UNIVERSITEIT
GENT

PITFALLS, METHODS and CRITERIA
in the ANALYSIS of RESIDUES and CONTAMINANTS
in BIOLOGICAL MATERIAL

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CONTENTS

ABBREVIATIONS

ADD	1,4-androstadiene-3,17-dione
a.m.u.	atomic mass unit
analyte	substance to be detected
And	Androsterone
CCα	Decision limit (2002/657/EC)
CCβ	Detection capability (2002/657/EC)
EGAs	Estrogens, gestagens and androgens
EI	Electron ionisation mode
FAVV	Federaal Agentschap voor de Veiligheid van de Voedselketen
g	gram
GC	Gas chromatography
GC-MS	Gas chromatography - mass spectrometry
$GC\text{-}MS^n$	Gas chromatography - tandem mass spectrometry
HPTLC	High performance liquid chromatography
I.D.	Internal diameter
IP	Identification point (2002/657/EC)
LC-MS	Liquid chromatography - mass spectrometry
$LC\text{-}MS^n$	Liquid chromatography - multiple mass spectrometry
LOD	Limit of detection
LR-MS	Low-resolution mass spectrometry
m	meter
M	Mole
mg	milligram (10^{-3} g)
min	minute
ml	milliliter (10^{-3} l)
mm	millimeter (10^{-3} m)
μm	micrometer (10^{-6} m)
μl	microliter (10^{-6} l)
MRL	Maximum Residue Limit
MRPL	Minimum Required Performance Limit (2002/657/EC)
MS	Mass Spectrometry
MSTFA	N-Methyl-N-(trimethylsilyl)-trifluoroacetamide

m/z	Mass-to-charge ratio
ng	nanogram (10^{-9} gram)
OD	Optical density
pg	picogram (10^{-12} gram)
PL	Permissible level (2002/657/EC)
ppb	parts per billion (μg/kg)
ppm	parts per million (mg/kg)
ppt	parts per trillion (ng/kg)
SPE	Solid phase extraction
SIM	Selected ion monitoring
S/N	Signal-to-noise ratio
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl

CHAPTER I: INTRODUCTION AND AIM

A question to be asked first is WHY????
Why should somebody at a blessed age (57 years old) after a doctorate (PhD) in chemistry and a "aggregaat hoger onderwijs in de analytische chemie van eetwaren" (some kind of second PhD in analytical chemistry of food), go for a doctorate again?

The reasons are multiple: first of all I want to express my affinity with the Faculty of Veterinary Medicine of Ghent University. Indeed it was the Faculty of Veterinary Medicine who offered me a job after my first doctorate in chemistry. With its ups and downs I was able to have a full career within the Faculty from assistent to full professor. I was able to make many friends from the Faculty staff, from relations of the Faculty and the laboratory with related services. Especially the cooperation with the inspection services (Institute of Veterinary Inspection, the ministry of Agriculture; now together in the FAVV (Federal Agency for the Safety of the Food Chain) was very strong. A lot of veterinary inspectors were closely encountered during the specialised course in veterinary food inspection.

Secondly, our rector, Andreas De Leenheer, is well known to be very research-minded. Strong impulses were given to encourage research at our University. However, scientific research is often measured in terms of number of doctorates only. For older post-docs, like me, this may give the impression that the research period is over after the doctorate - except for guiding other doctorates of course. The best way to prove that there is still research after a doctorate is making a doctorate again, this time in veterinary sciences. The current regulations in Flanders allow or did not forbid multiple doctorates.

Of course this will not be the doctorate of a young researcher, dedicated to one apparatus or a single subject over a limited period of time (4 – 6 years). It will more be like the long time work of a manager, responsible for the point of view and the final results of a laboratory. During the period covered (1990- 2003) the Laboratory of Chemical Analysis (LCA) of the department of Veterinary Food Inspection has grown from a small HPTLC unit to a laboratory using 5 modern MS^n instruments coupled to the neccessary clean-up procedures. Subsequently, the staff has grown from 4 to 16 people, all occupied with education, research and/or service to the community at a University level. The department is accredited under the ISO 17025 with - for LCA - a very flexible scope in analysis of residues and contaminants.

A trace is a substance, component, or analyte present in a small concentration in another material called matrix. There is no general agreement on the concentration level of a trace.

With increasing detection power of the analytical techniques this “trace” border continuously shifts to lower concentrations. In most cases the concentration range is expressed in ppm (μg/g) or ppb (ng/g) units or even lower (ppt (pg/g)).
A residue may be defined as a trace of a substance, which remains in a matrix after some kind of administration. Some examples are: an animal is injected with methyltestosterone. In meat residues of methyltestosterone may be detected.
Blood contains traces of testosterone (no residues of testosterone).
The formation of residues may be as well due to the production as to the manufacturing process. In most cases humans are the basic cause of residues and contaminants.

Residues may be divided in different groups in different ways. A simplified approach is given below.
A. Banned substances such as “hormones” (group A substances)
B. Veterinary Medicinal products such as antibiotics (group B substances)
C. Contaminants such as PCBs and dioxins (environmental contaminants)

Banned substances are mostly indicated by the word "hormones". This is too big a simplification. The growth promotors abused in animal fattening may be divided into four major groups :

Thyreostats (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 (e.g. Diethylstilbestrol, nortestosterone) increase weight gain and carcass quality. These substances are also called EGA’s (Estrogens, Gestagens and Androgens).

Beta-agonists or repartitioning agents (e.g. clenbuterol) cause also weigth gain in most slaughter animals. Moreover, the amount of muscle tissue (meat) increases and the amount of fatty tissue decreases.

Corticosteroids (e.g. dexamethasone) cause an extra weight gain most probably caused by water retention.

Veterinary Medicinal Products (VMP's) on the other hand are necessary for the treatment of animals in a veterinary practice. A number of these VMP's are extensively tested for food safety. On the website of EMEA (the European Agency for the Evaluation of Medicinal Products) (http://www.eudra.org) the MRLs (**M**aximum **R**esidue **L**imits) of VMP's in food may be found.
When the MRL is exceeded a "R" (Residue) label is put on a farm : during a certain period the farm is controlled extensively, on its own expenses. When a forbidden product is found a "H" (Hormone) label is given: the farm is controlled extensively but for a longer period.

Contaminants may be present in food on an unwanted or unforeseeable way. With illegal growth promotors and VMP's mostly an individual or a company is or may be responsible. In contrast, the detection of contaminants must be coupled to a responsibility of a community, e.g. towards the environment or the total food chain. Examples are the BSE (Bovine Spongiform Encephalopathy) and PCB (PolyChlorinated Biphenyls) crisis. It is not always clear, according to the circumstances, if one should speak about residues or contaminants or of residues of contaminants (e.g. the MPA crisis). Food crisisses, as the dioxine or PCB crisis may cause food scare with considerable economical consequences.

The problem of residues is a complex matter. More information may be found on the website of our department (http://allserv.ugent.be/~hdbraban) where easy links may be found to sites containing a lot of information. (e.g. the websites of the FAVV (Federaal Agentschap voor de Veiligheid van de Voedselketen) and BELTEST and of scientific organisations as BAMST (Belgian Association of Meat Science and Technology), WAVFH-Flanders (World Association of Veterinary Food Hygienists), KVCV-Food Division (Koninklijke Vlaamse Chemische Vereniging) and other websites as those of Quality Control.

Residue analysis is a key factor in the field of chemical contaminants in food of animal origin. The aim of this work is the production of analytical results for residues and contaminants in biological material. These results must be acceptable **"beyond any reasonable doubt"**.
Indeed, analytical results are challenged more and more by attorneys (lawyers, in most cases knowing very little of analytical chemistry). In 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 place for the "art of science" and the "analytical experience" of an individual.
A very common practice in challenging results is the second analysis of samples in a second independent laboratory. Contradictions between laboratories are mostly negative or even fatal for actions of inspections services, whatever the reason for that contradiction may be.

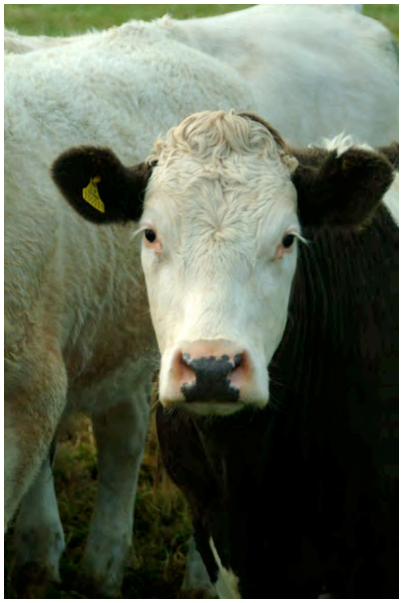
The first chapter of the body of this work consists therefore of pitfalls. A pitfall may be defined as: *a hidden or not easily recognized danger, error, or source of injury or destruction into which one that is unsuspecting or incausious may fall* (Merriam-Webster dictionary).
A first pitfall is pure mass spectrometry: isotope interference in selected ion monitoring. It shall be demonstrated that the large difference of concentration range between a residue (ppb?) and components of the matrix (ppm or higher) may cause interferences. Being aware of the possibility of this kind of interference is a "conditio sine qua non" for a lab using mass spectrometry.
A second pitfall concerns impurities in "bulk" chemicals. For most people a bottle, labelled with a name, contains only one product. However, commercial "bulk" chemicals (as pharmaceutical products) are seldom very pure. Some products may contain up to 5 % other mostly related chemicals. The chemicals may not interfere with the normal use of the product. However, they may interfere in residue analysis, as shall be demonstrated with an example (progesterone).
The third pitfall describes naturally present "thought to be exogenic" compounds. Nortestosterone serves here as an example but other molecules as boldenone are also under study these days. Nortestosterone was first found to be endogenic in the stallion during doping control. Later the boar (male pig), the pregnant cow and other animals followed. A recent case of a pitfall was the presence of endogenic nortestosterone in a pig intersex. The study of this phenomenon was a beautiful example of multidisciplinary cooperation between inspection services, morphologists and analytical chemists at our faculty. The results have been published recently in the Flemish Journal of Veterinary Sciences (Vlaams Diergeneeskundig Tijdschrift).

The knowledge of the existence of possible pitfalls is a "must" for an analyst of residues. Armed with that knowledge methods and criteria may be designed for avoiding "false positive" and "false negative" results and to improve the concordance of results between laboratories. The second chapter of the body therefore regards methods. Hereby the evolution of methodology between 1990 and 2002 is described. In 1973, when our laboratory was started by R. Verbeke, TLC (Thin Layer Chromatography) seemed the method of choice for the analysis of thyreostats and anabolics (now called EGA's: Estrogens, Gestagens and Androgens). The reasons therefore were the specificity, the simplicity and the possibility of reaching low limits of detection (magnitude 1 - 10 ppb (μg/kg)) for an acceptable budget. Very soon TLC was replaced by HPTLC (High Performance TLC). During the 90's more and more affordable GC-MS (Gas Chromatography – Mass Spectrometry) apparatus appeared on the market and the transition from TLC to GC-MS and further on the GC-MS-MS and even MS^n methods was ongoing.

In 1996 LC-MS was introduced in our laboratory. My co-worker, Katia De Wasch started this unit from "zero" and described parts of the results with LC-MS in her doctorate in 2001 (The use of LC-MS^n for the determination of residues of growth promotors and veterinary drugs). The aim of the chapter is showing how our lab has followed the ongoing evolution in analytical instruments and methods evolving from this.

The third chapter of the body is on analytical criteria. The basic criteria are described in the EC regulation 93/256/EEC of 14 april 1993 laying down methods to be used for detection residues of substances having a **hormonal** or **thyreostatic** action. However the continuous evolution in analytical possibilities made a revision of those criteria necessary. A EU expert group was formed and chaired by Prof. Francois André (Nantes, France). D. Courtheyn from the State Laboratory in Gentbrugge and myself were the two Belgian delegates in the European expert group who revised the criteria. The work of the expert committee was than adjusted by the Community Reference Laboratories (CRL's). Prof. R. Stephany (Utrecht, the Netherlands) played an important steering role in that process. The expert report was transformed to a real law by G. Gallhoff and her co-workers of the EU. This resulted, over a SANCO/1085/2000 document, with the publication in august 2002 of the 2002/657/EC. However, one must realise that with this publication, a new revison of the 2002/657/EC should start immediately.

Pitfalls, methods and criteria are clearly linked together. The aim of this work is to demonstrate the links and try to give some answers on avoiding pitfalls in the future.



CHAPTER II: PITFALLS IN RESIDUE ANALYSIS

2.1. PITFALLS IN MASS SPECTROMETRY

After

Pitfalls in Selected Ion Monitoring in GC-MS, a theoretical example

H.F. De Brabander, P. Batjoens, C. Vanden Braembussche, P. Dirinck, F. Smets, G. Pottie,

Anal Chim Acta, 275 (1993) 9-15.

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1. Introduction

For the routine analysis of residues of growth promotors in meat producing animals there is an increasing use of hyphenated techniques. In most cases a low resolution mass spectrometer coupled to a chromatographic separation is used (1, 2, 3, 4). In low resolution GC-MS two important apparatus may be distinguished: the Ultra-Trace Full-Scan GC-MS (e.g. the Ion Trap Mass Spectrometer (ITS40)) and most other quadrupole apparatus using full-scan for high amounts of analyte and the "Selected Ion Monitoring" (SIM) mode for detecting low amounts (< 1- 10 ng). Both techniques have their pro and cons and also their own fans (5).

With SIM a limited number of ions (2 - 4 ions) are monitored during a selected time interval. The presence of the analyte is determined by the presence of these "diagnostic" ions at the correct retention time and within the correct abundance ratio (between certain limits (6)). In the EEC guidelines the monitoring of 4 ions is advised (6). In practice, the monitoring of 4 ions at low concentrations (< 1 ppb) does not give satisfactory results (7). Since monitoring of only two ions is certainly not sufficient according to the quality criteria, a decision on 3 ions seems an acceptable analytical compromise (7). In this investigation SIM on 3 ions is taken as a rule.

With the Ion Trap Mass spectrometer (ITS40) the whole mass spectrum is stored for each point of the chromatogram (e.g. 1 spectrum per second). Afterwards, full-scan identification of the analyte by library search may be performed with the data system, while recording a new acquisition. The manufacurers of the ITS40 system claim a full-scan identification of components at (at least) the 10 pg level.

In extracts of biological materials (e.g. urine, meat) a large variety of components in a large variety of concentrations is present. Unknown and variable amounts of these matrix components are co-extracted with the analyte. With GC-MS analyses in the SIM mode these interferences are not observed by the highly selective use of the detector. Interference between these matrix components (present at relatively high concentrations (ppm range or higher)) and analytes (present at very low concentrations (ppb range)) should be avoided while using SIM.

In this investigation a theoretical example of a pitfall in SIM by isotope interference (^{13}C) from matrix components with the analyte is shown. This theoretical example is intended as a thinking exercise on the qualitative accuracy of SIM. It is the result of research about the origin of the positive signal for nortestosterone by RIA and SIM in the urine of pregnant cattle (8, 9). This signal, still open to discussion, could be caused by the aspecificity of the antibody against nortestosterone for the RIA analysis. For SIM analysis ^{13}C isotope interference with the high concentrations of estradiol, present in the urine of these animals could be possible.

2. Stable Isotopes: calculation of isotope peaks

The knowledge of the relative abundances of the stable isotopes is essential for the interpretation of mass spectra (10). However, a short review is given for those who are not familiar with this subject. Chlorine has two isotopes: ^{35}Cl and ^{37}Cl with an abundance ratio of 3 to 1 (mean atomic mass: 35,453). For a molecule containing one Cl atom two peaks separated by two amu and with an abundance ratio of 3 to 1 are observed (for the molecular ion and the fragments containing Cl). When a molecule contains 2 Cl atoms 3 peaks were observed in the mass spectrum. The total amount of isotope-peaks of Cl is given by (n+1) and their relative abundance by the formula: $(100)*(1 + 0.333)^n$ with n the number of Cl atoms. In Table 1 these isotope ratios for molecules containing 1 to 3 Cl atoms are summarized.

Table 1: Ratios of the isotope-peaks of Cl

n				
1	100	33		
2	100	66	11	
3	100	100	33	4

n = number of chlorine atoms

Isotope peaks may be very specific for the identification of residues (e.g. Cl containing growth promotors as Clenbuterol). They may also be important for the determination of the fragmentation pattern and metabolisation of these molecules.

3. Peaks of low relative abundance

For the other elements analogous calculations can be made. In Table 2 the most important atoms in organic molecules are given together with their most important natural isotopes.

Table 2: Most important elements in organic molecules and their natural isotopes

Element	MM	Isotopes *		
H	1.00797	1.00783 (99.985)	2.01410 (0.015)	
C	12.01115	12.00000 (98.89)	13.00335 (1.11)	
N	14.0067	14.00307 (99.63)	15.00011 (0.37)	
O	15.9994	15.99491 (99.76)	16.99913 (0.04)	17.99916 (0.20)
S	32.064	31.97207 (95)	32.97146 (0.76)	33.96786 (4.22)

*(Abundance in %)

Isotope interference may occur with any isotope with a relative high abundance. In this investigation ^{13}C is studied. Carbon has two natural isotopes: ^{12}C and ^{13}C with a ratio of 98.9 to 1.1 (the exact figures are rounded for simplicity). In residue analyses two other important parameters should be taken into account:

1) The very large difference in concentration between the analytes and the matrix components.
2) The analyte (an organic molecule) contains a relative high amount of carbon atoms. The components, co-extracted from the matrix may also contain a substantial amount of C atoms. These components may have analogous structures as the analytes (e.g. in anabolizing agents as steroids 20-30 carbon atoms are present and numerous steroids and metabolites with analogous structures are known).

The number of the isotope peaks produced by n carbon atoms is given by (n+1) and the relative abundance of these isotope peaks by the formula: $(100)*(1 + 0.0111)^n$
(with n the number of carbon atoms).
The general equation of this formula is given by the binomial distribution of Bernoulli:

$$(A+ B)^n = A^n + C^{n-1}_n * A^{n-1} * B + C^{n-2}_n * A^{n-2} * B^2 + ...C^1_n * A * B^{n-1} + B^n$$

For residue analysis the first three terms of this equation are the most important ones.
As an example, the equation is worked out for a component with 20 carbon atoms:

				Σ
First term:	the chance on "no" ^{13}C:	$(0.989)^{20}$	80.15 %	
2nd term:	the chance on "1" ^{13}C:	$C^{19}_{20}*(0.989)^{19}*(0.011)$	17.83 %	97.98 %
3rd term:	the chance on "2" ^{13}C:	$C^{18}_{20}*(0.989)^{18}*(0.011)^2$	1.88 %	99.86 %

The chances on "no", 1 and 2 ^{13}C in a molecule containing 20 carbon atoms are equivalent to the relative abundances of the peaks A, A+1 and A+2. Here A stands for the mass calculated on ^{12}C only. The ratios of these peaks to the "parent" or "generating" peak are 100/22 /2.3.
A mass spectrum of a component with 20 carbon atoms is given in Fig 1.

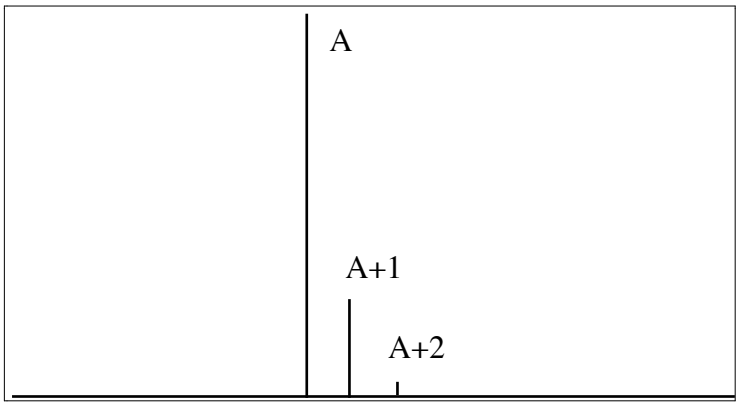

Fig 1: Spectrum of a component (ion) with 20 carbons

The sum of the first three peaks yields 99.9 % of the total abundance. The ion A+3 has a very low abundance for moderate carbon numbers (< 0.2 %) and is neglected here together with all the other peaks. Theoretically, the number of carbon atoms of a component could be calculated from the number of the isotope peaks and their relative intensities.

In Table 3 the results of the calculation of the relative abundances of the "A+1" and "A+2" ion in function of the number of C atoms of a molecule are given.
In this table it should be noticed that the relative abundance of the ion " A+1" is linearly related to the number of C atoms n multiplied by 1.11. The relative intensity of the " A+2 " ion in function of the number of carbon atoms is not linear and given by n*(n-1)*0.00616. This relationship is expressed in Fig 2. This graph shows clearly that the relative abundance of "A+2" increases considerably at high carbon numbers.

Table 3: Relative abundances of the isotope peaks to the generating ion A in function of the number of C atoms (%, rounded).

n	A	A+1	A+2
1	100	1	0
5	100	6	0.1
10	100	11	0.6
15	100	17	1.3
20	100	22	2.3
25	100	28	3.7
30	100	33	5.4

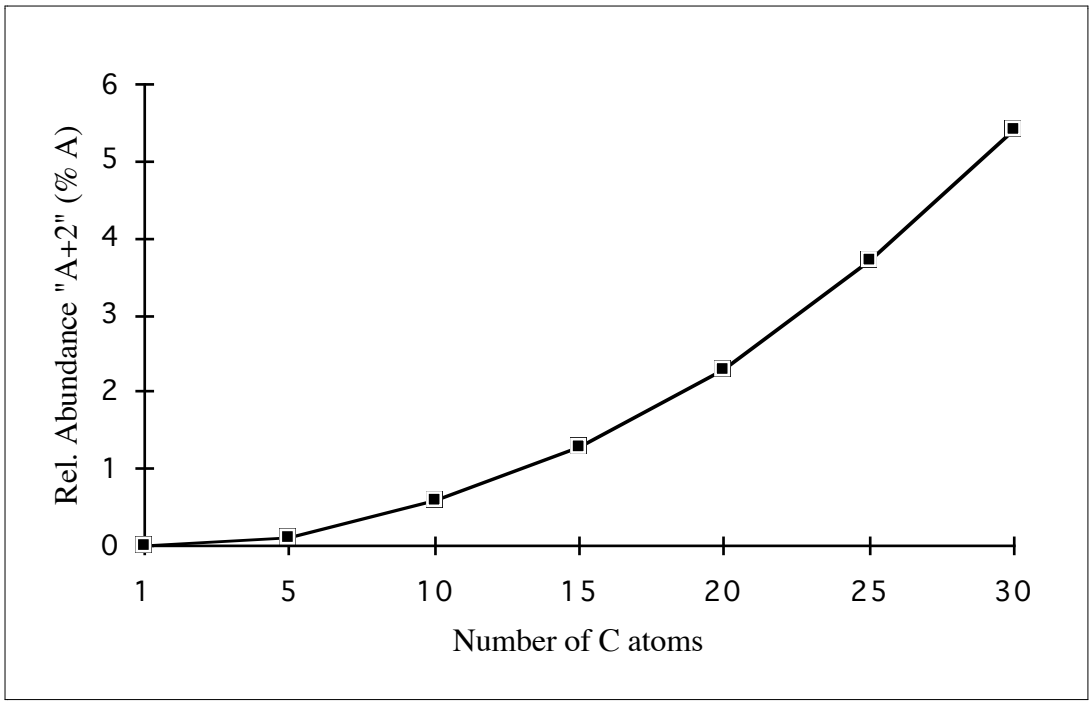

Fig 2: Relative abundance of the ion " A+2 " to the generating ion " A " in function of the number of carbon atoms n

4. Theoretical examples in residue-analysis

The presence of interferences, co-extracted with the analyte from the matrix, can cause the following phenomena: false positive and negative results and wrong quantification. The examples, given below are purely theoretical. However, the possibility of their occurrence is not imaginary and may be demonstrated with practical examples.

4.1. False positive results

A laboratory wants to determine the analyte NT in the matrix U with GC-MS at the 0.1- 1 ppb level. This is a very realistic situation: different authors claim to be able to determine nortestosterone at this level with GC-MS in the SIM mode (7, 8). In this investigation only a theoretical example is worked out.
The (theoretical) characteristics of the GC-MS SIM analysis are: retention time of the analyte = approx. 15 min. Three ions are followed during the time interval 14 -16 min: 418 (100 %), 403 (20 %), 328 (35 %). The mass spectrum of the analyte NT, reduced to the three diagnostic ions is given in Fig 3A.

In the matrix U a component E (an interference) is present in concentration of 10 ppm (10^4 - 10^5 times higher than the concentration of the analyte). The time of retention of the interference E is nearly that of NT (e.g. 14 min 58 sec) The mass spectrum of this component is given in fig 3B. In this mass spectrum only the ions 416, 402 and 326 are important for isotope interference with the analyte. The ion 402 has a very low abundance (ca 3 % of 416) and is nearly invisible in figure 3B. This ion is of no importance for the mass spectrum of E. However, its influence on the analysis of NT is not negligible and will be demonstrated below.

The exact number of C atoms of the interference E is not important, only the exact number of C atoms of the fragment ions is taken into calculation. These (theoretical) numbers of carbon atoms and the relative intensities of the isotope peaks generated are given in Table 4 (as derived from Table 3 and Fig 2). The bold figures represent the abundances, which are important for this example.

Table 4: Number of C atoms of the fragments and the relative abundance of the isotope peaks generated by these fragments (relative to generating peak)

ion	C number (n)	A+1	A+2
416	28	31	**4.7***
402	27	**30***	4.3
326	22	24	**2.9***

*only the figures in bold are important for this example

Since the interference E has a concentration of 10 ppm and the fragmention 416 forms ca 10 % of the total spectrum this ion is attributed a relative concentration equivalent to 1000 ppb. The ions 402 and 326 show relative concentrations of resp 28 and 500 ppb (their ratio to 416 is resp 2.8 % and 50 %). From these three ions isotope peaks are generated according to the ratios given in Table 4 and shown in Fig 4.

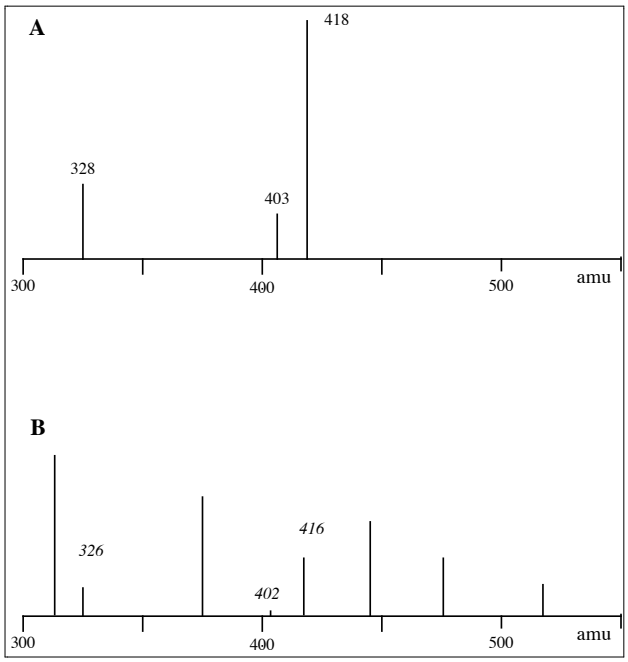

Fig 3: Mass spectrum of the analyte NT and the interference E in the full-scan mode

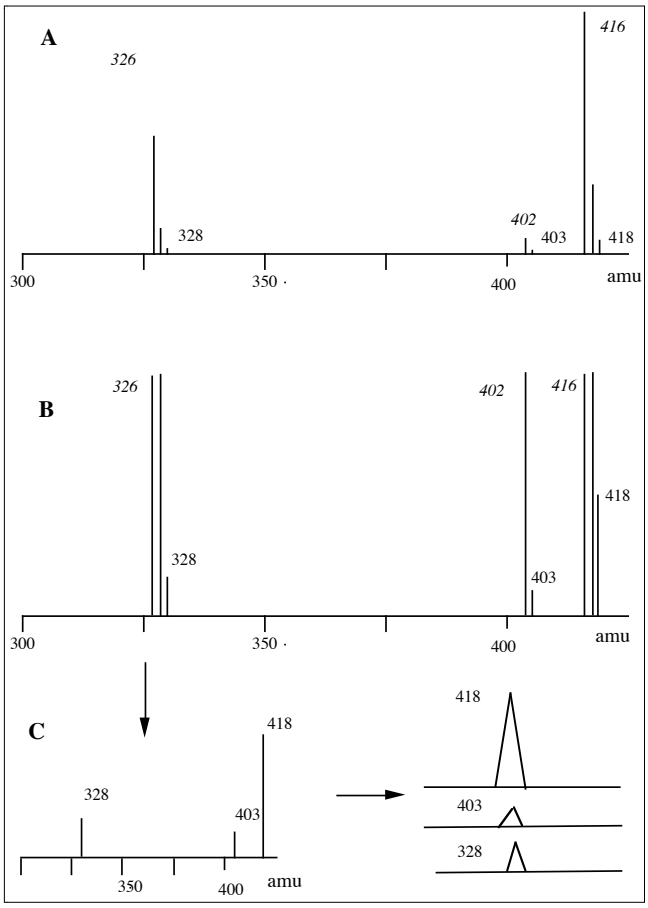

Fig 4: Isotope peaks of NT generated by E
A: Full-scan mode 416 = 100 % B: Full-scan mode, amplified C: SIM signal

In the SIM mode only the ions 418, 403 and 328 are selected. The relative concentration of these ions is resp 46.6, 8.5 and 14.2 ppb (ratio 100 / 18/ 31). Using the SIM method correctly, the analyst wil therefore conclude to the presence of NT with a concentration equivalent to 10 -50 ppb (depending on the ion used for quantification) since the 3 ions are present within the correct retention time windows and with the correct ratios.

Ions, with a low relative abundance in a mass spectrum of an interference, may be important when SIM is used. Next to this theoretical example, more analogous examples may be calculated. The interfering isotope peaks may also be generated by several interferences simultaneously or by stable isotopes of other elements. The hypothesis that nortestosterone (NT) could be present in the urine of pregnant cows (8,9) (in contrast to its natural presence in the urine of the stallion and the boar) (11,12,13)) is based on the positive signal obtained both with RIA and SIM. The signal for RIA could be caused by the aspecificity of the antibody against nortestosterone. For SIM analysis ^{13}C isotope interference with the high concentrations of estradiol, present in the urine of these animals could be possible.
This phenomenon was studied with full-scan ultra-trace mass spectrometry (14). It was shown clearly that α-estradiol and β-nortestosterone were not well separated in the chromatographic conditions used. The three peaks at 418, 403 and 328 amu at the correct

retention time and intensity ratios could be due to the high (and strongly variable) concentration of estradiol in urine of pregnant cows. In Fig. 5 the ion chromatogram at 418 amu of a urine extract of a pregnant cow, spiked with 2 ppb β-nortestosterone is shown. This ion chromatogram demonstrates clearly that a high level of estradiol (main ion 416) generates a 418 amu signal in the high ppb level by ^{13}C isotope interference.

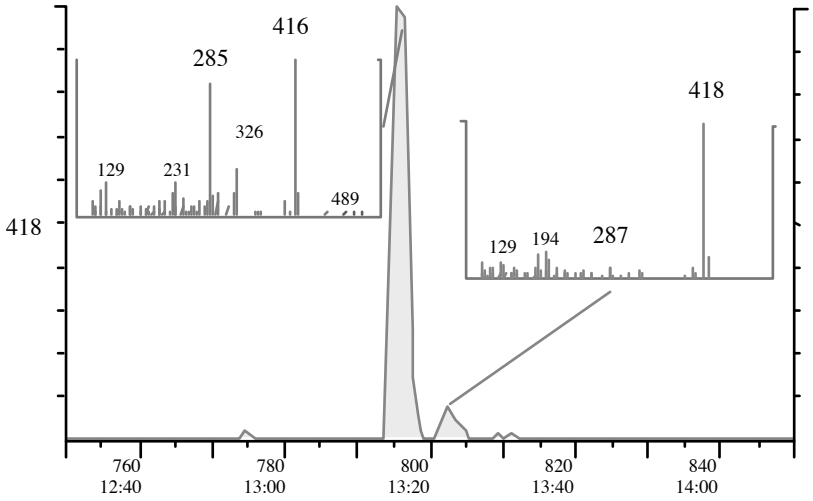

Fig. 5: Ion chromatogram at 418 amu of a urine extract of a pregnant cow, spiked with 2 ppb nortestosterone.

4.2. False negative results

In a laboratory C the analyte EE2 in the matrix M is determined with GC-MS at the 2-10 ppb level. In a slaughter animal an injection site, containing EE2 was found. In the matrix M of the same animal an EE2 value of 3 ppb was found (quantification on ion 425). Also this example is very realistic: ethinylestradiol may be determined at this level with GC-MS in the SIM mode (16).

The characteristics of the GC-MS SIM analysis are: time of retention = ca 17 min. Three ions are followed during the time interval 16-18 min: 440 (45 %), 425 (100 %), 300 (45 %). The mass spectrum of EE2 reduced to the three diagnostic ions is given in Fig 6A.

The matrix M contains a component TT in a concentration of 2 ppm with a retention time of 17 min. In the mass spectrum of this component an ion 439 with a very low abundance is present (ca 1 % of the total spectrum). This ion generates an isotope peak at 440 amu with a relative intensity of 1.2 ppb (the calculation is analogous as shown above). This 440 intensity adds to the intensity of 440 from the analyte (1.35 ppb). The "3 ion" spectrum after isotope interference is shown in Fig 6B. The "3 ion" spectrum is clearly distorted by the isotope peak and according to the rules of SIM the sample should be considered as negative although all 3 peaks are present and in the injection site, cut from the same animal EE2 was found.

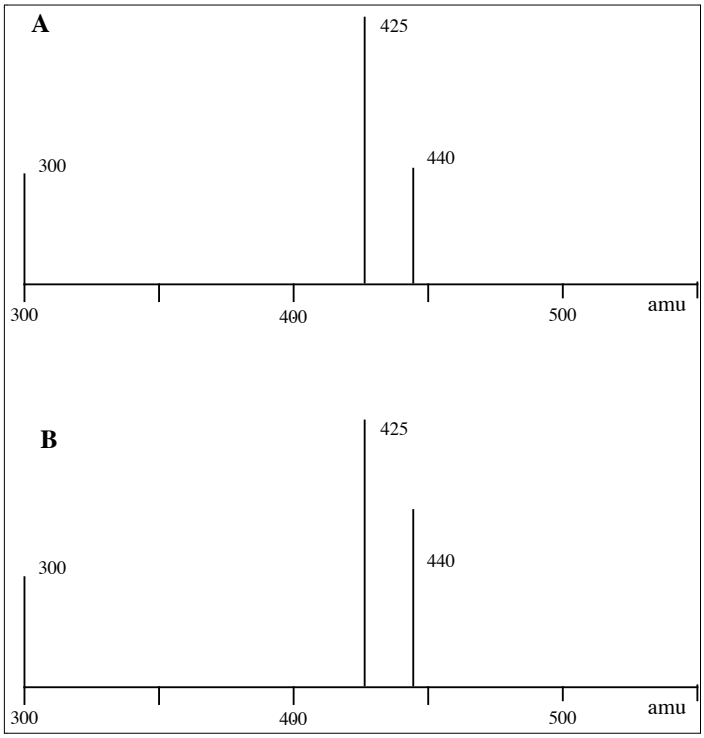

Fig 6: Mass spectrum of: A: EE2 standard
B: EE2 disturbed by isotope peak of 439

4.3. Wrong quantification

Quantification of residues may be very important, especially in the neighbourhood of the decision limit. In an analogous way as shown above interferences may influence both the analyte ions or the internal standard ions.

5. Conclusion

When SIM is used for the determination of residues of analytes by GC-MS at the ppb level, isotope interference should always be kept in mind. Isotope interference could generate the following three effects:

1) False positive results by the presence of three diagnostic ions at the correct retention time and in the correct ratio windows. However, these 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 transparant to the analyst when using the GC-MS in the SIM mode.

2) False negative results by disturbance of the normal peak ratios of the ions from the analyte by one or more isotope peaks from one or more interferences. This effect may even be of more importance than the generation of false positive results since the possibility of its occurrence is higher. 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.

3) Wrong quantification by disturbance of the ions of the analyte or the internal standard.

The reasonings, mentioned above, show that the possibility of isotope interference should be taken into account by the use of SIM in residue analysis. This isotope interference may be avoided by using apparatus capable of operating in the full-scan mode at low concentration levels. The absence of substantial concentrations of isotope peak generators in the full-scan mass spectrum has to be considered as a quality criterium.
With quadrupole apparatus, which are not able to take a full-scan at low concentration the following strategy could be recommended: in the case of a positive result a second full-scan run on the same sample is performed in order to exclude the presence of isotope generating peaks at the retention time of the analyte. SIM could also be used for screening purposes only and suspect samples rechromatographed and fully identified with another system.

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2.2. PITFALLS THROUGH IMPURITIES IN CHEMICALS

After

By-products of steroid synthesis: a cause of interferences in Thin-layer Chromatography Residue Analysis.

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The Analyst 119 (12) (1994) 2571-2576

1. Introduction

In Belgium seven field laboratories perform the control on the illegal use of anabolics.
Biological samples from animal origin such as urine, meat, kidney fat, injection sites, plasma and faeces are analysed by using appropriate methods, i.e., thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), immunoassay (IA) and gas chromatography-mass spectrometry (GC-MS). An important type of sample is kidney fat samples, analysed by TLC as screening and confirmation technique, used in conjunction with GC-MS in some laboratories[1].
In most instances TLC is the last step in the detection and identification in the anabolic residue analysis procedure. It is important that the results are reliable. This means that possible interfering compounds have to be removed via a good clean-up procedure. Because of the complexity of the biological materials, the purification step is of a great importance.
A solid phase extraction method (SPE) was developed by the National Reference Laboratory (NRL) and some field laboratories. The method is used for screening purposes[2].
More specificity was obtained by using HPLC purification in combination with 2D-HPTLC. This became the recommended method for confirmation[3,4].
To obtain the most reliable result by using TLC, the well defined quality criteria described previously have to be fulfilled [2, 3].
The two most important criteria discussed are:

1. the R_F value of the analyte should agree with the R_F value characteristic of the standard material (a variation of 3% is accepted)
2. the visual appearance of the analyte should be indistinguishable from that of the standard material.

Lately the laboratories involved in HPTLC control of hormonal residues in kidney fat, regularly detected a spot with a similar R_F to that of methyltestosterone (MT) that exhibited an almost identical fluorescence color. Depending on the concentration, a slightly different color and Rf was observed and research was started to determine the exact identity of this spot. In this paper we report on a technique performed to isolate and identify this interference, first called "***the false methyl***", in addition to performing the GC-MS method.

2. Experimental

Apparatus

A homogenizer (e.g., Ultra Turrax, 20 000 rpm), a water-bath, a centrifuge equipped with centrifugation tubes of 450 ml (e.g., Beckman), a mechanical extractor (e.g., Stomacher, form Sheward Medical, London, UK), a rotary vacuum evaporator (e.g., Rotavapor, from Buchi, Flawil, Switzerland), a N_2 evaporator (e.g., Vapotherm from Labor Technik Barkey, Beilefeld, Germany), extraction flasks of 100 ml and 250 ml, a solid-phase extractor (e.g.,

Baker, Deventer, The Netherlands), chromatographic tanks and a UV transilluminator (l = 366 nm) were used. The sample applicator used was a semi-automatic Camag Linomat IV, Camag, Muttenz, Switzerland. The LC system consisted of a Series 4 pump (Perkin-Elmer, Norwalk, CT, USA), an ISS-100 autoinjector (Perkin-Elmer), an automatic switching valve, a Model 440 UV detector (Waters, Milford, MA, USA), and a Model 202 fraction collector (Gilson, Worthington, OH, USA). The gas chromatograph-mass spectrometer was an ITS 40 ion trap (Finnigan MAT, San José, CA, USA).
HPTLC plates were obtained from Merck (Darmstadt, Germany).

Reagents, reference compounds and solutions

All solvents were of analytical-reagent grade or LC grade from Merck (Darmstadt, Germany), diethyl ether was obtained from Gifrer & Barbezat (Decines, France). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Düren, Germany) and iodotrimethylsilane (ITMS) and dithioerythritol (DTE) from Pierce (Rockford, IL, USA).
The progesterone standard was obtained from Sigma (St. Louis, MO, USA) and commercial progesterone powders were obtained from Diosynth (The Netherlands), Akzo (The Netherlands) and Profarma (Belgium). The methyltestosterone (MT) standard and the internal standards equilinine and spironolactone were obtained from Steraloids (Wilton, NY).

Solutions

Stock standard solutions were prepared in ethanol at a concentration of 1 mg ml^{-1}. For routine control purposes, working standard solutions were prepared by dilution of the stock standard solutions to 50 ng μl^{-1}.
Buffer solutions of sodium acetate (0.04 M; pH: 5.2) and sodium carbonate (10%) pH ≤ 10.25 were used.
The following solvent systems were used to develop the HPTLC plates:
1 = *n*-hexane-diethyl-ether-dichloromethane (25 + 45 +30);
2 = chloroform-acetone (90 + 10);
3 = cyclohexane-ethyl-acetate-ethanol (60 + 40 + 2.5)
4 = chloroform-*n*-hexane-acetone (50 + 40 +10);
5 = toluene-methanol-Aq dest. (5 + 75 + 20);
6 = *n*-hexane-dichloromethane-acetonitrile (80 + 20 + 10).

LC-columns

A semi-preparative C_{18}, Ultraphere ODS (pore size 80 Å (8 nm), particle size 5 μm) column (250 mm x 10 mm i.d.) was obtained from Beckman Instruments (San Ramon, CA, USA), C_{18} pellicular ODS (particle size 37-53 μm) guard column (30 mm x 4.6 mm i.d.) from Whatman (Maidstone, UK) and a C_{18} MCH-10 cartridge (particle size 10 μm) pre-column (30 mm x 4.6 mm i.d.) from Varian (Harbor City, CA, USA). Silica (Si) and Aminopropyl (NH_2) SPE columns (Bond Elut columns) were obtained from Varian.
Before use, all these columns were conditioned with the appropriate solvents, as described later.

Solid-phase extraction (screening procedure):

The overall scheme of the method used as screening is given in Fig. 1.
Kidney fat is cut into small pieces, 25g are weighed into a 250 ml polypropylene flask and 50 ml of sodium acetate buffer (0.04 M, pH 5.2) are added. The fat sample is than melted in a microwave oven (1 min on high power, 4 min on low power).
The sample is homogenized by using or Ultra Turrax (1 min on high speed, 1 min on low speed) or Waring Blendor (1 min on high speed, 1 min on low speed) or in the appropriate recipient using a Stomacher (5 min). A 125 ng amount of Equilenine and 50 ng of Spironolactone (internal standards) are added.
After extraction with 50 ml of hot methanol, the mixture is centrifuged at 13 000 g for 10 min and the supernatant is filtered through cotton wool in a separating funnel. De-fatting of the methanolic extract is carried out using 1 x 25 ml of hexane and the hexane phase is later discarded. Further extraction is performed using 1 x 100 ml of diethylether. This phase is washed with 15 ml of H_2O and then evaporated to dryness.
The SPE Si-column is preconditioned with 1 x 25 ml hexane. The dry ether extract is dissolved in 500 μl of chloroform and 5 ml of hexane are added just before the extract is loaded onto the column.
The Si column is washed with 5 ml of hexane. Next, the Si column is put on top of a NH_2-column and the combined columns are washed with 5 ml hexane. A 5 ml volume of chloroform-acetone (4 + 1) is used to elute the column. The eluate is evaporated to dryness under a stream of N_2 at 60 °C.

High-Performance Liquid Chromatography as clean up.

For confirmatory purposes a second purification procedure can be used. The primary methanolic extraction of the kidney fat and the liquid-liquid extractions are similar to those in the screening method but to achieve a sufficient degree of separation a gradient elution on a semi-preparative column was applied. Full details of this procedure are described in a previous paper [4, 7].

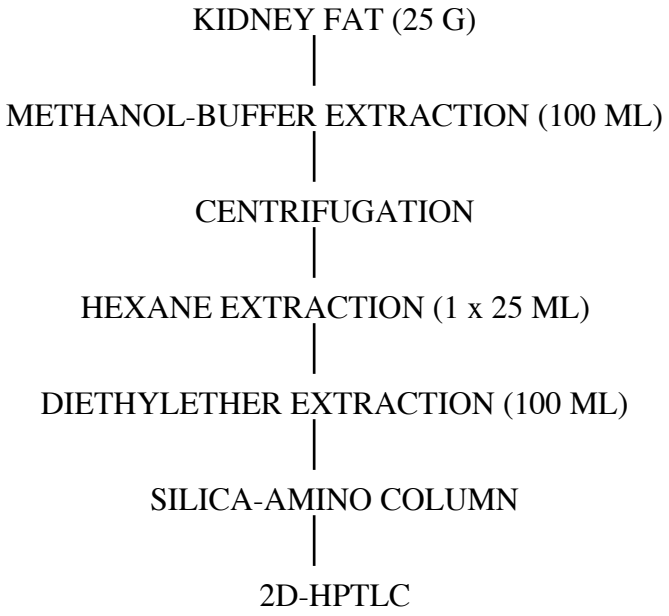

Fig. 1. : Over-all scheme of the screening procedure.

High-performance thin-layer chromatography.

For screening purposes the extract is chromatographed on a precoated silica gel plate (10 x 10 cm) following the 4 x 4 elution technique[1].
The dry residue obtained after SPE, is dissolved in 30 μl of ethanol and 10 μl is spotted onto the plate. Chromatographic development is performed with the solvent systems 1 and 2 or 3 and 4 (listed earlier).
After fluorescence induction, the spots are detected by viewing by transillumination under UV radiation (λ = 366 nm) and were identified by comparing the R_F values and fluorescence colors with those of the reference standards.

Gas chromatography - mass spectrometry.

The derivatization reagent mixture used is MSTFA-ITMS-DTE (1000/2/2,v/v/v). Extracts should be evaporated to dryness and then stored in a desiccator prior to derivatization.
To the tube containing the extract, 25 μl of the reagent mixture are added and heated at 60 °C for 15 min. The tube is allowed to cool and then 1 μl is injected (in the splitless mode) into the GC-MS instrument.

The analyses were carried out on a Finnigan ITS 40 ion trap in the full-scan mode. The GC column used was DB-5 fused silica (30 m x 0.25 mm i.d.) with a 0.25 μm film thickness and a carrier gas (helium) flow-rate of 1 ml min^{-1}. The temperature settings used are as follows: injector, 260 °C; transfer line, 300 °C ; oven, programmed from 100 to 200 °C at

16.7 °C min^{-1} and from 250 to 300°C at 4 °C min^{-1}, the final temperature of 300 °C being maintained for 3.5 min.

3. Results and Discussion

SPE-TLC

It has already been shown in an earlier study[7] that the results are influenced by the kind of matrix that is analysed and also by the method that is used for the detection of anabolic residues in biological samples. HPLC, for instance results in a better purification of the extract, enhancing the possibility of detecting more residues of interest and thus the method becomes more specific.

As this technique was very time-consuming for screening purposes and in some cases the HPLC column was blocked by the extract, the described SPE method was elaborated. With SPE there is less risk of column blockage, and only one fraction is eluted for further separation by TLC. It was in using this SPE-TLC screening technique that over the last few years the field laboratories regularly detected a false MT spot.

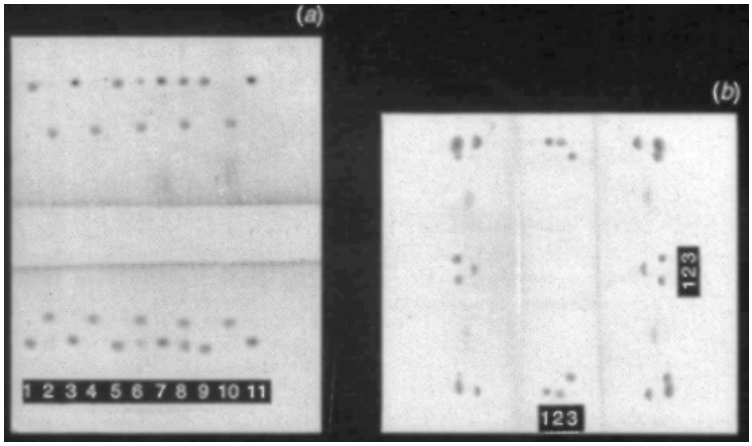

Fig. 2.: A: Silica HPTLC of the different standards.
lane 1, 5, 9: MT standard; lane 3, 7, 11: PA4; lane 2: $P_{Diosynth}$; lane 4: P_{Akzo} ; lane 6: $P_{Profarma}$; lane 8: $P_{illegal}$; lane 10: P_{Sigma}

B: Reversed phase C18 2D-co-chromatography of an illegal cocktail: standard lanes 1: 20ß-dihydroxyprogesterone; 2: Progesterone; 3: Methyltestosterone

Indeed, depending on the concentration and the TLC-elution circumstances, this spot can be very similar to methyltestosterone, even after 2D-co-chromatography. The first effort to obtain a differentiation between the two spots by performing reversed phase TLC on C_{18} plates was succesfull. The MT spot and the unknown spot were well separated in the first direction (see Fig. 2B).

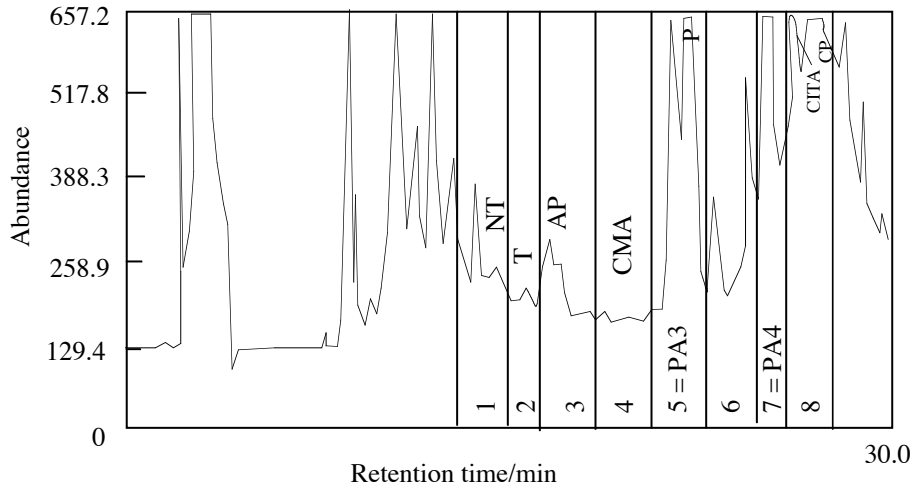

Fig. 3.: UV-chromatogram (l = 254 nm) of a kidney fat sample with selected windows.

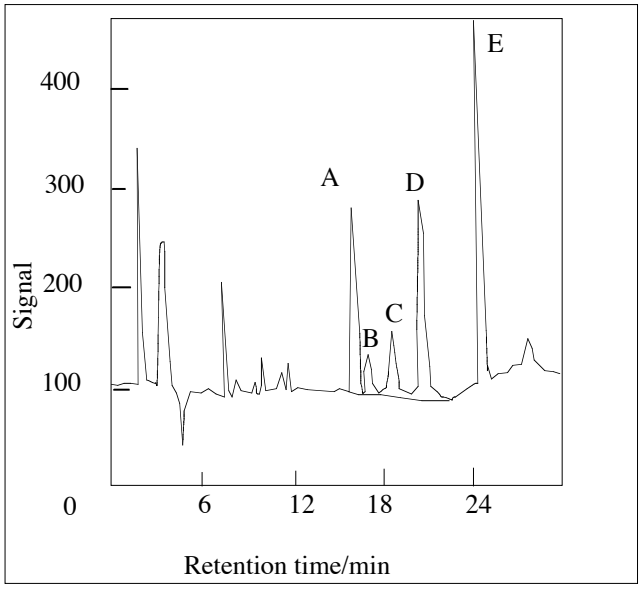

Fig. 4.: UV-chromatogram of the HPLC elution of some standards of interest.

One of the control labs related the false methyl to the progesterone standard they used as a reference. Every laboratory involved in the regulatory control was asked to supply the NRL (National Reference Laboratory) with their standard. This resulted in a series of various analytical progesterone standards in addition to the three commercial powders and an unknown powder used on the illegal market, which was sampled by the inspection.

The TLC results are shown in Fig. 2A.

Two of the commercial powders showed the false methyl spot in different concentrations and the unknown powder showed the same spot at a much higher concentration. The next step in our research was the isolation and identification of this compound.

HPLC-TLC

The extract of an appropriate fat sample was purified by using HPLC, and fraction collection was performed as shown in Fig. 3. Eight different fractions where collected so that the most important part of the elution could be analysed further. The same elution procedure was performed for the different standards (see Fig. 4) and also for an illegally used oilly suspension (cocktail) containing a high concentration of progesterone and the second compound (see Fig. 5). In fraction 7 we isolated and succesfully detected the green fluorescent spot, and confirmed this by comparison with the same fractions of the injected standard and dilution of the cocktail.

GC-MS

The HPLC fraction that we identified by TLC as the "second P-spot" was analysed by GC-MS after derivatisation with MSTFA. As for the TLC detection we analysed that same fraction of a false positive MT fat sample, a P-standard containing the second spot and the earlier mentioned cocktail. We isolated the appropriate fraction and obtained the mass spectrum, as shown in Fig. 6, which was the same in the three samples.

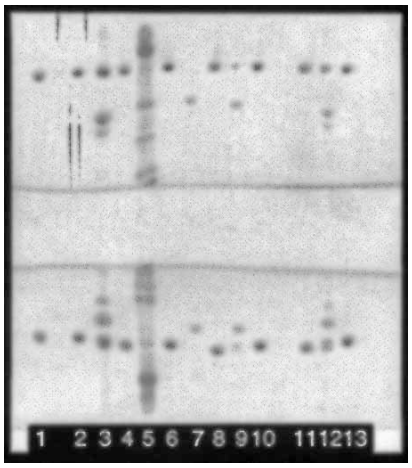

Fig. 5.: HPTLC of a cocktail, PA4 standard and MT-standard.
lane 3, 12: illegal cocktail; lane 2: PA4 standard; lane 4: MT-standard.
(Reversed figure)

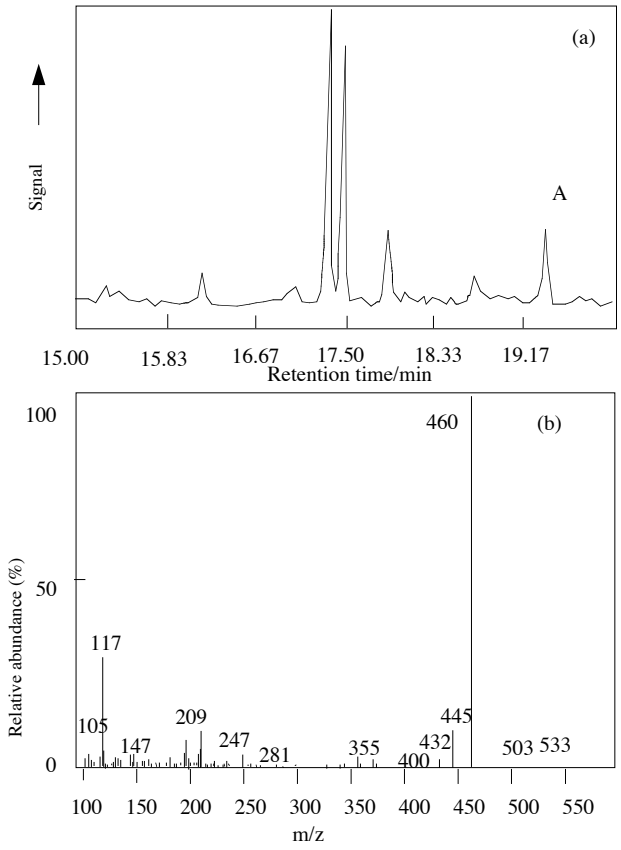

Fig. 6.: GC-MS results of a compound eluted in HPLC-fraction 7.

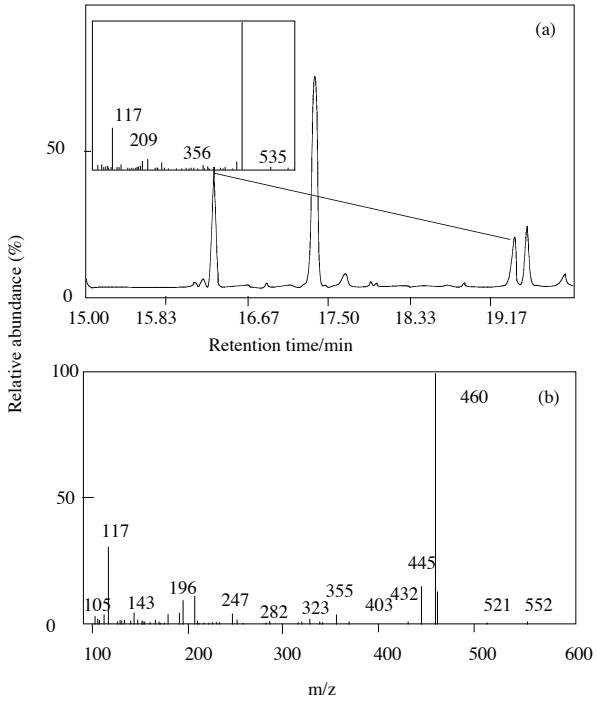

Fig. 7: GC-MS results of the PA4 standard and the corresponding fraction of a kidney fat sample.

Until now, the only conclusion that we could make was that the second P-spot was probably an impurity of the progesterone standard. The unknown compound has a M^+ ion of 460 after silylation with MSTFA. If a di-TMS was formed, the molecular mass of the unknown must be 316. After a dBase search in our list of standards we selected 20 compounds with *M* 316. After further research into the chemical structure (to find progesterone analogues), five products which we had as reference material in our laboratory were selected (see Table 1).

Fig. 7 shows the GC-MS results of the PA4 standard and the corresponding spectrum in a fat sample. The only product that corresponds to the HPLC, as well as the TLC and the GC-MS results was 20ß-hydroxyprogesterone. These results were confirmed in different kidney fat samples and also in some meat samples.
The conclusion that the interference was probably a by-product of progesterone is therefore confirmed because a relatively high concentration of progesterone was detected in all samples.

Table 1: Chemical nomenclature of the selected P-analogs.

P-Analogue	Systematic name
PA1:	5α-dihydroprogesterone = 5α-pregnane-3,20-dione
PA2:	5ß-dihydroprogesterone = 5ß-pregnane-3,20-dione
PA3:	20α-dihydroxyprogesterone = 4-pregnen-20α–ol-3one
PA4:	20ß-dihydroxyprogesterone = 4-pregnen-20ß-ol-3one
PA5:	pregnenolone = 5-pregnen-3ß-ol-20-one

4. Conclusions

The problem caused by the by-product of progesterone was first detected in the control laboratories after using the SPE-TLC as screening method. By using a HPLC clean up procedure prior to HPTLC we could increase the specificity of the technique and differentiate between **methyltestosterone** and the "**false methyl**".
Identification and confirmation of the results, obtained by using the other techniques, could be performed using GC-MS.
The first conclusion of this study is that all control laboratories must be aware of the by-products of anabolics used as illegal preparations in cattle fattening. These by-products can, as in the case of progesterone, be resorbed by the organism, and produce interfering problems in the residue analysis of biological samples. Moreover this "false methyl" discussed may be an indication of illegal treatment with natural hormones.

Secondly, the use of a combination of different techniques, for purification as well as for detection, is the best way to obtain the most reliable result. In our study, the HPLC clean-up procedure prior to HPTLC, in combination with GC-MS, was the tool with which we solved the lack of specificity of the screening method.

The final and significant conclusion is that the exposed problem of possible interferences caused by commercial steroids by-products, reminds us the problem of not only the purity but also the availability of certified reference standards and reference materials. This study is the result of the collaboration of five laboratories, all members of the working group “analysis of anabolics in kidney fat”.

5. References

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2.3. PITFALLS DUE TO ENDOGENIC SUBSTANCES

After

Endogenic nortestosterone in cattle?

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The Analyst 119 (12) (1994) 2581-2586

1. Introduction

Once upon a time Laurabolin, Decabolin, Durabolin, etc. all containing nortestosterone fatty acid esters were circulating on legal and illegal markets and were used throughout in cattle fattening, horse racing and athletes. When residues of nortestosterone (NT) (nandrolone) were found in urine of cattle, race horses or body builders, exogenous administration was thought to be proven. In literature no records were found of the endogenic presence of this molecule. Tuinstra et al[1] described in 1986 17α-nortestosterone (17αNT) and the estranediols as the most important metabolites of nortestosterone in cattle (Fig 1). These products were only detected in animals treated with nortestosterone or nortestosterone derivatives, typically esters.

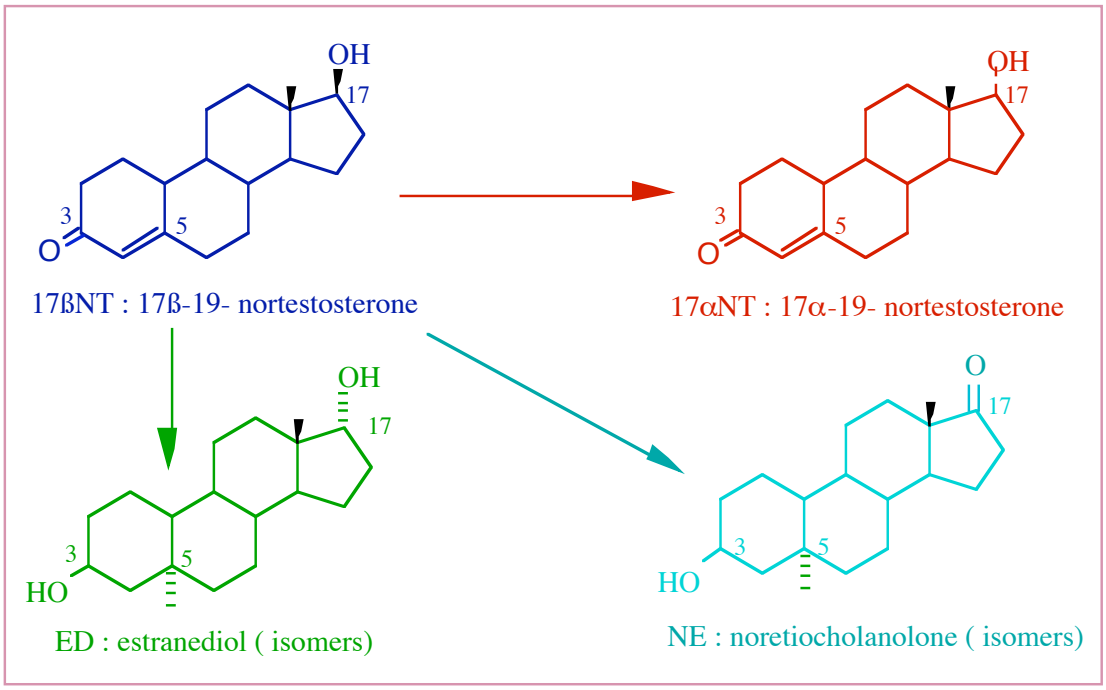

Fig 1: Metabolic pathways of nortestosterone (NT)

In the horse racing world, Houghton[2] and Courthot[3] found in 1984 that NT is normally present in the urine of the stallion. Belgian and Dutch researchers found that 17βNT is present in the urine and edible parts of the intact male pig (boar). These observations were presented firstly in working groups (e.g. the Veterinary Inspection working group and the BENELUX working group) and later published by Maghuin-Rogister (1988)[4], Van Ginkel et al (1989)[5] and Debruykere et al (1990)[6]. Rizzo et al (1992)[7] reported the endogenic origin of

nortestosterone by analysis of boar testis. Since then, for meat inspection purposes boars were no longer sampled for nortestosterone analysis. As another consequence the presence of NT in pig meat (and pig meat products) may no longer be controlled in trade between EC countries.

The first suspicion that NT would also be endogenic in the urine of male veal calves was based on an analytical artefact (confusion between 17α−testosterone and 17αNT ; De Ridder[8]). This problem did not exist in those labs that used HPLC purification prior to HPTLC. Meyer et al[9] reported in 1988 that NT could be present in non treated veal calves by consumption of contaminated milk replacers. Vandenbroeck et al (1991)[10] suggested for the first time the endogenic presence of NT (ßNT but not the α form) in the urine of the pregnant cow.

However, this could not be confirmed in other laboratories: by studies at the Dr. L. Willems-Institute (R1) and the UGent, Dept. of Veterinary Public Health & Food Safety, laboratory of Chemical Analysis (D1) a limited number of scientific experiments on pregnant cattle were carried out, following the publication of Vandenbroeck et al (1991)[10].

The results are summarised below. More details of these studies may be found in the thesis of Van Den Braembussche (1991)[11].

During regulatory control 25 female animals were sampled: 2 not pregnant and 23 in different stages of pregnancy. Eighteen urine samples, taken during the last 4 months of pregnancy were analysed by RIA (at R1). The stage of pregnancy was estimated. NT-RIA-responses were found as described by Vandenbroeck et al (1991)[10] and within the same range (2-10 ppb). The highest RIA responses were found during the last 2 months of pregnancy.

*Table 1: RIA responses of NT in the urine of pregnant cattle (ppb NT-equivalents)**

Pregnancy status*	sample number	NT -mean	Individual NT values
P-120	6	0.7	**0.1** / .0.2 / 0.4 / 0.7 / 1.2 / **1.5**
P-90	1	2.4	2.4
P-60	3	4.9	**1.3** / 3.1 / **10.2**
P-30	8	3.9	**1.0** / 2.4 / 3.1 / 3.8 / 4.2 / 4.7 / 5.4 / **6.3**

* partus = 0 (taken as zero point)

Table 1 and the results of Vandenbroeck proved clearly that during the last months of pregnancy there is a rise of the response to the NT antibody used (Antibody: lab d'Hormonology, Marloie Belgium). However, the presence of NT in the urine samples could not be confirmed by HPLC-HPTLC and HPLC-GC-MS. With these methods neither 17βNT nor 17αNT could be detected in the 25 samples by both laboratories. Possibly, the RIA NT response could be due to other products (other steroids probably) excreted during this phase of pregnancy.

However, very high concentrations of α-estradiol were detected in the urine of animals in the last stage of pregnancy. It was found that these high concentrations of α-estradiol could generate false positive β-NT results using selected ion monitoring (SIM) without using a full-scan chromatogram to trace interfering substances (Leyssens et al (1991)[12]. The principle of these analytical pitfalls in SIM was worked out further as a theoretical example by De Brabander et al (1992)[13]. For the 3 labs, involved in these experiments (D1, R1 and IHE) the detection of ßNT in the urine of pregnant cattle was considered as an analytical artefact caused by isotope interference.

During the international symposium on hormone and veterinary drug residue analysis, Ghent, 1992, Meyer (1993)[14] reported the presence of NT (in the 17αNT form) in relatively high amounts in the urine of the cow peri-partum and the neonatal calf. Since these observations are in contradiction with the results of our limited experiment and may have important consequences for veterinary inspection in the EC, it was decided to set up a larger scale experiment in co-operation with the EC Community Reference Laboratory (RIVM).

2. Experimental

The methods described were validated before[15,16,17] use. A short summary follows:

By IHE and D1 the samples were analysed with HPLC/GC-MS[15]. Urine (25 ml) is hydrolised with Helix Pomatia Juice and extracted with ether. The crude extract is cleaned up with HPLC with fraction collection (Smets en Vandewalle, 1984)[15]. The fractions are derivatised to TMS enol-TMS ether (trimethylsilyl) derivatives with MSTFA/ TMSI/ DTE (N-methyl-N-trimethylsilyltrifluoroacetamide/trimethylsilyl iodide/dithiotreitol) and analysed with full-scan MS on a Finnigan MAT Magnum (ITS40) system. The column used was a fused silica HP-Ultra 2 of 25 m x 0,2 mm with 0.25 μm film thickness. For quantification an internal standard ((16,16,17β-$^{2}H_{3}$)nortestosterone (NT-d3) was used. The detection limit of this method was 0.5 μg/l.

By R1 a combination of solid phase extraction on 3 columns (C18, silicagel and amino column) and HPLC purification was used as clean up of 50 ml of urine (Leyssens et al)[16]. The samples were enzymatically deconjugated (Helix Pomatia) after the first column. GC-MS on a part of the pooled HPLC fractions was performed with ethoxime-trimethylsilyl derivatives (EO-TMS). The GC-MS was a Varian Saturn I system. The column used was a fused silica DB-5 MS, 30 m x 0,32 mm with 0.25 μm film thickness. For quantification equilenin (d-1,3,5(10),6,8-estrapentaene-3-ol-17-one) and androsterone (5α-androstan-3α-ol-17-one) were used as internal standards. The detection limit of this method was 0.5 μg/l.

In the RIVM[17] urine (5 ml) is extracted with an automated (ASPEC) procedure on a C18- and an amino-SPE cartridge. Prior to extraction the internal standard NT-d3 was added and the samples were enzymatically deconjugated (Helix Pomatia). The primary extracts obtained after SPE procedure are further cleaned up by immuno affinitychromatography (IAC). After removal of the solvent, the dry residue is derivatised with heptafluorbutyric acid anhydride (HFBA) and the diHFB-NT derivatives are determined with GC-MS (HP5890A gas chromatograph with a fused silica DB1 column of 30 m X 0.25 mm with

0.25 μm film thickness and HP5989A MS Engine). The limit of detection was 0.2 μg/l but a limit of decision of 0.5 μg/l was used in this study.

In Fig 2 some examples of chromatograms and mass spectra obtained with the 3 different methods are given for the reader to make up his/her own mind.

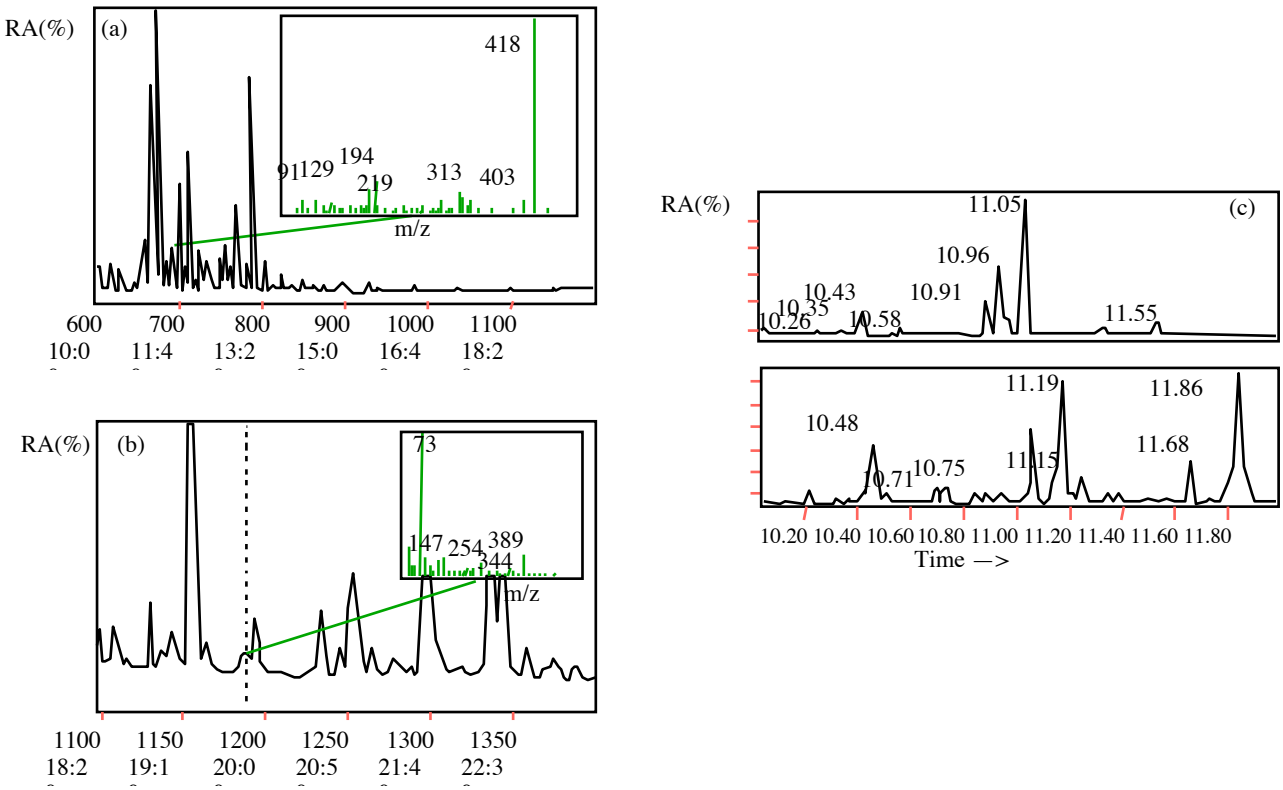

Fig 2: Examples of chromatograms and mass spectra obtained with the 3 different methods

a) IHE and D1: chromatogram and full-scan mass spectrum of NT-di TMS in a urine sample;

b) R1: chromatogram and full-scan mass spectrum of NT-EO-1 TMS-1 in a urine sample;

c) RIVM: quantitative determination of 17αNT-di HFB in a urine sample (17αNT : m/z =666 ; t = 10.91 , internal standard NT-d3: m/z 669 ; t= 11.19)

3. Results and discussion

Urine sampling

One of the problems within this project was the possibility of obtaining a large number of urine samples in sufficient quantities of non-treated pregnant animals. Obviously these samples could not be obtained from routine sampling.

Moreover, the stage of pregnancy of the animal sampled, should be known as accurately as possible. We learned that estimation of the stage of pregnancy by visual inspection (the "*occulus veterinarius*") only is very difficult and the absence of the exact knowledge of the stage of pregnancy in the preliminary experiments was considered as a weak point. The normal duration of pregnancy in cattle is 285 days (9.5 months of 30 days) but in practice, random variations may occure next to variations between breeds. In Table 2 some data for Belgian breeds are summarized.

Table 2: Some pregnancy data of cattle (Belgian breeds)

Breed	mean*	SD**	number of animals
white-blue	282	6	2 344
red pied	280	5,8	27 197
red	283	5,8	6 376
black pied	280	5,5	83 185
white-red	282	5,6	123 235

*days of pregnancy (mean) ** standard deviation (pregnancy days)

In this investigation the reference point for pregnancy was put on the day of the partus (P=0), which could be measured accurately. All further data were expressed in function of this zero point.

Experiments checking Meyer's observations.

The first 40 samples, in our large scale experiment, were taken on a dairy cow farm with a fairly good warranty that no nortestosterone was abused (the abuse has also no meaning for

this type of farming). The animals were normally grazing on the pasture and fertilisation took place by natural mating. From up to 2-3 weeks before partus (rough estimation by the farmer) the animals were brought into the stables and could be sampled more easily. Sampling plan: the date of partus was estimated and a urine sample was taken regularly from day **"P-20"** on (20 days before partus). These samples were frozen at -18° C and analysed after knowledge of the exact date of partus. After partus, 3 more urine samples were taken in the period up to 8 days. Unfortunately, some animals were sold during the experiment and only a rough estimation of the partus day was obtained. These samples are not included in Table 3 but only used as additional information.

The samples were analysed in one laboratory (D1) in order to preselect interesting samples. The samples were then distributed to the 3 other laboratories and analysed quantitatively on NT without pre-knowledge of the stage of pregnancy of the animal.

The results are given in Table 3.

Table 3: Results of analyses on α -nortestosterone (ppb 17α NT))

Pregnancy stage versus partus		Animal	Lab 1	Lab 2	Lab 3	Lab 4
-21	#	5455	Pos	2.3	4.6	-
-18		8007	Pos	-	5.4	5.6
-9	**	8315	Pos	1.4	3.9	0.8
-6	*	8890	Pos	2.4	5.8	3.8
-4		1960	Pos	3.8	5.0	5.1
-4		9805	Pos	2.8	6.5	4.8
-1		1585	Pos	4.7	7.2	3.3
0	*	8890	Pos	4.0	6.3	2.2
0	*	8890	Pos	1.5	4.3	0.7
0		8515	Pos	4.0	5.4	3.2
0	#	5455	-	-	4.6	9.3
0	#	5455	-	Neg	Neg	Neg
1	*	8890	Neg	Neg	Neg	0.9
1	**	8315	Neg	Neg	Neg	-
1	**	8315	Neg	Neg	Neg	Neg
1	#	5455	Neg	Neg	Neg	0.6
2		5805	Neg	0.7	0.6	Neg
2	*	8890	Neg	Neg	Neg	-
3	*	8890	Neg	Neg	Neg	Neg
3	**	8315	Neg	Neg	Neg	Neg
3	**	8315	Neg	-	Neg	Neg
4	*	8890	Neg	Neg	Neg	Neg
5		1960	Neg	Neg	Neg	Neg
5		9805	Neg	Neg	Neg	Neg
6	*	8890	Neg	Neg	-	Neg
6	*	8890	Neg	Neg	Neg	Neg
8		1585	Neg	Neg	Neg	Neg

(-4: means 4 days before the partus; P+2: 2 two days after partus; P = 0 : samples taken at different hours, see later) (Neg: NT ≤ 0.5)

In Table 3 it can be seen that all 4 laboratories detect 17αNT in samples taken shortly before partus (period of 21 days before partus) in a concentration range of 1- 10 ppb (only 3 labs did a quantitative analysis). In addition to the data given in Table 3 the 5 other samples,

with only a rough estimation of the day of partus give analogous results: the between laboratory reproducibility of the results is 49 % (% relative standard deviation). The agreement between the range of the results of the 3 laboratories, which produced quantitative data is acceptable regarding that the within laboratory reproducibility at the limit of detection is determined as approximately 25 % by the EC Community Reference Laboratory (RIVM). After the partus, negative (≤ 0.5 ppb) or very low 17 αNT results (< 1 ppb) were found. The data of animals 8890 (marked by *) and 8315 (marked by **), taken at different stages of pregnancy are of particular interest. These samples illustrate very well that NT is detected before and not after partus

Also very interesting are the urine samples of animal 8890 (marked by **) and animal number 5455 (marked by #) taken at different hours shortly after the partus (Fig 2).

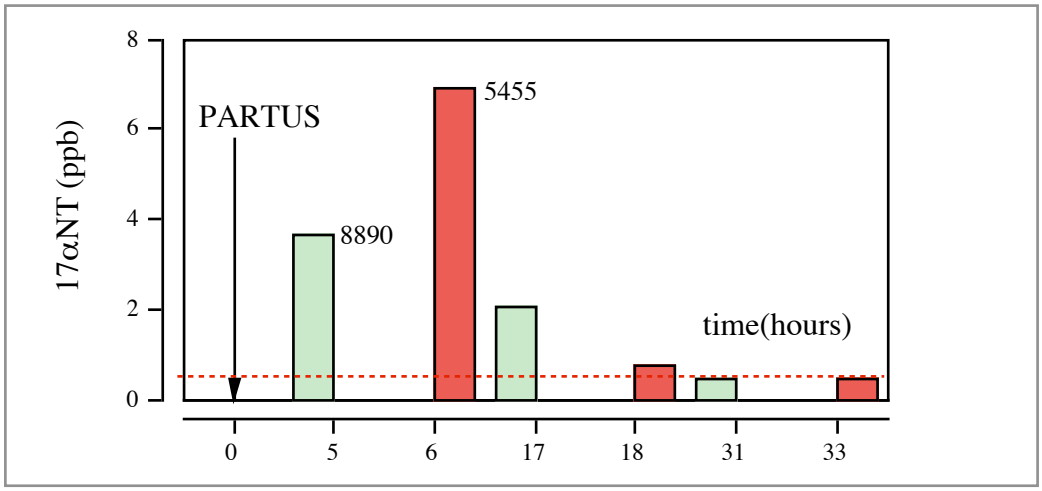

Fig 3: Nortestosterone values of urine samples taken shortly after the partus (animals 8890 and 5455)

Fig 2 clearly shows that the NT concentration falls below 0.5 ppb within 24 hours after partus. It should be kept in mind that the urine collected 6 hours after partus is (or may be) formed before partus. So excretion (and thus also production) of NT ceases quickly at partus. This should indicate that the presence and the evolutive status of the placenta is very important in the production of nortestosterone.

The form in which nortestosterone is present in the urine is 17α-nortestosterone as mentioned by Meyer et al. No labs could detect 17βNT in concentrations greater than 0.5 ppb (considered as an acceptable detection limit) as mentioned by Vandenbroeck et al[10].

Qualitatively our results confirm and complete the results of Meyer et al[14]: 17αNT may be present in the urine of pregnant cattle from ca 20 days before partus (P-20). This phenomenon is not dependant on the sex of the newborn calf and neither is it influenced by the parity of the cow (the number of calves she had)

In table 4 Meyer's data[14] are converted to our units (ppb, partus as zero point). From this Table and Table 3 it can be observed that quantitatively significant differences are found. The concentration of nortestosterone found by Meyer et al[14] is much higher than that in our experiments. However it should be kept in mind that HPLC IA and GC-MS results are compared. Possibly also metabolites of the NT metabolism cross react with the HPLC IA method used by Meyer[14] for quantification.

Table 4: NT values found by Meyer (1993) (αNT (ppb) determined by HPLC IA)

Day	Cow 1(F)*	2(F)	3(M)	4(M)	5(M)
P-18	-	29	-	-	-
P-12	27	25	-	18	-
P-7	35	20	28	21	-
P-1	-	-	25	20	-
P+1	4	1	18	-	11
P+11	2	1	3	4	1

* = gender of calf

Another important difference between our and Meyer's results[14] is the rate of disappearance of NT in the urine after partus. If our hypothesis of the NT producing placenta after a certain maturation stage is right, it should be verified on which time the cow discarded the placenta. Otherwise we must accept that Meyer[14] with his HPLC IA assay is measuring also something more than 17αNT.

Further experiments

The first question which rose after the confirmation of Meyer's observations was the length of the period during which 17αNT could be detected in urine. Therefore it was tried to extend foreward the sampling dates. However, the further away the sampling from partus date, the more difficult the accurate P-X can be determined. The strategy using one well known dairy farm, followed in the first experiment, could not be used. Therefore data from an artificial insemination station were used. On basis of artificial insemination (AI) data the date of partus of a large number of dairy cows were calculated (Fig 3). The farms of the animals are located and farmers were requested for sampling permission. Samples were taken and analysed as described before. Of these samples also the creatinine concentration and the density were determined. The parity of the cow and the sex of the veal calf was noted each time.

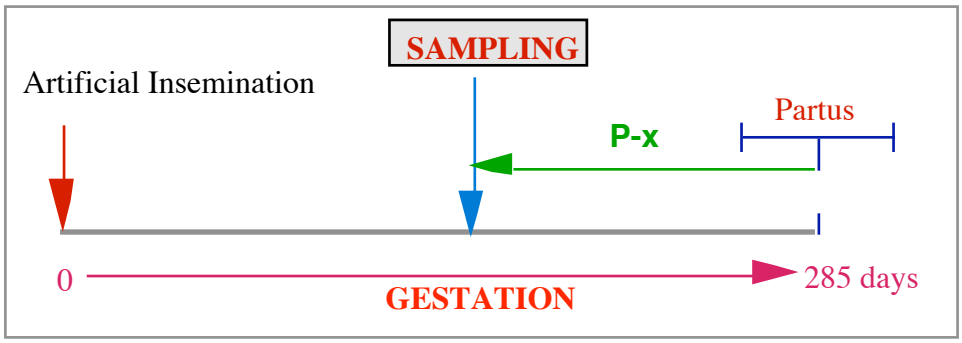

Fig 4: Estimation of the date of the partus from artificial insemination data

Hitherto 186 urine samples were taken. 93 samples were analysed in some labs only. Samples are divided over the labs but only a limited number of results are available and so the results have to be regarded as "non confirmed by interlaboratory experiments".

A review of the results is given in Table 5.

Table 5: Preliminary results of the additional experiments

Days before partus	number of analysis	17αNT detected
220-170	2	0
140-100	5	5
100-70	11	8
70-50	25	22
50-40	35	23
29-20	15	12

In the period P-140 to P-20 17αNT was detected in 70 out of 91 urine samples. In the 2 samples most far away from partus (P-173 and P- 223) no 17αNT could be detected.

Discussion

From the data, given above it is concluded that 17αNT is present in most of the urine samples taken in the last half period of pregnancy. So it is confirmed that the steroid is secreted or produced in the pregnant cow. In this context an old habit of Flemish farmers to improve the quality of cow meat by pregnancy (slaughter of the cow after 4 months pregnancy) could possibly be explained by the anabolizing effect of the steroids generated.
However, it stays an open question why concentrations of 2-9 ppb 17αNT were not detected during the first experiment following the article of Vandenbroeck[10]. So far, it is a complete riddle why 3 labs, with a lot of experience in steroid analysis could not detect 17αNT in the 23 samples taken during regulatory control and later without problems detect 17αNT in samples taken at dairy farms. Moreover, in this period, no significant improvement in analytical methods or detection limit has occured.
Another riddle: in the slaughterhouse, a veterinary inspector took a urine sample of a cow, in which a 7 month old calf foetus was found: no 17αNT could be detected.

The explication of the riddles, given above shall possibly not be for tomorrow. The clinical estimation of the stage of pregnancy may play an important role in that phenomenon. Also lactation and the breed of the animals could play a role.

4. Conclusion

From a large number of samples of pregnant non-treated cows, analysed by GC-MS in 4 different labs, it is proved clearly that 17αNT may indeed be present in the urine of pregnant cows from at least 4 months before the partus. In the samples, hitherto analysed 17ßNT was not detected in concentrations greater than 0.5 ppb. The experiment has to be continued, also for other species because in other experiments we found also 17αNT in the urine of pregnant sheep, goats and deer.

Furthermore the sampling strategy of the EC has to be reviewed if the presence of endogenous NT in urine of cows in gestation is confirmed in a sufficient number in other member states.

The research on nortestosterone, mentioned above, has been worked out in further publications of our laboratory, in multi-disciplinary co-operation with other scientists. a.o.

Consequence of boar edible tissue consumption on urinary profiles of nandrolone metabolites.
Mass spectrometric detection and quantification of 19-norandrosterone and 19-noretiocholanolone in human urine.
B. Le Bizec, I. Gaudin, F Monteau, F. Andre, S. Impens, K. De Wasch, H.F. De Brabander
Rapid commun. Mass spectrom., 14 (2000) 1-8

Interseksualiteit bij een varken: casuïstiek en implicaties voor hormonenonderzoek.
Van Cruchten S., De Wasch K., Impens S., Lobeau P., De Smet I., Simoens P., De Brabander H.
Vl. Diergen. Tijdschrift (2002), 71, 411 - 418

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CHAPTER III: METHODS FOR RESIDUE ANALYSIS

3.1. THIN LAYER CHROMATOGRAPHY

After
High Performance Thin Layer Chromatography for Residue Analysis
H. F. De Brabander, K. DeWasch
in **Residue Analysis in Food-Principles and Applications** p 145-175
Ed M. O'Keeffe, Harwood Academic publishers, 2000

1. Introduction

In thin-layer chromatography (TLC) the stationary phase is applied as a thin layer on a support. Since the prepared plate is essentially flat the name "planar chromatography" is also used. The first description of TLC dates from 1938. During the decade 1950-1960 TLC was transformed into an analytical technique. Stahl (1969) developed the necessary standardisation of the procedure. In the decade 1970-1980 a more modern and sophisticated version of TLC, high performance thin layer chromatography (HPTLC) was introduced. HPTLC has achieved a place in the list of important chromatographic techniques, next to GC and HPLC. However, maybe the time has come to consider if the prefix "HP" (for high performance) is still meaningful for HPTLC - or for HPLC. The major differences between TLC and HPTLC are only the physical dimensions of the plate and the size distribution of the stationary phase particles. Therefore, in this chapter generally TLC and HPTLC are used interchangeably.

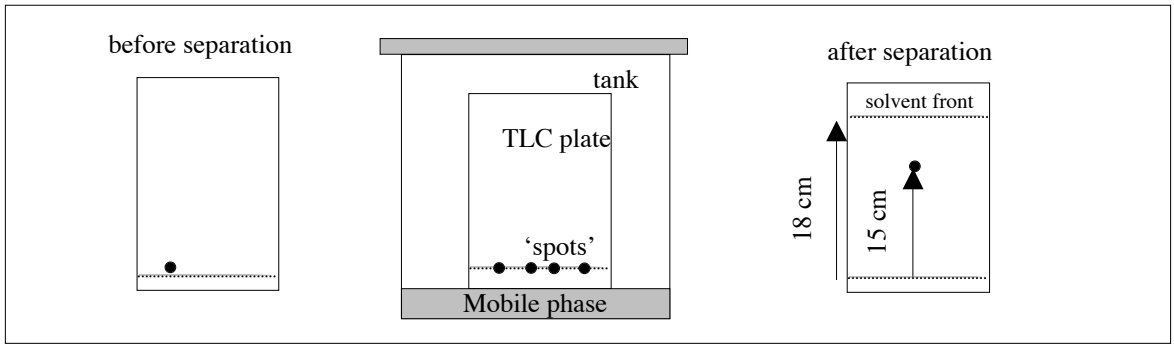

Fig. 1. A simple form of TLC separation

TLC has its own terminology, which is somewhat different from that used in GC and HPLC. In Fig. 1 an example of a simple TLC separation is given. The sample is applied as a spot on the origin (or origin line) and developed with a mobile phase. The different components of the mixture are separated and may be detected by viewing the plate in daylight, if they are coloured, or, otherwise, visualisation techniques must be used (see Section 2.3.1). Corresponding reference substances are used for identification. The distance of migration of the spot is expressed as the **R**atio to the **F**ront (R_F value).

R_F = migration distance of the spot / migration distance of the solvent front

For TLC a number of handbooks have been published (e.g. Bertsch *et al.* 1980; Hamilton, Hamilton 1987; Sherma, Fried 1996; Stahl 1969; Touchstone *et al.* 1979, 1980, 1982, 1990, 1992; Zlatkis, Kaiser 1977). Some of these works are introductions to TLC, others are laboratory handbooks and still others are dedicated to a special item. It is not the intention of this chapter to create another handbook on TLC. On the contrary, the authors have been

asked to write this chapter from their experience of the practical use of TLC in residue analysis. For theoretical points, for more detailed treatment of TLC and for applications of the technique in other fields, these other handbooks should be consulted.

2. The four steps of TLC

2.1. Application

The application to the thin layer plate is the equivalent of the injection in GC or LC. The sample is applied generally at a certain small distance from the edge of the plate. The exact positions for application are linked to the manner of development, which is discussed in Section 3 (*Some special features of TLC*). In contrast with GC and HPLC, "spotting" of a TLC plate may vary from being very simple and cheap to being very complicated and expensive. The simplest way of spotting small volumes is by use of a capillary (0.5 to 2 μl). The capillary containing the sample is placed gently on the stationary phase and the sample is transferred. In that way a "spot" is created. The art in TLC is the application of as small a spot as possible. Commercial apparatus make use of a magnetic holder for the capillary; by manipulating the electric power as a function of time the capillary is placed against the plate several times during a reproducible time period. Higher volumes may be applied with an adapted syringe; the flat needle of the syringe is placed just above or on the stationary phase and the syringe is emptied slowly. This can be done manually or with a motor. Commercial apparatus using this principle are available. A third, but not often used, possibility is "contact spotting"; the sample is applied in a small well formed in a special plastic sheet. The solvent is evaporated under nitrogen and the well in the sheet is reversed pushing the semi-dry sample against the adsorbent. These procedures are illustrated in Fig. 2.

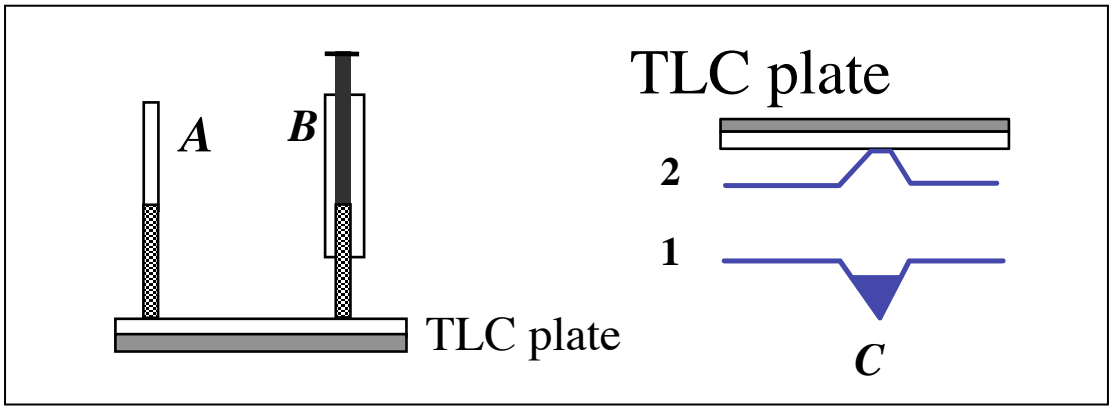

Fig 2. Different ways of application (A: with a capillary, B: with a syringe, C: contact spotting)

The solvent in which the sample is dissolved is also of great importance. If the solvent has a large displacement capacity for the analytes on the particular stationary phase it will be difficult to obtain small spots (e.g. alcohols on silicagel plates). The smallest spots on

silicagel are obtained using apolar solvents (e.g. hexane). On the other hand the analyte must be highly soluble in the solvent. A compromise between those two aspects needs to be reached.

The solvent in which the sample is dissolved is also of great importance. If the solvent has a large displacement capacity for the analytes on the particular stationary phase it will be difficult to obtain small spots (e.g. alcohols on silicagel plates). The smallest spots on silicagel are obtained using apolar solvents (e.g. hexane). On the other hand the analyte must be highly soluble in the solvent. A compromise between those two aspects needs to be reached.

In TLC all standards must be applied together with the samples in the same run. This is an important difference from GC and HPLC where external standards are injected at regular intervals between the sample runs.

2.2. Development

The development of a TLC plate may vary from being very simple to complicated. With a mono-dimensional linear development (as shown in Fig. 1) several samples and standard solutions may be analysed simultaneously. In most cases standard, vertically oriented developing chambers are used. There are also other tanks such as the so-called “horizontal developing chambers” where the TLC plate is horizontal and the plate is developed simultaneously from both edges. These chambers have the advantage of very small solvent consumption. For specialised purposes (e.g. testing out solvents) circular and anti-circular development may be used (Fig. 3).

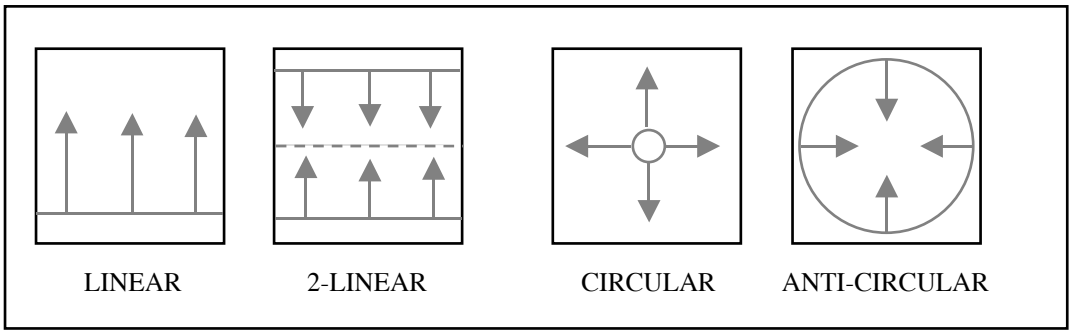

Fig. 3. Different ways of mono-dimensional development of a TLC plate

In residue analysis, mono-dimensional TLC is suitable for screening purposes only. For confirmation, two-dimensional TLC is mandatory (see Section 3.1). A very important factor in development is the state of saturation of the chamber. For many separations the chamber and/or the TLC plate are pre-saturated by using filter paper lining the walls of the developing chamber, or twin tanks, one for pre-saturation of the plate and the other as the developing chamber. In other applications an unsaturated chamber gives the best separation.

2.2.1. The stationary phase

In TLC the stationary phase is usually called the adsorbent. Many materials may be used. Silicagel is the adsorbent of choice for the majority of separations. Other phases, such as aluminium oxide, cellulose, polyamide and modified silicas (e.g. RP-2, RP-8) are used also. The nature of these stationary phases is analogous to those used in HPLC and will not be discussed here in detail.

The preparation (coating) of a thin layer plate is carried out by means of a spreader, which is available in various designs. Some manufacturers (e.g. Merck, Whatman, Schleicher & Schulz, etc.) offer a great variety of pre-coated plates (Fertigplatten). These plates generally are firmer, more reproducible and of better quality than home-prepared plates. To the stationary phase the manufacturer may add other substances such as binders or fluorescent indicators.

HPTLC is an upgraded version of TLC, which was introduced in 1976 (Zlatkis, Kaiser 1977). The success of HPTLC is based on the size of the stationary phase. In comparison with TLC, smaller and more uniform particles are used (5 μm). This permits a better contact between the stationary phase, the sample and the solvent, thereby improving separation. Also, the thickness of the layer is reduced from 0.25 mm to 0.2 mm. Recently, ultra-thin plates of 100 μm have been introduced for some types of residue analysis. A practical difference between HPTLC and TLC is the dimension of the plates (TLC: 20 x 20 cm; HPTLC: 5 x 5 cm or 10 x 10 cm). In most cases a HPTLC run uses only 3 - 5 cm (instead of 15 - 18 cm in TLC). The **H**eight **E**quivalent to the **T**heoretical **P**late (HETP) in HPTLC is of the magnitude of 0.01 mm (approximately 1000 theoretical plates per cm). The price of HPTLC plates is higher than TLC ones but there is a gain in time, space, resolution and consumption of solvents (Hamilton, Hamilton 1987). Recently, Empore TLC sheets were introduced. They combine inert PTFE fibres and chemically bonded silica particles into a sheet suited for TLC.

2.2.2. The solvents

The solvents used in TLC are analogous to those used in HPLC. However, since TLC is an open bed system, solvent toxicity may play an important role in the choice of solvent. The chromatographic background to the separation is analogous to HPLC (see Chapter 8) and will not be discussed in this chapter. Because TLC is mostly isocratic, complicated “home-designed” solvent mixtures are sometimes used or needed to obtain specific separations (e.g. n-hexane-diethylether-dichloromethane, 4:3:2, (v/v/v) for the separation of a number of steroids (Verbeke 1979). The basic composition of these mixtures arises mainly from the scientific literature or experience and is adapted by trial and error to suit particular applications.

2.2.3. Automated Multiple Development (AMD)

In most cases TLC is used isocratically and the solvent mixture is constant during development. With **A**utomated **M**ultiple **D**evelopment (AMD) (Burger 1984) a reproducible elution gradient may be employed on a thin layer plate (Fig. 4). AMD makes the separating power of a HPTLC layer comparable with that of a HPLC column. The main principles of AMD are that:

- the chromatogram is developed repeatedly in the same direction,
- each partial run uses a slightly longer solvent migration distance than the one before, and
- the developing solvent for each successive partial run has a lower polarity (elution power) than the one used before.

AMD is sometimes called "focusing" chromatography because of the good separations that may be obtained. AMD is mostly performed with specially designed commercially available equipment. However, even with normal tanks, but with more labour, excellent results may be obtained with manual multiple development for some residues (e.g. sulphonamides (Van Poucke *et al*. 1994)).

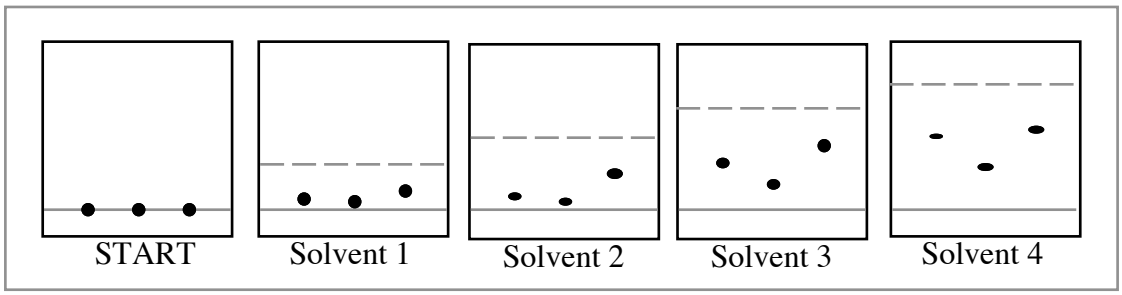

Fig. 4. Automated Multiple development (AMD)

2.3. Detection

The word detection indicates that step in which the components are located on the plate and conclusions are drawn from observing the plate. Detection consists of 2 steps: visualisation and documentation.

2.3.1. The visualisation

For coloured samples no detection step is needed; the plate may simply be viewed under daylight. If the analyte is fluorescent (by itself or by derivatisation) it may be visualised by irradiation with UV-light (Fig. 5). Standard equipment, therefore, is an UV-cabinet with three sources: white light and UV-light of 254 and 366 nm wavelengths. Also, transilluminators are often used in residue analysis. With this type of apparatus the analyte on the plate is irradiated through the stationary phase, which enhances in most cases the detection limit. Transillumination cannot be used with pre-coated TLC plates on aluminium support.

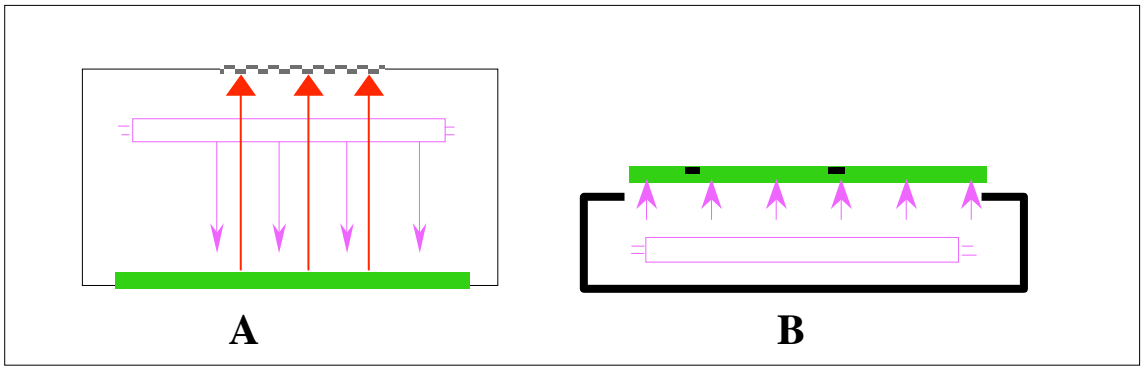

Fig. 5. Visualisation by irradiation with UV light (A: UV cabinet; B: transilluminator)

If the analyte is not fluorescent, but absorbs UV light, a plate with a fluorescent indicator may be used. In this type of plate the stationary phase is mixed with a product, which emits light when irradiated with UV light of 254 nm. The analytes appear as black spots on a green or white background. Alternatively the plate may be sprayed with a fluorescent product such as fluorescamine or 2,7-dichlorofluoresceine. In Table 1 some examples of typical detection limits on thin layer plates for some important residues are given. In Section 6 (*Examples of TLC methods in residue analysis*) more details about these methods are given.

Table 1. Some examples of detection limits on HPTLC plates

Class	Component	Limit of Detection		Reference
		On plate (ng)	In matrix (ppb)	
Thyreostats	Methylthiouracil	0.3	50	De Brabander, Verbeke 1975
	Tapazole	0.4	100	
Anabolics	Diethylstilbestrol	1	0.5	Verbeke 1979
	Trenbolone	3	0.2	
	Progesterone	20	10	
Beta-agonists	Clenbuterol	1	1	Courtheyn *et al.* 1988a,b
Corticosteroids	Dexamethasone	10	1	Courtheyn *et al.* 1992

A large number of chemical reagents may be used for the induction of colour or fluorescence (Stahl 1969). Visualisation with iodine is often used. When a thin layer plate is in contact with iodine vapours the spots appear brown on a white background. By using iodine low detection limits may be obtained (Medina 1986). Spraying with water is also used; lipophilic components will be observed as white spots with transillumination. With some commercial plates (e.g. amino plates : -NH_2 Merck 15 647) fluorescence of components, such as steroids and corticosteroids, can be induced by simply heating the plate at 200 °C. Some of the most useful reagents may be purchased also in commercially available spray bottles. Dipping of a thin layer plate is an alternative to spraying. With "dipping" the plate is immersed in a reagent solution. This may be done manually or automatically. The advantages of dipping are a more uniform dispersion of the reagent solution, no need for a fume-hood, etc.

2.3.2. The documentation

Ever-increasing legal considerations require accuracy of documentation. In contrast with GC and HPLC, where a hard copy of the chromatogram or a computer file is the result of the process, the TLC plate is not always stable over time. In many cases the colours on the plate fade away after some days and may not be redeveloped. Therefore, some kind of permanent record is necessary. In some cases the plates are stable for years if kept in the dark (e.g. thyreostats, see Section 6.1.1). In the case of coloured spots (e.g. corticosteroids, see Section 6.1.4), a permanent record of the plate may be obtained by taking a photocopy or using a scanner. Taking a photograph of an interesting TLC plate has been a common practice for many years. However, when a large number of 2D-TLC plates have to be documented this may become too expensive.

It is our experience that an excellent image of a TLC plate can be taken with a modern video camera (e.g. an 8 mm (Hi 8) or, even better, a digital camera). Moreover, these video cameras usually have excellent optics and so precise details on the plate may be documented. Although at first sight the use of a video camera looks expensive, this strategy is the cheapest solution; approximately 900 TLC plates can be stored on a tape of 90 min (at 6 sec for each plate). The cost of storage is at least 10 times lower than for photographs. Moreover, the camera may be coupled easily to a multimedia computer, the images transferred as pictures or as a movie and stored on a **W**rite **O**nce **R**ead **M**any (WORM) medium, such as a compact disc. In Fig. 6 an image of such a plate is given. Another possibility is the use of a camera that may be coupled directly to a computer.

Apart from its use for documentation, a video camera coupled to a computer may also be used for image analysis and quantification of TLC plates (see Section 2.4) (e.g. Jansen *et al.* 1989). Recently, video documentation has been expanded with commercial hard- and software becoming available for image analysis. Images may be stored, retrieved, printed, e-mailed and analysed by converting images to chromatographic peak data.

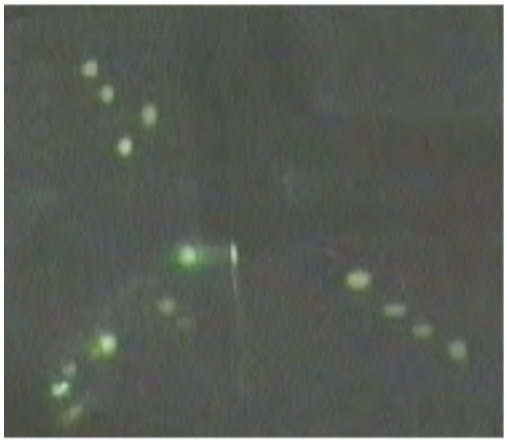

Fig. 6. Digitalised image of a TLC plate, taken by a video camera.

2.4. Quantification

If quantitative data are needed, TLC may be quantified in two ways: (1) Elution of the components from the plate and measurement by another method (e.g. spectrometric), (2) Quantification *in situ* on the thin layer plate which is done mostly by densitometry. Nowadays several modern and sophisticated densitometers (TLC scanners) are commercially available. All apparatus are capable of measuring absorption and fluorescence either in reflectance or transmission modes and transforming a mono-dimensional TLC into a "normal" chromatogram (Fig. 7). The densitometers are coupled to a computer (see Section 2.3) and may be used also for scanning of electrophoretograms.

However, in certain cases strict quantification is not necessary and qualitative methods may be used (De Brabander *et al.* 1996). Qualitative TLC may be used for analysis of residues having an "action limit" (e.g. the determination of anabolics with an action limit of 2 ppb). This action limit is not a legal value, such as a maximum residue limit (MRL), but an agreement based on the general performance of the testing laboratories. Qualitative TLC methods always contain a semi-quantitative aspect through comparing and estimating fluorescence intensities visually. It is our experience that, after some training, comparison of fluorescence intensities with the naked eye works very well. For instance, for the determination of anabolics the intensity of the fluorescence is compared to the fluorescence of a standard (of concentration corresponding to the action limit) chromatographed under the same conditions.

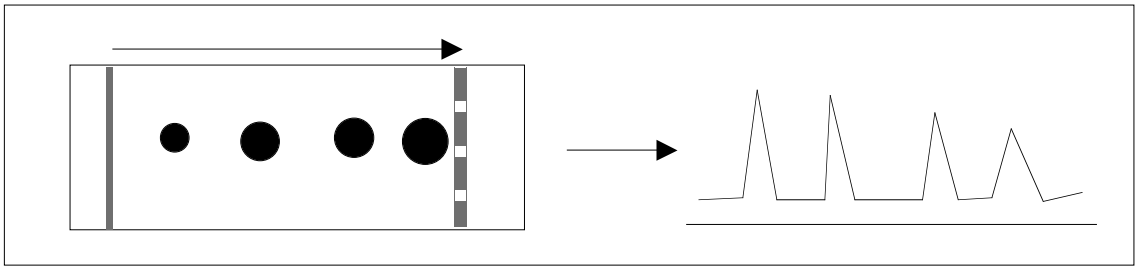

Fig. 7. Transformation of a TLC image into a "normal" chromatogram

Example: 25 g of a sample, being analysed for anabolics is extracted and cleaned-up with a yield of 80 %. Half of the final extract is spotted on the TLC plate. One can calculate, therefore, that the action limit of 2 ppb (2 ng/g x 25 g x 0.8 x 0.5) would be equivalent to 20 ng of the anabolic agent on the plate. 20 ng of standard is spotted on the plate and, if the intensity of the spot for the sample is greater than the intensity for the standard, then the concentration of the sample exceeds 2 ppb.

Other authors claim that it is possible to judge whether the concentration of some residues exceeds the MRL on the basis of TLC results alone (e.g. Abjean 1996).

3. Some special features of TLC

3.1. Two-dimensional TLC

In two-dimensional (2D-) TLC the sample is applied on one edge of the plate (Fig. 8). The standard mixtures are applied on the two adjacent tracks. The plate is then developed with solvent 1 in direction 1 (as far as the arrow). After drying, the plate is turned through 90° and developed in direction 2 with solvent mixture 2. The identification of the unknown component follows from comparison of the 2 R_F values in 2 solvent systems with those of the standards. With 2D-development, separations of complex mixtures that rival those of HPLC separations may be obtained. The whole area of the plate (instead of one line) is utilised for the separation of a single sample. This increases the separation power by almost a square of that obtained in mono-dimensional TLC. For residue analysis, 2D-TLC is mandatory for confirmation of results (see Section 4).

In performing 2D-TLC there are some small tricks that must be known and observed: e.g. between the two runs the plate must be dried very carefully, otherwise memory effects from traces of the first solvent may disturb the development with the second solvent. Also, the R_F value for a component in one of the two directions may not always be reproducible. Therefore, the original sample spot sometimes is overspotted with the standard mixture before the second development is carried out; this results in an exact comparison for R_F values.

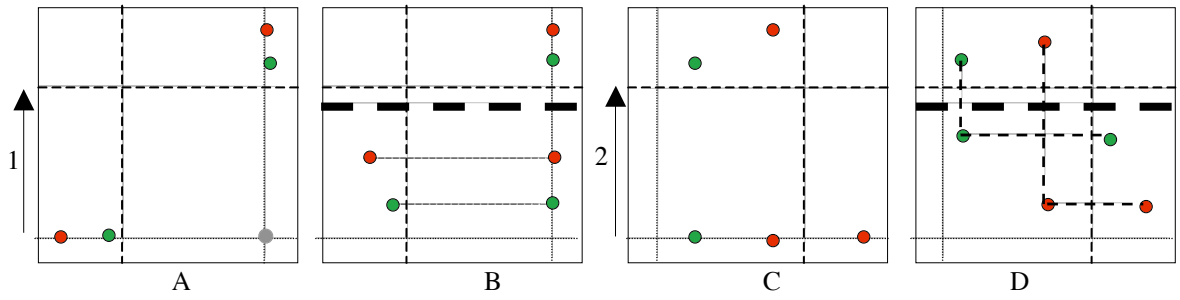

Fig. 8. Scheme for 2D-HPTLC

TLC is unique in the simplicity with which two-dimensional chromatograms may be performed. Difficult separations, needed in residue analysis, may be carried out at a relatively low cost. With other, non-planar techniques, special and sophisticated apparatus are needed to attain the same goal. However, the 2D technique is not well known and therefore not favoured by many analysts. As an illustration, in the handbook of Sherma, Fried (1996), containing approximately 1100 pages, only 1 page is dedicated to 2D-TLC.

3.2. "4X4"-TLC

A special application of 2D-TLC is the "4 x 4" mode (Fig. 9). In this mode, 4 samples and 4 standard mixtures are developed simultaneously on one TLC plate of 10 cm x 10 cm (De Brabander *et al*. 1988). In the "4 x 4" mode one reference series is used for each sample instead of 2 reference series as in the usual two-dimensional development. Also, for co-chromatography, "4 x 4"-TLC has advantages. The chromatogram and the co-chromatogram are developed under identical circumstances and may be compared easily with each other since they are side by side on the same plate; the chromatogram and the co-chromatogram are mirror images of each other (see S1 and S2, Fig. 9).

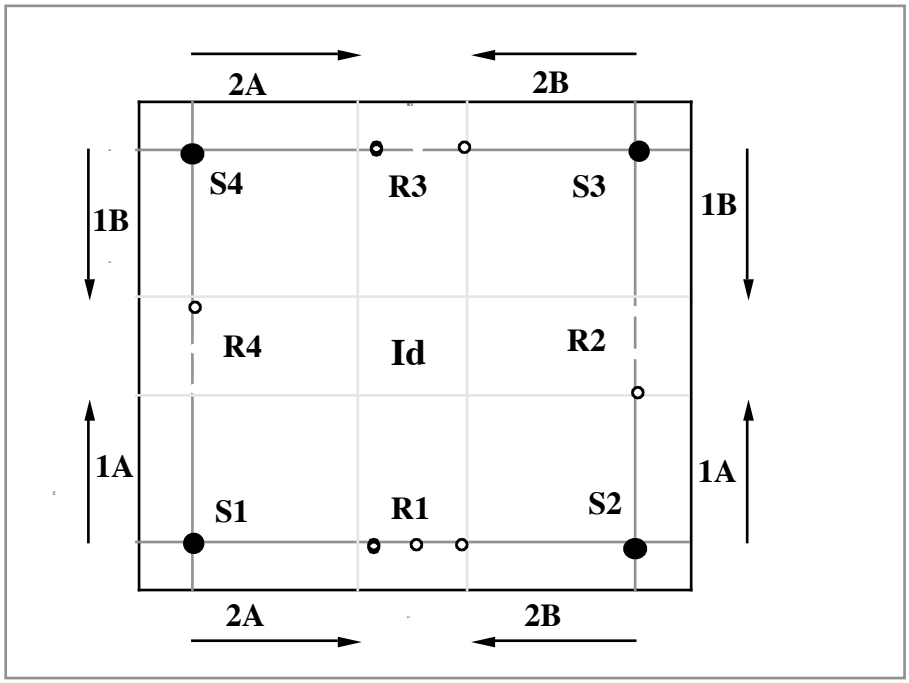

Fig. 9. Scheme for the "4 x 4 " 2D developing mode.

The "4 x 4" mode may be used with any sample applicator but the most efficient system is to use an adapted spotter where all four samples are applied simultaneously, thereby reducing the application time from approximately 10 - 15 minutes to 2 - 3 minutes per sample. For the "4 x 4" developing mode a special "4 x 4" spotter (Fig. 10) was constructed in the workshop of our laboratory (De Brabander *et al*. 1989b). With this spotter, four syringes of 10 μl are driven simultaneously by a metal plate, actuated by a stepper motor such that 4 samples of 10 μl each may be applied simultaneously at the 4 application sites of one TLC plate. For the evaporation of solvent during the application, 4 special nitrogen jets were constructed. The jets are formed from metal tubes of 3 mm ID and nitrogen is blown to the centre of the forming spot(s). The nitrogen jets are aligned to fit with the sample application spots. A secondary function of these nitrogen jets is to provide for exact positioning of the syringe needles.

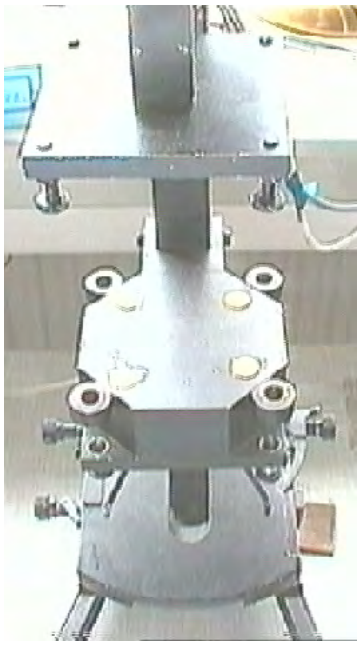

Fig. 10. Home designed "4X4" HPTLC spotter (model 2)

3.3. Anti-diagonal Development

Quantitative analysis is more difficult after two-dimensional TLC because the standards and the sample are not developed under the same conditions. There are two possible solutions to this problem, the first of which is the use of an internal standard (e.g. dimethylthiouracil (DMTU) in the analysis of thyreostats). Where use of external standards is required, these standards must be developed two-dimensionally also. With the anti-diagonal application technique, as described by Beljaars (1973), three standards of different concentration and one sample are developed simultaneously (Fig. 11). After the run, both the three standards and the sample are lying on one line and may be scanned in a similar way to a mono-dimensional development. Of course, with this technique only one analyte can be analysed on one plate.

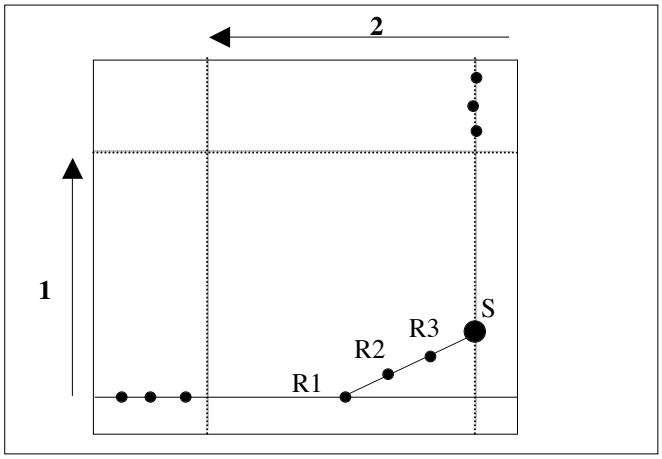

Fig. 11. Anti-diagonal developing technique (Beljaars et al. 1973)
(R1-3: standard series; S: sample)

3.4. Coupled Layers

On one TLC plate two (or more) stationary phases and two (or more) solvents may be combined. In that way difficult mixtures can be separated and the specificity and selectivity of the analysis may be improved (Mezetti *et al.* 1972). There are two (or more) adjacent layers, each of which exhibit different chromatographic properties and has an intrinsic function in the individual development. In Fig. 12 an example of the use of coupled layers in 2D development is given. A coupled layers plate may be made using a spreader that is divided into two parts by a plexiglas septum (Mezetti *et al.* 1972). Alternatively, two commercial TLC plates can be combined by cutting them carefully and pressing them together.

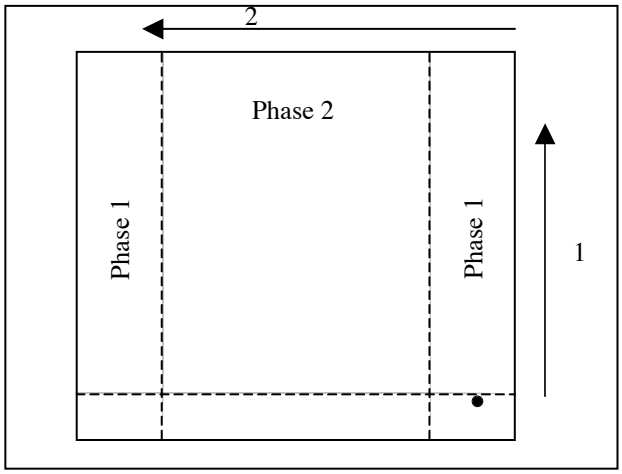

Fig. 12. An example of the use of coupled layers in 2-D development

A special (and commercially available) form of coupled layers is the “concentration” zone. With these plates (e.g. Merck No. 11845) the normal phase, such as silicagel, is coupled to an inert phase on which the analyte is not retarded. The sample may be applied roughly as a broad spot or band on this "concentration" zone. A preliminary development is used to concentrate the analytes as a band at the border between the two phases, before normal development begins. A scheme for the procedure is given in Fig. 13.

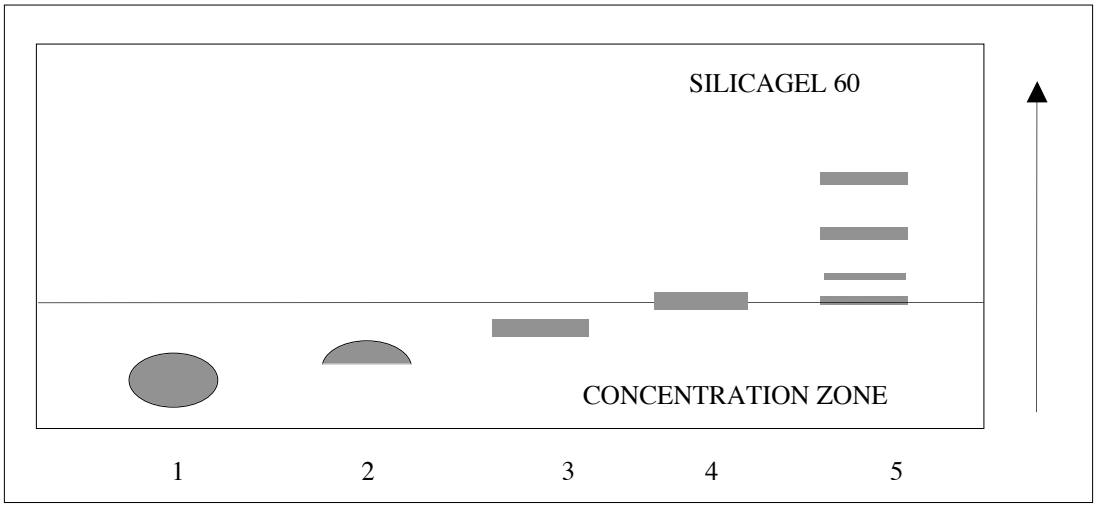

Fig. 13. Application with a concentration zone (1: start; 2,3: concentration to a band; 4: band reaches normal phase; 5: separation of the components starts)

The use of a concentration zone is a very simple and cheap method for application of sample in the form of a band; there is no need for special equipment and the results are reproducible. In our experience, very crude samples, such as urine, may be applied directly to the concentration zone.

A variation on the *coupled layers* are the “kissing layers” (De Brabander et al. 1995a). This expression refers to the development of two plates with their thin layers pressed against each other.

3.5. Reaction TLC

Since it is an "off-line" method there is the simple option of carrying out reactions on the TLC plate itself. It was observed that some reactions, such as some enzymatic reactions (Verbeke, De Brabander 1980), proceed better on a plate than in a reaction vessel.

3.5.1. Derivatisation at the application site

One of the most important reasons for reaction-TLC is the formation of a derivative of the analyte (thereby achieving better separation, detection, etc.). The derivatisation is carried out usually before application but reasons for performing the reaction "on plate" are (a) some reactions are faster on the adsorbent, (b) less reagent and analyte are needed, (c) it is simpler. An example is the formation of fluorescamine derivatives of amines by overspotting the sample spot with a fluorescamine solution at the application site.

3.5.2. Other reactions at the application site

Other examples of reactions at the application site are hydrolysis (e.g. esters of hormones in injection site sample extracts are treated with sodium methylate in order to liberate the

parent molecule) or deconjugation (e.g. overspotting a sample with *Helix pomatia* juice results in a lower consumption of the expensive enzyme).

3.6. Some Additional Advantages of TLC

TLC is unique as a disposable technique; the chromatographic system is thrown away after each analysis. This is particularly interesting in residue analysis because very dirty samples may ruin a GC or HPLC column. TLC can be used also for samples in which the concentration of residue is unknown and which can vary greatly (e.g. injection sites). It is a good practice to screen injection sites by TLC before any injection into another chromatographic system is made.

TLC is also a very good starting technique for those laboratories lacking funds (e.g. laboratories in developing countries). It is also an "off-the-shelf" method; a TLC method may be validated, put on the shelf and put into practice again later when the method is needed, without heavy investment in apparatus.

4. Quality criteria for the use of TLC in residue analysis

4.1 Introduction

In the EU, minimum quality criteria for the identification of residues using different analytical techniques have been published (EC Directive 1989). For the use of TLC the Commission has specified the following quality criteria:

1. The R_F value of the analyte should agree with the R_F value characteristic for the standard 8material. This requirement is fulfilled when the R_F value of the analyte is within 3 % of the R_F value of the standard material, under the same conditions.
2. The visual appearance of the analyte should be indistinguishable from that of the standard material.
3. The centre of the spot nearest to that due to the analyte should be separated from it by at least half the sum of the spot diameters.
4. For identification, additional co-chromatography in the TLC step is mandatory. As a result, only the spot presumed to be due to the analyte should be intensified; a new spot should not appear, and the visual appearance should not change.
5. For confirmation two-dimensional TLC is mandatory.

In Belgium the EC criteria were transformed into the following quality criteria for the use of TLC in residue analysis of growth promoters (Pottie *et al*. 1993). They are summarised as follows (Note that criteria 4 and 5 have been switched):

1. The R_F of the analyte should agree with the R_F obtained for the standard within 3 %.
2. The visual appearance of the analyte should be indistinguishable from that of the standard.
   The amount of standard to be applied on the TLC plate is agreed for all laboratories.
3. The centre of the nearest spot should be separated from **the centre** of the spot for the analyte by at least half the sum of the spot diameters.
4. For analysis of growth promoters, two-dimensional TLC **is mandatory.**
5. For additional information, co-chromatography **may be applied.**

4.2. Discussion of the Quality Criteria

1) Quality criterion number 1 is the same for any chromatographic procedure: likewise, in GC and HPLC the retention times of the two peaks, formed by the analyte and the standard, should correspond. Otherwise the analyte clearly differs from the standard. A window of 3% is a reasonable quality criterion. The centre of the spot of the analyte eluted, for example, for 2 cm may differ from the centre of the standard within a radius

of 0.6 mm (Fig. 14). A deviation outside of this radius is easily noticed by the analyst. Where a greater deviation occurs co-chromatography may be used (see paragraph 5).

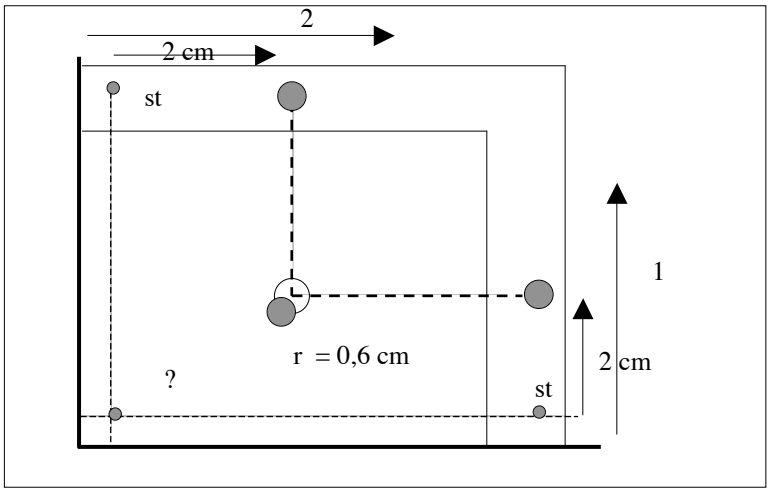

Fig. 14. Interpretation of the correspondence of R_F values

2) Criterion number 2 requires that the visual appearance of the spots for the analyte and the standard should be indistinguishable. In this respect TLC may offer more specificity and selectivity for particular analyses (e.g. residues of anabolics) than GC and HPLC (except where more specific detectors such as a Diode Array Detector or a Mass Spectrometer are used). Not only the R_F value but also the reaction of the analyte with certain reagents, the colour of the spot when illuminated with different sources (UV at 254 and 366 nm and visible light, for instance) may be used. The concentration of the analyte on the thin layer plate may be important; depending on the concentration a different fluorescence or colour may be obtained. Moreover, TLC is not restricted to two R_F values only. The same sample may be chromatographed simultaneously on different stationary phases (e.g. silicagel 60, RP-18 and CN for the analysis of anabolics in injection sites (Smets *et al.* (1991)). The use of more than two R_F values may be considered as additional or specific quality criteria.

3) Criterion number 3 requires a resolution of one between the two spots. However, this quality criterion is not clearly described in the EC document. The exact text should be: “separated from **the centre of** it by at least”. Otherwise the text could be interpreted as "the border" of the spot of the analyte which is clearly different. In practice this quality criterion means that the spots may touch each other, no matter how big or small the spots are (Fig. 15A). Here the question might be put whether the criterion should only be required for spots with the same visual appearance. For example, a green analyte may in practice be distinguished perfectly from a red interference even if the spots are partially overlaid (Fig. 15B). This situation is comparable with a diode array file where chromatograms at different wavelengths may be visualised.

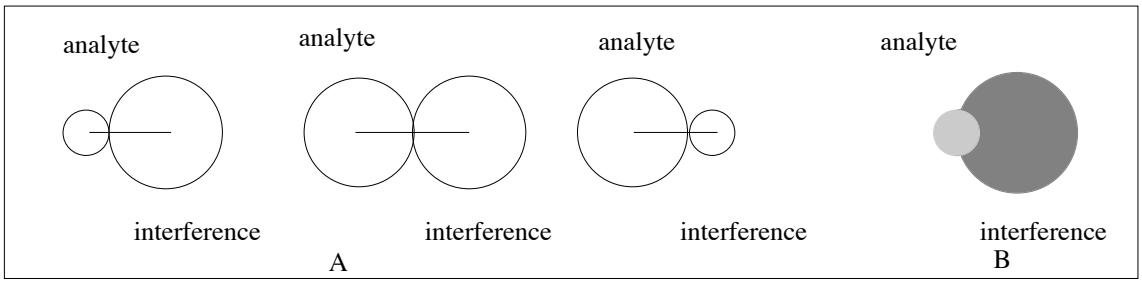

Fig. 15. Spots with the same visual appearance may touch each other

4) In criterion number 4 (*EC, number 5*) 2D-TLC is mandatory for confirmation of the identity of an analyte. It should be emphasised that two-dimensional thin layer chromatography is not the same as performing two 1D-TLCs in two different solvents. The resolving power and the identification power of 2D-TLC surpasses two 1D-TLCs. This is illustrated with a theoretical example in Fig. 16. It can be seen that an unknown product interferes with analyte 1 in the first direction. In cases of bad luck (Murphy's Law!), the same or another product interferes with analyte 2 in the second direction. Using two mono-dimensional chromatograms neither of the two analytes will fulfil the minimum required by quality criterion number 3. With two-dimensional TLC both of the analytes are separated clearly from this interference.

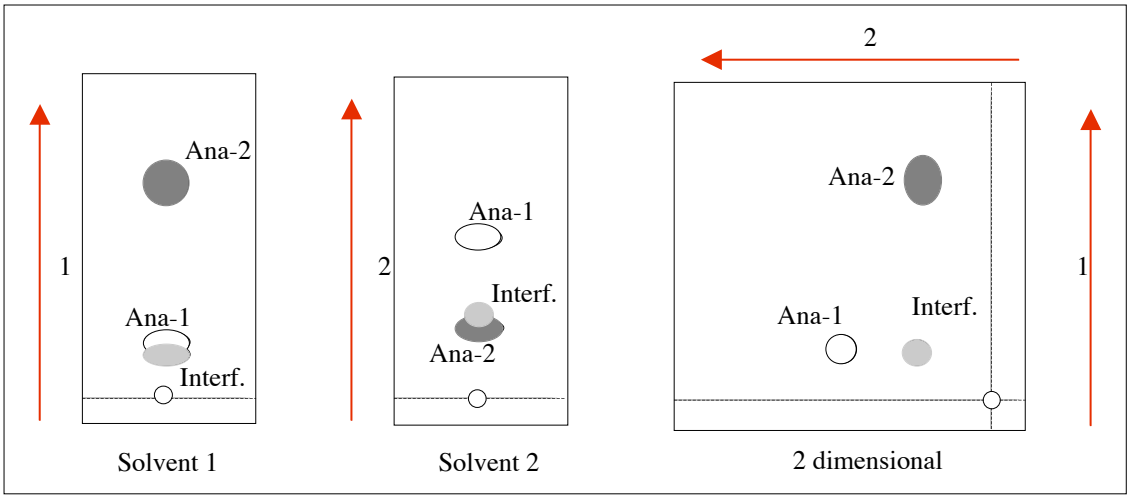

Fig. 16. Comparison between 2D-TLC and two 1D-TLCs, theoretical example

5) In criterion number 5 (*EC, number 4*) co-chromatography is required for proper identification of an analyte. This criterion is also mandatory for GC and HPLC (even for GC-MS). However, the application of co-chromatography in TLC is much easier than for the other chromatographic techniques because TLC is an off-line technique. It is important that the concentration of standard analyte added is of the same magnitude as that of the

sample. The usefulness of co-chromatography may be questioned; co-chromatography for (HP)TLC may prove that the spot in question is not the analyte but not that the spot is certainly the analyte. There is always the possibility of an interference. The products used in the illegal market are often "crude" (i.e. not of analytical grade) and impurities of pharmaceutical grade products may cause interferences in the analysis. As an example, a by-product of progesterone synthesis, 4-pregnen-20β-ol-3-one, was found to interfere with the TLC analysis of methyltestosterone and was called *le faux methyl* (the false methyl) (Smets *et al*. 1994). This interference has exactly the same fluorescence and exactly the same R_F values as the analyte methyltestosterone itself on silicagel plates. However, the two products are very well separated on reversed-phase plates.

5. Comparison of TLC with other methods for residue analysis

This comparison is based on practical use of the methods only; it is assumed that all the methods fulfil all the necessary quality criteria. The methods must be reliable and show an adequate detection limit. The equipment used should also be appropriate to the importance of the results; “it is not necessary to use a cannonshot to kill a fly”.

Stephany (1989a,b) published an interesting Table which gives an indication of costs for the analysis of residues of anabolic agents in urine and meat of slaughter animals (Table 2). The idea of calculating the cost of analysis by the cost/analyte ratio is, of course, correct. However, the costs given in Table 2 are only valid under conditions of a continuous supply of samples and Table 2 still contains a lot of unknowns.

Table 2. Indication of costs for routine residue analysis of anabolics (based on Stephany 1989a,b)

Method	analytes per sample	cost per sample*	cost per analyte (%)*
RIA	1	1	100
TLC	10	6.3	63
GC-MS	100	8.8	8.8

**Costs are quoted as relative to the cost of one RIA for one analyte as the unit (1 or 100%)*

Table 3. Indication of costs for routine residue analysis of anabolics (reviewed)

Method	analytes per sample	cost per sample*	cost per analyte (%)*
RIA	1	1	100
TLC	20	8	40
GC-MS	30	12	40

**Costs are quoted as relative to the cost of one RIA for one analyte as the unit (1 or 100%)*

In Table 3 a recalculated scheme is given. In our opinion a greater cost difference between TLC and GC-MS should be applied; the difference in equipment cost is high. Moreover, cost and performance of GC-MS apparatus may vary widely. The cost of an analysis with a mass selective detector (MSD) would not be the same as with a more expensive magnetic sector instrument. Also the number of analytes which could be separated by TLC is larger than 10 (the Fig. mentioned by Stephany). For example, with HPLC clean-up and the "4 X 4" method up to 20 anabolics are screened simultaneously in urine by TLC (Smets *et al.* 1984; De Brabander *et al.* 1988). The simultaneous identification of 100 components of interest by GC-MS in one single run is notional. It is no problem to separate 100 or more

components on a capillary column but they are not always components of interest. The real problem is finding the proper method for simultaneous extraction and clean-up of 100 analytes prior to GC-MS and separating those components in one run. Therefore, we reduced this Fig. to 30, which is still high. Moreover, the client is not always interested in 100 components. Comparing these figures, the GC-MS cost is of the same magnitude as a TLC analysis. It all depends on the exact number of analytes, which have to be screened (for example, the EC require testing for only a limited number of anabolics). If that number is less than 30, GC-MS will be more expensive than TLC. It should also be emphasised that variations in the cost of a single analysis are of minor importance if the number of analytes is very high. Of course, the parameters covered in Table 3 are not the only ones that should be considered by a laboratory manager. Quick results, for example, are sometimes of major importance. In this respect, TLC is a very robust technique and is not as prone to instrument failure as are complex instruments like GC- and LC-MS.

6. Examples of TLC methods in residue analysis

6.1. TLC Methods for Illegal Growth Promoters

6.1.1. Thyreostatic drugs

The use of these drugs in cattle results in a spectacular weight gain, arising mainly from an increased filling of the gastro-intestinal tract and an augmented water retention. Moreover, the quality of the meat is disimproved. In our laboratory a specific TLC method for the determination of thiouracil and analogous compounds has been established (Fig. 17). The method is based on TLC separation of the 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-) derivatives of the thyreostatics with fluorescence detection (De Brabander, Verbeke 1975).

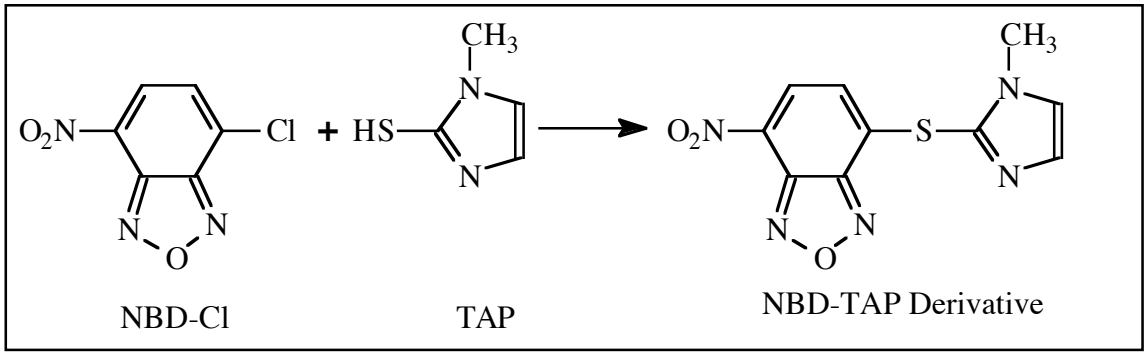

Fig. 17. Reaction of tapazole (MM = 114.2) with NBD-Cl (MM = 199.5) to form a TAP-NBD-Cl derivative (MM = 277)

The method was adopted by the EC for qualitative analysis of these drugs at the 50 ppb level. The clean-up of the samples was performed with a mercury column. The HPTLC method could be coupled to a GC-MS method for confirmatory analysis by removing the spot from the plate, transferring the silicagel to a vial and injecting it after derivatisation with N-Methyl, N-trimethylsilyl-trifluoroacetamide (MSTFA) (De Brabander *et al.* 1992). The method could be coupled also to multiple mass spectrometry (MSn) by injecting the remainder of the extract into the APCI interface of a mass spectrometer (see Section 7 (De Wasch *et al.* 1997)).

6.1.2. Anabolic steroids

The use of anabolic steroids as growth promoters in the fattening of animals is prohibited in all EU member-states. For regulatory control at farm level, urine and/or faeces of animals may be sampled. At the retail level, or in case of import/export of meat, sampling is restricted to tissue only. Where illicit administration has occurred the identity of the anabolics is unknown. From the analysis of injection sites it is known that cocktails of different anabolics are used (Smets *et al.* 1991). In order to protect the consumer, screening of the samples at a 2 ppb action level by a multi-residue TLC method is used. However, the

original clean-up described (Verbeke 1979) was too time-consuming for routine analysis. Therefore, modifications of the method of Verbeke have been described (De Brabander *et al.* 1989, 1990, 1993; Smets 1990; Hendriks *et al.* 1993). These methods are based on fluorescence induction by reaction of the steroids with sulphuric acid (Fig. 18).

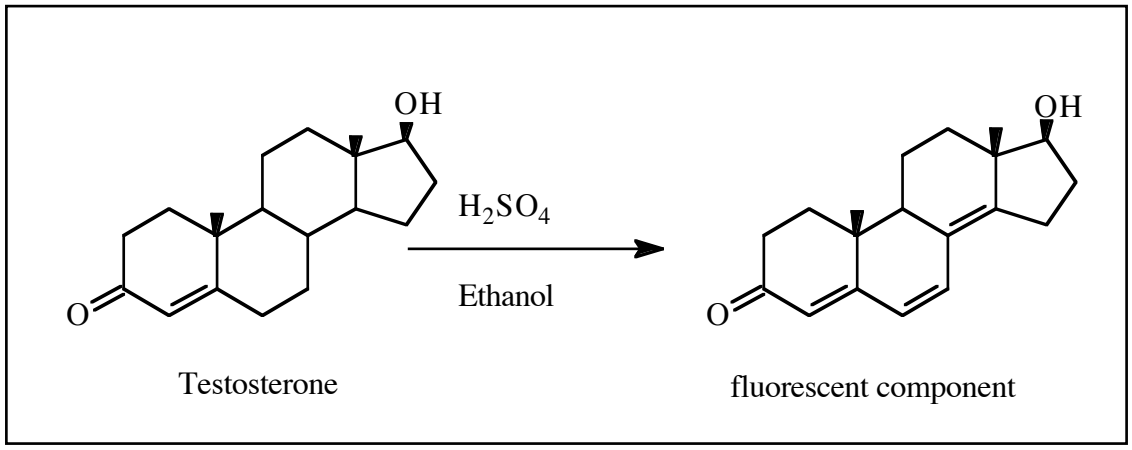

Fig 18. Fluorescence induction under influence of sulphuric acid/ethanol (Kober reaction)

These procedures could also be combined with an additional clean-up by HPLC with column switching as described by Smets (1984). By fraction collection, very "clean" extracts are obtained. Each fraction has only to be examined for a limited number of compounds eluting within that specific retention time. However, the use of HPLC also increases the cost of analysis. The reduction of matrix components improves considerably the interpretation of the TLC plates (Smets *et al.* 1997). The TLC results could be confirmed with GC-MS by derivatisation of the remainder of the extract (De Brabander *et al.* 1993).

6.1.3. Beta-agonists

A new line of drugs, the beta-agonists, found illegal application in animal breeding during the 1980s. Beta-agonists are products with a structure similar to that of epinephrine. At higher doses than the therapeutic one, these drugs were found to produce extra weight gain in the animals together with a repartition between muscle and fatty tissue. Therefore, they are also called repartitioning agents. A TLC method, for the determination of clenbuterol and cimaterol, was described by Courtheyn (1988a, b). Urine (18 ml) or an extract of meat is made alkaline (pH 12) and extracted through a ChemElut column with a mixture of toluene and dichloromethane. The beta-agonists are re-extracted from the organic phase with a minimum amount of acid (100 μl). This concentrate is analysed by 2D-TLC on silicagel 60. The beta-agonists are detected by spraying with a modified Ehrlich's reagent or a diazotisation mixture. The detection limit is approximately 1 ng, corresponding to 1 ppb (μg/kg) detection in urine. However, for beta-agonists other methods such as ELISA, HPLC

with diode array, post-column derivatisation, GC-MS and LC-MSn are described which produce quantitative results.

6.1.4. Corticosteroids

Recently corticosteroids have been abused in cattle fattening. The use of low doses of dexamethasone has been described as producing increased liveweight gain. This weight gain is probably due to secondary effects of the corticosteroids such as water retention. For the group selective detection of corticosteroids alkaline tetrazolium blue yields blue spots permitting a detection limit of 10 ng (Courtheyn *et al.* 1993) (Fig. 19). Other reagents, such as naphtol-sulphuric acid, yield analyte-specific fluorescence colours. Vanoosthuyze *et al.* (1993) selected a resorcylaldehyde spray as the most selective reagent for the analysis of corticosteroids in injection sites. These techniques permit the analysis of corticosteroids in faeces at a level of 2 ppb (Courtheyn *et al.* 1994).

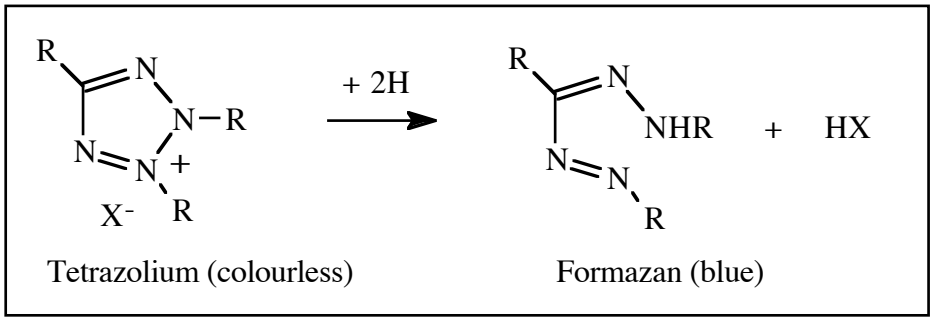

Fig. 19. Reduction of tetrazolium blue to a formazan.

6.2. TLC Methods for Antibacterials

In most EU member states slaughter animals are screened for residues of antibacterials by microbial inhibition tests on kidney tissue. However, inhibition tests lack sensitivity for some analytes and, in the case of a positive test, the identity and concentration of the substance should be determined to establish if the residue level is above the MRL. An overview of the methods -used for the determination of antibiotics used in agriculture- is given by Oka *et al.* (1995). In this section TLC analysis of some important families of antibacterials is discussed briefly.

6.2.1. Sulphonamides

Several TLC methods for the determination of sulphonamides have been described (Haagsma *et al.* 1984; Van Poucke *et al.* 1994; Posyniak *et al.* 1996; Steinbeck *et al.* 1996). In our laboratory a variation of the method developed by the Central Veterinary Laboratory (CVL), Great Britain (Heitzman 1992) was evaluated (Okerman *et al.* 1998). For that method TLC plates (silicagel 60) with a concentration zone were used. The plates were developed with a manual three-step method using different mixtures of 2% ammonia in methanol with methylene chloride ranging from 30 % to 5% (Van Poucke *et al.* 1994). The sulphonamides were detected by dipping in a 0.01 % fluorescamine in acetone solution.

6.2.2. Tetracyclines

A number of scientists have reported TLC methods for the determination of residues of tetracyclines (e.g. Oka *et al.* 1983; Oka *et al.* 1984; Oka, Suzuki 1984). Ligands have to be added to the chromatographic system (stationary or mobile phase) to prevent extreme tailing due to particular properties of the tetracyclines. For normal phase plates ethylenediamine tetra acetic acid (EDTA) is recommended. For reversed-phase plates, oxalic acid is added to the mobile phase. A good separation of most tetracyclines can be obtained. Many reagents have been described for the detection of tetracyclines. The most sensitive detection is performed through inducing fluorescence by spraying the plates with magnesium chloride. Most of these properties are analogous to the HPLC determination of tetracyclines with post-column derivatisation (Croubels *et al.* 1997).

6.2.3. Polyether antibiotics

Polyether antibiotics are particularly effective in poultry and may also act as growth promoters for cattle by increasing feed conversion efficiency. These components have a low polarity compared to other antibiotics and are unstable in acidic conditions. TLC separation of polyether antibiotics is described (Owles 1984) but mostly for their detection in animal feed. One of the main problems is selective detection of the analytes after the TLC separation.

6.2.4. Macrolide antibiotics

Macrolide antibiotics are a very important class of antibacterials used in veterinary medicine. Although they may be separated and detected by TLC, the technique is only occasionally used for analysis of these antibiotics. The main reason is that LC (-MS) methods are better suited for obtaining the quantitative data needed for the control of antibiotics having a MRL. The combination of TLC with microbial inhibition tests is worth mentioning. In this so-called TLC-bioautography, developed TLC plates are brought into contact with a bacterial growth medium. After incubation, the location of the inhibition zone is used for identification of the antibacterial (e.g. Salisbury *et al.* 1989).

6.3. TLC Methods for Other Residues

TLC methods have been described for many other residues. For pesticides, for instance, several multi-residue methods capable of detecting a large number of pesticides (100-200) by using different solvent and detection systems were reviewed (e.g. Fodor-Csorba 1996). From the presentations at two of the most important recent symposia on residues (*The International Symposium on Hormone and Veterinary Drug Residue Analysis* and *EuroResidue*) the following methods using TLC have been selected:

- screening for ivermectin at the level of 5 ppb (Abjean 1993),

- screening residues of albendazole, an antiparasitic drug, in cattle liver by TLC (Abjean 1996),
- screening of quinolone residues at 5-15 ppb in pork muscle (Juhel-Gaugain 1996),
- determination of thiamulin, a semi-synthetic derivative of the diterpene antibiotic pleuromutilin in feed and animal tissues (Posyniak *et al*. 1996),
- determination of 5-nitrofuran compounds in meat (Juszkiewicz *et al*. 1993),
- hygromycin B in bovine plasma and swine serum (Medina *et al*. 1993),
- determination of malachite green residues in fish (Munoz *et al*. 1993).

7. Is there a future for TLC in residue analysis?

When the first author of this chapter was appointed to the Laboratory for Chemical Analysis of Food of Animal Origin in 1973, TLC was our method of choice for residue analysis because it was the only technique we could afford which permitted us to assay at the ppb level. The only alternative was gas chromatography with electron capture detection. Nowadays, a lot of (relatively) affordable instruments are available (GC-MS, GC-MS-MS, LC-MSn) which compete with TLC. Most of these instruments are equipped with autoinjectors and may work around the clock. The future role of TLC in residue analysis will depend largely on two factors. Firstly, TLC has to be automated. Secondly, the detection limit and specificity of TLC has to be improved in order to enhance quantitative accuracy and precision. Automation and equipment for increasing specificity will, however, also increase the capital investment and the cost of analysis.

7.1. Automation in TLC

Automation in TLC is poor in comparison with the other chromatographic techniques. Application is a very time-consuming step (up to one third of the total analysis time). This problem may be solved by using “4 x 4” spotters or semi-automated spotters. Commercial TLC samplers are capable of applying several samples (e.g. 32) automatically from a sample rack. Unfortunately, the software for this spotter is designed mostly only for mono-dimensional TLC. Fig. 20 shows a scheme for what an automatic 2D-spotter should be able to do; application of 16 samples on four “4 X 4” TLC plates (4 samples to each of 4 “10 cm X 10 cm” TLC plates) and a number of standard solutions at the 16 reference positions, all in one run without the involvement of an analyst. Also, the development and detection step of TLC should be automated. A possible way to do this is through the use of laboratory robots. Only with the development of automated systems capable of handling different solvent mixtures, drying, spraying or dipping, etc. will TLC be able to compete with other chromatographic techniques already taking full advantage of autosamplers.

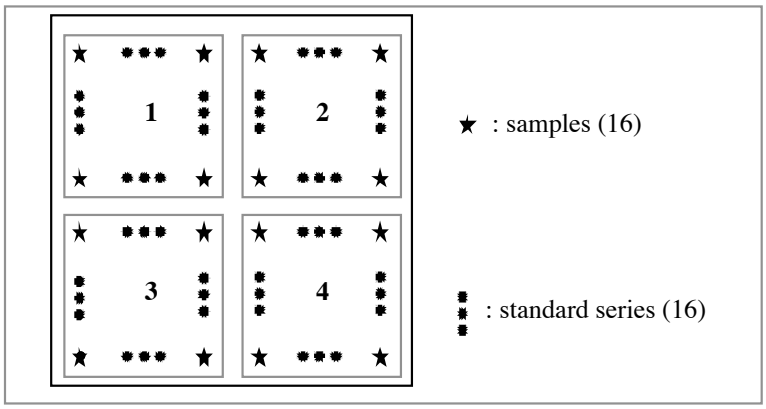

Fig. 20. Scheme for semi-automatic spotting of four "4X4" HPTLC plates containing 16 (4 plates of 4) samples and standard series.

7.2. Enhancing Specificity in TLC

In 2D-TLC the identity of an unknown analyte is determined on the basis of two R_F values. In addition, the specific reaction of the analyte with a reagent (e.g. sulphuric acid with anabolics) with formation of typical colours (illumination at 366 nm and visible light) gives an extra confirmation. Still, there is always the possibility that an unknown product (an interference) survives the clean-up, shows the same two R_F values and produces the same specific reactions as the standards. However, the probability is high that such an interference has a very similar structure to the analyte (e.g. the “faux methyl” described in Section 4).

The future of TLC in residue analysis depends largely upon the possibilities for enhancing the specificity and the detection limit of the technique. When lower amounts of analyte can be detected the amount of matrix used for analysis can be reduced. Limits of Detection (LOD) in fluorescence (now approximately 1 ng), and specificity could be enhanced by using strong excitation sources of the correct excitation wavelength. For the visible inspection of TLC plates there is a need for strong (trans-) illumination sources with variable wavelength. Coupling of TLC to spectrometric systems (UV-visible and fluorescence TLC scanners) is commercially available. Taking spectra of the suspect spots and comparing them with standard spectra could enhance specificity analogous to HPLC. However, UV-VIS spectra are more valuable for quantitative than for qualitative analysis. The coupling of TLC to FT-IR could contribute considerably to enhanced specificity since IR spectra can produce a characteristic fingerprint of a molecule. However, FT-IR usually needs more analyte than is available on a thin layer plate.

7.3. TLC/MS

TLC can be used as a form of screening technique following which the results have to be confirmed with another method (e.g. GC-MS, by derivatisation of the remainder of the extract (De Brabander *et al.* 1993)). This way of working is very useful (combination of quality criteria: 2 R_F values + GC retention time + mass spectrum) but the direct connection between a spot on the plate and a mass spectrum is lost. This connection could be very important for the identification of new "suspect" analytes detected on a plate.

7.3.1. Direct TLC/MS

Several systems for the coupling of TLC with MS (or MS-MS) have been described. Off-line mass spectra of TLC spots, have been described by several authors (Chang *et al.* 1984) using Fast Atom Bombardment (FAB) probes. The spot is transferred to the source of the MS apparatus. A FAB probe for automatic scanning of a small mono-dimensional TLC plate (10 mm x 65 mm) is commercially available. Unfortunately, the mass spectrometer required is so expensive that this probe is only of theoretical value for routine residue analysis. Busch (1996) has written an excellent review of TLC/MS. Busch (1987) and

Stanley *et al.* (1987) used Secondary Ion Mass Spectrometry (SIMS) with a home-designed apparatus. With this technique the molecules are sputtered from the plate and a two-dimensional mass map of a TLC plate can be produced. Unfortunately, the apparatus is not commercially available but the results show the goal, which could be attained. A fantasy for the future, based on the results of Busch (1987) is given in Fig. 21.

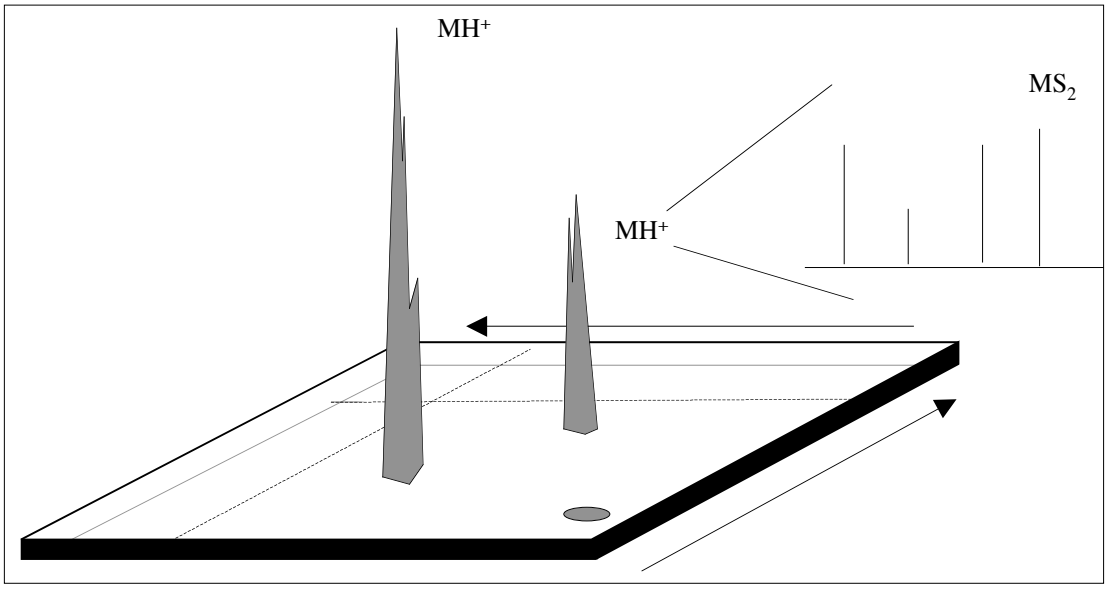

Fig. 21. 2D-TLC/MS^n: wishes for the future based on the work of Busch (1987)

7.3.2. TLC with additional hyphenated MS techniques

TLC results for suspect samples could also be confirmed by carrying out additional analysis with hyphenated MS techniques. An example of our attempts at obtaining more analytical information for the identification of thyreostats is given below (De Brabander *et al.* 1992, De Wasch *et al.* 1997).

TLC/GC-MS. By spraying the TLC plate with cysteine the thyreostats were converted to their original form because cysteine reacts better with NDB-Cl (Fig. 17). The suspect spot was removed from the plate. The silicagel was transferred to a vial, derivatised with MSTFA and injected into the GC-MS. This method was adopted by the Community Reference Laboratory (CRL) and demonstrated to the European Reference Laboratories during a workshop in Bilthoven, The Netherlands (Van Ginkel et al. 1997).

TLC/MS^n By use of multiple tandem mass spectrometry, in function of time, MS^n spectra (in practice MS^1, MS^2 and MS^3) could be taken. Since the machine is tuned by direct infusion of a calibration mixture of known molecular masses into the ESI interface we tried to infuse also the derivatised standards of thyreostats directly (ether extracts). To our surprise, excellent MS^{1,2,3} spectra of all thyreostats were obtained in that way. In Fig. 22 an example of these spectra for the tapazole-NBD derivative are given.

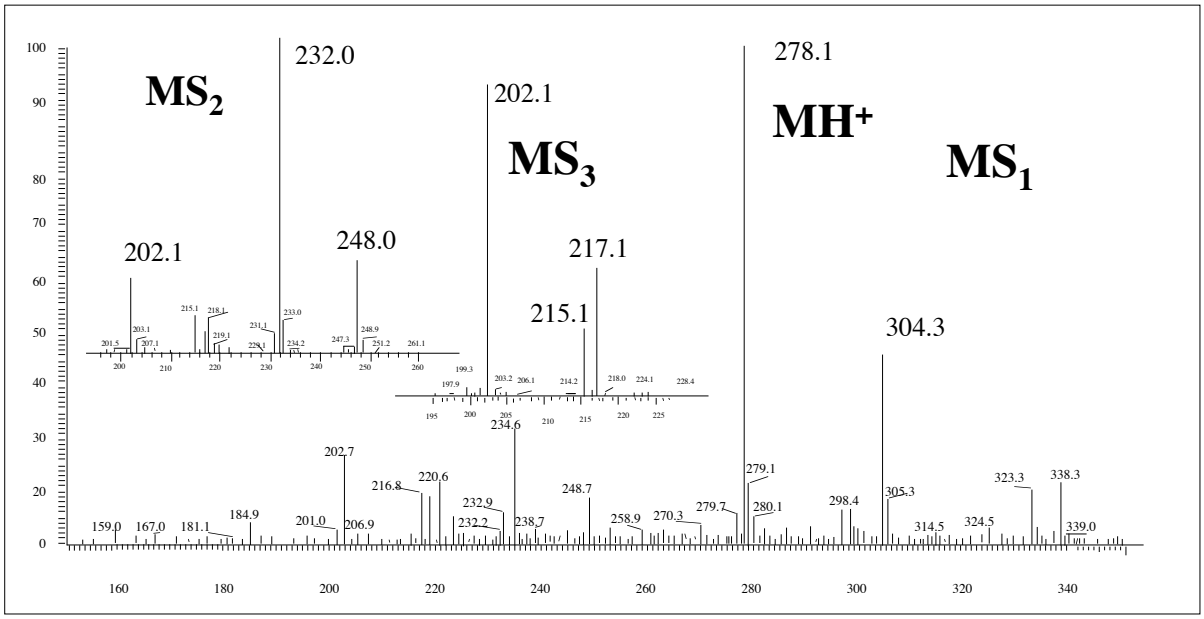

Fig. 22. MS^{1,2,3} spectra of tapazole-NBD derivative

Afterwards we infused the remainder of a number of TLC extracts from routine control. In all cases the added internal standard (DMTU) showed up in the MS^1 mode as a small peak for the MH$^+$ ion at 320 amu next to a large peak for an ion at 282.5 amu of unknown origin. From that MH$^+$ ion the correct MS^{2,3} spectra of the internal standard could be obtained, with nearly no interferences. Subsequently, we received some suspect thyroids from the CRL in Bilthoven. On TLC analysis, a small fluorescent spot at the correct R_F values for TAP was obtained. On infusion into the MS, a small peak for the MH$^+$ ion at 278 was seen in MS^1 and correct MS^{2,3} spectra were obtained (Fig. 23). In addition to the quasi-molecular ion, at least 6 diagnostic ions were obtained.

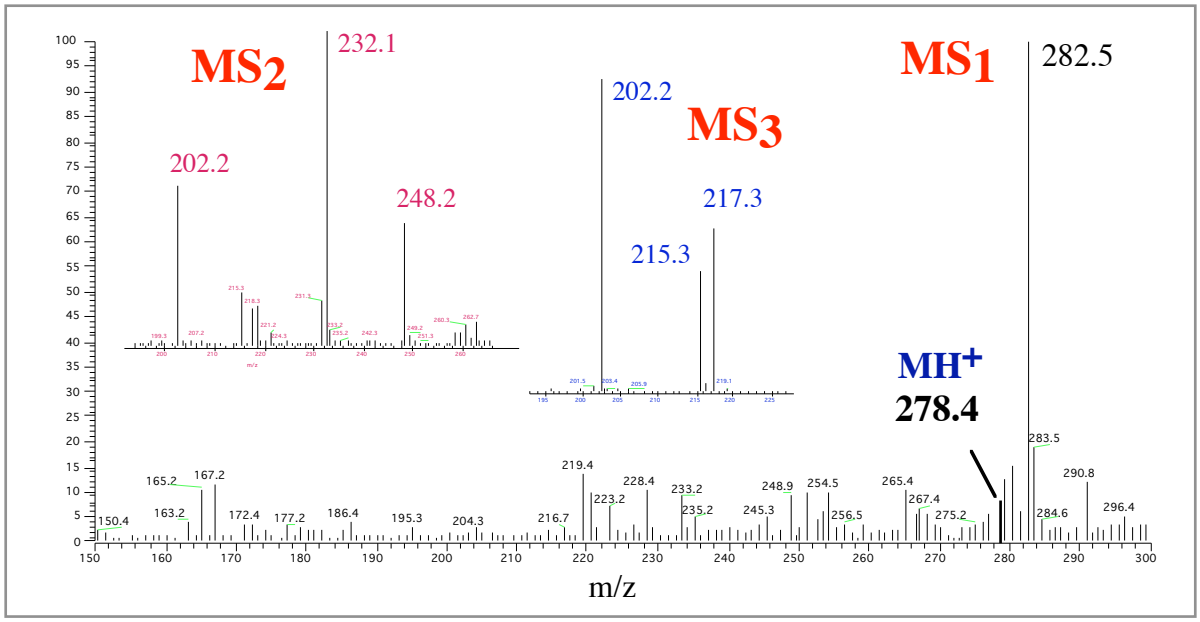

Fig. 23. MS[1,2,3] spectra of a suspect thyroid showing the correct MS[2,3] spectra of the quasi-molecular ion of the tapazole-NBD derivative.

The examples shown above demonstrate that the (old) TLC methods are still very useful for screening purposes on condition that the detection limit is low enough to be in accordance with the pharmacological properties of the drug. For the fewer suspect samples more information may be obtained by combining TLC with a MS technique.

8. Conclusion

TLC is a reliable and interesting analytical technique in residue analysis with unique possibilities not easily found in other chromatographic methods. For beginners or low budget laboratories TLC offers simplicity and low-priced primary equipment. Excellent qualitative results may be obtained quickly at a low cost and without high investment. The experienced planar chromatographer may like to use sophisticated apparatus for semi-automatic and/or highly specific work. However, special features of TLC, like the specificity and possibilities of 2D-TLC, are not always well known and therefore not favoured by many analysts.

In the years 1975-1985 TLC was the method of choice for the analysis of residues of veterinary drugs in biological matrixes. The main reason for the choice of this technique was the relatively low detection limits that could be obtained by the use of fluorescence induction (approximately 1 ng). Also, the relatively low cost of TLC in comparison with GC-MS apparatus was important. Moreover, for routine analysis TLC is very robust and nearly independent of instrument failure.

Nowadays the demands for specificity, speed and turnover are continuously increasing. Therefore, the future of TLC in residue analysis will depend largely upon the possibilities for automation and specific detection at low detection limits. However, many laboratories that have lost TLC experience will realise, sooner or later, that it was a bad move.

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3.2. USING GC-MS

After

Using GC-MS to identify of residues of illegal growth promoters

H.F. De Brabander, P. Batjoens, D. Courtheyn, K. De Wasch

LC*GC, The Magazine of separation science (1998) 16, 3, 252-256.

1. Introduction

In Europe the word "hormones" has a very bad reputation because of the possible danger for public health of residues of some of these products in foodstuffs of animal origin. Toxicologists have demonstrated that DES (diethylstilbestrol, a synthetic estrogen) is a potential carcinogen (Ferrando et al, 1974) (Marcus, 1993). In human medicine analogous experiences with DES were found (the so called DES-daughters) (Melnick et al, 1987). Recently, several cases of poisoning have occurred in Spain and France due to the consumption of liver of animals treated with clenbuterol (J.F. Martinez-Navarro, 1990; C. Pulce et al., 1991). Moreover, some environmentally persistent alkylphenolic compounds (as nonylphenol) and maybe other chemicals show estrogenic activity (R. White et al., 1994). These "environmental" estrogens are brought up in relation to the decreasing quality of human sperm and regarded as an assault on the Male (anonymous, 1993)

A residue may be defined as a trace of a component, which is present in a matrix after some kind of administration. The matrix may be anything in which a residue may be present, trapped or concentrated (meat, urine, faeces, liver etc.). There is no general agreement on the concentration level of a trace. However the ppb level (μg/kg) in which residues of illegal growth promoters are present may certainly be considered as a trace or even an ultra-trace level.

A residue analysis procedure consists of three distinct steps: first the analyte has to be extracted from the matrix. Then the extract is freed from as much interfering products as possible. The third step is the identification of the analyte (and eventually the quantification of the residue).

In modern multi-residue analysis of illegal growth promoters chromatographic techniques are very important. Next to HPTLC (High Performance Thin Layer Chromatography) methods (e.g. Verbeke, 1979 ; De Brabander et al, 1990, 1993) GC-MS (gas chromatography- mass spectrometry) is the technique most used. In low resolution GC-MS, two important types of apparatus may be distinguished. The major difference between them is the way of detection and recording of the chromatogram. Many quadrupole apparatus, as the well known MSD, use **S**elected **I**on **M**onitoring (SIM) for the determination of traces.

For the recording of a full-scan spectrum relative high amounts of analyte is needed (> 1-10 ng) with this type of apparatus. Systems based on Ion Trap technology record a mass spectrum in full-scan in pg concentrations.

With SIM a limited number of ions are monitored during a selected time interval of the chromatogram. The presence of the analyte is determined by the presence of these "diagnostic" ions at the correct retention time and within the correct abundance ratio (EEC criteria, 1989).

With systems based on Ion Trap technology the whole mass spectrum is stored for each point of the chromatogram. Afterwards, full-scan identification of the analyte by library search on general or dedicated (home made) libraries may be performed with the data system. The result of this search is expressed as a figure, which reflects the fit between the standard and the sample spectrum.

In this paper the different ways of identification of residues are discussed. Possible causes of interferences and ways to avoid them are given.

2. Possible interferences

Gas chromatography Mass spectrometry is often considered as a technique by which no identification mistakes could be made (apart from cross contamination). This may be true or nearly true in major and minor components analysis but this is certainly not the case in residue analysis of illegal growth promoters.

In extracts of biological material (e.g. urine, meat, faeces) a large variety of components in a large variety of concentrations is present. An unknown and variable amount of these matrix components are co-extracted with the analyte and introduced into the chromatographic system and the mass spectrometer. Interference between these matrix components, possibly present at relatively high concentrations (ppm range or higher) and analytes, present at very low concentrations (ppb range), is possible and should be avoided.

Interferences mostly result from co-eluting peaks or from background noise. The mass spectrum obtained is a mixture of two mass spectra and false interpretation is possible. Isotope interference is another possibility (De Brabander et al, 1993). This phenomenon may occur with any isotope but ^{13}C is a very good example. Carbon has two natural isotopes : ^{12}C and ^{13}C with a ratio of 98.9 to 1.1 (the exact figures are rounded for simplicity). In residue analyses three other important parameters should be taken into account:

1) The very large difference in concentration between the analytes and the matrix components.
2) The analyte or interference is mostly an organic molecule containing a relative high amount of carbon atoms.
3) Interferences may have analogous structures as the analytes.

In anabolizing agents as steroids 20-30 carbon atoms are present and numerous steroids and metabolites with analogous structures are known.

In a quadrupole using the SIM mode, many interferences are not observed by the highly selective use of the detector. In an Ion Trap high concentrations of co-eluting molecules may influence the ionisation time of analytes and so the detection limit.

This may cause the following phenomena: false positive and negative results and incorrect quantification.

3. False negative results

In SIM a number of diagnostic ions of the analyte are followed during a time window around the expected retention time. These diagnostic ions must be present in the correct relative intensities (± 20 % (CI) of ± 10 % (EI)). There is still a discussion about the number of diagnostic ions which must be followed. Two ions is certainly not enough: false positive results may be generated (see later). Four or more ions should be ideal from a theoretical point of view but are not practicable at lower concentrations: from ring tests it was observed that the relative intensities of the ions in most laboratories do not remain constant enough when the concentration decreases (E Daeseleire and C. Van Petegehem, 1996). The margin on the relative intensities must be increased to 20, 30 % or more with increasing chance on false positives (see later).

The higher the number of ions, the higher the specificity of the methods (less false positives) but also the higher the chance on false negative results when the identification criteria are strictly applied. Taking a decision on three ions seems to be an acceptable compromise at this moment.

The relative intensity of the ions may be disturbed by background noise and co-eluting substances. This is shown in Fig 1. In this figure the ion 440 is partly due to the analyte and partly due to an interference. In the standard the relative ratios of the ions are 425(100), 440(46) and 300(41). In the sample the relative ratios are 425(100), 440(68) and 300(41). The ratio between 440 and 425 is out of range (normal range 41 to 51) and according to the rules (the quality criteria) the sample has to be declared negative although the analyte is present.

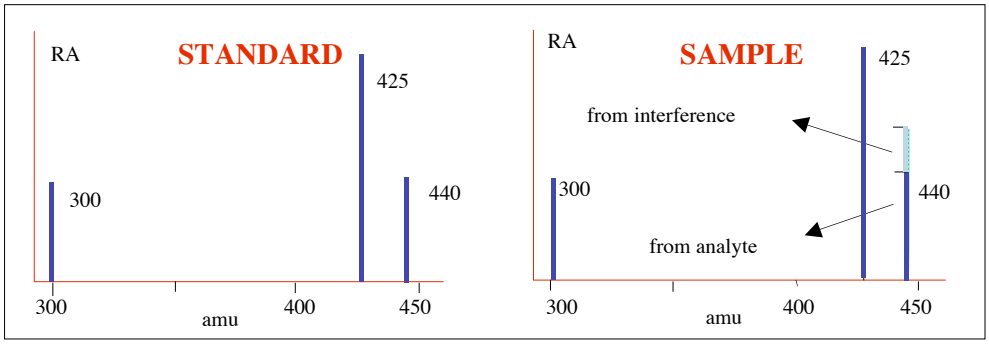


Fig 1: Formation of false negative results in SIM by interferences, which disturb the relative ratios of the diagnostic ions

The interference in Fig 1 may be due to a molecule having a molecular or fragment ion equal to one of the diagnostic ions of the analyte. However, interference is also possible with molecules containing ions, which are one or two amu units lower than the diagnostic ions. In the case of Fig 1 a molecule will also interfere when the ions 439 and 438 but also the ions 424, 423, 298 and 299 are present in a much higher concentration. All these ions will generate isotope peaks disturbing the ratio of the diagnostic ions. This phenomenon is of particular interest on shoulder peaks as is demonstrated in Fig 2. Here the huge peak 1 contains an ion 438, which has isotope peaks at 439 and 440. The ion 440 will interfere with the analyte in peak 2 when a diagnostic ion 440 is used.

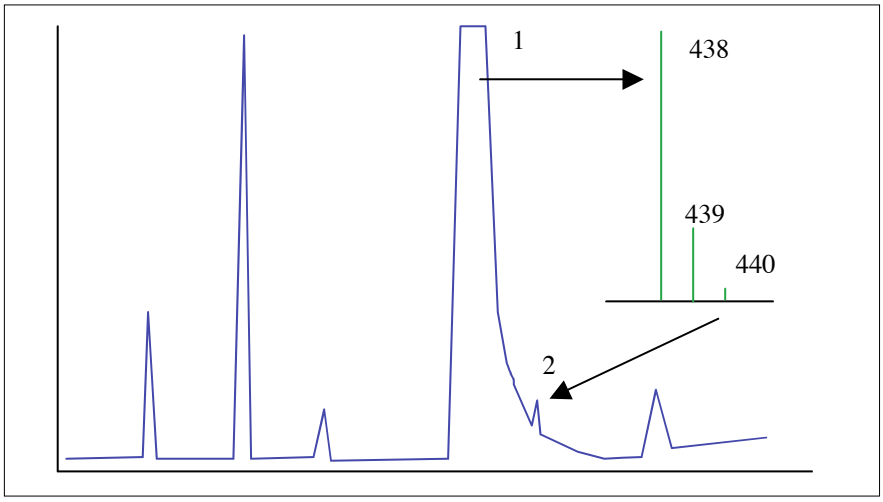


Fig. 2: Isotope interference from high amounts of molecules with small amounts of analyte.

4. False positive results

This is illustrated with an example. Nortestosterone is an anabolic steroid used in cattle fattening. The ß form is the active component of this hormone. Estradiol is the female hormone and the α form is present naturally in relative high fluctuating concentrations in female animals. It was shown that the disilyl derivatives of α-estradiol and ß-nortestosterone were not well separated in the chromatographic conditions used in most laboratories. The formulas of the di-trimethylsilyl derivatives of both components are given in Fig 3. It is observed that the molecular masses (MM) of these two components differ only 2 amu. Since the structures are similar, fragmentation may also be similar.

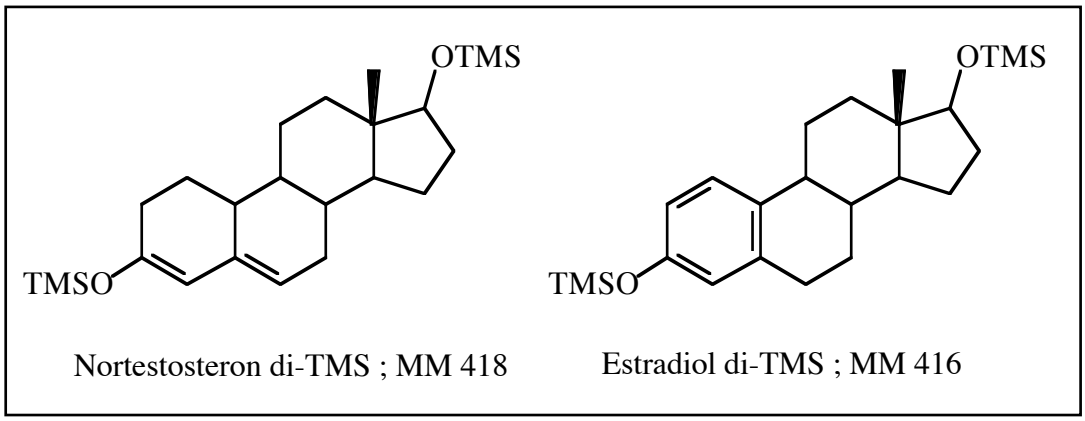


Fig 3: Formulas and molecular masses (MM) of nortestosterone (di-TMS) and estradiol (di-TMS) showing the similarity of the molecules.

For the determination of nortestosterone three ions are monitored : 418 (100 %), 403 (20 %), 328 (35 %). In the urine of pregnant cows α estradiol is present in concentrations 10^4 - 10^5 times higher than the concentration of nortestosterone found after illegal application. The mass spectrum of estradiol contains the ions 416 (molecular ion) and the fragment ions 402 and 326. It was calculated and demonstrated that the isotope peaks of estradiol may generate a SIM signal for nortestosterone (De Brabander et al, 1994). Using the SIM method according to the book, the analyst will therefore conclude to the presence of NT: the 3 ions are present within the correct retention time windows and with the correct ratios. The interfering ions may also be generated by several interferences simultaneously or by stable isotopes of other elements.

5. Improving qualitative accuracy

When GC-MS is used for the determination of residues of analytes, illegal growth promoters at the ppb level in particular, the possibility of interference should always be kept in mind. Moreover, the consequences for the owner of the animal of false positive results and for the inspection services of false negative results are considerable. However, caution and investing time (and money) into the analysis may prevent the analyst from taking a wrong decision.

5.1. Avoiding false negatives

False negatives by the loss of the analyte during the clean-up, derivatisation or injection should be monitored by using internal standards. Deuterated analogues of the illegal growth promoters are most suited for that purpose and could be used at the same time for quantification. However, their availability as well in number as in quantity is limited. Other internal standards should be used as an alternative. In Belgium for example equilenine (a hormone typical for the horse) is used as internal standard for analysis of estrogens, androgens and gestagens in matrices from other animals. Almost as important is the possibility to prove that a certain sample does not contain a certain illegal growth promoter. The best way to do this is the use of internal standards (e.g. deuterated hormones) added to the sample at the normal level of detection and monitor the recovery of these standards.

False negative results by disturbance of the normal peak ratios of the ions from the analyte by one or more interferences should be dealt with in another way. The analyst should be aware that the statistical possibility of its occurrence is high. Instead of declaring a sample immediately negative because the ratio of one of the ions is not within the range proposed by the quality criteria, other elements should be added to the analysis. Possibilities are: re injecting the same derivative on another column or GC-MS or using GC-MS-MS. Using other derivatisation reagents or techniques, performing a second analysis with a different method etc. The GC-MS data could for instance be combined with HPTLC results. At this moment, the identity of a "hormone" in a suspect sample may follow from 2 R_F values in 2D-HPTLC, a characteristic fluorescence after sulphuric acid induction, a retention window in HPLC, a retention time in GC^2 and mass spectrometric data. Although each technique on

its own does not fullfil exactly the quality criteria (e.g. disturbed ratios) the combination may give sufficient analytical accuracy.

5.2. Avoiding false positives

In SIM false positives may result from the presence of three diagnostic ions at the correct retention time and in the correct ratio windows. However, these 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 transparent to the analyst when using the GC-MS in the SIM mode.

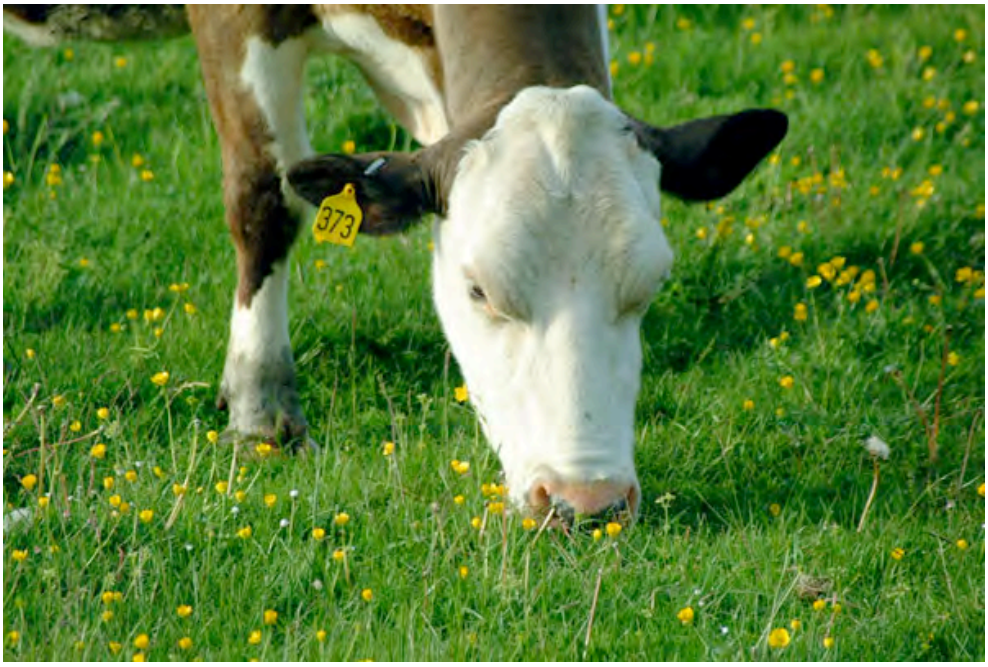
With quadrupole apparatus, which are not able to take a full-scan at low concentration the following strategy could be recommended: in the case of a positive result a second full-scan run on the same sample is performed in order to exclude the presence of isotope generating peaks at the retention time of the analyte. The absence of substantial concentrations of isotope peak generators in the full-scan mass spectrum has to be considered as a quality criterion.

These isotope interferences may be avoided by using apparatus capable of operating in the full-scan mode at low concentration levels. The quality criteria (3 ions) may be extended by using full-scan spectrum matches between the sample spectrum and (home made) library spectrum. One of the problems with systems based on Ion Trap technology is the difference of the total spectrum obtained on a Trap and that obtained on a quadrupole. In the most recent Ion Trap machines, as the Polaris Q, this problem is solved. Moreover, these instruments offer the analyst the power of MS-MS. In the near future MS-MS on benchtop machines will prove to be a powerful weapon for obtaining enhanced analytical accuracy.

6. Conclusions

It is a mistake to consider GC-MS as an ***error-less*** technique with no identification mistakes.

As in any other analytical technique false positive and false negative results could be obtained. However, when the analyst is aware of the possible cautions of these errors, the application of some simple rules and the investment of a little more time in analyses will prevent most of these mistakes.



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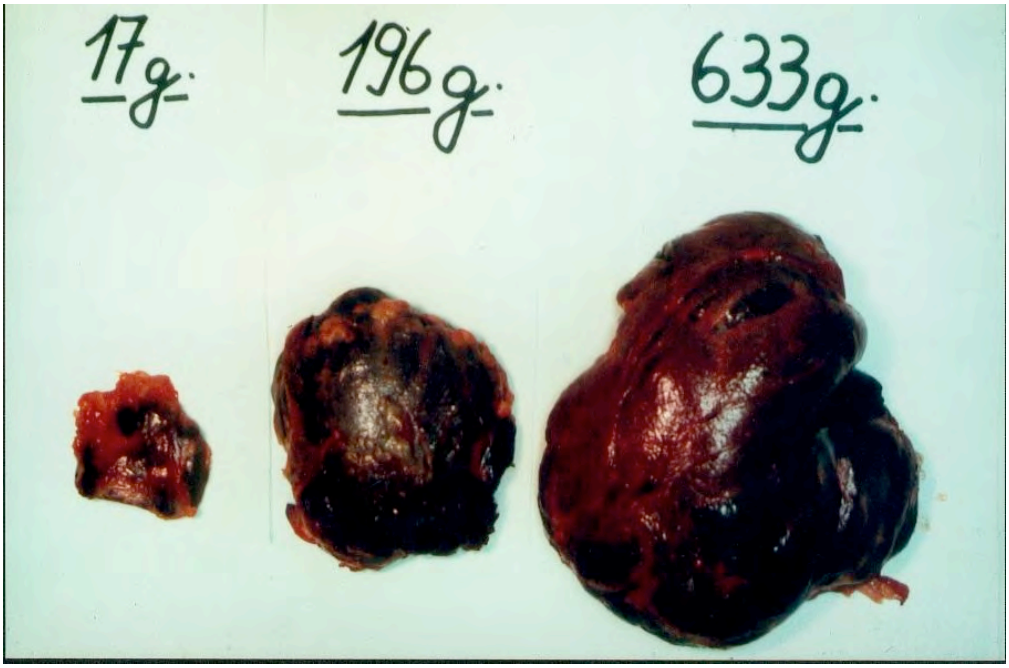
THYREOSTATS



Normal bovine thyroid (17 g) ; goiter under influence of MTU (196 g) ; super goiter (633 g)

3.3. DETERMINATION OF THYREOSTATIC DRUGS BY TLC/GC-MS

After

HPTLC of thyreostatic drugs with GC-MS confirmation

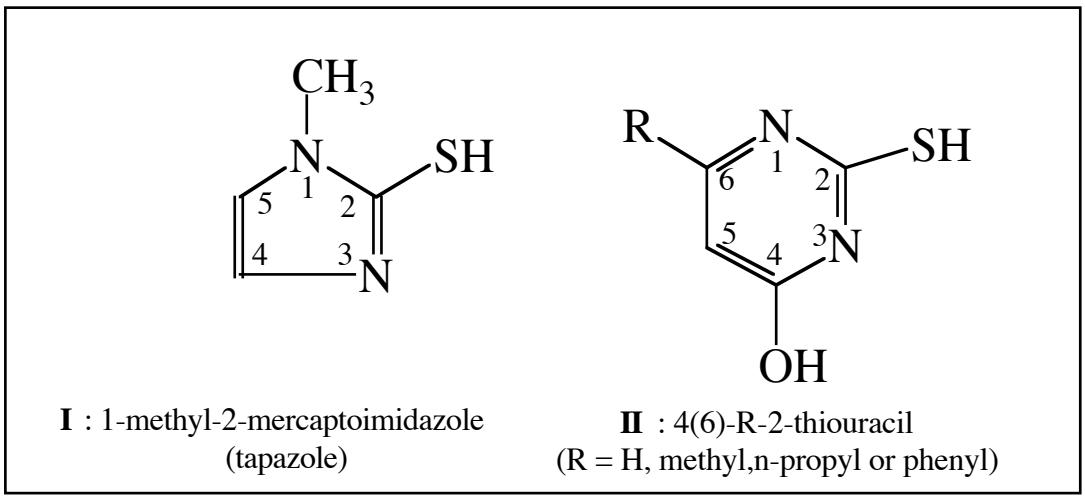
H.F. De Brabander, P. Batjoens, J. Van Hoof

J. Planar Chromatogr., 5 (1992) 124-130.

1. Introduction

The use of the thyreostatic drugs as growth promotors for animals is prohibited in all EEC member-states. In contradiction with anabolic agents there is a general agreement on the ban of these drugs: thyreostatic drugs may be harmful for human health and the meat, derived from animals treated with the drugs may be of inferior quality. The weight gain obtained with thyreostats consists mainly from an increased filling of the gastro-intestinal tract and an higher water retention of the animal. In Belgium the control on the illicit application of growth promotors is carried out by the Federal Agency for the Safety of the Food Chain (FAVV) according to the EEC directives [1,2].

Treatment of cattle with thyreostatic drugs may be detected by the residues present in plasma, excreta, meat or organs of the animal. In regulatory control at the farm plasma, urine and/or faeces of the animals may be sampled. At the retail level (butchershop, supermarket) or in case of import/export sampling is restricted to tissue only. At the slaughterhouse, tissue as well as excreta can be sampled. Moreover the inspection of the thyroid gland (e.g. the weight of the gland) may give an indication of the possible administration of anti-thyroid drugs. Finally, all kinds of matrixes (powders, fluids, feed) circulating on the (black) market have to be analysed for presence of thyreostatic drugs.



I : 1-methyl-2-mercaptoimidazole (tapazole)

II : 4(6)-R-2-thiouracil (R = H, methyl,n-propyl or phenyl)

Fig.1: Structural formulae of thyreostatic drugs (I: 1-methyl-2-mercapto-imidazole (tapazole)
II: 4(6)-R-thiouracil

The most important and powerful thyreostatic drugs, hitherto used are thiouracil and analogous compounds (especially methylthiouracil (MTU) and tapazole (TAP) see fig.1). Specific detection procedures for the detection of this group of drugs has been described previously [3,4,5]. These methods are based on the fluorescence induction of the NBD-derivatives (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) 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. These methods were adopted by the BENELUX [6] and EEC [7] for qualitative analysis of these drugs at the 50 ppb level. The sample clean-up with the mercurated affinity column was also used by Schilt [8] in a GC-NPD method. However, most of the literature cited above [4-7] is only of limited access. Moreover, since the control on anabolic agents was intensified during the last years thyreostatic drugs risk to be used again.

In this investigation the modifications of the NBD-Cl method, scientific details and some quantitative results are presented. In order to improve the qualitative accuracy of the method, the HPTLC results were confirmed by analysis of the suspect HPTLC "spots" with GC-MS with positive chemical ionisation.

2. Experimental

2.1. Apparatus

The following apparatus was used: homogenisator (e.g.Ultra-turrax), centrifuge (e.g. Sorvall), waterbath, extraction flasks (10 - 20 ml), nitrogen evaporator (e.g. Techni Dry Block), chromatographic columns and tanks, UV source of 366 nm with contrast filter, home made single sample applicator [5, 9] or "4 X 4" sample applicator [10], Tri Carb liquid scintillation counter (Packard, La Grange, Ill U.S.A) Zeiss KM 3 TLC scanner in the fluorescence mode (Zeiss, Oberkochen, D) and Ion Trap Mass Spectrometer ITS40 with positive chemical ionisation (isobutane)(ThermoFinnigan, San Jose, CA., U.S.A).

2.2 Reagents and standard solutions

Reagents:

Buffer pH 8: 94.5 ml 0.2 M Na_2HPO_4 is mixed with 5.5 ml 0.2 M KH_2PO_4. The pH is controlled and eventually adjusted to 8.

NBD-Cl solution (25 μM/ml): dissolve 5 mg NBD-Cl in 1 ml methanol. (Prepare fresh daily and keep in a cool dark place).

HPTLC plates were obtained from Merck (5547)(Darmstadt, D).

Spray solutions: I: mix 50 ml denaturated ethanol with 50 ml propan-2-ol. Add 2 ml 25 % ammonia. II: dissolve 0.6 g cysteine hydrocloride in 20 ml water

Immediately before use mix 2 ml solution II with 100 ml solution I

Standard solutions:

Stock solution of the thyreostatic drugs (TU, MTU, PTU PhTU and TAP) were prepared in methanol at a concentration of 20 mg /100 ml (200 ng /ml). A working solution is obtained by 100 X dilution in methanol (2 ng /ml).

Internal standard solution: dissolve 20 mg DMTU (4(5,6)-dimethyl-2-thiouracil) in 100 ml methanol (200 ng /ml). A working solution is obtained by 100 X dilution in methanol (2 ng /ml).

2.3 Preparation of the mercurated resin

Dowex 1x2 (50-100 mesh, analytical grade) is washed successively with 10 bed volumes distilled water, 0.5 N NaOH, dist. water, 0.5 N acetic acid and dist. water. The wet anion-exchanger (10 ml) is shaken with an aqueous solution of 2,7-dibromo-4-hydroxy mercurifluorescein (250 mg dissolved in 100 ml water) during 24 hours. The mercurated resin is washed with water until the eluate is colourless. Afterwards the resin is treated with 100 ml 0.1 HCl in 0.5 M NaCl, washed with 500 ml dist. water, treated with 100 ml 0.1 N NaOH and finally washed with 500 ml dist. water. The mercurated resin is stored in the dark.

2.4 Micro-column for the clean-up of thyreostatic drugs.

A diagram of the chromatographic micro-column, used in the 1980's for the clean-up of thyreostatic drugs, is given in fig. 2. A detailed description is given elswere [5]. Alternatively columns designed for immunoaffinity chromatography (e.g. Bio-rad Econo columns) or solid phase columns may be used.

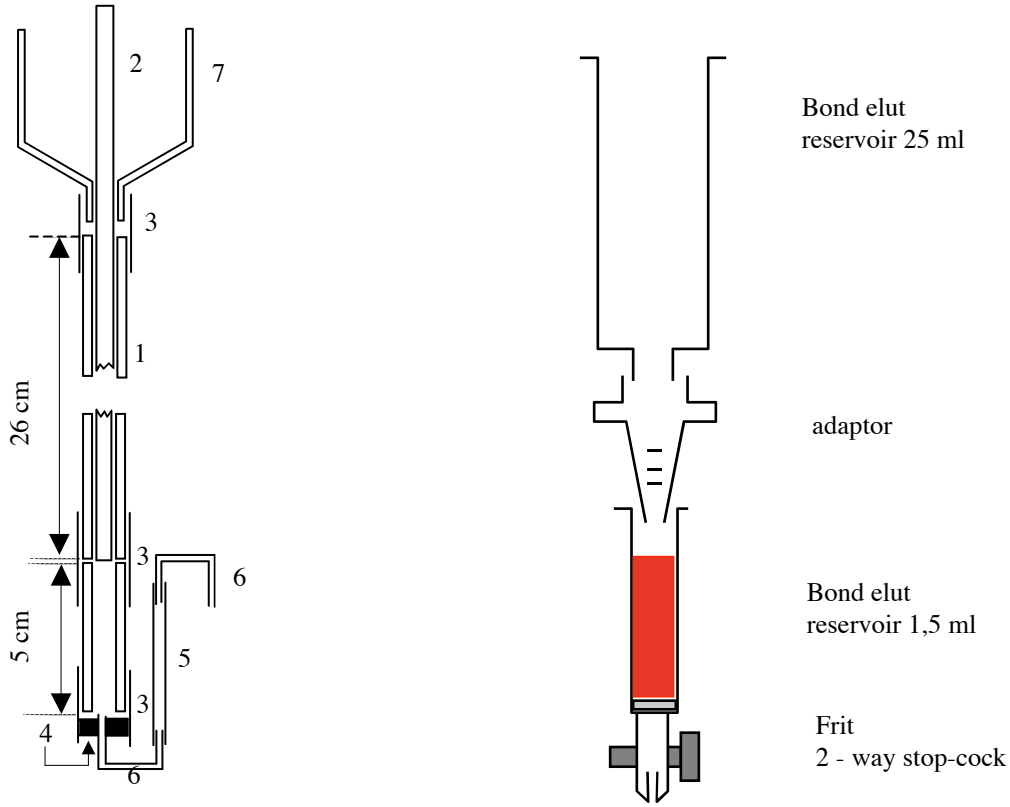


Fig 2: Micro-affinity column for clean-up of thyreostatic drugs

The micro-column is prepared as follows: the column is filled with water and the glass rod is removed. Approximately 0.6 ml mercurated resin is suspended in water and added to the glass funnel. After sedimentation of the resin in the glass column to a height of 5 cm, the excess of resin is removed. After depositing the glass rod on the resin bed, the column is ready for use. The final column volume is ca. 50 mm X 4 mm (0.6 ml).

2.5. Analytical procedures

General procedure: 2 g of tissue, 2 ml urine, plasma or skim milk are homogenised in 10 ml methanol using an ultra-turrax. The internal standard solution (4(5,6)-dimethyl-2-thiouracil (DMTU), 100 μl, equivalent to 100 ppb) is added and the homogenate is centrifuged at 10000 rpm (12000 g) during 10 minutes. The supernatant is decanted and percolated through the mercury column. The column is washed with dist. water and the thyreostatic drugs are eluted with 5 ml elution solution (0.5 M NaCl; 0.1 N HCl; pH = 1). One ml buffer pH 8 is added and the eluate is neutralized and adjusted to pH 8. A methanolic NBD-Cl

solution (0.1 ml) is added and the reaction allowed to proceed in the dark at 40°C during one hour. Thereafter, the reaction mixture is adjusted to pH 3-4 by adding 0.2 ml 6 N HCl. The NBD-derivatives are then extracted with successively 3, 2 and 2 ml diethylether. The combined ether extracts are dried over sodiumsulphate and concentrated under a jet of nitrogen, according to the concentration range investigated, to a volume of 0.1 - 1 ml.
Short procedure for urine: 0.1-0.5 ml urine is mixed with 5 ml buffer pH 8 and 100 ml internal standard (equivalent to 0.4 - 2 ppm). The solution is derivatised with NBD-Cl and extracted as described above.

Short procedure for solid matrixes (e.g. fodder): 2 g (or less) powder or fodder is mixed with 4 ml methanol in an ultrasonic bath. After centrifugation at 10000 rpm (12000 g) during 10 minutes the supernatant is mixed with 5 ml buffer pH 8 and derivatised with NBD-Cl and extracted as described above. [11]

2.6. HPTLC

A fraction (5 - 10 μl) of the concentrated extract (50 - 200 ml) is applied on a HPTLC plate by a home designed sample applicator, described elsewere [5,9]. As an alternative a faster "4 X 4" developing mode and a " 4 X 4 " sample applicator can be used [10,12].

Qualitative determination: The extracts are analyzed by two-dimensional chromatography on precoated silica gel 60 nanoplates (HPTLC plates: 10 x 10 cm or 5 x 5 cm)(e.g. Merck 5547). TLC on 20 x 20 cm plates may also be used but the application volumes have to be adapted. Development is carried out in non-saturated tanks. At the starting point of the nanoplates up to 10 μl of the extract is spotted. Appropriate concentrations of the reference mixtures are applied in the side lanes. Chromatographic development is carried out over a distance of 3 - 4 cm using methylenechloride: methanol 98:2 v/v. The plate is air dried and the starting point of the sample is overspotted with 20 ng of the thyreostatic drugs presumed to be present. This procedure is useful for additional RF-comparison between an unknown spot and the reference after the second development. The plate is then turned over 90° and run in the second direction, using methylenechloride: propionic acid 98/2 v/v. As an alternative a "4 X 4" developing mode can be used. In this mode 4 samples are developed in two dimensions on one HPTLC plate [12].
After drying fluorescence is induced by spraying or dipping with the alkaline cysteine solution (spray solution I + II). The identity of the spots follows from Rf comparison with derivatised standard solutions.
Derivatised standard solutions: 0.1 ml stock solution (= 20 mg) is mixed with 5 ml buffer pH = 8. After derivatisation and extraction the ether volume is reduced to 1 ml yielding a 20 ng/ml solution.
1 μl is applied on the HPTLC plate as reference.

Quantitative determination: the relative fluorescence intensities of the thyreostatic drug derivatives are measured against the internal standard derivative (DMTU) by thin-layer scanning in the fluorescence mode.

2.7. GC-MS Confirmation

The suspect spot is removed from the thin layer plate and transferred into an autosampler vial (100 ml). MSTFA (25 μl) is added, the contents are mixed and the vial is heated at 100 ° C during 15 min. After sedimentation of the silicagel 1-2 μl of the clear supernatant is injected into the GC (splitless injection).

GC-MS conditions:injection at 100 °C,2 min ISO at 100 °C, to 150 °C at 5 °C/min, to 250 °C at 20 °C/min, ISO at 250 °C during 3 min.(total program 20 min)

Column: J & W DB-5 (30 m X 0.25 mm ID., film thickness 0.25 mm).

Aquisition method: 1 scan/second during 20 min in a mass range of 90 to 400 amu.(filament, multiplier delay 600 sec).

The retention times and the ions used for tracing the peaks after full scan aquisition are summarized in table 1.

Table 1: Retention times and most important tracer ions of TMS derivatives of thyreostatic drugs

Thyreostat	Tr *	ion 1	ion 2	ion 3(MH^+)
TAP	13.2	171	186	259
TU	13.4	257	273	345
MTU	14.3	271	287	359
DMTU	15.2	285	301	373
PTU	15.6	299	315	387

* indicative

Preparation of standards: a mixture of thyreostatic drugs is prepared from the standard solutions at a concentration of 20 ng/ml. 10 ml of this standard mixture is transferred into an autosampler vial, evaporated to dryness and reacted with 50 μl MSTFA (15 min at 60°C). One μl is injected into the GC-MS (equivalent of 4 ng). Identification of an unkown is performed with full-scan fit search using a home made library.

3. Results and discussion

3.1. Mercurated affinity column

The extension to quantitative HPTLC of our first clean-up procedure [3] was not successful. It was found that the recoveries of thyreostatic drugs added to samples were significantly lower than those of standard solutions. Recoveries were reproducible within the same sample but very variable between samples (especially within urine samples). Obviously biological material contains unknown products in variable concentration which may inhibit the reaction of NBD-Cl with thyreostatic drugs. This is illustrated in fig. 3 by the decrease in extraction yield of ^{14}C- thiouracil, added to increasing amounts of urine.

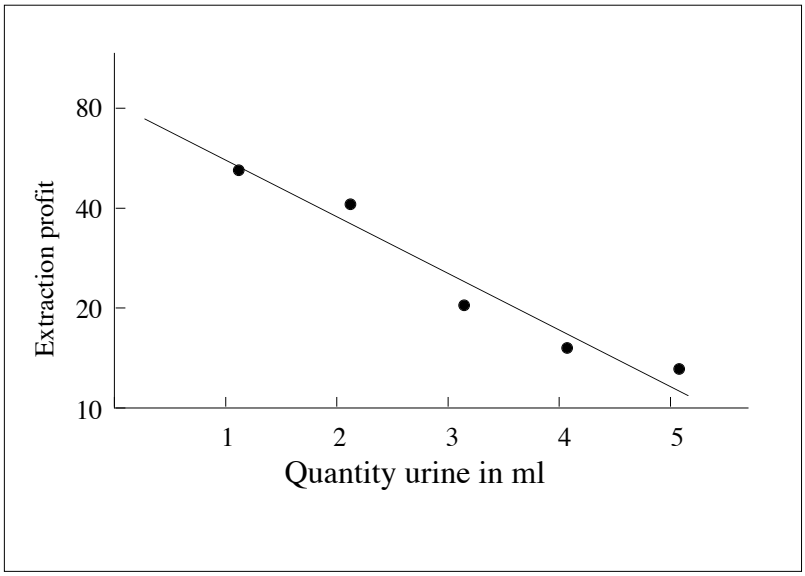


Fig 3: Decrease of extraction yield of ^{14}C -thiouracil with increasing amounts of urine

Therefore a new clean-up was searched which could avoid this problem. The name mercaptans (an old name for thiols) is derived from the phenomenon that -SH groups form very specific complexes with Hg. Several attempts were made to use this specific complex formation for a clean-up. It was found that strong anion-exchangers (e.g. DOWEX 1) strongly bind mercurial dyes (e.g. 2,7-dibromo-4-hydroxymercurifluorescein (DBMF)). Neither strong acids as 0.5 M HCl nor strong bases as 0.5 M NaOH are capable to strip off the dye from the resin. The strong bound between a mercurated dye and DOWEX-1 was already described by ZAK [13]. The binding characteristics of DBMF with DOWEX-1 resins of various cross-linkages were tested out and described previously [5]. DOWEX 1x2 was selected for clean-up of thyreostatic drugs. This resin binds 25 mg DBMF/ml resin, equivalent to 6.7 mg (33 μM) Hg^{++}/ml wet resin. This binding capacity is 2500 (PTU) to 2700 (TU) mg thyreostatic drug. Hitherto the highest concentration ever found in a thyroid gland was 200 ppm (400 mg /2 g sample). So, the binding capacity may be considered as sufficiently high.

3.2. Clean-up of thyreostatic drugs on DBMF columns

The adsorption of thiouracil on DBMF columns was studied using ^{14}C-thiouracil. After washing the column with distilled water the drug was eluted with an acid salt (0.5 M NaCl) solution. The elution yields of TU, at different pH values, in function of the elution volume, are given in fig. 4.

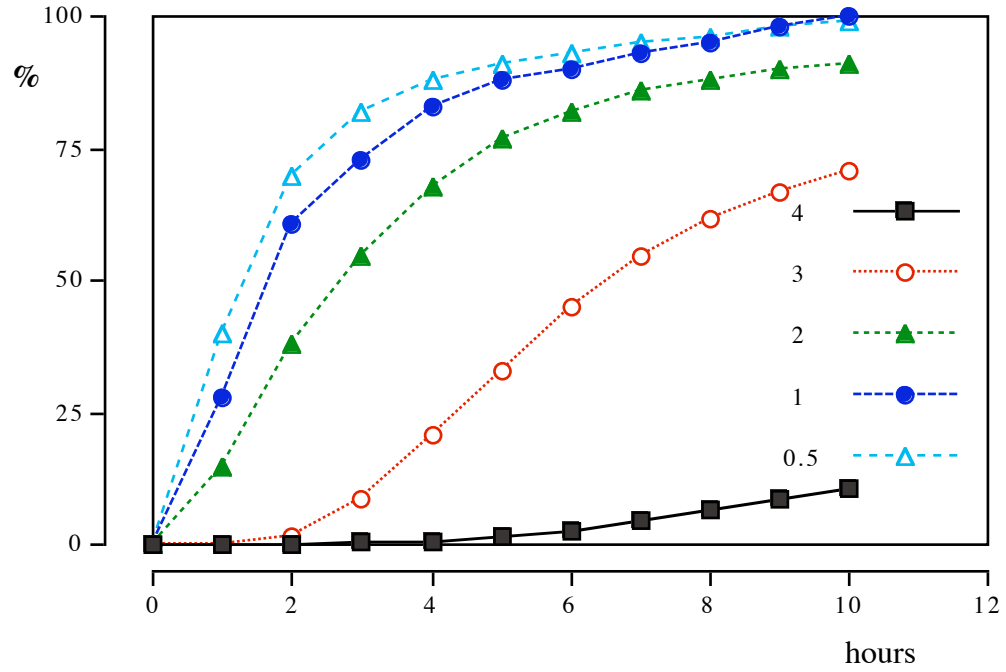


Fig 4: Elution of TU from a 0.6 ml DBMF column in function of volume eluens at different pH values (n: pH = 4; O: pH = 3; s: pH = 2; l: pH = 1; Δ: pH = 0.5)

A salt solution, with a pH value equal to 1, results in an optimal elution of thiouracil from the column.

The adsorption and elution yields of TU and other thyreostatic drugs are summarized in table 2.

Table 2:Yields of thyreostatic drugs on DBMF columns (column 0.4 cm x 5 cm = 0.6 ml) (pH=1).

thyreostatic drug	TU	MTU	PTU	DMTU	PhTU	TAP
not adsorbed (%)	3.8	0.5	1.5	0.5	8.0	0.5
elution yield: 5 ml (%)	82.0	80.0	78.0	79.0	17.0	60*
: 10 ml (%)	96.0	92.0	94.0	95.0	24.0	80*

(*): partially oxidised

The adsorption of the drugs from a methanol-water (80:20, v/v) extract (10 ml) on micro-DBMF columns (0.6 ml) is practically quantitative. By elution with 5 ml of 0.5 ml M NaCl solution (0.1 N HCl, pH 1) most of the thyreostatic drugs studied (TU, MTU, PTU, DMTU) are recovered in a 80% yield. On the contrary, lower recoveries were noted for PhTU. The interaction of the phenyl group with the polystyrene matrix of the resin may explain the strong adsorption of PhTU. The elution yield of TAP was also lower (60 %); Moreover a part of the molecules were oxidised on the column. The degree of oxidation may vary with the batch of DOWEX-1 used. By using DOWEX-1 of analytical grade the degree of oxidation is reduced but the exact reason for the oxidation is still unknown. This phenomenon restricts the quantitative analysis of thyreostatic drugs to TU, MTU and PTU.

3.3. Recovery and Reproducibility

The recovery and reproducibility of the determination of TU in meat measured using ^{14}C-thiouracil is given in table 3.

Table 3: Reproducibility of thyreostatic drugs analysis in meat (TU)

procedure step	n	mean ± SD	C.V.(%)
column elution	26	81 ± 3.9	4.8
derivatization	26	76 ± 5.0	6.6
HPTLC	22	88 ± 4.7	5.4
total procedure	22	55 ± 6.9	12.6

n = number of determinations

In each step a yield of ca. 80 % was obtained giving a total recovery of 55 %. The coefficient of variation of the total procedure amounted to 13%. The recovery of MTU, TU and PTU, at the 100 ppb level, in meat, plasma and milk using DMTU as internal standard is given in table 4.

Table 4: Recovery of the thyreostatic drugs in various media using the internal standard procedure (DMTU)

biological material	concentration added(ppb)	concentration found ± SD		
		PTU	MTU	TU
meat (3)[1]	100	106 ± 5.5	102 ±13.7	97 ± 21.8
plasma (3)[1]	100	84 ± 6.2	98 ± 5.0	76 ± 5.7
milk (3)[1]	100	88 ± 6.9	105 ± 3.6	85 ± 8.0

1: number of determinations

Recoveries for meat were quantitative for the drugs studied. Recoveries in plasma and milk were quantitative for MTU but substantially lower for PTU and TU.

3.4. GC-MS Confirmation

According to the Belgian law a so-called "positive" result on one sample, obtained in one laboratory can be (and often is) challenged by the owner of the animal. A second analysis in a second independant laboratory should be carried out. Also at export/import, discussions between different countries about the reliability of the results of residue analysis arise.
In contradiction with the fluorescence induced in steroids, where all kinds of colours are formed, all NBD- derivatives of the thyreostatic drugs are yellow. To improve the qualitative accuracy of the analysis it was tried to couple the HPTLC method to a GC-MS method and to obtain a mass spectrum of the "suspect spot" located at the correct two Rf values. So, the identity of the analyte could be proved with more analytical security.

Thyreostatic drugs could be derivatised with different reagents. MSTFA was tried first because it was also used for the derivatisation of steroids. It was found that the most important TS could easily be derivatised with MSTFA to di-TMS (TAP) and tri-TMS (TU, MTU, DMTU, and PTU) derivatives. These derivatives could be separated on an apolar column (e.g. DB-5) as is illustrated in fig 5. All the mass spectra (taken with chemical ionisation) show at least 2 characteristic ions together with the pseudo-molecular ion. These ions are listed in table 1.

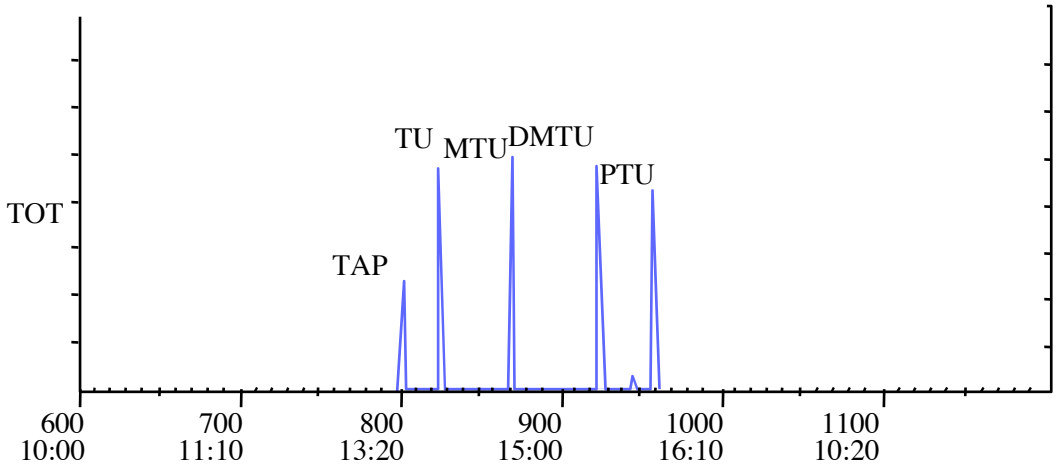


Fig 5: Gaschromatogram of TMS derivatives of thyreostatic drugs (standard solution)

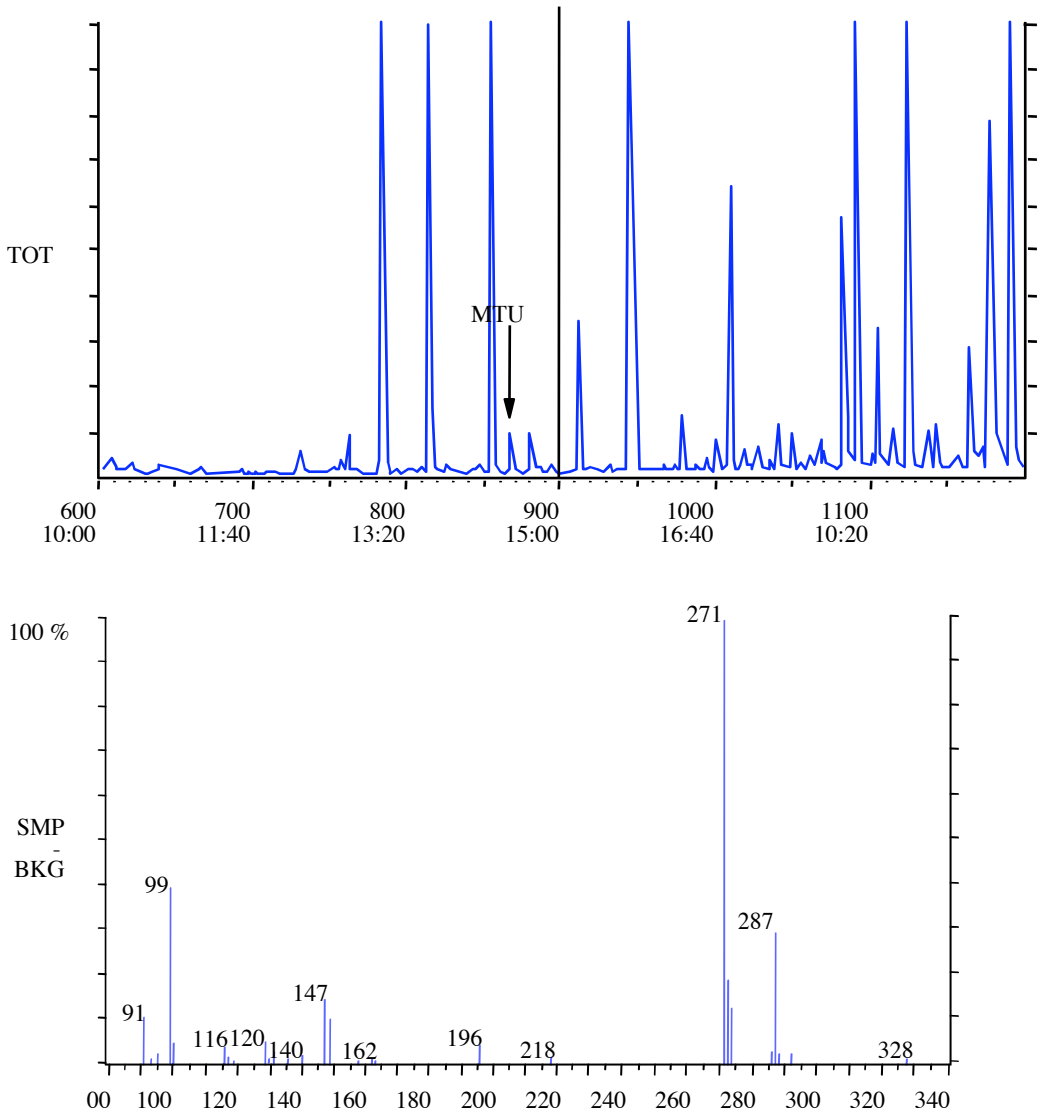


Fig 6: Gaschromatogram of MTU standard from a HPTLC plate with corresponding mass spectrum

It was also found that the identity of a certain spot on the HPTLC plate (corresponding with a thyreostatic drug), could be confirmed by scratching off the spot from the plate after fluorescence induction,transferring the silicagel into an autosampler vial and derivatizing the contents with MSTFA (at a higher temperature than the standards). This is not surprising since the TS-NBD derivatives are exchanged with cysteine-NBD derivatives for fluorescence induction. The thyreostatic drugs themselves are free again on the plate.

In Fig. 6 a chromatogram and a mass spectrum of MTU from a thin layer plate is shown. As can be seen the chromatogram contains a considerable amount of peaks, due to the background of the thin layer plate. The peak of MTU is separated from the background and a library searchable mass spectrum can be obtained. However there is a tendency for the pseudo-molecular ion to disappear at lower concentrations (scratched from the plate).

3.5. MTU concentrations in organs and muscular tissues

The concentration of MTU in the thyroid, the kidney and some muscles was determined in 5 animals, obtained from regulatory control. The results are summarized in table 4.

Table 5: Comparison of the MTU concentrations (ppm) in thyroid, kidney and some muscles of slaughtered animals taken from regulatory control.

number of the animal	1	2	3	4	5
tissue analysed.					
thyroid	30.60	48.00	53.2	41.5	37.5
kidney	0.30	0.81	2.1	2.3	2.4
M.Long.Dorsi (LD)	0.16	0.51	1.0	2.3	1.8
M. Psoas (P)	0.21	0.63	1.7	2.3	1.3
Cervical Muscle (C)	0.22	0.40	1.2	1.7	1.5
M. Gastrocnemius (G)	0.15	0.79	1.8	1.6	0.8
M. Trapezius (T)	0.17	0.54	1.0	1.7	1.2
M.Solius (S)	0.18	0.46	3.0	1.7	1.2
Diaphragma (D)	0.19	0.59	1.7	2.0	1.3

As expected, the highest concentration of MTU was found in the thyroid. The MTU concentration in the thyroid was 20-100 times the mean muscle concentration (0.2-1.9 ppm). The concentration of MTU in the kidney was significantly higher ($p < 0.005$) than the concentration found in muscular tissues. A significant difference ($p < 0.005$) in MTU

content was found between the M. Psoas (P), the diaphragma (D) and the M.Trapezius (T) (non parametric test of Wilcoxon). Classification of the different tissues in descending order of MTU concentration yields:

thyroid > kidney > P = D = LD > C > S > G > T

From these figures it can be concluded that the thyroid is the matrix of choice for the determination of residues of thyreostatic drugs. When the thyroid is absent (import / export) the diaphragma may be used as target matrix.

3.6. Effect of cooking on the MTU concentration of meat

Most of the meat consumed is prepared by heating. The fate of MTU during heating of meat was investigated using muscles of animals, taken from regulatory control at the slaughterhouses.A meat cut was divided into two portions: one portion was analysed directly. The other portion was heated in a plastic bag at 90°C during one hour. The cooked meat and the drip were separated. All fractions were analysed for their MTU content. In total, 4 different muscles have been analysed using the procedure described.

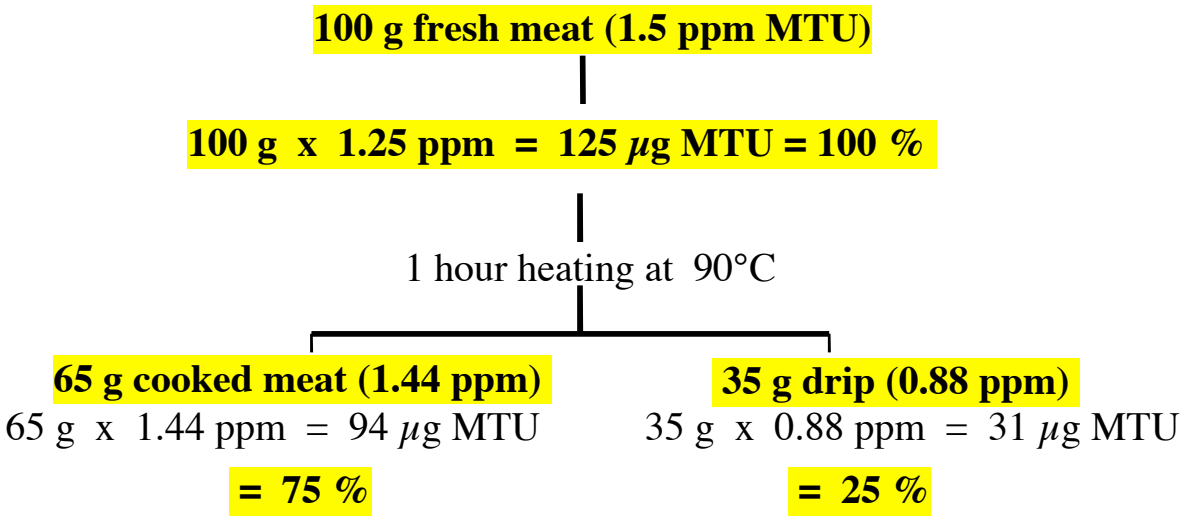


Fig 7: Effect of cooking on the residue concentration of MTU in meat

Typical results are given in fig. 7. Our measurements show that MTU is not substantially destroyed in meat after prolonged heating. Since only 25% of the total content is recovered in the drip (= 35% of the muscle weight), the MTU residues are concentrated in the cooked meat.

4. Conclusion

The use of mercurated affinity columns has several advantages in comparison with the first clean-up procedure: it permits the quantitative analysis of MTU, TU and PTU. So residue concentrations of these drugs in slaughter animals could be studied. These figures may be valuable for veterinary inspection. Unfortunately the partial oxidation of TAP on the mercury column inhibits hitheretoo the quantitative analysis of this drug. In beef carcasses, obtained from regulatory control, the residue levels in the thyroid were 20-100 times higher than in the corresponding muscular tissues. Cooking experiments demonstrated that MTU residues in meat are not substantially destroyed by heating.

Also for qualitative analysis, as needed in EEC routine control, this rapid clean-up procedure, coupled with the specific fluorescence detection after HPTLC-chromatography is important: the speed and selectivity of analysis is improved and the final extracts are cleaner and thus easier to evaluate. Next to MTU, TU and PTU the method could also be used for routine control of tapazole but with a higher detection limit (100 ppb instead of 25 ppb)

The coupling of the HPTLC method to a GC-MS method improves the qualitative accuracy of the analysis. The identity of the residue follows from two Rf values on a HPTLC plate, one retention time on a capillary column and a full-scan mass spectrum. With this strategy quality criteria for residue analysis could be fulfilled [14]. Moreover this coupling also permits mono-dimensional HPTLC to be used as screening method without the need of 2D-HPTLC: suspect spots could be analysed directly with GC-MS. For routine control at the farm, the short procedure for urine with direct derivatisation, coupled to GC-MS can be very useful.

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3.4. DETERMINATION OF THYREOSTATS BY GC-MS

After

Rapid and high performance analysis of thyreostatic drug residues in urine using GC-MS.

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J. Chromatogr. 750 (1996) 127-132

1. Introduction

Thyreostatic drugs inhibit the thyroid function: the decreased production of thyroid hormones reduces basal metabolism, lowers gastro-intestinal motility and favour extracellular water retention. The use of these substances thus allows a considerable increase in live weight gain, although this mainly results from an increased filling of the gastro-intestinal tract and augmented water retention in the slaughter animals [1, 2, 3].
In contradiction with anabolic agents there is a general agreement in the EU on the ban of these drugs: thyreostatic drugs may be harmful for human health and the meat, derived from animals treated with the drugs may be of inferior quality. In Belgium regulatory control on the illicit application of growth promotors is coordinated by the Federal Agency for the Safety of the Food Chain (FAVV) according to the EEC directives [4, 5].
The most important and powerful thyreostatic drugs, hitherto used are thiouracil and analogous compounds (especially methylthiouracil (MTU) and tapazole (TAP))

Specific procedures for the detection of this group of drugs have been described previously [6, 7, 8]. Chromatographic techniques have the advantage of not only detecting abuse of thyreostatic drugs but are also able to identify the different molecules or groups of substances.
HPTLC is used as the official method for the qualitative and quantitative determination of residues of thyreostatic drugs in biological material. The method is based on the fluorescence induction of the NBD-derivatives (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) of the drugs with cysteine after HPTLC separation. The clean-up is performed with a rapid and selective extraction procedure, based on a specific complex formation of the drugs with mercury ions bound in an affinity column. These methods have been adopted by the Benelux [9] and the EU member states [10]. Schilt et al. [11] used also the mercurated affinity column for sample clean-up in combination with a GC-NPD method.
The HPTLC method may also be coupled to an additional confirmation of the suspect "spots" by GC-MS with positive chemical ionisation.[12]. The abuse of these substances could be drastically reduced by regulatory control. Many years passed without any observation of thyreostatic residues.
Moreover, since the control on anabolic agents was intensified during the last years thyreostatic drugs risk to be used again. The last few years routine analysis of some feed samples revealed the presence of Tapazole. Urine samples and thyroid gland samples, however, were mostly free of Tapazole residues using HPTLC. Therefore a more sensitive method was developed using the hyphenated technique of GC-MS. Thyreostatic drugs are easily detected by GC-MS with positive chemical ionisation and electron impact.
Besides the confirmation of the suspect spots on HPTLC by GC-MS and now even GC-MS-MS, a rapid extraction method specific for Tapazole is evaluated using the power of tandem MS

2. Experimental

2.1 GC-MS apparatus and conditions.

GC-MS analysis was performed with an Magnum Ion Trap Mass Spectrometer (Finnigan MAT, San Jose, CA, USA) consisting of : Magnum Ion Trap System (Finnigan Mat., San Jose, CA., U.S.A) consisting of : Finnigan MAT A200S GC Autosampler, Varian 3400 GC with 1077 capillary split/splitless injector, Finnigan MAT Magnum Ion Trap Mass Spectrometer with electron impact and Advanced positive chemical ionisation and fitted with a 25 m x 0.20 mm i.d. column coated with a 0.11 μm film (HP ultra 2).

GC-MS conditions: initial column temperature: 100°C, to 200°C at 15°C/min, to 300°C at 30°C, ISO at 300°C during 3 min. (total program ca 13 min). Injector temperature: 260°C, transfer-line: 300°C: carrier gas: Helium.

Acquisition method: 1 scan/s during 10 min in a mass range of 80 to 400 amu., filament multiplier delay: 300 s., ionisation by electron impact.

GC-MS-MS was carried out with the GCQ (Finnigan Mat., San Jose, CA., U.S.A) consisting of Finnigan MAT A200S GC Autosampler, Finnigan MAT/Tremetrics high performance capillary GC, capillary split/splitless injector with electronic pressure control, Finnigan MAT Quadrupole Ion Trap Mass Analyzer.

GC-MS conditions: GC temperature programmation: identical as for the Magnum. Column: SGE BPX-5 (25 m x 0.22 mm ID., film thickness 0.25 µm).

MS mode : cfr. Magnum.

Tandem MS mode: 1 scan/s, mass range from 50 to a mass 1 amu unit higher than the parent ion selected (the parent ion is mostly the base peak of the full scan spectrum of the molecule), collision energy for fragmentation of the parent ion: 0.7 - 3.0 V.

2.2 Reagents and standard solutions

2.2.1 Reagents

Chloroform (E. Merck, Darmstadt, Germany) and dichloromethane (E. Merck, Darmstadt, Germany) were used as possible extraction fluids. MSTFA (N-Methyl-N-trimethylsilyl-trifluoroacetamide) is from Macherey-Nagel (Düren, Germany).

2.2.2 Standard solutions

Stock solution of the thyreostatic drugs (TU: thiouracil, MTU: methylthiouracil, PTU: propionylthiouracil and TAP: tapazole)(Fluka Chemie, Bornem, Belgium) were prepared in methanol at a concentration of 20 mg /100 ml (200 ng /µl). A working solution is obtained by 100x dilution in methanol (2 ng /µl).

Internal standard solution: dissolve 20 mg DMTU (4(5,6)-dimethyl-2-thiouracil) in 100 ml methanol (200 ng /µl). A working solution is obtained by 100 x dilution in methanol (2 ng /µl).

2.3 Analytical procedures

2.3.1 Extraction method for all thyreostatic drugs in urine:

Two milliliter urine were mixed with 10 ml methanol. An amount equivalent to 100 ppb of DMTU was added as internal standard and the solution was allowed to percolate through a mercury column. After washing with distilled water, the thyreostatic drugs were eluted with a solution of hydrochloric acid (0.1 N) in sodium chloride solution (0.5 M, 5 ml, pH 1). Buffer solution (pH 8, 1 ml) is added to the eluate. The eluate was then neutralized and was, after adjusting to pH 8, derivatized with NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) solution. The formed NBD-derivatives were extracted with diethyl ether and the concentrated extract (by evaporation) was spotted on the HPTLC plate.

2.3.2 Extraction method for tapazole in urine:

Two ml urine was brought in a separation funnel of 100 ml and 5 ml chloroform or dichloromethane were added.

The 2 phases were manually shaken intensively for 2 minutes. Afterwards the 2 layers were let at ease during 30 s to obtain optimal separation. The solvent layer was collected in a lab designed glass tube (Fig. 1). This extraction procedure was repeated on the upper layer, consisting of the urine sample.

The combined extraction phase (± 10 ml) was dryed completely in a Savant SpeedVac SC210A vacuum concentrator.

2.3.3 Derivatisation.

The dry residue was derivatised with MSTFA (50 μl) to form mono-TMS derivatives. [12] and brought over to an autosampler vial.

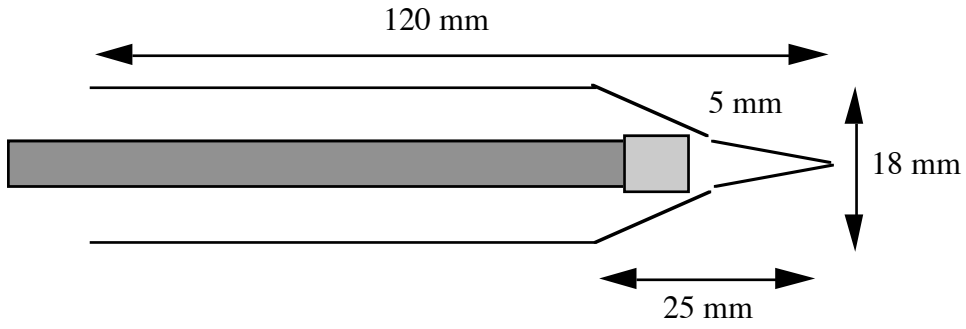

Fig 1: Lab designed special residue tube

Derivatisation of samples could be carrried out in autosampler vials after transfer of the sample and further drying under a nitrogen stream.

Alternatively the special residue tubes could be used to avoid unnecessary transfers of solvents (fFg 1). The diameter of these tubes (18 mm) was chosen to fit the tube holders of the Speedvac. Other tube containing apparatus (fraction collector of HPLC, sample preparation unit) are adapted to hold the same tubes. The length of the tube was adapted (shortened) to the syringes used for HPTLC spotting. These tubes have a maximum capacity

of 17 ml solvent (10 ml in this application) but volumes as small as 20-30 µl could be handled (e.g. derivatisation with 50 μl MSTFA). These "all purpose" residue tubes could be used to collect relative large amounts of solvents (15 ml), evaporate automatically and redissolve the analyte in small amounts (μl) of (another) solvent without tube transfer. This saves a lot of time.

Direct derivatisation for GC-MS could also be performed in the residue tubes. In that case a small reaction chamber is formed by introducing a glass rod covered at one side with teflon.

The external standard consisted of 4 ng TAP/μl and was prepared by evaporation of 10 μl of the TAP working solution (20 ng/μl) in an autosampler vial, untill complete dryness and derivatisation (15 min; at 60 °C) with MSTFA (50 μl).

3. Results and discussion

3.1 GC-MS detection of thyreostatic drugs

Different reagents were possible as derivatisation agent for thyreostatic drugs. MSTFA was tried first because it was also used for the derivatisation of steroids. It was found that the most important thyreostats could easily be derivatised with MSTFA to mono-TMS (TAP) and di-TMS (TU, MTU, DMTU and PTU) derivatives. These derivatives could be separated on an apolar column (e.g. HP Ultra 2, SGE BPX-5). This was very important for the routine operation of the GC-MS because the MSTFA derivatives could be injected right away without evaporation and redissolving in any solvent. So the column that was used for other purposes (with MSTFA) could be used. As can be seen from table 1 the 5 thyreostatic drugs of importance are all well chromatographed under these conditions with retention times varying between 5 and 8 minutes. All the mass spectra show at least 2 characteristic ions. These ions are listed in table 1.

Table I: Retention times and diagnostic ions of thyreostatic drugs in electron impact (MS and MS^2)

	Retention time*	Diagnostic ions	
		Electron impact	Electron impact MS2
TU	5:53	257	99
		99	147
		272	75
TAP	5:55	171	113
		186	130
		113	171
MTU	6:18	271	99
		99	149
		285	75
DMTU	6:55	285	113
		113	75
		299	147
PTU	7:23	299	99
		99	147
		149	75

*indicative

Therefore GC-MS-MS could be the solution to obtain more diagnostic ions to increase the specificity of the detection. The MS2 spectra were obtained by colliding the base peak of the MS spectrum which resulted in 3 usable product ions. The MS2 spectra in EI mostly contained 2 to 3 ions in the lower mass range. For the five different molecules only two different base peaks were obtained i.e. 99 for TU, MTU and PTU and 113 for TAP and DMTU.

The intensities of the different ions varied for the different molecules, but the diagnostic ions were for the 2 groups practically the same.

3.2 Comparison of the 2 organic solvents as extraction fluid for fast determination of tapazole.

Based on data from literature (13, 14) the extraction yield of TAP was estimated using chloroform ($CHCl_3$) and another organic solvent (dichloromethane (DCM)).

The recovery of TAP was obtained by adding known constant amounts of TAP to blank urine (1000 ng) and treating them in a similar manner to the unknown urine samples. One μl

out of fifty μl of the derivatised sample was injected into the GC-MS (20 ng). The yield was calculated by comparison of the respons after extraction of the spiked urine with DCM and $CHCl_3$ respectively with the respons of 20 ng TAP standard.
Quantitative analysis was performed using the external standard method, based on the area of the peak of the 3 combined diagnostic ions of TAP (171, 186, 113).

The extraction yield obtained with the 2 solvents is summarized in table II.

Table II: Extraction yield of TAP from urine, extracted with DCM and CHCl3.

	Mean value (ng)	Yield (%)
20 ng TAP standard	20	100
Spiked urine, DCM extraction	4.07 ± 0.26	20
Spiked urine, CHCl3 extraction	10.65 ± 1.46	53

The results in table II show some variation within each solvent recovery, but $CHCl_3$ offered a better recovery (53 %) compared with the extraction with DCM (20%). Therefore chloroform was chosen for the further experiment on the performance of the GCQ as rapid detection method for TAP in bovine urine. Fig 2 presents a Chromatogram of Tapazole residues in a urine sample 9 hours after administration of 1 g Tapazole.

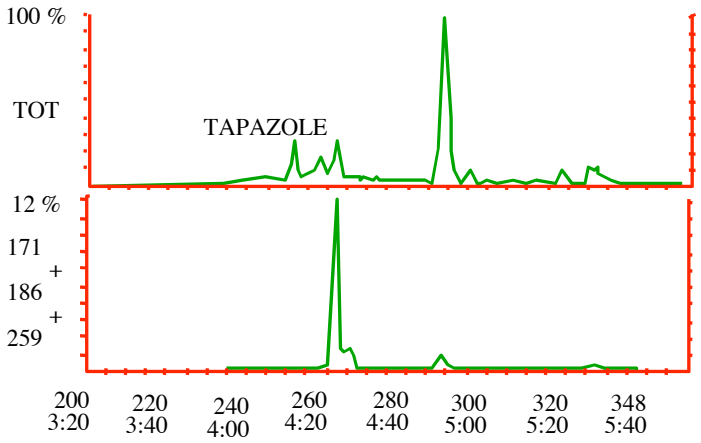

Fig 2: Chromatogram of TAP in a urine sample 9 hours after administration of 1 g TAP.

3.3 Tandem MS for tapazole detection in urine.

Urine samples spiked at 500, 250, 100 and 50 ppb were extracted with chloroform according to the method described before. Descending to 100 ppb this simple method was able to detect tapazole residues at 2 or 3 diagnostic ions in MS or tandem MS respectively. Fig. 3 shows the EI-spectra of tapazole in MS and MS2 (standard and urine sample spiked at 500 ppb).

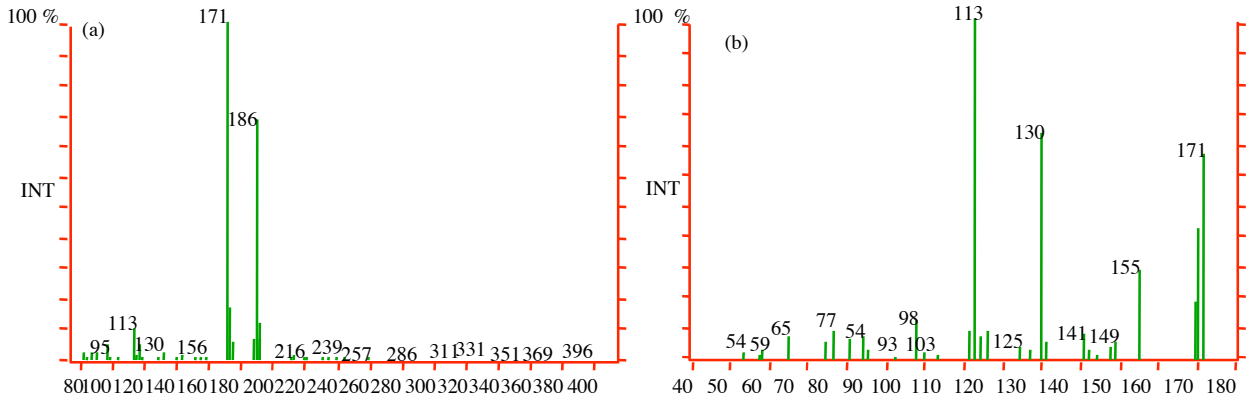

Figure 3: Electron impact spectra of Tapazole in spiked sample (500 ppb)[MS (a) and MS^2 (b)]

The combination of the rapid extraction method for Tapazole and the use of tandem MS for detection resulted in a higher specificity. On the other hand, no improvement of detection capability was achieved as could normally be expected in tandem MS.

4. Conclusion

From the two organic solvents studied, chloroform and dichloromethane(DCM), the first offered the best recovery of about 53 % compared with the extraction yield of only 20 % for DCM. A recovery rate of 53 % could hardly be called splendid, but compared with the limit of detection of the HPTLC method for tapazole (100 ppb) this very simple and rapid extraction method can be used in the analysis of urine samples as a supplementary to the official HPTLC method.

The theoretical gain of detection capability in tandem MS-MS as seen for the analysis of anabolic steroids is not accomplished for the analysis of thyreostatic drugs. Tandem MS-MS results of the last mentioned are situated in the lower mass ranges and therefor less specific. Besides this, there is also the loss of specificity between the different molecules (only 2 different base peaks). Another remarkable finding was the lack of reproducibility in the MS-MS spectra. Between different analyses, different settings of the collision energy were needed for obtaining the same comparable daughter spectra.

Nevertheless, it can be concluded that this chloroform extraction is a fast and easy to perform and is compatible for GC-MS analysis of TAP residues in bovine urine down to less than 100 ppb.

The research on thyreostats with GC-MS^n was closed after the introduction of LC-MS^n as methods of choice for this group of substances:

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EGA's
(ESTROGENS, GESTAGENS and ANDROGENS)

3.5. HPTLC OF ANABOLICS IN MEAT AND KIDNEY FAT

After

HPTLC analysis of residues of anabolics in meat and kidney fat

H. F. De Brabander, J. Van Hoof

J. Planar Chromatogr 3 (1990) 236-242

1. Introduction

The use of the steroids as growth promotors in the fattening of animals is prohibited in all EEC member-states. In Belgium the control on the illicit application of anabolics is carried out by the Institute of Veterinary Inspection (IVK-IEV) according to the EEC directives [1,2].

In regulatory control at the farm urine and/or faeces of the animals may be sampled. In urine substantial amounts of the anabolics and/or of their metabolites are present after treatment with the steroids. For the analysis of residues of anabolics in urine a routine HPTLC method has been described previously [3]. This procedure may be combined with an additional clean-up by HPLC with column switching as described by F.Smets [4,5]. However the use of an automated HPLC will increase considerably the cost of the analysis.

At the retail level (butchershop, supermarket) or in case of import/export sampling is restricted to tissue only. At the slaughterhouse tissue as well as excreta can be sampled. Some anabolics (e.g.the gestagens medroxyprogesterone acetate, megestrol acetate and chloormadinone acetate) are concentrated in kidney fat which is to be considered as the target tissue for the tracing of these group of drugs. After illicit administration the identity of the anabolics is not known. From the analysis of injection sites it is known that cocktails of different anabolics are used [6]. In order to protect the consumer, screening of the samples by a multi-residue method should be carried out. HPTLC is a valuable multi-residue method for the determination of residues of steroids [7,8,9]. In our laboratory a HPTLC method with fluorescence detection was developed by R. Verbeke [10]. Using this method anabolics could be detected at the 0.5 -10 ppb level. However the original clean-up described [10] is time consuming and may lead to bottlenecks in routine analysis. Therefore modifications of the method of Verbeke have been described [11] or are used in regulatory control in Belgium [12].

In this paper an improved column chromatographic clean-up procedure of anabolics in meat and kidney fat is presented. This method allows routine HPTLC detection of anabolic residues within a reasonable time interval. During application in regulatory control the method has been proved to be particularly useful for the detection of residues of gestagens.

This faster clean-up can also be used in addition with a clean-up by HPLC. By fraction collection very "clean" extracts are obtained. The reduction of matrix components improves considerably the interpretation of the two-dimensional HPTLC analysis. However, the use of an HPLC also increases the cost of analysis.

2. Experimental

2.1. Apparatus

The following apparatus was used: homogeniser (e.g. Waring Blendor with reservoir of 250 ml),microwave oven (e.g. Zanker), centrifuge equipped with metal centrifuge tubes of 300 ml (e.g. Sorvall),rotary vacuum evaporator, waterbath, extraction flasks (250 and 500 ml), ten placed vacuum manifold (e.g.Vac-Elut), empty reservoirs of 8 ml and corresponding frits of 20 mm (Analytichem International, Harbor City,CA,USA), nitrogen evaporator (e.g. Techni Dry Block), chromatographic columns and tanks, UV-transilluminator (366 nm) (e.g. c-62; U.V. Products Inc., San Gabriel, Calif., U.S.A.),white light trans-illumination source (local supplies shop), home made single sample applicator [10] or "4 X 4" sample applicator [13,14,15], LKB 1219 Rack beta liquid scintillation counter (Bromma,Sweden) and Zeiss KM 3 TLC scanner in the fluorescence mode (Zeiss, Oberkochen, D)

The HPLC used was a Perkin Elmer LC-5 (Norwalk,U.S.A) equipped with an automatic injector and coupled to a Frac 100 fraction collector (Pharmacia, Bromma, Sweden)

2.2. Reagents and reference compounds

Glucuronidase-sulfatase enzyme suspension (*Helix pomatia juice*: 100 000 Fishman units / ml ß glucuronidase + 1 000 000 Roy units / ml sulfatase) was obtained from I.B.F. (Clichy, France).

Most reference steroids were obtained from Steraloids (Wilton, N.Y., U.S.A.). Zeranol, zearalenone and medroxyprogesterone (acetate) was a gift from Upjohn (Puurs, Belgium). Trenbolone, trenbolone acetate, (6,7-^{3}H) trenbolone and (6,7-^{3}H)-trenbolone acetate were gifts from Roussel-Uclaf (Paris, France). Other radioactive steroids, (monoethyl-^{3}H) diethylstilboestrol, (2,4,6,7-^{3}H) - or (4-^{14}C) estradiol, (4-^{14}C)-testosterone, (4-^{14}C)progesterone and (^{3}H) zeranol were purchased from Amersham (Bucks, Great Britain).

Silica gel thin-layer plates or HPTLC plates without fluorescence indicator and reversed phase plates (RP-18) were obtained from E. Merck (Darmstadt, D) (Cat No 5721, 5631 and 5914, respectively), polyclar AT (a water-insoluble polyvinylpyrrolidone for binding phenols) [16] from Serva (Heidelberg, GFR) and phenylsilane bonded to silica from Analytichem International (Harbor City, CA,USA).

All other reagents (n-hexane, dichloromethane, chloroform, acetone, benzene, cyclohexane, ethylacetate) were reagent-grade products from E. Merck. Diethylether, free from peroxides was obtained from Gifrer & Barbezat (Decines, France).

Pretreatment of polyclar AT. In a glass-stoppered flask (250 ml), 20 g resin was swelled overnight in distilled water. Small particles were removed by sedimentation in a 500 ml graduated cylinder for 10 min and elimination of the supernatant. This procedure was repeated three times. The resin was washed with 250 ml 0.3 M Na_3CO_3 in 30 % methanol and finally with distilled water to neutral pH. The resin can be stored in water and is ready for use.

Pretreatment of phenylsilane: the commercial bonded phase was used without pretreatment.

2.3. Solutions

Stock solutions of the hormones in methanol were prepared at a concentration of 100 mg/ml.

Chromatographic solvent systems:

1 = chloroform - acetone (9:1, v/v);

2 = chloroform - acetone-n-hexane (25:5:20, v/v/v);

3 = chloroform - benzene - ethanol (9:1:0.25, v/v/v);

4 = n-hexane - diethylether - dichloromethane (4:3:2, v/v/v);

5 = cyclohexane - ethylacetate- ethanol (60:40:2.5, v/v/v);

6 = tetrahydrofurane-n.hexane(35:65,v/v)

2.4. Columns

Polyclar column : Columns provided with a PTFE stopcock and a female B14 glass joint (upper) and a male B14 glass-joint (lower) are used. The columns were filled with the stored resin up to a height of 5 cm (1 x 5 cm). No further treatment was necessary.

Phenylsilane column : 500 mg phenylsilane was filled into an empty reservoir of 8 ml provided with a porous frit of polyethylene (as an alternative pre-packed columns can be used) Before use the bonded phase was solvated by percolating the columns with 10 ml of methanol followed by 20 ml of distilled water. The phenylsilane (lower) column is coupled to the polyclar (upper) column by the lower male B14 joint.

2.5. Extraction procedure

An overall scheme of the extraction procedure is given in figure 1. In the description of the extraction procedure the quantities of solvents used for meat samples are different from those for kidney fat. The quantities of solvent for kidney fat are given between brackets.
Meat or (kidney) fat is cut into small pieces. 50 g is weighed into a glass flask and 40 ml dist. water is added. The fat samples are melted in a waterbath at 80°C (or in a microwave oven). Afterwards the content is homogenised with a Waring Blendor for one minute (30 seconds at low and then 30 sec at high speed). The homogenate is transferred into the original flask and a drop of chloroform (as anti-bacterial agent) and 10 ml sodium acetate buffer (0.2 M, pH 5.2) are added to the filtrate. The pH is checked and adjusted to pH 5.2, if necessary. After addition of 400 ml glucuronidase-sulfatase enzyme suspension the hormone conjugates are hydrolysed during 2 hours at 62 °C (or overnight at 37 °C). After hydrolysis the contents of the flask is shaked with 180 ml methanol (100 ml for kidney fat) and centrifuged during 10 minutes at 9000 rpm (13000 g). The supernatant is extracted twice with 50 ml hexane for the removal of the triacylglycerols. Afterwards the anabolics are extracted into 150, 90 and 90 ml dichloromethane (for the analysis of fat 90, 75 and 75 ml). The combined dichloromethane phases are washed with 50 ml 0,05 M Na_2CO_3 followed by 50 ml distilled water (this washing step is omitted in analysis needing optimum yield for zeranol). The dichloromethane phases are evaporated to "nearly dry" on a rotary evaporator.

2.6. Group fractionation of hormones

The evaporated extract is taken up in 1 ml of methanol and then diluted with 10 ml of distilled water. The extract is ready for a one-step passage through the coupled polyclar/phenylsilane columns to separate estrogens from androgens + gestagens. The meat or fat extract is quantitively transferred to the top of the polyclar column and allowed to drain into the coupled columns using the Vac-Elut coupled to a vacuum source. The evaporation flask is rinsed once, first with 1 ml of methanol and then diluted with 10 ml of distilled water. The combined rinsings are also transferred to the top of the polyclar column. After the rinsing had drained into the surface, the columns are uncoupled.

Estrogen fraction: the estrogens are eluted from the polyclar column with 15 ml of methanol. The eluate is evaporated to dryness and transferred to a fibrinogen tube with 2, 1 and 1 ml of ethanol. The extract is concentrated to 100 ml under a flow of nitrogen.

Androgen + gestagen fraction: the phenylsilane column is washed with 10 ml 30% methanol. The elution of androgens + gestagens from the phenylsilane column is performed with 10 ml methanol. The eluate is evaporated to dryness and the residue is transferred to a fibrinogen tube with 2, 1 and 1 ml of ethanol. The extract is concentrated to 100 ml under a flow of nitrogen.

2.7. HPLC purification

A spironolactone solution (2 mg/100 ml ; 10 ml) is added to the estrogen and androgen fraction. The (relative) retention times of the most important steroids are determined against spironolactone (internal standard) with standard solutions by UV detection. Spironolactone is a product with a structure similar to an androgen but exempt of anabolic activity. These retention times are given in Table 1

Table 1: Retention time of the most important steroids (min)

Estrogens		Androgens	
17ß-estradiol	5.6	trenbolone	5.2
ethinylestradiol	6.3	nortestosterone	6.5
dienestrol	7.6	testosterone	7.8
DES	8.0	methyltestosterone	9.6
I.S.*	9.2	I.S.*	9.2

gestagens	
chloormadinonacetate	14.9
megestrolacetate	15.0
medroxyprogesteronacetate	16.0
*spironolactone	

The chromatographic conditions are as follows: column, RPC18 column (5 m ROSIL 15 cm, Alltech, Eke Belgium); elution: acetonitrile-water (50:50, V/V). The different fractions are collected and evaporated to dryness under a stream of nitrogen. The fractions are taken up in 100 μl ethanol. During elution the retention time of spironolactone is recorded to control and adjust the window setting for fraction collection.

2.8. Sample application

A fraction (5 - 10 μl) of the concentrated extract (50 - 100 μl) of the estrogen or androgen + gestagen or HPLC fraction is applied on a HPTLC plate by a home designed sample applicator, described before [10]. As an alternative, a faster "4 X 4" developing mode and a " 4 X 4 " sample applicator could be used [13,14,15].

2.9. High Performance Thin Layer Chromatography

The extracts are analyzed by two-dimensional chromatography on precoated silica gel 60 nanoplates (HPTLC plates :10 x 10 cm or 5 x 5 cm). TLC on 20 x 20 cm plates could also be used but the application volumes should be adapted. Development is carried out in non-saturated tanks. At the starting point of the nanoplates up to 10 μl of the extract is spotted. Appropriate concentrations of the reference mixtures are applied in the side lanes. The reference mixtures contain 2 - 20 ng of the steroids presumed to be present in the sample.

Chromatographic development is carried out over a distance of 3 - 4 cm using solvent 5 for the androgens + gestagens and solvent 3 for the estrogen fraction. The plate is air dried and the starting point of the sample is overspotted with 5-10 ng of the steroids presumed to be present. This procedure is useful for additional R_F-comparison between an unknown spot and the reference after the second development. The plate is then turned over 90° and run in the second direction, using the appropriate solvents (androgens + gestagens = solvent 1; estrogens = solvent 4). As an alternative a "4 X 4" developing mode can be used. In this mode 4 samples are developed in two dimensions on one HPTLC plate [13,14].

2.10. Fluorescence detection

Before dipping the nanoplates are dried at 95 °C during 15 min. Fluorescence is induced by dipping in a 5% sulphuric acid-ethanol solution during 30 seconds. The plates are viewed under UV-light (366 nm) for fluorescent spots (e.g. trenbolone) and then incubated at 95°C during 10 min. The fluorescence is observed under trans-illumination at 366 nm and with visible (white) light.

The identity of the hormones is evaluated by comparing the R_F-values and the fluorescence (and visible) colours of the reference substances (table 3) with those of unknown spots under transillumination. Confirmation of the identity of the hormones is obtained by co-chromatography: the sample is overspotted with a known amount of the anabolic on a second plate. After development and fluorescence reaction, the anabolics in the sample must coincide exactly with the reference compounds added to the sample. If necessary, an additional confirmation of the identity of the steroids may be obtained by HPTLC on reversed phase plates (RP-18);

first dimension: methanol-dist.water-toluene (75:20:5,V/V/V);

second dimension: n-hexane-dichloromethane-acetonitrile (40:10:5, V/V/V).

3. Results and discussion

3.1. Extraction

Initially the extraction procedure used was the same as described by Verbeke [10]. During the investigations it was found that the use of a microwave oven (instead of a waterbath) for melting the fats may improve the speed of the analysis. The time for melting one fat sample (50 g) in a waterbath (80 ° C) is ca 30 minutes. In a microwave oven the same fat sample is melted within ca 7 minutes. However the melting time in a microwave oven increases with the number of samples placed into the oven. So, microwave melting is carried out during the preparation of a second sample. In examining a large number of tests no degradation of residues of steroids in meat and kidney fat samples has been observed after micro-wave melting

3.2. Group fractionation

A group fractionation into estrogens and androgens + gestagens was obtained by passing the extract through a coupled polyclar/ phenylsilane column. The phenolic anabolics (estrogens) are quantitatively retained by the polyvinylpyrrolidone column (=polyclar) as described for the analysis of bovine urine [3]. Polyclar is a water insoluble polyvinylpyrrolidone for binding phenols [16]. The 3-keto steroids (androgens + gestagens) pass through the upper column and are selectively bound to the phenylsilane colum. The polyclar adsorbent is packed into a glass column described before [3]. The phenylsilane column is a commercial solid phase column. A commercial ten placed vacuum manifold (Vac-Elut) is used for the collection of the eluates.

Elution of polyclar column. All estrogens were quantitatively eluted from the polyclar column with methanol (table 2). In comparision with urine samples [3], the interference of matrix components from meat and kidney fat samples leading to "dirty" chromatograms is much smaller. So, a more polar solvent could be used for elution (100 % methanol instead of 0,2 M Na_2CO_3 in 15% methanol). In comparision with urine also the elution volume could be reduced (15 ml instead of 90 ml). This improves the speed of analysis

Elution of phenylsilane column. During a first series of experiments the cyanopropyl column, used for the clean-up of urine [3] was tested out for meat and kidney fat extracts. It was found that the androgens and gestagens present in a meat extract could not quantitatively be retained on a cyanopropyl column. After passage of an extract the bonded phase material was very contaminated (visual observation). Even the use of larger amounts of bonded phase material was unsuccessful. Consequently a phenylsilane column was tested out and with more success. After washing with 30 % methanol the androgens were quantitatively eluted with methanol. In comparision with urine analysis also a smaller elution volume was obtained (10 ml versus 50 ml).

In table 2 the elution yields of some labelled steroids from polyclar and phenylsilane columns are shown.

Table 2: recovery of labelled anabolics in the different clean-up steps (n: number of individual experiments)

substance	PC-column*	Recovery phenyl-column*
[^{3}H] DES	69 ± 9.0 (6)	-
[^{14}C] 17ß-estradiol	76 ± 3.6 (6)	-
[^{14}C] nortestosterone	-	75 ±8.6 (5)
[^{14}C] testosterone	-	82 ± 7.0(8)
[^{14}C] progesterone	-	70 ± 9.3(4)

* mean ± S.D.(n)

3.3. HPLC purification.

This faster clean-up may also be coupled to an additional clean-up by HPLC with fraction collection. The first intention of the use of this extra clean-up procedure was to facilitate the interpretation of the HPTLC plates. Meat and kidney fat extracts obtained by the procedure described above still are "crude" extracts, containing a lot of matrix components. Due to the presence of other fluorescent spots these HPTLC plates can only be evaluated by experienced analysts. The HPTLC plates obtained after the HPLC purification are much cleaner and interpretation is facilitated.

Moreover, some "close" anabolics (e.g. nortestosterone and testosterone) are collected in different fractions. Therefore confusion between those two drugs is avoided. This is very important since testosterone is a natural steroid whereas nortestosterone is xenobiotic (at least for bovine samples).

3.4.HPTLC and sulphuric acid induced fluorescence.

For the optimum two-dimensional chromatography of the androgen+ gestagen and estrogen fractions different solvent combinations had to be tested out. With the solvents selected (table 3 and 4) the best separation between the steroids and the matrix components were obtained. The solvent combinations used for two-dimensional HPTLC of HPLC fractions are less critical. After dipping the plates in 5 % sulphuric acid in ethanol a detection limit of 1 - 10 ng of individual steroids was obtained as described by Verbeke[10]. The anabolics were identified by comparing the relative R_F - values and the different fluorescence colours (and colours under white light transillumination). The relative R_F-values(for androgens to methyltestosterone R_{F_1}: 0,53; R_{F_5}:0,46; for estrogens to 17 ß-estradiol R_{F_3}: 0,23; R_{F_4}: 0,30) were measured in triplicate for different solvent combinations and are given in table 3 and table 4.

Table 3: relative R_F-values of some estrogens on hptlc plates after sulphuric acid induced fluorescence.

Solvent systems:
3 = chloroform-benzene-ethanol (9:1:0.25, v/v/v)
4 = n-hexane-diethylether-dichloromethane (4:3:2, v/v/v)
Abbreviations : BE = blue; BN = brown; BT = bright; GN = green; LT = light; OE = orange; PU = purple; RD = red; YW = yellow).

Substance	Relative R_F -value* in solvent		Colour in U.V. (366 nm)
	3	4	
Benzestrol	0.77	1.46	GY
Dienestrol	0.82	1.60	RD
trans-diethylstilboestrol	0.99	1.81	RD
cis-diethylstilboestrol	0.36	1.02	RD
17ß-estradiol	1.00	1.00	YW
Estriol	0.09	0.02	YW-BN
Estrone	1.77	1.80	YW
Ethinylestradiol	1.27	1.53	YW
Hexestrol	0.86	1.68	GN
Mestranol	2.68	2.30	YW
Zearalenone	1.36	1.52	GN-YW
Zeranol	0.82	0.54	GN-YW

* relative to 17 ß-estradiol (R_{F3}: 0,23; R_{F4}: 0,30)

Table 4 : relative R_F -values of some anabolics of the"androgen" fraction on HPTLC plates after sulphuric acid induced fluorescence.

Solvent systems
1 = chloroform-acetone (9:1, v/v)
5 = cyclohexane-ethylacetate-ethanol (60:40:2.5, v/v/v).
(For abbreviations see table 3).

substance	relative R_F -value* in solvent		colour in UV (366 nm)
	1	5	
1 dehydrotestosterone	0.65	0.67	OE-BN
5α- dehydrotestosterone	1.15	1.27	BE-PU
epitestosterone	0.83	0.95	YW-BN
ethinyltestosterone	1.23	1.35	YW-BN
medroxyprogesterone	1.14	1.23	BE-PU
medroxyprogesteroneacetate	1.61	1.26	YW
melengestrolacetate	1.61	1.26	YW-BN
17α-methyltestosterone	1.00	1.00	YW-GN
4,9,11-methyltestosterone	1.02	1.02	YW-GN
19-nortestosterone	0.80	0.79	YW-LT
progesterone	1.61	1.36	BE-GN
testosterone	0.88	0.89	YW
trenbolone	0.80	0.84	BE-BT
trenbolone acetate	1.63	1.48	BE-BT
vinyltestosterone	1.28	1.34	YW-GN
megestrolacetate	1.66	1.23	YW
chloormadinoneacetate	1.66	1.1	YW

* relative to 17α-methyltestosteron (R_{F_1}: 0,53; R_{F_5}: 0,46)

By combination of solvents 5 and 1 (androgens) an insufficient separation between medroxyprogesterone acetate (MPA), and chloormadinoneacetate (CAP) was found. This problem could be solved by adding n-hexane to the chromatographic solvent (instead of 1:2 = chloroform-acetone-n-hexane (25:5:20,V/V/V)) Megestrolacetate (MEGA) and medroxyprogesteroneacetate (MPA) could not be separated with the normal solvent systems used. The differentiation between those two drugs is carried out on basis of the colour produced by illumination with white light. MEGA produces a blue colour (like CAP) while MPA is "dirty" green. Recently it was found that smaller amounts of MPA could be masked by larger amounts of MEGA. A separation between the two drugs could be achieved by using solvent 2 (first dimension) and 6 (in the second dimension).
At routine analysis sometimes a very unpleasant phenomenon occurs during the induction of fluorescence by dipping with ethanol-sulphuric acid. For some batches of HPTLC plates the thin layer loosens from the glass plates and cracks during heating. Plates showing this phenomenon are very difficult to handle and to evaluate. In most cases the analysis should be restarted. The problem was solved at first by purchasing "good" batches from the manufacturer on basis of the lot number. However, recently it was found that this phenomenon could be avoided in two ways: 1) by heating the plates before dipping or 2) by dipping the plates first into an ethanol solution (before the final dip into the ethanol-sulphuric acid solution). The first solution was included in the description of the procedure. The exact reason for this phenomenon is unknown. The occurrence of small amounts of water "trapped" between the thin layer and the glass plate could produce an exothermic reaction at contact with sulphuric acid. However, this is only a hypothesis.

3.5. Overall recovery.

The recovery of the anabolics through the entire procedure was measured with blank meat and fat samples spiked with hormones in the concentration range of 0.5-10 ppb. The amount of the anabolics in the final extract was determined by TLC scanning in the fluorescence mode using the anti-diagonal technique of Beljaars et al [17].

Table 5: Overall recovery of hormones added to meat and kidney fat.
n: number of individual experiments
** : concentration range added and determined by TLC-scanning.*

Substance	Recovery (%)	Concentration range*
Estrogens	(mean ± S.D.)	added(mg/l)
Dienestrol (5)	74 ± 9.0	1 - 3
Diethylstilboestrol (6)	80 ± 5.1	0.5 - 2
17ß-estradiol (9)	79 ± 5.0	1 - 2
Ethinylestradiol (8)	76 ± 7.4	2 - 4
Equilinin (3)	70 ± 1.5	2 - 3
Hexestrol (9)	75 ± 5.1	2 - 10
Zeranol (5)	29 ± 7.7	6 - 10
Androgens + Gestagens		
1-dehydrotestosterone (3)	78 ± 3.6	2 - 8
5-dihydrotestosterone (3)	77 ± 5.6	1 - 3
Medroxyprogesterone (4)	74 ± 11.9	2 - 4
Medroxyprogesterone acetate(5)	78 ± 3.5	1 - 4
Melengestrol acetate (4)	75 ± 3.3	2 - 3
17 methyltestosterone (8)	76 ± 10.4	1 - 3
4,9,11-methyltestosterone (4)	79 ± 6.3	1 - 2
19-nortestosterone (7)	73 ± 10.2	1 - 5
Testosterone (5)	81 ± 6.8	1 - 2
Trenbolone (8)	73 ± 9.8	1 - 2
Vinyltestosterone (4)	78 ± 5.9	1 - 2
Chlormadinoneacetate (8)	81 ± 3.3	1 - 2
Megestrolacetate (5)	73 ± 8.9	1 - 5
6-dehydroprogesterone (3)	73 ± 4.0	2 - 10

In Table 5 the recoveries of the unlabelled steroids are shown. The recoveries obtained for the most important anabolics are high (70 - 90 %). The lower recovery for zeranol is caused by the washing step of the dichloromethane extracts with 0.05 M Na_2CO_3. The sensitivity of the method for zeranol may be improved by omitting that step. However, this increases the interference of the matrix components with the other anabolics.

4. Conclusions

The method, described above permits the multi-residue analysis of anabolics in meat and kidney fat at residue levels of 0.5 - 10 ppb. The results obtained with this procedure were comparable with those obtained with the original method described by R. Verbeke [10,18]. However, the present method is considerably faster : one analyst is able to clean up at least 20 samples a week.

By combination of the faster clean-up with the faster "4 X 4 " HPTLC developing mode [13,14,15] the delay between sampling and the first results is considerably shortened. This permits the release of "negative" carcasses for the meat trade on a shorter time interval. Moreover, more time could be spent for additional tests (e.g. HPLC clean-up or MS confirmation) on "suspect" samples with the remainder of the extract.

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3.6. ANABOLICS IN KIDNEY FAT : FROM TLC TO GC-MS

After

Recent advances in the analysis of anabolic residues in kidney fat

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Proc. Euroresidue II , Veldhoven 3-5/5 (1993) 211-216

1. Introduction

As required by the EC directives, in Belgium the control on the illicit application of anabolics is carried out under the responsibility of the Institute of Veterinary Inspection (IVK-IEV; now FAVV). Kidney fat is considered as the tissue of choice for the tracing of the gestagens (e.g. medroxyprogesterone acetate, megestrol acetate and chloormadinone acetate). In 1979 the first multi-residue method for anabolics in animal tissue at the ppb level, using HPTLC with fluorescence detection was published by R. Verbeke (1). Using this method anabolics could be detected at the 0.5 -10 ppb level.

However the original clean-up was time consuming and might lead to bottlenecks in routine analysis. Therefore faster modifications of the method of Verbeke, especially for the clean-up, have been described and are used in regulatory control in Belgium (2,3). Also HPLC fractionation prior to HPTLC has been described (4). Using (slightly) different methods may lead to different detection limits for some analytes and so to contradiction in results.

To improve interlaboratory reproducibility and to come, if possible, to one validated standard operating procedure, used by the official labs; a working group: "Analysis of anabolics in kidney fat" under coordinance of the national reference lab, the IHE was installed

In this paper the most recent advances of the work of the active members of this group are presented. A method is described for the faster and still reliable clean-up of anabolic residues in kidney fat prior to routine HPTLC analysis. This faster clean-up can be used on its own or in addition with a clean-up by HPLC.

This faster clean-up can also be combined with GC-MS. In practice GC-MS in the full-scan mode may be carried out on the remainder of the extract used for HPTLC. However, combining HPTLC with HPLC or GC-MS increases both the time and the cost of analysis.

2. Experimental

2.1. Apparatus

The following apparatus were used: homogener (e.g. Waring Blendor with reservoir of 250 ml, Stomacher , Ultra-Turrax), microwave oven , centrifuge equipped with centrifuge tubes of 300 ml , rotary vacuum evaporator, waterbath, extraction flasks (250 and 500 ml), vacuum manifold (e.g.Sample Preparation Unit, Analytichem International, Harbor City, CA, USA), nitrogen evaporator (e.g. Techni Dry Block) or other type of evaporator (e.g. Speedvac SVC 200, SC 210A, Howe Gyrovap), chromatographic columns and tanks, UV-transilluminator (366 nm) (e.g. C-62; U.V. Products Inc., San Gabriel, CA, U.S.A.), white light source (local supplies shop), commercial sample applicator (e.g. Camag Linomat) or "4 X 4" sample applicator (5)

2.2. Reagents and reference compounds

Most reference steroids were obtained from Steraloids (St) (Wilton, N.Y., U.S.A.) or Sigma.(Si) (St-Louis, MO, U.S.A.). Internal standards are equilenin (St E400) and spironolactone (St S200 or Si S3378). Other steroids were gifts from various sources.
Silicagel thin-layer plates or HPTLC plates without fluorescence indicator and reversed phase plates (RP-18) were obtained from E. Merck (Darmstadt, G.F.R.)
Stock solutions of the hormones in methanol were prepared at a concentration of 100 μg/ml.
Chromatographic solvent systems are described before. (3)
Silica column and amino column (Varian Separation Products nr 1210-2037 resp 1210-2014) coupled by a Bond Elut adaptor.

2.3. Extraction procedure

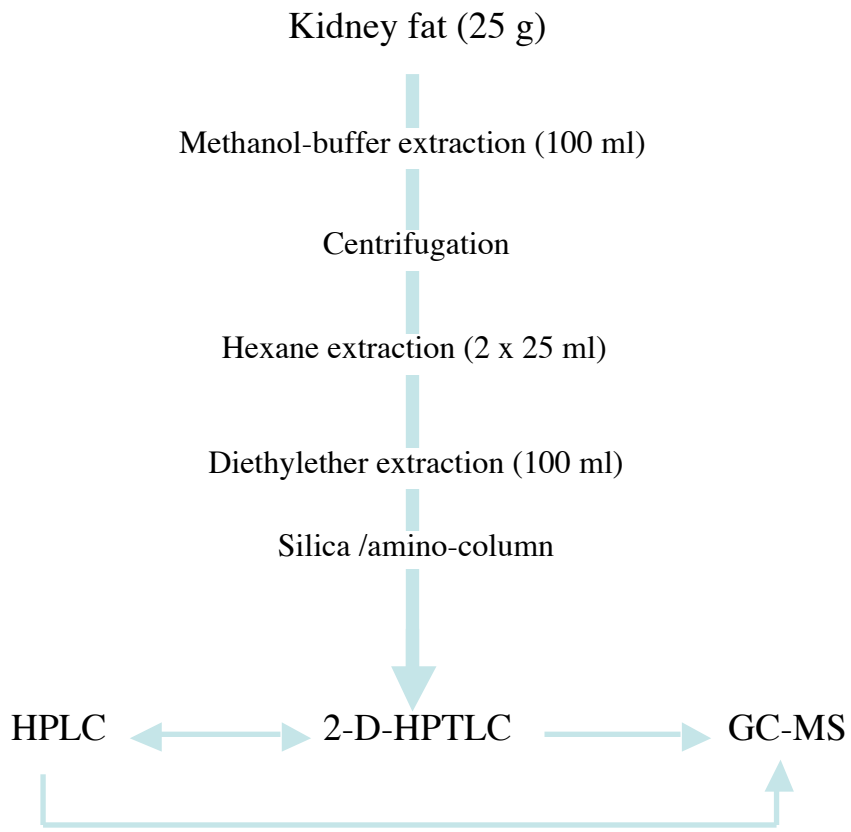

Fig 1: Overall scheme of the extraction procedure

An overall scheme of the extraction procedure is given in Fig. 1. Kidney fat is cut into small pieces . Twinty-five g is weighed into a glass flask, 10 ml 0.2 M NaOAc buffer and 40 ml water is added. The fat samples are melted in a microwave oven, (melting method depending on type of microwave oven). Internal standards: equilenine (5 ppb) and spironolactone (2 ppb) are added. Afterwards the content is homogenised (e.g. with a Waring Blendor during 2 minutes (1' low and then 1' high speed), ultra-turrax or stomacher). Fifty ml methanol is added and both phases are homogenised by shaking thoroughly.

The content is transferred to centrifugation tubes and centrifuged at 13000 g (e.g. 10 minutes at 9000 rpm or 40 min at 3000 rpm). The supernatant is transferred into a separation funnel over a funnel with a plug of cotton wool (**important** for elimination of lumps of fat) and extracted twice with 25 ml hexane for the removal of the remaining triacylglycerols. Afterwards the anabolics are extracted into 100 ml diethylether. The diethylether phase is washed with 15 ml water.
The diethylether phase is evaporated to "**completely** dry" on a rotary evaporator or equivalent.

The dry evaporated extract is taken up in 500 µl of chloroform and then diluted with 5 ml of hexane (just prior to SPE). This crude extract is ready for a passage through the coupled silica/amino columns. The silica column is conditioned with 2 X 2.5 ml hexane (still uncoupled). The extract is quantitatively transferred to the top of the silica column and allowed to drain into the column using a sample preparation unit coupled to a vacuum source. The silica column is washed with 5 ml hexane and allowed to run dry (still uncoupled). The silica column is now coupled to an amino column and the coupled columns are washed again with 5 ml hexane. The anabolics are eluted from the coupled columns with 5 ml of chloroform/acetone (4:1,v/v). The eluate is evaporated to dryness and retaken in 30 μl ethanol.

2.4. HPLC /HPTLC / GC-MS

A fraction (5 - 10 μl) of the concentrated extract (30 μl) is analysed by "4X4" 2D-HPTLC as described before (3). In case of *suspect* samples the remainder of the extract may be fractionated by HPLC (4) and rechromatographed by HPTLC. Alternatively the remainder of the extract may be analysed directly with GC-MS in the full-scan mode. TMS or ethoxime-silylethers are used (6,7).

3. Results and discussion

In the working group the steps were discussed one by one in order to come to one validated standard operation procedure

3.1. Extraction

The use of a microwave oven (instead of a waterbath) for melting the fats was generally accepted by all the labs. Also it was agreed to omit the use of glucoronidase-sulphatase (the use of Hélix pomatia juice (IBF biotechnics, France) was standard in some labs).

In order to speed up the procedure a mixture of buffer and methanol was used for primary extraction. In this phase also 2 internal standards (equilenine and spironolactone) were added to the homogenate. These standards are used for quality control and must be detected on the final HPTLC plate. No differences could be found between homogenisation of the fat with the water-methanol using a Waring-Blendor, Ultra-Turrax or Stomacher. Using a Stomacher the homogenisation must be carried out in two steps.
Centrifugation was studied intensively: no differences between temperature and centrifuge model could be found. However it was found very important to exclude any lumps of fat of entering the extraction funnel. Therefore a funnel with a small plug of cotton wool was used.
All the labs agreed to use diethyether for the extraction of the anabolics from the aqeous-methanol phase (instead of original dichloromethane extraction described by Verbeke (1)). The most important reasons were : elimination of halogenated solvents (hazardous waste), diethylether is also used for extraction of anabolics from urine , high extraction yield, economically interesting.

In an earlier investigation a coupled polyclar/phenylsilane column was used to separate the estrogens from the androgens (3). Later it was found that the polyclar column could be omitted. However, the final extracts obtained were not always as clean as the analysts wished and the HPTLC plates could not so easily be read in all labs. Later on a 3-column system (C18, silica, amino) was presented instead of the phenylsilane column by one member of the group (6). Although this system works very well, the C18 column complicates the procedure and negatively influences the yield of some anabolics (e.g. ClTA). This 3-column system was modified to a 2-column system again by another member of the group (7) and after some cross experiments accepted by the original inventor. The main problem with the 2-column system was the presence of a small amount of solid material in the final extract (fat). This problem was solved by filtering the supernatant through cotton wool after centrifugation and performing an extra hexane washing of the 2-column system.

3.2. HPLC purification.

A discussion on the pro and cons of HPLC fractionation prior to HPTLC was held. HPLC fractionation prior to HPTLC undoubtably improves qualitative accuracy: some anabolics which are not easy to separate by HPTLC (e.g. progesterone and methyltestosterone) are in different HPLC fractions. Therefore the IHE, as a reference lab strongly defends the use of HPLC. However, the labs working in the field of practical routine analysis are constantly put under pressure from the slaughterhouses to speed up analysis. HPLC fractionation seriously limits speed. Moreover, the crude diethylether extract is not always fit to routine HPLC fractionation as in the case of diethylether extract from urine samples (4,6,7). Regularly problems with the HPLC occurred due to overpressure (too dirty samples). Therefore it was proposed to use the 2 column clean-up-HPTLC as a routine screening technique : release the negative samples and submit only the suspect samples to HPLC fractionation. Alternatively, the remainder of the extract of a suspect sample is analysed by GC-MS.

3.3. GC-MS confirmation

Some members of the group (6,7) systematically confirm HPTLC results with GC-MS. The final extract, obtained after the 2 column system is sufficiently clean to be injected in a GC-MS with splitless injection. The 2 labs and the IHE use a Ultra Trace Full-scan mass spectrometer (Finnigan MAT ITS40, Finnigan MAT Magnum or Varian Saturn). For confirmation, the remainder of the extract is evaporated to dryness in a vial for an automatic injector, derivatised with either $MSTFA^{++}$ (formation of TMS-enol ethers; MSTFA-TMSI-DTE (1000+2+2))(7), or EOX-TMS (6) (formation of ethoxime-silylethers) and injected in an apolar column (e.g. Ultra-2, DB-5).
In Fig. 2 some results are shown from samples taken out of routine inspection.

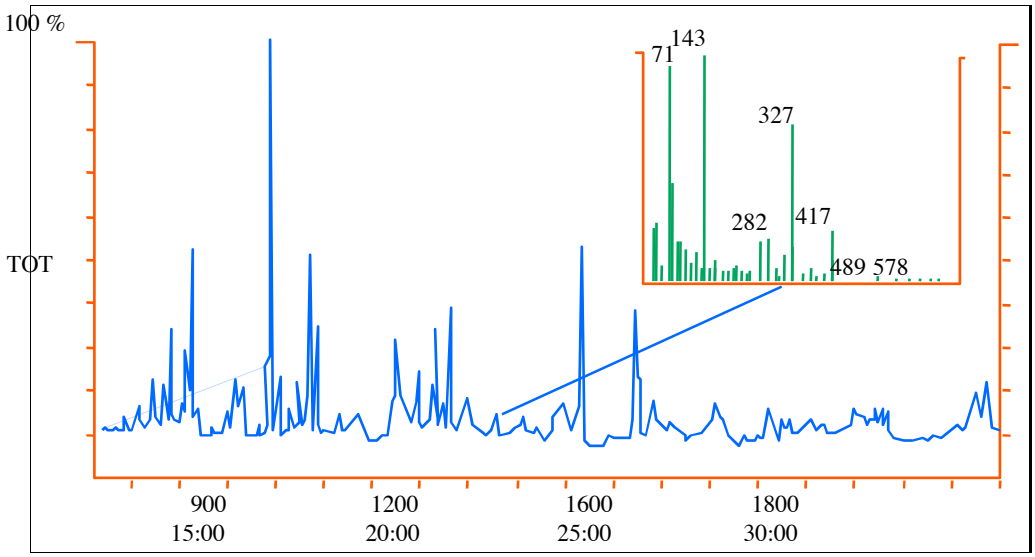

Fig 2A : chromatogram of a kidney fat extract with residues of methyltestosterone (ca 2 ppb) (6)(EOX-TMS ethers)

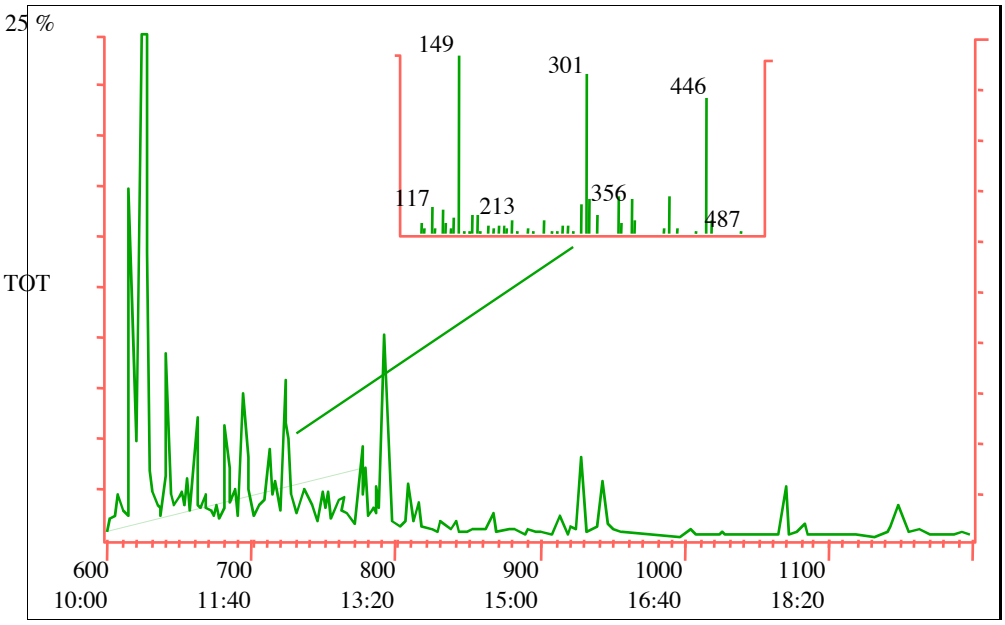

Fig 2B : chromatogram of a kidney fat extract with residues of methyltestosterone (ca 2 ppb) (7)(TMS-enol ethers)

3.4. Overall recovery.

The overall recovery of the anabolics through the entire procedure have not been measured quantitatively. However, blank kidney fat samples are spiked with hormones in the concentration range of 2-10 ppb and the analytes could be recovered at this concentration magnitude.

4. Conclusions

The method, described above permits the multi-residue analysis of anabolics in kidney fat at residue levels of 2 ppb. So, the fast 2 column-HPTLC method could be used for screening purposes and the identity of the anabolic residue confirmed with the GC-MS. Additional information could be obtained by fractionation by HPLC.

It is not the intention of the working group to replace 2D-HPTLC for GC-MS at this stage of research. On the contrary, it is found important to obtain as much information of the sample as possible.

At this moment, the identity of an analyte in a suspect sample may follow from 2 Rf values in 2D-HPTLC, a characteristic fluorescence after sulphuric acid induction , a retention window in HPLC, a retention time in GC^2 and a full mass spectrum. This combination must fullfil the most stringent quality criteria.

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3.7. ENHANCING SPECIFICITY BY HPLC CLEAN-UP

After

Enhancing the specificity of residue analysis of anabolics using HPLC clean-up

F. Smets, H. F. De Brabander, G. Pottie.

Arch Lebensmittelh. 48 (1997) 30-34

1. Introduction

For routine inspection kidney fat, urine and faeces samples have to be analysed for residues of estrogens, androgens, gestagens and related compounds. At this moment the number of target anabolics in Belgium is 28. This implicates the use of some multi-residue methods. The selection of such methods is not easy because each standard operating procedure has to be validated and all quality criteria have to be fulfilled for each analyte.

In document 93/256/EEC[1] general requirements for the methods to be used for detecting residues of substances having a hormonal or thyreostatic action are stated. One of the aspects of these quality criteria is the specificity of a method. Specificity is defined as the ability of the method to distinguish between the analyte being measured and other substances. This means that in this case the method must have the ability to distinguish between the analyte being measured at trace or ultra-trace concentrations and other substances possibly present at $10\text{-}10^3$ times higher concentration.

Two general approaches are possible for choosing the best method:

1. If available, a very specific detection technique (GC-MS, MS-MS, high-resolution GC-MS) could be chosen as detection technique. This could result in a reduction of sample pre treatment which is, at first sight, an advantage. However, it is not conceivable for a control lab to use these techniques in routine analysis: higher instrument cost, lower column lifetime and very likely quicker contamination of the system by using dirty extracts. As the number of substances of different parts to screen is high and not limited, one detection technique will never be even specific for all compounds.

2. A more extensive, but automated clean up procedure is the other option. HPLC fractionation, for instance results in several purified fractions each containing a limited number of anabolic compounds and matrix components. Each fraction may be analysed with a specific technique and if necessary with different techniques.

In this study two clean up techniques based on Liquid Chromatography: SPE (Solid Phase Extraction) and HPLC (High Performance Liquid Chromatography) are compared with each other. These are combined with two detection systems: HPTLC (High Performance Thin Layer Chromatography) and GC-MS (Gas Chromatography - Mass Spectrometry).

SPE is used in many multi-residue screening procedures[2-5]. Groups fractionation with a combination of SPE columns is a more selective method in routine detection of various anabolics[6]. The use of the higher separation efficiency of HPLC[7] as clean up has the

advantage that specific fractions for one analyte or a selected group of analytes can be isolated.

2. Experimental

2.1. Apparatus

A homogenizer (e.g., Ultra Turrax, 20 000 rpm), a water-bath, a centrifuge equipped with centrifugation tubes of 450 ml (e.g., Beckman), a mechanical extractor (e.g., Stomacher), a rotary vacuum evaporator (e.g., Rotavapor), a N_2 evaporator (e.g., Vapotherm from LaborTechnik Barkey), extraction flasks of 100 ml and 250 ml, a solid-phase extractor (e.g.,Baker), chromatographic tanks and a UV transilluminator (λ = 366 nm) were used. The sample applicator used was a semi-automatic Linomat IV (Camag). The LC system consisted of a Series 4 pump (Perkin-Elmer), an lSS-100 autoinjector (Perkin-Elmer), an automatic switching valve MUST (Chrompack), a Model 440 UV detector (Waters) and a Model 202 fraction collector (Gilson). The Gas chromatograph - Mass spectrometer was an ITS 40 ion trap (Finnigan MAT, USA).
HPTLC plates were obtained from Merck (Darmstadt, Germany).

2.2. Reagents, reference compounds and solutions

All solvents were of analytical reagent grade or LC grade from Merck (Darmstadt, Germany), diethyl ether was obtained from Gifrer & Barbezat (Decines, France). N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Duren, Germany) and iodotrimethylsilane (ITMS) and dithioerythritol (DTE) from Pierce (Rockford, IL).
All hormonal standards were obtained from Steraloids (Wilton, NY, USA).

2.3. Solutions

Stock standard solutions were prepared in ethanol at a concentration of 1 mg ml^{-1}. For routine control purposes, working standard solutions were prepared by dilution of the stock standard solutions to 50 ng ml^{-1}. Buffer solutions: sodium acetate (0.04M) pH: 5.2; sodium carbonate (10%) pH ≤ 10.25. Glucuronidase-sulfatase enzyme suspension (Helix pomatia juice: 100,000 Fishman units/ml ß-glucoronidase + 1,000,000 Roy units/ml sulfatase) was obtained from I.B.F. (Clichy, France) .The following solvent systems were used to develop the HPTLC plates: 1 = n-hexane-diethyl-ether-dichloromethane (25 + 45 +30); 2 = chloroform-acetone (90 + 10); 3 = cyclohexane-ethyl-acetate-ethanol (60 + 40 + 2.5) 4 = chloroform-n-hexane-acetone (50 +40+10).

2.4. LC-Columns

Two semi-preparative C_{18}, Ultrasphere ODS columns (80 Å pore size, particle size 5 μm , 50 mm L x 10 mm l.D.; 250 mm Lx 10 mm l.D.) were obtained from Beckman Instruments. A C_{18} pellicular ODS guard column (particle size 37-53 μm, 30 mm L x 4.6 mm l.D.) from Whatman and a C_{18} MCH-10 cartridge precolumn (particle size 10 μm, 30 mm L x 4.6 mm l.D.) from Varian. SPE - columns "Bond Elut" were obtained from Varian Analytichem;

Silica (Si) -3cc and Aminopropyl (NH2)-1 cc. Before use, all these columns were conditioned with the appropriate solvents.

2.5. Solid phase extraction as clean up.

For the regulatory control the SPE method in combination with 2D-HPTLC is used as screening procedure for the kidney fat samples. The overall scheme of the procedure is shown in Fig. 1A and described before[2,8] . Confirmation of the suspect samples follows from GC-MS analysis of the remainder of the extract or 2D-HPTLC or GC-MS analysis after HPLC clean-up (on one or two columns, see 2.6)

2.6. HPLC as clean up.

The aim of this technique is to purify the primary extract by the isolation of different specific fractions. Each fraction contains a limited amount of analytes and also a limited amount of interferences. The fractionation windows are determined after injection of the appropriate standards.

HPLC on one column:
For confirmatory purpose one dimensional HPLC is recommended for the analysis of kidney fat samples . The overall scheme of this procedure is shown in Fig. 1 B.
The detailed methodology is described before[9,10]

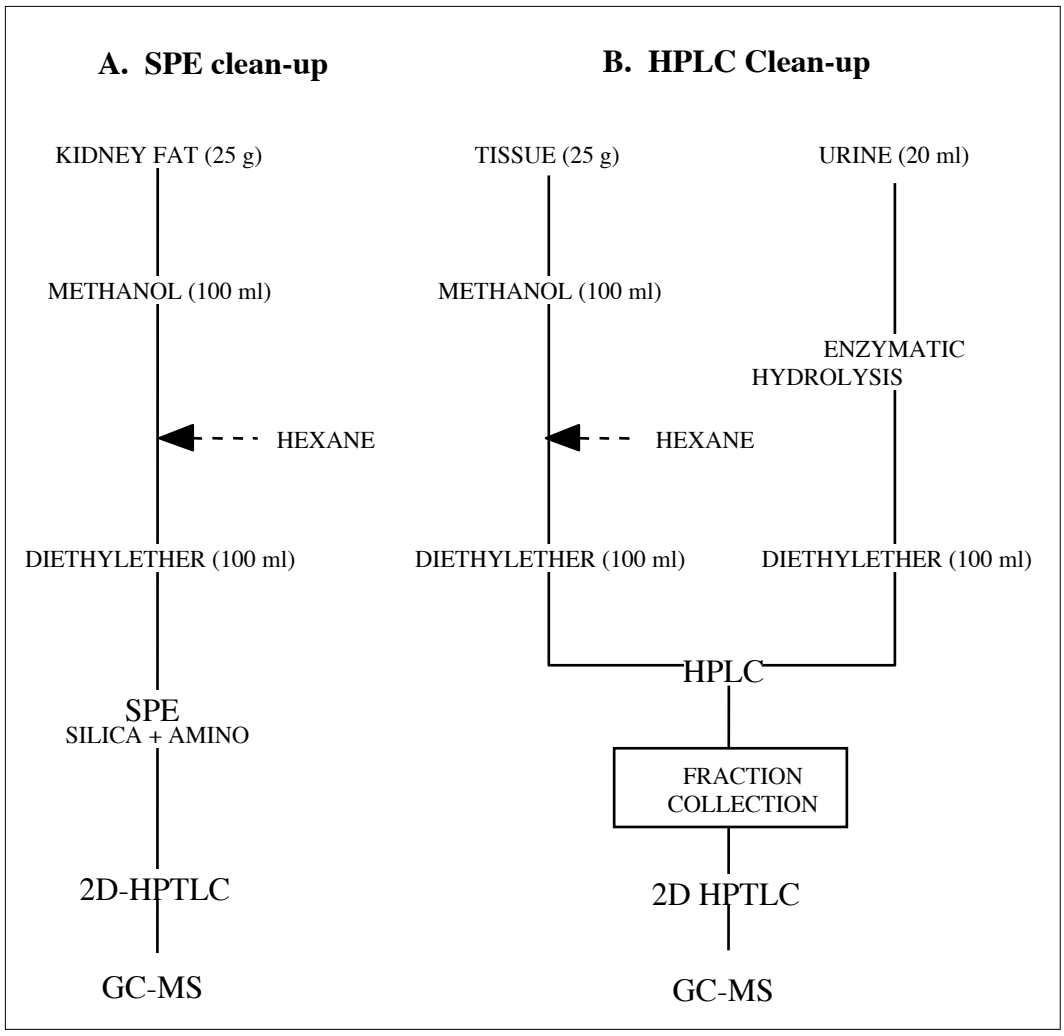


*Fig 1: Overall scheme of **A**: the SPE Clean-up and **B**: the HPLC clean-up procedure*

HPLC on two columns in series:

For urine samples SPE clean-up an also gradient elution on one single column is mostly not sufficient to achieve the specificity needed to fulfil all the identification criteria.

Therefore two HPLC columns were used in combination with a MUST switching valve-system in the heart-cutting mode. In this mode only a selected fraction (containing the anabolics of interest) of the eluate of the first column, is diverted to the second column for further elution. This second chromatography results in much cleaner fractions which are collected for further detection on the presence of anabolic residues.

2.7. HIGH-PERFORMANCE THIN-LAYER CHROMAT0GRAPHY

For screening purposes the extract is chromatographed on a precoated silica gel plate (10 cm x 10 cm) following the 4 x 4 elution technique[11].

The dry residue obtained after SPE or HPLC, is dissolved in 30 μl ethanol and 10 μl is spotted on the plate. Chromatographic development is performed with the solvent systems as described in "Solutions".

After fluorescence induction on the plate the spots are visualised by transillumination under UV radiation (λ= 366 nm) and identified by comparing the Rf values and the colours with those of the reference standards. Identification criteria for the qualitative determination of an analyte by TLC, in accordance with the EU-Directive 93/256/EEC:

1. The R_F value of the analyte should agree with the R_F value of the standard.
2. The visual appearance(color) of the analyte should be indistinguishable from that of the standard.
3. The centre of the spot nearest to that due to the analyte should be separated from it by at least half the sum of the spot diameters.
4. Two-dimensional development is obligatory.
5. Two-dimensional co-chromatography can give supplementary information and is obligatory in Belgium when there is an R_F variation greater than 3%.

2.8. GAS CHROMATOGRAPHY - MASS SPECTROMETRY.

The derivatization reagent mixture is MSTFA-TMSI-DTE (1000+2+2). Extracts should be evaporated to dryness prior to derivatization. To the tube containing the extract, 25 µl of the reagent mixture are added and heated at 60°C for 15 min. The tube is allowed to cool and then 1 µl is injected in the splitless mode into the GC-MS instrument.
The analyses were carried out on a Finnigan ITS 40 ion trap in the full-scan mode. The GC column used was a DB-5 fused silica (30 m x 0.25 mm I.D.) with a 0.25-mm film thickness and a carrier gas (helium) flow-rate of 1 ml min^{-1}. The temperature settings were as follows: injector, 260°C; transfer line, 300°C; oven, programmed from 100 to 200°C at 16.7°C min^{-1} and from 250 to 300°C at 4°C min^{-1}, the final temperature of 300°C being maintained for 3.5 min.

Identification criteria for the qualitative determination of an analyte by GC-MS.

1. The analyte has the same retention time as the reference.
2. The analyte shows a number of specific fragment ions:

-minimum three ions with a S/N ≥ 3, intensity ratio's equal to those of the reference and a minimum FIT-value of 800.

FIT=the degree to which the library spectrum is included in the sample mass spectrum.
PURITY=similarity between the sample mass spectrum and the library mass spectrum. This parameter gives an idea of the coeluting peaks that are important to consider when analysing extracts of biological samples.

3. Results and discussion

Kidney fat samples

Several kidney fat samples from regulatory control are analysed with the two clean up procedures (SPE purification and HPLC fractionation) combined with two different detection techniques (HPTLC and GC-MS). The results obtained for two of them are summarised as an example in table 1

Table 1 : Results of the analysis of two kidney fat samples analysed by different methods. (concentration in parts per billion)

	SPE clean-up		HPLC clean-up	
Sample nr	HPTLC	GC-MS	HPTLC	GC-MS
IHE/93/164	MT **(>5) AP (>4) MPA(<2)	MT (>5)	MT(>2) AP(>4) MPA(<2) , bNT(<2) bT(>2) a-T(<2)	MT AP MPA b-NT b-T a-T
IHE/93/165	MT(>2) AP(>5) MPA(<2)	MT (>2) AP	MT (>2) AP(>5) MPA (trace*) CMA (trace) b-NT(<2) b-T(<2) a-T(<2)	MT AP MPA b-NT b-T a-T

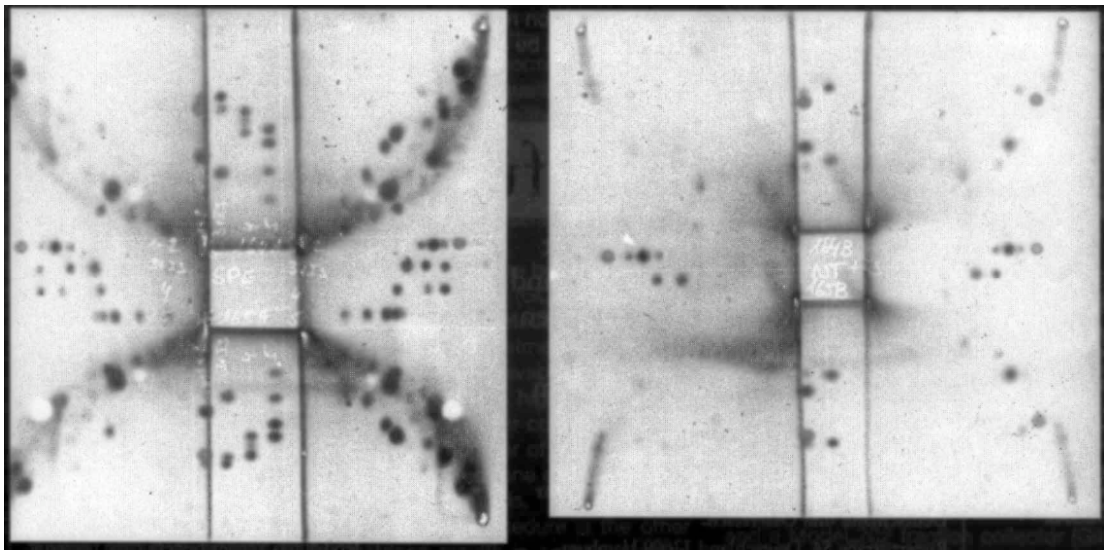
*(trace means that the quality cirteria are not completely fulfilled)

**MT: Methyltestosterone; AP: Acetoxyprogesterone; MPA: Medroxyprogesteroneacetate
NT: Nortestosterone; T: Testosterone; CMA: Chlormadinoneacetate

Thin layer chromatography:

After SPE purification only one fraction has to be chromatographed. This is only an advantage when relatively high residue concentrations are present: methyltestosterone (MT) and acetoxyprogesterone (AP)(> 5 ppb) are detected both after SPE and HPLC purification (table 1). A concentration greater than 5 ppb is considered as relatively high for anabolics but depends upon the analyte (detection limit , R_F values etc): medroxyprogesteroneacetate (MPA) for instance is detected after both purifications at concentrations smaller than 2 ppb. However, at lower concentrations the effect of interferences in some fractions becomes too important for other anabolics. In Fig.2 it is shown that some anabolics as Nortestosterone (NT) and Testosterone (T) are detected in the two analysed samples only after HPLC clean up (on one column). In the SPE-eluates too many interferences were present on the final thin

layer chromatogram. These interferences disturb the chromatography and the signals from those analytes.



A **B**

Fig 2 : Two- dimensional co-chromatography on silica plates of the extracts of the two fat samples
A : after SPE clean-up ; B : after HPLC fractionation (only early eluting fraction is shown)

Gas Chromatography-Mass Spectrometry:

The HPTLC results are completely confirmed by GC-MS for the extract purified by HPLC. This is not the case after SPE clean-up: in one sample only MT (in the other sample MT and AP) was confirmed by GC-MS (Table 1.). This is most probably due to the observation that gas chromato-graphy of the silyl derivatives of gestagens (as MPA and AP) is more easily disturbed by dirt than of other anabolics.

Moreover, according to the identification criteria used, the result of methyltestosterone as shown in Fig.3 is a result "on the limit". Due to the co-elution of interferences, the library search gives a low fit value of 807 and a very low purity factor of 193. In the spectrum of the HPLC fraction, fit- and purity values of respectively 982 and 707 are recorded. This means that the analyst is in a much more comfortable position to evaluate this latter result.

Besides, in other HPLC fractions of that same sample, the presence of additional anabolic compounds (NT,T) was detected. These analytes were not found after SPE purification because of the effect of interferences.

Urine samples

Our experience in analysing real urine samples from the regulatory control is that the results obtained on fractions eluted from one HPLC column were not always satisfactory. Especially the first eluting fractions still contain too much impurities.

To improve these results we used a double chromatography combining two HPLC columns containing the same stationary phase (C18) but with different dimensions. The columns were coupled by a multiport switching valve system giving the possibility of heart-cutting as explained before.

Fig.4 shows the GC-MS results of the same sample purified in the two modes. It can be seen that spectrum B is a mixture of different co-eluting peaks, including Nortestosterone. All specific ions (418, 403, 287, 182) are present but also a lot of noise because of the interferences. The interpretation of the result will become problematic when a lower concentration of this substance is present. Then the specific ions with the lower intensities will be dominated by the noise and a false negative result will be produced.

In Fig.4C we see that a pure spectrum is obtained after purification on a coupled column HPLC system. Lower limits of detection can be expected in proceeding this way.

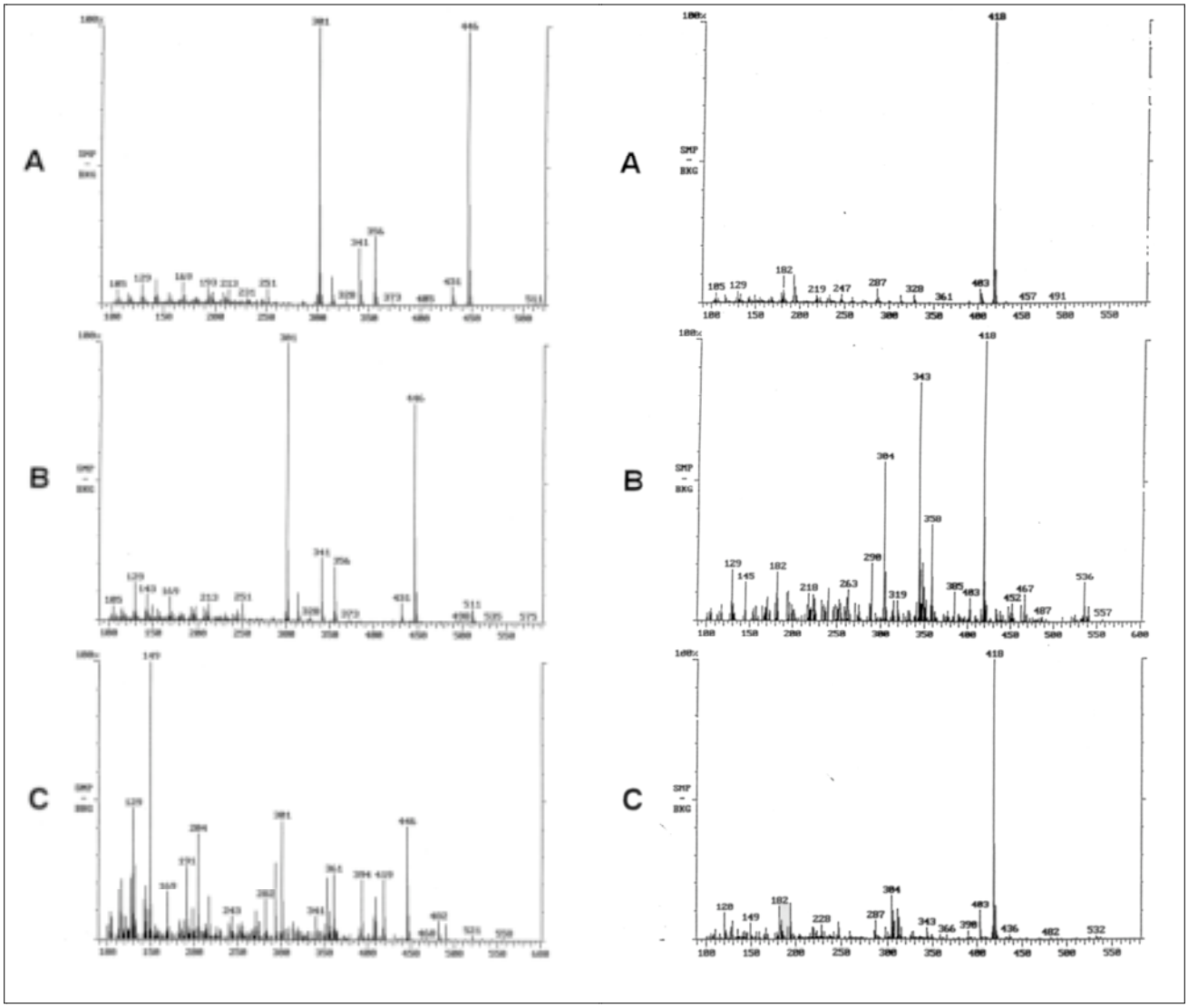


Fig 3: GC-MS spectrum of methyltestosterone

A: standard ;
B: Fat sample purified by HPLC ;
C: the same fat sample as B but after SPE clean-up

Fig 4: GC-MS spectrum of nortestosterone

A: standard;
B: Urine sample purified on one HPLC column;
C: the same urine sample purified on a two column system

4. Conclusions

The ability to distinguish the analyte, present in even trace or ultra-trace concentrations from interfering substances at much higher concentrations is an important parameter in the validation of an analytical procedure for residue analysis in biosamples.

In this paper it is demonstrated that the clean-up of the primary extract needs special attention and adds a considerable value to the specificity of the method. Using HPLC with fraction collection as clean-up step of the primary extract obtained from fat samples, it is proved that the interference effect obtained with these fractions is importantly smaller: SPE clean up, even the combination of different specific columns didn't eliminate interfering matrix substances as well as HPLC clean-up. For urine samples HPLC clean-up on one column is not satisfactory: a two column system is necessary. This observation was valid for both TLC as MS detection. For laboratory economics the same two-column HPLC system could of course be used for both kidney fat samples and urine samples.

HPLC purification adds a considerable value to the specificity of the method and influences in a positive sense the reliability of the results. By minimising the interference effects the exact amount of sample introduced into the detection system may be enlarged and the limit of detection substantially decreased. In Belgium the use of HPLC clean-up prior to detection of anabolics in excreta is mandatory.

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3.8. GC-MS OF ANABOLICS IN INJECTION SITES

After

GC-MS Confirmation of Anabolic Compounds in Injection Sites.

P. Batjoens, H. F. De Brabander, F. Smets, G. Pottie.

The Analyst 119 (12) (1994) 2607-2610

1. Introduction.

In the European Union (E.U.), the use of hormones as growth promoters is forbidden (1,2). Despite an intensive regulatory control program, the abuse of these compounds is still present in Belgium as well as in several other E.U.-member states.
Mixtures of endogenic and exogenic anabolic steroids, the so called cocktails, can be injected intramuscularly. This results in injection sites with a high concentration of residues of these products (3).
Consumption of these highly contaminated injection sites (mainly in minced meat) can be a considerable threat for human health.
In routine analysis these injection sites are analysed with a HPTLC method in the 4 x4 mode (4). Using this technique, the esters of the anabolic compounds are not always identified individually, but more as a group of the esters of a specific compound. To identify the esters with a greater certainty one is obliged to hydrolyse these compounds and to perform a second HPTLC analysis. The absence of the spots after hydrolysis confirms the earlier presence of esters of certain anabolic steroids. Because this is a rather complicated and time consuming way of analysing injection sites, it would be handy doing the analysis by GC-MS. GC-MS data also provide results with a greater certainty of the identity of a compound (T_R, mass spectrum).
Therefore we studied a large number of injection sites for the presence of anabolic steroids and their esters and tried to identify them, comparing their mass spectrum with the spectra of several standards of esterified anabolic compounds.

2. Experimental.

2.1. Apparatus

The following apparatus were used: extraction flasks (10 - 20 ml), nitrogen evaporator, reacti-term heating module, autosampler vials (e.g. Chromacol 07-CPV (A)).
Magnum Ion Trap System (Finnigan Mat., San Jose , CA., U.S.A) consisting of : Finnigan MAT A200S GC Autosampler, Varian 3400 GC with 1077 capillary split/splitless injector, Finnigan MAT Magnum Ion Trap Mass Spectrometer with electron impact.

2.2. Reagents and reference components.

Most of the reference compounds were obtained from Steraloids (Wilton, NY, USA) or Sigma (St. Louis, MO, USA).
MSTFA (N-Methyl N-trimethylsilyl-trifluoroacetamide) is from Macherey-Nagel (Düren, G.F.R.) and TMSI (Iodotrimethylsilane) from Janssen Chimica (Geel, Belgium). DL-Dithiothreitol is purchased from Sigma-Chemie (Brussels, Belgium).
MSTFA^{++} is prepared by dissolving 1‰ TMSI and a tip of a spatula point of a reductans dithiothreitol in MSTFA.(4)
All solvents used were analytical grade from Merck (Darmstadt, Germany).

2.3. Solutions.

Stock solutions of anabolic steroids were prepared at 200 ng/μl in ethanol. Ten-fold dilutions of these stock solutions result in working solutions at a concentration of 20 ng/μl.

2.4. Derivatisation and GC-MS conditions.

Anabolic steroids are derivatised with MSTFA^{++} : the sample or 10 μl standard solution (200 ng) is transferred into an autosampler vial (700 μl) and dried under a nitrogen stream. MSTFA^{++} (50 μl) is added, the contents are mixed and heated for half an hour at 60 °C. One μl (4 ng) is injected into the GC.
GC-MS conditions : initial: 100 °C, to 275 °C at 20 °C/min, to 320 °C at 30 °C/min, ISO at 300 °C, 10 min.(total program ca 35 min). Injector temperature : 260 °C, transfer-line : 300 °C.
Column: Hewlett-Packard Ultra-2 (25 m X 0.20 mm ID., film thickness 0.11 mm).
Aquisition method: 1 scan/sec during 25 min (mass range: 80-650 amu, filament-multiplier delay 600 sec).

3. Results and discussion.

The combination of a HPTLC method with a GC-MS in order to obtain a mass spectrum of suspect material in spots with the right colour and the correct Rf value, as performed for the confirmation of thyreostatic drugs, could not be used in the same way for steroids. Thyreostatic drugs are released in their original form after dipping of the TLC plate for visualisation of the residues. So, it was very easy to hyphenate HPTLC to GC-MS by scratching the suspected spots from the plate and transferring them into an autosampler vial for derivatisation (5). Otherwise, visualisation of steroids occurs after dipping in a H_2SO_4 solution. This reaction results in a fluorescence of the conjugated steroids. Therefore it was not possible to extrapolate the procedure described for thyreostatic drugs towards the steroids. Confirmation of HPTLC results of steroids had to be done starting from the extract of the sample.

Identification of anabolic steroids is based on their specific colour after dipping of the plate and the Rf-values as seen after the two-dimensional development. Since GC-MS is not yet introduced as an official analysis method for steroids in Belgium, only the positive results from HPTLC are confirmed with a GC-MS method. So we get a second confirmation based on the retention time of the GC-analysis and the full-scan mass spectra. This hyphenation gives the ultimate results a very high security.

Besides this confirmation, GC-MS data can be used for identification of new illicit residues or their metabolites.

In comparison with the short chain esters (methyltestosterone for instance), the long chain esters of the anabolic steroids (testosterone, estradiol and nortestosterone) do not leave the column within the time table of the normal temperature programmation of the GC.

Twelve standards of long chain esters of anabolic steroids were analysed by GC-MS. It is striking to observe that for all of the esters the molecular ion after derivatisation is present as base peak in their spectra with the exception for estradiol-benzoate, estradiol-diacetate and estradiol-dipropionate. Table 1 shows the molecular ions and the diagnostic ions of these 12 esters.

Table 1: Molecular masses and diagnostic ions of the 12 available standards in our laboratory.

Name	MM	MM (after derivatisation)	Diagnostic ions (Intensity)
E_2-Benzoate	376	448	105(100), 358(25), 448(12)
E_2-Cypionate	396	468	468(100), 327(11), 454(1)
E_2-Diacetate	356	428 ?	314(100), 254(9), 297(2)
E_2-Dipropionate	384	456 ?	328(100), 254(11), 311(2)
E_2-Valerate	356	428	428(100), 327(12), 413(3)
NT-Benzoate	378	450	450(100), 105(40), 436(4)
NT-Decanoate	429	501	501(100), 329(6), 194(5)
T-Acetate	330	402	402(100), 388(12), 343(8)
T-Benzoate	392	464	464(100), 105(47), 450(10)
T-Cypionate	412 (413)	484 (485)	485(100), 470(17), 343(14)
T-Decanoate	442 (443)	514 (515)	515(100), 343(5), 501(4)
T-Propionate	344	416	416(100), 402(10), 343(4)

Because the standards of the esters, mostly found in injection sites (3,6) were not all available in our laboratory, their identities were deducted from the molecular ion after derivatisation.(Table 2).

Table 2: The deducted molecular masses of some testosterone-esters.

Name	MM	MM (after derivatisation)
T-Butyrate	358	430
T-Valerate	372	444
T-Caproate	386	458
T-Enanthate	400	472
T-Caprylate	414	486
T-Fenylpropionate	420	492
T-Cyclohexylpropionate	426	498
T-Nonanoate	428	500
T-Tosylate	442	514
T-Undecylenate	456	528
T-Laurate	470	542
T-Hexyloxyfenylpropionate	520	592

The molecular masses given in table 2, were derived from the spectra of the available standards seeing them as compounds of homologue series. For all of the testosterone-esters available the derivatised molecular mass was found in their spectra as the base peak, but this was not the case for some esters of estradiol. The molecular mass after derivatisation is determined as the molecular mass of the original compound enlarged with the mass of 1 TMSI group (mass = 72). This procedure is extrapolated from the available standards towards the other esters of testosterone by taking account of the different substituted chains.

According to the length of the chain, a longer retention time could also be expected. These data have to be interpreted with the awareness that they are a guide-line for further investigation with the standards of the concerned compounds. One can only be sure of the identity of a residue when a proper standard is available to compare with.
Twenty-five percent of all the injection sites analysed in our laboratory in 1993 were negative for residues of anabolic compounds. This leaves 75 % positive samples. The most commonly found anabolic residues are summarized in table 3. Eighty-five percent of the positive injection sites contained one or more esters of testosterone and/or estradiol.

Table 3: Prevalence of anabolic residues in injection sites.

Name	N (%)	Name	N (%)	Name	N (%)
βT	32 (35)	E_2B	17 (18)	βNT	3 (3)
βT-E	32 (35)	STAN	14 (15)	EE_2	2 (2)
βE_2	28 (30)	MT	9 (10)	MPA	2 (2)
ClTA	27 (29)	FMT	6 (6)	βTB	2 (2)
βE_2-E	26 (28)	Mebol	5 (5)	CP	1 (1)
PG	22 (24)	DhPA	3 (3)	αTB	1 (1)

βT:beta-testosterone, βT-E: beta-testosterone esters, βE_2: beta-estradiol,
ClTA: chloro-testosterone acetate, βE_2-E: beta-estradiol esters, PG: progesterone,
E_2B: estradiol benzoate, STAN: stanozolole, MT: methyltestosterone, FMT: fluoxymesterone,
Mebol: methylboldenone, DhPA: algestone acetophenide, βNT: beta-nortestosterone,
EE_2: ethinylestradiol, MPA: medroxyprogesterone acetate, βTB: beta-trenbolone,
CP: caproxy-progesterone, αTB: alpha-trenbolone.

Thirty-five extracts of injection sites, positive for esters with HPTLC, were examined with GC-MS. The results are summarized in table 4.

Table 4: GC-MS confirmation and identification of HPTLC results for T- and E_2-esters.

HPTLC (N samples)	GC-MS (%)
positive for T + E (32)	T-Cypionate (94 %) T-Decanoate (50 %) T-Acetate (12 %) T-Propionate (12 %) T-Fenylpropionate (6 %)
positive for E_2-E (26)	E_2-Benzoate (92 %) E_2-Valerate (35 %) E_2-Cypionate (4 %) E_2-Dipropionate (4 %)

It is obvious that testosterone-cypionate was far most found in injection sites positive for testosterone-esters (94 %), followed by testosterone-decanoate, present in 50 percent of all T-E positive samples by HPTLC. The other esters were less represented. Estradiol-benzoate represented 92 % of the analyses positive for esters of estradiol, followed by estradiol-valerate with 35 % of all estradiol-ester positive samples. The occurrence of a couple of other estradiol-esters was a whole lot less (4 %).

The absence of some other esters such as testosterone-butyrate, -enanthate, -tosylate and several other, could be due to the lack of a proper standard.

The spectra of some esters identified in this paper (table 4) are summarised in fig. 2.

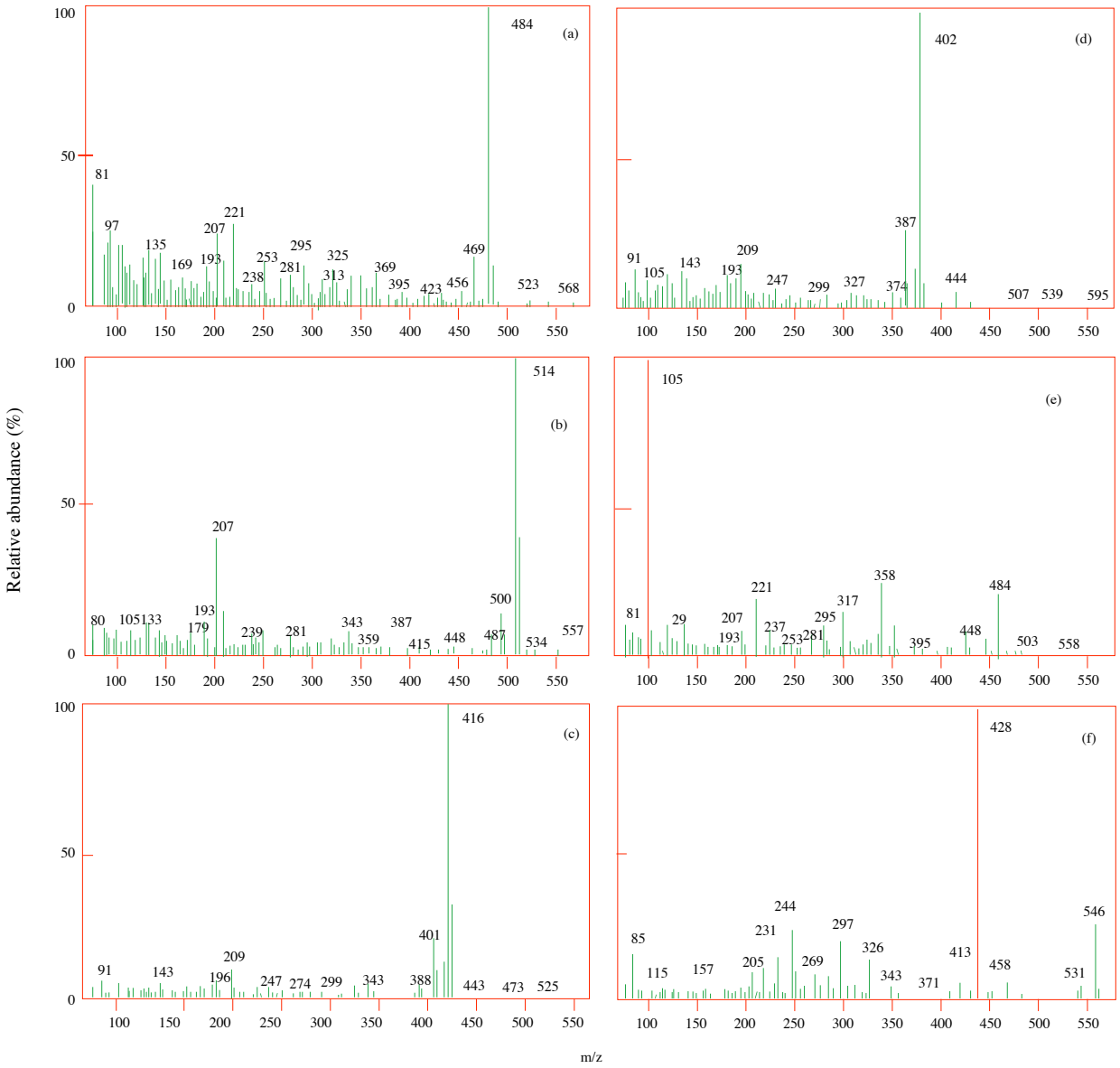

Figure 2: Spectra of some identified esters.

(a) Testosterone cypionate, (b) Testosterone decanoate, (c) Testosterone propionate (d) Testosterone acetate, (e) Estradiol benzoate, (f) Estradiol valerate

4. Conclusions.

As could be expected, it was not possible to hyphenate HPTLC with GC-MS for steroids confirmation in the same easy way as for thyreostatic drug residues. GC-MS analysis had to be performed after derivatisation of the extract.

The hyphenation of GC-MS to HPTLC was not only interesting for confirmation purposes but also gave the opportunity to identify unknown products as residues of recently introduced growth promoters or their metabolites. Besides the specific colour and Rf-values on the plate, additional information was obtained in the form of a second retention time value and the specific full-scan mass spectrum to get maximal security of the analysis results.

This paper concentrated on the occurrence of long chain esters of the natural anabolic steroids in injection sites. Eighty-five percent of the positive injection sites (HPTLC as well as GC-MS) contained esters of estradiol and/or testosterone. Estradiol-benzoate (92 %) and testosterone-cypionate (94 %) were predominantly present in the positive samples. Other esters found in smaller percentages were estradiol-valerate, -cypionate, -dipropionate and testosterone-decanoate, -acetate and -propionate.

Seventy-five percent of all the injection sites analysed during the last year were positive for one or more residues of anabolic steroids. This illustrates that the abuse of hormonal growth promoters is still very actual. The use of GC-MS is certainly a progress in the battle against the illicit use of growth promoters in livestock production, not only for confirmation purposes but also for identification of recently introduced compounds.

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3.9. COMPARISON OF GC-MS AND GC-MS^2

After

Comparison of the possibilities of GC-MS and tandem mass spectrometry systems for the analysis of anabolics in biological material

H.F. De Brabander , P. Batjoens, D. Courtheyn, J. Vercammen, K. De Wasch

J. Chromatogr. 750 (1996) 105-114

1. Introduction

In Europe the word "hormones" has a very bad reputation because of the possible danger for public health of some of these products, which are mostly anabolic steroids. Moreover, the consumer does not wish that animals are treated with anabolic steroids although toxicologists have declared that some are safe under certain application conditions. The Veterinary Food Inspection has to follow the legislation. However, a law without a good functioning control organism is not of much value. Analytical laboratories have key functions in the control mechanisms because no legal action can be taken before the presence of residues of these products are proved with a high qualitative accuracy.

Chromatographic techniques play the most important role in modern multi-residue analysis of anabolic steroids, both for screening and for confirmatory analysis. In Belgium many HPTLC (High Performance Thin Layer Chromatography) methods are used mostly in combination with a clean-up by SPE (Kidney fat) or HPLC (urine or faeces)[1-6]. However, most Belgian laboratories will confirm their HPTLC results systematically with GC-MS (gas chromatography- mass spectrometry)[7-9]. GC-MS apparatus of different manufacturers are used in Belgium. The major difference between them is the way of detection and recording of the chromatogram. Many apparatus use Selected Ion monitoring (SIM) for the determination of low concentrations (< 1 ng). Systems based on Ion Trap technology as the Finnigan MAT Magnum and the Varian Saturn record in full-scan to even pg concentrations.

Recently, the latest and completely revised model of the Finnigan Ion trap was released: the GCQ. This combination of quadrupole technology with the ultra-sensitive ion trap mass analyser gives the analyst new possibilities. The "tandem in time" system of this benchtop apparatus provides the added selectivity of MS-MS where selected ions can be fragmented and the results analysed further. Our labs ordered the first GCQ's in Europe and are building up experience in routine control. A first remark is that the nomenclature MS^2 is preferred to MS-MS because in the future also further developments as MS^3 (MS-MS-MS), MS^4 etc. will be part of the possibilities of these types of machines.

In this investigation the pros and cons of Magnum Ion Trap GC-MS and GCQ MS and MS^2 for the analysis of anabolics in biological material are compared using spiked samples. This comparison is focussed on qualitative accuracy: what criteria have to be fulfilled before the analyst may declare a sample positive. Possible quality criteria for GC-MS^2 are discussed. Quantitative analysis is of secondary importance for these illegal substances which have a so-called zero tolerance: it is impossible to quantify without a proper qualification. Moreover, in practice the residue levels found are often below the limit of quantification. However, in the near future quantification in GMS^n will become more and more important. In our laboratories projects for proper quantification at the (sub) ppb level were started up.

2. Experimental

2.1. Apparatus

The following apparatus were used: homogenisor (e.g. Waring Blendor with reservoir of 250 ml, Stomacher, Ultra-Turrax), microwave oven , centrifuge equipped with centrifugation tubes of 300 ml , rotary vacuum evaporator, waterbath, extraction flasks (250 and 500 ml), vacuum manifold (e.g. Sample Preparation Unit, Analytichem International, Harbor City, CA, USA), nitrogen evaporator (e.g. Techni Dry Block) or other types of evaporators (e.g. Speedvac SVC 200 ,SC 210A, Howe Gyrovap) , chromatographic columns and tanks, autosampler vials (e.g. Chromacol 07-CPV (A)).

2.2. Reagents and reference components

Most reference steroids were obtained from Steraloids (St) (Wilton, N.Y., U.S.A.) or Sigma.(Si) (St-Louis, MO, U.S.A.). Internal standards are equilenin (St E400) and spironolactone (St S200 or Si S3378). Other steroids were gifts from various sources. All recent standards were obtained through our NRL (National Reference Laboratory, IHE, Brussels) to ensure that all the field laboratories use the same standards [10]. The most important steroids used in this investigation are boldenone (BOL ; 1,4-androstadien-17ß-ol-3-one; St A200 ; MM 286,4), norgestrel (NG ; 18,19-dinor-13ß-ethyl-17ß-hydroxy-4-pregnen-20-yn-3-one ; Si N2260 ; MM 312,4), ethinylestradiol (EE2 ; 17α−ethynyl-1,3,5(10)-estratriene-3,17ß-diol ; Si E 4876; MM 296,4), fluoxymesterone (FMT ; 9α-fluoro-11ß-hydroxy-17α−methyltestosterone; Si F7751; MM 336,4) and norethandro-lone (NE ; 19-nor-4-androsten-17α-ethyl-17ß-ol-3-one ; St E3500 ; MM 302,4)

MSTFA (N-Methyl N-trimethylsilyl-trifluoroacetamide) is from Macherey-Nagel (Düren, Germany) and TMSI (Iodotrimethylsilane) from Janssen Chimica (Geel, Belgium). DL-Dithiothreitol is purchased from Sigma-Chemie (Brussels, Belgium).

MSTFA^{++} is prepared by dissolving 1‰ TMSI and a tip of a spatula point of a reductant dithiothreitol in MSTFA [9].

All solvents used were analytical grade from Merck (Darmstadt, Germany).

2.3. Solutions

Stock solutions of anabolic steroids were prepared at 200 ng/μl in ethanol. Ten-fold dilutions of these stock solutions result in working solutions at a concentration of 20 ng/μl.

2.4. Methods

All clean-up methods used were described and validated before [9,11,12]. A short summary is as follows: Urine (25 ml) is hydrolised with Helix Pomatia Juice and extracted with ether. The crude extract is cleaned up with HPLC with fraction collection. Kidney fat (25 g) is extracted with methanol and after extraction of most of the fat with hexane, cleaned up with a two column SPE system. Faeces (20 g) is extracted with diethylether and after a two column SPE purification also cleaned up with HPLC with fraction collection.

2.5. Derivatisation

The final SPE extract or HPLC fractions are derivatised to TMS enol-TMS ether (trimethylsilyl) derivatives with MSTFA^{++}: the sample or 10 μl standard solution (200 ng) is transferred into an autosampler vial (700 μl) and dried under a nitrogen stream. MSTFA^{++} (25-50 μl) is added, and the contents are mixed. One μl (equivalent to 4 ng standard) is injected into the GC.

2.6. GC-MS apparatus and conditions

Magnum Ion Trap System (Finnigan Mat., San Jose , CA., U.S.A) consisting of : Finnigan MAT A200S GC Autosampler, Varian 3400 GC with 1077 capillary split/splitless injector, Finnigan MAT Magnum Ion Trap Mass Spectrometer with electron impact and Advanced positive chemical ionisation.

GC-MS conditions : initial: 100 °C , to 250 °C at 17 °C/min, to 300 °C at 2 °C/min, ISO at 300 °C, 3 min.(total program ca 37 min). Injector temperature : 260 °C, transfer-line : 300 °C.

Column: SGE BPX-5 (25 m X 0.22 mm ID., film thickness 0.25 μm).

Aquisition method: 1 scan/sec during 25 min (mass range: 80-650 amu, filament-multiplier delay: 600 sec).

Finnigan MAT GCQ (Finnigan Mat., San Jose , CA., U.S.A) consisting of : Finnigan MAT A200S GC Autosampler, Finnigan MAT/Tremetrics high performance capillary GC, capillary

split/splitless injector with electronic pressure control, Finnigan MAT Quadrupole Ion Trap Mass Analyzer.

GC-MS conditions : GC temperature programmation: identical as for the Magnum Ion Trap. Acquisition method: 1 scan/sec (mass range: 50-650 amu, filament-multiplier delay 600 sec) Tandem MS mode: 1 scan/sec, mass range from 100 to a mass, 1 amu unit higher than the parent ion selected (the parent ion is mostly the base peak of the full scan spectrum of the molecule), collision energy for fragmentation of the parent ion: 0.7 - 3.0 V.

3. Results and discussion

3.1. GC-MS of anabolics: present situation

In Belgium anabolics are determined in different matrices: injection sites, excreta as urine and faeces and tissue samples as kidney fat and meat. For all analytes the clean-up used for HPTLC is compatible for GC-MS (gas chromatography-mass spectrometry) (splitless injection). In most cases the remainder of the final is derivatised with $MSTFA^{++}$ (formation of TMS-enol ethers) [9].

In the labs using SIM a number of diagnostic ions of the analyte are followed during a time window arround the expected retention time. These diagnostic ions must be present in the correct relative intensities (± 20 % (CI) of ± 10 % (EI)). There is still a discussion about the number of diagnostic ions which must be followed. Two ions is certainly not enough: false positive results may be generated by isotope interference [13]. Four or more ions should be ideal from a theoretical point of view but are not practicable at lower concentrations : from ring tests it was observed that the relative intensities of the ions do not remain constant enough when the concentration decreases [14]. Taking an objective analytical decision on three ions seems to be an acceptable compromise at this moment.

The higher the number of ions the higher the specificity of the methods but also the higher the chance on false negative results when the identification criteria are strictly applied. The relative intensity of the ions may be disturbed by background noise and co-eluting substances and the sample has to be declared negative although the analyte is present.

Quality criteria for full-scan identification of low concentrations are not yet officially described. In most cases a computer algorithm compares the 16 most important peaks of the mass spectrum of the unknown to those of the standard. In our lab a fit criterium of 800 (80 % match) is used: the degree to which the library spectrum (standard) is included in the sample mass spectrum. For anabolics this is mostly fulfilled for higher concentrations (≥ 2 ppb). When the FIT is that high in full-scan apparatus, SIM conditions are mostly fulfilled in SIM machines. At lower concentrations the analyte may disappear in a wood of interfering ions (fig 1) which lowers the FIT although the SIM criteria may still be valid. This wood of

interfering ions is caused by column bleeding and components from reagents and the matrix which are co- eluting with the analyte.

The situation presented in the theoretical example in fig 1 will happen with any analyte when its concentration is decreased. However, depending upon several circumstances this effect may occur at different concentration levels.

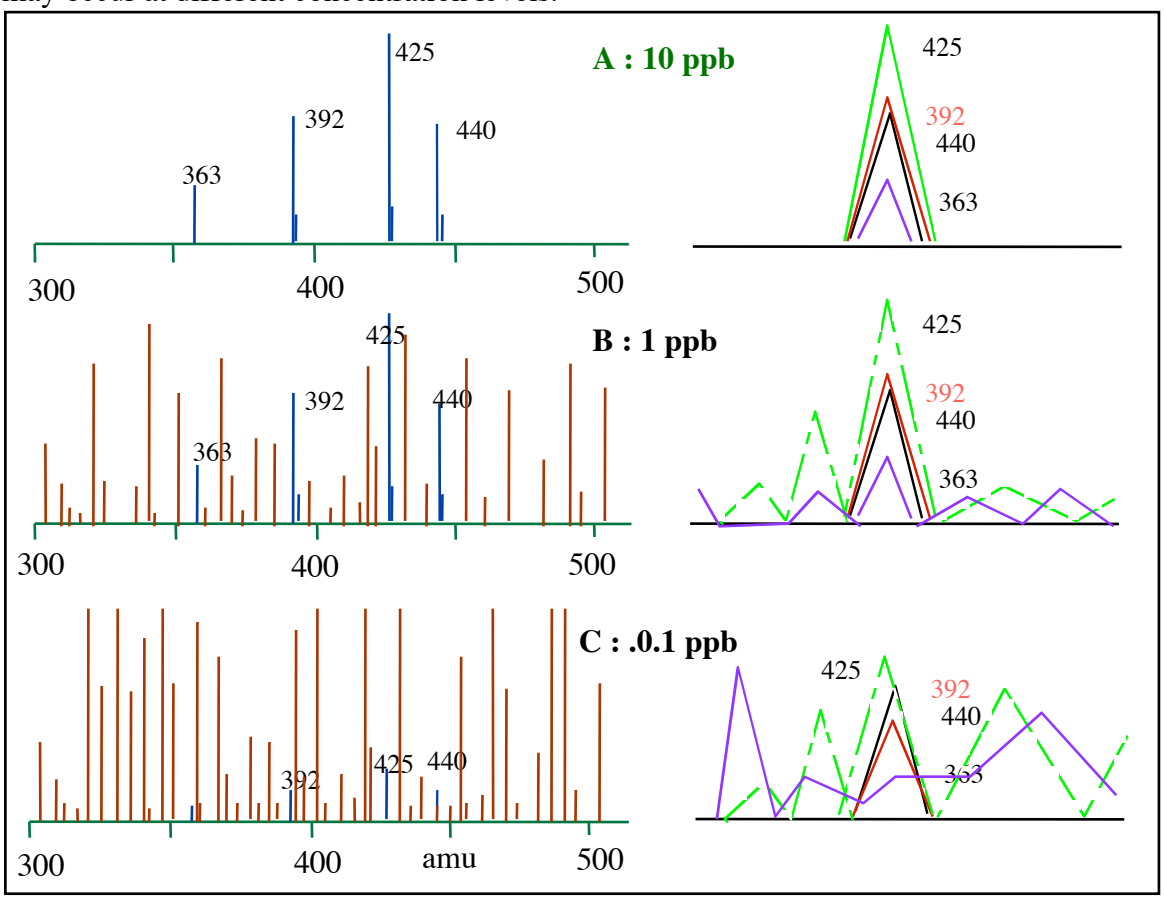

Fig 1 : Theoretical example : full-scan spectra and SIM signals of an analyte at different concentration magnitudes

A recent trend in GC-MS is the improvement of the detection limit also in classical quadrupole apparatus. According to some manufacturers lower concentrations may be measured in full scan so that both techniques may grow to each other in the future.

The present detection limits for anabolics in biological material with GC-MS may be calculated as follows: In the Finnigan Magnum the detection limit in full-scan of the derivative of an analytical standard is approximately 100 pg (0.1 ng). In the GC ca 1 μl of 20

μl final extract can be injected. This corresponds with 2 ng extracted from 20 g matrix. This corresponds with 0,1 ppb at a 100 % yield of the clean-up. In practice in full-scan a detection limit of ca 0.5 ppb may be attained.

In SIM much lower amounts of the same analytical standard may be detected (ca 10 pg or 0.01 ng). With an injection of ca 1 μl of 20 μl this corresponds with 0.2 ng, extracted from 20 g matrix. The corresponding theoretical detection limit is 0.01 ppb (at a 100 % yield of the clean-up). In SIM GC-MS methods start from 2 g material that brings the limit of detection to ca 0.1 ppb. However, in the determination of the real detection limit in GC-MS different other factors than the detection limit of standards are important: the mass range of the apparatus, the derivatisation and ionization method used (electron impact, positive or negative ionization) etc. It is not easy to compare apparatus of different manufacturers for different analytes in different matrixes and in different modes.

In the preparation of the quality criteria for SIM and full scan GC-MS, mentioned above most attention has been paid on obtaining as high a qualitative analytical accuracy as possible for a positive sample. In some cases (e.g. discussions, a second analysis etc.) it is also very convenient to prove the absence of an analyte in a matrix. Next to the analysis of blank and spiked samples the best way is the determination of the recovery of deuterated standards added to the sample on the concentration level of the decision limit (e.g. 2 ppb). Also in quantification deuterated products are very useful. The big problem is the availability of these standards.

The GC-MS data may also be combined with other results (e.g. HPTLC results). The identity of an analyte in a suspect sample may be based on 2 R_F values in 2D-HPTLC, a characteristic colour or fluorescence after induction, a retention time window in HPLC used as clean-up, a retention time in GC^2 and a mass spectrum (full or diagnostic ions). These combinations of methods must fulfil the most stringent quality criteria.

3.2. Comparison of the Magnum and the GCQ

This comparison is based on the analysis of urine samples, spiked with some anabolics of current interest at the level of the decision limit (2 ppb). The samples were cleaned-up and derivatised in routine as described before [9,11,12]. They were injected in both the Magnum

and the GCQ within a short time interval. In table I the results for the quality parameters are summarized.

Table 1: Comparison of quality parameters observed for some anabolics in the Magnum and the GCQ

Analyte	FIT (s/n)* obtained		Ions	Magnum (rel abund)		GCQ (rel abund)	
	Magnum	GCQ		Standard	Sample	Standard	Sample
BOL	954 (52)	966 (126)	206	100	100	100	100
			430	63	11	80	95
			325	44	16	38	41
NG	773 (32)	790 (48)	456	100	100	100	100
			316	60	37	21	30
			301	36	32	11	64
EE2	781 (99)	934 (184)	425	100	100	100	100
			285	41	49	29	32
			440	7	8	47	46
FMT	907 (12)	904 (78)	552	100	100	100	100
			462	75	69	32	33
			407	45	42	16	20
NE	915 (24)	971 (66)	446	45	51	100	100
			287	100	100	57	59
			356	27	29	51	48

*FIT = the results of the algoritm which measures how the 16 most important peaks of the standard spectrum are included in the peaks of the sample spectrum ; s/n = signal to noise ratio, measured on the sum of the 3 diagnostic ions of the anabolics

As can be seen in table I the FIT values (the results of the algoritm which measures how the 16 most important peaks of the standard spectrum are included in the peaks of the sample spectrum) obtained in the Magnum are in most cases equal to or greater than 800 (our tentative criterium). In the GCQ the FITS obtained are mostly higher than those found in the Magnum. This reflects a better match of the spectra with those of the spiked samples in the GCQ. The signal to noise ratio, measured on the sum of the 3 diagnostic ions of the anabolics is 2 to 3-fold higher in the GCQ as in the Magnum.

The SIM parameters (the relative abundances of the 3 diagnostics peaks) measured on the background substracted spectra of the Magnum are not always in accordance with 10 % limit

rule. In the GCQ the difference between the ratios of the diagnostics peaks in sample and standard is less than in the Magnum although some values still fall outside the theoretical limits (e.g. BOL ; ion 430 ; 80 (for standard) -95 (for sample)).

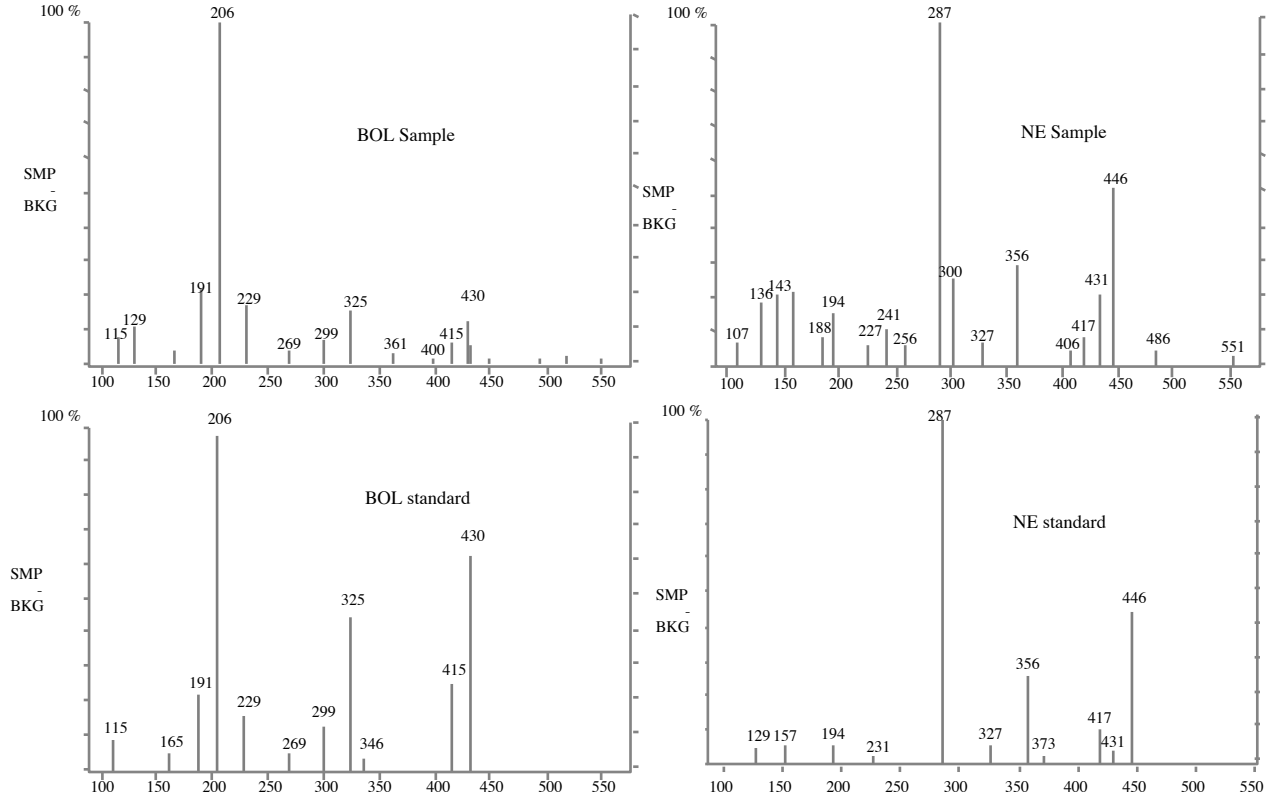

Fig 2: Mass spectra obtained for standards and urine samples spiked with boldenone and norethandrolone on the Magnum (2 ppb level)

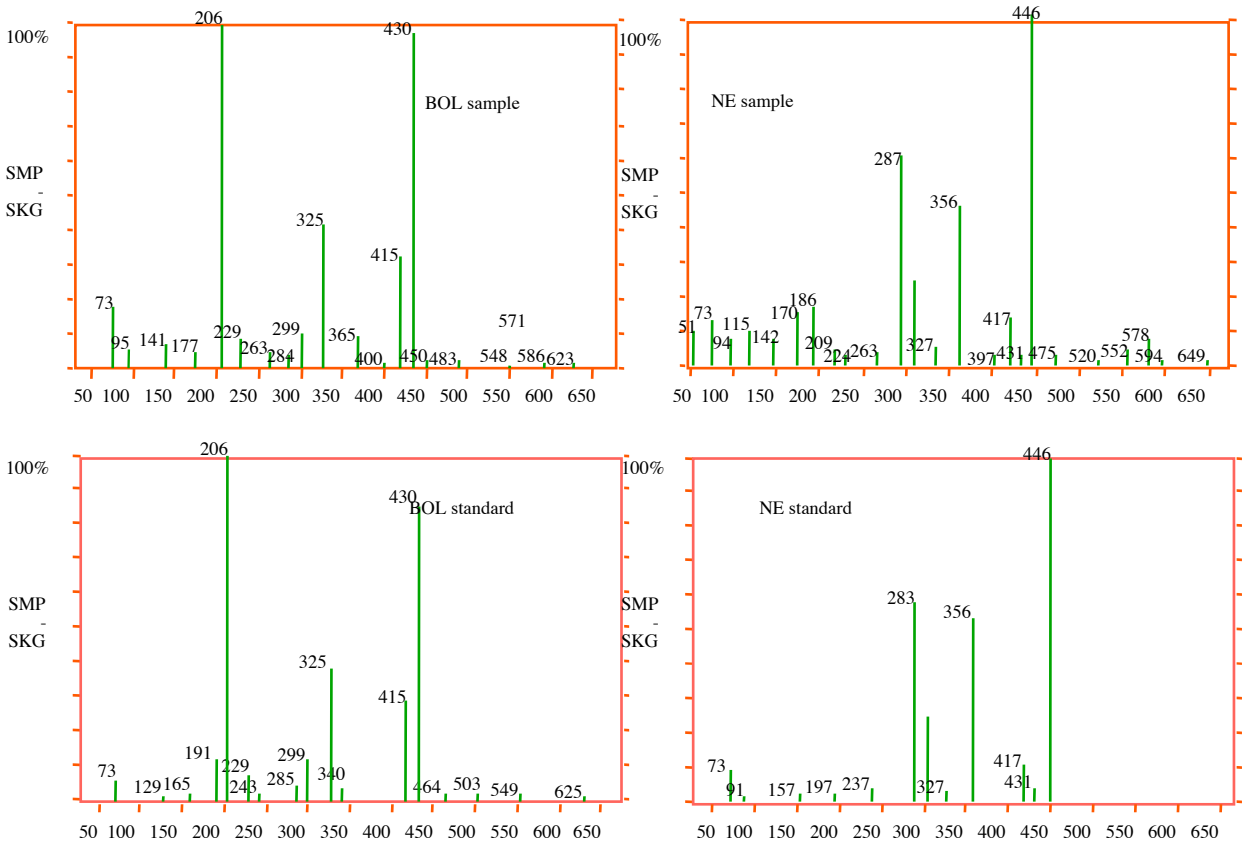

Fig 3: Mass spectra obtained for standards and urine samples spiked with boldenone and norethandrolone on the GCQ (2 ppb level).

Two anabolics are studied in more detail: boldenone (BOL) and norethandrolone(NE). In fig 2 the mass spectra obtained at the correct retention time for the standard and the spiked urine sample for BOL and NE on the Magnum are shown. The same spectra for the GCQ are presented in fig 3. The relative abundances for all the significant peaks of both anabolics on both apparatus are given in table II.

For the Magnum (fig 2) the spectra of both standards are easily recognized in the spectrum of the spiked sample: all the ions of the standard BOL and NE are present in the sample (see also table II.). For BOL the relative abundances of the ions 325, 415 and 430 are much lower in the sample than in the standard. This results in SIM criteria which are not fulfilled (although the analyte is present) but FIT criteria which were intact. The FIT algoritm is more sensitive to the presence of peaks that match by mass rather than by intensity [15].

In the GCQ the relative ratios of all the peaks are much more in concordance with each other resulting in a nearly perfect fit between standards and spiked samples.

For norethandrolone the biggest difference between standard and sample in the Magnum is for the non diagnostic ion 431. Also the value for 446 is just outside the limit. In the GCQ a nearly perfect fit of the standard and the sample spectrum was found (with the exception of the ion 300).

Table 2 Comparison of the abundances of all the (important) ions of the spectra of boldenone (BOL) and norethandrolone (NE)

BOL					NE				
Ion	Magnum		GCQ		Ion	Magnum		GCQ	
	St	Sa	St	Sa		St	Sa	St	Sa
206	100	100	100	100	**287**	100	100	57	59
229	12	12	9	8	300	24	26	20	24
269	4	4	1	–	327	6	5	5	5
299	11	7	12	10	**356**	27	29	51	48
325	44	16	38	41	417	7	8	11	13
415	26	6	29	32	431	4	20	3	3
430	63	11	80	95	**446**	45	51	100	100

The values presented above are measured manually on "background subtracted" spectra. They may differ from values obtained on crude spectra. Also small variations may occur between successive background subtractions.

3.3. GC-MS-MS in residue analysis.

GC-MS-MS, tandem MS or MS^2 exists since some time on the “bigger” apparatus. However, in most cases these machines are too expensive for use in field labs performing residue analysis. Two years ago a benchtop MS-MS on basis of a modification of an Ion Trap was introduced: the Varian Saturn. Instead of the classical MS-MS in space which consists of 3 mass spectrometers in series (one for the first mass analysis; one for a dissociation step and one for the second mass analysis) the whole MS-MS story takes place in one trap, in function

of time, controlled by a so-called MS-MS option. One ion (a precursor ion) is isolated in time and stored in the Ion Trap. Afterwards the dissociation of the precursor ion and the storage of product ions occurs in the same trap, but at a later time. The product ions are scanned from the trap at a third time resulting in a product ion spectrum. In this way smaller and also cheaper apparatus may be constructed. However, this MS-MS system does not have all the capabilities of the bigger machines (e.g. parent ion and neutral loss scan). On the other side Ion Traps theoretically allow MS^n : a mass spectrum from an ion of a mass spectrum from an ion of a mass spectrum etc.. could be obtained in one run. Therefore we prefer the nomenclature MS^2 instead of MS-MS.

In April 1994 Finnigan MAT also announced a MS-MS Magnum Performance Package upgrade for the Magnum Ion Trap. However, this upgrade was not released on the market. Instead of an upgrade, a new and completely revised GC-MS-MS was presented: the GCQ. This apparatus is a combination of the well-known quadrupole technology with the ultra-sensitive ion trap mass analyser. Potentially, this apparatus could be a very powerful weapon in the battle against illegal abuse of anabolics in cattle fattening.

3.4. GC-MS^2 of anabolics on the GCQ

In our laboratory GC-MS^2 of anabolics is not yet used as a technique on its own but only in relation to GC-MS. So the aim of GC-MS^2 spectra is to gain additional information to that already obtained in a previous GC-MS run. In fig 4 the MS^2 spectra of standards of BOL and NE and spiked urine samples taken at the correct retention time are presented.

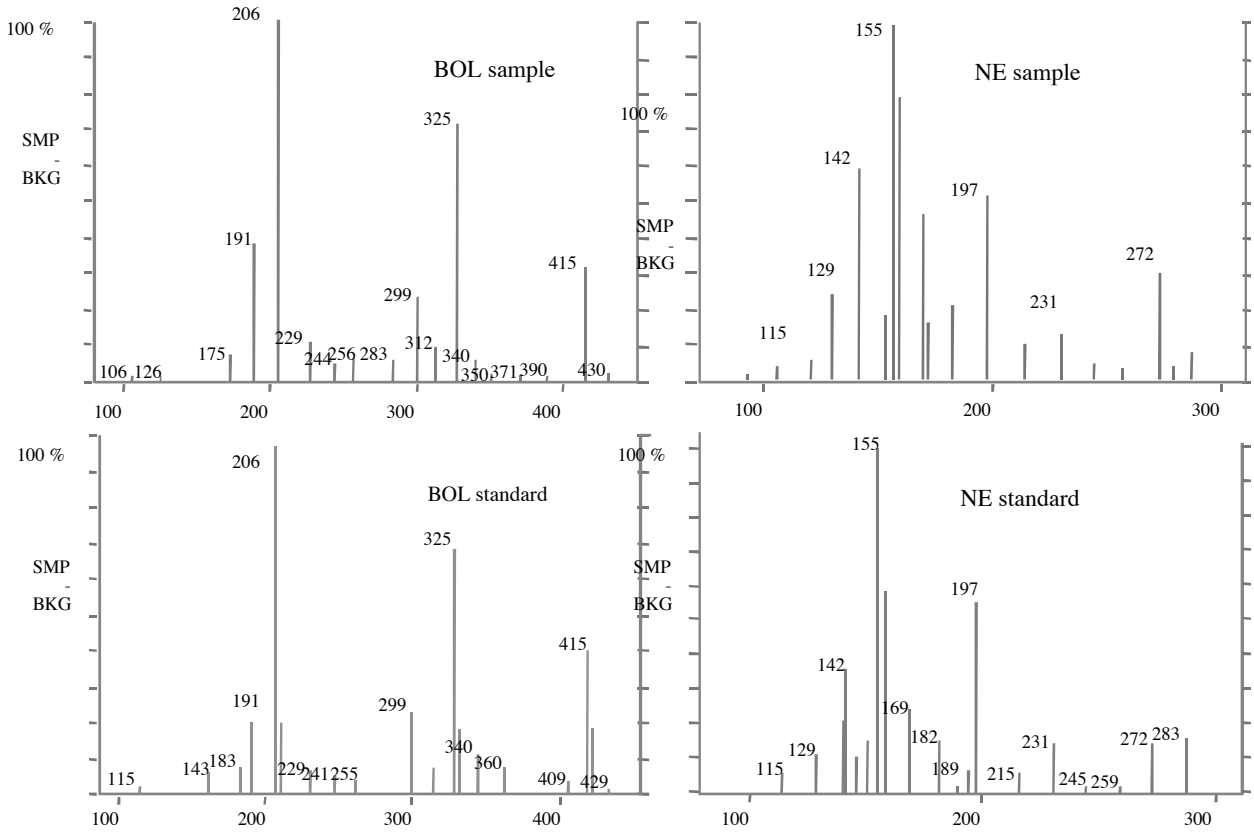

Fig 4: MS² spectra obtained for standards and urine samples spiked with boldenone and norethandrolone (2 ppb level)

For BOL the MS^2 spectrum on parent ion 430 shows 5 important ions which are the same as in the MS spectrum (with a slight difference between 191 and 189). However, less background, especially in the lower mass range is observed. For NE the MS^2 spectrum on parent ion 287 shows 8 important ions, which are different from those in the parent ion spectrum. Both MS^2 spectra taken on the sample match the standard MS^2 spectra taken up under the same conditions.

This is also demonstrated in table III where the intensities of all the peaks are given.

Table 3: Comparison of the abundances of all the (important) ions of the MS^2 spectra of boldenone (BOL, parent ion 430) and norethandrolone (NE, parent ion 287)

Ion	**BOL**		Ion	**NE**	
	ST	Sa		ST	Sa
415	42	31	272	14	30
325	69	71	231	13	13
299	24	24	197	53	53
206	100	100	182	16	21
191	21	21	169	24	46
			155	100	100
			142	36	61
			129	12	24

However, a MS^2 spectrum could be taken on various parent ions. In fig 5 MS^2 spectra for BOL and NE on the 3 diagnostic ions are shown.

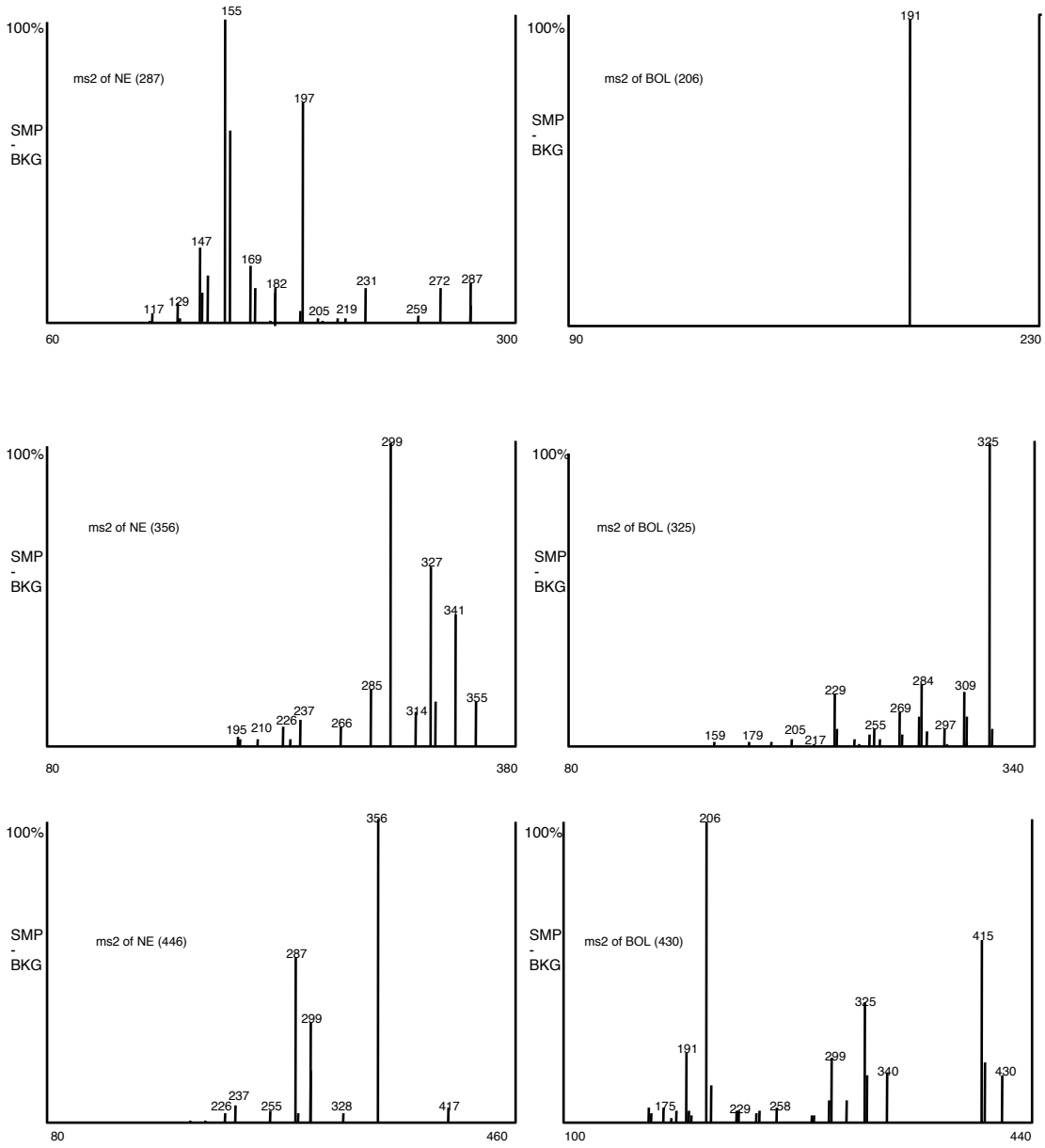

Fig 5: MS^2 spectra of the 3 diagnostic ions of boldenone and norethandrolone (standards)

It is obvious that in that way a lot of additional qualitative information of the analyte may be obtained. If all the MS^2 spectra of the sample match the MS^2 spectra of the standard the risk of interference by co-eluting substances is nearly negligable. On the GCQ a chromatographic run for taking up each MS^2 spectrum on different parent ions is needed. In the future MS^n will be able to do the job in one single run even with more specificity: the succesive MS^n spectra result from a previous daughter ion while in in fig 4 all daughter spectra were obtained on the parent spectrum.

4. Conclusion

The evolution in the possibilities of analytical equipment, in interrelationship with the very fast developments in electronics and computers seems to go faster and faster. This forces the laboratories to continuous investments, both in equipment and in skilled personnel. For laboratory economics it seems inevitable that the time used for clean-up has to be decreased and the time spent for the final detection to be increased. GC-MS^2 may play an important role in that philosophy. In this investigation a part of the comparison between the Finnigan Magnum and the GCQ was presented. The GCQ is not only capable of replacing successfully the Magnum but adds the identification power of MS^2 . Next to that power, new features as the extended mass range, NCI, ultra-SIM etc. have to be explored. Anyway, it looks as if the use of a GCQ will lead us to shorter and more reliable analysis which may be repeated within an acceptable time limit.

Finnigan Magnum

Finnigan Polaris (successor of GCQ)

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CHAPTER IV: CRITERIA IN RESIDUE ANALYSIS

4.1. QUALIFICATION OR QUANTIFICATION?

After

Qualitative or quantitative methods for residue analysis?

H.F. De Brabander, P. Batjoens, K. De Wasch, D. Courtheyn, G. Pottie, F. Smets

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1. Introduction

The importance of results in the field of residue analysis in veterinary food inspection is increasing. This is mainly due to the more severe legislation for some residues. Therefore the highest performance is required from the control methods.

Sometimes, a different way of interpretation of analytical results has grown between inspection services and laboratories. Inspection services are interested mainly in a YES/NO answer: has this animal been illegaly treated with anabolics?, is the concentration higher than a certain value (MRL, DtL)? etc. In fact all questions may be amalgamted to one: has the law been violated? When the answer is YES, actions are taken: the animal is rejected, animals are seized on the farm etc. Different kind of actions may be performed.

Laboratories evaluate crude analytical data on the basis of pre-defined criteria for all relevant parameters (e.g. S/N ratios, deviations from observed and target values for reference materials etc). For residue analysis on anabolic compounds such minimum quality criteria are part of EU legislation (1). Based on these criteria they come to YES/NO answers. The discussion on the kind of quality criteria for the convertion of analytical results into YES/NO answers is not yet finished. Therefore, in most countries a system of first and second analysis is used. When the final answer of the first analysis is YES, the legal action is suspended until the results of the second analysis, if any, is known.

If there is a contradiction between the first and the second analysis, the final result will be negative (NO answer) in the case of screening (first) and confirmatory (second) analysis since the confirmatory is, in terms of reliability, superior to the screening method. In the case of equivalent (confirmatory) methods the agreement of the criteria for qualification (identification) and quantification becomes extremely important. This will be even more the case if there would be a contradiction between EU member states in which case arbitration becomes necessary.

2. Qualitative Analysis

Qualitative methods may be used for illegal analytes having a so called zero tolerance. If the analyte is detected, the concentration can be determined or estimated and an Action Limit (AL) is used to convert the results in YES (positive) and NO (negative) answers. The AL is an agreement between inspection services and laboratories. Based on this limit (legal) actions are taken.

Even though different laboratories, for very practical reasons, as a rule use different methods in all the labs, the presence and the identity of an analyte must meet the right criteria: retention times or Rf values, specific colours, (mass) spectrometric data etc. The quality criteria accepted by the EU are described earlier (1,2,3,4). (e.g. for mass spectrometry four ions with correct relative intensities are needed). In order to fulfil all of these criteria, to see a spot or to have a signal out of the noise, a minimum amount of analyte will be necessary corresponding to a minimal mass content in the original sample. (this is not necessarily 2 μg/kg, the Action Limit that is proposed for anabolics in the EU at the moment). Therefore, qualitative methods have an intrinsic (semi) quantitative character.

When working under a quality system, e.g. based on EN 45001 (ISO 17025), validation of the standard operating procedures (SOPs) at the level of the Action Limit is mandatory. The relation between the Action Limit (AL) and the several analytical (and other) definitions of limits are not unequivocal. In analytical chemistry the practical limit of detection (sometimes called decision limit) and the theoretical limit of detection (mostly called limit of detection) are defined as respectively background +1.64s and +3s (s= standard deviation) (5). Moreover, in EU documents (1) the decision limit is defined as background + 6s. The Limit of Determination is defined as the smallest quantity which may be detected according to all the quality criteria especially for specificity (1,6). Inspection services sometimes define a decision limit as the limit on which a decision is taken. These different definitions and points of view make the dialogue between labs and inspection services difficult. A scheme of the situation for the owner, the laboratory and the inspection services is given in fig 1.

The Limit of Determination is analyte and matrix dependent mostly in a low concentration range (e.g. 0.5 or 2 ppb). If below the action limit, it may vary from lab to lab and from analyte to analyte without influencing the YES/NO answer to the inspection services. In the follow up of contradictions between field laboratories the NRL has to take this phenomena into account. There is a possibility that the first lab, with very sensitive equipment has given a result below the AL. However, this result may not be reproduced by a second lab of which the method is validated down to the AL but because of less sophisticated equipment not to the Limit of Determination of the first laboratory.

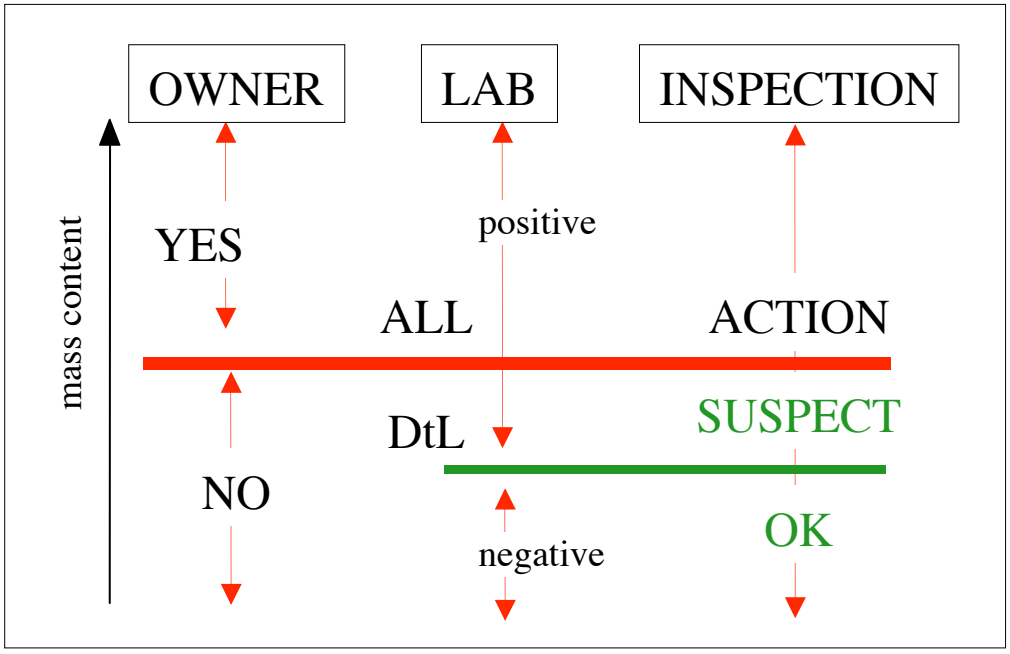

Fig. 1: The impact of the Action Limit (AL) and the Limit of Determination (DtL) on the owner of the animal and the Inspection services.

73. Quantitative Analysis

Quantitative analysis is necessary for residues with a Maximum Residue Limit (MRL). The method used must have a limit of quantification lower than the MRL. Recently consensus was reached on the fact that analytical methods to be used for controlling an MRL should have a limit of quantification of (at least) 0.5 MRL. For the values (much) lower than the MRL, positive qualitative errors do not play a role in the NO answer. For values obtained in the relatively high concentration area of the MRL (e.g. 500 ppb) quantitative accuracy and reproducibility is of major importance. If the result lies above the MRL qualitative criteria must be taken into account for quantification.

The difference between a MRL and an Action limit (AL) is 3-fold. First of all the MRL is mostly in a higher concentration range than an AL (e.g. MRL for some veterinary drugs = 100 ppb while AL for anabolics = 2 ppb). Secondly MRL's are only applicable to residues of registered veterinary drugs in those edible matrices which are not prone to temporary fluctuations in concentration (there are MRL's for liver, meat, milk but no MRL's for urine!; there are no MRL's for illegal substances).Thirdly a MRL is a legal value based on toxicological data while the AL is a value which is mostly an agreement based on the current analytical capabilities.

Three possible cases must be discussed.

1) For the values (much) lower than the MRL, positive quantitative errors do not play a role in the taking of an action. However negative quantitative errors may be important. If a screening method is used (e.g. the four plate method for antibiotics, an IA for beta-agonist) some analytes may escape the law because the method has no or a much lower response to that analyte. However, this problem must be studied in the strategy of screening and confirmatory methods.

2) If the result obtained lies above the MRL then qualification and quantification is very important. For surveillance of registered veterinary drugs often immunological methods are used. However, these are prone to cross reaction which may influence the result. It is therefore important to confirm and quantify the results of IA methods with an independent (chemical) method.

3) For values obtained in the neighbourhood of the MRL, quantitative accuracy and precision becomes of extreme importance. Next to point 2, described above, a laboratory must provide a so-called "safety zone" depending upon the precision of the methods used in their and in all possible second labs. In fact, a kind of secondary action limit (AL2) is created: $AL2 = MRL + 1{,}64\ (s / \sqrt{n})$ (n= number of experiments). Moreover, it may be questioned in what way the quantitative result should be converted into YES/NO answers

by rounding: will 1.56 ppb become 1.6 ppb (NO) or 2 ppb (YES). By most scientists the significant figures (all certain figures + the first uncertain figure) are given. The number of significant figures must reflect the precision of the analysis.

Anyway, it should be clear that qualitative criteria must be fulfilled during quantification. Moreover the way of quantification is very important. When mass spectrometry is used the quantitative result may be calculated by using the full signal of the sample versus those of the internal standard (calibrated against a series of standards) (fig 2). Alternatively the sum of the 3 (or 4) diagnostic ions, the most important ion or an algorithm including the correct ratios of the ions may be used. All possibilities will give different results and can be the cause of contradictions. In Fig. 2 peak 2 of the sample is distorted by an interference: taking the blind sum of all peaks will result in false quantification. A correction for the correct peak ratios should be made.

In real -life situations different laboratories will always use different methods (for calculation) and obtain different results: therefore a “safety zone” is necessary.

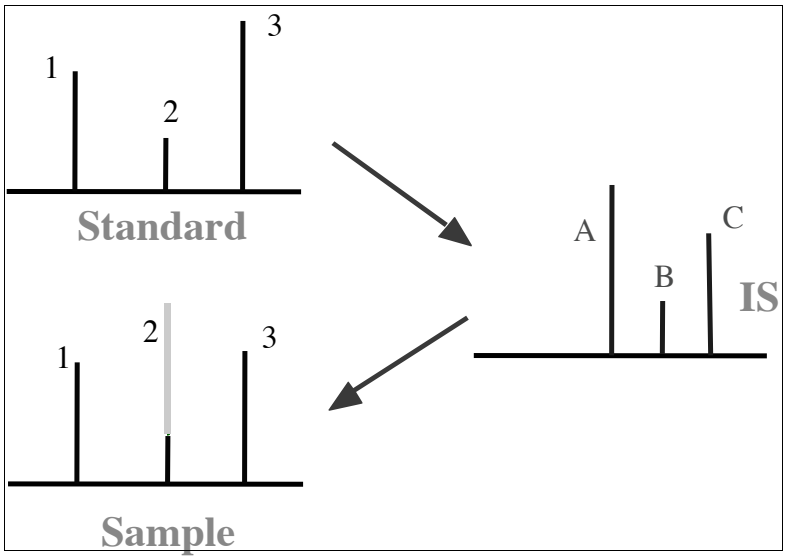

Fig 2: Ways of quantification with MS: blind sum of the peaks 1,2,3 in standard and sample to those of the Internal standard A,B, C or the use of correct peak ratios (in the sample peak 2 is distorted).

4. Interpretation of results

The problems described above are illustrated by the following example: in a farm 20 samples of urine are taken for analysis of anabolics (= illegal substance, thus no MRL but an AL). In 16 out of 20 samples lab A detects the presence of an anabolic XG. However, in only one sample the concentration of XG exceeds the Action Level of 2 ppb for anabolics although for all 16 samples a fitting full spectrum or 4 diagnostic ions at the correct ratios were obtained.
Which strategy should be followed ?

1) A YES (positive) answer on one sample because the concentration is above the AL (2 ppb)
2) A YES (positive) answer on all 16 samples (because quality criteria are fulfilled)

In strategy 1, which is the less probable, the analysis may be contested because 19 animals were declared negative and only one positive. The farmer may declare that all animals belong to one box and that he does not understand why only that particular animal should be positive. In court (some years later) lawyers may focus on the 19 negative results and try to find a alternative cause for the one positive. First of all it should be emphasised (to non analyst) that the meaning of the word "negative" or a NO answer does not necessarily mean that the analyte was absent. On our analysis reports the meaning of the abbreviation "neg" is explained on the basis of the EU regulations: neg = analyte absent or concentration lower than the "action limit"(1).

In strategy 2 the analysis could be contested by the owner by selecting some animals (e.g. 5) with the lowest concentration of XG and demanding a second analysis in an independent lab (the owner mostly knows very well the pharmacological history of his animals).
The following possibilities may occur.

1) In the second analysis the 2° laboratory detects XG in all samples in a concentration range above the AL: this is a quantitative but not a qualitative contradiction with no consequences.
2) The second lab did not detect XG. This may be due to a qualitative error in the first analysis (false positive) or because the concentration of XG is below the Limit of Determination of the second lab. This is a quantitative and/or qualitative contradiction.
3) In a third case XG is detected by the second lab but in a concentration below the action limit (e.g. 0.4 ppb). According to the rules, the lab may decide that the sample is negative because it is below the AL. This is also a qualitative contradiction for the inspection services.

Contradictions of the second kind must be handled by the NRL (**N**ational **R**eference **L**aboratory). In the EU it is the task of the NRL's to control the results of the field laboratories.

Contradictions of the third kind could be avoided by introducing a double action limit system. This is represented in fig 3.
A laboratory carrying out a first analysis should make a decision based to the action limit (e.g. 2 ppb). In samples, containing analytes at a higher concentration than the action limit the analytes found (but not the concentration) are mentioned. Samples below action limit are declared as NO answers in the light of administrative agreements (= the risk of a negative result at second analysis becomes to high) The concentration range between the Action Limit and the Limit of Determination may be used as a warning or suspect zone: inspection services may be informed (off the record?) that illegal substances are detected but have a concentration below the Action Limit (fig 1). The inspection services may use that information for future sampling.

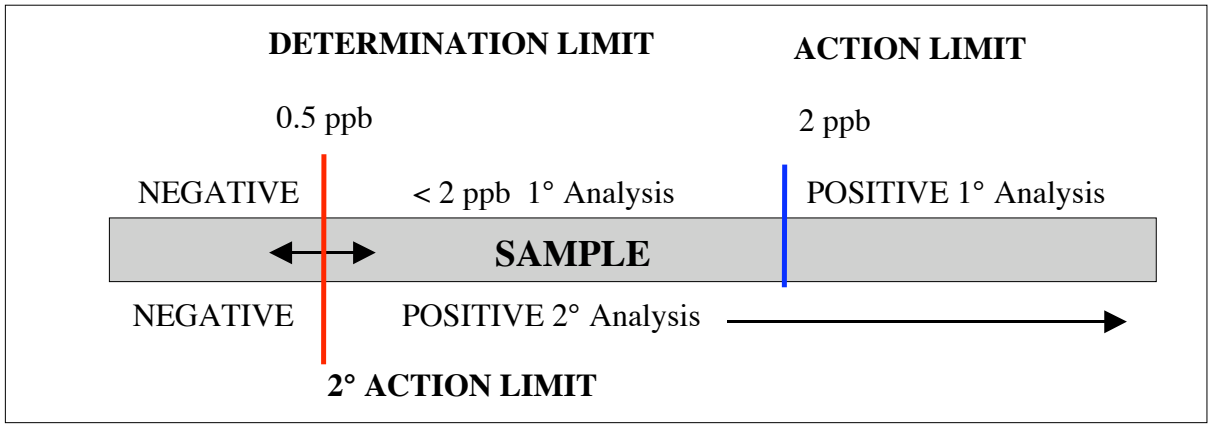

Fig 3: Presentation of a double action limit system in residue analysis. The (first) Action Limit is fixed (e.g. 2 ppb). The second Action Limit may vary with the Determination Limit of the second Laboratory.

In a second analysis the confirmatory method with the lowest Limit of Determination of the second lab is used as an Action Limit. Those second Action Limit is identical to a scientific parameter (DtL), which is laboratory dependant and not a consensus like the first AL.
A system with double Action Limits as described in fig 3 is only applicable to illegal substances with zero tolerance and thus not to MRL's.

5. Conclusions

Because of the Action Limit, qualitative results could not be produced without some form of quantification. Also in typical qualitative methods as HPTLC some kind of semi-quantitative observation must be made.

The production of quantitative results without qualification is unappropriate. The analyst should always be sure of the identity of the molecule(s) for which the figure is produced. So, in our opinion the answer to the question: Qualitative or quantitative methods for residue analysis? may not be given that easily but will depend upon the legislation, the matrix and the analyte.

6. References

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4.2. IDENTIFICATION POINTS

After

Identification points: a new approach to quality criteria

H.F. De Brabander, K. De Wasch, L.A. van Ginkel, R. Schilt, D.Courtheyn, S. Impens

Proc. Euroresidue IV, Veldhoven 7-10/5 (2000) 248- 254

1. Introduction

The European Commission decisions were published to regulate routine methods (i.e. 87/410 EC, 93/256 EC), confirmatory methods (i.e. 89/610 EC, 93/257 EC) and specific contaminants (i.e. 90/515 EC). Most of these decisions refer to Council Directive 86/469 EEC, concering the examination of animals and fresh meat for the presence of residues. The decisons have to be systematically revised in order to take into account the scientific knowledge and the technical improvements. The quality criteria for the analysis of residues of veterinary drugs are described in the 93/256 EC. In May 1998, on the request of DG VI B II 3 veterinary legislation (W. Schuller), a working group was formed to update the 93/256 EC under the supervision of F. André (Nantes, F) (1). The draft version has been elaborated by François André (chairman) (F), Yves Bonnaire (F), Hubert De Brabander (B), Dirk Courtheyn (B), Peter Furst (D), Petra Gowik (D), Glenn Kennedy (UK), Thomas Kuhn (Au), Jean-Pierre Moretain (F), Maurice Sauer (UK), Robert Schilt (NL) and Leendert Van Ginkel (NL).

By a BENE group (BElgium, the NEtherlands) the concept of **I**dentification **P**oints (IPs) for setting up quality criteria for qualitative methods (or the qualitative part of quantitative methods) was introduced, discussed and accepted by the working group. The basic idea of IPs is that a laboratory is allowed to use any spectrometric technique or combination of techniques in order to earn a minimum number of IPs, necessary for proper identification of a component. At this moment, the minimum number of points to be earned for group A components is four. This number corresponds with the classical 4 ions (in correct ratios) of EI mass spectrometry. However, a laboratory should not necessarily restrict itself to those four points. The main advantage of the IP system is that new techniques are very easily introduced in a system of quality criteria. In this paper some of the aspects and backgrounds of the use of IPs are given.

2. 93/256 EC: problems

The criteria for LRMS of the 93/256 EC are found under 2.4.4.2.2 and 2.4.4.2.3.

2.4.4.2.2. For use of GC-LRMS as a confirmatory method, preferably the intensities of at least four diagnostic ions should be measured. If the compound does not yield four diagnostic ions with the method used, then identification of the analyte should be based on the results of at least two independent GC-LRMS methods with different derivatives and/or ionization techniques, each producing two or three diagnostic ions. The molecular ion should preferably be one of the diagnostic ions selected.

2.4.4.2.3. The relative abundances of all diagnostic ions monitored from the analyte should match those of the standard analyte, preferably within a margin of ± 10 % (EI mode) or ± 20 % (CI mode).
The most important problems with these rules are threefold: 1) the criteria are not applicable to all components, e.g. some anabolics and ß-agonists. Moreover, the 96/23 differentiates between two groups of components (A and B) with another approach for identification criteria 2) the criteria of the 93/256 can be interpreted in various ways, e.g. the ratio intensity of the peaks. 3) the criteria are only applicable to GC-MS. Since 1993, modern techniques as MS-MSn were introduced.

2.1. The criteria are not applicable to all components

In this respect there are 2 problems : firstly not all (group A) components, as some ß-agonists, show 4 suitable diagnostic ions in EI or CI MS. According to the structure of the molecules some analytes show only 2 or 3 diagnostic ions. A lot of time is spent for testing out new derivatives in order to obtain the necessary ions for proper identification.
Secondly, If a component shows 4 diagnostic ions identifcation at relatively high (> 2 ppb) concentration shows no problem. However, when the concentration of the analyte is decreased the less abundant ions may disappear, creating false negative results according to the criteria. This is easily demonstrated using a series of spiked samples.

2.2. The criteria may be interpreted in various ways

The most important factor is the intrepretation of the tolerance of the ratio of the peaks. As an example: some analysts have interpreted the tolerances of ion ratios as absolute (± 10 %). As an example a spectrum consisting of one major diagnostic ion and 3 minor ones (as given in Fig 1) is used. When the tolerances are interpreted as absolute the ion 384 may vary between 1 and 21 %. These tolerances are so wide that a spectrum as given in Fig 1 will easily match criteria when the major peak (410) is present. Noise may deliver the peaks of lower intensity.

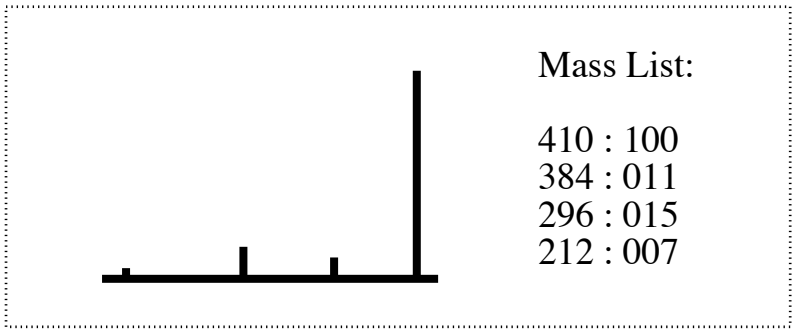


Fig 1: example of a mass spectrum

2.3. The criteria are only applicable to GC-MS

Mass spectrometric detection can be carried out by recording full mass spectra as MS-MS^n techniques (as in Ion Traps) or Selected Ion Monitoring (SIM) and Selected Reaction Monitoring (SRM) (as in quadrupoles) or other MS or MS-MS^n techniques in combination with appropriate ionisation modes (GC ar LC-M). The criteria for mass spectrometry are not really applicable given the technical advances that have occurred recently.

3. Some "new" definitions influencing Quality Criteria and IPs

3.1. New definitions of analytical parameters

In the revision of the 93/256 EC a series of definitions may influence the use of IPs and are discussed briefly. They include clearer definitions of quantitative and qualitative methods: a method is only considered as quantitative if criteria for accuracy (sum of trueness and precision) are fullfilled. It has also been highlighted that identification of compounds has to be done before their quantification. The definitions of the analytical limits are based on detection capability (CCß)(instead of detection limit) and of decision limit (CCα) (instead of limit of determination). Some terms have been changed, "violative result" replacing "positive result" and "non violative result" instead of negative result. In addition, a new regulatory limit has been established, the "Minimum Required Performance Limit", in order to establish more officially the level at which European control has to be made for banned substances.

3.2. Definitions of Ion recognition

These must be defined in full scan or mass fragmentography

3.2.1. Full scan

If mass spectrometric determination is performed by the recording of full scan spectra the presence of all measured diagnostic ions, with a relative intensity of more than 10% in the reference spectrum of the standard analyte, is obligatory.

3.2.2. Mass fragmentography

If mass spectrometric determination is performed by fragmentography, the molecular ion should preferably be one of the selected diagnostic ions. The selected diagnostic ions should not exclusively originate from the same part of the molecule. The signal-to-noise ratio for each diagnostic ion must be ≥ 3:1. The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion or transition, must correspond to those of the standard analyte, either from calibration standards or from spiked samples, at comparable concentrations, measured under the same conditions, within the tolerances given in table I :
The relative intensities of the diagnostic ions and/or precursor/product ion pairs have to be identified by comparing spectra or by integrating the signals of the single mass traces. Whenever background correction is applied, this must be applied uniformly throughout the batch.

Table 1: Maximum permitted tolerances for relative ion intensities

Rel. Int.*	EI-GC-MS**	Other techn. ***
>50 %	± 10 %	± 20 %
> 20% - 50%	± 15 %	± 25 %
> 10% - 20%	± 20 %	± 30 %
≤ 10%	± 50 %	± 50 %

a) Relative intensity (in % of base peak); **(relative)
b) *** CI-GC-MS, GC-MS-MS^n , LC-MS, LC-MS-MS^n (relative)

3.3. Interpretation of mass spectral data.

Mass spectrometric methods are suitable for consideration as confirmatory and/or reference methods only following either an on-line or an off-line chromatographic separation.

3.3.1. Full scan

If full scan spectra are recorded in single MS, a minimum of four ions must be present with a relative intensity of ≥ 10 % of the base peak. The molecular ion should be included if it is present in the reference spectrum with a relative intensity of ≥ 10%. Computer-aided library searching may be used. In this case, the comparison of mass spectral data in the test samples to that of the standard analyte must exceed a critical match factor. This factor shall be determined during the validation process for every analyte on the basis of spectra for which the criteria described below are fulfilled. Variability in the spectra caused by the sample matrix and the detector performance should be checked.

3.3.2. Fragmentography

If mass fragments are measured IPs may be applied (see 4). However, 3.3.1 may be difficult to implement in full scan : spectra could be considered to consist of mass fragments and IPs on (at least) four peaks may be used instead.

4. Quality criteria and IPs

If mass fragments are measured a system of IPs shall be used to interpret the data. For the confirmation of Group A and B substances a minimum of 4 respectively 3 IPs are required. Table II shows the number of IPs that each of the basic mass spectrometric techniques can earn. However, in order to qualify for the IPs: a **minimum** of at least one ion ratio must be measured, **all measured ion ratios** must meet the criteria described above, and **a maximum** of 3 separate techniques can be combined to achieve the minimum number of IPs.

Table 2: The relationship between a range of classes of mass fragment and IPs earned.

MS technique	IPs 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

For the counting of IPs the following remarks should be taken into account: of course each ion may only be counted once. GC-MS using EI is regarded as being a different technique to GC-MS using CI. Different chemical derivatives of an analyte can be used to increase the number of IPs only if the derivatives employ different reaction chemistries. For substances in Group A, if the following techniques are used in the analytical procedure: HPLC coupled with full-scan diode array spectrophotometry (DAD); or HPLC coupled with fluorescence detection; or HPLC coupled to an immunogram; or two-dimensional TLC coupled to spectrometric detection; they may contribute a maximum of one IP, providing that the relevant criteria for these techniques are fulfilled. Transition products include both daughter and granddaughter products.

In table 3 some examples of the number of IPs earned for a range of techniques and combinations thereof are given.

Table 3. Examples of the number of identification points earned for a range of techniques and combinations thereof (n = an integer).

Technique	Number of ions	IPs
GC-MS (EI or CI)	n	n
GC-MS (EI and CI)	2 (EI) + 2 (CI)	4
GC-MS (2 deriv)	2 (Derivative A) + 2 (Derivative B)	4
LC-MS	n	n
GC-MS-MS	1 precursor and 2 daughters	4
LC-MS-MS	1 precursor and 2 daughters	4
GC-MS-MS	2 precursor ions, each with 1 daughter	5
LC-MS-MS	2 precursor ions, each with 1 daughter	5
LC-MS-MS-MS	1 precursor, 1 daughter and 2 granddaughters	5.5
HRMS	n	2 n
GC-MS and LC-MS	2 + 2	4
GC-MS and HRMS	2 +	4

The practical application of those IPs may be applied by computer programs such as Excalibur. In fig 2 an example is given of how a datasheet of a violative result may show.

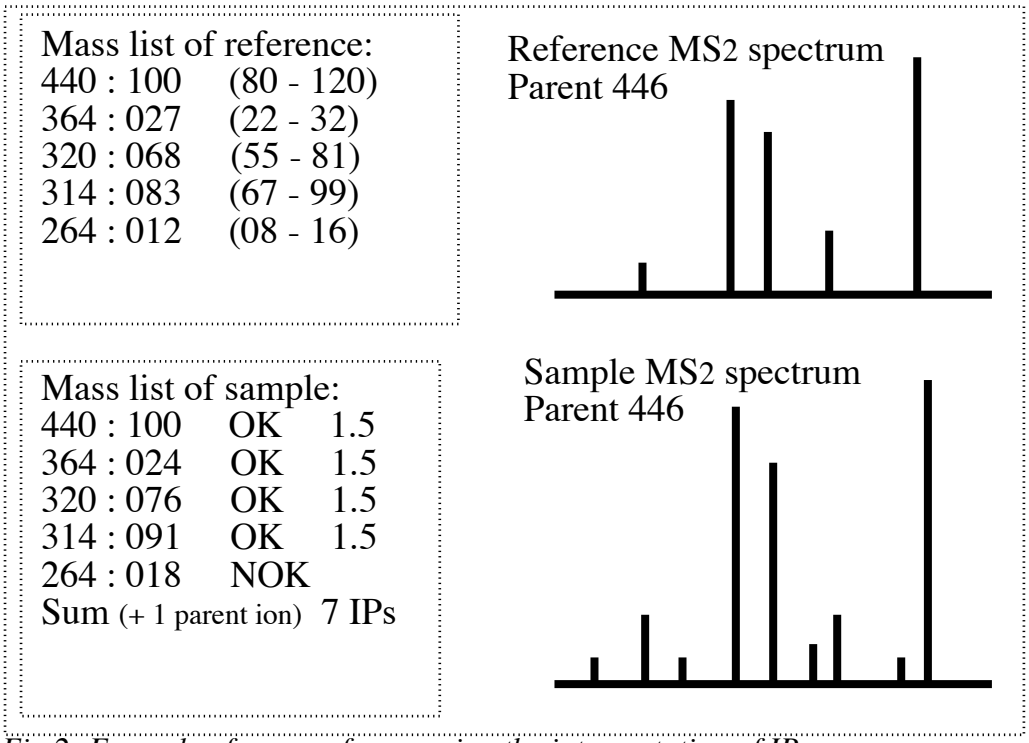
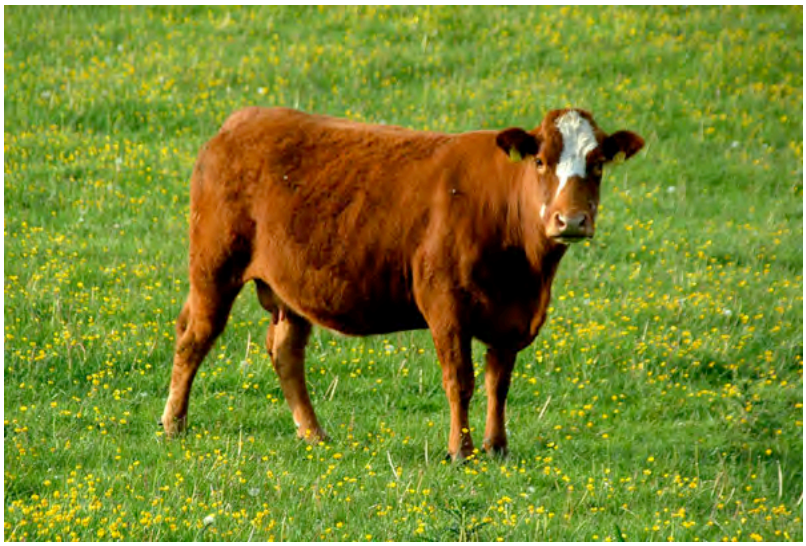


Fig 2: Example of a way of expressing the interpretation of IPs

5. Conclusion

The use of identification points (IPs) is a new approach to set up quality criteria for qualitative methods or the qualitative part of quantitative methods. The authors hope that this contribution may help in the general understanding and acceptance of this principle.



The principles, mentioned above, are worked out in publications of the expert group

Trends in the identification of organic residues and contaminants: EC regulations under revision
F. André, K.K.G. De Wasch, H.F. De Brabander, S.R. Impens, L.A.M. Stolker, L. van Ginkel, R.W. Stephany, R. Schilt, D. Courtheyn, Y. Bonnaire, P. Furst, P. Gowik, G. Kennedy, T. Kuhn , J-P Moretain, M. Sauer
Trends in Anal. Chem. 20 (2001) 435-445

and further taken over in the

Commission Decision of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC)
Off. J. Eur.Communities L 221 (2002) 8-36

Later some practical experiences with some new analytical aspects of the decision 2002/657/EC are discussed and published in the proceedings of EuroFoodChemXII, Bruges, 2003:

The commission decision 2002/657/EC: discussion on some new analytical aspects.
K. De Wasch, H.F. De Brabander, D. Courtheyn, N. Van Hoof, S. Poelmans, H. Noppe
Proc. EuroFoodChem XII, Brugge, Belgium 24-26/9/2003 45-48

CHAPTER V: GENERAL DISCUSSION

It is a “contradictio in terminis” that the general perception of the population on the safety of food seemed to decrease in the same period that the general medical observation of public health seems to increase. Indeed, along medical observations, the Belgian population belongs to the healthiest in the world, directly followed by the Dutch. These “winged” sentences were put on the world by my young, but very tall colleague and friend from our sister faculty in Utrecht: Aldert Bergwerff. Even being very critical to the medical observations of health it is a fact that since the 19th century people grow older and older and stay active during a longer period in their life. An example: a man of 57 was very, very old in the dark middle ages, while now he can still be writing a doctorate.

Who is responsible for this phenomenon? I fear that we must admit very humbly: analytical chemistry, at least, partly. Analysts can measure now “what could not be measured ever before” and so components, which were never considered as a problem may turn into a problem nowadays. Don’t look and you will not find was an expression, sometimes used by critics on regulatory control. On the other hand, the analytical instruments were sometimes overclassed by biological detectors: the PCB crisis was detected by chickens, BSE scare by cattle, the MPA contamination by pigs. Only later, instruments and methods were able to measure what chickens, cattle and pigs didn’t feel or were not affected by any more.

In the analysis of residues and contaminants, analysts were not only confronted with very low concentrations but also with a lot of different and sometimes difficult matrices from different animal species and genders. The analytes have to be determined in edible tissues as meat, liver etc., slaughter offal as kidney fat, the thyroid, the eyes and also in excreta as urine (most European countries), faeces (Belgium almost exclusively) or bile (in the UK). However also other matrices as hair, animal feed, drinking water and all kinds of bottles and other recipients with all kinds of fluids and solids have to be examined.
Injection sites represent a special class as a matrix: the concentration of the active and inactive components in this mostly very small and limited amount of matrix may be very high. On the other hand injection sites may end on the plate of the consumer and can thus be considered as meat or edible tissue.

This complexity of analytes and matrices forms an excellent brewing place for pitfalls. The red wire in these pitfalls is the combination of low limits of detection of the analytical instruments

and strong differences in concentration between the analyte and matrix components. Examples demonstrated in chapter III are isotope interferences using SIM, minor components in (crude) chemicals and differences in naturally present components in different animal species and genders. An analyst operating in the field of residues does need a knowledge of anatomy and physiology in order to understand the background of his analysis.

In addition to these problems, most modern methods are using more and more instruments working with analytical (eye) blinkers: so called target analysis. With other words, the instrument is focused on one or a limited series of analytes by using very selective or specific filters (e.g. in MS-MS an ion of an ion of a chromatographic peak). An analogous active component, present in a thousand fold higher concentration than the analyte may be completely missed (false compliant result, "compliant" = 2002/657EC expression replacing "negative") or, even worse, an innocent component may generate a signal for the analyte (false non-compliant result, "non-compliant" = 2002/657EC expression replacing "positive"). This is one of the disadvantages of modern MS^n apparatus (next to the - of course - many advantages).

The "dark side" of the abuse of growth promoters and veterinary drugs may cleverly play upon this phenomenon. Adding or deleting a group (e.g. a methyl or ethyl group) in a molecule may not change (very much) the action wanted (= weight gain) but may let the molecule escape completely from regulatory control. A typical example is clostebolacetate (chlorotestosterone acetate; ClTA). After study of the metabolism of this molecule during research projects in Belgium and France, molecules as norclostebolacetate (NClTA) and even methyl-norclostebolacetate (MClTA) appeared on the black market. Another example are the thyreostats of the thiouracil series: thiouracil, methyl- and propylthiouracil are not only commercially available, but also relatively cheap components. The ethylthiouracil derivative on the other hand is not. This phenomenon is not exclusively the case for molecules related to the safety of the food chain but also linked to other areas as body-building and doping control. A "good chemist" on the "bad side" of the law may design new molecules more easily than analytical chemists on the right side of the law may be able to detect them in a complex matrix.

The internet plays also an important role in the "dark trade" of components. From behind a computer all kinds of growth promoters and anabolic agents may be studied and purchased. It is an inept that official laboratories involved in the control of these substances must invest a lot of time, paper work and money in obtaining the necessary allowances for purchasing milligram amounts of standards while on the illegal market kilograms of new products may be bought on-line. Here the law is counteracting the application of another law. Accreditation according to BELTEST is meant to obtain and maintain a high quality standard in the laboratories. On the other hand the high demands of the EN 17025 for validation of methods requires also a considerable effort in time and money for the laboratories. This results in the first instance in a

limited and rigid scope: it takes a long dead time before a lab is able to respond to new products or new situations. The flexible scope is an answer to that problem: laboratories, which are very experienced in a certain field may, under certain conditions, extend their scope without previous intervention of a technical auditor. Our laboratory participated in the preparation of documents for the flexible scope.

Not only modern methods but also analytical criteria for those methods were needed inside and outside of Europe. These criteria have to be adapted to possible pitfalls and the demands and possibilities of modern analytical instrumentation. Our laboratory participated in the development of the Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (the so-called 2002/657/EC criteria). This participation was within the working group but also by scientific publications.

One of the major changes to the directive 93/256/EEC is the way of expressing a result. Before (in the 93/256EEC) the words “positive” and “negative” were used for the presence and absence of components. However, in normal language positive means “good” and negative equals “bad”. In residue analysis however, negative means “good” and “positive” is bad. In the 2002/657/EC these expression where replaced by compliant (for negative) and non-compliant (for positive). These expressions leaves more space for laboratories and inspection services and are more generally acceptable and applicable, also to MRL substances. The presence of beta-nortestosterone in a sample derived from a boar (an intersex or a chryptorch) may be considered as compliant but not really as negative for that molecule. The same counts for alfa-nortestosterone in urine of a pregnant cow and for alfa-boldenone in cattle in general. Some BELTEST auditors had problems with a “negative” result in these and analogous cases.

Other significant changes in terminology are for limits. First of all, the use of MRPL’s (Minimum Required Performance Levels) for banned substances. MRPL means minimum content of an analyte in a sample, which at least has to be detected and confirmed. It is intended to harmonize the analytical performance of methods for substances for which no permitted limited has been established. MRPL’s are not based on toxicological basis, as MRL’s but merely on the performance of methods and the knowledge of excretion of the component and/or its metabolites.

The definitions CCalfa and CCbeta, respectively for decision limit and detection capability are not really new (they can be compared with a “practical” and a “theoretical” limit of detection) but are more general and open new ways of validation of methods hitherto unexplored. An

example: Europe asks a MRPL of 0.3 ppb (ng/g; 300 ppt) for chloramphenicol (CAP). Laboratories of the member states have to prove that the methods used for import control at least meet this demand. In this case CCbeta could be validated by spiking (at least) 20 samples at a level below 300 ppt (e.g. 100 ppt) and be able to detect CAP in more than 95% of the spikes, according to the identification criteria. Since CCalfa is always lower than CCbeta the validation conditions for limits are fulfilled.

The knowledge of and lessons from previous pitfalls, the regular upgrade to new methods and the evaluation and application of criteria are key factors for the laboratories involved in the control of residues. Routine control on residues may never stand on its own. It should always be coupled to research in the same area. University laboratories, in open cooperation with state laboratories, are the best solution for fulfilling that purpose.

From the latest food scares: BSE, PCB, MPA etc…it can be learned that no approach, no agency, no government whatever will be able to protect the population completely from so called “food scares”. However, it is the duty of inspection services and scientists to learn from these crisises and try to avoid the next one. In that respect, regulatory control and scientific research should grow together in mutual respect.

SUMMARY

It is a “contradictio in terminis” that the general perception of the population of the safety of its food seemed to decrease in the same period that the general medical observation of public health seems to increase. Who is responsible for this phenomenon? I fear that we must admit very humbly: analytical chemistry, at least, partly. Analysts can measure now “what could not be measured ever before” and so substances, which were never a problem may turn into a problem nowadays because they are detected at decreasing residue levels (ppb, ppt…). On the other hand, analytical instruments were sometimes surpassed by biological detectors: the PCB crisis was detected by chickens, BSE scare by cattle, the MPA contamination by pigs. Only later, instruments and methods were able to measure what chickens, cattle and pigs didn’t detect or were not affected by any more.

Residue analyse is a key factor in this residue problematic. The aim is the production of analytical results for residues and contaminants in biological material, which are acceptable **“beyond any reasonable doubt”**. Indeed, analytical results are challenged more and more. In court, a result must be able to survive all scientific and non-scientific arguments brought in against it.

Unlike most doctorates, dedicated to a single subject over a limited period of time (4 – 6 years) this manuscript describes the work of a manager, responsible for the point of view and the final results of a laboratory over a longer period (1990- 2003). During that period the Laboratory of Chemical Analysis (LCA) of the department of Veterinary Food Inspection of the Faculty of Veterinary Medicine of Ghent University has grown from a small HPTLC unit to a laboratory using 5 modern MS^n instruments coupled to the neccessary clean-up procedures. Subsequently, the staff has grown from 4 to 16 people, all occupied with education, research and/or service to the community on a University level. The department is accredited under the ISO 17025 with - for LCA - a very flexible scope in analysis of residues and contaminants.

In the analysis of residues and contaminants, analysts were not only confronted with very low concentrations but also with a lot of different and sometimes difficult matrices from different species and genders of animals. This complexity of analytes and matrices forms an excellent brewing place for pitfalls. The red wire in these pitfalls is the combination of low limits of detection of the analytical instruments and strong differences in concentration between the analyte and matrix substances. An analyst, working in the field of residues does need a basic knowledge of anatomy and physiology in order to understand the background of his analysis.

The first chapter of this manuscript consists therefore of pitfalls. A pitfall may be defined as: *a hidden or not easily recognized danger, error, or source of injury or destruction into which one that is unsuspecting or incausious may fall* (Merriam-Webster dictionary).

A first pitfall is pure mass spectrometry: isotope interference in selected ion monitoring. The large difference of concentration range between a residue (ppb, ppt) and components of the matrix (ppm or higher) may cause interferences. Being aware of the possibility of this kind of interference is a "conditio sine qua non" for a lab using mass spectrometry. Isotope interference could generate the following three effects:
False positive (*non-compliant*) result obtained by the presence of the diagnostic ions with correct ratios at the correct retention time. However, these ions do not originate from the analyte but are generated by interferences, present at high concentration in the final extract. False negative (*compliant*) results by disturbance of the normal peak ratios of the ions from the analyte by one or more isotope peaks from one or more interferences. Finally, wrong quantification by disturbance of the ions of the analyte or the internal standard.

A second pitfall concerns impurities in "bulk" chemicals. Commercial "bulk" chemicals (as pharmaceutical products) are seldom very pure. Some products may contain up to 5 % other mostly related chemicals. The chemicals may not interfere with the normal use of the product. However, they may interfere in residue analysis, as demonstrated with an example (progesterone). From this study it can be concluded that analysts must be aware of the by-products of anabolics used as illegal preparations. The use of a combination of different techniques, for purification as well as for detection, is the best way to obtain a reliable result.

The third pitfall describes naturally present "thought to be exogenic" compounds. Nortestosterone serves here as an example but other molecules as boldenone are also under study these days. Nortestosterone was first found to be endogenic in the stallion during doping control. Later the boar (male pig) followed. From a large number of samples of pregnant non-treated cows, analysed by GC-MS in 4 different labs, it is proved clearly that 17αNT may indeed be present in the urine of pregnant cows from at least 4 months before the partus.

The second chapter of this manuscript regards methods. Hereby the evolution of methodology between 1990 and 2002 is described. In 1973, when our laboratory was founded, TLC (Thin Layer Chromatography) seemed the method of choice for the analysis of thyreostats and anabolics. The reasons therefore were the specificity, the simplicity and the possibility of reaching low limits of detection for an acceptable budget. During the 90's more and more affordable GC-MS (Gas Chromatography – Mass Spectrometry) apparatus appeared on the market and the transition from TLC to GC-MS and further on the GC-MS-MS and even MS^n methods was ongoing. The aim of the chapter is: showing how our lab has followed the ongoing evolution in analytical instruments and methods evolving from this.

The third chapter of the body is on analytical criteria. The basic criteria are described in the EC regulation 93/256/EEC, laying down methods to be used for detection residues of substances having a **hormonal** or **thyreostatic** action. However the continuous evolution in analytical possibilities made a revision of those criteria necessary. D. Courtheyn from the Food&Feed Laboratory of the FAVV in Gentbrugge and myself were the two Belgian delegates in the European expert group who revised the criteria. This resulted, with the publication in august 2002 of the 2002/657/EC.

One of the major changes to the 93/256/EEC is the way of expressing a result. Before the words "positive" and "negative" were used for the presence and absence of components. However, in normal language positive means "good" and negative equals "bad". In residue analysis however, negative means "good" and "positive" is bad. In the 2002/657/EC these expression where replaced by compliant (for negative) and non-compliant (for positive). These expressions leaves more space for laboratories and inspection services and are more generally acceptable and applicable. Other significant changes in terminology are for limits. First of all, the use of MRPL's (Minimum Required Performance Levels) for banned substances. MRPL means minimum content of an analyte in a sample, which at least has to be detected and confirmed. It is intended to harmonize the analytical performance of methods for substances for which no permitted limited has been established. MRPL's are not based on toxicological basis, as MRL's but merely on the performance of methods and the knowledge of excretion of the component and/or its metabolites.

The knowledge of and lessons from previous pitfalls, the regularly upgrade to new methods and the evaluation and application of criteria are key factors for the laboratories involved in the control of residues. Routine control on residues should always be coupled to research in the same area. University laboratories, in open cooperation with food and feed laboratories, are the best solution for fulfilling that purpose.

From the latest food scares: BSE, PCB, MPA etc…it can be learned that no approach, no agency, no government whatever will be able to protect the population completely from so called "food scares". However, it is the duty of inspection services and scientists to learn from these crisises and try to avoid the next one. In that aspect, regulatory control and scientific research on its problems should grow together in mutual respect.

SAMENVATTING

Het is een “contradictio in terminis” dat de algemene perceptie van de veiligheid van ons voedsel lijkt te dalen terwijl de volksgezondheid in het algemeen stijgt. Wie of wat is verantwoordelijk voor dit fenomeen? Ik vrees dat wij nederig moeten toegeven: analytische chemie, tenminste gedeeltelijk. Analysten kunnen nu “vroeger niet meetbare” substanties bepalen en sommige substanties kunnen nu een probleem worden omdat ze in steeds lagere concentratie kunnen worden bepaald (ppb, ppt niveau…). Analytische instrumenten worden echter soms overtroffen door biologische detectoren: de PCB crisis werd gedetecteerd door kippen, BSE door runderen, MPA contaminatie door varkens. pas later, waren de instrumenten en methoden in staat te meten wat kippen, runderen en varkens niet meer konden detecteerden (of door werden beïnvloed).

Residu analyse is de koningin van de residu problematiek. Het doel is de productie van analytische resultaten voor residuen en contaminanten in biologisch materiaal. Deze resultaten moeten aanvaardbaar zijn **“buiten elke redelijke twijfel”**. Inderdaad, resultaten worden meer en meer betwist. In de rechtbank, dient een resultaat in staat te zijn alle argumenten die men ertegen kan inbrengen te overleven.

In tegenstelling tot de meeste doctoraten, die handelen over een gespecialiseerd onderwerp over een gelimiteerde periode (4-6 jaar) wordt in dit manuscript het werk van een manager, verantwoordelijk voor het standpunt en de finale resultaten van een laboratorium over een langere periode (1990-2003), beschreven. Gedurende deze periode is het Laboratorium voor Chemische Analyse van de vakgroep Veterinaire Volksgezondheid & Voedselveiligheid (Fac. Diergeneeskunde, Universiteit Gent) uitgegroeid van een kleine HPTLC eenheid tot een lab met 5 moderne MS^n instrumenten, gekoppeld aan de noodzakelijke clean-up procedures. Het personeelsbestand is uitgegroeid van 4 tot 16 personen en is betrokken bij onderwijs, onderzoek en wetenschappelijke dienstverlening op Universitair niveau. De vakgroep is geaccrediteerd volgens ISO 17025, met een flexibele scoop in de analyse van residuen en contaminanten.

Bij de analyse van residuen en contaminanten worden de analisten niet alleen geconfronteerd met zeer lage concentraties, maar ook met een aantal verschillende en soms moeilijke matrices van verschillende species en verschillend geslacht. Deze complexiteit van substanties en matrices vormt een uitstekende broedplaats voor valkuilen. De rode draad doorheen deze valkuilen is de combinatie van lage detectielimieten en de sterke verschillen in concentratie tussen analyten en andere substanties aanwezig in de matrix. Een analist, die op het gebied van residuen werkt heeft een basiskennis van anatomie en fysiologie nodig om de achtergrond van de analyses te begrijpen.

Het eerste hoofstuk handelt over valkuilen. Een valkuil wordt gedefinieerd als: een verborgen of niet eenvoudig herkenbaar gevaar of fout, waarin iemand, die er niet op bedacht is terecht kan komen (vrij vertaald uit de Merriam-Webster dictionary).

Een eerste valkuil is puur massaspectrometrisch: isotoop interferentie in "Selected Ion Monitoring". Het grote concentratieverschil tussen een residue en de matrix componenten kan interferenties veroorzaken. Het "zich bewust zijn" van de mogelijkheid van dit type interferentie is een "conditio sine qua non" voor een laboratorium dat MS in gebruik heeft. Isotoop interferentie kan drie effecten genereren:
Vals positieve (*niet-conforme*) resultaten, bekomen door de aanwezigheid van diagnostische ionen in de juiste verhoudingen bij de correcte retentietijden. Deze ionen zijn echter niet afkomstig van het analyt maar worden gegenereerd door interferenties, die in het finale extract in hoge concentratie aanwezig zijn. Vals negatieve (*conforme*) resultaten, worden bekomen door verstoring van de normale piekverhouding van de ionen van het analyt, door één of meerdere isotoop pieken van één of meerdere interferenties. Finaal, kan foutieve kwantificatie optreden door verstoring van ionen van het analyt of de inwendige standaard.
Een tweede valkuil betreft onzuiverheden in chemicaliën. Commerciële "bulk" chemicaliën (zoals sommige farmaceutische producten) zijn zelden zeer zuiver. Sommige kunnen ca 5 % andere, meestal aanverwante substanties bevatten. Deze zullen (mogen) niet interfereren bij het normale gebruik. Ze kunnen echter wel interfereren bij residu analyse, zoals aangetoond met een voorbeeld (progesteron). Uit deze studie kan worden besloten dat de analisten zich moeten bewust zijn van bij-producten van bv. Anabolica, gebruikt in illegale preparaten. Het gebruik van een combinatie van verschillende technieken, zowel voor opzuivering, als voor detectie is de beste garantie voor het bekomen van een betrouwbaar resultaat.
Een derde valkuil betreft natuurlijk voorkomende substanties die (vroeger) beschouwd werden als exogeen. Nortestosteron (NT) dient hierbij als voorbeeld maar andere substanties (zoals boldenone) worden nog bestudeerd. Gedurende doping controle werd gevonden dat NT endogeen is bij de hengst. Later volgde het aantonen van NT bij het mannelijk varken. Door de GC-MS analyse, in vier verschillende laboratoria, van een groot aantal stalen van niet-behandelde koeien, werd duidelijk bewezen dat 17αNT ook aanwezig kan zijn in de urine van drachtige runderen vanaf (ten minste) 5 maanden dracht.

Het tweede hoofdstuk gaat over methodes. Hierin wordt de evolutie van de analyse methodologie tussen 1990 en 2002 beschreven. In 1973, wanneer ons laboratorium werd opgericht, leek TLC de meest geschikte methode voor de analyse van thyreostatica en anabolica. De redenen daartoe waren de specificiteit, de eenvoud en de mogelijkheid om lage detectielimieten te halen voor met aanvaardbaar budget. Gedurende de jaren '90 verschenen meer en meer betaalbare GC-MS apparatuur op de markt en gebeurde de transitie van TLC naar GC-MS, GC-MS-MS en zelfs MS^n methodes. Het doel van dit hoofdstuk is aan te tonen hoe ons

laboratorium de evolutie van analytische instrumenten en de daaruit voortvloeiende methodes heeft gevolgd.

Het derde hoofdstuk handelt over analytische criteria. De basis criteria werden beschreven in de EC verordering 93/256/EEC. De continue evolutie van de analytische mogelijkheden maakte echter een revisie van deze criteria noodzakelijk. D. Courtheyn van het Federaal Voedingslaboratorium, Gentbrugge en ikzelf waren de twee Belgische vertegenwoordigers in de Europese experten groep voor het herzien van deze criteria. Dit resulteerde in de publicatie van de 2002/657/EC in augustus, 2002.

Een van de belangrijkste wijzigingen tov de 93/256/EEC is de wijze van uitdrukken van een resultaat. Vroeger werden de woorden “positief” en “negatief” gebruikt voor het aanduiden van de aanwezigheid of afwezigheid van een substantie. In de normale spreektaal, betekent positief: goed en negatief: slecht. In residu analyse betekent negatief echter “goed” en “positief” slecht. In de 2002/657/EC worden deze uitdrukkingen vervangen door conform (voor negatief) en niet conform (voor positief). Deze manier van uitdrukken laat de laboratoria en de inspectiediensten meer ruimte en is meer algemeen toepasbaar. Voor limieten zijn er eveneens significante veranderingen in de terminologie. Eerst en vooral, het gebruik van MRPL’s (Minimaal Vereiste Performantie Niveau’s) voor verboden substanties. MRPL betekent: het minimale gehalte van een analyt in een monster dat aangetoond en bevestigd moet worden. Dit is bedoeld om de analytische prestaties voor stoffen waarvoor geen toelaatbaar gehalte is vastgesteld, te harmoniseren. MRPL’s zijn niet gebaseerd op een toxicologische basis, zoals MRL’s, maar op de performantie van de methodes en de kennis van de excretie van een substantie en/of van zijn metabolieten.

De kennis van, en de lessen uit vorige valkuilen, het regelmatig upgraden naar nieuwe methodes en de evaluatie en applicatie van criteria zijn kernfactoren voor de laboratoria betrokken bij de controle van residuen. Routine controle op residuen, dient altijd gekoppeld aan research in hetzelfde vakgebied. Universitaire laboratoria, in een open samenwerking met federale voedingslaboratoria, zijn het best geplaatst om dit doel te bereiken.

Uit de laatste voedselcrisissen: BSE, PCB, MPA enz …kan worden geleerd dat geen enkele aanpak, geen enkel agentschap, geen enkele regering in staat zal zijn de bevolking volledig te beschermen tegen een ploseling opkomende “voedselpaniek”. Het is echter de plicht van de inspectiediensten en wetenschappers te leren uit deze crisissen en zo te trachten een volgende te vermijden. Daarom dienen routine controle en wetenschappelijk onderzoek samen te groeien in wederzijds respect.

CURRICULUM VITAE

De Brabander Hubert was born on May 21 1946 in Ghent, Belgium.

He graduated at Ghent University as Master in Chemistry (Licentiaat in de Scheikunde, 1968), PhD in Chemistry (Doctor in de Wetenschappen, groep Scheikunde, 1974) and Master in Environmental Sciences (Licentiaat in de Milieusanering, 1974). He promoted as PhD in Analytical Chemistry of Food (Geaggregeerde voor het Hoger Onderwijs in Analytische Chemie van Eetwaren) in 1984.

He started his career at the Faculty of Sciences, Ghent University (1968-1973) and then shifted to the Faculty of Veterinary Medicine, Ghent University. He is a full professor in the Department of Veterinary Public Health & Food Safety and director of the Laboratory of Chemical Analysis.

His main working area is chemical analysis of food, with as major subject of research and service to the community, the analysis of residues and contaminants in biological material.
He is author or co-author of more than 100 scientific publications and technical reports.
He is member of boards, scientific organisations, working groups and committees (a.o. member of the board of Ghent University, president of the Food Division of the Royal Flemish Chemical Society (KVCV), president of the Belgian Association of Meat Science and Technology (BAMST), member of the Belgian Society for Mass Spectrometry (BSMS) etc.)
He is also president of the institute Quality Control, a consumer label.

More info could be found on http://allserv.ugent.be/~hdbraban/

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