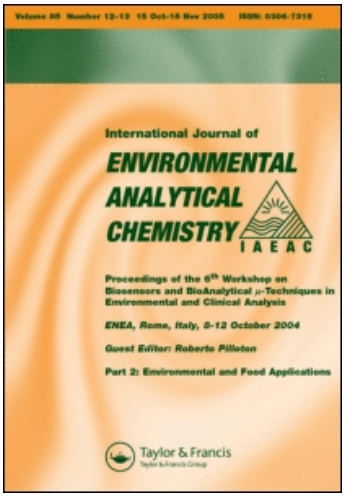

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Benzidine and Congeners: Analytical Chemical Properties and Trace Analysis in Five Substrates

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Benzidine and Congeners: Analytical Chemical Properties and Trace Analysis in Five Substrates

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KEY WORDS: benzidine and congeners, carcinogens, trace analysis, spectrophotofluorimetry, analytical properties.

Spectrophotofluorimetric (SPF) methods are described for the trace analysis of benzidine, 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, and their dihydrochloride salts in microbiological growth media, waste water, potable water, human urine, and rat blood. The salient elements of the methods for these known or suspected carcinogens are: extraction of the residues as the free amine with benzene, rapid cleanup on an alumina column, and quantification of the free amine in methanol via SPF. Potable water solutions of the salts are diluted with buffer (pH-4) and quantified directly by SPF. Ancillary analytical information concerning the solubility and stability of these compounds in water, p-values, gas chromatographic analysis of the free amines, and thin layer chromatographic data in ten solvent systems are also presented.

INTRODUCTION

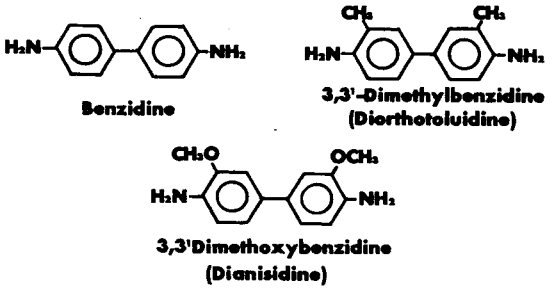
Urinary bladder tumors in human subjects exposed to chemicals utilized in the synthesis of dyes were first reported by Rehn[1] in 1895. Rehn called the lesions "aniline tumors", however, subsequent research proved that aniline was not the causative agent and other aromatic amines, such as benzidine and its congeners, were then indicated. The toxicological effects of compounds in the benzidine family have been widely studied for several years, and in 1974, the U.S. Department of Labor called for the regulation of benzidine, 3,3′-dichlorobenzidine, and 4-aminobiphenyl by placing them on the list of chemical compounds known to, or suspected to, cause human cancer from occupational exposure.[2] A comprehensive overview of the literature

and problems associated with the use of benzidine and its congeners was recently reported by Haley[3].

The National Center for Toxicological Research (NCTR) is currently initiating long term, low-dose, chronic feeding studies of aromatic amines (benzidine, dianisidine, and diorthotoluidine) to develop protocols for long term bioassays as well as to determine their carcinogenic effects in mice and other experimental animals. Therefore, analytical chemical methods for these compounds were required to: (a) verify purity, proper dosages, and chemical stability of the compounds administered via the animal's drinking water; (b) monitor the clothing, work areas, and urine of personnel to signal any accidental spillage or exposure to the chemicals; (c) monitor waste water resulting from decontamination of the test areas; (d) develop methods for cleaning up the waste; (e) determine the level of compounds in the blood of the test animals; and (f) evaluate microbiological systems currently being sought to destroy the carcinogenic effects of the chemicals. The formulas of the three compounds are shown in Figure 1. In order to improve their water solubility and chemical stability and reduce volatility, the chemicals are administered to the animals as aqueous solutions of dihydrochlorides; therefore, analytical methods for these salts were also required.

Several colorimetric methods for benzidine, based on diazotization and coupling reactions, have been reported[4–7]; also several researchers[8–11] have employed Chloramine-T reagent to determine benzidine and its congeners. Clayson *et al.*[12] reported a qualitative method for benzidine and its metabolites based on reversed phase paper chromatography. Rinde[13] used 2,4,6-trinitrobenzenesulfonic acid and fluorescamine to detect benzidine on thin layer chromatographic plates; spectrophotometric and fluorimetric methods employing these reagents were also used to quantitate benzidine excretion following feeding of benzidine dyes. None of these methods, however, provide the sensitivity, specificity, accuracy, and precision required for our use. For example, in the colorimetric method for human urine (sensitive to about 50 ppb) described by Sciarini and Mahew[9] employing Chloramine-T reagent, the yellow meriquinone complex begins to decompose after two minutes.

This paper describes spectrophotofluorimetric (SPF) procedures for the analysis of benzidine, 3,3′-dimethylbenzidine (diorthotoluidine), 3,3′-dimethoxybenzidine (dianisidine), and their dihydrochloride salts in potable water, waste water, microbiological media, human urine, and rat blood. Monitoring procedures for detecting trace level contamination of our work areas by these chemicals are discussed, and ancillary analytical information, concerning p-values, solubility and stability in water, gas chromatographic (GC) analysis of the free amines, and thin layer chromatographic (TLC) data for ten systems, is also presented.



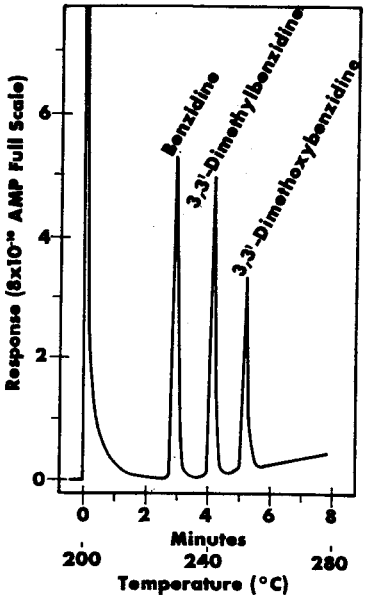


FIGURE 1 Top. Formulas of benzidine; 3,3′-dimethylbenzidine; and 3,3′-dimethoxybenzidine. Bottom. A typical temperature-programmed chromatogram of 500 ng amounts of the three compounds injected in 5 μl of chloroform.

EXPERIMENTAL

Test materials and other chemicals

Benzidine (mp 127–129°C) was purchased from Fisher Scientific Co., and the benzidine·2HCl was Matheson, Coleman, Bell chemical No. B-260.

The 3,3'-dimethylbenzidine (mp 129–131°C) and 3,3'-dimethoxybenzidine·2-HCl were purchased from Pfaltz and Bauer Co., Flushing, NY The 3,3'-dimethylbenzidine·2HCl was prepared from the corresponding amine (2 g in 50 ml benzene) by bubbling an excess of anhydrous HCl through the solution to precipitate the salt; the 3,3'-dimethoxybenzidine (mp 137–139°C) was prepared from an aqueous solution of the corresponding salt by making it strongly alkaline with NaOH and extracting the free amine with benzene. All six chemicals were vacuum dried overnight at 60°C prior to use. The high purity of the amines was demonstrated by the absence of extraneous GC peaks; the salts, after conversion to the free amines, were also free of extraneous GC peaks and yielded the correct amount of product.

The adsorbent (basic alumina, Brockman Activity I) from Fisher Scientific Co. (No. A-941) was used as received. Sodium sulfate was anhydrous; all reagents were CP grade; and all solvents were pesticide grade. Ingredients for the microbiological culture media were purchased from Difco Laboratories, Detroit, MI., and the media were prepared in accordance with the instructions supplied by the manufacturer. The TLC plates (20 × 20 cm, Fisher No. 6–601A), precoated with Silica Gel GF (250 microns thick), were activated in an oven at 130°C for 1 hour and allowed to cool in a desiccator prior to use. All culture tubes were of borosilicate glass and equipped with Teflon-lined screw caps. The buffer (pH-4, potassium biphthalate, 0.05 M) was Fisher Chemical No. SO-B-98.

Preparation of samples for SPF analysis

Microbiological Growth Media Ten ml of the trypticase soy dextrose (TSD) or brain heart infusion (BHI) medium was added to a 30-ml culture tube and 1 g NaCl, 0.2 ml 10 N NaOH and 10 ml of benzene were added. The tube was sealed, shaken vigorously for 2 min, and centrifuged for 10 min at 2000 rpm. The benzene layer was carefully withdrawn by using a syringe and cannula and percolated through a plug of sodium sulfate (25 mm diam. × 10 mm thick) in tandem with a glass column (12 mm i.d., Kontes No. 420,000) prepared by adding successively, a plug of glass wool, 5 g sodium sulfate, 1 g of basic alumina, and 5 g of sodium sulfate. The medium was extracted with two additional 10-ml portions of benzene, which were successively percolated through the plug and column. Finally, the column was washed with 10 ml of dichloromethane-10% methanol to ensure complete elution of the free amine residue from the column. The combined eluates, after the addition of one drop of diethylene glycol to serve as a "keeper" were evaporated just to dryness by using water pump vacuum and a 60°C water bath. The dry residue was dissolved in an appropriate amount of methanol for SPF analysis as the free amine.

Waste Water One hundred ml of the sample, 2 g NaCl, and 0.5 ml of 10 N NaOH were added to a 160 ml culture tube. The sample was then shaken, centrifuged, cleaned-up, and prepared for analysis as described for media {Exception: Three 15-ml portions of benzene were used for the extraction.}

Urine Procedures utilizing alkaline hydrolysis or acid hydrolysis prior to extraction, as well as no hydrolysis, were tested: (a) No hydrolysis-analyses were performed exactly as described for waste water; (b) Alkaline hydrolysis-analyses were performed exactly as described for waste water except the alkaline solution in the tube was sealed, heated in a water bath at 80°C for 2 hr, then cooled prior to the extraction with benzene; (c) Acid hydrolysis—these analyses were also performed as described for waste water, except that 0.5 ml of concentrated HCl was substituted for the 10 N NaOH, then the mixture was heated at 80°C for 2 hr, cooled and made alkaline with 1 ml of 10 N NaOH prior to extraction with benzene.

Blood Procedures employing no hydrolysis of the sample prior to extraction, as well as alkaline or acid hydrolysis, were tested: (a) No hydrolysis—six ml of distilled water, 1 ml of 10 N NaOH and 1 g of NaCl were added to a 20-ml culture tube containing 1 ml of whole rat blood. The contents were mixed, and then extracted with three 10-ml portions of benzene, cleaned-up and prepared for SPF analysis as described for media; (b) Alkaline hydrolysis—samples were analyzed as for no hydrolysis except that the mixture in the sealed tube was held in an 80°C heating block for 2 hr with frequent shaking, then cooled prior to extraction with benzene; (c) Acid hydrolysis—analyses were performed as for no hydrolysis except that 1 ml of concentrated HCl was substituted for the NaOH and the sealed tube was then heated at 80°C for 2 hr, cooled, made alkaline by adding 2 ml of 10 N NaOH. The mixture was then extracted, cleaned-up and prepared for SPF analysis as described.

Potable Water Aqueous solution of the dihydrochloride salts slated for use as the animals' drinking water to administer the proper dosage of test chemical are assayed for proper concentration after sequential dilutions with water containing 2% (v/v) of buffer (pH-4). The fluorescence of the diluted sample (e.g. 1 ppm) is compared directly with that of a standard solution prepared in the same manner.

Recovery Experiments Triplicate samples of both biological media were separately spiked with 100 μl of methanol (or water) containing the appropriate amount of the free amine (or its salt) to produce residues of 0, 0.1, and 1.0 ppm. The samples were allowed to stand in the refrigerator (5°C) overnight prior to extractions. Samples (1 ml) of whole rat blood were

spiked in the same manner at 0 and 10 ppm, held at 5°C overnight, and analyzed as described. Waste water and urine samples (100 ml) were spiked at 0 and 20 ppb by adding 1 ml of methanol (or water) containing the appropriate amount of the test compound, held at 5°C overnight, and analyzed as described.

Stability of aqueous solutions of the test compounds

Aqueous solutions of the three amines (ca. 50 ppm) and of their dihydrochloride salts (ca. 50 and 500 ppm) were prepared for use in tests to determine the chemical stability of the test compounds under simulated animal test conditions. The animal drinking water dispenser for each cage (4 mice per cage) consisted of a glass bottle (500 ml, 62 mm square × 180 mm high) fitted with a No. 8 rubber stopper and a stainless steel "sipper tube" (8 mm o.d. × 90 mm long) containing a steel ball to serve as a valve. Triplicate dispensers, each containing 375 ml of the various test solutions, were placed in cages and exposed to ambient conditions (25±2°C and continuous fluorescent lighting) in the animal room. Samples (10 ml) were taken from each dispenser immediately and 1, 2, 4, 8, and 16 days later. The pH of each sample was determined and solutions of the salts were diluted and analyzed as described for potable water. Solutions of the free amines were diluted with methanol to an appropriate concentration (e.g. 0.5 μg/ml) and the fluorescence related to standards of the free amines diluted in the same manner.

Gas chromatographic analysis

A Hewlett–Packard Model 5750 gas chromatograph equipped with a flame ionization detector (FID) was fitted with a 100-cm glass column (4 mm i.d., 6 mm o.d.) containing 10% OV-101 (w/w) on Gas Chrom Q (80–100 mesh) and operated with a helium carrier flow of 100 ml/min. The injection port and detector temperatures were 275 and 290°C, respectively. The column oven was operated isothermally for the quantitative analysis of the individual free amines (e.g. p-value determinations) as follows: benzidine (215°C); 3,3′-dimethylbenzidine (235°C); and 3,3′-dimethoxybenzidine (240°C); under these conditions, their retention times (t_R) were 2.35, 2.25, and 2.70 min, respectively. In assays concerning the GC purity of the free amines, the oven was temperature programmed from 200 to 280°C at the rate of 10°C/min. A typical temperature-programmed chromatogram of the three compounds is shown in Figure 1.

Spectrophotofluorimetric analysis

An Aminco–Bowman instrument (American Instrument Co., Silver Spring,

MD.), equipped with a xenon lamp and a 1P28 detector, was used with 1-cm square cells and a 2-2-2-mm slit program to measure fluorescence. All dilutions of the free amines were freshly prepared in methanol and those of the salts were in water-2% buffer (pH-4). Excitation (λ_{Ex}) and emission (λ_{Em}) wavelengths (nm) and relative intensities (RI) were:

Compound	λ_{Ex}(nm)	λ_{Em}(nm)	RI/μg/ml
Benzidine	295	396	33.5
Benzidine·2HCl	302	410	7.45
3,3′-Dimethylbenzidine	300	384	51.8
3,3′-Dimethylbenzidine·2HCl	310	410	12.2
3,3′-Dimethoxybenzidine	312	380	64.3
3,3′-Dimethoxybenzidine·2HCl	318	422	8.25

The instrument was frequently calibrated to produce a RI of 5.0 with a dilution of 0.3 μg quinine sulfate/ml of 0.1 N sulfuric acid (λ_{Ex} = 350, λ_{Em} = 450). Readings were corrected for solvent blanks and RI was plotted vs concentration of the six compounds on log-log paper to produce a standard curve.

To ensure that the RI was within the linear range of the standard curve and thus unaffected by concentration quenching, samples were diluted with an equal volume of solvent to ascertain whether the RI was about half of the undiluted solution. If it was not, dilution was continued until RI was halved by dilution. Extracts of waste water and urine, medium, and blood for SPF analysis were first diluted to contain 10, 1, and 0.2 gram equivalents of sample per ml, respectively; further dilutions were made as required to be certain the RI was within the linear range of the standard curve. The RI of the untreated control samples was then subtracted from that of the unknown and concentration in μg/ml was determined from the standard curve. In the instances where salts are assayed as the free amines, the analytical results for benzidine, 3,3′-dimethylbenzidine, and 3,3′-dimethoxybenzidine are multiplied by the factors 1.40, 1.34, and 1.30, respectively to express them on the proper basis.

Solubility, p-Value, and TLC Determinations

The solubilities of benzidine, 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine and their salts in water at 25$\pm$2°C were determined by SPF as described by Bowman and King.[14]

Extraction p-values for the three amines were determined in several solvent systems by GC in the manner described by Bowman and Beroza.[15,16]

The TLC determinations were made by using a Gelman Model 51325-1 apparatus and activated glass plates precoated with silica gel GF. The plates were spotted with 5 μl (5 μg) of methanol solutions of the free amines or anthracene (reference compound). After the developing solvent had ascended 13 cm above the spotting line (ca. 25 min), the plates were removed and the solvent allowed to evaporate. The spots were made visible by viewing them under ultraviolet light (254 nm) and the R_f values calculated.

RESULTS AND DISCUSSION

In preliminary studies of the analytical chemical properties of these compounds, the free amines were found to gas chromatograph well with minimal column conditioning; the three compounds were also readily separated (Figure 1) and quantified. GC analysis was therefore employed for p-value determinations and purity assays, however the FID lacked the sensitivity and specificity required for trace analysis of residues in a variety of substrates. The possibility of oxidizing the free amines to their corresponding dinitro analogs was investigated, since these derivatives might then be assayed by electron-capture GC with higher sensitivity and specificity. The use of *m*-chloroperbenzoic acid, hydrogen peroxide, and potassium permanganate under various reaction conditions, however, produced no more than a 20% yield of the derivative. The portion of the free amines not derivatized remained unchanged or was converted to products that did not emerge from the gas chromatograph, depending on the severity of the oxidation reaction.

Tests pertaining to the SPF properties of the compounds revealed that all six fluoresce strongly. The RI values of the free amines in methanol were about five times greater than those of the salts in aqueous buffer. Limits of detection and linearity of the amines and salts were about 2 and 10 ng/ml, respectively. As expected, the SPF response of aqueous solutions of the salts varied with pH and assays of these compounds diluted with water alone were not reproducible. This problem was overcome by using an aqueous solution containing 2% buffer (v/v, pH-4) for dilutions and SPF measurements. Excitation and emission spectra and standard curves for the six compounds are presented in Figures 2, 3, and 4. SPF was selected for subsequent assays because of its high sensitivity and the specificity afforded of the characteristic excitation and emission maxima of each compound. Determination of the three amines or the three salts in admixture was attempted by measuring the RI at the excitation and emission maxima for each compound and calculating the amount of each constituent by using

simultaneous equations. However, this was not successful because of the large differences in specific RI and extensive overlap of the spectra.

Results from the assays of microbiological growth media are presented in Table I. Recoveries averaged 68 and 80% for samples spiked with 0.10 and

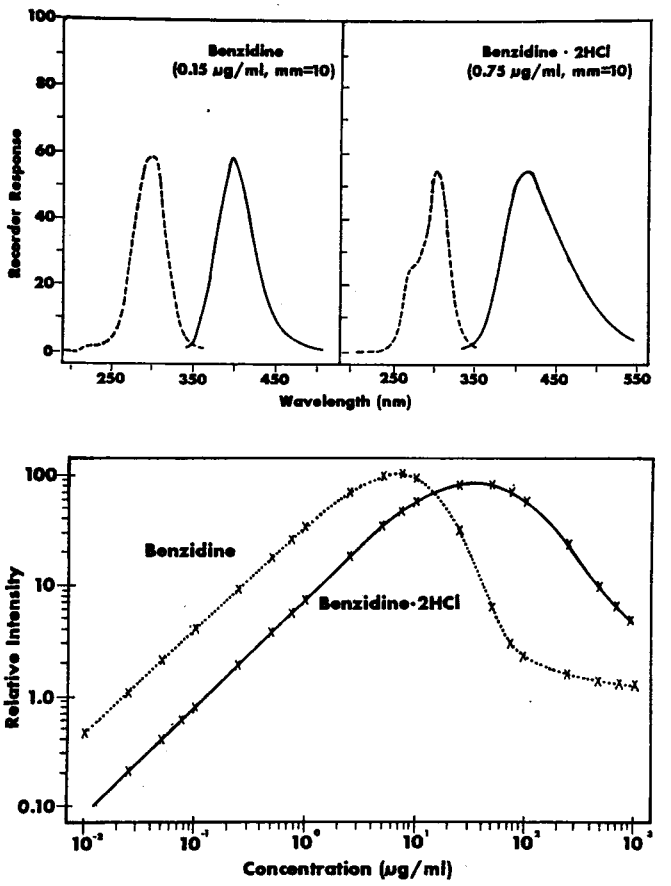


FIGURE 2 Top. Excitation (dotted line) and emission (solid line) spectra of benzidine and its dihydrochloride salt. Bottom. Standard curves for the two compounds.

1.0 ppm, respectively. The SPF background for unspiked BHI and TSD media was about 0.04 and 0.02 ppm. Lowest recoveries were obtained with 3,3'-dimethoxybenzidine and its salt. Assays of samples of waste water (collected from the decontamination of control animal cages) separately spiked with 20 ppb of each compound gave recoveries averaging 75%

(Table II). The precision was excellent and the control background was about 4 ppb.

Table III lists the results of SPF assays of whole rat blood spiked with 10 ppm of the six compounds. Recoveries without hydrolysis of the sample and after alkaline or acid hydrolysis averaged about 14, 19, and 63%,

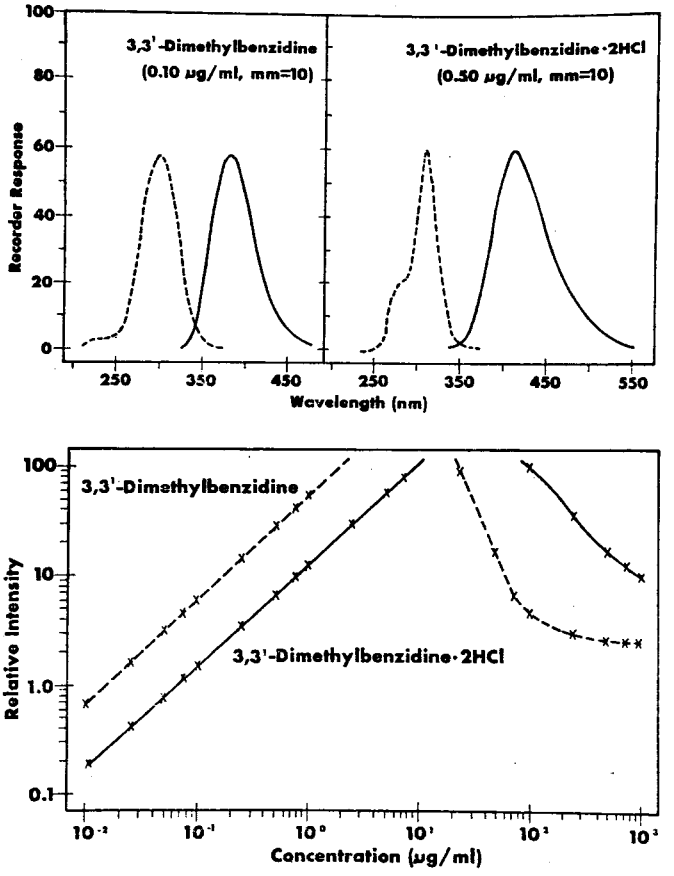


FIGURE 3 Top. Excitation (dotted line) and emission (solid line) spectra of 3,3′-dimethylbenzidine and its dihydrochloride salt. Bottom. Standard curves for the two compounds.

respectively. Acid hydrolysis is therefore, required to recover a substantial portion of the residue; control background was about 0.15 ppm. Recovery of the compounds from blood varied inversely with their polarity.

Data from human urine spiked with 20 ppb of the compounds are presented in Table IV. Recoveries of the free amines from urine without hydrolysis

and after alkaline hydrolysis were 90 and 68%, respectively; those for the salts were 38 and 57%. It is apparent that the procedure employing no hydrolysis should be used when residues of the free amines are sought, since alkaline hydrolysis diminished the recovery by about 20%. On the

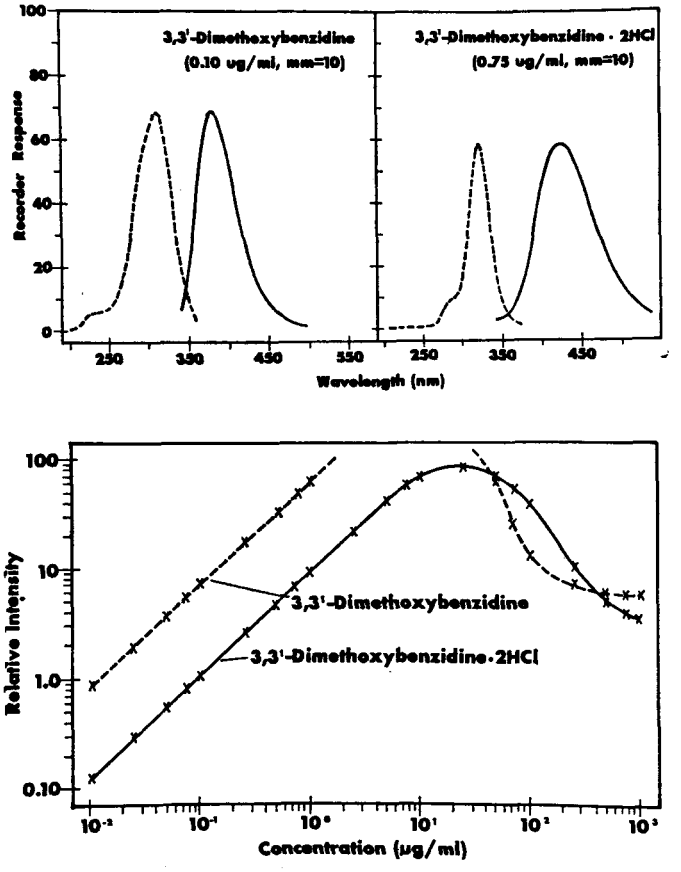


FIGURE 4 Top. Excitation (dotted line) and emission (solid line) spectra of 3,3'-dimethoxybenzidine and its dihydrochloride salt. Bottom. Standard curves for the two compounds.

other hand, alkaline hydrolysis enhanced the recovery of the salts by about 20%; the reason for this behavior is not fully understood. Acid hydrolyses of urine were also performed, however, formation of a purple-colored product in both control and spiked samples prevented the assay by SPF.

TABLE I

Analysis of two biological growth media spiked with benzidine, two congeners, and their salts at 0, 0.10, and 1.0 ppm

Compound	Medium	Added[a] ppm	Added[a] μg	Recovered ($\bar{x} \pm$ SE)[b] ppm	Recovered ($\bar{x} \pm$ SE)[b] %
Benzidine	BHI	0.0	0.0	0.040+0.000	—
		0.10	1.0	0.070±0.000	70.0±0.0
		1.00	10.0	0.780±0.004	78.0±0.4
	TSD	0.0	0.0	0.019±0.000	—
		0.10	1.0	0.075±0.000	75.0±0.0
		1.00	10.0	0.787±0.012	78.7±1.2
Benzidine·2HCl	BHI	0.0	0.0	0.040±0.000	—
		0.10	1.0	0.073±0.002	73.0±2.0
		1.00	10.0	0.795±0.000	79.5±0.0
	TSD	0.0	0.0	0.019±0.002	—
		0.10	1.0	0.073±0.001	73.0±1.0
		1.00	10.0	0.778±0.004	77.8±0.4
3,3′-Dimethylbenzidine	BHI	0.0	0.0	0.037±0.001	—
		0.10	1.0	0.074±0.005	74.0±5.0
		1.00	10.0	0.839±0.010	83.9±1.0
	TSD	0.0	0.0	0.029±0.004	—
		0.10	1.0	0.073±0.002	73.0±2.0
		1.00	10.0	0.902±0.009	90.2±0.9
3,3′-Dimethylbenzidine·2HCl	BHI	0.0	0.0	0.045±0.004	—
		0.10	1.0	0.070±0.002	70.0±2.0
		1.00	10.0	0.777±0.006	77.7±0.6
	TSD	0.0	0.0	0.036±0.003	—
		0.10	1.0	0.064±0.001	64.0±1.0
		1.00	10.0	0.710±0.003	71.0±0.3
3,3′-Dimethoxybenzidine	BHI	0.0	0.0	0.031±0.003	—
		0.10	1.0	0.078±0.001	78.0±1.0
		1.00	10.0	0.923±0.007	92.3±0.7
	TSD	0.0	0.0	0.019±0.001	—
		0.10	1.0	0.055±0.000	55.0±0.0
		1.00	10.0	0.829±0.008	82.9±0.8
3,3′-Dimethoxybenzidine·2HCl	BHI	0.0	0.0	0.030±0.003	—
		0.10	1.0	0.057±0.005	57.0±5.0
		1.00	10.0	0.715±0.002	71.5±0.2
	TSD	0.0	0.0	0.027±0.003	—
		0.10	1.0	0.052±0.001	52.0±1.0
		1.00	10.0	0.770±0.006	77.0±0.6

[a]Per 10 ml of sample.

[b]Mean and standard error from triplicate assays; spiked samples are corrected for controls. Controls and 0.10 ppm samples contained 1 g equivalent of medium/ml for SPF reading; the 1.0 ppm samples contained 0.2 g equivalents/ml.

Results of 16-day stability studies with aqueous solutions of benzidine (50 ppm) and its salt (50 and 500 ppm) under simulated animal test conditions are presented in Table V. The solutions of the salt were essentially stable with a decrease in concentration of less than 2% during the test period; whereas the free amine declined about 11%. In similar tests, 3,3'-dimethylbenzidine and its salt declined about 9%, and 3,3'-dimethoxybenzi-

TABLE II

Analysis of waste water spiked with benzidine, two congeners, and their salts at 0 and 20 ppb

Compound	Added[a]		Recovered ($\bar{x} \pm$ SE)[b]	
	ppb	μg	ppb	%
Benzidine	0	0.0	4±1	—
	20	2.0	17±0	85
Benzidine·2HCl	0	0.0	4±1	—
	20	2.0	15±1	75
3,3'-Dimethylbenzidine	0	0.0	4±1	—
	20	2.0	15±0	75
3,3'-Dimethylbenzidine·2HCl	0	0.0	3±1	—
	20	2.0	13±1	65
3,3'-Dimethoxybenzidine	0	0.0	3±0	—
	20	2.0	16±0	80
3,3'-Dimethoxybenzidine·2HCl	0	0.0	3±0	—
	20	2.0	14±1	70

[a]Per 100 ml of sample.

[b]Mean and standard error from triplicate assays; spiked samples are corrected for controls. Samples contained 10 g equivalents of water/ml for SPF reading.

dine and its salt declined about 64 and 9%, respectively. The results in Table V illustrate the excellent precision of the SPF procedure for the analysis of these compounds in potable water.

Partition values (the fraction of solute partitioning into the nonpolar phase of an equivolume immiscible binary solvent system) are useful in developing extraction and cleanup methods and for confirmatory tests.[15,16]

TABLE III

Analysis of whole rat blood spiked with benzidine, two congeners, and their salts at 0 and 10 ppm

Compound	Hydrolysis	Added[a]		Recovered ($\bar{x} \pm$ SE)[b]	
		ppm	μg	ppm	%
Benzidine	None	0.0	0.0	0.051±0.013	—
		10.0	10.0	0.668±0.216	6.7±2.2
	Alkaline	0.0	0.0	0.063±0.013	—
		10.0	10.0	2.52 ±0.34	25.2±3.4
	Acid	0.0	0.0	0.048±0.005	—
		10.0	10.0	7.39 ±0.09	73.9±0.9
Benzidine·2HCl	None	0.0	0.0	0.045±0.007	—
		10.0	10.0	0.563±0.108	5.6±1.1
	Alkaline	0.0	0.0	0.086±0.028	—
		10.0	10.0	2.49 ±0.31	24.9±3.1
	Acid	0.0	0.0	0.052±0.008	—
		10.0	10.0	7.57 ±0.125	75.7±1.2
3,3′-Dimethylbenzidine	None	0.0	0.0	0.152±0.052	—
		10.0	10.0	1.58 ±0.13	15.8±1.3
	Alkaline	0.0	0.0	0.276±0.044	—
		10.0	10.0	2.16 ±0.10	21.6±1.0
	Acid	0.0	0.0	0.223±0.006	—
		10.0	10.0	6.55 ±0.09	65.5±0.9
3,3′-Dimethylbenzidine·2HCl	None	0.0	0.0	0.152±0.052	—
		10.0	10.0	2.85 ±0.19	28.5±1.9
	Alkaline	0.0	0.0	0.276±0.044	—
		10.0	10.0	2.11 ±0.06	21.1±0.6
	Acid	0.0	0.0	0.223±0.006	—
		10.0	10.0	6.04 ±0.37	60.4±3.7
3,3′-Dimethoxybenzidine	None	0.0	0.0	0.128±0.049	—
		10.0	10.0	0.464±0.098	4.6±1.0
	Alkaline	0.0	0.0	0.236±0.034	—
		10.0	10.0	1.19 ±0.47	11.9±4.7
	Acid	0.0	0.0	0.192±0.006	—
		10.0	10.0	5.35 ±0.06	53.5±0.6
3,3′-Dimethoxybenzidine·2HCl	None	0.0	0.0	0.128±0.049	—
		10.0	10.0	2.10 ±0.20	21.0±2.0
	Alkaline	0.0	0.0	0.236±0.034	—
		10.0	10.0	1.01 ±0.18	10.1±1.8
	Acid	0.0	0.0	0.192±0.006	—
		10.0	10.0	4.64 ±0.14	46.4±1.4

[a]Per ml of sample.

[b]Mean and standard error from triplicate assays; spiked samples are corrected for controls. Control and spiked samples contained 200 and 20 mg equivalents of blood/ml, respectively for the SPF readings.

TABLE IV

Analysis of human urine spiked with benzidine, two congeners, and their salts at 0 and 20 ppb

Compound	Hydrolysis[c]	Added[a]		Recovered ($\bar{x} \pm$ SE)[b]	
		ppb	μg	ppb	%
Benzidine	None	0	0.0	5±0	—
		20	2.0	18±1	90
	Alkaline	0	0.0	6±1	—
		20	2.0	13±1	65
Benzidine·2HCl	None	0	0.0	5±0	—
		20	2.0	7±1	35
	Alkaline	0	0.0	6±1	—
		20	2.0	11±1	55
3,3′-Dimethylbenzidine	None	0	0.0	5±0	—
		20	2.0	18±1	90
	Alkaline	0	0.0	6±1	—
		20	2.0	14±1	70
3,3′-Dimethylbenzidine·2HCl	None	0	0.0	5±0	—
		20	2.0	8±1	40
	Alkaline	0	0.0	6±1	—
		20	2.0	11±2	55
3,3′-Dimethoxybenzidine	None	0	0.0	4±1	—
		20	2.0	18±2	90
	Alkaline	0	0.0	5±1	—
		20	2.0	14±1	70
3,3′-Dimethoxybenzidine·2HCl	None	0	0.0	4±0	—
		20	2.0	8±1	40
	Alkaline	0	0.0	5±1	—
		20	2.0	12±1	60

[a]Per 100 ml of sample.

[b]Mean and standard error of triplicate assays; spiked samples are corrected for controls. Control and spiked samples contained 10 g equivalents of urine/ml for the SPF readings.

[c]Development of purple-colored product upon acid hydrolysis of unspiked urine prevented the assay via SPF.

TABLE V

Stability of aqueous solutions of benzidine and its dihydrochloride after exposure to simulated animal test conditions for 16 days

Sampling Interval (days)	Concentration and pH of Solution Indicated[a]					
	Benzidine		Benzidine·2HCl			
	50 ppm Solution		50 ppm Solution		500 ppm Solution	
	ppm	pH	ppm	pH	ppm	pH
0	54.0±0.0	6.70±0.10	49.7±0.2	3.95±0.01	507±3	3.30±0.02
1	52.0±0.2	6.35±0.15	49.6±0.2	3.95±0.01	496±2	3.26±0.01
2	53.8±2.9	6.65±0.12	49.7±0.3	4.00±0.21	493±3	3.25±0.02
4	52.8±0.5	6.45±0.10	49.4±0.5	4.07±0.02	498±4	3.34±0.02
8	50.2±0.7	6.35±0.30	49.9±0.1	4.10±0.02	504±4	3.35±0.05
16	47.8±0.7	6.25±0.18	48.9±0.2	3.98±0.07	497±4	3.35±0.03

[a]Mean and standard error from triplicate assays.

The following values were obtained for the three free amines by using gas chromatography:

Solvent System	p-Value		
	Benzidine	3,3′-Dimethyl-benzidine	3,3′-Dimethoxy-benzidine
Hexane-Acetonitrile	0.02	0.01	0.03
Hexane-80% Acetone (20% Water)	0.08	0.16	0.15
Chloroform-Water	1.0	1.0	1.0
Chloroform-60% Methanol (40% Water)	0.85	0.96	0.97
Chloroform-Aqueous NaOH (5.0, 0.5, or 0.05 N)	1.0	1.0	1.0
Chloroform-Aqueous HCl (5.0, 0.5, or 0.05 N)	0.00	0.00	0.00
Hexane-Dimethylformamide	0.00	0.00	0.00

Solubility data for the three amines and their dihydrochloride salts were required before analytical method development or formulation of the spiked animal drinking water could be undertaken; precise values could not be found in the literature. Results of our determinations via SPF were:

Compound	Solubility in water (mg/ml) at 25±2°C
Benzidine	0.52
Benzidine·2HCl	61.7
3,3′-Dimethylbenzidine	1.3
3,3′-Dimethylbenzidine·2HCl	76.7
3,3′-Dimethoxybenzidine	0.06
3,3′-Dimethoxybenzidine·2HCl	41.4

TLC R_f values for the three free amines and anthracene (included as a reference compound) in ten solvent systems are reported in Table VI. These data are useful in the development of cleanup procedures and for the separation and identification of the compounds in admixture. The three compounds

TABLE VI

TLC R_f values of benzidine, 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, and anthracene in ten solvent systems

Solvent System (v/v)	R_f values (×100) of compound indicated			
	Benzidine	3,3′-dimethyl-benzidine	3,3′-dimethoxy-benzidine	Anthracene
Chloroform	10	14	11	100
Chloroform-Ethyl Acetate (9/1)	20	29	29	92
Chloroform-Diethyl Ether (9/1)	21	32	32	100
Chloroform-Acetone (9/1)	24	33	38	83
Chloroform-Methanol (9/1)	70	80	85	97
Benzene	2	2	2	69
Benzene-Ethyl Acetate (9/1)	9	11	13	73
Benzene-Diethyl Ether (9/1)	6	10	11	75
Benzene-Acetone (9/1)	16	21	26	76
Benzene-Methanol (9/1)	33	45	50	81

were separated by using 10% acetone or methanol in chloroform or benzene. Aqueous solutions of the salts spotted and developed as described, gave R_f values identical to those of the free amines in all of the solvent systems tested.

At the onset of animal tests with benzidine·2HCl-treated drinking water, routine monitoring of the air and work areas was also initiated to signal any accidental exposure of our personnel to traces of the compound. Air samples were collected by using a Model No. EMWL-2000H High Volume Air Sampler (General Metal Works, Inc., Cleves, OH) equipped with a No. 3000 (20 × 25 cm) fiberglass filter (retains 99.9% of particles larger than 0.3 microns in diameter) and operated continuously during the work day with an air flow of 1.42 m^3/min; the filter was removed weekly for chemical analysis and a new one was installed. For analysis, the filter was cut into small pieces, mechanically shaken with 250 ml of 0.1 HCl for 1 hr, filtered, made alkaline with 5 ml of 10 N NaOH, and extracted three times with 25-ml portions of chloroform. The combined chloroform extracts successively percolated through a plug of sodium sulfate were evaporated to dryness in the presence of 1 drop of keeper; the dry residue was dissolved in benzene for cleanup and analysis as described for waste water. The SPF background of a new filter is equivalent to about 0.4 μg of benzidine, whereas, after a week in the sampling device, it averages about 10 μg. This background fluorescence has not exhibited the characteristic excitation and emission maxima of the compound sought and, thus far, all filters except those spiked in the laboratory have contained no detectable residues of the test chemical.

The monitoring of work areas (cages, floors, benches, apparatus, etc.) suspected of being contaminated with benzidine·2HCl (or benzidine) is accomplished by using kits consisting of a cotton applicator and a 5-ml culture tube containing exactly 2 ml of the aqueous buffer. The applicator is moistened with the aqueous buffer and used to swab a specific area then the applicator is vigorously stirred in the buffer after each of several subsequent swabbings of the same area. The tube and contents are then centrifuged at 2000 rpm for 10 min to remove any suspended material. One ml of the supernatant is either analyzed directly or appropriately diluted as described for the analysis of potable water. Background fluorescence is generally equivalent to 0.10 μg of benzidine; areas contaminated with as little as 0.30 μg of the salt are readily detected and the identity of the chemical confirmed by its characteristic excitation and emission maxima.

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References

1. L. Rehn, *Arch. Klin. Chirugie* **50,** 588 (1895).
2. *Federal Register* **39,** 3756 (1974).
3. T. L. Haley, *Clin. Toxicol.* **8,** 13 (1975).
4. L. J. Sciarini and J. W. Meigs, *A. M. A. Arch. Industr. Health* **18,** 521 (1958).
5. R. K. Baker and J. G. Deighton, *Cancer Res.* **13,** 529 (1953).
6. L. A. Elson, F. Goulden and F. L. Warren, *Brit. J. Cancer* **12,** 108 (1958).
7. M. A. El-Dib, J. *Ass. Offic. Anal. Chemists* **54,** 1383 (1971).
8. J. M. Glassman and J. W. Meigs, *A. M. A. Arch. Ind. Hyg. Occup. Med.* **4,** 519 (1951).
9. L. J. Sciarini and J. A. Mahew, *A. M. A. Arch. Ind. Hyg.* **11,** 420 (1955).
10. G. Ghetti, *Med. Lavoro* **51,** 102 (1965).
11. T. Akiyama, *Jikei, Med. J.* **17,** 1 (1970).
12. D. B. Clayson, E. Ward and L. Ward, *Acta Unio. Intl. Contra. Concerum* **15,** 581 (1959).
13. E. Rinde, Thesis, New York Univ. (1974).
14. M. C. Bowman and J. R. King, *Biochem. Med.* **9,** 390 (1974).
15. M. C. Bowman and M. Beroza, *Anal. Chem.* **38,** 1544 (1966).
16. M. Beroza and M. C. Bowman, *Anal. Chem.* **37,** 291 (1965).