

Note

Determination of aniline derivatives by high-performance liquid chromatography with fluorescence detection*

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Aromatic amines** are introduced into the environment either directly in industrial effluents or indirectly as breakdown products of herbicides, dyestuffs and pharmaceuticals. In our institute the aromatic amines are determined by capillary gas chromatography after derivatization with heptafluorobutyric anhydride (HFBA)¹. However, there is increasing interest in the direct determination of aromatic amines by high-performance liquid chromatography (HPLC)^{2–5}. Trace analysis, however, needs sensitive and selective detection and consequently a derivatization procedure is required and/or pre-concentration techniques must be applied.

Derivatization of aromatic amines has been reported as both pre-column⁶ and post-column⁷ techniques in which dansyl chloride⁶ and fluorescamine⁷ served as the fluorescent label. Sample pre-treatment based on liquid–solid sorption techniques has been shown⁸ to be very useful for the pre-concentration of environmental samples in liquid chromatography. Both off-line and on-line methods are widely used. One should realize, however, that related organic compounds are often simultaneously sorbed on and eluted from the pre-column. Goewie *et al.*⁹ demonstrated that with a combination of a metal-loaded pre-column and a C₁₈ pre-column prior to reversed-phase LC with UV detection, the simultaneous detection of fourteen phenylurea herbicides in water samples at the micrograms per litre concentration level is possible without interference from anilines. Nielen and co-workers^{10,11} described a micro-processor-controlled valve-switching unit for on-line group separation and trace enrichment on small pre-columns packed with C₁₈, PRP-1 and cation-exchange materials. The polar anilines were concentrated on the strongly acidic cation exchanger with sub-micrograms per litre detection limits for real samples when LC with electrochemical detection was used. Although both selective concentration without losses of aromatic amines and separation of complex mixtures have been demonstrated, until now no LC method has met the requirements of the Dutch Water Action Programme 1985–1989¹² for the chlorinated aromatic amines, *i.e.*, 1 $\mu\text{g l}^{-1}$ for the median concentration of all aromatic amines and 0.5 $\mu\text{g l}^{-1}$ for the median concentra-

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** Throughout this paper, “aromatic amines” refers to aniline and some of its chloro and methyl derivatives (see Table II for a list of the compounds studied).

tion of individual aromatic amines. In this work, the aim was to study the potential of dansylation for all aromatic amines mentioned in the Dutch Water Action Programme. Special emphasis was placed on the reaction conditions of the dansylation procedure. From the literature, a gain in sensitivity was expected with respect to the detectability of the derivatives by derivatization rather than by enhancing the extraction efficiency on solid surfaces.

EXPERIMENTAL

Reagents

HPLC-grade hexane, tetrahydrofuran (THF), isopropyl alcohol, acetone, methanol and water and also analytical-reagent grade EDTA, potassium monohydrogenphosphate, perchloric acid, sodium hydroxide and sodium carbonate were obtained from J. T. Baker (Deventer, The Netherlands). All aromatic amines except 2,5-dichloroaniline (Chem Service, West Chester, PA, U.S.A.), 5-dimethylamino-1-naphthalenesulphonyl chloride (Dns chloride) and heptafluorobutyric anhydride were obtained from Fluka (Buchs, Switzerland).

Stationary phases and columns

Spiked surface water samples were pre-concentrated on respectively (a) a 25 × 2.3 mm I.D. PRP-1 (16–20 μm) spherical chromatographic resin guard column, manufactured by Hamilton (Reno, NV, U.S.A.), (b) a 10 × 3 mm I.D. silica-based cation exchanger (40 μm) manufactured by Chrompack (Middelburg, The Netherlands) or (c) a 10 × 3 mm I.D. (empty Chrompack pre-column) spherical resin-based Aminex A-7 (7–11 μm) (Bio-Rad Labs., Richmond, CA, U.S.A.) sulphonic acid cation exchanger. The last column was packed manually with a microspatula.

Before sampling, the pre-columns were activated by flushing first with 30 ml (2 ml min⁻¹) of water and then with 30 ml (2 ml min⁻¹) of 0.05 mol l⁻¹ sodium hydroxide (PRP-1 column) or 0.02 mol l⁻¹ perchloric acid (cation-exchanger column). Conditioning was performed by flushing the pre-column with 30 ml (2 ml min⁻¹) of 0.001 mol l⁻¹ sodium hydroxide (PRP-1 column) or 0.001 mol l⁻¹ perchloric acid (cation-exchanger column). A new pre-column was used in each experiment in this study.

Separations were carried out at ambient temperature on a 200 × 3 mm I.D. glass column, pre-packed with LiChrosorb Si (7 μm) (Chrompack) using hexane-THF (90:10) at a flow-rate of 0.8 ml min⁻¹ as the mobile phase. Every month the guard column was replaced. The injection volume was varied between 10 and 50 μl.

Apparatus

The HPLC equipment included an LKB (Bromma, Sweden) Model 2150 pump, a Spark (Emmen, The Netherlands) Promis autoinjector, a Merck Hitachi (Darmstadt, F.R.G.) Model F 1000 fluorescence detector and a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3392 integrator. The excitation and emission wavelengths for the fluorescence detection were 350 and 500 nm, respectively.

Gas chromatographic analyses to determine the fractions collected in the breakthrough experiments and the apparatus have been described elsewhere¹.

Procedures

Stock solutions of the aromatic amines were prepared by weighing followed by dissolution in dichloromethane. These solutions were diluted with dichloromethane to obtain standard solutions and mixed standard solutions. Saturated solutions of Dns chloride (*ca.* 2 g l^{-1}) in HPLC-grade acetone were ultrasonically prepared, centrifuged and kept in brown vials in a refrigerator.

Dansylation method A involves the following steps: (1) mixing 1 ml of standard solution with 1 ml of Dns chloride solution in a brown vial with a PTFE-lined screw-cap, adding *ca.* 20 mg of sodium carbonate and setting aside for 30 min at 45°C ; (2) cooling to room temperature, evaporating under a gentle stream of nitrogen to *ca.* 0.1 ml and setting aside for 30 min at 45°C ; and (3) evaporating to dryness under a gentle stream of nitrogen and dissolving in hexane. Dansylation method B is the same as method A except for reaction (2), which proceeds for 16 h (overnight) at 45°C .

Simultaneous enrichment of the aromatic amines was carried out on PRP-1, Chrompack cation-exchange and Aminex A-7 pre-columns.

Surface waters were spiked with all the aromatic amines investigated (see Table I), each at a concentration of $50 \mu\text{g l}^{-1}$, placing 1 ml of mixed standard solution in an empty flask, carefully evaporating the solvent and adding 1 l of surface water. This solution was adjusted to the desired pH with perchloric acid or sodium hydroxide. The flow-rate during the experiments was 2 ml min^{-1} and the pre-column eluate fractions collected were 20, 20, 20, 20, 20, 50 and 100 ml.

To determine the breakthrough volumes, these fractions were analysed according to the procedure outlined elsewhere¹, which involves extraction with dichloromethane at pH 9, derivatization with HFBA and gas chromatographic analysis.

To minimize interferences from heavy metal ions, EDTA complexation was applied by adding 2.5 ml EDTA solution (20 g l^{-1}) to 250 ml of surface water at pH 7. This solution was filtered and adjusted to pH 3.0 with perchloric acid.

RESULTS AND DISCUSSION

Pre-concentration

Liquid-solid extraction procedures for polar compounds such as aromatic amines are more complex than pre-concentration procedures for low-polarity compounds. Low breakthrough volumes or even no enrichment is the result. To overcome this situation, several workers^{9,13,14} have investigated different types of adsorbents possessing a higher affinity for the compounds to be analysed. These adsorbents included metal-loaded surfaces, resins (methacrylic ester polymers) and graphitized carbon black.

The experiments in this study were started with a resin-based sorbent, PRP-1. It was expected that with this material aromatic amines would be concentrated easily from an alkaline sample, resulting in amines that are uncharged and so their adsorption on the hydrophobic adsorbent might be maximal. Moreover, a high pH favours selective concentration as acidic compounds are not retained, metals are precipitated as hydroxides and amines are released (the latter, however, are easily bound to humic materials).

TABLE I

SOME DISSOCIATION CONSTANTS AND APPROXIMATE BREAKTHROUGH VOLUMES OF AROMATIC AMINES IN SURFACE WATER SPIKED AT THE $50 \mu\text{g l}^{-1}$ LEVEL

Breakthrough volumes are within the volume ranges indicated. Minimum volume tested, 20 ml; maximum volume tested, 250 ml.

Compound	Breakthrough volume (ml)						
	Resin-based PRP-1 (16 μm) column (25 \times 2.3 mm I.D.)			Resin-based cation-exchange Aminex A-7 (9 μm) column (10 \times 3 mm I.D.)			
	pH 9.2	pH 11.5	pH 12.5	pH 3.0	pH 2.5	pH 3.0*	pK_a
Aniline	0-20	0-20	0-20	40-60	60-80	80-100	4.63
2-Methylaniline	0-20	0-20	0-20	40-60	60-80	80-100	4.44
3-Methylaniline	0-20	0-20	0-20	40-60	60-80	80-100	4.73
4-Methylaniline	0-20	0-20	0-20	60-80	80-100	150-250	5.08
2-Chloroaniline	0-20	0-20	20-40	100-150	100-150	150-250	2.65
3-Chloroaniline	0-20	0-20	20-40	0-20	0-20	0-20	3.46
4-Chloroaniline	0-20	0-20	20-40	0-20	0-20	40-60	4.15
2,3-Dichloroaniline	0-20	60-80	100-150	40-60	80-100	100-150	
2,4-Dichloroaniline	0-20	60-80	100-150	40-60	80-100	100-150	2.05
2,5-Dichloroaniline	0-20	80-100	100-150	0-20	80-100	100-150	
2,6-Dichloroaniline	20-40	150-250	250	0-20	150-250	150-250	
3,4-Dichloroaniline	0-20	60-80	100-150	40-60	40-60	100-150	3.2
3,5-Dichloroaniline	0-20	80-100	100-150	0-20	60-80	150-250	2.5
2,4,5-Trichloroaniline	20-40	250	250	0-20	60-80	150-250	

* After EDTA complexation (see Experimental).

From the data in Table I it can be seen that at pH 9.2, where the anilines would be expected to have been converted into their conjugated bases, no adsorption takes place except for 2,6-dichloro- and 2,4,5-trichloroaniline. At pH 11.5, all dichloro- and trichloroanilines investigated are retained and at least 60-80 ml could be concentrated, but again aniline and its monomethyl and monochloro derivatives do not show any retention. At the high pH of 12.5 the situation improves further, including the monochloroanilines, but from the results in Table I it is clear that the resin-based material is not capable of concentrating all aromatic amines. Considering the retention mechanism with the resin-based material, *i.e.*, high retention of uncharged conjugated bases (or acids), this was not expected. The behaviour, however, might be explainable, as aniline itself and its methyl derivatives are the strongest bases of the aromatic amines investigated and as such most prone to aqueous elution by complexation with (heavy) metal ions as Lewis acids. Moreover, the relatively high concentration of individual anilines ($50 \mu\text{g l}^{-1}$) and the composition of the surface water (complexation by Lewis acids such as metal ions of the conjugate aniline bases and competitive adsorption of organic components in the surface water sample) may explain further the decreased extraction efficiency of PRP-1 in our experiments.

The second experiment was carried out using a silica-based cation exchanger. After careful wetting and conditioning of the material, the results at pH 3.0, 2.5 and 2.0 were disappointing. No concentration at all was observed owing to the low capacity of the cation exchanger and therefore the results are not included in Table I.

With the resin-based cation exchanger (Aminex A-7), for most of the aromatic amines concentration is effected at pH 3.0. From Table I it can be seen that at pH 3.0 only 3- and 4-chloroaniline are not retained at all. On decreasing the pH to 2.5 the situation did not deteriorate and for most compounds even an increase in breakthrough volume was recorded. Following Nielsen *et al.*¹¹, we introduced an EDTA complexation as outlined under Experimental. With respect to the results at pH 3.0 and 2.5, this step effects an increase in breakthrough volume in all instances but one (3-chloroaniline). From Table I it can be seen that under these circumstances at least 50 ml (except for 3-chloroaniline) can be concentrated from a real surface water sample with a relatively high spike concentration. Undoubtedly the breakthrough volume depends on the composition of the sample solution and may be the concentration and sorption properties of the organic constituents, or complexing metal ions may have a major influence on the effective capacity of cation-exchange columns. This capacity might be increased by insertion of a C₁₈ or PRP-1 filter as outlined by Nielsen and co-workers^{10,11}.

Considering procedures for the off-line pre-concentration of surface water, which involves the extraction of 50–250 ml of sample, one can conclude that simultaneous trace enrichment of the aromatic amines studied here on solid surfaces is possible, except for 3-chloroaniline.

Pre-column dansylation

It is well known that the fluorescence intensity of dansyl derivatives increases as the dielectric constant of the solvents decreases^{15,16}. It may also be expected that hydrolysis of the reagent [to form the unreactive but highly fluorescent sulphonic acid (Dns-OH), being a major reason why only pre-column dansylation has been adopted] will be low in a non-aqueous medium. From this point of view, in preliminary experiments we started with a simple derivatization procedure, *i.e.*, 1 ml of aniline stock solution in dichloromethane and 1 ml of Dns chloride solution in acetone, 30-min reaction at 45°C, evaporation to dryness and dissolution in the mobile phase. Applying this procedure, aniline and 2-, 3- and 4-chloro-, 3,4- and 3,5-dichloro- and 2-, 3- and 4-methylaniline showed fluorescence yields; however, UV detection at 245 nm still showed unreacted anilines, indicating incomplete conversion.

To optimize the reaction conditions a study was carried out with 2-chloro- and 4-chloroaniline as test compounds. From this, it appeared that the fluorescence yield increased 5-fold when *ca.* 20 mg of solid sodium carbonate was present in the reaction medium to scavenge the hydrogen chloride evolved. Increasing the reaction time at 45°C to 2 h did not have much effect. Increasing the temperature to 60–70°C led to an increase in fluorescence yield but unfortunately the reproducibility at this high temperature was poor, in contrast to the results at 45°C. During the experiments, one of the vials was not closed well, which resulted in a reduction of the reaction volume to *ca.* 0.1 ml by evaporation of solvent.

After the derivatization reaction at the resulting high reagent concentration, the fluorescence yield was very high. This appeared like evaporative derivatization

TABLE II
DETECTION LIMITS FOR DANSYLATION METHODS A AND B

Compound	Detection limit (pg)	
	Method A	Method B
Aniline	40	20
2-Methylaniline	40	20
3-Methylaniline	40	20
4-Methylaniline	40	20
2-Chloroaniline	2000	200
3-Chloroaniline	200	50
4-Chloroaniline	50	20
2,3-Dichloroaniline	35 000	1000
2,4-Dichloroaniline	7500	300
2,5-Dichloroaniline	35 000	1000
2,6-Dichloroaniline	*	*
3,4-Dichloroaniline	500	100
3,5-Dichloroaniline	500	100
2,4,5-Trichloroaniline	*	*

* 300 ng not detected.

as employed by Weinberger *et al.*¹⁷, who evaporated their sample during the dansylation procedure at 60°C under reduced pressure (50–60 cmHg).

When dealing with volatile compounds in the mixture, one should take special care. Fortunately, the more volatile anilines react rapidly with Dns chloride (see above) this led us to develop dansylation method A. Method A essentially consists in a "pre-derivatization" of the more volatile (easily derivatizable) components followed by a subsequent reaction at very high reagent concentrations [to achieve complete conversion and dansylation of the more difficult (sterically hindered) components].

From Table II, it can be concluded that with method A detection limits in the low picogram range are easily obtained for aniline itself, all monomethyl-substituted anilines and 4-chloroaniline. Apparently both the position and number of chlorine substituents in the aromatic ring determine whether the dansylation reaction is complete or not. This agrees well with the results of Lantos *et al.*⁶, who reported fluorescence yields for various Dns-anilines. From Table II it can also be concluded that the fluorescence yields are influenced by quenching due to heavy (chlorine) atom effects. These effects are most predominant with an *ortho* substituent and stronger for a *meta* than a *para* substituent. The variation in the results due to these effects is very high.

Other effects, *e.g.*, kinetic and/or thermodynamic effects, in the dansylation reaction may also play a role. Increasing the reaction time, after reduction of the reagent volume, to 16 h (overnight) gave a very large increase in fluorescence yields, indicating that kinetic effects in the dansylation reaction are important. This increase compensated for the chlorine quenching effects and resulted in less variation of the detection limits.

From Table II (method B), it can be seen that the influence of the position and

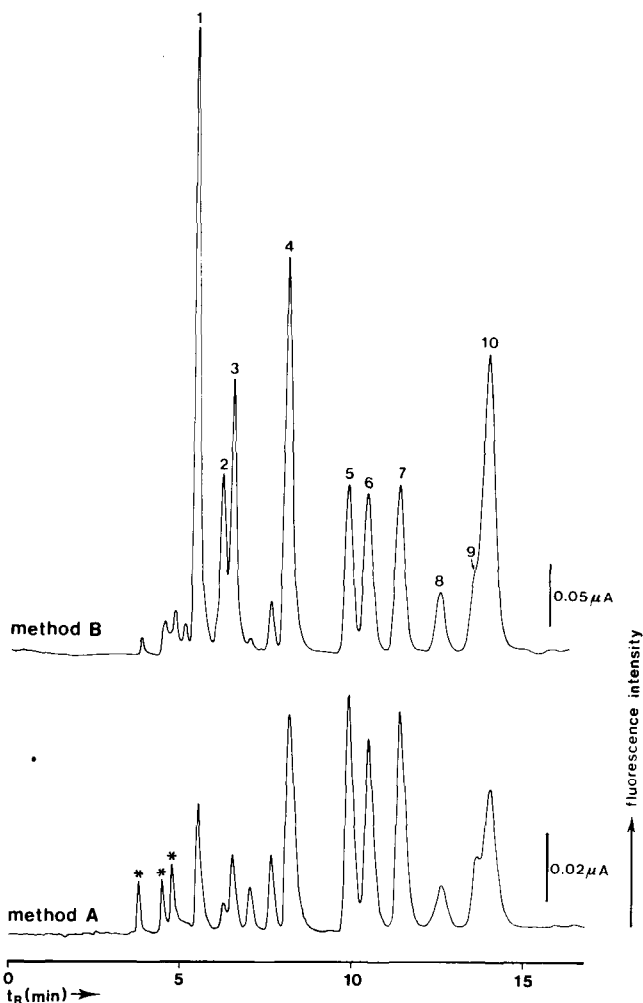


Fig. 1. Normal-phase HPLC of ten aniline dansyl derivatives using methods A and B. System: LiChrosorb Si with hexane-THF (90:10) as eluent; fluorescence detection, $\lambda_{ex} = 350$ nm, $\lambda_{em} = 500$ nm. Peaks: 1 = 2,4-dichloro-; 2 = 2,5-dichloro-; 3 = 2-chloro-; 4 = 2-methyl-; 5 = 3-methyl-; 6 = 4-methyl-; 7 = aniline; 8 = 3-chloro-; 9 = 4-chloro-; 10 = 3,4-dichloro-. Reagent peaks are marked with asterisks.

number of chlorine substituents was smaller than with method A except for 2,6-dichloro- and 2,4,5-trichloroaniline, for which even an injection of 300 ng, based on the starting compound, did not result in a peak in the chromatogram.

The optimal amount of reagent solution required for the derivatization is not very critical, and with 1 ml of Dns chloride solution (2 mg ml^{-1}) linear calibration graphs over at least two orders of magnitude of concentration were obtained in the nanogram range. The dansyl derivatives are stable for at least a few weeks at 4°C . Reagent blank peaks did not interfere with the Dns-aniline peaks and were always less intense (see Fig. 1).

From these results, it can be calculated that the detection limits for the anilines investigated are 1–50 ng l⁻¹ in surface water samples. For the most sensitive Dns-anilines these detection limits are comparable to those obtained using gas chromatography¹ and well below the requirements of the Dutch Water Action Programme.

HPLC separation

The experiments described above were performed using HPLC with a LiChrosorb Si 60 cartridge column and elution with hexane–THF (90:10). Initial studies showed that a mobile phase consisting of hexane containing 0.5–1% of isopropyl alcohol resulted in poor selectivity. Hexane–dichloromethane mixtures containing 0.1–0.4% of isopropyl alcohol gave good separations but the reproducibility of the retention times was poor. Hexane–THF (90:10), even without further optimization, gave the best selectivity. An HPLC trace obtained with this eluent is shown in Fig. 1.

Dissolving the Dns-anilines in hexane, instead of the mobile phase, gave some improvement in the separation of the dansyl derivatives of 4-chloro- and 3,4-dichloroaniline but one of the derivatives should not be present in a dominant concentration.

The use of a Spherisorb Si column with the mobile phases mentioned above gave poorer selectivity in all instances.

CONCLUSIONS

Most of the polar anilines mentioned in the Dutch Water Action Programme can be determined simultaneously down to the required concentration level with modern liquid chromatographic techniques. Liquid–solid sorption techniques on various materials (PRP-1 and a resin-based cation exchanger) showed sufficient retention for most of these compounds from surface water. The best results were obtained with the resin-based cation exchanger Aminex A-7. Enrichment of at least 50 ml (except for 3-chloroaniline) was effected at pH 3.0 after EDTA complexation. In general, however, enrichment is difficult to predict when dealing with surface waters with fluctuating chemical composition. A simple dansylation procedure has been developed to derivatize almost all of the anilines investigated (except 2,6-dichloro- and 2,4,5-trichloroaniline). In combination with solid-phase extraction, most of the anilines mentioned in the Dutch Water Action Programme can be well separated and detected down to the required levels.

REFERENCES

- 1 R. B. Geerdink, *Technical Report*, Institute for Inland Water Management and Wastewater Treatment, Ministry of Transport and Public Works, Lelystad, No. 87.041x, 1987.
- 2 E. M. Lores, D. W. Bristol and R. F. Moseman, *J. Chromatogr. Sci.*, 16 (1978) 358.
- 3 E. M. Lores, F. C. Meekins and R. F. Moseman, *J. Chromatogr.*, 188 (1980) 412.
- 4 F. A. Maris, A. van der Vliet, R. B. Geerdink and U. A. Th. Brinkman, *J. Chromatogr.*, 347 (1985) 75.
- 5 A. de Kok, R. B. Geerdink and U. A. Th. Brinkman, *Chromatographia*, 16 (1982) 237.
- 6 J. Lantos, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.*, 292 (1984) 117.
- 7 A. H. M. T. Scholten, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.*, 218 (1981) 3.
- 8 R. W. Frei and U. A. Th. Brinkman, *Anal. Chem.*, 1 (1981) 45.

- 9 C. E. Goewie, P. Kwakman, R. W. Frei, U. A. Th. Brinkman, W. Maasfeld, T. Seshadri and A. Kettrup, *J. Chromatogr.*, 284 (1984) 73.
- 10 M. W. F. Nielen, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 317 (1984) 557.
- 11 M. W. F. Nielen, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, 57 (1985) 806.
- 12 *Dutch Water Action Programme 1985-1989*, Staatsdrukkerij, 's-Gravenhage, 1986.
- 13 H. A. Stuber and J. A. Leenheer, *Anal. Chem.*, 55 (1983) 111.
- 14 C. Borra, A. DiCorcia, M. Marchetti and R. Samperi, *Anal. Chem.*, 58 (1986) 2048.
- 15 S. Uchiyama and M. Uchiyama, *J. Chromatogr.*, 153 (1978) 135.
- 16 H. C. Thompson, Jr., C. L. Holder and J. R. Althaus, *Talanta*, 30 (1983) 251.
- 17 R. Weinberger, T. Koziol and G. Millington, *Chromatographia*, 19 (1984) 452.