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Note

Residue analysis of the herbicides cyanazine and bentazone in sugar maize and surface water using high-performance liquid chromatography and an on-line clean-up column-switching procedure

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In order to decide about the acceptability of the application of the herbicides bentazone (3-isopropyl-1*H*-benzo-2,1,3-thiadiazin-4-one 2,2-dioxide; CAS No.25057-89-0) and cyanazine [2-(4-chloro-6-ethylamino-1,3,5-triazin-2-ylamino)-2-methylpropionitrile; CAS No. 21725-46-2] to sugar maize in The Netherlands, a field study was carried out in which maize fields were treated with both herbicides. In this paper a high-performance liquid chromatographic (HPLC) method, involving on-line clean-up by means of column switching, is described for the simultaneous determination of these herbicides together with the 6- and 8-hydroxy metabolites of bentazone. Generally this type of work requires limits of determination (LOD) of 0.1 mg/kg.

Cyanazine, a triazine herbicide, can be determined by gas chromatography (GC)[1,2] and also by reversed-phase HPLC (UV detection, 224 nm) using a basic eluent (pH 8)[2,3]. Bentazone can be determined by GC after derivatization with diazomethane[4] or pentafluorobenzoyl chloride[5] or by reversed-phase HPLC (UV detection, 229 nm), using an acidic eluent (pH 3)[6]. In our Institute, a mobility study has been conducted in order to study the behaviour of bentazone and its two metabolites, 6- and 8-hydroxybentazone, in soil columns. The three compounds were analysed by reversed-phase HPLC (UV detection, 229 nm) using an acidic eluent[7]. Liquid–liquid extraction from the acidified effluent was carried out with dichloromethane. Probably owing to adsorption effects, the 6- and 8-hydroxy bentazone metabolites could not be recovered after addition to a soil column.

Cyanazine has been determined in grain[1], soil[2] and surface water[3], after extraction into an organic solvent (both methanol and dichloromethane have been described) followed by subsequent column chromatography over silica gel, Florisil and Bio-Beads. The LOD after HPLC analysis was typically 0.01 mg/kg[2].

Akerblom and Gunborg[8] isolated bentazone from soil and crops using methanol. The extract was cleaned by a complex liquid–liquid extraction procedure, involving three different washing steps, followed by ion-pair extraction of the analyte using tetrabutylammonium hydroxide in dichloromethane. The LOD of the HPLC procedure was 0.02 mg/kg.

From the literature survey, we concluded that the method of choice for the simultaneous determination of the compounds mentioned above is HPLC. In line with

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our previous work[9–11], we decided to develop a clean-up procedure involving precolumn switching for the extracts, in order to increase the speed and reproducibility of the sample clean-up step.

EXPERIMENTAL

Equipment

The HPLC system consisted of the following components: a PROMIS (Spark Holland, Emmen, The Netherlands) autosampler, equipped with a time-programmable high-pressure switching valve, type 7010, one low-pressure switching valve, Type 5300, and an electropneumatic unit, Type 7163 (Rheodyne, Cotati, CA, U.S.A.), two HPLC pumps (Series 10, Perkin-Elmer, Norwalk, CT, U.S.A.) a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 770 UV detector, set at 229 nm, a Kipp (Delft, The Netherlands) recorder and an LDC/Milton Roy (Co. Clare, Ireland) Cl-10 integrator. The precolumn was a 15 × 3.2 mm I.D. Brownlee (Santa Clara, CA, U.S.A.) New Guard cartridge packed with 7-μm RP-18 and the analytical column was a 150 × 4.6 mm I.D. stainless-steel column, packed in-house with 5-μm Hypersil ODS (Shandon, Runcorn, U.K.). The analytical column was kept at 22°C by means of a water jacket.

Chemicals

Cyanazine and bentazone, both of >99% purity, were obtained from BASF (Ludwigshafen, F.R.G.); 6- and 8-hydroxybentazone were a gift from Dr. N. Drescher (BASF). Dichloromethane of analytical quality was bought from Merck (Darmstadt, F.R.G.) and methanol (UV spectrometry grade) and acetonitrile (HPLC quality) from Baker (Deventer, The Netherlands). HPLC water was obtained by filtration of doubly distilled water over a Millipore (Bedford, MA, U.S.A.) Norganic filter, Type CC15 120 00. Anhydrous sodium sulphate and hydrochloric acid (6 *M*) were bought from Baker and orthophosphoric acid (89% pure) and Titrisol buffer (pH 4) from Merck.

HPLC eluents were mixed by volume, and subsequently filtered and degassed by vacuum suction over a 0.5-μm filter (Millipore).

Extraction procedure for maize

Maize kernels were stripped from the cobs and collected. After mixing of the kernels, 50 g were weighed in the cup of a Waring blender and 200 ml of dichloromethane, 4 ml of 6 *M* hydrochloric acid and 100 g of sodium sulphate were added. The mixture was blended for 3 min, then the homogenate was transferred into a tube and centrifuged for 5 min at 7000 *g*. A 100-ml volume of the dichloromethane phase was dried over sodium sulphate and concentrated to 10 ml in a Kuderna Danish apparatus. A 1-ml volume of the dichloromethane extract, containing 2.5 g ml of maize, was pipetted into a calibrated tube, which was placed on a warm water-bath (*ca.* 80°C) and, with the use of a gentle stream of nitrogen, the solvent was removed. The residue was dissolved in hexane, which had been previously saturated with acetonitrile, 1 ml of acetonitrile was added and the tube was shaken for 1 min. The upper layer was removed by pipetting and the acetonitrile was evaporated on a water-bath. The residue was dissolved in 100 μl of methanol, then 1.9 ml of water was added. Aliquots of 100 μl of a solution of the extract in methanol–water (5:95), were further processed automatically by injection on to the precolumn.

Extraction procedure for drinking and surface water (LOD 0.5 μg/l)

A sample of 100 ml of water was placed in a 250-ml round-bottomed flask and concentrated with a rotary film evaporator at 70°C to *ca.* 0.5 ml. About 0.5 ml of distilled water was added and the flask was placed in an ultrasonic bath for 2 min. The concentrate was transferred to a calibrated tube by pipetting. The flask was rinsed with 0.5 ml of water and the final volume in the tube was brought to 2 ml with distilled water. Aliquots of 100 μl of this concentrate were injected on to the precolumn.

Extraction and partial clean-up procedure for drinking water, raw water and rain (LOD 0.01 μg/l)

A 200-ml volume of water was adjusted to pH 10 by dropwise addition of 1 *M* sodium hydroxide solution. The sample was concentrated in a rotary film evaporator at 80°C to *ca.* 3 ml. The sample was transferred to a calibrated tube and, if necessary, the pH was adjusted to 10 by dropwise addition of 1 *M* sodium hydroxide solution. A 2-ml volume of *n*-hexane was added and the tube was shaken vigorously for 30 s. The hexane layer was removed by pipetting. Another 2 ml of *n*-hexane were added and the shaking was repeated. The *n*-hexane was pipetted off and the aqueous layer was acidified by addition of 1.5 ml of 4 *M* hydrochloric acid. Bentazone was extracted with 2 × 2 ml of dichloromethane. The dichloromethane layer was filtered over sodium sulphate into a calibrated tube. The tube was placed on a warm water-bath (*ca.* 60°C) and the solvent was evaporated with a gentle stream of nitrogen. The residue was dissolved in 5% methanol by first adding 20 μl of methanol followed by 180 μl of water. The solution was then pipetted into an autosampler vial and processed further by precolumn switching HPLC.

Precolumn clean-up and HPLC procedure

The set-up for the automated sample clean-up and subsequent HPLC analysis is shown in Fig. 1. The precolumn flushing solvent, A, consisted of methanol–0.03 *M* phosphate buffer (pH 2.70) (5:95), at a flow-rate of 1 ml/min. The eluent for the analytical column, E, was methanol–0.02 *M* phosphate buffer (pH 2.70) (35:65) at a flow-rate of 1 ml/min.

Aliquots of 100 μl were injected by the autosampler (AS) on to the precolumn (PC), using 2.5 ml of flushing solvent for water concentrates and 4.0 ml for maize extracts. The sample was transferred to the analytical column, using 0.5 ml of eluent, by switching the precolumn temporarily in-line with the analytical column.

While the analysis takes place, the precolumn is washed with 5 ml of methanol (B) and reconditioned for the next injection with 10 ml of flushing solvent (A). After every fifth sample injection, an external standard (std. vial) was analysed.

RESULTS AND DISCUSSION

HPLC procedure

Optimization of reversed-phase analysis was carried out on a C_{18} column using methanol and acetonitrile as modifiers and, because of the acidic nature of bentazone (pK_a 3.2), using an acidic aqueous buffer (initially 0.02 *M* phosphate, pH 2.8) to suppress ionization. We observed symmetrical peaks for the analytes when using methanol and serious peak tailing with acetonitrile as the modifier, and therefore continued to work with methanol.

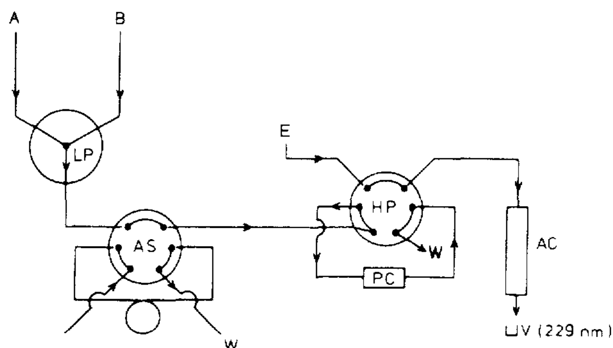

Fig. 1. Schematic representation of the eluent streams and switching valves used for the HPLC analysis. A = Methanol–0.03*M* phosphate buffer (pH 2.70) (5:95); B = 100% methanol; E = methanol–0.03 *M* phosphate buffer (pH 2.70) (35:65); LP = low-pressure three-way selection valve; HP = high-pressure six-port switching valve, equipped with a C_{18} precolumn (PC); AS = autosampler with a 200-μl sample loop; AC = analytical column; W = waste; UV = UV detector.

We studied the behaviour of the analytes and that of a potential interferent, present in maize extracts (see Experimental), as a function of the methanol content of the eluent. The results are presented as ln *k vs.* percentage modifier in Fig. 2. It can clearly be seen that the separation of the analytes is easy, but that an unknown component will interfere with either bentazone or cyanazine. Calculation of the R_s values[12] of each of the two peak pairs showed that, in the system used, the resolution is optimal (R_s = 0.91) at 35% methanol.

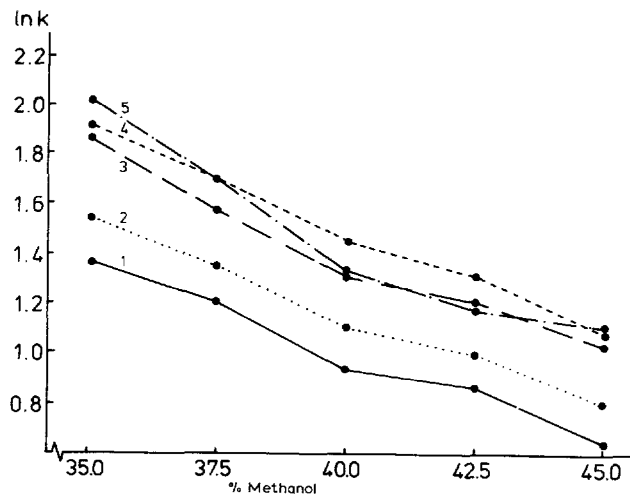

Fig. 2. Logarithmic plot of the capacity factors (*k*) of (3) cyanazine, (4) bentazone, (1) 6- and (2) 8-hydroxybentazone and (5) a unknown matrix compound from maize as a function of the percentage of methanol in the mobile phase. Column: 150 × 4.6 mm I.D. Hypersil ODS. Eluent: methanol in 0.02 *M* phosphate buffer (pH 2.70) (35:65) at a flow-rate of 1 ml/min. For further details, see Experimental.

In order to improve the resolution, we varied the pH of the eluent, keeping the organic modifier composition constant. In this experiment it appeared that pH had a major influence on the behaviour of bentazone and its metabolites. At this stage two new potential interferences appeared in the chromatograms of the maize extract blanks, and these peaks were therefore included in the optimization study.

Fig. 3 shows a plot of the resolution, R_s, as a function of the pH of the buffer in the eluent. It can be seen that no condition can be found where the resolutions for all components is good (*i.e.*, have a value of *ca.* 1.2 or larger). However, as the two interferences are relatively small (corresponding to 0.05 mg/kg of each of the metabolites of bentazone), we decided not to pay much attention to the resolution between the metabolites and the matrix interferences and to select, as a compromise, the conditions that give a good separation between the analytes of interest and some separation between the metabolites and the matrix.

Precolumn clean-up procedure

Initially, we studied the possibility of using disposable cartridges (Baker-10) for clean-up. We tested the ability of cartridges filled with different adsorbents (200 mg of C_8, C_{18} and CN material) to sorb the best retained analyte, bentazone, from an aqueous solution of pH 2.70. None of the cartridges could retain this analyte.

On normal phase material (Baker-10, 500 mg silica) the situation was the opposite: all analytes were very strongly retained. In order to desorb bentazone, 1% of water had to be added to the eluent (methanol–dichloromethane, 50:50). Such a solvent cannot easily be evaporated and therefore this method was rejected.

Analogous to previous work[9–11], we decided to clean redissolved extracts on a reversed-phase precolumn. As the flushing solvent we used the same aqueous buffer

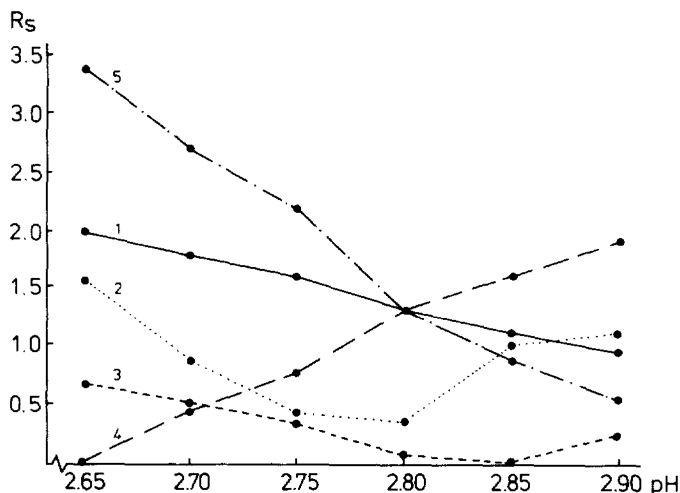

Fig. 3. Resolution (R_s) plotted against pH of the mobile phase for the five peak pairs in Fig. 2. Column: 150 × 4.6 mm I.D. Hypersil ODS. Eluent: methanol in 0.02 *M* phosphate buffer (35:65) at a flow rate of 1 ml/min. For further details see Experimental. 1,6-Hydroxybentazone–8-hydroxybentazone; 2, cyanazine–bentazone; 3,6-hydroxybentazone–interferent 1; 4,8-hydroxybentazone–interferent 2; 5, bentazone–interferent 2.

of pH 2.70 as was used in the HPLC eluent. In order to keep lipophilic matrix residues in solution we added 5% of methanol to this solvent. The flushing solvent was used to transfer the sample from the vial to the precolumn and for clean-up.

We tested three different packing materials in the precolumn: PRP-1 (a divinylbenzene–styrene copolymer), RP-8 and RP-18. We determined the breakthrough volumes of the first-eluted compound, 6-hydroxybentazone, from the flushing solvent [methanol–0.03 *M* phosphate buffer, pH 2.70 (5:95) at a flow-rate of 1 ml/min], and of the last-eluted compound, bentazone, from the transfer or desorption solvent [=eluent of the analytical column: methanol–0.02 *M* phosphate buffer, pH 2.70 (35:65) at a flow-rate of 1 ml/min]. All desorption experiments were carried out in the forward flush mode.

The results are given in Table I. The peak volumes eluted from the precolumn were measured on a recorder as their UV absorbance at 229 nm. In this context, V_{start} and V_{end} are the corresponding starting and end points of the recorder peak in the chromatogram. It can be seen from Table I that on RP-8 there is little retention, and desorption takes place fast; on PRP-1 there is good retention but desorption is slow, which will give rise to undesirable band broadening during analysis. With RP-18, one can concentrate the analytes with 3 ml of flushing solvent, without breakthrough, and desorption can take place with as little as 0.4 ml of eluent.

We used the described procedure with RP-18 to analyse maize extracts spiked with cyanazine, bentazone and the two metabolites. The extracts were found not to contain the metabolites. Further study showed that the concentrations of the metabolites, added to partially cleaned maize extracts, quickly decrease and can no longer be measured after standing for a few hours. Therefore, we concluded that a matrix effect occurs, as had also been observed by Loch[7] in soil, and that attempts to measure the metabolites of bentazone in maize are useless. Our efforts were therefore directed towards the optimization of the analysis of the two herbicides. Clean-up on the precolumn could now be carried out with 4 ml instead of 3 ml of flushing solvent, which made the clean-up in the first part of the chromatogram more efficient. The result is shown in Fig. 4.

TABLE I

BREAKTHROUGH VOLUMES AND ELUTION PROFILES OF 6-HYDROXYBENTAZONE[a] AND BENTAZONE[b] FROM THREE DIFFERENT 7-μm BROWNLEE NEWGUARD (15 × 3.2 mm I.D.) PRECOLUMNS

Packing material in precolumn	*Breakthrough volume of 6-hydroxybentazone (ml)*		*Elution profile of bentazone (ml)*	
	V_{start}	V_{end}	V_{start}	V_{end}
PRP-1	5	>20	0.1	~2
RP-8	0.5	2.5	0.1	0.3
RP-18	3.2	7.0	0.15	0.40

[a] The precolumn flushing solvent was methanol–0.03 *M* phosphate buffer (pH 2.70) (5:95) at a flow-rate of 1 ml/min.

[b] The desorption eluent was methanol–0.02 *M* phosphate buffer (pH 2.70) (35:65), at a flow-rate of 1 ml/min.

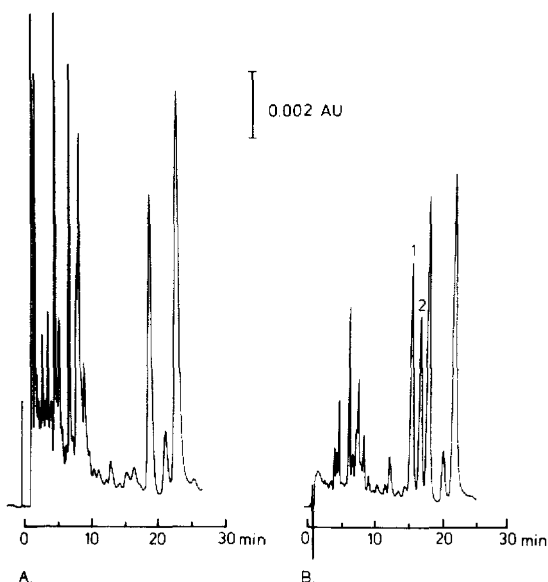

Fig. 4. Effect of the on-line clean-up step described under Experimental; analytical procedure. (A) 125 mg of maize blank analysed without precolumn clean-up; (B) 125 mg of maize with standard addition at a level of 0.46 mg/kg of (1) cyanazine and (2) bentazone, analysed using precolumn clean-up with a 4-ml flushing step.

Quantitative analysis of maize samples

The efficiency of the chromatographic system, including the precolumn switching procedure, was measured with bentazone. The number of theoretical plates, N, was 5200 and the peak asymmetry, measured at 10% peak height ($A_{0.1}$), was 1.1.

The linearity of the calibration graph [standard solutions of compounds in methanol–water (5:95)] was good, with correlation coefficients of over 0.999 for five data points in the range 10–1500 ng injected for each of the four analytes. The repeatablity of the responses of 50-ng injections of standard solutions with the system mentioned above was good. The coefficients of variation were 2.3% for 6-hydroxybentazone, 2.2% for 8-hydroxybentazone, 1.1% for cyanazine and 3.2% for bentazone ($n=8$).

The recoveries of cyanazine and bentazone added to maize at concentrations of 0.1 and 1 mg/kg were excellent (93–97%), as can be seen from Table II. As mentioned above, the two metabolites of bentazone could not be recovered on addition to the raw product, possibly owing to a matrix effect.

The reproducibility of the method was determined during five days, using spike levels of 1.2 mg/kg of each compound. The mean recovery of cyanazine was 91% and for bentazone 98% with corresponding coefficients of variation of 2.9 and 6.7%, respectively ($n=5$).

The LOD of the procedure (calculated from three times the peak-to-peak noise level in the chromatograms) was 0.02 mg/kg for both cyanazine and bentazone. With this procedure, eight samples of maize, four treated with cyanazine and four with bentazone in a supervised trial, were analysed together with four blank samples. In none of the samples could residues of the herbicides be detected.

TABLE II

RECOVERIES OF BENTAZONE AND CYANAZINE ADDED TO MAIZE

Compound	*Spike level (mg/kg)*	*n*[a]	*Mean recovery (%)*	*Standard deviation (%)*
6-Hydroxybentazone	0.096	6	–	–
	0.96	7	–	–
8-Hydroxybentazone	0.16	6	–	–
	1.6	7	–	–
Cyanazine	0.12	6	95	5.8
	1.2	6	93	2.9
Bentazone	0.12	6	97	9.1
	1.2	7	95	5.5

[a] Number of independent analyses.

Water analysis

The method described for maize was applied to the analysis of surface and drinking water which had been concentrated 50-fold by evaporation (see Experimental). The fatty residue in water samples is negligible compared with maize extracts so the liquid–liquid partitioning with *n*-hexane–acetonitrile as a first clean-up step was omitted. For surface water, the limit of determination was 1 μg/l (ppb) and for

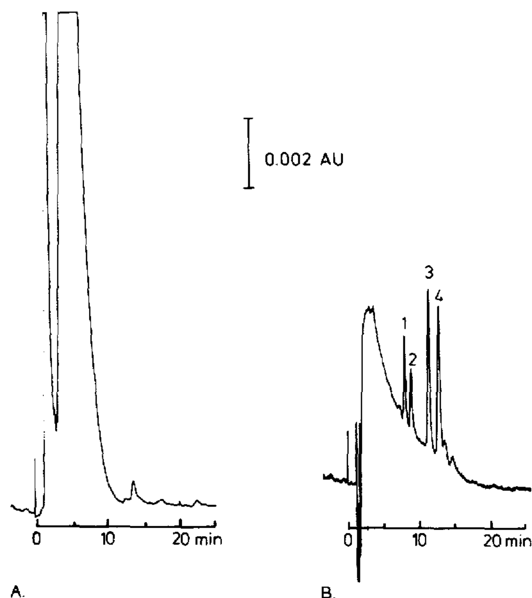

Fig. 5. HPLC of 5 ml of drinking water; (A) Blank, without precolumn clean-up. (B) spiked drinking water with precolumn clean-up at a level of (1) 2.0 μg/l 6-hydroxybentazone, (2) 3.2 μg/l 8-hydroxybentazone and 2.3 μg/l of (3) cyanazine and (4) bentazone. For procedure, see *Extraction procedure for drinking water (LOD 0.5 μg/l)*.

drinking water 0.5 μg/l for cyanazine and bentazone. The two hydroxymetabolites of bentazone can be determined at the higher limit of 2 μg/l, owing to a large tail of interferences present in surface water, as can be seen in Fig. 5.

At the 2 μg/l spiking level, the mean recoveries and corresponding coefficients of variation from drinking water were 84 ± 6% for 6-hydroxybentazone, 81 ± 5.2% for 8-hydroxybentazone, 98 ± 2.7% for cyanazine and 99 ± 4.5% for bentazone ($n=6$ for each analyte). At the 10 μg/l level these recoveries were 97 ± 2.7% for 6-hydroxybentazone, 96 ± 2.5% for 8-hydroxybentazone, 100 ± 1.9% for cyanazine and 98 ± 2.1% for bentazone ($n=5$ for each analyte). The recoveries for surface water (Poelpolder, The Netherlands) were determined at spike levels of 10 μg/l and were 44 ± 2.5% for 6-hydroxybentazone, 51 ± 2.2% for 8-hydroxybentazone, 97 ± 3.9% for cyanazine and 97 ± 3.2% for bentazone ($n=5$ for each analyte). The low recoveries for the two polar metabolites of bentazone are probably caused again by matrix compounds, as observed with soil and maize.

In another study, we had been asked by the drinking water authorities to confirm the presence of bentanone in raw water and "purified" water used for the preparation of drinking water and originating from the (polluted) river Rhine, and in rain, both at levels of 0.01 μg/l. In this instance, the method without liquid–liquid partitioning as a clean-up step was insufficient. We therefore introduced an acid–base separation procedure, as described under Experimental.

Bentazene recoveries were determined in "purified" water at spike levels of 0.05–0.15 μg/l to be 73 ± 9.5% ($n=4$) and at 1.1 μg/l to be 77 ± 6.6% ($n=4$) and in rain to be 81 ± 3.1% ($n=6$). In five out of six water samples we found bentazone at levels of 0.1–0.7 μg/l. Our results were also confirmed by gas chromatography–mass spectrometry, using negative ion chemical ionization, after derivatization with pentafluorobenzoyl bromide.

CONCLUSION

An HPLC method for the simultaneous determination of cyanazine and bentazone in sugar maize has been developed using precolumn switching for the automated on-line clean-up of extracts. The method makes use of a reversed-phase HPLC system with UV detection at 229 nm. Compared with the existing methods[2,8], the main advantage is the increase in sample throughput due to the automated process. Because of the reconditioning of the precolumn during the HPLC analysis on the analytical column, many samples (more than 200) can be cleaned on one precolumn, reulting in a cost-effective sample clean-up.

The method is also applicable to the residue analysis of these herbicides in surface and drinking waters at levels down to at least 1 μg/l (ppb). For the specific determination of bentazone in drinking water and rain, the limit of determination was further lowered to 0.01 μg/l (ppb).

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