MASS SPECTROMETRY/MASS SPECTROMETRY AS A TOOL FOR THE IDENTIFICATION AND QUANTITATION OF MYCOTOXINS

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ABSTRACT

Mass spectrometry/mass spectrometry (MS/MS) is a useful technique for the identification and quantitation of mycotoxins at low levels directly from crude extracts with minimal chemical or chromatographic clean-up. In MS/MS the mycotoxin is identified by selecting its molecular ion or other representative ion with the first mass filter and producing daughter ions from the collision of that selected ion with a neutral target gas. The daughters are then mass analyzed by the second mass filter. The resulting daughter spectrum can be used to identify the mycotoxin and determine the amount present in the sample. Because the technique often relies on chemical ionization (CI) and the entire mouldy grain extract is entering the mass spectrometer source, it is possible that this complex mixture may significantly disturb normal CI reaction conditions. This may alter the yield of ions for the small amount of mycotoxin present in the sample. Matrix effects, these deviations from expected response for the target component, can be significant and affect quantitation. Therefore careful selection of standards and control of sample ionization are important parameters for successful quantitative work.

The widespread occurrence of mycotoxins, toxic secondary metabolites produced by fungi, is an important problem both from economic and health perspectives. A great deal of research has been directed toward the development of analytical methodology to identify and quantitate mycotoxins in food and feed. Mycotoxins are a large group of chemically unrelated compounds which are linked only by their production by fungi and some toxic effect in man or animals. Therefore the isolation, identification and quantitation of each individual mycotoxin presents its own unique set of problems. These problems are often compounded by the fact that mycotoxin detection in food or feed is often required in the low part-per-billion concentration range.

Mass spectrometry (MS) is a sensitive and highly selective technique of choice for the identification and quantitation of
target compounds at low levels. In practice, MS is generally coupled with gas chromatography (GC) to separate the target component (or a derivative) from the rest of the sample prior to ionization in the mass spectrometer. However, if the mycotoxin is not amenable to GC/MS, direct analysis by mass spectrometry can be a difficult analytical problem. Because naturally contaminated samples often contain only low levels of mycotoxins along with many other extraneous components, mass spectra of simple extracts of the sample yield spectra with some signal at virtually every mass-to-charge value. These signals, which interfere with the detection of almost any trace component are referred to as "chemical noise". The use of a soft ionization technique such as chemical ionization (CI) helps to minimize noise, but samples must still be extensively cleaned-up before analysis by conventional MS. Figure 1, the isobutane CI mass spectrum of zearalenone, shows the protonated molecule (m/z 319) as the predominant feature of the mass spectrum. Figure 2

is the CI mass spectrum of the extract of a corn sample that is contaminated with 1 part-per-million of zearalenone. A signal is observable at m/z 319 from the protonated molecule of zearalenone, but it is insignificant among the chemical noise. Obviously a clean-up step would be required for acceptable identification of zearalenone by conventional MS. By contrast, mass spectrometry/mass spectrometry (MS/MS) can greatly enhance
detection limits and improved signal-to-noise over conventional MS. First the protonated molecule is selected and separated from the chemical noise with the first mass filter. This ion is then collided with a neutral target gas (usually argon) in a collision cell to produce daughter fragments. The daughters can be mass analyzed by the second mass filter, and the resulting daughter spectrum can be used to identify the mycotoxin. Figure 3a shows the daughter spectrum from m/z 319 in the zearalenone contaminated corn extract while figure 3b shows the daughter spectrum from pure zearalenone. These two spectra are virtually identical. The detection limit for zearalenone in corn by this method.
is less than 100 ppb (Plattner and Bennett, 1983). The experiment is rapid, requiring only about 2-3 minutes per analysis.

Unlike zearalenone, aflatoxins are not easily analyzed by GC/MS. Therefore mass spectrometry has not been as useful as HPLC and TLC for the detection of aflatoxins because of the extensive clean-up needed for MS. Negative CI/MS under resonance capture conditions has been suggested as the method of choice to confirm aflatoxin B1 replacing the bioassay (Nesheim and Bruml, 1981). Negative CI was selected because many of the matrix contaminants do not respond in the resonance capture mode and are transparent to the analysis. Still the method requires clean-up by two dimensional TLC for positive confirmation of aflatoxin B1 at the 20 ppb level. Figure 4 shows the negative CI spectrum of aflatoxin B1.

Fig. 4. Negative Chemical Ionization/MS

one of the test samples used in the collaborative study of this method. This sample is contaminated at 20 ppb with aflatoxin B1. It has undergone clean-up by the AOAC CB method (Official Methods of Analysis, 1980). The noise in the spectrum is much more intense than the signal from the aflatoxin. Pure aflatoxin B1 is also shown on this figure. To actually confirm aflatoxin B1 in this sample, it must be further purified by two dimensional TLC before mass spectrometry. An alternative is to
use a MS/MS daughter experiment (Plattner, Bennett and Stubblefield, 1984). The molecular anion of aflatoxin B1 (m/z 312) is selected by the first mass filter. It is decomposed in the collision cell with argon collisions and the resulting daughters are mass analyzed by the second mass filter. This experiment (figure 5) allows the aflatoxin B1 to be identified directly from the sample without the TLC step.

**MS/MS Daughters of m/z 312**

![MS/MS Daughters of m/z 312](image)

Fig. 5.

The principle use of mass spectrometry in the analysis of mycotoxins generally has been as a tool to definitively identify a mycotoxin and/or confirm its presence in some sample matrix. The preceding two examples illustrate the power of MS/MS to reduce signal-to-noise, allowing unambiguous identification of target mycotoxins in much cruder samples than possible by conventional MS. MS/MS can also provide valuable quantitative information. However, reliable quantitative data requires careful control of instrumental parameters. Because the entire mouldy grain extract, a complex milieu, may be put into the mass spectrometer source, it is possible that operating conditions will deviate significantly from normal CI conditions. This may alter
the yield of ions for the small amount of mycotoxin present in
the crude sample from that observed without the presence of the
complex extract. This deviation from expected response for a
target component has been called a matrix effect. In the absence
of careful work with spiked blank samples or suitable internal
standards, matrix effects can lead to erroneous results because
the target compound is not behaving the same in the crude samples
as it is with the standards.

One of the advantages of CI is that it usually produces
relatively simple spectra with most of the information contained
in a protonated molecular ion (MH+). Some structural information
is often also present from fragmentation. The extent of fragment-
ation can be quite variable because it is controlled by the
exothermicity of the reaction between the sample molecule and the
reactant gas. Nonselective reactant ions are present in many CI
gas plasmas. The concentration of these ions can be quite
variable with changes in source temperature and pressure.
Furthermore, the presence of water vapor in the source can change
the composition of the major reactant ions giving H3O+ leading to
significant changes in the amount of fragmentation. These
changes can significantly lower the yield of the parent ion that
is selected for a NS/NS experiment causing a negative matrix
effect (Kallos, et al., 1982). If this loss is not corrected by
a stable isotope labeled analog, quantities of mycotoxin can be
underestimated if crude samples are referenced to pure standards,
not spiked blanks.

We have identified a second matrix effect. This time in the
positive direction when compounds with strong electron capture
cross sections enter the source, the lifetime of positive ions in
the source is increased. (Rudewicz and Munson, 1984) The mag-
nitude of this electron capture effect varies with the analyte and
the source conditions. This positive matrix effect is illus-
trated in figure 6. Methyl stearate is admitted into the CI
source with the standard probe. The probe was not heated and the
methyl stearate level in the source remained approximately
constant throughout the experiment. One hundred ng of ethylene
dibromide (EDB) was injected into the source through the
capillary gas chromatograph inlet. When the EDB eluted, even
though the amount of methyl stearate was constant, the yield of
the MH+ ion at m/z 299 was vastly increased. After the EDB
that the sample may have a component with a high electron capture cross section. This will cause an increase in the yield of the positive parent ions for a target mycotoxin and may lead to an over estimate of its level.

The best method to minimize matrix effects is to incorporate internal standards for quantitative work. Stable isotope labeled analogs of the target mycotoxins will give the most reliable corrections from matrix effects. Related compounds may or may not react the same as the target compound to matrix effects. Unfortunately, stable isotope labeled internal standards for many important mycotoxins are not available. In these cases one must maintain careful control of sample size and matrix composition and monitor quantitative work through the use of spiked blanks and samples. With these procedures, useful quantitative results can be obtained.

For example, in the case of aflatoxin B1 contamination, the "CD" clean-up method was used and the analysis aliquot was limited to the extract equivalent to 0.2 mg of contaminated corn or less. Good recoveries and linear response over levels ranging from 0.5 to 1000 pph were obtained (figure 7). This data is from selected reaction monitoring of the decomposition of the negative molecular anion (m/z 312) to the phenoxide anion (m/z 297).
Signal-to-noise at 0.5 ppb was about 5 to 1. The coefficient of variation was typically about 20–25%. In a preliminary experiment, the addition of aflatoxin G1 as an internal standard did not markedly improve the precision of the analysis.

Another class of important toxic compounds that are of interest as mycotoxins but not readily amenable to gas chromatography are the ergopeptine alkaloids that are produced by the fungus *Claviceps purpurea* and associated with ergotism. The ergopeptine alkaloids are a family of a dozen tripeptide amides of lysergic acid with differing substituents. These alkaloids are difficult to separate and analyze by conventional methods.

Tall fescue is grown extensively in the United States as a pasture for cattle. Although the grass has many desirable agronomic features, it is frequently toxic to cattle. The most severe form of the toxicity, fescue foot, is a gangrene of the animal's extremities. These symptoms are strikingly similar to ergotism, but they occur in the absence of *Claviceps* ergot. Tall fescue is often infected by an endophytic fungus (*Epichloë typhina* or *Acremonium coenophialum*) also a member of the family Clavicipitaceae. A family of ergopeptide alkaloids cause the symptoms of ergotism in animals. The endophyte has been shown to produce these compounds in culture, but they had not previously been demonstrated present in fescue grass samples.
This class of compounds produces intense negative ion spectra with detection limits below 1 pg (Plattner, Yates and Porter, 1983). The tripeptide anion, the major ion in the negative CI spectrum, still contains both sidechains that differentiate members of the family. MS/MS daughter experiments on this ion undergo losses that completely identify the compound. Ergopeptide alkaloids are easily detected in simple chloroform extracts of fescue samples at the part-per-billion level by MS/MS. Full scan MS/MS daughter spectra that are virtually identical to those from standards can be obtained from aliquots of extract equivalent to 1 mg of plant containing 10 picograms of alkaloid. Using selected reaction monitoring, the detection limit for the major daughter fragment is near 50 fg.

The profile of members of the ergopeptide alkaloids present in ergot and endophyte infected fescue plants is significantly different. In ergot sclerotia and ergot infested plant extracts, a major alkaloid is ergotamine. It is not detected in endophyte infected plant extracts. Therefore ergotamine could be spiked into fescue samples and used as an internal standard to monitor recoveries and help identify any matrix effect problems. Endophyte free plants contain no detectable alkaloids, while in infected plants the levels range from a few ppb's in lightly infected pastures (<5%) to a few ppm's in toxic fields that are highly infected.

CONCLUSIONS

The above examples demonstrate that MS/MS is well suited to the analyses of mycotoxins, especially when the mycotoxins are not amenable to GC/MS. The ability of the MS/MS technique to remove "chemical noise" makes possible simple MS/MS analyses of crude or partially purified extracts of suspected mycotoxin-contaminated samples without the rigorous purification needed for conventional mass spectrometry. It is this sample clean-up requirement, a completely different problem for each mycotoxin and matrix, that has limited the applicability of mass spectrometry as a primary quantitative tool for mycotoxin quantitation. If suitable internal standards are not available, care must be exercised over the conditions of analysis, but useful quantitative data can be obtained. Even with mycotoxins that are amenable to GC/MS, the speed of analysis by MS/MS can significantly increase sample
throughput. MS/MS analyses for the presence of a mycotoxin, accomplished by direct-probe introduction of an aliquot of a crude extract, typically take only a minute or two. The ability to achieve both high sample throughput and highly definitive toxin identification can make MS/MS a cost-effective approach compared to other techniques, in spite of its high initial cost.

REFERENCES


