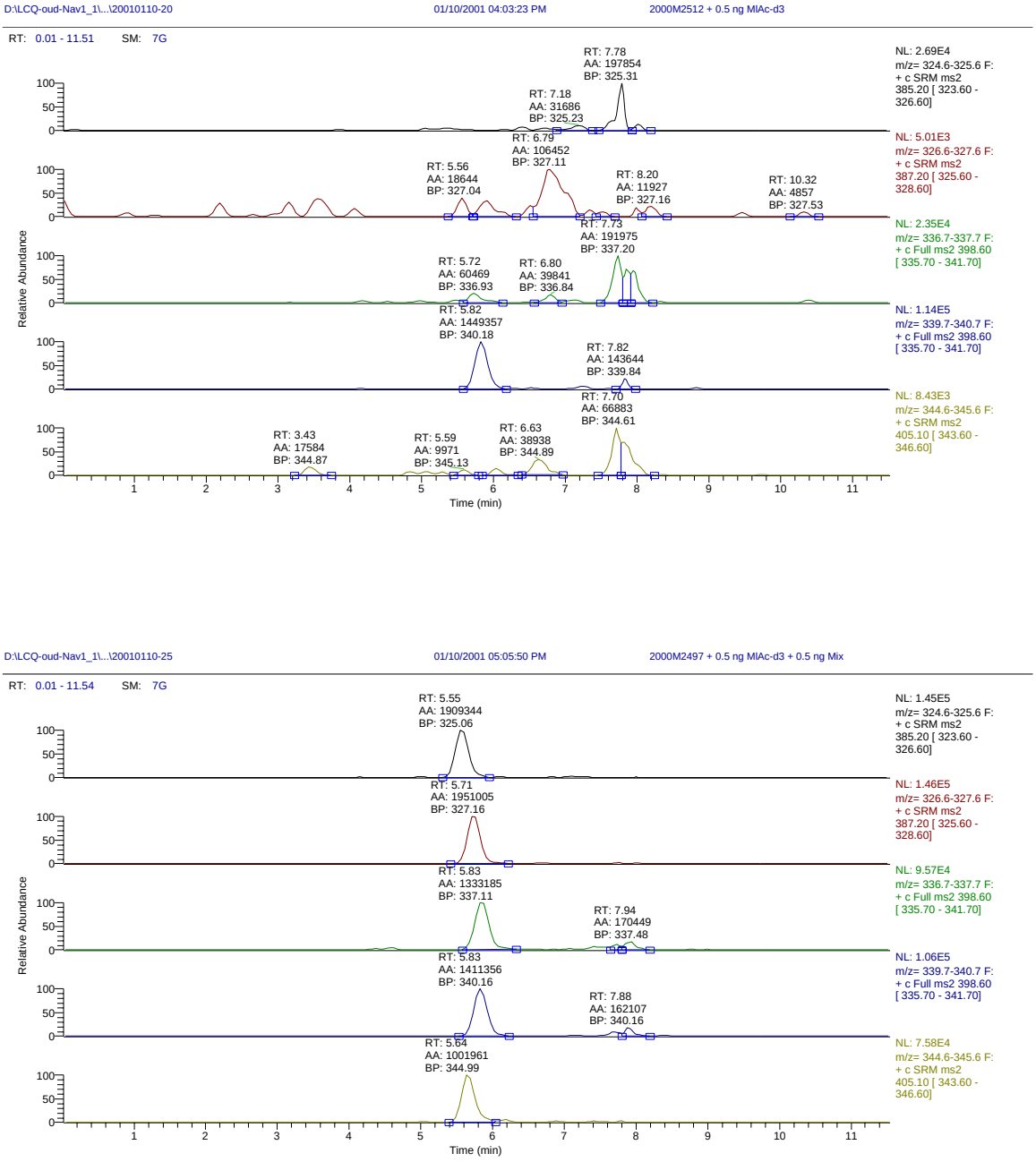


From top to below, Medroxyprogesterone (deuterated analogue) and Megestrol (deuterated analogue)



From top to below, Melengestrol (deuterated analogue) and Chlormadinone (Cl^{37} analogue)

Typical chromatograms obtained for a sample of kidney fat. Upper panel, blank sample with deuterated internal standard (melengestrol acetate-d_3), lower panel spiked with 0.5 µg/kg of each of the acetylated gestagens



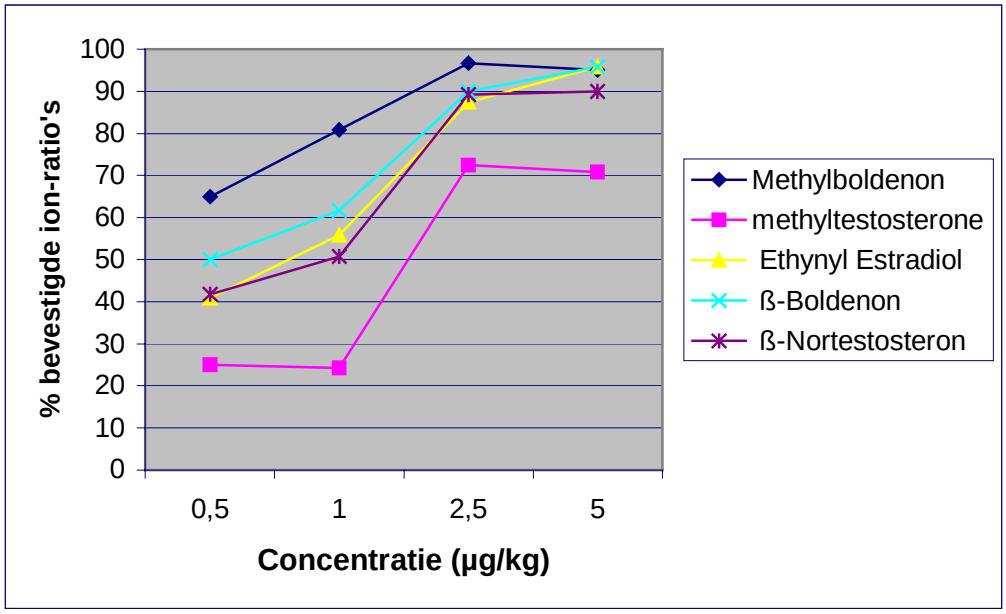
Chromatograms, from top to bottom;
Megestrol acetate (MS^2-ion 325, transition product of $[M+H]^+$ with m/z = 385
Medroxyprogesterone acetate (MS^2-ion 327, transition product of $[M+H]^+$ with m/z = 387
Melengestrol acetate (MS^2-ion 337, transition product of $[M+H]^+$ with m/z = 385
Melengestrol acetate-d_3 (MS^2-ion 340, transition product of $[M+H]^+$ with m/z = 385
Chlormadinon acetate (MS^2-ion 345, transition product of $[M+H]^+$ with m/z = 405

Stategies for confirmation[4]

The result of the first analysis is a semi quantitative result for an individual; analyte, based on the detection of a single (fragment) ion, in conjunction with a signal of the (corresponding) internal standard. For final confirmation of the identity additional measurements will be necessary. The number and nature of these measurements strongly depend on the technique selected. For residues of gestagens, all methods for confirmation are based on Mass Spectrometry (MS). The critical difference is the choice between GC-MS and LC-MS.

However, frequently a single measurement will not be able to confirm the identity of the analyte at the Limit of Detection as applicable for the semi quantitative first measurement. In theory the Limit of Identification is equal to the limit of detection of the weakest signal of the analyte.

To illustrate this phenomenon, the figure below gives a graphical representation of a study performed on the validation of a multi residue method for steroids in muscle tissue



At low ppb levels the number of confirmed ratios is significantly less than 100% for known positive samples. Additional measurement, combining the results obtained with different techniques, are necessary for full confirmation.

The revised criteria document describes the conditions under which the combination of results is allowed.

[4] Based on document SANCO 1805/2000 rev 1

Definitions

High-resolution mass spectrometry (HRMS). The resolution, R, should typically be greater than 10,000 for the entire mass range at minimum 10% valley.
Diagnostic ions. Diagnostic ions are: the molecular ion, characteristic adducts of the molecular ion, characteristic fragment ions, and all their isotope ions.

Chromatographic separation. For GC
MS procedures, the gas chromatographic separation must be carried out using capillary columns. For LC
MS procedures the chromatographic separation must be carried out using suitable LC columns. In any case, the minimum acceptable retention time for the analyte under examination is twice the retention time corresponding to the void volume of the column. The retention time (or relative retention time) of the analyte in the test portion must match that of the standard analyte within a specified retention time window. The retention time window should be commensurate with the resolving power of the chromatographic system. The ratio of the chromatographic retention time of the analyte to that of the internal standard, i.e. the relative retention time of the analyte, should correspond to that of the calibration standard at a tolerance of ± 0.5% for GC and ± 2.5% for LC.

Mass spectrometric detection.
Mass-spectrometric detection can be carried out by employing MS-techniques such as recording of full mass spectra (full scans) or Selected Ion Monitoring (SIM), as well as MS-MS^n techniques such as Selected Reaction Monitoring (SRM), or other suitable MS or MS-MS^n techniques in combination with appropriate ionisation modes.
Ion recognition.
Full scan: If mass spectrometric determination is performed by the recording of full scan spectra, the presence of all measured diagnostic ions, with a relative intensity of more than 10% in the reference spectrum of the standard analyte, is obligatory.
SIM: If mass spectrometric determination is performed by fragmentography, the molecular ion should preferably be one of the selected diagnostic ions. The selected diagnostic ions should not exclusively originate from the same part of the molecule. The signal-to-noise ratio for each diagnostic ion must be ≥ 3:1.
Full scan and SIM: The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion or transition, must correspond to those of the standard analyte, either from calibration standards or from spiked samples, at comparable concentrations, measured under the same conditions, within the following tolerances:

Table 4. 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 %

Interpretation of mass spectral data. Mass spectrometric methods are suitable for consideration as confirmatory and/or reference methods only following either an on-line or an off-line chromatographic separation.

The relative intensities of the diagnostic ions and/or precursor/product ion pairs have to be identified by comparing spectra or by integrating the signals of the single mass traces. Whenever background correction is applied, this must be applied uniformly throughout the batch (3.2.2) and must be clearly indicated.

Full scan: If full scan spectra are recorded in single MS, a minimum of four ions must be present with a relative intensity of ≥ 10 % of the base peak. The molecular ion should be included if it is present in the reference spectrum with a relative intensity of ≥ 10%. At least four ions must lie within the maximum permitted tolerances for relative ion intensities (Table 4). Computer-aided library searching may be used. In this case, the comparison of mass spectral data in the test samples to that of the standard analyte must exceed a critical match factor. This factor shall be determined during the validation process for every analyte on the basis of spectra for which the criteria described below are fulfilled. Variability in the spectra caused by the sample matrix and the detector performance should be checked.

Sim: If mass fragments are measured using other than full-scan techniques, a system of identification points shall be used to interpret the data. For the confirmation of substances listed in Group A of Annex I of Council Directive 96/23/EC, a minimum of 4 identification points are required. For the confirmation of substances listed in Group B of Annex I of Council Directive 96/23/EC, a minimum of 3 identification points are required. The Table, below, shows the number of identification points that each of the basic mass spectrometric techniques can earn. However, in order to qualify for the identification points required for confirmation and the sum of identification points to be calculated:
a) A ***minimum*** of at least one ion ratio must be measured, and

b) ***The measured ion ratios*** must meet the criteria described above, and

c) ***A maximum*** of three separate techniques can be combined to achieve the minimum number of identification points.

Table 5. The relationship between a ranassge of classes of mass fragment and Identification Points earned.

MS technique	**Identification Points earned per**
Low resolution mass spectrometry (LR)	1.0
LR-MS^n Precursor ion	1.0
LR-MS^n Transition products	1.5
High resolution mass spectrometry (HR)	2.0
HR- MS^n Precursor ion	2.0
HR-MS^n Transition products	2.5

Footnotes:

1) Each ion may only be counted once.

2) GC-MS using Electron Impact ionisation is regarded as being a different technique to GC-MS using Chemical ionisation.

3) Different chemical derivatives of an analyte can be used to increase the number of identification points only if the derivatives employ different reaction chemistries.

4) For substances in Group A of Annex 1 of Council Directive 96/23/EC, if the following techniques are used in the analytical procedure: HPLC coupled with full-scan diode array spectrophotometry (DAD); or HPLC coupled with fluorescence detection; or HPLC coupled to an immunogram; or two-dimensional TLC coupled to spectrometric detection; they may contribute a ***maximum*** of one identification point, providing that the relevant criteria for these techniques are fulfilled.

5) Transition products include both daughter and granddaughter products

Confirmation of gestagens

Gas chromatography mass spectrometry.

For confirmation by GC-MS derivatives have to be formed. The derivative obtained should yield four diagnostic ions with sufficient abundances to allow adequate detection and quantification of the signal. A number of different procedures have been used. However, in our experience, derivatisation with HFBA in acetone is the preferred technique.

Starting from the free base molecules, in most cases mono-HFB derivatives are being formed. Due to extensive isomerisation during derivatisation, however, multiple isomers are formed. These isomers show similar mass spectra with different relative abundances though.

Formation of HFB-derivatives from the free base molecules.

	Molecular weight	Nominal weight	-HFB Derivative	High mass ion	M/z
Medroxyprogesterone	344.5	344	540 (mono)	$M^{+\cdot}$ - H_2O-C_2H_3O	479
Megestrol	342.5	342	538 (mono)	$M^{+\cdot}$ - H_2O	520
Melengestrol	354.5	354	746 (di)	$M^{+\cdot}$ - H_2O	728
Chlormadinone	362.9	362	558 (mono)	$M^{+\cdot}$ - H_2O	540

Table 1: Diagnostic ions monitored during confirmation analyses, HFB-derivatives.

	HFBA ions most suitable derivative			
Medroxyprogesterone	147	317	331	479
Megestrol	381	421	477	520
Melengestrol	281	343	383	447
Chlormadinone	401	462	497	540

Melengestrol is the only compound forming diHFB derivatives. The fragmentation, however, is extensive resulting in poor fragment-ion abundances. The acetylated gestagens, however, can also be derivatised forming in all cases monoHFB-derivatives

Formation of HFB-derivatives from the esters.

	Molecular weight	Nominal weight	-HFB Derivative	High mass ion	M/z
Medroxyprogesterone acetate	386.5	386	582	$M^{+\cdot}$	582
Megestrol acetate	384.5	384	580	$M^{+\cdot}$	580
Melengestrol acetate	396.5	396	592	$M^{+\cdot}$	592
Chlormadinone acetate	404.9	404	600	$M^{+\cdot}$	540

Diagnostic ions monitored during confirmation analyses, HFB-derivatives of the acetates.

	HFBA ions most suitable derivative			
Medroxyprogesterone acetate	331	479	522	582
Megestrol acetate	381	477	520	580
Melengestrol acetate	367	381	489	592
Chlormadinone acetate	133	462	497	600

Unfortunately, the yield is poor. The favourable exception though is melengestrol acetate. In fact, this compound is most reliably detected and confirmed as the acetate. Unfortunately, this poses a problem in multi-residue testing.

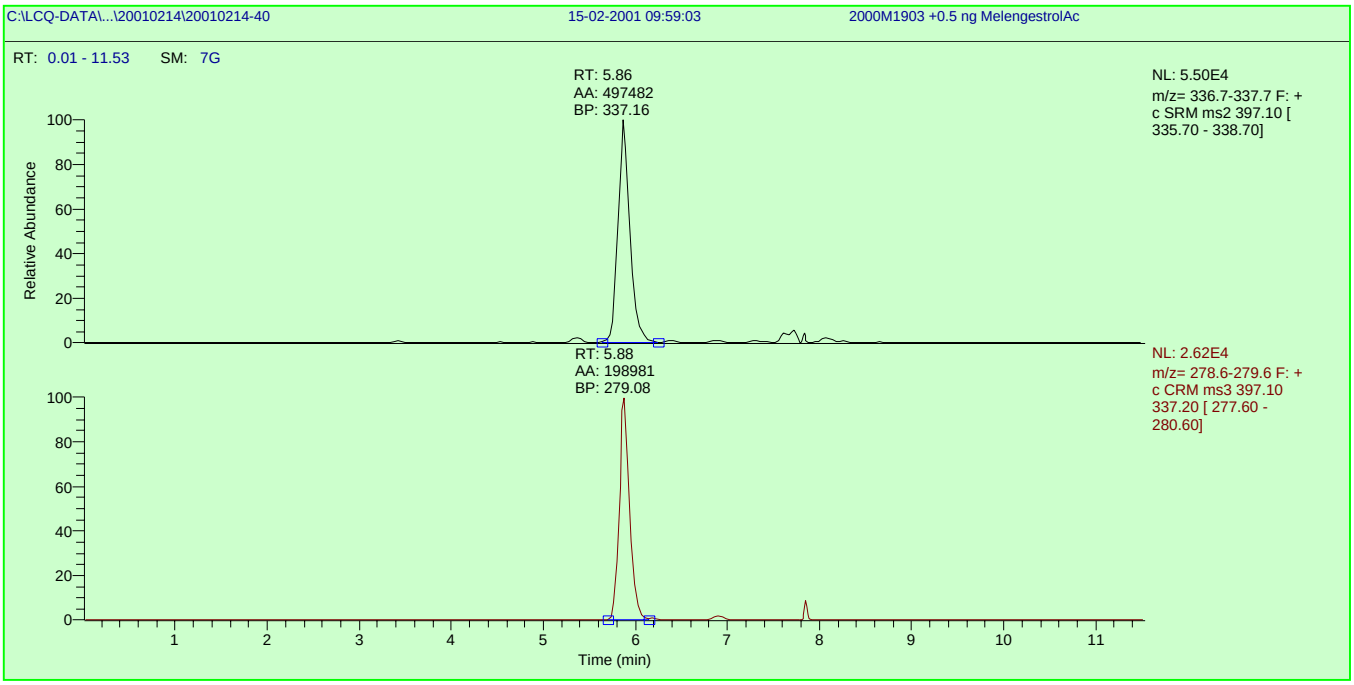
Liquid chromatography mass spectrometry.

Confirmation with LC-MS^n is based on the detection of at least two transition ions, inclusive the ratio between these two ions. One transition ion yields 1.5 identification points. However, also the presence of the precursor ion can, implicitly, be assumed. Since this ion yields an additional identification point, the total comes to the minimum of 4 identification points, necessary for confirmation of illegal compounds.

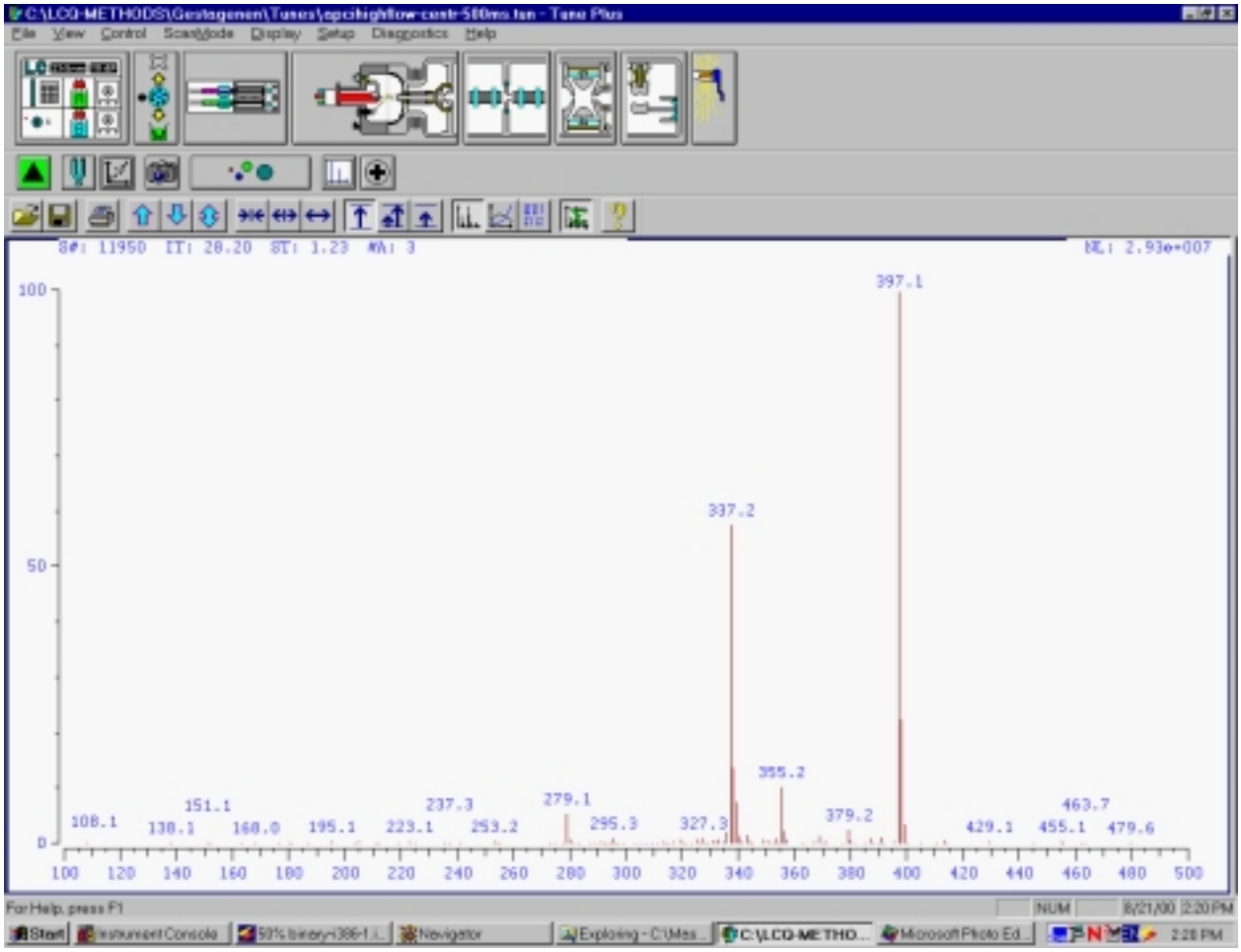
MS Selected diagnostic ions for LC-MS^n of acetylated gestagens.

	Molecular weight	Nominal weight	MS^1 Selected $[M+H]^+$	MS^2 Measured $[M+H-C_2H_5O_2]^+$	MS^3 Measured
Medroxyprogesterone acetate	386.5	386	387	327	285/309
Megestrol acetate	384.5	384	385	325	267
Melengestrol acetate	396.5	396	397	337	261/279
Chlormadinone acetate	404.9	404	405	345	309

LC-MS^n confirmation chromatogram of a sample of fat fortified with 0.5 µg/kg Megestrol acetate upper track MS^2 ion m/z 337. Lower track MS^3 ion m/z 279.



The Figures on the nex two pages show the MS^1 to MS^4 mass spectra for Melengestrol acetate.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237.3
151.1
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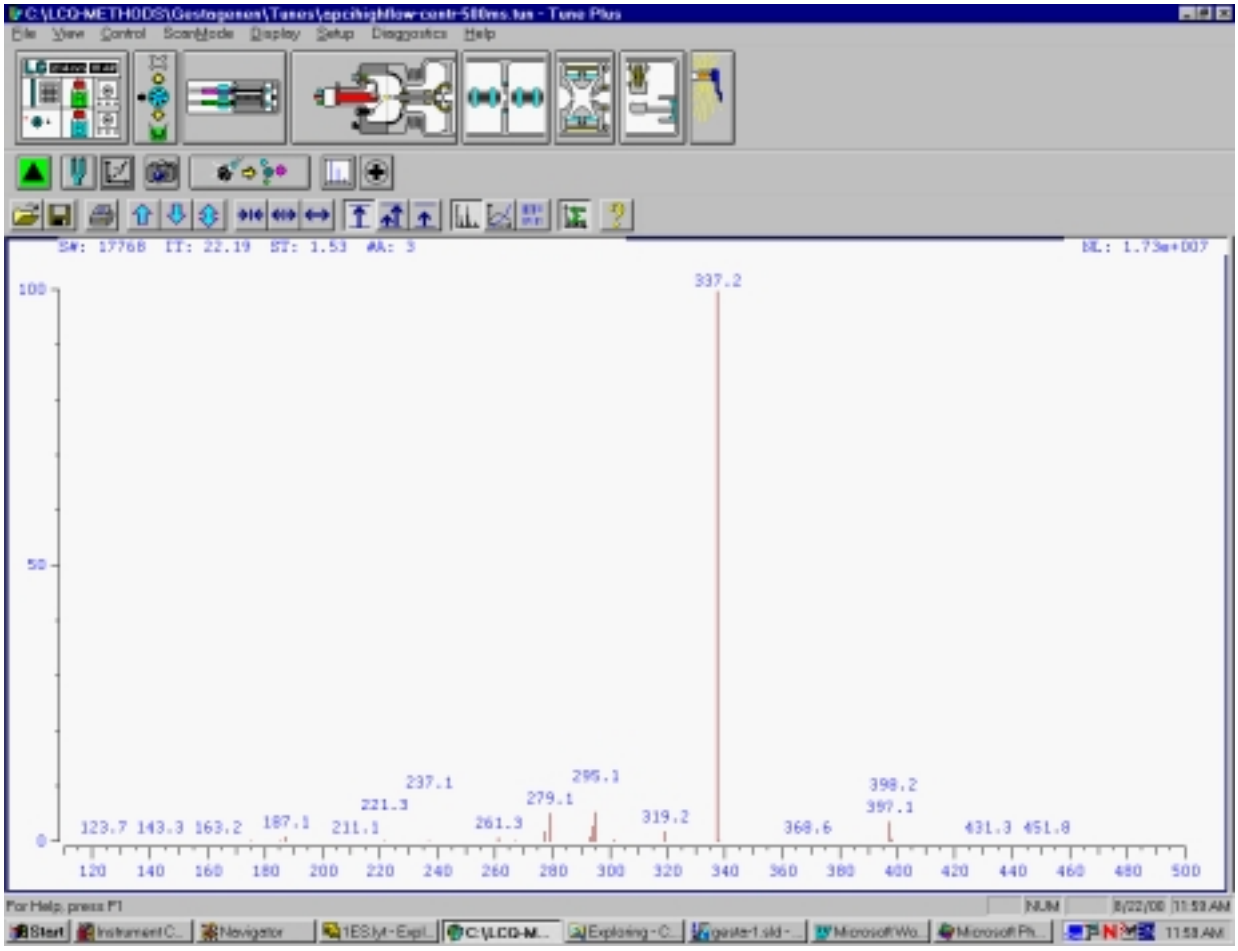
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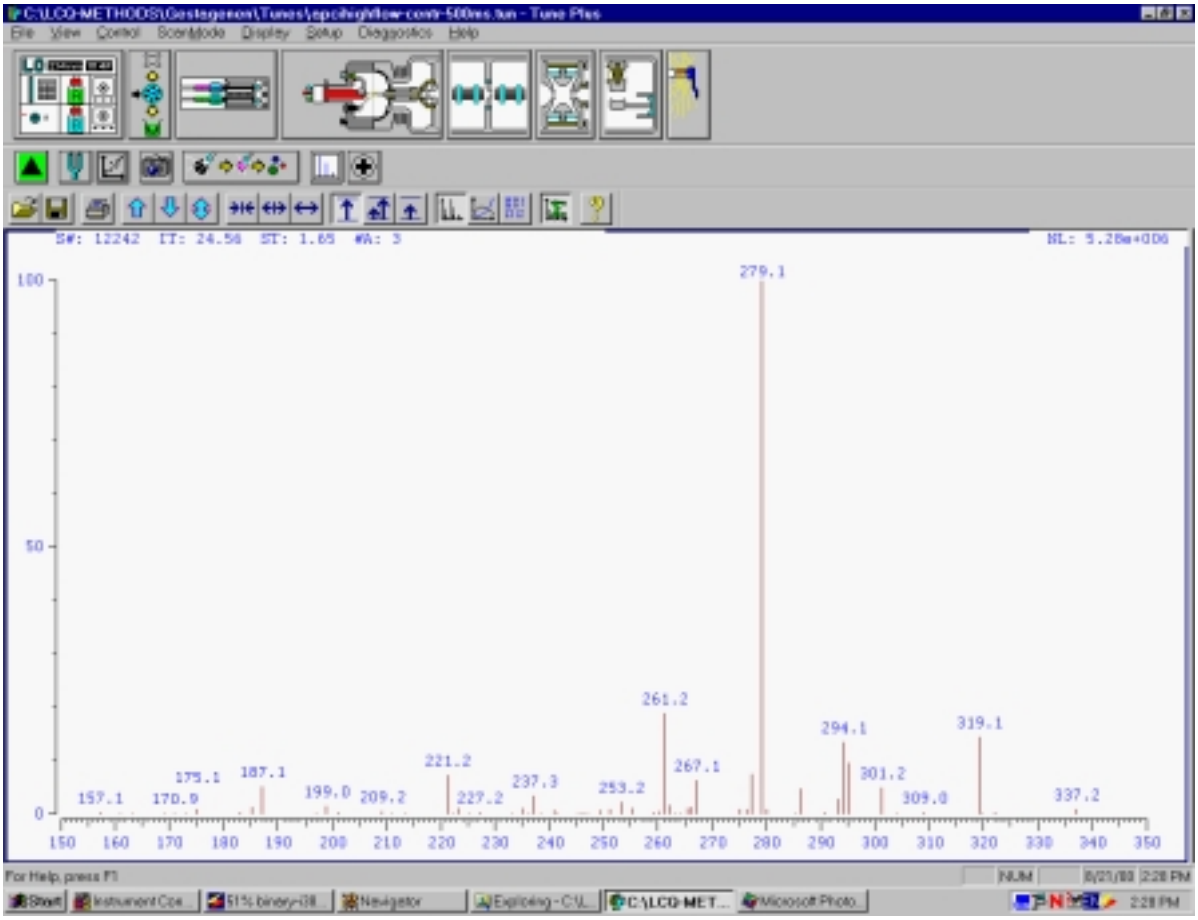
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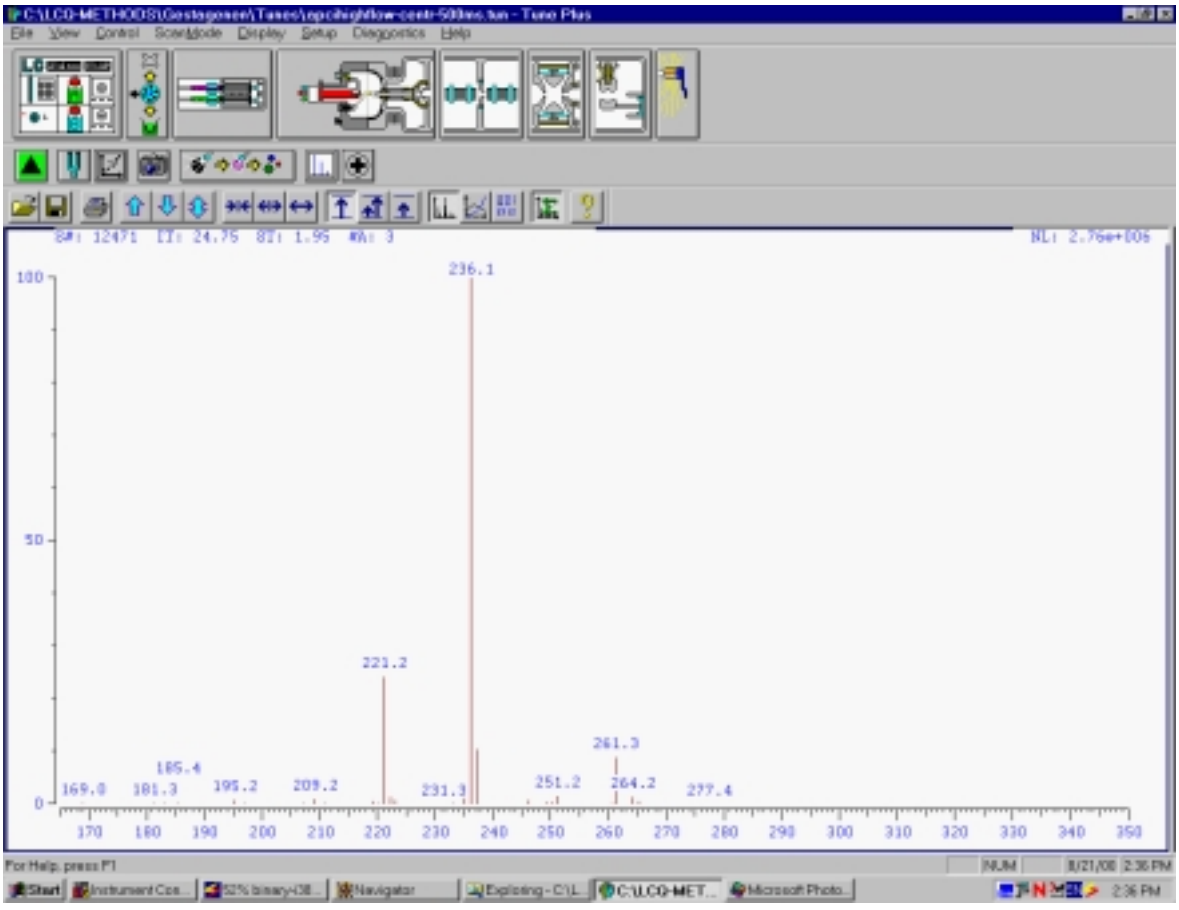
301.2
237.3
253.2
187.1
175.1
199.0
209.2
227.2
157.1
170.9
309.0
337.2
100
50
0
150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350
For Help, press F1
NUM
8/21/03 2:28 PM
Start
Instrument Con...
51% binary-03...
Navigator
Exploring - C:\L...
C:\LCQ-MET...
Microsoft Photo...
2:28 PM


C:\LCQ-METHODS\Gestagenen\Tunes\epcaheighflow-contr-500ms.tun - Tune Plus
File View Control Scan Mode Display Setup Diagnostics Help
S#: 12471 IT: 24.76 ST: 1.95 #A: 3
NL: 2.76e+006
236.1
221.2
261.3
185.4
169.0
181.3
195.2
209.2
231.3
251.2
264.2
277.4
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170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350
For Help, press F1
NUM
8/21/03 2:36 PM
Start
Instrument Con...
52% binary-03...
Navigator
Exploring - C:\L...
C:\LCQ-MET...
Microsoft Photo...
2:36 PM

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P.O. Box 1, 3720 BA Bilthoven, The Netherlands