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Quantitative assessment of the contribution of high resolution mass spectrometric analysis to the reliability of compound confirmation

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ABSTRACT

Applications of high resolution mass spectrometry (HRMS) in food safety and residue analysis have increased remarkably over the last few years. The high resolution detection of ions reportedly enhances the assay selectivity but quantitative assessment of HRMS contribution to the assay selectivity has not yet been undertaken. We devised a method to assess the impact of instrument resolution on the probability that a spectral assignment to a given compound was made in error. The method allows for evaluating the quality of a spectral assignment based on resolution and the number of fragmentation stages. It thus provides a firm basis for comparing analytical methods performed on very different mass spectrometric instrumental platforms as well as in the context of the current regulatory framework.

1. Introduction

In recent years, the need to cover a large number of analytes together with budget constraints have lead to the adoption of multi-residue analytical methods. Instrumental platforms are expected to handle complex mixtures where matrix effects, or co-eluting nearly isobaric compounds, present considerable challenges. Technologies such as ultra-high performance liquid chromatography (UHPLC) and fast-switching triple quadrupole instrumentation have made a considerable impact in the field. Recent developments in the field of high resolution mass spectrometry (HRMS), especially the robustness, ease of use, and a relative affordability, mean that these systems are beginning to play a significant role that will no doubt increase in near future [1–3].

To date, the most established experimental setup for targeted qualitative and quantitative analysis in food safety application area is the use of a triple quadrupole system running in selected reaction monitoring (SRM) mode, where the first (selection) quadrupole is parked on a single parent mass, the second quadrupole (collision cell) dissociates that mass, and the third (selection) quadrupole is parked on a selected fragment ion. A minimum of two transitions is required to fulfill the regulatory requirements for a reliable confirmation under CD 2002/657/EC [4]. SRM is known to be highly selective. On the other hand, the number of analyzed compounds is clearly limited by the dwell time which determines the number of transitions achievable within a particular time segment of the method. Also, the mass tolerance window for the isolation of the precursor (and fragment) ion is usually set to 1 u which means that potential false positive identifications could (and do) occur due to the appreciable complexity of the samples being analyzed. But the major inherent limitation of this targeted approach is simply the fact that it is 'targeted'. Compound(s) not being considered when setting up a particular SRM method will not be detected. If information on additional compound(s) is required *a posteriori*, the sample, worse case, must be re-collected, re-prepped and re-analyzed, and, best case, just re-analyzed. Either way, this represents a significant complication and loss of productivity/revenue.

The inability to detect residues not known *a priori* is the main reason why mass spectrometric approaches based on a full scan analysis seem so attractive. They offer the possibility of simultaneous analysis of a really large number of compounds limited solely by the peak capacity of the system. Detection and

Abbreviations: HRMS, high resolution mass spectrometry; SRM, selected reaction monitoring; UHPLC, ultra high performance liquid chromatography; FWHM, full-width at half-maximum peak height; SAs, sulfonamides; NSAIDs, nonsteroidal anti-inflamatory drugs; LC/MS, liquid chromatography coupled to mass spectrometric detection; LRMS, low resolution mass spectrometric analysis; CD 2002/657/EC, Commission Decision 2002/657/European Community

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quantification of several hundred different pesticides in complex matrices by a single analytical method using high mass resolution and accuracy have been reported [5,6]. There are also publications describing multi-analyte methods for detecting residues of veterinary drugs and hormones in biological samples [7–11]. The acquisition of full scan mass spectrometric data allows for a post-acquisition data processing. The data files can be 'mined' for both qualitative and quantitative information about any compound or its metabolite without *a priori* knowledge.

The full scan approach to the detection and confirmation of contaminants is, however, only practically feasible with high resolution data that ensures sufficiently reliable accurate mass measurement of compounds. Very narrow mass tolerance settings can then be used to reconstruct specific mass chromatograms for the compounds of interest, effectively minimizing or even eliminating the background. The importance of mass resolution for a successful identification of residues or contaminants in complex samples was clearly demonstrated by Nielen and coworkers [12]. In their samples high amounts of matrix co-extracts caused significant deviations in exact mass measurements when insufficient resolution settings were used. That was also the case for steroid ester determination in hair samples, where using a relatively low resolution of 10 000 full width at half peak height (FWHM) provided by a time-of-flight analyzer was insufficient to resolve analyte ions and co-eluting nearly isobaric compounds. The resulting mass measurements for compounds of interest deviated often by more than 5 ppm from the expected values [13].

This phenomenon is illustrated in Fig. 1. A mixture of 150 pesticide, fungicide and veterinary drug standards was spiked into a horse feed matrix and analyzed at various resolving power settings. At resolution 15 000 (defined as the full-width at half-maximum peak height; FWHM) the pesticide Sulcotrione ($C_{14}H_{13}ClO_5S$; $[M+H]^+$ 329,02450) was not detected using a mass tolerance window of 5 ppm. Re-analysis at resolution 50 000 enabled its confident detection. In summary, increased resolution thus significantly improves the assay selectivity. Ignoring the uncertainty in mass accuracy introduced by co-eluting matrix components can lead to false-compliant (false negative) results.

The use of high resolution full scan analysis as an alternative to SRM-based assays in food safety and environmental analysis seems very appealing. The regulatory framework in its current form, however, does not reflect on the recent technology advances. For instance, the CD 2002/657/EC stipulating requirements for validated analytical methods considers sector instrumentation when mentioning HRMS; a stipulated resolution of 10 000 thus corresponds to the 10% valley definition. That translates to resolution 20 000 expressed as FWHM, a definition all time-of-flight and Fourier transform-based analyzers are using. Moreover, there are no criteria for mass accuracy described in CD 2002/657/EC, even though the mass accuracy and the precision of its determination are strongly dependent on sample matrix and interfering compounds. Additional criteria have been therefore proposed for the confirmation of known analytes or identification of unknowns, whereby higher point values were gained for an increased resolution and for mass deviation smaller than 5 mu [12]. These empirically derived criteria remain, however, open to questioning. For instance, what would happen if mass deviation were smaller or larger? Why the point values for analysis at resolution settings in excess of 10 000 and 20 000 are 1.5 and 2.5, respectively? How do these values reflect the contribution of resolution to the reliability of compound confirmation?

Several other groups attempted to perform a direct comparison of selectivity achieved by SRM and HRMS-based approaches [14–18]. These studies mostly concluded that HRMS scored similarly to SRM in terms of detection limits, recoveries and repeatability and matrix effects. Remarkably better performance was reported in the case of benzophenone contamination of foodstuffs [18] and anthocyanin analysis in tissue extracts [14] where better selectivity and more than 200-fold higher sensitivity compared to SRM methods were noted, respectively. Albeit very interesting and hard to dismiss as 'anecdotal' evidence, the need to provide systematic evaluation remained.

First such systematic comparison of the selectivity provided by SRM and HRMS was recently undertaken by Kaufmann et al. who determined the HRMS resolution required to produce a selectivity corresponding to SRM (so-called 'crossover point') [19]. The authors used artificial 'dummy' transitions and exact masses to monitor blank samples by respective LC–MS instrumentation platforms. By monitoring large enough number of transitions/masses they provoked a large number of endogenous matrix

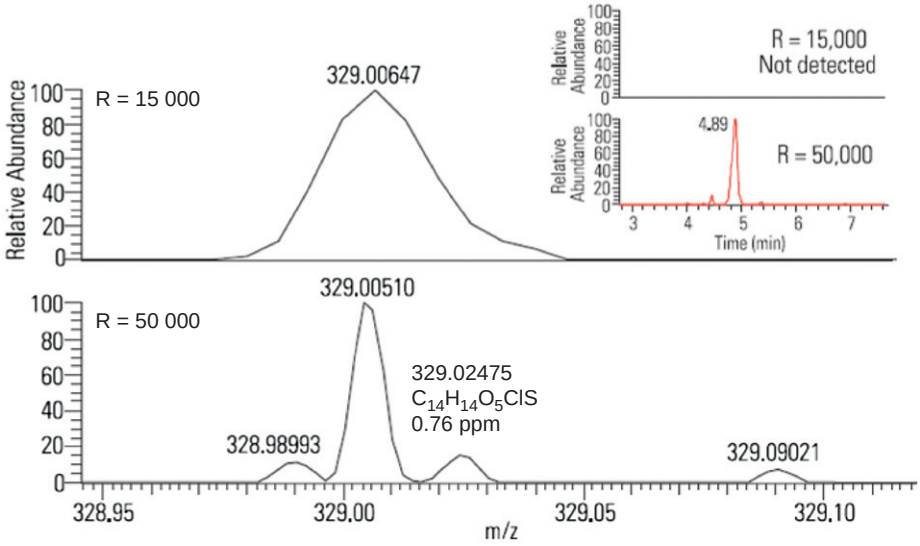

Fig. 1. High resolution prevents a false negative result. Pesticide Sulcotrione ($C_{14}H_{13}O_5ClS$; $[M+H]^+$ 329,02450) was measured in a mixture with another 149 pesticides and food toxins in a horse feed matrix. The mass deviation at a resolution of 15 000 is higher than 5 ppm set by the user due to the presence of an interference (top pane) giving a false negative result (insert, top trace). Sulcotrione can be detected with mass deviation of less than 1 ppm at 50 000 resolution (bottom pane) leading to a confident identification and quantitation (insert, bottom trace). Figure courtesy of Markus Kellmann, Thermo Fisher Scientific.

compounds to produce measurable chromatographic peaks. The instrument responses were standardized and authors concluded that resolution 50 000 marked the crossover point where HRMS provided same or higher selectivity than SRM approach. Even though well justified and highly competently realized, it could be objected that a more representative experiment might be needed to answer the general question of HRMS vs. SRM selectivity comparison.

We tried to address this issue by developing a statistical method to evaluate the quality of a spectral assignment based upon resolution and the number of fragmentation stages. This method quantitatively assesses the impact of instrument resolution on the probability that a spectral assignment to a given compound was made in error. We present here the rationale for such an approach and demonstrate its practical application and utility using our own experimental data as well as data sets published in the literature.

2. Material and methods

2.1. Analytes

Substances (99% purity) belonging to the class of sulfonamides (SAs): sulfathiazole, sulfadiazine, sulfaquinoxaline, and nonsteroidal anti-inflamatory drugs (NSAIDs): diclofenac, carprofen, tolfenamic acid, were purchased from Sigma-Aldrich, Seelze, Germany. The compounds were dissolved in methanol (1 mg/mL) and analyzed via infusion into a mass spectrometer. In the second batch of experiments, the same compounds were isolated from spiked matrix such as muscle, liver and milk, purified with SPE, and analyzed with liquid chromatography coupled to mass spectrometric detection (LC/MS).

2.2. Mass spectrometric analysis

The LCQ Deca ion trap mass spectrometer (Thermo Scientific) coupled to a Surveyor LC (Thermo Scientific) consisting of a binary pump, an autosampler, and a column heater, was used for the analysis of the first batch of samples. Compounds were separated on Synergi Polar column (150/2 mm, 4 μm d.p.) kept at 40 °C with a flow rate 300 μL/min using a gradient of acetonitrile (0.1% formic acid) and water (0.1% formic acid) as a mobile phase. The gradient started with 5% acetonitrile, linearly increased to 95% acetonitrile over 12 min, held for 4 min, and then brought back to 5% acetonitrile initial condition within 0.1 min followed by a 3-min re-equilibration prior to the next injection. Full scan mass spectra were acquired at resolving power 1000. Source parameters were 50 and 10 arbitrary units for the sheath and auxiliary gas (nitrogen) flow rates, respectively. The spray voltage and the capillary temperature were set at 3 kV and 300 °C, respectively, with a tube lens voltage kept at 20 V. For MS^2 experiments, the chromatographic run time was divided into several segments, so that a maximum of three parent masses of interest were monitored in each segment. Ten injections of each sample were made. Fragmentation spectra of SAs and NSAIDs were acquired at 20% and 35% normalized collision energy values, respectively. An isolation window of 1.5 u was used in the experiments.

For high resolution analysis, sulfathiazole, sulfadiazine, sulfaquinoxaline, diclofenac, carprofen, and tolfenamic acid standards were dissolved in methanol (0.1 mg/mL), centrifuged to remove any remaining undissolved particulate material, and infused at 3 μL/min into the LTQ Orbitrap XL (Thermo Scientific). The spray voltage was 4.3 kV, capillary voltage and temperature were set at 30 V and 275 °C, respectively, with a tube lens voltage kept at 75 V. Both full scan and fragmentation spectra were detected at 100 000 resolution settings (defined as FWHM at m/z 400). Fragmentation up to level MS^6 was successfully attempted for most compounds.

3. Results and discussion

The identification point system implemented within the framework of the CD 2002/657/EC is empirically derived. That means the point values being assigned to different methods were not determined quantitatively.

Using this point system, we can see that SRM methods fulfill criteria for a compound confirmation with a combination of a precursor ion and two product ions. On the other hand, an analyte detected solely in a full scan, albeit with high resolution, earns just 2.0 points for its molecular ion. Applying the criteria given by the CD 2002/657/EC regulatory framework, such an analyte confirmation would not be considered reliable. This might appear as a paradox which could easily be resolved by having some measure of quantitative assessment for the contribution of mass resolution to the compound confirmation.

3.1. Probability of erroneous spectral assignment

To resolve this issue, we have adapted an approach originally proposed for determining the reliability of compound identification in complex mixtures analyzed LC/MS [20,21]. The method considers mass spectra as maps in which characteristic ions (parent or fragments) and their respective relative intensities are used for identification. A formula quantitatively assessing the contribution of individual parameters to the reliability of a compound confirmation is as follows:

$$P^k_{m,n} = \frac{(n-k)!k!}{m \times n!} \quad (1)$$

where $P^k_{m,n}$ is the probability that two spectra would overlap ('probability of erroneous assignment'). Further, n represents a maximum number of ions which can be fully separated within an applied mass range, hereafter referred to as a peak capacity of a mass spectrum. Value k refers to the number of characteristic ions having a relative abundance m. This formula enables reliable comparison of selectivity of spectral assignment across different LC/MS platforms.

The systematic error (accuracy) and the statistical or random error (precision) of mass measurements should be taken into consideration in order to establish the smallest possible mass tolerance window that can be used to distinguish individual m/z values of the analytes. The precision and accuracy of a mass measurement can be assessed by making multiple independent measurements at the mass of interest and performing statistical analysis of the data. In order to test if two characteristics (in our case two neighboring ions at particular m/z's) are different (i.e., they can be distinguished), one needs to take into account individual standard deviations σ of the measurements. Neighbour ions A and B will be separated with 95% confidence when the difference between their masses is equal to $(2\sigma_A+2\sigma_B)$. For neighbour ions with similar intensities the values σ_A and σ_B are approximately equal, therefore a mass interval $4\sigma_A$ is going to be used in our calculations.

In our study, when using our own data obtained in routine quantitative LC/MS laboratory practice, we considered ten or more measurements when establishing σ_A values. In addition, to illustrate the general applicability of the quantitative assessment approach described herein, we used the mass measurement precision and accuracy values published in the literature [13].

3.2. Determining characteristics of mass spectra

3.2.1. Peak capacity

The maximum number of ions n that can be fully separated within the applied mass range corresponds to the peak capacity of the spectrum. Its value is obtained by dividing the effective mass range used for the analysis by the confidence interval 4σ. The mass range of full scan analysis is tailored to the compound being analyzed; as molecular weight of SAs used in our experiment did not exceed 450 Da, the analysis was performed over the mass range m/z 50–450. Using an ion trap instrument, we shall consider the confidence interval of ion trap measurements to be approximately 1 u. Consequently, the peak capacity of that measurement, n, equals to 4.00 10^2 [$=(450-50)/1$].

When analysed on an LTQ Orbitrap instrument at resolution 100 000 (defined as FWHM at m/z 400) over a mass range 50–450 Da, the $[M+H]^+=256.02089$ u of sulfathiazole ($C_9H_9N_3O_2S_2$) was determined with $\sigma=0.000025$ u) and the m/z interval at 95% confidence was 0.0001 u. At this value, the peak capacity, n, was equals to 4.00 10^6 [$=(450-50)/0.0001$].

Similarly, considering the results obtained by Van der Heeft and co-workers [13] for testosterone acetate ($C_{21}H_{30}O_3$; $[M+H]^+$ 331.22677) the 95% confidence based on fifty consecutive measurements using time-of-flight instrument was 3.2 ppm, which corresponds to m/z confidence interval 0.00106 u [0.00106/331.22677=3.2 ppm]. The 95% confidence level obtained for the same compound using the Orbitrap analyzer was 0.88 ppm which corresponds to confidence mass interval 0.00029 u [0.00029/331.22677=0.88 ppm]. Performing a full scan analysis (at 10 000 resolution) over a mass range 100–400 Da, the time-of-flight instrument would fully separate $n=2.83$ 10^5 [$=(400-100)/0.00106$] ions. Analogously, full scan analysis with the Orbitrap analyzer operated at 60 000 resolution over the same mass range would completely separate $n=1.03$ 10^6 [$=(400-100)/0.00029$] ions. Much higher selectivity of the analysis carried out at higher resolving power is reflected in the experimental finding of the authors who confirmed testosterone acetate in a complex sample using the Orbitrap analyzer but failed to do so using a lower resolution time-of-flight instrument [13]. This example demonstrates that the increased peak capacity of true HRMS is a clear advantage.

3.2.2. Relative abundance of characteristic ions

The abundance of an ion and its reproducibility are the main factors which determine the m value in Eq. (1). The overlap of the characteristic ions with ions of other compounds or impurities decreases the reproducibility of the ion abundance measurement, especially for trace residues.

Modern mass spectrometers have an analytical dynamic range of four to five orders of magnitude. This means that an ion with a signal abundance 0.1% of a base peak can be measured reproducibly [22]. In our routine work at concentrations around 1 mg/kg we usually observe actual value of standard deviation for relative ion abundances being below 5%. The roughly $\pm 10\%$ tolerance regarding the normalized signal abundance corresponds to $\pm 2\sigma$ confidence interval and the m value then becomes 5 [$=100\%/(2\times 10\%)$].

3.2.3. Number of characteristic ions

Three decades ago, Schon argued that three structurally related ions would be required to provide a proof of the presence of an analyte in an analyzed sample [23]. This assumption was based on a statistical approach using an extensive MS data base as a model of a universal (but not exhaustive) repository of organic compounds. According to CD 2002/657/EC, the minimum required identification points for confirmation of substances of Group A (prohibited) and Group B (controlled), are 4 or 3, respectively. This can be achieved with three characteristic ions (molecular ion and two transitions), thus k equals 3.

3.3. Probability for distinguishing mass spectra

As there is no measure for a quantitative evaluation of the reliability of identification/confirmation, we used the requirements of CD 2002/657/EC for reliable confirmation as a base for our comparisons. In this way, we were able to compare the contribution of different types of mass spectrometric analyses and their characteristics to selectivity of analysis.

3.3.1. Full scan mass spectrometric analysis

Full scan mode of analysis provides only one characteristic of an analyte—its m/z which can be used to derive a compound's molecular weight. Therefore, the k value equals 1. As it is impossible to use the (normalized) abundance of the molecular ion as another analyte characteristic, the m value becomes 1 as well. When $m=1$ and $k=1$, Eq. (1) transforms to:

$$P_n = 1/n \tag{2}$$

Applying this simplified formula to an example of sulfonamide analysis as described above (using a mass range 50–450 Da and a unit mass accuracy instrumentation giving us the maximum number of fully separated ions of $n=400$) the probability of erroneous spectral assignment, P_n, would be ${}^{LR}P_n=1/(4.00\ 10^2)=2.50\times 10^{-3}$.

Analogously, employing the example of testosterone acetate given above, the probability of erroneous spectral assignment for an analysis carried out with a time-of-flight system operating at 10 000 resolution ($n=2.73\times 10^5$) would be ${}^{HR}P_n=1/(2.83\times 10^5)=3.53\times 10^{-6}$. The probability of erroneous spectral assignment using the Orbitrap analyzer operated at 60 000 resolution ($n=1.00\times 10^6$) would be ${}^{HR}P_n=1/(1.00\times 10^6)=1.00\times 10^{-6}$. These calculations demonstrate that the compound confirmation by full scan MS depends largely on the resolving power used.

3.3.2. Selected reaction monitoring at unit mass accuracy

It is well accepted that fragmentation spectra offer high selectivity. Eq. (1) gives an opportunity to quantify the effect employing MS^2 would have on the selectivity compared to that of conventional full scan MS analysis. The resolution of the second (and of any subsequent) fragmentation step is independent of the resolution used for full scan analysis. The combined probability of erroneous spectral assignment P^{MSx2} would be a product of respective probabilities for individual MS stages used in the experiment. For illustration, assuming a full scan and MS^2 analysis, the probability of erroneous spectral assignment becomes:

$$P^{MSx2} = P^{I} \times P^{II} \tag{3}$$

where P^I and P^{II} denote the probability of erroneous spectral assignment for full scan and MS^2 analysis, respectively.

A peculiarity exists regarding the determination of the spectral peak capacity value n for MS^2 spectra. Values m/z of daughter ions are always smaller than the mass of a parent ion because they are obtained by a loss of fragment(s) from the parent ion (we are considering singly charged precursors only). Since the smallest fragment which the molecular ion loses is a methyl group ($\Delta m/z$ 15 u), the highest value of the mass range in any subsequent level of MS analysis would be [$(m/z)_{\text{parent ion}}$—15].

This is illustrated in the following example: ion trap analysis of sulfadimidine ([$M+H^+$]=279; Fig. 2). The full scan detection was carried out over m/z interval 250 (defined by higher and lower

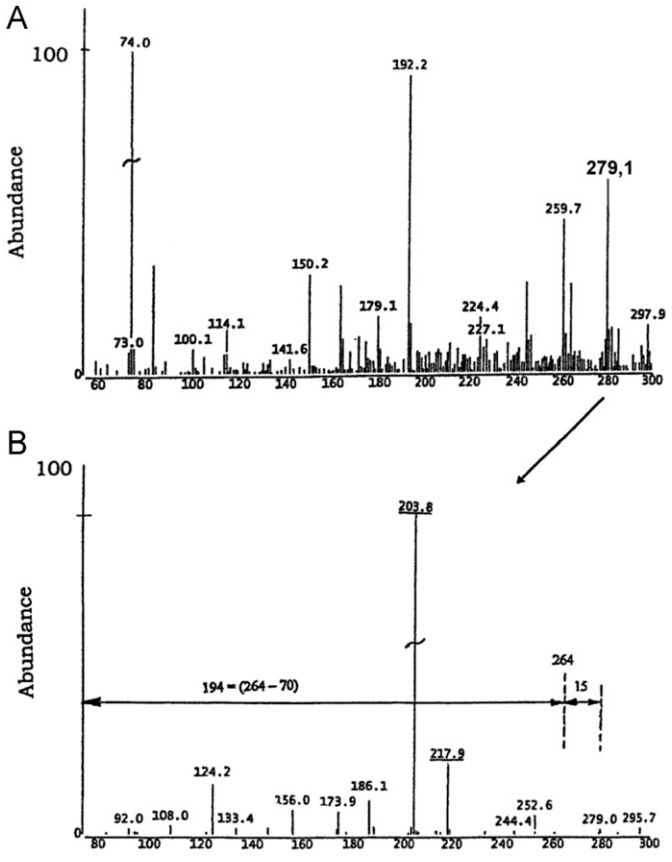

Fig. 2. Confirmation of sulfadimidine in meat extract using unit mass accuracy instrumentation. Full scan MS of an extract from meat containing sulfadimidine (*m/z* 279.1) (A), and its MS/MS spectrum with two characteristic fragment ions (highlighted), (B). Data acquired on the LCQ Deca ion trap.

scan limits being 300 and 50, respectively), while fragment ions were detected over *m/z* interval 214 [=(279−15)–50]. The probability of erroneous spectral assignment relying on three characteristic ions (*m/z* 279, 203 and 217) as prescribed by CD 2002/657/EC assuming unit mass accuracy is:

$P^I = 1/n = 1/250 = 4.00 \times 10^{-3}$; where in $n=250$.

$$P^{II} = \frac{(n-k)!k!}{m \times n!} = \frac{(214-2)!2!}{5x214!} = 8.78 \times 10^{-6};$$

where in $n=214$, $m=5$ (at concentration level 1 mg/kg, see Section 3.3.2), and $k=2$

$$P^{MSx2} = (4.00 \cdot 10^{-3}) \times (8.78 \cdot 10^{-6}) = 3.51 \times 10^{-8}$$

The probability of erroneous spectral assignment of sulfathiazole ($[M+H]^+=256$) with low resolution mass spectrometric analysis (LRMS) was:

$$[P^{MSx2}]_{LR} = [P^I \times P^{II}]_{LR} = \left(4.00 \cdot 10^{-3}\right) \times \left(1.10 \cdot 10^{-5}\right) = 4.40 \times 10^{-8}$$

where in $[P^I]_{LR} = 1/n = 1/(2.50 \cdot 10^2) = 4.00 \cdot 10^{-3}$;
$n = (300-50)/1 = 2.50 \cdot 10^2$

and

$$[P^{II}]_{LR} = \frac{(191-2)!2!}{5x191!} = 1.10 \cdot 10^{-5};$$ where in
$n = [(256-15)-50]/1 = 191; \quad m = 5; \quad k = 2$

The value for the combined probability of erroneous spectral assignment P^{MSx2} relying on three characteristic ions obtained by SRM operated at unit mass accuracy is approximately two orders of magnitude lower than the probability values obtained for the Orbitrap high resolution full scan MS analysis. This means that full scan analysis operated at resolution up to 60 000 is not complying with the requirements for reliable confirmation as set out by CD 2002/657/EC.

3.3.3. MS^2 and higher levels of fragmentation using HRMS

The following example illustrates in detail the calculation of probability of erroneous spectral assignment based on data obtained in full scan, MS^2 and higher levels of fragmentation (MS^n), all acquired at resolution 100 000 using the LTQ Orbitrap analyzer. Sulfathiazole ($C_9H_9N_3O_2S_2$; $[M+H]^+$ 256.02089) was measured in a full scan with a 95% confidence level of 0.5 ppm (which corresponds to confidence interval 0.0001 u). When acquiring a full scan over the mass range *m/z* 50–300 Da, the probability of erroneous spectral assignment for the first stage of MS analysis was calculated as follows:

$P^I = 1/n = 1/(2.50 \cdot 10^6) = 4.00 \cdot 10^{-7}$;
$n = (300-50)/0.0001 = 2.50 \cdot 10^6$

Five measurements of sulfathiazole fragment at *m/z* 156 yielded the mean value 156.01113, with standard deviation (σ) 0.000022 u, defining the confidence interval of 0.000088 u, or approximately 0.0001 u. The probability of erroneous spectral assignment for the second stage (MS^2) analysis was:

$$P^{II} = 1/n = 1/\left(1.91 \cdot 10^6\right) = 5.2 \cdot 10^{-7};$$

where in $n = [(256-15)-50]/0.0001 = 1.91 \cdot 10^6$

The probability of erroneous spectral assignment for both levels of analysis thus became:

$$P^{MSx2} = P^I \times P^{II} = (4.00 \cdot 10^{-7}) \times \left(5.2 \cdot 10^{-7}\right) = 2.08 \cdot 10^{-13}$$

When considering two fragment ions in MS^2 spectra, $k=2$ and the probability of erroneous spectral assignment in MS^2 spectra would be:

$$P^{II} = \frac{(n-k)!k!}{m \times n!} = \frac{(1{,}910{,}000-2)!2!}{5x1{,}910{,}000!} = 1.09 \cdot 10^{-13};$$ where in n
$= [(256-15)-50]/0.0001 = 1.91 \cdot 10^6; \quad m = 5; \quad k = 2$

That gives us the combined probability of erroneous spectral assignment:

$$P^{MSx2} = P^I \times P^{II} = (4.00 \cdot 10^{-7}) \times (1.09 \cdot 10^{-13}) = 4.36 \cdot 10^{-20}$$

When three fragment ions were used for identification, $k=3$ and the probability of erroneous spectral assignment in MS^2 spectra would be:

$$P^{II} = \frac{(1910{,}000-3)!3!}{5x1{,}910{,}000!} = 2.09 \cdot 10^{-19}$$

which gives the combined probability of erroneous spectral assignment:

$$P^{MSx2} = P^I \times P^{II} = (4.00 \cdot 10^{-7}) \times (2.09 \cdot 10^{-19}) = 8.36 \cdot 10^{-26}$$

The next example discusses how the probability of erroneous spectral assignment reflects the contribution of multiple levels of fragmentation with detection of both full scan spectrum and all fragmentation spectra at high resolution. Tolfenamic acid was subjected to multiple levels of fragmentation in an ion trap and both parent and fragment ions were then analyzed using a resolving power 100 000 in the Orbitrap detector (Fig. 3). The following fragment ions were monitored: *m/z* 262.06330 in full scan MS, *m/z* 244 in MS^2, *m/z* 209 in MS^3, and *m/z* 180 in MS^4. The combined probability considering four consecutive fragmentation steps of mass spectral analysis P^{MSx4} is given by:

$$P^{MSx4} = P^I \times P^{II} \times P^{III} \times P^{IV}$$

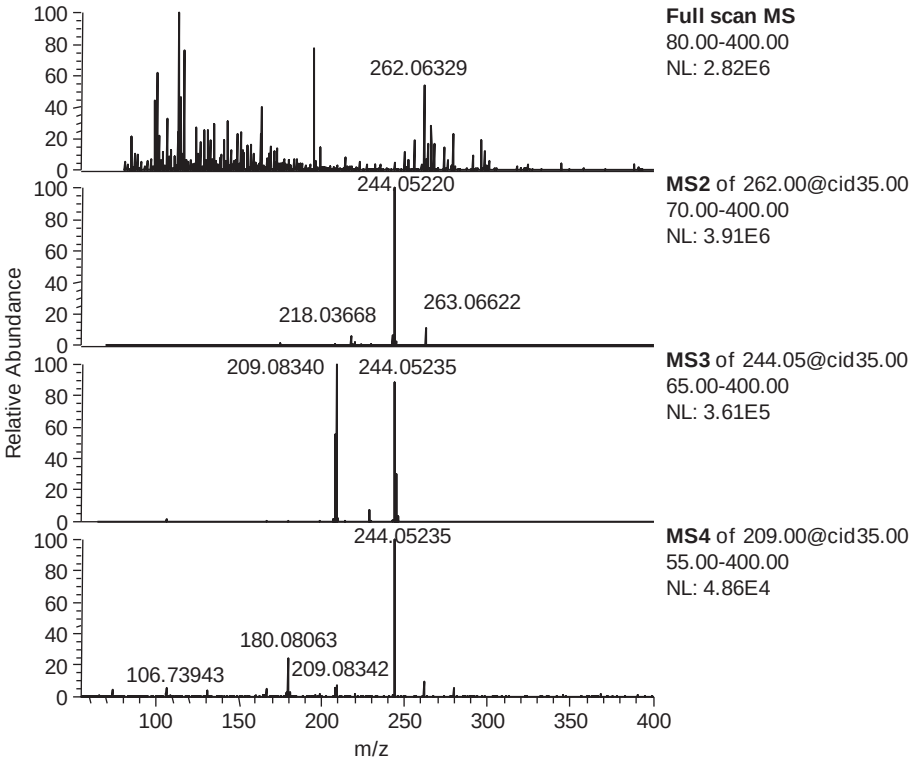

Fig. 3. Multiple levels of fragmentation. Data for tolfenamic acid acquired in infusion experiment with the LTQ Orbitrap at a resolution setting of 100 000 FWHM.

The contributions of individual fragmentation steps were:

$$P^{I} = 1/n_{I} = 1/\left(2.50 \times 10^{6}\right) = 4.00 \times 10^{-7};$$

$$n = (300-50)/0.0001 = 2.50 \times 10^{6}$$

$$P^{II} = 1/n_{II} = 1/\left(1.98 \times 10^{6}\right) = 5.05 \times 10^{-7};$$

$$n = [(263-15)-50]/0.0001 = 1.98 \times 10^{6}$$

$$P^{III} = 1/n_{III} = 1/\left(1.79 \times 10^{6}\right) = 5.59 \times 10^{-7};$$

$$n = [(244-15)-50]/0.0001 = 1.79 \times 10^{6}$$

$$P^{IV} = 1/n_{IV} = 1/\left(1.44 \times 10^{6}\right) = 6.94 \times 10^{-7};$$

$$n = [(204-15)-50]/0.0001 = 1.44 \times 10^{6}$$

Thus P^{MSx4} became:

$$P^{MSx4} = 4.00 \cdot 10^{-7} \times 5.05 \cdot 10^{-7} \times 5.59 \cdot 10^{-7} \times 6.94 \cdot 10^{-7} = 7.84 \cdot 10^{-26}$$

The above example of tolfenamic acid analysis demonstrates the exceptional selectivity achievable when employing high resolution and multiple levels of fragmentation. Aproximately the same selectivity would be obtained in MS^2 using three characteristic ions ($P^{MSx2} = 8.36 \times 10^{-26}$). The choice of an appropriate approach would depend on the fragmentation behaviour of the analyte. If its fragmentation tends to produce just a single ion in MS^2, then another level (e.g., MS^3 or MS^n) or another type (e.g., higher collision energy dissociation) of fragmentation should be used.

Approximately the same selectivity would be obtained in MS^2 using three characteristic ions ($P^{MSx2} = 8.36 \times 10^{-26}$). But the above example of tolfenamic acid analysis by multiple fragmentations demonstrates the exceptional selectivity because the consecutive cleavage of neutral losses from the molecular ion elucidates the structure of the analyte. This approach, combined with nitrogen and ring plus double bonds rules proved very useful at identification and structural determination of the related compounds of Tilmicosin [24].

Clearly, the probability of erroneous spectral assignment $P^{I} = 4.00 \times 10^{-7}$ obtained with only one (molecular) ion in full scan analysis at 100 000 resolution is higher than the one calculated for low resolution SRM approach ($P^{MSx2} = 4.40 \times 10^{-8}$, see Section 3.3.2). This means that also in this example the compound confirmation relying solely on a full scan analysis, albeit carried out at resolution 100 000, is not sufficient to fulfill the regulatory requirements. Recently published work has stated that UHPLC coupled to the Orbitrap analyzer operated at sufficient resolution was especially powerful, resulting in selectivity comparable or better than traditional SRM-based approaches [25]. Our previous study ascertained that the contribution of the column efficiency to the reliability of confirmation was of secondary importance, and that MS resolution was the main factor determining selectivity of LC/MS analysis [26]. The conclusion of these authors is thus contrary to the outcome of our theoretical analysis which states that even at resolving power 100 000 the selectivity of a full scan analysis is lower than the one of a unit mass accuracy SRM-based assay.

Nevertheless, with just a single fragment ion in MS^2 the probability of erroneous spectral assignment $P^{I} \times P^{II}$ becomes $(4.00 \times 10^{-7}) \times (5.05 \times 10^{-7}) = 2.2 \times 10^{-13}$. This is five orders of magnitude lower than for low resolution SRM ($P^{MSx2} = 4.40 \times 10^{-8}$). This suggests that a reliable identification in accordance with the requirements of CD 2002/657/EC could be achieved with just a precursor and one fragment ion measurement at sufficiently high resolution. As it is usually possible to obtain at least one daughter ion performing the fragmentation even on a small quantity of analyte, this would hint on an increased sensitivity of such an approach.

An area where this could be highly relevant is the analysis of compounds that produce one major fragment ion accompanied by others at low abundances (e.g., benzimidazoles and NSAIDs).

For illustration, benzimidazoles such as flubendazole, fenbendazole, or oxfendazole produce fragments whose intensity difference within the MS^2 spectrum exceeds 3 orders of magnitude. Normally, large quantities of these substances are needed to obtain two daughter ions in order to acquire the necessary 3 or 4 identification points fulfilling CD 2002/657/EC requirements. Alternatively, a consecutive fragmentation of the most abundant ion from the MS^2 spectrum could be performed if an instrument capable of multiple levels of fragmentation is available, but that also requires larger quantities of analyte than just a straightforward MS^2 analysis.

Our calculations demonstrate the following:

a. Mass resolving power and multiple levels of fragmentation are key factors impacting the selectivity of analysis.
b. Fragmentation step has a greater impact on the analytical selectivity than resolving power.
c. Combining high resolution detection of parent ion with that of one of its fragment ions brings about a significant decrease in probability of erroneous spectral assignment. The resulting selectivity is high enough to make such an analysis fully compliant with the regulatory requirements of CD 2002/657/EC.
d. The corollary of the preceding point is that compounds predominantly yielding a single fragment ion, when analyzed at high enough resolution, could thus fulfill the criteria for confident confirmation set out in CD 2002/657/EC.
e. HRMS combined with multiple levels of fragmentation realizes truly phenomenal selectivity exceeding, by far, the selectivity stipulated by CD 2002/657/EC. This fact was intuitively acknowledged by Nielen [12].

3.3.4. *Relying solely on high resolution full scan analysis*

The facts summarized in the section above give rise to an interesting question: What resolution settings should be used if relying solely on full scan analysis in order to fulfill the selectivity requirements of the CD 2002/657/EC?

It has already been determined (Section 3.3.2 above) that a reliable identification of sulfathiazole in accordance with the CD 2002/657/EC relying on a precursor and two fragment ions in a standard low resolution SRM approach is characterized by a probability of erroneous spectral assignment equal to 4.40×10^{-8}. Let us suppose that an analyte, e.g., tolfenamic acid, would be analyzed in a full scan over the same *m/z* range as the one used in SRM analysis (a range 250 u determined by the lower and higher limits of scanned mass range being 50 Da and 300 Da, respectively). To fulfill the regulatory requirements, the instrument resolution must be able to discriminate 2.27 10^7 ($=1/4.40 \times 10^{-8}$) compounds which should then all be fully separated. If the number of compounds which must be separated were divided with the scan range, we arrive to the number of compounds to be separated over an interval of 1 u, here $(2.27 \times 10^7)/250 = 9.08 \times 10^5$. This number then corresponds to the resolution of a mass spectrometer which would ensure, in full scan analysis, the same selectivity as the unit mass accuracy SRM approach. In other words, the full scan high resolution MS could be used to confirm an analyte such as tolfenamic acid if performed at resolving power approximately 1 million. While such a resolution is certainly achievable [27], it is hardly practical in the present routine laboratory analysis settings.

3.4. *The effect of analyte's molecular weight*

Until now we have not taken into consideration the fact that when the molecular weight of an analyte increases, the number of potential elemental compositions fitting the given mass tolerance (defined by the resolution employed in the analysis) increases exponentially. For example, the number of molecular formulas for the 11 most common elements at 1000 Da was reported to be more than 350 millions [28]. Of course, many of these compounds are hypothetical, nevertheless, the number of potential compounds in biological samples is very large.

The likelihood that an instrument operating at a particular resolution setting (defining a particular mass tolerance) could separate all elemental compositions possible corresponding to a mass of 300 Da would be much larger than for compounds with mass 600 Da or 1000 Da. Fig. 4 shows the number of elemental compositions which will not be separated relative to the mass accuracy and the dramatic effect as you go up in molecular weight (based on only considering the elements of carbon, hydrogen, nitrogen and oxygen) [29].

At *m/z* 300 an instrument operating at resolution defining mass tolerance equal to or better than 5 ppm will not be able to separate compounds with approximately five different elemental composition formulas from each other. Under the same experimental conditions a compound at *m/z* 1000 could have one of 140 possible elemental compositions.

The slope of the relationship between mass accuracy and number of unresolved elemental composition suggestions is relatively shallow for a compound at *m/z* 300, and the number of undistinguishable elemental composition suggestions is practically independent from mass accuracy (and hence mass resolution) within the assessed range of 0–5 ppm. Resolution, however, plays an important role for the analytes at higher masses. For instance, assuming *m/z* 1000 the number of unresolved elemental composition suggestions at mass tolerance 5 ppm is 140 while it decreases to 55 at mass tolerance 2 ppm. Therefore, the criteria of CD 2002/657/EC for compound confirmation must take into account the mass of an analyte.

Another observation made from Fig. 4 is that an accuracy needed for unambiguous assignment of elemental composition is very high, corresponding to a mass deviation smaller than 0.1 ppm. This is in agreement with the statements of Fiehn, who postulates the need for employing additional criteria, such as isotope abundances or nitrogen rule, when identifying unknown compounds based on their accurate molecular weight measurement [30,31].

Using isotope abundances for increased confidence of identification in this type of analysis is certainly a working proposition as employing high effective resolution enables observation of fine isotopic structures, providing valuable information regarding elemental composition possibilities. Fig. 5 provides a detailed

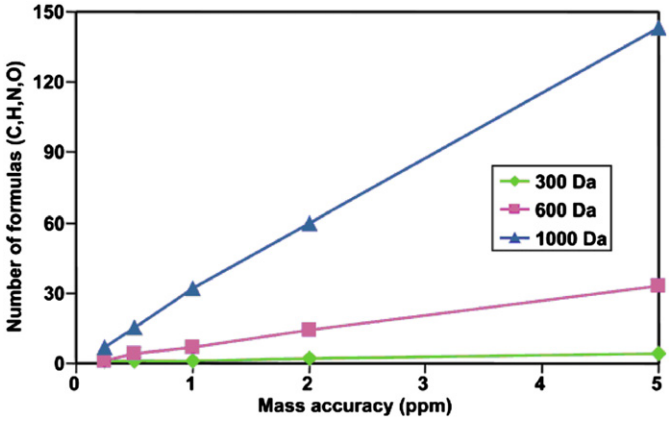

Fig. 4. Number of possible elemental compositions for a given mass tolerance increases considerably with the mass of an analyte. Only C, H, N, O elements considered in this example. Figure courtesy of Eric Milgram, Mike Greig and Ben Bolaños.

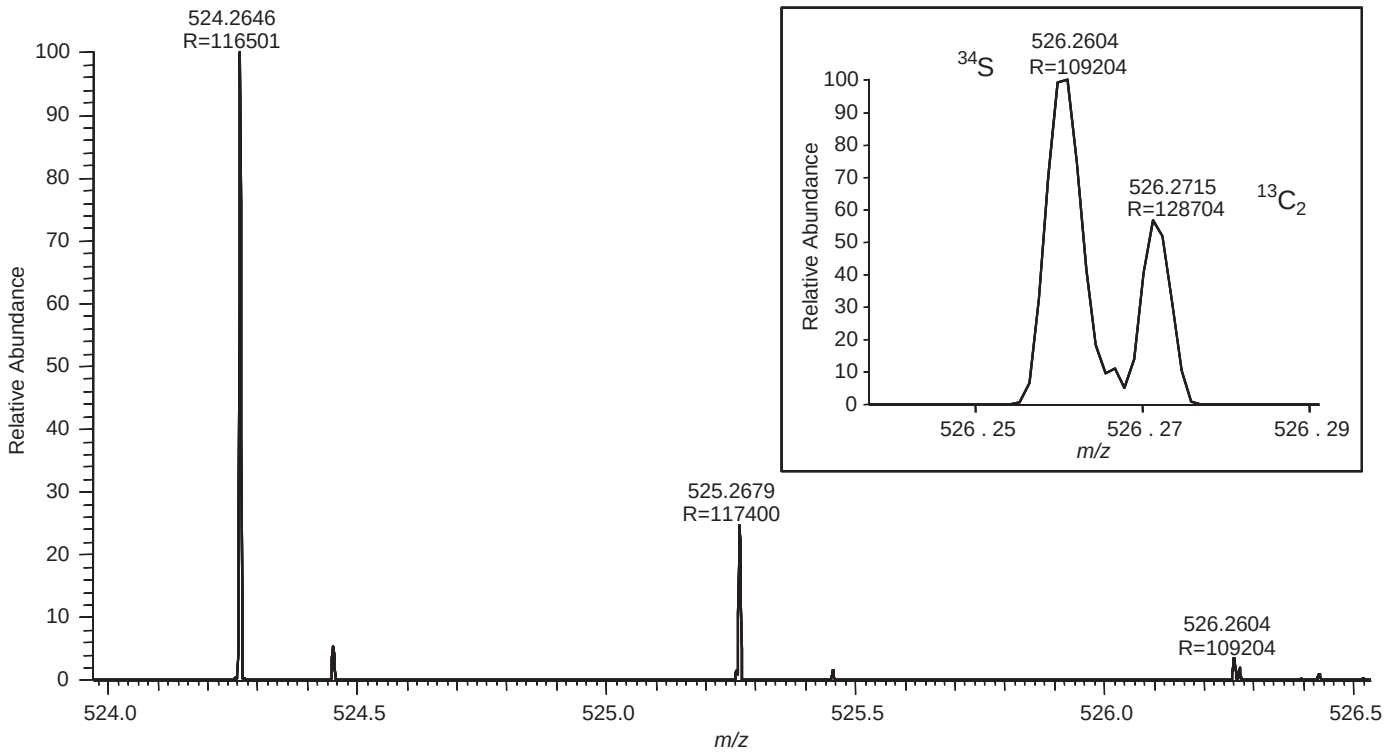

Fig. 5. Resolving fine isotopic structures. Peptide (sequence MRFA) measured at 100 000 resolution settings (defined as FWHM at *m/z* 400) contains one sulfur atom within its amino acid methionine. A closer look at the (A+2) isotopologue (insert) reveals presence of two peaks corresponding to the presence of two different species, $C_{23}H_{38}N_7O_5^{34}S$ with $[M+H]^+$ 526.2607 and $^{13}C_2C_{21}H_{38}N_7O_5S$ with $[M+H]^+$ 526.2716. Mass difference between the two species is $\Delta m = 0.0109$ u. The minimum resolution required to discern these two species in the same spectrum is 48 000.

look into the region of the second isotope (A+2) of peptide with MRFA sequence. The presence of sulfur in amino acid methionine is reflected by detecting two different isotopologues containing either ^{34}S or $^{13}C_2$. The mass difference between these two species is 0.0109 u, and effective resolution required for discerning them is 48 000. Though isotope abundances can ultimately play a critical role in the confidence of identification, it was not taken into account for this particular study. The above rationale corroborates the fact that the selectivity of a full scan analysis alone is not sufficient, and additional information, such as fragmentation spectrum and/or elemental composition restrictions based on fine isotopic structure observation, must be considered.

4. Conclusions

We present a statistical method for evaluating the quality of a spectral assignment based upon resolution and the number of fragmentation stages The method allows for comparison of various modes of mass spectrometric analysis such as high resolution, SRM, full scan MS/MS or MS^n acquisition, against the criteria set out in CD 2002/657/EC. The outcomes of our study can be summarized as follows:

1. The described method enables to quantify the contribution of increased resolution to the selectivity of an assay, both for parent ion and fragments.
2. In order to fulfill the requirements of CD 2002/657/EC while relying solely on the detection of a parent ion to provide the same degree of selectivity as that obtained by unit mass accuracy SRM methods, resolution in excess of 1 million would be required. While such high resolution is certainly achievable with some types of mass spectrometric instrumentation, it is hardly practical in routine laboratory settings at present.
3. Adopting high resolution detection for both parent and fragment ions impacts very positively on the confidence of confirmation. This is of particular significance for compounds producing just a single product ion upon their fragmentation, as even such compounds would then fulfill the criteria for reliable confirmation under CD 2002/657/EC.
4. This method could be extended to evaluate any confirmation/identification criteria within the regulatory framework. For example, it is important to take into account that when the molecular mass of an analyte increases, the number of possible elemental composition suggestions grows exponentially and, as a result, an appropriate much higher mass resolution is required.

Knowing the minimum required resolving power is useful since it is directly linked to the selectivity (reliability of confirmation) and thus to the validation of analytical methods. In the course of the routine qualitative or quantitative work each laboratory has available the values of standard deviations for the compounds analyzed. It is worth pointing out that applying the calculations outlined in this paper to assess the reliability of confirmation does not require any additional sample measurements or data processing by the analyst, just a straightforward use of the given formula. We hope that the proposed method designed to assess the impact of instrument resolution on the probability of erroneous spectra assignment provokes further discussions surrounding the use of high resolution mass spectrometry ultimately leading to an update of the existing regulatory framework.

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