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HPLC/Colorimetry Combination Method for Quantitative Analyses of Sclerotiorin Produced by *Penicillium sclerotiorum*

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ABSTRACT

In this study, quantitative analysis of sclerotiorin from *Penicillium sclerotiorum* was established by using HPLC/colorimetry combination method at the condition of unavailability of authentic sclerotiorin compound. Mycelium was extracted with methanol, then analyzed by HPLC (a C18 reverse phase column with acetonitrile/water = 65/35, v/v as mobile phase and detected at 370 nm) and simultaneously measured OD_{370} with spectrophotometers. The limit of quantitation (LOQ) was 0.5 μg/mL. Recovery studies were performed using the lyophilized mycelia, with each spiked with sclerotiorin at 1 and 10 mg, respectively. The recoveries were found in the range of 97~109%. The coefficients of variation were less than 3.8% with this HPLC / OD_{370} combination method.

Key words: *Penicillium sclerotiorum*, sclerotiorin, HPLC/colorimetry combination method, quantitative analyses

INTRODUCTION

In the recent years, many studies concerning the antioxidant and anti-tumor effects of natural colorants such as carotenoids[(1)], anthocyanins[(2)], curcumins[(3)], and monascus[(4)] have been reported. This indicates that the researchers and consumers are becoming interested in pursuing "natural and healthy" products and thus increase the demand for natural food colorants[(5)]. In terms of production efficiency, microorganisms are good sources for producing natural food colorants[(6,7)]. In addition, many secondary metabolites of microorganisms showed antibacterial, anti-tumor, and hypotriglyceridemic activities[(8,9)]. Therefore, microorganisms become one of the most potential biological resources.

Penicillum sclerotiorum can produce yellow pigments, which structurally are classified as azaphilones[(10)]. The major constituent in those pigments is sclerotiorin [(11,12,14)]. The main characteristic of azaphilones is its affinity to ammonia. The oxygen atom in azaphilone ring is likely to be substituted by NH group. Azaphilones are therefore called "nitrogen loving"[(13)]. Monascus, which has long been used in Asian countries, is also categorized as azaphilones. Sclerotiorin possesses various biological activities: chlamydospore-like cell-inducing activity[(13)], endothelin receptor binding activity[(14)], and cholesteryl ester transfer protein inhibition activity[(15)]. It also can act as a phospholipase A2 and lipase inhibitor[(16,17)].

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Sclerotiorin standard is not commercially available and causes the difficulty in quantitation. The purpose of this study was to develop an HPLC/colorimetry combination method for quantitative analysis of sclerotiorin produced from microorganism under the condition of lacking commercial sclerotiorin standard.

MATERIALS AND METHODS

I. *Materials*

(I) *Strains and culture media*

P. sclerotiorum BCRC 32017 was purchased from Bioresources Collection & Research Center at Food Industry Research and Development Institute (Hsinchu, Taiwan). The strain culture medium containing 1.5% potato dextrose agar (PDA) was obtained from Difco Laboratories (Detroit, MI). The strain was cultivated in PDA slant at 27˚C for 5 days until the surface of the medium was fully covered with spores. The medium was then stored at 4˚C in a refrigerator ready for use. The liquid culture used for producing yellow pigment was potato dextrose broth (PDB), which was obtained from Difco Laboratories (Detroit, MI).

(II) *Reagents*

The HPLC grade organic solvents, acetonitrile, methanol, ethanol, ethyl acetate, isopropanol, acetone, and

n-hexane were purchased from Merck Co. (Darmstadt, Germany).

II. *Methods*

(I) *Cultivation of P. sclerotiorum BCRC 32017 and production of yellow pigment*

Sterile water containing 0.1% Tween 80 (TCI, Tokyo, Japan) was added to culture medium which was covered with *P. sclerotiorum* BCRC 32017 spores. After a series of dilution, the numbers of spores were counted using a hemacytometer and a spore suspension (1×10^7 CFU/mL) for inoculation was prepared. The above suspension (0.1 mL) was spiked into a 500-mL flask containing 100 mL of culture medium. The flask was then capped with a gas permeable silicon and cultured in a shaker at 120 rpm, 27˚C for 10 days until the orange-yellowish mycelia appeared. The mycelia was collected by filtration, washed twice with distilled water, freeze-dried (lyophilized mycelia, LM), and then kept at -20˚C freezer ready for use.

(II) *Isolation and purification of sclerotiorin*

Isolation of yellow pigment, sclerotiorin, was according to the method of Negishi *et al*.[17] with slight modification. The lyophilized mycelia (LM) were extracted with 20-fold (v/w) ethyl acetate at room temperature for 1 hr. After filtration, the solution was collected and the residue was extracted again with 20-fold ethyl acetate. The above procedure was repeated. After extraction for 3 times, the filtrates were combined and evaporated to dryness under reduced pressure to obtain a crude yellow pigment, which was then purified with a silica gel column. A 10% (w/v) pigment solution was prepared by dissolving crude pigment (10 g) in 100 mL of ethyl acetate. Fifty grams of silica gel (70-230 mesh) was added to and mixed with the above solution. The organic solvent was evaporated under a reduced pressure. The pigment-adsorbed silica gel was then loaded onto a column (30 cm × 9 cm), which was prepacked with 250 g of silica gel (70-230 mesh) in *n*-hexane. The pigment was eluted with 2.5 L of *n*-hexane/ethyl acetate (5/1, v/v) solution. The eluate was evaporated to dryness and the residue was dissolved in methanol. The yellow needle-like crystalline sclerotiorin was obtained after re-crystalization at 0˚C for 3 times in methanol. The chemical structure of this needle-like crystalline compound was determined by analyzing the following parameters: melting point, molecular weight, UV/VIS, IR, and ^{1}H and ^{13}C NMR. The analytical instruments used in this study were Fisher-Johns melting apparatus (Fisher Scientific Co. Pittsburgh, PA, USA), Hewlett-Packard 5970 mass spectrometer, Hitachi 2000 spectrophotometer for full wavelength absorption scanning, Perkin Elmer system 2000 FT-IR spectrometer and Bruker DMX-300 MHz FT-NMR spectrometer. The analytical data for this compound are as follows: EI-MS (%): 390 (M^+, 100) and 392 (M^++2, 35). UV: 1720, 1636, and 1528 cm^{-1}. ^{1}H-NMR ($CDCl_3$, δ): 7.91 (^{1}H, s), 7.06 (^{1}H, d, J = 16Hz), 6.62 (^{1}H, s), 6.08 (^{1}H, d, J = 16Hz), 5.70 (^{1}H, d, H = 10Hz), 2.51 (^{1}H, m), 2.15 (^{3}H, s), 1.82 (^{3}H, s), 1.23-1.54 (^{2}H, m), 1.00 (^{3}H, d, J = 6Hz), 0.87 (^{3}H, t, J = 7Hz). ^{13}C-NMR ($CDCl_3$, δ) 191.7, 186.0, 170.1. 158.1, 152.7, 148.8, 142.9, 138.7, 132.0, 115.7, 114.6, 106.4, 84.6, 35.1, 30.0, 22.5, 20.2, 20.1, 12.4, 12.0. The recrystallized sclerotiorin was used as a standard in this study and the HPLC chromatogram of this compound shows single peak. Its chemical structure is shown in Figure 1.

(III) *Quantification of sclerotiorin by HPLC*

An adequate quantity of *P. sclerotiorum* BCRC 32017 wet mycelia (WM) and lyophilized mycelia (LM) was taken and extracted with 60-fold (v/w) methanol twice. The solution was poured into a 100-mL volumetric flask and methanol was then added to the constant volume. The final solution was assayed by HPLC after filtered through a 0.45-μm membrane. The sclerotiorin calibration curve was plotted with the peak areas against concentrations made by a series of dilution of 0.1% (w/v) sclerotiorin standard solution in methanol. A Hitachi L-6200 (Tokyo, Japan) HPLC system equipped with an UV-VIS Hitachi L-4200 (Tokyo, Japan) at 370 nm was used. The analytical column was LiChrospher® 100 RP-18e column (4mm i.d. × 250 mm, 5 μm) (Merck, Damstadt, Germany). The mobile phase was acetonitrile/water (65/35) pumped with a flow rate of 1.0 mL/min. Injection volume was 20 μL. The limit of quantitation (LOQ) was defined as the value with a 15% CV (coefficient of variation) of three repeated analysis[18-19].

(IV) *Quantification by HPLC/OD_{370} combination method*

An adequate quantity of *P. sclerotiorum* BCRC 32017 wet mycelia (WM) and lyophilized mycelia (LM) was taken and extracted with 60-fold (v/w) methanol twice. The solution was poured into a 100-mL volumetric flask and methanol was then added to the constant volume. This pigment solution was analyzed by HPLC as described above and measured using a spectrophotometer (Hitachi 2000, Tokyo, Japan) at OD_{370} after an adequate dilution was made. The content of sclerotiorin was calculated according to the following equation:

$$\text{Scelerotiorin (mg/g)} = [(A_{scl} / A_{total} \times OD_{370} \times D.F. \times 100 \times 390) / \varepsilon] / W \quad \text{.....(1)}$$

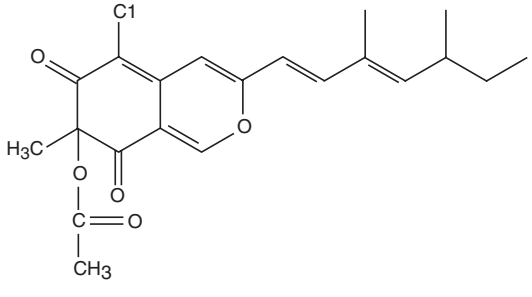

Figure 1. Structure of sclerotiorin.

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Where

A_{scl}: Peak area of sclerotiorin in HPLC chromatogram.
A_{total}: Total peak area in HPLC chromatogram excluding the solvent peak.
D.F.: Dilution factor.
390: Molecular weight of sclerotiorin.
ε: Molar extinction coefficient of sclerotiorin (L $mole^{-1}$).
W: Sample weight (g).

The molar extinction coefficient of sclerotiorin was determined according to the equation: A = εbc. Sclerotiorin standard (0.01g) was dissolved with 100 mL of methanol. After dilution, the absorption of this pigment solution was detected at OD_{370}.

A sclerotiorin standard solution (0.1%, w/v) was prepared. The limitation of quantification (LOQ) of sclerotiorin was determined under such analytical condition after detection of a series of dilution of 0.1% standard solution.

(V) *Recovery test*

One gram of LM was spiked with 1 mL or 10 mL of 0.1% (w/v) sclerotiorin standard solution. After mixing, the mixtures were extracted with 60-fold (v/w) methanol twice and put into a 100-mL volumetric flask to make to the constant volume. The above solution was diluted and assayed by HPLC after filtration through a 0.45-μm membrane. The final solution was also measured with a spectrophotometer at OD_{370}. Each recovery test was done in triplicate. Blank test was also performed at the same time.

(VI) *Determination of the relative error of the mean (REM)*

Two concentrations (5 and 100 μg/mL) of sclerotiorin standard solution were prepared, and injected to HPLC after filtration through a 0.45-μm membrane. The absorbance at 370 nm was also detected with a suitable dilution. One intraday and three interday analyses were conducted. Samples were tested in triplicate. The standard deviation (SD) and coefficient of variation (CV%) of results were calculated to determine the precision of analytical method. The relative error of the mean (REM) was calculated for the determination of accuracy according to the following equation:

REM (%) = [(measured value - mean value) / mean value] × 100% .....(2)

RESULTS AND DISCUSSION

I. *Quantification of Sclerotiorin by HPLC*

The maximum absorption of sclerotiorin in methanol was found to be at 370 nm that was based on the results of UV/VIS scanning in the range of 200-800 nm. The absorbance at 370 nm was thus used as the reference for sclerotiorin content determination. In general, chemical concentration in sample can be determined by spectrophotometric method, in which the absorbance is measured and the Beer's law is applied. The molar extinction coefficient (ε) can be found in Merck Index. However, the pigments produced by microorganisms belong to the secondary metabolites, which are a group of analog compounds. In the case of sclerotiorin, the fermented product from *P. sclerotiorum*, the sclerotiorin content would be overestimated if it was determined solely by the spectrophotometric method. Thus, HPLC is required to determine the specific constitute in metabolites. In this study, a 0.1% sclerotiorin standard was prepared. After a series of dilution, 20 μL of which was injected to HPLC and detected at 370 nm. Results showed the LOQ was determined to be 0.5 μg/mL (Table 1). A satisfactory linearity with regression coefficient (R^2) > 0.999 (data not shown) was achieved at the range of 1-100 μg/mL. The HPLC chromatograms of re-crystallized sclerotiorin standard and LM extracts are shown in Figure 2. The LOQ was also determined to be 0.5 μg/mL while using HPLC/OD_{370} combination method, in which the analytical data was calculated according to equation (1) (data not shown).

II. *Effect of Solvents on Sclerotiorin Extraction*

The lyophilized mycelia were extracted with 60-fold valume of six different solvents including methanol, ethanol, isopropanol, acetone, ethyl acetate, and *n*-hexane. After filtration, 1 mL of filtrate was filtered through a 0.45-μm membrane prior to HPLC injection (20 μL) for quantification of sclerotiorin. The remaining filtrate was evaporated to dryness under vacuum and then weighed. The extraction yields were calculated. Table 2 shows that the extraction yields obtained by using methanol, ethanol, isopropanol, acetone, ethyl acetate, and *n*-hexane were 18.2, 13.0, 12.7, 10.9, 9.2, and 5.6%, respectively. Among them, extraction with methanol yields the highest amount of sclerotiorin (6.77 mg/g). Methanol, therefore, was used as the extraction solvent for quantitative analysis of sclerotiorin in LM.

Table 1. The LOQ of sclerotiorin by HPLC with UV/VIS detector at 370 nm

Sclerotiorin concentration (μg/mL)	Recovery (%)[a]	CV (%)[b]
50.0	103.5 ± 1.7	1.6
10.0	101.7 ± 3.6	3.5
5.0	98.7 ± 1.2	1.2
2.0	102.1 ± 0.7	0.7
1.0	98.8 ± 3.9	3.9
0.5	109.2 ± 9.5	8.7
0.1	111.2 ± 17.9	16.1
0.05	131.5 ± 27.1	20.6

[a]Average of triplicate analyses.
[b]Coefficient of variation (CV%).

III. *HPLC/Colorimetry Combination Method for Quantification of Sclerotiorin*

Sclerotiorin, a yellow pigment, is not commercially available, leading to the difficulty in sclerotiorin quantification. So far, there is no reliable analytical method for sclerotiorin analysis. In this study, our aim was to develop an HPLC/spectrophotometric combination method to quantify sclerotiorin without any commercial standard. The mycelium was extracted with methanol and analyzed with HPLC/OD_{370} combination method. The sclerotiorin content was calculated according equation (1). This study used the re-crystallized sclerotiorin as the standard to evaluate this method. Its molar extinction coefficient (ε) was determined to be 30130, which was similar to the finding (30200) by Nigish *et al*.[17]. The ε value 30130 was introduced to equation (1) to get the sclerotiorin concentration. The sclerotiorin contents from several batches of fermentation were measured by HPLC and the above HPLC/colorimetry combination methods, as shown in Table 3. The sclerotiorin contents measured by HPLC/colorimetry method was 1.05~1.12 times (about 7% higher) the value obtained by HPLC method. Therefore, equation (1) was modified to equation (3) with regression calibration, enabling the data obtained by HPLC/colorimetry to be in agreement with that obtained by the HPLC method. As a result, calculations using equation (3) showed no significant statistical difference from those obtained by the HPLC method (Table 3).

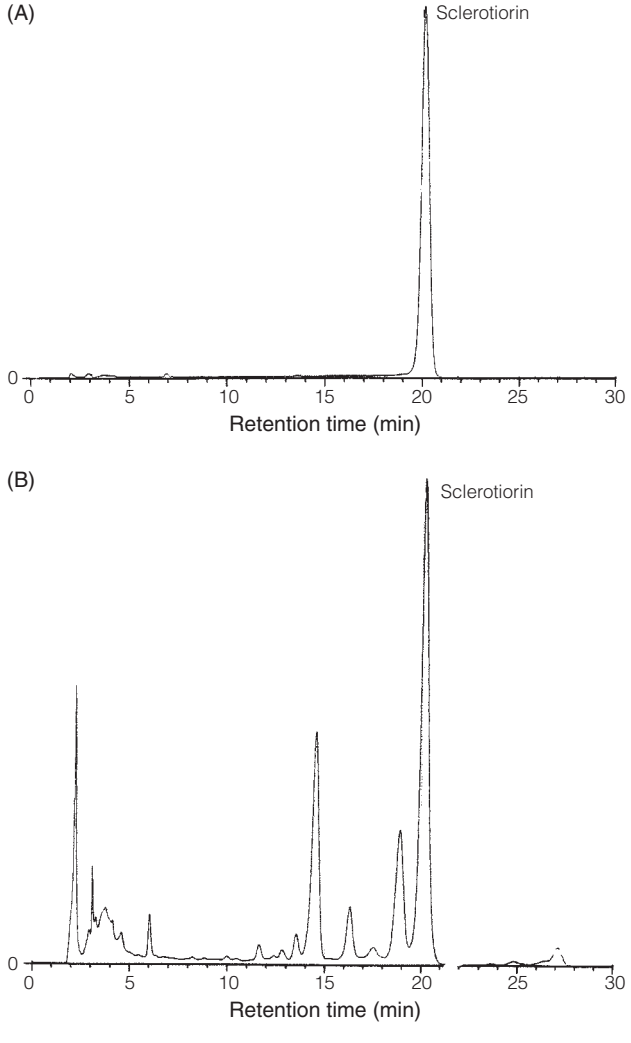

Figure 2. HPLC chromatograms of (A) sclerotiorin crystal; (B) methanol extracts of lyophilized mycelia (LM) of *P. sclerotiorum* BCRC32017.

Table 2. Effect of solvent types on extraction of sclerotiorin in lyophilized mycelia of *P. sclerotiorum* BCRC 32017

Extraction solvent[a]	Extraction yield (%)[b]	Sclerotiorin content mg/g[c]
n-Hexane	5.6	4.24 ± 0.07
Ethyl acetate	9.2	5.61 ± 0.09
Acetone	10.9	5.92 ± 0.09
Isopropanol	12.7	6.01 ± 0.09
Ethanol	13.0	6.38 ± 0.09
Methanol	18.2	6.77 ± 0.10

[a]100 mL solvent / g lyophilized mycelia (LM).
[b]Yield: weight of crude pigment extracted / LM × 100%.
[c]Average of triplicate analyses. Sclerotiorin in LM.

Table 3. Comparison of sclerotiorin contents obtained by HPLC and HPLC/colorimetry combination method

Method	Sample[a]	
	LM sclerotiorin content (mg/g)	WM sclerotiorin content (mg/g)
HPLC	6.77 ± 0.10	0.58 ± 0.02
HPLC/OD_{370}[b]	7.26 ± 0.15	0.63 ± 0.03
HPLC/OD_{370}[c]	6.75 ± 0.09	0.59 ± 0.02

[a]LM: lyophilized mycelia, WM: wet mycelia.
[b]Determined by equation (1).
[c]Determined by equation (3).

$$\text{Sclerotiorin (mg/g)} = 0.93 \times [(A_{scl}/A_{total} \times OD_{370} \times D.F. \times 100 \times 390)/\varepsilon] / W \quad (3)$$

IV. *The Relative Error of the Mean*

Table 4 shows the recovery of sclerotiorin from the lyophilized mycelium (LM). After 1 mg and 10 mg of sclerotiorin were spiked into 1 g of LM, recoveries were 97-109% and 95-108% and the coefficient of variation was less than 3.9% when quantified by HPLC/OD_{370} and HPLC methods, respectively. On the other hand, the intraday and interday (three days) analytical data revealed that the coefficients of variation (CV%) were 2.1-4.2% with spiking of 5 μg/mL sclerotiorin, and were 2.7-4.3% with spiking of 100 μg/mL sclerotiorin as determined by HPLC/OD_{370} method. While using HPLC method, recoveries were 2.0-2.8% and 2.8-3.8%, respectively. These results indicate that both HPLC and HPLC/OD_{370} methods were capable of producing a satisfactory precision in quantitation. The relative errors of the mean (REM) of intraday and interday analyses using HPLC/OD_{370} method were in the range of -4.0~-1.9% and -6.0~-1.3%, respectively, whereas that of HPLC methods were in the range of -2.0~-3.9% and 1.7~4.0%, respectively, indicating that a good accuracy was

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Table 4. Recoveries of sclerotiorin in spiked lyophilized mycelia by HPLC and HPLC/Colorimetry methods

Method	Blank[a](mg) (A)	Amount spiked (mg)(B)	Amount found (mg) (C)[b]	Recovery (%)[c]	CV (%)[d]
HPLC					
	6.77 ± 0.10	1	7.85 ± 0.31	108.0	3.9
	6.77 ± 0.10	10	16.26 ± 0.39	94.9	2.4
$HPLC/OD_{370}$					
	6.75 ± 0.09	1	7.84 ± 0.30	109.0	3.8
	6.75 ± 0.09	10	16.49 ± 0.35	97.4	2.1

[a]Sclerotiorin in 1 g of lyophilized mycelia (LM).
[b]Average of triplicate analyses.
[c]Recovery (%) = (C–A) / B × 100%.
[d]Coefficient of variation (CV%).

Table 5. Precision and accuracy of intraday and interday validation for sclerotiorin concentration ranging from 5 to 100 μg/mL by HPLC and HPLC/Colorimetry methods

Method	Concentration (μg/mL)	Intraday (n = 3)[a]		Interday (n = 9)[b]	
		Precision Mean ± SD (CV%)	Accuracy REM (%)[c]	Precision Mean ± SD (CV%)	Accuracy REM (%)
HPLC					
	5	4.9 ± 0.1 (2.0)	- 2.0	5.2 ± 0.2 (3.8)	4.0
	100	96.1 ± 2.7 (2.8)	- 3.9	101.7 ± 2.8 (2.8)	1.7
$HPLC/OD_{370}$					
	5	4.8 ± 0.2 (4.2)	- 4.0	4.7 ± 0.2 (4.3)	-6.0
	100	101.9 ± 2.2 (2.1)	- 1.9	98.7 ± 2.7 (2.7)	-1.3

[a]n = 3, three repeated injection on the same day.
[b]n = 9, three repeated injection each day for three days.
[c]REM = relative error of the mean.

achieved (Table 5). This analysis is simple. It takes only 30 min for sample preparation, including extraction of yellow pigment with methanol from sample (wet mycelia or lyophilized mycelia), filtration and dilution.

CONCLUSIONS

In this study, an $HPLC/OD_{370}$ combination method was developed to quantify sclerotiorin contained in *P. sclerotiorum* mycelium with no chemical standard was available. Sample was extracted with 60-fold (v/w) methanol twice. The extract was filtered and analyzed by HPLC using a C18 reverse-phase column in combination with spectrophotometric measurement at OD_{370}. The sclerotiorin content in mycelia was thus determined. The results showed that the $HPLC/OD_{370}$ combination method without a sclerotiorin standard is comparable to the HPLC method with sclerotiorin standard, suggesting that the currently developed method could be used as a routine method for the determination of sclerotiorin for studying the production of sclerotiorin by *P. sclerotiorum*.

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