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FLUORESCENCE DETECTION OF CHLOROANILINES IN LIQUID CHROMATOGRAPHY USING A POST-COLUMN REACTION WITH FLUORESCAMINE

COMPARISON OF REACTOR TYPES AND MIXING TEES

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SUMMARY

The fluorescamine (Fluram) reaction for primary amines has been used for the post-column derivatization of three chloroanilines at relatively low pH values. Separation was carried out on a CN-modified bonded phase with acetonitrile–water (pH 3) as the mobile phase. Various types of reactors (tubular, packed-bed and air-segmented) and mixing tees have been studied and compared for their suitability in post-column reactor systems.

Under optimal conditions, detection limits for the derivatized chloroanilines are in the 100-pg range, which is about 10-fold better than with UV detection. The linearity of the calibration graphs is excellent (r = 0.9999–1.0000) over a concentration range of 2–3 orders of magnitude.

INTRODUCTION

Halogenated anilines, especially chloroanilines, can be released into the environment either directly as industrial effluents or indirectly as breakdown products of phenylcarbamates and phenylurea herbicides, and materials such as paints. Over the years, many papers on the determination of halogenated anilines have been published (see, *e.g.*, refs. 1–4 and the literature cited therein). Here, we call attention to a paper by Sherma and Marzoni[5], who separated these anilines by thin-layer chromatography and quantitated them by fluorescence detection after spraying with fluorescamine {4-phenylspiro[furan-2(3*H*),1′(3′*H*)-isobenzofuran]-3,3′-dione}. Fluorescamine (Fluram) is a well known reagent for the detection of primary and secondary amino groups[6,7] in a variety of compounds such as amino acids[8,9], polyamines[10], peptides[11,12], proteins[13], cephalosporin[14], phenelzine[15], ampicillin[16] and fluvoxamine and clovoxamine[17]. The non-fluorescent reagent reacts with the primary amines to form pyrrolinones which, upon excitation at 390 nm, emit strong fluorescence at 475–500 nm; in the quoted work both pre- and post-column derivatization were used. Several thorough investigations on the selection of suitable conditions for

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the reaction between amines and Fluram have been published[6,13,18–21]. It has been shown that, in general, reaction must take place at pH 7–9 and that in organic solvents the reaction proceeds much more slowly than it does in an aqueous medium. Under non-aqueous conditions, 50% conversion into the Fluram derivative typically takes from several minutes[21] to several hours[20], compared with seconds in an aqueous solution.

Hydrolysis of Fluram, which takes place parallel to the derivatization reaction, can be a serious interference, particularly for kinetically slow reactions. According to Stein *et al.*[20], who studied the use of various aqueous–organic solvent mixtures, the rate of hydrolysis of Fluram increases with increasing pH, and depends strongly on the nature of the organic co-solvent and the buffer selected. The authors also state that the product of hydrolysis is non-fluorescent. According to our observations, however, and also those of Castell *et al.*[13], a fluorescence signal appears upon hydrolysis. The latter workers suggested two possible hydrolysis reactions, *viz.*, a reversible one, in which merely the lactone ring of Fluram is opened, and one which results in the formation of a yellow (fluorescent) end-product.

In this paper, the separation of three of the more important chloroanilines (3- and 4-chloroaniline and 3,4-dichloroaniline) by high-performance liquid chromatography (HPLC) and their subsequent determination by post-column derivatization with Fluram at relatively low pH values is described. The system has been used to compare the potential of a tubular reactor with that of a packed-bed reactor and a reactor based on the air-segmentation principle. Also, the dependence of band broadening and, thus, system performance, on the construction of mixing tees has been evaluated.

EXPERIMENTAL

Chromatography

The experimental arrangement is shown in Fig. 1. The system consisted of an Altex (Berkeley, CA, U.S.A.) Series 100 pump, a Valco (Houston, TX, U.S.A.) six-port injection valve with a 20-µl sample loop, a 10 cm × 4.6 mm I.D. stainless-steel column packed with Polygosil 60-10 CN (Macherey, Nagel & Co., Düren, G.F.R.), a Perkin-Elmer (Norwalk, CT, U.S.A.) LC 55 variable-wavelength UV detector set at 243 nm, a post-column reaction system (see below) and a Perkin-Elmer Model 204A or 3000 fluorescence spectrophotometer with 10-nm slits operated at $\lambda_{ex.}$ = 398 nm and $\lambda_{em.}$ = 498 nm. The signals were recorded with Kipp (Delft, The Netherlands) BD-8 recorders.

Post-column reaction system

For reagent addition, a home-made syringe pump with a capacity of 300 ml was used. With this constant-pressure pump a regular and pulse-free reagent stream could be added to the mobile phase, with a pressure limit of about 200 bar. In the air-segmentation experiments, a Technicon (Tarrytown, NY, U.S.A.) AutoAnalyzer pump was installed in the system. Routinely, the reagent stream and mobile phase were mixed in a stainless-steel tee-piece, in which the capillaries (0.25 mm I.D. and 1/16 in. O.D.) made a 120° angle. In order to prevent the formation of tiny air bubbles in this tee-piece, which often occurred, a piece of stainless-steel capillary or Tygon

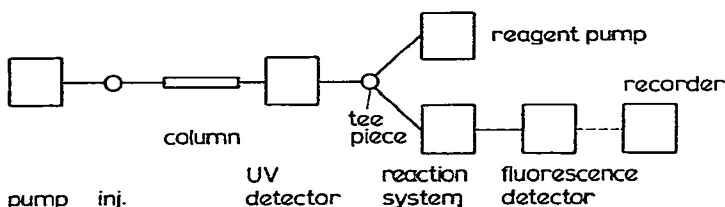


Fig. 1. Instrumental arrangement of the chromatographic system.

tubing, which will give a back-pressure of a few bars, was installed at the outlet of the detector flow-cell. In such cases, the Perkin-Elmer Model 3000 fluorospectrometer had to be used for analysis, as its flow-cell can withstand back-pressures of up to 35 bar.

Three types of reactor were used: (1) the packed-bed reactor consisted of a 10 cm × 4.6 mm I.D. stainless-steel column packed with 15-μm glass beads (Euroglas, Delft, The Netherlands); (2) the tubular reactor was a stainless-steel capillary of varying length (6–18 m) and 0.3 mm I.D. with a helix diameter of 3 cm; (3) for the air-segmentation system a 2-m long PTFE capillary purchased from Omnifit (Biolab, Cambridge, Great Britain) of 0.8 mm I.D., 1/16 in. O.D. and a 5-cm helix diameter was used as the reactor. Air was added via a Technicon A-10 glass tee-piece at a flow-rate of 0.6 $ml \cdot min^{-1}$. A modified[22] tee-piece of 1/16 in. I.D. in order to allow insertion of the PTFE capillary was used as a phase separator; 50–80 % of the liquid stream passed through the phase separator to the flow cell of the detector.

Reagents

Fluram and 3- and 4-chloroaniline were obtained from Fluka (Buchs, Switzerland) and 3,4-dichloroaniline from Merck (Darmstadt, G.F.R.). Acetonitrile and all other chemicals were of analytical-reagent grade from Merck. Aqueous buffer solutions were prepared using appropriate amounts of sodium acetate and acetic acid; the total acetate concentration was 0.05 *M*.

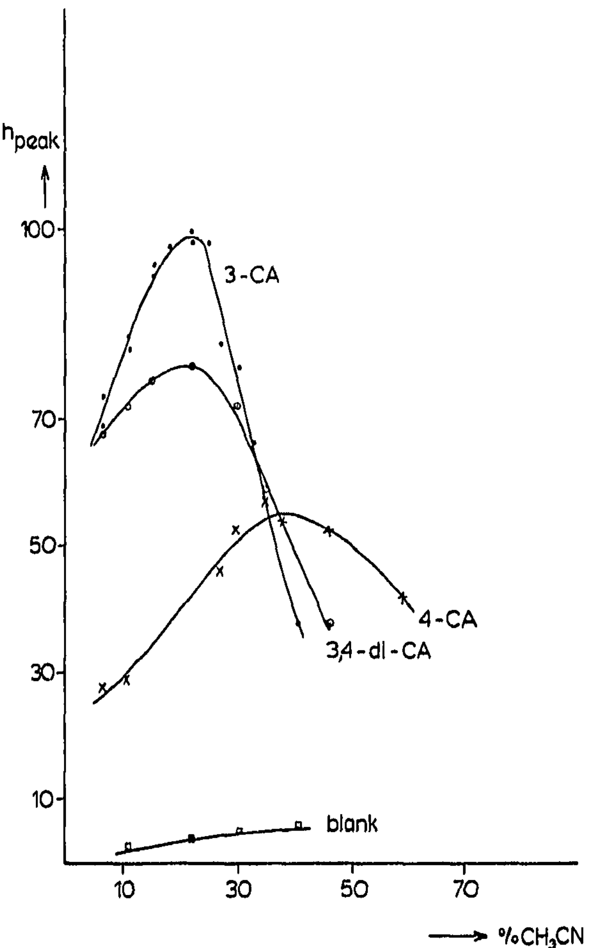
Stock solutions of the anilines were prepared in the mobile phase and kept in the dark. Solutions of Fluram in acetonitrile were degassed in an ultrasonic bath for 10 min. Mobile phases were degassed under vacuum for 2 min.

RESULTS AND DISCUSSION

HPLC conditions

HPLC on chemically bonded stationary phases is often carried out with methanol–water or acetonitrile–water mixtures as the mobile phase. Acetonitrile was preferred as the organic solvent, as methanol (and other alcohols) are known to effect a considerable increase in the time of reaction between Fluram and amino groups because of reversible hydrolysis of Fluram by the alcohol[20]; consequently, the net fluorescence is low.

Initially, 10-μm LiChrosorb RP-18 (Merck) was used as the stationary phase. A good separation of 3- and 4-mono- and 3,4-dichloroaniline with capacity ratios (k') of 1–3 was obtained with acetonitrile–0.05 *M* sodium acetate (4:6) as the mobile phase. One should realize, however, that Fluram has to be added to the mobile phase



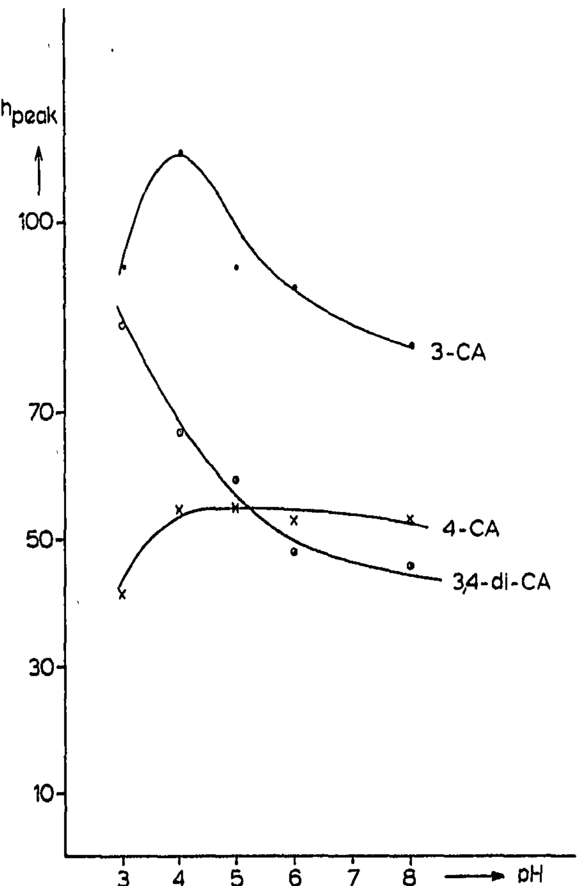


Fig. 2. Influence of the percentage of acetonitrile on the fluorescence of the Fluram derivatives of 3-chloroaniline (3-CA), 4-chloroaniline (4-CA) and 3,4-dichloroaniline (3,4-di-CA) in batch experiments. Conditions: 0.05 *M* sodium acetate–acetic acid buffer (pH 4); reaction time, 10 min.

Fig. 3. Influence of pH on fluorescence intensity in batch experiments. For conditions and symbols, see Fig. 2.

as a solution in acetonitrile. This implies that the post-column reaction medium contains 50–60% or more of acetonitrile. Such high proportions of organic solvent are known[12,13] to have a negative effect on the formation of the fluorescent reaction products. This was confirmed in a series of batch experiments (Fig. 2) in which the optimal percentage of acetonitrile was found to vary from 20% for 3,4-di- and 3-chloroaniline to 35% for 4-chloroaniline.

As a consequence of the above, with a flow-rate of 1.0 ml $\cdot$ min^{-1} through the HPLC column and even with a reagent flow-rate as low as 0.2 ml $\cdot$ min^{-1} (see below) the mobile phase had to consist of an organic–aqueous solvent mixture containing about 95% of water. The use of more polar packing materials such as LiChrosorb RP-2 and Polygosil 60-10 CN as the stationary phase therefore had to be studied. Excessive retention could be avoided only by using the CN-modified bonded phase. Unfortunately, however, at an aqueous phase pH of 5 or higher, the resolution between 3- and 4-chloroaniline was negligible, and a 95:5 mixture of a pH 3 acetate buffer and acetonitrile had to be used to create well resolved peaks. Under these conditions, the capacity ratios were: 3-chloroaniline 1.7, 4-chloroaniline 1.4 and 3,4-dichloroaniline 4.4. The results of a subsequent batch study (10 min reaction time

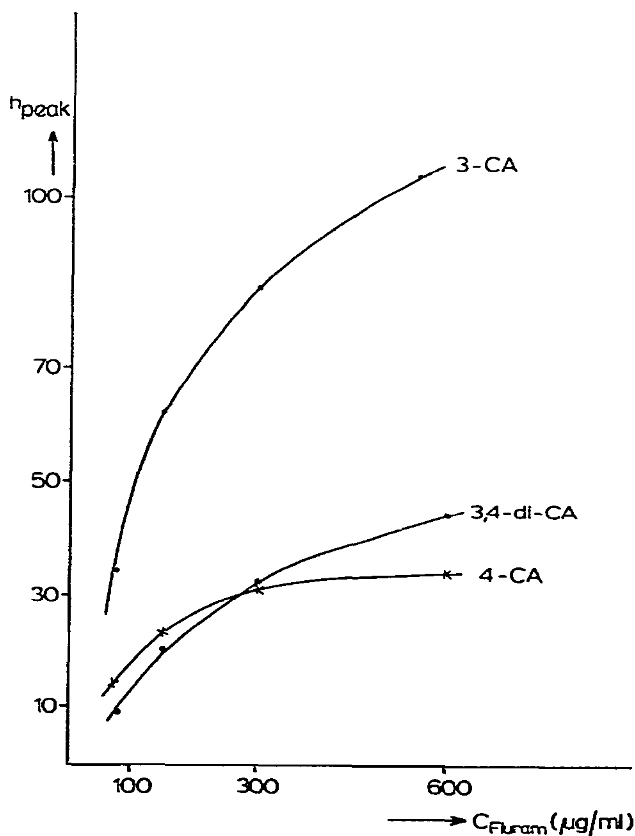


Fig. 4. Influence of Fluram concentration on fluorescence intensity, measured in the dynamic system at pH 3. Reaction time, 60 sec; further conditions and symbols, see text and Fig. 2.

with optimal acetonitrile–acetate conditions) on the influence of pH on fluorescence intensity are shown in Fig. 3. A pH of about 4 is seen to be the best overall choice; however, the decrease in signal intensity observed for 3-mono- and 3,4-dichloroaniline on lowering the pH to 3 certainly is not dramatic.

Fluram concentration and reaction time

Fig. 4 shows the dependence of fluorescence peak height on Fluram concentration, recorded at pH 3 under dynamic conditions. The time of reaction was 60 sec and the composition of the reaction system corresponded to the optimal conditions established in the previous section. For all further work, a concentration of 600 $\mu g \cdot ml^{-1}$ of Fluram was used; higher concentrations were found to result in precipitation of Fluram and clogging of the capillaries on mixing of the reagent and carrier stream.

The influence of the reaction time on fluorescence yield was studied by using different lengths of capillary, each 6 m × 0.3 mm I.D. capillary causing a 30-sec residence time in the post-column reactor. An increase in the reaction time from 30 to 60 sec resulted in a 10–40% increase in peak height, the actual percentage depending on the chloroaniline tested. A further increase to 90 sec led to a diminution of the peak heights, although the peak areas still showed an increase. As the resolution was found to deteriorate considerably on increasing the capillary length, a 30-sec residence time was deemed to offer the best compromise as regards band broadening and fluorescence peak height.

Linearity and detection limits

Calibration graphs were constructed using the following conditions: mobile phase, acetonitrile–pH 3 acetate buffer (5:95) at a flow-rate of 1 $ml \cdot min^{-1}$; reagent stream, 600 μg of Fluram per millilitre of acetonitrile at 0.2 $ml \cdot min^{-1}$; reaction time, 30 sec. Over the concentration ranges investigated the linearity was excellent, as is evident from the following data: 3-chloroaniline, $r = 1.0000$ (0.03–4.1 $\mu g \cdot ml^{-1}$); 4-chloroaniline, $r = 0.9999$ (0.1–12.7 $\mu g \cdot ml^{-1}$); 3,4-dichloroaniline, $r = 0.9999$ (0.09–11.8 $\mu g \cdot ml^{-1}$).

The detection limits for the Fluram derivatives of the chloroanilines, calculated for a signal/peak-to-peak noise ratio of 2:1, were 60 pg for 3-chloroaniline and 130 pg for 4-mono- and 3,4-dichloroaniline. The UV detection limits, recorded at 243 nm, were 0.6 ng for the former and 1 ng for the latter two compounds. It should be emphasized that under the experimental conditions used, the fluorescence background signal of hydrolysed Fluram was relatively high. Consequently, especially when working at high sensitivities, a pulse-free pumping system had to be used in order to avoid undue baseline fluctuations.

Reactor design

Comparison of reactors. The work reported so far was carried out with a coiled tubular reactor. On the basis of literature data, it could not be expected that, for the reaction times used (30 sec), a segmented system can compete with a tubular or a packed-bed reactor. Recently, we have calculated[22], however, that the use of a segmented instead of a non-segmented system should be beneficial for fast reactions. For example, it was found that with reaction times of over about 8 sec in a capillary of 0.25 mm I.D., and a time of over 19 sec in a 10 cm × 4.6 mm I.D. reactor packed with

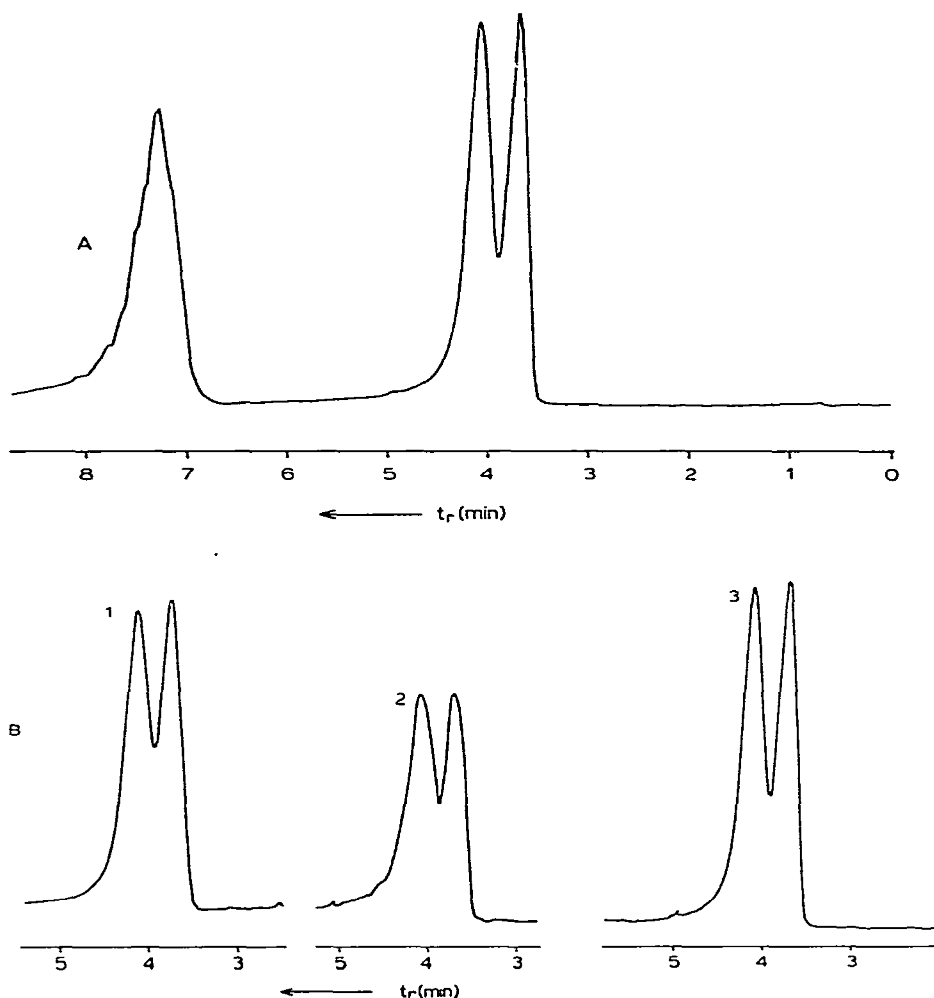


Fig. 5. (A) HPLC trace for air-segmented system. (B) Resolution of peaks due to Fluram derivatives of 3- and 4-chloroaniline: 1, tubular non-segmented reactor; 2, packed-bed reactor; 3, air-segmented reactor. For conditions and explanation, see text and Table I.

15-μm glass beads, a segmented reactor should be preferred. The three types of reactor were therefore compared. Typical chromatograms are shown in Fig. 5. Further results are summarized in Table I; the data on band broadening and resolution are average values from five measurements.

The main conclusion from the data in Table I is that the air-segmented system shows a distinctly smaller total band broadening of $\sigma^2_{v,tot}$ = 8500 μl^2 compared with the systems in which a coiled tubular or a packed-bed reactor was inserted ($\sigma^2_{v,tot}$ = 18,000 μl^2). This corresponds to σ_v values of *ca.* 90 and 130 μl, respectively, a difference which is reflected in the considerably improved resolution of the peaks due to 3- and 4-chloroaniline, *viz.*, from 0.69–0.71 to 0.89. It is disappointing that the band broadening for the packed-bed reactor system is as high as that for the tubular

TABLE I

COMPARISON OF PERFORMANCES OF COILED-TUBULAR REACTOR, PACKED-BED REACTOR AND AIR-SEGMENTED SYSTEM

Reactor type	t_r *(sec)*	φ_{cell} *($\mu l \cdot sec^{-1}$)*	σ_t *(sec)*	$\sigma^2_{v,tot}$ *(μl^2)*	R_s
Coiled tubular (1 capillary)	30	18	7.4	18,000	0.71
Packed-bed (15-μm glass beads)	41	18	7.4	18,000	0.69
Air-segmented	38	14.7	6.3	8600	0.39
UV signal	—	16	5.1	6700	1.14

t_r = Retention time; φ_{cell} = flow-rate through the flow-cell; R_s = resolution.

reactor system (which is at least partly due to the relative large variance contributions caused by the other parts of the HPLC system). Still, the experimental results agree satisfactorily with calculated band-broadening data (σ_v = 125, 115 and 100 μl for systems involving the tubular reactor, packed-bed reactor and segmented reactor, respectively). Obviously, also when using a system as complicated as the present post-column reaction system, our previous conclusions hold true, *i.e.*, segmentation is an experimentally sound principle to suppress band broadening even with relatively short reaction times.

Detection limits determined with the air-segmented reaction system were 30–40% better than those obtained with the tubular reactor. Such a relatively minor improvement is to be expected on the basis of the σ_t results quoted in Table I. An attempt to substitute liquid for air segmentation (with *n*-hexane as segmentation liquid) was only partly successful. Peak shapes were much the same in both instances; however, the signal of the Fluram derivative of 3-chloroaniline was seriously reduced, possibly owing to dissolution of the compound in hexane.

Mixing tees. In the literature, information on the proper construction of mixing tees, and especially on the contribution of these devices to band broadening, is rather scarce. A short study was therefore made using three different home-made tee-pieces (for constructional details, see Fig. 6), in which complete mixing was shown to occur by means of an indicator technique. Two further series of experiments were performed. In one, two purely aqueous streams were mixed; in another series, ac-

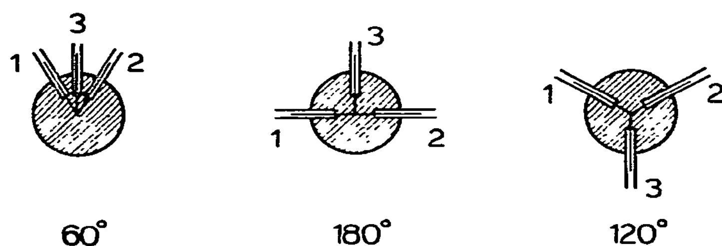


Fig. 6. Home-made stainless-steel tee-pieces tested (diameter, 2 cm). 1 = Eluent stream; 2 = reagent stream; 3 = to detector. Capillaries: 0.25 mm I.D. and 1/16 in. O.D.; channels: 0.25 mm I.D.

TABLE II

DEPENDENCE OF BAND BROADENING ($\sigma_{v,tee}$, μl) OF VARIOUS TEE-PIECES ON FLOW-RATE

Conditions: water–water system with 20 μl of sodium nitrate solution injected in the "eluent" stream; for tee-pieces, see Fig. 6.

Eluent flow-rate, φ_1 (ml/min)	*Tee-piece*	*Reagent flow-rate, φ_2 (ml/min)*			
		0.5	*1.0*	*2.0*	*3.0*
0.5	60°	28	44	66	90
	120°	26	36	65	83
	180°	26	38	64	84
1.0	60°	20	24	41	60
	120°	17	15	37	48
	180°	20	24	42	55
2.0	60°	21	22	31	40
	120°	16	18	26	32
	180°	19	22	31	40
3.0	60°	11	17	22	33
	120°	<1	4	20	25
	180°	20	24	34	42

etonitrile–water (15:85) mixtures were mixed with pure acetonitrile. Measurements were performed by injecting 20 μl of a sodium nitrate solution into the pure water and a *p*-aminobenzoic acid solution into the acetonitrile–water stream.

In order to calculate the variance contribution of the tee-piece, $\sigma^2_{v,tee}$, experimentally determined and/or calculated values of the variance contributions for the injector, connective tubing, coupling pieces and detector flow cell were subtracted from the total band broadening of the system, $\sigma^2_{v,tot}$. The results so obtained for the water–water system are shown in Table II. With the acetonitrile–water/acetonitrile system, the $\sigma^2_{v,tee}$ values showed the same trend, but they generally were up to 30% lower than for the purely aqueous system.

From the data in Table II two main conclusions can be drawn. First, there appears to be little difference in performance between the three geometrical configurations tested. The 120° angle device gives slightly better results; however, one should consider that, for each configuration, only a single tee-piece was tested. Secondly, $\sigma^2_{v,tee}$ is seen to increase with increasing dilution of the mobile phase by the reagent stream (horizontal rows from left to right; vertical columns from bottom to top). This implies that, in practice, the use of a relatively low flow-rate of the reagent stream has to be recommended. As for an interpretation, one should realise that $\sigma^2_{v,tee}$ is made up from contributions due to (1) the dilution which inevitably occurs upon mixing and (2) band broadening inside the tee-piece itself. As regards the dilution effect, it seems reasonable to assume that, if the solute zone emerging from a HPLC column is n-fold diluted [$n = (\varphi_1 + \varphi_2)/\varphi_1$; *cf.*, Table II] by the addition of a reagent stream, the variance due to injection, $\sigma^2_{v,inj} = V^2_{inj}/4$, will increase n^2-fold. In other words, the variance contribution due to dilution, $\sigma^2_{v,dil}$, can be written as

$$\sigma^2_{v,dil} = (n^2 - 1)\, V^2_{inj}/4 \qquad (1)$$

Use of this equation allows the rapid calculation of $\sigma^2_{v,dil}$, and thus also of the actual contribution of the tee-piece, for any set of experimental (V_{inj}; n) conditions. Using this procedure, we have calculated from the data in Table II that, except with impractical values of n greater than about 4, $\sigma_{v,tee}$ is largely (80–95%) caused by band broadening inside the tee-piece, with a relatively minor (20–5%) contribution due to dilution.

CONCLUSION

The potential of a post-column reactor for the fluorescent detection of chloroanilines after their derivatization with Fluram at low pH values (<5) and short reaction times (30 sec) has been demonstrated. Hydrolysis of Fluram causes a relatively high fluorescence background; for optimal performance a pulseless reagent pump is therefore required. The use of the Fluram reactor instead of direct UV detection of the substituted anilines increases both the sensitivity and selectivity; the latter aspect has been demonstrated in a previous paper[23] on the determination of the phenylurea herbicide diuron in soil samples.

The Fluram reaction has been used to compare three reactor designs, *viz.*, a tubular non-segmented, a tubular (air-)segmented and a packed-bed reactor. Even for the short reaction time mentioned above, the segmented system has been shown to give less band broadening and, thus, higher resolution. As for the miniaturized tee-pieces used in this work, their design does not appear to be very critical. Under normal operating conditions, their contribution to band broadening is mainly due to the tee-piece proper, with a minor contribution from dilution effects.

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