available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/aca

Review article

Novel analytical methods for the determination of steroid hormones in edible matrices

H. Noppe[a,*], *B. Le Bizec*[b], *K. Verheyden*[a], *H.F. De Brabander*[a]

[a] *Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Research Group of Veterinary Zoonoses, Lab Chemical Analysis, Salisburylaan 133, 9820 Merelbeke, Belgium*

[b] *LABoratoire d'Etudes des Résidus et Contaminants dans les Aliments (LABERCA), Ecole Nationale Vétérinaire de Nantes, Atlanpôle de Chantrerie, Route de Gachet, BP 50707, 44307 Nantes Cedex 03, France*

ARTICLE INFO

Article history:
Received 30 September 2007
Received in revised form
22 January 2008
Accepted 22 January 2008
Published on line 7 February 2008

Keywords:
Steroid hormones
EU-criteria
Residue analysis
Edible matrices
Gas chromatography
Liquid chromatography
Mass spectrometry
Isotope ratio MS
Phytosterols

ABSTRACT

This paper reviews recently published multi-residue chromatographic methods for the determination of steroid hormones in edible matrices. After a brief introduction on steroid hormones and their use in animal fattening, the most relevant EU legislation regarding the residue control of these substances is presented. An overview of multi-residue analytical methods, covering sample extraction and purification as well as chromatographic separation and different detection methods, being in use for the determination of steroid hormones (estrogens, gestagens and androgens), is provided to illustrate common trends and method variability. Emphasis was laid on edible matrices and more specifically on meat, liver, kidney, fat and milk. Additionally, the possibilities of novel analytical approaches are discussed. The review also covers specific attention on the determination of natural steroids. Finally, the analytical possibilities for phytosterols, naturally occurring steroid analogues of vegetable origin and a specific group of steroid hormones with a hemi-endogenous status are highlighted.

Contents

* *Corresponding author*. Tel.: +32 92647462; fax: +32 92647492.
E-mail address: Herlinde.Noppe@UGent.be (H. Noppe).

0003-2670/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2008.01.066

1. General Introduction to steroid hormones

Steroid hormones: estrogens, androgens, gestagens and corticosteroids are steroids which act as hormones. Although the use of this large group of compounds for animal fattening purposes has been described since the early 1950s, it has been prohibited in the European Union nowadays. Consequently, the increased public concern and the launch of legislative strategies within the framework of method validation and risk evaluation induced the need for the development of highly sensitive and specific analytical methods for the determination of steroid hormones in edible matrices.

Steroid hormones are a group of lipophilic, low-molecular weight, biologically active compounds that act as hormones. The parent compound from which all steroids are derived is cholesterol. As shown in Fig. 1, cholesterol consists of 3 hexagonal carbon rings and 1 pentagonal ring, generally arranged in a 6-6-6-5 fashion. Besides this cyclopentaperhydro[*a*]phenanthrene (also called gonane) skeleton steroid hormones can also have a skeleton derived there from, differing by one of more bond scissions or ring expansions or contractions. Two angular methyl groups are present at position C_{18} and C_{19}. Removal of a part of the side chain of cholesterol results in the C_{21} compounds, the gestagens and the corticosteroids, whereas the total removal of this side chain produces the C_{19} steroids, including the androgens. Additional removal of the 19-methyl group by aromatization, resulting in the conversion of the first hexagonal ring into a phenolic structure, yields the estranes, to which the estrogens belong. Steroids can vary by the functional groups attached to these ring structures or the oxidation state of the rings. Moreover, functional groups can be oriented either in the equatorial or axial position, resulting in a great number of stereoisomers which is very important for the biological activity. Besides that, the orientation of the hexagonal rings also affects the biological activity f.i. 17β-estradiol in comparison with 17α-estradiol (10 times less active regarding estrogen receptor).

According to their biological activity and pharmacological effects, steroid hormones can be divided into 2 important groups. At first, the sex steroids, a subset of hormones producing sex differences or supporting reproduction. They include the estrogens, gestagens and androgens. The second group, the corticosteroids, includes glucocorticosteroids and mineralocorticosteroids. Glucocorticosteroids regulate many aspects of metabolism and immune function, while mineralocorticosteroids regulate blood volume and electrolyte content. Steroid hormones can also be classified upon their endo- or exogenous origin. Steroid hormones biosynthetically present in the body are called endogenous hormones and are the chemical messengers from one cell (or group of cells) to another (f.i. estradiol). Xenobiotic or exogenous steroids are foreign compounds, naturally or synthetically produced (f.i. methyltestosterone, norethandrolone). Besides the classification of the steroid hormones upon their endo- or exogenous origin, they can also be classified by chemical structure and/or pharmacological effects. Using this, steroid hormones can be, in general, divided into three principal groups: estrogens, gestagens and androgens, or also called the EGAs.

Estrogens (alternate oestrogens or C_{18}-steroids), of which 17β-estradiol is the most active compound, are a group of steroid compounds, named for their importance in the estrous cycle. For this reason, they are called the female sex hormones. They are naturally occurring substances formed out of androgen precursors such as 4-androstenedione through the action of the enzyme aromatase in the ovaries, in the adipose tissue of the adrenal glands as well as in other organs. These C_{18} (estrane) steroids stimulate the development of female reproductive structures and secondary sexual characteristics. In combination with gestagens they influence the menstrual cycle. Estrogens are also effective in the management of menopausal disorders. Besides their function in the reproduction they also play an important role in the mineral-, fat-, sugar- and protein metabolisation. They also affect intestinal motility, blood coagulation, cholesterol metabolisation and sodium and water conservation by the kidneys.

Because of their anabolic effects, estrogens have been used in animal fattening. The endogenous estrogens (estrone, estradiol, estriol) and the hemi-synthetic analogues (f.i. estradiol-3-benzoate) are less oral active compared to the synthetic estrogens (f.i. ethinylestradiol, the synthetic counterpart of estradiol). Stilbenes (f.i. diethylstilbestrol, dienestrol, hexestrol) and zeranol are xenobiotic non-steroidal compounds imitating estrogenic effects by structural similarities with estradiol.

Pregnane (C_{21}-steroids) is the basic chemical structure for the gestagens, also called progestins or progestagens. Either they are of natural origin, or they are synthetic derivates of progesterone or 17-hydroxyprogesterone. These hormones produce effects similar to the endogenous progesterone, the only natural progestagen. This is the main hormone secreted by the *corpus luteum* in the ovary of cycling females, the testes, the adrenal glands and placenta. For animal fattening purposes, gestagens are frequently employed as esters (f.i. melengestrol-acetate) in hormonal contraceptive preparations, either alone or in combination with estrogens.

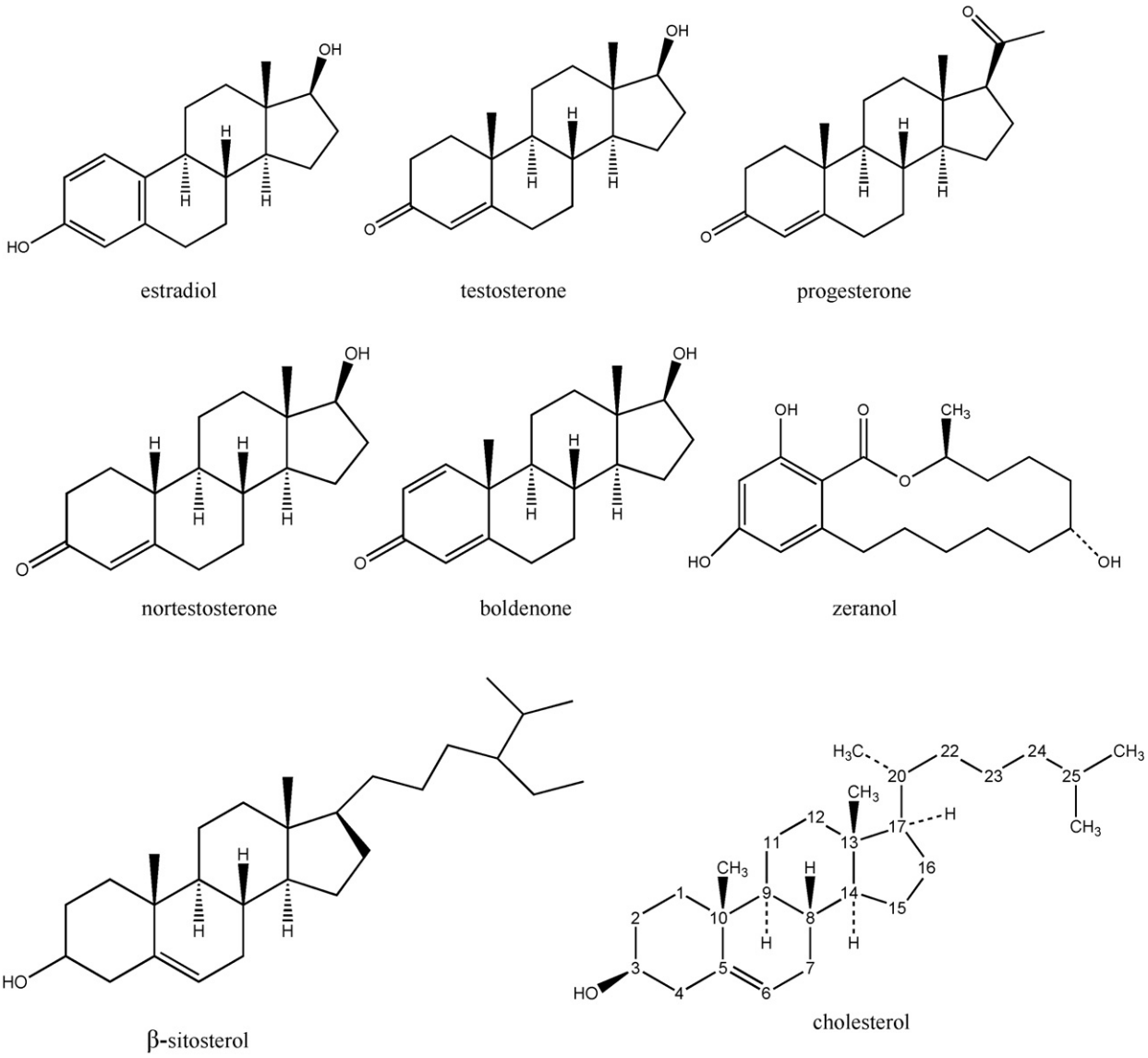

Fig. 1 – Examples of steroid hormones and related compounds (estradiol, testosterone, progesterone, nortestosterone, boldenone, zeranol, β-sitosterol) and their parent compound cholesterol.

Androgen (C_{19}-steroids) is the generic term for any natural or synthetic compound, usually a steroid hormone, which stimulates or controls the development and maintenance of masculine characteristics. Androgens, also called androgenic hormones, are the most often used anabolic steroids on the black market. The primary and most well known androgen is testosterone. Other important members of the group of the androgens are testosterone related compounds such as 4-androstenedione and (3α,5α)-3-hydroxyandrostan-17-one (androsterone) and the synthetic androgenic substances like 17α-methyltestosterone and testosterone esters [1].

Besides the endogenous corticosteroids (f.i. cortisol, cortisone) and those suspected to be endogenous (f.i. prednisone, prednisolone), there are the synthetic exogenous corticosteroids (f.i. dexamethasone, betamethasone), developed because of their anti-inflammatory properties.

Hemi-synthetic androgens (f.i. esterification of the 17-hydroxylgroup) are more active than the endogenous structures and their action is prolonged in time due to a slower release into the circulation compared to non-esterified steroids. Anabolic androgenic steroids exert two effects, an androgenic and an anabolic effect. Well-known examples of anabolic androgenic steroids are 19-nortestosterone (also known as nandrolone), 17α-methyltestosterone, boldenone and trenbolone. Besides these, also a lot of other analogues have been synthesized f.i. stanozolol, 4-chlortestosterone, norethandrolone and fluoxymesterone [1–3]. Finally, there are also the so-called 'designer drugs', all kind of new drugs regularly being introduced in the black market and on the Internet. In most cases, these substances are variations of 'old' structures. Well-known examples of 'designer drugs' with a steroid structure are norbolethone, tetrahydrogestrinone (THG) and desoxymethyltestosterone (DMT) [4].

An important group of steroid look-alikes are the phytosterols. These plant sterols are naturally occurring steroid alcohols and have a chemical structure which is similar to that of cholesterol. They are made up of a tetracyclic cyclopenta[α]phenanthrene ring and a long flexible side chain at the C_{17} carbon atom (Fig. 1.) [5–7]. More than 200 different types of phytosterols have been reported in plant species, the most abundant being β-sitosterol (24α-ethylcholesterol), campesterol (24α-methylcholesterol) and stigmasterol (22,24α-ethylcholesterol). In general, vegetable oils and products derived from oils are regarded as the richest natural sources of sterols, followed by cereal grains, nuts and vegetables [5–13].

Apart from the structural analogy of phytosterols to steroids, their transformation into natural steroids is suggested by several authors [14,15]. Microbial transformation of plant sterols into androsta-1,4-diene-3,17-dione (ADD), the precursor of boldenone and androst-4-ene-3,17-dione (AED), precursor of testosterone is frequently described [16–22]. Metabolisation of plant sterols has been demonstrated in higher organisms [23–25]. The physiological effects of phytosterols and mainly their cholesterol lowering properties have increased interest in their occurrence in food products and diets. Consequently, there is growing interest in their determination in foods, in which phytosterols can be either intrinsic or added. Since the bovine spongiform encephalopathy (BSE) and dioxin crisis, animal fat has been banned out of animal feed and is replaced by vegetable material. This implicated that, instead of cholesterol, phytosterols are the main kind of sterols in animal feed [26].

2. The use of steroid hormones in animal fattening

Steroid hormones are legally used in veterinary medicine under veterinary prescription by the law of 15 July 1985 concerning the use of substances with hormonal, anti-hormonal, beta-androgenic or stimulating function. Besides their use under regulated conditions, their use for growth promotion is forbidden. Nevertheless, synthetic hormone-like substances such as stanozolol, 17-methyl-testosterone, trenbolone, hexestrol, diethylstilbestrol, ethinylestradiol, nandrolone and others are still offered on the 'black' market for animal fattening purposes.

Steroid hormones are used in animal fattening because of their capacity to increase 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 ability to reduce nitrogen retention and to increase the water retention and fat content have been reported in literature. Also corticosteroids can be illegal used in animal fattening. This because it has been described that they may have a synergetic effect when combined with f.i. anabolic steroids or β-agonists [2,27–31].

Usually, steroid hormones are implanted in the animal's ear so that the active substance can be released over a long period of time in the bloodstream [32]. Additionally, illegal growth promoters can also be injected, resulting in injection sites in which high concentrations (mostly esters) can be found [33,34]. Also via feed, animals can be treated with EGAs [1,32]. In conclusion, it needs to be stressed that the improper use of both legal and illegal formulations of steroid hormones may lead to residues in edible matrices.

For several years now, the use of anabolic steroids in animal fattening is prohibited in the European Community because of their possible toxic effects on public health. Although toxicologists have declared that certain growth promoters are safe under conventional application conditions, these results are under discussion and sometimes scientifically controversial [35].

3. Legislation and regulation

The European Union issued many regulations concerning the use of certain substances having a hormonal action in livestock breeding. In this paragraph, the most relevant legislation concerning steroid hormones and edible matrices of animal origin is presented.

In 1981 (with Directive 81/602/EEC [36]), the EU prohibited the use of substances having a hormonal action (17β-estradiol, testosterone, progesterone, zeranol, trenbolone acetate and melegestrol acetate (MGA)) for growth promotion of farm animals.

Directive 88/146/EEC [37] was promulgated prohibiting the administration of both synthetic hormones (trenbolone acetate and zeranol) as well as the administration of natural hormones (estradiol, progesterone and testosterone) for growth promotion or fattening purposes. Trade in meat and meat products derived from animals treated with such substances for therapeutic or zootechnical purposes were regulated by 88/299/EEC [38]. Council Regulation 2377/90/EC [39] regulates the use of veterinary drugs by describing a procedure for the establishment of maximum residue limits (MRLs) for veterinary products in foodstuff of animal origin. These MRLs mean the maximum concentration of a residue of a substance, that may be present as the result of the use of this product and which may be accepted to be legally permitted or recognized as acceptable in food. For this reason, the occurrence of a wide range of steroid hormones has to be monitored. The revision of the Directives 81/602/EEC [36], 88/146/EEC [37], 88/299/EEC [38] and 2377/90/EC [39]; Council Directive 96/22/EC [40] stated that administration of substances with thyreostatic, estrogenic, androgenic and gestagenic action in husbandry and aquaculture is forbidden. Moreover, it stated that member states have to prohibit import of meat from treated animals from third countries. Council Directive 96/23/EC [41] regulates the residue control (monitoring and surveillance) of veterinary drugs, growth promoting agents and specific contaminants in live animals and animal products. This directive comprises the residue control of a large group of veterinary medicinal products for food-producing animals as well as for their primary products such as meat, eggs and honey. This Directive divides all residues into Group A compounds, which comprises prohibited substances (in conformity with 96/22/EC [40] and annex IV of 2377/90/EC [39]) and as such the steroid hormones. Group B comprises all authorized veterinary medicinal products in conformity with annex I and III of 2377/90/EC [39]. All this is implemented through surveil-

lance according to the National Plans of the individual Member States.

So far, for group A substances 'zero tolerance' levels had to be applied. For the compounds considered in this review, only one MRPL has been set by the EU, 1 μg kg^{-1} for medroxyprogesterone acetate (MPA) in kidney fat.

Council Directive 2003/74/EC [42] amended Council Directive 96/22/EC to reduce the circumstances under which 17β-estradiol may be administered under strict veterinary control for purposes other than growth promotion (treatment of foetus maceration/mummification, uterus disease of cattle for animal welfare reasons and estrus induction).

In order to ensure the harmonized implementation of Directive 96/23/EC [41], Directive 2002/657/EC [43] replaces the former Decisions 93/256/EEC [44] and 93/257/EEC [45] and regulates the implementation of the analytical methods and the interpretation of the results by giving performance criteria and instructions for the validation. Moreover, the European Criteria 2002/657 [43] establish common criteria for the interpretation of test results and introduces a procedure to progressively establish minimum required performance limits (MRPL) for analytical methods employed to detect substances for which no permitted limit (maximum limit) has been established. This is in particular important for compounds which use is not authorized or is specifically prohibited in the EU. Within the Commission decision EC/2002/657 [43], a system of identification points (IPs) is introduced in order to interpret the obtained data (chromatograms, spectra) when detection methods are used other that full-scan techniques. This system is based on the number and the ratio of the ions in the obtained MS spectrum. For the confirmation of the banned substances (96/23/EC, group A [41]) a minimum of four IPs is required [30,32,43,46,47]. Since the implementation of the 2002/657/EC [43] criteria, a number of studies describe the applicability of these guidelines for determination of steroid hormones in tissue, fat and milk. Parameters that need to be evaluated during the validation procedure are selectivity, specificity, linearity, trueness, recovery, applicability, ruggedness, stability, repeatability, reproducibility and decision (CCα) and detection (CCβ) limits.

4. Monitoring of steroid hormones in edible matrices

In recent years, there have been concerns about the presence of steroid hormones in edible matrices, covering a wide range of physical types of matrix, from muscle and organ (liver and kidney) tissue to fat and milk. Consequently, there was a need for continuous development of improved multi-residue, multi-matrix and multi-technique analytical methods. First of all, these methods must be sensitive enough to cover not only the legislative limits but also allow the determination of banned, forbidden or unknown compounds following misuse or unintentional use that may lead to residues in matrices of animal origin. Secondly, the determination of residues in matrices of animal origin requires the development of extraction and clean-up methods prior to detection. This is due to the matrix complexity and the low concentrations (ng up to μg kg^{-1}) that should be detected.

Urine, manure and hair are mostly used to monitor the illegal use of steroid hormones because they are available before slaughtering. After slaughtering, liver, kidney, fat or muscle tissues are collected. Consumable parts of the animal like liver, kidney and muscle tissue are the target tissues for residue analysis. Moreover, steroid hormones are lipophilic compounds and as such they may accumulate in fat; progestagens are known for this particular property. Commonly, kidney fat is taken at slaughterhouse level because it is the easiest matrix to take and thereby the lowest detrimental for the carcass [1,32].

Based on the available literature, with emphasis on multi-residue methods for steroids in meat, a number of analytical methods have been developed and are described [29,48–54]. Fewer methods are described for kidney fat [50], kidney [29], liver [29] and milk [29,55]. De Brabander et al. [30] has extensively reviewed the possibilities of mass spectrometry in the determination of residues of banned substances (amongst other things EGAs and cortisosteroids) in matrices of meat-producing animals.

4.1. *Sample extraction and/or purification*

Matrix complexity, the broad range of EGAs and related compounds and the often low levels (ng up to μg kg^{-1}) that should be measured, make residue analysis of animal matrices for steroid hormones a challenging task. In order to detect residue levels, sample pre-concentration is necessary but it will also lead to the concentration of potential interfering matrix contaminants. Moreover, the more intensive the extraction and clean-up procedures, the greater the potential for obtaining lower recoveries.

Conventionally, solid samples (f.i. muscle, fat, kidney and liver) are extracted with organic solvents based on liquid solid partition, normally preceded by grinding and/or freeze-drying and homogenizing, followed by a multi-step clean-up using liquid–liquid extraction (LLE) and/or solid phase extraction (SPE). Based on available literature, liquid solid extraction (LSE) is the commonly used extraction technique for steroid hormones, most of the time performed in the form of solid phase extraction [29,50,52,54,56], whereas few papers report lipid removal by freezing filtration [53] and HPLC-fractionation [49,57]. Alternatively, novel approaches for the extraction using accelerated solvent extraction (ASE) or supercritical fluid extraction (SFE) are developed [58,59].

Most of the methods described for edible matrices are based on the determination of free steroids involving hydrolysis using *Helix pomatia* juice, containing β-glucuronidase and aryl sulphatase [49,52,54] although the usefulness of this technique for edible tissues is controversial and is discussed since the portion of cleavable conjugated forms of steroid hormones in tissue are described to be very low [29]. Buisson et al. [60] discussed the possibilities of both enzymatic (with different sources of enzyme, f.i. *H. pomatia*, *Escherichia coli*) and chemical (solvolysis) for the sulpho-conjugated steroid hormones. Hartmann and Steinhart [61] reported the possibility of production of a distortion of the hormone patterns due to enzymatic hydrolysis, f.i. the conversion of pregnenolone to progesterone, caused by side activities of *H. pomatia*.

Table 1 – Multi-residue methods for the determination of steroid hormones in edible matrices using gas chromatography (GC) (LSE, liquid solid extraction; SPE, solid phase extraction; EI, electron impact; QqQ, quadrupol; IT, ion trap; MS, mass spectrometry; SIM, selected ion monitoring)

Matrix	Sample preparation	GC	Derivatization	Limits ($\mu g\,kg^{-1}$)	Reference
Meat	LSE C_8 SPE Si-NH_2 SPE	GC–EI-QqQ-MS (SIM)	MSTFA + TMIS	0.02–0.1	[48]
Meat	LSE HPLC-fractionation	GC–EI-QqQ-MS (SIM)	HFBAA MSTFA + DTE	0.1–4.6	[49]
Liver	Lyphilization + LSE	HRGC–QqQ-MS	MSTFA + I_2	5–100	[51,52]
Meat	Envi-ChromP SPE Si-NH_2 SPE		MSTFA + TMIS + DTE MSTFA MTBSTFA + TBDMSCl		
Kidney fat Meat	LSE Si-NH_2 SPE	GC–EI-IT-MS^n	$MSTFA^{++}$	0.5–5	[50]
Kidney fat	Melting + LSE CN SPE	GC–EI-IT-MS^n	$MSTFA^{++}$	1–6	[65]
Meat	LSE C_{18} SPE	GC–EI-IT-MS^n	MSTFA + TMIS + DTE	0.1–0.4	[56]
Meat	LSE Freezing lipid filtration C_8 SPE Si-NH_2 SPE	GC–QqQ-MS (SIM)	MSTFA + NH_4I + DTE	0.1–0.4	[53]

In nearly all studies described, methanol was used for extraction of steroid hormones out of tissue samples [29,48,50,52,53], however, Fuh et al. [56] and Blasco et al. [54] stated that acetonitrile gives cleaner extracts. Daeseleire et al. [49] performed extraction with diethylether.

As can be noticed in Tables 1 and 2, different sorbents, like C_8, Envi-ChromP, Si-NH_2, C_{18} and Oasis HLB were used for SPE and/or purification. Clean up of the primary extract adds a considerable value to the specificity of the method because it results in several purified fractions, each containing a limited number of target and matrix compounds. Each fraction may be analysed with a specific technique f.i. GC–MS-MS or even a combination of different techniques. By using specific solvents for SPE, different fractions of steroid hormones can be separated [52,62]. Automated clean-up procedures, like high

Table 2 – Multi-residue methods for the determination of steroid hormones in edible matrices using liquid chromatography (LC) (ASE, accelerated solvent extraction; LSE, liquid solid extraction; SPE, solid phase extraction; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; QqQ, quadrupol; IT, ion trap; MS, mass spectrometry; MeOH, methanol; ACN, acetonitrile)

Matrix	Sample preparation	LC	Mobile phase	CCβ or LOQ ($\mu g\,kg^{-1}$)	Reference
Kidney fat	SFE	LC–APCI(+)-IT-MS^n	MeOH:H_2O (50:50) MeOH MeOH:Ethanol (50:50)	≤0.5	[105]
Kidney fat	ASE C_{18} SPE	LC–ESI(+)-QqQ-MS^n	0.5% Formic acid in H_2O/ACN (9:1) 0.5% Formic acid in H_2O/ACN (1:9)	<2	[59]
Meat	Enzymic digestion	LC–ESI(+)-QqQ-MS^n	MeOH (androg) or ACN (estrog)	Porcine meat: 0.007–0.3	[29]
Kidney Liver	LSE HLB SPE		H_2O	Chicken meat: 0.003–0.06 Liver: 0.05–0.3	
Milk	Si SPE NH_2 SPE			Kidney: 0.003–0.3 Milk: 0.004–0.15	
Meat	Enzymic digestion	LC–ESI (+/−)-QqQ-MS^n	(+) MeOH:H_2O (65:35) + 0.3% formic acid (−) MeOH:H_2O (80:20) + 0.5% ammonia	<0.5	[54]

performance liquid chromatography (HPLC)-fractionation, were applied for the determination of steroid hormones in kidney fat [57] and in meat [49]. Both were based on separation on a C_{18} column using a methanol:water gradient.

To avoid lipids that are usually co-extracted with steroid hormones from matrices of animal origin, a small number of methods are described. Seo et al. [53] developed a novel approach based on the solubility of steroid hormones in organic solvents. To extract samples, a methanol–water solvent extraction was used, followed by lipid removal by freezing filtration. The latter is based on the difference in freezing points between lipids (below about 40 °C) and growth hormones (127–282 °C) in methanol (−98 °C) which makes separation of the lipids easy by centrifugation at below 4 °C in cold methanol solution. C_8 SPE was used for sample purification. This technique replaces former techniques for lipid removal like liquid–liquid partition of the lipids in the obtained extracts using hexane or cyclohexane [63,64]. Downscaling the sample size combined with hexane extraction and saponification is also described to minimize lipid interferences [65] (see Fig. 2).

Finally, it needs to be pointed that it is surprising that only few papers have published analytical methods for the determination of steroid hormones in milk within the framework of residue analysis. Besides that, although the online coupling of automated solid phase extraction and chromatographic systems are described frequently for environmental analysis [66,67], no approaches are described so far for the determination of steroid hormones in matrices of animal origin.

4.2. *Advanced extraction and purification techniques*

Former methods usually are time consuming, demanding a lot of organic reagents and are labour-intensive. Moreover, these methods are used for the determination of a single or a small group of steroids. In this section, the possibilities of novel extraction and clean-up techniques are highlighted.

Many applications of Soxhlet extraction are described for some groups of steroid hormones in food analysis and environmental chemistry (f.i. in soils, sediments and suspended solids). It is based on the repeated percolation of organic solvent (usually hexane or petroleum ether) to extract solid samples for certain lipophilic target compounds or to remove oil and fat from solid material. Soxtec® extraction is automated Soxhlet extraction, reducing extraction time and boiling, rinsing and solvent recovery is performed automatically. So far, no applications in the residue analysis of steroid hormones in edible matrices are described.

Accelerated solvent extraction, also called pressurized liquid extraction (PLE), extracts solid samples under high pressure and at high temperatures. It is a technique with a lot of applications in the environmental chemistry and food analysis. The most important advantages are the reduction in solvent use and the speed-up of the extraction process when a large number of samples need to be analyzed. Moreover, Soxhlet methods can be easily converted to PLE using the same extraction solvent. Based on literature, this technique is utilized in environmental chemistry, f.i. for extraction of estrogens from sediments and suspended solids [68]. So far, not many applications of ASE in the field of steroid hor-

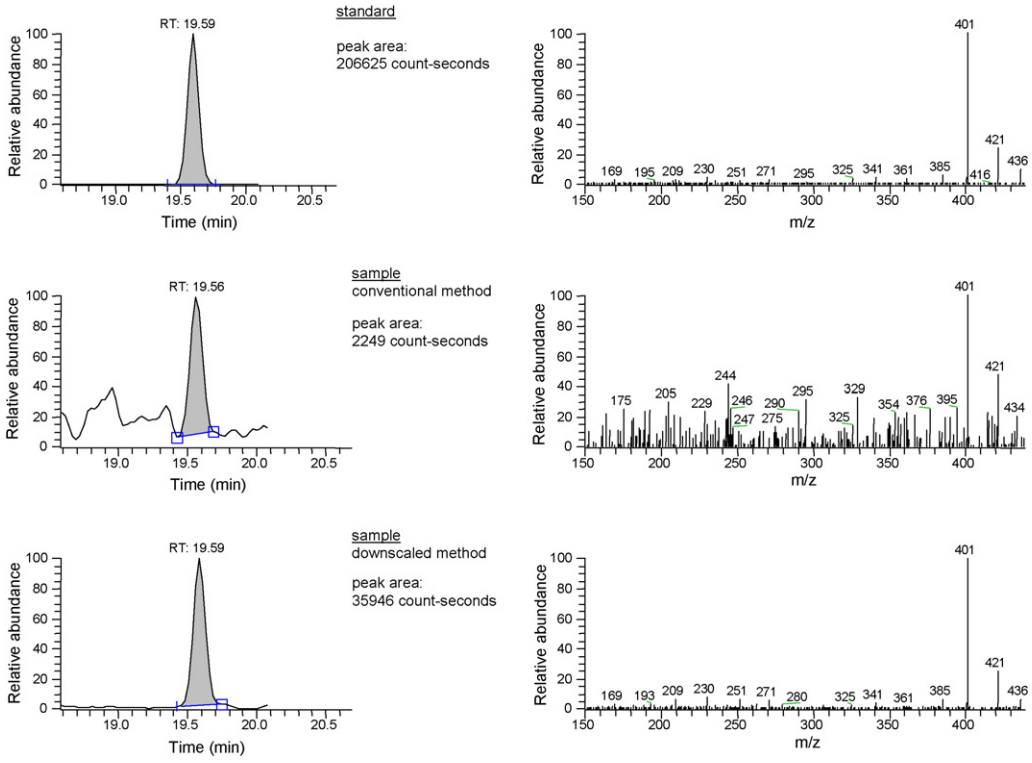

Fig. 2 – Effect of downscaling of the sampling size to the detection of ClTA (ChloroTestosterone-Acetate) using fortified samples at 5 μg kg^{-1} (adapted from ref. [65]).

mones and residue analysis are published, although this seems a very promising technique. Hooijerink et al. [59] developed an analytical approach for screening of 6 gestagens (flurogestone acetate, delmadinone acetate, megestrol acetate, chloromadinone acetate, melengestrol acetate and medroxyprogesterone acetate) in kidney fat using ASE. For this application, ASE vessels were filled with alumina, anhydrous sodium and melted kidney fat. In the ASE apparatus, samples were defatted with hexane before the gestagens were trapped on the alumina. Finally, the alumina was online extracted with acetonitrile followed by freezing of the extract to precipitate the remaining fat. This extract was purified with C_{18} SPE. As can be noticed, PLE offers a lot of possibilities important during extraction like, addition of modifiers to the extraction solvent, filling the extraction cells with sorbents with specific properties or repeated PLE with different solvents to separate groups of compounds. Moreover, to allay concerns about possible cross-contamination between samples, automatic rinsing steps can be programmed between samples.

Supercritical fluid extraction also may resemble Soxhlet or Soxtec® extraction except that the used solvent is a supercritical fluid, which is a substance above its critical temperature and pressure. As such, SFE may be regarded as an alternative for organic solvent extraction methods in general. The main advantages are the good solvating power, the high diffusivity, the low viscosity and the minimal surface tension. Additional possibilities are manipulating the pressure and/or temperature or the use of modifiers into the obtained fluid which changes the solvating power of the supercritical fluid. Carbon dioxide (CO_2) is the most frequently used supercritical fluid [58,69]. Few papers described the use of SFE for residue analysis of steroid hormones. Din et al. [70] used this technique for the extraction of trenbolone from beef and Huopalahti and Henion [71] applied it for the extraction of seven estrogenic and anabolic agents from bovine tissues. Stolker et al. [58] developed a multi-analyte (megestrol acetate, medroxyprogesterone acetate, chlormadinone acetate, melengestrol acetate), multi-matrix (skin, meat and fat) method for the routine determination of steroids in animal tissues coupling SFE to SPE. Kurečková et al. [69] concluded that SFE is suitable as a sample preparation technique for monitoring trace levels of corticosteroids in animal tissue. As reviewed by Ridgway et al. [72] one of the main problems with SFE is the robustness of the method compared to other extraction techniques. Moreover, additional clean-up of the extracts is needed. It needs also to be pointed that available automated systems are mainly aimed at the environmental area, rather than the trace analysis in f.i. foods.

Solid phase microextraction (SPME) is a process allowing analytes to be adsorbed onto the surface of a small fused-silica fiber coated with suitable polymeric phase, placed in a syringe-like protective holder. Subsequently, analytes are desorbed into a suitable apparatus for separation and determination. This technique is based on the distribution of analytes between an extraction phase (polymer) and the matrix [69]. As reviewed by Ridgway et al. [72] the main advantages of SPME compared to solvent extraction are the reduction in solvent use, the combination of sampling and extraction and the ability to examine smaller sample sizes. Kurečková et al. [69] showed the great potential of SPME for the isolation of corticosteroids from water and urine. So far, no applications for f.i. milk or extracts from solid matrices of animal origin are available for the steroid hormones.

Microwave-assisted extraction (MAE) agitates and heats the sample during extraction which augments the extraction efficiency for solid samples. It can replace former techniques such as Soxhlet extraction because extraction times are shorter and less solvent is needed. However, additional extraction and/or purification steps are needed. This technique is used a lot for extraction of environmental samples for a variety of compounds such as estrogens, herbicides, phenols, polycyclic aromatic hydrocarbons and polychlorinated biphenyls in environmental matrices as described by Liu et al. [73], but so far this technique has no applications for steroid hormones in edible matrices. Hermo et al. [74] used this technique for the extraction of quinolone residues in pig muscle.

The combination of molecularly imprinted polymers (MIPs) and solid phase extraction is reviewed by Qiao et al. [75]. Molecular imprinting is a rapidly developing technique for the preparation of polymers having specific recognition properties. During the last few years, MIPs have appeared as new selective sorbents for SPE of organic compounds in complex matrices like herbicides and drugs, which can be selectively extracted from samples such as beef-liver extract, blood serum and urine. Although MIPs is a promising technique which allows specific analytes to be selectively extracted from complex matrices, applications in the residue analysis for steroids in edible matrices are, based on current literature, not existing. However, Dong et al. [76] developed a MIP polymer with specific affinity towards β-estradiol, most applications are described for pesticides (f.i. triazine herbicides), and not for multi-residue methods for steroid hormones in edible matrices. Besides that, nearly all described studies using MIPs are methods for the extraction of small groups of compounds [75]. On the other hand, Van Hoof et al. [77] developed a multi-residue method for the determination of β-agonists in urine using MIPs. In general, it can be concluded that MIPs is a very promising technique, but so far, there are still some features that need to be investigated for the application in the clean-up of f.i. extracts of edible matrices for the detection of steroid hormones. Based on the available literature, no applications were described so far for other selective sorbents, like Restricted Access Media (RAM) or immunosorbents for the determination of steroid hormones in edible matrices.

A novel particular effective approach for sample fractionation is size exclusion chromatography (SEC), which is a chromatographic technique in which particles are separated based on their size, or in more technical terms, their hydronamic volume. When an organic solvent is used to transport the sample through the column, the name gel permeation chromatography (GPC) is used. Because this is a quite new sample purification technique, few applications are described for steroids. Houtman et al. [78], for example, described the use of GPC after extraction of sediment samples with ASE or Soxhlet extraction for a certain group of estrogens that are considered to act as endocrine disruptors.

4.3. *Methods for steroid hormone detection*

Over the last years, the determination of steroid hormones in edible matrices has been dominated by chromatographic separation methods (GC or LC) coupled to sensitive and specific detection systems such as MS. Generally, GC–MS or GC–MS-MS has been the most employed technique. In recent years, liquid chromatography coupled to MS has gained in popularity. These chromatographic devices replace the screening assays using immunochemical based methods and thin layer chromatography.

In the past, immunological techniques like radio immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) were used for screening of a limited number of steroid hormones depending on the antibodies used. Nowadays, multi-residue screening methods are developed based on immunochemical techniques. Scippo et al. [79] developed a multi-analyte detection assay for the detection of compounds with estrogenic, gestagenic, androgenic or glucocorticoidic activity using recombinant receptors. This technique allowed the detection of steroid hormones at the action limit of $2\,\mu g\,kg^{-1}$. Based on recent literature, there are a lot of publications on the application of immuno-assays for the detection of steroid hormones in urine [80–84] but routine application for steroid hormones in edible matrices of animal origin is, based on available literature, to date sparse. It also has to be stressed that binding assays represent potential screening methods but need to be confirmed by chromatographic separation methods such as f.i. gas or liquid chromatography coupled to f.i. mass spectrometric detection. In the case of natural hormones, background concentrations of these compounds have to be taken into account [79]. As the list of steroid hormones in animal fattening is ever-changing and the analytical requirement, including validation procedures, are increasingly stringent, specific immunoassays [80,85], which allow the determination of a single compound or a small group of steroid hormones are now being replaced by multi-residue screening methods using chromatographic separation [52].

In 1979, high performance thin layer chromatography (HPTLC) with fluorescence detection was introduced for the multi-residue screening and confirmation of EGAs at the $\mu g\,kg^{-1}$ (ppb) level in animal tissue [86]. With this method most EGAs could be detected at the 0.5 up to $10\,\mu g\,kg^{-1}$ level. Later on, this method was modified to be used in the routine analysis and to be applied for the regulatory control in Belgium [87–91]. This method was based on fluorescence induction by reaction of the steroids with sulphuric acid. In combination with HPLC fractionation, reduction of the matrix components improved the interpretation of the TLC plates considerably [57]. De Brabander et al. [90] combined HPTLC and confirmation with GC–MS for the detection of anabolic residues in kidney fat within the concentration range of 2 ppb. Batjoens et al. [92] used HPTLC screening in the 4×4 mode (4 samples and 4 standard mixtures are developed simultaneously on one TLC plate [87]) in combination with confirmation with GC–MS for the identification of anabolic compounds in injection sites. Today, the former technique is used less because of its low sensitivity and the difficulties encountered in satisfying the quality control criteria required. It is replaced by gas chromatography (GC) or liquid chromatography (LC) resulting in a higher selectivity and specificity. Only elucidation of the identity of 'difficult molecules', such as f.i. α- or β-trenbolone, HPTLC may still be useful.

When resuming the different multi-residue approaches that are described in literature for the separation and detection of steroid hormones in edible matrices, gas chromatography coupled to single or multiple mass spectrometry (MS(-MS)) is the common used detection technique (Table 1) [48–52,53,56,65]. Due to the poor thermal stability and volatility of steroid hormones, derivatization is required. In literature, different derivatization reagents in combination with catalysers are described for derivatization of steroid hormones which contain hydroxyl- and/or keto-groups. In nearly all described cases, the trimethylsilylation derivatization was applied with MSTFA (*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide) as derivatization reagent. Daeseleire et al. [49] used heptafluorobutyric acid anhydride (HFBAA) derivatization, but MSTFA for confirmation. Alternaively, MSTFA^{++} (also called MSTFA/TMIS) can be applied to obtain suitable extracts for GC–MS-(MS) analysis for anabolic compounds [50,51,93]. This derivatization reagent contains MSTFA, ammonium iodide (NH_4I) as catalyst and ethanethiol as antioxidant. On the other hand, also dithioerythritol (DTE) was described as antioxidant. Seo et al. [53] used a derivatization mixture containing MSTFA/NH_4I and DTE. This was also used by Hartmann and Steinhart [48] and Fuh et al. [56], however, NH_4I was replaced by trimethylsilytrifluoroacetamide (TMIS), which reacts as catalyst. Besides that, the formation of additional unexpected derivatives or by-products (artifacts) following derivatization of steroid hormones was discussed [94–96].

For separation of steroid hormones, most often capillary columns containing a 5% phenyl liquid phase (phenyl polysilphenylene-siloxane or phenyl methylpolysiloxane) are used [48–50,56,65]. Seo et al. [53] utilized a capillary column with an 1% dimethylpolysiloxane liquid phase. Alternatively, Bizec et al. [51] and Marchand et al. [52] applied 2 different columns for different groups of steroid hormones, one fused silica or polysiloxane polymer column respectively for all steroids except trenbolone and one column with a 100% dimethylsiloxane liquid phase for trenbolone. As can be seen in Table 1, nearly in all cases detection was performed by Electron Impact ionization followed by ion trap (IT) or quadrupole (QqQ) mass spectrometry (MS).

Since the introduction of the newer API interfaces, such as electrospray (ESI) and atmospheric pressure chemical ionization (APCI) and since the possibility to couple LC to ion trap or quadrupole MS, more and more applications for the determination of steroid hormones are described for LC. Today, high performance liquid chromatography, coupled to a wide variety of detection systems has gained in popularity for residue analysis due to its high selectivity, specificity and sensitivity. The most important reason is that there is no need for derivatization. Moreover, it is known that the lack of a universal derivatization reagent for the large group of compounds (f.i. trenbolone) that needs to be considered strongly stimulated the development of methods based on liquid chromatography. However, many LC–MS methods have been developed to measure steroid hormones and hormone-like substances in environmental samples [97–102] or urine [103,104] the application to edible matrices of animal origin are rather

limited [29,54,59,105] compared to the number of detection methods based on gas chromatographic separation and mass spectrometric detection. Liquid chromatography is the preferred technique for trenbolone-like steroids (thermolabile), for stanozolol (strong adsorption in GC due to the pyrazole ring), for steroid conjugates (high polarity) and progestagens (strong non-polarity) [30]. As can be seen in Table 2, both ESI and APCI are applied for steroid hormones. Mass analyzers are mainly ion trap or quadrupole technologies. Single MS is today replaced by multi-dimensional techniques.

4.4. *Advanced techniques*

Liquid chromatography coupled to different MS techniques (f.i. ion trap or time of flight MS) makes it possible to screen for 'novel unknown' residues. By accelerating ions with a known electrical charge and unknown mass using an electrical field of known strength results in a separation of ions based on the time it takes to reach the detector. As such, from this time and the known experimental parameters, one can find the mass-to-charge-ratio of the particle. Touber et al. [106] developed a multi-detection method using ultra performance liquid chromatography (UPLC) coupled to time of flight (ToF) MS for the determination of 40 corticosteroids and β-agonists in calf urine. The good selectivity in complex sample matrices is provided by the ToF-MS characterized by high resolution and accurate mass capabilities. The extra resolution provided by the UPLC system reduces the risk of non-detection of potentially important co-eluting analytes. Time of flight-MS also seems very promising in the case of edible matrices of animal origin; however, based on current evidence, no applications for the screening of steroid hormones in edible matrices of animal origin exist up to date. Alternatively, highly sophisticated ion trap-based accurate mass Fourier transform mass spectrometry (FTMS), such as ion cyclotron resonance (ICR) or Orbitrap MS, might be applied for the identification of known and unknown steroid hormones. However, criteria like EC/2002/657 [43] are missing for this application, it is expected that accurate mass LC–ToF-MS screening of target residues and accurate mass confirmation of known and identification of unknown residues will expand the coming years [107].

5. Detection of natural hormones

5.1. *Natural steroid hormones*

In illegal preparations used for growth promotion the natural hormones have become popular ingredients [2]. Detection of misuse of natural hormones is problematic since these steroid hormones are naturally synthesized by food producing animals. Besides that, the concentration of naturally occurring steroids in food products of animal origin depends on the type of animal product, the species and its gender, the feed, castration, gestation, disease, age, medication and physiological condition [35,62]. In general, tissue from adult bovine cattle can reach higher testosterone and progesterone concentrations than calves, where the latter may show comparatively high amounts of estrogens (except in comparison with pregnant adults). The hormone patters of male and female cattle differ with heifers showing higher levels of progesterone but lower levels of testosterone than male animals. Natural hormone levels in cattle liver resemble those in muscle tissue, whereas fatty tissue accumulates lipophilic hormones. In pig tissue, a similar steroid pattern as in ruminants was observed, with a predominance of the metabolic intermediates and lower concentrations of hormonally active steroids. In contrast to cattle, no accumulation of hormones in fat was found. Between gilts (female pigs) and barrows (castrated males) no remarkable differences were found. Reports about the contents of steroid hormones in poultry are rare Hartmann et al. As reviewed by Hartmann et al. [62] concentrations of natural hormones in milk depend on the fat content and as such correlated to the concentration level of progesterone.

Above this, the occurrence of unchanged steroid hormones is higher in plasma and tissues from non-excretory organs in comparison with f.i. urine and faeces (f.i. conjugates and sulphates).

As such, one of the most challenging tasks for the analyst in the field of chemical residue in food is currently to draw the distinction between residues resulting from an endogenous production and as a consequence of an exogenous administration. The conventional mass spectrometric approaches permit quantitative assessment of hormone levels in biological matrices, but because of intra- and inter-individual physiological variability, this approach can only be used for screening purposes. Traditionally, there have been 2 approaches for the diction of the abuse of endogenous steroids: (1) the measurement of the absolute concentration of the steroid or its metabolite(s) or (2) the determination of the ratio between the amount of the analyte and a second steroid which is not a metabolite of the first. However, none of these approaches is capable of discriminating directly between endogenous and illegally administrated steroids [2,18].

Definitive proofs can be given by advanced techniques based on $^{13}C/^{12}C$ determination of steroid metabolites in cattle biological fluids and tissues. Indeed, steroid carbon isotopic composition depends strongly on its origin. When endogenously produced by the organism, estrogens and androgens derive from cholesterol so that their $^{13}C/^{12}C$ is directly dependant on the animal diet. Expressed by reference to an international reference [Vienna Pee Dee Belemnite (V-PDB) or Pee Dee Belemnite (PDB)], endogenous isotopic deviations ($\delta_{VPDB}‰$) in cattle are in the range −15 to −26‰ (corrected values), depending on the feeding (from maize to hay, respectively).

Synthetic steroid sources (f.i. estradiol, testosterone or progesterone and/or esters) are characterized by more depleted values contained in between −28 and −34‰. Gas chromatography–combustion/isotope ratio mass spectrometry (GC–C/IRMS) is the technique of choice for the measurement of such low isotopic differences. Whereas 17α-estradiol is the main metabolite allowing to detect 17β-estradiol misuse in ruminants, etiochalonolone and 5α-androstane-3β,17α-diol (AAdiol) are the main indicators of testosterone administration in bovine. In both cases, dehydroepiandrosterone (DHEA) and 5-androstene-3β,17α-diol (AEdiol) are used as endogenous reference compounds (ERC).

Demonstration of the usefulness of the technique has already been done for steroid residues measurement mainly for urine samples [60,108–112]. The sensitivity of instruments is often reported as one of the main limitations of this technique; indeed, 10 ng steroids are necessary to fulfil the minimum analytical criteria (with the linearity range, i.e. >1 nA) and to be confident into the measured isotopic deviation value. This characteristic made theoretically impossible the measurement of steroids in edible tissues as the concentrations of these biological compounds remain extremely low, i.e. often below the $ng\,g^{-1}$. When considering realistic sample size (f.i. 20 g muscle), the limit of detection in matrix is limited to $100\,ng\,g^{-1}$ when considering a global recovery yield of target analytes (10–20% due to the multiple steps of the analytical process) and the incomplete injection of the extracted sample in the GC (1/5th at best). It means that the only feasible control in edible tissue is summarised to injection sites or samples in the neighbourhood. An exam-

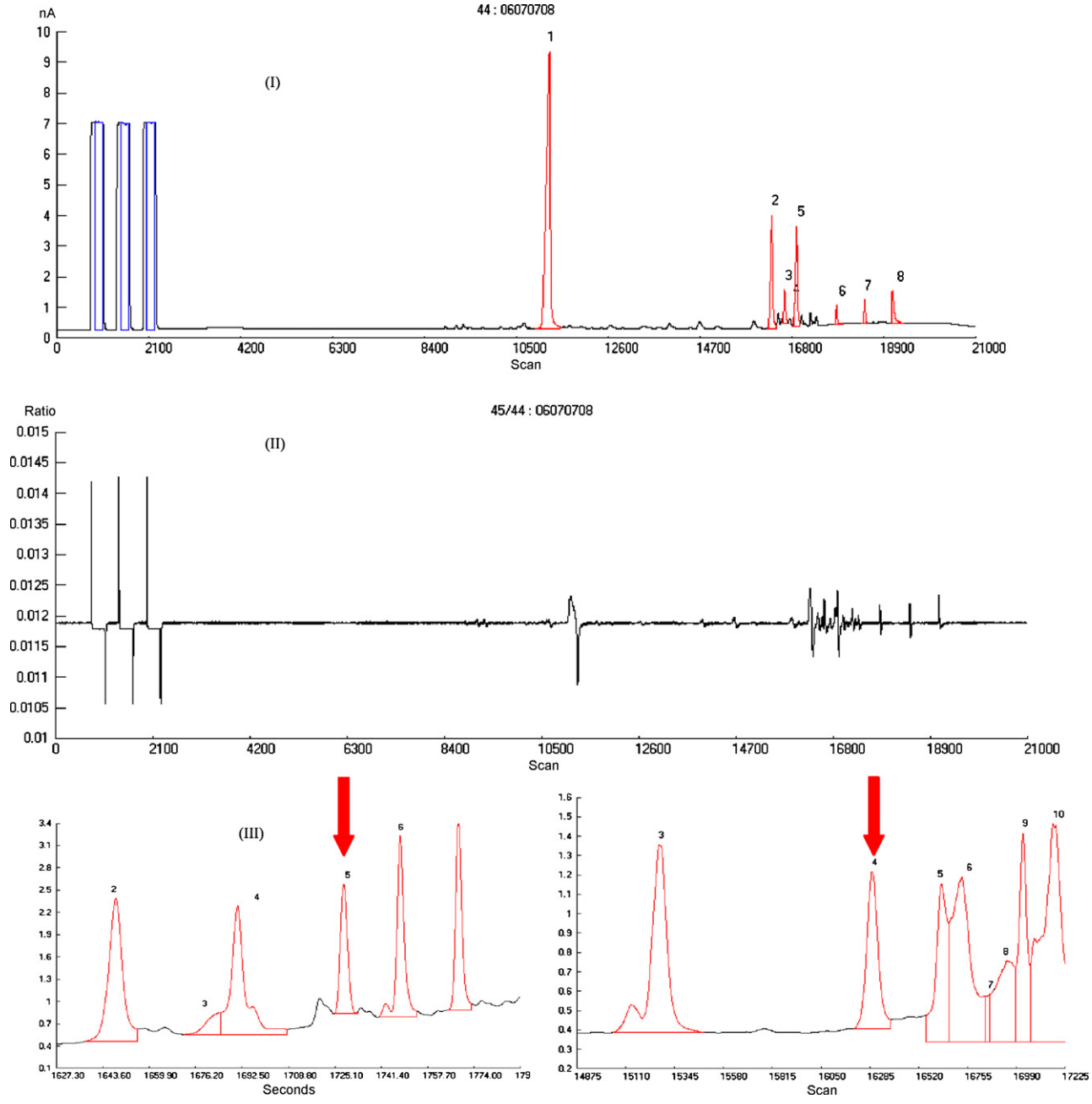

Fig. 3 – GC–C/IRMS chromatograms ((I) *m/z* 44 and (II) 45/44 ratio) of acetylated 5-androstene-3β,17α-diol (peak no. 2, Endogenous Reference Compound, uncorrected values −26.02‰), and 5β-androstane-3,17-dione (peak no. 1, Internal Standard, uncorrected values −33.18‰). Left of the chromatogram (I), three pulses of CO_2 to check the stability of the system, ratio 45/44 = 1.177E−02) is given in (II). (III) GC–C/IRMS chromatograms (*m/z* 44) of a muscle extract (sample size 400 g, acetylation). 17β-Estradiol is peak no. 5 on the left chromatogram; 17β-testosterone is peak no. 4 on the right chromatogram.

ple is given on Fig. 3; 17β-estradiol and 17β-testosterone were detected in muscle (sample size 400 g). In all analysed samples, the method permitted GC–C/IRMS measurements of the low concentration (2 ppb) of added internal standard (MT-d_3). The purity of the analyte after clean up was controlled by GC–MS and compared to the pure standard by full scan acquisition: the contamination factor was proved to be 0% with 99% peak quality. The low spiking level (2 ppb) required a drastic reduction of the final volume (no more than 10 μL hexane) before GC–C/IRMS injection. An increased injection volume of 3 μL was found necessary to increase the signal intensity above 1 nA into the linearity range of the instrument. GC–C/IRMS trace modus (mass to charge ratio of 44) proved the efficiency of the method: the signal intensity of 2 ppb was above 1 nA without interferences at the expected retention time of the analyte. The δ^{13}C-values of the MT-d_3 in the sample extracts ($-30.52 \pm 0.83‰$, $n = 16$, each sample injected in duplicate) and reference standards ($-30.60 \pm 0.36‰$, $n = 19$) were compared and agreed well. These results show a promising repeatability between two consecutive injections and an acceptable standard deviation while the signal intensity does not exceed 2 nA. The comparison of the two $^{13}C/^{12}C$ averages shows no isotopic difference after extraction. Eight muscle samples were extracted before GC–C/IRMS characterisation; the target analyte 17β-estradiol could not be integrated correctly in most of the muscle samples, only one value could be measured with a good level of confidence (Fig. 3). For 17β-testosterone, the clear separation of the analyte from co-eluting substances and a sufficient peak height (>1 nA) allowed measurements in three samples. The samples from hormone treated males fed with maize showed depleted δ^{13}C-values (-28 to $-30‰$) for 17β-testosterone compared to untreated male ($-15.5‰$). They all agreed well with corresponding analytes in urine samples for maize fed animals (average etiocholanolone in untreated male measured at $-15.7‰$, and treated animals $-25.1‰$).

As a drawback of this technique, it is worth mentioning that, although routine methods exist for urine and hair [60,112], so far no routine methods using stable carbon isotope analysis are described for edible matrices of animal origin based on currently available literature. Moreover, no criteria for analytical methods using stable isotope analysis are described and included in the EC/2002/657 [43], the European criteria that establish criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories.

5.2. *Phytosterols*

It must be pointed out that, to date, no official reference methods for the determination of phytosterols in sterol-enriched food products are acknowledged [6,8]. Some international reference methods exist for the determination of sterol fractions of fats and oils, such as ISO 6799, IUPAC methods 2.401 and 2.403, ISO 12228 and AOCS Ch 6-91. Also, Codex Stan 210 refers to ISO 6799 and IUPAC 2.403 methods [8]. In complex food matrices phytosterols may occur as free sterols, steryl esters, steryl glycosides and acylated steryl glycosides [6]. For this reason, reliable analytical methods for their extraction, saponification, clean up and detection are required. A comprehensive review of detection methods described for the determination of sterols in food products was carried out by Abidi [7] and Lagarda et al. [8].

Typically, determination of phytosterols involves extraction of the lipid fraction, followed by saponification and extraction of the unsaponifiable matter, clean up of the extract by thin layer chromatography (TLC) or solid phase extraction, the formation of sterols derivatives and their detection by capillary gas chromatography [6,7,113]. The nature of the sample source largely determines the most appropriate extraction technique. Sterols can be isolated from plant tissues or oilseeds by solvent extraction, supercritical fluid extraction or supercritical fluid fractionation (SFF) [7]. In cereal products, determination of phytosterols was performed by lipid extraction with toluene and an additional acid hydrolysis, alkaline saponification or a combination of the latter two [8]. Extraction of lipids from vegetables using surpercritical carbon dioxide, followed by isolation of the sterols and saponification or SFF was reported by Lagarda et al. [8] and Lu et al. [114]. Since isolation of the unsaponifiable material by solvent extraction followed by evaporation of the solvent to dryness, is characterized by some disadvantages, the use of solid phase extraction is preferred [8]. A SPE method using neutral alumina cartridges was applied for the extraction of free and esterified sterols from oils and fats [8]. SPE has proved to hold a proper purification during sample preparation and has proved to be a viable alternative for chromatographic purification techniques for sterol analysis of vegetable oil [6–8]. For biological samples, solid phase microextraction has become a widely used extraction and purification method [8]. Nevertheless, a wide variety of chromatographic techniques including column chromatography (CC), gas chromatography, TLC, normal phase HPLC, reversed phase HPLC, capillary electrochromatography (CEC) and supercritical fluid chromatography (SFC) are reported in literature for purification and separation of sterols [6–8]. Although HPLC methods offer a non-destructive alternative to GC methods, the latter remain the most frequently used techniques for the determination of sterols [6,7]. In a typical sterol analysis GC is coupled to flame ionisation detection (FID) or to MS, when focusing on structural identification and quantification by selected ion monitoring (SIM) or multiple ion monitoring (MIM) [6,8]. Other detection methods than FID and MS include UV detection, photodiode array detection (DAD), refractive index (RI) detection, evaporative light scattering detection (ELSD), infrared detection (IR) and nuclear magnetic resonance (NMR) detection [7,8,11,115,116]. A comprehensive review on this topic was carried out by Abidi [7].

Early data on sterol analysis of food products were mainly obtained using enzymatic and spectrophotometric methods. Those methods though struggled with matrix interferences and a lack of specificity [8]. Recently, liquid chromatography coupled to MS with atmospheric pressure chemical ionization was also effectively applied for the determination of sterols in different sample matrices [114–116]. For lipophilic compounds, EI ionization in particular and the recently developed atmospheric pressure photoionization (API) technique are more sensitive and effective alternatives. Furthermore, Lu et al. [114] recently developed a sensitive, selective and reliable UPLC APCI MS method, using SIM mode, for the individual determination of diversiform sterols in food matrices. In addi-

tion, a sensitive LC method with fluorescence detection was found to be effective for the simultaneous determination of cholesterol and sitosterol in food products and other biological samples [12]. Nonetheless, GC–MS with electron impact (EI) ionization of trimethylsilyl ether or acetate derivatives of sterols remains the method of choice for sterol analysis [6]. In most laboratories, packed columns have nowadays been replaced by capillary columns, since the former could not offer effective separation as needed in plant sterol analysis [6].

6. Detection of hormones with a hemi-endogenous status

Besides the endogenous hormones (f.i. estradiol, testosterone, progesterone) there are also synthetic substances that have achieved a hemi-endogenous status. The most famous hormones from which their 'dual' nature is discussed are nandrolone (also called nortestosterone), norandrostenedione and boldenone (Fig. 1). Besides these there is zeranol, which is an anabolic compound of which it was shown that may be formed in vivo from the mycotoxin zearalenone (Fig. 1). Next to the pure analytical side of the problem also the knowledge about the natural occurrence of some of these substances in some species in certain circumstances is important for monitoring and surveillance of residues of these substances. Because of the number of non-compliant cases in the last years and because of the emphasis on edible matrices, special interest is given in the trace level determination of the anabolic steroids nandrolone or nortestosterone (NT) and boldenone (Bol) and the zeranol. In what follows, it will be demonstrated that, even with novel analytical approaches, it is difficult to elucidate the difference between the natural occurrence of these compounds and the possible illegal administration.

Nandrolone or 19-nortestosterone is a steroid of which the β-form has been described as one of the most powerful androgenic anabolic androgens. In the past, when residues of NT were found in urine of cattle, racehorses or bodybuilders, exogenous administration was thought to be proven. Later on, it was found that NT is naturally present in the urine of male horses [117]. Recently, this was also proven for urine of pregnant cows (in the α form) [118] and of bulls due to stress prior to slaughter [M. O'Keeffe, G. Kennedey, personal communication]. In 1989, Belgian and Dutch researchers found that NT (in the β form) and norandrostenedione are also present in the urine and edible parts of the intact male pig [119–121]. Several researches demonstrated that eating organs of intact male pigs might lead to false positive results in doping analysis for sportsmen [122–127]. Based on the recent literature, most novel analytical methods are described for urine of different species, whereas for meat, the conventional immunoassays [128,129] or chromatographic techniques like liquid chromatography [127,129] or gas chromatography [127,130,131] are applied.

17β-Boldenone, also called 1-dehydrotestosterone or androsta-1,4-diene-17β-ol-3-one, differs from 17β-testosterone by only one double bond at the 1-position. Since 2000, boldenone has been detected in an increased number of biological samples (mostly veal calves) in different EU Member States. However, also the increased analytical capabilities in function of time need also to be taken in account. The question arose whether this number of boldenone findings was due to the illegal treatment of animals, or whether, in some circumstances, boldenone could be of endogenous origin. Several studies were devoted to the search for possible sources of Bol, with variable success [14,132,131]. At this moment only the presence of 17β-boldenone conjugates in urine from veal calves (at any level) is considered as a proof of illegal treatment [133]. As reviewed by De Brabander et al. [14] described methods are for detection of boldenone in urine or faeces. So far, no methods are described for detection of boldenone in edible matrices.

Zearalenone is an estrogenic mycotoxin, produced by several *Fusarium* species [134] in corn, barley, maize, wheat and sorghum. Among the different metabolites of zearalenone, the non-steroidal oestrogenic α-zearalanol (zeranol) has been marketed as a growth promoter [135–137] with estrogenic activity with the commercial name "Ralgro". Thus, the finding of zeranol in an animal might, on its own, be an in sufficient proof that malicious abuse of zeranol has occurred. Within the EU, this steroid hormone is a banned substance. The control on the abuse of zeranol is hindered by the presence of the structurally related mycotoxins α-zearalenol, β-zearalenol and zearalenone which exist in a metabolic relationship with zeranol and its metabolites [138]. In order to distinguish zeranol abuse from *Fusarium* toxin contamination in food animals Launay et al. [137] concluded that both zeranol and the Fusarium toxins (α-zearalenol, β-zearalenol and zearalenone) must be measured in the case of natural occurrence. As reviewed by Stolker et al. [32], several analytical approaches for tissue samples (liver, muscle and kidney) are described, all of them using the conventional techniques, such as liquid or gas chromatographic coupled to mass spectrometry [137–140].

7. Conclusions

Due to the increasing production pressure, farmers are pushed towards more intensive production systems and consequently towards the, legal or illegal, use of veterinary medicinal products, including the steroid hormones. Since the use of these compounds is strictly controlled within national and international legislative frameworks, a considerable number of analytical approaches are developed for the detection of steroid hormones in edible matrices.

In general, it can be concluded that the use of liquid solid extraction under the form of solid phase extraction and gas chromatography coupled to Mass Spectrometry are the state-of-the-art. Recently, there has been a shift towards the use of liquid chromatography coupled to mass spectrometry. Besides that, more sophisticated extraction and detection techniques, like supercritical fluid extraction, time of flight and combustion isotope ratio mass spectrometry are nowadays employed. The use of comparable novel techniques, f.i. accelerated solvent extraction, selective sorbents and others is to date rather limited in residue analysis. Main reasons for that are probably the ever changing group of known and 'new' steroid hormones, the cost of the equipment and the time-consuming optimization of techniques.

REFERENCES

[1] S. Impens, The use of gas chromatography–tandem mass spectrometry (GC–MS-MS) for optimization of targeted research on residues in matrices of animal origin, Ph.D. Thesis, Ghent University, Ghent, Belgium, 2002, p. 201, ISBN 90-5864-025-6.
[2] D. Courtheyn, B. Le Bizec, G. Brambilla, H.F. De Brabander, E. Cobbaert, M. Van de Wiele, J. Vercammen, K. De Wasch, Anal. Chim. Acta 473 (2002) 71.
[3] S. Poelmans, Application of GC– and LC–MS in the analysis and metabolization studies of steroids in livestock and aquatic invertebrates, Ph.D. Thesis, Ghent University, Ghent, Belgium, 2006, p. 196, ISBN 90-5864-094-9.
[4] H. Noppe, S. Poelmans, K. Verheyden, H.F. De Brabander, N. Van Hoof, Vlaams Diergen. Tijds. 74 (2005) 340.
[5] P. Fernandes, J.M.S. Cabral, Bioresour. Technol. 98 (2007) 2335.
[6] V. Piironen, D.G. Lindsay, T.A. Miettinen, J. Toivo, A.-M. Lampi, J. Sci. Food Agric. 80 (2000) 939.
[7] S.L. Abidi, J. Chromatogr. A 935 (2001) 173.
[8] M.J. Lagarda, G. García-Llatas, R. Farré, J. Pharmaceut. Biomed. Anal. 41 (2006) 1486.
[9] H.S.M. Ahmida, P. Bertucci, L. Franzò, R. Massoud, C. Cortese, A. Lala, G. Federici, J. Chromatogr. B 842 (2006) 43.
[10] S. Rozner, N. Garti, Colloid Surf. A 282–283 (2006) 435.
[11] R. Santos, E. Limas, M. Sousa, M. da Conceição Castilho, F. Ramos, M.I.N. da Silveira, Food Chem. 102 (2006) 113.
[12] Y.-T. Lin, S.-S. Wu, H.-L. Wu, J. Chromatogr. A 1156 (2007) 280.
[13] R.E. Ostlund, Curr. Opin. Lipidol. 15 (2004) 37.
[14] H.F. De Brabander, S. Poelmans, R. Schilt, R.W. Stephany, B. Le Bizec, R. Draisci, S.S. Sterk, L.A. Van Ginkel, D. Courtheyn, N. Van Hoof, A. Macrí, K. De Wasch, Food Addit. Contam. 21 (2004) 515.
[15] M.M. Ros, S.S. Sterk, H. Verhagen, A.F.H. Stalenhoef, N. De Jong, Food Addit. Contam. 24 (2007) 679.
[16] K. Kieslich, J. Basic Microb. 25 (1985) 461.
[17] S.B. Mahato, S. Garai, Steroids 62 (1997) 332.
[18] O.V. Ergorova, S.A. Gulevskaya, I.F. Puntus, A.E. Filonov, M.V. Donova, J. Chem. Technol. Biot. 77 (2002) 141.
[19] P. Fernandes, A. Cruz, B. Angelova, H.M. Pinheiro, J.M.S. Cabral, Enzyme Microb. Technol. 32 (2003) 688.
[20] K. Sarangthem, T.N. Singh, Curr. Sci. 84 (2003) 1544.
[21] M.V. Donova, O.V. Ergorova, V.M. Nikolayeva, Process Biochem. 40 (2005) 2253.
[22] C.-L. Huang, Y.-R. Chen, W.-H. Liu, Enzyme Microb. Technol. 39 (2006) 296.
[23] R. Draisci, R. Merlanti, G. Ferretti, L. Fantozzi, C. Ferranti, F. Capolongo, S. Sefato, C. Montesissa, Anal. Chim. Acta 586 (2007) 171.
[24] S. Poelmans, K. De Wasch, Y. Martelé, R. Schilt, N. Van Hoof, H. Noppe, T. Verlsycke, C.R. Janssen, D. Courtheyn, H.F. De Brabander, Proceedings EuroFoodChem XIII, Bruges, Belgium, 2003, p. 74.
[25] K. Verheyden, H. Noppe, V. Mortier, J. Vercruysse, E. Claerebout, F. Van Immerseel, C.R. Janssen, H.F. De Brabander, Anal. Chim. Acta 586 (2007) 163.
[26] M.J. Tikkanen, Handb. Exp. Pharmacol. 170 (2005) 215.
[27] J.-P. Antignac, B. Bizec, F. Monteau, F. Poulain, F. André, J. Chromatogr. B 757 (2001) 11.
[28] J.-P. Antignac, F. Monteau, J. Négrolli, F. André, B. Le Bizec, Chromatographia 59 (2004) 1.
[29] B. Shao, R. Zhao, J. Meng, Y. Xue, G. Wu, J. Hu, X. Tu, Anal. Chim. Acta 548 (2005) 41.
[30] H.F. De Brabander, B. Le Bizec, G. Pinel, J.-P. Antignac, K. Verheyden, V. Mortier, D. Courtheyn, H. Noppe, J. Mass Spectrom. 42 (2007) 983.
[31] H. Noppe, K. Verheyden, W. Gillis, D. Courtheyn, P. Vanthemsche, H.F. De Brabander, Anal. Chim. Acta 586 (2007) 22.
[32] A.A.M. Stolker, U.A.Th. Brinkman, J. Chromatogr. A 1067 (2005) 15.
[33] K. De Wasch, H.F. De Brabander, D. Courtheyn, C. Van Peteghem, Analyst 123 (1998) 2415.
[34] K. De Wasch, N. Van Hoof, S. Poelmans, L. Okerman, D. Courtheyn, A. Ermens, M. Cornelis, H.F. De Brabander, Anal. Chim. Acta 483 (2003) 387.
[35] A. Daxenberger, D. Ibarreta, H.H.D. Meyer, Hum. Reprod. Update 7 (2001) 340.
[36] Directive 81/206/EEC, Off. J. Eur. Comm., L222 (1981) 32.
[37] Directive 88/146/EEC, Off. J. Eur. Comm., L70 (1988) 16.
[38] Directive 88/299/EEC, Off. J. Eur. Comm., L128 (1988) 36.
[39] Commission Regulation 2377/90, Off. J. Eur. Comm., L224 (1990) 1.
[40] Council Directive 96/22/EC, Off. J. Eur. Comm., L125 (1996) 3.
[41] Council Directive 96/23/EC, Off. J. Eur. Comm., L125 (1996) 10.
[42] Council Directive 2003/74/EC, Off. J. Eur. Comm., L262 (2003) 17.
[43] Commission Decision 2002/657/EC, Off. J. Eur. Comm., L221 (2002) 8.
[44] Commission Decision 93/256/EEC, Off. J. Eur. Comm., L118 (1993) 64.
[45] Commission Decision 93/257/EEC, Off. J. Eur. Comm., L118 (1993) 75.
[46] F. André, K. De Wasch, H.F. De Brabander, S. Impens, L.A.M. Stolker, L. Van Ginkel, R. Stepahny, R. Schilt, D. Courtheyn, Y. Bonnaire, P. Fürst, P. Gowik, G. Kennedey, T. Kuhn, J.-P. Moretain, M. Sauer, Trends Anal. Chem. 20 (2001) 435.
[47] J.-P. Antignac, B. Le Bizec, F. Monteau, F. André, Anal. Chim. Acta 483 (2003) 325.
[48] S. Hartmann, H. Steinhart, J. Chromatogr. B 704 (1997) 105.
[49] E. Daeseleire, R. Vandeputte, C. Van Peteghem, Analyst 123 (1998) 2595.
[50] S. Impens, K. De Wasch, M. Cornelis, H.F. De Brabander, J. Chromatogr. A 970 (2002) 235.
[51] B. LeBizec, P. Marchand, C. Gadé, D. Maume, F. Monteau, F. André, Proceedings of the EuroResidue IV Conference, Veldhoven, 2000, p. 226.
[52] P. Marchand, B. Le Bizec, C. Gade, F. Monteau, F. André, J. Chromatogr. A 867 (2000) 219.
[53] J. Seo, H. Kim, B.C. Chung, J. Hong, J. Chromatogr. A 1067 (2005) 303.
[54] C. Blasco, C. Van Poucke, C. Van Peteghem, J. Chromatogr. A 1154 (2007) 230.
[55] H. Malekinejad, P. Scherpenisse, A.A. Bergwerf, J. Agric. Food Chem. 54 (2006) 9785.
[56] M. Fuh, S. Huang, T. Lin, Talanta 64 (2004) 408.
[57] F. Smets, H.F. De Brabander, G. Pottie, Arch. Lebensmittelhyg. 48 (1997) 30.
[58] A.A.M. Stolker, P.W. Zoontjes, L. VanGinkel, Analyst 123 (1998) 2671.
[59] H. Hooijerink, E.O. Van Bennekom, M.W.F. Nielen, Anal. Chim. Acta 483 (2003) 51.
[60] C. Buisson, M. Hebestreit, A. Preiss Weigert, K. Heinrich, H. Fry, U. Flenker, S. Banneke, S. Prevost, F. André, W. Schaenzer, E. Houghton, B. Le Bizec, J. Chromatogr. A 1093 (2005) 69.
[61] S. Hartmann, H. Steinhart, Arch. Lebensmittelhyg. 48 (1997) 111.
[62] S. Hartmann, M. Lacorn, H. Steinhart, Food Chem. 62 (1998) 7.

[63] F. Smets, Ch. Vanhoenackere, G. Pottie, Anal. Chim. Acta 275 (1993) 9.
[64] L. Van Look, P. Deschuytere, C.V. Van Peteghem, J. Chromatogr. B 489 (1989) 213.
[65] S. Impens, D. Courtheyn, K. De Wash, H.F. De Brabander, Anal. Chim. Acta 483 (2003) 269.
[66] M.J. López de Alda, D. Barceló, J. Chromatogr. A 938 (2001) 145.
[67] M.J. López de Alda, D. Barceló, J. Chromatogr. A 911 (2001) 203.
[68] H. Noppe, T. Verslycke, E. De Wulf, K. Verheyden, E. Monteyne, P. Van Caeter, C.R. Janssen, H.F. De Brabander, Ecotoxicol. Environ. Safe. 66 (2007) 1.
[69] K. Kurečková, B. Maralíková, K. Venture, J. Chromatogr. B 770 (2002) 83.
[70] N. Din, K.D. Bartle, A.A. Clifford, L. Castle, J. High Resol. Chromatogr. 19 (1994) 465.
[71] R.P. Huopalahti, J.D. Henion, J. Liquid Chromatogr. Relat. Technol. 19 (1996) 69.
[72] K. Ridgway, S.P.D. Lalljie, R.M. Smith, J. Chromatogr. A 1153 (2007) 36.
[73] R. Liu, J.L. Zhou, A. Wilding, J. Chromatogr. A 1038 (2004) 19.
[74] M.P. Hermo, D. Barrón, J. Barbosa, Anal. Chim. Acta 539 (2005) 77.
[75] F. Qiao, H. Sun, H. Yan, K.H. Row, Chromatographia 64 (2006) 625.
[76] H. Dong, A. Tong, L. Li, Spectrochim. Acta A 59 (2003) 279.
[77] N. Van Hoof, D. Courtheyn, J.-P. Antignac, M. Van de Wiele, S. Poelmans, H. Noppe, H.F. De Brabander, Rapid Commun. Mass Spectrom. 19 (2005) 2801.
[78] C.J. Houtman, P.E.G. Leonards, W. Kapiteijn, J.F. Bakker, A. Brouwer, M.H. Lamoree, J. Legler, H.J.C. Klamer, Sci. Total Environ. 1–3 (2007) 134.
[79] M.L. Scippo, C. Van De Weerdt, P. Willemsen, J.M. François, F. Rentier-Delrue, M. Muller, J.A. Martial, G. Maghuin-Rogister, Anal. Chim. Acta 473 (2002) 135.
[80] M.L. Scippo, P. Gaspar, G. Degand, F. Brose, G. Maghuin-Rogister, P. Delahaut, J.P. Willemart, Anal. Chim. Acta 275 (1993) 57.
[81] M.L. Scippo, G. Degand, A. Duyckaerts, G. Maghuin-Rogister, P. Delahaut, Analyst 119 (1994) 2639.
[82] C.T. Elliot, K.S. Francis, H.D. Shortt, W.J. McCaughey, Analyst 120 (1995) 1827.
[83] T.L. Fodey, C.T. Elliot, S.R.H. Crooks, W.J. McCaughey, Food Agric. Immunol. 8 (1996) 157.
[84] C.J.M. Arts, M.J. Van Baak, C.T. Elliot, S.A. Hewitt, J. Cooper, K. Van De Velde-Fase, R.F. Witkamp, Analyst 123 (1998) 2579.
[85] C.J.M. Arts, M.J. Van Baak, Proceedings of Euroresidue, Noordwijkerhout, The Netherlands, 1990, p. 105.
[86] R. Verbeke, J. Chromatogr. 177 (1979) 69.
[87] H.F. De Brabander, F. Smets, G. Pottie, J. Planar. Chromatogr. 1 (1988) 369.
[88] H.F. De Brabander, J. Van Hoof, J. Planar Chromatogr. 3 (1990) 236.
[89] H.F. De Brabander, F. Smets, G. Pottie, J. Planar Chromatogr. 4 (1991) 207.
[90] H.F. De Brabander, L. Hendriks, F. Smets, P. Delahaut, P. Batjoens, L. Leyssens, G. Pottie, Proceedings Euroresidue II, Veldhoven, 1993, p. 211.
[91] L. Hendriks, D. Courtheyn, B. Gielen, L. Leyssens, J. Raus, Proceedings Euroresidue II, Veldhoven, 1993, p. 367.
[92] P. Batjoens, H.F. De Brabander, F. Smets, G. Pottie, Analyst 119 (1994) 2607.
[93] D. Maume, B. Le Bizec, P. Marchand, M.-P. Montrade, F. André, Analyst 123 (1998) 2645.
[94] J.L. Little, J. Chromatogr. A 844 (1999) 1.
[95] D.H. Van De Kerkhof, R.D. Van Ooijen, D. De Boer, R.H. Fokkens, N.M.M. Nibbering, J.W. Zwikker, J.H.H. Thyssen, R.A.A. Maes, J. Chromatogr. A 954 (2002) 199.
[96] V. Meunier-Solère, D. Maume, F. André, B. Le Bizec, J. Chromatogr. B 816 (2005) 281.
[97] C. Baronti, R. Curini, G. D'Ascenzo, A. Di Corcia, A. Gentili, R. Samperi, Environ. Sci. Technol. 34 (2000) 5059.
[98] M. López de Alda, D. Barceló, J. Chromatogr. A 892 (2000) 391.
[99] M. López de Alda, S. Díaz-Cruz, M. Petrovíc, D. Barceló, J. Chromatogr. A 1000 (2003) 503.
[100] M.S. Díaz-Cruz, M.J. López de Alda, D. Barceló, Trends Anal. Chem. 22 (2003) 340.
[101] V. Ingrad, G. Herry, J. Beausse, M. De Roubain, J. Chromatogr. A 1020 (2003) 99.
[102] T. Isobe, H. Shiraishi, M. Yasuda, A. Shinoda, H. Suziki, M. Morita, J. Chromatogr. A 984 (2003) 195.
[103] C. Van Poucke, M. Van De Velde, C. Van Peteghem, J. Mass. Spectrom. 40 (2005) 731.
[104] E.N.M. Ho, D.K.K. Leung, T.S.M. Wan, N.H. Yu, J. Chromatogr. A 1120 (2006) 38.
[105] A.A.M. Stolker, P.W. Zoontjes, P.L.W.J. Schwillens, P.R. Kootstra, L.A. Van Ginkel, R.W. Stephany, U.A.Th. Brinkman, Analyst 127 (2002) 748.
[106] M.E. Touber, M.C. Van Engelen, C. Georgakopoulus, J.A. Van Rhijn, M.W.F. Nielen, Anal. Chim. Acta 586 (2007) 137.
[107] M.W.F. Nielen, M.C. Van Engelen, R. Zuiderent, R. Ramaker, Anal. Chim. Acta 586 (2007) 122.
[108] P.M. Mason, S.E. Hall, I. Gilmour, E. Houghton, C. Pillinger, M.A. Seymour, Analyst 123 (1998) 2405.
[109] V. Ferchaud, B. Le Bizec, F. Monteau, M.P. Montrade, F. André, Analyst 12 (1998) 2617.
[110] V. Ferchaud, B. Le Bizec, F. Monteau, F. André, Rapid Commun. Mass Spectrom. 14 (2000) 652.
[111] G. Balizs, A. Jainz, P. Horvatovich, J. Chromatogr. A 1067 (2005) 323.
[112] M. Hebestreit, F. André, S. Banneke, C. Buisson, U. Flenker, H. Fry, K. Heinrich, S. Hird, M. Lang, B. Le Bizec, A. Preiss-Weigert, W. Schaenzer, C. Von Kuk, J. Agric. Food Chem. 54 (2006) 2850.
[113] P. Laakso, Eur. J. Lipid Sci. Technol. 107 (2005) 402.
[114] B. Lu, Y. Zhang, X. Wu, J. Shi, Anal. Chim. Acta 588 (2007) 50.
[115] L.A. Riddle, G. Guiochon, J. Chromatogr. A 1137 (2006) 173.
[116] B. Cañabate-Díaz, A. Segura Carretero, A. Fernández-Gutiérrez, A. Belmonte Vega, A. Garrido Frenich, J.L. Martínez Vidal, J. Duran Martos, Food Chem. 102 (2007) 593.
[117] E. Houghton, J. Copsy, M.C. Dumassia, P.E. Haywood, M.S. Moss, P. Teale, Biomed. Mass Spectrom. 11 (1984) 96.
[118] H.F. De Brabander, J. Van Hende, P. Batjoens, L. Hendriks, J. Raus, F. Smets, G. Pottie, L. Van Ginkel, W. Stephany, Analyst 199 (1994) 2581.
[119] G. Maghuin-Rogister, A. Bosseleire, P. Gaspar, C. Dansas, G. Pelzer, Ann. Med. Vet. 132 (1988) 437.
[120] L.A. Van Ginkel, R.W. Stephany, H.J. Van Rossum, H. Van Blitterswijks, P.W. Zoontjes, R.C.M. Hooijschuur, J. Zuydendorp, J. Chromatogr. 489 (1989) 95.
[121] G. Debruykere, C. Van Peteghem, H.F. De Brabander, M. Debackere, Vet. Quart. 12 (1990) 247.
[122] L. Dehennin, Y. Bonnaire, P. Plou, J. Chromatogr. B 721 (1999) 301.
[123] B. Le Bizec, F. Monteau, I. Gaudin, F. André, J. Chromatogr. B 723 (1999) 157.
[124] B. Le Bizec, I. Gaudin, F. Monteau, F. Andre, S. Impens, K. De Wasch, H.F. De Brabander, Rapid Commun. Mass Spectrom. 14 (2000) 1.
[125] B. Le Bizec, F. Bryand, I. Gaudin, F. Monteau, F. Poulain, F. André, J. Anal. Toxicol. 26 (2002) 43.

[126] B. Le Bizec, F. Bryand, I. Gaudin, F. Monteau, F. Poulain, F. André, Steroids 67 (2002) 105.
[127] K. De Wasch, B. Le Bizec, H.F. De Brabander, F. André, S. Impens, Rapid Commun. Mass Spectrom. 15 (2001) 1442.
[128] C.J. Van Peteghem, C.J. Van Look, Anal. Chim. Acta 205 (1988) 223.
[129] E. Daeseleire, A. Deguesquiere, C. Van Peteghem, J. Chromatogr. B 564 (1991) 445.
[130] R. Bagnatie, T. Ranelli, J. Chromatogr. 547 (1991) 325.
[131] S. Poelmans, K. De Wasch, H. Noppe, N. Van Hoof, S. Van Cruchten, B. Le Bizec, Y. Deceuninck, S. Sterk, H.J. Van Rossum, M.K. Hoffman, H.F. De Brabander, Food Addit. Contam. 22 (2005) 808.
[132] S. Sterk, M.H. Blokland, L.A. Van Ginkel, R. Schilt, E. van der Vlis, P. Boshuis, M.J. van Baak, M.W.F. Nielen, J.A. van Rhijn, D. Samson, H.J. Keukens, R.W. Stephany, Proeedings of the EuroResidue V Conference, Noorwijkerhout, 2004, p. 900.
[133] B. Le Bizec, F. Courant, I. Gaudin, E. Bichon, B. Destrez, R. Schilt, R. Draisci, F. Monteau, F. André, Steroids 71 (2006) 13.
[134] P. Zöllner, B. Mayer-Herlm, J. Chromatogr. A 1136 (2006) 123.
[135] D.G. Kennedey, J.D.G. McEvoy, W.J. Blanchflower, S.A. Hewitt, A. Cannavan, W.J. McCaughey, C.T. Elliot, J. Vet. Med. B 42 (1995) 509.
[136] D.G. Kennedey, S.A. Hewitt, J.D.G. Currie, A. Cannavan, W.J. Blanchflower, C.T. Elliot, Food Addit. Contam. 15 (1998) 535.
[137] F.M. Launay, L. Ribeiro, P. Alves, V. Vozikis, S. Tsitsamis, G. Alfredsson, S.S. Sterk, M. Blokland, A. Iitia, T. Lövgren, M. Tuomola, A. Gordon, D.G. Kennedey, Food Addit. Contam. 9 (2004) 833.
[138] J. Jodlbauer, P. Zöllner, W. Lindner, Chromatographia 51 (2000) 681.
[139] M. Horie, H. Nakazwa, J. Chromatogr. A 882 (2000) 53.
[140] X.M. Fang, J.H. Chen, D.H. Guo, J. AOAC Int. 85 (2002) 841.