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# RESIDUE ANALYSIS OF METHOXURON AND ITS BREAKDOWN PRODUCT (3-CHLORO-4-METHOXYANILINE) BY THIN-LAYER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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## SUMMARY

Dns derivatization prior to fluorescence detection of phenylurea herbicides (after hydrolysis) and their aniline metabolites was further developed for residue analytical purposes with high-performance (HPLC) and thin-layer chromatography (TLC). Metoxuron and 3-chloro-4-methoxyaniline (CMA) were used as model compounds. An increased fluorescence intensity in TLC was obtained by dipping the plates into paraffin-hexane (2: 1). A simple derivatization procedure, *viz.*, addition of Dns chloride, evaporation and dissolution, plus overnight reaction, in the acetonitrile-water mixture used as the mobile phase, was developed for HPLC. The fluorescence intensity of Dns-anilines decreases with increase in the number of halogen substituents, and increases if a methoxy substituent is present. The detection limit of Dns-CMA is about 1 ng in both TLC and HPLC.

Residue analyses of CMA and metoxuron in water, potato and soil samples involved acetone or dichloromethane extraction, silica gel clean-up, hydrolysis on a TLC plate (for metoxuron), Dns derivatization and HPLC or TLC evaluation. Retention data and recoveries are presented. The detection limits for CMA are 0.001 mg/kg in water, 0.02 mg/kg in potato and 0.2 mg/kg in soil.

## INTRODUCTION

The identification and quantitation of residue levels of urea herbicides and their major metabolites, substituted anilines, is difficult. Recently, methods have been proposed that make use of heat treatment' or catalytic hydrolysis[2–4] of ureas to anilines prior to further analysis, rather than classical hydrolysis techniques. The resulting anilines can then either be derivatized to make them suitable for gas chromatography with an electron-capture detector[4], or fluorescent derivatives suitable for liquid chromatographic analysis can be formed[2,3]. The advantage of derivatization with 5-dimethylaminonaphthalene- 1 -sulphonyl (Dns) chloride, prior to thin-layer (TLC) or high-performance liquid chromatography (HPLC) and fluorescence detection has been stressed for various types of compounds such as organophosphorus

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pesticides[5–7], carbamates[8,9] and ureas[2,3]. Often fluorigenic labelling prior to the chromatographic step will enhance the clean-up potential of a sample-handling step and result in overall simpler sample treatment procedure[10]. The improved sensitivity and selectivity will help in determining trace amounts of pesticides and metabolites in complex matrices.

In this work, it was our intention to study further the potential of the earlier proposed in situ catalytic hydrolysis of urea herbicides and Dns derivatization of the resulting anilines, with final analysis by TLC or HPLC. Special emphasis was placed on the Dns derivatization of substituted anilines, as these are more toxic than the parent compounds and are often more abundant in the environment. Metoxuron and its major metabolite 3-chloromethoxyaniline (CMA) were chosen as model compounds to demonstrate the potential of the approach to residue analyses of water, soil and plant material.

## EXPERIMENTAL

### *Reagents*

Commercially available 20 × 20 cm thin-layer plates pre-coated with a $C_{18}$ chemically bonded phase ($KC_{18}F$; Whatman, Clifton, NJ, U.S.A.) or silica (Kieselgel 60 $F_{254}$; Merck, Darmstadt, F.R.G.) were cut into 10 × 10 cm pieces. For column chromatographic clean-up, Kieselgel 60 Reinst (70–230 mesh; Merck) was activated at 165°C overnight and cooled to room temperature before use.

Solutions of 2.5 mg/ml of Dns chloride (Merck) in distilled acetonitrile were kept in brown vessels and stored in a refrigerator. Metoxuron and the anilines mentioned in Table I were dissolved in analytical-reagent grade acetone to give 1 mg/ml solutions; dilutions for separation and detection limit studies were made with acetone or dichloromethane.

Acetone, benzene, dichloromethane, ethyl acetate and methanol were pro analysi quality solvents from Baker (Deventer, The Netherlands). These solvents and all further reagents were used as received. Acetonitrile-water and methanol-water mixtures for HPLC and TLC (with 3% sodium chloride) were prepared with Millipore water. The paraffin used was "Baker" grade; melting point 52–54°C (Baker Chemicals B.V., Deventer, The Netherlands).

### *Apparatus*

TLC was carried out in a Camag (Muttenz, Switzerland) linear HPTLC chamber. A Zeiss (Oberkochen, F.R.G.) PMQ II spectrophotometer with a densitometer attachment was used to evaluate the TLC plates, using 350 nm as the excitation wavelength and an FL 46 emission filter. A scanning slit width of 4 mm, a scanning speed of 20 mm/min and an attenuation of 10 × 10 were used.

The HPLC equipment included an Altex 100 pump, a Valco (Houston, TX, U.S.A.) six-port injection valve with a 20-$\mu$l loop, a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 204 A fluorescence spectrophotometer and a Kipp (Delft, The Netherlands) Model BD 8 recorder. Separations were carried out at ambient temperature on a 15 cm × 4.6 mm I.D. stainless-steel column packed with 5-$\mu$m Spherisorb ODS (Phase Separations, Queensferry, U.K.), using acetonitrile-water and methanol–water mixtures at a flow-rate of 0.5 ml/min as the mobile phase. The excitation and emission wavelengths for fluorescence detection were 350 and 535 nm, respectively.

## PROCEDURE

### *Sample preparation and extraction*

*Water.* A 500-ml volume of tap water or surface water (river Amstel, Amsterdam) was spiked with 1 ml of a standard solution of CMA or metoxuron in acetone in a l-l separating funnel. The pH was adjusted to 8 by adding dilute sodium hydroxide solution. Next, 20 ml of saturated sodium chloride solution were added and the aqueous sample was subsequently extracted with 100, 80 and 60 ml of dichloromethane, using a 2-min shaking period each time. The extracts were dried over anhydrous sodium sulphate in an Erlenmeyer flask and filtered. A S-ml volume of benzene was added and the combined extracts were evaporated at 30°C in a rotavapor to remove all dichloromethane. The concentrated extracts were transferred into a 10-ml conical centrifuge tube and divided into two halves. One half was further evaporated to 0.5 ml and used for TLC; the other was used for direct Dns derivatization and HPLC determination of CMA.

With the water samples, no additional clean-up was required.

*Potato.* The extraction procedure was taken from Ambrus *et al.*[1], but the pH of the aqueous acetone was adjusted to 8 with dilute alkali. A 50-g sample was placed in a Waring blender homogenizer, spiked with 1 ml of a standard CMA or metoxuron solution and homogenized for 3 min with 100 ml of acetone. The homogenate was filtered through a Büchner funnel and the homogenizer jar and plant residue were rinsed with 50 ml of acetone. The extract was transferred into a l-l separating funnel, 20 ml of saturated sodium chloride solution and 450 ml of distilled water were added and the pH was adjusted to 8. Liquid-liquid extraction, drying and evaporation were carried out as for water samples. The final benzene extract was evaporated to 2 ml before clean-up in a silica column.

*Soil.* A 50-g sample of air-dried soil was spiked, by spraying, with 1 ml of a standard solution of CMA or metoxuron in dichloromethane in a Waring blender homogenizer. The sample was extracted three times with 100 ml of dichloromethane, using a shaking time of 2 min, and the extracts were filtered through a double layer of filter-paper in a Büchner funnel. A 5-ml volume of benzene was added, all the dichloromethane was evaporated, the concentrated extract was transferred into a conical centrifuge tube and the volume adjusted to 10 ml with benzene.

All extracts were stored in a refrigerator until clean-up (potato, soil) or analysis (water).

### *Clean-up*

A 20 × 0.5 cm I.D. glass column packed with 1 g of activated silica was used for clean-up. A l-ml volume of sample extract was applied to the top of the column and, after removal of any air bubbles present, part of the compounds interfering in TLC or HPLC analysis was removed by elution with 9 ml of dichloromethane. Elution was continued with ethyl acetate, 12 ml being sufficient to collect both metoxuron and CMA. After evaporation to 2–2.5 ml, the purified extract was ready for final analysis.

### *Analysis*

*TLC.* A 10-μl Hamilton syringe was used to apply 5 (potato, soil) or 10 μl

(water) of sample and standard solutions on a pre-coated plate; the spot distance was 8 mm. The spot size was limited to diameters of less than 2 mm by using a Camag spotter. For quantitative analysis, two blank samples, four or five standards (in the range of l-50 ng for CMA and 5-50 ng for metoxuron), and four or five spiked samples were spotted on each plate.

In aniline determinations, the applied spots were overspotted with 4 μl of 0.25% Dns chloride solution. In order to improve the efficiency of the Dns derivatization reaction, after applying the Dns chloride the chromatogram was immediately developed over a length of about 2 mm with acetone. Next, the thin-layer plate was covered with a clean glass plate and stored in the dark. After a reaction time of *ca.* 45 min, trace amounts of acetone were removed with a gentle stream of nitrogen and the silica TLC plate was developed with dichloromethane over a distance of 4 cm. To preserve and increase the intensity of the fluorescence of the Dns-aniline, after development the TLC plate was immediately (*i.e.*, without letting the dichloromethane evaporate) immersed for 10 sec in paraffin–hexane (2:1). The excess of this mixture was removed by letting it drip. After 20–30 min, the plates were inspected under 365-nm light or evaluated by means of densitometry.

With metoxuron, a catalytic hydrolysis step[2,7] preceded the Dns derivatization. To achieve hydrolysis, the TLC plate was covered with a clean glass plate and heated in an oven at 160°C for 20 min. After cooling, the plate was subjected to the treatment reported above for aniline determination.

*HPLC.* The solution of a standard in an organic solvent (l-50 ng per 20 μl) or the ethyl acetate fraction from the clean-up over silica was evaporated to 1 ml under a stream of nitrogen. Next, 120 μl of a 0.25% solution of Dns chloride in acetonitrile were added and, after mixing, the solution was evaporated to dryness at 70–80°C on a water-bath under a stream of nitrogen. To prevent sudden boiling, the centrifuge tube was regularly shaken during this step. After addition of 4 ml of acetonitrile-water (3: 1) and mixing (30 sec), the tube was stored overnight in the dark at room temperature, Reversed-phase HPLC was carried out with acetonitrile-water (3:1) as the mobile phase.

## RESULTS AND DISCUSSION

### *TLC*

Previous work[3] has shown that anilines can be derivatized with Dns directly on a TLC plate. With phenylurea herbicides, hydrolysis is required prior to derivatization; essentially the same procedure as in ref. 2 was used in this work for both normal-phase and reversed-phase stationary phases. Visual inspection of the plates (under 254-nm UV light) showed that silica is more efficient than a $C_{18}$-modified silica; with the former material much less unreacted aniline remained after Dns derivatization.

The $hR_F$ values of the anilines and their Dns derivatives are summarized in Table I; the solvent systems were selected on the basis of a short optimization study. From the data it is evident that Dns chloride and Dns sulphone can easily be separated from the Dns-anilines in both normal- and reversed-phase systems. In the silica system, two further fluorescent spots originating from Dns chloride were noted. These were, however, also separated from the Dns-anilines.

TABLE I

CHROMATOGRAPHIC DATA OF PARENT COMPOUNDS AND Dns DERIVATIVES

$t_R$ = Retention time.

| *Compound* | *Aniline: $hR_F$* | | *Dns compounds* | | |
|---|---|---|---|---|---|
| | *I** | *II*** | *$hR_F$* | | *$t_R$ (min)**** |
| | | | *I** | *II*** | |
| Dns sulphone | | | 0 | 90 | 1.35 |
| 3-Chloro-4-methoxyaniline | 29 | 79 | 40 | 62 | 2.80 |
| Aniline | 36 | 79 | 45 | 68 | 2.13 |
| 4-(4-Methoxyphenoxy)aniline | 43 | 69 | 47 | 58 | 3.10 |
| 4-Chloroaniline | 42 | 70 | 48 | 58 | |
| 3,4-Dichloroaniline | 52 | 68 | 48 | 51 | |
| 4-Bromoaniline | 42 | 70 | 49 | 58 | 2.95 |
| 3-Chloro-4-bromoaniline | 56 | 66 | 49 | 53 | 2.77 |
| 3-Chloroaniline | 48 | 71 | 49 | 58 | 2.88 |
| 4-Trifluoromethylaniline | 52 | 74 | 49 | 58 | |
| 3,5-Dichloroaniline | 58 | 66 | 51 | 48 | 2.91 |
| 4-(4-Chlorophenoxy)aniline | 33 | 58 | 51 | 50 | 3.70 |
| 3-Chloro-4-methylaniline | 22 | 66 | 52 | 57 | 3.15 |
| Dns chloride | | | 80 | 44 | |

* I, Kieselgel 60 $F_{254}$/dichloromethane methanol (99: I).
** II, Whatman $KC_{18}F$/acetonitrile–water (85: 15) 3% sodium chloride.
*** 5 $\mu$m Spherisorb ODS (15 cm × 4.6 mm)/methanol–water (3:1); flow-rate, 0.5 ml/min.

In reversed-phase TLC, problems were encountered when using methanol–water mixtures as mobile phase. Methanol apparently reacts with Dns chloride and a blue fluorescent streak appears on the TLC plate in the $hR_F$ range occupied by the Dns-anilines. Similar problems were observed during HPLC analysis. They were absent, however, in both TLC and HPLC when freshly distilled acetonitrile was used instead of methanol.

For all further work, silica was preferred to alkyl-modified silica because of efficient Dns derivatization (see above) and easy spotting.

From the literature, it is known that the fluorescence intensity in TLC of, amongst other compounds, Dns derivatives increases if detection is carried out in the presence of (trace amounts of) the mobile phase. Treatment of the developed chromatogram with highly viscous liquids has been used[2,11] successfully to achieve this end. In this work, spraying of the TLC plates with isopropanol-triethanolamine (8:2) did increase the fluorescence intensity; however, the reproducibility deteriorated. This is probably due to the fact that part of the fluorescent compounds migrates rapidly (within several minutes) into the polar viscous liquid[12].

After several series of tests with mixtures involving paraffin, glycerol, triethanolamine, isopropanol and hexane, it was concluded that immersion of the TLC plate is to be preferred to spraying because of easier handling and better reproducibility. Good results were obtained when using paraffin-hexane (2:1); migration of the spots was negligible even after several hours, while the fluorescence intensity increased 20–30-fold.

Plots of peak heights *versus* amount of CMA and metoxuron are shown in Fig. 1. The detection limits on different plates were 0.5-2 ng for CMA and l-5 ng for metoxuron. The curved plot observed for the herbicide, but not the aniline, is probably caused by greater losses of aniline during hydrolysis at relatively low concentrations. In order to obtain reproducible results, it is advisable to work at a level of at least 10 ng of metoxuron.

*Pre-column Dns derivatization and HPLC separation of anilines*

The initial selection of a reversed-phase HPLC solvent system was based on earlier work[3]. These experiments showed that after Dns derivatization of CMA on a silica gel column a broad "solvent peak" appeared in the reversed-phase system when methanol was used as a mobile phase component; however, Dns-CMA gave a well separated peak with good reproducibility. With our experimental conditions the "solvent peak" and Dns-CMA did not separate. Similar phenomena were observed in our work with some other anilines; probably, the presence of an interfering peak is due to a side-reaction of Dns chloride with methanol or an impurity in methanol[13]. In order to circumvent this problem, methanol was replaced with acetonitrile as a polarity modifier in the mobile phase. However, under these conditions the reproducibility of the peak height became poor.

For this reason, experiments were carried out with different Dns derivatization techniques using hexane, acetone, acetonitrile, aqueous acetone and aqueous acetonitrile as reaction media, and the effects of temperature, reaction time and the presence of sodium carbonate, with or without an evaporation step, were investigated. This study led to the conclusion that the derivatization can be carried out in a reproducible way using the method described under Experimental. Linear calibration graphs in the range l-100 ng of CMA were obtained under these circumstances.

Both evaporation to dryness under a stream of nitrogen at 70–80°C and dissolution of the residue in the acetonitrile water (3: 1) mobile phase are essential steps in this method. If the evaporation step is omitted, reaction times of up to 48 h are needed in order to obtain stable and reproducible peaks. It is also important to run

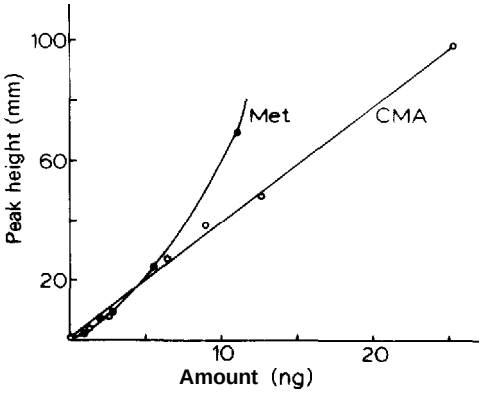


Fig. 1. Calibration graphs for Dns-CMA and Dns-CMA obtained after hydrolysis of metoxuron (Met), Dns derivatization and hydrolysis were carried out on the TLC plate. For TLC system and fluorescence detection conditions, see text.

a reagent blank before the actual sample. The optimal amount of reagent solution required for the derivatization of CMA is not very critical and is in the range 100–400 $\mu$l of 0.25% Dns chloride. Similar amounts were used successfully for the other anilines investigated. The use of 50 $\mu$l of reagent resulted in distinctly lower peaks.

Reproducible results were also obtained for the other anilines but their linearity range was not investigated. The relative fluorescence signals for the various anilines are indicated in Fig. 2. If we can assume that complete reaction to the Dns derivative has taken place, it is the type, number and position of substituents on the aniline moiety that govern the results. Not surprisingly, then, relatively high yields are obtained for the methoxy-substituted species methoxyphenoxyaniline and chloromethoxyaniline (CMA), whereas halogen-substituted anilines are more strongly quenched owing to heavy-atom effects. As expected, this quenching is stronger for a *meta* than a *para* substituent and more pronounced for a bromo than a chloro substituent. Similar results have been reported earlier[13].

Retention times in HPLC for most of the Dns-anilines are presented in Table I.

### *Recovery studies and analysis of real samples*

Urea-type pesticides degrade to aniline metabolites, which have toxicities similar to or greater than those of the parent compounds. It is therefore advisable to determine both types of compounds, especially with environmental samples or during a degradation study. For this reason. it is preferable if at least partly the same procedure can be used for their determination.

***Extraction.*** Previous results showed' that different classes of pesticides, in-

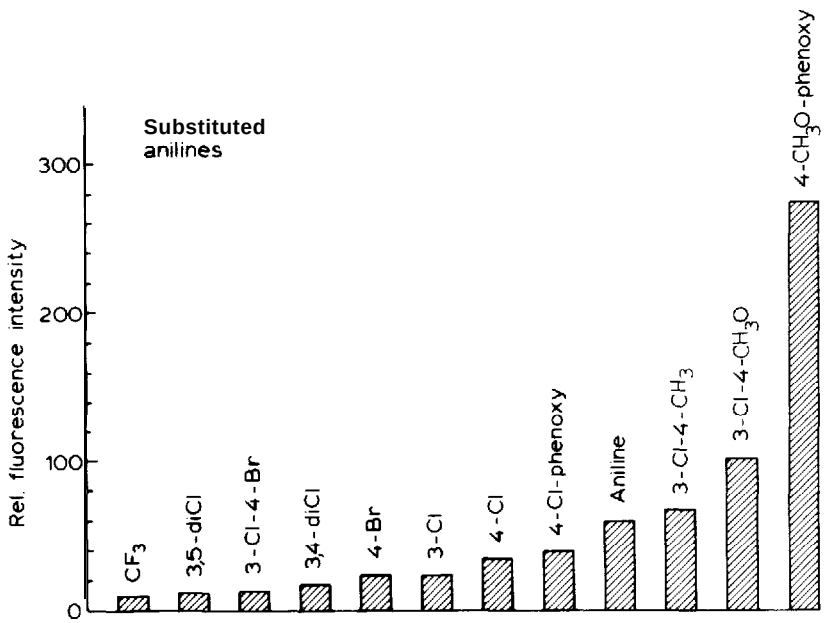


Fig. 2. Relative fluorescence intensity of twelve Dns-anilines. For derivatization procedure and HPLC analysis, see text.

cluding ureas, can be extracted from plant or soil samples using a procedure that involves maceration and homogenization of the sample in acetone followed by filtration, dilution with an aqueous salt solution and extraction into dichloromethane. In this study it was of interest to know whether the same procedure could be used for aniline metabolites. As the anilines are basic, it seemed reasonable that the pH of the solution should be adjusted to neutral or basic conditions in order to extract them.

Preliminary results with the model solutes metoxuron and CMA showed that after adjusting the pH of aqueous acetone to 8 complete recovery can be achieved, whereas a recovery of only a few percent was observed at pH 3. The direct evaluation of an aqueous blank by such an extraction procedure did not result in interfering fluorescence in either normal-phase TLC or reversed-phase HPLC systems.

The acetone extraction of soil (with a high organic content) did not give acceptable recoveries. Three consecutive extractions of air-dried soil samples with dichloromethane yielded about a 50% recovery and reproducible results for CMA determination. The acetone extraction of potato samples did not present any problems.

*Clean-up and sample analysis.* Previous results suggested that residue analysis based on Dns derivatization and fluorimetric detection does not require complicated clean-up procedures because of the inherent selectivity of the derivatization system. In our studies, a clean-up step over silica was found to be necessary with the soil and potato samples, whereas no clean-up was required with the water samples.

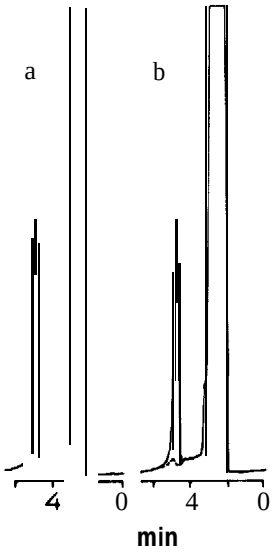


Fig. 3. HPLC of (a) 12.5 ng of CMA. (b. broken line) a 1250-mg water blank and (b, continuous line) 1250 mg of water spiked with 0.01 ppm of CMA. For experimental conditions, see text.

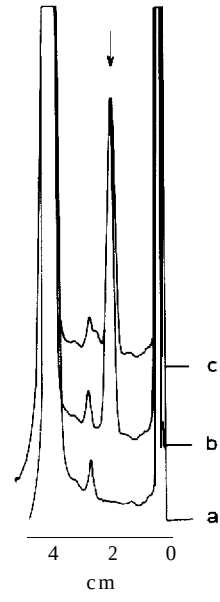


Fig. 4. TLC of (a) a 5000-mg water blank, (b) a SO-ng metoxuron standard and (c) 5000 mg of water spiked with 0.01 ppm of metoxuron. For densitogram conditions. see text.

TABLE II

SUMMARY OF EXPERIMENTAL RESULTS

| *Compound* | *Sample* | *Spiking level (mg/kg)* | *HPLC* | | *TLC* | |
|---|---|---|---|---|---|---|
| | | | *Recovery (%)* | *Detection limit (mg/kg)** | *Recovery (%)* | *Detection limit (mg/kg)** |
| CMA | Water | 0.01 | 92 ± 2 | 0.001 | 73 ± 12 | 0.001 |
| | Potato | 0.10 | 87 ± 5 | 0.02 | 93 ± 20 | 0.02 |
| | Soil | 1.00 | 54 ± 2 | 0.20 | 46 ± 10 | 0.20 |
| Metoxuron | Water | 0.01 | | | 101 ± 10 | 0.001 |

* Detection limit at a signal-to-noise ratio of 2: 1.

For residue analysis of tap and surface waters, Fig. 3 shows HPLC traces for a CMA standard and a typical water sample without and with added aniline. Results for the determination of metoxuron in aqueous samples by means of TLC are presented in Fig. 4. Table II summarizes spiking levels, mean recoveries (three spiked


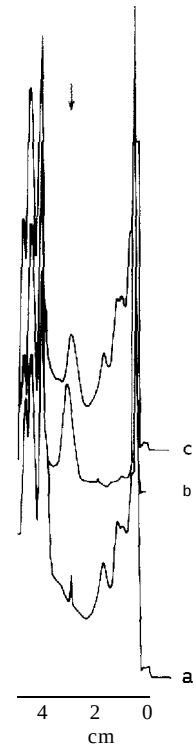


Fig. 5. TLC of (a) a 50-mg potato blank, (b) a 5-ng CMA standard and (c) 50 mg of potato spiked with 0.1 ppm of CMA. For densitogram conditions, see text.

Fig. 6. TLC of (a) a 12.5-mg soil blank, (b) a 10-ng CMA standard and (c) 12.5 mg of soil spiked with 1 ppm of CMA. For densitogram conditions. see text.

samples each) and detection limits (calculated on the basis of peak heights).

Soil and potato samples require clean-up before chromatographic analysis, partly because of interferences due to the presence of fluorescent compounds and also because of difficulties encountered during spotting on the silica TLC plate. Clean-up on a silica column was found to be suitable. Elution with dichloromethane removes most of the fluorescent interferences and also some plant pigments, and the herbicide and/or its corresponding aniline can then be eluted with ethyl acetate. This simple procedure is appropriate for both HPLC and TLC with CMA-spiked potatoes, and for TLC with CMA-containing soil samples. In the latter instance, HPLC requires an additional step, *viz.*, changing the solvent from ethyl acetate to acetonitrile–water. This causes some colouring materials that dissolve freely in ethyl acetate but not in the partly aqueous mixture to precipitate on the wall of the centrifuge tube.

Fig. 5 shows TLC data for a CMA standard and a blank and a spiked potato sample. TLC densitograms and HPLC traces relating to the determination of CMA in soil are given in Figs. 6 and 7, respectively. Data on spiking levels, mean recoveries and detection limits are given in Table II. It is interesting that here, as with the water samples (see above), the precision obtained in HPLC is distinctly better than that in TLC. Occasionally, the baseline in TLC is much poorer than that in HPLC (compare Figs. 6 and 7).

Finally, two remarks should be made. (1) In order to obtain reproducible results, one should limit the sample size to 1500–6000, 50 150 and 10–25 ng for water, potato and soil, respectively, in both TLC and HPLC. (2) The determination of metoxuron in potatoes by TLC was also shown to be possible. With soil samples, however, poor results were obtained owing to high backgrounds; further work on the clean-up procedure is indicated here.

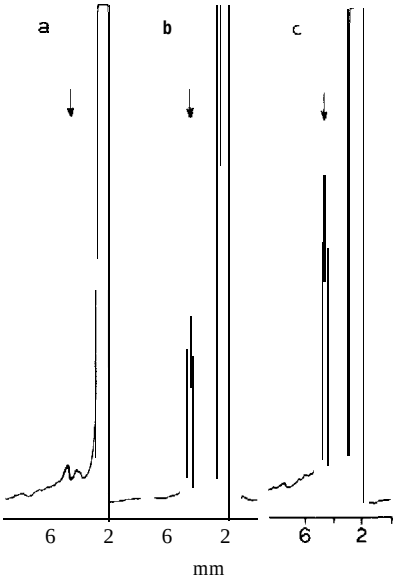


Fig. 7. HPLC of (a) a 25-mg soil blank. (b) a IO-ng CMA standard and (c) 25 mg of soil spiked with 1 ppm of CMA. For experimental conditions, see text.

## CONCLUSION

A simple procedure can be used to derivatize anilines in batch prior to chromatographic analysis. The potential of such a technique has been demonstrated with CMA in soil, potato and water samples. using HPLC or TLC for the final analysis. The latter technique has the advantage that Dns derivatization of the anilines can be carried out directly on the plate by overspotting the aniline sample spot with Dns chloride solution and reaction on the plate followed by separation of the derivatives. In HPLC such an "on-column" technique is difficult to perform,

In the analysis of a phenylurea herbicide, TLC on plain silica plates offers the advantage of *in situ* catalytic hydrolysis of the compound to its aniline followed by *in situ* Dns derivatization. This possibility has been demonstrated with the determination of metoxuron residues in water samples and is potentially also feasible with other matrices. For HPLC, on-column hydrolysis of urea herbicides is possible, but only under conditions that are detrimental to further processing of the eluate with Dns chloride, and further study will be requited in order to solve this problem.

In this study, most of the work was carried out with CMA and metoxuron as model compounds. It has also been shown. however, that other anilines can be handled similarly. The fluorescence yields of the Dns-anilines are dependent on the type, number and positions of the substituents on the phenyl ring, but good sensitivity can be expected for all of them.

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