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DETECTION OF PRIMARY ARYLAMINES ON THIN-LAYER CHROMATOGRAMS BY DIAZOTISATION AND COUPLING

COMPARISON OF A NEW REAGENT WITH EXISTING METHODS

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SUMMARY

Most primary arylamines can be conveniently detected by spraying with a stable mixture of pentyl nitrite (3% v/v) and formic acid (3% v/v) in diethyl ether; subsequent coupling with 2-naphthol in aqueous alkali gave colours ranging from orange-yellow to purple. Under these conditions the diazonium salts did not diffuse randomly on the plates. The specificity of this new modification was compared with existing techniques (examination in ultraviolet light; glucose–phosphoric acid; vanillin; salicylaldehyde; salicylaldehyde–alkali; Folin–Ciocalteu; Folin–Ciocalteu and carbonate; ferric ferricyanide; acidified dichromate; phenol–hypochlorite; atmospheric oxidation; sodium 1,2-naphthoquinone-4-sulphonate and carbonate; 2,3,5,6-tetrachlorobenzoquinone; phthalaldehyde); in addition, the specificities of these and other methods for primary arylamines were reviewed.

INTRODUCTION

This study provided a background for the preparation and purification of certain primary aromatic amines. These particular compounds, which are for the most part carcinogenic, are used as intermediates in the syntheses of pure tetrazolium salts for purposes of quantitative cytochemistry; purification of the salts themselves or of the corresponding formazans may not be a straightforward process. The required standards of purity are necessarily high.

Chromatography has been valuable not only in revealing the presence of impurities, but also in giving information regarding their chemical nature. The scope of such information is limited by the specificity of the reagents employed. An extensive range of tests is available for the localization of primary aromatic amines. Some of these, such as examination under UV light, can detect a wide variety of

compounds (for example, see refs. 1–4). Reagents which depend on oxidising reactions (acidified dichromate[5]; ceric sulphate[6]; silver-catalysed persulphate[7]; ammoniacal silver nitrate[8–12]; ferric ferricyanide[2,13]) fall in a similar category. Likewise, 2,3,5,6-tetrachlorobenzoquinone (chloranil) reacts with secondary and tertiary amines, phenols[14,15] and thiols[14]; 4-dimethylaminobenzaldehyde, in addition to giving colours with primary arylamines[3,4,16–27], can react with substituted hydroxylamines[21,23] and hydrazines[28,29], indoles[26,29,30], certain hydroxyindoles[26], ureides[26], azulenes and proazulenes[6], pyrroles[25,31], certain hydroxyphenols[8] and urea[29], depending on the conditions. The high specificity for and sensitivity towards primary amines offered by prior condensation with 1,4-dimethoxyfurfural is partially offset by the subsequent use of 4-dimethylaminobenzaldehyde to detect the resulting pyrrole derivative[32]. The Folin–Ciocalteu reagent, followed by aqueous sodium carbonate[33], also reacts with substituted hydroxylamines, N-hydroxyacylamides[21], hydroxyphenols and reducing agents[33]; 2,3-dichloro-1,4-naphthoquinone can react with secondary and tertiary arylamines[25] and N-alkylvinylamines[34]; Rhodamine B also detects secondary and tertiary arylamines[35]. Treatment with diazonium salts (prepared from 2,4-dinitroaniline[24], 4-nitroaniline[4,9,11,12,22,29,30,36,37], sulphanilic acid[9,18,20,22,30,36–39] or 4-anisidine[40]) or tetrazonium salts (prepared from benzidine[1] or *o*-dianisidine[36]) can reveal a variety of compounds in addition to primary arylamines, depending on the choice of salt. Other reagents, of limited or little-known reactivity towards aromatic amines, include vanillin (for a few amino acids[41]; see also refs. 1 and 6) and the Folin reagent sodium 1,2-naphthoquinone-4-sulphonate[6,17,18]. The sensitivity, specificity and general applicability of some seven reagents in the detection of primary, secondary and tertiary alkyl- and arylamines have been thoroughly and carefully assessed by BARNEY *et al.*[25]; four (4-dimethylaminobenzaldehyde, 2,3-dichloro-1,4-naphthoquinone, salicylaldehyde and furfural) were recommended, while three (cupric copper–carbon disulphide, 2-thiobarbituric acid, and *o*-tolidine–bromine–cyanide) were not. In addition, the reactivities of certain carcinogenic arylamines and of some of their derivatives towards the Folin–Ciocalteu reagent, ammoniacal silver nitrate, 4-dimethylaminobenzaldehyde, 2,6-dichloroquinone chloroimide, sodium amminoprusside and ferric chloride have been investigated[21].

In theory, an ideal method of detection would involve diazotisation followed by coupling to give a stable azodye. This technique, with aqueous nitrous acid as the diazotising reagent, has been used fairly extensively[2,6,11,16,18–20,22,39,42], but does have certain drawbacks. First, some amines fail to give diazonium salts. *Ortho*-substituted diamines yield benzotriazoles[43–45], and therefore cannot form azodyes; moreover, aminonaphthalene sulphonic acids diazotise with difficulty[46]. Second, elution of amines and diazonium salts by the initial reagent can occur[46]. RATNEY[46] attempted to overcome this problem by diazotising with nitrosyl fluoborate, but resorted to gaseous nitrogen dioxide. Despite their toxicity, nitrogen dioxide or nitrous fumes have been used by other workers[3,24,27]. The aim of the present work has been to devise a diazotising solution of low polarity and toxicity, and to compare the specificity of the azodye method of detection with those of certain other techniques, some of which do not appear to have been used extensively with primary arylamines. In the course of this study the high specificity of the glucose–phosphoric acid reagent of MICHEEL AND SCHWEPPE[47] has become apparent.

It has not been an aim of this work to investigate optimal conditions for the

running of amines, because the versatile petroleum ether–acetone[21] and alcoholic ammonium hydroxide[1,24,30,39] systems have proved to be perfectly adequate. The question of separation has, moreover, been studied in detail by a number of investigators (for example, see refs. 2, 3, 17, 24, 27, 35, 48, and 49).

EXPERIMENTAL

Individual solutions of a total of thirty-four aromatic compounds (see Tables I and II) in ethanol or water were applied to commercially-prepared glass plates coated with Silica Gel F_{254} (DC-Fertigplatten Kieselgel F_{254}; E. Merck AG, Darmstadt, G.F.R.); 3,3'-dicarboxybenzidine and 4-aminobiphenyl were dissolved in dimethylsulphoxide and chloroform, respectively. Loadings were 200 ± 50 μg per spot. In the cases of chromatograms which were later run, the substances were applied along lines approximately 5 mm in length; in other cases spots were applied as circles 5 ± 1 mm in diameter. Most primary arylamines were run on plates measuring 40 × 50 mm in a mixture of light petroleum (40–60° fraction)–acetone (7:3) (ref. 21). Greater or smaller R_F values could be obtained by increasing or decreasing, respectively, the proportion of acetone in this solvent mixture. On the other hand, most compounds which bore carboxylic or sulphonic acid substituents showed a tendency to tail, and were run more effectively in a system composed of equal volumes of *n*-propanol, *tert.*-butanol and 0.880 ammonium hydroxide (compare refs. 1, 24, 30, and 39).

Methods of detection

(1) Diazotisation and coupling. The diazotising reagent consisted of a solution of 3% v/v freshly-prepared[50] pentyl nitrite in diethyl ether, to which 98% formic acid (3%) was added. Plates were liberally sprayed (1 ml to 10 sq. cm), and dried at room temperature in a current of air for about 10 min before cautiously spraying with the coupling reagent (1% 2-naphthol dissolved in 5% aqueous sodium hydroxide).

(2) Ultraviolet light. Chromatograms were examined in light of wavelength 254 nm.

(3) Glucose–phosphoric acid. Plates were sprayed with a solution of 0.2 g of glucose dissolved in a mixture of 4 ml of water, 3 ml of ethanol, 3 ml of *n*-butanol and 0.9 ml of orthophosphoric acid, sp.gr. 1.75, before heating at 115° for 10 min (ref. 47).

(4) Vanillin. The initial spray reagent was 2% vanillin in *n*-propanol; after allowing to dry at room temperature the plates were sprayed with 1% ethanolic potassium hydroxide, and heated at 110° for 10 min (after CURZON AND GILTROW[41]).

(5a) Salicylaldehyde alone. The chromatograms were sprayed with a solution of 1% salicylaldehyde in ethanol which contained 1% glacial acetic acid (after refs. 51 and 52), and heated at 115° for 10 min.

(5b) Salicylaldehyde and alkali. As (5a), with the modification that plates were sprayed with 1% ethanolic potassium hydroxide prior to heating.

(6a) Folin–Ciocalteu alone. Chromatograms were sprayed with the undiluted reagent (BDH Chemicals Ltd., Poole, Dorset, Great Britain).

(6b) Folin–Ciocalteu and carbonate. As (6a); plates were then air-dried before spraying with 20% aqueous sodium carbonate[33].

TABLE I

R_F VALUES OF PRIMARY ARYLMONOAMINES, WITH COLOURS GIVEN IN RESPONSE TO VARIOUS DETECTING AGENTS

NC = no colour. S_1 = Light petroleum (40–60°)–acetone (7:3); S_2 = *n*-propanol–*tert.*-butanol–0.88 ammonium hydroxide (1:1:1).

	R_F		Method of detection		
	S_1	S_2	*Diazotisation and coupling*	*Glucose–phosphoric acid*	*Vanillin*
4-Aminoazobenzene	0.60		Carmine	Red	Light orange
2-Aminobenzoic acid		0.50	Red	Brown	Yellow
4-Aminobenzoic acid		0.46	Red	Brown	Yellow
4-Aminobiphenyl	0.73		Red	Pale brown	NC
4-Amino-4′-dimethylaminoazobenzene	0.54		Brownish mauve	Pink	Orange
4-Aminodiphenylamine	0.45		Black	Brownish grey	Orange
1-Amino-2-hydroxynaphthalene-4-sulphonic acid		0.40, 0.45[a]	Negligible	Faint purple	Negligible
2-Aminophenol	0.48		Brown	Light brown	Yellow
2-Aminopyridine	0.25		NC	NC	Very faint yellow
4-Anisidine	0.52		Mauve-red	Light brown	Faint yellow
1-Naphthylamine	0.65		Brownish purple	Light brown	Negligible
2-Naphthylamine	0.59		Brownish red	Light brown	Faint yellow
2-Nitroaniline	0.65		Orange	Brown	NC
3-Nitroaniline	0.61		Orange-yellow	Brown	NC
4-Nitroaniline	0.51		Orange-red	Brown	Negligible
Sulphanilic acid		0.45	Orange-red	Brown	Yellow
4-Toluidine	0.66		Orange-red	Brown	Yellow

[a] This particular sample contained two major spots.
[b] As the hydrochloride.

(6c) Carbonate and Folin–Ciocalteu. Chromatograms were sprayed with 20% aqueous sodium carbonate, air-dried, and sprayed with the Folin–Ciocalteu reagent diluted to one-quarter strength with water[6].

(7) Ferric ferricyanide. Equal volumes of 0.1 *M* aqueous ferric chloride and 0.1 *M* aqueous potassium ferricyanide were mixed just before use, and sprayed directly[2].

(8) Dichromate–sulphuric acid. Plates were sprayed with a solution of 5% potassium dichromate in 40% sulphuric acid[5].

(9) Phenol–hypochlorite. The plates were sprayed with 5% ethanolic phenol, followed by 0.2% sodium hypochlorite in 15% aqueous sodium hydroxide[16].

(10) Atmospheric oxidation. Chromatograms were allowed to remain on the bench.

(11) Folin reagent. After spraying with a freshly-prepared solution of 0.02% sodium 1,2-naphthoquinone-4-sulphonate in 5% aqueous sodium carbonate[6,53], chromatograms were heated at 115° for 5 min.

(12) 2,3,5,6-Tetrachlorobenzoquinone. The spray reagent consisted of 0.2% quinone in monochlorobenzene[14].

Salicyl-aldehyde	*Salicyl-aldehyde and alkali*	*Folin–Ciocalteu alone*	*Folin–Ciocalteu and carbonate*	*Ferric ferricyanide*	*Dichromate–sulphuric acid*	*Phenol–hypochlorite*
NC	NC	Red	Olive green	Blue	Negligible	NC
Yellow	Yellow	Faint green	Dirty grey	Blue	Faint brown	Pale grey
NC	Pale yellow	Negligible	Dirty grey	Blue	Faint purple	NC
Yellow[b]	NC	Negligible	Negligible	Faint blue	Purple	NC
Pale pink	NC	Reddish brown	Dirty green	Negligible	NC	Light brown
Brown	Brown	Blue	Dark blue	Blue	Blue	Brown
NC	NC	Negligible	Negligible	Blue	NC	Brown
Negligible	Yellow	Grey	Dirty green	Blue	Purple	Brownish black
NC	Negligible	NC	Pale yellow	NC	NC	NC
Yellow[b]	Yellow	Blue	Dark blue	Purple	Light blue	Bluish brown
Light brown	NC	Blue	Blue	Blue	Purple	NC
Light brown	Pale yellow	Steel blue	Dark blue	Blue	Brown	NC
NC	NC	NC	NC	NC	Negligible	NC
NC	NC	NC	NC	NC	Negligible	NC
NC	NC	NC	NC	NC	Negligible	NC
NC	NC	NC	NC	NC	Faint purple	NC
Yellow	Yellow	Grey	Blue	Blue	Purple	Pale pink

(13) Phthalaldehyde. Plates were sprayed with 1% phthalaldehyde in xylene, and heated at 110° for 10 min (after TURNER AND WIGHTMAN[54]).

RESULTS AND DISCUSSION

The behaviour of each compound with the more useful reagents for detection is shown in Tables I and II. Not all substances were run on chromatograms; for those that were, R_F values are stated in the tables. These compounds were each tested with procedures (1), (2), (3), (4), (6a) and (6b), (7), (8) and (10), both as test spots and after running on chromatograms; the other techniques were only used on test spots which had not been run.

(1) Diazotisation and coupling. In the elaboration of this technique, the concentrations of formic acid and pentyl nitrite in the diazotising reagent were varied from 1–4% and 1–5%, respectively; initially, the coupling solution was 1% 2-naphthol dissolved in 2% aqueous sodium hydroxide. Most of these diazotising mixtures worked effectively, but dye formation was not always satisfactory at the higher concentrations of formic acid; the wet plates were sometimes acidic to pH paper even after

TABLE II

R_F VALUES OF CERTAIN AROMATIC COMPOUNDS, WITH COLOURS GIVEN IN RESPONSE TO VARIOUS DETECTING AGENTS

NC = no colour. S_1 = Light petroleum (40–60°)–acetone (7:3); S_2 = *n*-propanol–*tert.*-butanol–0.880 ammonium hydroxide (1:1:1).

	R_F		*Method of detection*		
	S_1	S_2	*Diazotisation and coupling*	*Glucose–phosphoric acid*	*Vanillin*
Diamines					
Benzidine	0.30		Dirty mauve	Light brown	Yellow
o-Dianisidine	0.35		Purple	Brown	Yellow
3,3'-Dicarboxybenzidine		0.32	Purple	Brown	Brick red
o-Phenylenediamine	0.53		Light brown	Light brown	Yellow
p-Phenylenediamine	0.20		Brownish mauve	Light brown	Yellow
Substituted hydrazines					
2,2'-Dicarboxyhydrazobenzene	0.07		Negligible	Light brown	Orange-red
Hydrazobenzene	0.71		Negligible	Brown	Negligible
Phenylhydrazine hydrochloride			Very pale brown	Negligible	NC
Secondary amines					
Diphenylamine			NC	Light grey	Negligible
Monomethylaniline			NC	NC	NC
Tertiary amines					
Dimethylaniline			NC	NC	NC
2,2'-Dipyridyl			NC	NC	NC
8-Hydroxyquinoline			Negligible	NC	NC
Tetramethyldiamino-benzoquinone			NC	NC	NC
Phenols and naphthols					
2-Naphthol			Negligible	Negligible	NC
Orcinol			NC	Negligible	NC
Resorcinol			Dirty green	NC	NC

excessive spraying with the coupling reagent. The problem was overcome by increasing the concentration of sodium hydroxide to 5%; however, if the plates were oversprayed with this solution, very slight leaching of those azodyes which bore carboxylic groups took place. The final concentrations of formic acid and pentyl nitrite selected for the diazotising reagent were each 3%. Dipping plates in this mixture gave good results with most compounds, but the simpler monoamines displayed a limited tendency to run; the procedure is not generally recommended. The reagent is stable for at least two months. The solution of 2-naphthol, however, tends to discolour, and is best prepared at monthly intervals.

All primary arylamines tested reacted strongly to give azodyes in a multiplicity of colours, with the exception of 1-amino-2-hydroxynaphthalene-4-sulphonic acid, which reacted weakly, the refractory 2-aminopyridine, which failed to react at all,

Salicyl-aldehyde	*Salicyl-aldehyde and alkali*	*Folin–Ciocalteu alone*	*Folin–Ciocalteu and carbonate*	*Ferric ferricyanide*	*Dichromate–sulphuric acid*	*Phenol–hypochlorite*
Pale yellow	NC	Mauve	Dark blue	Dirty purple	Yellow	Dirty mauve
Yellow	Orange-yellow	Brownish mauve	Dark blue	Dirty purple	Red	Mauve
Brown	Orange	Deep blue	Dark blue	Blue	Dirty yellow	Pale purple
Yellow	Pale orange	Purple	Dark green	Dark blue	Purple	Brown
NC	Orange, fluorescent	Blue	Dark blue	Blue	NC	Purple
Orange-brown	Pale brown	Steel-blue	Dark blue	Faint blue	Brown	NC
NC	NC	Steel-blue	Steel-blue	Blue	Dirty yellow	NC
NC	NC	Blue	Blue	Blue	Pale brown	NC
NC	NC	Green	Blue	Blue	Blue	NC
NC	NC	Negligible	Blue	Blue	Faint brown	NC
NC	NC	Light blue	Blue	Blue	Faint brown	Blue
NC	NC	Pale yellow	Yellow	Pink	Negligible	NC
NC	NC	NC	Blue	Blue	Light brown	NC
NC	NC	NC	Greenish blue	Blue	Negligible	NC
NC	NC	Pale blue	Pale bluish mauve	Blue	Dirty blue	NC
NC	Pale brown	Light grey	Blue	Blue	Brown	Brown
NC	NC	Light grey	Dark blue	Blue	Brown	Olive green

and *o*-phenylenediamine (Tables I and II). On treatment with pentyl nitrite, the latter compound turned light brown, probably forming benzotriazole[44,45]; the brown colour remained unaffected by the coupling reagent. Differentiation was readily made on the basis of colour; in general, monoamines formed red dyes of various shades, while the diamines yielded purple colours of different hues. These colours are stable, changing only slightly over a period of months. One minor aesthetic drawback is that within a day a light brown speckled background sometimes developed; this did not interfere with recognition of the spots. The specificity of the technique is high. Of the other compounds tested, only two yielded colours. Dimethylaniline gave a green colour on treatment with pentyl nitrite, but this was discharged by alkali; similarly, resorcinol gave a brown colour, which was turned by the coupling agent to an atypical green.

(2) Ultraviolet light. All compounds showed up as purplish-blue spots against a greenish-yellow fluorescent background.

(3) Glucose–phosphoric acid. This reagent is highly specific (Tables I and II). Apart from diphenylamine, which initially gave a weak reaction, darkening to blue with time, and the hydrazobenzenes, only primary amines reacted positively. It was felt that conversion of hydrazobenzenes to benzidines might occur on the chromatograms as a result of heating to 115° in the presence of phosphoric acid. The possibility was investigated by spraying samples of the compounds spotted on to plates with a dummy reagent from which glucose had been omitted, heating, and testing with the diazotisation–coupling technique. This revealed that treatment with the phosphoric acid reagent had effectively generated primary arylamino groups from both hydrazobenzene and, 2,2′-dicarboxyhydrazobenzene. No colour was obtained with 2-aminopyridine. The spots faded slightly after a week or so; certain other compounds, notably diphenylamine, phenylhydrazine and orcinol, slowly developed colours within the same period of time.

(4) Vanillin. This reagent is known to give a positive result with a very limited number of amino acids[41], but its use has been suggested for the detection of primary arylamines[1,6]. Although the three nitroanilines and 4-aminobiphenyl failed to react, most primary amines showed up strongly. No other compound gave a colour, except 2,2′-dicarboxyhydrazobenzene, which was surprising, since hydrazobenzene itself failed to react. When the dicarboxy derivative was subjected to a dummy run from which vanillin had been omitted, and then tested by diazotisation and coupling, it was found, not surprisingly, that the procedure had failed to generate primary arylamino groups within the molecule. The colours produced by this technique were stable, but somewhat difficult to see when only trace amounts were present. Only six amines gave colours in the cold.

(5) Salicylaldehyde. MILUN[62] noted that while all classes of aliphatic amines reacted with salicylaldehyde, condensation with primary aliphatic amines took place only in the presence of acetic acid. For this reason the reagent contained 1% of the acid. Colours were obtained with less than half the amines tested, and also with 2,2′-dicarboxyhydrazobenzene (Tables I and II). Treatment with alkali would appear to offer no substantial advantage. As in the case of vanillin, differentiation on the basis of colour is difficult; the value of the technique for arylamines appears to be limited.

(6) Folin–Ciocalteu. The Folin-Ciocalteu phosphotungstic acid reagent, when used on its own (a), detected most amines, giving strong stable colours, mostly blue, against a light yellow background. The solution is well known for its capacity to react with other classes of compounds, notably phenols. When used in conjunction with 20% aqueous sodium carbonate (b), a few more spots appeared and certain colour changes occurred. However, both definition and the weaker spots tended to be lost; moreover, the colours were less stable, and a blue background of variable intensity developed. When the order of reagents was reversed and the Folin–Ciocalteu reagent was diluted (c)[6], the results were quite useless; the colours were notably less intense, and tended to dissolve out of the chromatograms.

(7) Ferric ferricyanide. Apart from the nitroanilines, 2-aminopyridine and sulphanilic acid, all compounds gave a strong positive reaction (Tables I and II). Overspraying produced a blue background, which deepened on exposure to light. Although the reaction appears to have been used largely to identify primary aryl-

amines[2], a good case for using the reagent to detect other categories of compounds might be made on the basis of the present results.

(8) Dichromate–sulphuric acid. In general, where compounds reacted positively, the colours were faint and blotchy, and tended to alter rapidly with time. Moreover, 4-aminoazobenzene, 2-aminopyridine and the nitroanilines gave reactions only after 24 h. It is perhaps unfair to criticise this technique on the basis of these unsatisfactory results because the original suggestion[5] was that the reagent be used on paper, and that the chromatograms be heated to 150°. However, aliphatic acids have been found to react well under the present conditions in the absence of heat[55].

(9) Phenol–hypochlorite. As in the case of salicylaldehyde, only about half the amines gave a positive reaction (Table I), but the two hydroxyphenols also yielded colours (Table II).

(10) Atmospheric oxidation. Most amines which had been run on chromatograms tended to develop colours on standing. Spots were often faintly visible after 24 h, and well marked in some cases after a week. This technique is not recommended for standard use on account of the time involved.

The separate use of sodium 1,2-naphthoquinone-4-sulphonate, 2,3,5,6-tetrachloroquinone, and phthalaldehyde was disappointing in all cases. Too small a proportion of the amines reacted; the specificity of the reagents was poor; in addition, with a few exceptions the colours were not well marked. The application of heat in the case of sodium 1,2-naphthoquinone-4-sulphonate was not recommended by the original author[53], but produced colour changes in a few individual cases. These were, however, insufficient to redeem the usefulness of the reagent. Phthalaldehyde has been used successfully in the detection of alkylamines[54]; modifications of this technique have also proved useful with histamine[56].

CONCLUSION

A few general points emerged in the course of this study. First, certain amines, once applied to the plates, diffused rapidly of their own accord. This was especially true of the methylanilines and 4-toluidine; aniline had been selected as a test compound in early experiments, but its use was soon discontinued because the extent of diffusion was unacceptable. Detection procedures which involved heating tended to produce somewhat more diffuse spots than those carried out at room temperature. Second, the colours obtained were not entirely reproducible in all cases. Various factors are thought to be responsible; these include subjectivity on the part of the experimentalist, and variations in loading and in the extent of spraying. On occasion, depending on the reagent and the compound, colours were found to have altered overnight, or on prolonged keeping. Changes of this kind have not been described in detail because, in most investigations, emphasis is laid on the rapid location and identification of spots. For the same reason, the stabilities of the colours produced by different reagents have been mentioned only in broad outline. Third, the recommendation is made that at least two chemical detection procedures be used, preferably in conjunction with the non-specific but sensitive method of examination under UV light, because not all primary arylamines gave positive reactions with any one single chemical test.

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