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Accurate Determination of Oosporein in Fungal Culture Broth by Differential Pulse Polarography

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A simple and accurate differential pulse polarographic method has been developed for the determination of oosporein in the culture broth of the fungus *Beauveria brongniartii*. This hydroxybenzoquinone derivative is the only major secondary metabolite secreted by this entomopathogenic fungus, which is used as biological pest control agent (BCA) against *Melolontha melolontha* larvae. It can be found in the host organism as well as in the formulated product. The polarographic behavior of oosporein was examined in various buffer systems over the pH range 3−10. In Britton−Robinson buffer/methanol solution (3:7 v/v, pH 5.5) the differential pulse polarograms exhibited reproducible peaks at $E_p = -0.18$ V vs silver/silver chloride/potassium chloride (3 M). Under these conditions, a plot of peak height vs concentration of oosporein was found to be linear over the range 5.9×10^{-7} to 2.5×10^{-5} M (0.18−7.74 μg mL^{-1}; $r = 0.9998$). The detection limit was calculated to be 54 ng mL^{-1}. To evaluate the concentration of oosporein, the standard addition method was applied. The analysis of oosporein in the culture broth led to a mean value of 524.9 μg mL^{-1} broth with a relative standard deviation (S_{rel}) of ±2.6%. The proposed polarographic method is accurate, not time-consuming, and it is of low cost because no separation steps are necessary.

KEYWORDS: Oosporein; *Beauveria brongniartii*; differential pulse polarography; DPP

INTRODUCTION

Beauveria brongniartii (Sacc.) Petch is known to be a very selective and highly virulent entomopathogenic soil fungus used in the control of *Melolontha melolontha* (cockchafer) (*1*). The symmetrical hydroxybenzoquinone derivative oosporein (**Figure 1**) is the only major secondary metabolite secreted into the culture filtrate by commercially used *B. brongniartii* strains (e.g., IMBST 95041, production strain of Melocont Pilzgerste, Kwizda Austria) (*2*).

The red oosporein, which is a widespread secondary metabolite of soil-dwelling fungi (*3*, *4*), showed moderate antibiotic activity (*5*, *6*), no antifungal activity (*6*, *7*), and no phytotoxic activities (*8*, *9*) at levels below 1 mM. Choice feeding and non-choice-feeding experiments with *M. melolontha* and other nontarget insect larvae proved that oosporein does not seem to be the active principle of *B. brongniartii* (*10*, *11*). The principle is not yet identified. So far, there has been no report of oosporein having any negative effect on humans and nontarget organisms even though *Beauveria* control agents have been deployed extensively in specific crop production systems. However,

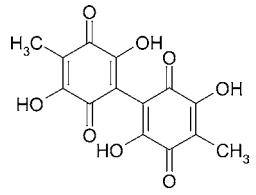

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Figure 1. Structure of oosporein.

pronounced nephrotoxic activities of oosporein were found in feeding experiments using purified oosporein in artificial media (LD_{50} about 5−10 ppm, 16−32 μM) (*8*, *12*, *13*). Nevertheless, when feed mixtures (in general ground corn preparations) with crystalline oosporein added were used, the activities were much lower. No lethal effect was observed at <500 ppm in turkey (<1.63 mM (*14*)) and at <200 ppm in broiler chicken (<0.65 mM (*12*, *13*)). As can be seen, there is some confusion in the literature. Even the spare data on the nephrotoxicity of oosporein reported in the literature are not congruent.

Oosporein can be regarded as a marker substance for the presence of the *Beauveria* because the metabolite is secreted in copious concentrations into the culture broth and was also found on sterilized barley kernels used as biocontrol agent (Melocont Pilzgerste, Kwizda Austria) and inside mycosed *M. melolontha* larvae (*2*).

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There is significant interest in defining the criteria for registration of fungal biological control agents (BCAs) and global harmonization of registration procedures. Nevertheless, fungal BCAs have to be evaluated in terms of hazard and risk assessment for man and the environment because they are chemical plant protection products (see Council Directive 91/414/EEC). In analogy to chemicals, the major metabolites secreted by fungal BCAs must be determined by using sensitive analytical methods and tools. At present, for analyzing the quantity of oosporein in biological matrixes, HPLC-methods are used (*2*, *10*).

The favorable, electrochemically active characteristics of quinone systems offer the application of a differential pulse polarographic (DPP) method to the determination of oosporein in complex matrixes, e.g., culture broth as an alternative approach. Therefore, the aim of this study was to develop an analytical method that is able to measure oosporein in complex matrixes and does not require an arduous separation procedure. To develop the suitable analytical conditions, the electrochemical behavior of oosporein using a dropping mercury electrode (DME) was investigated. In addition, the sample preparation and the polarographic parameters for the analysis were improved and optimized.

Voltammetric methods for the determination of oosporein using various electrodes (glassy carbon electrode and gold electrode) were also investigated. But the best results concerning reproducibility and selectivity were approached using the DPP method.

MATERIALS AND METHODS

Materials. Oosporein (98% purity, 2,2′,5,5′-tetrahydroxy-4,4′-dimethyl[bi-1,4-cyclohexadien-1-yl]-3,3′,6,6′-tetron) and *Beauveria brongniartii* (IMBST 95041) culture broth were produced in a 14 L stirred tank reactor (BioEngineering NLF22) with 10% v/v inoculation density containing S2G medium, pH 6.0, and an antifoam agent, 0.05% v/v Silicon 289 antifoam (Sigma). The aerated culture was stirred (350−400 rpm) for 4 days at 23 °C to ensure a dry biomass greater than 7 g L^{-1} dry weight (*2*). All reagents were of Suprapur and/or ProAnalysis grade (E. Merck, Darmstadt, BRD). Distilled water was purified with a Milli-Q Nanopure (Millipore, Bedford, MA) system and was stored in Nalgene containers. The nitrogen used was 99.9995% pure, while the mercury was 99.999% pure (Ögussa, Graz, Austria). Britton−Robinson (BR) buffer solutions (0.1 M) pH 2−10, acetate buffer (0.1 M), pH 4, and McIlvaine buffer (0.1 M), pH 4, were used as supporting electrolytes for fundamental polarographic tests. The calibration curve and culture broth analyses were performed in a BR buffer, pH 4.1, mixed with methanol (3:7 v/v). This methanolic buffer solution shows a pH of 5.5 and is made up fresh once a week. Stock solution of oosporein (1×10^{-3} M) was prepared by transferring 6.2 mg of oosporein into a 20 mL volumetric flask, dissolving it in the methanolic BR buffer solution (pH 5.5, 3:7 v/v), and bringing the mixture to volume. This solution contains 0.31 μg μL^{-1}, and the content remains the same throughout the week. Further standard solutions were prepared by appropriate dilution of the stock solution with methanol and making it fresh when required.

Instruments and Apparatus. Differential pulse polarographic measurements were carried out by using the polarographic analyzer/stripping voltammeter model 264 A (EG&G, PARC, NJ) in combination with a polarographic stand model 303 A SMDE (EG&G, PARC). This electrode stand consists of a dropping mercury electrode (DME) as working electrode, an Ag/AgCl/3 M KCl reference electrode, and a platinum wire as an auxiliary electrode. For preparation of the calibration graph and for analysis of the culture broth, the analyzer was operated under the following parameters: mode, DPP; drop size, M; drop time, 0.5 s; potential range, −0.05 to −0.4 V; scan rate, 5 mV s^{-1}; pulse amplitude, 25 mV; current sensitivity, 1−5 μA.

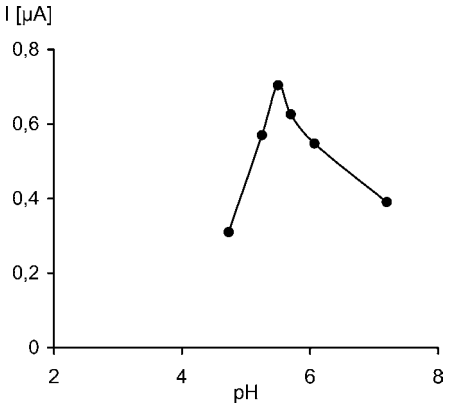

Figure 2. Peak current versus pH in BR buffer/methanol (3:7 v/v) at a concentration of 0.8 μg mL^{-1}.

Evaluation was performed with computer assistance, using the tangents method (ECAR computer interface and software from ACE, Heidelberg, Germany).

The pH of the solutions was adjusted employing a Metrohm pH meter model 632 and a glass electrode model 6.0202.000 (Metrohm AG, Herisau, Switzerland). All measurements were carried out at room temperature.

Calibration Graph of Oosporein. Transfer 10.0 mL of BR buffer/methanol solution (3:7 v/v, pH 5.5) to a polarographic cell and purge with nitrogen for 8 min for deoxygenation. After determination of the blank value, aliquots (20 μL each) of oosporein stock solution are added successively to the cell, and the cell is purged after each addition with nitrogen for another 30 s. The polarograms are recorded with the instrumental parameters described above. The data are evaluated by applying the tangents method, considering the increase of volume. By use of proper standard solutions, it is possible to determine oosporein content in the range 0.18−7.74 μg mL^{-1}.

Procedure for Oosporein Determination in Culture Broth. Transfer 10 mL of BR buffer/methanol solution (3:7 v/v, pH 5.5) to the polarographic cell, and after purging with nitrogen for 8 min, record this polarogram as the blank solution. Dilute the fungal culture broth (1:1 v/v) with BR buffer/methanol (3:7 v/v, pH 5.5). An aliquot (50 μL) of the so diluted culture broth is transferred to the cell, and after purging for another 30 s with nitrogen, read the polarogram. To determine the concentration of oosporein, the standard addition method is applied by adding successively three aliquots (20 μL each) of the stock solution.

RESULTS AND DISCUSSION

Appropriate Polarographic Conditions for the Determination of Oosporein. The DPP analyses using BR buffer showed that oosporein is reduced at the dropping mercury electrode with a single well-defined peak in a pH range 4.7−7.2. In the acidic range the peak was beyond the potential window limited by the supporting electrolyte and/or the mercury. At higher pH values, an unemployable splitting up of the peak was registered.

The peak occurred in a potential range of −0.13 to −0.31 V depending on the pH value of the solution (4.7−7.2). The linear correlation between peak potential (E_p in V) and pH has the following function:

$$E_p = -(0.0679)(\text{pH}) + 0.188 \qquad (R = 0.999;\ \text{pH } 4.7-7.2)$$

The intensity of the peak current (I_p) of oosporein was influenced by both the type of buffer system (i.e., acetate buffer, McIlvaine buffer, BR buffer) and the pH value. It was found that the peak current was reproducible with optimal sensitivity in Britton−Robinson buffer/MeOH solution (3:7 v/v) at pH 5.5 (**Figure 2**). It was necessary to add methanol to the buffer solution as a

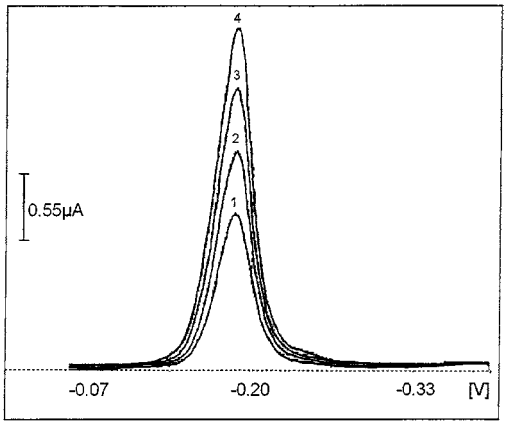

Figure 3. Differential pulse polarograms of oosporein in BR buffer/methanol solution (3:7 v/v, pH 5.5). The concentrations of oosporein employed were (1) 1.5, (2) 2.2, (3) 2.8, and (4) 3.5 μg mL^{-1}.

Table 1. Overview of the Validation Parameters

validated parameters	from DPP method	from HPLC method[a]
linearity (M)	5.9×10^{-7} to 2.5×10^{-5}	*b*
LOD (M)	1.8×10^{-7}	1.3×10^{-5}
intraday accuracy (%)	±2.0	*b*
intraday bias (%)	−1.3	*b*
interday accuracy (%)	±1.4	*b*
interday bias (%)	−2.7	*b*

[a] Strasser et al. (*2*). [b] Not given.

solubilizer. The peak potential (E_p) of oosporein at pH 5.5 was found to be −0.18 V vs Ag/AgCl in accordance with the above equation.

Under these optimized conditions, strict linearity between peak height and concentration of oosporein in the range 0.18−7.74 μg mL^{-1} (5.9×10^{-7} to 2.5×10^{-5} M) was observed.

The determination of the calibration curve led to the following linear function:

$$I_p\,(\text{in } \mu\text{A}) = (0.5851)(C, \text{ in } \mu\text{g mL}^{-1}) + 0.0293 \qquad (R = 0.9998;\ n = 3)$$

From an analytical point of view, a validation of the proposed method is appropriate. So the accuracy of the polarographic method during 1 day was tested on a solution of oosporein with a concentration of 1.50 μg mL^{-1} by means of the standard addition method (**Figure 3**). This analysis revealed a mean of 1.48 ± 0.03 μg mL^{-1} (n = 5), corresponding to a relative standard deviation of ±2.0%. A recovery of 98.7% was achieved. In addition, interday accuracy was analyzed by measuring five solutions of oosporein (1.50 μg mL^{-1}) on five different days. The analyses showed a mean of 1.46 ± 0.02 μg mL^{-1}, corresponding to a relative standard deviation of ±1.4% with a recovery of 97.3%.

According to the Analytical Methods Committee (*15*), the limit of detection (LOD) is defined as the mean value of the intercepts (blank mean y_b) of the calibration curves plus 3 times the standard deviation of the intercepts (blank s_b). This led to an LOD of 0.054 μg mL^{-1}. The limit of quantitation (LOQ) is estimated similarly to the LOD value but with $y_b + 10s_b$. This resulted in an LOQ of 0.18 μg mL^{-1}. In addition, the validated parameters are summarized in **Table 1**.

Oosporein Content in Fungal Culture Broth. *Beauveria brongniartii* BIPESCO 2 (IMBST 95041) was used for submerged batch cultivation on Sabouraud 2% glucose (S2G medium, Merck 108339), and the fungus was harvested by centrifugation. The culture broth was tested for its oosporein content.

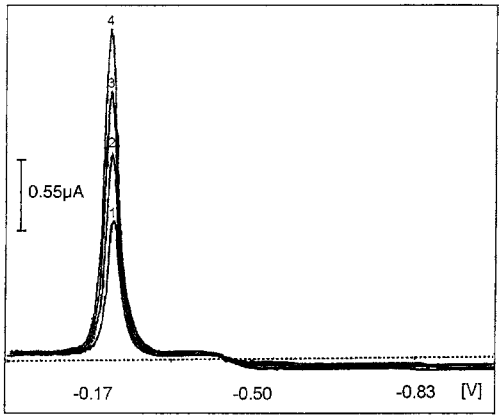

Figure 4. Determination of oosporein in fungal culture broth. Polarograms of (1) fungal culture broth/BR buffer methanol solution (1:1 v/v), 50 μL, and (2–4) addition of oosporein stock solution, 20 μL each.

To evaluate the content of oosporein in the culture broth, the sample preparation had to be optimized. Several rates of dilution were examined. The best results were obtained by mixing the culture broth with BR buffer/methanol (3:7 v/v, pH 5.5) in a ratio of 1:1. Since the culture broth might cause disturbances resulting in incorrect peak heights, the standard addition method had to be applied (**Figure 4**). Following the analysis of the culture broth as described in the working procedure, a mean value of 524.9 μg mL^{-1} broth was obtained, with a relative standard deviation of ±2.6%. The presented DPP method was compared with the HPLC method (*2*). The HPLC result of 505 μg mL^{-1} broth is in good agreement with the DPP method.

To check the specifity of the proposed method, a distinct amount of oosporein was added to the culture broth. As a result of these investigations, the recovery rate was found to be 95.1%.

CONCLUSION

In summary, a simple differential pulse polarographic method has been developed for the determination of oosporein, especially in fungal culture broth. The significant advantage of this DPP method is that the analysis requires neither extensive separation of the culture medium nor extraction of oosporein, with the result that the method is selective without being time-consuming. The relative standard deviation of 2.6% for the culture broth indicates a reproducible and precise method. The resulting oosporein content was compared with an HPLC method using the same culture broth. The excellent correlation between the polarographic and the chromatographic values confirms the accuracy of the proposed method. Thus, the established DPP method is robust, not expensive, and rapid and is therefore suitable for routine analysis of oosporein in complex matrixes.

The determination of oosporein in soil and/or finish products using this method requires extended investigations and is part of our ongoing research efforts.

LITERATURE CITED

(1) Strasser, H. Beurteilung der Wirksamkeit des biologischen Pflanzenschutzpräparates MELOCONT-Pilzgerste zur Maikäferbekämpfung. *Förderungsdienst* **1999**, *5*, 158−164.

(2) Strasser, H.; Abendstein, D.; Stuppner, H.; Butt, T. M. Monitoring the distribution of secondary metabolites produced by the entomogenous fungus *Beauveria brongniartii* with particular reference to oosporein. *Mycol. Res.* **2000**, *104*, 1227−1233.
(3) Roberts, D. W. Toxins of entomopathogenic fungi. In *Microbial Control of Pests and Plant Diseases 1970−1980*; Burges, H. D., Ed.; Academic Press: New York, 1981; pp 441−463.
(4) Strasser, H.; Vey, A.; Butt, T. M. Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium, Tolypocladium* and *Beauveria* species? *Biocontrol Sci. Technol.* **2000**, *10*, 717−735.
(5) Brewer, D.; Jen, W. C.; Jones, G. A.; Taylor, A. The antibacterial activity of some naturally occurring 2,5-dihydroxy-1,4-benzoquinones. *Can. J. Microbiol.* **1984**, *30*, 1068−1072.
(6) Strasser, H.; Abendstein, D. Oosporein, a fungal secondary metabolite with antimicrobial properties. *IOBC/WPRS Bull*. **2000**, *23*, 113−115.
(7) Brewer, D.; Maass, W. S. G.; Taylor, A. The effect on fungal growth of some 2,5-dihydroxy-1,4-benzoquinones. *Can. J. Microbiol*. **1977**, *23*, 845−851.
(8) Cole, R. J.; Kirksey, J. W.; Cutler, H. G.; Davis, E. E. Toxic effects of oosporein from *Chaetomium trilaterale*. *J. Agric. Food Chem.* **1974**, *22*, 517−520.
(9) Abendstein, D.; Pernfuss, B.; Strasser, H. Evaluation of *Beauveria brongniartii* and its metabolite oosporein regarding phytotoxicity on seed potatoes. *Biocontrol Sci. Technol.* **2000**, *10*, 789−796.
(10) Abendstein, D. Der entomopathogene Pilz *Beauveria brongniartii* in Oberflächen und Submerskultur: Morphologie, Wachstum und ökotoxikologische Charakterisierung des Sekundärmetaboliten Oosporein. Ph.D. Thesis, University of Innsbruck, Austria, 2001.
(11) Abendstein, D.; Schweigkofler, W.; Strasser, H. Effect of the fungal metabolite oosporein on feeding behaviour and survival of *Melolontha melolontha*—and *Galleria mellonella*—larvae. *Laimburg J.*, in press.
(12) Pegram, R. A.; Wyatt, R. D. Avian gout caused by Oosporein, a mycotoxin produced by *Chaetomium trilaterale*. *Poult. Sci*. **1981**, *60*, 2429−2440.
(13) Manning, R. O.; Wyatt, R. D. Comparative toxicity of *Chaetomium* contaminated corn and various chemical forms of Oosporein in broiler chicks. *Poult. Sci.* **1984**, *63*, 251−259.
(14) Pegram R. A.; Wyatt R. D.; Smith T. L. Oosporein toxicosis in turkey poult. *Avian Dis*. **1982**, *27*, 47−59.
(15) Analytical Methods Committee. Recommendations for the definition, estimation and use of the detection limit. *Analyst* **1987**, *112*, 199−204.

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