

**APPENDIX A TO PART 136  
METHODS FOR ORGANIC CHEMICAL ANALYSIS OF MUNICIPAL AND  
INDUSTRIAL WASTEWATER**

**METHOD 605—BENZIDINES**

**1. Scope and Application**

- 1.1 This method covers the determination of certain benzidines. The following parameters can be determined by this method:

Parameter	Storet No.	CAS No.
Benzidine . . . . .	39120	92-87-5
3,3'-Dichlorobenzidine . . . . .	34631	91-94-1

- 1.2 This is a high performance liquid chromatography (HPLC) method applicable to the determination of the compounds listed above in municipal and industrial discharges as provided under 40 CFR Part 136.1. When this method is used to analyze unfamiliar samples for the compounds above, identifications should be supported by at least one additional qualitative technique. This method describes electrochemical conditions at a second potential which can be used to confirm measurements made with this method. Method 625 provides gas chromatograph/mass spectrometer (GC/MS) conditions appropriate for the qualitative and quantitative confirmation of results for the parameters listed above, using the extract produced by this method.
- 1.3 The method detection limit (MDL, defined in Section 14.1)<sup>1</sup> for each parameter is listed in Table 1. The MDL for a specific wastewater may differ from those listed, depending upon the nature of the interferences in the sample matrix.
- 1.4 Any modification of this method, beyond those expressly permitted, shall be considered as a major modification subject to application and approval of alternate test procedures under 40 CFR Parts 136.4 and 136.5.
- 1.5 This method is restricted to use by or under the supervision of analysts experienced in the use of HPLC instrumentation and in the interpretation of liquid chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method using the procedure described in Section 8.2.

**2. Summary of Method**

- 2.1 A measured volume of sample, approximately 1-L, is extracted with chloroform using liquid-liquid extractions in a separatory funnel. The chloroform extract is extracted with acid. The acid extract is then neutralized and extracted with chloroform. The final chloroform extract is exchanged to methanol while being concentrated using a rotary evaporator. The extract is mixed with buffer and separated by HPLC. The benzidine compounds are measured with an electrochemical detector.<sup>2</sup>

2.2 The acid back-extraction acts as a general purpose cleanup to aid in the elimination of interferences.

### **3. Interferences**

3.1 Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in chromatograms. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in Section 8.1.3.

3.1.1 Glassware must be scrupulously cleaned.<sup>3</sup> Clean all glassware as soon as possible after use by rinsing with the last solvent used in it. Solvent rinsing should be followed by detergent washing with hot water, and rinses with tap water and distilled water. The glassware should then be drained dry, and heated in a muffle furnace at 400°C for 15-30 minutes. Some thermally stable materials may not be eliminated by this treatment. Solvent rinses with acetone and pesticide quality hexane may be substituted for the muffle furnace heating. Volumetric ware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with aluminum foil.

3.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

3.2 Matrix interferences may be caused by contaminants that are co-extracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. The cleanup procedures that are inherent in the extraction step are used to overcome many of these interferences, but unique samples may require additional cleanup approaches to achieve the MDL listed in Table 1.

3.3 Some dye plant effluents contain large amounts of components with retention times closed to benzidine. In these cases, it has been found useful to reduce the electrode potential in order to eliminate interferences and still detect benzidine. (See Section 12.7.)

### **4. Safety**

4.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified<sup>4-6</sup> for the information of the analyst.

- 4.2 The following parameters covered by this method have been tentatively classified as known or suspected, human or mammalian carcinogens: benzidine and 3,3'-dichlorobenzidine. Primary standards of these toxic compounds should be prepared in a hood. A NIOSH/MESA approved toxic gas respirator should be worn when the analyst handles high concentrations of these toxic compounds.
- 4.3 Exposure to chloroform should be minimized by performing all extractions and extract concentrations in a hood or other well-ventilated area.

## 5. Apparatus and Materials

- 5.1 Sampling equipment, for discrete or composite sampling.
- 5.1.1 Grab sample bottle—1 L or 1 qt, amber glass, fitted with a screw cap lined with Teflon. Foil may be substituted for Teflon if the sample is not corrosive. If amber bottles are not available, protect samples from light. The bottle and cap liner must be washed, rinsed with acetone or methylene chloride, and dried before use to minimize contamination.
- 5.1.2 Automatic sampler (optional)—The sampler must incorporate glass sample containers for the collection of a minimum of 250 mL of sample. Sample containers must be kept refrigerated at 4°C and protected from light during compositing. If the sampler uses a peristaltic pump, a minimum length of compressible silicone rubber tubing may be used. Before use, however, the compressible tubing should be thoroughly rinsed with methanol, followed by repeated rinsings with distilled water to minimize the potential for contamination of the sample. An integrating flow meter is required to collect flow proportional composites.
- 5.2 Glassware (All specifications are suggested)
- 5.2.1 Separatory funnels—2000, 1000, and 250 mL, with Teflon stopcock.
- 5.2.2 Vials—10-15 mL, amber glass, with Teflon-lined screw cap.
- 5.2.3 Rotary evaporator.
- 5.2.4 Flasks—Round bottom, 100 mL, with 24/40 joints.
- 5.2.5 Centrifuge tubes—Conical, graduated, with Teflon-lined screw caps.
- 5.2.6 Pipettes—Pasteur, with bulbs.
- 5.3 Balance—Analytical, capable of accurately weighing 0.0001 g.
- 5.4 High performance liquid chromatograph (HPLC)—An analytical system complete with column supplies, high pressure syringes, detector, and compatible recorder. A data system is recommended for measuring peak areas and retention times.
- 5.4.1 Solvent delivery system—With pulse damper, Altex 110A or equivalent.

- 5.4.2 Injection valve (optional)—Waters U6K or equivalent.
- 5.4.3 Electrochemical detector—Bioanalytical Systems LC-2A with glassy carbon electrode, or equivalent. This detector has proven effective in the analysis of wastewaters for the parameters listed in the scope (Section 1.1), and was used to develop the method performance statements in Section 14. Guidelines for the use of alternate detectors are provided in Section 12.1.
- 5.4.4 Electrode polishing kit—Princeton Applied Research Model 9320 or equivalent.
- 5.4.5 Column—Lichrosorb RP-2, 5 micron particle diameter, in a 25 cm x 4.6 mm ID stainless steel column. This column was used to develop the method performance statements in Section 14. Guidelines for the use of alternate column packings are provided in Section 12.1.

## **6. Reagents**

- 6.1 Reagent water—Reagent water is defined as a water in which an interferent is not observed at the MDL of the parameters of interest.
- 6.2 Sodium hydroxide solution (5 N)—Dissolve 20 g of NaOH (ACS) in reagent water and dilute to 100 mL.
- 6.3 Sodium hydroxide solution (1 M)—Dissolve 40 g of NaOH (ACS) in reagent water and dilute to 1 L.
- 6.4 Sodium thiosulfate—(ACS) Granular.
- 6.5 Sodium tribasic phosphate (0.4 M)—Dissolve 160 g of trisodium phosphate decahydrate (ACS) in reagent water and dilute to 1 L.
- 6.6 Sulfuric acid (1+1)—Slowly, add 50 mL of H<sub>2</sub>SO<sub>4</sub> (ACS, sp. gr. 1.84) to 50 mL of reagent water.
- 6.7 Sulfuric acid (1 M)—Slowly, add 58 mL of H<sub>2</sub>SO<sub>4</sub> (ACS, sp. gr. 1.84) to reagent water and dilute to 1 L.
- 6.8 Acetate buffer (0.1 M, pH 4.7)—Dissolve 5.8 mL of glacial acetic acid (ACS) and 13.6 g of sodium acetate trihydrate (ACS) in reagent water which has been purified by filtration through a RO-4 Millipore System or equivalent and dilute to 1 L.
- 6.9 Acetonitrile, chloroform (preserved with 1% ethanol), methanol—Pesticide quality or equivalent.
- 6.10 Mobile phase—Place equal volumes of filtered acetonitrile (Millipore type FH filter or equivalent) and filtered acetate buffer (Millipore type GS filter or equivalent) in a narrow-mouth, glass container and mix thoroughly. Prepare fresh weekly. Degas daily by sonicating under vacuum, by heating and stirring, or by purging with helium.

- 6.11 Stock standard solutions (1.00 µg/µL)—Stock standard solutions may be prepared from pure standard materials or purchased as certified solutions.
- 6.11.1 Prepare stock standard solutions by accurately weighing about 0.0100 g of pure material. Dissolve the material in methanol and dilute to volume in a 10 mL volumetric flask. Larger volumes can be used at the convenience of the analyst. When compound purity is assayed to be 96% or greater, the weight can be used without correction to calculate the concentration of the stock standard. Commercially prepared stock standards can be used at any concentration if they are certified by the manufacturer or by an independent source.
- 6.11.2 Transfer the stock standard solutions into Teflon-sealed screw-cap bottles. Store at 4°C and protect from light. Stock standard solutions should be checked frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them.
- 6.11.3 Stock standard solutions must be replaced after six months, or sooner if comparison with check standards indicates a problem.
- 6.12 Quality control check sample concentrate—See Section 8.2.1.

## 7. Calibration

- 7.1 Establish chromatographic operating conditions equivalent to those given in Table 1. The HPLC system can be calibrated using the external standard technique (Section 7.2) or the internal standard technique (Section 7.3).
- 7.2 External standard calibration procedure
- 7.2.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask and diluting to volume with mobile phase. One of the external standards should be at a concentration near, but above, the MDL (Table 1) and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.
- 7.2.2 Using syringe injections of 5-25 µL or a constant volume injection loop, analyze each calibration standard according to Section 12 and tabulate peak height or area responses against the mass injected. The results can be used to prepare a calibration curve for each compound. Alternatively, if the ratio of response to amount injected (calibration factor) is a constant over the working range (<10% relative standard deviation, RSD), linearity through the origin can be assumed and the average ratio or calibration factor can be used in place of a calibration curve.
- 7.3 Internal standard calibration procedure—To use this approach, the analyst must select one or more internal standards that are similar in analytical behavior to the compounds of interest. The analyst must further demonstrate that the measurement of the internal

standard is not affected by method or matrix interferences. Because of these limitations, no internal standard can be suggested that is applicable to all samples.

- 7.3.1 Prepare calibration standards at a minimum of three concentration levels for each parameter of interest by adding volumes of one or more stock standards to a volumetric flask. To each calibration standard, add a known constant amount of one or more internal standards, and dilute to volume with mobile phase. One of the standards should be at a concentration near, but above, the MDL and the other concentrations should correspond to the expected range of concentrations found in real samples or should define the working range of the detector.
- 7.3.2 Using syringe injections of 5-25  $\mu\text{L}$  or a constant volume injection loop, analyze each calibration standard according to Section 12 and tabulate peak height or area responses against concentration for each compound and internal standard. Calculate response factors (RF) for each compound using Equation 1.

*Equation 1*

$$\text{RF} = \frac{(A_s) (C_{is})}{(A_{is}) (C_s)}$$

where:

$A_s$  = Response for the parameter to be measured.

$A_{is}$  = Response for the internal standard.

$C_{is}$  = Concentration of the internal standard ( $\mu\text{g}/\text{L}$ ).

$C_s$  = Concentration of the parameter to be measured ( $\mu\text{g}/\text{L}$ ).

If the RF value over the working range is a constant ( $<10\%$  RSD), the RF can be assumed to be invariant and the average RF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios,  $A_s/A_{is}$ , vs. concentration ratios  $C_s/C_{is}$ .

- 7.4 The working calibration curve, calibration factor, or RF must be verified on each working day by the measurement of one or more calibration standards. If the response for any parameter varies from the predicted response by more than  $\pm 15\%$ , a new calibration curve must be prepared for that compound. If serious loss of response occurs, polish the electrode and recalibrate.
- 7.5 Before using any cleanup procedure, the analyst must process a series of calibration standards through the procedure to validate elution patterns and the absence of interferences from the reagents.

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\*This equation corrects an error made in the original method publication (49 FR 43234, October 26, 1984). This correction will be formalized through a rulemaking in FY97.

## 8. Quality Control

- 8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and an ongoing analysis of spiked samples to evaluate and document data quality. The laboratory must maintain records to document the quality of data that is generated. Ongoing data quality checks are compared with established performance criteria to determine if the results of analyses meet the performance characteristics of the method. When results of sample spikes indicate atypical method performance, a quality control check standard must be analyzed to confirm that the measurements were performed in an in-control mode of operation.
- 8.1.1 The analyst must make an initial, one-time, demonstration of the ability to generate acceptable accuracy and precision with this method. This ability is established as described in Section 8.2.
- 8.1.2 In recognition of advances that are occurring in chromatography, the analyst is permitted certain options (detailed in Sections 10.9, 11.1, and 12.1) to improve the separations or lower the cost of measurements. Each time such a modification is made to the method, the analyst is required to repeat the procedure in Section 8.2.
- 8.1.3 Before processing any samples, the analyst must analyze a reagent water blank to demonstrate that interferences from the analytical system and glassware are under control. Each time a set of samples is extracted or reagents are changed, a reagent water blank must be processed as a safeguard against laboratory contamination.
- 8.1.4 The laboratory must, on an ongoing basis, spike and analyze a minimum of 10% of all samples to monitor and evaluate laboratory data quality. This procedure is described in Section 8.3.
- 8.1.5 The laboratory must, on an ongoing basis, demonstrate through the analyses of quality control check standards that the operation of the measurement system is in control. This procedure is described in Section 8.4. The frequency of the check standard analyses is equivalent to 10% of all samples analyzed but may be reduced if spike recoveries from samples (Section 8.3) meet all specified quality control criteria.
- 8.1.6 The laboratory must maintain performance records to document the quality of data that is generated. This procedure is described in Section 8.5.
- 8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.
- 8.2.1 A quality control (QC) check sample concentrate is required containing benzidine and/or 3,3'-dichlorobenzidine at a concentration of 50 µg/mL each in methanol. The QC check sample concentrate must be obtained from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory in Cincinnati, Ohio, if available. If not available from that source, the QC check sample concentrate must be obtained from another external source. If not

available from either source above, the QC check sample concentrate must be prepared by the laboratory using stock standards prepared independently from those used for calibration.

- 8.2.2 Using a pipet, prepare QC check samples at a concentration of 50 µg/L by adding 1.00 mL of QC check sample concentrate to each of four 1 L aliquots of reagent water.
  - 8.2.3 Analyze the well-mixed QC check samples according to the method beginning in Section 10.
  - 8.2.4 Calculate the average recovery ( $\bar{X}$ ) in µg/L, and the standard deviation of the recovery (s) in µg/L, for each parameter using the four results.
  - 8.2.5 For each parameter compare s and  $\bar{X}$  with the corresponding acceptance criteria for precision and accuracy, respectively, found in Table 2. If s and  $\bar{X}$  for all parameters of interest meet the acceptance criteria, the system performance is acceptable and analysis of actual samples can begin. If any individual s exceeds the precision limit or any individual  $\bar{X}$  falls outside the range for accuracy, the system performance is unacceptable for that parameter. Locate and correct the source of the problem and repeat the test for all parameters of interest beginning with Section 8.2.2.
- 8.3 The laboratory must, on an ongoing basis, spike at least 10% of the samples from each sample site being monitored to assess accuracy. For laboratories analyzing one to ten samples per month, at least one spiked sample per month is required.
- 8.3.1 The concentration of the spike in the sample should be determined as follows:
    - 8.3.1.1 If, as in compliance monitoring, the concentration of a specific parameter in the sample is being checked against a regulatory concentration limit, the spike should be at that limit or one to five times higher than the background concentration determined in Section 8.3.2, whichever concentration would be larger.
    - 8.3.1.2 If the concentration of a specific parameter in the sample is not being checked against a limit specific to that parameter, the spike should be at 50 µg/L or one to five times higher than the background concentration determined in Section 8.3.2, whichever concentration would be larger.
    - 8.3.1.3 If it is impractical to determine background levels before spiking (e.g., maximum holding times will be exceeded), the spike concentration should be (1) the regulatory concentration limit, if any; or, if none (2) the larger of either five times higher than the expected background concentration or 50 µg/L.
  - 8.3.2 Analyze one sample aliquot to determine the background concentration (B) of each parameter. If necessary, prepare a new QC check sample concentrate (Section 8.2.1) appropriate for the background concentrations in the sample. Spike a second sample aliquot with 1.0 mL of the QC check sample concentrate and



analyze it to determine the concentration after spiking (A) of each parameter. Calculate each percent recovery (P) as  $100(A-B)/T$ , where T is the known true value of the spike.

- 8.3.3 Compare the percent recovery (P) for each parameter with the corresponding QC acceptance criteria found in Table 2. These acceptance criteria were calculated to include an allowance for error in measurement of both the background and spike concentrations, assuming a spike to background ratio of 5:1. This error will be accounted for to the extent that the analyst's spike to background ratio approaches 5:1.<sup>7</sup> If spiking was performed at a concentration lower than 50 µg/L, the analyst must use either the QC acceptance criteria in Table 2, or optional QC acceptance criteria calculated for the specific spike concentration. To calculate optional acceptance criteria for the recovery of a parameter: (1) Calculate accuracy (X') using the equation in Table 3, substituting the spike concentration (T) for C; (2) calculate overall precision (S') using the equation in Table 3, substituting X' for X; (3) calculate the range for recovery at the spike concentration as  $(100 X'/T) \pm 2.44 (100 S'/T)\%$ .<sup>7</sup>
- 8.3.4 If any individual P falls outside the designated range for recovery, that parameter has failed the acceptance criteria. A check standard containing each parameter that failed the criteria must be analyzed as described in Section 8.4.
- 8.4 If any parameter fails the acceptance criteria for recovery in Section 8.3, a QC check standard containing each parameter that failed must be prepared and analyzed.
- NOTE:* The frequency for the required analysis of a QC check standard will depend upon the number of parameters being simultaneously tested, the complexity of the sample matrix, and the performance of the laboratory.
- 8.4.1 Prepare the QC check standard by adding 1.0 mL of QC check sample concentrate (Section 8.2.1 or 8.3.2) to 1 L of reagent water. The QC check standard needs only to contain the parameters that failed criteria in the test in Section 8.3.
- 8.4.2 Analyze the QC check standard to determine the concentration measured (A) of each parameter. Calculate each percent recovery (P<sub>s</sub>) as  $100 (A/T)\%$ , where T is the true value of the standard concentration.
- 8.4.3 Compare the percent recovery (P<sub>s</sub>) for each parameter with the corresponding QC acceptance criteria found in Table 2. Only parameters that failed the test in Section 8.3 need to be compared with these criteria. If the recovery of any such parameter falls outside the designated range, the laboratory performance for that parameter is judged to be out of control, and the problem must be immediately identified and corrected. The analytical result for that parameter in the unspiked sample is suspect and may not be reported for regulatory compliance purposes.
- 8.5 As part of the QC program for the laboratory, method accuracy for wastewater samples must be assessed and records must be maintained. After the analysis of five spiked wastewater samples as in Section 8.3, calculate the average percent recovery ( $\bar{P}$ ) and the standard deviation of the percent recovery ( $s_p$ ). Express the accuracy assessment as a percent recovery interval from  $\bar{P} - 2s_p$  to  $\bar{P} + 2s_p$ . If  $\bar{P} = 90\%$  and  $s_p = 10\%$ , for example, the

accuracy interval is expressed as 70-110%. Update the accuracy assessment for each parameter on a regular basis (e.g., after each 5-10 new accuracy measurements).

- 8.6 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to assess the precision of the environmental measurements. When doubt exists over the identification of a peak on the chromatogram, confirmatory techniques such as HPLC with a dissimilar column, gas chromatography, or mass spectrometer must be used. Whenever possible, the laboratory should analyze standard reference materials and participate in relevant performance evaluation studies.

## **9. Sample Collection, Preservation, and Handling**

- 9.1 Grab samples must be collected in glass containers. Conventional sampling practices<sup>8</sup> should be followed, except that the bottle must not be prerinsed with sample before collection. Composite samples should be collected in refrigerated glass containers in accordance with the requirements of the program. Automatic sampling equipment must be as free as possible of Tygon tubing and other potential sources of contamination.
- 9.2 All samples must be iced or refrigerated at 4°C and stored in the dark from the time of collection until extraction. Both benzidine and 3,3'-dichlorobenzidine are easily oxidized. Fill the sample bottles and, if residual chlorine is present, add 80 mg of sodium thiosulfate per liter of sample and mix well. EPA Methods 330.4 and 330.5 may be used for measurement of residual chlorine.<sup>9</sup> Field test kits are available for this purpose. After mixing, adjust the pH of the sample to a range of 2-7 with sulfuric acid.
- 9.3 If 1,2-diphenylhydrazine is likely to be present, adjust the pH of the sample to 4.0 ±0.2 to prevent rearrangement to benzidine.
- 9.4 All samples must be extracted within seven days of collection. Extracts may be held up to seven days before analysis, if stored under an inert (oxidant free) atmosphere.<sup>2</sup> The extract should be protected from light.

## **10. Sample Extraction**

- 10.1 Mark the water meniscus on the side of the sample bottle for later determination of sample volume. Pour the entire sample into a 2-L separatory funnel. Check the pH of the sample with wide-range pH paper and adjust to within the range of 6.5 - 7.5 with sodium hydroxide solution or sulfuric acid.
- 10.2 Add 100 mL of chloroform to the sample bottle, seal, and shake 30 seconds to rinse the inner surface. (Caution: Handle chloroform in a well ventilated area.) Transfer the solvent to the separatory funnel and extract the sample by shaking the funnel for two minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration of the emulsion through

glass wool, centrifugation, or other physical methods. Collect the chloroform extract in a 250 mL separatory funnel.

- 10.3 Add a 50 mL volume of chloroform to the sample bottle and repeat the extraction procedure a second time, combining the extracts in the separatory funnel. Perform a third extraction in the same manner.
- 10.4 Separate and discard any aqueous layer remaining in the 250 mL separatory funnel after combining the organic extracts. Add 25 mL of 1 M sulfuric acid and extract the sample by shaking the funnel for two minutes. Transfer the aqueous layer to a 250-mL beaker. Extract with two additional 25 mL portions of 1 M sulfuric acid and combine the acid extracts in the beaker.
- 10.5 Place a stirbar in the 250 mL beaker and stir the acid extract while carefully adding 5 mL of 0.4 M sodium tribasic phosphate. While monitoring with a pH meter, neutralize the extract to a pH between 6 and 7 by dropwise addition of 5 N sodium hydroxide solution while stirring the solution vigorously. Approximately 25-30 mL of 5 N sodium hydroxide solution will be required and it should be added over at least a two-minute period. Do not allow the sample pH to exceed 8.
- 10.6 Transfer the neutralized extract into a 250-mL separatory funnel. Add 30 mL of chloroform and shake the funnel for two minutes. Allow the phases to separate, and transfer the organic layer to a second 250-mL separatory funnel.
- 10.7 Extract the aqueous layer with two additional 20 mL aliquots of chloroform as before. Combine the extracts in the 250-mL separatory funnel.
- 10.8 Add 20 mL of reagent water to the combined organic layers and shake for 30 seconds.
- 10.9 Transfer the organic extract into a 100-mL round bottom flask. Add 20 mL of methanol and concentrate to 5 mL with a rotary evaporator at reduced pressure and 35°C. An aspirator is recommended for use as the source of vacuum. Chill the receiver with ice. This operation requires approximately 10 minutes. Other concentration techniques may be used if the requirements of Section 8.2 are met.
- 10.10 Using a 9 in. pasteur pipette, transfer the extract to a 15-mL, conical, screw-cap centrifuge tube. Rinse the flask, including the entire side wall, with 2 mL portions of methanol and combine with the original extract.
- 10.11 Carefully concentrate the extract to 0.5 mL using a gentle stream of nitrogen while heating in a 30°C water bath. Dilute to 2 mL with methanol, reconcentrate to 1 mL, and dilute to 5 mL with acetate buffer. Mix the extract thoroughly. Cap the centrifuge tube and store refrigerated and protected from light if further processing will not be performed immediately. If the extract will be stored longer than two days, it should be transferred to a Teflon-sealed screw-cap vial. If the sample extract requires no further cleanup, proceed with HPLC analysis (Section 12). If the sample requires further cleanup, proceed to Section 11.

10.12 Determine the original sample volume by refilling the sample bottle to the mark and transferring the liquid to a 1000-mL graduated cylinder. Record the sample volume to the nearest 5 mL.

## **11. Cleanup and Separation**

11.1 Cleanup procedures may not be necessary for a relatively clean sample matrix. If particular circumstances demand the use of a cleanup procedure, the analyst first must demonstrate that the requirements of Section 8.2 can be met using the method as revised to incorporate the cleanup procedure.

## **12. High Performance Liquid Chromatography**

12.1 Table 1 summarizes the recommended operating conditions for the HPLC. Included in this table are retention times, capacity factors, and MDL that can be achieved under these conditions. An example of the separations achieved by this HPLC column is shown in Figure 1. Other HPLC columns, chromatographic conditions, or detectors may be used if the requirements of Section 8.2 are met. When the HPLC is idle, it is advisable to maintain a 0.1 mL/min flow through the column to prolong column life.

12.2 Calibrate the system daily as described in Section 7.

12.3 If the internal standard calibration procedure is being used, the internal standard must be added to the sample extract and mixed thoroughly immediately before injection into the instrument.

12.4 Inject 5-25  $\mu\text{L}$  of the sample extract or standard into the HPLC. If constant volume injection loops are not used, record the volume injected to the nearest 0.05  $\mu\text{L}$ , and the resulting peak size in area or peak height units.

12.5 Identify the parameters in the sample by comparing the retention times of the peaks in the sample chromatogram with those of the peaks in standard chromatograms. The width of the retention time window used to make identifications should be based upon measurements of actual retention time variations of standards over the course of a day. Three times the standard deviation of a retention time for a compound can be used to calculate a suggested window size; however, the experience of the analyst should weigh heavily in the interpretation of chromatograms.

12.6 If the response for a peak exceeds the working range of the system, dilute the extract with mobile phase and reanalyze.

12.7 If the measurement of the peak response for benzidine is prevented by the presence of interferences, reduce the electrode potential to +0.6 V and reanalyze. If the benzidine peak is still obscured by interferences, further cleanup is required.

## **13. Calculations**

13.1 Determine the concentration of individual compounds in the sample.

- 13.1.1 If the external standard calibration procedure is used, calculate the amount of material injected from the peak response using the calibration curve or calibration factor determined in Section 7.2.2. The concentration in the sample can be calculated from Equation 2.

*Equation 2*

$$\text{Concentration } (\mu\text{g/L}) = \frac{(A) (V_t)}{(V_i) (V_s)}$$

where:

- A = Amount of material injected (ng).
- V<sub>i</sub> = Volume of extract injected (μL).
- V<sub>t</sub> = Volume of total extract (μL).
- V<sub>s</sub> = Volume of water extracted (mL).

- 13.1.2 If the internal standard calibration procedure is used, calculate the concentration in the sample using the response factor (RF) determined in Section 7.3.2 and Equation 3.

*Equation 3*

$$\text{Concentration } (\mu\text{g/L}) = \frac{(A_s) (I_s)}{(A_{is}) (RF) (V_o)}$$

where:

- A<sub>s</sub> = Response for the parameter to be measured.
- A<sub>is</sub> = Response for the internal standard.
- I<sub>s</sub> = Amount of internal standard added to each extract (μg).
- V<sub>o</sub> = Volume of water extracted (L).

- 13.2 Report results in μg/L without correction for recovery data. All QC data obtained should be reported with the sample results.

## 14. Method Performance

- 14.1 The method detection limit (MDL) is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the value is above zero.<sup>1</sup> The MDL concentrations listed in Table 1 were obtained using reagent water. Similar results were achieved using representative wastewaters. The MDL actually achieved in a given analysis will vary depending on instrument sensitivity and matrix effects.
- 14.2 This method has been tested for linearity of spike recovery from reagent water and has been demonstrated to be applicable over the concentration range from 7 x MDL to 3000 x MDL.<sup>10</sup>

- 14.3 This method was tested by 17 laboratories using reagent water, drinking water, surface water, and three industrial wastewaters spiked at six concentrations over the range 1.0-70 µg/L.<sup>11</sup> Single operator precision, overall precision, and method accuracy were found to be directly related to the concentration of the parameter and essentially independent of the sample matrix. Linear equations to describe these relationships are presented in Table 3.

## References

1. 40 CFR Part 136, Appendix B.
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9. "Methods 330.4 (Titrimetric, DPD-FAS) and 330.5 (Spectrophotometric, DPD) for Chlorine Total Residual," Methods for Chemical Analysis of Water and Wastes, EPA-600/4-79-020, U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, March 1979.
10. "EPA Method Study 15, Method 605 (Benzidines)," EPA 600/4-84-062, National Technical Information Service, PB84-211176, Springfield, Virginia 22161, June 1984.
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**Table 1—Chromatographic Conditions and Method Detection Limits**

Parameter	Retention time (min)	Column capacity factor (k')	Method detection limit (µg/L)
Benzidine . . . . .	6.1	1.44	0.08
3,3'-Dichlorobenzidine . . . . .	12.1	3.84	0.13

HPLC Column conditions: Lichrosorb RP - 2, 5 micron particle size, in a 25 cm x 4.6 mm ID stainless steel column. Mobile Phase: 0.8 mL/min of 50% acetonitrile/50% 0.1M pH 4.7 acetate buffer. The MDL were determined using an electrochemical detector operated at +0.8 V.

**Table 2—QC Acceptance Criteria—Method 605**

Parameter	Test conc. (µg/L)	Limit for $s$ (µg/L)	Range for $\bar{X}$ (µg/L)	Range for P, $P_s$ (%)
Benzidine . . . . .	50	18.7	9.1 - 61.0	D - 140
3,3'-Dichlorobenzidine . . . . .	50	23.6	18.7 - 50.0	5 - 128

$s$  = Standard deviation of four recovery measurements, in µg/L (Section 8.2.4).

$\bar{X}$  = Average recovery for four recovery measurements, in µg/L (Section 8.2.4).

P,  $P_s$  = Percent recovery measured (Section 8.3.2, Section 8.4.2).

D = Detected; result must be greater than zero.

**NOTE:** These criteria are based directly upon the method performance data in Table 3. Where necessary, the limits for recovery have been broadened to assure applicability of the limits to concentrations below those used to develop Table 3.

**Table 3—Method Accuracy and Precision as Functions of Concentration—Method 605**

Parameter	Accuracy, as recovery, $X'$ ( $\mu\text{g/L}$ )	Single analyst precision, $s_r'$ ( $\mu\text{g/L}$ )	Overall precision, $S'$ ( $\mu\text{g/L}$ )
Benzidine . . . . .	$0.70C + 0.06$	$0.28\bar{X} + 0.19$	$0.40\bar{X} + 0.18$
3,3'-Dichlorobenzidine . . . . .	$0.66C + 0.23$	$0.39\bar{X} - 0.05$	$0.38\bar{X} + 0.02$

$X'$  = Expected recovery for one or more measurements of a sample containing a concentration of  $C$ , in  $\mu\text{g/L}$ .

$s_r'$  = Expected single analyst standard deviation of measurements at an average concentration found of  $\bar{X}$ , in  $\mu\text{g/L}$ .

$S'$  = Expected interlaboratory standard deviation of measurements at an average concentration found of  $\bar{X}$ , in  $\mu\text{g/L}$ .

$C$  = True value for the concentration, in  $\mu\text{g/L}$ .

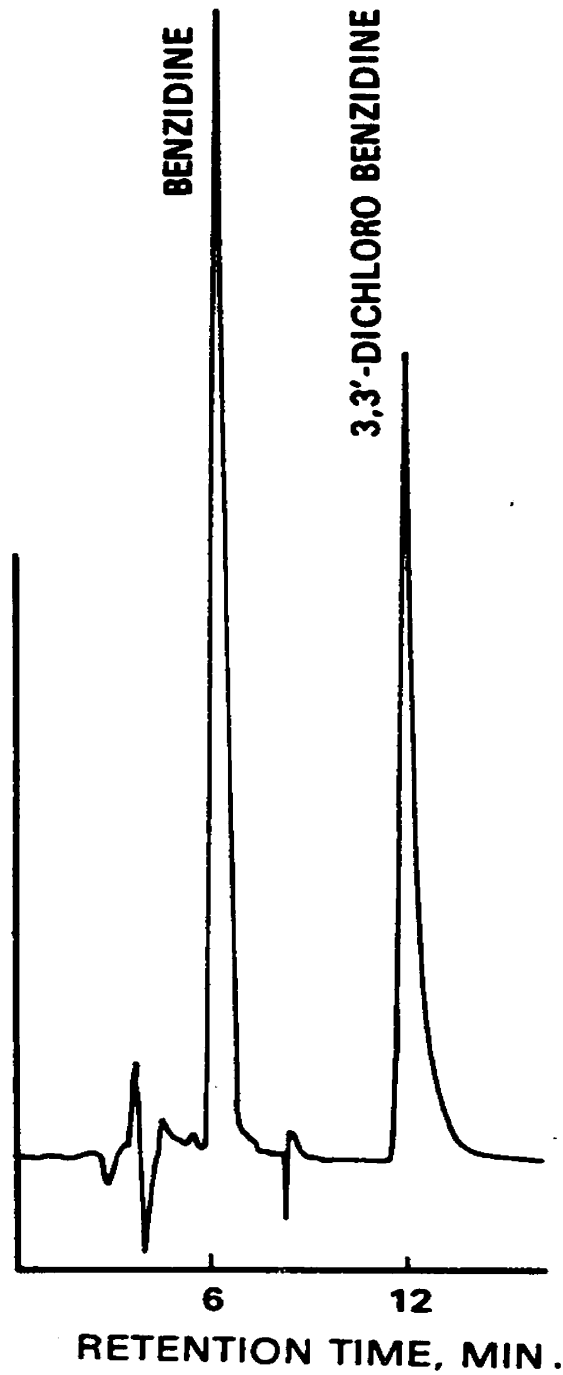
$\bar{X}$  = Average recovery found for measurements of samples containing a concentration of  $C$ , in  $\mu\text{g/L}$ .



**COLUMN: LICHROSORB RP-2**

**MOBILE PHASE: 50% ACETONITRILE IN ACETATE BUFFER**

**DETECTOR: ELECTROCHEMICAL AT + 0.8 V**



**Figure 1. Liquid chromatogram of benzidines.**