Tetrahedron Vol. 45, No. 8, pp. 2251 to 2262, 1989
Printed in Great Britain.

0040-4020/89 $3.00+.00
Pergamon Press plc

Analysis of Trichothecene Mycotoxins by Mass Spectrometry and Tandem Mass Spectrometry

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(*Received in South Africa* 16 *June* 1988)

abstract

To illustrate tandem mass spectrometry (MS/MS) as an alternative to GC/MS for the analysis of trichothecenes in solid and liquid cultures, diacetoxyscerpenol (DAS) levels were measured by GC/MS and by three MS/MS protocols. GC/MS had the lowest detection limit and better precision than the MS/MS procedures; however, GC/MS required 20-30 minutes per analysis. MS/MS was more rapid, requiring less than two minutes per analysis. DAS could be measured accurately at low nanogram levels directly from filtrates of liquid cultures. Extraction of culture filtrates simplified the identification by effectively removing an interfering matrix component which had an ion at the same m/z as the protonated DAS molecule. Use of the GC as an inlet for MS/MS was an effective way to minimize physical manipulations necessary for sample introduction. All three MS/MS procedures had similar detection limits and precisions. Occurrence levels of three trichothecenes, T-2 toxin, neosolaniol, and DAS were measured in a single analysis by sequential selection of parents for each trichothecene.

INTRODUCTION

Historically, mass spectrometry (MS) has rarely been used for analysis of trichothecene mycotoxins. This was due, in part, both to various technical difficulties in applying the technique to trichothecenes and to the relatively high cost of obtaining and maintaining the necessary instrumentation. However, the past decade has witnessed a rapid improvement in instrument capabilities and significant reductions in the cost of obtaining and supporting a mass spectrometer. Today MS is often the technique of choice for the identification and quantitation of trichothecene mycotoxins. MS offers the high selectivity and sensitivity required to identify and quantitate target mycotoxins at the levels in which they often occur.

Analysis of trichothecenes by MS is complicated by the fact that these mycotoxins are often only trace components in a complex sample matrix. When introduced into the mass spectrometer, extraneous constituents in the sample produce interfering signals in the mass spectrum; these signals are called "chemical noise". In most cases, chemical noise, not sensitivity, is the limiting factor in the analysis of a particular mycotoxin by MS. Therefore, in practice, MS is generally coupled with gas chromatography (GC) in order to separate the target mycotoxin, or a derivative, from the rest of the sample prior to ionization in the mass spectrometer.

An alternative method of reducing chemical noise, when a mycotoxin is

not amenable to GC, is tandem mass spectrometry (MS/MS). MS/MS has rapidly gained acceptance in the analytical community over the past few years, and instruments with MS/MS capabilities are now available in many laboratories. The MS/MS technique had its roots in structure elucidation problems and in studies of ion structure using metastable decompositions. However, the rapid rise in popularity of MS/MS has been primarily due to recognition that the technique is particularly well suited for providing sensitive and selective analyses of complex mixtures. There are several excellent reviews describing MS/MS and its potential for the determination of trace components in complex mixtures (1-5).

In a typical MS/MS procedure, a crude mycotoxin-containing sample is introduced into the mass spectrometer source and ionized from a solids probe. Mass spectra of all components present in the crude sample are thus generated simultaneously. The mycotoxin of interest is separated from most other components in the sample matrix by selection of a structurally significant ion of the target compound with the first mass filter. This selected ion is then induced to decompose by collision with a neutral target gas (usually argon) in a collision cell. Daughter ions produced by these conditions may then be mass analyzed with the second mass filter, and the resulting daughter spectrum can be used to identify or to quantitate the target mycotoxin.

The signal recorded for a MS/MS daughter ion is inherently of lower intensity than would be recorded for the parent ion in conventional MS, however, interference from chemical noise in the MS/MS scan is generally greatly reduced. Because the detection limit for MS analysis with the solids probe is often limited by chemical noise, overall sensitivity, defined as signal-to-noise ratio for the analysis, is frequently improved using MS/MS. This is particularly true in cases where the mycotoxin in question is only a trace component in the sample analyzed. Detection of the mycotoxins zearalenone and deoxynivalenol at sub part-per-million levels in Fusarium-infected grains using probe inlet MS/MS has been reported (6). The only sample clean-up involved in the analysis was a solvent extraction of the grain sample. Similarly, aflatoxins have been detected in simple grain extracts at the low part-per-billion level (7).

Three basic elements are common to all quantitative MS analyses; these are sensitivity, selectivity and time required for completion of the analysis. Tandem MS experiments offer the analyst a great deal of freedom in selecting from among many possible ways of performing a targeted analysis and allows the analyst to optimize any specific analysis with respect to these three elements. In developing an MS/MS targeted analysis the operator makes selective trade-offs between these three elements to obtain an acceptable blend of speed, sensitivity and selectivity appropriate to the problem at hand. In this paper, selected mycotoxin analysis problems from the authors' laboratory are used to illustrate available choices and how the variables stated above can affect each analysis.

RESULTS AND DISCUSSION

Studies of trichothecene biosynthesis in our laboratory encompass several approaches, which require rapid methods to measure trichothecene levels produced in liquid cultures of several Fusarium species. One approach uses a genetic system for Gibberella pulicaris (Fusarium sambucinum) to investigate the inheritance of trichothecene production, as well as other traits (8). A second approach has been the generation of UV-induced mutant strains that are altered in their ability to biosynthesize trichothecenes (9). Both of these approaches have created a need to obtain information about trichothecene levels present in large numbers of samples. A preliminary study was conducted in which we evaluated several MS-based methods for measuring trichothecene levels in fungal liquid cultures, in order to select appropriate analytical methods for use in these studies.

Several MS- and MS/MS-based analytical protocols were examined in a study of diacetoxyscerpenol (DAS) production by strain MB1716. MB1716 is a mutant strain of Fusarium sporotrichioides NRRL 3299 that has been altered in its ability to produce T-2 toxin and, instead, accumulates diacetoxyscerpenol (DAS) (9). Trichothecene levels were determined by conventional GC and by GC/MS. Three tandem MS protocols were examined. First, trichothecenes were measured by a direct MS/MS analysis of one microliter of culture filtrates without sample work-up. Secondly, the culture filtrates were extracted with ethyl acetate, and aliquots of the ethyl acetate extracts equivalent to one microliter of the initial culture filtrate were analyzed by direct MS/MS. Thirdly, samples were measured by a rapid GC/MS/MS procedure.

In our studies with solid and liquid cultures of Fusarium species it should be noted that trichothecenes were often produced at much higher levels than are usually encountered in naturally contaminated grain samples that are only partially infected with the fungus. Furthermore, the culture medium may be a relatively simple mixture in liquid cultures. The fungus may still produce a complex array of primary and secondary metabolites, but trichothecenes are found almost exclusively in the culture media and are easily concentrated by filtration from the fungal mycelia. These factors greatly simplify analytical requirements for trichothecene analysis of liquid cultures. Thus, it is likely that analyses of liquid cultures, solid cultures, and naturally contaminated grain samples will have different requirements, which will lead the analyst to different choices for each specific analysis.

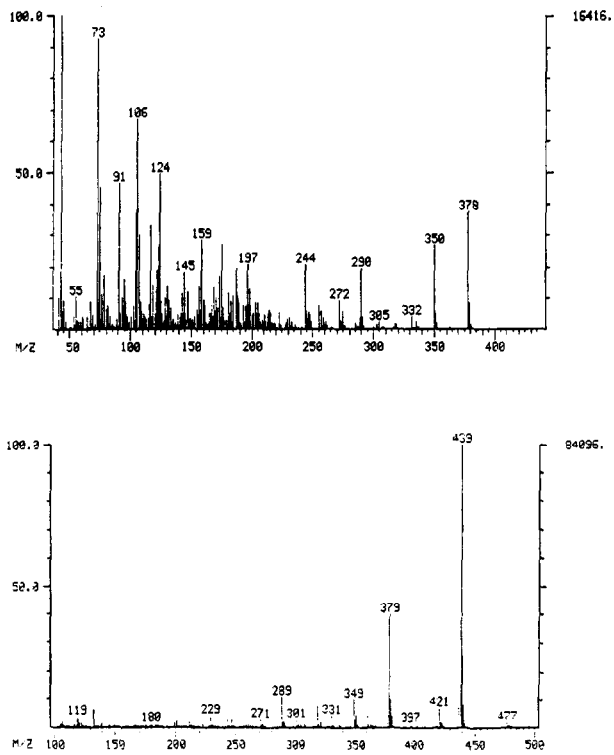
GC AND GC/MS OF DERIVATIZED TRICHOTHECENES

Most naturally occurring trichothecenes have one or more free hydroxyl groups and, for best GC results, samples should be derivatized prior to analysis. Conversion of free hydroxyl groups to trimethylsilyl (TMS) ethers generally results in compounds of suitable stability and

chromatographic properties. TMS ethers of trichothecenes may be analyzed by GC and detected with a flame ionization detector. Trichothecene derivatives are easily detected at low nanogram levels when injected onto a modern gas chromatograph equipped with a fused silica capillary column. The limits of detection, however, are affected by the complexity of the sample matrix. Since the flame ionization detector is a universal detector, other matrix components may elute from the GC near the elution time of the target compound and these components can severely interfere with positive identification of the various trichothecenes.

By coupling the outlet of the GC column to a mass spectrometer, each component produces a multidimensional response, a mass spectrum, rather than the one-dimensional intensity response of conventional GC detectors. Thus GC/MS is superior to GC in its ability to deal with interference from sample background. Electron impact (EI), the traditional ionization mode for MS, produces fairly complicated spectra for trichothecenes. Figure 1a shows the EI mass spectrum of the TMS derivative of DAS. No molecular ion is observed at m/z 438. The greater proportion of ions produced are the less diagnostically useful lower mass fragments. The three most useful higher mass fragments are m/z 378 (M-60), m/z 350 (M-60,-28), and m/z 290; these three ions, combined, carry only about 5% of the total ion intensity.

Figure 1. Electron ionization (a) and chemical ionization (b) mass spectra of the TMS derivative of DAS.



For identification of unknown components, complete mass spectra are usually recorded. However, several approaches are possible for quantitative analyses. Full scans may be acquired and the total mass spectrum at the appropriate GC retention time can be compared with a standard spectrum for positive identifications. This approach provides the greatest certainty for positive identifications. Because the mass spectrometer is continually scanned across the entire mass range for this method, only a small portion of the total analysis time is actually spent measuring the most important mass-to-charge ratios (m/z 290, 350, and 378 for DAS); therefore, this method is relativly low in sensitivity. Many mass spectrometers can also operate in the selected ion mode (SIM). In the SIM mode, the mass spectrometer only measures signals at selected m/z values. The SIM technique improves sensitivity of ion abundance measurements (typically by a factor of 50 to 100) but the procedure also has its negative aspects. In SIM, instead of a complete mass spectrum for identity confirmation of the mycotoxin, only relative intensities of the selected ions are available. It is debatable how many correct ion ratios are necessary to positively identify a component by GC/MS, but three ions are generally regarded as a sufficient number (10).

Usable full-scan EI spectra of TMS derivatives of deoxynivalenol, DAS, neosolaniol and T-2 toxin require 1-20 ng of the compound. In the SIM mode, DAS can be positively identified and quantitated in the 50-100 pg range. Liquid cultures of Fusarium sporotrichioides mutant MB1716 accumulate DAS in the medium. GC/MS analysis of an aliquot of derivatized ethyl acetate extract equivalent to 1 microliter of culture filtrate gave reasonable full-scan spectra for DAS at levels down to a few ng/ul. DAS was quantitated by using the response of three ions, m/z 378, 350, and 290 and a calibration curve prepared from injections of known amounts of pure DAS-TMS derivative. The use of SIM to improve sensitivity was not required. Typical coefficients of variation obtained with this procedure were on the order of 7-15% and DAS levels down to a few ng/µl could be routinely quantitated. Interference from the matrix increased considerably with extracts of solid cultures and was dependent on substrate. This increase in chemical noise resulted in poorer full-scan performance. Injection of an aliquot equivalent to 10 mg of solid culture using SIM (m/z 378, 350, and 290) provided routine quantitation of DAS down to the 10 ng/g level.

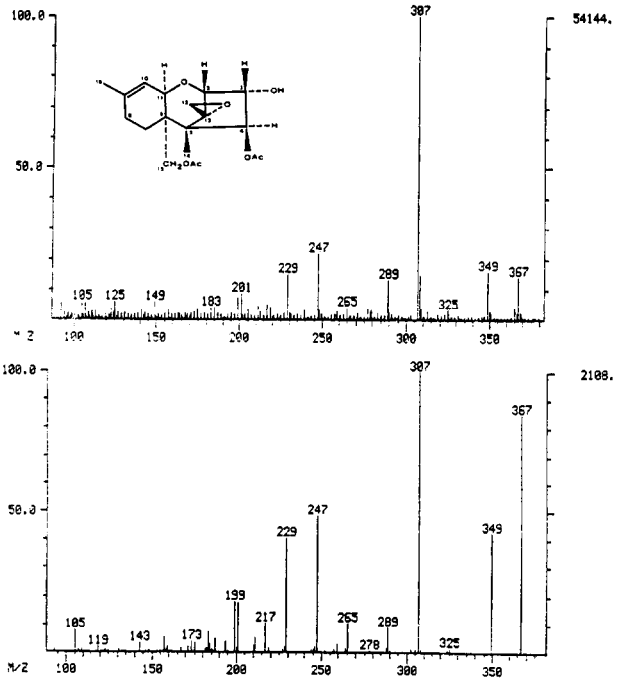
Chemical ionization (CI) is an alternate form of ionization available on many mass spectrometers. CI is a softer ionization mode that is accomplished by collision of sample molecules with a reagent gas ion; this procedure protonates many organic molecules. For many compounds, little or no fragmentation is observed. However, trichothecenes do produce varying amounts of fragmention in CI depending on their structures. Most fragments arise from loss of substituents as neutral molecules; but a substantial portion of the total ion current is normally carried by the protonated molecule (MH^+). Figure 1b shows an isobutane CI spectrum of the TMS derivative of DAS. The MH^+ at m/z 439 and the fragment ion at

m/z 379 account for most of the ion current. Since CI is a softer ionization mode, chemical noise from the matrix also produces fewer fragments and, therefore, is often a less severe problem. Total ion responses for CI and EI are approximately equivalent but, since a greater portion of the total ions generated in CI are the more diagnostic higher mass fragments, detection limits for trichothecenes are somewhat better. Overall performance, equivalent to or better than EI, can easily be achieved with CI. With solid culture samples, CI identification and quantitation of DAS in the full-scan mode can be accomplished at levels that require SIM for measurement in the EI mode.

MS/MS OF CULTURE FILTRATES

Tandem MS has the potential to greatly reduce times required to conduct analyses. GC/MS provides a sensitive method for analysis of trichothecenes, but its cost is time. High resolution separation of sample components by capillary GC requires from 20-30 minutes per analysis. In contrast, samples may rapidly be inserted into the mass spectrometer, when analyses are made using the solids probe, and total sample analysis times are on the order of a minute or so.

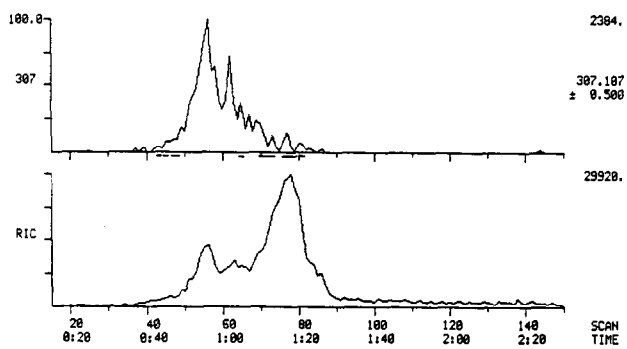
Figure 2. Chemical ionization mass spectrum of DAS (a) and its MS/MS daughter spectrum of m/z 367, the protonated molecule (b).



Trichothecenes produce abundant MH^+ ions in CI that can be used in MS/MS experiments. For example, DAS can be determined in liquid cultures of MB1716 by direct MS/MS analysis of an aliquot of culture filtrate. Figure 2a shows the CI mass spectrum of DAS and figure 2b is the tandem MS daughter spectrum of the protonated DAS molecule (m/z 367). Intense daughters are observed from the loss of water (at m/z 349), and acetic acid (at m/z 307). Additional fragments are observed from consecutive losses of combinations of water and acetic acid as neutral molecules from the parent ion. This daughter pattern is quite similar to the fragmentation observed in the CI spectrum of DAS itself.

For tandem MS analysis, a 1 µl aliquot of culture filtrate is placed in a probe vial, evaporated to dryness, inserted into the mass spectrometer and then ionized. The first mass filter is set to pass only m/z 367, the parent ion of DAS. These mass-selected ions are then collided with argon (1 mtorr and -20V), and the resulting daughters are mass analyzed by Q3. Scanning Q3 from m/z 90 to 400, once each second, produces a profile of eluting daughters of 367 as the probe is heated. Figure 3b shows the total ion profile obtained from a 1 µl aliquot of culture filtrate from a four day old culture of MB1716. The profile of the selected daughter (m/z 307) shown in figure 3a elutes at about the same time (temperature) as a pure sample of DAS.

Figure 3. MS/MS elution profile of daughters of m/z 367 from 1 microliter of culture filtrate from four day old culture of strain MB1716. A) response of m/z 307 daughter. B) total daughter ion response.



A full daughter scan obtained at this time (figure 4) is virtually identical with a daughter spectrum from the pure standard (figure 2b), while the full daughter scan of later eluting material (figure 5) is quite different. M/z 307 is not an intense daughter fragment in this spectrum. The latter is the daughter spectrum of an unknown component of the culture filtrate that also has an ion at m/z 367 in isobutane CI. This component is fortuitously less volatile than DAS and was separated from DAS by fractional distillation from the solids probe. DAS may be quantitated by measuring the intensity of the m/z 307 daughter signal and comparing it to standards.

Figure 4. MS/MS daughter spectrum of scan 54 of analysis shown in fig. 3.

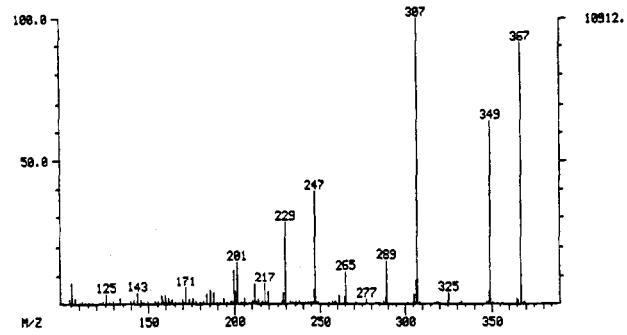
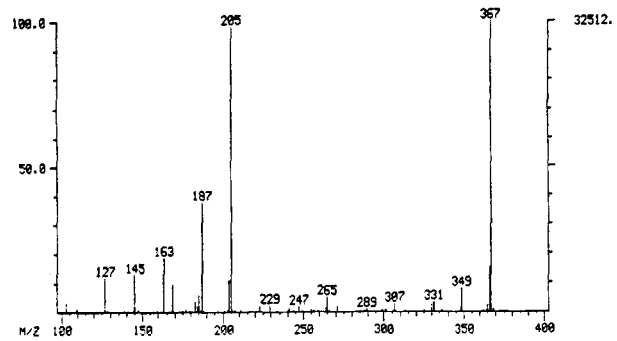


Figure 5. MS/MS daughter spectrum of scan 78 of analysis shown in fig. 3



MS/MS OF CULTURE FILTRATE EXTRACTS

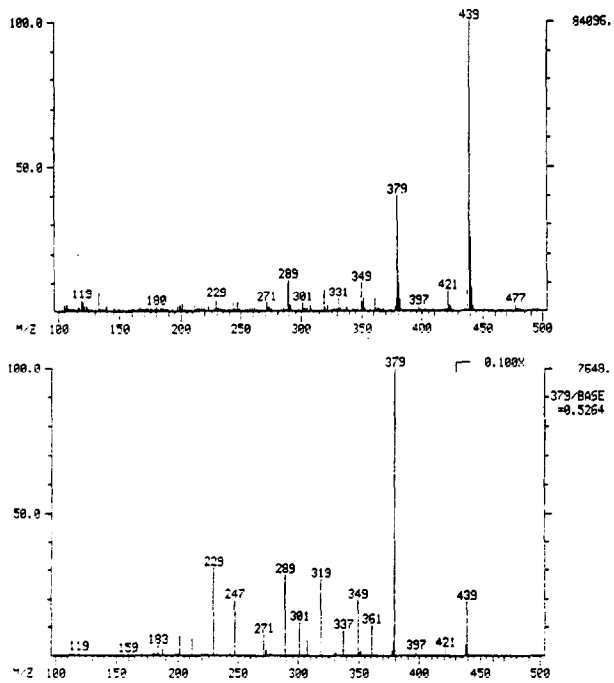
Ethyl acetate extraction of culture filtrates effectively removes most of the later eluting component because this contaminant is not efficiently extracted into ethyl acetate. This simple purification step simplifies the analysis. Response for DAS using this MS/MS procedure was lower than that obtained by probe MS, both because ion transmission in MS/MS is not 100% and because the parent selected (MH^+, m/z 367) represented only about 10% of the ions generated in the source for DAS. Full-scan daughter spectra required about 50-100 ng of DAS. Sensitivity can be improved in MS/MS experiments by measuring only selected daughter ions rather than obtaining full daughter scans. This procedure, called selected reaction monitoring (SRM), is analogous to SIM in conventional MS; it is more specific because both a parent and a daughter ion are defined. With SRM of the parent (m/z 367) and daughters at m/z 349,307,289,247, and 229; daughter spectra with good signal-to-noise and the proper ratios of all daughters were obtained at the 10 ng level. This method was sufficiently sensitive to detect DAS in liquid cultures of MB1716 after 24 hours incubation (i.e. when toxin accumulation had just begun) using only a 1-2 µl aliquot of culture filtrate extract.

GC/MS/MS OF DERIVATIZED CULTURE FILTRATE EXTRACTS

MS/MS analysis of culture filtrate extracts using the solids probe is simple and rapid enough to be useful for analysis of large numbers of samples but analysis of samples using the solids probe is fairly tedious. The operator must place each sample vial on the probe, mount it, evacuate the inlet, open a valve, slide the probe into the source, heat the sample, cool the probe, slide it out and close the valve for each sample (one analysis requires about two minutes). A sample inlet system that requires fewer physical manipulations would greatly simplify this procedure. One alternative is use of the GC as the sample inlet system for MS/MS. Since MS/MS is being used to detect DAS, complete separation on the column is not an important factor for identification and, therefore, short columns and higher temperatures can be used to introduce samples into the mass spectrometer source rapidly.

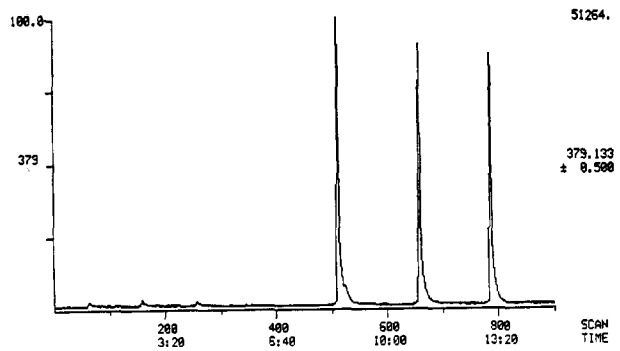
DAS could be introduced via the GC into the mass spectrometer for MS/MS without derivatization, however, reproducability problems and losses were encountered in preliminary experiments with underivatized T-2 toxin and neosolaneol. Therefore, TMS derivatives were chosen for MS/MS experiments using the GC inlet in order to avoid problems. Figure 6 shows the CI spectrum of DAS (6a) and the daughter spectrum of the MH^+ (6b).

Figure 6. MS/MS daughter spectrum of m/z 439, the protonated molecule of the TMS derivative of DAS.



As in the daughter spectrum of underivatized DAS, the major daughter arises from a neutral loss of acetic acid and the daughter spectrum closely parallels the CI spectrum. Response for the TMS derivative in the MS/MS experiments was slightly better than for underivatized DAS because the parent, m/z 439, carried a higher percentage of the total ionization. Acceptable full scan daughter spectra were obtained on 20-50 ng of sample. SRM of m/z 439 and daughters at m/z 379,319,289, and 229 gave the proper daughter ratios from injections of a few nanograms. Figure 7 shows typical signals for the m/z 379 daughter of the protonated DAS molecule (m/z439) for three replicate injections of 25 ng and 250 ng. The signal to noise ratio for the 25 ng injection was greater than 30/1. SRM conditions may be modified to measure only the m/z 439 parent to m/z 379 daughter; this lowered the detection limit about an order of magnitude, but at the expense of selectivity.

Figure 7. MS/MS response for the m/z 379 daughter of the parent m/z 439 for six injections of DAS (3x 25ng and 3x 250ng).



Data presented above was for the detection of one trichothecene, DAS, in cultures of the mutant MB1716. However, MS/MS may also be used to analyze several different trichothecenes simultaneously. Fusarium sporotrichioides, NRRL 3299, the parent strain from which MB1716 was derived, produces high levels of T-2 toxin, as well as lower levels of DAS and neosolaniol. Cultures of NRRL 3299 may be analyzed for these three trichothecenes in a single analysis by alternatively selecting each of the protonated molecules as parents for MS/MS. In chemical ionization MS, neosolaniol and T-2 toxin (underivatized and as the TMS derivatives) both fragment much more than DAS. Therefore, the yield of protonated molecules for the MS/MS scan, and thus the response for these trichothecenes, are about 10 fold less when the protonated molecule is selected for the MS/MS scan. None-the-less, MS/MS provided a useable method for simultaneous analysis of these three trichothecenes in liquid cultures of NRRL 3299 with detection limits and precisions similar to those obtained for DAS in MB1716.

STRENGTHS AND LIMITATIONS OF MS/MS vs. GC/MS

MS/MS can be a more rapid alternative to GC/MS, however, several limitations must be kept in mind. First, because of the possibility that the ion selected in the first mass filter may arise from components other than the target compound, a MS/MS procedure must be shown to be valid in the matrix analyzed. Identifications are most reliable when full daughter spectra are recorded, and when they are identical to the daughter spectra of standards. Daughter ion(s) selected for quantitation must be specific for the target mycotoxin. Care must be exercised when selecting an ion other than the protonated molecule for a MS/MS daughter scan to insure that the parent fragment is unique to the target trichothecene and that it is not a common structural fragment of other related trichothecenes or matrix components. When compounds are quantitated by MS/MS it must be remembered that the conditions of ionization used are far from ideal. A relatively large amount of matrix is introduced into the ion source and one must establish that the parent ion selected for the target component responds the same way in the presence of this sample matrix as it did in the reference standard, or internal standards must be added for reliable quantitation.

MS/MS experiments provide a rapid analysis method for DAS in liquid culture samples. DAS can be separated from the matrix in the mass spectrometer and the time consuming full GC separation is not necessary. Analysis of aliquots equivalent to 1-5 µl of cultures, by either direct analysis of culture filtrates or of ethyl acetate extracts, was found to provide reliable quantitative data compared to external standards. MS/MS results agreed well with other methods of analysis. Coefficient of variation for MS/MS analyses using external standards was slightly poorer (typically about 20-30%) than was observed with GC/MS. Under these MS/MS conditions, internal standards were not necessary for accurate quantitative data, but the addition of an internal standard could significantly improve precision. The external standard procedure is suitable for a rapid screening type method requiring only 1-2 minutes per analysis.

Mutants were selected from NRRL 3299, in part, because a competitive inhibition enzyme-linked immunoassay (CIEIA) was available to detect mutant cultures in which there were significant changes in the level of T-2 toxin biosynthesis (9). Cultures were screened for altered T-2 toxin production by growing small liquid cultures in microtiter plates and using CIEIA. The MS/MS procedure is more flexible as it is capable of measuring trichothecenes, other than T-2, that are not detected by CIEIA. Our rapid tandem MS procedure is a potential alternative screen for mutants which have altered biosynthesis of trichothecenes for which no antibodies are available.

Isomeric compounds, such as monoacetoxyscerpenol (MAS) isomers can present problems for MS/MS. The three possible MAS isomers all have identical molecular weights and all show the same daughter fragments on

CID of the protonated molecule. Their spectra differ only in relative intensities of the daughter ions. Therefore, these isomers must be separated, prior to ionization, in order to reliably measure levels of these compounds when the occur in the same sample.

Design of a quantitative MS/MS analysis involves choices of conditions for sample introduction, ionization, and mass analysis to detect and measure target compounds at predetermined levels in a specific matrix. If these choices are made carefully and correctly, MS/MS can be a rapid and flexible alternative to GC/MS for the analysis of trichothecenes.

EXPERIMENTAL

MS and MS/MS procedures. All MS and MS/MS experiments were performed on a Finnigan TSQ-46 triple quadrupole mass spectrometer. In chemical ionization, isobutane was the reagent gas at a pressure of 0.25 torr. Source temperature was maintained at 100C and electron energy was 70 eV. Argon was the target gas in the Q_2 collision cell for CID experiments. The pressure in the collision cell was approximately 1 mtorr as measured by the Hastings gauge. The collision energy was set at either -12 or -20V. Samples were introduced either via the direct insertion probe, which was heated by the ballistic ramp heater or via the gas chromatograph. For GC/MS/MS, either a 3 foot x 2mm packed column coupled to the mass spectrometer via a glass jet separator, or a six foot DB-1 fused silica capillary directly coupled to the source, was used to inlet the samples. Samples were injected, splitless, onto the column at 250 C.

Cultures, media and culture conditions. Cultures were maintained on V-8 juice agar (11) slants at 4C and as conidial suspensions in 10 to 15% glycerol at -70C. Liquid cultures were inoculated at a starting density of 0.5 x 10^5 to 1 x 10^5 conidia per ml of media [YEPD-5G (9)] with conidia washed from strains grown on V8 agar plates for 7 to 10 days. Liquid cultures were grown in Erlenmeyer flasks in a volume of medium equal to one-half the volume of the flasks for 6 to 7 days at 28 C on a rotary shaker operated at 180 to 200 rpm. At selected intervals, aliquots of the cultures were removed and filtered. Filtrates were stored at -20C until analyzed, or extracted with ethyl acetate (equal volumes, 2 times, combined) and stored until analyzed. For GC analyses, portions of ethyl acetate extracts were cleaned up using a charcoal column procedure (8). The clean-up procedure was omitted for GC/MS analyses. For preparation of TMS-derivatives a fraction of the sample equivalent to a 1-ml portion of the original sample was evaporated to dryness at 80C under nitrogen, reacted with 100 µl of trimethyl silylating reagent (Tri-Sil/TBT; Pierce Chemical Co., Rockford, Ill) for 1 hour at 80C, and brought to 1 ml by addition of 900 µl of hexane.

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