Method development for the trace analysis of the highly polar fungal hydroxybenzoquinone derivative oosporein

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INTRODUCTION

Oosporein is the major secondary metabolite secreted by different soil dwelling fungi, amongst these the selective and virulent entomopathogenic *Beauveria brongniartii*. Although its bioactivity profile is well investigated, its role in the process of fungal attack has not been unveiled. Since *B. brongniartii* is the active ingredient of registered biological pest control agents (BCAs) used in the control of *Melolontha melolontha* (chockchafer), the legal authority (directive 91/414EEC) demands data on the concentration and fate (e.g. adsorption, breakdown) of oosporein in the environment (BCA, soil, and crop).

In the course of HPLC-DAD method development, two problems arose: a SAX-SPE protocol for the enrichment from potato tubers failed due to the loss of oosporein (10 ppm) to the sample matrix and the loss of oosporein (< 5 ppm) from methanolic HPLC reference solitions was observed. Therefore, new strategies had to be developed permitting reproducible trace analysis of oosporein in biological matrices.

Photographs by H. Strasser, F. Hadacek, T. Längle, U. Griesser, and http://www.inra.fr/Internet/Produits/HYPPZ/RAVAGEUR/6melmel.htm

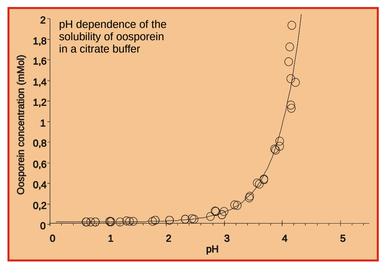
RESULTS

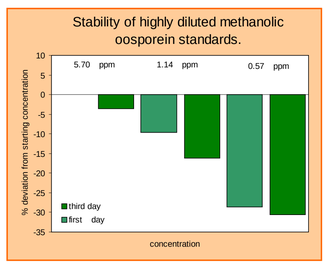
- The adsorption of the analyte to polar surfaces was prevented by establishing a new sample solvent system based on the Britton-Robinson universal buffer system.
- A sensitive (LOQ = 50 ppb) and robust (reproducibility at 0.12 ppm = 4.9 % RSD) HPLC-DAD method for the quantification of oosporein was realized.
- The applicability of this method was proven for different biological matrices, amongst these potato tubers and barley kernels.
- The oosporein content in BCA formulations (Melocont®-Pilzgerste and Melocont®-WG) was determined as 7.4 ppm and 38.2 ppm, respectively.

REFERENCES

Michelitsch A., Rückert U., Rittmannsberger A., Seger C., Strasser H. & Likussar, W. (2004) *Journal of Agriultural and Food Chemistry,* 52 1423.
Mongay C. & Cerda V. (1974) *Annali di Chimica,* 64, 409.

PHYSICO-CHEMICAL PARAMETERS





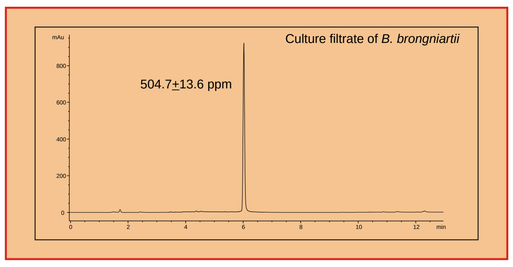
- Oosporein is an organic acid.
- The water solubility rises with the pH.
- Oosporein is soluble in organic solvents at low pH (free acid).
- 1st deprotonation pKa = 2.42.
- Oosporein is instable at alkaline pH.

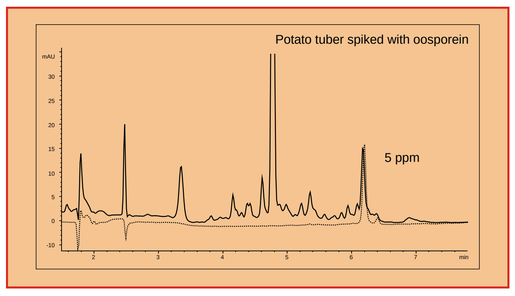
APPLICATIONS

In all application cases, the sample material was diluted with BR5.5/MeOH buffer to reduce matrix interferences. Spiking experiments were carried out in all cases to obtain the recovery rates. LOD and LOQ concentrations were raised with the dilution factor.

Application example 1

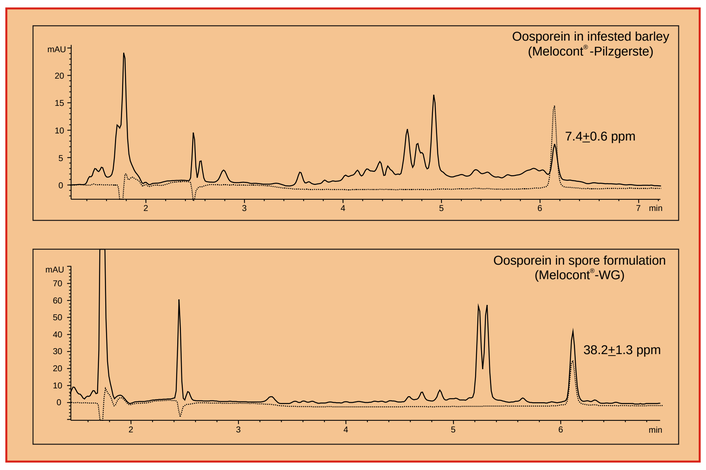
Oosporein in the culture filtrate.
Recovery rate: 94.3±2.0 %
Dilution factor = 20.





Application example 3

Oosporein spiked potato tubers.
Recovery rate: 98.2±1.3 %
Dilution factor = 5.

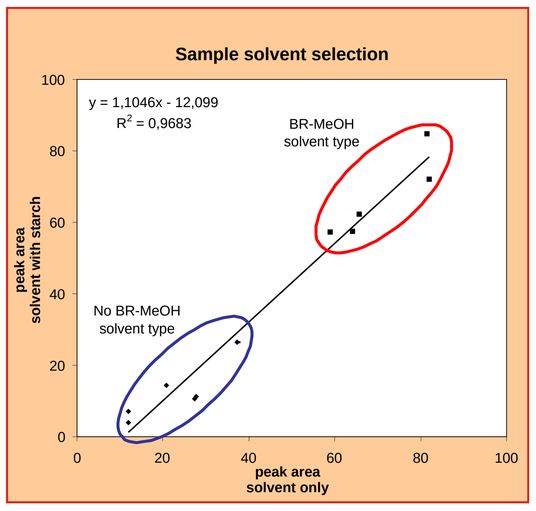


Application example 2

Oosporein in BCA formulations. Full line: BCA chromatogramm.
Dashed line: Oosporein spiking solution (top: 14 $\mu g g^{-1}$ BCA, bottom: 23 $\mu g g^{-1}$ BCA).

Recovery rate from Melocont®-Pilzgerste: 99.1±7.5 % Dilution factor = 20.
Recovery rate from Melocont®-WG: 91.6±2.7 % Dilution factor = 50.

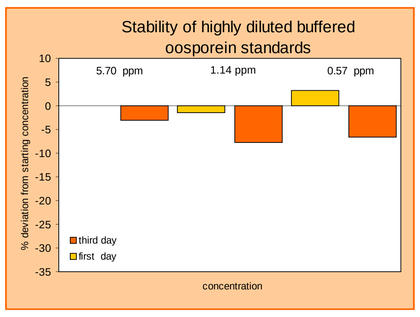
SAMPLE PREPARATION - SOLVENT SELECTION



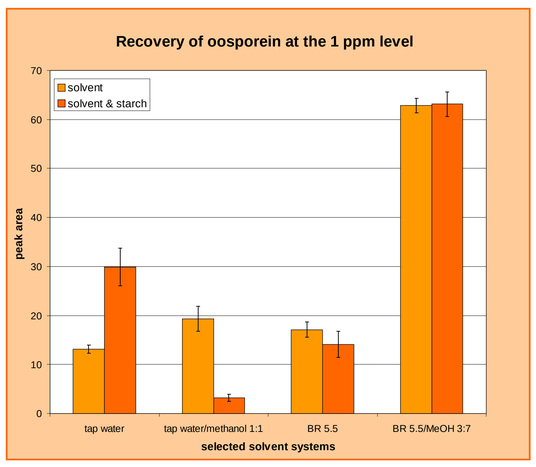
BRITTON ROBINSON sample buffer (BR5.5/MeOH)

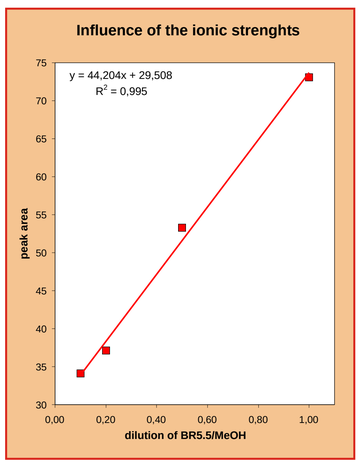
- 2.3 gL^{-1} sodium hydroxide
- 1.7 gL^{-1} acetic acid
- 2.8 gL^{-1} phosphoric acid
- 1.8 gL^{-1} boric acid

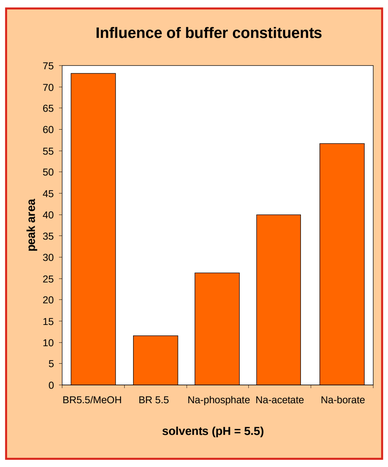
Ajust pH to 5.5 with sodium hydroxide or phosphoric acid. This buffer has an ionic strength of approximately 0.57 mMol (Mongay & Cerda 1974). Dilute with methanol in the ratio 3:7.



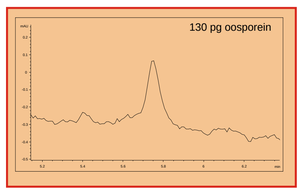
The adsorption of oosporein to surfaces (glass, glass + starch) was tested for several solvent systems. In all cases, combinations of Britton-Robinson buffer systems with organic modifiers (Michelitsch et al. 2004) did prove to be superior. The recovery of oosporein at a level of 1 ppm was reproducible and the solutions remained (in contrast to methanolic solutions) stable over days. The influence of the ionic strength was tested and found to be linear. The use of methanol as organic modifier was mandatory and a pH dependence (pH 2 to pH 6) was not observed. Boric acid is the most active buffer component.

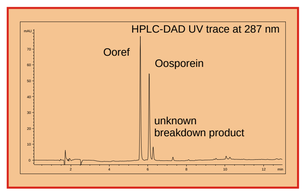


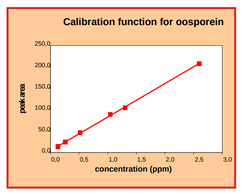


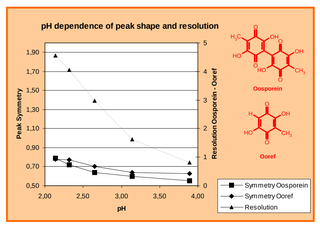


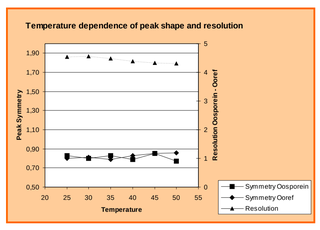
METHOD DEVELOPMENT AND VALIDATION

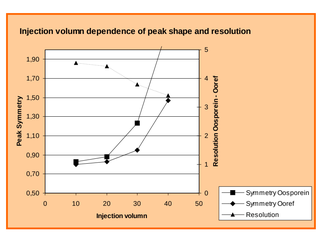












HPLC-DAD METHOD

Instrumentation: Agilent HP1050.
Column: Phenomenex Synergi-Hydro RP 80A 150x4.6 mm, 4 µm particles.
Solvent: H_20(A)-ACN(B); both with 0.1% acetic acid and 0.9% formic acid.
Elution profile:
t= 0 min 5% B, t = 6 min 60 % B,
t= 8 min 98 % B, t = 13 min 98 % B
7 min post time - total 20 min.
Detection: UV-DAD 287 nm.
Injection volume 10 µl.
Flow rate: 1 ml/min, room temperature (24-28 °C)

VALIDATION DATA

Calibration function: Y(peak area) = 63.96 x C(µgmL^{-1})+12.86; R^2 = 0.9999
Calibration range: 0.13 - 26.40 µgmL^{-1}
LOD: 150 pg on column = 15 ppb = 0.049 µMol
LOQ: 500 pg on column = 50 ppb = 0.163 µMol
Repeatability (at 0.12 ppm): < 2.9% RSD
Reproducibility (at 0.12 ppm): < 4.9% RSD

OPTIMIZATION REMARKS

* Replacing 0.1 % AcOH/ 0.9 % HCOOH (pH=2.10) with 0.1% TFA (pH=1.89) lowers the resolution.
* Replacement with H_2SO_4 or H_3PO_4 (pH ~ 2) is possible.
* Rising the temperature has no influence on peak width and symmetry.
* Rising the pH leads to losses in peak symmetry and resolution to ooref.
* The injection volumn has to be < 20 µL.
* The proposed internal standard Ooref is degrading oosporein.

ACKNOWLEDGEMENT

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