Improving LC–MS/MS Analyses in Complex Food Matrices, Part I — Sample Preparation and Chromatography

Peter Zöllner,[a] Alexander Leitner,[b] Diana Berner,[b] Martina Kleinova,[b] Justus Jodlbauer,[b] Bernhard X. Mayer[c] and Wolfgang Lindner.[b]

[a]Bayer Cropscience GmbH, Product Technology, Frankfurt, Germany,
[b]Institute of Analytical Chemistry, University of Vienna, Austria,
[c]Department of Clinical Pharmacology, University School of Medicine, Vienna, Austria.

In Part I of this article, the authors highlight some of their recent experiences with high performance liquid chromatography–(tandem) mass spectrometry as an analytical tool for determining trace amounts of xenobiotics (mycotoxins and antibiotics) in a variety of food matrices and biological fluids. Possibilities and limitations of the technique are outlined and special attention is paid to the impact of sample preparation and chromatography on the ionization efficiency of analytes isolated from complex food matrices.

Introduction

Modern atmospheric pressure interfaces (API), such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), offer clear advantages of robustness and ease of use, and allow the development of routine and reliable liquid chromatography–mass spectrometry (LC–MS) instrumentation for high sample throughput.[1] In contrast to gas chromatography–mass spectrometry (GC–MS), LC–(API)MS is not limited to a small number of analytes with sufficient volatility. The technique can be applied to analytes of diverse polarities and molecular masses. A further distinct advantage of LC–(API)MS is the fact that time-consuming and error-prone derivatization steps are seldom necessary.

API ion sources are most often used in combination with quadrupole technology.[2] Tandem mass spectrometers (triple quadrupole instruments) with facilities for collision-induced fragmentation offer, in principle, the possibility to eliminate all potential interferences from the sample matrix, and from the mobile and stationary phases. Continuous instrument and software developments have resulted in robust and easy-to-use instruments for high sample throughput, especially in the fields of drug discovery (combinatorial chemistry), drug development (metabolism studies and pharmacokinetics) and biomolecule structure elucidation (e.g., peptides and proteins).[1] Although ESI is preferred for most applications because of its superior sensitivity, APCI offers the advantage of

The need for LC–MS methods has been further increased because unambiguous analyte identification and accurate quantification are prerequisites in food and drug analysis, according to recent national and international laws and regulations.

being more useful for analytes with low to medium polarity and lower molecular mass.[2] However, ESI should be evaluated for neutral compounds instead of APCI when analyte degradation or significant background signals occur in the APCI mode. Furthermore, ion recording principles, such as selected ion monitoring (SIM) or multiple reaction monitoring (MRM) with tandem mass spectrometers, guarantee a high degree of selectivity as well as additional sensitivity to quantify analytes of diverse polarities at trace levels in complex mixtures.[3]

A major problem surrounding the analysis of compounds in complex biological matrices is the need for laborious, time-consuming and occasionally error-prone sample preparation strategies. The excellent sensitivity and high selectivity of MS detection offer a powerful approach to reduce or even omit sample preparation, and simultaneously to enhance the sensitivity of the analytical method. In this respect, MS may be regarded as a universally applicable detection system that guarantees unambiguous analyte identification and detection.

LC–MS has recently attracted increasing attention, especially in food, drug and environmental analysis, because the demands of sensitive and selective analyte detection in complex biological, environmental and food matrices are met by this technique.[4–7] The need for LC–MS methods has been further increased because unambiguous analyte identification and accurate quantification are prerequisites in food and drug analysis, according to recent national and international laws and regulations. Tandem mass spectrometry provides the highest degree of certainty in analyte identification and, therefore, may be employed in accordance with recent European Union guidelines to obtain data with relevant unambiguity (European Commission Council Directive SANCO/1805/2000). In addition, extensive sample clean-up strategies may be considerably reduced by using selective LC–MS detection so that higher sample throughput can be achieved. Consequently, numerous LC–MS(/MS) applications have been developed in these fields or increasingly used to replace more laborious and time-consuming GC–MS methods. Limits of detection (LOD) and limits of quantification (LOQ) in the low ppb and even ppt range can be achieved for many different analytes. Linear ranges over three orders of magnitude can be easily established.

Despite these numerous advantages, limitations of the technique have to be considered and suitable measures have to be applied to reach the highest possible selectivity, sensitivity and data accuracy. Therefore, we feel that it is worthwhile to report some recent experiences with LC–MS/MS method development for the determination of trace amounts of xenobiotics in a variety of food matrices. Some methodological possibilities and limitations are outlined and a special emphasis is put on the impact of sample preparation and chromatography towards the ionization efficiency of analytes from complex food matrices. This includes aspects such as ion suppression phenomena, the application of internal standards and chromatographic separation, and how they affect the accuracy of quantitative results.

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). The system was either equipped with an electrospray interface (for ochratoxin A analysis) or an atmospheric pressure chemical ionization interface (for zearalenone and metabolites analysis). Depending on the analytes, chromatographic separations were either performed on a 125 × 3 mm i.d. Superspher RP-18 endcapped column (Merck, Darmstadt, Germany), a 150 × 3 mm i.d. HP/HPV Shield RP-8 column (Waters, Milford, Massachusetts, USA) or a 100 × 4.6 mm i.d. Chromolith Performance RP-18 endcapped column (Merck). Different mixtures of methanol, acetonitrile and aqueous 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 (for ochratoxin A analysis) or negative ion mode (for zearalenone and metabolites analysis) using MRM. The precursor/product ion combinations are listed in the respective figures. Nitrogen was used as the collision gas and the collision energy was set at 32.5 eV (for ochratoxin A analysis) and 30 eV (for zearalenone and metabolites analysis).

The clean-up of liquid samples and extracts of solid samples was based on a solid-phase extraction (SPE) step. Depending on the analyte/matrix combination, 100 mg RP-18 columns (Phenomenex, Torrance, California, USA), 60 mg Oasis HLB columns (Waters) or immunoaffinity columns (Vicam, St. Watertown, Massachusetts, USA) were used for this purpose. Further details on instrumentation, materials and sample preparation have been described previously.[8–11]

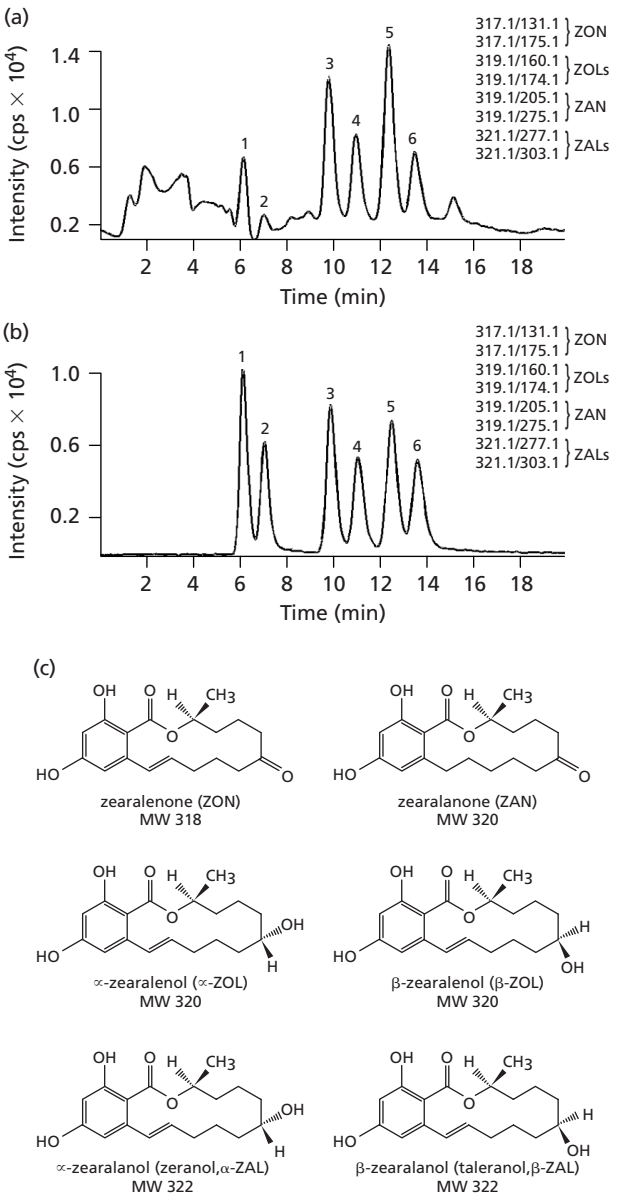
Results and Discussion

Ion-suppression phenomena (matrix effects): It has been recognized that LC–MS(/MS) practically guarantees specificity, the highest possible level of selectivity, for a given analyte. From this point of view, it seems that sample preparation and chromatographic separation may be simplified or even eliminated to achieve the highest possible sample throughput as, for example, in drug screening, pharmacokinetics and other areas of the life sciences. In contrast to this common perception, it has been shown quite frequently that the gas-phase basicity or acidity of co-eluting matrix components may be higher than that of the analyte of interest, and thus proton transfer probably occurs in the ionization process (ESI and APCI), decreasing the ion intensity of the analyte.[12]

Several authors have shown that ion-suppression phenomena in complex biological matrices are directly related to insufficient sample clean-up and/or chromatographic separation, reducing the sensitivity and accuracy of pharmacokinetic assays.[3,13–15] Not surprisingly, non-polar compounds with weak basicity or acidity seem to be, in general, more sensitive to the influence of matrix effects, because their capacity to carry positive or negative charges is limited compared with polar matrix compounds. Besides, it has been reported that ion suppression is more likely to occur in ESI than in APCI.[14]

Because of the high complexity and variability of food matrices, ion-suppression phenomena are very likely to occur in the analysis of food samples[8–10] irrespective of whether single

Figure 1: (a) Total ion LC–(MRM)MS/MS chromatogram of a blank pig liver sample spiked with 5 µg/kg zearalenone and its major metabolites. Final concentration after sample clean-up in the injected solution: 50 µg/L of each analyte; (b) total ion LC–(MRM)MS/MS chromatogram of a matrix-free standard solution of 50 µg/L zearalenone and its major metabolites and (c) structures of compounds involved. (Reproduced with permission from *J. Agric. Food Chem.*, **50**, 2494–2501 (2002). Copyright 2002 Am. Chem. Soc.)



Peaks: 1 = β-zearalanol, 2 = β-zearalenol, 3 = α-zearalanol, 4 = α-zearalenol, 5 = zearalanone, 6 = zearalenone.

MS or tandem MS instruments are used. This is well illustrated by an LC–(APCI)MS/MS chromatogram of a pig liver sample (Figure 1) that was spiked after sample preparation (extraction followed by SPE[10]) with 5 µg/kg of the estrogenic mycotoxin zearalenone[16] and five of its possible metabolites. Highly abundant matrix compounds, especially at the front of the LC–MS chromatograms, reflect the low selectivity and efficiency of the sample clean-up. Unambiguous analyte identification and quantification is possible because all analytes are chromatographically well separated from each other and, especially, from "visible" matrix compounds that have the same molecular masses and/or fragment ion masses. Nevertheless, it is apparent from a comparison of the LC–MS chromatograms of mixtures containing each analyte at a final concentration of 50 µg/L in a solution derived from a liver sample (Figure 1(a)) and a standard solution (Figure 1(b)), that the ionization efficiency of the first two analytes, β-zearalenol and taleranol is negatively influenced by co-eluting "invisible" matrix compounds. This causes a distinct decrease in signal intensity for β-zearalenol and taleranol in comparison to all other analytes. An obvious consequence of this ion suppression is the significantly lower detection and quantification limits of both compounds (LOD 0.3/1 µg/kg and LOQ 1/3 µg/kg) compared with the other analytes (LOD 0.1 µg/kg and LOQ 0.5 µg/kg), together with a decreased accuracy of the assay for taleranol (relative standard deviation 9% compared with 2–4% for the other analytes).

These observations, together with other reports,[3,13–15] clearly support the need to establish calibration curves for each analyte in the presence of the sample matrix and not in standard solutions, to obtain good method accuracy and linearity. However, this measure is not always sufficient to compensate for considerable ion suppression, as illustrated by the analysis of zearalenone in grains.[8] Though calibration curves were established in the presence of the grain matrix, agreement between expected and observed values was poor when no internal standard protocol was applied (2–24% deviation). The slopes of regression curves obtained in diverse grains varied considerably also revealing changing influences of co-eluting "invisible" matrix compounds on the analyte signal (Figure 2(a)). It is evident that no general regression curve for different grain matrices could be used to obtain reliable data. Instead, calibration for each individual grain sample (standard addition protocol) would be necessary to reach this goal, which would be extremely time-consuming.

Application of internal standards: In principle, the easiest way to eliminate matrix effects, without any further laborious method development in sample clean-up and/or chromatographic separation prior to MS detection, is to add a chromatographically co-eluting compound of known concentration. The ionization efficiency of this reference compound could be influenced by co-eluting matrix compounds in the same way as the target analyte. The usefulness of such an internal standard protocol to increase the accuracy of LC–MS data has been shown for the determination of zearalenone in grain.[8] The accuracy of results is strikingly improved by application of zearalanone as internal standard. Deviations between expected and observed values are reduced below 3%. Also, the variations of the regression curve slopes in different grain matrices are minimized such that only one calibration curve can be used for all (Figure 2(b)).

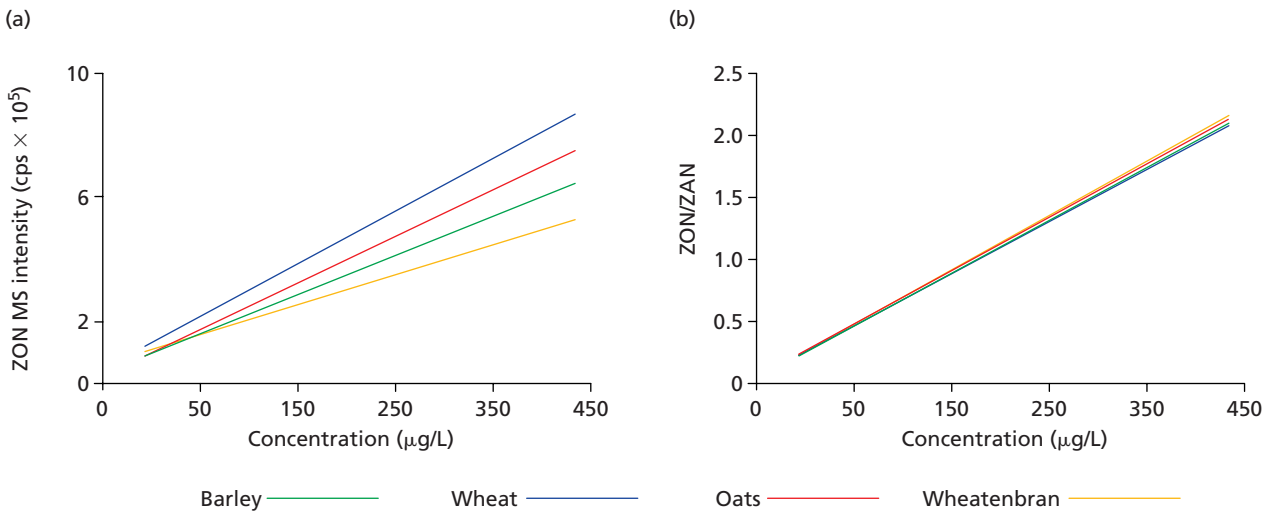
Apart from ionization suppression, the use of internal

standards is always recommended to compensate for any detector response variations that frequently occur because of changes in the interface performance over longer (sample) sequences. If the internal standard is added before sample clean-up, analyte losses during sample preparation may also be compensated for.

Physical/chemical properties of a proper internal standard for LC–MS analysis: Ideally, the structural, physical and chemical properties of an internal standard, together with its chromatographic behaviour, should be identical to the analyte of interest; otherwise both compounds may have different ionization properties in the presence of co-eluting components resulting in an insufficient compensation of matrix effects. These requirements are met by stable isotope-labelled compounds because compounds with identical LC behaviour but with different masses can be distinguished by mass spectrometry. Unfortunately, stable isotope-labelled internal standards are not always easily available and suitable synthesis is often complicated or even made impossible by insufficient isotopic purity and contamination of the labelled compound with unlabelled material. Consequently, structurally similar compounds that should not occur in the sample matrix are frequently used as internal standards.[8,9] These compounds are often obtained by chemical modification of the target analytes. A major problem with this strategy may be the necessity to achieve chromatographic co-elution of internal standard and target analyte, thus enabling maximum compensation for matrix effects. Unfortunately, slight changes in the molecular structure often result in dramatic and unpredictable changes in retention behaviour.

The significance of structural similarity between analyte and internal standard for the accuracy of quantitative analytical data became apparent during LC–MS/MS method development for the determination of the nephrotoxic mycotoxin,

Figure 2: Determination of zearalenone in different grains. (a) Calibration curves obtained without an internal standard, and (b) calibration curves obtained with the internal standard zearalanone.



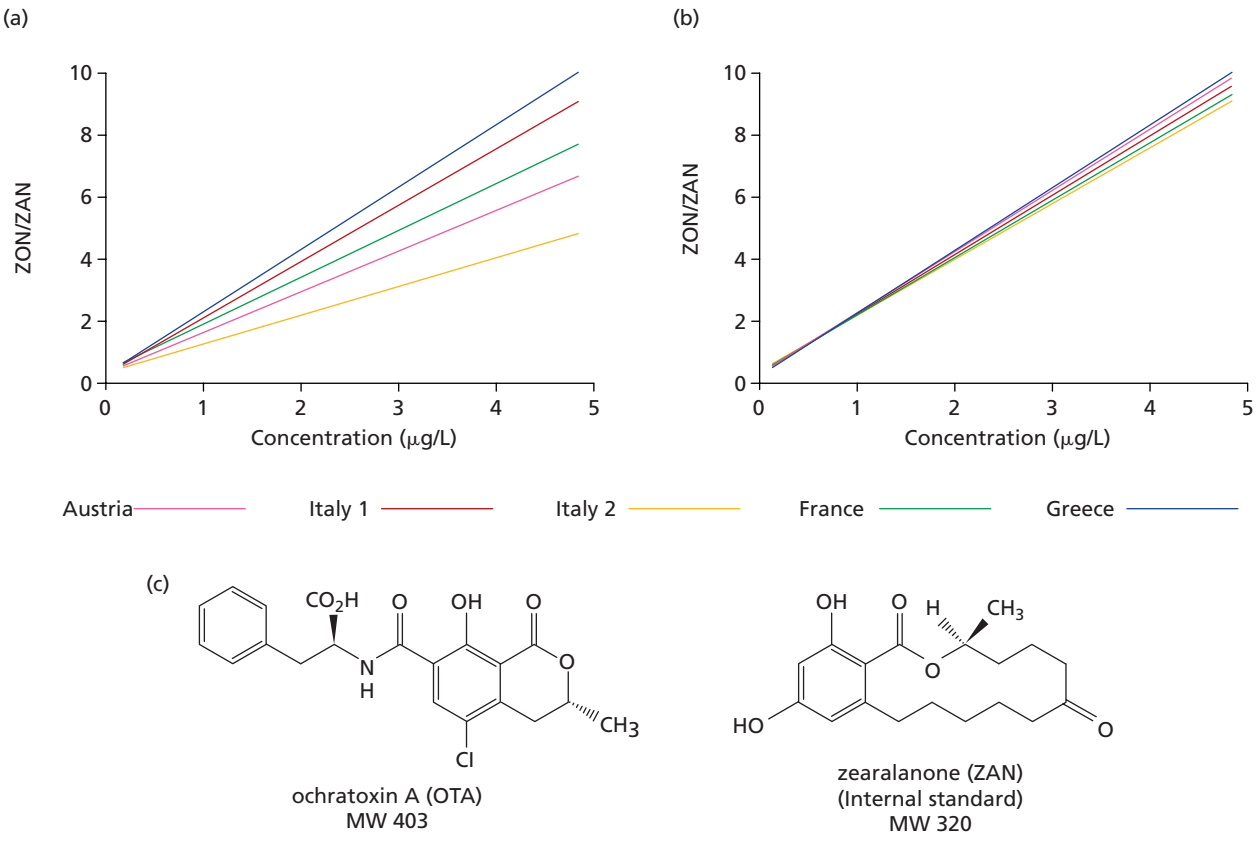
ochratoxin A,[16,17] in red and white wine. Applying a standard addition protocol, accuracy of data and linearities of calibration curves were shown to be excellent, with correlation coefficients of ≥0.999 (sample clean-up: RP-18 solid-phase extraction.)[11,17] In contrast, the slopes of calibration curves obtained for the individual samples exhibit a standard deviation of 12% for 18 different investigated wine samples (Figure 3(a)) with deviations of up to 25% between the concentration values obtained by the standard addition method and those derived from a general calibration curve. This observation reveals that the internal standard protocol using zearalanone can indeed compensate for matrix effects within each sample but not between different wine matrices, even though zearalanone virtually co-elutes with ochratoxin A (Figure 4(a)). Structural, physical and chemical differences between zearalanone and ochratoxin A, however, induce different ionization suppression phenonema on both compounds in the presence of co-eluting matrix components.

Structural similarity of internal standards and analyte does not, however, always guarantee sufficient compensation of matrix effects when internal standards elute so far away from the target analytes that both compounds are influenced by different matrix components during the ionization process. For example, for the LC–MS/MS analysis of zearalenone and its metabolites α- and β-zearalenol in different beer samples[9] a general calibration curve for different beer matrices could not be established, even though analytes and internal standard have very similar structures because the analytes do not co-elute with the internal standard zearalanone[9] (Figure 4(b)). This observation clearly demonstrates that an internal standard eluting at a different time from the LC column is influenced by other matrix components than the analytes, and is thus not able to compensate properly for ion-suppression effects. The fact that a general calibration curve can be also established for the determination of zearalenone in different grains (see earlier, Figure 2), proves that both prerequisites — structural similarity and chromatographic co-elution — are fulfilled (Figure 4(c)).

Consequently, a further problem is evident for multi-analyte determination in one LC–MS run. In this instance, one internal standard for each analyte would be ideal. As this is almost always impractical, an internal standard is needed that elutes between the analytes or, at least, co-elutes with the early eluting ones, because matrix effects of salts and other polar components are more likely to occur at the beginning of a chromatographic separation (on reversed-phase columns). Generally, a protocol designed to cover a long chromatographic elution zone with one internal standard will not be able to compensate for the matrix effects of all analytes. In such an instance at least one additional internal standard with a suitable chromatographic retention behaviour is necessary to generate a robust and accurate LC–MS method.

Improving sample clean-up and chromatographic separation prior to MS detection: Another measure to avoid quantification problems is the removal of interfering matrix compounds. This

Figure 3: Determination of ochratoxin A in different red wine samples: calibration curves (internal standard protocol) were established in the presence of the wine matrix. (a) After RP-18 SPE sample clean-up; (b) after immunoaffinity SPE sample clean-up and (c) structures of ochratoxin A and zearalanone.

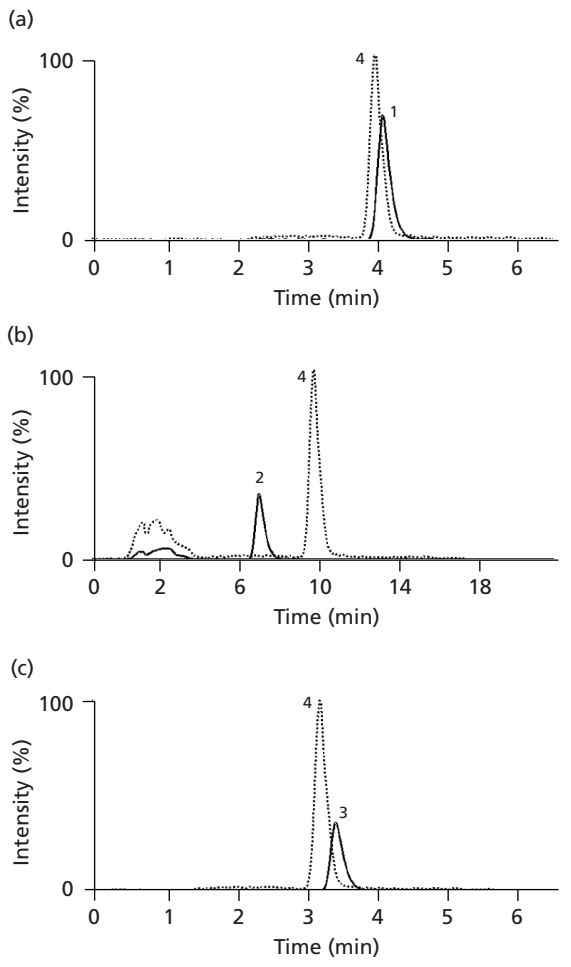


can either be achieved by improving the selectivity of sample clean-up prior to the final LC–MS(/MS) run[10,11,13] or (and) by enhancing the efficiency of the LC separation to avoid co-elution of analytes and matrix compounds.[14,18] The positive effect of an improved sample clean-up procedure on the quality of quantitative data can also be demonstrated for the above mentioned ochratoxin A determination in wine. By applying an ochratoxin A–selective immunoaffinity SPE material, standard deviations of the calibration curve slopes obtained in different wine samples were reduced from 12% to about 5% (Figure 3(b)), because matrix compounds could be almost completely removed from the samples.[11]

Improving the LC selectivity of the final analysis system might be an even more straightforward and reliable way to remove matrix interferences. However, this often implies a significant increase in chromatographic run times. Using short HPLC columns of ≤50 mm length at high flow-rates may help to reduce this, especially when many analyses are required.[19] However, merely decreasing analysis time by decreasing the retention factor 'k' may lead to inconsistent quantitative results in complex biological samples because of insufficiently separated matrix compounds.

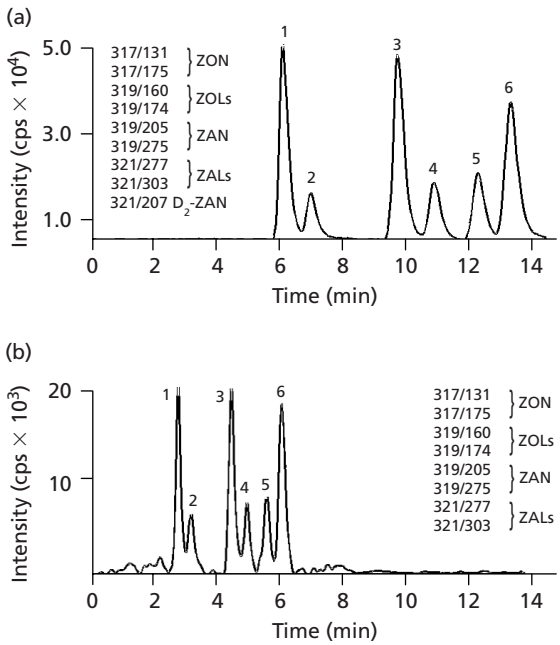
In this respect, the use of modern monolithic LC columns seems to be an interesting approach. These HPLC columns can be run at high flow-rates while maintaining the high separation efficiencies of conventional analytical HPLC columns. The resulting shorter retention times make them suitable for high-throughput LC–MS applications. This is demonstrated in Figure 5, in which zearalenone and some of its metabolites are separated on a conventional RP-18 125 × 3.0 mm column (Figure 5(a)), and a RP-18 Chromolith column[20] (100 × 4.6 mm; Merck KGaA, Darmstadt, Germany; Figure 5(b)). While the separation efficiency is comparable on both columns, run times are reduced by a factor of almost three on the monolithic column. The usefulness of this type of column for

Figure 4: Extracted ion LC–(MRM)MS/MS chromatogram of (a) a wine sample spiked with 5 µg/L ochratoxin A and 100 µg/L internal standard zearalanone; (b) beer sample spiked with 3.1 µg/L of β-zearalenol and 31 µg/L internal standard zearalanone, and (c) a maize sample spiked with 123 mg/kg zearalenone and 400 µg/kg internal standard zearalanone. (Figure 4(a) reproduced with permission from *Anal. Chim. Acta.*, **453**, 33–41 (2002). Copyright 2002 Elsevier.)



Peaks: 1 = ochratoxin A, 2 = β-zearalenol, 3 = zearalenone, 4 = internal standard (zearalanone).

Figure 5: Total ion LC–MS/MS chromatogram of zearalenone and of its metabolites. (a) On a Superspher 100 RP-18 endcapped, 125 × 3 mm i.d. column (Merck, Darmstadt, Germany); flow-rate: 0.5 mL; mobile phase: acetonitrile/methanol/water (10:45:45, v/v/v) with 15 mM ammonium acetate; column temperature: 35 °C. Injected amount: 5 ng per analyte, and (b) on a Chromolith Performance RP-18 endcapped column, 100 × 4.6 mm i.d. (Merck); flow-rate: 1.6 mL; mobile phase: acetonitrile/methanol/water (10:45:45, v/v/v) with a concentration of 15 mM ammonium acetate; column temperature: 35 °C. Injected amount: 250 pg per analyte.



Peaks: 1 = β-zearalanol, 2 = β-zearalenol, 3 = α-zearalanol, 4 = α-zearalenol, 5 = zearalanone, 6 = zearalenone.

food analysis has already been demonstrated for the determination of ochratoxin A in wine,[17] although the narrow peak widths (≤0.1 min) might negatively affect the accuracy of analytical results in the ppb to ppt range.[17]

Conclusions

LC–MS sensitivity and data accuracy is closely related to the sufficient removal of interfering components by suitable sample clean-up protocols and/or LC separation. This is because co-eluting matrix compounds may influence the ionization efficiency of analytes, significantly reducing MS data accuracy and MS sensitivity, particularly if matrix interferences change from sample to sample. Furthermore, suitable internal standards should always be used to compensate for any remaining matrix effects or other MS detector variations. In this respect, identical or similar physical/chemical properties (structural similarity) and LC behaviour of the internal standard and analyte(s) should be considered as important prerequisites for successful LC–MS method development.

It is apparent that all these features must be considered to guarantee the full potential of LC–MS in terms of accuracy and sensitivity, not only in food analysis but also in pharmaceutical, environmental and biological applications.

References

1. W.M.A. Niessen, *Liquid Chromatography-Mass Spectrometry 2nd ed.* (Marcel Dekker, New York, USA, 1999), 233–549.
2. E. De Hoffmann, J. Charette and V. Stroobant, *Mass Spectrometry, Principles and Applications* (John Wiley & Sons, New York, USA, 1996), 21–33.
3. F.M. Lagerwerf et al., *Trends Anal. Chem.*, **20**, 418–427 (2001).
4. T.A. Ternes, *Trends Anal. Chem.*, **20**, 519–532 (2001).
5. T. Reetsma, *Trends Anal. Chem.*, **20**, 533–542 (2001).
6. M.W. Trucksess, *J. Assoc. Off. Anal. Chem.*, **80**, 119–136 (1997).
7. M. Careri, A. Mangia and M. Musci, *J. Chromatogr. A*, **794**, 263–297 (1998).
8. P. Zöllner, J. Jodlbauer and W. Lindner, *J. Chromatogr. A*, **858**, 167–174 (1999).
9. P. Zöllner et al., *J. Chromatogr. B*, **738**, 233–241 (2000).
10. P. Zöllner et al., *J. Agric. Food Chem.*, **50**, 2494–2501 (2002).
11. A. Leitner et al., *Anal. Chem. Acta*, **453**, 33–41 (2002).
12. P. Kebarle and L. Tang, *Anal. Chem.*, **65**, 972A–985A (1993).
13. D.B. Buhrmann, P.I. Price and P.J. Rudewicz, *J. Am. Soc. Mass Spectrom.*, **7**, 1099–1105 (1996).
14. B.K. Matuszewski, M.L. Constanzer and C.M. Chavez-Eng, *Anal. Chem.*, **70**, 882–889 (1998).
15. J. Yamaguchi et al., *Rapid. Commun. Mass Spectrom.*, 15, 629–636 (2001).
16. J.D. Miller and H.L. Trenholm (Eds.), *Mycotoxins in Grain. Compounds other than Aflatoxin* (Eagan Press, St. Paul, USA, 1997)
17. P. Zöllner et al., *Chromatographia*, **52**, 818–820 (2000).
18. M. Nelson and J.W. Dolan, *LC•GC Eur.*, **15**, 73–79 (2002).
19. L.A. Romanyshyn and P.R. Tiller, *J. Chromatogr. A*, **928**, 41–51 (2001).
20. K. Cabrera et al., *Trends Anal. Chem.*, **17**, 50–53 (1998).

Peter Zöllner was employed as university assistant and research assistant until February 2002 at the Institute of Analytical Chemistry, University of Vienna where he worked on the analyses of food, biological and pharmaceutical samples by LC–MS, MALDI-MS and GC–MS. Currently, he is responsible for the mass spectrometry facility in the R&D department of Bayer Cropscience GmbH (Product Technology-Analytics Frankfurt, Industriepark Höchst, G 836, D-65926 Frankfurt am Main – Germany; e-mail: peter.zoellner@bayercropscience.com). His research interests are focused on the chromatographic and mass spectrometric analyses of small bioactive molecules in complex biological and food matrices.

Alexander Leitner, Diana Berner and **Martina Kleinova** performed their master theses about LC–MS method development in food analysis between 1999 and 2002 under the supervision of Peter Zöllner and Wolfgang Lindner. Currently, they are all PhD students working on biological mass spectrometry or on related subjects in different research groups at the University of Vienna.

Justus Jodlbauer received his PhD in chemistry from the University of Vienna in 2001. His thesis was focused on method development and sample preparation in mycotoxin analysis. He is currently employed in the pharmaceutical industry.

Bernhard X. Mayer is employed in the Department of Clinical Pharmacology at the Vienna University School of Medicine, where he is head of the analytical laboratory. His research experience includes the development of stationary phases in high-temperature gas chromatography and the application of HPLC and CE in different fields.

Wolfgang Lindner is Professor for Analytical Chemistry at the University of Vienna and Head of the Department of High Performance Separation Techniques and Materials. His research activities include the development of new separation materials and their application in HPLC, CE and CEC analyses of pharmaceutical and biological samples.