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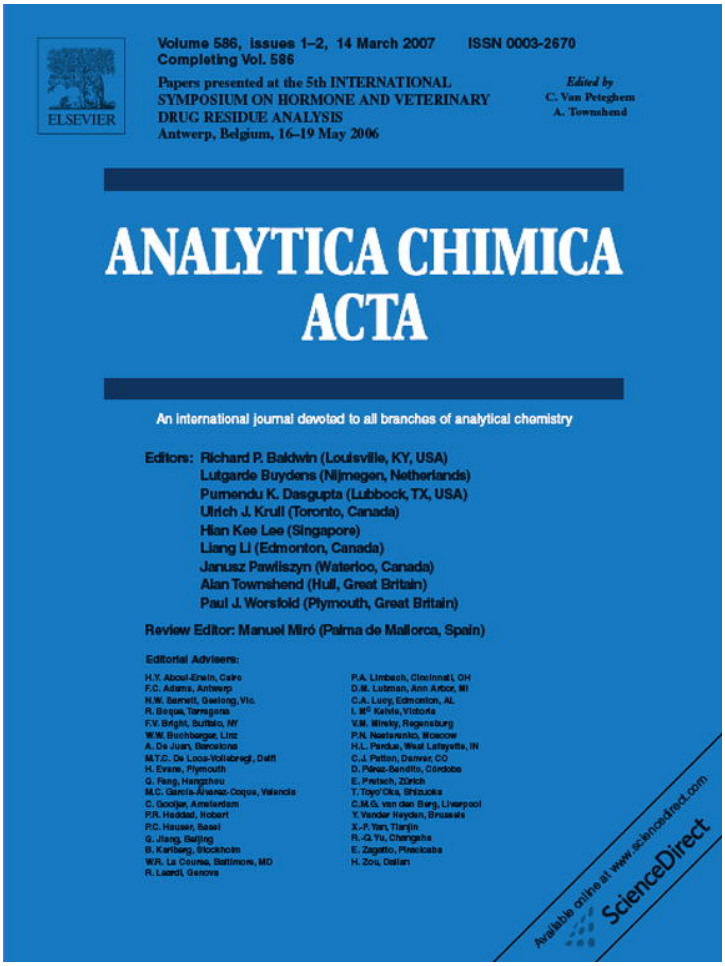

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Multi-analyte approach for the determination of ng L^{-1} levels of steroid hormones in unidentified aqueous samples

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Abstract

Since the 1970s, many analytical methods for the detection of illegal growth promoters, such as thyreostats, anabolics, β-agonists and corticosteroids have been developed for a wide range of matrices of animal origin, including meat, fat, organ tissue, urine and faeces.

The aim of this study was to develop an analytical method for the determination of ng L^{-1} levels of estrogens, gestagens, androgens (EGAs) and corticosteroids in aqueous preparations (i.e. drinking water, drinking water supplements), commercially available on the 'black' market. For this, extraction was performed with Bakerbond C_{18} speedisk, a technique commonly used in environmental analysis. After fractionation, four fractions were collected using a methanol:water gradient program. Gas chromatography coupled to electron impact multiple mass spectrometry (GC–EI-MS^2) screening for the EGAs was carried out on the derivatized extracts. For the detection of corticosteroids, gas chromatography coupled to negative chemical ionization mass spectrometry (GC–NCI-MS) was used after oxidation of the extracts. Confirmation was done by liquid chromatography coupled to electrospray ionization multiple mass spectrometry (LC–ESI-MS^2). The combined use of GC and LC coupled to MS enabled the identification and quantification of anabolics and corticosteroids at the low ng L^{-1} level. This study demonstrated the occurrence of both androgens and corticosteroids in different commercial aqueous samples.
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Keywords: Gas chromatography; Liquid chromatography; Multiple mass spectrometry; Steroids; Corticosteroids; Anabolics; Residue analysis

1. Introduction

Steroid hormones are steroids which act as hormones. They can be divided into different groups: corticosteroids (glucocorticosteroids, mineralocorticosteroids) and estrogens, gestagens and androgens (EGAs) [1,2]. This large group of estrogenic compounds is legally used in human and veterinary medicine. However, besides their use under regulated conditions, they are also illegally used in animal fattening. Steroid hormones have the possibility to increase the weight gain and to reduce the feed conversion ratio, which is the average feed intake in relation to the weight gain. In addition, their synergetic effects and their possibility to reduce nitrogen retention and to increase the water retention and fat content were also reported in literature [1,3–5]. Illegal growth promoters are mostly injected, resulting in injection sites in which high concentrations (mostly esters) can be found [6,7]. Also via the feed, animals can be treated with EGAs [8].

The improper or illegal use of steroid hormones may result in drug residues in food products produced of these animals. To protect consumer's health, the European Union requires that all veterinary drugs are evaluated [9], and establishes maximum residue limits (MRLs) for these compounds in specific edible matrices, i.e. muscle, fat, organ tissue, milk and eggs. The illegal use of steroid hormones in livestock breeding and aquaculture is banned within the European Union as described by 96/22/EC [10]. Surveillance for the presence of residues of veterinary drugs in food-producing animals and foods is regulated by 96/23/EC [11]. Consequently, the Federal Agency for the Safety of the Food Chain (FAVV-AFSCA) controls the illegal

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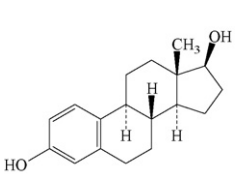
use of these compounds. For analytical method validation and interpretation of the results, criteria are established as described in the European Criteria EC/2002/657 [12].

Nowadays, the presence of steroid hormones in matrices of animal origin is not a new issue. The illegal use of veterinary medicines is monitored both by injections sites as by analysis of urine, faeces, fat, muscle and organ tissue (e.g. kidney, liver, thyroid gland). In this sense, the need to develop highly sensitive and specific analytical methods for the determination of these compounds in a wide variety of animal matrices has increased due to the wide variety of illegal applications of steroid hormones. As reported in literature, many novel approaches have been developed for the detection of steroid hormones in matrices of animal origin like faeces, urine, liver, meat, fat, hair, milk, feed and injection sites [3,4,7,13–17].

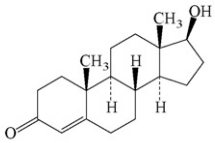
Recently, there has been a shift towards the use of unknown aqueous preparations, e.g. drinking water or drinking water supplements with suspected very low concentrations of compounds with growth promoting properties. These preparations seem to be available on the 'black' market. Due to the 'unknown' status of these aqueous preparations, target analysis is not always possible. For this reason, the development of multi-analyte and multi-disciplinary approaches is required. Using the combination of both *in vitro* and *in vivo* techniques and different analytical techniques, the residue analysis may cope with the ever-changing environment of legal and illegal veterinary medicine.

The present study was based on a previously described multi-disciplinary approach for the detection of estrogens in water samples [18]. The major goal was to develop and to apply a multi-disciplinary strategy to identify and quantify a large

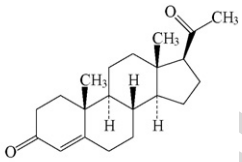
Table 1
Structures and diagnostic ions of the investigated anabolic steroids (internal standards are marked in italic)



Estradiol (E2)



Testosterone (T)



Progesterone (P)

Compound	M_W	Full scan MS	Precursor ion	Product ions MS^2	Spike (ng L^{-1})
β-Zeranol (bZ)	322.4	307–335–389–433	433	295–309–323–337–389–415	50
Hexestrol (HEX)	270.4	163–179–191–207	207	163–179–191	50
Diethylstilbestrol (DES)	268.4	217–383–397–412	412	217–383–396–397	50
Dienestrol (DE)	266.3	379–381–395–410	410	379–381–395	50
β-Boldenone (bBOL)	286.4	206–325–415–430	206	163–175–183–191	75
α-Boldenone (aBOL)	286.4	206–325–415–430	206	163–175–183–191	75
Ethinyl estradiol (EE2)	296.4	232–285–425–440	425	193–231–281–283–303–323–407	50
Fluoxymesterone (FMT)	336.4	319–407–462–552	552	319–407–462	125
α-Zeranol (aZ)	322.4	307–335–389–433	433	295–309–323–337–389–415	50
17β-Nortestosterone (bNT)	274.4	182–194–403–418	418	182–247–287–313–327–328–403	50
Methyl boldenone (MeBol)	300.4	206–339–429–444	444	191–206–283–297–312–339–354–429	75
17α-Nortestosterone (aNT)	274.4	182–194–403–418	418	182–247–287–313–327–328–403	50
Norgestrel (NG)	312.4	194–301–316–456	456	301–316–337–366–427	50
Chlorandrosteendione (ClAD)	320.4	429–449–456–464	464	234–339–359–429–449	125
Methyl testosterone (MT)	302.4	301–341–356–446	446	251–301–314–341–356	50
Methanedriol (MAD)	304.5	253–268–343–358	253	155–169–183–197–211	125
Acetoxy progesterone (AP)	372.5	208–366–441–456	456	208–351–366–428–441	2500
Norethandrolone (NE)	302.4	287–300–356–446	446	287–299–300–356	50
Methyl androstandiol (MeAD)	306.5	255–270–345–435	435	199–213–255–345	125
Ethyl estrandiol (EED)	306.0	157–241–331–421	331	145–185–199–241	50
Medroxyprogesterone acetate (MPA)	386.5	222–380–455–470	470	222–237–365–380–455	500
Melengestrol acetate (MeLA)	396.5	375–467–480–482	482	337–376–377–454–467	2500
Megestrol acetate (MeGA)	384.5	363–453–468–470	468	323–363–440–453	1250
Chlormadinon acetate (CMA)	404.9	437–453–473–488	488	363–383–437–453–473	500
Caproxy progesterone (CP)	428.6	208–366–441–456	456	208–351–366–428–441	2500
Chlortestosterone acetate (CITA)	364.8	401–421–436–438	436	230–385–401–421	2500
Androstadiendione (*ADD*)	284.0	206–323–413–428	428	191–206–222–323–413	125
Equilenine (*EQ*)	266.3	280–305–395–410	410	280–294–305–320–381–395	125
Ethinyl testosterone (*ET*)	312.4	301–316–441–456	456	299–301–316–351–441	125
Methyl nortestosterone (*MeNT*)	288.4	287–342–417–432	432	285–287–300–342	125
1-Dehydroprogesterone (*1-DhP*)	312.4	235–351–441–456	456	206–235–250–351–441	125
6-Dehydroprogesterone (*6-DhP*)	312.4	171–351–441–456	456	171–249–351–366–441	125
16β-Methyl progesterone (*16b-MeP*)	328.5	171–367–457–472	472	171–302–367–382–457	125
Androsterone (*And*)	290.4	239–329–419–434	434	239–329–344–419	125

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number of steroid hormones in aqueous samples and to use this method for the detection of ng L^{-1} levels of these compounds in a wide variety of 'unknown' aqueous preparations. For this, different chromatographic techniques, i.e. GC and LC coupled to MS were used.

2. Methodology

2.1. Chemicals

Standards of the natural and synthetic hormones were purchased from Sigma–Aldrich (St. Louis, MO, USA), Steraloids (Newport, RI, USA) or were gifts from various sources. All solvents used for extraction and clean-up of the samples were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Acros (Acros organics, Fairlawn, New Jersey, USA).

Primary stock standard solutions of the targeted steroid hormones were prepared individually in ethanol (EtOH) at a concentration of 200 ng μL^{-1}. The working solutions of the mixtures at various concentrations were prepared by appropriate dilution of the stock solutions in ethanol and were used for subsequent spiking of the aqueous preparations. All standard solutions were stored at 4 °C in the dark following the quality assurance instructions of Belac accreditation (EN17025).

2.2. Chemical analysis

The targeted steroid hormones in this study, their structure, molecular weight, precursor and product ions are summarized in Tables 1 and 2. This selection was based on the extended experience of the laboratory of chemical analysis with residue analysis of these compounds in matrices of animal origin.

2.2.1. Sample extraction and clean-up

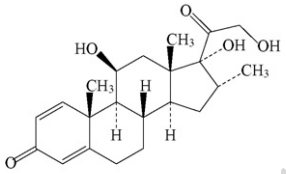
Of the aqueous preparations 100–500 mL (depending on the characteristics of the sample) were diluted to 1 L with ultrapure water, and subsequently spiked with internal standard (see Tables 1 and 2, 125 ng L^{-1} for EGAs and 40 ng L^{-1} for corticosteroids). When needed, samples were filtered through Whatman filter paper (GF/C Ø 47 mm, Merck, Darmstadt, Germany) prior to extraction in order to avoid clogging of the sorbent. Filters were extracted with methanol (MeOH) to prevent for losses of the compounds of interest. The extraction procedure carried out in this study was based on a method developed for the extraction of estrogens from environmental water samples using Bakerbond Speedisk^{TM} Octadecyl-bonded silica (C_{18}XF), 50 mm (J.T. Baker, Deventer, The Netherlands) as previously described by the authors [18,19]. In short, disks were preconditioned with MeOH and water. After loading of the sample to the disk elution was performed with acetone and MeOH.

2.2.2. Analytical procedure EGAs

After fractionation of the obtained extracts using a water:methanol gradient programme [after 18], samples for the analysis of EGAs were derivatized with a mixture of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), ethanethiol and ammoniumiodide [2]. Chromatographic analysis for the EGA's was carried out by gas chromatography coupled to ion trap multiple mass spectrometry in the electron impact mode (GC–EI-MS^2).

All chromatographic and spectrometric analyses were performed using a Trace GC 2000 Gas Chromatograph fitted with a Polaris ion trap mass spectrometer (Thermo Finnigan, Austin, TX, USA) with a Carlo Erba autosampler AS2000 (Thermo Finnigan, Austin, TX, USA). Helium (99.99% purity, Air Liquide, France) was used as carrier gas at a flow rate of 1 mL min^{-1}. FC43 (Perfluorotributylamine) (Ultra Scientific, North Kingstown, USA) was used as calibration gas. A volume of 1 μL was injected (spit flow 60 mL min^{-1}, splitless time 1 min). Separation of the target analytes was performed on a BPX-5 (SGE Inc., Austin, TX, USA) (25 m × 0.22 mm i.d.) fused silica capillary column with 5% phenyl liquid phase (film thickness 0.25 μm). Injector, ion source and transfer

Table 2
Chemical structure and diagnostic ions of the investigated corticosteroids (isoflupredone = internal standard)



Dexamethasone (Dxm)

Compound	M_W	GC–NCI full scan MS	LC–ESI	
			Full scan MS	Product ions MS^2
Dexamethasone (Dxm)	392.5	295–310–311–312	451	361–391
Betamethasone (Btm)	392.5	295–310–311–312	451	361–391
Prednisolone (prolon)	360.4	177–297–298–299	419	329–359
Methyl prednisolone (Mprolon)	374.5	177–312–313–314	433	343–373
Flumethasone (Flm)	376.5	313–314–328–329	469	379–409
Fluorometholone (Fml)	410.5	295–310–311–330	435	255–355–375
Isoflupredone (IFP)	378.4	281–282–296–297	437	347–377

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line temperature were, respectively, 250, 200 and 275 °C. Temperature program: initial 100 °C; ramp at 17 °C min^{-1} to 250 °C; ramp at 2 °C min^{-1} to 300 °C (hold 1.30 min). The spectra were obtained in electron impact (EI) mode at 70 eV.

2.2.3. Analytical procedure corticosteroids

For the chromatographic analysis of the corticosteroids, extracts were after fractionation analysed by gas chromatography coupled to ion trap mass spectrometry in the negative chemical ionization mode (GC–NCI-MS) and if necessary confirmed by liquid chromatography coupled to multiple ion trap mass spectrometry in the electrospray ionization mode (LC–ESI-MS^2). For the GC analysis, the targeted extract was taken to dryness and reconstituted in a mixture of 50 μL acetonitrile and 50 μl of a solution consisting of 1 g potassiumdichromate and 10 mL of 10% aqueous sulphuric acid. Subsequently, extraction was performed using 100 μL aqueous sodium carbonate (10%), 800 μL water and 3 mL *n*-hexane–dichloromethane (2:1). This mixture was centrifuged and frozen. The organic layer was taken to dryness and reconstituted in 50 μL toluene.

Gas chromatographic analyses were carried out in negative chemical ionization mode with a Finnigan trace gas chromatograph coupled to a PolarisQ ion trap mass spectrometer and a Finnigan MAT A200S autosampler (Thermofinnigan, Austin Texas, USA). Separations were conducted on a BPX-35 fused silica capillary column, 25 m × 0.22 μm i.d.; 0.25 μm film thickness, 35% phenyl liquid phase (SGE Inc., Austin Texas, USA). A volume of 1 μL of sample was injected with a split–splitless injector (split flow 20 mL min^{-1}, splitless time 1 min). The column was held at 90 °C (1 min), ramped at 90 °C min^{-1} to 270 °C, ramped at 3 °C min^{-1} to 300 °C (1 min). The injector, the ion source and transfer line temperature were, respectively, 250, 200 and 275 °C. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. FC43 was used as a calibration gas. The ion trap was equipped with the variable damping gas option that provided a control of the helium damping gas and the ammonium (NH_3 VLSI 0.2 kg × 0.4 S Din 8, quality 5.2, Air products, Vilvoorde) gas flow in the ion trap. This flow was set at respectively, 0.3 and 1.4 mL min^{-1}. Spectra were obtained in the full scan mode.

For the LC–ESI-MS^2 analysis, the targeted extract was taken to dryness and reconstituted in 100 μL of 0.2% aqueous acetic acid and 0.2% acetic acid in acetonitrile (20:80%).

The LC system consisted of a Finnigan surveyor autosampler plus and a Finnigan surveyor MS pump plus coupled to a Finnigan LTQ linear ion trap mass spectrometer equipped with an electrospray ionization (ESI) source, which was in the negative mode (Thermo Electron, San José, CA, USA). Chromatographic separation was achieved using a Thermo hypercarb

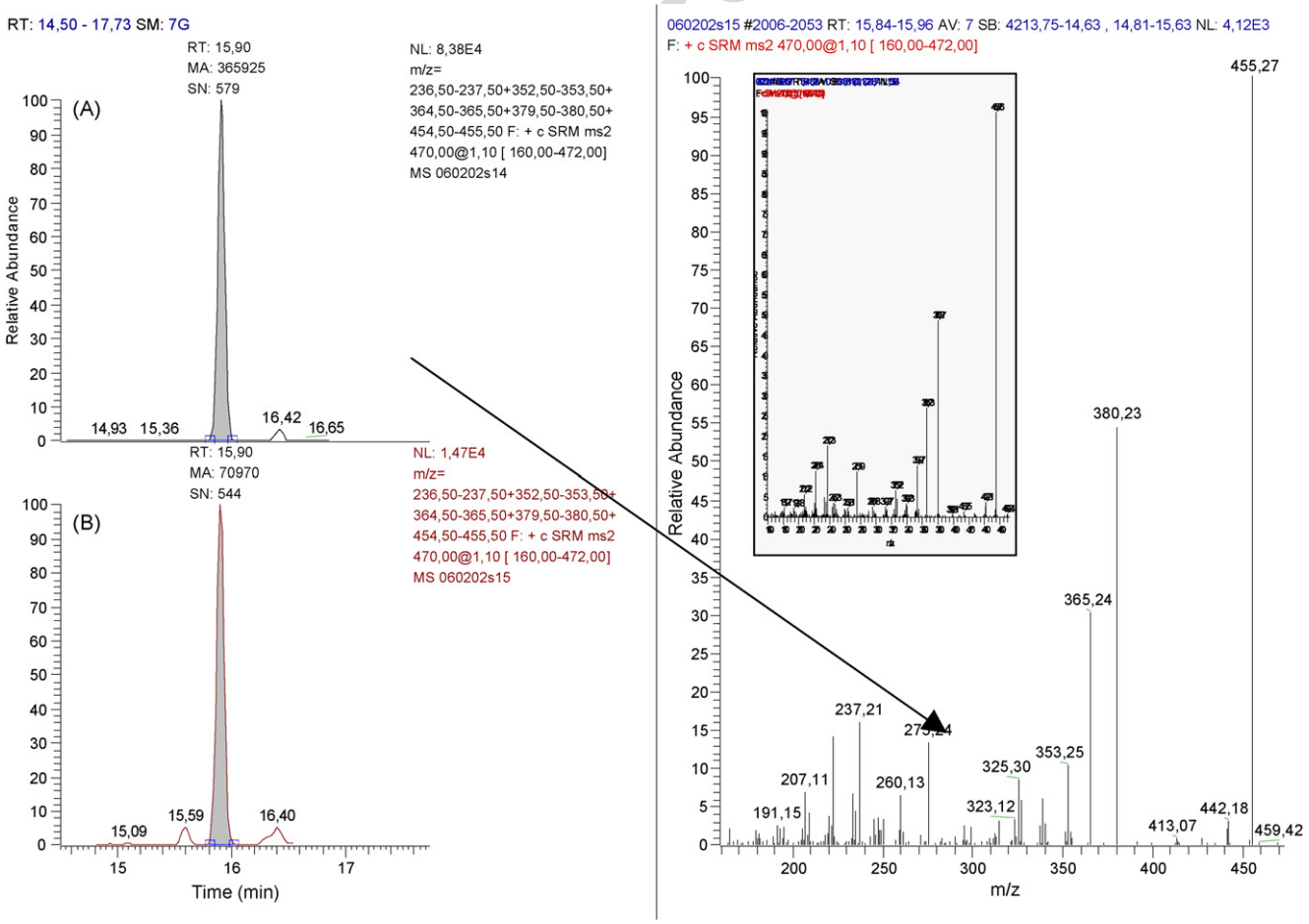

Fig. 1. Chromatograms (shaded zones = peak area) and spectrum of medroxyprogesterone acetate (MPA) in (A) an unknown water sample (40 ng L^{-1}) and (B) standard mixture (2 ng on column).

column (100 mm × 2.1 mm, 5 μm particle size, Thermo electron, San José, CA, USA). The mobile phase consisted of 0.2% aqueous acetic acid (A) and 0.2% acetic acid in acetonitrile (B). The gradient started with 20% A:80% B for 18 min and subsequently increased to 100% B. At 22.10 min the initial gradient conditions were restored until 26 min. Mobile phase flow was set at 0.3 mL min^{-1}. The sample tray was maintained at 15 °C, whereas the column was maintained at 35 °C. Spectra were obtained using the multiple MS scan mode. A sample volume of 10 μL was injected.

2.2.4. Data interpretation

Prior to sample analysis standard mixture of the targeted compounds was injected in order to check the operation conditions of the chromatographic devices. All data were processed using Xcalibur® software (Thermo Electron, San José, CA, USA).

3. Results and discussion

3.1. Extraction and clean-up procedure

Due to the 'unknown' status of the aqueous preparations and the suspected low concentration levels, sample volumes as large as possible, depending on the characteristics of the sample, were processed in order to attain the preconcentration factors needed for a quantitative analysis. For this, up to 500 mL sample (or a certain amount diluted to 1 L with ultrapure water) was used for speedisk extraction, a technique commonly used in environmental analysis [19].

Also due to the 'unknown' state of these samples, fractionation was performed in order to obtain clean extracts that can be used for chromatographic analysis. As described earlier by the authors [18], fractionation of the extracts is an advisable approach to get rid of interfering peaks and background noise in the chromatogram. Based on the extended experience of the laboratory with the detection of hormone steroids in animal matrices (i.e. faeces, urine, meat, fat) and the use of fractionation as clean-up technique for extracts of these matrices, it was known that of the four fractions obtained, the targeted corticosteroids (see Table 2) were within the first collected fraction and the targeted EGAs (see Table 1) were collected within the other three fractions.

3.2. Method validation

Because no guidelines for the analysis of 'unknown water samples' exist, the European Criteria 2002/657, which are the criteria for analytical residue methods for matrices of

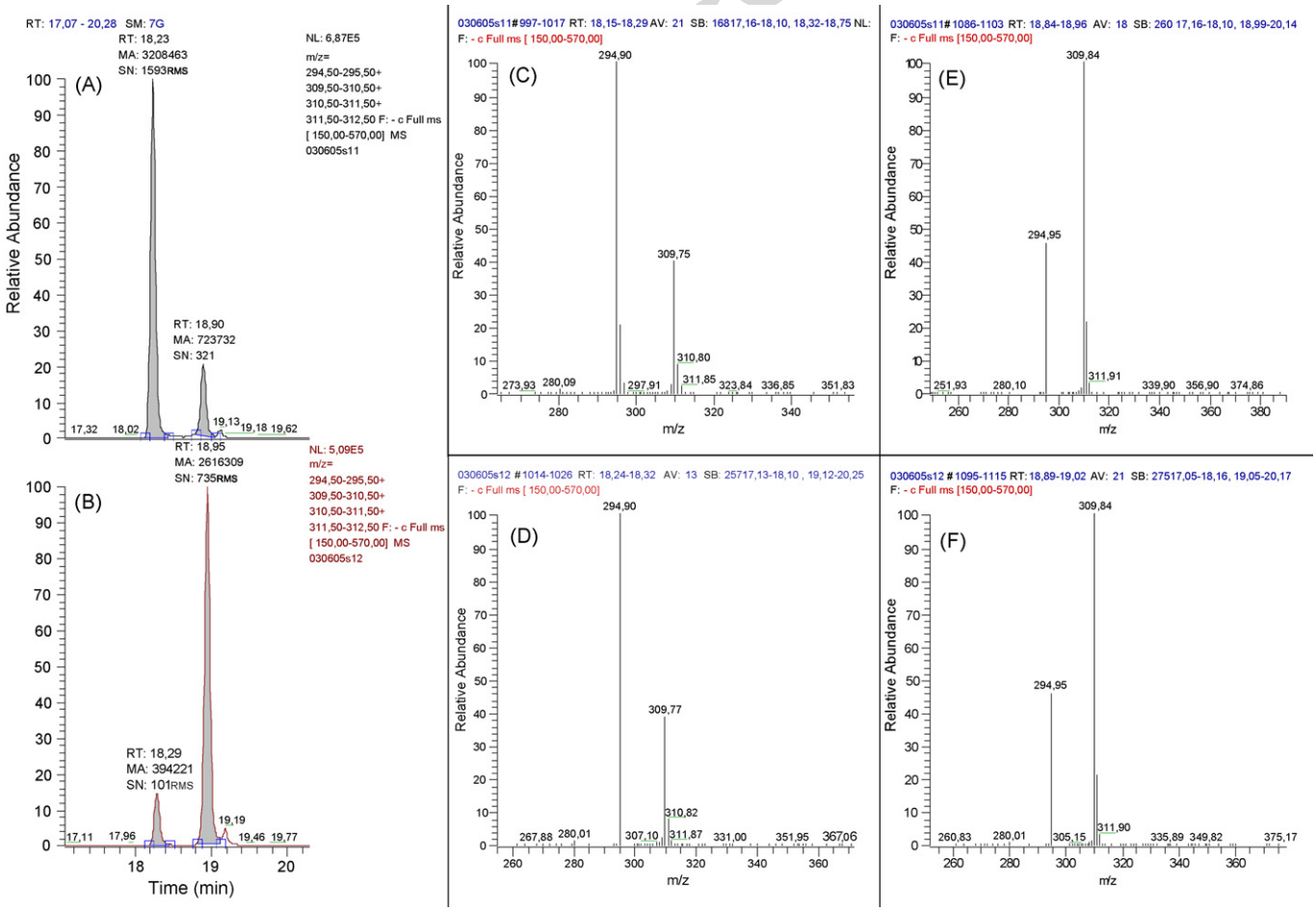

Fig. 2. Chromatograms (shaded zones = peak areas) of a standard mixture of (A) dexamethasone (Dxm) and (B) betamethasone (Btm) and spectra of both peaks of Dxm (C and E) and of Btm (D and F).

animal origin were used in the present study. Compounds were identified based on relative retention time and the ion ratio of the precursor/product ions in the obtained spectrum. The described multi-residue method for the detection of steroid hormones in 'unknown water samples' is a semi-quantitative method. Because no blank 'unknown water sample' was available, the specificity of this method was assessed by the analysis of blank and fortified ultrapure (which was used when the samples were diluted) and tap water samples. For this, blank water samples were fortified with steroid hormones in the range of 50–2500 ng L^{-1} (see Table 1), depending on the target compound and based on preliminary experiments. No interferences could be observed using both GC–NCI-MS and LC–ESI-MS^2. According to the European Criteria 2002/657 the minimum number of identification points (IPs) for steroid hormones is set at four. For the targeted EGAs, each precursor ion counts for 1 IP and each product ion counts for 1.5 IPs. As can be seen in Table 1 each targeted EGA has at least teo product ions. For the targeted corticosteroids using GC–NCI-MS, four precursor ions (isotope ions included) were selected each counting for 1 IP. When the samples were analysed with LC–ESI-MS^2, one precursor ion and at least two product ions were selected each counting for respectively, 1 and 1.5 IPs. When the criteria for both the relative retention time and the ion ratio (IPs) were fulfilled, the concentration of the steroid compound was estimated using standard mixture injections or fortified blank samples.

3.3. Chromatographic analysis

3.3.1. EGAs

Fractions of EGAs were analysed using GC–EI-MS^2 in the electron impact mode. It should be added that, for screening purposes for EGAs, tandem MS is preferred above MS. Although the latter results in a higher intensity, the selectivity is insufficient when taken into account the possible matrix interferences and the low levels of interest in veterinary or water matrices. Fig. 1 shows the chromatogram and spectrum obtained from the extraction of 1 L of an unknown aqueous sample. In this sample medroxyprogesterone acetate (MPA) or 6α-methyl-3,20-dioxopregn-4-en-17-yl acetate was detected (>4 IPs), which is a synthetic progestagen. The detected concentration of 40 ng L^{-1} was determined using standard mixture injections.

3.3.2. Corticosteroids

In the first place, GC–NCI-MS was used to analyse the fraction for the targeted corticosteroids because it is a better technique when matrix interference is expected (see Table 2 for

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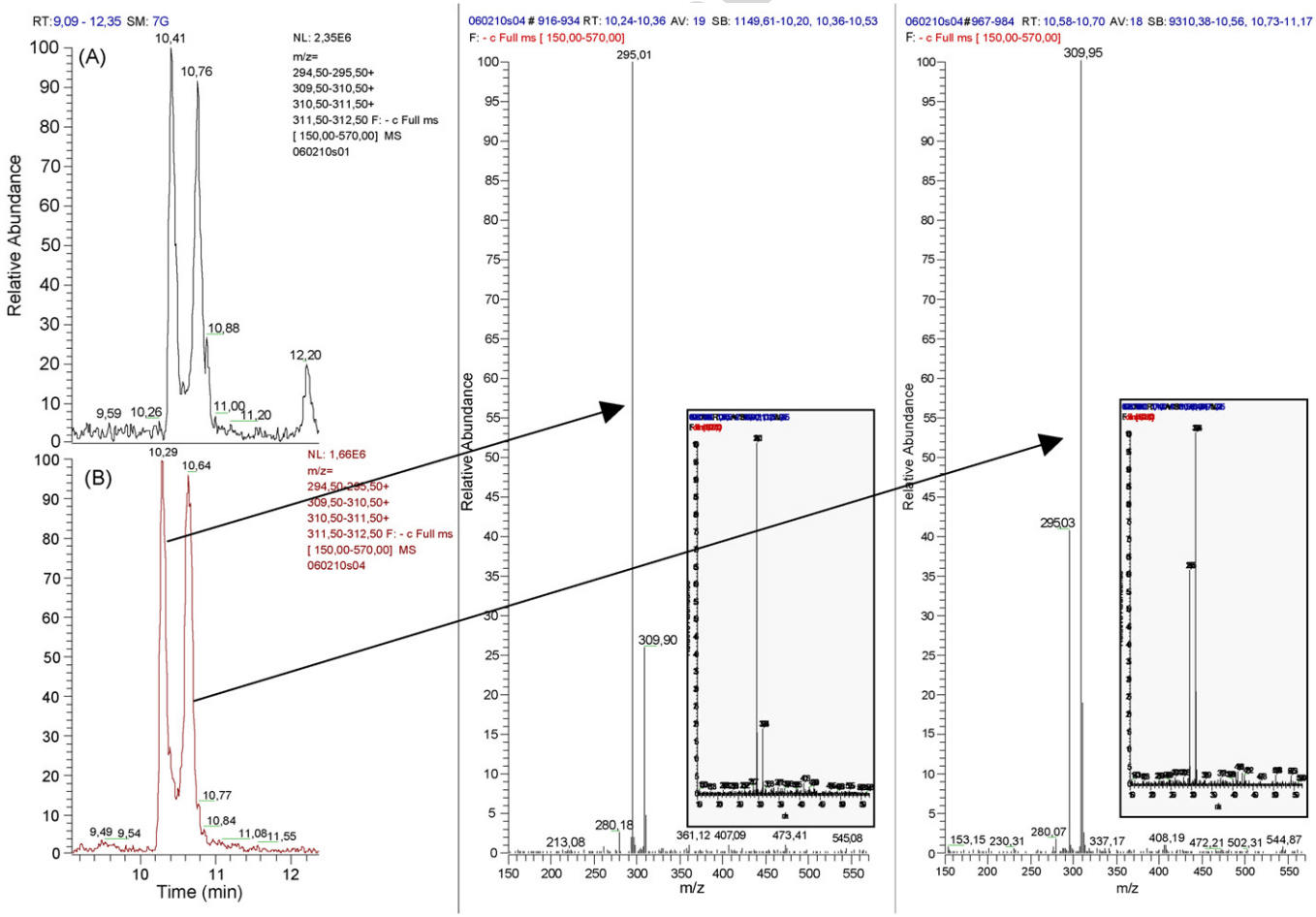

Fig. 3. Chromatograms and spectra of dexamethasone (Dxm) and betamethasone (Btm) in (A) standard mixture (1 ng on column) and (B) an unknown water preparation after analysis with GC–NCI-MS. Insets are standard mixture spectra.

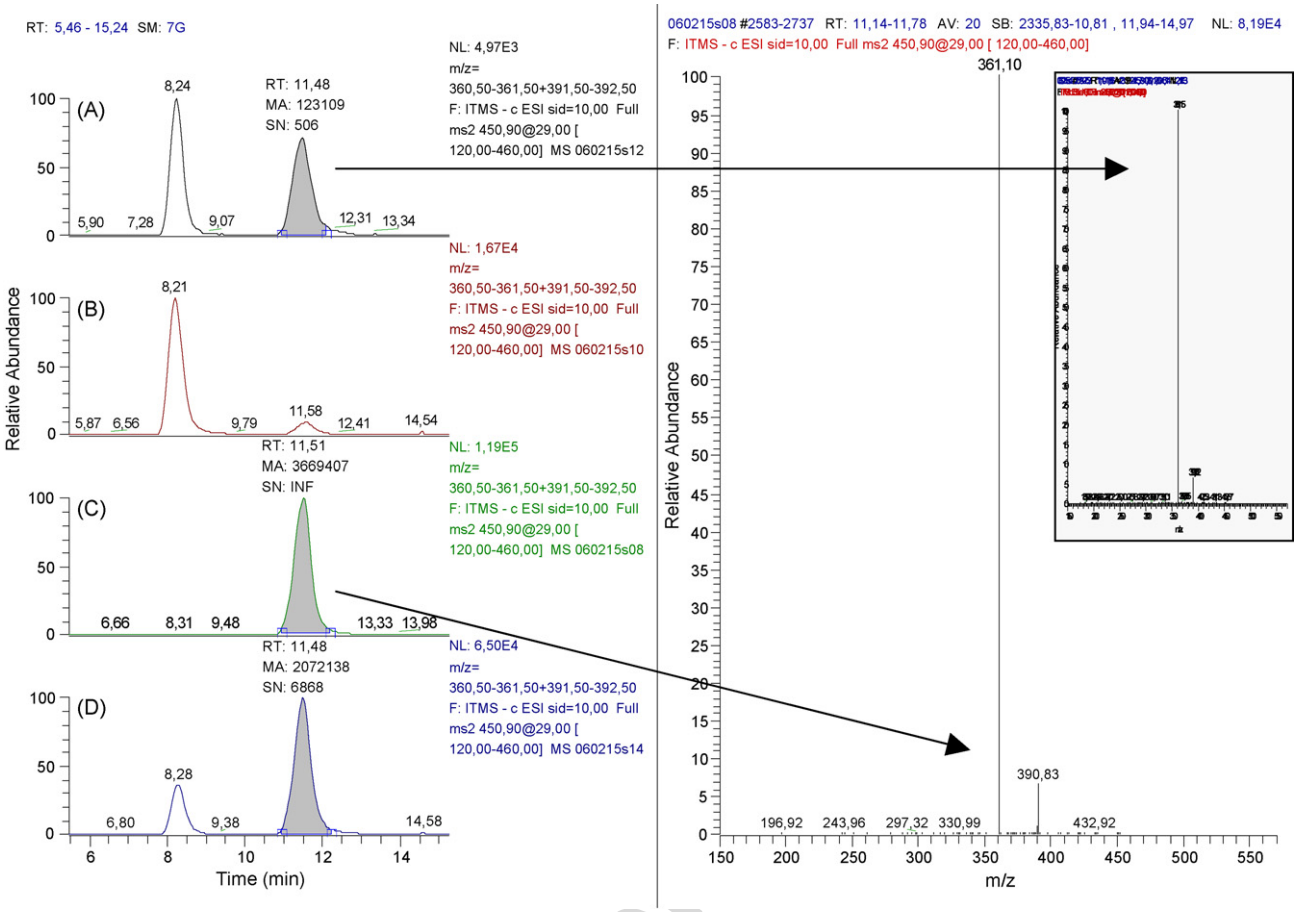

Fig. 4. Chromatograms of (A) standard mixture (1 ng on column), (B) a fortified ultrapure water sample (40 ng L^{-1}), (C) an unidentified aqueous water sample (50 ng L^{-1}) and (D) the same extract fortified with Dxm. On the right side the spectrum of betamethasone (Btm) of the 'unidentified' water sample (inset is the spectrum of Btm from the standard mixture).

precursor and product ions). However, using GC–NCI-MS, it is known that by-products and interfering compounds can complicate proper interpretation of the chromatographic analysis and less complex sample preparation [4,16]. It is also known that the differentiation between dexamethasone (Dxm) and betamethasone (Btm), which differ only in the configuration of the methyl group on C_{16}, is not always clear [20–22]. Only when there is no matrix interference or apparatus contamination, distinction between Dxm and Btm can be made through the ratio of both peaks as shown in Fig. 2. As can be seen in Fig. 3 for an 'unknown' water preparation, two peaks were obtained in the chromatogram, both with the same relative retention time and with the same product ions and ion ratios in their corresponding spectrum in comparison with the standard injection. This indicates that the field of application of GC–NCI-MS is limited to screening purposes because different compounds, e.g. Dxm and Btm can lead to the same derivative. In addition, LC does not require a derivatization step and as such, enables direct measurements of corticosteroids [4]. For this, to obtain better selectivity in order to confirm the unambiguous identity of suspected Dxm or Btm, a second injection on a new device, LC coupled to a LTQ linear ion trap MS was performed. This device offers more sensitivity due to the novel ion trap, dual detector and ion ejection technologies.

Interpreting the results of the GC–NCI-MS analysis (Fig. 3) it can be concluded that Dxm or Btm is suspected; however, a clear distinction between these two compounds is not possible. After addition of Dxm to the sample and LC–ESI-MS^2 confirmation analysis (Fig. 4) it was concluded that the sample contained Betamethasone (9α-fluoro-11β, 17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) at a concentration of 50 ng L^{-1} (4 IPs). This synthetic glucocorticosteroid has a widespread application in human and veterinary medicine.

4. Conclusions

In this investigation, a routine multi-analyte approach for the screening of estrogens, gestagens and androgens (EGAs) and corticosteroids in unidentified aqueous preparations is described. With this method, a large group of steroid hormones can be detected at ng L^{-1} levels, which fits into the inspection services strategy to control the abuse of EGAs and corticosteroids for animal fattening purposes.

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