# Improving LC–MS/MS Analyses in Complex Food Matrices, Part II — Mass Spectrometry

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**The mass spectrometric response in complex biological samples is often directly related to the influence of co-eluting compounds on the ionization efficiency of analytes. This can lead to decreased sensitivity, selectivity and accuracy of liquid chromatography–mass spectrometry (LC–MS) analyses, especially when matrix composition varies from sample to sample. By applying certain sample clean-up and/or chromatographic separation strategies LC–MS method performance can be improved. In contrast to these approaches, this article will discuss other simple and effective strategies to improve LC–MS method performance. The usefulness of these approaches will be exemplified with our recent experiments in the trace analysis of mycotoxins and antibacterial agents in food matrices and biological fluids.**

## Introduction

Modern liquid chromatography–mass spectrometry (LC–MS) instrumentation equipped with atmospheric pressure interfaces offers robustness, ease of handling and high sample throughputs.[1] In contrast to gas chromatography–mass spectrometry (GC–MS), LC–MS is not limited to the analysis of a small number of analytes with sufficient volatility and can be applied to analytes with a wide variety of polarities and molecular masses. In this context, ion recording principles such as single ion monitoring (SIM) or multiple reaction monitoring (MRM) with tandem mass spectrometers guarantees not only a high degree of selectivity but also additional sensitivity to quantify analytes at trace levels in complex mixtures.[2,3] Because of the excellent sensitivity and high selectivity of MS detection, LC–MS has attracted increasing attention as a technique for analyte detection in complex biological, environmental and food matrices, especially in view of recent national and international laws and regulations.[4–6]

It is thought that extensive sample clean-up strategies in trace analysis (ppb to ppt range) may be considerably reduced or even omitted by the use of selective LC–MS detection, in order to achieve higher sample throughputs and increased linear ranges of calibration curves. However, this common perception overstates the potential of the technique, as co-eluting matrix compounds can influence the ionization efficiency of analytes (matrix effects). This results in decreased data accuracy and MS detection sensitivity, particularly if matrix interferences change from sample to sample.[7–9] In this context, we have recently shown that LC–MS sensitivity and data accuracy are closely related to sample clean-up protocols and/or LC separation. Furthermore, the usefulness of internal standards for successful LC–MS method development has been demonstrated and implications of their chemical nature on data accuracy have been discussed.[10]

In this article we focus on MS features that might improve selectivity and sensitivity for the determination of compounds

**Applying tandem MS instrumentation adds further selectivity to the MS detection of compounds in complex biological samples.**

in complex biological sample surroundings. This includes the selection of fragment ions for MRM recording, and the use of derivatization reactions to enhance analyte ionization efficiencies and induce more compound-specific fragmentation pathways for a higher selectivity of tandem MS detection. It will be shown that these measures represent a very simple and effective way to improve the performance of LC–MS/MS methods without the risk of further error-prone sample preparation steps.

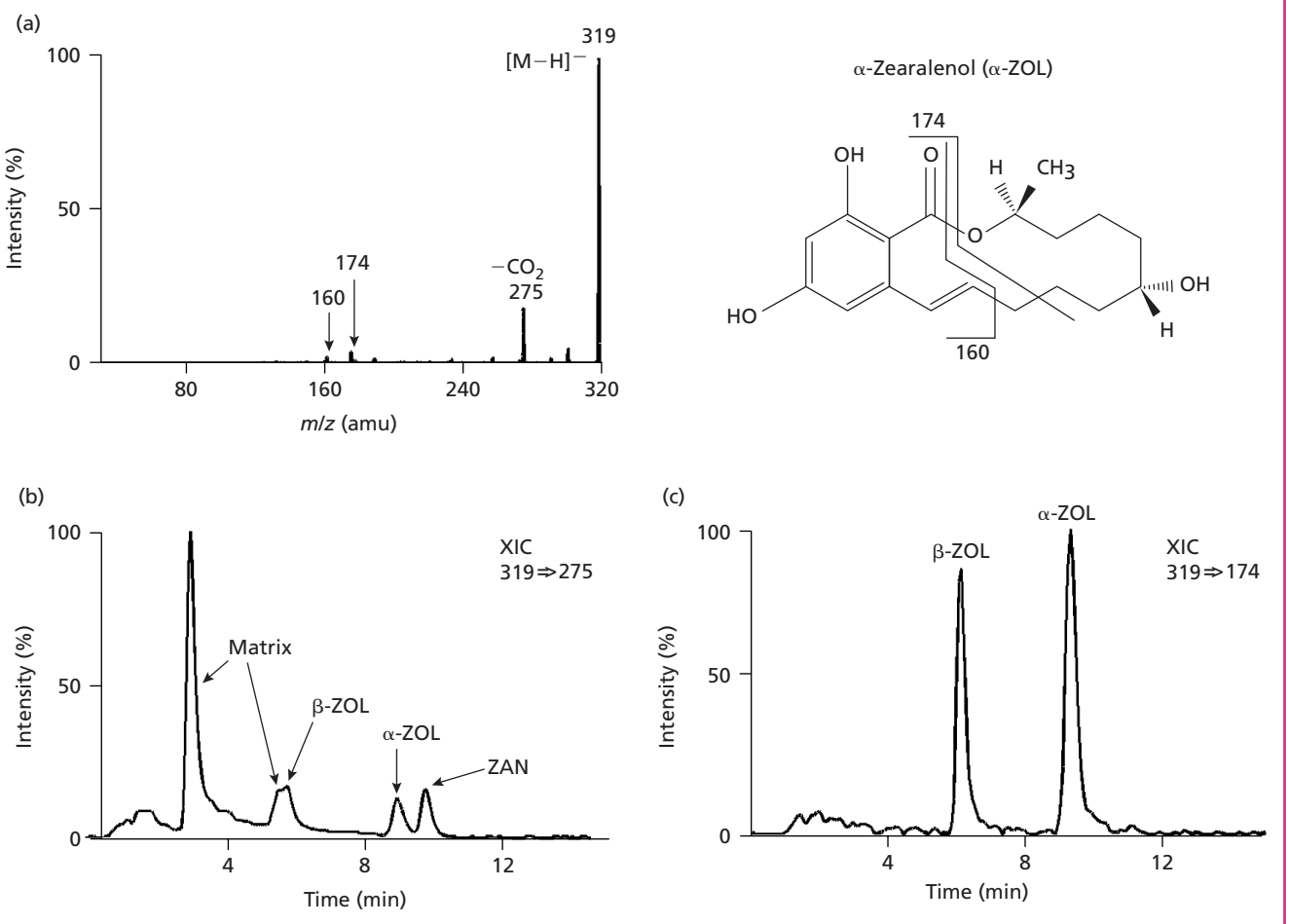
## Experimental

All LC–MS/MS analyses were performed on an API 365 triple quadrupole mass spectrometer (PE Sciex Instruments, Thornhill, Canada) connected to an 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany). This system was either equipped with an electrospray interface (for nitrofuran antibiotics analysis) or an atmospheric pressure chemical ionization interface (for the analysis of zearalenone and its metabolites). Depending on the analytes, chromatographic separations were either performed on a 150 × 3 mm i.d. Inertsil®, ODS-3 column (GL Sciences Inc., Tokyo, Japan) (nitrofurans) or on a 125 × 3 mm i.d. Superspher RP-18 endcapped column (Merck KGaA, Darmstadt, Germany) (zearalenone and its metabolites). Mixtures of methanol, acetonitrile and water with ammonium acetate were used in the isocratic mode as mobile phases. When the electrospray interface was used, the solvent flow was split in a ratio of 1:50 prior to MS detection.

MS detection was either performed in the positive ion mode (nitrofurans) or negative ion mode (zearalenone and metabolites) using MRM. The precursor/product ion combinations are listed in the respective figures. Nitrogen was used as collision gas and the collision energy was set to 15 eV (nitrofurans) and 30 eV (zearalenone and metabolites).

Clean-up of liquid samples and of extracts of solid samples was based on a solid-phase extraction (SPE) step. Depending on the analyte/matrix combination, 100 mg RP-18 columns

**Figure 1:** (a) Product ion mass spectrum of α-zearalenol (negative ion mode); (b) α-Zearalenol (α-ZOL) selective extracted reaction monitoring chromatogram (XIC) of a pig urine sample spiked with zearalenone and its metabolites (5 µg/L of each analyte). Recorded fragmentation pathway: 319 u > 275 u; $[M-H]^- - CO_2$. ZAN: zearalanone, β-ZOL: β-zearalenol; (c) α-Zearalenol selective extracted reaction monitoring chromatogram (XIC) of the same pig urine sample. Recorded fragmentation pathway: 319 u > 174 u. (Figure 1(a), (b) and (c) reproduced with permission from *Chromatographia*, **51**, 681–687 (2000). Copyright 2000 Friedrich Vieweg.)



(Phenomenex, Torrance, California, USA) or 200 mg LiChrolut®, EN SPE columns (Merck) were used for this purpose. Further details on instrumentation, materials and sample preparation have been previously described.[11,12]

## Results and Discussion

**Selection of suitable MRM ions:** Applying tandem MS instrumentation adds further selectivity to the MS detection of compounds in complex biological samples. A crucial point in the development of multidimensional mass spectrometric methods (MS/MS) is the suitable selection of ion pairs, especially for MRM. Normally, the most abundant fragment ions are selected from preliminary product ion experiments, followed by adjustment of relevant MS/MS parameters, such as the collisional energy, to reach highest sensitivity for the selected ion pairs and, consequently, for a given analyte.

Unfortunately, low-energy collisions in triple quadrupole instruments predominantly induce non-specific losses of small stable neutrals (e.g., water and carbon dioxide) in a lot of organic compounds. This is exemplified with a product ion mass spectrum of α-zearalenol, a metabolite of the estrogenic mycotoxin zearalenone,[13] in which the major product fragment ion at *m/z* 275 reflects the non-specific loss of carbon dioxide (Figure 1(a)). Other more compound-specific fragment ions in the range of *m/z* 150–250 are distinctly less abundant, and even an increase in the collisional energy does not result in higher relative intensities of these ions.

Non-specific product ions, if applied to MRM in the analysis of complex biological matrices, may result in severe problems in terms of MS sensitivity and selectivity. This is demonstrated in Figure 1(b). High abundance and interfering matrix peaks in the reaction monitoring chromatogram of α-zearalenol (319.1 ➔ 275; $[M\text{-}H]^-$-CO2), isolated by SPE from a spiked urine sample, show that the loss of carbon dioxide is common to a large number of unknown matrix compounds but also to other structurally similar analytes (zearalanone, ZAN, shows up in the reaction monitoring chromatogram of α-zearalenol, Figure 1(b)). Furthermore, the high baseline noise decreases the signal-to-noise ratio and consequently the sensitivity of the MS detection.

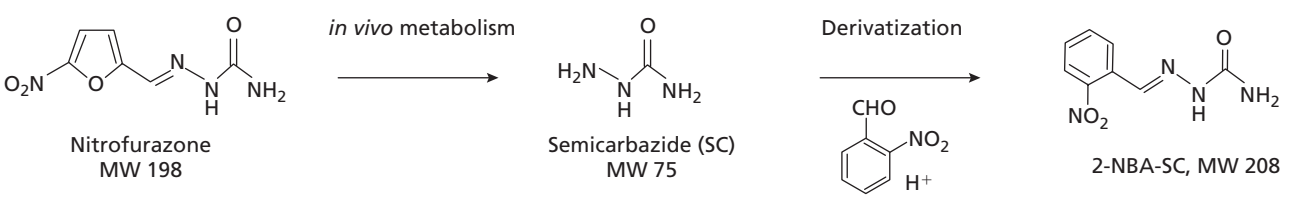
In other words, a non-selective fragmentation pattern of an analyte used for MRM both increases the probability of false positive MS signals (because of the underlying ions stemming from the chromatographically unresolved and interfering matrix components) and simultaneously reduces the MS sensitivity. In principle, this necessitates an improvement in the selectivity of sample preparation and/or LC separation to separate the analytes of interest from both interfering matrix compounds and structurally similar compounds, and thus to compensate for the relatively low MS selectivity.

A distinctly less time-consuming approach would be the correct selection of other more compound-specific fragment ions for MRM. As depicted in Figure 1(c), the reaction monitoring chromatogram of α-zearalenol is free from any interfering compound signals (except for the well resolved diastereomer β-zearalenol) when the compound-specific fragment ions at *m/z* 174 and 160 (Figure 1(a)), which reflect bond cleavages and rearrangements in the macrocyclic ring system, are used for MRM. Though both ions are significantly less abundant than the ion at *m/z* 275 (non-specific loss of carbon dioxide), the signal-to-noise ratio is dramatically increased because MS background noise is strikingly reduced. This enhances the overall method sensitivity by a factor of 10 to 0.1 µg/L in urine,[11] showing the advantage of careful selection of specific ions for MRM during method development.

**Suitable derivatization of the analyte:** Analytes with molecular masses ≤150 g/mol and relatively high polarities pose a general problem to LC–MS(MS) sensitivity and selectivity. These analytes very often possess no or only unfavourable sites to stabilize positive and negative charges by protonation or deprotonation reactions, and thus frequently possess poor ionization efficiencies with low MS sensitivity. Furthermore, highly abundant MS background noise in this low-mass range has a further severe negative impact on MS sensitivity towards low mass analytes. This is especially relevant for MS instruments that are used for the high-throughput analysis of "dirty" food samples with a permanently varying sample matrix complexity. Even the use of MS/MS techniques does not offer a solution to this problem because of non-specific fragmentation behaviour of such analytes, predominantly with losses of small neutral molecules (e.g., ammonia, water or carbon dioxide).

This is demonstrated with semicarbazide (Figure 2), a major metabolite of the antibacterial agent nitrofurazone, which has been used as a food additive for the treatment of gastrointestinal infections in cattle, pig and poultry, but which has now been banned by the European Union. Because of its low molecular mass (75 g/mol) the MS detection sensitivity is relatively poor, as it is not well ionized by electrospray ionization. Furthermore, highly abundant MS background noise in this mass range significantly reduces the signal-to-noise ratios and consequently the MS sensitivity to the low-ppm level. Also, LC selectivity is strikingly reduced, because semicarbazide offers little retention on reversed-phase HPLC columns, preventing separation from matrix compounds as it elutes almost in the void volume. Besides, fragmentation is dominated by non-specific losses of ammonia and carbon monoxide (Figure 3(a)). Consequently, a sufficient overall method detection limit in the low µg/kg range could neither

**Figure 2:** Nitrofurazone: major metabolitization pathway to semicarbazide and derivatization with 2-nitrobenzaldehyde.

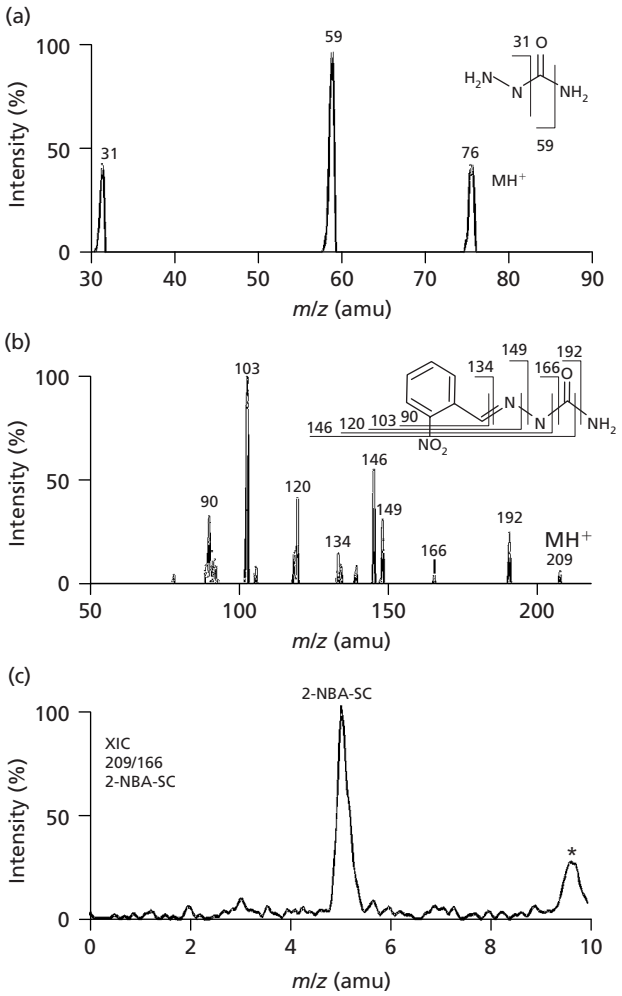


be achieved by MS nor MS/MS experiments.[12]

It has been repeatedly demonstrated that derivatization of analytes prior to LC separation may sometimes be useful to efficiently enhance sensitivity using UV and fluorescence detection,[14,15] but also to distinctly improve MS sensitivity and selectivity of low-mass analytes.[12,14]

This is well documented with the nitrofuran metabolite semicarbazide. Derivatization of the free amino group was performed with 2-nitrobenzaldehyde (Figure 2), yielding an imine derivative that was previously used to improve UV and MS detection of similar nitrofuran antibiotics.[14] The molecular mass of the analyte is increased by a factor of 3 to 208 g/mol moving the molecular ion out of the mass range of typical MS background noise. The ionization efficiency of the derivatized semicarbazide is strikingly improved indicating that positive charges are better stabilized on the 2-nitrobenzyl derivative than on the free semicarbazide. Furthermore, the selectivity of the LC–MS/MS detection is enhanced, because the retention on reversed-phase HPLC materials is increased by the reduced polarity of the derivatized analyte. This enables a better separation from tissue matrix compounds and other analytes. In addition, the fragmentation pattern (Figure 3(b)) offers more compound-specific fragment ions for MRM, which eliminates interfering matrix compound peaks (muscle tissue of pig after clean-up with SPE) and reduces MS background noise (Figure 3(c)). The signal-to-noise ratio, and consequently the MS sensitivity, is improved by a factor of 300 reaching a detection limit of 3 µg/kg.

**Figure 3:** (a) Product ion mass spectrum of semicarbazide (M = 75 g/mol); (b) Product ion mass spectrum of its 2-nitrobenzylimine derivative (M = 208 g/mol); (c) Semicarbazide (as its 2-nitrobenzaldehyde, 2-NBA-SC) selective extracted ion chromatogram (XIC) of a spiked pig muscle sample (10 µg/kg semicarbazide). Matrix peaks are marked with asterisks. Recorded fragmentation pathway: 209 > 166 u. (Figure 3 reproduced with permission from *Journal of Chromatography A*)[16]




It is apparent from these findings that a single-step, though time-consuming chemical modification of low-mass analytes, may dramatically increase MS sensitivity and selectivity. Consequently, in some instances derivatization should be considered as a possible alternative to the development of laborious, extensive and more selective sample clean-up/enrichment protocols.

## Conclusions

LC–MS sensitivity and data accuracy are closely related to the removal of interfering components by suitable sample clean-up protocols and/or LC separation. However, MS sensitivity is not just a matter of ionization efficiency for a given analyte, but is often directly related to the selectivity of MS detection (signal-to-noise ratio of analyte and matrix background), which depends on the individual combination of matrix and analyte. In this respect, the reliable selection of analyte-specific fragment ions for MRM recording is a valuable and comparably simple tool to efficiently enhance MS selectivity and sensitivity. In addition, derivatization is sometimes another interesting approach to enhance ionization of low-mass analytes and/or to induce more compound-specific fragmentation for MRM detection.

In conclusion, maximum sensitivity can be achieved without further time-consuming sample preparation and chromatography, when suitable MS principles are applied.

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