JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2007; **42**: 983–998
Published online in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/jms.1272

JMS

SPECIAL FEATURE: PERSPECTIVE

Past, present and future of mass spectrometry in the analysis of residues of banned substances in meat-producing animals

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Received 14 May 2007; Accepted 26 June 2007

A residue is a trace (μg kg^{-1}, ng kg^{-1}) of a substance, present in a matrix. Banned substances, such as growth promoters, which are abused in animal fattening and where this article is focused on, may be divided into four major groups: thyreostats, anabolics or anabolic steroids, corticosteroids and beta-agonists or repartitioning agents. The combination of chromatographic techniques with mass spectrometry (GC-MS^n, LC-MS^n, etc.) plays a key role in the production of specific results in residue analysis. In this review, the past, present and future of mass spectrometry in this area are discussed in the light of the impact of these substances on human health and the reliable production of analytical results, ready for challenge in a court. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: residues; banned substances; growth promoters; animal production; mass spectrometry

INTRODUCTION

A residue is a trace of a substance, present in a matrix after some kind of administration. The matrix can be anything in which a residue may be present, trapped or concentrated (meat, urine, faeces, hair, liver, etc.). There is no general agreement on the concentration level of a trace. However, the ppb (μg kg^{-1}) and ppt (ng kg^{-1}) levels in which residues are present may certainly be considered as trace level. In Europe, banned substances indicated by council directive 96/23/EC[1] as group A substances (substances having anabolic effect and unauthorized substances) are sometimes indicated by the word 'hormones'. This simplification is not correct. The growth promoters abused in animal fattening, where this article focusses on, may be divided into four major groups. *Thyreostats* (group A2; e.g. methylthiouracil) may cause a considerable weight gain in a short time. However, that weight gain consists mainly of increased filling of the gastro-intestinal tract and increased water retention in the animal. *Anabolics* or *anabolic steroids* (group A1, A3, A4; e.g. diethylstilbestrol, nortestosterone) increase weight gain and improve carcass quality. These substances are also called estrogens, gestagens and androgens (EGAs). *Beta-agonists* or *repartitioning agents* (group A5; e.g. clenbuterol) cause also weight gain in most slaughter animals. Moreover, the amount of muscle tissue (meat) increases and the amount of fatty tissue decreases. *Corticosteroids* (group B2f; e.g. dexamethasone) cause extra weight gain, most probably due to water retention. Corticosteroids, however, are not group A substances but belong to the class of veterinary drugs (group B).

In Europe, the word 'hormones' has a very bad connotation for the consumer. The background for this bad reputation is the possible danger for public health of the residues of these products in meat. Toxicologists have demonstrated that DES (commonly known as diethylstilbestrol, a synthetic estrogen, the cattle growth-promoting properties of which were discovered in 1954) is a potential carcinogen.[2,3] In human medicine, analogous experiences were found with DES (the so-called DES-daughters).[4]

The consumer has extended the bad reputation of DES to all hormones, and considers all growth promoters as dangerous substances. Other toxicologists have declared that some substances may safely be used in animal production

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under certain conditions (e.g. the natural hormones).[5] This point of view is adopted in the US and some other countries, but in Europe the consumer does not accept that meat animals may be treated with 'hormones' even if some toxicologists have declared that some are safe. The debate between the US, Canada and Europe on the safety of some substances and their impact on the environment is still going on. Since they are banned in Europe, Directive 96/23/EC[1] has laid down the measures to monitor these substances. Analytical laboratories have key functions in this mechanism. The impact of analytical results of these banned substances is increasing because the consequences of the results (higher fees, etc.) are increasing. Therefore, the highest specificity is required for the confirmatory methods used and their performance, and the interpretation of the results is described in the Commission decision 2002/657/EC[6]

The specificity needed is obtained only by the combination of chromatographic techniques with mass spectrometry (GC-MS^n, LC-MS^n, etc.). However, as the number of substances to be screened and confirmed is high and not limited, in future one technique will never be sufficient to detect all compounds. In this review the past, present and future of mass spectrometry in residue analysis is described. In the 1970s thin layer chromatography (TLC) appeared to be the method of choice for the analysis of thyreostats and anabolics (the two most important groups of substances at that point). The reasons therefore were the specificity, the simplicity and the possibility of reaching low limits of detection on an acceptable budget. During the 1990s, more and more affordable gas chromatography-mass spectrometry (CG-MS) apparatus appeared on the market, and the transition from TLC to GC-MS and further on to GC-MSMS and even MS^n methods was ongoing. Somewhat later on, LC-MS^n became more and more the standard equipment of a residue-testing laboratory. In Fig. 1 a time evolution of the methods used in residue analysis of banned substances is given.

In Fig. 2 the evolution of detection capability of mass spectrometric instruments as a function of time is given. In this figure the standardized limits of detection (LOD; S/N = 3) of standard substances on different instruments are plotted.

As can be seen, the LOD in GC-MS has decreased by a factor of 100 from 1989 to 2003. In LC-MS, a decrease of the LOD by even a factor 1000 was observed in the period 1997 to 2004. If we extrapolate to the year 2010, an LOD of approximately 100 ag will be obtained. Of course, this is a very rough estimation and the following question may be asked: is this value low and where will this end? From basic chemistry and Avogadro's number, we learn that 100 ag

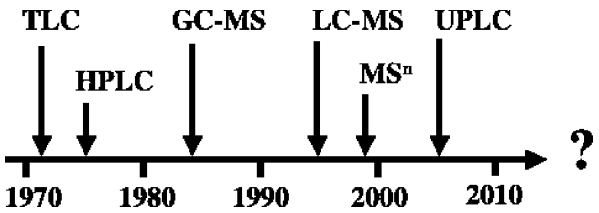


Figure 1. Time evolution of the methods used in residue analysis of banned substances.

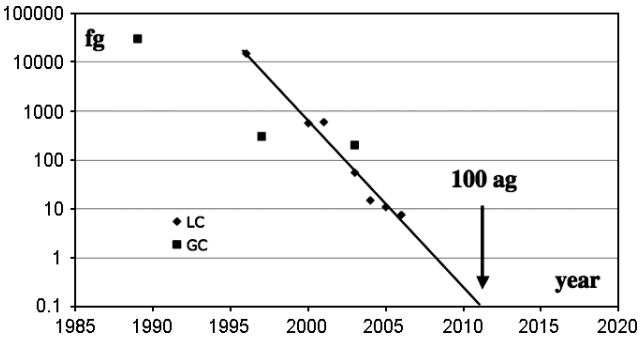


Figure 2. Trends in the detection capability of instruments as a function of time.

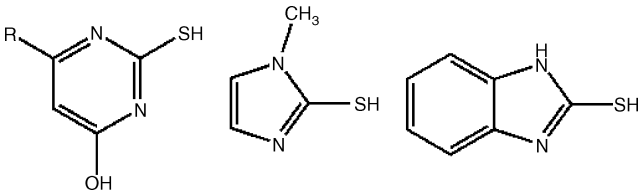
still corresponds to 200 000 molecules (in the case of steroids with a molecular mass of approximately 300 amu). So, the decrease of the LOD may still go on for some time. The improved MS techniques may reveal the natural presence of substances formerly considered to be of exogenous origin (e.g. nortestosterone, boldenone, thiouracil). This generates some grey areas in which the border between illegality and natural conditions may not be clear enough.

Next to the detection capability of the instruments, the clean-up of the sample prior to instrumental analysis must also be kept in mind. The better the clean-up, the better the results of the hyphenated technique used. Solid-phase extraction (SPE), immuno affinity chromatography (IAC) and molecular imprinted polymers (MIP) are often used as clean-up methods. Another option is HPLC fractionation. This procedure results in several purified fractions, each containing a limited number of analytes and matrix components. Each HPLC fraction may be analysed with a specific technique (e.g. GC-MS) and if necessary with different techniques.

The choice of the analytical strategy must also and always be seen in the light of the interpretation of the analytical results. Inspection services are interested mainly in a YES/NO answer: has this animal been treated with illegal substances? Is the concentration of the residue higher than a certain value, etc.? In fact, all questions may be reduced to one major question: is the law violated? When the answer to that question is YES, actions are taken. Therefore, the analytical results must be acceptable 'beyond any reasonable doubt'. This is an expression used in courts, and, indeed, analytical results are challenged more and more by attorneys. In a court, a result must be able to survive all scientific and non-scientific arguments brought in against it. In most cases, these arguments are based on laws and regulations, leaving little space for the 'art of science' and the 'analytical experience' of an individual researcher.

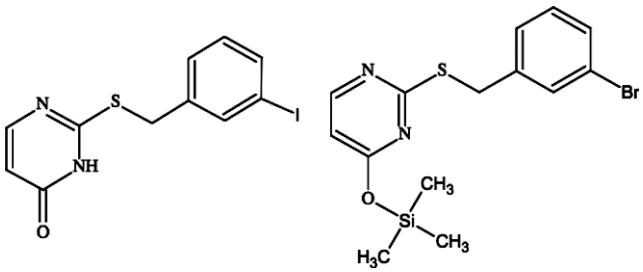
MASS SPECTROMETRY AND THE ANALYSIS OF THYREOSTATS (GROUP A2)

Thyreostatic drugs are banned in the EU and are considered worldwide as unwanted and dangerous substances. They have been applied illegally to animals to obtain a higher live weight gain. This gain is mainly due to higher water retention in the edible tissue and increased filling of the gastro-intestinal tract. The first effect gives rise to, next



(A) (B) (C)

Figure 3. The most important thyreostats (A: 4(6)-*R*-2-thiouracil; B: tapazole; C: mercaptobenzimidazole).



(A) (B)

Figure 4. Thiouracil derivatized with (A) 3-IBBr and (B) 3-BrBBr/MSTFA.

to the fraudulent higher weight ('water instead of meat'), a reduction in the meat quality.[7,8] The most important thyreostats are given in Fig. 3.

In Spain, the consumption of meat contaminated with thyreostats has caused an increased incidence of aplasia cutis, a characteristic scalp defect.[9]

Specific detection procedures for the detection of these groups of drugs with HPTLC have been described a long time ago.[10–12] These methods are based on the fluorescence induction of the 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD) derivatives of the drugs with cysteine, combined with a rapid and selective extraction procedure, based on a specific complex formation of the drugs with mercury ions. Later, in order to improve the qualitative accuracy of the method, the HPTLC results were confirmed by analysis of the suspect HPTLC 'spots' with mass spectrometry (GC-MS). In 1997, when advances in ion trap technology resulted in the possibility of multiple MS, also called MS^n, the suspect samples were subjected to a confirmatory method by infusion of the remainder of the extract into an ion trap mass spectrometer operating in the MS^n mode.[13] Ionization is performed by the electrospray interface, and fragment ions are acquired up to MS^3. Previously, methods for the determination of thyreostats did not include mercaptobenzimidazol (MBI). Through the European Union Reference Laboratory (CRL, Bilthoven, the Netherlands) we were informed in 2000 that a 'new' thyreostatic compound was illegally used and not yet monitored. Therefore, the extraction procedure was optimized, which allowed since then the detection of all thyreostats in the thyroid tissue including MBI down to a level of 20 $\mu g\ kg^{-1}$.[14] Other matrices (urine, faeces, animal feed) were tested with this method but further development was needed. Other authors have also studied the GC and LC-MS analysis of thyreostats.[15,16]

In order to increase the performances of thyreostat detection in various biological matrices and to extend the monitoring to a wide range of compounds, further work was carried out in 2005 that focused on the derivatization step prior to LC-MS/MS or GC-MS/MS analysis. The aims of the derivatization are numerous, and include the stabilization of the compounds, the reduction of their polarity to improve their separation as well as the increase of their molecular weight to allow lower detection capabilities and limits of decision.[17] For this purpose, different derivatives (Fig. 4.) have been studied and compared with thyreostats in terms of extraction, specificity, quality of the chromatographic separation, ionization efficiency and specificity of the fragmentation.

3-Iodo-benzylbromide (3-IBBR) has been selected as the most efficient derivatization reagent when detection is performed in MS^2 by LC-MS/MS (triple quadrupole) after negative electrospray ionization (ESI) (Fig. 5(a)).

The protocol has been successfully applied to biological matrices (urine, tissues, faeces and hair) and the multi-residue method was validated according to the EU criteria (2002/657/EC Decision), leading to improved performances of identification and quantification (in the $\mu g\ l^{-1}$ range).[17] Once derivatized with 3-bromobenzylbromide (3-BrBBr) and *N*-Methyl-*N*-(trimethylsilyl)-trifluoro-acetamide (MSTFA), thyreostats are also easily analysed in MS^2 by GC tandem quadrupole MS/MS after electron impact (EI) ionization (Fig. 5(b)).[18] The use of an improved method has revealed the 'natural' presence of thiouracil in the urine of a non-treated bovine.[19] Further research is needed for the discrimination between abuse and the natural presence of this, and maybe other thyreostats.

THE ANALYSIS OF RESIDUES OF EGAS (GROUPS A1, A3, A4)

For several years now, the use of anabolic steroids, such as EGAs in animal fattening is prohibited in the European Community because of their possible toxic effect on public health. Although toxicologists have declared that certain growth promoters are safe under conventional application conditions, most consumers do not want to eat meat coming from animals that were treated with these drugs. The large number of steroids (Fig. 6) complicates the control of their illegal use.

Initial screening of animals for steroid abuse is performed using chemical or immunochemical methods,[20,21] followed by the complete chemical confirmation of steroids in suspect samples by MS.[22–26] Urine and hair samples are popular since they are readily available at both slaughterhouse and farm levels. Analytical strategies for sample preparation are generally based on at least two-stage SPE columns. C_{18}, C_8, NH_2 and SiOH are among the most frequently used stationary phase columns, while strong anion exchange (SAX) columns are applied for direct isolation of steroid conjugates. Conjugates are usually hydrolysed either by enzymatic (glucuronidase) or chemical approaches (solvolysis or methanolysis).

DOI: 10.1002/jms

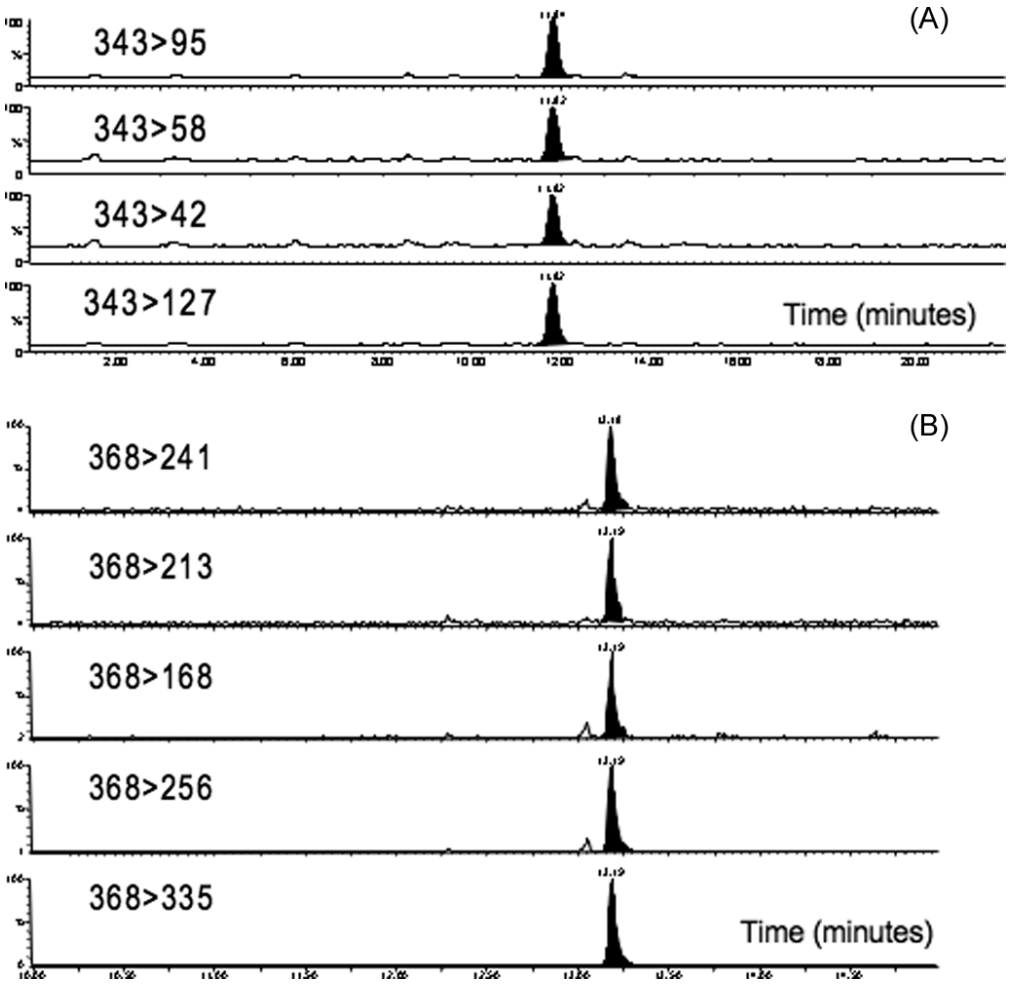


Figure 5. SRM ion chromatograms for (A) standard sample (TU 10 μg l^{-1}) (3-IB derivative), LC(ESI-)MS-MS analysis and (B) urine sample spiked with TU (10 μg l^{-1}) (3-BrB/TMS derivative), GC(EI^+) MS-MS analysis.

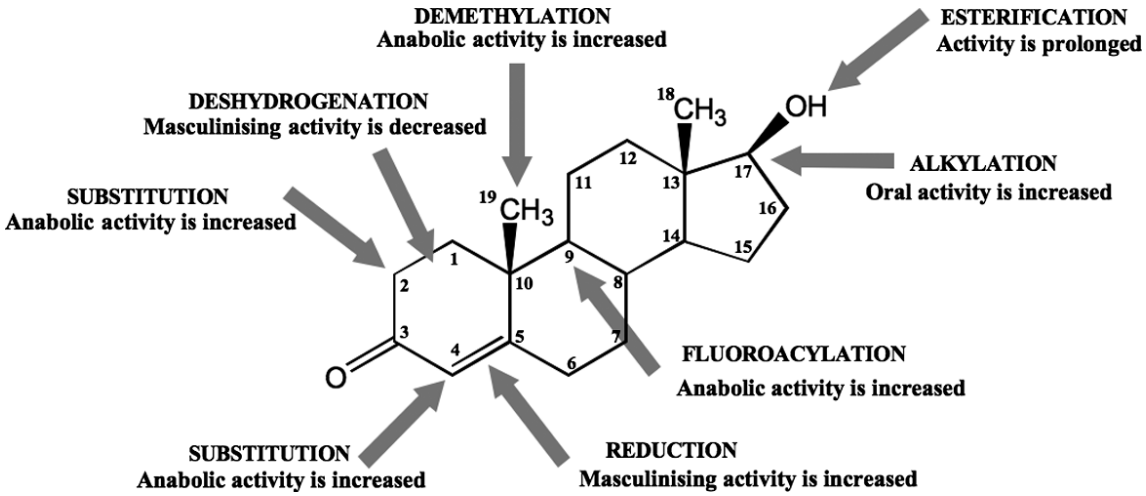


Figure 6. Possible changes on the steroid nucleus leading to a large number of steroids.

For long time, the standard analytical technique for steroid analysis has been GC-MS. This required the derivatization of the steroids. The most popular approach incontestably has been silylation. MSTFA is often used as the silylating agent in combination with ammonium iodine (NH_4I) or trimethyliodosilane (TMIS) as catalyst and dithiothreitol (DTE) as antioxidant. EI ionization is the most traditional ionization technique, except when fluoroacylation derivatization is used; in this case, the high sensitivity of electron capture negative chemical ionization (EC-NCI) is preferred. Examples of different mass spectra observed for hexestrol in various ionization conditions are given in Fig. 7.

After trimethylsilylation, this stilbene is drastically fragmented leading to a single m/z 207, as a consequence

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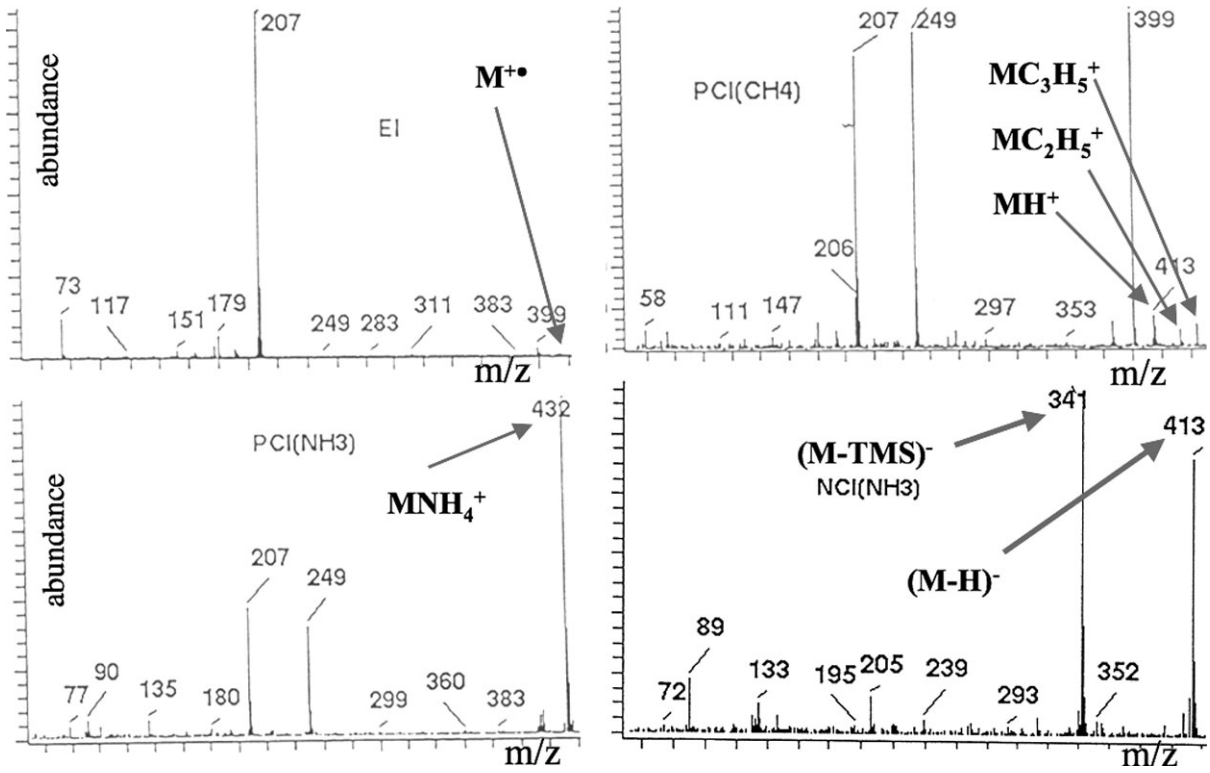

Figure 7. Influence of the ionization technique on the mass spectrum of hexestrol (PCI(CH_4): Positive chemical ionization with methane; PCI(NH_3): Positive chemical ionization with ammonia; NCI(NH_3): Negative chemical ionization with ammonia).

of the benzyllic fragmentation. Positive chemical ionization (PCI) permitted limiting the fragmentation and observation of pseudo-molecular ion information as well as adducts with the reagent gas. Owing to its high proton affinity, ammonia led to the formation of an abundant $[M + NH_4]^+$ ion, whereas methane generated a more fragmented mass spectrum characterized as well by an intense pseudo-molecular ion $[M + H]^+$, and two adducts $[M + C_2H_5]^+$ and $[M + C_3H_5]^+$. In the negative mode, a proton abstraction led to an intense $[M - H]^-$ at m/z 413.

LC-MS is preferred for trenbolone-like steroids (thermolabile), stanozolol (strong adsorption in GC due to the pyrazole ring), steroid conjugates (high polarity) and progestagens (strong non-polarity). Liquid chromatography is operated in the positive or negative (steroid conjugates) ESI mode. Atmospheric pressure chemical ionization (APCI) or atmospheric pressure photon ionization (APPI) are preferred for non-polar steroids such as progestagen esters or estrogens, respectively. Mass analysers are mainly quadrupoles and ion trap technologies. Single MS is today replaced by multi-dimensional techniques based either on triple quadrupoles, ion trap detectors or hybrid systems such as Q-TRAP.

The strategy generally relies on the selection of a precursor ion representing a significant percentage of the MS^1 total ionic current (TIC). This can be done by reduction of the fragmentation either by mild ionization conditions (chemical ionization, lower electron energy in EI) or stabilization of the molecule by specific derivatization (Fig. 8).

Then when a QqQ system is used, the fragmentation in the collision cell must be limited but should permit generation of at least two product ions for better sensitivity on the final transitions. When these principles are respected, the performance of this approach can be tremendous as shown on Fig. 9, where 1 ng g^{-1} of 17α-methyltestosterone could be characterized on four transitions even in 100 mg of bovine hair sample (10 pg on-column).

High-resolution mass spectrometers (HRMSs) are mainly electromagnetic instruments operated at resolutions equal or better than 10 000 (10% valley); they are used in confirmatory processes (control laboratories). This technique is particularly useful for steroids characterized by a mass defect; the increase in resolution on such an instrument can produce an efficient mass clean-up, as shown in Fig. 10.

Illustration of the influence of resolution on the signal-to-noise ratio is given for 4-chlorotestosterone in a urine sample (50 ng l^{-1}). One can see that the optimal resolution for the measurement of this steroid in urine is in between 5000 and 10 000. Below this value, the mass clean-up is limited; above, the signal decreases rapidly even if no interfering compounds are visible.

THE ANALYSIS OF CORTICOSTEROIDS (GROUP B2F) WITH MASS SPECTROMETRY

Fifty years after the discovery of natural corticosteroid hormones (CoSTs) and their anti-inflammatory properties, many synthetic derivatives of these molecules are available today. Their legal use in human and veterinary medicine is strictly regulated, with withdrawal periods between treatment and slaughtering and the establishment of a maximum residue limit (MRL) in edible biological matrices for some compounds. These substances have also been used illegally

DOI: 10.1002/jms

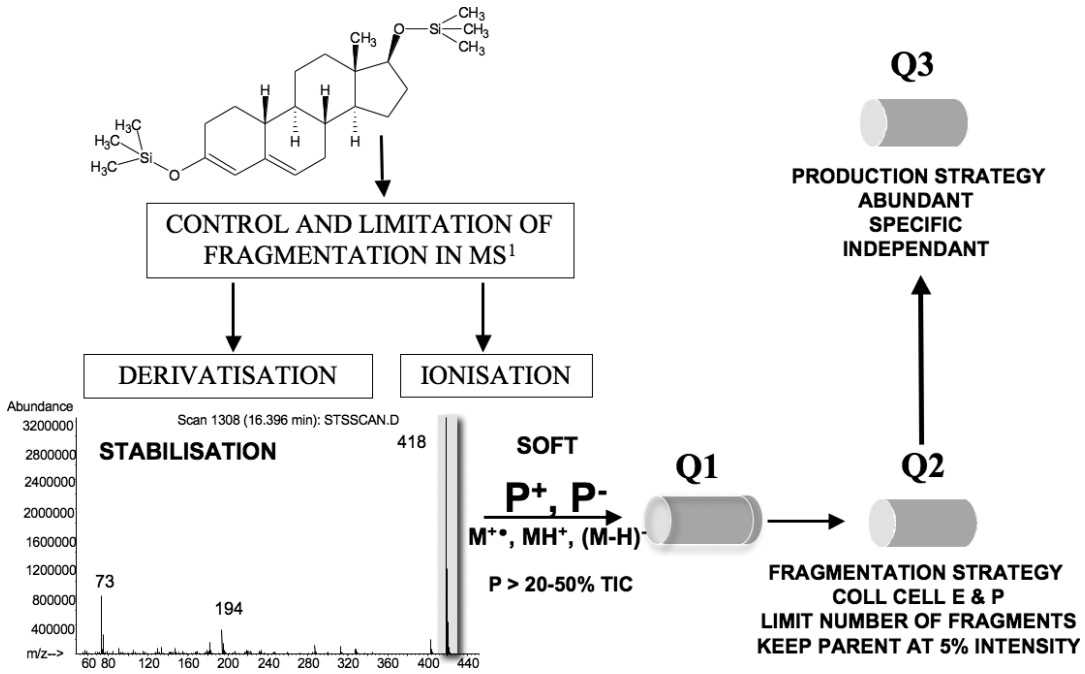

Figure 8. Strategy of multi-dimensional techniques.

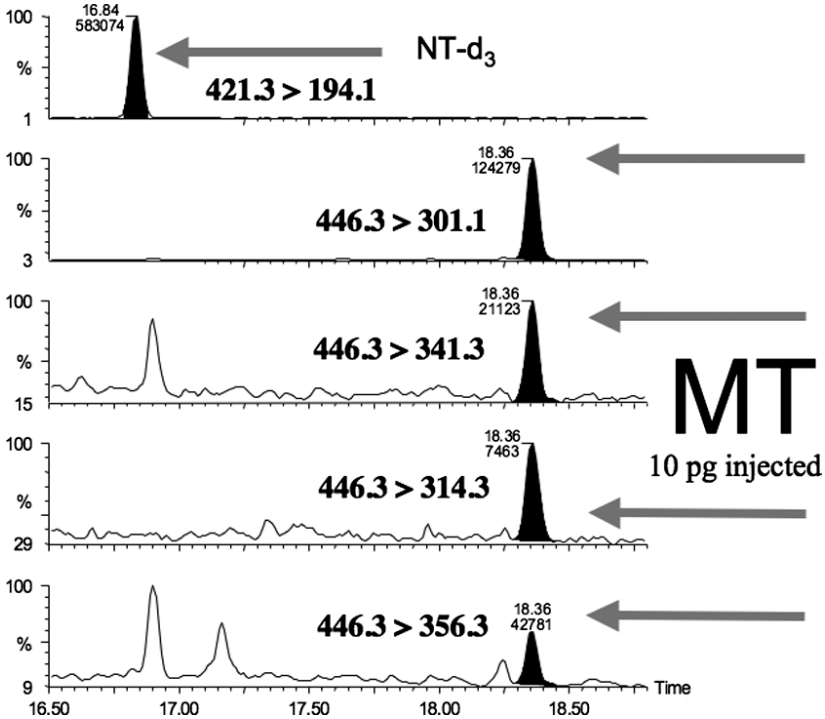

Figure 9. Characterization of 17α-methyltestosterone on four transitions in 100 mg of bovine hair sample.

as growth promoters in cattle. Indeed, low concentrations of CoSTs are known to increase weight gain, reduce feed conversion ratio, and have a synergetic effect with other molecules like β-agonists or anabolic steroids.[27–29] For many years, various analytical methods have been proposed for the identification of CoST residues in various biological matrices. During the 1980s and early 1990s, the main detection methods used for CoSTs were radioimmunology,[30,31] fluorimetry (after HPLC or TLC)[32,33] or liquid chromatography with UV detection.[34–38] Nowadays, mass MS coupled to GC or LC is the method of choice for the unambiguous identification of these compounds at trace level in biological matrices. GC-MS with E EI was the first mass spectrometric technique to be applied for those compounds.[39–49] Isotopic ratio mass spectrometry (GC-C-IRMS) was also used to distinguish natural endogenous CoSTs from their exogenous analogues.[50] But further developments in LC-MS[51–54] and LC-MS^n[55–64] with API techniques permitted significant improvements in terms of sensitivity and specificity, as well as kinetic and metabolism studies.[65] The recently

DOI: 10.1002/jms

introduced ultra-performance/fast liquid chromatography systems (UPLC) should be an ultimate reason for considering LC-MS^n as the actual and future standard for corticosteroid analysis.

GC *versus* LC separation

Owing to the moderate polarity of CoSTs, their GC analysis requires a preliminary derivatization step. Currently, chemical oxidation followed by negative chemical ionization (NCI), or classical silylation followed by positive electronic impact ionization remains the most used technique. On the contrary, LC allows direct measurement of CoST. However, the separation efficiency obtained in LC is clearly lower than that obtained in GC, but when coupled to MS or MS^n, the specificity of the MS permits overcoming this problem. LC is also well adapted to the analysis of corticosteroid phase I and phase II metabolites, as well as their esterified forms. Last but not least, very recent developments in the field of UPLC will probably lead to the imposition of LC-based systems for corticosteroid analysis.

EI *versus* CI *versus* ESI ionization

A silylation derivatization reaction (penta-TMS derivative) usually leads to a very low intensity molecular ion and to a large fragmentation of the derivative, with poor final sensitivity. Consequently, this silylation derivatization followed by EI^+ ionization can be used for screening purposes on the basis of full scan or SIM acquisitions and library databases. However, the main limitations of this technique are the derivatization time and its delicate extension to a large number of corticosteroids.

In order to avoid the multiple reaction products observed with silylation derivatization, some authors have proposed the protection of these functional groups with oxime formation before silylation.[49,66] Methoxylamine (MOX) is currently the most used reagent permitting to replace the C═O functions by C═N–OCH_3 groups. Its advantageous is to produce four diagnostic ions with high masses.

Another alternative derivatization procedure is by the use of boronic esters. The produced derivative is very stable, leading to an intense molecular ion for α-γ diol compounds. However, its main disadvantage is once again an insufficient sensitivity for the analysis of trace residue levels.

The last chemical reaction permitting the GC-MS analysis of CoST is the elimination of the polar C_{17} side chain by chemical oxidation and the subsequent oxidation of residual hydroxyl groups to ketone functions. This technique was the most commonly used one before the development of LC-MS^n systems.[31,40,41,44–46,66] In Fig. 11 GC-MS chromatograms of dexamethasone (Dxm) and betamethasone (Btm) and their spectra are shown.

In the NCI mode, two diagnostic ions are detected, $[M - HF]^-$ and $[M - HF - CH_3]^-$. Chemical oxidation coupled to NCI appears to be the method of choice for the detection of CoSTs in GC-MS, as it presents advantages in terms of sensitivity. Nevertheless, the formation of isomeric products, the delicate application to all CoST compounds and the need for unambiguous identification made this technique less interesting compared to the more recent developments in LC-MS^n, at least for confirmatory purposes.

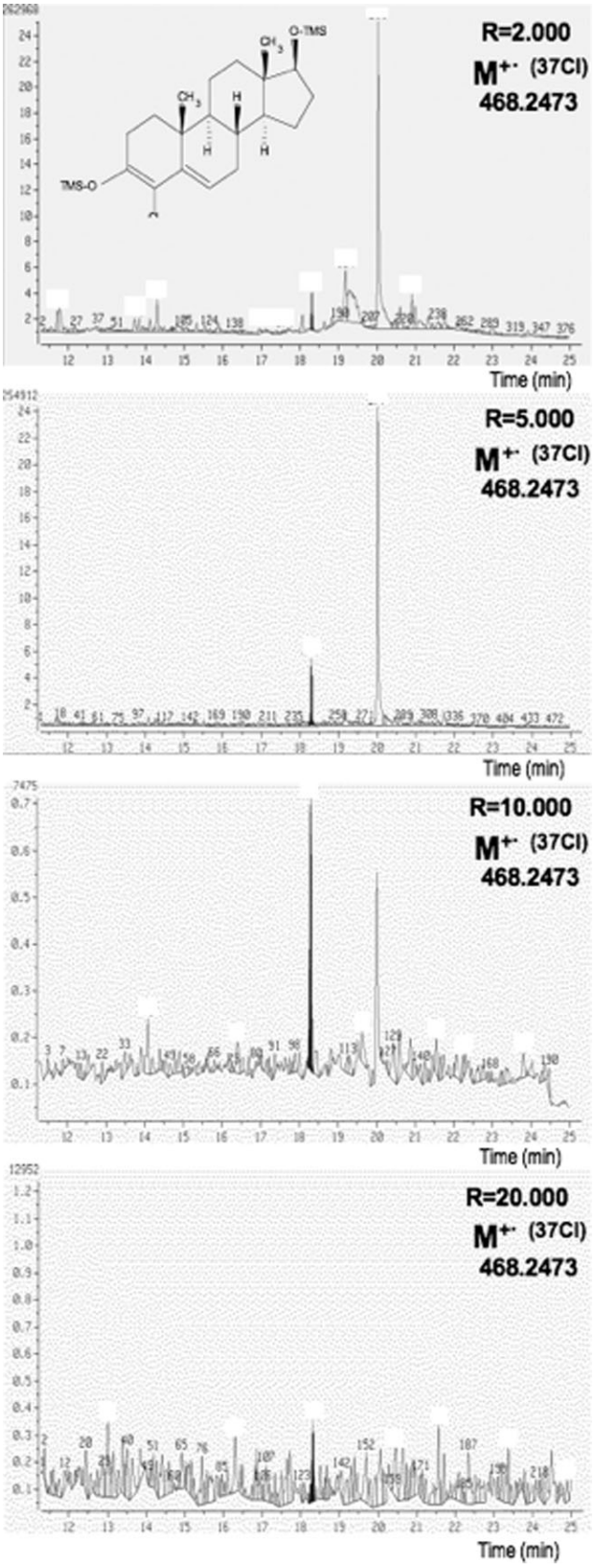

Figure 10. Efficient mass clean-up by increase in resolution of the MS (*R* varying from 2 000 (top) to 20 000 (bottom)).

ESI as well as APCI are clearly well adapted for the analysis of CoST. Under slightly acidic conditions,

DOI: 10.1002/jms

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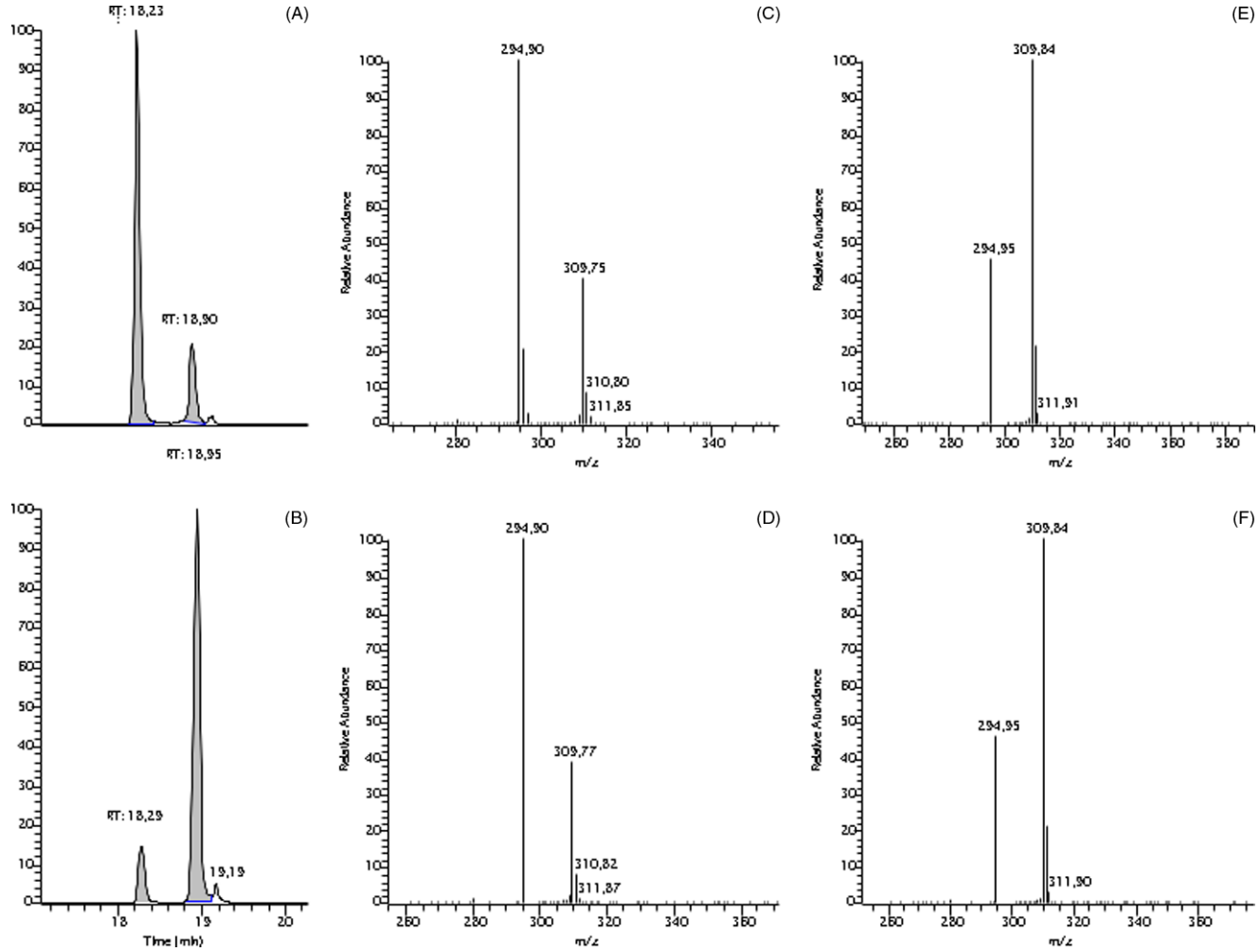


Figure 11. Chromatograms (GC-MS) (shaded zones = peak areas) of (A) dexamethasone (Dxm) and (B) betamethasone (Btm) and spectra of both peaks of Dxm (C and E) and both peaks of Btm (D and F).

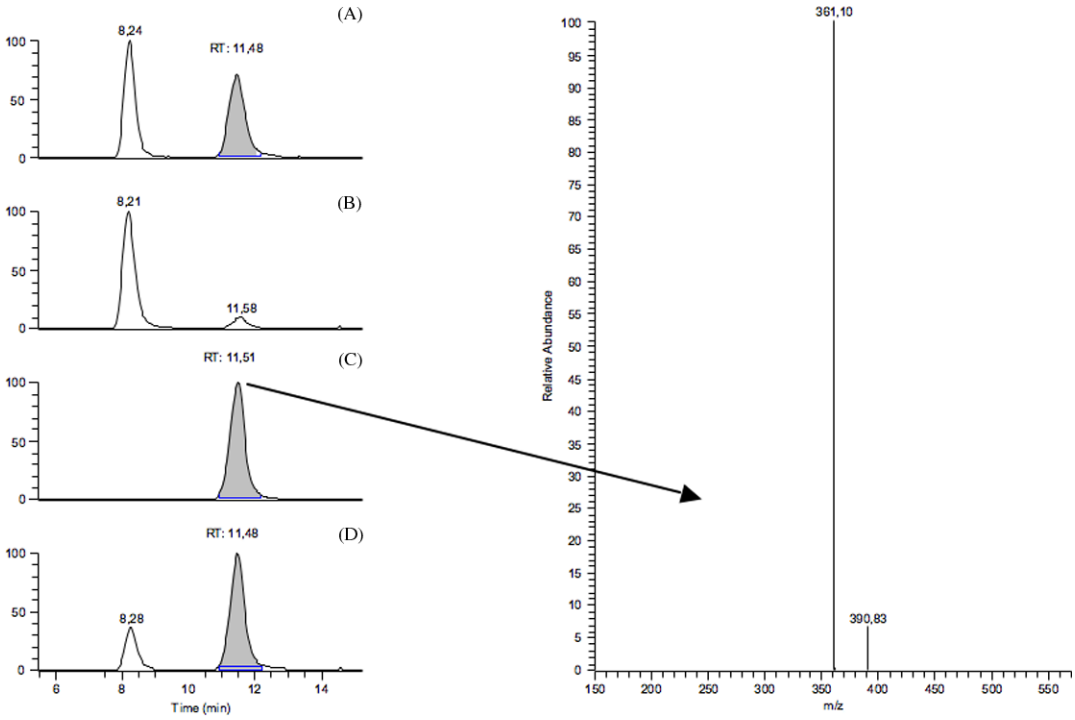


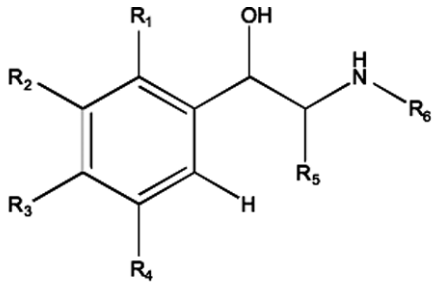
Figure 12. Chromatograms (LC-MS) of (A) standard mixture (1 ng on-column), (B) a fortified ultrapure water sample (40 ng l^{-1}), (C) an unidentified aqueous water sample and (D) the same extract fortified with Dxm. On the right side, the spectrum of betamethasone (Btm) of the 'unidentified' water sample is given.

DOI: 10.1002/jms

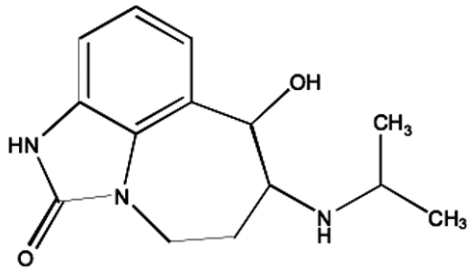
these relatively polar compounds give an intense pseudo-molecular ion $[M+H]^+$ in ESI^+, and an adduct with the conjugated base of the used organic acid $[M+base]^-$ in ESI^-.[55,67] These diagnostic ions are suitable to be selected as precursor ions for further fragmentation in MS.[2] In ESI^+, numerous but not very specific fragment ions can be observed, which correspond to loss of water molecules and/or halogen atoms, as well as to some other minor cleavages within the B and C rings. In ESI^-, the fragmentation is reduced to two ions: the pseudo-molecular ion $[M-H]^-$ and the fragment corresponding to a cleavage of the side chain correlated with loss of formaldehyde $[M-CH_2O-H]^-$. This fragmentation pathway appears extremely efficient for the measurement of a large number of CoSTs at the trace residue level. Finally, APCI techniques operating in the negative mode represent today the technique of choice for the ionization and fragmentation of CoSTs because of its better sensitivity and specificity compared to all other ionization techniques. The chromatographic separation of isomers such as dexamethasone and betamethasone may remain an ultimate analytical challenge. Some specific stationary phases permitting this separation are available today.[68] In Fig. 12 the LC-MS separation of dexa- and betamethasone is illustrated.

But the relative intensities of diagnostic ions produced in ESI can be another way to distinguish these two isomers, using a conventional model[69] of multi-dimensional statistical approach.[70]

Future analytical challenges linked to CoST analysis in the field of food safety includes continuous improvement in terms of sensitivity and target analyte range, as well as structural elucidation of potentially new compounds appearing in the black market. The sensitivity question is probably a less critical issue, considering the very high performances of the more recent mass filters (latest generation of 3D ion trap or triple quadrupoles, new systems like 2D ion trap or Orbitrap, etc.). Regarding mass spectrometric approaches for detecting new compounds, the combined use of LC-API-MS^n and GC-EI-MS^n techniques is certainly the key to success. Indeed, LC-based systems allow very quick and efficient comparative analysis, permitting the detection of



	R_1	R_2	R_3	R_4	R_5	R_6
Cimaterol	H	CN	NH_2	H	H	$CH(CH_3)_2$
Salbutamol	H	CH_2OH	OH	H	H	$C(CH_3)_3$
Terbutaline	H	OH	H	OH	H	$C(CH_3)_3$
Clenproperol	H	Cl	NH_2	Cl	H	$CH(CH_3)_2$
Ractopamine	H	H	OH	H	H	$CH(CH_3)$-$(CH_2)_2$-PhOH
Clenbuterol	H	Cl	NH_2	Cl	H	$C(CH_3)_3$
Tulobuterol	Cl	H	H	H	H	$C(CH_3)_3$
Mabuterol	H	Cl	NH_2	CF_3	H	$C(CH_3)_3$
Brombuterol	H	Br	NH_2	Br	H	$C(CH_3)_3$
Isoxsuprine	H	H	OH	H	CH_3	CH(CH3)-CH2-O-Ph
Fenoterol	H	OH	H	OH	H	$CH(CH_3)$-CH_2-PhOH
Hydroxymethyl clenbuterol	H	Cl	NH_2	Cl	H	$C(CH_3)_2$-CH_2-OH
Mapenterol	H	Cl	NH_2	CF_3	H	$C(CH_3)_2CH_2CH_3$



Zilpaterol

Figure 13. Structures of the most important beta-agonists.

DOI: 10.1002/jms

the presence of potential analytes of interest, while the GC-based system leads to more abundant structural data for unambiguous identification. The distinction between natural (cortisol, cortisone) CoSTs or those suspected to be endogenous (prednisolone, prednisone) and their exogenous homologs, based on carbon isotope ratio mass spectrometry (C-IRMS) or metabolic profile is also an emerging challenge for the future.

BETA ADRENERGIC AGONISTS (GROUP A5) AND THEIR ANALYSIS WITH MASS SPECTROMETRY

Beta adrenergic agonists (BAAs) have been a class of (new) illegal growth promoters since about 1986. These substances act as repartitioning agents because they decrease the fat content of the carcass in favour of a higher percentage of muscle. In the EU these substances are banned, but other countries like the USA, Mexico and South Africa have licensed some of them for animal fattening.[71] Therefore, multi-residue methods are necessary to monitor the abuse of beta-agonists. In Fig. 13 the structures of the most important BAAs are given.

As can be seen, a large variety of BAAs have to be monitored: owing to a small change in the molecule, new illegal BAAs such as clenproperol and mapenterol are brought into the black market. The minimum required performance limit (MRPL) for most of these substances is under preparation by the EU (Table 1).

The requirements for unambiguous identification of the BAAs have led to the large utilization of mass spectrometry as the confirmatory method. GC-MS was historically the most widely used, but today LC-MS appears to be the method of choice. However, after an initial period of great enthusiasm, some problems related to this LC-MS-related technique have started to be reported. One main source of the pitfall was the existence of matrix effects in general, and the ion suppression phenomenon in particular.[72] This phenomenon affects many aspects of the performance of the method, such as detection capability, repeatability and accuracy. The cause of ionization suppression is a change in the droplet solution properties of the spray arising from the

Table 1. MRPL values for β-agonists in urine proposed by the EU Reference Laboratory

Analyte	Matrix	Proposed MRPL ($\mu g\ l^{-1}$ or $\mu g\ kg^{-1}$)
Clenbuterol	Plasma/muscle	0.2
Brombuterol	Kidney/drinking water	–
Hydroxymethylclenbuterol	Urine/lever/faeces	–
Mabuterol	Eyes	2
Mapenterol	Retina/hair	2
Tulobuterol	–	–
Clenpeterol	–	–
Clenproperol	–	–
Chlorbrombuterol	–	–
Cimaterol	Plasma/muscle/	0.5
	kidney/drinking water	–
	urine/liver/	–
	faeces	–
Cimbuterol	Eye	5
Isoxuprin	Retina/hair	5
Fenoterol	–	–
ß-Isoprenaline	–	–
Ritodrin	–	–
Ractopamine	–	10
Clencyclohexerol	Plasma/muscle/kidney/drinking water	1
Salbutamol	urine/liver/faeces	–
Salmeterol		–
Zilpaterol	Eye	5
Orciprenalin	Retina	5
Metaproterenol = orciprenaline	Hair	
Terbutalin	Urine	3
	Retina/liver/muscle	10
	Urine/liver	3
	retina	10
	Muscle/kidney	3
	Drinking water/faeces	3
	Hair	3

DOI: 10.1002/jms

presence of co-eluting non-volatile or less volatile solutes. Polar compounds such as BAAs seem to be particularly susceptible to ion suppression.

Therefore, one should observe an actual tendency to reconsider the necessity of an improved sample preparation before LC-MS analysis in order to minimize these types of problems.

Clean-up methods described for LC-MS analysis of BAAs are mainly based on mixed phase SPE.[73–81] These SPE procedures have been found to be selective not only for BAAs but also for other basic drugs. The use of MIPs for the sample clean-up of beta-agonists has been investigated, demonstrating the importance of clean-up prior to sophisticated hyphenated techniques.[82,83]

A mixture of 15 beta-agonists was spiked into blank calf urine at their proposed MRPL concentrations. With Clean Screen Dau (CSD) SPE columns all beta-agonists could be detected at the MRPL level, but the signals for zilpaterol and terbutaline were weak and subjected to significant interferences. After clean-up with MIP columns, all the beta-agonists could be detected at the MRPL level according to the 2002/657/EC decision criteria (Fig. 14). Recoveries for the different beta-agonists using MIP clean-up are in the range 40%–70%, except for zilpaterol, salbutamol and terbutaline which have recoveries below 40%.

The evaluation of ion suppression in LC-MS^n is carried out as follows: a standard solution containing the analyte of

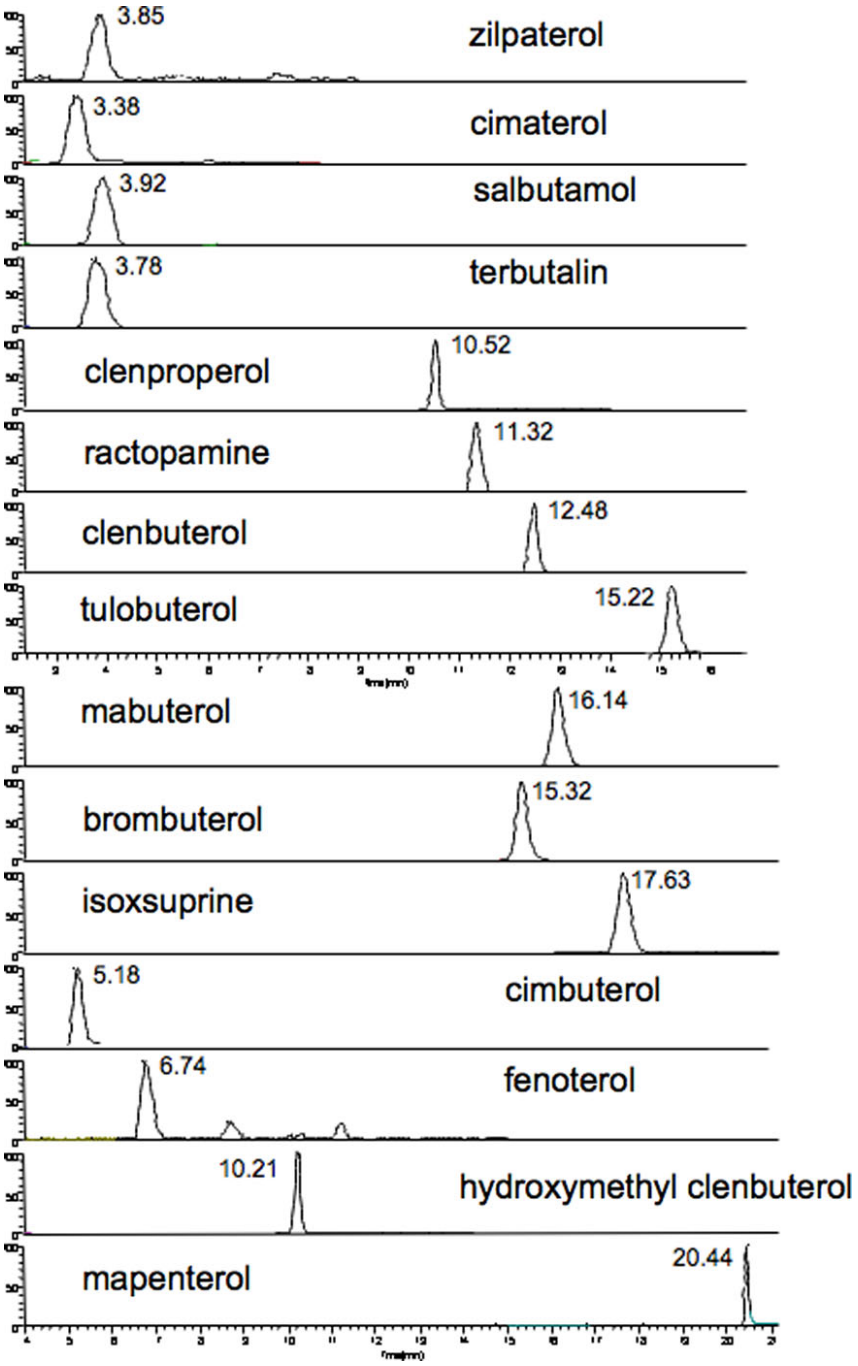


Figure 14. Ion chromatograms of the different beta-agonists at MRPL concentration in calf urine using MIP clean-up.

DOI: 10.1002/jms

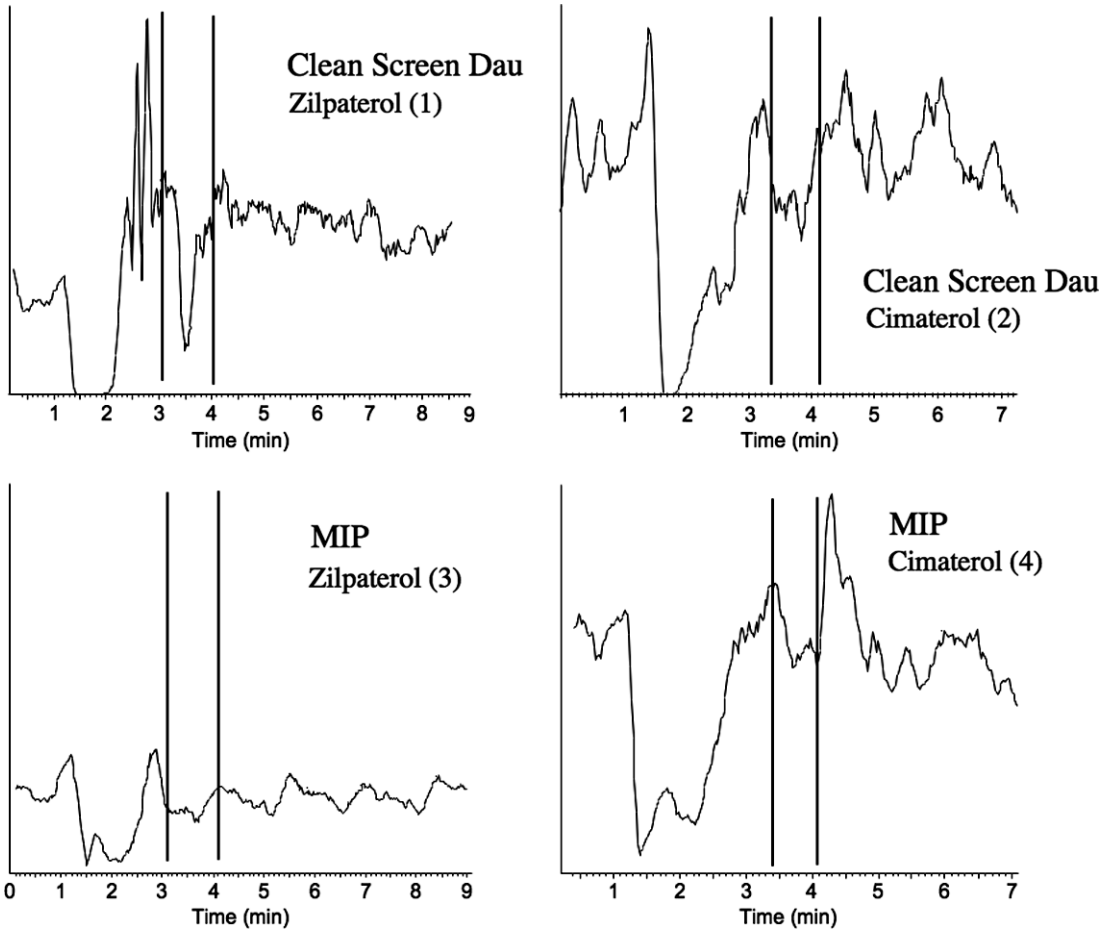


Figure 15. MS/MS signals for zilpaterol (1 and 3) and cimaterol (2 and 4) detected using continuous infusion of zilpaterol or cimaterol and LC injection of blank calf urine samples after clean-up using either CSD (1 and 2) or MIP (3 and 4).

interest is continuously infused through a T-coupling system, mixed with the LC eluate and passed into the mass spectrometer interface. The resulting signal recorded by the mass spectrometer is the net result of these two solutions. Because the analyte is introduced into the mass detector at a constant rate, a constant ESI response should be observed. This is the case when a pure mobile phase is injected into the LC. When an extract of blank urine is injected into the LC system, the resulting TIC increases because of the new material arriving in the interface, and the product signal of the analyte decreases in certain retention time regions as a result of the negative influence of interfering compounds eluting at these retention times.

As an example, an experiment is described for the beta-agonists zilpaterol and cimaterol. These BAAs elute at around the same retention time. The signals for zilpaterol and terbutaline are weak after clean-up with CSD. First, the standard solution and a spiked urine sample at the proposed MRPL concentration were injected to obtain the retention time of the analytes. Subsequently, the pure mobile phase was injected while the analyte was continuously infused. Finally, to evaluate ion suppression blank urine was injected while the analyte was infused. Figure 15 shows the data obtained by the injection of blank urine extracts obtained after clean-up with CSD and after clean-up with MIP while continuously infusing zilpaterol and cimaterol.

After clean-up with CSD no significant suppression was observed for the product signal of cimaterol near its expected retention time (RT = 3.1 min). However, severe ion suppression appeared for zilpaterol (RT = 3.6 min), i.e. in the time window in which zilpaterol elutes there was a serious decrease of the zilpaterol signal due to the interfering compounds that also eluted in this retention time window. In contrast, after clean-up with MIP there was no significant suppression of the signals for either zilpaterol or cimaterol in the time windows in which each analyte elutes.

This experiment shows that CSD sample clean-up could lead to underestimation of the concentrations of some BAAs and could lead to a potential risk of false compliant results. A possible solution to overcome false compliant results is the use of an adequate internal standard, preferably an isotope-labelled internal standard, in order to correct for the ion suppression effect. Of course, this is possible only when the ion signal is not suppressed completely.

This example illustrates that MIPs are very promising for sample clean-up for BAAs and that an adequate sample clean-up is necessary for fully validated quantitative assays even with modern hyphenated techniques.

CONCLUSIONS

The objective of this paper was to review the past, present and future of mass spectrometry in the analysis of residues

DOI: 10.1002/jms

Table 2. Number of identification points that each of the mass spectrometric techniques can earn (2002/657/EC)

MS technique	Identification points earned per ion
Low-resolution mass spectrometry (LR)	1.0
LR-MS^n Precursor ion	1.0
LR-MS^n Transition products	1.5
High-resolution mass spectrometry (HR)	2.0
HR-MS^n Precursor ion	2.0
HR-MS^n Transition products	2.5

of banned substances in meat-producing animals. This is a very broad area and therefore this paper is restricted to methods for illegal growth promoters ('hormones'), such as thyreostats, estrogens, gestagens and androgens, corticosteroids and beta-agonists. The legislation as well as the attitude of the consumers towards these substances is totally different in Europe and in the US and some other countries. In the US, some of these substances are allowed in animal fattening and so little research is being carried out on analytical methods for the detection of the residues. The debate on the safety of some of these substances is still going on but a very recent publication (2007) shows that in the US sperm concentration of boys is inversely related to the mothers' beef meals.[84] These data suggest that maternal beef consumption, and possibly xenobiotics in beef, may alter a man's testicular development *in utero* and adversely affect his reproductive capacity. Therefore reliable analytical methods for the detection of residues of 'hormones' are more than necessary.

Mass spectrometry, hyphenated with a separation technique, is a very powerful tool for the analysis of these substances. During the 1990s the classical selected ion monitoring (SIM) technique was more and more complemented by other techniques such as full scan at low concentrations (as in ion trap systems), MS-MS, MS^n and HRMS. Since most of the positive (non-compliant) hormone cases end up in court, there is a need for internationally recognized criteria for the presence (or absence) of banned substances.

In the Commission decision of August 12, 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC), a system of identification points (IPs) is introduced to interpret the data when mass fragments are measured using methods other than full-scan techniques.[6,85] For the confirmation of banned substances (Group A) a minimum of four IPs are required. In Table 2 the number of IPs that each of the mass spectrometric techniques can earn is given. A minimum of at least one ion ratio shall be measured, and all relevant measured ion ratios shall meet the criteria for relative ion intensities (Table 5 in 2002/657/EU). A maximum of three separate techniques can be combined to achieve the minimum number of IPs. When full-scan spectra are recorded in single mass spectrometry, also at least four ions shall lie within the maximum permitted tolerances for relative ion intensities. The 2002/657/EC offers the analyst objective criteria for the demonstration of the presence of a banned substance.

However, even while using this criterion it was found very dangerous to consider GC or LC-MS as absolute, error-free techniques.[86,87] As in any other analytical technique, false negative (compliant) and false positive (non-compliant) results as well as wrong quantification can be obtained. False negative (compliant) results may arise from the disturbance of the normal peak ratios of the ions from the analyte by one or more isotope peaks from one or more interferences. In the study of contradictory results (in a second analysis in a second lab) this effect must always be considered; by the use of slightly different methods (different columns, reagents, etc.) different interferences from the same matrix may be present in the final extract. When all the ions corresponding to an analyte are present, but one or more ratios are out of range, additional tests (e.g. other chromatographic conditions, other clean-up) must be performed. False positives (non-compliants) may result when the diagnostic ions do not originate from the analyte but are generated by one or more interferences present at high concentration in the final extract. The fact that the correct ion ratios can be produced from the interfering (endogenous) compounds is obvious to the analyst when using the GC-MS in the SIM mode. False positives (non-compliants) may also be obtained in the MS-MS mode when the precursor is not correctly selected (e.g. an abundant fragment ion with lower mass). In that aspect the 2002/657/EC is clear: when mass spectrometric determination is performed by fragmentography, the molecular ion shall preferably be one of the selected diagnostic ions and the selected diagnostic ions should not exclusively originate from the same part of the molecule. Especially, when derivatization is used, care should be taken on that aspect of the identification criteria. Third, wrong quantification may occur by disturbance of the ions of the analyte or of the internal standard. Quantification of residues may be very important, especially in the neighbourhood of a decision limit.

The improvement of the limit of detection of MS methods has revealed the natural presence of substances formerly considered of exogenous origin (e.g. nortestosterone, boldenone, thiouracil)[19,86] and may do so in the future. This complicates and extends the detection of the abuse of natural hormones (e.g. testosterone, estradiol). The discrimination between 'natural-natural' substances and 'synthetic-natural' ones needs other MS techniques such as C-IRMS. Another approach to screen for the abuse of hormonal substances is the establishment of 'normal' hormonal profiles (or so-called 'standard' animals). The perturbation of this profile may indicate the presence of a hidden substance, and in quality systems (labelling) such animals could be rejected until the owner is able to prove otherwise. However, this is much more complicated than a YES/NO answer from an analytical technique only.

When the analyst is aware of and able to accept the possible causes of these errors, the application of some simple rules and the investment of a little more time in analyses may prevent most of these pitfalls. A golden strategy is to consider a first 'non-compliant' result as a 'suspect' result and to repeat the complete analysis immediately with (preferably) another technique (within the laboratory

DOI: 10.1002/jms

or between laboratories). Only when the two successive results match, qualitatively as well as in the magnitude of the concentration, the result is totally reliable. Even then, an open mind for the follow-up of the results of a second analysis in an independent laboratory is necessary. An analytical result obtained by mass spectrometry complying with all aspects of the international criteria (e.g. 2002/657/EC) and confirmed in a second laboratory is ready for a trial in a court.

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