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Journal of Chromatography A, 1031 (2004) 11–16
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Quantitative assessment of the reliability of identification by high-performance liquid chromatography–mass spectrometry

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Abstract

At present, mass spectrometry (MS) is the most reliable method for identification but there is not yet a quantitative equation describing this fact. In this investigation an approach to the quantitative assessment of the reliability of identification by MS is proposed which is useful for determination of the selectivity and the validation of analytical methods. Mass spectra of the analytes are presented as maps in which the characteristic ions and their intensities are used for identification. A formula for the quantitative expression of the significance of these parameters to the reliability and the identification is given. The contribution of the resolution of MS instruments or their possibilities of a multiple fragmentation to the reliability of the identification is shown. This approach makes it possible to compare the reliability of identification with different MS instruments. Despite the small contribution of the separation of the chromatographic column compared to the MS separation, the role of the column in the identification is very important to distinguish isomers because their MS spectra are similar.

Keywords: Mass spectrometry; Reliability

1. Introduction

The selectivity of an analytical method is defined as its ability to assess an analyte unequivocally in the presence of all possible components, which may be present in the sample. The fulfillment of this requirement guarantees an unambiguous identification of the analyte. However, how can an analyst estimate which compounds are present in the sample and what is their number? Because the answers of these questions are not known there may be an accidental overlapping of the identifying characteristics of the analyte with those of some compounds in the sample. The consequence is an uncertain identification. For example, overlapping peaks occur often in LC or even in capillary GC and the identification by the relative retention times is uncertain. At present the analyst can apply powerful methods for identification as UV–Vis, diode array detection or MS. However, again the question concerning the probability of coincidental overlapping of the spectra exists and the analyst asks himself: is the identification correct and what is the probability of wrong identification?

Recently, a system of identification points for data from analytical methods was accepted [1]. Four identification points must be earned for identification of an analyte. The number of the identification points per ion, that each MS technique can earn, is represented in Table 1.

It is well accepted that high-resolution (HR) multiple mass spectrometry (MS^n) is more informative than low-resolution (LR) MS^n, but there is no explanation of why the former earns twice as many identification points. It is also not explained why four points guarantee reliable identification; why their number is not more or less than four and what is the possibility of a wrong identification when four points are earned.

In this study, a quantitative assessment is proposed for the reliability of the identification obtained by different MS techniques. This approach makes it possible to quantitatively present and compare the selectivity of the various analytical methods.

2. Theory

Let us represent a MS spectrum as a map with n and m strips along the abscissa (mass-to-charge ratio, m/z) and the ordinate (abundance), respectively (Fig. 1). The

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doi:10.1016/j.chroma.2003.12.060

Table 1
Values of the identification points per ion obtained with different MS techniques

MS techniques	Identification points
Low-resolution (LR) MS	1.0
LR–MS^n precursor ion	1.0
LR–MS^n transition product	1.5
High-resolution (HR) MS	2.0
HR–MS^n precursor ion	2.0
HR–MS^n transition products	2.5

characteristic ions, k, are arranged on the abscissa by their increased m/z value. We refer to this specific arrangement of the ions and their abundance as a "finger print" of the analyte. The overlapping of the pattern spectrum of a "standard" object with that of the analyte means that they are identical in respect to the m/z and abundance values of the characteristic ions. The number of strips along the abscissa n, is determined by the capability of the MS instrument to separate ions. The number of strips, m, along the ordinate depends on the reproducible measurement of the ion's abundance and the sensitivity of the MS instrument.

If there is only one characteristic ion in one MS spectrum, for example, the molecular ion of the analyte, the probability $P_{n,m}$ that this ion is in any quadrant of the map is:

$$P_{n,m} = \frac{1}{n \times m} \tag{1}$$

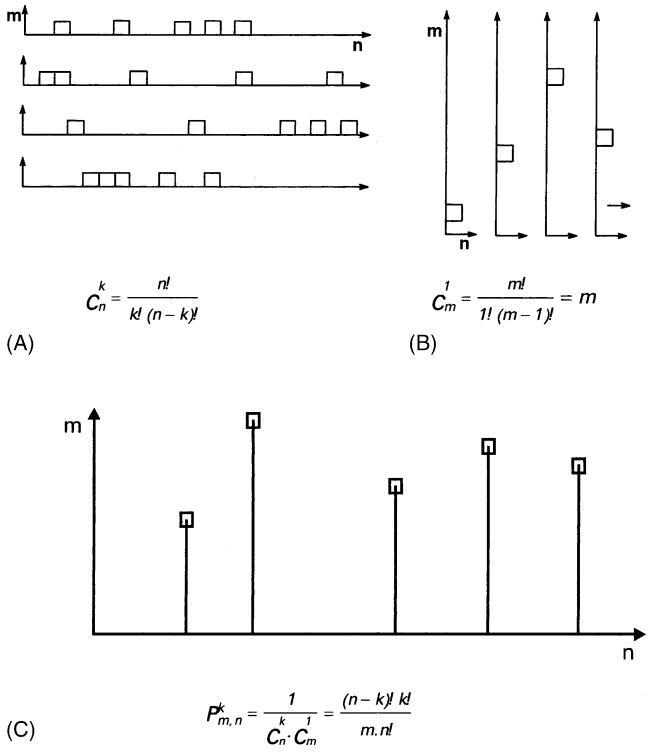

Fig. 1. Combinations of n elements from kth class toward the abscissa (A), combinations of m elements from 1st class toward the ordinate (B) and all arrangements at k number of separated strips (C).

The following combinations C_n^k of n elements from kth class exist towards the abscissa for a k number of characteristic ions with progressively increasing m/z values (Fig. 1):

$$C_n^k = \frac{n!}{k!(n-k)!} \tag{2}$$

Towards the ordinate, the combinations C_m^1 will consist of m elements from 1st class:

$$C_m^1 \frac{m!}{1!(m-1)!} = m \tag{3}$$

The number of all arrangements at k number of characteristic ions of the spectrum and n number of separated strips will be:

$$C_n^k \times C_m^1 = \frac{n!}{k!(n-k)!} m \tag{4}$$

The probability of that one and the same arrangement of these k points in the map will be:

$$P_{m,n}^k = \frac{1}{C_m^1 \times C_n^k} = \frac{(n-k)!k!}{m \times n!} \tag{5}$$

This value of the probability is accepted as a threshold value for distinguishing the analytes by their MS spectra and can be used as a measure of the identification of the analytes. Below this value the analytes will not be distinguished; nevertheless some of them are different.

In order to test if two numbers (mass-to-charge ratios or detector's signals) are different considering their individual standard deviations, i.e. they can be distinguished, a two-sided t-test can be performed. Commonly a 95% confidence level is used and the uncertainty, α, is $(100 - 95)/100 = 0.05$. In applying the two-sided t-test for the comparison of two means, $\bar{X}_A$ and $\bar{X}_B$, in five runs and looking up the result in the t-test table, the value of the difference, u, is evaluated from:

$$u = 1.03\sqrt{\sigma_A^2 + \sigma_B^2} \tag{6}$$

Here σ_A^2 and σ_B^2 are the variances of the values of A and B being compared, respectively. If the absolute value of the difference in the means, $|\bar{X}_A - \bar{X}_B|$, is greater than u, then the values are considered different at the 95% confidential level, and if $|\bar{X}_A - \bar{X}_B|$ is less than u, then X_A and X_B are not distinguishable.

3. Experimental

The Surveyor LC/Finnigan LCQ Deca MS system was used in this investigation. The efficiency of the ODS-2 column (150 × 2.1 mm, 5 μm, Alltech) was 12 600 theoretical plates, measured with sulfadimidine at a capacity factor of 6. The mobile phase was a mixture of acetonitrile–acetate buffer (20 mM, pH 6.0) (30:70). Electrospray ionization (ESI) and MS–MS were applied for the identification of the analyte.

Acetonitrile (HPLC gradient grade) and sulfadimidine were supplied from Merck. Water was purified and deionized with a Milli-Q system.

4. Results and discussion

4.1. Determination of the n value

The number of strips *n*, is closely related to the resolution of the MS instrument. Mass resolution, which is needed for the separation of two ions with masses M and $M + \Delta M$, is defined as the ratio of mass to peak width, hence, mass resolution increases with mass. This statement is somewhat misleading as can be seen from the following example: mass resolution for *m*/*z* 65 at $\Delta M = 0.1$ is 130 (65/0.5 = 130) while that for *m*/*z* 650 is 1300. The real performance of the instrument is gauged from the width of the ion signal produced upon ion ejection. Upon decreasing the scan rate, it was found that the peak width was reduced so that for a given mass, the resolution was increased [2]. Although in research instruments peak widths less than 0.003 u have been observed, the narrowest peak widths in commercial instruments are ≈0.2 u [3], so that for *m*/*z* 2000 a mass resolution 10 000 is achieved. It means that this instrument is able to distinguish statistically 10 000 ions with masses progressively increasing by 0.2 u. For this reason, the values of the technical characteristics of the instruments were used for determining the possibility of distinguishing MS spectra.

Low-resolution mass spectrometers are accepted as unit-mass resolution instruments for unequivocally determining masses, although they possess the ability to separate ions with $\Delta M = 0.5$. For example, Varian Saturn 4D GC–MS scans within the mass range 15–650 *m*/*z* at a rate of 5555 u is with 6.308 steps of the digital-to-analog converter (DAC) per unit, with a standard deviation of 0.15 DAC and variance of 0.47 DAC. Using Eq. (6) a value of 0.69 DAC was determined for the uncertainty *u*, and this instrument was able to distinguish two ions with difference of mass 0.11 u (6.308/0.69 = 9.1 → 9; (1/9) u = 0.11 u). Despite this low value, the manufacturer accepts this mass spectrometer as an instrument with a unit mass resolution. Taking into account the mass range of this mass spectrometer and the accepted unit resolution value, the number of strips *n*, determined by Eq. (5) is 635 [(650 − 15)/1.0 = 635].

The Finnigan LCQ Deca mass spectrometer possesses a mass range of 20–2000 and unit resolution. The zoom-scan function of this instrument makes it possible to increase the resolution to 0.1 u in the zoomed range. Besides the easier estimation of the molecular structure by the increased accuracy of the *m*/*z* values, the last one gives an opportunity for more reliable identification. Here the number of strips *n* is determined by the zoom range, which is a 0.1 u resolution. Of course, the zoom range is smaller than this at one unit resolution (20–2000 u), but the spectrometrist is able to apply the zooming consequently in the whole *m*/*z* range and by this way he/she will embrace the whole 20–2000 *m*/*z* range. In this case, the number of strips *n* will be 19 800 [(2000 − 20)/0.1 = 19 800].

Table 2
Value of the number of strip *m*, determined by the ion abundance and permeated tolerance[a]

Abundance (*A*) (%)	Tolerance (*t*)[a] (%)	$A \pm t$	u[b]	m (= *A*/*u*)
100	10	100 ± 1	20.0	5.0 → 5
>9	30	9 ± 2.7	5.4	1.7 → 1
3.4–9	39	9 ± 3.5	7.0	1.3 → 1
1.0–3.4	46	3.4 ± 1.6	3.2	1.1 → 1
0.24–1.0	51	1.0 ± 0.5	1.0	1.0 → 1
<0.24	71	0.24 ± 0.2	0.4	0.6 → 1

[a] According to Ref. [9].
[b] $u = t \times 2$; $u = |\bar{X}_A - \bar{X}_B|$.

4.2. Determination of the m value

The abundance of an ion and its reproducibility are the main factors, which determine the *m* value in Eq. (5). Overlapping of the characteristic ions with ions of other compounds or impurities decreases the reproducibility of the abundance and consequently the *m* value.

Modern mass spectrometers have an analytical dynamic range of five orders of magnitude [4] and an ion abundance of <0.1% can be measured reproducibly [5]. For the most abundant ion in the mass spectrum of an unknown compound, $a \pm 10\%$ tolerance is accepted [6]. The "window" tolerance corresponds to the difference *u*, in Eq. (6). On the basis of the abundance of a large number of ions, different values of tolerance have been statistically determined as the probability based matching (PBM) system [7–9] (Table 2). For example, for ions with abundance 3.4–9%, the accepted tolerance is 39% and the permeated "window" of the abundance is 7.0% (0.09 × ±39% = ±3.5% × 2 = 7.0%). The number of strips *m* will be 14 (100%/7.0% = 14.4 → 14). Despite the increased value of the tolerance of ions with low abundance, the width of the "window" decreases and as a result the number of strips *m* increases. For example, at an ion abundance of 1.0% and tolerance ±51% the "window" is 1% and the value of strips *m* is 1 [(0.01 × 51%) × 2 = 1%; 1%/1% = 1]. This high value of *m* is a result of the small absolute value (magnitude) of the "window". For the most abundant ion the accepted tolerance is only ±10% but the "window" of abundance is 20% and *m* is 5. If we accept lower values of the abundance "window", the most abundant ions will not be distinguished because their abundance "windows" are larger. By this reason a value of $m = 5$ was accepted in this investigation.

4.3. Determination of the k value

In the PBM system, 15 ions of the analyte ordered by decreasing confidential levels are checked by the reference

spectra for the presence of the base peak; for the most abundant isotopic peak in the molecular ion ($M^{\bullet+}$) cluster, and for a peak or two peaks (if $M^{\bullet+}$ is not present) corresponding to the neutral loss(es) of 18, 20, 27, ..., 64 u. If some of the 15 peaks are not present, they are flagged; if the number of missing peaks exceeds the number of allowed flagged peaks, the searching proceeds to the next reference spectrum. Four classes of criteria were introduced at the evaluation of the *m*/*z* value of peaks: (a) the probability of the "uniqueness" of the peaks, (b) the abundance value of the peak as it appears in the reference spectrum, (c) the "window factor", which is a measure of the agreement required between the abundance of the reference peak and an unknown, and (d) the "dilution factor" for mixed spectra, due to the presence of other components, mainly impurities. In this way the PBM system using 15 characteristic ions permits matching of an unknown spectrum against a large base of data, which is not restricted to spectra taken under the same experimental conditions. Accepting 15 characteristic ions ($k = 15$) at $m = 5$ and $n = 635$, 1850 and 18 500 the probabilities $P^k_{m,n}$, Eq. (5), will be: 2.81×10^{-31}, 2.72×10^{-38} and 2.9×10^{-53}, respectively. Taking into account these very low values of a coincidental overlapping, every one of the 220 000 "perfect" spectra (370 000 spectra of the 1999 Wiley/US National Institute of Standards and Technology (NIST) databases, corrected by Matching Quality Index [10]), will be distinguished unambiguously. And what is more, instruments with unit resolution give an opportunity for distinguishing all the spectra of NIST, even if their number is increased many times.

More than two decades ago, Schon [11] argued that a minimum of three structurally related ions would be necessary to provide proof of the presence of an analyte in an analyzed sample. This assumption was based on a statistical approach using an extensive MS data base as a model of an universal repository containing all possible organic compounds. Since, different purification methods can be used, it is impossible to determine the number of the organic compounds, which can be present as impurities in one sample.

4.4. Calculation of the probability of distinguishing MS spectra

To improve the criteria for confirmation, the relative abundance ratios were required to be within 5%, when compared with a reference standard recorded under similar conditions. In this case, the value of *m* is 10 instead of 5. Doubling the abundance reproducibility doubles the certainty for distinguishing spectra despite the magnitude of the resolution. Applying Eq. (5) at $m = 10$ and $n = 635$ and 1850 the values of overlapping $P^3_{10.635}$ and $P^3_{10.1850}$ are 2.35×10^{-9} and 9.50×10^{-11}, respectively, and their ratio is 24.8. This means that the probability for coincidental overlapping of two different spectra in an instrument with resolution 2000 is approximately 25 times lower than one with a resolution of 650. It is obvious that distinguishing two analyte is determined mainly by the resolution and the number of the characteristic peaks and less than by the reproducibility of the abundance. Recently, some mass spectrometrists have accepted five characteristic ions as proof for the presence of an analyte. If we look again at Table 3 the reliability at $k = 5$ is 2.0×10^4 time higher than one at $k = 3$ ($P^3_{5.635}/P^5_{5.635} = 2.0 \times 10^4$). When an instrument with higher resolution is used the reliability is increased 9×10^4 times ($P^3_{5.1850}/P^5_{5.1850} = 9 \times 10^4$). That is why if an analyst is not able to work with a high-resolution instrument, he/she must increase the number of characteristic peaks in order to obtain the desirable reliability of identification. However, if the low- and the high-resolution instruments have the same sensitivity, the former needs a bigger quantity of analyte to obtain more characteristic ions. For example, the probability of an instrument with unit resolution and mass range of 650 to distinguish mass spectra with five characteristic ions ($k = 5$), is approximately equal to one of an instrument with 0.1 unit resolution and mass range of 2000 when the latter uses three characteristic ions [$P^5_{5.636}(= 2.35 \times 10^{-13}) \approx P^3_{5.2000}(= 1.91 \times 10^{-13})$] (Table 3). This means that Eq. (5) makes it possible to convert the sensitivity to selectivity, i.e. the reliability of identification.

It is well accepted that MS–MS is a technique with excellent sensitivity and high specificity, which becomes increasingly important because the samples get more complex. Eq. (5) makes it possible to calculate the effect of increasing the selectivity of MS–MS compared to that of conventional MS. The second stage of resolution of MS–MS is independent of the first MS resolution. Therefore they are orthogonal, and the combined probability to distinguishing of spectra, $P^{\mathrm{MS-MS}}$, will be:

$$P^{\mathrm{MS-MS}} = P^{(\mathrm{I})} \times P^{(\mathrm{II})}. \tag{7}$$

A peculiarity exists in the determination of the number of *n* strips: the *m*/*z* values of the daughter ions are always smaller than that of the parent ion because they are obtained by the loss of some fragment(s) from the parent ion. If the smallest fragment which the molecule or ion loses at fragmentation is a methyl group ($m/z = 15$), the highest value of the mass range at the next step of MS^n will be [$(m/z)_{\mathrm{parent\ ion}}$—15]. For example, the molecular ion of sulfadimidine, *m*/*z* 279 (in fact $M+1$), arises at the first step of MS–MS, Fig. 2 and the value of the mass range will be 229 ($279 - 50 = 229$). The probabilities for distinguishing of spectra at three characteristic ions (*m*/*z* 74, 192 and 279) or seven ions (*m*/*z* 74, 150, 179, 192, 224, 260 and 279) at resolution 1.0 ($n = 229$) or resolution 0.1 ($n = 2290$) are represented in Table 4. At the second stage, several daughter ions arise by collision of the molecular ion accepted as a parent ion. The value of the highest limit of the mass range in which the daughter ions arise is 264 ($279 - 15 = 264$). The used Finnigan Deca instrument automatically determines at the

Table 3
Influence of the number of characteristic ions (k) and the resolution of the MS instrument on the probability for distinguishing the mass spectra, $P^k_{m,n}$

Resolution (R)	k					
	15	12	8	5	3	
					$m = 5$	$m = 10$
$R = 1.0$; $n = 635$[a]	2.81×10^{-31}	2.48×10^{-26}	3.19×10^{-19}	2.35×10^{-13}	4.70×10^{-9}	2.35×10^{-9}
$R = 1.0$; $n = 1850$[b]	2.72×10^{-38}	6.18×10^{-32}	5.97×10^{-23}	2.11×10^{-15}	1.90×10^{-10}	9.50×10^{-11}
$R = 0.1$; $n = 18500$[c]	$2\ 59 \times 10^{-53}$	5.99×10^{-47}	5.89×10^{-32}	1.11×10^{-20}	1.91×10^{-13}	9.55×10^{-14}

$k = 15$ (all 15 peaks are used); $k = 12$ (3 peaks are flagged); $k = 8$ (7 peaks are flagged), etc.
[a] A mass range 15–650.
[b] Mass range 150–2000.
[c] Sum of zoom scans at resolution of 0.1 in the range 150–2000.

applied collision dissociation the lowest limit of the trapping frequency of the ion with m/z 70. That is why the mass range in which the daughter ions will arise is 194 ($264 - 70 = 194$) (Fig. 2). In this case, with unit resolution, the number of n strips will be 194. The probability for overlapping $(P^k_{m,n})^{II}$ will be $(P^3_{5.194})^{II} = 1.7 \times 10^{-7}$. At zoom scan, with a 0.1 unit resolution, the value of the probability $(P^3_{5.1940})^{II}$ is 1.7×10^{-10}, i.e. the identification at zoom scan is 10^3 times more reliable then one scanning with unit resolution (Table 4). If the analyst accepts seven characteristic ions (the first most abundant ions m/z 124, 156, 174, 186, 204, 217 and 253), the probabilities at 1.0 and at 0.1 unit resolution

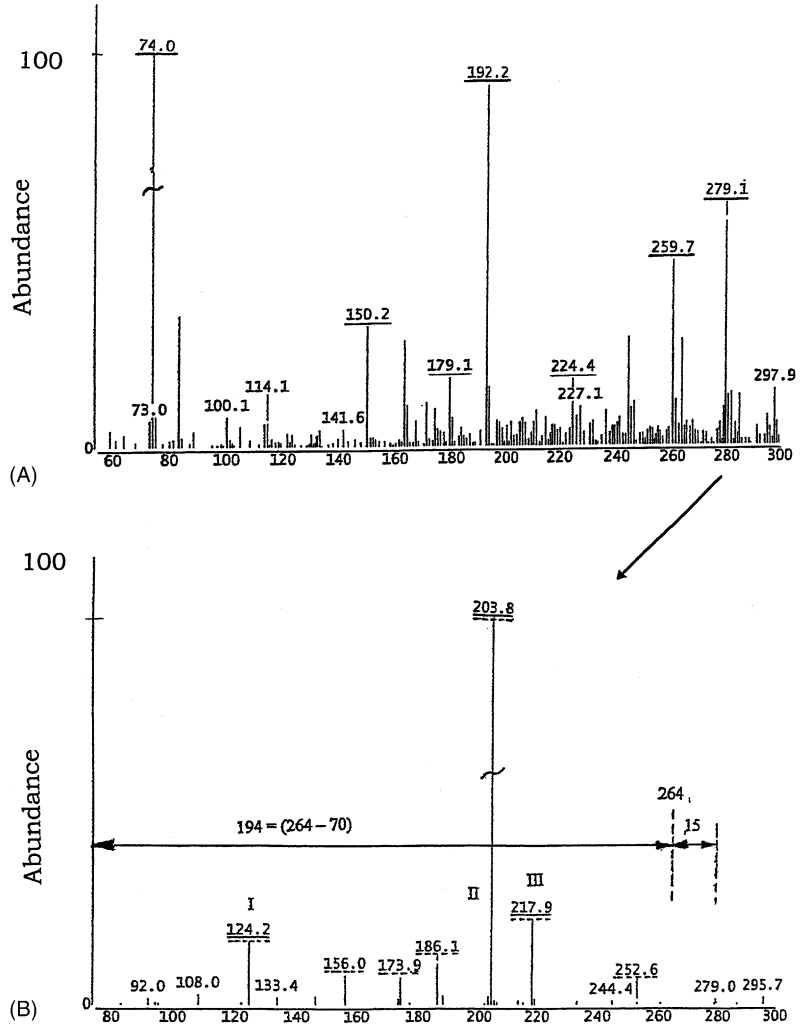

Fig. 2. MS spectrum of sulfadimidine (A) and MS–MS spectrum (B); the molecular m/z 279 is accepted as a parent ion; seven characteristic ions are pointed by dashed line. x-axes: m/z.

Table 4
The probability of overlapping of mass spectrum of Sulfadimidine at LC–MS–MS[a]

k	Resolution	MS^I	LC–MS	MS^{II}	$MS^I \times MS^{II}$	LC–MS–MS
3	1.0	1.0×10^{-7}	2.1×10^{-10}	1.7×10^{-7}	1.7×10^{-14}	3.6×10^{-17}
	0 1	1.0×10^{-10}	2.1×10^{-13}	1.7×10^{-10}	1.7×10^{-20}	3.6×10^{-23}
7	1.0	3.4×10^{-14}	7.1×10^{-17}	5.8×10^{-14}	2.0×10^{-26}	4.2×10^{-29}
	0.1	$1.6 \times 10-^{20}$	3.3×10^{-23}	5.8×10^{-2}	9.3×10^{-41}	1.9×10^{-43}

Contribution of LC, to distinguishing is $P_{r,s} = 2.1 \times 10^{-3}$.
[a] $k = 3$ or 7, $m = 5$ and resolution 1.0 or 0.1.

will be $P^7_{5.194} = 5.8 \times 10^{-14}$ and $P^7_{5.1940} = 5.8 \times 10^{-21}$, respectively.

The very low values of the probability with tandem mass spectrometry, $P^{MS–MS}$ compared to the published with single mass spectrometry demonstrates the increasing reliability of the identification—above 7 or 14 orders of magnitude. That is why tandem mass spectrometry MS^n, besides its increased sensitivity as a result of low signal/noise at ion trapping, is a powerful tool for the identification and structural characteristics of the analytes.

Supporting evidence provided by the GC or LC retention time, as well as appropriate sample preparation and clean up, has been recognized as fundamentally important [12] at GC–MS or LC–MS, especially for the analysis of isomers or enantiomers [13]. The probability for overlapping of chromatographic peaks $P_{r,s}$ is determined by the efficiency of the column [14] and the selectivity and the sensitivity of the detector [15]. The separation of analytes by chromatographic methods and the separation of their ions by mass spectrometry are independent events. That is why the combined probability for overlapping P, can be expressed as:

$$P = P_{r,s} \times P^k_{m,n} \tag{9}$$

The number of strips r, corresponding to the retention time, was determined by the peak capacity n_c:

$$n_c = 1 + \frac{\sqrt{N}}{4} \ln(1 + k_{max}) \tag{10}$$

At $N = 12\,600$ theoretical plates and $k_{max} = 30$, $n_c = 96$, i.e. the column will separate statistically 96 peaks. Accepting ±10% (i.e. $m = 5$), reproducibility of the detector signal, which in fact is the applied MS, the contribution of the chromatographic separation to distinguishing the analytes is $P_{r,s} = 2.1 \times 10^{-3}$ ($1/96 \times 5 = 2.1 \times 10^{-3}$). The combined probabilities $P_{r,s} \times P^{MS}$ or $P_{r,s} \times P^{MS–MS}$ are represented in Table 4. The contribution of the separation to the identification is not as important as that of mass spectrometry, especially MS–MS. However, the contribution of the chromatographic separation to distinguishing isomers and enantiomers is invaluable because their MS spectra are similar.

References

[1] Council Directive 96/23/EC, Directorate General For Agriculture, European Commission, Brussels, OJL 125, 23.5, 1996, p. 10.
[2] J.C. Shwartz, J.E. Syka, I. Jardine, J. Am. Soc. Mass Spectrom. 2 (1991) 198.
[3] Analytical News ThermoQues, 1100 Tap Deca comp.table, 15 May 2000, p. 2.
[4] LCQ Deca MS, Thermo Finnigan News, March 2001.
[5] R.E. March, J. Mass Spectrom. 32 (1997) 351.
[6] F.W. MacLafferty, F. Turecek, Interpretation of Mass Spectra, University Science Books, 1993, p. 3.
[7] F.W. McLafferty, R.H. Hertel, R.D. Villwock, Org. Mass Spectrom. 9 (1974) 7.
[8] G.M. Pesyna, F.W. McLafferty, R. Venkataraghavan, H.E. Dayriger, Anal. Chem. 47 (1975) 1161.
[9] G.M. Pesyna, R. Venkataraghavan, H.E. Dayringer, F.W. McLafferty, Anal. Chem. 48 (1976) 1362.
[10] F.W. McLafferty, D.A. Stauffer, S.Y. Loh, Ch. Wesdemiotis, J. Am. Soc. Mass Spectrom. 10 (1999) 1229.
[11] A.J. Sphon, J. Assoc. Off. Anal. Chem. 61 (1998) 1247.
[12] T. Cairns, E.G. Siegmund, J.J. Stamp, J. Mass Spectrom. Rev. 8 (1989) 93.
[13] S.R. Needham, T. Wehr, LC·GC 4 (2001) 244.
[14] G. Stoev, J. Chromatogr. A 845 (1999) 3.
[15] G. Stoev, A. Mihailova, J. Chromatogr. A 869 (2000) 275–284.