HPLC-MS/MS Method for Analysis of Isoproturon in Difficult Matrix: Poppy Seeds

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Abstract

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While several validated methods have been developed for analysis of phenylurea herbicides in staple food plants, analytical procedures suitable for their quantification in minor crops such as poppy seeds are not available. For the registration of isoproturon use in this crop, the documentation of dynamics of its residues following treatment was requested. To accomplish this task, HPLC-MS/MS method was developed. Extraction of residues was realised by methanol-water mixture, Supelclean LC-18 SPE cartridges were used for purification of crude extracts. For HPLC separation of isoproturon SPE fraction Lichrospher C18 column (25 cm × 4 mm, 5 μm) was employed. For detection and quantification of target analyte, mass spectrometer with ion trap analyser operated in positive atmospheric pressure chemical ionisation (APCI) and MS/MS mode was used. Following performance parameters of method were obtained: detection limit 0.01 mg/kg, recovery 84%, and repeatability 7%.

Keywords: HPLC-MS/MS; isoproturon; method development; SPE; poppy seeds

Isoproturon (*N,N*-dimethyl-*N'*-[4-(1-methylethyl)phenyl]urea) (Figure 1) is a selective systemic herbicide. Following application, the absorption occurs by the roots and leaves. It is used for the pre- and post-emergence control of annual grasses (*Alopecurus myosuroides*, *Apera spica-venti*, *Avena fatua* and *Poa annual*) and many annual broad-leaved weeds in spring and winter wheat (except durum wheat), spring and winter barley, winter rye, and triticale. Typical application rate used for treatment is in range 1.0–1.5 kg/ha (Tomlin 1997).

In general, GC determination of phenylureas residues in major crops requires derivatisation step to prevent the degradation of target analyte in hot injector and/or GC columns. For this purpose, either alkylation by iodomethane or iodoethane in the presence of sodium hydride (acting as proton extracting agent) or acetylation by heptafluorobutyric anhydride were typically used (Bűchret & Løkke 1975; Karg 1993; Scott 1993; Gerecke *et al*. 2001).

Currently, high performance liquid chromatography (HPLC) represents preferred method of choice for determination of isoproturon and others similar phenylureas extracted from examined matrix by water or its mixture with miscible solvent, since no laborious derivatisation of analyte is needed. However, an extensive clean-up of crude extract has to be accomplished, when using this separation technique coupled with conventional

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detectors such as UV/DAD. Unfortunately, this "classic" detector is neither sensitive nor selective enough, to allow determination trace levels of target analytes in complex matrices (Sannino 1998; Hajšlová & Zrostlíková 2003).

Utilisation of HPLC separation hyphenated with tandem mass spectrometric detection (HPLC-MS/MS) represents in particular case the optimal solution. However, in spite of high detection selectivity, removing of partially co-isolated matrix components from a crude extract obtained from oily poppy seeds is a crucial step limiting reliable sample analysis. This paper describes optimisation and validation of procedure enabling accurate determination of isoproturon in difficult matrix.

MATERIAL AND METHODS

Chemicals and material. Pesticide residues grade solvents (acetonitrile, methanol) were obtained from Merck (Germany). Acetone was obtained from Penta (Chrudim, Czech Republic). Deionised water for preparation of a mobile phase and the extraction mixtures was produced by Milli-Q apparatus (Millipore, Germany). Supelclean LC-18, 500 mg/3 ml (Supelco, USA) solid phase extraction (SPE) cartridges were used for purification of crude extracts. Within the method development following SPE cartridges were also tested: (*i*) Oasis HLB 3cc/60 mg, 30 µm (Waters, USA), (*ii*) Oasis MCX 3cc/60 mg, 30 µm (Waters, USA). Poppy seeds samples with incurred residues were obtained from field experiment, in which Tolkan-Flo preparation was used for crops treatment. Blank samples were obtained from organic farm. Samples (approx. 200 g) of poppy seeds were ground using of laboratory mill (Porkert, Czech Republic).

Pesticide standard solutions. The isoproturon standard (purity 99.0%) was purchased from Dr. Ehrenstrofer (Germany). Pesticide stock solution (1250 µg/ml) prepared by dissolving of solid standard material in methanol was used for preparation of: (*i*) solution for setting up HPLC-MS/MS conditions and MS-tuning (50 µg/ml in methanol), (*ii*) spiking solutions (1–10 µg/ml) and (*iii*) calibration solutions (0.01–1 µg/ml in methanol).

For calibration, two types of matrix-matched standards were prepared from blank poppy seeds: (*i*) The residue after evaporation of SPE fraction was dissolved in 1 ml of isoproturon standard corresponding to the spiking level (0.04 and 0.4 mg/kg) and (*ii*) aliquots of crude extracts obtained within optimisation of extraction step were after filtration evaporated to dryness and the residue was re-dissolved in isoproturon standard 0.1 µg/ml, corresponding to 0.4 mg/kg.

Method development and optimisation. 12.5 g of ground blank poppy seeds were weighted into extraction plastic cuvette and spiked by 1 ml of 5 µg/ml isoproturon standard (i.e. contamination level 0.4 mg/kg). It was incubated for 20 min and then 20 ml of tested solvent/solvents mixture: (*i*) methanol, (*ii*) acetonitrile, (*iii*) acetone, (*iv*) methanol:water (1:1, v/v) and (*v*) acetone:water (1:1, v/v) were added. Suspensions were shaken (300 RPM) by a shaker (IKA Werke, Germany) for 1 h and then solid particles were separated by centrifugation at 6000 RPM for 10 min using of centrifuge (Hettich, Germany). The supernatant was transferred into the volumetric flask. The suspension was twice rinsed by 10 ml of extraction solvent. The final volume of combined supernatants was made up to 50 ml by the extraction solvent (the matrix content corresponded to 0.25 g/ml). 5 ml of crude extract was filtered through 5 µm PTFE microfilter (Carl Roth, Germany) and 1 ml of the filtrate was evaporated by a stream of nitrogen. The residue was re-dissolved in 1 ml of methanol, and transferred into a vial (2 ml) and analysed by HPLC-MS/MS. For calibration, matrix-matched standards were prepared (see chapter Chemicals and material). For determination of the amount of co-extracted matrix 25 ml of crude extract obtained by particular solvent were evaporated and the residue was weighed. All experiments were carried out in three parallel determinations.

For choosing both, optimal SPE cartridge and, respective procedure, 4 ml of isoproturon standard (0.1 µg/ml) in 20%, 50% methanol and 20% acetonitrile in water (v/v) were loaded on to the tested SPE cartridge. Prior to isoproturon standard loading, cartridges were conditioned by: (*i*) elution solvent and (*ii*) loading solvent. After the isoproturon standard loading, cartridges were washed. Final step of SPE procedure was elution of target analyte (for details see Table 1). All steps (conditioning, loading, washing, and eluting) were performed at a constant flow rate 0.5 ml/min.

HPLC separations were carried out using 1100 Series liquid chromatograph (Agilent, USA) equipped by reversed phase Lichrocart C18 column (25 cm × 4 mm, 5 µm) (Merck, Germany). HPLC conditions were as follows: methanol-water was used as a mobile phase, gradient program was:

Table 1. SPE procedures employing for different sorbents for method development/optimisation

SPE sorbent	Loading	Washing	Elution A	Elution B
LC18	acetonitrile:water (2:8, v/v)	8 ml of ACN:water (2:8, v/v)	4 ml of methanol	4 ml of acetonitrile
	methanol:water (2:8, v/v)	8 ml of MeOH:water (2:8, v/v)		
	methanol:water (1:1, v/v)	8 ml of MeOH:water (1:1, v/v)		
HLB	acetonitrile:water (2:8, v/v)	8 ml of ACN:water (2:8, v/v)		
	methanol:water (2:8, v/v)	8 ml of MeOH:water (2:8, v/v)		
	methanol:water (1:1, v/v)	8 ml of MeOH:water (1:1, v/v)		
MCX	acetonitrile:water (2:8, v/v)	8 ml of ACN:water (2:8, v/v)	4 ml of 1M ammonium acetate in methanol	4 ml of 1M ammonium acetate in methanol
	methanol:water (2:8, v/v)	8 ml of MeOH:water (2:8, v/v)		
	methanol:water (1:1, v/v)	8 ml of MeOH:water (1:1, v/v)		

0–3 min: linear from 70 to 80% methanol (v/v), 3–10 min: linear from 80 to 100% methanol (v/v), 10–15 min: isocratic 100% methanol (v/v). Total analysis time was 15 min, additional 7 min post run time was required to re-condition the column to initial conditions. Flow rate 0.7 ml/min, column temperature 25°C and the injection volume 20 µl were used in all experiments. Mass spectrometric detection/quantification was performed using LCQ Deca ion trap instrument (Finnigan, USA) with atmospheric pressure chemical ionisation (APCI). Experimental conditions were as follows: capillary temperature 170°C, heater temperature 300°C and spray voltage 5 kV in positive ionisation mode. Automated gain control (AGC) was set to fill the ion trap with ions up to 2×10^7. Maximum inject time was 250 ms. Ion optics parameters and capillary voltage were tuned automatically for the target compound. MS/MS transitions were performed with parent ion 207 *m/z* and daughter ion 165 and 72 *m/z*. Data processing was carried out using Xcalibur software (Finnigan, USA).

Recovery and repeatability information were obtained by analysing of spiked blank poppy seeds samples. Validation study was carried out using optimised method employing methanol:water mixture (1:1, v/v) for extraction. Crude extracts were purified using Supelclean LC-18, 500 mg/3 ml cartridges. Prior to sample loading it was conditioned by (*i*) 6 ml of methanol and (*ii*) 4 ml of methanol: water mixture (1:1, v/v). After sample loading (4 ml) cartridges were washed by 8 ml of methanol:water mixture (1:1,v/v). Target analyte was eluted by 4 ml of methanol. After evaporation of elution fraction, the residue was dissolved in 1 ml of methanol.

Method validation. Validation of method was realised at two concentrations levels – 0.04 mg/kg and 0.4 mg/kg (i.e. 0.04 µg/ml and 0.4 µg/ml in the final purified sample). Ground poppy seeds (12.5 g) were spiked with 0.5 ml of spiking solution (1 or 10 µg/ml, respectively) and incubated 20 min prior to extraction procedure. Six replicate determinations of recovery were performed for both concentration levels. Matrix-matched standards were used for construction of calibration curve. Repeatability, expressed as relative standard deviation (RSD, %), was used for uncertainty estimation. The limit of detection (LOD) and limit of quantification (LOQ) were estimated as an analyte concentration, for which minimal signal to noise ratio 3 and 9, respectively, were achieved.

RESULTS AND DISCUSSION

While in most cases a wide range of analytical methods can be found in literature for analysis of various pesticide residues potentially occurring in major crops for which respective preparations containing particular active ingredients were registered, procedures for minor crops, such as poppy seeds, are mostly unavailable. It should be noted, that with respect to requirements of EU (SANCO/3029/99 and SANCO/10232/2006), generic approach could be only applied for analysis of these crops provided there is not a significant difference in matrices composition. Unfortunately, this is not case of poppy seeds investigated in this study. This crop may contain up to 40% of lipids and in addition to polar lipids and soluble proteins, phenolic acids could be also partially

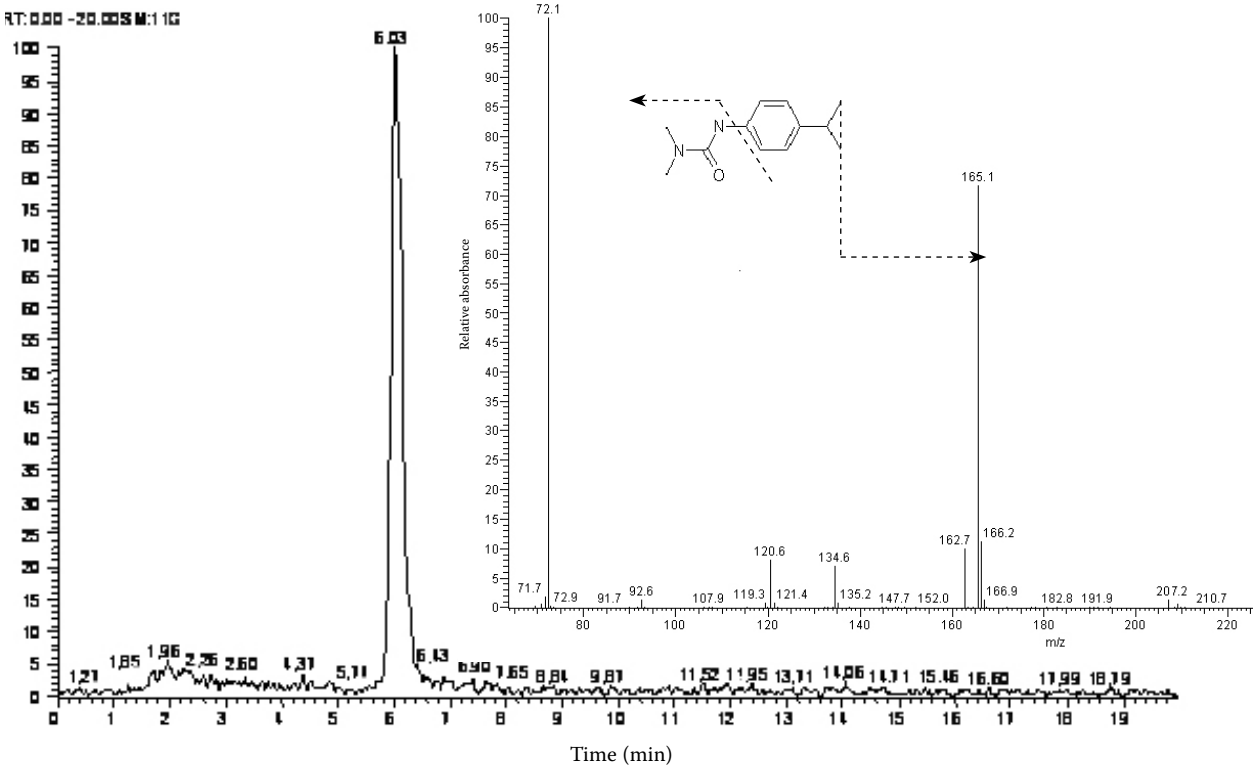
co-extracted, when using water miscible solvents for isoproturon isolation. These components can in a negative way influence the processes taking place in APCI. For minimisation of these adverse effects clean-up step employing SPE was involved into the developed method.

HPLC-MS/MS

Using Supelco Lichrocart C18 column, good retention and peak shape of isoproturon was obtained. The chromatogram of real poppy seeds sample at level 0.1 mg/kg is shown in Figure 1. Isoproturon provided the most abundant molecular ion ($[M + H]^+$ = 207 *m/z*) with characteristic daughter ions 165 and 72 *m/z* that were selected for SRM monitoring. MS/MS spectrum together with structural formula are shown in Figure 1.

Optimisation of method performance characteristics

To obtain acceptable recovery of relatively polar isoproturon (K_{ow} = 2.5) from oily matrix and to ensure reasonable compatibility of sample preparation step with reverse phase separation system, water miscible polar solvents were employed within extraction optimisation. Selectivity of this step was another considered issue. In addition to the assessment of target analyte extraction efficiency, the amount of co-isolated matrix was measured. Recoveries of isoproturon achieved by various extraction solvents together with the amount of co-extracted matrix components (parameter indicating the extraction selectivity) are shown in Figure 2. When comparing results obtained by acetone, acetonitrile, methanol, acetone-water and methanol-water mixture, the last one was identified as the best option as regards recoveries of isoproturon. This extraction mixture was selected for further experiments. Nevertheless, the amount of matrix components was rather higher than acetonitrile and/or methanol, they could be removed easily in the following clean-up step realised by SPE that is widely used for sample preparation prior to HPLC analysis of various pesticides (Hennion 1998, 1999; Rossi & Zhang 2000). The results of SPE clean up optimisation



Isoproturon ($[M–H]^+$ = 207 *m/z*) provides in MS/MS most abundant daughter ions *m/z* 165 and 72

Figure 1. HPLC-MS/MS chromatogram of isoproturon in spiked poppy seeds sample at the concentration level 0.1 µg/ml (i.e. corresponding to 0.1 mg/kg)

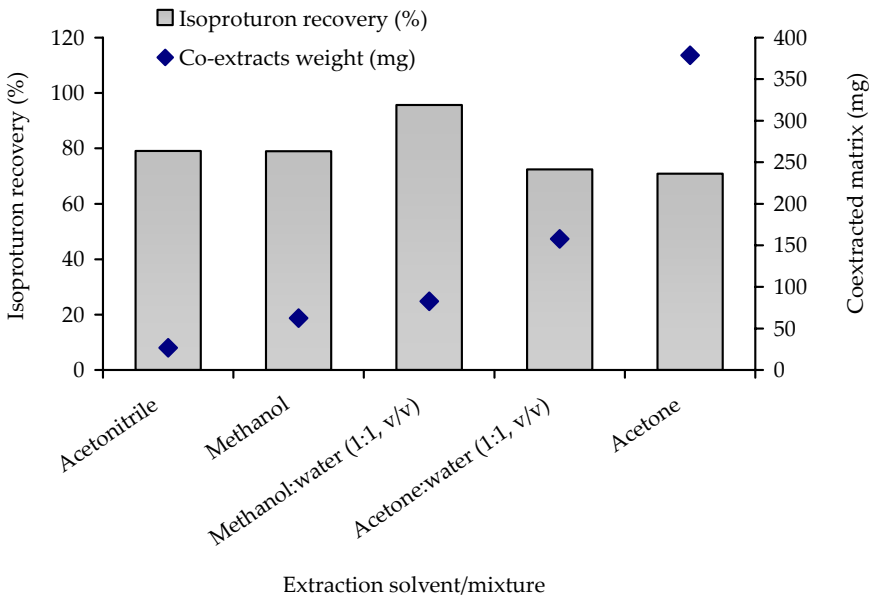


Figure 2. Amount of matrix components in crude extract co-extracted by various solvents and recoveries of isoproturon in spiked poppy seeds (at concentration level 0.4 mg/kg)

experiments are summarised in Table 2. Since satisfactory recovery of isoproturon (96%) was obtained by Supelco Supelclean LC18 cartridge when standard solution was loaded in methanol: water mixture (1:1, v/v) and the possibility of direct crude extract loading, these cartridges were selected as optimal for further experiments.

Optimisation and method performance characteristics

Although SPE clean-up may results in analyte loses and adds more sample handling effort, employing this step in HPLC-MS/MS residue analysis is so often the only way to reduce/minimalise adverse influence of matrix effects. As far as crude sample extract is analysed, matrix components co-eluting with target analyte may in some extent suppress the ionisation process occurring in APCI, and consequently the limit of quantitation is increased. (Kebarle & Tang 1993; Barnes *et al.* 1997; Choi *et al.* 2001; Zrostlíková *et al.* 2002, 2003; Hajšlová & Zrostlíková 2003). Attempting to analyse crude poppy seeds extract, response suppression as low as 20% was observed as compared to area of isoproturon standard in neat solvent. A significant improvement was achieved by SPE clean-up, since no response suppression occurred when analysing purified sample (i.e. matrix effect was 98%). Therefore isoproturon

Table 2. Recoveries of SPE using different SPE cartridges

SPE sorbent	Loaded in	Recovery of isoproturon (%)			
		loading	washing	elution A	elution B
LC18	20% ACN	7	5	85	1
	20% MeOH	0	0	97	1
	50% MeOH	0	0	96	0
HLB	20% ACN	0	0	75	21
	20% MeOH	0	0	91	1
	50% MeOH	15	12	65	1
MCX	20% ACN	1	73	19	0
	20% MeOH	0	0	95	0
	50% MeOH	8	0	90	0

standard in neat solvent could be used for calibration. Elimination of the need to prepare matrix-matched standard compensated, in some extent, labour demand associated with SPE step. Identical recovery of isoproturon (84% and 85%, respectively) was achieved at both concentrations levels with good repeatability (7% and 5%, respectively). Achieved LOD (0.01 mg/kg) and LOQ (0.03 mg/kg) of isoproturon were comparable to other published methods for isoproturon determination in major crops (Herrera *et al*. 1998; Hetherton *et al*. 2004; Hernández *et al*. 2006). Calibration curve, for which construction data were measured in 6 consecutive calibration sets (within the same analytical sequence) of spiked poppy seeds extracts, shows an excellent linearity (correlation coefficient, $R^2 = 0.9984$) over the whole calibration range (0.03–1 μg/ml).

CONCLUSIONS

Developed method employed Supelclean LC18 SPE cartridges for clean-up of crude methanol: water (1:1, v/v) extract and HPLC-MS/MS examination of purified sample. Validated method was found as rapid and robust, since no laborious changing of crude extract solvent was needed. Although involved SPE procedure resulted in lower isoproturon recovery and more sample handling effort, it was compensated by the use of automated SPE and calibration standard in neat solvent. LOD (0.01 mg/kg) as well as other method performance characteristics were fully in accordance with requirements for documentation of isoproturon residue dynamics.

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