

# Androstadienetrione, a boldenone-like component, detected in cattle faeces with $GC-MS^n$ and $LC-MS^n$

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#### Abstract

Boldenone (1,4-androstadiene-17-ol-3-one, Bol) has been the subject of a heated debate because of ongoing confusion about its endogenous or exogenous origin when detected in one of its forms in faecal or urine samples from cattle. An expert report was recently written on the presence and metabolism of Bol in various animal species. Androstadienedione (ADD) is a direct precursor of  $17\beta$ -boldenone ( $\beta$ Bol). It is a 3,17-dione;  $\beta$ Bol is a 17-ol-3-one. Not much is published on 1,4-androstadiene-3,17-diol, which is a 3,17-diol (ADL). If animals were exposed for a longer period to one of these analytes, a metabolic pathway would be initiated to eliminate these compounds. Similar to recent testosterone metabolism studies in the aquatic invertebrate *Neomysis integer*, ADD,  $\beta$ Bol and ADL could also be eliminated as hydroxymetabolites after exposure. The presence of 11-keto-steroids or 11-hydroxy-metabolites in faecal samples can interfere with a confirmation method by gas chromatography-negative chemical ionization mass spectrometry (GC-NCI-MS), after oxidation of corticosteroids with a double bond in the A-ring (e.g. prednisolone or its metabolite prednisone). The presence of androstadienetrione (ADT) in faecal samples of cattle has never been reported. The origin of its presence can be explained through different pathways, which are presented in this paper.

Keywords: Prednisolone, corticosteroid, metabolite, androstadienedione, boldenone, androstadienetrione, GC-MS<sup>n</sup>, LC-MS<sup>n</sup>

#### Introduction

Residue analysis of steroidal compounds in animals used for food production is of great importance.  $17\beta$ -boldenone ( $\beta$ Bol, a 17-ol-3-one) is a steroid with strong anabolic activity. An expert report on its presence and metabolism in various animal species was presented (De Brabander et al. 2004). Androstadienedione (ADD, a 3,17-dione) is the direct precursor of  $\beta$ Bol. Androstadiene-3,17-diol (ADL, a 3,17-diol) has been called '1,4-androdiol' and is used by bodybuilders as a muscle enhancer. Because of the presence of a double bond at the 1-position, it could have high oral activity. There is a lot of suspicion that these analytes could also be used as growth promoters in cattle. Residue laboratories face difficulties confirming the origin of the analyte. Are they endogenous or have they been administered illegally? There is still an unexplained excretion in untreated animals.

The metabolism of testosterone  $(17\beta$ -hydroxyandrost-4-en-3-one), which differs only in a double bound with  $\beta$ Bol, has been studied intensively. We have previously used testosterone as a substrate for metabolic studies with the aquatic invertebrate *Neomysis integer* (Crustacea; Mysidacea) (Verslycke et al. 2002). These studies demonstrated the presence of cytochrome P45011B1 (CYP11B1) in N. integer, which is the enzyme responsible for the hydroxylation in the 11-position. This enzyme is also present in mammals, and thus in cattle. We have found interesting similarities between steroid metabolism in the invertebrate N. integer and vertebrate models (De Wasch et al. 2002). 11-Ketosteroids have been described to play a role in the reproductive cycle of fish (Eckstein et al. 1982; Manire et al. 1999). An example of an 11-ketosteroid is adrenosterone (4-androstene-3,11,17-trione). It is an adrenal steroid derived mainly from the reversible conversion of  $11\beta$ -hydroxy-androstenedione. Adrenosterone may also be derived directly from adrenal metabolism of cortisone. Adrenosterone is a weak androgen but levels are elevated in hirsute female and decreased in hypogonadic males.

Besides the above-mentioned anabolic compounds, another group of steroidal compounds, the glucocorticosteroids, are of importance in the residue analysis. Prednisolone (Prolon) is a synthetic glucocorticosteroid. In veterinary medicine, it (as the free alcohol) is included as an ingredient in a number of antibiotic preparations, which are indicated for intramammary administration for the treatment of bovine mastitis (EMEA/MRL/629/99 1999). Prolon can be metabolized to prednisone (Pron), 20ß-dihydroxyprednisolone and 20ß-dihydroprednisone. There is an interconversion of prednisone and prednisolone (Meikle et al. 1975; Garg and Jusko 1994). Maximum residue limits (MRLs) have been described in bovine species in muscle, fat, liver, kidney and milk at concentrations of 4, 4, 10, 10 and  $6 \,\mu g \, kg^{-1}$ , respectively, (EMEA/MRL/629/99 1999).

Recently, a number of faecal samples from bulls as part of a monitoring programme were analysed for corticosteroids with gas chromatography-negative chemical ionization-mass spectrometry (GC-NCI-MS). In this procedure, the target analyte is oxidized with potassium dichromate under acidic conditions at  $60^{\circ}$ C for 10 min (Courtheyn et al. 1998). After HPLC fractionation, the presence of the derivatization product of prednisolone was identified in a variety of concentrations in most of the faecal samples. An important detail in this case is that  $\alpha$ -boldenone ( $\alpha$ Bol), and rostenedione (AED) and progesterone (P) were also identified in these faecal samples with GC-MS electron impact (EI). The EC criteria 2002/657/EC (European Commission 2002) were checked and fulfilled for all identified analytes. After a second analysis with the same extraction and detection method, the same results were obtained. A third analysis was performed for the identification of Prolon and Pron with a different detection method: liquid chromatography atmospheric pressure chemical ionization multiple mass spectrometry (LC-APCI-MS<sup>2</sup>) in positive-ionization mode. No Prolon and Pron were identified with this technique.

In an attempt to explain the discrepancy between the results of the used detection methods, different possible pathways were investigated. The objective of this study was to investigate the identity of a compound that was detected as an derivatized oxidized product by GC-NCI-MS.

## Materials and methods

#### Chemicals

All chemicals used for extraction were of analytical grade from Merck-Eurolab (Overijse, Belgium). The derivatization procedure (oxidation) was described by Courtheyn et al. (1994).

Standards were obtained from Steraloids (Wilton, NY, USA): Prolon (prednisolone, P0650-000), Pron (prednisone, P0300-000), IFP (isoflupredone, P0553-000), ADT (androstadienetrione, A0230-000), 11βOH-ADD (11β-hydroxy-1,4-androstadiene-3,17-dione, A0170-000) and  $\beta$ Bol ( $\beta$ -boldenone, A0200-000). Only  $\alpha$ Bol  $(\alpha$ -boldenone) was obtained from the Rijksinstituut voor Volksgezondheid en Milieu (RIVM; National Institute for Public Health and the Environment, Bilthoven, the Netherlands).

## $GC-MS^n$ analysis

GC-NCI-MS. For GC-NCI-MS analysis, an iontrap mass spectrometer PolarisQ (ThermoFinnigan, Austin, TX, USA) coupled to a Trace GC 2000 (ThermoFinnigan) was used. A Finnigan Mat A200S autosampler was used to inject the samples. Analyses were performed using a non-polar 35% phenyl-polysilphenylene-siloxane SGE BPX-35 GC-column  $(25 \text{ m} \times 0.22 \text{ mm} \text{ i.d.}, 0.25 \mu\text{m} \text{ film})$ thickness) (SGE, Inc., Austin, TX, USA). Glass injector liners  $(10.5 \text{ cm} \times 3 \text{ mm})$  were supplied by SGE. The injector, ion source and transferline temperature were, respectively, 250, 200 and 275°C. A temperature gradient was used starting at 90°C and increasing to 270°C in steps of  $90^{\circ}$ C min<sup>-1</sup>. In a second step the temperature was increased to 298°C in steps of 3°C min<sup>-1</sup>. A last ramp of  $50^{\circ}$ C min<sup>-1</sup> to  $300^{\circ}$ C was performed. Helium was used as carrier gas at a flow of  $1 \text{ mlmin}^{-1}$ . A total of  $1 \mu \text{l}$  of  $25 - \mu \text{l}$  sample was injected with a split-splitless injector (split flow  $20 \,\mathrm{ml}\,\mathrm{min}^{-1}$ , splitless time 1 min). Negative chemical ionization mode (NCI) was used to ionize the analytes, with ammonia as the reaction gas.

*GC-EI-MS*. A trace GC 2000 coupled to an ion-trap mass spectrometer Polaris (both ThermoFinnigan) was used for GC-EI-MS analysis. A Carlo Erba AS2000 (ThermoFinnigan) autosampler was used to inject. Analyses were performed using a non-polar

5% phenyl-polysilphenylenesiloxane SGE BPX-5 GC-column  $(25 \text{ m} \times 0.22 \text{ mm i.d.}, 0.25 \text{ µm film})$ Glass injector thickness) (SGE). liners  $(10.5 \text{ cm} \times 3 \text{ mm})$  were supplied by SGE. The injector, ion source and transferline temperature were, respectively, 250, 200 and 275°C. The temperature gradient started at 100°C and increased by  $17^{\circ}$ C min<sup>-1</sup> to 250°C. Further, the temperature increased to 273°C in steps of 2°C min<sup>-1</sup> and was finally ramped at  $30^{\circ}$ C min<sup>-1</sup> to  $300^{\circ}$ C. Helium was used as carrier gas at a flow of  $1 \text{ mlmin}^{-1}$ . A total of 1 µl of 25-µl sample was injected with a split-splitless injector (split flow  $20 \text{ ml min}^{-1}$ , splitless time 1 min). Electron ionization (EI) was used in the positive-ion mode, MS full-scan acquisition.

# $LC-MS^n$ analysis

 $LC-APCI-MS^2$  in positive-ion mode method 1 (Federal Feed and Food Laboratory). Chromatographic separation was achieved using an Alltima C18 5 µm column ( $250 \text{ mm} \times 3.0 \text{ mm}$  i.d.) (Alltech Associates, Deerfield, IL, USA). The HPLC apparatus consisted of an Agilent 1100 system with quaternary gradient pump, degasser, column oven and autosampler. The mobile phase consisted of a gradient of 1% acetic acid in water and methanol at a flow rate of  $0.5 \,\mathrm{ml}\,\mathrm{min}^{-1}$ . The mass spectrometrical analysis was performed using an LCQdeca ion-trap mass spectrometer (ThermoFinnigan). ADT and the internal standard (deuterated cortisol) were measured in fullscan and in MS<sup>2</sup>. The pseudo molecular ion of ADT 299 was isolated with an isolation width of 2.5 amu and fragmented with a relative energy of 26% (absolute energy 0.85V).

LC-APCI-MS<sup>2</sup> in positive-ion mode method 2 (Veterinary Public Health and Food Safety). Chromatographic separation was achieved using an YMC column J'Sphere ODS M-80 (Achrom, Zulte, Belgium). The HPLC apparatus comprised of a TSP P4000 pump and an AS3000 autosampler (Thermo Separation Products, San Jose, CA, USA). The mobile phase consisted of a mixture of 45% acetonitrile and 55% water and was pumped at a flow rate of  $0.5 \text{ ml} \text{ min}^{-1}$ . The gradient was changed from 5 to 20 min to 100% acetonitrile. In between samples, there was an equilibration time of 5 min at initial conditions. The mass spectrometrical analysis was performed using an LCQdeca ion-trap mass spectrometer (ThermoFinnigan). The ions were acquired after atmospheric pressure chemical ionization (APCI) in positive-ion mode MS<sup>2</sup>. In a one-time segment five scan events were performed. The pseudo-molecular ions of Prolon, Pron, ADT and 11βOH-ADD; 361, 359, 299 and

301, respectively, were isolated with an isolation width of 3 amu, with the exception of Prolon, which had an isolation width of 3.5 amu. All components were fragmented with a relative energy of 30% (absolute energy, dependent on mass component: Pron: 0.86V, ADT and 11 $\beta$ OH-ADD: 0.79V), except again Prolon, which was fragmented with 25% of relative energy (absolute energy: 0.72V). In addition, a full-scan mass spectrum was acquired.

# Extraction procedure

The extraction procedure of corticosteroids from faeces has previously been described by Courtheyn et al. (1994). In short, faecal samples were diluted with water and extracted with diethyl ether. Further clean-up comprised evaporation of the extracts and redissolving in methanol. This solution was purified by a solid-phase extraction and HPLC fractionation. The derivatization before GC-MS analysis was dependent on the ionization method. NCI (negative chemical ionization) analysis was preceded by derivatization with potassium dichromate, while EI (electron impact) needed MSTFA<sup>++</sup> derivatization. MSTFA<sup>++</sup> is a mixture of MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide), NH<sub>4</sub>I (ammonium iodide) and ethanediol.

The procedure used for the extraction of (cortico)steroids from N. *integer* with ethylacetate was previously by Verslycke et al. (2002).

# Results

# Mass spectral analysis, GC-NCI-MS

Blank faecal samples and blank faecal material spiked at  $2 \mu g k g^{-1}$  Prolon and IFP (internal standard) were analysed by GC-NCI-MS and used as quality control samples. The diagnostic ions of Prolon (177, 297, 298, 299) eluted at a relative retention time (relative to the internal standard IFP) of 1.09 min  $\pm 8.41E-4$  min. The ion ratios and tolerance levels, as mentioned in Commission Decision 2002/657/EC (European Commission 2002), of the derivatization product of Prolon are given in Table I. For ions with a relative intensity >50, >20–50,

Table I. Relative ion intensities of Prolon in the spike and the tolerance levels, calculated as prescribed in Commission Decision 2002/657/EC (European Commission 2002).

| Diagnostic<br>ions | Relative<br>intensity<br>(% of base peak) | Lower<br>limit (%) | Upper<br>limit (%) |
|--------------------|---|--------------------|--------------------|
| 177                | 82  | 66                 | 99                 |
| 297                | 16  | 11                 | 21                 |
| 298                | 100                                       | 80                 | 120                |
| 299                | 20  | 15                 | 25                 |

>10–20% as a per cent of the base peak, the tolerances are, respectively,  $\pm 20$ ,  $\pm 25$  and  $\pm 30\%$ .

In the blank faeces samples no signal was observed for the oxidation product of Prolon (Figures 1 and 2). In almost 70% of the batch of 40 samples, the signal corresponding with the derivatization product of Prolon was found. The relative retention time was within the required limits and the relative abundance of the diagnostic ions was situated within the tolerance levels, according to Commission Decision 2002/657/EC (European Commission 2002). The extraction and detection procedure was repeated and the same results were obtained. Since the detection method used for the faecal samples involved a derivatization procedure in which the 17-side chain was removed and all hydroxyl-groups were oxidized



Figure 1. Structure of Prolon, ADT and  $11\beta$ OH-ADD.

to keto-groups, the results had to be interpreted cautiously. Different compounds can result in the same derivatization product. An LC-MS<sup>2</sup> procedure was used to confirm the previous results.

#### Mass spectral analysis, $LC-MS^n$

At first an LC-APCI-MS<sup>2</sup> method in positive-ion mode, created at the Federal Feed and Food Laboratory, was used to confirm the presence of Prolon and its major metabolite Pron in samples giving GC-NCI-MS detection for the derivatization product of Prolon. The presence of Prolon or Pron could not be confirmed in these samples. Both the components were identified in the quality control samples spiked at the level of 2 ppb.

In a next stage, the Department of Veterinary Public Health and Food Safety developed an LC-APCI-MS<sup>2</sup> method in positive-ion mode to detect Prolon, Pron, ADT and  $11\beta$ OH-ADD. The differences between both LC-MS<sup>2</sup> methods were because the samples were analysed in two different laboratories.



Figure 2. (left) Trace 1 standard Prolon, trace 2 spiked faeces  $2 \mu g kg^{-1}$ , trace 3 non-compliant faeces sample and trace 4 blank sample; (right) spectrum of Prolon oxidation product with m/z 177, 297, 298 and 299.

#### Discussion

Three pathways were investigated for the explanation of the presence of the derivatization product of Prolon, described in a screening procedure with GC-NCI-MS. First, the possibility of the presence of another corticosteroid, with the same derivatization product as Prolon, in the samples is described. Second, metabolic pathways of oxidized boldenone are considered. Third, the possible formation of ADT, derived from oxidized phytosterols, is discussed.

The results obtained after using a derivatization procedure, which oxidizes the 17-side chain of compounds, need to be interpreted with caution. Different analytes leading to the same derivatization product could be the cause of the interference detected with screening method GC-NCI-MS. Therefore, possible analytes leading to the same derivatization product as Prolon were searched for in The Merck index (ChemOffice, CambridgeSoft Corp., Cambridge, MA, USA). The search was performed using the basic structure of Prolon, without any substitution on the 17-position. Fortysix analytes were selected from the Merck index (Table II).

The analytes containing a fluor or chlorine were first eliminated since the Prolon derivatization product does not contain fluor or chlorine. Compounds that cannot be oxidized to the Prolon derivatization product because of the presence of a substitution are formebolone (2-COH), methylprednisolone (6-CH<sub>3</sub>), prednylidene (16-CH<sub>2</sub>) and rimexolone (16-CH<sub>3</sub>, 17-CH<sub>3</sub>). Twelve analytes

were retained for further investigation: budesonide, ciclesonide, deflazacort, desonide, loteprednol etabonate, mazipredone, prednicarbate, prednimustine. prednisolone, prednival, prednisolone 21-diethylaminoacetate and prednisolone sodium phosphate. Figure 3 shows the chemical structure of the analytes. The clean-up procedure of the faecal samples contains an HPLC fractionation step. Prednisolone and prednisone both eluted in the same fraction that gave the positive response with GC-NCI-MS. Since the presence of both compounds was not confirmed with an LC-MS procedure, prednisolone and prednisolone esters (prednisolone 21 diethyl aminoacetate, prednisolone sodium phosphate, prednicarbate, prednimustine and prednival) could also be eliminated from the list. The esters could be eliminated because illegal administration of prednisolone-esters would result in the detection of prednisolone or prednisone in the faeces samples. From the remaining six analytes, we checked which could react to the same derivatization product as prednisolone and elute in the same HPLC fraction. Budesonide, ciclesonide and desonide are acetonides, deflazacort is an oxazoline. In theory, these products can react to the Prolon derivatization product. However, acetonides and oxazolines would elute later than prednisolone on a  $C_{18}$  column. This means that even though the acetonides and the oxazoline would react to form the same derivatization product as Prolon, they would not be captured in the correct HPLC fraction.

Mazipredone and loteprednol (etabonate) remained as last possible candidates responsible



Table II. Forty-six analytes containing the basic structure of Prolon without any substitution in the 17 position.



Figure 3. Chemical structures of analytes that can interfere with the detection of the oxidation product of prednisolone.



Figure 4. Possible formation of the derivatized oxidation product of Prolon from mazipredone and loteprednol etabonate.

for the positive GC-NCI-MS screening results (Figure 4), where the LC-MS confirmation method would be negative for Prolon. The presence of these analytes in the faeces samples could be confirmed with a specific LC-MS method and the use of standard solutions.

Besides the analytes that produced the same derivatization product of Prolon by oxidation, metabolism also needs to be considered. An important consideration is the link to the presence of boldenone in faeces samples. Boldenone, ADD and ADL (androstadienediol), all three having two double bounds in the A-ring, can metabolize to an 11-hydroxy metabolite. Cattle do have steroid  $11\beta$ -hydroxylases, by which the 11-position of steroids can be hydroxylated (Brown 1998; Görög 2003; Popinigis et al. 2004). The oxidized derivatization product of the 11-hydroxy metabolite should be the same as the derivatization product of prednisolone. In the review of De Brabander et al. (2004), the boldenone-containing samples originate from male animals (bulls). Similarly, the faeces samples described in the present paper were from male animals. Two standards were purchased, the oxidation product androstadienetrione (ADT) and 11 $\beta$ OH-ADD. The two compounds eluted in the same HPLC fraction as Prolon and prednisone, hence were highly suspected of causing the false screening signal for Prolon with GC-NCI-MS. This hypothesis of the production of 11-hydroxy metabolites of boldenone, ADD or ADL was indeed valid as  $\alpha$ Bol was detected in most of the suspect faecal samples. In addition, when  $\alpha$ Bol was detected in routinely screened faeces, the GC-NCI-MS procedure was used to detect the Prolon derivatization product. In most cases, the presence of the derivatization product was confirmed.

An attempt was made to identify the analyte present in the faeces samples. Spiked samples with ADT and 11BOH-ADD were investigated after dervatization with GC-NCI-MS and compared with the samples giving a signal for Prolon. The standards ADT and  $11\beta$ OH-ADD were also injected on column without preceding derivatization. The response of ADT with and without derivatization was nearly the same.  $11\beta$ OH-ADD on the other hand, showed a low response without oxidation and a 1000 times better response with derivatization. The response of the samples suspected for Prolon and the faeces spiked with ADT and  $11\beta$ OH-ADD were also compared with and without derivatization. The response of the Prolon suspected faeces and the ADT spike were better without oxidation, while 11 $\beta$ OH-ADD in faeces showed only a low response without oxidation. These experiments indicated that the most probable interfering analyte present in the Prolon suspected faeces was ADT. In order to confirm unequivocally the identity of the analyte, other methods were used for confirmation.

The faecal samples were also analysed by GC-MS electron impact (EI) method in which full-scan mass spectra were acquired to look for 'unknowns' and the formation of metabolites. The expected m/z values were calculated from the MSTFA<sup>++</sup> derivative. In none of the faeces could ADT be detected in high concentrations comparable with the high concentrations of the Prolon derivatization product screened with GC-NCI-MS. This anomaly could be explained by the fact that ADT does not derivatize very well

with MSTFA<sup>++</sup>. Therefore, an LC-MS<sup>2</sup> confirmation gave additional information. Using this LC-APCI-MS<sup>2</sup> method, ADT was clearly confirmed in the three examined Prolon suspected samples (Figure 5).

This is the first study to report the presence of ADT in faeces of cattle. In fish, however, the presence of 11-keto-steroids (especially 11-ketotestosterone) has been demonstrated. These 11-keto-steroids play a crucial role in the reproductive cycle (Eckstein et al. 1982; Manire et al. 1999). In vitro experiments have been performed with testicular tissue of the European eel in which tritiated androstenedione and progesterone were used as substrates. 11-Keto and 11-hydroxy steroids were produced. In addition, adrenosterone was identified (Eckstein et al. 1982). An additional 1(2) dehydrogenation of adrenosterone would form ADT. Microbial transformation of adrenosterone by Cephalosporium aphidicola produced three major metabolites; ADT,  $17\beta$ -hydroxyandrost-4-ene-3, 11-dione and 17  $\beta$ -hydroxyandrosta-1,4-diene-3, 11-dione (Musharraf et al. 2002).

The invertebrate N. integer can be used as an alternative model to vertebrates to study metabolic pathways (De Wasch et al. 2002). An exposure of N. integer to Prolon was performed and the produced metabolites were detected with LC-APCI-MS<sup>2</sup>. 11 $\beta$ OH-ADD was detected in a high concentration, prednisone was only detected in very low amounts and a trace of ADT was present. This indicates that the 17-side chain (-COCH<sub>2</sub>OH) was removed to form a major metabolite. The removal of the side chain is shown in Figure 6. Figure 7 shows the presence of the metabolites formed by N. integer after exposure to Prolon. With this knowledge, the misuse of Prolon can still be the cause of the suspected faeces. Knowing that the fraction of excreted dose of unchanged Prolon after intravenous administration to humans is very small and that elimination occurs mainly by metabolism (Habet and Rogers 1989), more research need to be performed to study the metabolite formation of Prolon in cattle.

An explanation for the presence of ADT in faeces is the oxidation of phytosterols. Phytosterols are plant sterols and are therefore present in the feed of herbivores, like cattle. Nowadays, phytosterols are present in higher concentrations since the use of animal fat is banned in animal feed. If phytosterols were oxidized at the 11-position by the steroid  $11\beta$ -hydroxylase, ADT could be formed. It has been proven by experiments with *N. integer* that the sidechain of the phytosterols can be removed by metabolization (Poelmans et al. 2003). The administration of  $\beta$ -sitosterol was responsible for the formation of  $\beta$ Bol and after exposure to stigmastanol



Figure 5. Chromatogram and spectrum of ADT detected by LC-APCI- $MS^2$  in a faeces sample.



Figure 6. Removal of the C17-side chain of Prolon to form 11 BOH-ADD and ADT by Neomysis integer.



Figure 7. Prolon (trace 1) metabolization by *Neomysis integer*. Formation of metabolites Pron (trace 2), 11OH-ADD (trace 3) and a trace of ADT (trace 4) confirmed by LC-APCI-MS<sup>2</sup>.



Figure 8. Transformation of a phytosterol to the oxidation products (11OH-ADD and ADT).

*N. integer* produced AED. The substitution of the side chain from the phytosterols by a keto-group was responsible for the formation of these steroids. 11OH-phytosterols, in which the removal of the C17-side chain and the oxidation at the C17-position occurs, could be an origin of the presence of the oxidation products (11 $\beta$ OH-ADD or ADT) in the faeces samples (Figure 8).

#### Conclusions

In faces of cattle, a new boldenone-alike substance, androstadienetrione (ADT), was detected. It can interfere with corticosteroid detection methods using a derivatization technique, where an important side chain of the corticosteroid structure is eliminated on the C17 position. Although the interpreted data matched all criteria as described in the 2002/657/EC decision, a false 'non-compliant' result could be obtained. Laboratories should be aware of this pitfall and use this GC-MS method only as a screening method. The results should be confirmed by a confirmation method.

Possible causes for the presence of the derivatization product screened with GC-NCI-MS were investigated and described here. First, the oxidation of other corticosteroids was discussed. Mazipredone and loteprednol etabonate are two corticosteroids that are potentially responsible for the presence of the derivatization product. Second, the presence of a triketon steroid (ADT) was investigated. This ADT could be present in the faeces samples through the metabolization of boldenone or through the conversion of phytosterols. The present authors previously demonstrated that the side chain of the phytosterols could be removed to form steroids by using an invertebrate model. Further research is needed to clarify the exact origin of ADT. The endogenous origin of ADT is not yet proven.

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