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# DETERMINATION AND QUANTIFICATION OF FOLPET IN BARLEY USING HPLC-DAD ANALYSIS

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**Abstract**

Although routine pesticide analyses are mostly performed using GC-MS, GC-MS/MS and/or LC-MS/MS, some pesticides especially those with relatively high MRLs and good detector response could also be precisely quantified with less sensitive equipment which gives the opportunity to some laboratories to expand their scope of work. The study focuses on HPLC-DAD method development for detection of folpet in barley grain. QuEChERS method was performed for extraction after which, additional clean-up was performed using PSA and polypropylene filtration. Pesticide separation was achieved using mobile phase of acidified ACN (pH 2.5) and water. The initial mobile phase was 60:40% (v/v), the elution gradient starts from 80:20% (v/v) to 100:0% (v/v) in 7 min and holds 100:0% (v/v) up to 8.5 min at a flow rate of 1 ml/min after which in the next 6.5 min the column was re-equilibrated to 40% phase-B. Calibration was performed using matrix-matched calibration standards. The obtained limits of detection (LOD) and quantification (LOQ) were 0.18 and 0.55 mg/kg, respectively, and the linear regression coefficient was 0.9973. Recovery, repeatability and reproducibility were investigated at three fortification levels (0.1 mg/kg, 0.6 mg/kg, and 1.6 mg/kg) and were found acceptable with relative standard deviation less than 10%. The method was applied for the analysis of three barley grain samples obtained from different producers and showed that folpet was present in one sample below the LOQ. Overall, the developed method is suitable for the determination of folpet in barley grains at levels below the established MRL of 1 mg/kg.

**Key words:** folpet, HPLC-DAD, QuEChERS, barley

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**Introduction.** Global climate changes increase the incidence of some plant pathogens and pests in agriculture production and contribute to their occurrence in new regions. Cereals are highly affected by these changes resulting in rapid occurrence of associated diseases such as rusts, fusarium head blight, and some pests [1–3]. This imposes the need for more intensive application of pesticides. Barley is a popular cereal which is used as one of the components in beer production and in domestic animal feed mixtures so pesticides applied during its production and storage may affect the safety and quality of the barley products and furthermore reach the final consumers.

Folpet is a broad spectrum Chloroalkylthiodicarboximide (Phthalimide) fungicide. It is effective against the common foliar pathogens in barley such as *Septoria* spp., *Ramularia* spp., *Pyrenophora* spp., *Rhynchosporium* spp., and it is also useful in reduction of yellow and brown rusts. Because of its unique multi-site mode of action, folpet is recommended in barley and wheat protection programmes not only because of its efficacy but also because of delaying the development of resistance in single-site fungicides, and of maximizing the yield potential of barley. To this purpose, maximum two applications are recommended with the last one up till the end of the booting stage (GS 49). Literature data shows that folpet may be found in barley grain in routine quality control analysis [4, 5]. According to its toxicity, the EU Commission set the MRL of 1 mg/kg for folpet in barley [6].

Adequate methodology is proposed for folpet determination in technical active substance, water, soil and air (Regulation (EU) No 528/2012, 2014) which employs GC-MS, GC-MS/MS and/or LC-MS/MS methods for analysis [7]. But the extraction of pesticides in cereal commodities like barley is quite a challenge for analysts due to its complex composition and the content of high molecular weight compounds like starch, fatty acids, organic acids, glucans, vitamins, etc., which can reduce the efficiency of the chromatographic separation [8]. Preparation, extraction and clean up analytical techniques for pesticide residue analysis differ regarding matrix composition, pesticide properties, and the equipment available in the laboratory. Since it was first published in 2003, the original QuEChERS method [9], was improved and modified to be applicable for various commodities and pesticides. The proposed official extraction method from EURL (CEN/TC 275 prEN 1556662:2007) which employs QuEChERS extraction of pesticides from cereal commodities uses acetonitrile extraction with citrate buffer – salt mixture followed by freezing at −80 °C to remove fats, and additionally clean up with poly sorbent amine (PSA) for no acidic pesticides and GC-MS, GC-MS/MS and/or LC-MS/MS analysis [10].

In this study a method for quantification of folpet in barley grain is proposed using QuEChERS extraction according to CEN/TC 275 prEN 1556662:2007 and High Performance Liquid Chromatography analysis coupled with Photo Diode Array detector (HPLC-PDA). To the best of our knowledge this is the first proposed method in the literature data for determination of folpet in barley grain using QuEChERS extraction followed by HPLC-PDA quantification.

**Experimental analysis.** ***Standard, reagents and samples.*** Pesticide standard of folpet (99.7%), was obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Information on the important properties of the investigated pesticide is given in Table 1. Acetonitrile (HPLC grade) and hexan (pesticide analysis-grade) were purchased from Sigma Aldrich. Ultrapure water (18 Ω) was obtained from the Milli-Q water purification system by Thermo Fisher Scientific. Barley grain (cv. Rex) for the method development was obtained from the local organic producer.

T a b l e 1

Folpet properties and method validation data ($n = 6$)

| Chemical formula | CAS No | Log P | pKa | Kow | MRL |
|---|---|---|---|---|---|
| $C_9H_4Cl_3NO_2S$ | 133-07-3 | 3.02 | nd | 2.85 | 1 ppm |

| Retention time $t_R$ | Detection wavelength | LOD | LOQ | Recovery | RSD |
|---|---|---|---|---|---|
| 6.69 min | 223 nm | 0.28 mg/kg | 0.84 mg/kg | > 80% | < 6.3 |

| Spike level 0.5 mg/kg | | | Spike level 1 mg/kg | | | Spike level 1.5 mg/kg | | |
|---|---|---|---|---|---|---|---|---|
| Mean | RSDr | RSDR | Mean | RSDr | RSDR | Mean | RSDr | RSDR |
| 80.2% | 4.5% | 6.3% | 82.9% | 4.0% | 6.0% | 95.3% | 3.1% | 3.5% |

pKa – dissociation constant at 25 °C; Log P – octanol-water partition coefficient at pH and 20 °C; nd – no dissociation; MPC – maximum permitted concentration in barley; RSD – Relative Standard Deviation; RSDr – repeatability; RSDR – reproducibility

***Pesticide standard solutions.*** Standard solution was prepared following the EU guide SANCO/12571/2013 [11]. Stock standard solution was prepared in HPLC-grade acetonitrile (ACN) in concentration of 1 mg/ml, and stored at −18 °C. Working solution was prepared in chromatographic mobile phase (60 v/v ACN:40 v/v $H_2O$) at concentration of 20 µg/ml. Working solution was stored at +4 °C and used for spiking of ground barley samples and preparing matrix-matched calibration standards. Calibration curves were prepared using matrix-matched calibration standard at levels of 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg and 2.5 mg/kg (Fig. 1). Stability of folpet in the matrix was checked using five blank barley samples in aliquot of 5 g and spiked at 1 mg/kg. One spiked sample was analyzed immediately and the others were stored at 4 °C and analyzed after 1, 3, 6, and 12 weeks. The concentration of folpet in the sample was shown to be stable up to 12 weeks.

***Extraction procedure.*** Pesticide from barley was extracted using buffered QuEChERS extraction method and poly sorbent amine (PSA) clean up (CEN/TC 275 prEN 1556662:2007). For that purpose, 50 g organically produced barley seeds were homogenized to flour by milling, and 5 g were transferred to 50 ml centrifugation tube. In the next step 10 ml ultrapure $H_2O$ was added and vortexed after which 10 ml of ACN were added and vortexed again for 1 min. A buffer

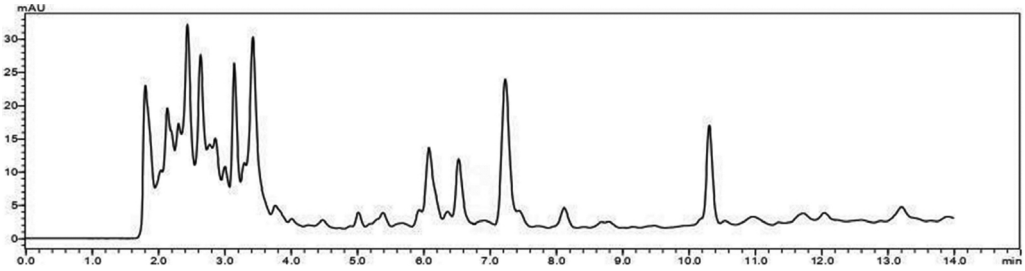

Fig. 1. HPLC–DAD chromatogram of non-spiked barley extract, measured at $\lambda$ 223 nm

salt mixture consisting of 4 g $MgSO_4$, 1 g NaCl, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogencitrate sesquihydrate were added. The solution was shaken and centrifuged at 4500 rpm for 10 min. After centrifugation 8 ml of the raw ACN were transferred to 15 ml centrifugation tube and stored at −80 °C for 1 h followed by centrifugation at 5 °C and 4500 rpm for 5 min. Then, 6 ml of cold acetonitrile extract was transferred to a PP-disposable centrifugation tube containing 150 mg PSA and 900 mg $MgSO_4$. The centrifugation tube was shaken vigorously for 30 s and centrifuged for 5 min at 4500 rpm. An aliquot of 4 ml was transferred to another 15 ml centrifugation tube and acidified with 40 µl of 5% formic acid solution in ACN. Before analysis, 1 ml of acidified extract was filtered through the polypropylene filter with pore size of 0.20 µm (CHROMAFIL O-20/3), transferred to autosampler vials and analyzed by HPLC-PDA.

***HPLC-PDA system and operating conditions.*** Pesticides were qualitatively and quantitatively determined by reverse phase High performance liquid chromatography (RP HPLC) using a Shimadzu Prominence System modular HPLC apparatus (Shimadzu Co. Ltd., Kyoto, Japan) equipped with Shimadzu model LC-20AT pumps, Shimadzu diode array detector model SPD-M20A, and the software LC-Solution was connected to a Shimadzu processor model CBM-20A using Shimadzu Shim-pack GIST C-18 reverse-phase analytical cartridge column (4.6 mm × 250 mm × 5 µm). For the separation of analytes, the column was equilibrated in 60% phase A (100% ACN acidified to pH 2.5 with $H_3PO_4$) and 40% phase B (100% ultrapure $H_2O$) with a flow rate of 1 ml/min. The mobile phase was then changed to 20% B in 3 min, and to 0% B in 8.5 min. During the next 6.5 min, the column was re-equilibrated in 40% phase-B. The injection volume was 10 µl and the total run time of the method including cleaning was 15 min.

***Method validation.*** Validation of the method was done according to EPA 525.2 [12]. The values for LOD and LOQ were calculated by the ordinary least square regression data analysis using the dispersion characteristics of the regression line of the chromatographic peak area against concentration [13]. LOD corresponds to the analyte amount for which the area is 3.3 times the standard deviation, and LOQ corresponds to the analyte amount for which this area is 10 times the standard deviation. The recovery and precision studies were performed based

on six replicates, at three fortification levels (0.4, 0.8, and 1.6 µg/mg) by spiking organically produced and pesticide free ground barley grain. Concentrations were calculated by the equation:

$$\text{C (mg/kg)} = \frac{k \cdot V}{m},$$

where C is the concentration of folpet in barley, $k$ is the concentration of folpet in the sample extract ($k = (A - b)/a$), $V$ is the volume of the extraction solvent, and $m$ is the sample size.

**Results and discussion.** Folpet is a non acidic phthalimide fungicide. According to its log P value it is well suited for reverse phase chromatography. The highest UV absorption maximum appears at 223 nm. As mentioned before, the separation method was carried out with gradient elution on a Shimadzu Shimpack GIST C-18 RP column. The blank barley extract, measured at the band maxima of the UV spectra did not show any interfering peaks at the same retention time with the time of folpet elution, which indicates that the proposed extraction, clean-up procedure and HPLC operation conditions may be suitable for determination of folpet (Fig. 1).

Barley is a dry commodity with around 88% of dry matter with starch amount up to over 70% of the dry weight [14]. Therefore, a suitable amount of water is necessary to facilitate the extraction of the target compounds. In the proposed QuEChERS extraction, the remaining co-extracted low soluble substances like fats are removed with freezing, after the first step of extraction with ACN. Organic acids, sugars, fatty acids and lipids are removed with poly sorbent amine (PSA). The study showed greater than 80% recovery of folpet by this method (Table 1).

Calibration was performed using matrix-matched calibration standards. To that purpose, a blank extract of the barley grain is obtained following the previously described extraction procedure. The blank matrix extract, was fortified with the appropriate volumes of standard working solution to obtain five different levels (0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, and 2.5 mg/kg) of concentration (Fig. 2). The calibration data obtained are shown in Table 1. The linearity of the response was good at concentrations within the tested interval, with linear coefficient of determination of 0.9878 (Fig. 3).

Accuracy of the present method was evaluated at three fortification levels (0.5 mg/kg, 1 mg/kg, and 1.5 µg/ml). Folpet showed acceptable recovery within the mentioned validation intervals between 80.2 and 95.3%. The mean recovery of folpet ($n = 6$) for each fortification level is shown in Table 1. Method precision was calculated using the double measurement of peak area of folpet in the matrix. It was determined by repeatability and reproducibility studies, and expressed by the relative standard deviation. The repeatability RSDr was measured by comparing standard deviation of the recovery percentages of spiked barley samples run at the same day. The reproducibility RSDR was determined by analyzing spiked barley

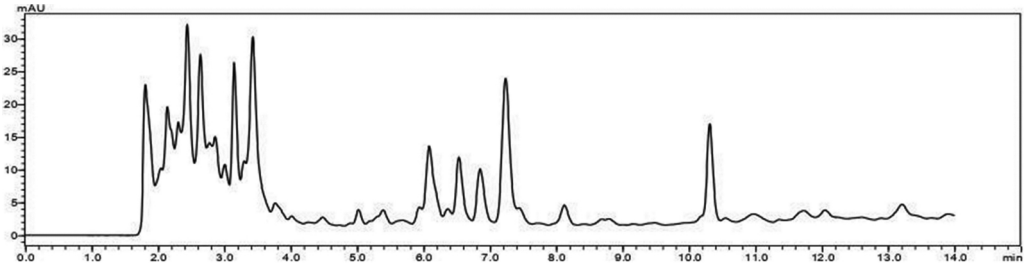


Fig. 2. HPLC-DAD chromatogram after extraction of a spiked barley sample measured at $\lambda$ 223 nm. Folpet $t_R$ 6.69 min

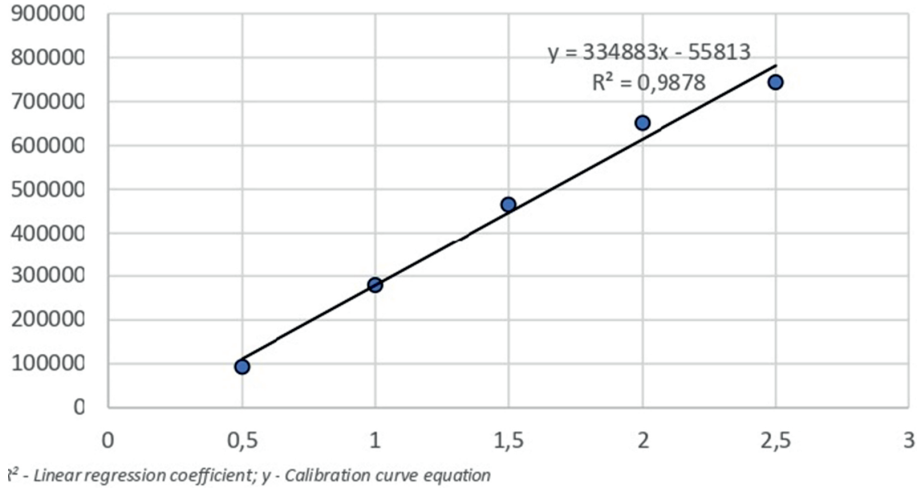


Fig. 3. Matrix matched calibration curve of folpet

samples for five alternate days. The method was found to be precise (RSD $< 10\%$) at all spiking levels (Table 1). The LOD and LOQ values obtained were 0.28 and 0.84 mg/kg, respectively.

After validation, the method was applied to investigate the barley grain samples obtained from three conventionally produced barley grain samples. The result did not show the presence of folpet in concentration above the LOD.

**Conclusions.** In this paper a fast and easy determination of folpet in barley samples using HPLC-DAD is described. The extraction procedure does not require any pretreatment of the barley grain except milling and does not require any preconcentration of the matrix. Although barley is a very challenging commodity, no interfering peaks were present at the retention time of the folpet elution. The method showed satisfactory validation parameters in terms of reproducibility, sensitivity, accuracy and precision. The calculated LOQ (0.84 mg/kg) was lower than the estimated MRL (1 mg/kg) established by the European legislation. Due to the lower toxicity of the new generation of pesticides their MRLs have higher

values which makes them possible to be analyzed by the less sensitive equipment such as HPLC-DAD. Likely this method could be extended to other cereals and cereal flours, and may also be used as an alternative for HPLC-MS when lower detection limits could be achieved. Furthermore, the method could be implemented by laboratories lacking GC-MS, GC-MS/MS, and/or LC-MS/MS to expand their scope of work.

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