Original

Rapid and simultaneous determination of tetra cyclic peptide phytotoxins, tentoxin, isotentoxin and dihydrotentoxin, from *Alternaria porri* by LC/MS.

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Abstract

Tentoxin (**1**) and its related compounds, isotentoxin (**2**) and dihydrotentoxin (**3**), from *Alternaria porri* were determined simultaneously by APCI, ESI and FAB−MS. LC/APCI−MS was a most suitable method with sufficient sensitivity and selectivity for determination of tentoxins. Because, APCI−MS provided not only $[M + H]^+$ but also $[M + H - Gly - (N-Me-Ala)]^+$ and the intensity ratio of these ions of three tentoxins were similar ratio, 100 / 7, 100 / 10 and 100 / 7, respectively. Selective ion mass chromatogram provided by LC/APCI−MS gave quantitative information of tentoxins with high linearity from 100 ng/injection to 10 μg/injection. Furthermore, one assay can be performed within 15 min. Therefore, this method was applied for the determination of tentoxins in the culture liquid of *A. porri.*

Key words: tentoxin; isotentoxin; dihydrotentoxin; Alternaria porri; LC/APCI−MS

Introduction

In the course of our investigation on bioactive products of *Alternaria porri*, the causal fungus of black spot disease in the stone−leek and onion, we have reported the isolation of cyclic tetra peptide, tentoxin [cyclo−(L−leucyl−*N*−methyl−(*Z*)−dehydrophenylalanyl−glycyl−*N*−methyl−L−alanyl), **1**] [1]. Subsequently, we isolated and identified two tentoxin related cyclic peptide, isotentoxin [cyclo−(L−leucyl−*N*−methyl−(*E*)−dehydrophenylalanyl−glycyl−*N*

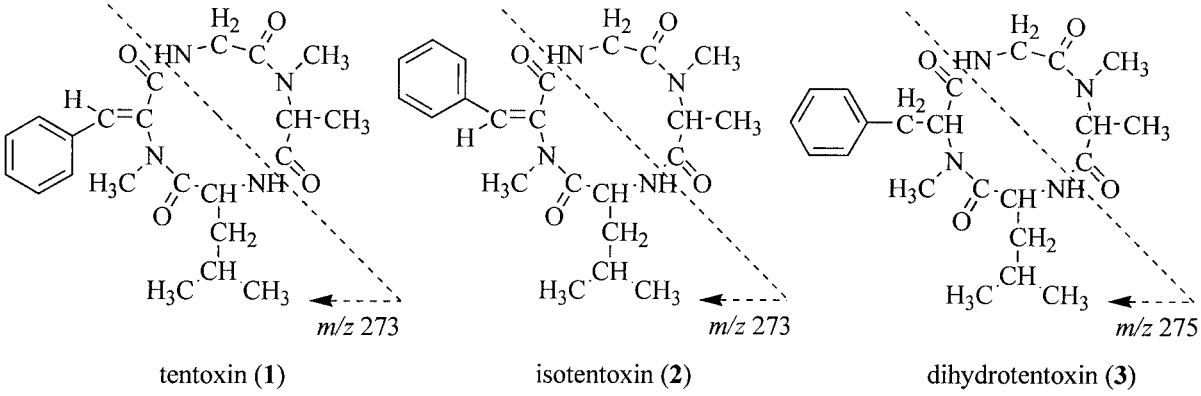

Figure 1. Structures and positive−ion APCI mass fragmentation patterns of tentoxin (**1**), isotentoxin (**2**) and dihydrotentoxin (**3**).

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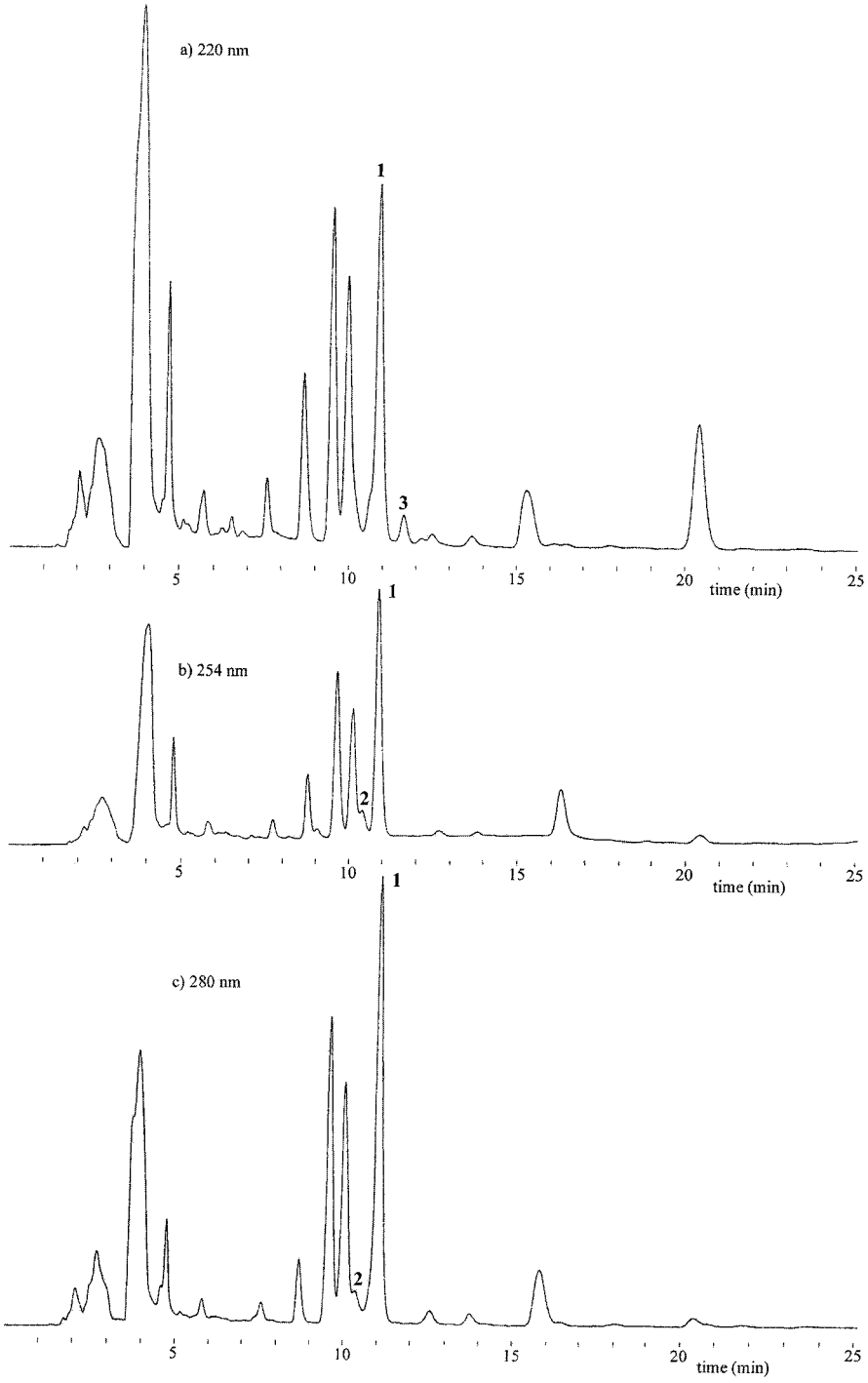

Figure 2. Chromatograms of the ethyl acetate extract from the culture liquid of *A. porri*. HPLC; column: Wakopak Wakosil−II 5C18 250 mm L. × 4.6 mm I. D., mobile phase: acetonitrile−water (2:3), flow rate: 1.0 ml/min, peak 1: tentoxin, 2: isotentoxin, 3: dihydrotentoxin. a) the chromatogram operating at 220 nm; b) the chromatogram operating at 254 nm; c) the chromatogram operating at 280 nm.

−methyl−L−alanyl), **2**] [2] and dihydrotentoxin [cyclo−(L−leucyl−*N*−methyl−L−phenylalanyl−glycyl−*N*−methyl−L−alanyl), **3**] [3, 4] from the culture liquid of *A. porri* (Figure 1).

Tentoxin (**1**) is produced in some *Alternaria* species [1−3, 5−9] and its many bioactivities have been reported. The important effect of **1** among them is its influence on the photophosphorylation by binding to the chloroplast coupling factor 1 (CF_1) [10]. Furthermore, **1** induces chlorosis in many species of higher plants, but

other plants are insensitive [6]. Therefore, it has been reported the possibility to use **1** as a potential biogenic herbicide [11]. Isotentoxin (**2**), the *E*−isomer of **1**, was converted from **1** by UV irradiation [12]. Moreover, it was reported that a conversion of **1** into **2** occurred during the isolation procedure from the culture liquid of *A. alternata* [12]. The chlorosis activity of **2** was weaker than that of **1**. On the other hand, the wilting activity against *Galium aparine* L of **2** was stronger than that of **1** [12]. Dihydrotentoxin (**3**) is the biosynthetic precursor of **1** [13]. The chlorosis activity of **3** was also weaker than that of **1** [3]. It has been reported that **1** and **3** increased membrane lipid fluidity on human erythrocytes [14]. Previously, we reported the analysis of tentoxin in the culture liquid of *A. porri* by reverse phase HPLC using UV (254 nm) monitoring [15]. This method was suitable for rapid analysis of **1** in the culture liquid of *A. porri.* However, it is difficult for simultaneous analysis of three tentoxins (**1**, **2** and **3**) by HPLC using single wave−length UV detection. Because, first, **2** and **3** were very minor components, second, absorption maximum of **3** is 60 nm shorter than that of **1** and **2**, third, there is potential for overlapped compound with tentoxins in HPLC (Figure 2). Therefore, the high selective and sensitive analysis methods for tentoxins are required. Currently, LC/MS with atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) are powerful techniques for the analysis of natural products. In this paper, APCI, ESI and fast atom bombardment (FAB) MS for identification of tentoxins and LC/MS analysis of tentoxins in the culture liquid of *A. porri* have been described.

Experimental

General.

The positive−ion APCI mass spectra were acquired by scanning from *m/z* 70 to 600 using a Hitachi M−1200 quadrupole mass spectrometer (Hitachi). The APCI interface section was set at a drift voltage of 60 V, a multiplier voltage of 1800 V, a needle voltage of 3000 V and a nebulizer temperature of 200℃. The positive−ion ESI mass spectra were obtained by API−III instrument (PE SCIEX) with an orifice plate voltage of 90 V, an interface plate voltage of 650 V and an ion spray voltage of 5300 V. The FAB mass spectra were measured by a JMS−HX/HX110A mass spectrometer (JEOL) with *m*−nitrobenzyl alcohol as a matrix. HPLC was performed on a Shimadzu LC−6A liquid chromatograph with a UV detector (Shimadzu SPD−6AV) and an integrator (Shimadzu C−R3A). The solvent system was acetonitrile−water (2:3). The column was Wakopak Wakosil−Ⅱ 5C18 250 mm L. × 4.6 mm I. D. (Wako). The mobile phase flow rate was 1.0 ml/min.

Fungus.

The strain of *A. porri* used in this experiment was purchased from IFO (Institute for fermentation, Osaka), strain number 9762.

Culture condition.

A. porri was cultured in the onion decoction medium for 35 days. The composition of the medium was as follows (g/L): sucrose (20.0) and onion decoction (1.0 L, onion 30.0%). The medium was sterilized in an autoclave at 121℃ for 70 min.

Procedure for HPLC and LC/APCI−MS.

After culturing, the 40 L culture liquid was filtrated by gauze. The culture filtrate was extracted with ethyl acetate. The ethyl acetate extract was treated with an evaporator, so as to evaporate to dryness. The ethyl acetate extract gave a yield of 3.292 g from 40 L of the culture liquid. The 0.5 w/v % methanol solution for **1** and **3** and the 5 w/v % methanol solution for **2** of the dry ethyl acetate extract were applied for HPLC and LC/APCI−MS. The sample of 10 μl was injected onto the column.

Preparation of tentoxin (**1**), *isotentoxin* (**2**) *and dihydrotentoxin* (**3**) *from A. porri.*

Three tentoxins were obtained by HPLC using JAIGEL−ODS−S343−15 (Japan Analytical Industry Co.) with a solvent of acetonitrile−water (2:3) and LiChrospher 100 RP−18 (MERCK) with a solvent of acetonitrile−water (3:7) from the dry ethyl acetate extract. The extract gave a colorless solid of **1** in a yield of 34.2 mg, a colorless solid of **2** in a yield of 0.1 mg and a colorless solid of **3** in a yield of 8.2 mg from 40 L of the culture liquid.

Tentoxin (**1**).

Physicochemical properties of **1** have been reported [1].

Isotentoxin (**2**).

^{1}H−NMR data of **2** was identical with that reported in the literature [2] and a NOE correlation showed between the methine proton in the *N*−methyldehydrophenylalanyl moiety and the methyl proton in *N*−methyldehydrophenylalanyl moiety [12].

Dihydrotentoxin (**3**).

^{1}H and ^{13}C NMR of **3** were identical with those reported in the literature [3].

Results and Discussion

APCI , ESI and FAB MS of tentoxins.

APCI−MS.

Positive ion APCI mass spectra of tentoxins were shown in Figure 3. Not only protonated molecules $[M + H]^+$ but also characteristic fragment ion $[M + H - Gly - (N-Me-Ala)]^+$ at *m/z* 273 for **1** and **2** and at *m/z* 275 for **3** were observed. $[M + H]^+$ was obserbed as base peak for all tentoxins. Moreover, the intensity ratio of $[M + H]^+$ to $[M + H - Gly - (N-Me-Ala)]^+$ of **1**, **2** and **3** were similar

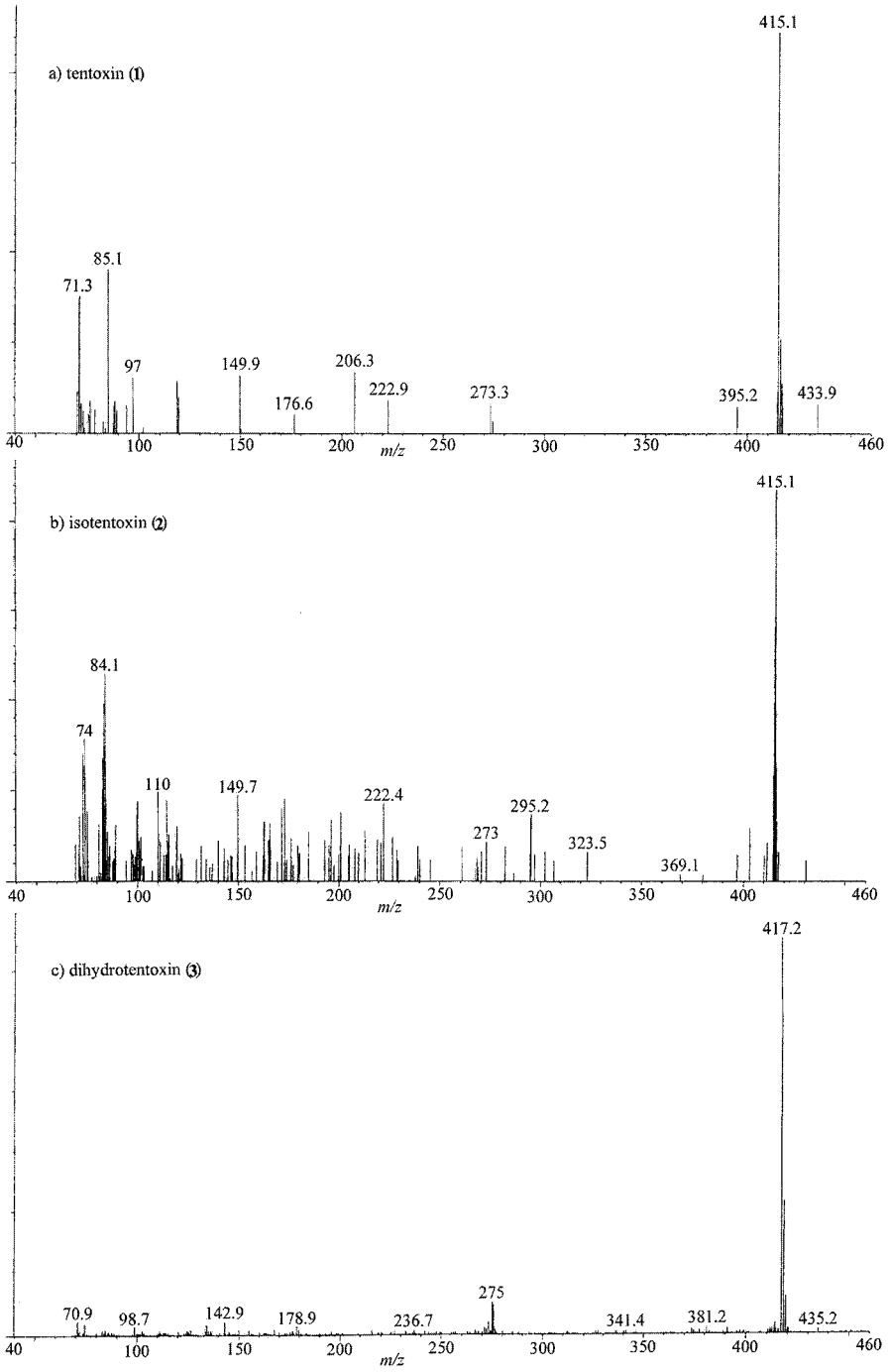

Figure 3. Positive−ion APCI mass spectra of tentoxin (**1**), isotentoxin (**2**) and dihydrotentoxin (**3**). a) tentoxin (**1**); b) isotentoxin (**2**); c) dihydrotentoxin (**3**).

ratio, 100 / 7, 100 / 10 and 100 / 7, respectively. These ions and intensity ratio were the useful information for the identification of **1**, **2** and **3**.

ESI−MS.

Nevertheless, no addition of alkaline metal in sample solution, the positive ion ESI mass spectra of tentoxins provided sodium adduct ion $[M + Na]^+$ as a base peak and $[M + K]^+$. However, $[M + H]^+$ were observed as weak intensity, relative intensity 8.9% for **1** and 25.8% for **3** (Figure 4). The method using $[M + H]^+$ as a target has difficulty for determination of tentoxins.

FAB−MS.

The positive−ion FAB mass spectra of **1**, **2** and **3** provided not only molecular−related ions such as $[M + H]^+$ and $[M + Na]^+$ but also characteristic fragment ion $[M + H - Leu - (N-Me-Ala)]^+$ at

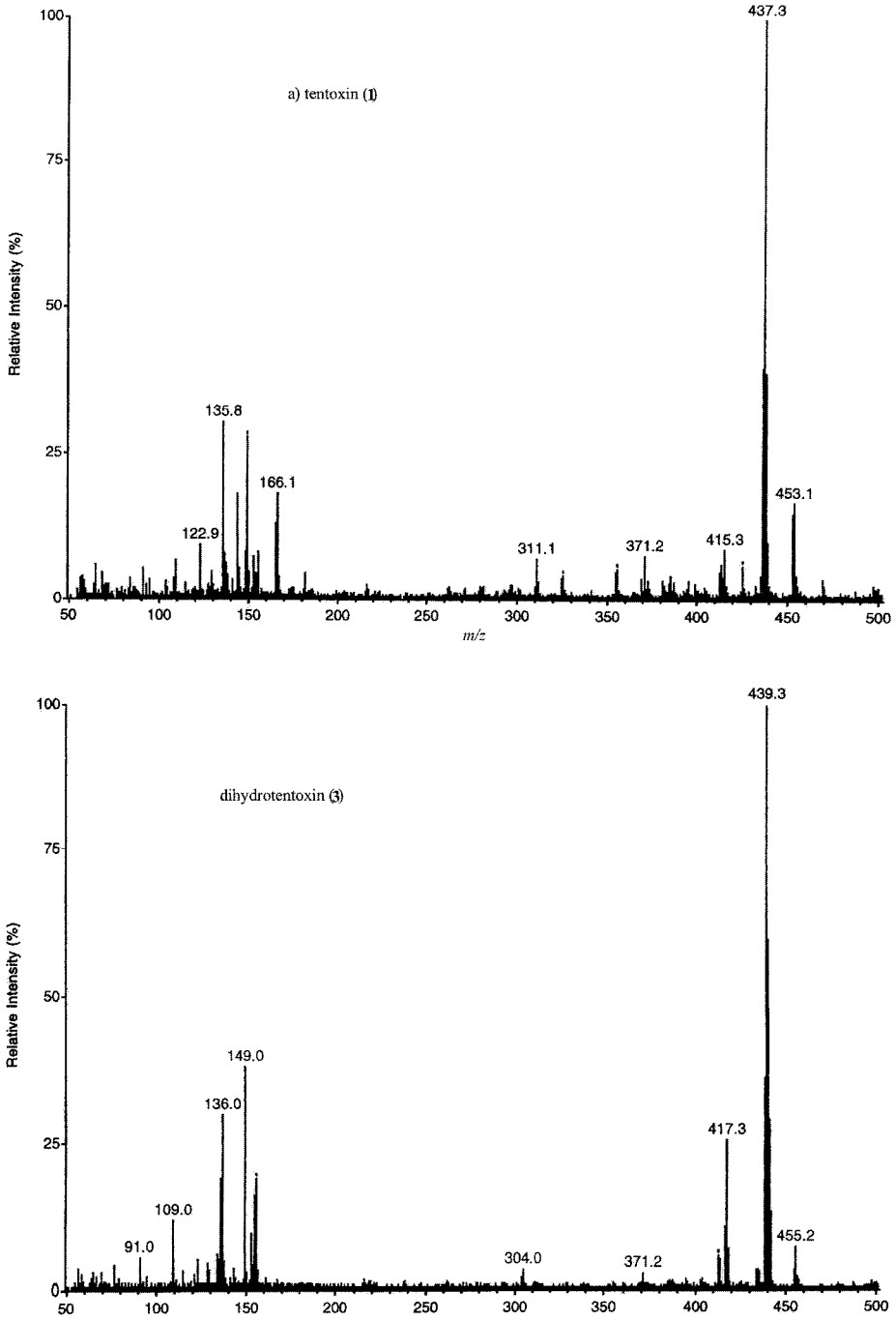

Figure 4. Positive−ion ESI mass spectra of tentoxin (**1**) and dihydrotentoxin (**3**).
a) tentoxin (**1**); b) dihydrotentoxin (**3**).

m/z 217 for **1** and **2** and at *m/z* 219 for **3** (Figure 5). However, the intensity ratio of $[M + H]^+$ to $[M + H - Leu - (N-Me-Ala)]^+$ of **1**, **2** and **3** were slightly different ratio, 100 / 6, 100 / 11 and 100 / 3, respectively. Furthermore, LC/FAB−MS is not so facile method for routine analysis. Therefore, we have selected the positive−ion APCI−MS for detection method of HPLC for analysis of tentoxins in the ethyl acetate extract.

Application of LC/APCI−MS for analysis of tentoxins in the culture liquid of A. porri.

Figure 6 showed the total ion and SIM (*m/z* 415 and 417) chromatograms of the ethyl acetate extract of the culture liquid obtained by the positive−ion mode LC/APCI−MS. HPLC systems used on LC/APCI−MS were the same conditions as used in UV detection method.

In the case of UV (220 nm) detection, it is difficult to identify

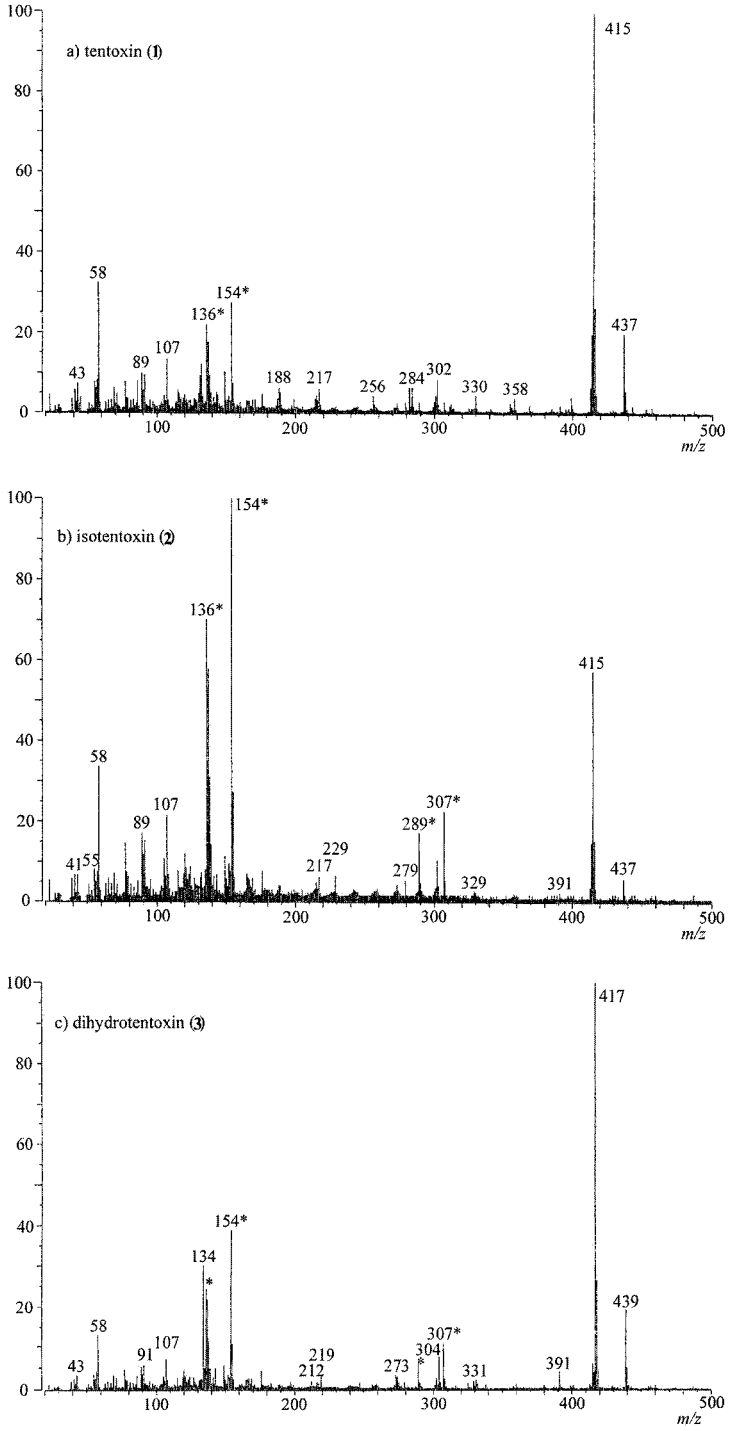

Figure 5. Positive−ion FAB mass spectra of tentoxin (**1**), isotentoxin (**2**) and dihydrotentoxin (**3**). a) tentoxin (**1**); b) isotentoxin (**2**); c) dihydrotentoxin (**3**).
*These peaks come from *m*−nitrobenzyl alcohol.

and quantify **2**, because the peak of **2** was overlapped with impurity as shown in Figure 2. On the other hand, the SIM (*m/z* 415) chromatogram could selectively determine tentoxins as shown in Figure 6. The SIM (*m/z* 415, $[M + H]^+$ of **1** and **2**) chromatogram showed the peak of **1** at 9.7 min and that of **2** at 8.6 min, respectively (Figure 6). The SIM (*m/z* 417, $[M + H]^+$ of **3**) chromatogram showed the peak of **3** at 10.4 min (Figure 6). By comparing the retention time in HPLC and $[M + H]^+$, the same characteristic fragment ion $[M + H - Gly - (N-Me-Ala)]^+$, the fragment pattern and the intensity ratio of $[M + H]^+$ to $[M + H - Gly - (N-Me-Ala)]^+$ in

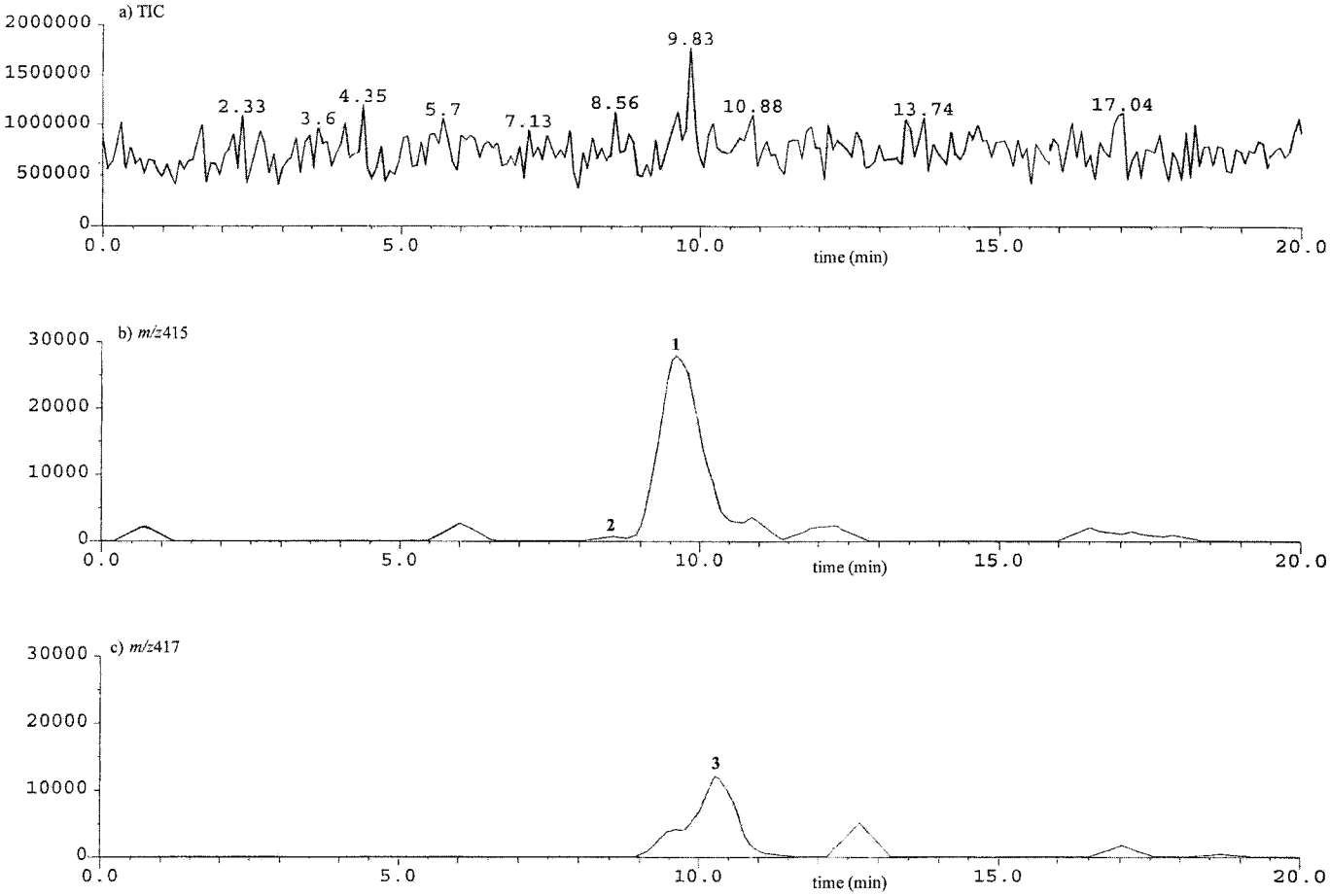

Figure 6. The total ion chromatogram and SIM (*m/z* 415 and 417) chromatograms of the ethyl acetate extract from the culture liquid of *A. porri*. HPLC; column: Wakopak Wakosil−II 5C18 250 mm L. × 4.6 mm I. D., mobile phase: acetonitrile−water (2:3), flow rate: 1.0 ml/min, peak 1: tentoxin, 2: isotentoxin, 3: dihydrotentoxin. a) the total ion chromatogram; b) the SIM chromatogram operating at *m/z* 415; c) the SIM chromatogram operating at *m/z* 417.

positive−ion LC/APCI−MS, **1**, **2** and **3** in the ethyl acetate extract were identified. Furthermore, three tentoxins in the extract were determined by using this method, because the concentrations of them existed in the quantitative range. A calibration curve with linearity was obtained for three tentoxins from 100 ng/injection to 10 μg/injection; y = 14.950 x (r^2 = 0.9983) for **1**, y = 14.661 x (r^2 = 0.9991) for **2**, y = 15.641 x (r^2 = 0.9993) for **3**. The quantitative analysis of tentoxins in the culture liquid of *A. porri* was performed by this selective ion monitoring LC/APCI−MS.

The concentrations of three tentoxins in the culture liquid of *A. porri* were found to be 1.202 μg/ml for **1**, 0.0255 μg/ml for **2** and 0.466 μg/ml for **3**.

Conclusion

The method of LC/APCI−MS allows **1**, **2** and **3** to be characterized by retention time in HPLC and APCI−MS spectral data so that it offers the simultaneous, rapid and accurate identification from the culture liquid of *A. porri*. The procedure for the analysis is very simple and easy. One assay can be performed within 15 min. Therefore, this method was useful for the quantitative analysis of tentoxins in culture liquid of different fungi. Moreover, it might be possible to determine them in the pathogenic tissue of black spot disease in the stone−leek.

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