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Structure Elucidation and Quantification by HPLC Ion-Trap Multiple Mass Spectrometry

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Abstract. High-performance liquid chromatography combined with ion-trap mass spectrometry allows now to record mass spectra in the full-scan mode with sample quantities around 1 ng or less. In addition, the on-line generation of multiple daughter-ion spectra has become possible. This has revolutionised organic trace analysis. Small polar and/or thermolabile molecules such as biotoxins can be detected in the trace range and their structure unequivocally identified. In addition, a complete or partial structure elucidation of related structures is possible. This overview demonstrates the possibilities and limitations of HPLC-MS^n with ion traps. Examples shown are the structure elucidation of aconitum alkaloids, trichothecenes and photochemically formed carbonyl compounds. This survey discusses also problems caused by system contamination and adsorption effects. Finally, the achievable performance of quantification in the trace range is briefly presented.

1. Introduction

Ion-trap mass spectrometry allowing on-line multiple fragmentation processes is a rather new technique which was commercially introduced in the beginning of 1996 [1]. Traditional MS/MS techniques induce the fragmentation process of ions by their passage through a collision cell. Fragments formed first decay further by new collision-induced energy transfer resulting in more complex mass spectra. In ion traps, ions with a selected mass are collected and further fragmented by a highly specific energy transfer from the quadrupole field [2]. In this way, fragmentation can be controlled. Furthermore, one of the formed fragments can be trapped again and the process repeated.

By varying the transferred energy, a consecutive loss of functional groups can be induced followed by a more extensive fragmentation of the carbon skeleton. In this way, a lot of information for structure elucidation can be gained. This process is also abbreviated as multiple mass spectrometry (MS^n). *Fig. 1* shows the differ-

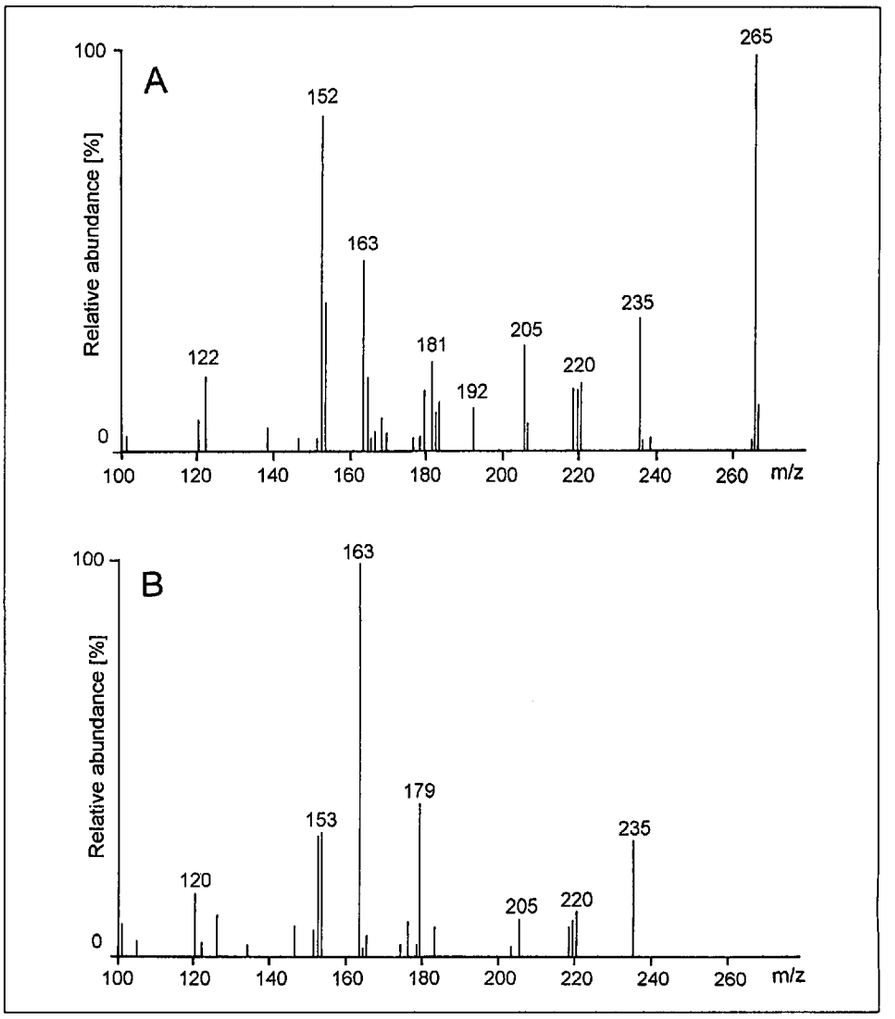

Fig. 1. *Low-pressure octapole* (A) *and ion-trap-induced* (B) *fragmentation of the deprotonated molecular ion* $[M–H]^-$ *of 1 ng of the 2,4-dinitrophenylhydrazone derivative of isovaleraldehyde*

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ence between fragmentation induced at low pressure in an octapole in front of an ion trap and inside the trap. Much less fragments are observed in the ion-trap MS/MS.

Due to the storage function of the trap, the sensitivity can be increased by collecting ions over a longer period. This results in detection limits in the full-scan mode which are 1–2 orders of magnitude lower than for quadrupole systems. Sample amounts around 1 ng can be traced by MS^{3-4}. Since such multiple MS/MS processes do not require more than a few seconds, they can be carried out on-line during an HPLC separation. *Fig. 2A* shows the ion-current trace of an on-line MS^n experiment where the base ion was further fragmented, and *Fig. 2B* displays a separation of a few ng of some trichothecenes (biotoxins mainly formed by *fusarium* fungus attack of, *e.g.*, wheat) in a wheat extract detected by atmospheric-pressure chemical ionisation (APCI) in the positive-ion mode. Mass chromatograms of selected ions allow to achieve detection limits in the range of low pg amounts [3]. HPLC ion-trap MS has therefore become a very suitable tool for trace analysis of thermolabile and polar compounds showing a comparable performance as GC-MS.

Our interest in HPLC ion-trap MS^n is mainly focused on the structure elucidation and quantification of small polar molecules (usually up to 500 u, occasionally up to 1000 u). Examples are biotoxins formed by plants, algae or different fungi, or polar photochemical degradation products in the atmosphere. Such compounds have often to be quantified in the low ng range or even below. As for quadrupole techniques, both electrospray ionisation (ESI) and APCI can be employed for primary ionisation. However, ESI is much more suited for molecules with 'preformed' charges, while APCI allows to ionise even rather non-polar molecules by charge transfer from charged species formed in the corona discharge.

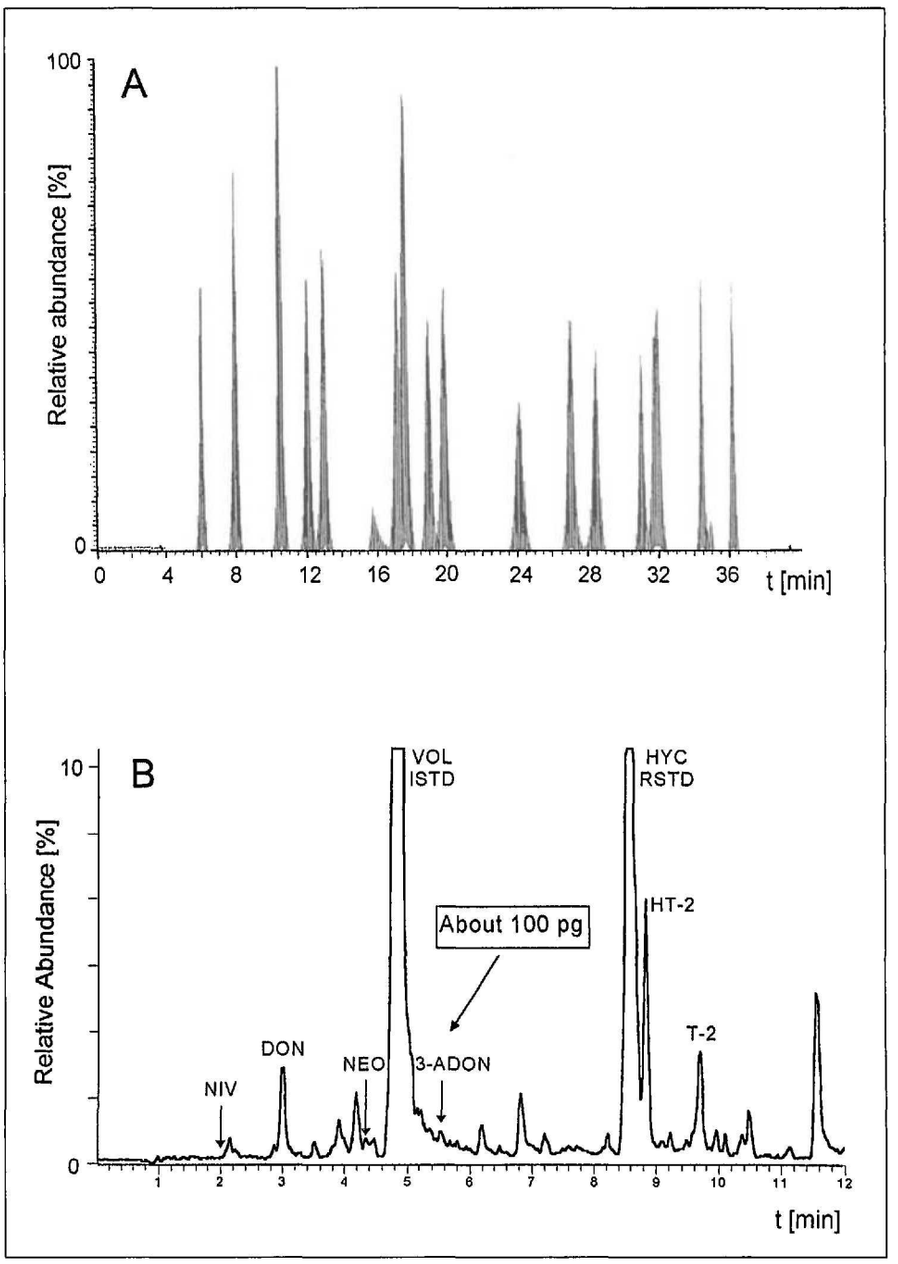


Fig. 2. A: *Total ion-current trace of an HPLC-MS^n run of the separation of 2,4-dinitrophenylhydrazone derivatives of C_1 to C_6 carbonyl compounds* (concentrations in the 10 ng range). Numerous experiments are possible during the elution of a compound (indicated by a collapse of the ion current). B: *Base-ion chromatogram* (scan range *m/z* 150 to 500) *of the separation of trichothecenes* (for the basic structure, see *Fig. 6*) *in a wheat extract.* For details, see [4]. Abbreviations: NIV, nivalenol; DON, deoxynivalenol; NEO, neosolaniol; VOL, verrucarol (internal standard); 3-ADON, 3-acetyldeoxynivalenol; HYC, hydrocortisone (recovery standard); HT-2, HT-2 toxin; T-2, T-2 toxin.

2. Selected Ionisation Techniques and Contamination Problems

APCI is in our case normally the method of choice due to the following reasons. It is well suited for quantification since only few ion adducts are formed. In the ESI mode, frequently the appearance of $[M+Na]^+$, $[M+K]^+$ or $[M+NH_4]^+$ ions or other adducts is observed. Their proportion on the total ion current is dependent on the salt background in the sample and the build-up of salt residues in the spray region of the ion source. Furthermore, the formed charges can be more easily stabilised by large molecules (proteins) being still present in the sample matrix. Then, small molecules present at low concentrations are no longer properly ionised.

However, the considerably improved sensitivity in the full-scan mode of ion-trap LC-MS has also its price. Due to the much lower detection limits, the cleanliness of the separation system is much more important. Even minute amounts of organic contaminants in the mobile phase (water and a solvent such as methanol) will lead to a high background or 'ghost' signals which might jeopardise the achieved gain in sensitivity [5]. *Fig. 3* shows the background chromatogram of a batch of HPLC-grade methanol. Though suitable for UV detection, numerous contaminants such as dioctylphthalate and polyethylene glycols are present. In gen-

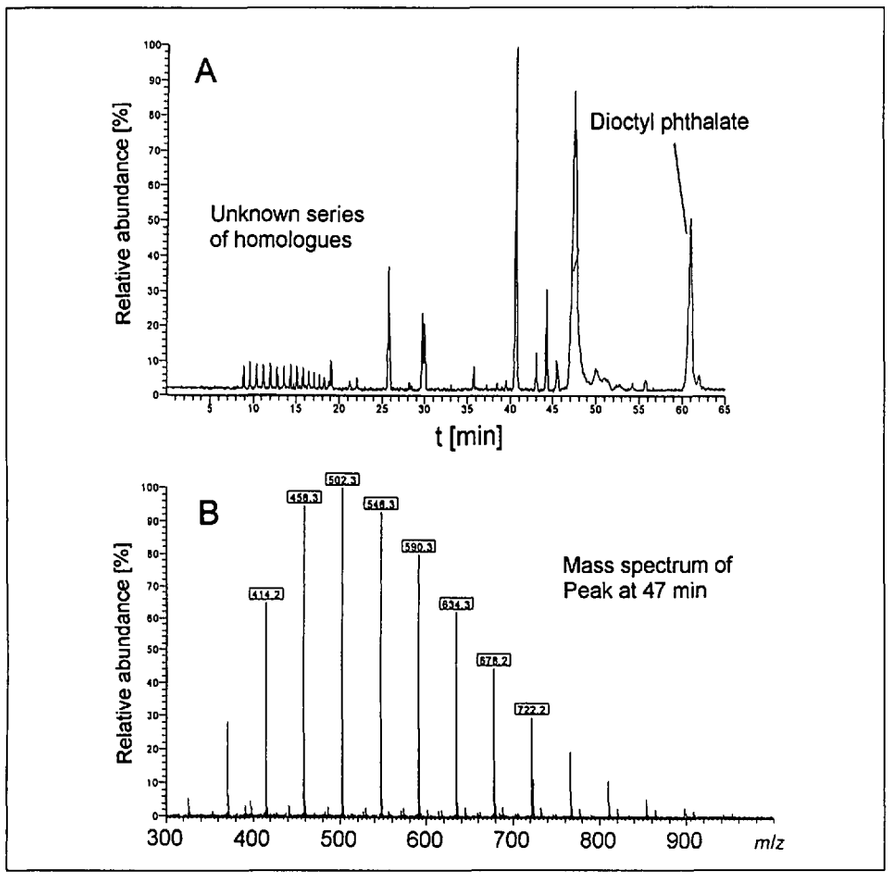


Fig. 3. A: *Base-peak HPLC-MS chromatogram of a blank run obtained with a water/methanol gradient using HPLC-grade methanol.* Numerous contaminants are visible, among them dioctyl phthalate (DOP) and a homologue series of polyethylene glycols as the mass spectrum at 47 min (B) shows.

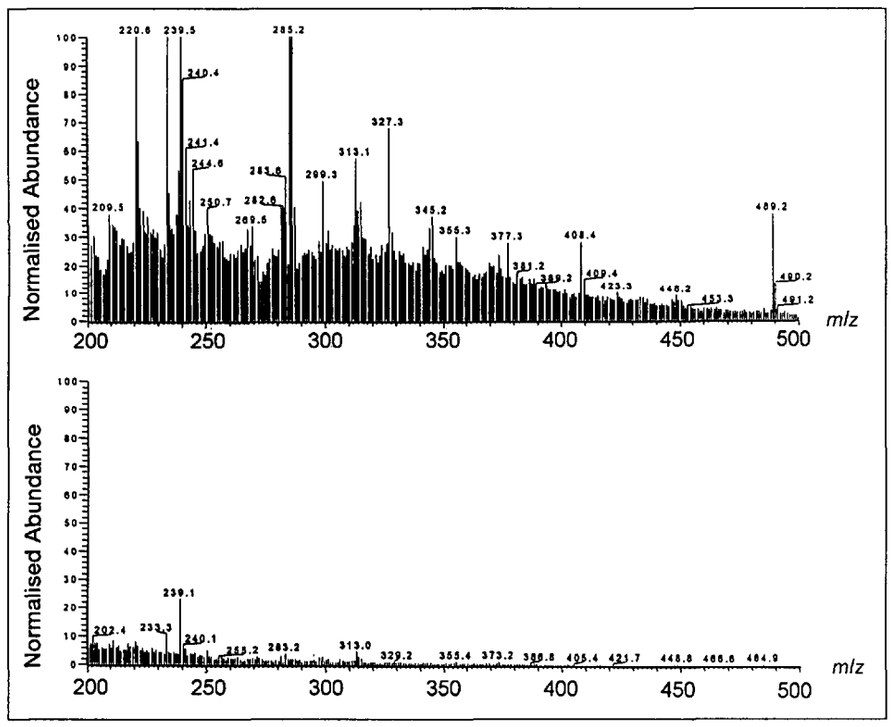


Fig. 4. *Background mass spectra obtained from two C_{18} reversed-phase columns* (125 mm length, 3 mm i.d., 5 mm particle size) *from different manufacturers with H_2O/CH_3CN* (50/50, *v/v*) *after 15 min rinsing with acetonitrile.* The best (bottom) and worst (top) example from a test of 20 columns is shown. The abundances normalised to the worst example (=100) are shown.

eral, pesticide-grade solvents are much purer than those only purified for maximum UV transmission.

Furthermore, the slow (always present) hydrolysis of reversed phases depends highly on the manufacturing process. Particularly in the APCI negative-ion mode, cleavage products of the alkyl-chain-modified surface are visible. Depending on the quality of the column, degradation products have to be removed by shorter or longer flushing with solvents after a longer standby period (*e.g.*, overnight). *Fig. 4* demonstrates an example of an extreme quality difference.

3. Experimental Conditions

The examples of HPLC-MS^n applications shown here were recorded under different separation and ionisation conditions. Further details can be found in the literature cited at the end of this article. However, the parameters in common can be briefly summarised as follows:

A low-pressure binary-gradient HPLC pump (*Rheos 4000, Flux Instruments*, Basel, Switzerland) was employed. The samples were injected with a *Valco Cheminert* valve equipped with a 5 µl to 20 µl loop. Reversed-phase HPLC separation was applied (*e.g., C18* normal-density phase (*Nucleosil*, pore size 120 Å, 3-µm particles, column length 125 mm, 2 mm i.d., *Macherey-Nagel*, Oensingen, Switzerland)). The flow rate of the mobile phase was around 250–500 ml/min. Binary gradients of water/methanol or water/acetonitrile were employed.

An ion-trap mass spectrometer (*LCQ, Finnigan MAT*, San Jose, CA, USA) was used in the positive- or negative-ion mode employing atmospheric-pressure chemical ionisation (APCI(+)). Mass spectra were registered in the full-scan mode (mass range 150 to 550 u). The following instrument parameters were applied: Heater temperature, 230–450°; nitrogen sheath-gas flow, 25–40 arbitrary units (corresponding to *ca.* 250–400 ml/min); ionisation current of corona discharge, 1.5–5 µA.

4. Examples of Structure Elucidation by MS^n

Aconitum alkaloids are present in most members of the plant family *aconitum*. So far, several hundreds of such compounds have been isolated and their structure elucidated in the conventional way by IR, NMR, and MS. They are easily ionised with ESI and no fragmentation is observed

[6]. The structure of one of these diterpene alkaloids is shown in *Fig. 5*. It was found as an impurity in a mixture of isolated compounds. In addition to those known to be part of this reference solution, one alkaloid was present with the protonated molecular mass $[M+H]^+$ *m/z* 752. Isolation of this mass in the trap and consecutive MS^n revealed a loss of 122 u (benzoic acid) in the MS/MS spectrum and a MS^3 spectrum identical to pseudaconitin which has instead of two benzoyl moieties only one, but an additional acetyl group. *Fig. 5* compares the MS^3 spectra of pseudaconitin and the hitherto unknown aconitum alkaloid. Due to the characteristic MS^n fingerprint for aconitum alkaloids, the compound could be unequivocally identified as an aconitum alkaloid, and information about functional groups was obtained. Of course, additionally, a final confirmation of the structure has to be carried out in the conventional way.

A similar approach can also be used for the identification of trichothecene toxins. Here, their ability to form ionic adducts helps to differentiate between the two main groups of A- and B-trichothecenes (for the basic structure, see *Fig. 6*) [4]. Both can have H-, HO- or acetyl (AcO-) substituents at R^2 to R^5. However, only B-trichothecenes have a carbonyl function at R^1 which can form an acetate ion adduct (addition to the α,β-unsaturated ketone) detectable by APCI(–) after post-column addition of NH_4OAc to the eluent. Furthermore, strong ammonium adducts determinable by APCI(+) are only formed when an acetyl group is present at C(15). So far, no sound explanation can be given for this particular behaviour.

Loss of functional groups as water or acetic acid *etc.* is visible in the MS and MS/MS spectra while the MS^3 spectra show the decay of the carbon skeleton forming a fragment sequence of losses of 15, 18, 28 and 42 u which is a fingerprint for a trichothecene. *Fig. 6* presents the MS to MS^3 spectra of deoxynivalenol as an illustration of this behaviour. This compound forms an abundant acetate anion in the APCI negative-ion mode, and the $[M+H]^+$ ion loses 18 u (H_2O, *m/z* 279), 30 u (CH_3OH from C(6), *m/z* 249), and 18 u again (*m/z* 231) which allows to identify all substitutents. After loss of the epoxide ring (*m/z* 203), the typical trichothecene sequence of –15 u (*m/z* 188), –18 u (*m/z* 185), –28 u (*m/z* 175) and –42 u (*m/z* 161) is found in the MS^3 spectrum. Combining all this information allows to identify an unknown compound unequivocally as a trichothecene, to elucidate the number and kind of functional groups as well as to

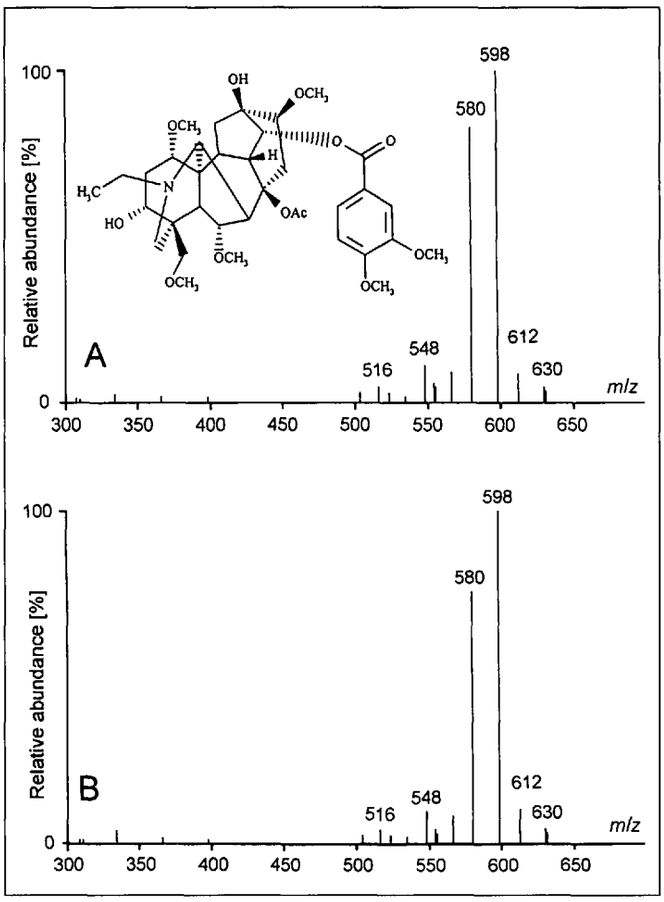


Fig. 5. *Structure of the aconitum alkaloid pseudaconitine and the ESI(+) MS^3 mass spectra of an unknown alkaloid in a mixture* (top, *m/z* 752 $[M+H]^+ \rightarrow$ *m/z* 630 → full scan) *and of pseudaconitine* (bottom, *m/z* 690 $[M+H]^+ \rightarrow$ *m/z* 630 → full scan). The only difference between both structures is the replacement of the acetyl by a further benzoyl group. After cleavage of this group by MS/MS, the resulting MS^3 spectra are identical.

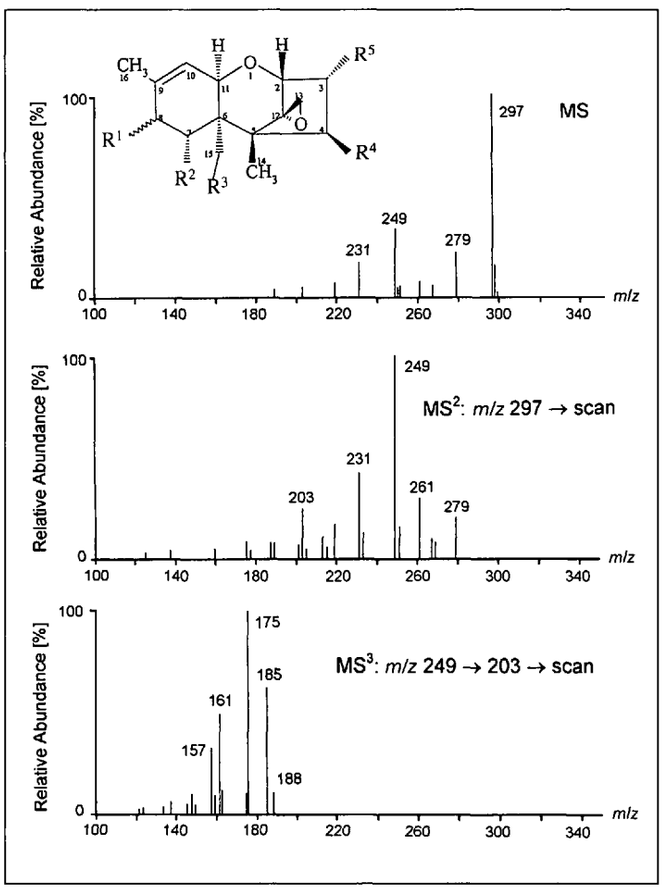


Fig. 6. *Basic structure of trichothecenes and MS, MS/MS as well as MS^3 mass spectra of deoxynivalenol* ($[M+H]^+$, *m/z* 297, R^1:=O, R^2, R^3 and R^5: OH, R^4: H) *recorded in the APCI(+) mode*

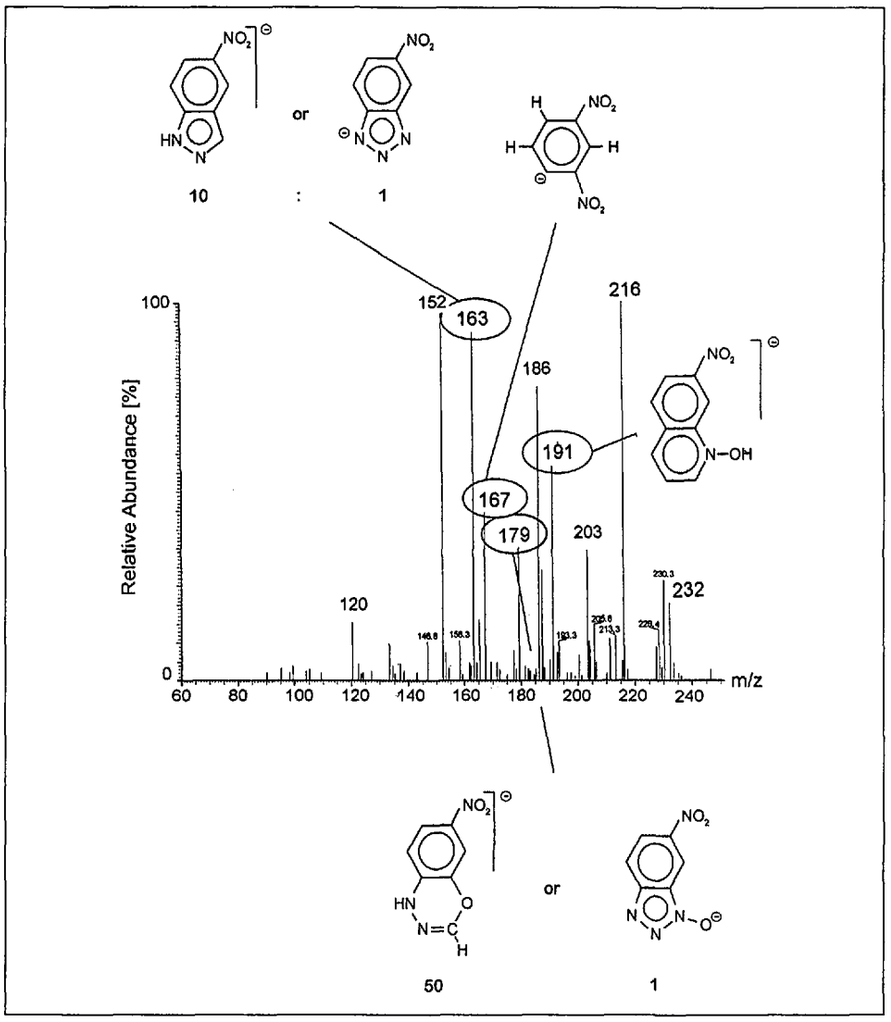


Fig. 7. *APCI(–) MS/MS-Spectrum of the [M–H]⁻ ion (m/z 263) of* trans-*2-pentenal.* The structure of some characteristic ions is assigned. An abundant *m/z* 163 is typical for an aldehyde, and *m/z* 167 is preferentially formed by unsaturated aldehydes.

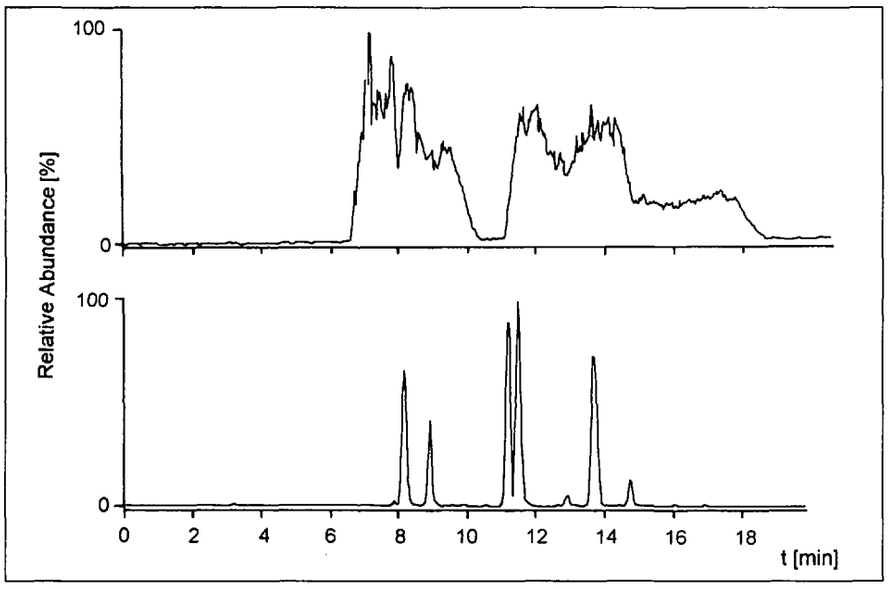


Fig. 8. *ESI(+) Base-peak chromatogram of a mixture of different aconitum alkaloids* (20 ng each, for a structure example, see *Fig. 5*). Top: Signals are disturbed due to adsorption to the surface of the transfer capillary to the MS made from fused silica. Bottom: Addition of 50 mM ammonium acetate to the water phase of the eluent (75 to 100% methanol in water within 10 min) solved the problem.

classify it as an A- or B-trichothecene. More details and applications of this identification procedure are found in [4].

Also for other compound classes, characteristic fragmentation schemes can be established. For example, the APCI(–) fragments of carbonyl compounds formed photochemically in the atmosphere and transferred into 2,4-dinitrophenylhydrazone derivatives during sampling, allow to identify structure elements. In the MS/MS mass spectra of aldehydes, an abundant *m/z* 163 is present (often base ion) while those of ketones show a low intensity. The structure of the *m/z* 163 fragment was identified by ^{15}N-labelling of the hydrazone N-atom and MS^{3-6} experiments.

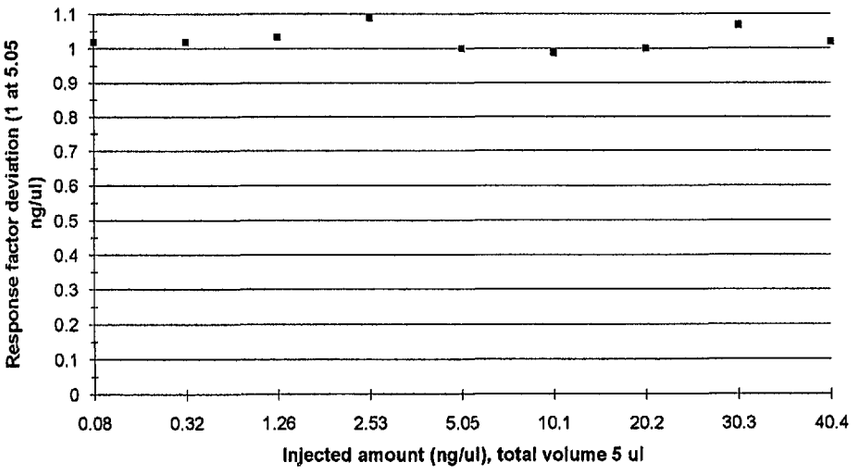
Fig. 7 shows the MS/MS spectrum of the $[M–H]^-$ ion of *trans*-2-pentenal and the structure of some fragments. Aromatic aldehydes, or those with a double bound in α-position, form an abundant $[M–H–47]^-$ fragment but no $[M–H–30]^-$, as saturated aldehydes do. Those having a double bound in α-position show an abundant *m/z* 167 (*Fig. 7*) which is not visible in the MS/MS spectra of aromatic aldehydes. Furthermore, positions of chain branching in aliphatic aldehydes can also be determined. Here too, the structure of unknown carbonyls can be partially or completely elucidated as shown in detail in [3].

Common to all examples of structure elucidation by MS^n is that some reference compounds have to be available to learn something about the structure-specific fragmentation behaviour of a compound class. For totally unknown substances, in most cases, only limited structure information can be obtained at present.

5. Quantification

Quantification by ion-trap MS has had a rather bad reputation. At low compound concentrations, the trap can be filled with background ions which might react further with compound molecules. This, in turn, might lead to non-linear calibration curves. However, compared to the more reactive ions generated by electron ionisation, this is much less a problem with APCI or ESI, since only robust ions of low reactivity are formed which do not easily react or decay further.

Before blaming the mass spectrometer for non-linear calibration curves, one should check whether adsorption effects in the HPLC system are the reason for problems. The sensitivity of ion traps in the full-scan mode allows to detect quantities around 1 ng or less with a good signal-to-noise ratio. At these levels, both



Detection Limits (Signal-to-Noise ≥ 3:1) *of LC-MS only in pg and of the Whole Method in ppb*

	NIV	DON	F-X	NEO	3-ADON	15-ADON	DAS	HT-2	T-2
DL [pg]	120	100	40	60	30	100	20	30	40
DL [ppb]	10	6	3	4	3	10	1	1	3

Recovery Rate in Wheat at a Spike Level of 500 ppb (n = 3) and at the Limit of Quantification (n = 3)

	NIV	DON	F-X	NEO	3-ADON	15-ADON	DAS	HT-2	T-2
500*	80	86	91	106	98	102	102	90	87
QL**	81	87	100	94	96	112	96	88	79

Relative standard deviation: *0.5-5%, ** 2–5.5%

Repeatabilities of the Whole Method at Different Levels (Three to Four Times Extraction and Clean-Up, Each Sample Three Times Quantified)

137 ± 11 ppb (8.3%, *n*=4); 2430 ± 35 ppb (1.4%, *n*=3)

Fig. 9. *Linear range for deoxynivalenol using verrucarol as internal standard, detection limits, repeatabilities and recoveries of a method for the determination of trichothecene toxins in wheat.* NIV, nivalenol; DON, deoxynivalenol; F-X, fusarenon-X; NEO, neosolaniol; 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; DAS, diacetoxyscirpenol; HT-2, HT-2 toxin; T-2, T-2 toxin [4].

the column surface and the transfer capillary might cause substantial compound losses. Such effects are easily observable, causing a deformation of the signal shape. However, a partial irreversible adsorption decreases only the signal height but does not affect the peak form. *Fig. 8* shows the adsorption of low-ng quantities of polar aconitum alkaloids on the surface of the transfer capillary, made from fused silica, between column and mass spectrometer. An addition of millimolar concentrations of ammonium acetate to the eluent saturates active sites and overcomes this problem.

As in GC-MS, quantification should be based on an internal standard added to the sample before extraction/sample clean-up or at a later stage. Together with a recovery standard added just before separation and quantification, this allows to detect losses during sample clean-up as well as to compensate for variable injection volumes and instrument responses. The perfect internal standard is the identical isotope-labelled compound, but in most cases a substance from the same compound class is suitable as well.

A careful method evaluation including elimination of fragmentation mechanisms and matrix interferences, as well as control of the linear range, compound recoveries, and repeatabilities, allows to obtain performance criteria which are comparable to those of GC-MS techniques, as the summary of a method validation in *Fig. 9* shows for the determination of trichothecenes in wheat. Response factors relative to the internal standard did not vary more than ± 10% from 80 pg/µl to 40 ng/µl injected.

6. Conclusions

HPLC-MS Techniques using ion traps are now so sensitive in the full-scan mode that mass spectra with quantities around 1 ng can be recorded. Furthermore, the possibility of on-line MS^n experiments with the same amount of compound allow to identify unequivocally compounds and to elucidate at least partially unknown structures belonging to the same compound class. Furthermore, quantification in the pg range is well achievable if the risk of adsorption in the separation system is minimised. The performance of HPLC-MS with ion traps is now comparable to GC-MS and makes it an attractive tool for the determination of thermolabile and/or polar compounds in organic trace analysis.

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