Report 389002 117

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

Meeting Report, Conclusions and Recommendations

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RIVM document 389002 117

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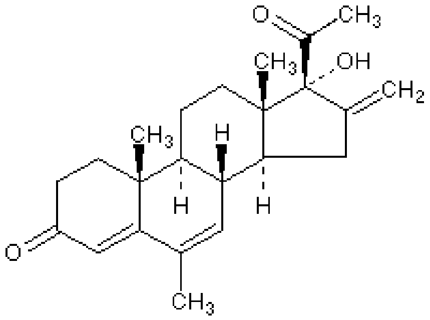

EU Community Reference Laboratory
National Institute of Public Health and the Environment
Laboratory for Residue Analysis
P.O. Box 1
3720 BA Bilthoven
The Netherlands
tel. ** 31 30 2742613
fax. ** 31 30 2744403
email: crl.aro@rivm.nl

Preface

This workshop report is the final document related to the 2001 EU NRL/CRL workshop organized in Bilthoven, The Netherlands. During this workshop, analytical methods for the detection and identification of acetylated gestagens were presented. Contrary to the usual approach in residue analyses, no integrated methods, but analytical modules were used. Combining modules for sample extraction, clean-up and detection results in full methods optimized for specified circumstances, defined by e.g. number and type of samples to be analyzed, available time and infrastructure.

At the moment, most, but not all, EU Member States have included the analyses of gestagens within their Annual National Plan. Several Member States have limited themselves to the validation of screening procedures. It is concluded that all Member States have the adequate knowledge and infrastructure for testing biological samples for the presence of residues of gestagens.

The participants prepared a series of conclusions and recommendations with respect to the topic of this workshop, several related topics and on some policy issues.

A series of SOPs for the analyses of actylated gestagens is included in CRL document 389002 116, also published in conjunction with this workshop. SOPs, however, are regularly updated. Information can be obtained by e-mail at crl.aro@rivm.nl.

This workshop was only possible with the help of a large group of colleagues from the CRL and the Laboratory for Residue Analysis, who enthusiastically worked on developing the program and making all the necessary arrangements. The financial contribution of the European Commission is acknowledged. Last, but certainly not least, the contributions of all participants were of essential importance for this workshop

Saskia S. Sterk
Rainer W. Stephany
Leendert A. van Ginkel

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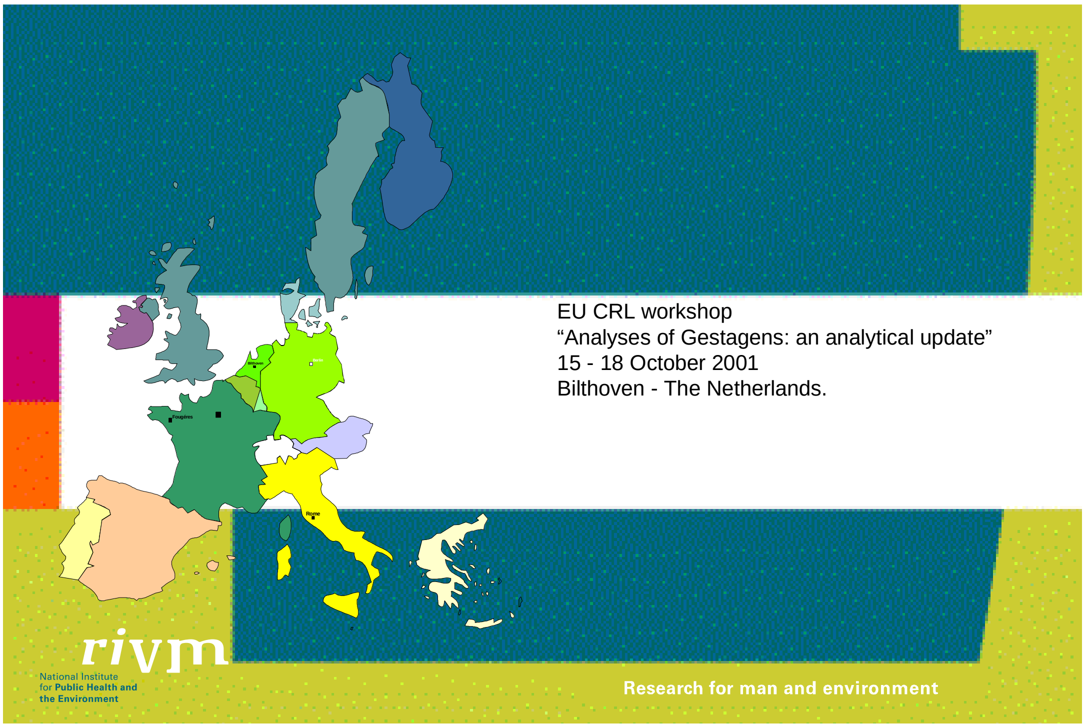
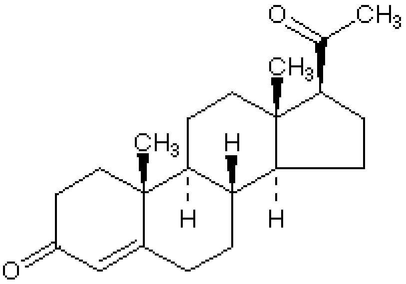


EU CRL workshop
“Analyses of Gestagens: an analytical update”
15 - 18 October 2001
Bilthoven - The Netherlands.



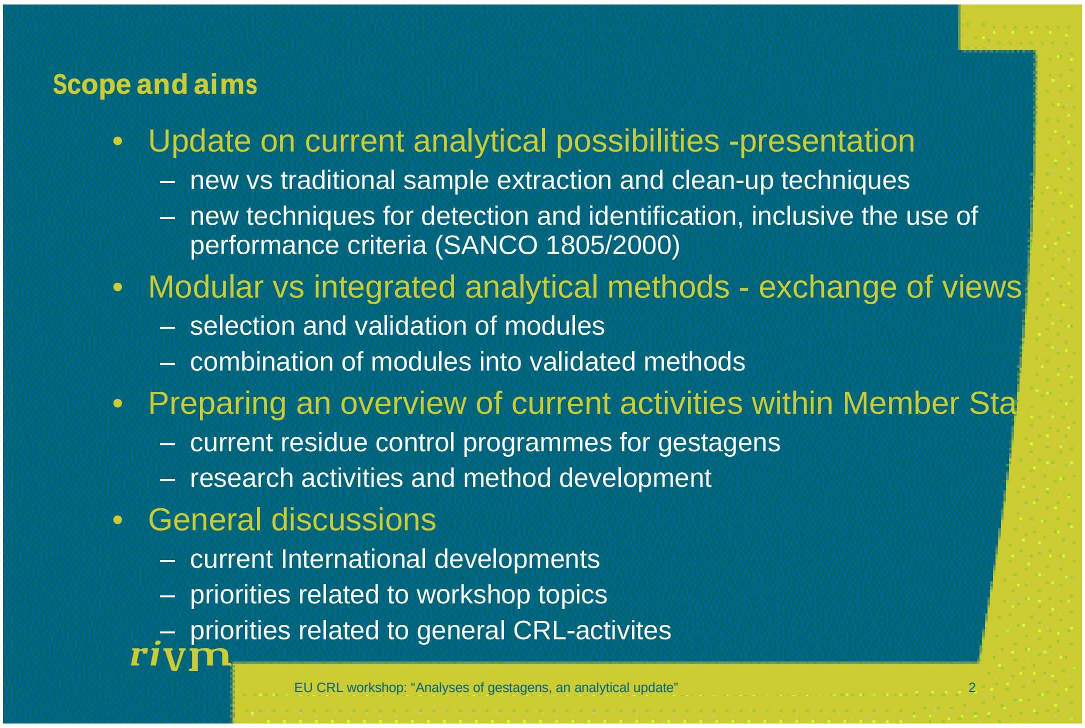
Progesterone

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 defatting procedures of the extracts obtained. Advances in Solid Phase Extraction (SPE) procedures and the increased use of Gas Chromatography Mass Spectrometry (GC-MS) significantly improved the possibilities for 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.



Scope and aims

- Update on current analytical possibilities -presentation
  - new vs traditional sample extraction and clean-up techniques
  - new techniques for detection and identification, inclusive the use of performance criteria (SANCO 1805/2000)
- Modular vs integrated analytical methods - exchange of views
  - selection and validation of modules
  - combination of modules into validated methods
- Preparing an overview of current activities within Member Sta
  - current residue control programmes for gestagens
  - research activities and method development
- General discussions
  - current International developments
  - priorities related to workshop topics
  - priorities related to general CRL-activites

EU CRL workshop: “Analyses of gestagens, an analytical update” 2

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 development of alternative approaches for sample extraction and detection, however, has consequences for analytical methods. Contrary to the traditional integrated analytical methods new, modular approaches are put into place where laboratories have a choice in combining modules into methods on basis of the available infrastructure and cost-benefit aspects. Consequently, the validation of individual modules and maintaining a good level of Quality Assurance and Quality Control (QA/QC) will be one of the key areas of discussion and development of clear guidelines in the near future. The key question will be:

When integrated validated analytical methods are replaced by ad hoc combinations of individual analytical modules, how will we assure proper validation and adequate QA/QC?

During this workshop this topic was discussed with the representatives.

Conclusions and Recommendations
EU CRL-NRL Workshop
Bilthoven, The Netherlands
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"Analyses of gestagens: An Analytical Update"

It was demonstrated that a variety of approaches for both the extraction of samples of kidney fat and muscle tissue, as well as for the detection and identification of residues of acetylated gestagens, are possible. For practical reasons, the use of LC-MS is preferred. The inclusion of alkaline hydrolysis complicates the analysis by GC-MS.

The establishment of a database with information on natural hormones; levels in biological matrices, analytical control strategies and methods of analysis is recommended.

Continuation and distribution of a database, including method descriptions and performance characteristics of methods of analysis is recommended. When ever possible, SOPs should be included. On line availability for NRLs, e.g. through the development of an Internet application, is recommended.

The identification criteria as layed down in document SANCO 1805/2000 are applied in NRLs on a regular basis. All attempts must be made to have this document formalised as soon as possible.

From a control point of view, kidney fat is the most appropriate matrix. However, muscle tissue is an alternative in case of imported meat.

All CRL activities related to proficiency testing and the development of reference materials and standards are of essential importance to the NRL/CRL network and its adequate performance.

Validation of individual analytical modules is an interesting approach which must be studied further worth in order to increase flexibility within an ISO 17025 based QA/QC system.

Workshops should allow sufficient time for the exchange of information between participants and operators. When necessary the total duration should be extended to four days.

Organisation of specific technical training could be added to the CRL activities

Workshop information should be made available electronically to NRLs to facilitate further dissemination to Routine (Field) Laboratories

List of participants

Austria

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

Belgium

M. De Winter
WIP-ISP
J. Wytsmannstraat 14
B-1050 BRUSSELS

Denmark

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

Finland

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

France

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

Germany

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

J. Polzer (obs)
BGVV/CRL
Diedersdorferweg 1
D-122 77 BERLIN

Greece

Th. Dabalis
G. Kaklamanos (obs)
Veterinary Laboratory of Serres
Terma Omonoias
GR-62110 SERRES

Ireland

Not represented

Italy

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

Luxembourg

Represented by Belgium

Portugal

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

Spain

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

Sweden

Not represented

The Netherlands

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

D. P. Samson (obs)
LRVV
Bornsesteeg 41
NL- 6708 PD WAGENIGEN

United Kingdom

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

List of abbreviations

AOAC	Association of Official Analytical Chemists
CM(A)	Chlormadinone (Acetate)
CRL	Community Reference Laboratory
DEL(A)	Delmadinaone (Acetate)
EC	European Commission
EI	Electron Impact
ELISA	Enzyme Linked Immunosorbend Assay
GC	Gas Chromatography
Hr	High Resolution
IAC	Immunoaffinity chromatography
LLE	Liquid Liquid extraction
LC	Liquid Chromatography
Lr	Low resolution
MEG(A)	Megestrol (Acetate)
MG(A)	Melengestol (Acetate)
MP(A)	Medroxyprogesterone (Acetate)
MS	Mass Spectrometry
MSPD	Matrix Solid Phase Dispersion
NRL	National Reference Laboratory
QA	Quality Assurance
QC	Quality Control
RIVM	Rijksinstituut voor Volksgezondheid en Milieu
SFE	Supercritical Fluid Extraction
SPE	Solid Phase Extraction
TBME	Tertiar Butyl Methyl ether

Participants and Laboratory Staff EU-CRL-NRL workshop RIVM 2001

From left to right:
Front row: J. Polzer, Th Dabilis, M. Kartasasmita, M. De Winter, S. Sterk, K. Wubs, P. Zoontjes.
Middle row: C. Marchiafava, P. Moreno, F. Delli Quadri, M. Pedersen, J. Barbosa, Y. Deceuninck, S. Berg, G. Kaklamanos, T. Kuhn, L. van Ginkel, R. Stephany.
Back row: P. Kootstra, C. Kuijpers, R. Lippold, E. van Bennekom, G. Balisz, D. Samson, S. Hewitt, H. van de Top, P. Schwillens.

Overview of gestagen analysis in EU Memberstates

Member State	Included in ANP	Analytical Method	Analytical Limits (µg/kg(l))	Matrices	Analytes
Austria	Yes, 150 samples/year	SPE/ELISA. For confirmation additional HPLC clean-up. *Future LC-MS planned.*	1 - 2	Kidney fat	MPA, CMA MEG
Belgium	Yes	GC-(lr/hr)MS after SPE and HPLC (multi-residue steroids)	10	Kidney fat	MPA, CMA, MEGA, MGA
Danmark	Yes 400 for gestagens, app. 300 for Progesterone	LLE/ELISA No confirmatory method. *Future GC-MS planned*	0.5 - 1 for plasma	Plasma, Kidney fat	MEGA, MPA, CMA
Finland	No, not at the moment	Automated SPE(silica) followed by HPLC-diode array	4 - 5	Kidney fat	MEGA, MGA, CMA, MPA and P
France	Yes	GC-MS (EI)	0.01 - 0.04	Edible tissue	CM, MG, MEG, MP, P
Germany	Yes	ELISA after SPE. Confirmatuion by GC-MS (not yet validated)	1	Kidney fat	MGA, MPA, MEGA
Greece	No	Methods under development			

Member State	Included in ANP	Analytical Method	Analytical Limits (µg/kg(l))	Matrices	Analytes
Ireland	Not represented				
Italy	Yes	SPE/LC-MSMS	0.1 - 0.5	Blood, urine and edible tissues	MPA, P
Luxemburg	Represented by Belgium				
Portugal	Yes	LLE followed by GC-MS	6 - 8, still being optimized and validated	Kidney fat and injection sites	MPA, MEGA, MGA, CMA, P
Spain	No	Methods under development			
Sweden	Not represented	ELISA after automated MSPD	1 - 4	Kidney fat	MPA, CMA, MEGA
The Netherlands	Yes	LC-MSMS after ASE	< 5	Kidney fat	Flu-CEL, MEGA, CLOS, CMA, MLG, MPA
United Kingdom	Yes	GC-(hr)MS after MSPD	1	Kidney fat	CHLO, DEL, FLU, MPA, MEGA, MGA, ALT

Analytical modules for the analyses of gestagens in samples of kidney fat

During the workshop the methodology was presented in a modular form

Liquid Liquid Extraction
with organic solvents,
defatting of the primary
extract with SPE on
Florisil

HPLC fractionation,
isolation of acetyl
gestagens

Alkaline hydrolysis,
extraction and chemical
derivatisation
Gas Chromatography Mass
Spectrometry

Mass Spectrometry
Liquid Chromatography

Supercritical Fluid
Extraction

Alkaline hydrolysis,
extraction and chemical
derivatisation
Gas Chromatography Mass
Spectrometry

Liquid Chromatography
Mass Spectrometry

Experimental work included the following combinations of modules.

Analysis of muscle tissue by SFE and GC-MS
Analysis of kidney fat by SFE and GC-MS
Analysis of kidney fat by SFE and LC-MS
Analysis of muscle tissue by LLE/HPLC clean-up and GC-MS
Analysis of kidney fat by LLE/HPLC clean-up and GC-MS
Analysis of kidney fat by LLE/HPLC clean-up and LC-MS

Analysis of muscle tissue by SFE and GC-MS

Summary. A sample portion of 5 gram was enzymatically digested, extracted with TBME. The TBME was mixed with extrelut and transferred to the SFE-cartridge. SFE was performed overnight, using in-line trapping on aluminiumoxide. After SFE the aluminiumoxide was eluted and the eluate was extracted with TBME. After removal of the solvent, an alkaline hydrolysis was applied, followed by derivatisation (HFBA). After derivatisation the reagent was removed and the residue was dissolved in iso-octane.

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

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

Ions for GC-MS analysis (screening)

Analyte	m/z	m/z internal standard
Medroxyprogesterone	479	482
Melengestrol	447	450
Megestrol	477	480
Chlormadinone	497	499

Formation of HFB-derivatives from the free base molecules.

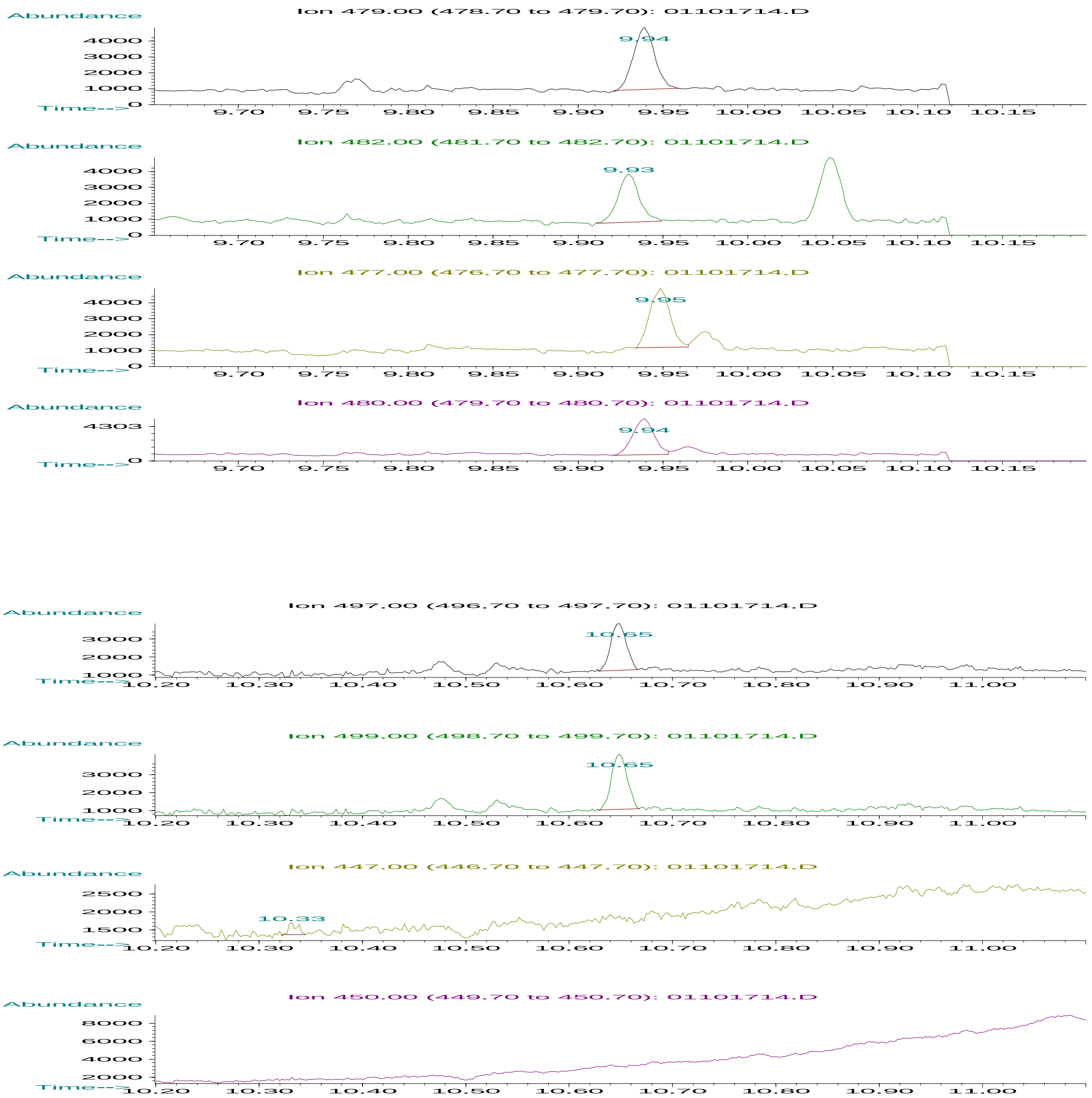
	Molecular weight	Nominal weight	-HFB Derivative	High mass ion	M/z
Medroxyprogesterone	344.5	344	540 (mono)	$M^{+\cdot}$ - H_2O-C_2H_3O	479
Megestrol	342.5	342	538 (mono)	$M^{+\cdot}$ - H_2O	520
Melengestrol	354.5	354	746 (di)	$M^{+\cdot}$ - H_2O	728
Chlormadinone	362.9	362	558 (mono)	$M^{+\cdot}$ - H_2O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

Diagnostic ions monitored during confirmatory analyses, HFB-derivatives.

	HFBA ions most suitable derivative			
Medroxyprogesterone	147	317	331	479
Megestrol	381	421	477	520
Melengestrol	343	383	447	728
Chlormadinone	401	462	497	540

Results.

One of the GC-MS chromatograms obtained is reproduced below. The first two tracks show Medroxyprogesterone-monoHFB (m/z 479 for MP and m/z 482 for MP-d3, r_t = 9.9 min.), track 3 and 4 show Megestrol-monoHFB (m/z 477 for MEG and m/z 480 for MEG-d3, r_t = 9.9 min.), track 5 and 6 show Chlormadinone-monoHFB (m/z 497 for CM and m/z 499 for CM-CL^{37}, r_t = 10.69 min.), track 7 and 8 show Melengestrol-diHFB (m/z 447 for MGL and m/z 450 for MG-d3, r_t = 10.3 min.)



GC-MS chromatograms of a sample of muscle tissue; 5 gram of muscle tissue spiked with 1 μg/kg of all acetylgestagens.

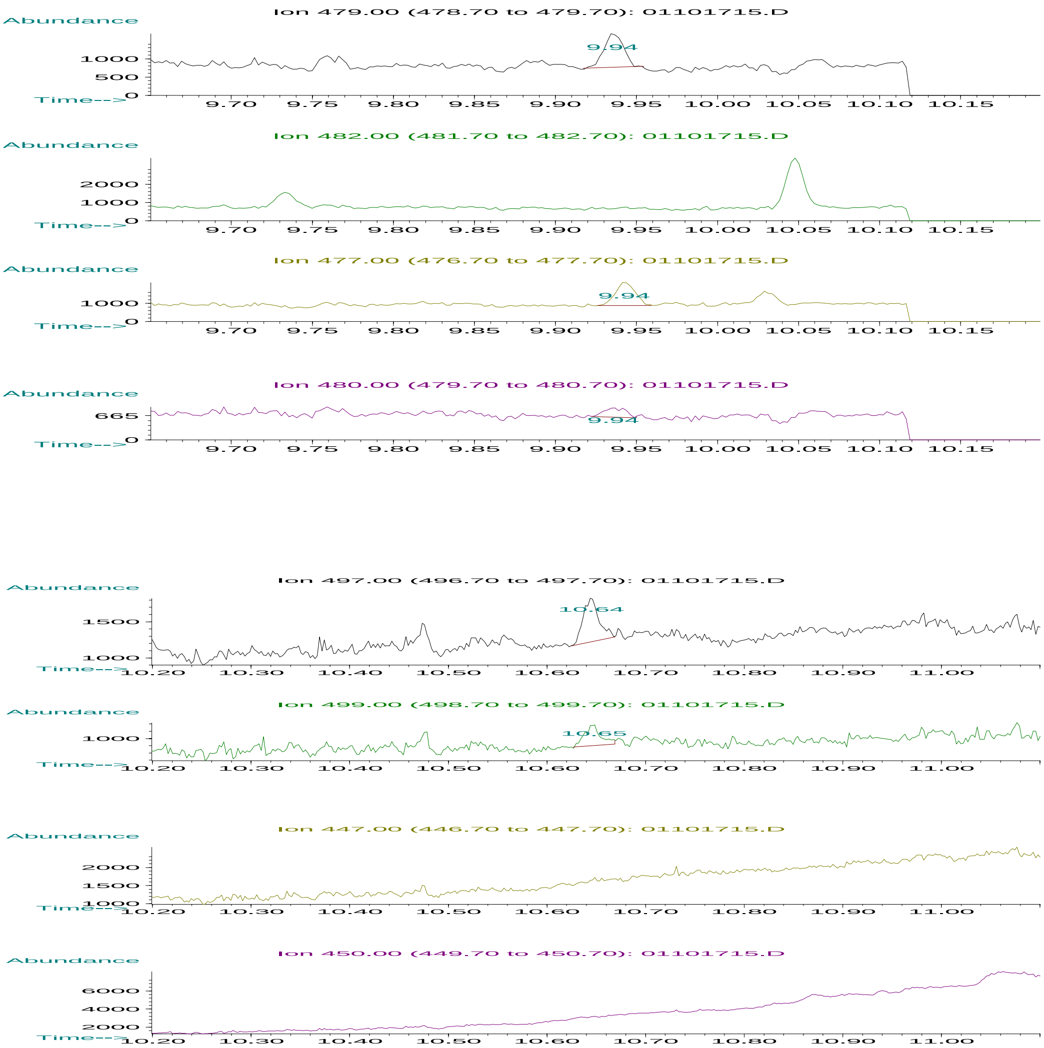
From the chromatograms it is concluded that the analyses of samples of muscle tissue, at a level of 1 μg/kg is possible for Medroxyprogesterone(acetae), Megestrol(acetate) en Chlormadinone(acetate). Melengestrol(acetate) cannot be effectively analysed at this level.

Analysis of kidney fat by SFE and GC-MS

Summary. A sample portion of 1 gram was mixed with extrelut and transferred to the SFE-cartridge. SFE was performed overnight, using in-line trapping on aluminiumoxide. After SFE the aluminiumoxide was eluted and the eluate was extracted with TBME. After removal of the solvent the residue was analysed as bescribed above: alkaline hydrolysis and chemical derivatisation.

Results.

One of the GC-MS chromatograms obtained is reproduced below. The first two tracks show Medroxyprogesterone-monoHFB (m/z 479 for MP and m/z 482 for MP-d3, r_t = 9.9 min.), track 3 and 4 show Megestrol-monoHFB (m/z 477 for MEG and m/z 480 for MEG-d3, r_t = 9.9 min.), track 5 and 6 show Chlormadinone-monoHFB (m/z 497 for CM and m/z 499 for CM-CL^{37}, r_t = 10.69 min.), track 7 and 8 show Melengestrol-diHFB (m/z 447 for MG and m/z 450 for MG-d3, r_t = 10.3 min.)



GC-MS chromatograms of a sample of kidney fat (1 gram of kidney fat spiked with 1 µg/kg of all acetylgestagens).

From the chromatograms it is concluded that the analyses of samples of kidney fat, at a level of 1 µg/kg is possible for Medroxyprogesterone (acetate) and Megestrol (acetate) Chlormadinone (acetate) and Melengestrol (acetate) cannot be analysed with this procedure at this level.

Analysis of kidney fat by SFE and LC-MS

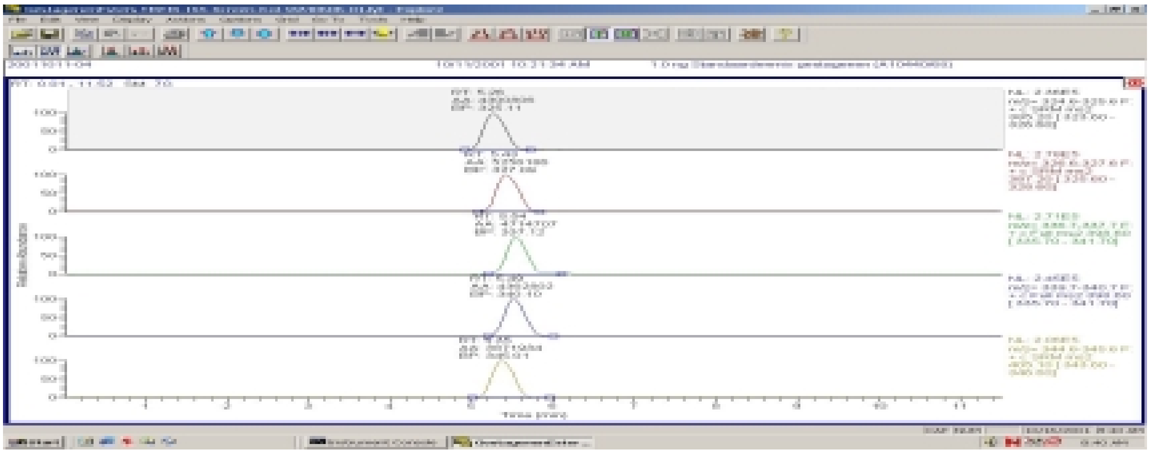
Summary. A sample portion of 1 gram of kidney fat was mixed with extrelut and transferred to the SFE-cartridge. SFE was performed overnight, using in-line trapping on aluminiumoxide. After SFE the aluminiumoxide was eluted and the eluate was extracted with TBME. After removal of the solvent the residue was dissolved in LC-eluens.

Ions for LC-MS, screening

analyte	m/z	m/z Internal Standard
Medroxyprogesterone acetate	327	
Melengestrol acetate	337	340
Megestrol acetate	325	
Chlormadinone acetate	345	

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

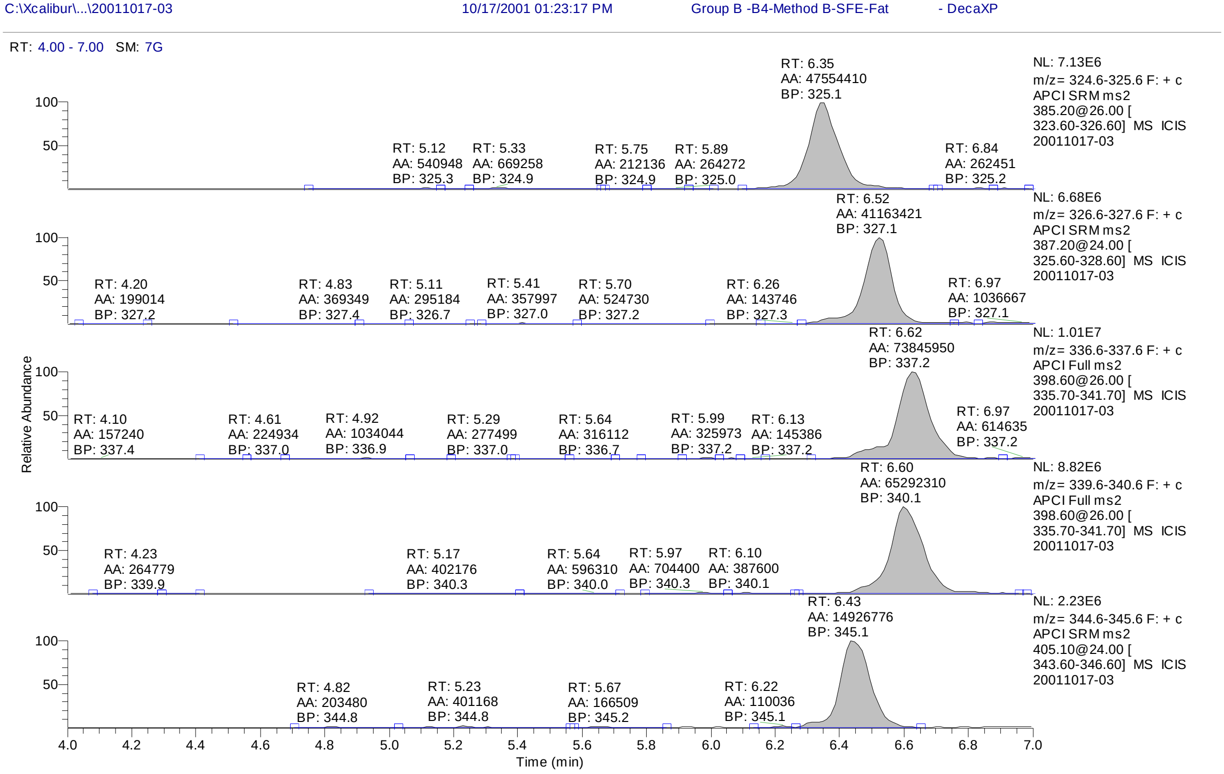


LC-MS chromatogram of a mixture of acetyl gestagens (standards, injection of 1 ng).

Results

One of the LC-MS chromatograms obtained on a LCQ-decaXP for a sample of kidney fat spiked at 1 μg/kg is reproduced below. 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 = 397)
Melengestrol acetate-d$_3$ (MS^2-ion 340, transition product of $[M+H]^+$ with m/z = 400)
Chlormadinone acetate (MS^2-ion 345, transition product of $[M+H]^+$ with m/z = 405)

Chromatogram for a sample of kidney fat spiked at 1 μg/kg



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

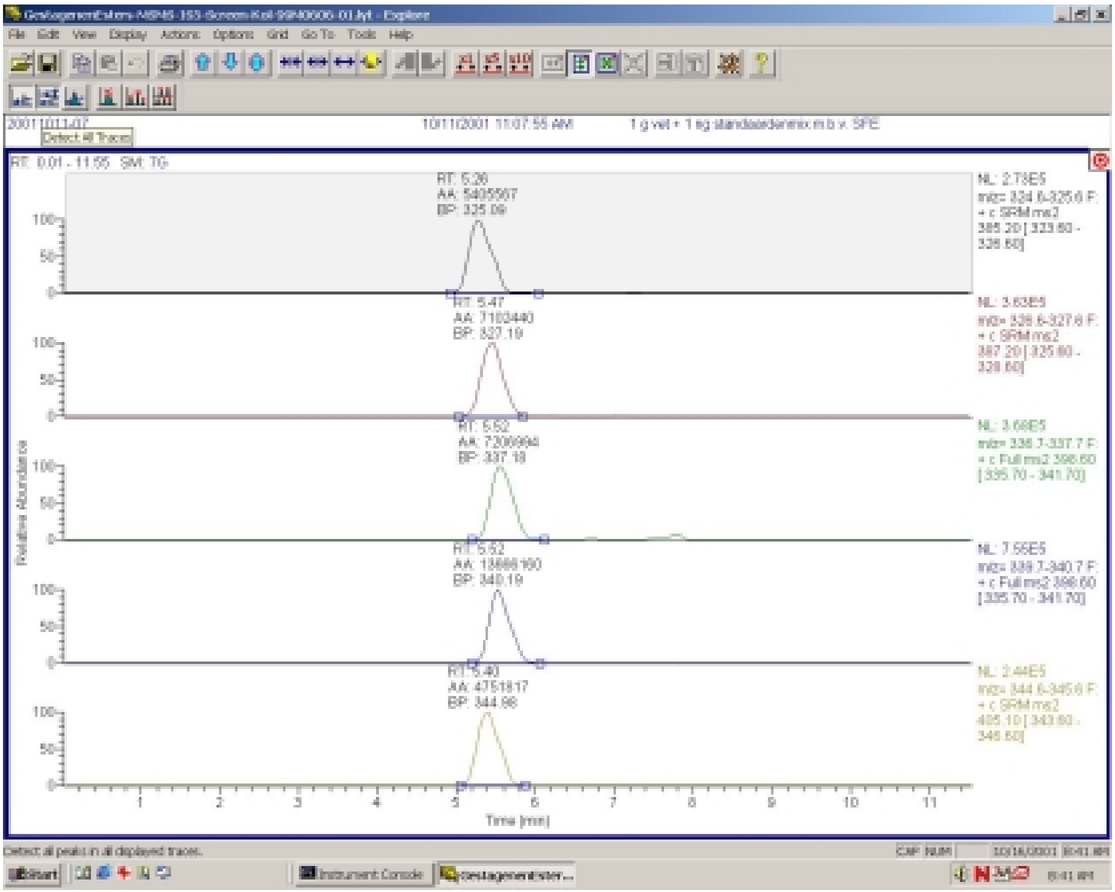
	Time (min)	Flow rate (mL/min)	%A	%B	%C
1:	0.00	0.90	100	0	0
2:	6.00	0.90	40	60	0
3:	6.01	0.90	0	0	100
4:	8.00	0.90	0	0	100
5:	8.01	0.90	100	0	0

MS Detector Settings:
Segments: 1

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

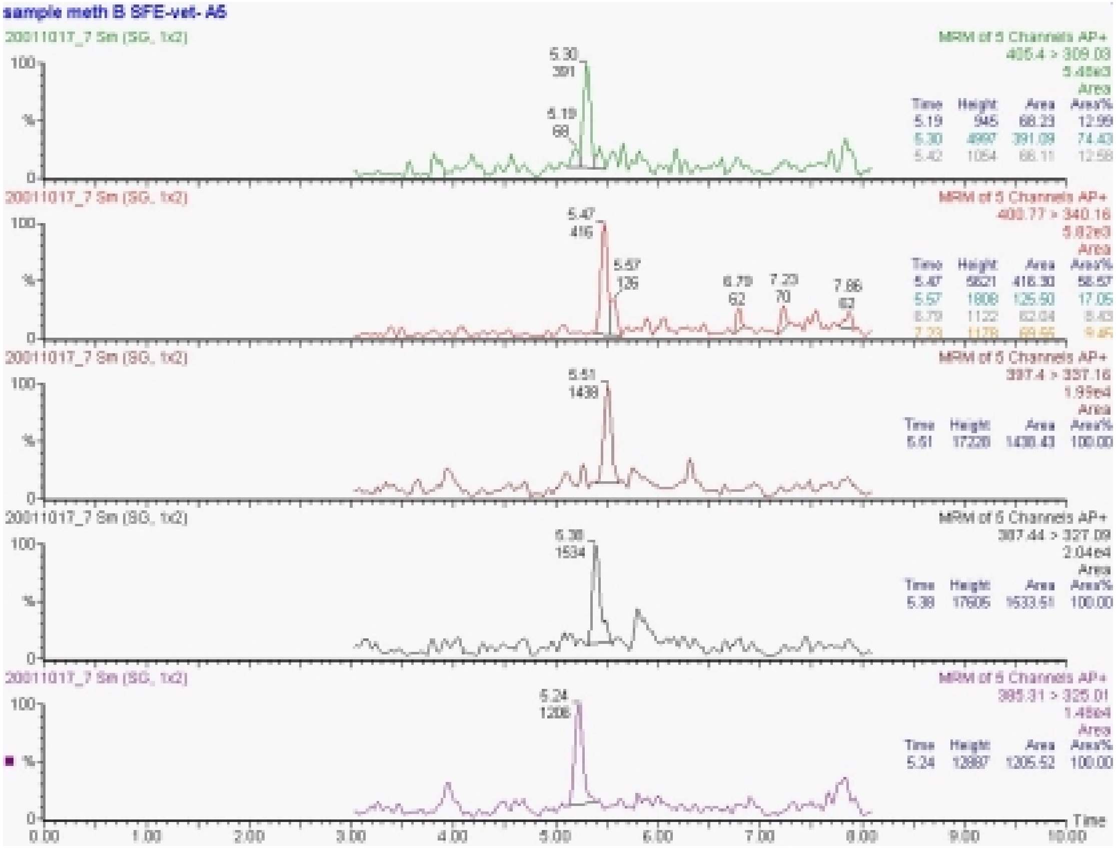
Segment 1 Scan Events:
1: Pos ·(385.2)->oS(323.6-326.6) CE = 20.0 % IsoW = 3.0
2: Pos ·(387.2)->oS(325.6-328.6) CE = 19.0 % IsoW = 3.0
3: Pos ·(398.6)->oS(335.7-341.7) CE = 20.0 % IsoW = 6.0
4: Pos ·(405.1)->oS(343.6-346.6) CE = 19.0 % IsoW = 3.0

A typical chromatogram, not produced during the workshop, as obtained with this procedure, the LCQ classic, is reproduced below.



Chromatograms obtained for a sample of kidney fat, spiked at 1 µg/kg with acetyl-gestagens, screening analyses. From tot 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 = 397)
Melengestrol acetate-d_3 (MS^2-ion 340, transition product of $[M+H]^+$ with m/z = 400)
Chlormadinon acetate (MS^2-ion 345, transition product of $[M+H]^+$ with m/z = 405)

Below the chromatogram as obtained on the Micromass Ultima LC-MSMS are reproduced. The sequence of signals differs from the sequence listed above



Chromatograms obtained for a sample of kidney fat, spiked at 1 μg/kg with acetyl-gestagens, screening analyses. From tot to bottom:
Chlormadinon acetate (MS^2*-ion 309, transition product of [M+H]*$^+$ *with m/z = 405)*
Melengestrol acetate-d$_3$ *(MS*^{2}*-ion 340, transition product of [M+H]*$^+$ *with m/z = 400)*
Melengestrol acetate (MS^2*-ion 337, transition product of [M+H]*$^+$ *with m/z = 397)*
Medroxyprogesterone acetate (MS^2*-ion 327, transition product of [M+H]*$^+$ *with m/z = 387)*
Megestrol acetate (MS^2*-ion 325, transition product of [M+H]*$^+$ *with m/z = 385)*

For Chlormadinon acetate the selected daughter ion differs from the ion selected for measurement on the LCQ.

From the results it is concluded that the method is suitable for the detection of all acetyl gestagens in samples op kidney fat at a level of 1 μg/kg. Moreover, the method is very robust. During the workshop results were obtained similar to those obtained during routine laboratory performance.

Analysis of muscle tissue by LLE/HPLC and GC-MS

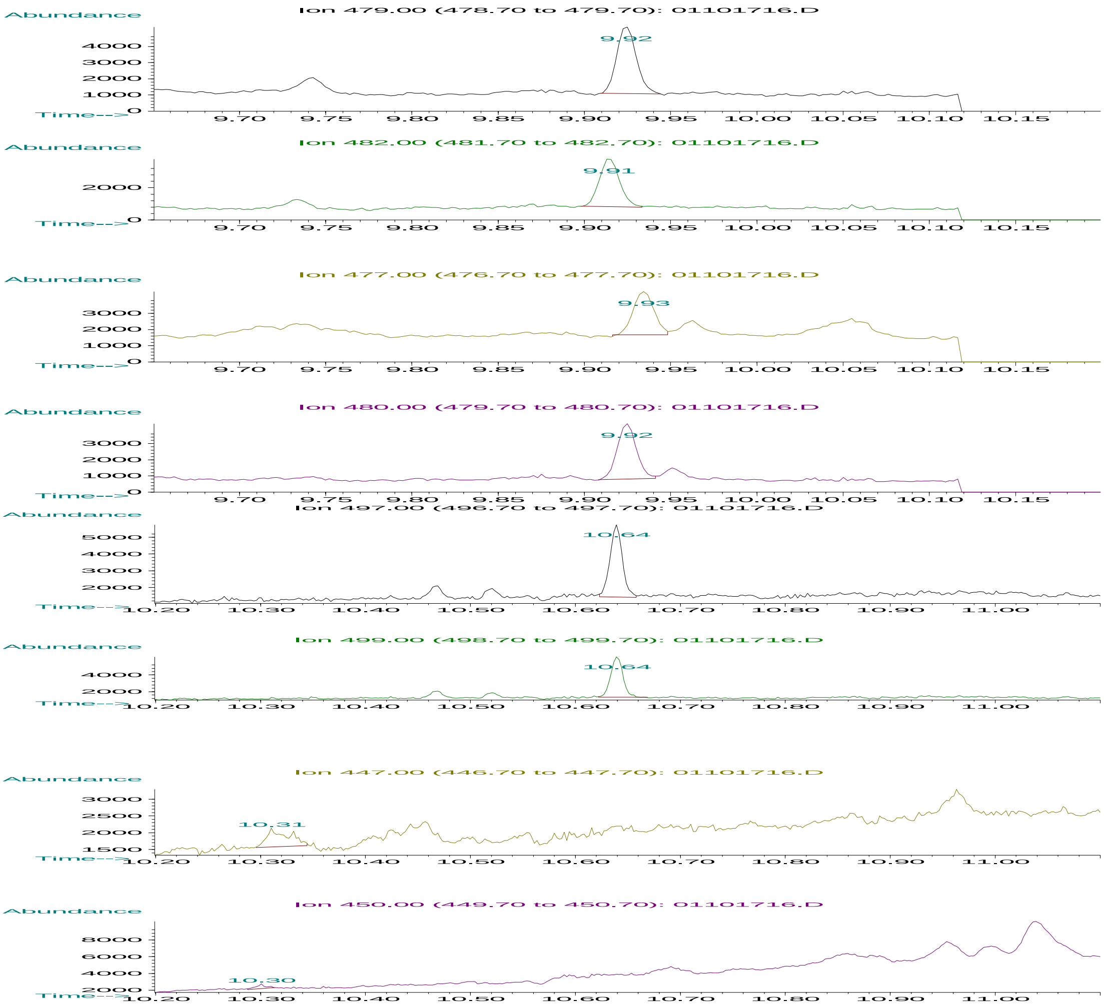
Summary. A sample portion of 5 gram of muscle tissue was enzymatically digested and extracted with TBME. The TBME was mixed with extrelut and transferred to the SFE-cartridge. SFE was performed overnight, using in-line trapping on aluminiumoxide. After SFE the aluminiumoxide was eluted and the eluate was extracted with TBME. After removal of the solvent, an alkaline hydrolysis was applied, followed by derivatisation (HFBA). After derivatisation the reagent was removed and the residue was dissolved in iso-octane. GC-MS conditions as specified above.

LC-conditions

pre-column	chromguard-reversed phase cartridge
analytical column	ODS-hypersil C18 (150 x 4.6 (mm x mm))
eluent	MEOH:H_2O-70:30
flow-rate	0.7 ml/min
detection	254 nm

Results

One of the GC-MS chromatograms obtained is reproduced below. The first two tracks show Medroxyprogesterone-monoHFB (m/z 479 for MP and m/z 482 for MP-d3, r_t = 9.9 min.), track 3 and 4 show Megestrol-monoHFB (m/z 477 for MEG and m/z 480 for MEG-d3, r_t = 9.9 min.), track 5 and 6 show Chlormadinone-monoHFB (m/z 497 for CM and m/z 499 for CM-CL^{37}, r_t = 10.69 min.), track 7 and 8 show Melengestrol-diHFB (m/z 447 for MG and m/z 450 for MG-d3, r_t = 10.3 min.).



GC-MS chromatograms of a sample of muscle tissue; 5 gram of muscle tissue spiked with 1 μg/kg of all acetylgestagens.

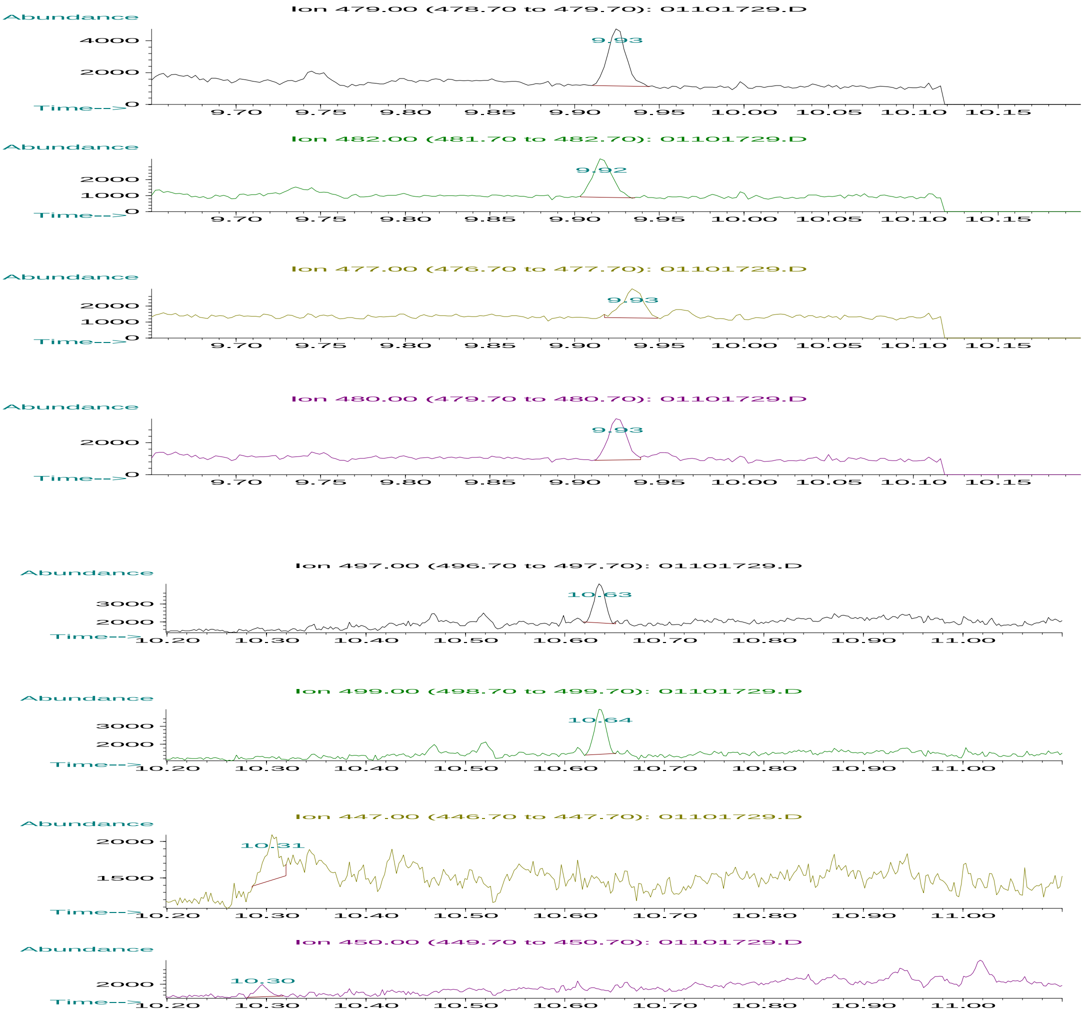
From the chromatograms it is concluded that the analyses of samples of muscle tissue, at a level of 1 μg/kg is possible for Medroxyprogesterone(acetaTe), Megestrol(acetate) en Chlormadinone(acetate). Melengestrol(acetate) cannot be analysed at this level.

Analysis of kidney fat by LLE/HPLC clean-up and GC-MS

Summary. A sample portion of 5 gram was extracted with TBME. After defatting the extract was purified by HPLC. After removal of the HPLC eluens of the isolated fraction containing the acetyl-gestagens an alkaline hydrolysis was applied, followed by derivatisation (HFBA). After derivatisation the reagent was removed and the residue was dissolved in iso-octane. LC and GC-MS conditions as specified above.

Results

One of the GC-MS chromatograms obtained is reproduced below. The first two tracks show Medroxyprogesterone-monoHFB (m/z 479 for MP and m/z 482 for MP-d3, r_t = 9.9 min.), track 3 and 4 show Megestrol-monoHFB (m/z 477 for MEG and m/z 480 for MEG-d3, r_t = 9.9 min.), track 5 and 6 show Chlormadinone-monoHFB (m/z 497 for CM and m/z 499 for CM-CL^{37}, r_t = 10.69 min.), track 7 and 8 show Melengestrol-diHFB (m/z 447 for MG and m/z 450 for MG-d3, r_t = 10.3 min.).



GC-MS chromatograms of a sample of muscle tissue (5 gram of kidney fat spiked with 5 µg/kg of all acetylgestagens).

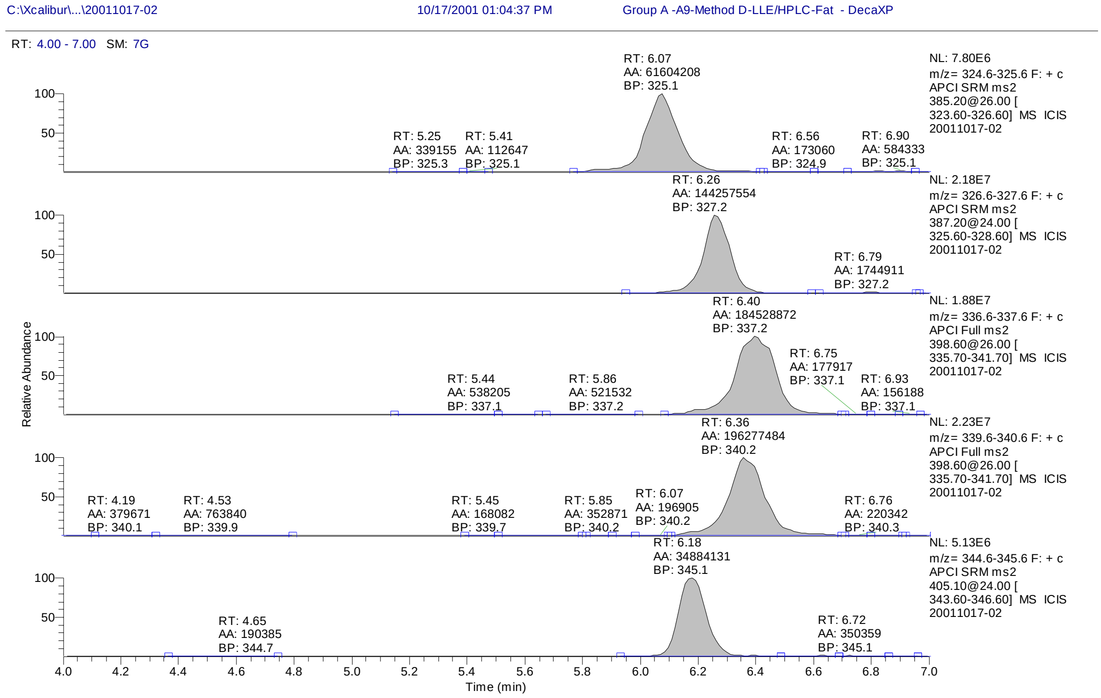
From the chromatograms it is concluded that the analyses of samples of kidney fat, at a level of 5 µg/kg is possible for Medroxyprogesterone(acetaTe), Megestrol(acetate) en Chlormadinone(acetate). Melengestrol(acetate) cannot be analysed at this level.

Analysis of kidney fat by LLE/HPLC clean-up and LC-MS

Summary. A sample portion of 5 gram was extracted with TBME. After defatting the solvent was removed and the dry extract dissolved in HPLC eluens. All other conditions as specified above.

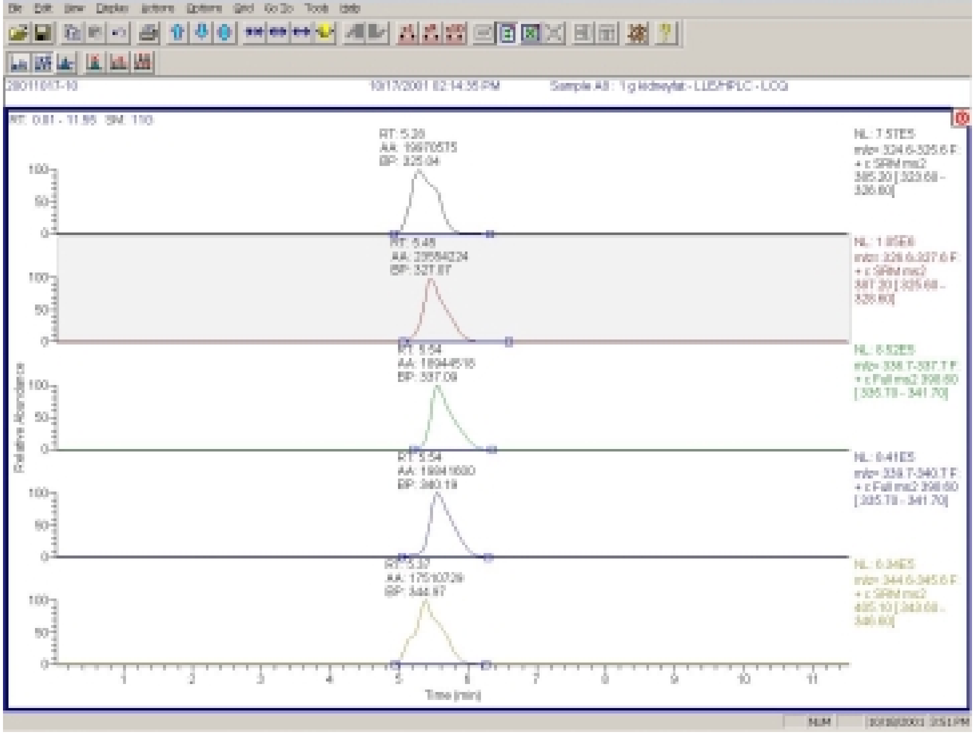
Results

LC-MS chromatograms obtained on a LCQ-decaXP and a LCQ classic respectively are reproduced below. 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 = 397)
Melengestrol acetate-d_3 (MS^2-ion 340, transition product of $[M+H]^+$ with m/z = 400)
Chlormadinone acetate (MS^2-ion 345, transition product of $[M+H]^+$ with m/z = 405)

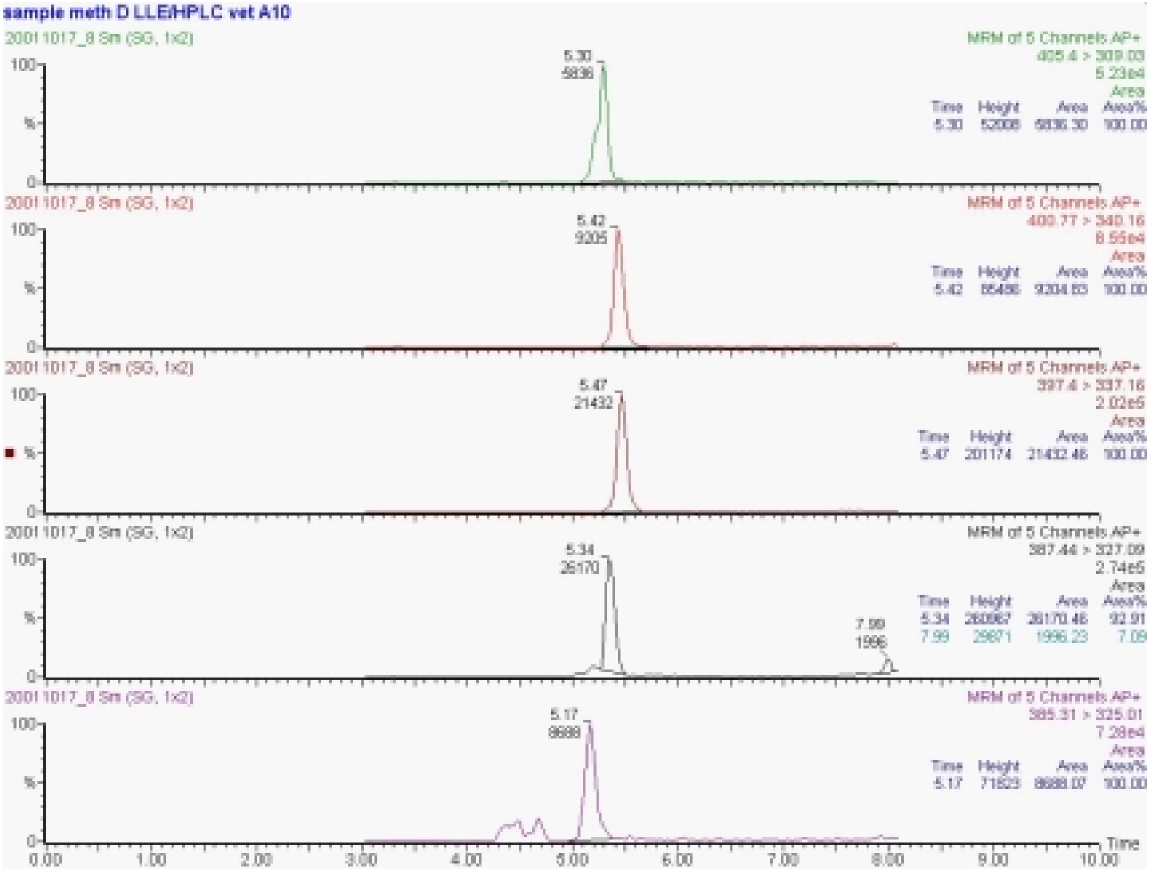


Results obtained for the LCQ-decaXP

From the results it is concluded that the method is suitable for the detection of all acetyl gestagens in samples op kidney fat at a level of 5 µg/kg. Moreover, the method is very robust. During the workshop results were obtained similar to those obtained during routine laboratory performance.



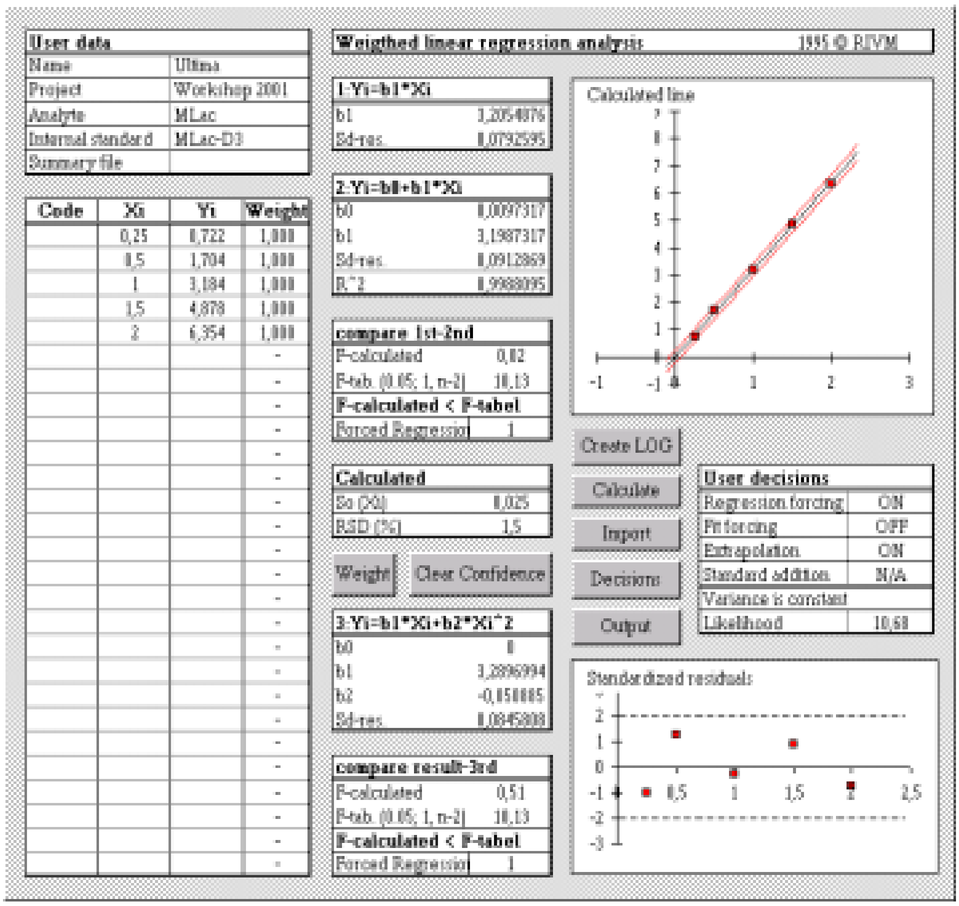
Results obtained on the LCQ classic.



Results obtained on the Micromass Ultima
Chromatograms obtained for a sample of kidney fat, spiked at 5 µg/kg with acetyl-gestagens, screening analyses. From tot to bottom:
Chlormadinon acetate (MS^2*-ion 309, transition product of [M+H]*$^+$ *with m/z = 405)*
Melengestrol acetate-d$_3$ *(MS*^{2}*-ion 340, transition product of [M+H]*$^+$ *with m/z = 400)*
Melengestrol acetate (MS^2*-ion 337, transition product of [M+H]*$^+$ *with m/z = 397)*
Medroxyprogesterone acetate (MS^2*-ion 327, transition product of [M+H]*$^+$ *with m/z = 387)*
Megestrol acetate (MS^2*-ion 325, transition product of [M+H]*$^+$ *with m/z = 385)*

Quantification with the Micromass Ultima

Quantification with LC-MSMS needs some special consideration. Analytical methods for detection and identification of residues of illegal compounds usually are presented as qualitative methods. The precise value, inclusive its uncertainty, seldom are of importance. The quantitative aspect of method validation therefore usually is limited to the lower limits (Limits of detection and identification). Nevertheless, quantification with the methods demonstrated is very well possible. Below the data obtained for two calibration curves obtained on the Micromass Ultima are given. The first figure shows the calibration curve for Melengestol acetate. For this compound the corresponding internal standard Melengestrol acetate-d3 was used. The second figure shows the curve obtained for Medroxyprogesgterone acetate. For this compound the same internal standard was used.



User data

Name	Ultima
Project	Workshop 2001
Analyte	MLac
Internal standard	MLac-D3
Summary file	

Code	Xi	Yi	Weight
	0,25	0,722	1,000
	0,5	1,704	1,000
	1	3,184	1,000
	1,5	4,878	1,000
	2	6,354	1,000
			-
			-
			-
			-
			-
			-
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			-
			-
			-
			-

Weigthed linear regression analysis 1995 © RIVM

1:Yi=b1*Xi	
b1	3,2054876
Sd-res.	0,0792595

2:Yi=b0+b1*Xi	
b0	0,0097317
b1	3,1987317
Sd-res.	0,0912869
R^2	0,9988095

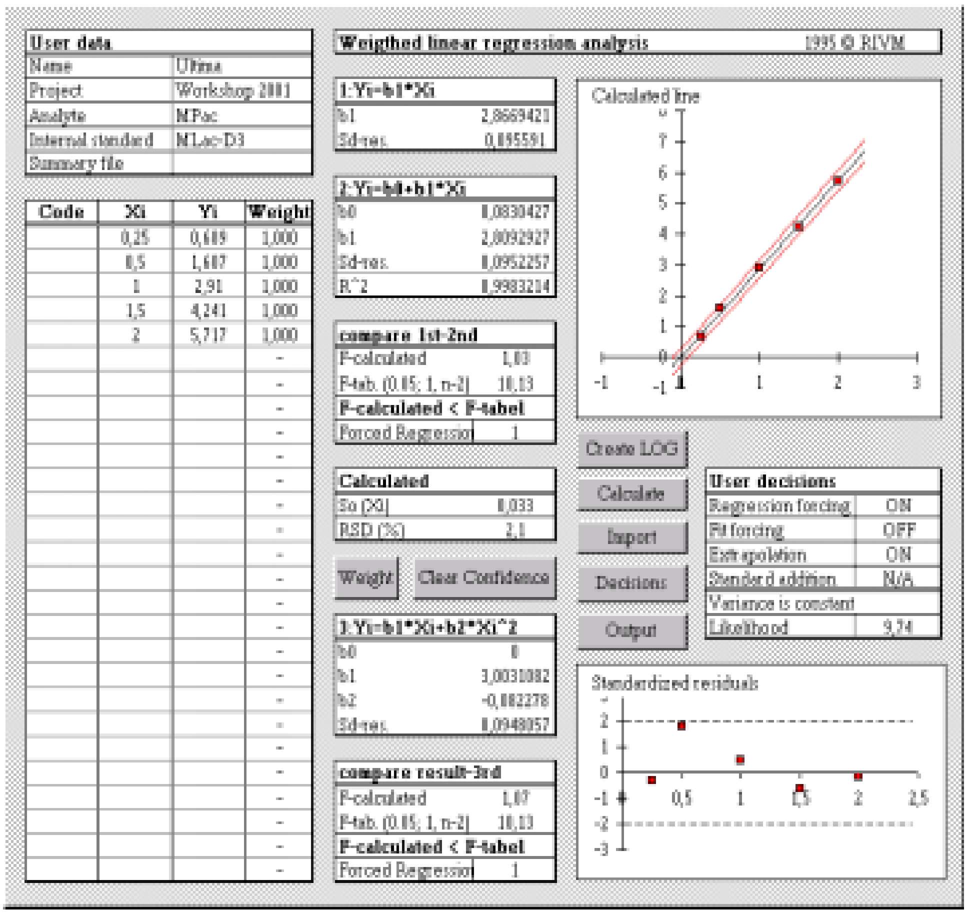
compare 1st-2nd	
F-calculated	0,02
F-tab. (0.05; 1, n-2)	10,13
F-calculated < F-tabel	
Forced Regressio	1

Calculated	
So (X0)	0,025
RSD (%)	1,5

3:Yi=b1*Xi+b2*Xi^2	
b0	0
b1	3,2896994
b2	-0,050885
Sd-res.	0,0845808

compare result-3rd	
F-calculated	0,51
F-tab. (0.05; 1, n-2)	10,13
F-calculated < F-tabel	
Forced Regressio	1

User decisions	
Regression forcing	ON
Fit forcing	OFF
Extrapolation	ON
Standard addition	N/A
Variance is constant	
Likelihood	10,68



User data

Name	Ultima
Project	Workshop 2001
Analyte	MPac
Internal standard	MLac-D3
Summary file	

Code	Xi	Yi	Weight
	0,25	0,689	1,000
	0,5	1,607	1,000
	1	2,91	1,000
	1,5	4,241	1,000
	2	5,717	1,000
			-
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			-
			-
			-

Weigthed linear regression analysis 1995 © RIVM

1:Yi=b1*Xi	
b1	2,8669421
Sd-res.	0,095591

2:Yi=b0+b1*Xi	
b0	0,0830427
b1	2,0092927
Sd-res.	0,0952257
R^2	0,9983214

compare 1st-2nd	
F-calculated	1,03
F-tab. (0.05; 1, n-2)	10,13
F-calculated < F-tabel	
Forced Regressio	1

Calculated	
So (X0)	0,033
RSD (%)	2,1

3:Yi=b1*Xi+b2*Xi^2	
b0	0
b1	3,0031082
b2	-0,082278
Sd-res.	0,0948057

compare result-3rd	
F-calculated	1,07
F-tab. (0.05; 1, n-2)	10,13
F-calculated < F-tabel	
Forced Regressio	1

User decisions	
Regression forcing	ON
Fit forcing	OFF
Extrapolation	ON
Standard addition	N/A
Variance is constant	
Likelihood	9,74

Overall discussion practical results

During the practical program two major parameters were compared. Sample extraction by Supercritical Fluid extraction (SFE) or by Liquid-Liquid Extraction followed by Liquid Chromatography (HPLC) and Liquid Chromatography Mass Spectrometry or Gas Chromatography Mass Spectrometry.

The Table below summarizes the different combinations and gives the page numbers where the respective chromatograms can be found.

Matrix	Clean-up procedure	Method for detection	Page No. chromatograms
Muscle tissue	SFE	GC-MS	16
Muscle tissue	LLE/HPLC	GC-MS	24
Kidney fat	SFE	GC-MS	17
Kidney fat	LLE/HPLC	GC-MS	225
Kidney fat	SFE	LC-MS	19,21,22
Kidney fat	LLE/HPLC	LC-MS	26,27,28

All combinations proved to be suitable for the detection of (acetyl) gestagens at the low µg/kg level. However, there clearly are differences. The traditional approach using LLE, defatting followed by HPLC , alkaline hydrolysis, derivatisation and GC-MS analysis, when based on a sample size of 5 gram, allows the detection of all (acetyl) gestagens at a level of 1 µg/kg. The problem, however, is the detection of Melengestrol acetate (MGA). This limit of detection for this compound is significantly higher than for the other compounds. The reason is the fact that Melengestrol forms, under the conditions used, melengestrol-diHFB. The fragmentation pattern (the spectrum is reproduced in the annex) is not favourable for sensitive detection.

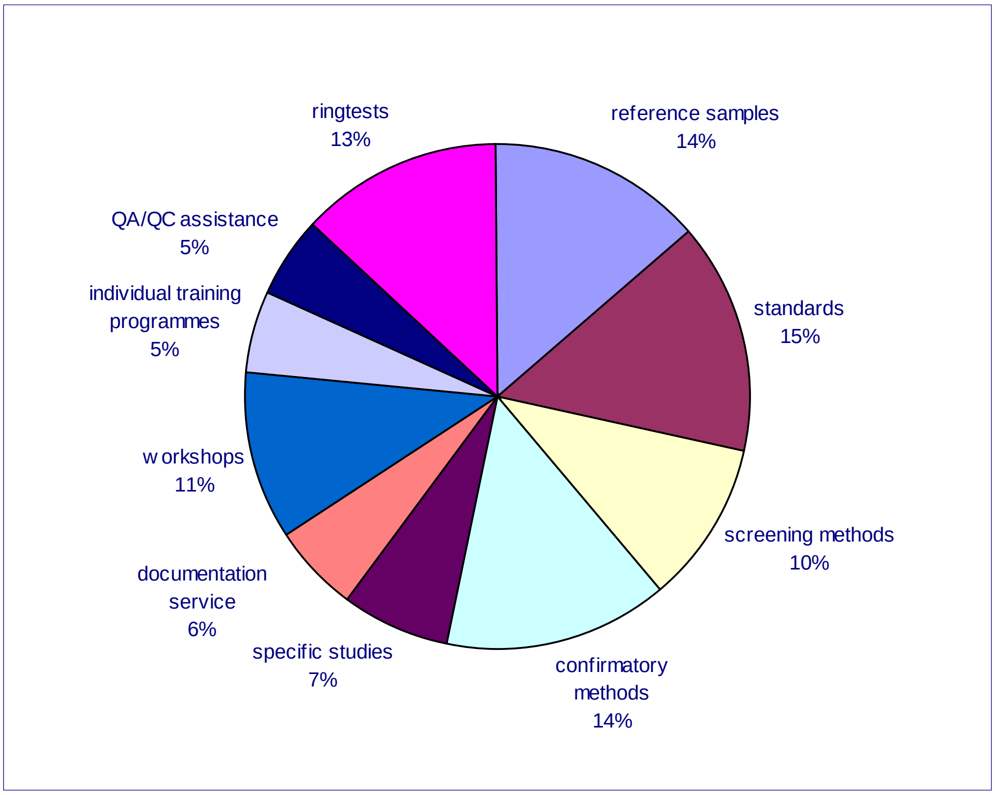
Shifting over to LC-MS has the advantage that the alkaline hydrolysis can be omitted. Subsequently, the detection for all compounds is possible at the level of 1 µg/kg. Additionally, confirmation according to the criteria described in document SANCO 1805/2000 rev 1 can be more readily fulfilled. The measurement of one additional transition-ion is sufficient, whereas for confirmation with GC-lrMS the measurement of three additional fragment-ions is necessary.

The second major parameter tested was the use of SFE. For muscle tissue the sample size was 5 gram, as it was for the extraction with LLE/HPLC. For kidney fat the sample size was reduced to 1 gram. The major advantage of automated SFE is the strongly reduced sample preparation time. However, when combined with GC-MS, the performance characteristics for kidney fat are relative poor. In combination with LC-MSMS, however, a very fast and efficient analytical procedure is possible.

Overall we conclude that for LC-MSMS is the preferred technique over GC-MS. The choice between LLE/GC-MS and SFE depends on the number of samples to be analysed and the available infrastructure.

Setting the priorities for future activities

Annually, the activities of the CRL are updated after discussions with the representatives of NRLs and the EC. The diagram below summarizes the most resent results from which it was concluded that the organisation of ring tests, in combination with the development of reference materials, is considered one of the most important CRL tasks.



Secondly the development of confirmatory and to a lesser extent screening methods are mentioned, followed by the organisation of workshops. The participants in this workshop were of the opinion that this distribution of resources still is in line with the current demands of NRLs.

During this workshop it was concluded that this distribution of activities still adequately reflects the current priorities as seen by the NRLs.

Workshop topics

The discussion on future research- and workshop topics focussed on the problems associated with the control for residues of natural hormones, including topics as databases with reference (back-ground) values and action levels in control situations. But also analytical topics like isotope ratio MS for the discrimination between endogenous and synthetic molecules was considered of interest. Further, the topic should also include compounds like boldenone, nortestosterone and Zeranol.

Current international developments

Criteria for method validation

Criteria for method validation are described in detail in the draft revision of Commission Decision 93/256, the by now well known SANCO document[1]. Internationally, however, there are similar discussions going on, resulting in alternative documents. With respect to general aspects of method validation documents are produced by EMEA[2] and Codex[3]. To a large extent these documents are similar to each other. Critical areas for discussion and harmonisation are those related to within laboratory reproducibility and especially, specificity. None of the documents other than the SANCO document give relevant details on identification criteria. This does not mean that there are no other groups discussing identification criteria. One most relevant example is a FDA document[4] on Mass Spectrometry. It is of the interest of all laboratories involved in regulatory residue analysis that international harmonisation will be achieved effectively.

One group, internationally leading in method validation, is the AOAC International. For many years, the Official Methods Programme® has been one of the most important sources of validated analytical methods, both for microbiological and for chemical methods. In recent years, however, AOAC was facing growing problems with maintaining this validation programme and this year it was decided to introduce a new system of 5 levels of method validation. The first three levels are reserved for AOAC *Official methods*, *Mutually Recognised Methods* and *Peer Verified Methods*. Within the European context category 4 and 5 are important, the *in house validated methods* and *ad hoc methods*. The *in house validated methods* are of a status similar to methods validated according to document SANCO 1805. The CRL, represented in the AOACinternational Official Methods Board, will actively try to harmonize the criteria for in house method validation. By the end of 2004, AOACinternational plans to make analytical methods of all five categories available through the internet.

Five level of method validation as defined by AOACi

Level	
1	Official AOACi Method
2	Mutually Recognised Method
3	Peer Verified Method
4	In House Validated Method
5	Ad Hoc Method

The CRL will actively contribute to the discussion concerning e.g. criteria for In House method validation. Further, guidelines will be developed for the validation of analytical modules, e.g. by sequential spiking experiments.

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[1] Document SANCO 1805/2000 rev. 1

[2] EMEA CVMP "Draft Volume 8" (EMEA/CVMP/573/00-FINAL)

[3] CODEX CX/RVDF01/12 "Review of performance based criteria for methods of analysis for veterinary drug residues in foods.

[4] FDA/CVM Draft Guidance for Industry 118. Mass spectrometry for confirmation of the identity of animal drug residues.

Annexes

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

Overview of compounds

Principle of SFE

Spectra of HFB-derivatives of gestagens

Isotope enriched internal standards: compressed datasheet Melengestol acetate-d3

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.

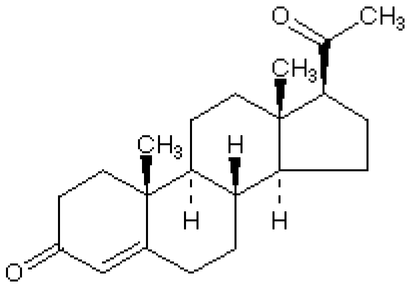
Thursday 18 October 2001

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

Overview of compounds

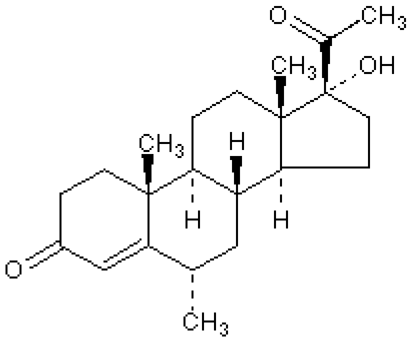
Progesterone.

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



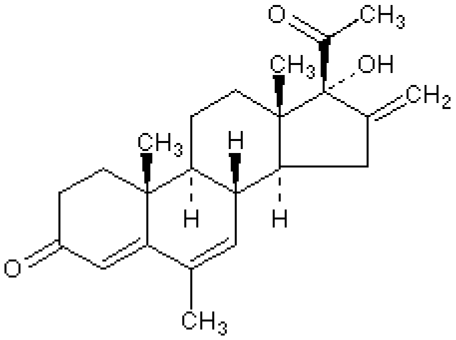
Medroxyprogesterone.

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



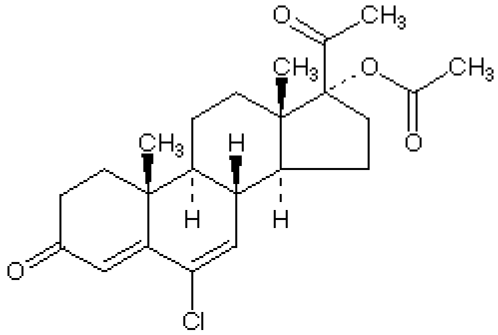
Melengestrol.

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



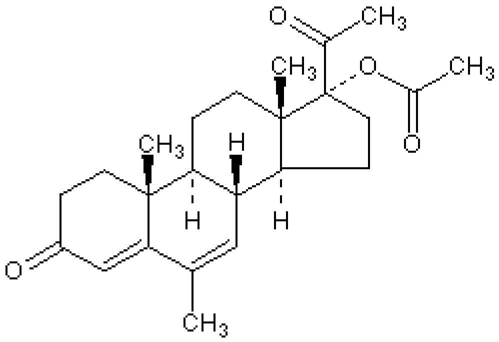
Chlormadinone Acetate.

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.



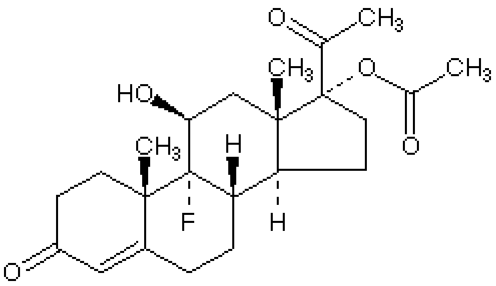
Megestrol Acetate.

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

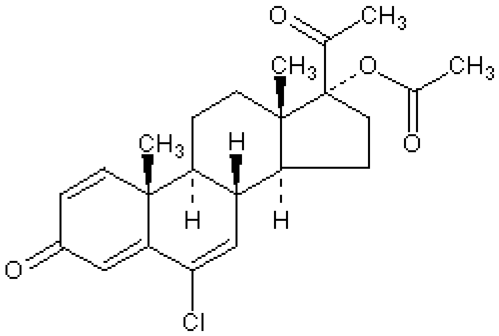


Flurogestone Acetate.

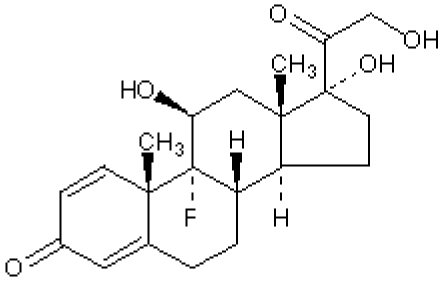
(11β)-17-(Acetyloxy)-9-fluoro-11-hydroxypregn-4-ene-3,20-dione: $C_{23}H_{31}FO_5$; mol wt 406.49.



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.



Isoflupredone
11β-9-Fluoro-11,17,21-trihydroxypregna-1,4-diene-3,20-dione: $C_{21}H_{27}FO_5$; mol wt 378.44.



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	$C_{22}H_{32}O_3$	71-58-9	$C_{24}H_{34}O_4$
Melengestrol	5633-18-1	$C_{23}H_{30}O_3$	2919-66-6	$C_{25}H_{32}0_4$
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		$C_{21}H_{29}FO_4$	2529-45-5	$C_{23}H_{31}FO_5$
Delmadinone	15262-77-8	$C_{21}H_{25}Cl0_3$	13698-49-2	$C_{23}H_{27}Cl0_4$
Isoflupredone	$C_{21}H_{27}FO_5$	$C_{21}H_{27}FO_5$		$C_{23}H_{29}FO_6$

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.

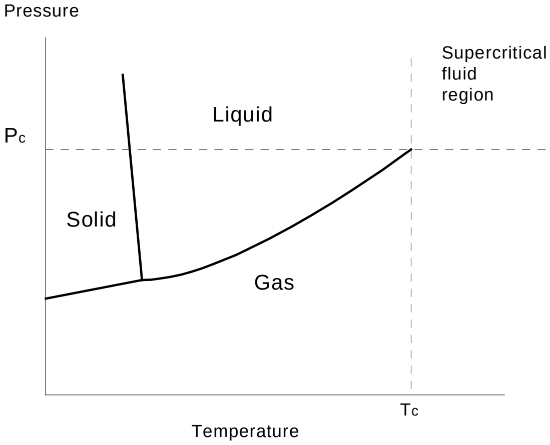


Figure 1 *Phase diagram for a liquid-gas supercritical fluid system*

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 T (°C)	Critical P (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.

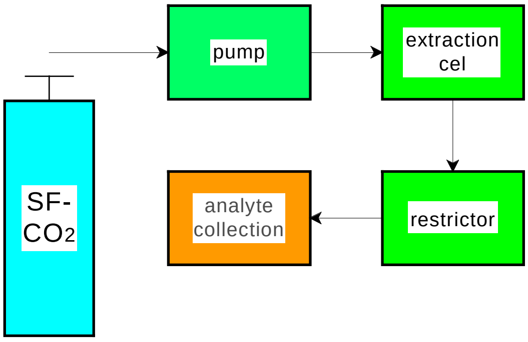


Figure 2 *Schematic representation of a SFE system*

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.

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.

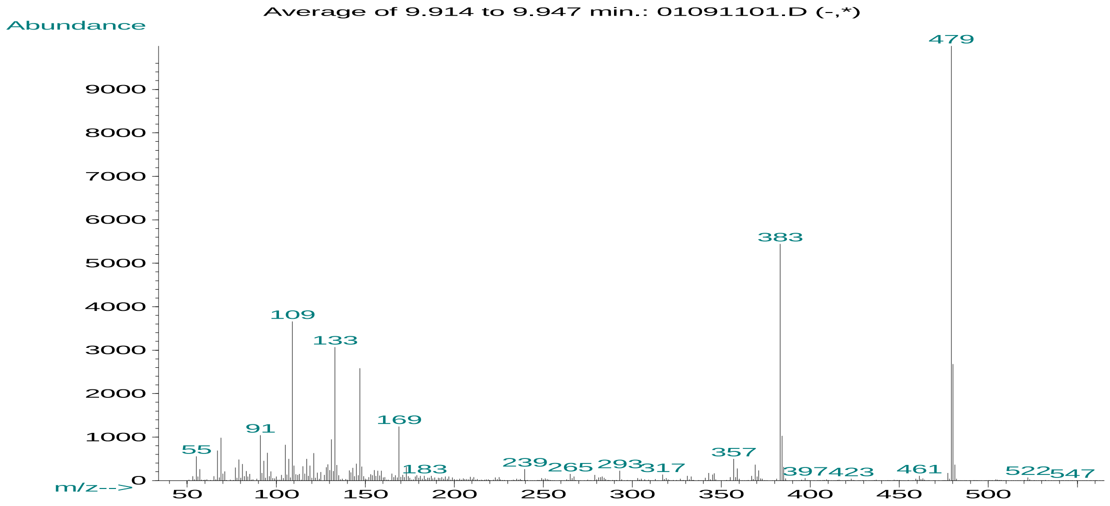
Automated SFE equipment

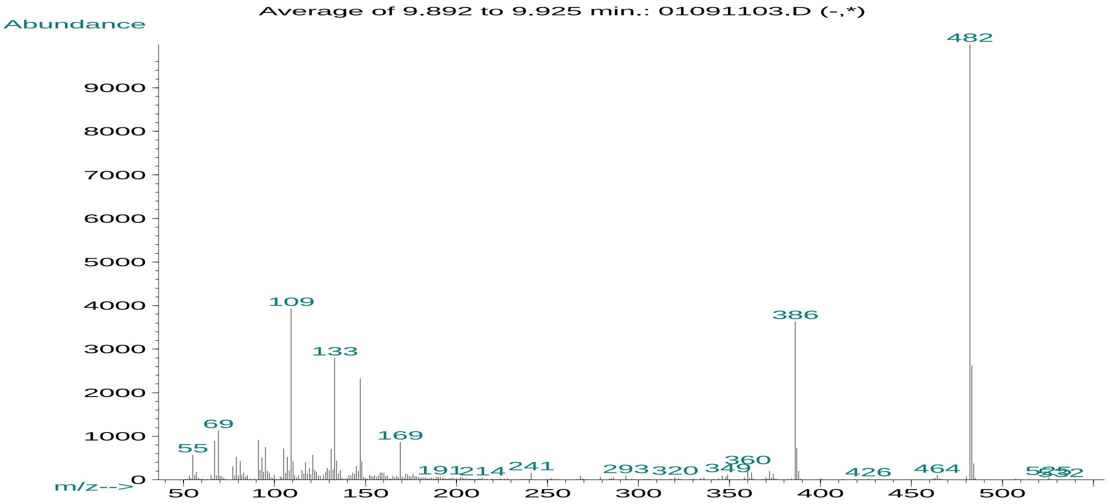
Thermal trapping is inherently simple since the SFE effluent is simply depressurized into a cooled vessel. Quantitative thermal trapping has been limited to non-volatile organic compounds since the high gas flow rate causes losses of moderately volatile analytes. Non-volatile analytes may also be lost 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

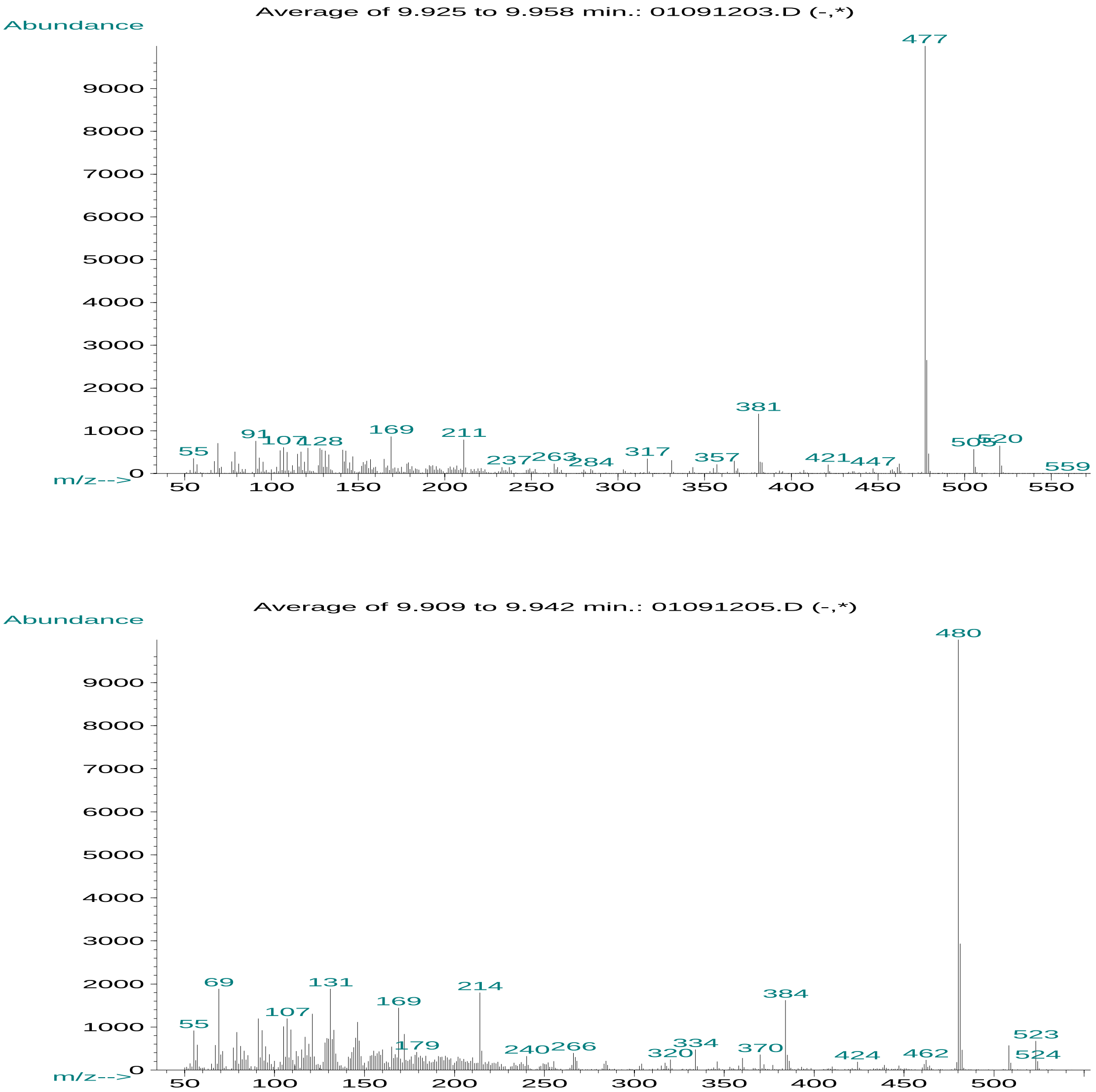
For example, an SFE extract could contain three compounds with different polarities, A, B and C, of which only B is of interest. Compound A could be removed by choosing a sorbent trap with low affinity for A, but high affinity for B and C, thus causing A to be lost through the trap during the SFE step. 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.





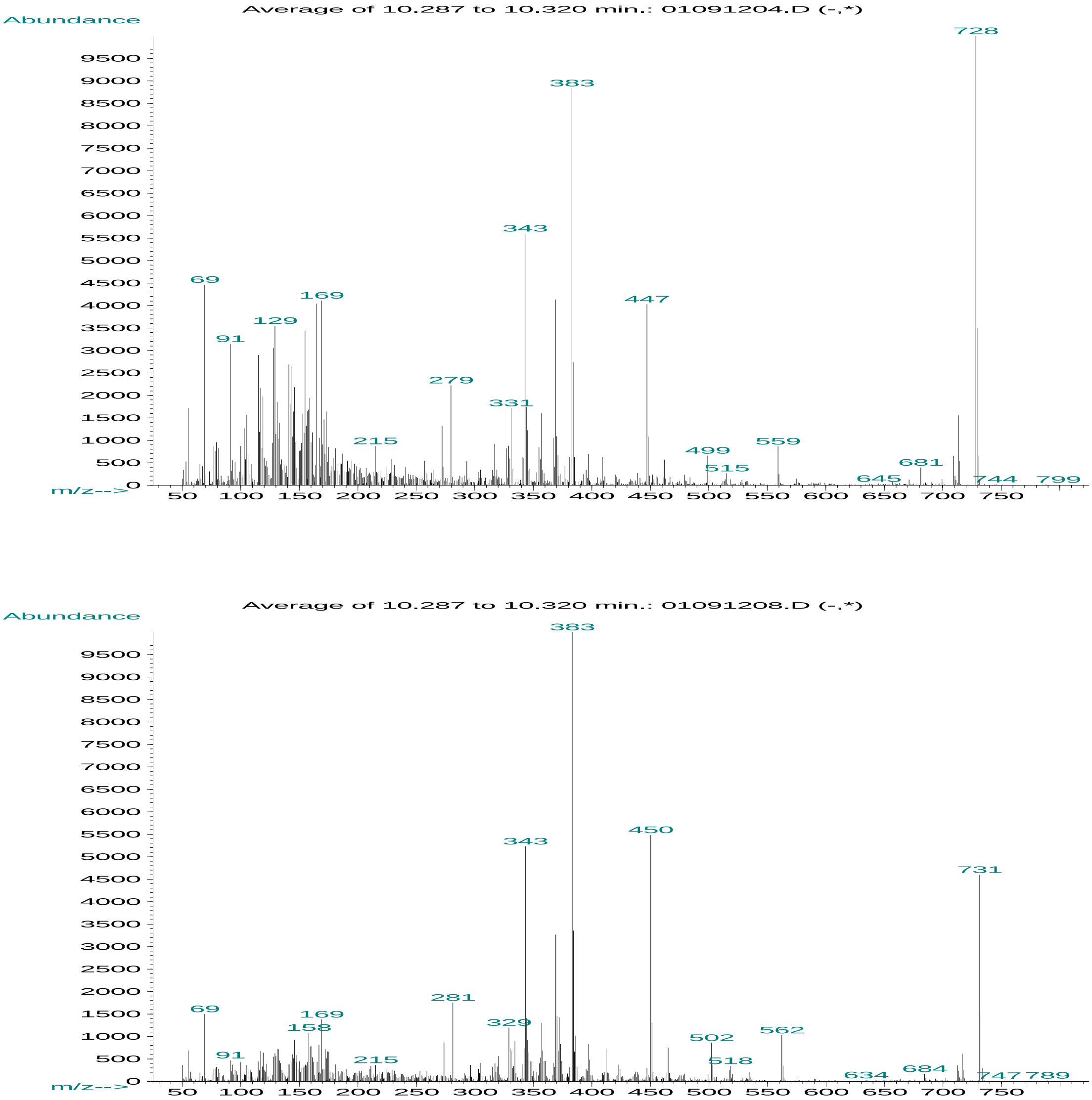
MS spectra of Medroxyprogesterone as mono-HFB derivative.
*Top Medroxyprogesteron, bottom Medroxyprogesteroned*3.
Agilent GC MSD type 5973N



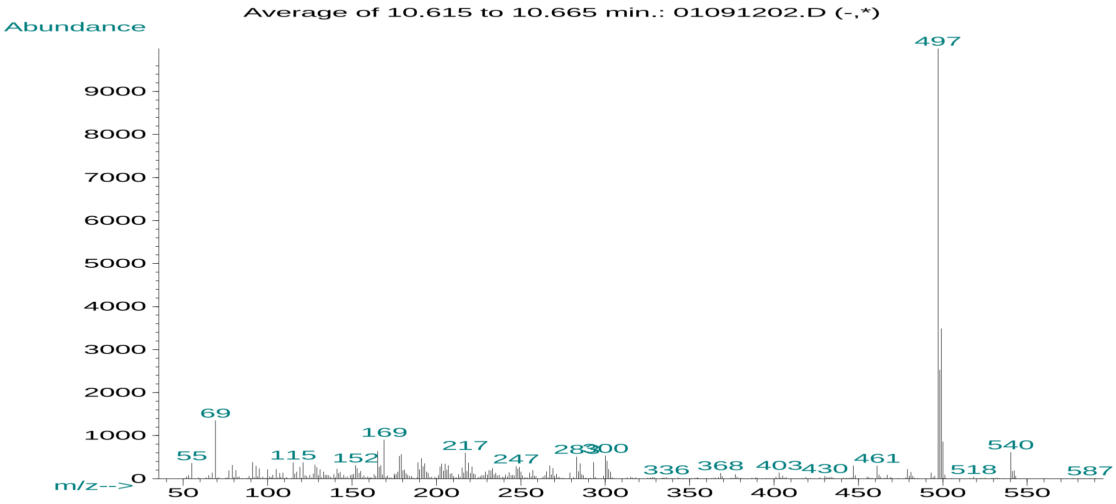
Average of 9.925 to 9.958 min.: 01091203.D (-,*)

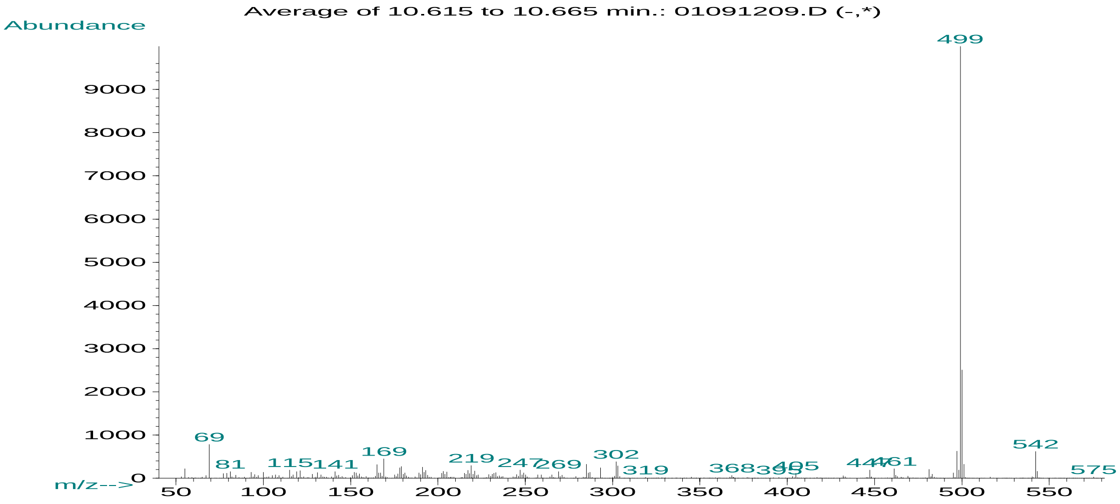
Average of 9.909 to 9.942 min.: 01091205.D (-,*)

MS spectra of Megestrol as mono-HFB derivative.
*Top Megestrol, bottom Megestrol-d*3.
Agilent GC-MSD type 5973N



MS spectra of Melengestrol as di-HFB derivative.
*Top Melengestrol, bottom Melengestrol-d*3.
Agilent GC-MSD type 5973N





MS spectra of Chlormadinone as mono-HFB derivative.
*Top Chlormadinone, bottom Chlormadinone-d*3.
Agilent GC-MSD type 5973N

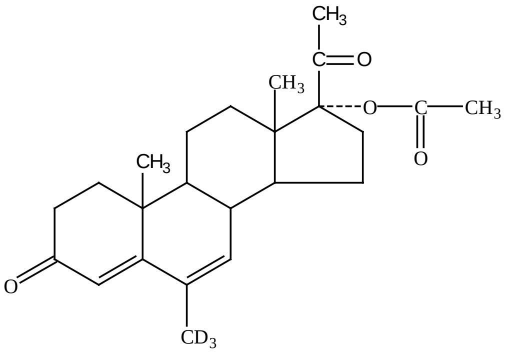
Isotope enriched internal standards

Th CRL has an extensive series of isotope enriched internal standards available, including triple deuteraqted analogues of Melengestrol, Megestrol and Medroxyprogesterone and Cl^{37}-Chlormadinone as well as of their respective acetates. Standards from this Bank of reference standards can be ordered at RIVM.

A compresed version of the datasheet for melengesttol acetate-d3 is attached

National Institute
for Public Health and
the Environment

Datasheet Megestrolacetate-D_3
Reference number : CEC/MAT : 13[5]
Date of preparation : 1994.05.16
date : 16 juli 2002
source : MAT - RIVM
"Bank of Reference Standards"



MEGESTROLACETATE- D3

Name : 17α-Hydroxy-6-trideuteromethylpregna-4,6-diene-3,20-dione acetate
Synonym : megestrolacetate - D_3
Molecular formula : $C_{24}H_{29}O_4D_3$
Cas # : not available
Molecular weight : 387.53

Long term stability tested on 1997.09.22 : 98.2 ± 2.3 %
(storage 4°C, analysis HPLC-UV, 6 tests on 2 ampoules)

Last update : 1998.01.06

<u>Method used for characterization</u>

I	IR spectroscopy
II	Mass spectrometry
III	HPLC-UV spectroscopy
IV	^{1}H-NMR spectrometry
V	Homogeneity and stability obtained with HPLC

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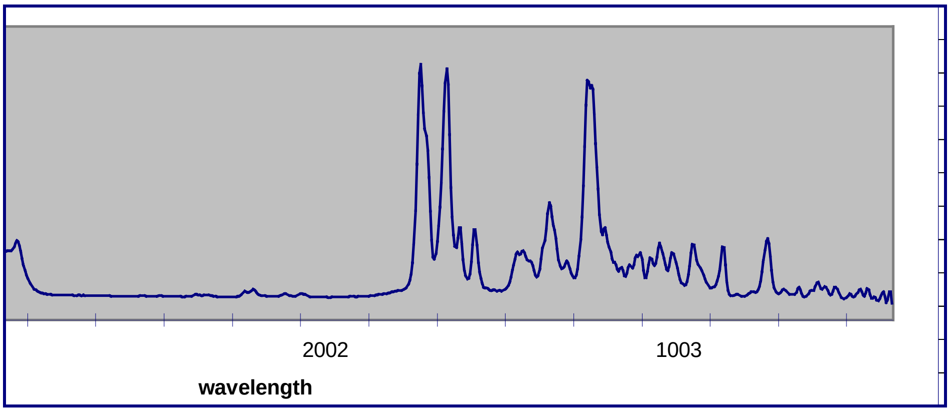
[5] The Bank of Reference Standards was financially supported by the European Commission
Directorate General "Science, Research and Development DG XII"
Contract MAT 1 - CT92 - 0020

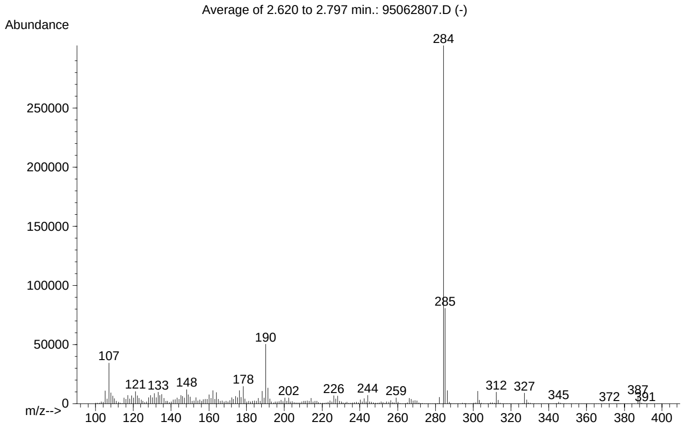
I IR-SPECTROSCOPY

Instrument: Bruker IFS-55 FTIR; detector DTGS
Sampling technique: KBr-tablet.



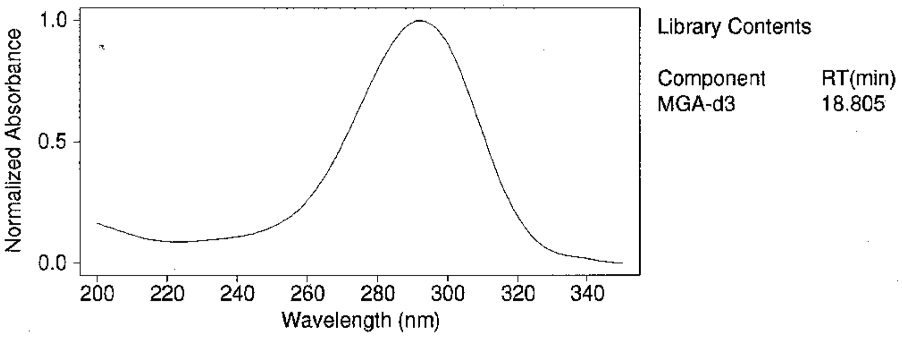
II MASS-SPECTROMETRY

Instrument: Hewlett Packard 5989 A MS
MS-spectrum, DIP = direct inlet probe



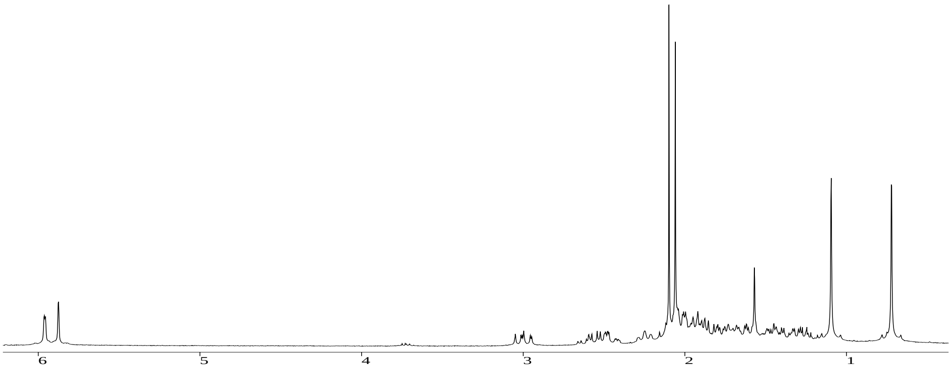
III HPLC-UV spectrum

Instrument:TSP spectrasystem UV2000; resolution 2nm.
HPLC eluens: methanol/water (70:30 $^{v}/_{v}$)
HPLC column: Lichrocart 125-4 hplc cartridge



IV ^{1}H-NMR SPECTROMETRY

Instrument: FT-NMR Jeol GSX; 270 MHz, 5 mm probe, solvent $CDCl_3$



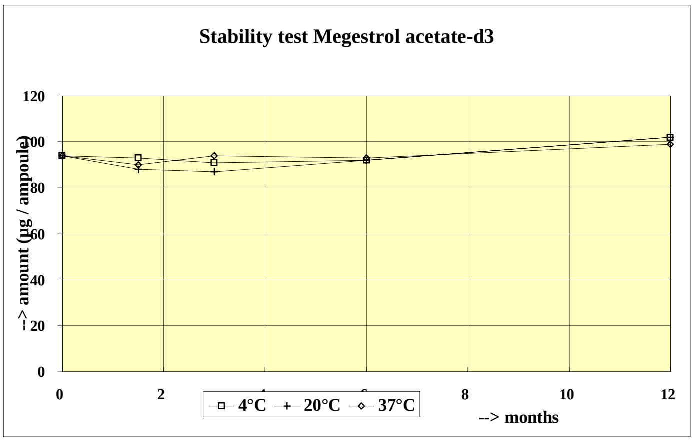
Chemical shifts (ppm)	Amount of protons (multiplicity)	Designation
0.75	3 (s)	18-CH_3
1.12	3 (s)	19-CH_3
2.08	3 (s)	21-CH_3
2.10	3 (s)	17-acetyl
5.92	2 (2s)	4-H en 7-H

V HOMOGENEITY AND STABILITY

Stability and homogeneity test of Megestrol-acetate-D_3

CEC/MAT: 13

temp.	t = 0 months homogeneity (n = 10) µg (m ± SD)	t =1,5 months (n = 2) µg (m ± SD)	t = 3 months (n = 2) µg (m ± SD)	t = 6 months (n = 2) µg (m ± SD)	t = 12 months (n = 2) µg (m ± SD)
4°C	(94 ± 4)	(93 ± 1)	(91 ± 4)	(92 ± 1)	(102 ± 3)
20°C		(88 ± 1)	(87 ± 1)	(92 ± 4)	(102 ±1)
37°C		(90 ± 1)	(94 ± 1)	(93 ± 1)	(99 ± 1)


Stability test Megestrol acetate-d3

Research for man and environment

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www.rivm.nl

Report 389002 117