Determination of Capsidiol in Tobacco Cells Culture by HPLC

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Abstract

Capsidiol is a bicyclic sesquiterpene, which accumulates extracellularly in plants, and has been isolated from many types of *Solanaceae*. It acts as a phytoalexin produced by *Nicotiana tabacum* in response to pathogens. Capsidiol has antifungal activity and is formed first in tobacco and pepper plants after infestation. The amount of capsidiol in tobacco cell suspension culture has been previously determined by solid-phase extraction and organic solvent extraction with thin-layer chromatography or gas chromatography analysis. A high-performance liquid chromatography method with UV detection at 210 nm on a C_8 column utilizing both extraction methods was developed to analyze capsidiol in suspension cell culture. The HPLC method was linear in the concentration range of 0.1–2.0 mg/L. The lower limit of quantitation was 0.1 mg/L. Organic solvent extraction and solid-phase extraction methods were compared. Both methods are generally similar in their overall efficiency (82% and 75%, respectively), but eliminations of interfering compounds are different. The relative standard deviation across five extractions of known amounts of capsidiol from plant sample was less than 5.1%. The relative standard deviation across five elicitations of cell cultures was less than 5.9%. Gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry analysis of capsidiol was performed, and corresponding mass spectra are presented.

Introduction

Many secondary compounds produced by plants play an important role in resistance to infection induced by bacteria, fungi, and nematodes. These compounds usually have antimicrobial effects and, on the basis of their time of production in plant, are divided into two classes: phytoanticipins and phytoalexins (1). Both classes of these secondary metabolites include a wide range of low molecular compounds with different chemical structures. Phytoanticipins are present in plant just before infection with the microorganism, or they are produced after infection but only from previously synthesized precursor components (2). On the other hand, phytoalexins are synthesized and accumulated in plants after infection with microorganisms (pathogens) (3,4). It is well-known that interaction between *Nicotina tabacum* plant and elicitins leads to the hypersensitive reaction defined by a rapid and localized necrosis of the cells surrounding the site of infection (5,6). Elicitins are a family of small proteins with conserved structure secreted by species of the genus *Phytophthora* (7). These proteins induce a defense response in certain plants of the families *Solanaceae* and *Cruciferae* (8). After elicitin recognition, a plant induces some specific physiological changes such as pH changes, ion fluxes across the plasma membrane, depolarization of the plasma membrane, oxidative burst, and cell death (9). Moreover, in tobacco plants, induction of systemic acquired resistance after elicitin uptake has been well-demonstrated (10,11). This resistance is characterized by accumulation of pathogenesis-related proteins at the site of infection, and subsequently, in all uninfected parts of a plant (12,13). In addition, a set of defense-related genes covering the enzymes responsible for synthesis of phytoalexins is activated during the defense response. Leaves and suspension cell cultures of *Nicotina tabacum* produce phytoalexins such as capsidiol, phytuberin, and phytuberol (14).

Capsidiol is a bicyclic dihydroxylated sesquiterpene that accumulates in the extracellular space in plants and has been isolated from many species of *Solanaceae* (15). Capsidiol has an antifungal activity and is synthesized as the principle phytoalexin in tobacco and pepper plants in response to fungal elicitation (16,17). It is derived from the isoprenoid pathway via its hydrocarbon precursor 5-epi-aristolochene, where the enzyme 5-epi-aristolochene synthase is responsible for the conversion of 5-epi-aristolochene to capsidiol (18,19).

In previous studies, capsidiol content was determinated by thin-layer chromatography or gas chromatography (GC) after extraction with dichloromethane or chloroform (14,20). Also, a solid-phase extraction (SPE) method was used with absorption to polyamide and C_{18} columns (21), and supercritical CO_2 extraction (22) has been used. Moreover, an high-performance liquid chromatography (HPLC) method employs cyanopropyl-bonded phase column for analysis of sesquiterpene phytoalexins such as

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capsidiol has been developed (23). In this work, we modified the extraction method for suspension cultures and leaves using polyamide and C_{18} columns, and we describe a new simple method for analysis and quantification of capsidiol content by reversed-phase HPLC on a C_8 column with diode array detector (DAD).

Experimental

Cultures

Tobacco suspension cells (*Nicotina tabacum* L.cv. Xanthi) were grown in 250-mL Erlenmeyer flasks at constant temperature (23°C) and humidity (55%), and shaken at 120 rpm in a Minitron (Infors-HT, Bottmingen, Switzerland). They were subcultured weekly in Chandler's medium (24) .

Chemicals

Cryptogein was isolated as previously described (8). Cryptogein was dissolved in water and was stored at –20°C. Methanol and dichloromethane were obtained from Sigma Aldrich (Prague, Czech Republic). Sorbents for SPE were obtained from Macherey-Nagel (Duren, Germany).

Elicitation

Suspension cultures after sub-culturing for three days were used for elicitation. These cells in the rapid growth phase at a density of 1 g fresh weight (FW) per 10 mL media gave the greatest capsidiol production in response to elicitor (20) . Aliquots (1 g FW) of the tobacco suspension cells were transferred to smaller Erlenmeyer flasks and were treated with a cryptogein (0.2 mg/L) (14). Flasks with elicited cells were cultivated under usual growth conditions. Cell suspensions were used for analysis after 20 h because production of capsidiol starts after 6 h and is maximal after 18–20 h (14). After elicitation, cells were separated from media by filtration on frit, cells were weighed, and the medium was used for extraction.

Organic solvent extraction

Organic solvent extraction (OSE) of capsidiol from the media was carried out using dichloromethane. Media (10 mL) were shaken three times with dichloromethane. The obtained extracts were concentrated on a rotary vacuum evaporator, and the evaporated extracts were dissolved in 0.75 mL dichloromethane and then were dried under a stream of nitrogen. The residues were dissolved in 0.2 mL of methanol. The samples prepared this way were used for analysis by HPLC.

SPE

SPE of capsidiol was carried out by adsorption on two sorbents in columns. Methanol was added to filtrates of elicited cells to give a 40% concentration. This mixture was applied first to the polyamide column (500 mg MN-polyamide CC 6.6 per column) for removal of cell residues. Capsidiol flowed through and was applied to the second C_{18} column (250 mg C_{18} per column). This sorbent retained capsidiol. Thereafter, the C_{18} column was washed with 5 mL of water. Capsidiol was then eluted with 3 mL of ethanol. The eluate of capsidiol was evaporated, and residues were redissolved in 0.2 mL methanol and analyzed by HPLC.

HPLC analysis of capsidiol

Capsidiol was analyzed by chromatography by reference to an authentic standard, which was provided by Dr. Milat's laboratory (Dijon, France). The quantitative analysis of capsidiol was carried out with a model HPLC HP 1100 liquid chromatography system (Agilent) equipped with a DAD detector set at 210 nm and with a reverse-phase column LC-8-DB (4.6 mm i.d. × 25 cm, particle size 5 µm) and stationery phase C_8 (Sigma Aldrich). Typical sample volume was 10 µL. The mobile phase was methanol–water. The gradient elution started at 70% methanol, and it was increased linearly from 70% to 80% over 16 min (flow rate 1 mL/min). The standard error of analysis was about ± 0.8%. Extraction yield (mg capsidiol/kg sample) was defined as the mass of extracted capsidiol divided by the mass of sample on a fresh basis.

LC–MS–MS analysis of isolated capsidiol

Liquid chromatography coupled to mass spectrometry (LC–MS–MS) experiments were performed on an HPLC system consisting of an UltiMate gradient pump (Dionex, Sunnyvale, CA), a Famos autosampler, and a Switchos column-switching device coupled online with an Esquire 2000 ion-trap mass spectrometer (Bruker Daltonik, Billerica, MA) equipped with an orthogonal electrospray ion source. Samples were concentrated and desalted using a PepMap C_{18} trapping column (300 µm × 5 mm, LC Packings). Typical sample volume was 10 µL. After washing with 0.1% formic acid, the compounds were eluted from the trapping column using an acetonitrile–water gradient (4 µL/min) onto a C_{18} fused-silica capillary column (320 µm i.d. × 185 mm) on which compounds were separated. This column was filled with 4 µm Jupiter Proteo sorbent (Phenomenex, Torrance, CA), according to a previously described procedure (25). Mobile phase A consisted of acetonitrile–0.1% formic acid (5:95 v/v) mixture, and mobile phase B consisted of acetonitrile–0.1% formic acid (80:20 v/v) mixture. The gradient elution started at 30% of mobile phase B, and after 2 min it was increased linearly from 30% to 90% over 28 min. The analytical column outlet was connected to the electrospray ion source via a 50-µm i.d. fused-silica capillary. Nitrogen was used as the nebulizing as well as the drying gas. The pressure of nebulizing gas was 8 psi. The temperature and flow rate of drying gas were set to 250ºC and 4 L/min, respectively, and the capillary voltage was 4.0 kV. The mass spectrometer was operated in the positive ion mode in a *m/z* range of 100–550 (target mass *m/z* 237). The compounds were fragmented using improved auto-MS–MS mode with the SmartFrag option, which enabled the ramping of the fragmentation energy automatically across a range of excitation amplitudes (0.3–2.0 V) during a fragmentation step. Helium was used as a collision gas. Extraction of the mass spectra from the chromatograms, mass annotation, and deconvolution of the mass spectra were performed using DataAnalysis 2.0 software (Bruker Daltonik).

GC–MS of isolated capsidiol

The qualitative identity was analyzed using GC–MS Trace 2000 (Finnigan/Thermo Scientific, Waltham, MA). Sample (5 µL) was

injected into the split/splitless (SSL) injector at 250°C with the split ratio 1:15 and the split flow 15 mL/min. The gas chromatograph was equipped with a capillary column DB5-MS (25 m × 0.25 mm × 0.25 µm) and stationery phase (5%-phenyl)–methylpolysiloxane (J&W Scientific/Agilent, Santa Clara, CA). The temperature program was isothermal at 190°C for 1 min followed by increase at 5°C/min to 225°C, where it was held for 10 min. The flow of the carrier gas (He) was constant at 1.0 mL/min. The MS was operated in scan mode (10–243 amu). The temperature of the ion source and the GC–MS interface was 250°C. Electron ionization was conducted at 70 eV with an emission current of 150 µA.

Results and Discussion

HPLC analysis

For quantitative analysis of capsidiol after extraction from media, a HPLC method on the C_8 column with DAD detection was developed. Elution with small gradient was preferred over isocratic elution for short analysis and clear recognition of capsidiol in plant sample. The retention time of capsidiol was 6.2 min. By this method, we were able to detect a minimum concentration of 0.1 mg/L. The capsidiol content in cell suspension cultures after elicitation is about 45 mg/kg FW (14,20,26), which corresponds to about 1 mg/L in the sample after isolation. Therefore, the linear calibration was constructed over the range 0.1–2 mg/L of capsidiol, and the corresponding equation was y = 597, 5 + 8, 3 based on linear regression (n = 6).

OSE and SPE

Capsidiol was extracted from the media by OSE using dichloromethane (14) or chloroform (20). Organic extracts were evaluated qualitatively by thin-layer chromatography and quantitatively by GC. Large amounts of capsidiol (greater than 10 mg/kg FW) were uniformly produced in elicitor-treated cultures (14,20). The maximal amount of capsidiol measured by GC was 50 mg/kg FW (20). Another method extracted capsidiol from the media by SPE using polyamide and C_{18} columns. Detection and quantification of capsidiol was performed by GC.

Therefore, the efficiency of OSE and SPE extraction procedures was tested by extraction of known amounts of capsidiol from media. On the basis of measured values, the yield and error of extraction were calculated. The average yields of extractions were 81.8% and 75.1% for OSE and SPE, respectively. The relative standard errors were 6.5% and 6.0% for OSE and SPE, respectively (Table I). These results are comparable with previous published work (22).

These results indicate that OSE had superior yields with similar relative standard error to the SPE method. On the other hand, the OSE method is very dependent on the intensity of shaking during extraction. This procedure could cause losses. Both, OSE and SPE procedures showed the comparable efficiency in isolation of capsidiol. The higher relative standard deviation (RSD) of both methods probably results from capsidiol instability.

Reproducibility of plant sample extraction

To test extraction efficiency, one batch (100 mL) of suspension cells culture was inoculated with the usual cryptogein concen-

Table I. Comparison of OSE and SPE

	OSE	SPE
n	5	5
$\bar{x}$ (%)	81.8	75.1
SD	5.3	4.5
RSD (%)	6.5	6.0

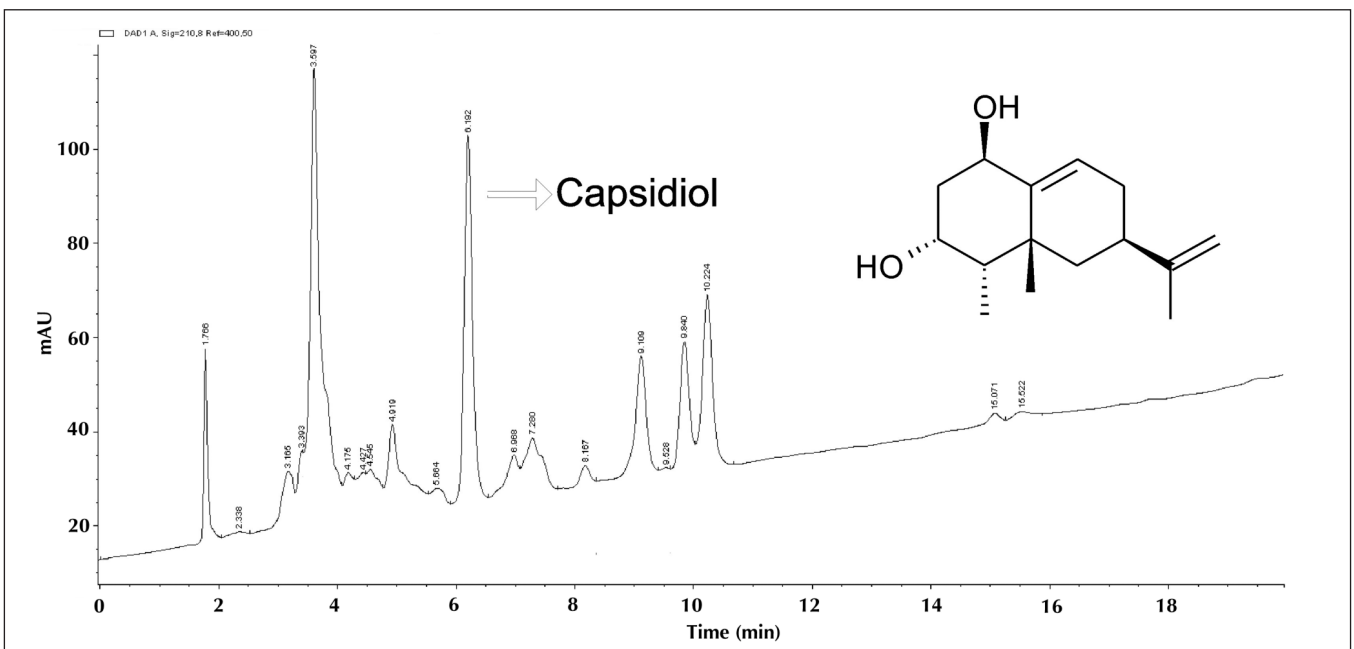

Figure 1. Representative elution profile of capsidiol in plant sample after OSE (λ = 210 nm), including capsidiol structure.

tration. After 20 h of cultivation, the medium from elicited suspension cells was divided into two identical parts. Extractions were carried out five times with 10 mL aliquots for either SPE or OSE method. Extracts were analyzed by HPLC–DAD. The chromatograms (Figure 1–2) show that OSE and SPE methods are generally similar in their overall efficiency of interfering compound elimination. On the one hand, the SPE method can more effectively remove the interfering compounds with a higher retention time. By contrast, the OSE method is more efficient in removing interfering compounds with lower retention times. Errors of extraction reproducibility were calculated (Table II). Yields of capsidiol were expressed relative to FW of cells. Results of extraction reproducibility from the plant samples show RSDs of about 5.1% and 4.9% for SPE and OSE, respectively. This value is lower than the RSDs for the extraction of capsidiol alone. The results indicate that capsidiol is probably more stable in the plant sample.

Table II. Plant Sample Extraction and Yields of Capsidiol After Extraction by SPE and OSE

	OSE	SPE
n	5	5
$\bar{x}$ (mg/kg)	27.6	24.5
SD	1.4	1.2
RSD (%)	5.1	4.9

Table III. Plant Sample Elicitation and Yields of Capsidiol After Extraction by SPE and OSE

	OSE	SPE
n	5	5
$\bar{x}$ (mg/kg)	25.3	23.7
SD	1.5	1.4
RSD (%)	5.9	5.9

Identification of capsidiol

The corresponding LC fraction from the plant sample (retention time 6.2 min) was subjected to GC–MS and LC–electrospray ionization-MS analyses. GC–MS spectrum of the compound (Figure 2) eluting in the fraction main peak was identical with those of the authentic capsidiol standard published previously (18). LC–electrospray ionization mass spectrum of the peak contained dominant ions of *m/z* 219 and 201 (Figure 2), which corresponds to the loss of one and two molecules of water, respectively, implying the presence of two hydroxyl groups and a molecular weight of 236; but the pseudomolecular ion $(M+H)^+$ (*m/z* 237) was not detected. Both dominant ions were fragmented by collision-induced dissociation in the ion trap. As expected, the ion of *m/z* 219 primarily lost a molecule of water (*m/z* of 201), and the ion of *m/z* 201 dissociated into fragments common for cleavage of a sesquiterpene skeleton (*m/z* 105, 107, 119, 131, 145, 159, 173).

Reproducibility of plant cell elicitation

Ten flasks containing 10 mL suspension culture were elicited with an identical cryptogein concentration. After 20 h of cultivation, the media from five flasks were used for SPE and five for OSE. Extracts were analyzed by HPLC–DAD. Yields of capsidiol were re-related to the FW of cells. Errors of reproducibility of elicitation were calculated (Table III). Comparison of elicitation reproducibility of OSE and SPE methods leads to the conclusion that they are similar. RSD was 5.9% for SPE and OSE. The higher RSD corresponds to the greater variability of cell culture experiments.

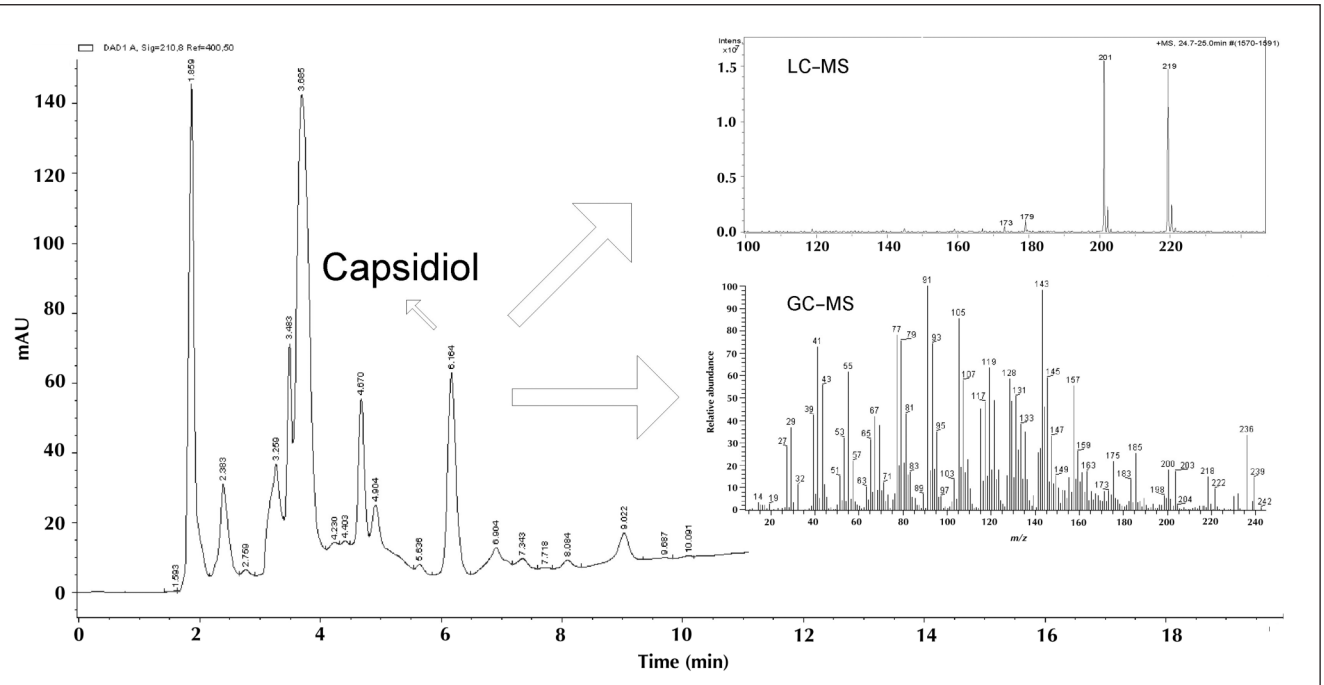

Figure 2. Representative elution profile of capsidiol in plant sample after SPE, including corresponding GC–MS and LC–MS spectra.

Conclusions

In the first part of this investigation, the liminal concentration of capsidiol and its retention time were determined, and the calibration curve was created on an HPLC–DAD system. Capsidiol was identified in corresponding LC fraction of plant samples by MS analysis. In the second part of this investigation, RSD of SPE and OSE extractions were determined. Yields of known amounts of capsidiol were 82% and 75% for OSE and SPE, respectively. In the final part of this work, the reproducibility of plant sample extraction and elicitation were determined. These results indicate that the method is suitable for analysis of capsidiol in plant samples.

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