A new GC-MS method for the analysis of ascaulitoxin, its aglycone and 4-aminoproline from culture filtrates of *Ascochyta caulina*

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Received 7th September 2009, Accepted 14th November 2009
First published as an Advance Article on the web 1st December 2009
DOI: 10.1039/b9ay00162j

A new GC-MS method was developed for the analysis of ascaulitoxin, its aglycone, and 4-amino-D-proline, which are phytotoxins with potential herbicidal activity produced by *Ascochyta caulina*. The method involved directly treating the lyophilized culture filtrate with a derivatizing reagent, converting the mixture of toxins in the filtrate to their corresponding trimethylsilyl derivatives, and consequent analysis by EI-MS. The method is rapid, sensitive and highly specific for the identification and analysis of the toxins in a complex sample matrix. Analysis of culture filtrates using this method suggested that phytotoxicity correlates with the level of ascaulitoxin in the culture filtrate. A new method for the purification of 2,4,7-triamino-5-hydroxyoctandioic acid, the aglycone of ascaulitoxin, is also described.

Introduction

Ascochyta caulina (P. Karst) v.d. Aa and v. Kest, is a promising biological agent for the control of *Chenopodium album*,[1] known as lambsquarter or fat hen, which is a common weed of arable crops worldwide, such as beet and maize.[2] *A. caulina* produce in liquid culture hydrophilic low molecular weight phytotoxins: ascaulitoxin, its aglycone and 4-aminoproline. These toxins have been chemically characterized by spectroscopic methods as the β-D-glucopyranoside of 2,4,7-triamino-5-hydroxyoctandioic acid (**1**), the acid itself (**2**) and *trans*-4-amino-D-proline (**3**) (Fig. 1), which are all non-protein amino acids.[3–5]

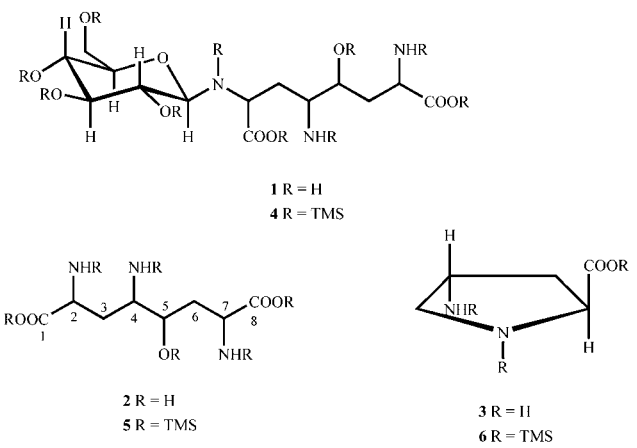

Fig. 1 Structures of ascaulitoxin (**1**), its aglycone (**2**) and 4-amino-D-proline (**3**), and their corresponding trimethylsilyl derivatives (**4–6**).

The relative stereochemistry of **1** was previously determined by NMR configurational analysis based on the observed homo ($^3J_{H-H}$) and hetero ($^3J_{C-H}$ and $^2J_{C-H}$) nuclear coupling constants, in combination with ROESY (Rotating Overhauser Effect Spectroscopy) data according to Murata method.[6] The absolute stereochemistry of **3** (2*R*,4*S*) was likewise previously achieved using chemical and spectroscopic methods. This phytotoxin was converted to its N^1,N^4-ditosyl methyl ester derivative. The derivative obtained showed spectroscopic and chromatographic behavior different from the derivative of *cis*-4-amino-L-proline. The latter was synthesized starting from *trans*-4-hydroxy-L-proline, a natural amino acid, according to the reaction sequence reported in Evidente *et al.*, 2000.[4] Phytotoxin **3** was determined as the D-enantiomer of *trans*-4-aminoproline because an optical rotation opposite to that reported for *trans*-4-amino-L-proline was recorded.

When assayed at 30 μg/droplet on punctured leaves of host and non host plants, including wild and cultivated, **1** showed very high phytotoxicity on *C. album* and also on other very noxious weeds and cultivated plants, with only very weak activity observed on some agrarian crops.[3] Similar results were obtained with **3** at 1 μg μl^{-1}, assayed using the same method and test plants. Unlike **1**, **3** appeared to have greater phytotoxic specificity towards dicots, being that it was nontoxic to several monocots.[4] These results, together with observed lack of activity against fungi and bacteria, lack of toxicity to brine shrimp larvae (*Artemia salina* L.), and high water solubility, make these amino acids good lead compounds for development as safe natural herbicides.[7]

A method based on cationic exchange chromatography was previously developed and optimized for obtaining a semi-pure mixture of **1–3** from fungal culture filtrates. The chromatographic method proved to be convenient and simple, and was utilized for the large-scale production of toxin mixture. In view of the practical application of using the toxin mixture as a safe

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herbicide, glasshouse trials were carried out with formulations achieved by adding to different combinations of *A. caulina* conidia, the phytotoxins and low doses of herbicides, selected adjuvants and nutrients.[8,9]

This paper describes the development of a rapid, sensitive and specific method for the qualitative and quantitative analysis of the phytotoxins **1**, **2** and **3** in fungal culture filtrates using GC-MS. On the basis of the results, this method appears useful also in the view of a potential practical application to the toxin mixture.

Experimental

General

N,*O*-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*,*N*-dimethylformamide (DMF) were purchased from Pierce (Rockford, IL, USA). All solvents used were HPLC grade, purchased from Fisher Scientific (Fairlawn, NJ, USA). Analytical and preparative TLC were performed on Silica Gel 60-F_{254} (Merck, Darmstadt, Germany). Ninhydrin solution (Sigma Aldrich, St. Louis, MO, USA) was used for detection.

A strain of *A. caulina* (P. Karst) v.d. Aa and v. Kest, isolated from a diseased leaf of *C. album*, was kindly supplied by Dr C. P. Scheepens (Department of Crop and Production Ecology, Wageningen University and Research Centre, The Netherlands). The fungus was maintained on potato-dextrose-agar medium as a single-spore culture in the Collection of Istituto Scienze delle Produzioni Alimentari, CNR Bari, Italy (ITEM 1058).

Production and purification of *A. caulina* toxins

A. caulina was grown in liquid culture under conditions previously reported.[8] Compounds **1** and **3** were purified from the fungal culture filtrates as previously reported.[3,4] Compound **2** was purified as follows: lyophilized culture filtrate (73 g, corresponding to 2 litres) was dissolved in 75 ml of formic acid (1 M) and fractionated by cation exchange chromatography as previously reported.[8] A toxin mixture was obtained as a pale yellow powder after lyophilization (700 mg). This mixture was subjected to TLC purification (0.5 mm thickness) using BuOH–H_2O–AcOH–Me_2CO (2 : 2.5 : 1 : 0.5) as developing solvent. After plate development a narrow strip was cut from the longitudinal side of the TLC plate, which was sprayed with nynhydrin solution and heated to 200 °C. A pink band (R_f 0.4) evidenced the presence of **2**. This band was scraped from the TLC plate and the scrapings were treated with EtOAc–MeOH (1 : 1) for 15 min and filtered. The filtrate was evaporated at reduced pressure. The identity of the compound obtained was confirmed by ^{1}H NMR analysis and compared with the spectrum previously recorded for the same toxin.[5]

Analysis of *A. caulina* toxins by gas chromatography-mass spectrometry (GC-MS)

Lyophilized culture filtrate (1 mg in a GC vial) was treated with l ml of BSTFAO–DMF (50 : 50), heated at 60 °C for 20 min and allowed to cool to room temperature, as previously reported by Rimando and Perkins-Veazie.[10] Standard samples of **1–3** were converted into the corresponding trimethylsilyl (TMS) derivatives (**4–6**) using the same conditions as those for the culture filtrate. The toxin derivatives and the derivatized fungal culture filtrate were analyzed by GC–MS [Agilent 6890 Series GC system (Agilent Technologies, Palo Alto, CA, USA), coupled to a JEOL GC Mate II mass spectrometer (JEOL USA, Peabody, MA, USA)] using a DB-5 capillary column (J&W Scientific, Folsom, CA, USA) 30 m × 0.25 mm I.D., 0.25 μm film, run under the following GC temperature program: initial 80 °C held for 3 min, raised to 310 °C at 10 °C min^{-1} rate and held at this temp for 4 min. The injector, GC interface, and ionization chamber were maintained at 250, 250, and 300 °C, respectively. The carrier gas was ultra high purity helium at 1 ml min^{-1} flow rate. The sample injection volume was 1 μl. The MS detector was a magnetic sector. Spectra were acquired in the positive, low resolution, total ion scan mode. Quantitative determination of the toxins was performed from a calibration curve of the TMS derivatives of the pure compounds, as external standards. In particular the calibration curves for TMS derivatives (**4–6**) of toxins **1–3** were obtained by measuring standard solution containing toxin concentration in the range 1.0–0.25; 0.25–0.0625 and 0.0625–0.0156 μg ml^{-1}, respectively, in triplicate for each concentration, with 14 to 19 points each for calibration (Table 1).

Results and discussion

The crude mixture of toxins obtained as eluate from *A. caulina* culture filtrate cation exchange chromatography purification procedure was used to optimize a TLC method to isolate ascaulitoxin aglycone. This new method is more convenient and rapid compared to the one previously used, which was a longer purification process (*i.e.*, gel-filtration column chromatography followed by reversed phase TLC)[5] and resulted to be accurate as the ascaulitoxin aglycone band was well separated from the other toxins and unequivocally detected.

A GC-MS method was developed for the analysis of the phytotoxins, individually and in the lyophilized culture filtrate, as their trimethylsilyl (TMS) derivatives. The conditions used to

Table 1 Analytical characteristics of calibration curves of TMS derivatives *A. caulina* toxins (**4–6**)[a]

Analyte	R_t/min	Range/μg	Slope (×10^5) (SD%)	Intercept (×10^6) (SD%)	SD y[b] (×10^5)	r^2	Number of data points	Detection limit/μg[c]
4	26.30	1.0–0.016	1.88 (1.8)	2.33 (4.1)	1.92	0.996	17	0.04
5	17.60	1.0–0.016	2.65 (2.2)	1.05 (15)	1.12	0.991	19	0.04
6	12.68	1.0–0.015	10.90 (1.54)	1.39 (6.3)	2.18	0.962	14	0.02

[a] Calculated in the form $y = a + bx$ (where y = chromatographic peak area and x = μg of analyte). [b] SD = standard deviation. [c] Calculated as 3 × SD y/S (S = analyte chromatographic peak area per μg of analyte injected for the lower calibration curve point).

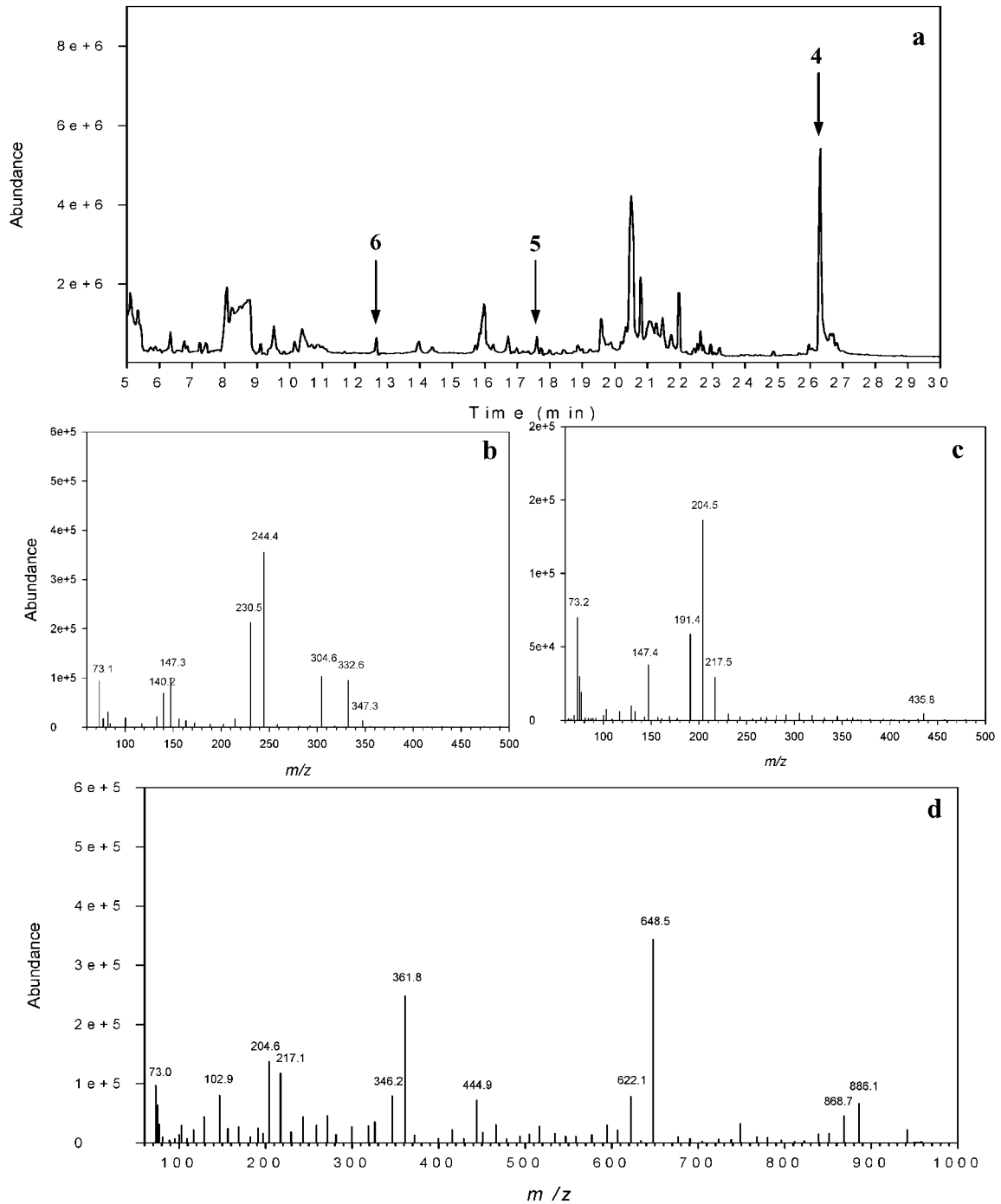

Fig. 2 GC profile of *A. caulina* culture filtrate treated with BSTFA–DMF (a) and EI-MS of the trimethysilyl-derivatives of 4-amino-D-proline, **6** (b), ascaulitoxin aglycone, **5** (c) and ascalitoxin, **4** (d) present in the mixture.

prepare the TMS derivatives **4–6** were very similar to those previously reported in the literature[10] and proved to be the most suitable to quantitatively convert the toxins as compared to those that others used, changing different parameters such as reaction temperature and time. The best chromatographic conditions obtained, described in the experimental section, resulted in well-resolved sharp peaks of the TMS derivatives of **1–3** (**4–6**, respectively). The retention times were 12.68, 17.60 and 26.30 min for **6**, **5** and **4**, respectively (Fig. 2a).

The mass spectrum of *trans*-4-amino-L-proline exhibited a peak at *m*/*z* 347, due to a protonated tri-TMS derivative (Fig. 2b). The other ion peaks observed in its mass spectrum could be explained from the fragmentation route presented in Fig. 3, which is in accordance with known fragmentation mechanism for pyrrolidides.[11,12] Cleavage of the C2–C3 bond, following α cleavage mechanism,[12] gave rise to the molecular species **a**, which undergoes two different fragmentation routes leading to fragments, which both showed *m*/*z* 230. Route **a1** gave

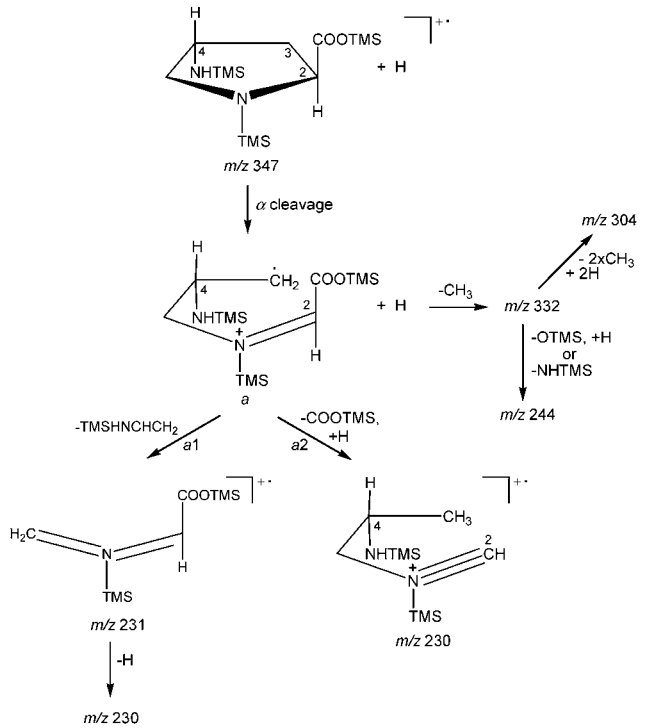

Fig. 3 Fragmentation of the tritrimethylsilyl derivative of 4-amino-D-proline (**6**).

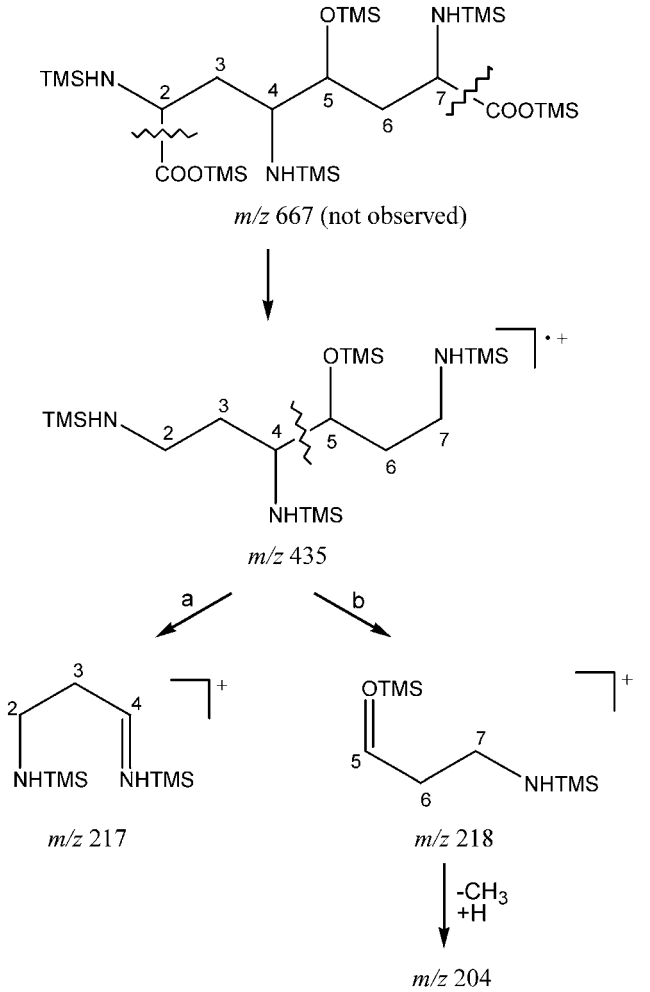

Fig. 4 Fragmentation of the hexatrimethylsilyl derivative of ascaulitoxin aglycone (**5**).

a fragment ion typically observed for pyrrolidides with concomitant loss of the C3–C4 neutral fragment. Removal of COOTMS fragment from species **a** (route **a**2) also gave rise to *m*/*z* 230. Loss of methyl group from species *a*, most likely from a TMS group, provided the ion at *m*/*z* 332, which through further fragmentation by loss of either OTMS or NHTMS gave the base peak at *m*/*z* 244.

The molecular ion peak of the TMS derivative of ascaulitoxin aglycone was not observed, but its mass spectrum showed a peak with the highest mass at *m*/*z* 435 (Fig. 2c), while the most abundant peak was observed at *m*/*z* 204. This represented a tetratrimethylsilylated fragment ion with the two carboxylic groups removed, as depicted in its fragmentation (Fig. 4). This fragment undergoes homolytic α-cleavage[13] with a breakage of the bond between C-4 and C-5. The fragment ion at *m*/*z* 217, due to *N*,*N*-dimethylsilyl-3-aminopropanimmine moiety (route **a**, Fig. 4), is one of the resultant complementary ions. The other complementary ion, theoretically *m*/*z* 218, was not observed but appears to have lost a methyl group that gave rise to a peak at *m*/*z* 204 (route **b**, Fig. 4). Loss of a methyl group from this fragment gives daughter ion with *m*/*z* 191, and yet further loss of three methyl groups gives the ion at *m*/*z* 147.

Like its aglycone, the molecular ion peak of ascaulitoxin was not observed in its mass spectrum. The highest mass peak displayed was at *m*/*z* 886 (Fig. 2d), representing a fragment ion with the two carboxylic groups, at C-2 and C-7, removed. Similar to the fragmentation of the aglycone, a homolytic α-cleavage occurred between C-4 and C-5 resulting in a fragment ion that showed a peak at *m*/*z* 217, as observed in Fig. 2d (see Fig. 5 for fragmentation of ascaulitoxin). The complementary ion, theoretically *m*/*z* 669, appeared to have lost a molecule of H_2O from the glucose residue and gave rise to the fragment ion peak at *m*/*z* 648 following fragmentation route **a**. Cleavage of the C3′–C4′ bond yielded a daughter ion at *m*/*z* 434, which upon loss of a TMS group gave rise to a fragment at *m*/*z* 361. The latter ion can also be obtained from fragmentation route **b**, *i.e.*, with removal of the glucose residue from *m*/*z* 886.

Quantitative analysis of the phytotoxins in the culture filtrate was performed on the reconstructed ion chromatograms of the peaks at 12.68, 17.60 and 26.30 min, corresponding to the TMS derivatives of 4-aminoproline (**6**), ascaulitoxin aglycone (**5**) and ascaulitoxin (**4**) (Fig. 2), from calibration curve of individual compounds used as external standards. The characteristics of the calibration curves and detection limits of the TMS derivatives of the three toxins are reported in Table 1. All TMS derivatives analyzed at amounts one order of magnitude lower than the minimum determined within the assay method still gave detectable peaks, but standard deviations of correlation parameters were greatly increased because of the reduced reproducibility of the area measurements.

Analysis of culture filtrates obtained from cultures of three different years (Table 2) showed that the levels of **1**–**3** consistently were in the order **1** > **3** > **2**. The levels of **2**–**3** were similar

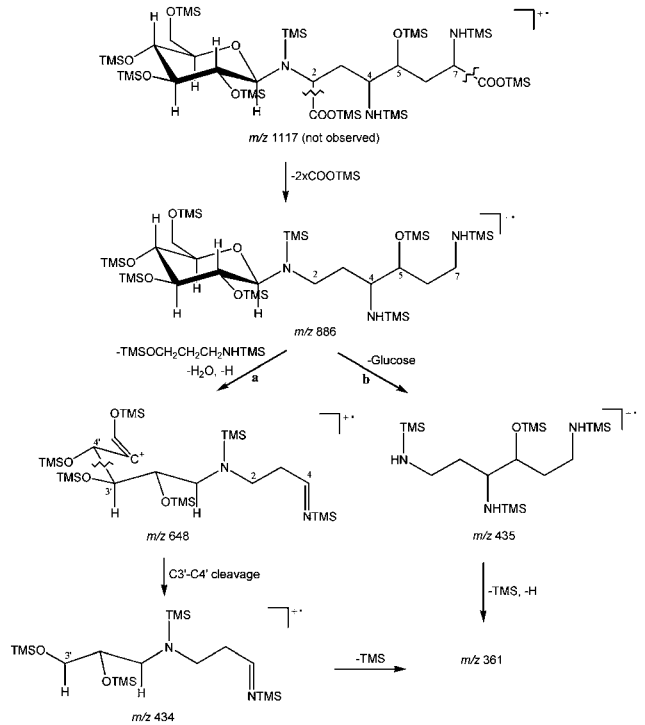

Fig. 5 Fragmentation of the decatrimethylsil derivative of ascaulitoxin (**4**).

Table 2 Levels of **1–3** in *A. caulina* culture filtrate[a]

Filtrate[b]	Ascaulitoxin (**1**)	SE[b]	Ascaulitoxin aglycone (**2**)	SE[b]	*trans*-4- amino-D-proline (**3**)	SE[c]
A	189.8	0.5	37.6	0.5	43.4	0.5
B	199.0	0.5	32.1	0.5	45.6	0.5
C	82.5	0.5	43.8	0.5	58.4	0.5

[a] Values are expressed in mg l^{-1}, analyzed in the TMS-derivatized culture filtrate. [b] A, B and C are culture filtrates from production of the same strain of *A. caulina* (ITEM 1058) in 2003, 2004 and 2005, respectively. [c] SE = Standard error.

for all the samples, but the level of **1** from the third year of production was lower compared to those from the first and second year filtrates. The lower level of **1** is not surprising, and may be due to the age of the inoculum. What is important to note is that the level of **1** correlated with the phytotoxicity of the filtrates; *i.e.*, the year 3 filtrate was the least phytotoxic (data not shown).

Conclusions

A GC-MS method was developed, which proved to be rapid, efficient and specific for the qualitative and quantitative analysis of three phytotoxins (**1–3**) produced by *A. caulina* in a complex sample matrix. The GC-MS method appears also to be simpler and provides a more accurate identification of the phytotoxins than the previous method used was based on the use of the high-performance anion exchange chromatography coupled with a pulsed amperometric detector.[5] In fact, the latter required use of very expensive column and solvents, and necessitated preliminary purification of the fungal culture filtrate to remove saccharose, an important component of medium growth, which gave a peak overlapping with that of ascaulitoxin. This new method also allowed for the distinction of the level of **1** as the possible determinant for phytotoxicity of *A. caulina* culture filtrates. A simpler method was also developed for the isolation of **2**. In view of the practical application of using a mixture of the toxins and need for their large-scale production, the new GC-MS method developed represents an important tool for the selection of the best strain(s) for fermentation, and can likewise be employed for monitoring toxin production during culture growth.

Acknowledgements

The authors thank Dr Maurizio Vurro, Istituto Scienze delle Produzioni Alimentari, CNR, Bari, Italy and Prof. Stephen O. Duke, USDA-ARS-NPURU, Oxford, MS, USA for their continuous interest and suggestions. This work was carried out within the project "Enhancement and Exploitation of Soil Biocontrol Agents for Bio-Constraint Management in Crops" (contract n. FOOD-CT-2003-001687), which is financially supported by the European Commission within the 6th FP of RTD, Thematic Priority 5 Food Quality and Safety. The information/opinions provided in the paper do not necessarily represent the official position/opinion of the European Commission. The research was also supported by grants from Regione Campania L.R. 5/02. Contribution DISSPAPA 204.

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