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Analysis on residues of estrogens, gestagens and androgens in kidney fat and meat with gas chromatography–tandem mass spectrometry

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Abstract

The use of estrogens, gestagens and androgens (EGAs) in animal fattening is prohibited in the European Community. Based on the general detection capabilities of Belgian laboratories, National Minimum Required Performance Limits (National MRPLs) for a number of EGAs have been imposed by the inspection services. Selective hyphenated techniques, e.g. GC–MS and GC–MS^2, with high detection capability are needed. β-Trenbolone, which is meant to be a ''problem'' molecule for GC–MS, can be detected at the 2 μg/kg level using GC–MS^2. Based on the National MRPLs in different matrices, our laboratory has divided the EGAs into a class system. In this set-up, analysis of EGAs in kidney fat and meat is discussed. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Kidney fat; Steroids; Estrogens; Androgens; Gestagens

1. Introduction

For several years now, the use of natural and synthetic anabolic steroids, indicated as estrogens, gestagens and androgens (EGAs), in animal fattening has been prohibited in the European Community because of their possible toxic effect on public health. Although toxicologists have declared that certain growth promoters are safe under conventional application conditions, most European consumers do not want to eat meat coming from animals that were treated with these drugs. In Belgium, the Federal Agency for the Safety of the Food Chain (FAVV—AFSCA), founded in 2000 and established to coordinate the five inspection services, leads the control on illicit administration of EGAs. According to the national residue plan, a number of EGAs must be monitored by analysis of different matrices: injection sites, excreta such as urine and faeces, and tissue samples such as kidney fat and meat. In order to harmonise the analytical performance of methods for EGAs (and other banned substances) across EU member states, Minimum Required Performance Limits (MRPLs) have to be established. MRPLs, established by the European Commission and Community Reference Laboratories after consultation of the National Reference Laboratories, are the mini-

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mum contents of analytes to be detected in a sample. Ahead of the European Community, Belgian inspection services have introduced National MRPLs, namely common performance limits for all Belgian field laboratories analysing EGAs.

Kidney fat is considered to be the tissue of choice for detection of EGAs at slaughterhouse level. In 1979, the first multi-residue method for screening and confirmation of EGAs at the μg/kg (ppb) level in animal tissue was published by Verbeke [1], thereby applying High Performance Thin Layer Chromatography (HPTLC) with fluorescence detection. With this method most EGAs could be detected at the 0.5–10 ppb level. However, the clean-up concerned was time consuming and might lead to bottlenecks in routine analysis. For that reason modifications of the original method, particularly to obtain a faster clean-up, have been developed for potential application in regulatory control in Belgium [2–6]. Methods, in which High Performance Liquid Chromatography (HPLC) fractionation prior to HPTLC is integrated [7,8], or combination with gas chromatography–mass spectrometry (GC–MS)—by injecting a proportion of the extract used for HPTLC [9]—have also been described. At first, most Belgian laboratories used full scan GC–MS only to systematically confirm suspected HPTLC results [10–12]. Afterwards, GC–MS was used both as screening and as confirmation method. Only some particular ''GC–MS difficult molecules'' such as α/β-trenbolone, were still screened with HPTLC. Confirmation of α/β-trenbolone suspected samples, which occurred only seldom, was performed with liquid chromatography–multiple mass spectrometry (LC–MS^n) or other specific derivatization techniques [13,14]. Also for stanozolol and its major metabolite 16-hydroxystanozolol, LC–MS^n methods were developed [13–16]. Since 2 years, suspected GC–MS samples are systematically confirmed (or not) by re-injection into a gas chromatographic–multiple mass spectrometric (GC–MS^n) system [17].

In this investigation, a routine GC–MS^2 method for screening and confirmation of EGAs in kidney fat and meat is described. Quality criteria for the proper identification according to the revision of the commission decision 93/256/EEC (SANCO 1805/2000 revision 2) are discussed [18,19].

2. Experimental

2.1. Reagents and reference components

All reagents and solvents used were of analytical grade quality and provided by Merck (Darmstadt, Germany). Most reference steroids were obtained from Steraloids (Wilton, NY, USA) or Sigma (St Louis, MO, USA). Other steroids were gifts from various sources. All recent standards were obtained through the National Reference Laboratory (WIV-LP, Brussels, Belgium) to ensure that all the field laboratories use the same standards [http://www.iph.fgov.be/phbr/food/fr/anabolis.html]. The internal reference standard used was 1,4-androstadiene-3,17-dione (ADD). The GC–MS^n reference standard used was androsterone.

2.2. Solutions

From the stock solutions (200 ng anabolic steroid/μl in absolute ethanol, stored at 4 °C), working solutions at a concentration of 20 ng/μl were prepared. A solution containing the EGAs for which a National MRPL has been imposed by the inspection services, was prepared.

The derivatization reagent $MSTFA^{2+}$, needed to obtain suitable extracts for GC–MS^n analysis, was prepared by dissolving 100 mg ammonium iodide (NH_4I) (Sigma, St Louis, MO, USA) and 0.2 ml ethanethiol (Acros, Geel, Belgium) in 5 ml *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, Germany), followed by dilution of 1.5 ml of this solution with 10 ml MSTFA.

2.3. Apparatus and materials needed for extraction and clean-up of samples

For extraction and clean-up, a series of devices was used: a balance, a homogenisation set-up (e.g. Waring Blendor with reservoir of 250 ml, Stomacher, Ultra-Turrax), a microwave oven, a minishaker, a centrifuge equipped with centrifugation tubes of 300 ml, a rotary vacuum evaporator, a water bath, a vacuum manifold (e.g. Adsorbex SPU, Sample Preparation Unit, Analytichem International, Harbor City, CA, USA), an agitation instrument (Agitelec AG6, J.

Toulemonde and Cie, Paris, France) and a nitrogen evaporator (e.g. Techni Dry Block) or other types of evaporators (e.g. Speedvac SVC 200, SC 210A Savant, Howe Gyrovap). Materials and recipients were selectively chosen to be suitable in each step of the procedure: Schott Duran pots (250 ml), Stomacher bags (180–300 mm), centrifugation tubes (300 ml), extraction flasks (250 ml), solid-phase extraction columns (Isolute Si sorbent—3 cc/500 mg, Isolute NH_2 sorbent—1 cc/100 mg, IST International, Mid Glamorgan, UK) and amber 0.7 ml autosampler vials.

2.4. GC–MS apparatus and conditions

2.4.1. Apparatus and materials

To obtain gas chromatographic and coupled mass spectrometric information, two low-resolution devices were used. On one hand, the ion trap used was a POLARIS ion trap mass spectrometer coupled to a ThermoQuest CE Trace GC gas chromatograph (ThermoFinnigan, Austin, TX, USA). Samples were injected using a Carlo Erba autosampler AS2000 (ThermoFinnigan, Austin, TX, USA). A hydrogen generator (Packard, Meriden, USA) was coupled to the gas chromatograph (GC) and hydrogen gas was used as GC carrier gas at a flow-rate of 1 ml/min.

On the other hand, GC–MS^2 analyses were carried out using a GCQ plus (ThermoFinnigan, Austin, TX, USA) consisting of a Finnigan GC coupled to GCQ ion trap mass spectrometer. Here samples were injected using a Finnigan MAT A200S autosampler. Helium gas was used as GC carrier gas at a flow-rate of approx. 1 ml/min.

In both systems, MS–MS measurements were performed using helium as collision gas in the ion trap at a supply pressure of 3 bar, the electron ionisation energy being 70 eV.

2.4.2. GC–MS^n conditions

Analyses were performed using a non-polar 5% phenyl-polysilphenylene-siloxane SGE BPX-5 GC-column (25 m×0.22 mm I.D., 0.25 μm) (SGE Incorporated, Austin, TX, USA). In Table 1, the gas chromatographic parameters are presented for both devices.

Full scan MS acquisition method parameters were identical for both MS systems: electron impact mode, three microscans; mass range 150–570 a.m.u.; ion source temperature at 200 °C; transfer line temperature at 275 °C. Also the full scan tandem MS (MS–MS) acquisition method parameters (three microscans; several scan segments with scan events depending on the EGAs to be analysed; mass range depending on the selected precursor ion; activating potential between 0.70 and 1.30 V) were the same.

2.4.3. GC–MS^n interpretation

Xcalibur™ software (ThermoFinnigan, Austin, TX, USA) version 1.2 was used to perform the interpretation of the analytical results.

Table 1
Gas chromatographic parameters to perform GC–MS^n analyses

Parameter	POLARIS	GCQ plus
Temperature program		
Initial temperature	100 °C (hold 1 min)	100 °C (hold 1 min)
Segment 1	250 °C (30 °C/min)	250 °C (17 °C/min)
Segment 2	290 °C (2.5 °C/min)	300 °C (2 °C/min)
Segment 3	300 °C (10 °C/min)	–
Isotherm segment	300 °C (hold 1.5 min)	300 °C (hold 1 min)
GC carrier gas	Hydrogen	Helium
Column flow	1 ml/min	0.91 ml/min
Injector (splitless mode)		
Temperature	250 °C	260 °C
Split flow	60 ml/min	60 ml/min

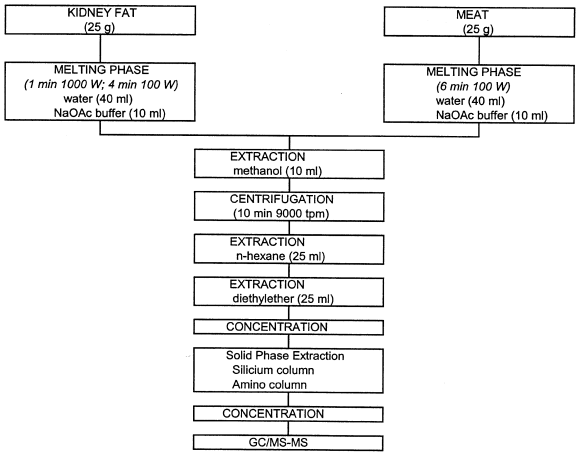

Fig. 1. Overall scheme for the extraction and clean-up procedure for anabolic components in kidney fat or meat.

2.5. Methods

2.5.1. Extraction

In Fig. 1, an overall scheme of the extraction procedure is given. A 25-g aliquot of kidney fat or meat was cut into small pieces and weighed into a glass flask. Sodium acetate buffer (10 ml, 0.2 *M*) and 40 ml ultrapure water were added. The fat or meat samples were molten in a microwave oven (melting method depending on the matrix type). Subsequently, 5 ng ADD per gram matrix (5 ppb ADD) was added. The content was homogenised and, after addition of 50 ml methanol and homogenisation by shaking thoroughly, the content was transferred to centrifugation tubes and centrifuged at 13 000 *g*. Subsequently, the supernatant was brought into a separation funnel, thereby excluding fatty lumps, and extracted twice with 25 ml *n*-hexane in order to remove the remaining triacylglycerols. Then the EGAs were extracted into 100 ml diethyl ether. The diethyl ether phase was washed with 15 ml ultrapure water. Finally, the diethyl ether phase was evaporated to ''completely dry'' using a rotary evaporator or equivalent device.

2.5.2. Clean-up

The evaporated crude extract was resolved in 0.5 ml chloroform and diluted with 5 ml *n*-hexane. Solid phase extraction (SPE), thereby using a coupled Silicium/amino (Si/NH_2) column system, could be established: the Si-column was conditioned with 2× 2.5 ml *n*-hexane (still uncoupled). The extract was quantitatively passed on the top of the Si-column and allowed to drain in, using a sample preparation unit coupled to a vacuum source. The Si-column was washed with 5 ml *n*-hexane and allowed to run dry. Subsequently, the Si-column was coupled to an NH_2-column and the whole system was washed with 5 ml *n*-hexane. Elution of the anabolic components from the two columns was carried out with 5 ml chloroform/acetone (4:1, v/v). The eluate was evaporated to dryness under a nitrogen flow, and resolved in 0.5 ml ethanol. Finally, the extract, to which 5 ppb androsterone was added, was evaporated to dryness once more.

2.5.3. Derivatization

The final SPE extract was derivatized to enol-trimethylsilyl ether derivatives with $MSTFA^{2+}$: the sample was transferred into an autosampler vial (0.7 ml) and evaporated to dryness under a nitrogen flow. A standard solution of which the concentration was equivalent to 4 ng analyte on column, was analysed along with the sample to verify the optimum status of the GC–MS device. Therefore, 10 μl working solution (20 ng/μl) was dried in another autosampler vial under the same conditions as the sample. A 25-μl aliquot of $MSTFA^{2+}$ was added to each vial and after closure the vials were mixed thoroughly using a vortex mixer. Then, 1 μl was injected into the GC.

3. Results and discussion

3.1. Analysis of EGAs: present situation

The control on the illicit use of EGAs nowadays is monitored by analysis of different matrices: injection sites, excreta such as urine and faeces, and tissue samples such as kidney fat and meat. In this investigation, kidney fat and meat are discussed. Until now, a multi-stage analytical clean-up strategy, compatible for HPTLC and GC–MS [4,9], was followed. Kidney fat and meat sample extracts were screened with HPTLC, the remainder of the extract was then analysed with GC–MS in full-scan MS

mode after derivatization with $MSTFA^{2+}$. Being suspected of containing an EGA, the derivatized extract was injected once more (in another GC–MS apparatus) to perform a full scan $GC–MS^2$ analysis in order to gain more analytical information and identification points [18]. If the presence of the illicit EGA could be established, the procedure was completely resumed before results were passed to the inspection services. Then, the owner of the ''violative'' animal had the right to demand a second analysis, in an accredited laboratory of his own choice.

In order to fit into the system of accreditation according to EN45001 (to be replaced by ISO EN-17025 from 2002 on), a system of intra-laboratory ''performance limits'' was validated to ensure the quality for this kind of analyses. Based on build-up experience, the lowest robust concentration level in samples (kidney fat or meat) was determined for each EGA. Subsequently, a large number of samples (e.g. $n=50$) were spiked with EGAs at the concerning ''performance limit'' and analyses were performed using the method mentioned above. If a component was found present—according to all quality criteria—in at least 95% of all the incurred samples, that particular concentration level was considered to be equal to or greater than its detection capability ($CC\beta$) [20]. This latter can be defined as the smallest content of analyte, identified by a specified set of identification parameters, that may be detected or quantified in a sample with an error probability of β (chance on a false negative decision; β-error=5%) [18]. Determination of a $CC\beta$ value guarantees detection certainty for suspected EGAs, at or above their $CC\beta$ value, with a probability of at least 95%. This approach is more preferable than the use of limits of detection, of which determination is based on the analysis of one blank meat or kidney fat sample, spiked at different concentrations. For this method, validation was carried out according to SANCO 1805/2000 (revision 2), meaning that the detection capability $CC\beta$, the decision limit $CC\alpha$, selectivity and specificity, and applicability, ruggedness and stability were successfully determined [18–20].

In order to harmonise the performance characteristics that Belgian accredited laboratories must achieve in their analytical methods for banned substances, the inspection services have introduced National MRPLs for EGAs as described in the revision of the commission decision 93/256/EEC [19]. All labs were invited to turn in their individual ''performance'' limits. These data were discussed in a working group and an official list, taking into account the analytical possibilities at that time, was made. The resulting National MRPLs for EGAs are given in Table 2.

Now that the National MRPLs are laid down, all Belgian accredited laboratories have to reach them, keeping in mind that those values are always temporarily and thus will decrease with increasing analytical possibilities. Under the supervision of the

Table 2
National Minimum Required Performance Limits for the determination of EGAs in kidney fat and meat samples

Component	National MRPL (μg/kg)	Component	National MRPL (μg/kg)
Diethylstilbestrol	2	Chlortestosterone acetate	50
Hexestrol	5	β-Trenbolone	2
Dienestrol	2	Trenbolone acetate	2
α/β-Zeranol	5	Norethandrolone	2
Ethinylestradiol	2	Medroxyprogesterone acetate	10
α/β-Nortestosterone	2	Chlormadinone acetate	10
Methyltestosterone	2	Megestrol acetate	10
α/β-Boldenone	5	Melengestrol acetate	10
Methylboldenone	3	Acetoxyprogesterone[a]	10
Norgestrel	5	Caproxyprogesterone[a]	10

[a] Caproxyprogesterone can be distinguished from acetoxyprogesterone only by HPLC fractionation.

inspection services, the Belgian accreditation organization BELTEST has to check the analytical capability of the laboratories by organizing audits (according to EN-45001/ISO EN-17025).

3.2. GC–MS^2 for the analysis of EGAs

In order to meet these National MRPLs, our laboratory has decided to withdraw HPTLC and full scan GC–MS from the routine screening program and to turn over to full scan GC–MS^2 to perform routine analyses.

When using full scan GC–MS^2, only one molecule-specific ion is held in the ion trap within the mass analyser. This precursor ion is then fragmented once again due to the applied activating potential, resulting in a series of fragment ions, so-called ''transition products''. In Table 3 the precursor ion and transition product ions of EGAs that are generally monitored in routine residue analysis are given. To illustrate that the precursor ion can be any of the trimethylsilyl (TMS) ether's diagnostic ions, the molecular mass ion of the TMS ether is also mentioned. In this set-up, two devices (ThermoFinnigan GCQ plus and POLARIS) were used. Though the GC carrier gas was different, no significant differences in the fragmentation pattern of the EGAs were noticed. It should be mentioned that using hydrogen as GC carrier gas will shorten analysis time, thus resulting in more analytical results in less time. Moreover, the chromatographic resolution of GC–MS analyses is much better compared to analyses performed with helium [21].

Using full scan GC–MS^2 has several advantages. Some molecules, e.g. β-trenbolone and trenbolone-acetate, which are mentioned to be difficult for screening in meat or kidney fat with full scan GC–MS, can be detected much more specifically at better CCβ values using full scan GC–MS^2. In 1991, it was mentioned that the TMS ether and fluoroacyl (FA) ester derivatives of trenbolone were not stable due to the formation of enol derivatives at the 3C-position in several tautomeric forms, which in their turn were not stable and lost two or four hydrogens under the conditions studied [13]. Conventional GC–MS procedures failed to detect trenbolone or its

Table 3
Specific diagnostic ions of the EGAs that are monitored in routine residue analysis (GC–MS^2)

Component	TMS-ether molecular mass ion GC–MS (m/z)	Precursor ion GC–MS^2 (m/z)	Transition product ions GC–MS^2 (m/z)
Diethylstilbestrol	412	412	217-383-396-397
Hexestrol	399	207	163-179-191
Dienestrol	410	410	379-381-395
α/β-Zeranol	433	433	295-309-323-337-389-415
Ethinylestradiol	440	425	193-231-281-303-323-407
α/β-Nortestosterone	418	418	182-287-313-327-328-403
Methyltestosterone	446	446	251-301-314-341-356
α/β-Boldenone	430	206	163-175-183-191
Methylboldenone	444	444	283-297-312-339-354-429
Chlortestosterone acetate	436	436	230-385-401-421
β-Trenbolone	414	414	283-298-309-324
Trenbolone acetate	472	412	323-337-355-370-383-397
Norethandrolone	446	446	287-299-300-356
Medroxyprogesterone acetate	470	470	222-237-365-380-455
Chlormadinone acetate	488	488	363-383-437-453-473
Megestrol acetate	468	468	323-363-440-453
Melengestrol acetate	482	482	337-376-377-454-467
Acetoxyprogesterone[a]	456	456	208-351-366-428-441
Norgestrel	456	456	301-316-337-366-427
Flurogestone acetate	562	562	208-347-367-437-457-562

[a] Caproxyprogesterone can be distinguished from acetoxyprogesterone only by HPLC fractionation.

metabolites due to derivatization problems. Therefore, methoxime-TMS ether or methoxime-FA ester derivatives, or LC–MS methods [13] were used to identify trenbolone. This resulted in analysing each sample twice, causing increasing personnel and instrument costs.

Rather surprisingly, GC–MS^2 can be a suitable solution to overcome the problems for trenbolone. In Fig. 2, chromatograms and mass spectra for β-trenbolone 17-TMS (a: full scan GC–MS; b: full scan GC–MS^2) are presented, in Fig. 3 the principal fragmentation mechanisms are illustrated. As mentioned in Table 3, the diagnostic ions for β-trenbolone are m/z 283, 298, 309 and 324. Performing full scan GC–MS, this molecule is assumed to be a ''problem molecule'' because its CCβ—and also that of trenbolone acetate—is not within the range of the other EGAs ($CC\beta = 18$ μg/kg). However, with full scan GC–MS^2, the $CC\beta$ can be turned down to 2 μg/kg, which is a great improvement and meets the National MRPL. Moreover, screening with HPTLC may be omitted.

Application of full scan GC–MS^2 in routine analysis also creates some pitfalls. Since lower concentration levels can be reached, interferences from background signals become more important. The precursor ion, which should be component-specific, can also be present in the background signal, leading to interferences in the GC–MS^2 results. The lower the concentration level of the component looked for, the higher this risk. This effect is illustrated in Fig. 4. A sample, suspected of the presence of β-trenbolone at a concentration = 0.2 μg/kg, was injected twice on the same GC–MS apparatus and the spectra were compared with a standard. Concerning the first injection results (middle) the criteria for the diagnostic ions could certainly not be fulfilled (= non-violative result). The

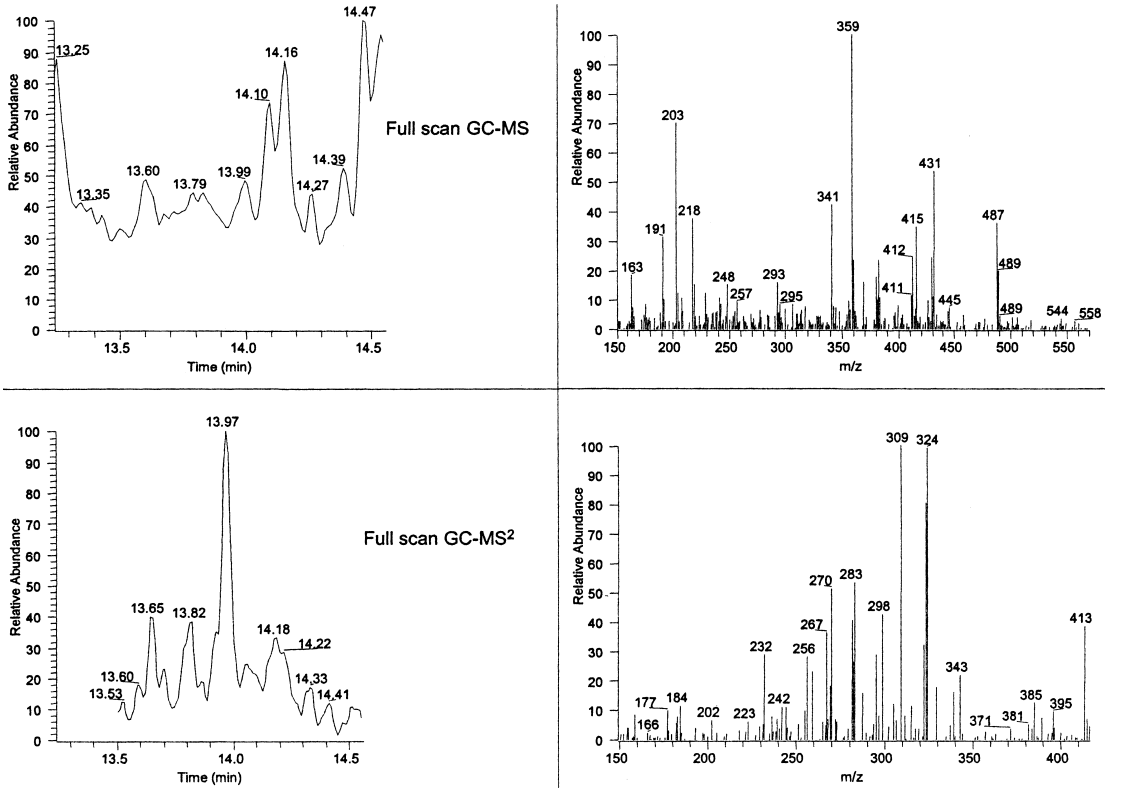

Fig. 2. Chromatograms and mass spectra for β-trenbolone 17-TMS (a: full scan GC–MS; b: full scan GC–MS^2) at (absolute) retention time 13.97 min.

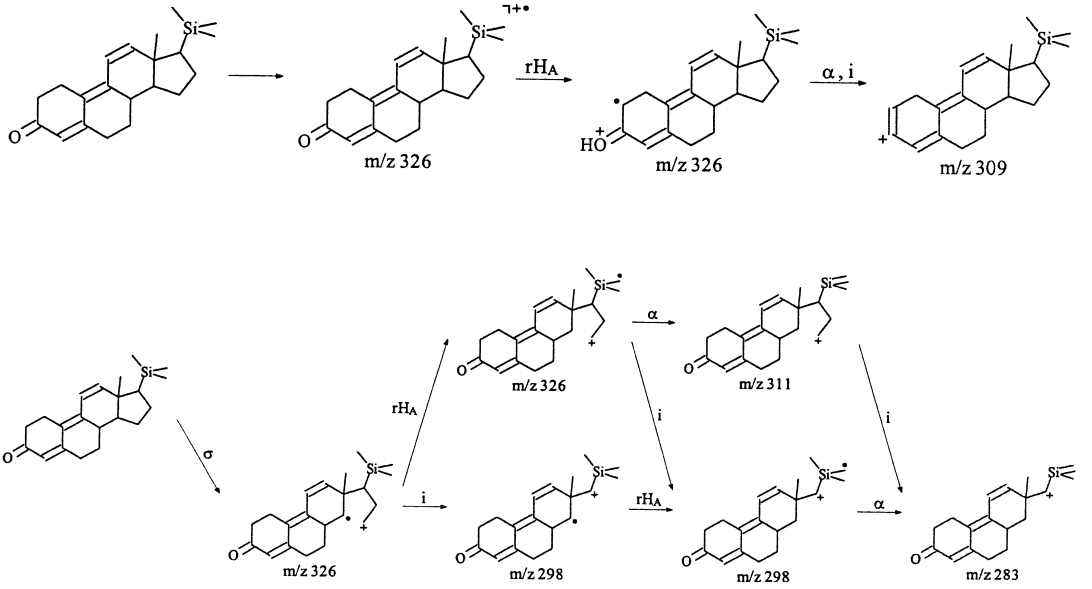

Fig. 3. Principal fragmentation mechanisms of β-trenbolone 17-TMS in electron impact mode.

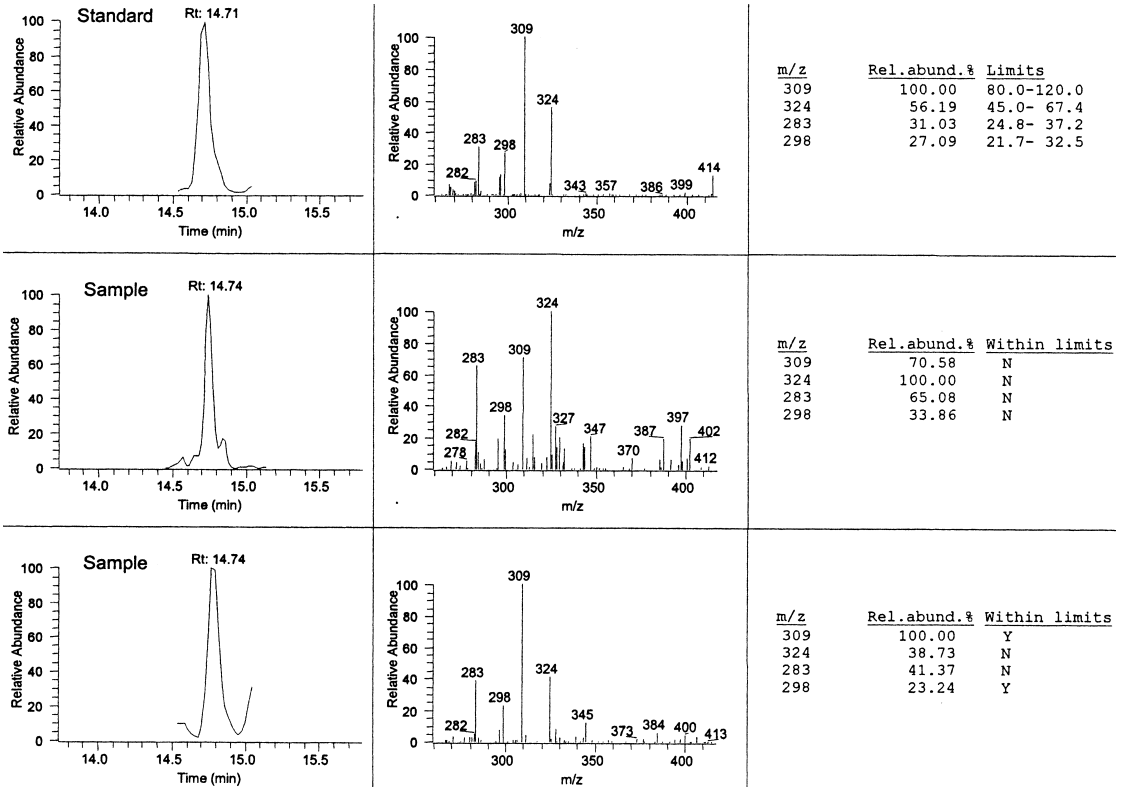

Fig. 4. Interferences of background signals using full scan GC–MS^2.

second time (below) criteria could be fulfilled by two diagnostic ions (309 and 298) thereby yielding four identification points: 1 IP for the precursor ion and 3 IP for the transition products (each transition product yields 1.5 IP) (=violative result (see below)). Moreover, in the second injection mass spectrum, the two other diagnostic ions are also present and the spectrum's visual appearance is similar to that of the standard. However, a careful analyst would still feel doubt towards the spectrum being generated by β-trenbolone. If possible, the analysis should be repeated to obtain additional information. To overcome this background signal problem, another clean-up procedure — pointed specifically to this component, e.g. HPLC fractionation in which a narrow fraction containing the molecule of interest is collected — another derivatization technique, or another detection mode such as LC–MS^n, can be used. Although GC–MS^2 has proven to be more selective than GC–MS, analysts should always be aware of these interference signals. Moreover, the analytical performance of the applied methods should always be in balance with the residue levels obtained after illicit administration. These are reflected in the MRPLs.

3.3. QA criteria for EGAs

As described earlier [18], the use of identification points (IPs) is a new approach to set up quality criteria for the identification of organic residues and contaminants in general. The system of IPs balances the identification power of the different analytical techniques and has the advantage that new techniques can be introduced easily. If analyses are performed using GC–MS^2, the relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion, must correspond to those of the reference analyte, either from calibration standards or from incurred samples, at comparative concentrations and measured under the same conditions, within the tolerances given in Table 4.

For the confirmation of the banned EGAs, a minimum of four IPs is required. Using low-resolution GC–MS^2, only the precursor ion (IP 1.0) and two transition product ions (each transition product yielding an IP 1.5) with suitable relative intensities are needed to fulfil the criteria. In Fig. 5 the IP system is illustrated by means of the mass spectra and relative ion intensities for 17-methyltestosterone (above), chlortestosterone acetate (in the middle) and dienestrol (below). Each GC–MS^2 fragmentation process resulted in four transition products. Seven IPs could be calculated for each component: 1 IP for the precursor ion and 1.5 IP for each transition product, resulting in $1+(4\times1.5)=7$ IPs.

Although the system of IPs is mentioned to be a ''golden rule'', some attention must be paid because the concentration of the analyte present in the sample plays an important role for interpretation of GC–MS^2 results. As mentioned above it is important to compare the sample results with those of a control sample that was spiked at the same concentration level because the fragmentation pattern of an analyte can vary as the concentration becomes lower than 1–2 ppb and thus reaches the detection capability. Full scan GC–MS^2 mass spectra and principal fragments of flugestone acetate 3,11,17-TMS in different concentration levels are demonstrated in Figs. 6 and 7, respectively. This figure shows that the relative mass intensities may have variable values due to the concentration level. Even, when a sample containing an analyte in very low concentration is analysed twice, variable results can be obtained. It is sometimes difficult to meet the quality criteria though the mass spectrum shows the presence of a banned analyte, leading to a risk of false negative

Table 4
Maximum permitted tolerances for relative ion intensities

Relative intensity (% of base peak)	Tolerance (% of peak intensity) (GC–EI-MS)	Other techniques (GC–CI-MS, GC–MS^n, LC–MS, LC–MS^n)
>50%	±10%	±20%
>20–50%	±15%	±25%
>10–20%	±20%	±30%
≤10%	±50%	±50%

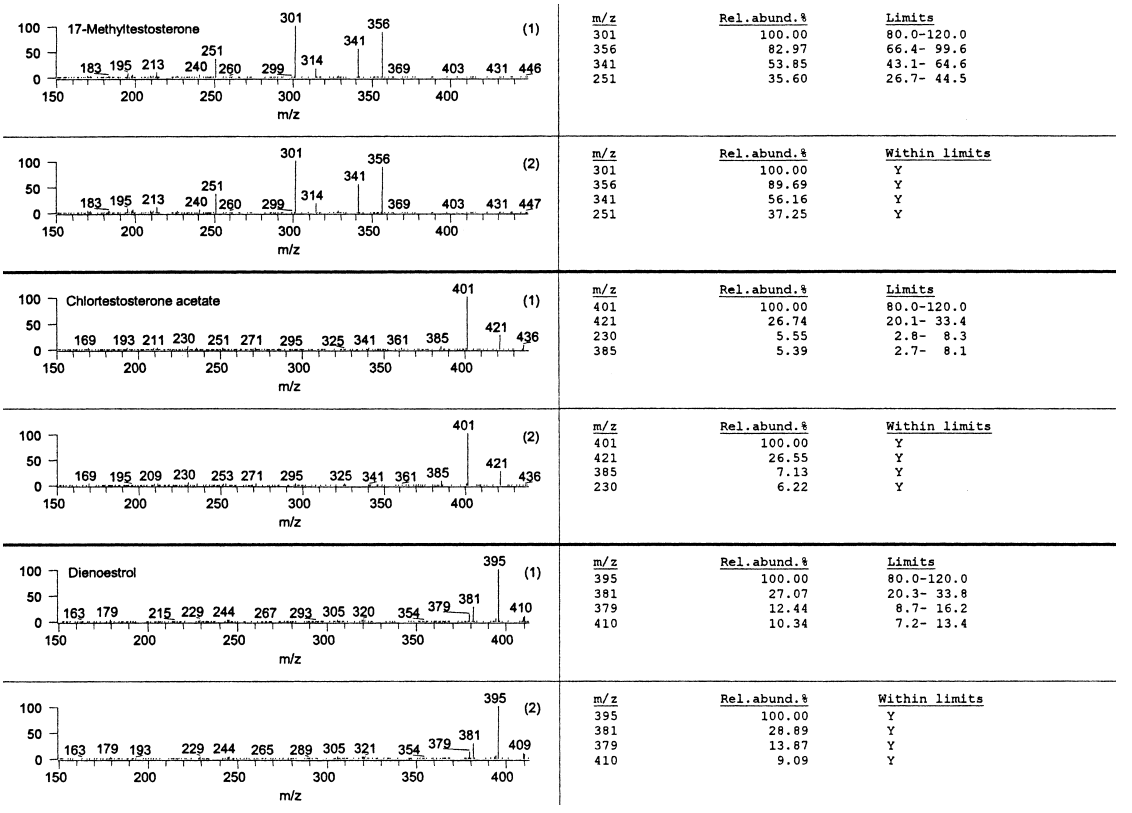

Fig. 5. Identification points to confirm a violative sample for 17-methyltestosterone (above), chlortestosterone acetate (middle) and dienestrol (below).

samples if the IP system is applied blindly. However, it can be concluded that GC–MS^2 is very useful for screening and confirmation of samples under routine conditions.

3.4. Strategy towards National MRPLs

In order to meet the National MRPLs within the laboratory, the EGAs were divided into three classes (Table 5). EGAs validated at a concentration below the National MRPL belong to class I. EGAs validated at the National MRPL itself are marked as class II substances. EGAs validated at a concentration above the National MRPL belong to class III. All the EGAs must belong to class I or class II to meet requisites of the inspection services. The strategy towards these three intra-laboratory classes is different. First of all, priority was given to move class III EGAs to (at least) class II. Afterwards, considerable effort has been made to improve the general detection capability in order to classify all EGAs in class I. In our laboratory most of the EGAs (with the exception of trenbolone and trenbolone acetate) are validated as class I substances. The intra-laboratory class system has the following function: if, during routine analysis, identification criteria cannot be fulfilled for the control sample—spiked with a class I EGAs concentration—the spike concentration level is increased to the class II (=National MRPL) level. The routine analysis then still meets the demands of the inspection services. However, the reason why the EGA cannot be detected anymore at the class I concentration level should be investigated. The problem has been solved only if the EGA can be detected at the same concentration level as before, by which all quality criteria are fulfilled and the same detection capability is reached. Afterwards, the spike concentration level

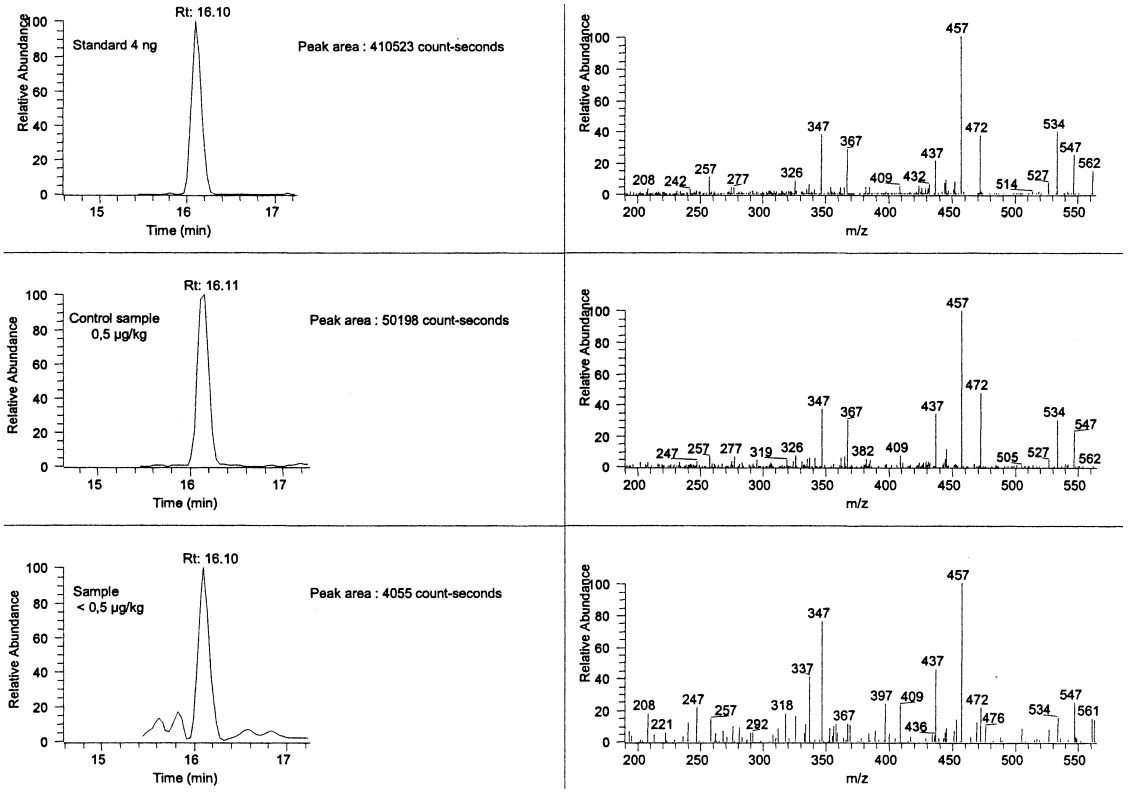

Fig. 6. Full scan GC–MS^2 mass spectra of flugestone acetate 3,11,17-TMS at different concentration levels.

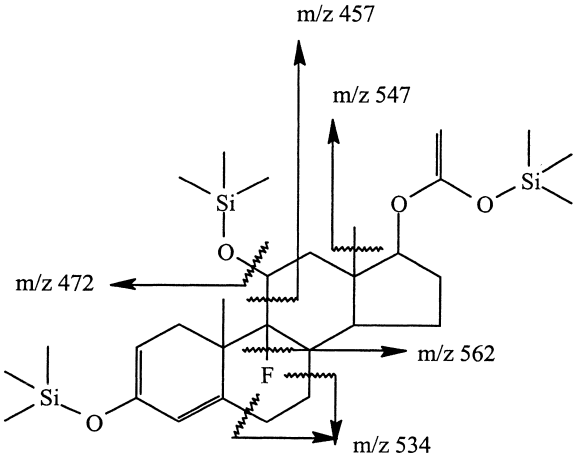

Fig. 7. Principal fragments of flugestone acetate 3,11,17-TMS in electron impact mode.

is brought back to class I. EGAs for which no National MRPL is imposed are automatically classified as class I components.

The class system can be useful under accreditation conditions, especially in the future. As mentioned above, the (National) MRPLs will be temporary and will thus decrease with increasing analytical possibilities. Our laboratory tries to be ahead of that phenomenon. However, having a better CCβ than needed also includes some problems, e.g. what a lab should do if an analyte is found—according to all quality criteria—at a concentration level (far) below the MRPL. The answer to this question has to be given by the inspection services themselves. Viewing an efficient control, the information obtained from the laboratories, may be divided into ''hard'' and ''soft'' information. Results above the National MRPL may be considered as violative and can be used for the rejection of carcasses or legal actions. Results (far) below the National MRPL should be

Table 5
Detection capabilities (CCβ) in our laboratory compared with National MRPLs

Component	National MRPL (μg/kg)	Class within the laboratory	CCβ within the laboratory $\leq x$ (μg/kg)
Diethylstilbestrol	2	I	0.5
Hexestrol	5	I	0.5
Dienestrol	2	I	0.5
α/β-Zeranol	5	I	0.5
Ethinylestradiol	2	I	0.5
α/β-Nortestosterone	2	I	0.5
Methyltestosterone	2	I	0.5
α/β-Boldenone	5	I	1
Methylboldenone	3	I	1
Norgestrel	5	I	0.5
Chlortestosterone acetate	50	I	5
β-Trenbolone	2	II	2
Trenbolone acetate	2	II	2
Norethandrolone	2	I	0.5
Medroxyprogesterone acetate	10	I	1.5
Chlormadinone acetate	10	I	2.5
Megestrol acetate	10	I	2.5
Melengestrol acetate	10	I	5
Acetoxyprogesterone	10	I	1.5
Caproxyprogesterone	10	I	2.5
Methandriol[a]	–	I	2
Fluoxymesterone[a]	–	I	2
Flurogestone acetate[a]	–	I	0.5

[a] No NMRPL in kidney fat and/or meat is laid down.

considered as soft information and can be mentioned as non-violative. However, they can be used to inform the inspection services to take other actions, e.g. sampling other matrices from the same production line in which the particular EGA is suspected to be present at a higher concentration level.

Another question can be asked: is it necessary to have such low CCβs in accordance with the expected residue levels in kidney fat and meat after illicit administration? The answer is yes. The CCβ level must be as low as possible since cocktails of synergetic EGAs are composed and administered with the purpose that the residue level of each individual EGA is that low that the EGA cannot be detected.

4. Conclusion

In this investigation, a routine GC–MS^2 method for screening and confirmation of estrogens, gestagens and androgens (EGAs) in kidney fat and meat is described. The use of this technique has improved significantly the detection capability (CCβ) and permits detection of all listed EGAs at or below their National Minimum Required Performance Limits (National MRPLs). Moreover, a number of other EGAs (without a National MRPL) such as fluoxymesterone, methandriol and flurogestone acetate, can be monitored at a concentration level of the same magnitude. This method fits into the inspection services strategy to control the abuse of EGAs in cattle fattening.

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