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Faster analysis of anabolic steroids in kidney fat by downscaling the sample size and using gas chromatography-tandem mass spectrometry

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Abstract

A rapid and easy-to-perform method for the screening and confirmation of estrogens, gestagens and androgens (EGAs) in kidney fat has been developed. In this investigation only 5 g of fat was needed. After extraction with acetonitrile the steroid phase was defatted using *n*-hexane. Followed by a saponification step, the sample extract was purified by solid phase extraction. After derivatisation as trimethylsilyl ether derivatives, the steroids in the final extract were analysed using gas chromatography-tandem mass spectrometry (GC-MS^2). With this method the Belgian National Minimum Required Performance Limits (National MRPLs) for EGAs could be fulfilled.

Keywords: Anabolic steroids; Kidney fat; Solid phase extraction; GC-MS^2

1. Introduction

In the European Community the use of steroidal hormones for cattle fattening purposes has been forbidden since 1988. Steroidal hormones can be classified in three subgroups: estrogens, gestagens and androgens (EGAs). Some natural and synthetic EGAs have been used as growth promoters in animal feeds or have been administered directly to the animals. Because of the danger of residues of these hormones being present in foods for human consumption, the use of EGAs as growth promoters has been banned in all the member states of the European Community. Also the sale and slaughter for consumption animals treated with EGA substances is subject to control [1]. Quality criteria for the identification [2] of banned substances according to the revision of the commission decision [3] and [4] have been imposed and inspection services in every European Union member state lead the control on illicit administration of EGAs. In Belgium, the Federal Agency for the Safety of the Food Chain (FAVV-AFSCA) has a national residue plan and illegal steroid administration is monitored by analysis of different matrices at various stages in the food chain. At farm level, feed and excreta, such as urine and faeces, are checked; at slaughterhouse level, injections sites, meat or fat tissue samples and organs

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(liver, kidney, bile) are analysed. Because many steroids are fat soluble, kidney fat is considered to be the tissue of choice for detection at slaughterhouse level.

In order to harmonise the criteria at which analytical laboratories must fulfil to be permitted to perform residue analyses, the European Commission has established Minimum Required Performance Limits (MRPLs). These are the minimum contents of analytes in a sample which at least have to be detected and confirmed [2,3]. In Belgium, the inspection services have introduced their own National MRPLs for hormonal substances in accordance with the European Commission revision 93/256/EC. To meet these National MRPLs a laboratory needs analytical methods with low limits of detection (LODs).

Since 1979, many extraction and detection techniques have been developed to screen and confirm steroid residues based on liquid-liquid extraction, immuno-affinity, solid phase extraction, high performance thin layer chromatography (HPTLC) with fluorescence detection, liquid chromatography (LC) and gas or liquid chromatography in combination with mass spectrometry (GC-MS^n or LC-MS^n) [5–22].

Sample size may be a key element in an analytical procedure. As for a sample being suspected of containing illicit EGAs, the procedure is completely resumed before results are passed to the veterinary inspection services, sample size often is a deciding factor. Moreover, the larger the test portion needed to carry out an analysis, the bigger the solvent volumes needed to perform the extraction procedure and the greater the personnel efforts. For any method developer it is a challenge to minimise solvent volumes and test portions and to shorten analysis time. The aim of this study was to develop an analytical procedure in which primarily the test portion was reduced, and simultaneously time and solvent consuming were decreased.

In this investigation, an extraction procedure for kidney fat is described. Here, only 5 g of molten fat was needed to perform a EGAs residue analysis. After extraction and clean up, the EGAs in the final extract were detected using gas chromatography-tandem mass spectrometry (GC-MS^2). Next to this, norclostebol acetate (NClTA), a newly found variant of chlortestosterone acetate (ClTA) that is used in bovine species, and its traceability in fat matrix is also discussed.

2. Materials and methods

2.1. Materials

2.1.1. Reagents and reference standard EGAs

All reagents and solvents used were of analytical grade quality and provided by Merck (Darmstadt, Germany). Most reference EGAs were obtained from Steraloids (Wilton, NY) or Sigma (St. Louis, MO). Other steroids were gifts from various sources. All recent standards were obtained through the National Reference Laboratory (WIV-LP, Brussels) to ensure that all the field laboratories use the same standards. The internal reference standard used was 1,4-androstadiene-3,17-dione (ADD). The GC-MS^n reference standard used was androsterone (And).

2.1.2. Method for preparation of the EGAs standard solution and the derivatisation reagent

From the individual stock solutions (200 ng of anabolic steroid μl^{-1} in absolute ethanol, stored at 4 °C) a working solution containing EGAs for which a National MRPL has been imposed by the inspection services was prepared. The concentration level of each EGA was equilibrated to its National MRPL (Table 1). Though no National MRPL has been imposed for fluorogestone acetate (FGA) and NClTA, both EGAs were integrated in the standard working solution since it is known that they can be misused for cattle fattening.

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

2.1.3. Test materials

Kidney fat of cow, calve and swine were collected. After testing for the presence of EGAs using the conventional analytical procedure, only the fats found to be free from EGAs were used for the experimental set-up.

Table 1
CCβ and CCα for the downscaled method compared to the Belgian National MRPLs for EGAs in kidney fat

Component	National MRPL ($\mu g\,kg^{-1}$)	CCβ within the laboratory $\leq x$ ($\mu g\,kg^{-1}$)	CCα within the laboratory $\leq x$ ($\mu g\,kg^{-1}$)
Diethylstilbestrol	2	2	1
Hexestrol	5	2	1
Dienestrol	2	2	1
α-Zeranol	5	5	3
β-Zeranol	5	2	1
Ethinylestradiol	2	2	1
α-Nortestosterone	2	2	1
β-Nortestosterone	2	2	1
Methyltestosterone	2	2	1
α-Boldenone	5	3	2
β-Boldenone	5	2	1
Methylboldenone	3	2	1
Norgestrel	5	2	1
Chlortestosterone acetate	50	5	3
β-Trenbolone	2	2	1
Trenbolone acetate	2	2	1
Norethandrolone	2	2	1
Medroxyprogesterone acetate	10	5	3
Chlormadinone acetate	10	10	6
Megestrol acetate	10	10	6
Melengestrol acetate	10	10	6
Acetoxyprogesterone	10	5	3
Caproxyprogesterone	10	10	6
Norclostebol acetate[a]	–	5	3
Flurogestone acetate[a]	–	2	1

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

2.1.4. Apparatus and materials needed for extraction and clean up

The following devices were used for extraction and clean up: a balance, a microwave oven, a mini-shaker, a centrifuge (e.g. Sorvall® Dupont Company, Newton, USA), a rotary vacuum evaporator (Büchi, Flawil, Switzerland), a water bath, a vacuum sample processing station (e.g. VacMaster®, IST International, Mid-Glamorgan, UK), and a nitrogen evaporator (e.g. Turbovap LV, Zymark Corporation, Hopkinton, USA) or other types of evaporators (e.g. Speedvac SVC 200, SC 210A Savant, Howe Gyrovap). Glassware and other recipients were selectively chosen to be suitable in each step of the procedure. The following specific material was used: *extraction*: Nalgene and Nunc tubes (Nalge Nunc International, Rochester, NY); *clean up*: solid phase extraction (SPE) columns (Isolute CN sorbent—500 mg 3 ml^{-1}, IST International) and amber 0.7 ml autosampler vials (Filter Service AG, Eupen, Belgium).

2.1.5. GC-MS apparatus

Gas chromatographic and coupled mass spectrometric information was performed on a POLARIS ion trap mass spectrometer coupled to a ThermoQuest CE Trace GC gas chromatograph (Thermo Finnigan, Austin, TX, USA) with a split/splitless injector. Samples were injected using a Carlo Erba autosampler AS2000 (Thermo Finnigan, Austin, TX, USA). Helium gas or hydrogen gas was used as GC carrier gas at a flow rate of 1 $ml\,min^{-1}$. When using hydrogen the carrier gas was prepared from ultrapure water using a hydrogen generator (Packard, Meriden, USA). Experiments were also performed using a GCQ plus (Thermo Finnigan) consisting of a Finnigan GC with split/splitless injector and 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 ca. 1 $ml\,min^{-1}$. 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.

2.1.6. GC-MS^2 conditions

Analyses were performed using a non-polar 5% phenyl-polysilphenylene-siloxane SGE BPX-5 GC-column (25 m × 0.22 mm ID 0.25 μm) (SGE Incorporated, Austin, TX).

Gas chromatographic parameters to perform the analyses were as following: split/splitless injector temperature on 250 °C (POLARIS) or 260 °C (GCQ plus) with a split vent flow of 60 ml min^{-1}, injection in splitless mode (split valve closed at −0.10 min, open at 1.00 min). Temperature program (hydrogen as carrier gas): initial 100 °C (hold 1 min); to 250 °C (30 °C min^{-1}); to 290 °C (2.5 °C min^{-1}); to 300 °C (10 °C min^{-1}) (hold 1.5 min). Temperature program (helium as carrier gas): initial 100 °C (hold 1 min); to 250 °C (17 °C min^{-1}); to 300 °C (2 °C min^{-1}) (hold 1 min).

Full scan MS acquisition method parameters were identical for both MS systems: 1 microscan (i.e. the number of microscans—consisting of a prescan followed by an analytical scan—the MS detector will average and display (or save to the datafile) for every scan); mass range 150–570 amu; ion source temperature at 200 °C; transfer line temperature at 275 °C. Also the tandem MS (MS/MS) acquisition method parameters (1 microscan; 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 V and 1.30 V) were the same.

2.1.7. GC-MS^2 interpretation

Xcalibur™ software (Thermo Finnigan) version 1.2 was used to perform the interpretation of the analytical results.

2.2. Methods

2.2.1. Sample pretreatment

A fat tissue sample of ca. 10 g was cut into very small pieces. Brought into a funnel with cotton wool, the fat was melted in a microwave oven at maximum power for 4 min and the rendered fat was collected. The fat test portion, 5 g of liquid fat, was weighed into a Nalgene tube. In case of routine analysis, the unknown samples, a blank and control sample spiked with the EGAs standard solution were prepared for analysis. Subsequently, the internal reference standard (5 μg kg^{-1} ADD) was added.

2.2.2. Extraction

The 12.5 ml of acetonitrile was added to the liquid test portion. If the fat was no longer fluid, the tube was put into a warm water bath to melt the fat. The fat–acetonitrile mixture was shaken vigorously and afterwards cooled in a stream of cold water. After centrifugation (9000 rpm for 10 min) the supernatant was decanted. The extraction was repeated on the precipitated fat portion and the supernatants were combined.

2.2.3. Clean up

The acetonitrile extract was washed with 7.5 ml of *n*-hexane. After discarding the *n*-hexane phase a second wash step was carried out and the acetonitrile extract was evaporated until dry using a rotavapor. The residue was redissolved in 10 ml of *n*-hexane and transferred to a Nunc tube. The 2.5 ml of 0.1 M NaOH and 1.25 ml of 1.0 M $MgCl_2$ were added successively to perform a saponification and thus precipitate the fat extracted along with the EGAs during the acetonitrile extraction. After 30 min incubation at 60 °C and centrifugation (3000 rpm for 5 min), the supernatant was evaporated until dry.

2.2.4. Solid phase extraction

The residue was resolved in 2.5 ml of *n*-hexane. SPE, by which a cyanopropyl (CN) column was used, was established: the CN-column was conditioned with 3 ml of ethyl acetate and equilibrated with 5 ml of *n*-hexane. The extract was quantitatively passed (at 1 ml min^{-1}) to the top of the column and allowed to drain in, thereby using a vacuum sample processing station (e.g. VacMaster®). The tube was rinsed twice with 1 ml of *n*-hexane. The SPE column was washed twice with 5 ml of *n*-hexane. The EGAs were eluted with 3.5 ml of ethyl acetate/*n*-hexane (90:10, (v/v)). After addition of the GC-MS^n reference standard (5 μg kg^{-1} And), the eluate was evaporated until dry under a nitrogen flow.

2.2.5. GC-MS^2 analysis

After transfer to an autosampler vial, the EGAs in the final SPE extract were converted into enol-trimethylsilyl ether derivatives with $MSTFA^{++}$. Since

a mixture of standard EGAs to be detected at an appropriate concentration with GC-MS was analysed along with the sample to verify the optimum status of the GC-MS device, an aliquot of the working solution was evaporated and derivatised under the same conditions as the test sample: 25 μl MSTFA^{++} was added to each vial and after closure the vials were mixed thoroughly using a vortex mixer. After incubation (60 min at 60 ± 2 °C), 1 μl was injected into the gas chromatograph.

3. Results and discussion

During the past two decades the control on illicitly administered EGAs has been monitored by analysis of different animal matrices. Intra-muscular or subcutaneous injections with EGAs have been traced by analysis of muscle tissue (injection sites); other ways of administration have been tracked down by analysis of urine, faeces and bile, organs such as liver and kidney, meat and kidney fat. At the slaughterhouse level, beside injection sites, kidney fat is considered to be the matrix of choice for detection since many EGAs are fat soluble. Methods for steroid analysis in kidney fat have been published [5–25], by which various extraction and detection techniques were proposed. Since nowadays cocktails of synergetic EGAs containing each EGA at a very low concentration level are administered, higher demands from the veterinary inspection services who lead the national control on illicit traffic of EGAs have forced analytical laboratories to develop more sensitive analytical procedures. As described earlier, application of chromatography with tandem mass spectrometry (LC/GC-MS^n) becomes necessary, as the Belgian National MRPLs have to be fulfilled [26].

As long as the analytical results are not known, the sampled animals are retained in the slaughterhouse and may not be commercialised. For that reason analytical procedures must be carried out as quickly as possible. Until now, with the conventional method, approximately one day passed between the start and end of the analysis (Fig. 1). Here, 25 g of kidney fat tissue was extracted with methanol and diethyl ether, followed by a clean up on a coupled Silicium/Amino SPE column system by which chloroform/acetone (40:10, (v/v)) was used to elute the EGAs fraction. The EGAs in the final extract were derivatised to enol-trimethylsilyl ethers and analysed by GC-MS^2. This method was quite efficient and enabled the laboratory to detect EGAs at the National MRPLs. Nevertheless, this procedure was solvent consuming. Moreover, when a sample had to be re-analysed because it was suspected of containing EGAs or the first procedure was for some reason not successful, it might be impossible to resume the whole process as most laboratory samples weigh scarcely 50–70 g. In order to overcome with this problem, downscaling became essential.

For the method described in this investigation only 5 g of molten fat was needed to perform one analysis (Fig. 1). Originally used for extraction of gestagens [27], acetonitrile was selected to extract EGAs in general from the fat matrix. Lipids extracted along with the EGAs during the acetonitrile extraction were eliminated with *n*-hexane. As EGAs have a greater affinity for acetonitrile than for *n*-hexane, no significant loss of EGAs due to this washing step could be observed. Saponification with NaOH and precipitation with $MgCl_2$ was integrated to get a cleaner and fat-free extract. It was tried to carry out the saponification part without the preceding *n*-hexane wash as *n*-hexane can be the cause of losses of gestagens, but then the final extract was not sufficiently clean to be injected on the GC-MS apparatus. For final clean up, SPE on a CN column was performed. A restricting factor of the conventional method was the loss of gestagens due to large *n*-hexane volumes. During new method development, various ratios of ethyl acetate and *n*-hexane were tested. An interference elimination step with exclusively *n*-hexane seemed optimal as combinations with ethyl acetate resulted in losses of androgens and estrogens. These tests also proved that an excess of ethyl acetate (ethyl acetate/*n*-hexane (90:10 (v/v)) should be recommended for elution of EGAs fraction. In that case EGAs were maximally eluted from the SPE column while the lipid fraction was not.

After concentration and derivatisation as enol-trimethylsilyl ethers, EGAs were analysed by GC-MS^2.

As described earlier [26], EGAs are detected nowadays by GC-MS^2 because of better detection capacities. Also, some EGAs that are mentioned to be “problem” molecules with full scan GC-MS, such as α/β-trenbolone and trenbolone acetate, can be detected much more selectively using tandem mass

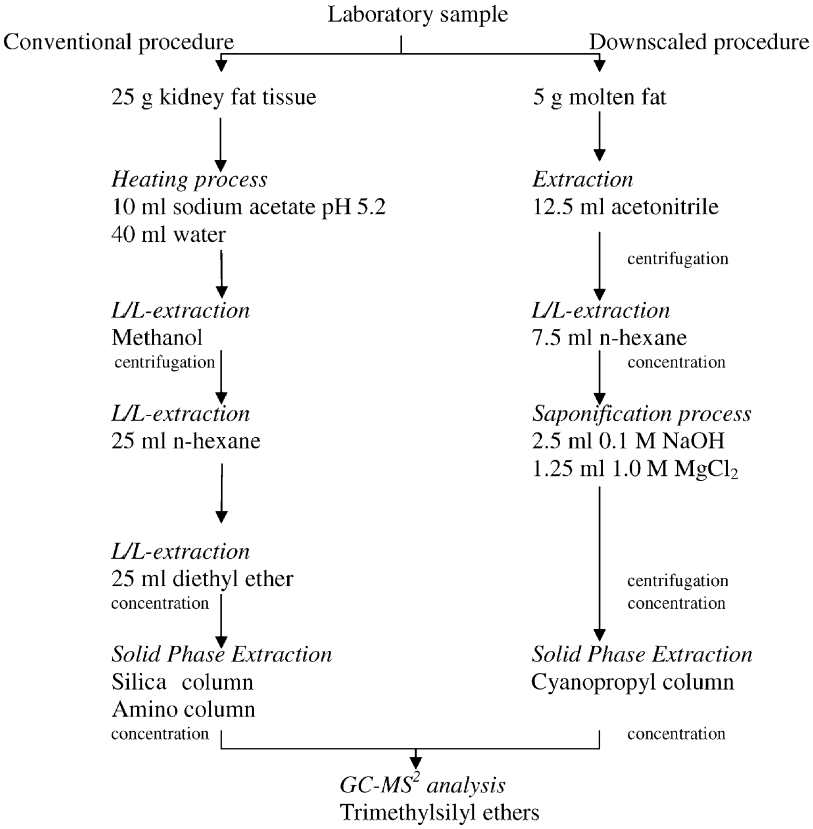

Fig. 1. Overview of the conventional and downscaled procedure for analysis on EGAs in kidney fat.

spectrometry. Moreover, interfering matrix peaks no longer cause difficulties during interpretation because with GC-MS^2 only one component-specific ion (precursor ion)—in most cases the molecular ion—is held within the ion trap where it is fragmented, resulting in a series of specific fragment ions (transition product ions), a reduction of chemical noise and achievement of a higher degree of confirmation (Fig. 2).

A big improvement of specificity is observed when using GC-MS^2 (Fig. 3). In the full scan mode, dienestrol (DE) and diethylstilbestrol (DES) are not separated chromatographically and the full scan MS spectrum at the correct retention time is a mixture of the diagnostic ions of both components (if both are present). Moreover, ions generated by the matrix (e.g. 407) may be present in the spectrum. Therefore it is difficult to unequivocally identify one of the two substances in the presence of the other using a full scan. (Fig. 3—above). In GC-MS^2, with the fragmentation of the precursor ions 410 and 412 for DE and DES respectively (isolation with ±0.5 amu), a different MS^2 spectrum for both EGAs could be obtained even in the presence of the other component and matrix components. (Fig. 3—middle and below). This MS^2 spectrum contains sufficient diagnostic ions to fulfil the identification criteria.

The method described in this investigation was considerably easier to use than the conventional method and analysis duration time, including chromatographic interpretation, could be reduced. Moreover, if GC-MS^2 analysis is carried out using hydrogen as the GC carrier gas, the duration time can be shortened even more, thus resulting in more results in less time [28]. And, as 5 g of molten fat which can be gained from ca. 10 g of fat tissue, was sufficient to start the analysis—instead of 25 g of fat tissue for

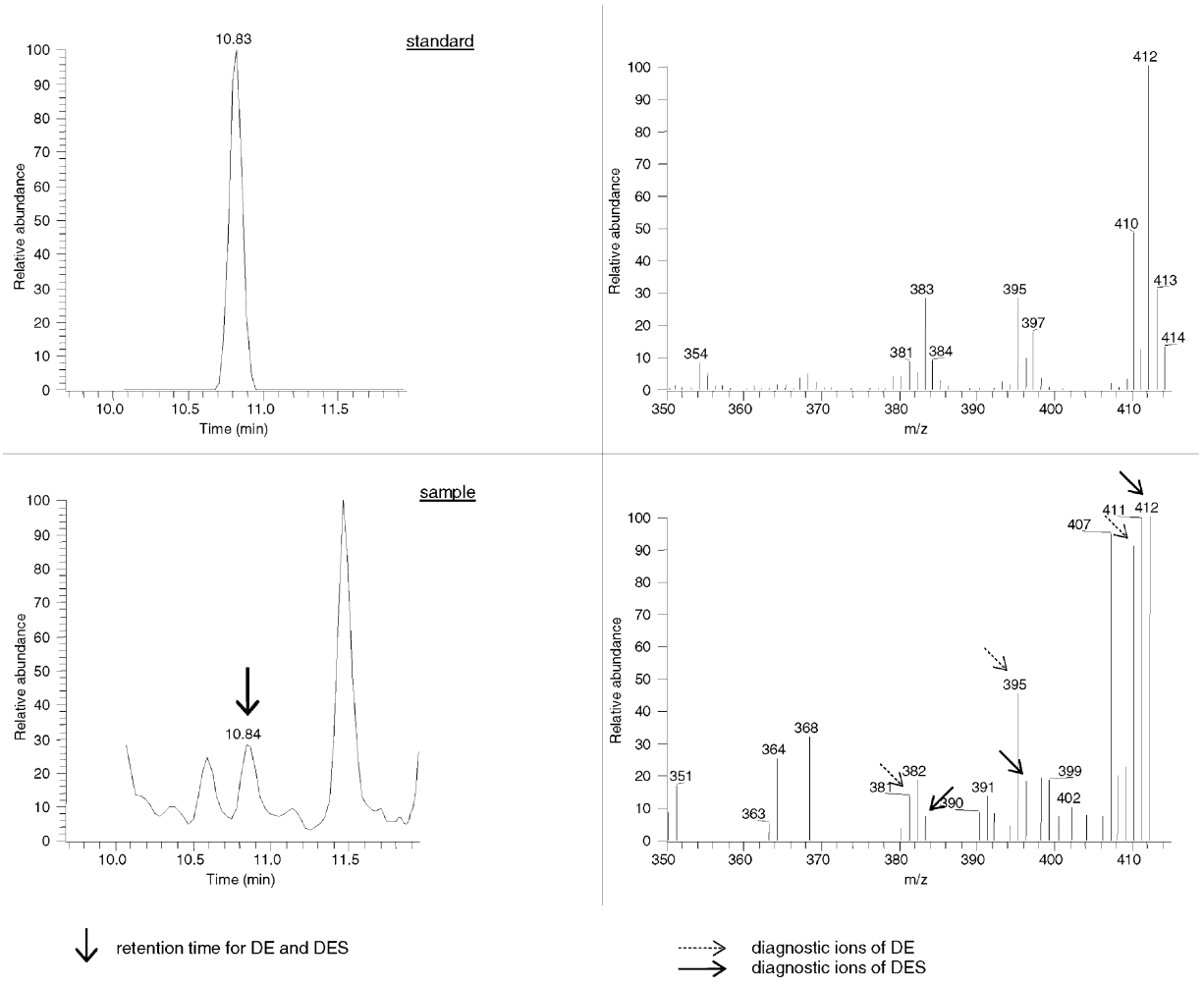

Fig. 2. Interference of matrix in full scan GC-MS.

the conventional method-, less solvent volumes and smaller recipients were needed thereby lowering analytical costs. In comparison with the conventional procedure, solvent costs could be decreased by 54%. Also, in order to fit into the system of accreditation according to ISO-17025, a blank matrix and control sample, spiked with a mixture of EGAs for which a National MRPL has been imposed, have to be analysed along with the unknown. Using the new method standard EGAs costs were five times lower than when using the conventional procedure.

The most important reason to use the downscaled method was the gain in detection capability. Using the conventional procedure, thus starting from 25 g of kidney fat, EGAs could be determined at or below their National MRPL level, depending on the kind of EGA [26]. On analysis with the downscaled method National MRPL levels could be reached for all EGAs according to the quality criteria [2] (Fig. 4). For some EGAs the detection capacity (CCβ)—in this case (for substances for which no permitted limit has been established) the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of $1-\beta$ and $\beta \leq 0.05$) was much better than the National MRPL: hexestrol, β-zeranol, α/β-boldenone, methylboldenone, norgestrel

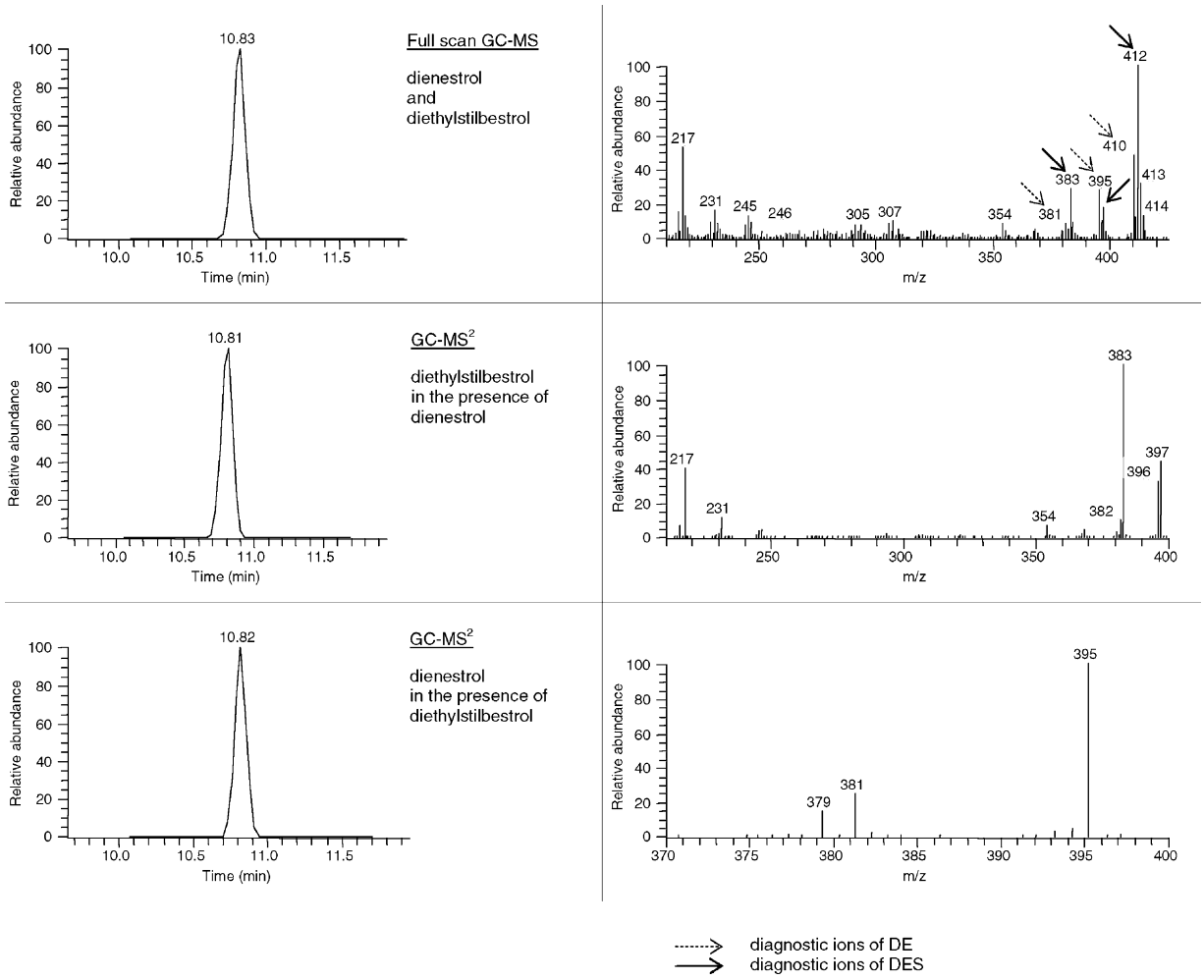

Fig. 3. Specificity of tandem mass spectrometry compared to full scan GC-MS.

and fluorogestone acetate could be detected with a CCβ value of 2 $\mu g\,kg^{-1}$ (Fig. 5), and the CCβ values for acetoxyprogesterone and medroxyprogesterone acetate were 5 $\mu g\,kg^{-1}$. In Table 1 an overview of CCβ and CCα values for the investigated EGAs is shown. CCα or the decision limit is the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. Keeping in mind that for the downscaled method absolute quantities of reference EGAs standards—used to fortify blank samples—were 5 times less than in the conventional procedure and that EGAs could also be detected at their national MRPL level or even lower, it could be concluded that the analytical sensitivity and recovery must be better than with the conventional procedure. In fact, keeping in mind that 5 g of rendered fat—fortified at national MRPL level after the melting phase—was gained from 10 g of fat tissue, the EGAs CCβ and CCα values mentioned above could be halved.

For ClTA, also known as clostebol acetate, an immense improvement of CCβ compared to the conventional method was observed. For this androgenic component a National MRPL value of 50 $\mu g\,kg^{-1}$ has been imposed. When analysed by the conventional method, the CCβ value for ClTA was 12.5 $\mu g\,kg^{-1}$ [26], but with the new method it could be decreased

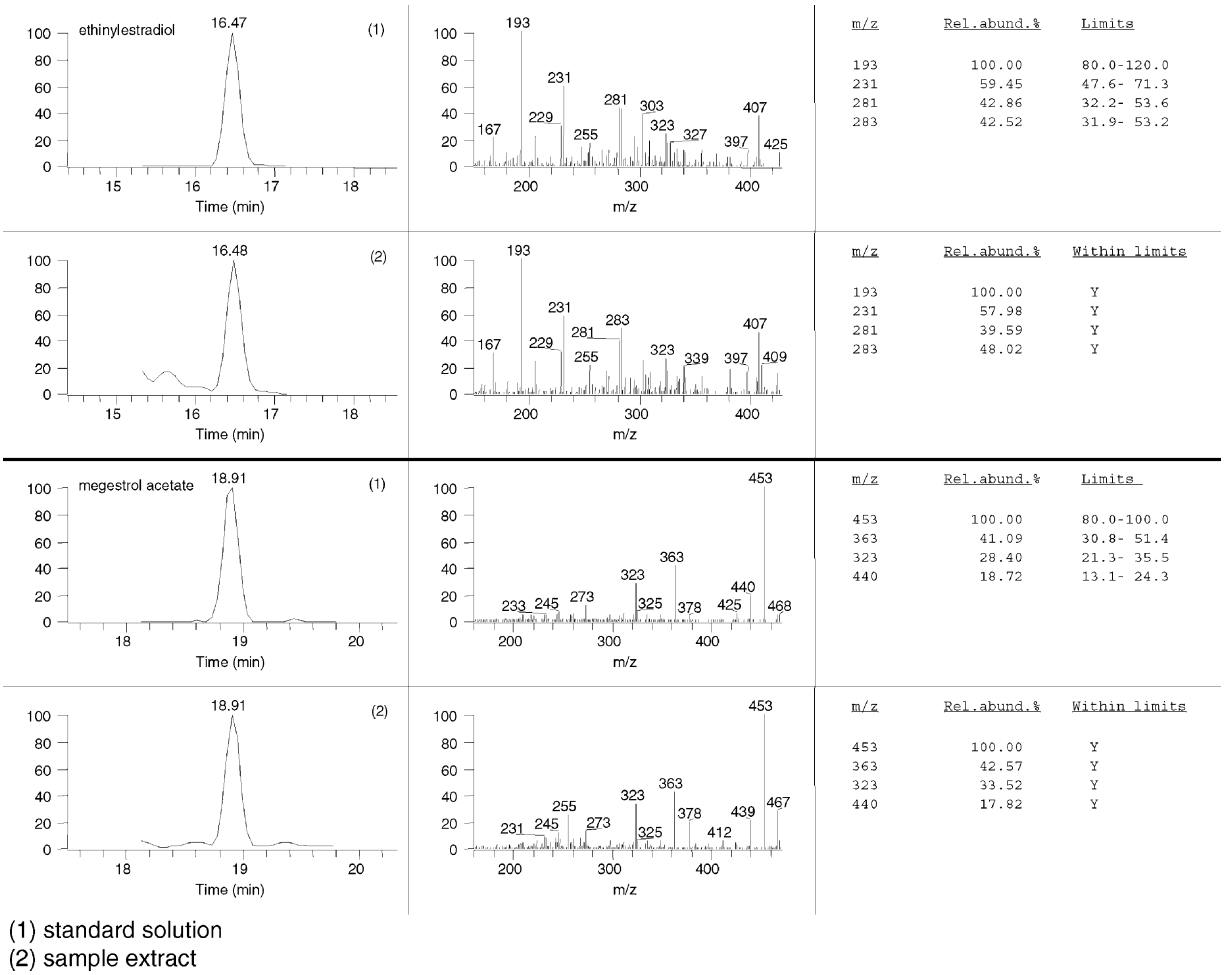

Fig. 4. Ethinylestradiol and megestrol acetate at National MRPL concentration level.

to 5 μg kg^{-1}. In Fig. 6, chromatographic data clearly illustrate the difference in analytical LODs between both methods for ClTA. Two samples were fortified with 5 μg kg^{-1} ClTA, one sample was analysed by the conventional method and the second one was tested with the downscaled method. The mass spectrum of a standard (2 ng μl^{-1} ClTA on column) was used as reference to compare both methods with each other. Using the conventional method (middle), the ClTA peak area is small and some background noise is disturbing the ClTA mass spectrum. On the other hand, ClTA could be identified unequivocally with the downscaled method (below).

For NClTA, a structural relative of ClTA that contains no methyl group on the ten-position, an analogous mass spectrum could be observed. In Fig. 7, the fragmentation pattern of NClTA is shown. As could be expected, diagnostic transition product ions' mass/charge (*m/z*) ratios for NClTA were 14 less than those for ClTA. Although a National MRPL level has not yet been imposed by the Belgian official authorities, monitoring of NClTA should be included in routine residue analysis as some research has shown that it might be (mis)used for cattle fattening.

Being short of incurred materials, the experimental set up was drafted with fortified blank samples. In

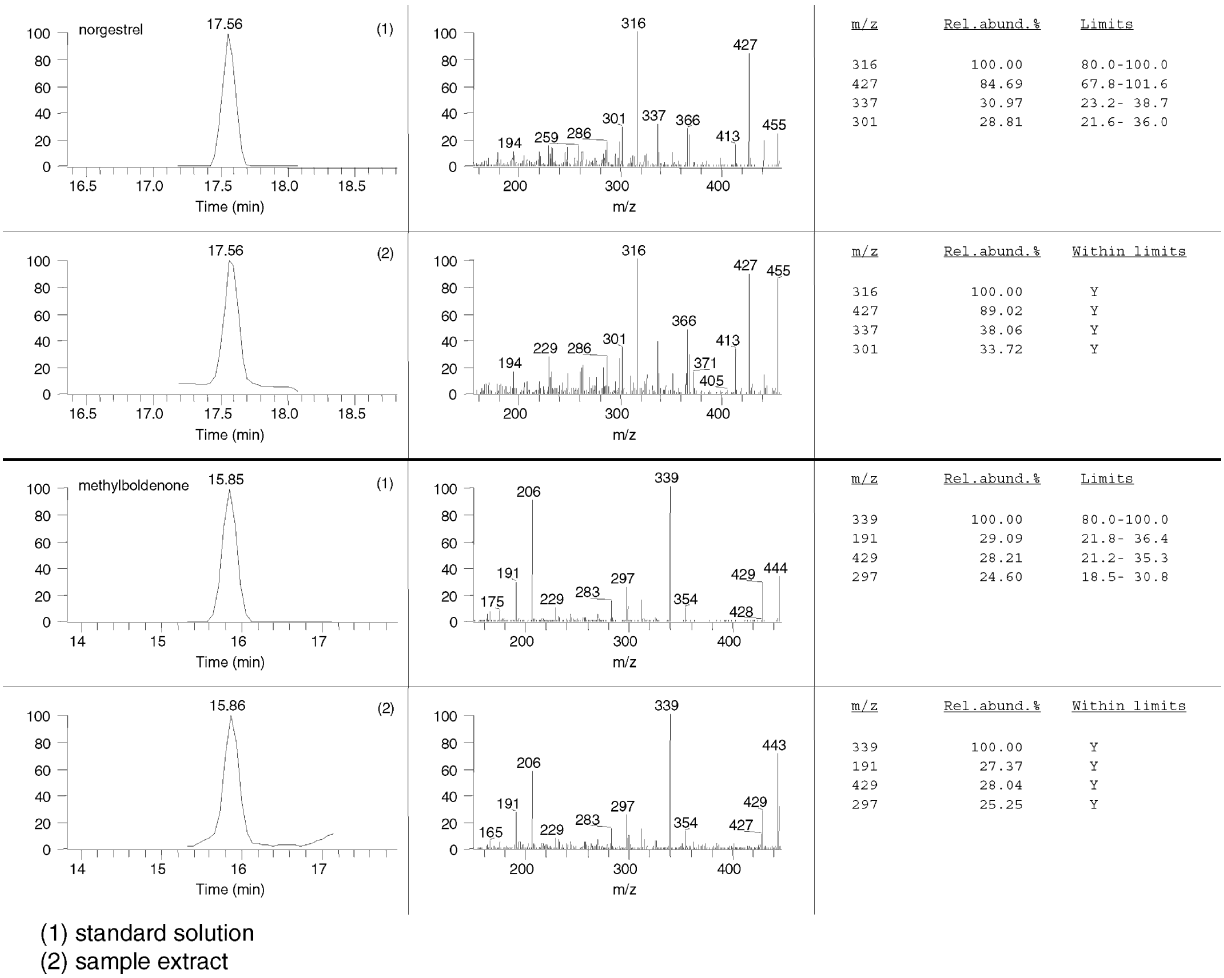

Fig. 5. CCβ of norgestrel and methylboldenone ($2\,\mu g\,kg^{-1}$).

order to prove whether the method was applicable for routine samples, regular kidney fat was analysed by both procedures. The presence of endogenous EGAs could be confirmed with both analytical methods.

To test the method's ruggedness minor changes were incorporated and their influence on the measurement results was observed: the parameters changed were the power of the microwave fat melting process (sample pretreatment), heating and cooling of the sample (extraction and saponification process), pH control (clean up/SPE), elution solvents and flow rates (SPE). Fat tissues from species other than bovine (pork, calf) were also tested. As mentioned above, the ethyl acetate/*n*-hexane ratio to elute the EGAs fraction from the cyanopropyl SPE column should consist of an excess of ethyl acetate, and an optimal flow rate during SPE was tested to be $1\,ml\,min^{-1}$. Another critical aspect was the matrix effect. This effect was not species related, but when using rather old, rancid kidney fat tissue, the final extracts still contained some fat residues. Therefore, if possible, freshly sampled laboratory samples should be analysed. To overcome the matrix effect, further research will be done.

Downscaling the test portion and as a consequence analytical detection of less absolute EGAs quantities intensifies the analytical instrument's demands as EGAs are detected at concentration levels in the middle and low picogram range. Besides improvement of

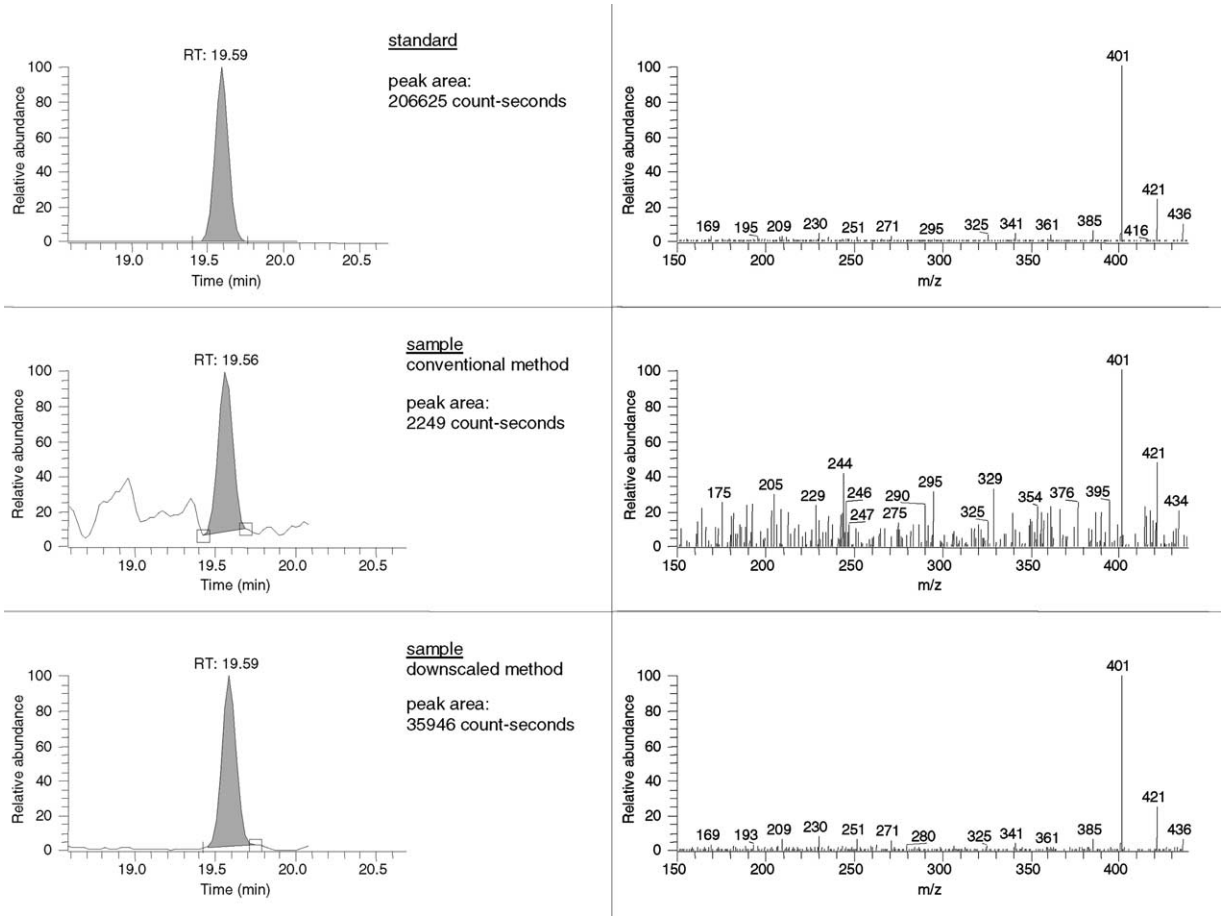

Fig. 6. A comparison between the conventional and downscaled method for ClTA at $5\,\mu g\,kg^{-1}$.

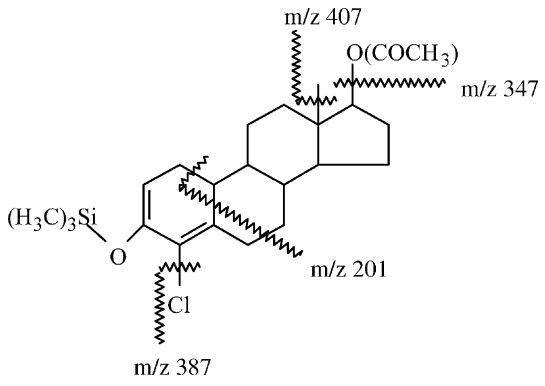

Fig. 7. Principal fragments of NClTA in the positive electron impact mode.

extraction and clean up, the analytical instrument's limits must be extended, or the use of other techniques such as low resolution quadrupole or high resolution instruments should be considered.

4. Conclusions

In this investigation, a GC-MS^2 method for screening and confirmation of steroid growth promoters in kidney fat was described. Downscaling the test portion from 25 g to 5 g enabled the laboratory to resume the analytical procedure without fear of having lack of sample. General analysis costs and use of reference EGAs standards were decreased significantly. This

extraction procedure in combination with GC/MS^2 has prove to be robust and sensitive enough to permit detection of EGAs at or below their National Minimum Required Performance Limits (National MRPLs). Especially for ClTA and NClTA an immense gain in detection capability could be achieved.

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