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Pitfalls in selected ion monitoring in gas chromatography–mass spectrometry: a theoretical example

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Abstract

For the routine determination of residues of growth promoters two important types of low-resolution gas chromatograph–mass spectrometer may be distinguished: the ultra-trace full-scan instrument [e.g., the ion trap mass spectrometer (ITS40)] and most other quadrupole apparatus using the selected ion monitoring (SIM) mode for detecting very small amounts (< 1–10 ng). In analysing biological extracts interference between matrix components, present at high concentrations, and analytes, present at low concentrations, should be avoided. In this investigation theoretical examples of pitfalls in SIM due to isotope interference (^{13}C) from matrix components with the analyte were considered. These interferences may lead to false-positive and -negative results and false quantification.

Keywords: Gas chromatography–mass spectrometry; Anabolic steroids; Biological samples; Interferences; Selected ion monitoring; Steroids

For the routine determination of residues of growth promoters in meat-producing animals there is increasing use of coupled techniques. In most instances a low-resolution mass spectrometer coupled to a chromatographic separation is used [1–4]. In low-resolution gas chromatography–mass spectrometry (GC–MS) two important types of apparatus may be distinguished: the ultra-trace full-scan instrument [e.g., the ion trap mass spectrometer (ITS40)] and most other quadrupole apparatus using full scan for large amounts of analyte and the selected ion monitoring (SIM) mode for detecting very small amounts (< 1–10 ng). Both techniques have advantages and disadvantages and also their own supporters [5].

With SIM a limited number of ions (2–4) 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 with the correct abundance ratio (between certain limits [6]). In the EC guidelines the monitoring of four ions is advised [6]. In practice, the monitoring of four ions at low concentrations (< 1 ng ml^{-1}) does not give satis-

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factory results [7]. As the monitoring of only two ions is certainly not sufficient according to the quality criteria, a decision on three ions seems an acceptable analytical compromise [7]. In this investigation, SIM on three ions was 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., one spectrum per second). Subsequently, full-scan identification of the analyte by a 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 material (e.g., urine, meat), a wide variety of components with a large range of concentrations are 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 (μg ml^{-1} range or higher), and analytes, present at very low concentrations (ng ml^{-1} range), should be avoided when using SIM.

In this investigation a theoretical example of a pitfall in SIM due to isotope interference (^{13}C) from matrix components with the analyte was considered. 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 radioimmunoassay (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. For SIM analysis ^{13}C isotope interference with the high concentrations of estradiol present in the urine of these animals could be possible.

STABLE ISOTOPES: CALCULATION OF ISOTOPE PEAKS

A knowledge of the relative abundances of stable isotopes is essential for the interpretation of mass spectra [10]. A brief explanation is given here for those unfamiliar with this subject. Chlorine has two isotopes, ^{35}Cl and ^{37}Cl, with an abundance ratio of 3:1 (mean atomic mass 35.453). For a molecule containing one Cl atom two peaks separated by 2 u and with an abundance ratio of 3:1 are observed (for the molecular ion and the fragments containing Cl). When a molecule contains two Cl atoms, three peaks are observed in the mass spectrum. The total number of isotope peaks of Cl is $n + 1$ and their relative abundance by the formula $100(1 + 0.333)^n$ where n is the number of Cl atoms. In Table 1 these isotope ratios for molecules containing one to three Cl atoms are summarized.

TABLE 1

Ratios of the isotope peaks of Cl

n [a]	Ratio
1	100:33
2	100:66:11
3	100:100:33:4

[a] Number of carbon atoms.

Isotope peaks may be very specific for the identification of residues (e.g., Cl-containing growth promotors such as clenbuterol). They may also be important for the determination of the fragmentation patterns and metabolization of these molecules.

PEAKS OF LOW RELATIVE ABUNDANCE

For the other elements analogous calculations can be made. In Table 2 the most important

TABLE 2

The most important elements in organic molecules and their natural isotopes

Element	M_r	Isotopes (abundance, %)
H	1.00797	1.00783 (99.985), 2.01410 (0.015)
C	12.01115	12.00000 (98.89), 13.0335 (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)

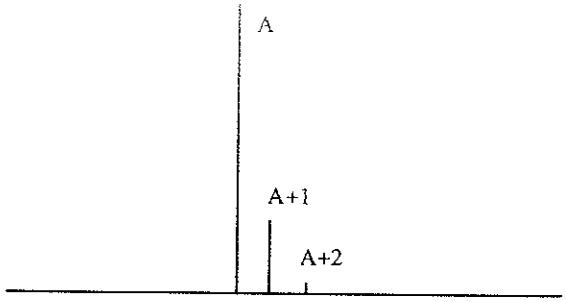

Fig. 1. Spectrum of a component (ion) with 20 carbon atoms.

atoms in organic molecules are given together with their most important natural isotopes.

Isotope interference may occur with any isotope with a relatively high abundance. In this investigation ^{13}C was studied. Carbon has two natural isotopes, ^{12}C and ^{13}C, with a ratio of 98.9:1.1 (the exact figures are rounded for simplicity). In residue analyses two other important parameters should be taken into account: the very large difference in concentration between the analytes and the matrix components, and the fact that the analyte (an organic molecule) contains a relatively large number of carbon atoms. The components co-extracted from the matrix may also contain a substantial number of C atoms, and these components may have similar structures to the analytes (e.g., in anabolizing agents such as steroids 20–30 carbon atoms are present and numerous steroids and metabolites with analogous structures are known).

The number of isotope peaks produced by n carbon atoms is $n+1$ and the relative abundance of these isotope peaks by the formula $100(1+0.011)^n$. The general equation for this formula is given by the Bernoulli binomial distribution:

$$(A+B)^n = A^n + C_n^{n-1}A^{n-1}B + C_n^{n-2}A^{n-2}B^2 + \ldots + C_n^1 AB^{n-1} + B^n$$

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

First term:	chance of no ^{13}C: 80.15%	$(0.989)^{20}$
Second term:	chance of one ^{13}C: 17.83%	$C_{20}^{19}(0.989)^{19}(0.011)$ Σ97.98%
Third term:	chance of two ^{13}C: 1.88%	$C_{20}^{18}(0.989)^{18}(0.011)^2$ Σ99.86%

TABLE 3

Relative abundances of the isotope peaks to the generating ion A as a function of the number of carbon atoms, n (%, 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

The chances of no, one and two ^{13}C atoms in a molecule containing 20 carbon atoms are equivalent to the relative abundances of the peaks A, $A+1$ and $A+2$, where A represents 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. 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 $A+2$ ions as a function of the number of carbon atoms in a molecule are given. The relative abundance of the ion $A+1$ is linearly related to the number of

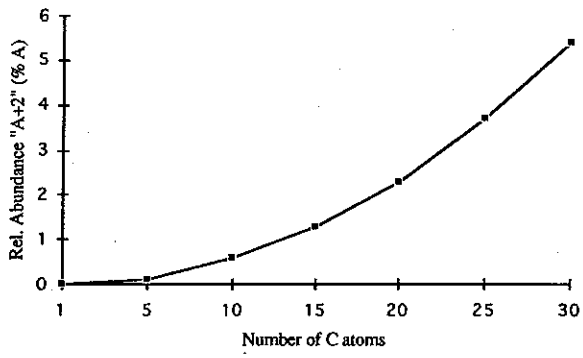

Fig. 2. Relative abundance of the ion $A+2$ with respect to the generation ion A as a function of the number of carbon atoms n.

carbon atoms n multiplied by 1.11. The relative abundance of the $A+2$ ion as a function of the number of carbon atoms is not linear and is given by $n(n-1)\times 0.00616$. This relationship is expressed in Fig. 2 which shows clearly that the relative abundance of $A+2$ increases considerably at high carbon numbers.

THEORETICAL EXAMPLES IN RESIDUE ANALYSIS

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

False-positive results

A laboratory wants to determine the analyte NT in the matrix U at the 0.1–1 ng ml^{-1} level by GC–MS. This is a very realistic situation: different workers claim to be able to determine nortestosterone at this level by 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 ≈ 15 min and three ions are followed during the time interval 14–16 min: m/z 418 (100%), 403 (20%) and 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 interferent) is present at 10 μg ml^{-1} (10^4–10^5 times higher than the concentration of the analyte). The retention time of the interferent E is nearly identical with that of NT (e.g., 14 min 58 s). The mass spectrum of this component is given in Fig. 3B. In this mass spectrum only the ions of m/z 416, 402 and 326 are important for isotope interference with the analyte. The ion of m/z 402 has a very low abundance (ca. 3% of m/z 416) and is nearly invisible in Fig. 3B. This ion of no importance for the mass spectrum of E. However, its influence on the determination of NT is not negligible, as will be demonstrated below.

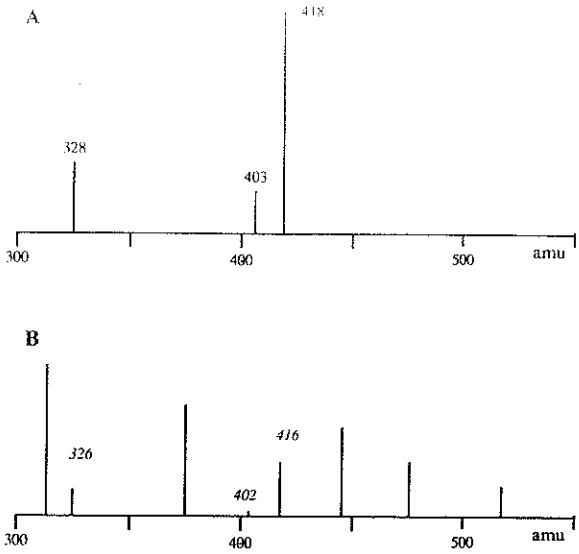

Fig. 3. Mass spectrum of the analyte NT and the interferent E in the full-scan model.

The exact number of carbon atoms in the interferent E is not important, as only the exact number of carbon atoms in the fragment ions is used in the calculation. These (theoretical) numbers of carbon atoms and the relative abundances of the isotope peaks generated are given in Table 4 (as described from Table 3 and Fig. 2). The values in italics represent the abundances that are important for this example.

As the interferent E has a concentration of 10 μg ml^{-1} and the fragment of m/z 416 forms ca. 10% of the total spectrum, this ion is attributed a relative concentration equivalent to 1000 ng ml^{-1}. The ions of m/z 402 and 326 show relative concentrations of 28 and 500 ng ml^{-1}, respectively (their ratios to m/z 416 are 2.8% and 50%, respectively). From these three ions isotope peaks are generated according to the ratios given in Table 4 and shown in Fig. 4.

TABLE 4

Number of carbon atoms of the fragments and the relative abundances of the isotope peaks generated by these fragments (relative to the generating peak)

Ion (m/z)	Carbon number (n)	$A+1$	$A+2$
416	28	31	*4.7*
402	27	*30*	4.3
326	22	24	*2.9*

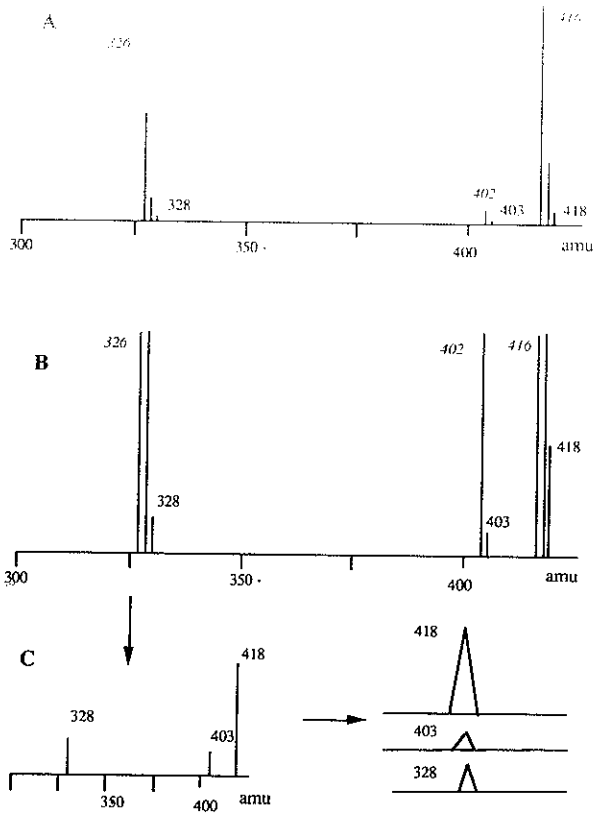

Fig. 4. Isotope peaks of NT generated by E. (A) Full-scan mode, m/z 416 = 100%; (B) full-scan mode, amplified; (C) SIM signal.

In the SIM mode only the ions of m/z 418, 403 and 328 are selected. The relative concentrations of these ions are 46.6, 8.5 and 14.2 ng ml^{-1}, respectively (ratio 100:18:31). Using the SIM method correctly, the analyst will therefore conclude that NT is present at a concentration equivalent to 10–50 ng ml^{-1} (depending on the ion used for quantification) as the three 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 interferent may be important when SIM is used. In addition to this theoretical example, more analogous examples may be calculated. The interfering isotope peaks may also be generated by several interferents 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 addition to its natural presence in the urine of the stallion and the boar) [11–13] is based on the positive signal obtained with both RIA and SIM. The signal for RIA could be caused by the aspecificity of the antibody against NT. 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 by full-scan ultra-trace mass spectrometry [14]. It was shown clearly that α-estradiol and β-nortestosterone were not well separated under the chromatographic conditions used. The three peaks at m/z 418, 403 and 328 at the correct retention times 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 m/z 418 of a urine extract of a pregnant cow, spiked with 2 ng ml^{-1} of β-nortestosterone, is shown. This ion chromatogram demonstrates clearly that a high level of estradiol (main ion of m/z 416) generates a signal at m/z 418 at high ng ml^{-1} level owing to ^{13}C isotope interference.

False-negative results

In a laboratory C the analyte EE2 in the matrix M is determined at the 2–10 ng ml^{-1} level by GC–MS. In a slaughtered animal an injection site containing EE2 was found. In the matrix M of the same animal an EE2 value of 3 ng ml^{-1} was found (quantification on the ion of m/z 425). This example also is very realistic: ethinylestradiol may be determined at this level by GC–MS in the SIM mode [15].

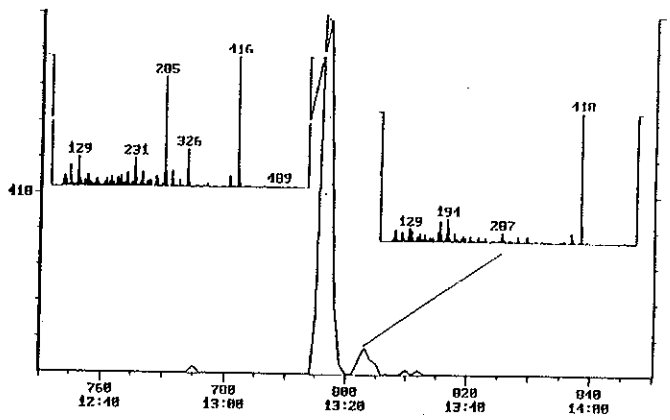

Fig. 5. Ion chromatogram at m/z 418 of a urine extract of a pregnant cow spiked with 2 ng ml^{-1} of nortestosterone.

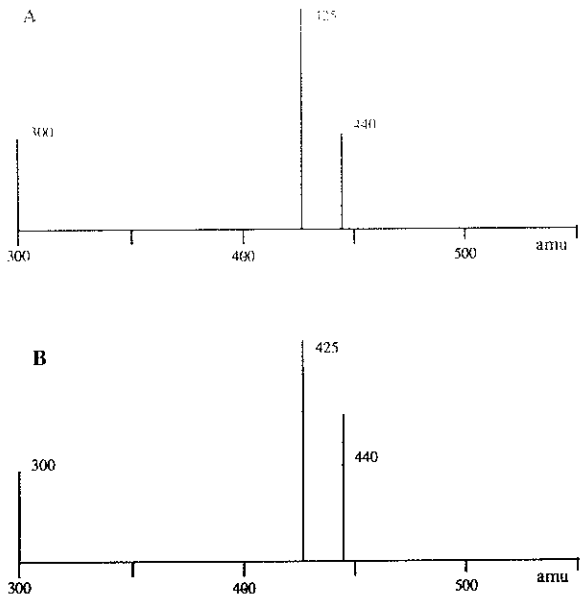

Fig. 6. Mass spectrum of (A) EE2 standard and (B) EE2 distorted by ^{13}C isotope peak of m/z 439.

The characteristics of the GC–MS SIM analysis are retention time $\approx$ 17 min and three ions are followed during the time interval 16–18 min : m/z 440 (45%), 425 (100%) and 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 at a concentration of 2 μg ml^{-1} with a retention time of 17 min. In the mass spectrum of this component an ion of m/z 439 with a very low abundance is present (ca. 1% of the total spectrum). This ion generates an isotope peak at m/z 440 with a relative abundance of 1.2 ng ml^{-1} (the calculation is analogous to that shown above). The abundance of this m/z 440 ion adds to that of the m/z 440 ion from the analyte (1.35 ng ml^{-1}). The "three-ion" spectrum after isotope interference is shown in Fig. 6B. This spectrum is clearly distorted by the isotope peak and according to the rules of SIM the sample should be considered as negative although all three peaks are present and EE2 was found in the injection site cut from the same animal.

False quantification

Quantification of residues may be very important, especially in the neighbourhood of the decision limit. In an analogous way to that shown above, interferents may influence both the analyte ions and the internal standard ions.

Conclusion

When SIM is used for the determination of residues of analytes by GC–MS at the ng ml^{-1} level, isotope interference should always be kept in mind. Isotope interference could generate the following three effects. First, false-positive results to 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 interferents 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 GC–MS in the SIM mode. Second, false-negative results owing to disturbance of the normal peak ratios of the ions from the analyte by one or more isotope peaks from one or more interferents. This effect may be of even more importance than the generation of false-positive results as the statistical possibility of its occurrence is higher. In the study of contradictory results (in a second analysis in a second laboratory) this effect must always be considered; using slightly different methods (different columns, reagents, etc.) different interferents from the same matrix may be present in the final extract. Finally, false quantification owing to disturbance of the ions of the analyte or the internal standard.

The above reasonings show that the possibility of isotope interference should be taken into account when using SIM in residue analysis. These 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 criterion. With quadrupole apparatus, which is 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 re-chromatographed and fully identified with the other system.

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