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Gas chromatographic method for determination of uracil herbicides in roots of *Echinacea angustifolia* Moench (Asteraceae)

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Abstract

A GC/NPD method and a rapid screening TLC method were developed for the simultaneous determination of uracil herbicide residues (bromacil, lenacil, terbacil) in the roots of *Echinacea angustifolia* Moench (Asteraceae). The uracil herbicide residues were extracted into acetone. After evaporation of acetone from the acetone-water extract the residue was dissolved in water-methanol (5:1 v/v). Cyclohexane was used for removal of the non-polar co-extractives in the sample matrix. After separation of the cyclohexane phase the uracil herbicide residues were extracted into chloroform. This extract was purified on a Florisil® column, and residues were eluted with dichloromethane-acetone (9:1, v/v). The cleaned up extract was analysed by the GC/NPD method on a capillary column DB-1 using atrazine as internal standard. A minimum recovery of 70% was attained for contamination levels of 0.02–0.40 mg kg^{-1}.

Keywords: Residual analysis; Uracil herbicides; *Echinacea angustifolia* Moench roots; GC/NPD

1. Introduction

The plant material of the *Echinacea* genus, (Asteraceae) is represented by nine species. A review [1] reports the therapeutic effects of this plant. *Echinacea* species have been indicated in the treatment of internal urological conditions, anti-inflammatory diseases and gynacological conditions and as a stimulator of the immune response. They are used externally to promote wound healing as it is believed to support the formation of granulation tissue. Preparations from *Echinacea* have also been reported to be effective against viruses (herpatic, influenza, poliomyelitis and variola).

For the protection of *Echinacea angustifolia* or *Echinacea purpurea* monocultures grown for pharmaceutical use herbicide formulations are needed. The uracil herbicides bromacil, lenacil and terbacil are still on chemical trial. Bromacil and diuron provide full season weed control and maintain phytotoxicity for up to 24 months in the soil [2]. The uracil herbicides are strong inhibitors

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of photosynthesis. Terbacil is a uracil herbicide widely used for the selective control of many annual and some perennial weeds in crops such as sugar cane, apples, peaches, citrus and peppermint (*Mentha piperita*) [3]. Residues of uracil herbicides can be analysed by GC with selective detectors (nitrogen-phosphorus detector, NPD or electron capture detector, ECD). Jarczyk employed GC/NPD for the determination of bromacil in strawberries [4] and for lenacil in sugar beet [5]. Cessna used a capillary GC/NPD for determination of the bromacil residues in asparagus [6]. The same method was used for determination of the lenacil residues in sugar [7]. GC/ECD was used for the analysis of bromacil [8] and terbacil [9] in milk. Bromacil and terbacil in various agricultural products were analysed by a GC/MS [10]. Draper [11] has improved the performance of the nitrogen-phosphorus detector for multiresidual pesticide analysis by gas chromatography. Liquid chromatography with a UV detector was used for the determination of bromacil in ground water [12] and drinking water [13], and for terbacil leached from soil [3].

However, apart from sophisticated instrumental methods, simple screening methods have also been used for analysis of herbicide residues. A thin layer chromatographic (TLC) method with determination based on the Hill reaction was developed by Kováč et al. [14,15]. This method, known as chronometric method was used for the determination of uracil herbicides residues in sugar [7] and in the green plants of *Melissa officinalis* [16].

The Hill reaction is one of the sequence of biochemical reactions constituting the process of photosynthesis in plants and for about 40% of all herbicides (uracils, phenyl ureas, triazines and some others) their weed-killing effects are based on their ability to interfere with this process. This reaction also takes place in isolated plant chloroplasts and can be carried out and visualised on a TLC chromatogram. The quantitation makes use of the observations that the inhibition is reversible within a certain concentration range of the herbicide and that the lifetime of the inhibition zone (spot) on a silica gel chromatographic plate is directly proportional to the amount of the herbicide in that spot [7,14–16].

For the isolation of uracil herbicide residues from plant material extraction into acetone [4,5,10,16] or into methanol [6] is routinely used. The resulting extract may be cleaned up by column chromatography using silica gel [5,6], alumina [7,15], Florisil® [6,16], or by gel permeation chromatography using Bio Beads SX-3 [10,17] or with mini-column silica gel chromatography.

The present work was directed to isolation and purification procedures for simultaneous determination of the bromacil, lenacil and terbacil residues in roots of *Echinacea angustifolia* Moench. The extract after purification was analysed by capillary GC with a selective detector (NPD) using the DB-1 column and by the TLC screening method.

2. Experimental

2.1. Chemicals

Bromacil (5-bromo-3-sec.butyl-6-methyluracil), lenacil (3-cyclohexyl-5,6-trimethyleneuracil), terbacil (5-chloro-3-sec.butyl-6-methyluracil), and atrazine (2-chloro-ethylamino-6-isopropylamino-1,3,5-triazine) were supplied by Labor Dr Ehrenstorfer (Augsburg, Germany) with declared purity of 99.4% Atrazine served as an internal standard.

Acetone, methanol, chloroform, *n*-hexane (analytical grade) were supplied by Lachema (Brno, Czech Republic); dichloromethane and cyclohexane (analytical grade) were supplied by Merck (Darmstadt, Germany). All solvents were distilled in a glass apparatus before use.

Florisil® (60–100 mesh) was obtained from Merck and anhydrous sodium sulphate was obtained from Lachema.

Florisil® was first washed with acetone, then activated at 110°C for 24 h and after cooling, homogenized with 5% (v/w) of water for 2 h. Sodium sulphate was dried at 600°C for 18 h.

2.2. Equipment for GC or TLC

A Hewlett Packard 5890 Series II Plus with EPC gas chromatograph equipped with a nitrogen-phosphorus detector (NPD) was used (Hewlett-Packard, Palo Alto, USA).

Table 1
Recovery of uracil herbicides added to the plant samples determination by GC/NPD and TLC method

Herbicide	Contamination level (mg kg^{-1})	Recovery mean ± R.S.D. (%)[a]	Method
Bromacil	0.4	88.0 ± 3.2	GC/NPD
	0.1	76.3 ± 5.3	
	0.02	82.2 ± 5.2	
Lenacil	0.4	71.5 ± 2.6	
	0.1	76.8 ± 5.6	
	0.02	70.5 ± 3.4	
Terbacil	0.4	89.1 ± 3.2	
	0.1	76.8 ± 5.9	
	0.02	79.3 ± 6.4	
Bromacil	0.5	82.5 ± 4.1	TLC
	0.1	73.8 ± 3.6	
	0.05	72.2 ± 3.8	
Lenacil	0.5	74.0 ± 3.7	
	0.1	74.4 ± 4.8	
	0.05	71.2 ± 4.0	
Terbacil	0.5	81.0 ± 2.4	
	0.1	80.4 ± 1.5	
	0.05	77.0 ± 1.3	

[a] $n = 5$; but for contamination levels of 0.4 and 0.5 mg kg^{-1}; $n = 3$.

A fused silica capillary column DB-1 (15 m × 0.32 mm I.D., 0.25 μm film thickness, J and W Scientific, USA) was employed with helium carrier gas for NPD, the flow rate was 2.51 ml min^{-1} and the input pressure was 6.80 p.s.i. (46.9 kPa).

Injection port and detector temperatures were maintained at 220 and 250°C, respectively. The oven temperature was kept at 65°C for 1 min, increased to 150°C at 30°C min^{-1}, and kept at the final temperature for 8 min. A 1-μl volume of sample was injected splitless with the split valve closed for 0.8 min.

Table 2
Chromatographic separation of uracil herbicides by GC and TLC

Herbicide	Gas chromatography		Thin-layer chromatography	
	t_R [min]	$t_{R,rel}$	hR_f	$R_{f,rel}$
Bromacil	7.67	1.217	52	0.81
Lenacil	10.09	1.589	41	0.64
Terbacil	6.89	1.094	57	0.89
Atrazine (IS)	6.30	1.000	64	1.000

A high speed mixer Ultra Turrax®, model T 25 (Janke and Kunkel, Staufen, Germany) was used.

Chromatographic plates Silufol®, 20 × 20 cm (Kavalier, Votice, Czech Republic), were prewashed before use by developing in acetone and air-dried.

2.3. Standard stock solution

Individual standard stock solutions were prepared by dissolving 10 mg of each herbicide in 100 ml of acetone. A series of standard solutions in acetone was prepared at concentrations of 0.1–1.0 μg ml^{-1} for each herbicide.

2.4. Model samples for the measurement of recovery of the method

Plant material (*E. angustifolia* Moench, roots 100 g) was homogenized on a laboratory homogenizer. Acetone solution of a uracil herbicide ($c = 1$ μg ml^{-1}) was used for modelling the contamina-

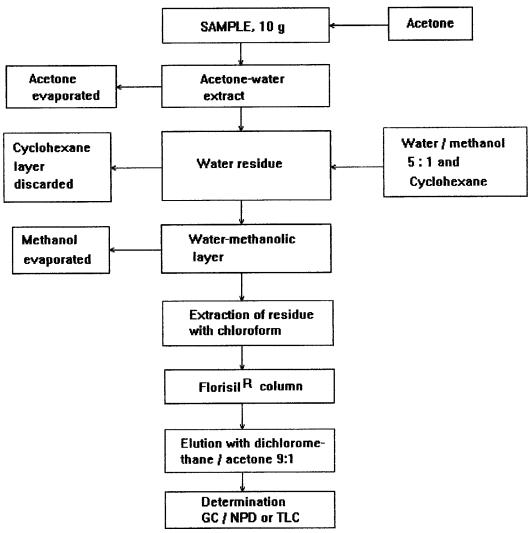

Fig. 1. A flow chart of the clean-up procedure

tion of 10 g matrix so as to give a contamination level of 0.02–0.5 mg kg^{-1}. Model samples were prepared individually for each herbicide. After thorough mixing and evaporation of acetone at ambient temperature the samples were analysed by a procedure described in the following paragraph.

2.5. Preparation of sample (clean-up procedure)

Plant roots were homogenized in a laboratory homogenizer and a 10 g aliquot was extracted in the high speed mixer with 2×100 ml portions of acetone; each extraction was conducted for 2 min at 6000–7000 r.p.m. The combined extracts were filtered through filter paper and the residue was washed with 20 ml of acetone. The filtrate was evaporated on a rotary vacuum evaporator (RVE) at 40°C to 4 ml; the residue was dissolved in 60 ml of water-methanol (5:1, v/v), transferred into a 100-ml separation funnel and shaken with 25 ml of cyclohexane or *n*-hexane. Hydrochloric acid (5 ml, $c = 1$ mol l^{-1}) was then added to obtain good separation of the two phases. The water-methanol layer was then separated and methanol removed on the rotary vacuum evaporator. The uracil herbicide residues were finally extracted with 5×15 ml of chloroform. The combined chloroform extracts were passed through a layer of anhydrous sodium sulphate and washed with 20 ml of chloroform. The total dried extract was evaporated at reduced pressure (RVE) to about 2 ml. This was cleaned up as follows: a glass tube (40×1 cm) with a needle Teflon® stopcock was filled with 2 g of Florisil® and topped with 1 g of anhydrous sodium sulphate, and the evaporated extract was poured on it. Subsequently, the uracil herbicides were eluted with 40 ml of dichloromethane—acetone (9:1, v/v). The resulting eluate was evaporated to dryness on the RVE and the residue was dissolved in 1 ml of acetone. The purified sample was analysed by GC or TLC method.

2.6. TLC detection reagent

To prepare the homogenate of chloroplasts for the detection reagent, 30 g of the leaves of bean plants (Phaseolus vulgaris), at the stage of two well-developed leaves, were washed with distilled water, dried quickly with filter paper and pulped in a small homogenizer. Glycine (3 ml) and distilled water ice (15 g) were then added and the mixture homogenised for 30 s. The homogenate was hand-pressed through four layers of dressing gauze and kept in darkness at 2°C. Under these conditions, the homogenated kept its activity for 5 days.

The detection reagent for TLC was prepared immediately before spraying by mixing one volume of the homogenate of chloroplasts with two volumes of a solution of 2,6-dichlorophenolindophenol sodium salt ($c = 0.4$ mg ml^{-1}) in a borax buffer solution (pH = 8.6). Of the spray reagent, 9 ml was needed for spraying each TLC plate [7,15].

2.7. TLC screening determination

Acetone solution (10 μl) of the cleaned up sample was spotted on a silica gel TLC plate using a glass capillary micropipette. On the same plate, a series of standard solutions with different concentrations, i.e., calibration series, were spotted. The plates were developed in the mobile phase benzene-ethyl acetate-acetone (4:1:1, v/v)

and subsequently dried at laboratory temperature. The air dried chromatogram was then sprayed with the detection reagent [7,14,15] and exposed to the light of two 40 W neon tubes at a distance of 20 cm, in a horizontal position. Within 1–2 min after exposure, dark blue inhibition zones appeared on a pale yellow-green back-ground. The time from the beginning of the exposure to the complete disappearance of the spot was measured with a stop-watch. The amount of herbicide residues in the sample was determined from the analytical curve of plotting the lifetime of the inhibition zones of the standard series against the concentrations of the standards in the zones.

3. Results and discussion

The capillary column DB-1 and proposed temperature program comply with all the requirements of simultaneous analysis of bromacil, lenacil, and terbacil residues by the GC/NPD method. The selective nitrogen-phosphorus detector is suitable for the analysis of all uracil herbicides.

The plant material samples (fresh roots) of *Echinacea angustifolia* Moench with contamination levels in the range of 0.02–0.4 mg kg^{-1} were analysed by gas chromatography or thin layer chromatography. The uracil herbicide residues were extracted from the sample with acetone. The extracts after the clean-up were analysed by both methods. Average recoveries ranged from 70.5 to 89.1% with the relative standard deviation (R.S.D.) between 2.6 and 6.4% for the GC/NPD. Good linearity was obtained for all the herbicides under study in the range 0.1–1.0 ng. Average recoveries ranged from 71.2 to 82.5% with R.S.D. between 1.3 and 4.8% for the screening method. The results of their determination are summarised in Table 1. Principal characteristics (t_R, R_f) for chromatographic methods (GC, TLC) are summarised in Table 2.

The lower limit of detection (LOD) of the proposed GC/NPD method was 0.01 mg kg^{-1} under the conditions as described for each uracil herbicide in the plant sample extract. The LOD for the screening method (TLC) using a selective detection method was 0.01 mg kg^{-1}. In both cases the signal-to-noise ratio was ($S/N = 3$).

The isolation and clean-up procedure (Fig. 1) is versatile and may be used without modification for the GC of TLC analysis. The simplicity and low demands on the instrumental equipment are advantages of the screening method. The advantage of GC/NPD is that it enables any type of the uracil herbicide to be analysed using atrazine as a common internal standard. A selective electron capture detector cannot be used for lenacil which does not contain a halogen atom in its molecule. The determination of pesticides by GC/NPD can be further improved by attention to the condition of the capillary column and the thermo-ionic ionisation source. Solvent cleaning [11] also reduces the chromatographic tailing of many polar pesticides (i.e. uracils). A methyl silicone capillary column and a selective nitrogen-phosphorus detector are suitable for the multiresidue analysis of uracil herbicide residues in *Echinacea angustifolia* Moench (roots).

Both methods can be used for routine analyses of uracil herbicides in *E. angustifolia*. The TLC method also provides a reliable confirmation of the identity of the uracil herbicides, being based on a sensitive and selective biochemical detection utilising the mechanism of action of the herbicides.

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