Short communication

High-performance liquid chromatography-diode array detection assay for the detection and quantification of the *Beauveria* metabolite oosporein from potato tubers

Christoph Seger [a,b,*], Tobias Längle [b], Barbara Pernfuss [b],
Hermann Stuppner [a], Hermann Strasser [b]

[a] *Institute of Pharmacy, Leopold Franzens University Innsbruck, Innrain 52, A-6020 Innsbruck, Austria*
[b] *Institute of Microbiology, Leopold Franzens University Innsbruck, Technikerstraße 25, A-6020 Innsbruck, Austria*

Abstract

A high-performance liquid chromatography-diode array detection (HPLC-DAD) assay is described for the detection and quantification of the secreted *Beauveria brongniartii* metabolite oosporein from potato tubers. Analyte recovery was achieved with a Britton–Robinson buffer system at pH 5.5 diluted with methanol 3:7 (v/v) (BR5.5-MeOH). An internal standard protocol using 2-iodobenzoic acid was established to minimize analytical error. The resulting assay, using a binary solvent gradient with acidic modifiers and detecting the metabolite at 287 nm, showed a limit of detection (LOD) of 2.4 mg oosporein/kg potato tubers. The oosporein content of potato tuber samples obtained from a field trial using the biological pest control *B. brongniartii* formulation Melocont-Pilzgerste in up to five-fold higher doses (250 kg Melocont-Pilzgerste/ha) as recommended per year was found to be below the established LOD.
© 2005 Elsevier B.V. All rights reserved.

Keywords: HPLC-DAD; *Beauveria brongniartii*; Oosporein; Biological pest control formulation; *Solanum tuberosum*; Potato tubers; Risk assessment

1. Introduction

Oosporein **1** (Fig. 1) is the major secreted secondary metabolite of the enthomopathogenic fungus *Beauveria brongniartii* (Sacc.) Petch (Ascomycota: Clavicipitaceae), which is used as selective and highly virulent bio-control agent (BCA) against *Melolontha melolontha* L. (Coleoptera: Scarabaeidae) (common European cockchafer) [1,2]. The current legal regulations regarding the registration and the use of BCA formulations within the EU (amended Council Directive 91/414/EEC) demand a hazard evaluation 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 the environment, e.g. in the biocontrol agent, in target or non-target species, in soil or water (if applicable), and in harvested crops. It has been shown recently, that the sample preparation protocol can be improved by using a Britton–Robinson type buffer solutions with methanol as organic additive. This allows overcoming analyte adsorption to sample and container matrices. As a result, it was possible to recover oosporein from diverse organic and inorganic sample matrices [3,4]. In this contribution the extension of this analytical approach to the detection and quantification of oosporein from potato tubers (*Solanum tuberosum*), which are a significant target of *M. melolontha* larvae, will be discussed.

2. Experimental

2.1. Reagents and buffers

Oosporein (**1**) [purity 98%; 2,2′,5,5′-tetrahydroxy-4,4′-dimethyl-(bi-1,4-cyclohexadien-1-yl)-3,3′,6,6′-tetron] was gained from *B. brongniartii* (strain BIPESCO 2 (DSM 15205) IMBST 95041 [3,5]. Acetonitrile, methanol (both gradient grade), acetic acid, boric acid, phosphoric acid, and sodium hydroxide (all analytical grade) were purchased from Merck

* Corresponding author. Tel.: +43 512 507 5344; fax: +43 512 507 2939.
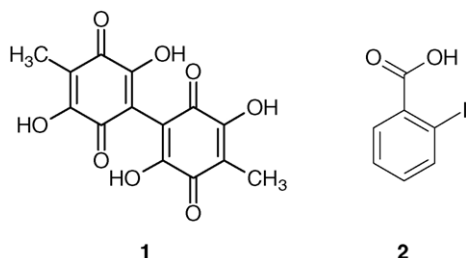 *E-mail address:* christoph.seger@uibk.ac.at (C. Seger).

Fig. 1. Structures of oosporein (**1**) and 2-iodo-benzoic acid (**2**).

(Darmstadt, Germany) and 2-iodobenzoic acid (**2**) (analytical grade) was obtained from Sigma–Aldrich (Sigma–Aldrich, Vienna, Austria). Water for the HPLC was produced by reverse osmosis followed by distillation. The Britton Robinson buffer system (BR5.5-MeOH) used as extraction solvent was prepared as given in Seger et al. [4]. The stock solutions of the buffer components (acetic acid, boric acid phosphoric acid, and sodium hydroxide) were combined and diluted with distilled water to a final volume of 300 ml. If necessary, the pH was adjusted to 5.5 using sodium hydroxide or phosphoric acid. This BR5.5 buffer was diluted with methanol (3:7, v/v) to give 1000 ml of the BR5.5-MeOH buffer. An oosporein stock solution was prepared by dissolving 24.4 mg oosporein (**1**) in 900 ml BR5.5-MeOH buffer. Oosporein solutions used for calibration were prepared from this stock by dilution with BR5.5-MeOH. The internal standard (IS) stock solution was prepared by transferring 125.4 mg 2-iodobenzoic acid (**2**) into a 5 ml volumetric flask and dissolving it with methanol. IS solutions used for calibration were prepared by serial dilutions in BR5.5-MeOH starting from 50 µl IS stock added to 100 ml BR5.5-MeOH. Solutions containing oosporein and IS in different weight ratios were prepared by adding 300 µl IS stock to 600 ml of the oosporein dilution series. The extraction solvent was obtained by adding 100 µl of the IS stock to 200 ml BR5.5-MeOH buffer prior to use.

2.2. Biological samples

Potato tubers (*S. tuberosum*, strain Ditta) were obtained from a field trial performed as part of the RAFBCA project (EC project No. QLK1-CT-2001–01391). The trial site was located at the Agro Trial Center, Gerhaus, Austria (16°51′43.7″ East, 48°03′20.4″ North, 150 m asl, soil type sandy loam), a research station accredited for GLP/GEP field tests. The trial design was compliant with international guidelines for a pesticide residue trial as well as for pesticide phytotoxicity assessment. Plots were laid out as a randomized block design with five treatments and four replicates in accordance with EPPO Standard PP 1/152 [6]. Treatments comprised the recommended (50 kg/ha), double (100 kg/ha) and five-fold (250 kg/ha) doses of Melocont-Pilzgerste (F. Joh. Kwizda, Austria); sterile barley kernels (33 kg/ha, equivalent of carrier material) and Agritox (F. Joh. Kwizda, Austria, 6 l/ha) as reference insecticide (active ingredient chloropyrifos) served as controls. Plots were planted with potatoes and sampling was done at harvest time following the recommendations of EU guidance documents [7] and US

Environmental Protection Agency (EPA) guidelines for residue field trials [8] as well as suggestions specific to fungal BCAs (RAFBCA Standard BCA 1/6 [9]. Three potato tubers were randomly selected from each plot and pooled to a 12 tuber sample for each treatment and control. Samples were frozen at −20 °C, transported to the analytical laboratory on dry ice, and kept at −20 °C until sample preparation, which was carried out according to EU recommendations [7].

2.3. Sample preparation

Frozen potato tubers of 200–400 g weight were chopped into pieces of 5–20 g. Aliquots of 35–45 g were carefully weighed and transferred to a kitchen blender (Moulinex Turbo Blender, Hausberger, Austria) containing 200 ml extraction solvent. The sample material was blended for 5 min. Ten milliliters of the resulting solution were transferred to polypropylene tubes and the matrix debris was pelleted by centrifugation (Hermle Z383 centrifuge from Hermle Labortechnik, Wehingen, Germany, at 3500 rpm, 30 min, and room temperature). One milliliter of the clear supernatant was transferred to a HPLC vial for HPLC-DAD measurement.

2.4. HPLC-DAD assay conditions

HPLC analyses were performed using a HP1100 liquid chromatograph (Agilent, Waldbronn, Germany) equipped with a diode array detector, an automatic injector, an auto sampler and a column oven [4]. Separations were performed on a Phenomenex Synergi Hydro-RP 80A column (150 mm × 2 mm), particle size 4 µm (Phenomenex, Torrence, USA). The Phenomenex SecurityGuard system equipped with a C_{18} cartridge (4 mm × 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 kept constant for a further 5 min at a flow rate of 0.3 ml/min. The column was kept at 23 °C (thermo stated). Between analyses, the column was re-equilibrated for 7 min. The injection volume was 2 µl, chromatograms were recorded at 287 nm.

2.5. HPLC-DAD assay validation

Analyte and IS loss during sample preparation (recovery rates) was evaluated with a series of spiking experiments. Forty grams potato tuber aliquots from the untreated field plots and 200 ml BR5.5-MeOH extraction solvent, fortified with different concentrations of the analyte (1, 3 or 9 mg oosporein/l) and identical amounts of the IS (12.5 mg 2-iodobenzoic acid/l), respectively, were used The DAD response (peak area) of the extracted samples was corrected for the carefully measured volume increase due to the sample volume (∼15%) and compared to responses of standard solutions with identical analyte concentrations in BR5.5-MeOH.

Calibration curves in the absence of sample matrix were obtained for a IS dilution series in BR5.5-MeOH, and an

oosporein dilution series with different weight ratios of oosporein and IS in the same solvent system. These curves were used to assess the linearity of IS response in BR5.5-MeOH, and the linearity of IS and oosporein response in the presence of each other and under buffer conditions. The calibration curve for the quantitative assay was obtained by extracting potato tuber aliquots (sample preparation see above) from untreated field plots with BR5.5-MeOH extraction solvent spiked with a dilution series of oosporein and 12.5 mg IS/l. From this experimental setup the validation parameters LOD, LOQ, precision, accuracy, intra- and inter-day repeatability of the method were obtained.

All calibration functions used were obtained by linear regression of the analyte concentration (c_A) against the peak area (A) resulting in $A = axc_A + b$. No weighting functions were used. The limits of detection (LOD) and the limits of quantification (LOQ) were calculated from the obtained calibration functions [4,5,10,11]. The LOD equals the value of the intercept b plus three times the standard deviation of the intercept, the LOQ was obtained by adding ten times the standard deviation to the intercept value.

Precision was determined as the relative standard deviation (% RSD) of the calibration levels and accuracy was calculated as deviation of the measured mean from the fortification level [expressed as percentage of the fortification level (% dev)]. Intra- and inter-day repeatability of the assay was measured three different days in 5 day intervals with 5 replications per day.

The average response factor RF for the quantitative assay was calculated as RF $= (A_{IS} \times C_{OO})/(A_{OO} \times C_{IS})$ with A_{IS} and A_{OO} being the peak areas of IS and oosporein and C_{IS} and C_{OO} as corresponding concentrations of the respective standard solutions.

3. Results and discussion

3.1. HPLC-DAD method validation

An almost quantitative recovery of oosporein (>94%, $n = 3$) was obtained between 5 and 135 mg oosporein/kg potato tuber. The recovery level of the IS was >97% ($n = 3$) over the whole oosporein calibration range. The chosen IS gave a linear signal response between 10 and 100% of the concentration used in the assay (12.5 mg/l BR5.5-MeOH extraction solvent). No interaction with oosporein (e.g. additional peaks in the chromatogram, signal loss of the analyte or the IS, loss of linearity) was observed in any experiment in the absence or presence of extracted potato starch matrix. At room temperature, extracted samples remained stable for at least 7 days. An average response factor RF $= 17.66 \pm 0.10$ (RSD $= 0.5\%$ for 30 measurements) was obtained over the investigated concentration range of the analyte (5 mg oosporein/kg potato to 135 mg oosporein/kg potato tuber). Although the sample matrix presented a multitude of chromatographic peaks under the chosen detection conditions, the peaks of IS and oosporein were well separated from other constituents (Fig. 2). The peak purity of both was checked with the HPLC software protocols and was found to be within the threshold limits.
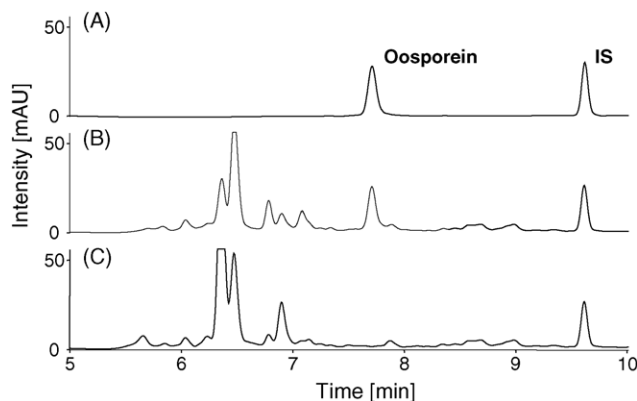

Fig. 2. Representative HPLC-DAD chromatograms recorded at 287 nm. (A) Extraction solvent BR5.5-MeOH spiked with internal standard (IS) 2-iodobenzoic acid (12.5 mg/l) and oosporein (1 mg/l). (B) Potato tuber aliquot (40 g) from an untreated field lot extracted with 200 ml BR5.5-MeOH spiked with 1 mg/l oosporein and 12.5 mg/l IS. (C) Potato tuber aliquot (38 g) from a field lot treated with 250 kg/ha Melocont-Pilzgerste extracted with 200 ml BR5.5-MeOH spiked with 12.5 mg/l IS.

The signal response of oosporein was linear between 5 mg oosporein/kg potato tuber and 135 mg oosporein/kg potato tuber. An associated calibration function of Y(Peak area) $= 26.05 \pm 0.29C$ (mg oosporein/kg tuber) $+ 121.28 \pm 20.86$ (four calibration levels, eight replications, $R^2 = 0.996$) was obtained. The derived LOD and LOQ of the HPLC-DAD assay were determined as 2.4 mg oosporein/kg tuber and 8.0 mg oosporein/kg tuber. Precision of the calibration levels was below 4% and the accuracy was ranging between -2 and 15% for concentration levels above or equal 15 mg oosporein/kg tuber. The loss of accuracy at the lowest calibration level of 5 mg oosporein/kg tuber (-64%) reflects the calculated LOQ.

3.2. Quantification of oosporein in treated field plots

The developed assay was used to screen potato tuber samples obtained from the treated field plots. A total of seven samples (four one tuber samples from the plots treated with the five-fold dose of Melocont-Pilzgerste, one pooled sample representing twelve tubers from the five-fold dosed plots and one sample each from the two-fold dosed and the single-dosed plots) were investigated. In none of the samples a traceable oosporein concentration was observed.

4. Conclusions

The developed internal standard supported HPLC-DAD assay enables to address the absence or presence of oosporein in potato tubers, one of the most important subterranean staple foods harvested in moderate climates. This metabolite is secreted by the fungal biocontrol agent *B. brongniartii*, which is targeting the grubs of *M. melolontha*, a soil dwelling pest feeding on plant roots and tubers. The non-biogenic salicylic acid congener 2-iodobenzoic acid was chosen as IS in this assay, since it has a pK_a value close to oosporein [12], a well detectable UV response at 287 nm and is commercially available at a high

degree of purity. Using the Britton–Robinson buffer for extraction [4] a high recovery of both analyte and internal standard was achieved. The assay allows monitoring oosporein in the lower ppm (mg/kg) concentration range. In none of the investigated samples, even at the five-fold dose of the BCA used, oosporein has been found in the investigated tubers.

With this method oosporein can now be quantitatively traced in fungal culture broth [4,5], the biocontrol agent [4], the target insect [6], and in one of the most important produce – potato tubers. The biology of the fungus itself is well understood [5,13] and both the knowledge about the physical-chemical properties of oosporein [12] and its mobility in the soil compartment [14] has recently been increased. Considering all these facts, the probability of oosporein to enter the food chain via subterranean crops can be estimated to be extremely low, since the *B. brongniartii* can hardly propagate in the soil compartment without the host and does not infect potato tubers, since it is not a phytopathogen [15]. Even if secreted into the infested host *M. melolontha*, oosporein will neither be spread in the soil compartment nor entering the ground water due to its charged character (adsorption nearly irreversible) and (in addition) its degradability at neutral to moderate alkaline conditions [12,14].

Acknowledgements

References

[1] H. Strasser, Förderungsdienst 5 (1999) 158.
[2] H. Strasser, A. Vey, T.M. Butt, Biocontrol Sci. Tech. 10 (2000) 717.
[3] A. Michelitsch, U. Rückert, A. Rittmannsberger, C. Seger, H. Strasser, W. Likkusar, J. Agric. Food Chem. 52 (2004) 1423.
[4] C. Seger, S. Sturm, T. Längle, W. Wimmer, H. Stuppner, H. Strasser, J. Agric. Food Chem 53 (2005) 1364.
[5] H. Strasser, D. Abendstein, H. Stuppner, T.M. Butt, Mycol. Res. 104 (2000) 1227.
[6] Method PP1/152(2): Design and Analysis of Efficacy Evaluation Trials, European and Mediterranean Plant Protection Organization, Paris, 2004.
[7] Guidelines for the generation of data concerning residues as provided in Annex II part A, section 6 and Annex III, part A, section 8 of Directive 91/414/EEC concerning the placing of plant protection products on the market. Appendix B: General recommendations for the design, preparation and realization of residue trials. Working Document 7029/VI/95 rev. 5: Commission of the European Communities, Brussels, 1997.
[8] Residue chemistry test guidelines. Crop field trials. OPPTS 850.1500, US Environmental Protection Agency, Cincinnati, 1996.
[9] Phytotoxicity and residue trials for the risk assessment of fungal BCAs, in: T. Längle (Ed.), RAFBCA Standard BCA 1/6, Ph.D. thesis, LFU Innsbruck, Innsbruck, 2005.
[10] Analytical Methods Commitee, Analyst 112 (1987) 199.
[11] C. Seger, S. Sturm, H. Stuppner, T.M. Butt, H. Strasser, J. Chromatogr. A 1061 (2004) 35.
[12] C. Seger, D. Erlebach, H. Stuppner, U.J. Griesser, H. Strasser, Helv. Chim. Acta 88 (2005) 802.
[13] D. Abendstein, W. Schweigkofler, H. Strasser, Laimburg J. 1 (2004) 273.
[14] M. Ottowitz, Diploma thesis, KFU Graz, Graz, 2004.
[15] D. Abendstein, B. Pernfuss, H. Strasser, Biocont. Sci. Technol. 10 (2000) 789.