Development of a Sensitive High-Performance Liquid Chromatography-Diode Array Detection Assay for the Detection and Quantification of the *Beauveria* Metabolite Oosporein from Submerged Culture Broth and Bio-Control Formulations

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A sensitive high-performance liquid chromatography−diode array detection (HPLC−DAD) assay is described for the detection and quantification of the *Beauveria* metabolite oosporein from fungal culture broth and two biocontrol agent formulations. In all cases, analyte recovery was achieved with a Britton−Robinson buffer system at pH 5.5 diluted with methanol 3:7 (v/v) (BR5.5-MeOH). The HPLC−DAD assay, using a binary solvent gradient with acidic modifiers and detecting the metabolite at 287 nm, showed linearity over 3 orders of magnitude and a limit of detection (LOD) of 6.0 ± 2.3 µg of oosporein/L of BR5.5-MeOH. The oosporein content of the representative fungal culture broth samples and two *Beauveria* formulations (Melocont-Pilzgerste and Melocont-WP) was found to be 504.7 ± 13.6 mg of oosporein/L of culture filtrate, 7.4 ± 0.6 mg of oosporein/kg of Melocont-Pilzgerste, and 38.2 ± 1.3 mg of oosporein/kg of Melocont-WP with recovery rates of 93 ± 2, 99 ± 8, and 92 ± 3%, respectively.

KEYWORDS: HPLC−DAD; *Beauveria brongniartii*; oosporein; hydroxybenzoquinone derivative; analyte adsorption; biological pest control formulations; risk assessment

INTRODUCTION

Oosporein is a C_2 symmetrical red 2,5-dihydroxybenzoquinone derivative biosynthesized by a broad variety of soil borne fungi and has been known for almost six decades (**Figure 1**) (*1*−*3*). It is the major secreted secondary metabolite of the enthomopathogenic fungus *Beauveria brongniartii* (Sacc.) Petch (*Ascomycota*: *Clavicipitaceae*), which is used as a selective and highly virulent biocontrol agent (BCA) against *Melolontha melolontha* L. (*Coleoptera*: *Scarabaeidae*) (common European cockchafer) (*4*, *5*). Because of its presence in the fungal culture broth, in the BCA formulation, and in the mycosed target organism, its occurrence can be used as an indication for the presence of *B. brongniartii* (*3*). The current legal regulations regarding the registration and the use of BCA formulations within the EU (amended council directive 91/414/EEC) demand an evaluation of the hazard and risk assessment for man and environment in analogy to chemical plant protection products. In this context, metabolites secreted by the BCA have to be monitored in BCA formulations and the environment using appropriate analytical tools and methods. Only a limited number
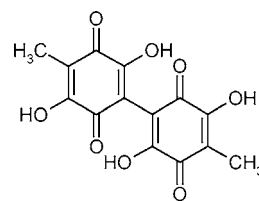

Figure 1. Structure of oosporein.

of analytical approaches meet these requirements today. Besides a sensitive electrochemical method presented recently with an intra-assay limit of detection (LOD) of 54 µg of oosporein/L (*6*), three high-performance liquid chromatography (HPLC) assays have been shown to be generally feasible to analyze oosporein. A high-performance liquid chromatography assay with diode array detection (HPLC−DAD) published by Strasser et al. (*5*) showed an intra-assay LOD of 4 mg/L of oosporein, whereas the two other contributions did only deal with the detectability of oosporein without going into methodological details of the metabolite quantification (*7*, *8*). Two further methods have been developed 15 years ago to monitor oosporein in poultry feed and corn. Ross et al. (*9*) utilized positive and negative ion chemical ionization mass spectrometry to identify oosporein in feed matrix. The reported detection limit was 5 mg/kg, but no sample preparation protocols have been disclosed.

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Rottinghaus et al. (*10*) used a strong anion-exchanger solid-phase extraction (SAX−SPE) procedure to enrich oosporein from ground feed samples extracted with acetonitrile/1% NaHCO$_3$ (1:1, v/v). The detection was carried out fluorometrically after diazomethane mediated methylation of oosporein and TLC separation. Detection limits of 0.5 and 1.0 mg/kg for corn and poultry feed, respectively, have been reported, and the recovery rate was exceeding 90% in the range between 5 and 100 mg/L. However, using this extraction scheme, we failed to recover oosporein from potato tubers and barley kernels at a concentration level of 14 mg of oosporein/kg of matrix. It was shown that this loss was not associated with binding to the stationary phase used or to the sample workup (data not presented). Furthermore, it could be demonstrated that diluted oosporein solutions in methanol (<5 mg/L) lost the analyte within 24 h, even if stored at −20 °C. Thus, it had to be concluded that oosporein is forming complexes with both sample matrixes and glass surfaces of the containers used for sample handling and storage. This hypothesis is supported by the chemical behavior observed for 2,5-dihydroxybenzoquinone (DHB), structurally closely related to oosporein, which may be regarded as methylated DHB dimer. DHB forms dianions at neutral pH (pK_a values are 2.73 and 5.18 in H$_2$O) (*11*) and easily forms metal ion complexes (*12, 13*). These di- and polynuclear complexes have been reported with various central ions and a broad variety of ligands including ortho-functionalized benzoic acids, ortho-quinones, and pyridine derivatives (*14−17*). Precipitation of these complexes has been observed as well as the oriented adsorption to metal surfaces (*12, 13, 18*).

On the basis of these facts, the aim of this study was to establish new methods allowing the isolation, handling, and quantification of the polar fungal analyte oosporein from biotic and abiotic matrixes at a sub-mg/kg level. In this paper, we will show how these goals were achieved by (i) improving the extraction and sampling solvent with the aid of adsorption experiments and (ii) by developing a sensitive HPLC−DAD assay. Finally, three application examples for the quantification of oosporein with the developed HPLC−DAD assay will be presented.

MATERIALS AND METHODS

Reagents and Buffers. Oosporein reference standard materials (purity 98%; 2,2′,5,5′-tetrahydroxy-4,4′-dimethyl-[bi-1,4-cyclohexadien-1-yl]-3,3′,6,6′-tetron) and culture filtrates were gained from *B. brongniartii* (strain BIPESCO 2 (DSM 15205) IMBST 95041, production strain of Melocont-Pilzgerste) submerse cultures (*5, 6*). BCA formulations were obtained from F. Joh. Kwizda GmbH Austria (Melocont-Pilzgerste) and from AgResearch, Lincoln, New Zealand (Melocont-WP) (*19, 20*). Acetonitrile, methanol (both gradient grade), acetic acid, boric acid, phosphoric acid, and sodium hydroxide (all analytical grade) were purchased from Merck (Darmstadt, Germany). Water for the HPLC was produced by reverse osmosis followed by distillation. Britton−Robinson buffer (5.13 mL of stock solution acetic acid, 8.40 mL of stock solution phosphoric acid, 53.1 mL of stock solution boric acid, and 6.84 mL of stock solution sodium hydroxide; distilled water was added to a final volume of 300 mL, and the pH was adjusted with sodium hydroxide or phosphoric acid if needed) was prepared from appropriate stock solutions (100 g/L for acetic acid, phosphoric acid, and sodium hydroxide and 10 g/L for boric acid) accordingly to the procedure given by Mongay and Cerda (*21*) and diluted with methanol (3:7, v/v) to give the final BR5.5-MeOH sample buffer. Typical stock solutions of oosporein were prepared by 1−2 mg of oosporein being transferred into a 10 mL volumetric flask and dissolved with the BR5.5-MeOH buffer. All spiking solutions were prepared by appropriate dilution of stock solution with BR5.5-MeOH buffer.

Adsorption Experiments. For each solvent system tested, three 25 mL volumetric flasks were used. Flask 1 was charged with 0.5 g of potato starch, 250 μL of the oosporein stock solution was added, and the flask was filled to the mark with the tested solvent system. Flask 2 was charged with 250 μL of the oosporein stock solution, and the flask was filled to the mark with the tested solvent system. Flask 3 served as a control and was charged with the tested solvent system. All flasks were agitated for 16 h on a flat bed shaker moving at 23 °C with 200 rpm. HPLC samples were drawn directly from the flasks after sedimentation of the starch. The HPLC method described next was used for the quantification of oosporein present in the solution.

HPLC−DAD Conditions. HPLC analyses were performed using a HP 1090 liquid chromatograph (Agilent, Waldbronn, Germany) equipped with a diode array detector (DAD), an automatic injector, an auto sampler, and a column oven. Separations were performed on a Phenomenex Synergi Hydro-RP 80A column (150 × 2 mm) with a particle size of 4 μm (Phenomenex, Torrence, CA). The Phenomenex SecurityGuard system equipped with a C18 cartridge (4 × 2 mm) was used as guard column. The binary elution gradient consisted of water (solvent A) and acetonitrile (solvent B), both containing 0.1% (v/v) acetic acid and 0.9% (v/v) formic acid. The course of the gradient was 5−60% B in 6 min, followed by 60−98% B in 2 min and was kept constant for a further 5 min at a flow rate of 0.3 mL/min. The column was kept at 23 °C (thermostated). Between analyses, the column was reequilibrated for 7 min. The injection volume was 2 μL, and chromatograms were recorded at 287 nm (sample bandwidth 4 nm, reference wavelength 450 nm, reference bandwidth 80 nm).

HPLC−DAD Calibration Curves and Assay Validation. Calibration curves were obtained for a BR5.5-MeOH dilution series of oosporein. All calibration levels were measured 5 times. Calibration functions were obtained by linear regression of the respective oosporein concentration (c_A) against the peak area (A) resulting in the calibration function $A = ac_A + b$. The limit of detection (LOD) and the limit of quantification (LOQ) for the HPLC−DAD method were calculated from the calibration function (*22*). The LOD equals the value of the intercept of this calibration curve (blank mean b_b) plus 3 times the standard deviation of the intercept (blank s_b), and the LOQ is obtained by adding 10 times s_b to b_b. LOD and LOQ have been established from a 1 mL sample volume. The precision of the method was determined as the relative standard deviation (% RSD) of the calibration levels, and the accuracy was determined as the deviation of the measured mean from the fortification level expressed as a percentage of the fortification level (% dev). Intra- and interday repeatability of the method was measured on three sub-ppm fortification levels of oosporein in BR5.5-MeOH buffer. Measurements were performed on three different days in five day intervals with five replications per day. All spiking experiments for the determination of the recovery rate from the matrixes are described next and have been performed in triplicate. All spiking levels used were adjusted to the oosporein concentration found in the respective matrix.

Quantification of Oosporein in Culture Filtrate. A culture filtrate sample (50 μL) was diluted with 950 μL of sample buffer (BR5.5-MeOH), transferred into a HPLC vial, and measured without further purification. The recovery rate of oosporein from the culture filtrate was determined with a spiking experiment using a BR5.5-MeOH sample buffer fortified with 16.5 mg of oosporein/L (equals 313.5 mg of oosporein/L of culture filtrate) and a culture filtrate sample with an analyte concentration of 493.2 mg/L.

Quantification of Oosporein in Melocont-Pilzgerste. Ten g of frozen (−20 °C) Melocont-Pilzgerste (*Beauveria* colonized barley kernels) was submersed in 200 mL of extraction solvent BR5.5/MeOH and milled in a kitchen blender (Moulinex Turbo Blender). Changes in the volume were carefully monitored and were found to be negligible. The debris of the homogenized material was pelleted by centrifugation (Hermle Z383 (Hermle Labortechnik, Wehingen, Germany) centrifuge at 3500 rpm, 30 min, room temperature), and an aliquot (1 mL) of the clear supernatant was transferred to a HPLC vial for measurement. Analyte identification and recovery rate were determined with a spiking experiment using BR5.5-MeOH extraction solvent fortified with 0.70 mg of oosporein/L (equals 14.0 mg of oosporein/kg of Melocont-

Pilzgerste) and a Melocont-Pilzgerste sample with an analyte concentration of 7.1 mg/kg.

Quantification of Oosporein in Melocont-WP. Melocont-WP (0.1 g of water dispensable powder) was suspended in the extraction solvent (BR5.5-MeOH) in a 5 mL volumetric flask and ultrasonicated (3×5 min). Changes in the volume were carefully monitored and were found to be negligible. Two mL of this solution was purified by centrifugation (Hermle Z383 (Hermle Labortechnik, Wehingen, Germany) centrifuge at 2500 rpm, 20 min, room temperature) over a M_r 10 000 cutoff membrane (Vivaspin2, CTA membrane, Sartorius, Göttingen, Germany) and used for HPLC analysis without further treatment. The recovery rate was determined with a spiking experiment using BR5.5-MeOH extraction solvent fortified with 0.46 mg of oosporein/L (equals 23.0 mg oosporein/kg Melocont-WP) and a Melocont-WP sample with an analyte concentration of 38.0 mg/kg.

RESULTS AND DISCUSSION

Sample Buffer Development. To overcome the loss of oosporein described in the Introduction, adsorption experiments were used to improve the sample solvent conditions. Chosen oosporein concentrations (0.91−1.22 mg of oosporein/L of sample solvent) were well below the LOD achieved with methanolic dilution series of oosporein. Experiments were carried out in the absence or presence of potato starch using regular analytical glassware. Starch was chosen as organic matrix model because analyte loss was observed in starch rich crops as potato tubers or barley kernels. Different solvent systems including tap water and organic solvents and buffer systems were used. Combinations of Britton−Robinson buffer systems with organic modifiers (acetonitrile or methanol) turned out to be superior to any other solvent systems tested. A linear relationship ($R^2 = 0.986$) was observed between experiments with and without starch added. Therefore, adsorption to the organic matrix is a pure additive effect and is independent from the solvent system used. The interaction of the analyte with the glass surface is predominant in the test setup. Thus, the encountered loss of oosporein was most likely due to complex formation with OH/O^- groups on any surface—independent of the protonation state of this hydroxyl function (starch OH groups can be considered protonated, whereas Si−OH groups can be considered mostly deprotonated at ambient pH). Further investigations into the Britton−Robinson buffer system did show that organic modifiers as acetonitrile or methanol are mandatory for oosporein recovery. Sodium borate turned out to be the most active single component of the buffer system. No influence of the pH of buffer compositions mixed accordingly to the tables given by Mongay and Cerda (*21*) was observed. At the pH values tested (pH 2−6), oosporein is certainly at least partially present in an ionic form since the pK_a of the first deprotonation step of the structurally related 2,5-dihydroxybenzoquinone was determined as 2.73 in water (*11*). Buffer dilution experiments did show that oosporein recovery drops linearly ($R^2 = 0.995$) with decreasing buffer concentration. This experimental evidence allowed concluding that the oosporein anion can be only stabilized in solution by complex formation with the Britton−Robinson buffer system. Organic buffer modifiers are needed for the stabilization of this complex, and a certain buffer concentration threshold is needed for oosporein recovery from inorganic and organic matrix surfaces. In analogy to DHB complexes described in the literature (see Introduction), ubiquitous cations such as sodium, potassium, or calcium can serve as central ions in these complexes. With this refined buffer system, oosporein handling and analysis is possible using regular glassware. No surface inactivation of the containers (e.g., by silanization) is needed. Furthermore, when using plastic materials for analyte handling and storage (e.g., Eppendorf cups), sufficient oosporein recovery was obtained using the Britton−Robinson buffer system (data not shown). Oosporein stability in the sample solvent is reflected by the interday repeatability measurements given later. No visible oosporein breakdown or analyte loss was observed within this period.

HPLC−DAD Method Development. The improvement of the assay was driven by the need of a rapid and sensitive method for the determination of oosporein in biological matrixes and was meant to replace the rather time-consuming (35 min including column equilibration) and insensitive method by Strasser et al. (*5*), which was more focused on possible lipophilic constituents. Because of the broad variability of polar matrix components to be expected, the development of a gradient method was favored over isocratic protocols. A combination of the Phenomenex Synergi Hydro stationary phase with a water/acetonitrile gradient proved to be superior to other systems tested. The use of an acidic mobile phase modifier for successful oosporein analysis has been already reported (*5*). The refinement process resulted in a combination of formic acid and acetic acid (ratio 9:1 v/v) added to both mobile phase constituents (1%), resulting in a pH of the water phase of 2.13 ± 0.05. This additive was superior to other modifiers (e.g., 0.1% trifluoroacetic acid) and still allows coupling to mass spectrometry (HPLC−DAD/MS), if wished. The UV cutoff is at approximately 240 nm, which is low enough to allow undisturbed detection of oosporein at its UV maximum of 287 nm. Replacement of the organic acids by inorganic acids without UV cutoffs <200 nm (e.g., phosphoric acid or sulfuric acid) did not change the chromatographic behavior of oosporein remarkably (data not shown). Thus, these acids are alternative modifiers if the use of a low detection wavelength is mandatory. To assess the robustness of the developed method, both the influence of system temperature and the mobile phase acidity on the peak symmetry of oosporein were addressed. Whereas a temperature increase does not influence the shape of the analyte peak, a remarkable decline in symmetry had to be observed for elevated pH values. Thus, keeping the mobile phase pH < 2.5 is a prerequisite for maintaining a reasonable peak shape of oosporein. Under the final chromatographic conditions, a retention time of $t_R = 6.09 \pm 0.02$ min and a peak symmetry of 0.82 ± 0.02 was obtained.

HPLC−DAD Method Validation. The developed HPLC−DAD method gave a linear signal response over a concentration range between 13 μg of oosporein/L and 29.6 mg of oosporein/L of BR5.5-MeOH buffer. The associated calibration function was calculated as Y(peak area) $= 68.60 \pm 0.13C$(mg of oosporein/L of BR5.5-MeOH) $+ 0.54 \pm 1.46$ (8 calibration levels, $R^2 = 0.9999$). Precision of the calibration levels was below 4.1%, and the accuracy ranged between −1.5 and 14.6% for concentration levels above or equal 41 μg of oosporein/L of BR5.5-MeOH buffer. The lowest calibration level (13 μg of oosporein/L) had a precision of 17.0% and an accuracy of 84.4%. Intra- and interday repeatability of the assay was assessed for three calibration levels covering a concentration range between 41 μg of oosporein/L and 365 μg of oosporein/L of BR5.5-MeOH buffer. Three experiments were performed in five day intervals. The assay precision was found to be below 5.5% for all intraday measurements and below 5.0% for the interday measurements. The intraday accuracy of all calibration levels was between −3.0 and 1.3%, and the averaged interday accuracy was between −1.2 and 0.5%. LOD and LOQ of the HPLC−DAD assay were calculated as 6.0 ± 2.3 μg of oosporein/L (12.0 ± 4.5 pg of oosporein on column) and 20.0 ± 7.6 μg of oosporein/L (40.0 ± 15.1 pg of oosporein on column) BR5.5-MeOH

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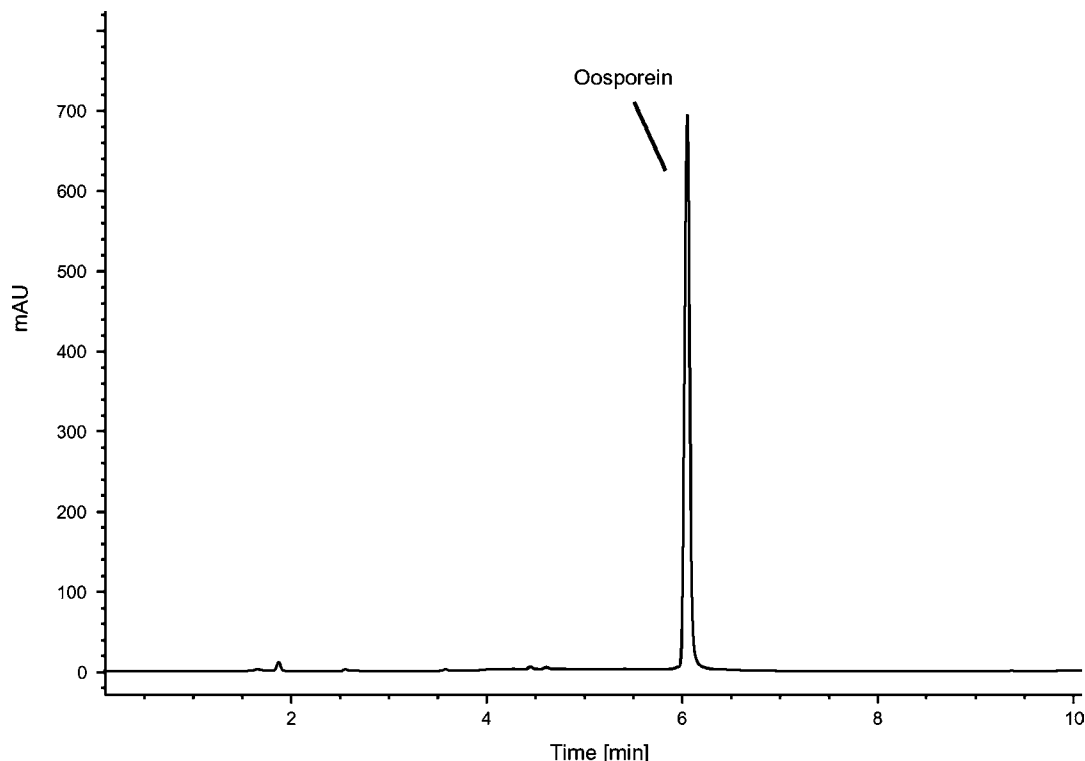*J. Agric. Food Chem.,* Vol. 53, No. 5, 2005 **1367**

Figure 2. Chromatogram of a *B. brongniartii* culture filtrate sample with an oosporein concentration of 504.7 ± 13.6 mg oosporein/L of fungal culture filtrate (recovery rate 93 ± 2%).
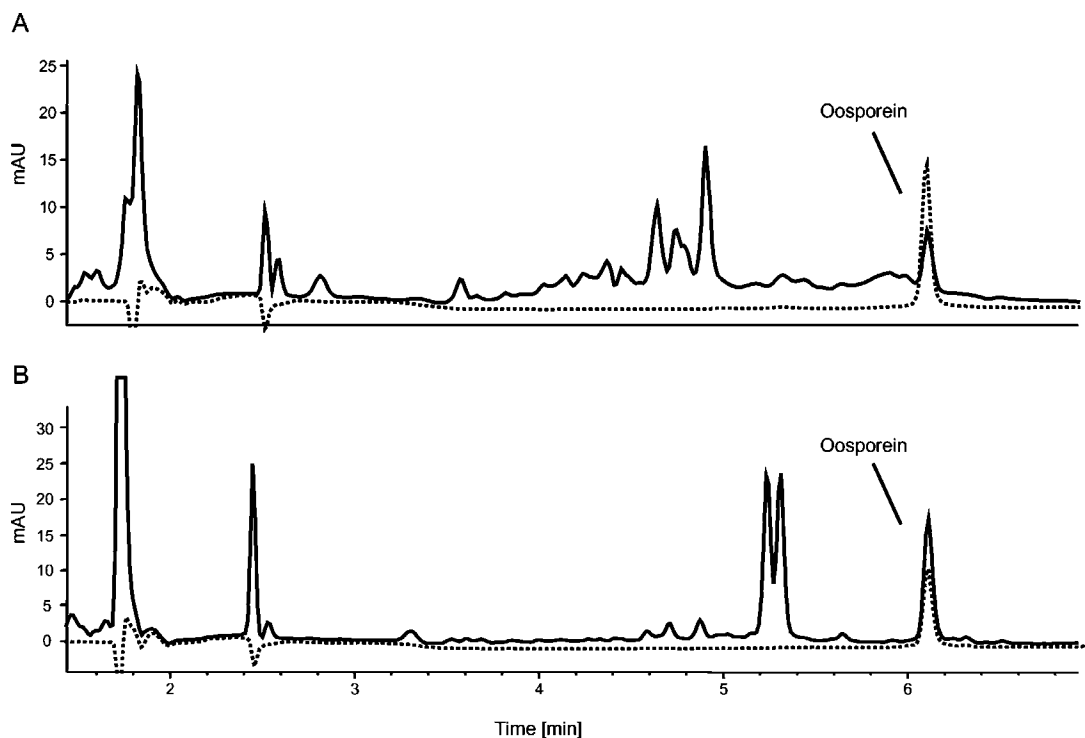

Figure 3. Representative HPLC–DAD chromatograms: (**A**) Oosporein content in Melocont-Pilzgerste. Full line: chromatogram obtained from a 10 g Melocont-Pilzgerste sample containing 7.4 ± 0.6 mg of oosporein/kg (recovery rate 99 ± 8%). Dashed line: oosporein solution in BR5.5-MeOH used as extraction solvent in the spiking experiment with an oosporein concentration equivalent to 14 mg of oosporein/kg of Melocont-Pilzgerste. (**B**) Oosporein (**1**) in Melocont-WP. Full line: chromatogram obtained from a 0.1 g Melocont-WP sample containing 38.2 ± 1.3 mg of oosporein/kg (recovery rate 92 ± 3%). Dashed line: oosporein solution in BR5.5-MeOH used as extraction solvent in the spiking experiment with an oosporein concentration equivalent to 23 mg of oosporein/kg of Melocont-WP.

buffer, respectively. The validation parameters are summarized in **Table 1**.

Quantification of Oosporein in Biological Matrixes. To quantify oosporein present in fungal culture broth, *B. brongn-*

iartii culture filtrates were diluted with BR5.5-MeOH buffer and measured without further purification. Under these conditions, a recovery rate of 93 ± 2% (*n* = 3) was achievable. Fully developed fungal cultures have oosporein contents of 0.3−0.5

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Table 1. Overview of the HPLC–DAD Method Validation Parameters[a]

parameter	
linearity range (μg/L)	13.0–29600
LOD (μg/L)	6.0 ± 2.3
LOQ (μg/L)	20.0 ± 7.6
intraday accuracy (%)	–3.0 to 1.3
intraday precision (%)	<4.2
interday accuracy (%)	–1.2 to 0.5
interday precision (%)	<5.0

[a] Intra- and interday repeatability measurements were carried out at three fortification levels between 41 mg of oosporein/L of BR5.5-MeOH buffer and 365 mg of oosporein/L of BR5.5-MeOH buffer.

g of oosporein/L of culture filtrate, and a representative chromatogram is given in **Figure 2**. This assay application has been used in our laboratories over months, and hundreds of culture filtrate samples have been quantified without any loss of the column performance. Standard samples measured at regular intervals ensured the performance of the method. The detection and quantification of oosporein from BCA formulations was carried out with the already marketed product Melocont-Pilzgerste and a technologically more advanced spore formulation (Melocont-WP) containing inorganic excipients. In the case of Melocont-Pilzgerste, the frozen organic matrix (barley kernels overgrown with fungal biomass) was homogenized in BR5.5-MeOH buffer and centrifuged. The supernatant was measured without further treatment, and 7.4 ± 0.6 mg of oosporein/kg of Melocont-Pilzgerste was found with a recovery rate of 99 ± 8% ($n = 3$) (**Figure 3A**). The water dispersible powder formulation (Melocont-WP) was ultrasonicated in BR5.5-MeOH buffer solution and filtrated over a M_r 10 000 cutoff membrane to remove any insoluble inorganic particles. In this case, the recovery rate was found to be 92 ± 3% (n =3), and an oosporein concentration of 38.2 ± 1.3 mg of oosporein/kg of Melocont-WP was measured (**Figure 3B**). Peak purities have been checked with the HPLC software protocols. Purity factors have been found to be within the calculated threshold limits. LODs and LOQs have not been determined for these applications since blank matrixes were not available for all three applications. However, the obtained high recovery rates showed that matrix interferences can be neglected under the sample workup schemes used. Therefore, the LOD and LOQ of these applications can be roughly estimated to be in the range of the intra-assay LOD and LOQ values multiplied with the respective dilution factor (20 in the case of culture filtrate ans Melocont-Pilzgerste; 50 in the case of Melocont-WP) used. Oosporein concentrations found in these matrixes clearly exceeded these thresholds.

Conclusion. An HPLC–DAD assay was developed, lowering the oosporein analysis time to 57% as compared to the previously used method (*5*). Sample preparation and storage was carried out in a Britton–Robinson buffer system (BR5.5-MeOH) successfully preventing the loss of oosporein to glass surfaces and biogenic matrixes. Assay validation did show detector linearity over 3 orders of magnitude, a LOD of 6.0 μg of oosporein/L of BR5.5-MeOH, and satisfactory intra- and interday repeatability. New oosporein extraction methods based on the developed BR5.5-MeOH sample solvent system allowed us to quantify oosporein from fungal culture broth (504.7 ± 13.6 mg of oosporein/L) and the bio-control formulations Melocont-Pilzgerste (7.4 ± 0.6 mg of oosporein/kg) and Melocont-WP (38.2 ± 1.3 mg of oosporein/kg).

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