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Standard Test Method for Biodegradability of Alkylbenzene Sulfonates[1]

This standard is issued under the fixed designation D2667; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

This standard has been approved for use by agencies of the Department of Defense.

1. Scope

1.1 This test method[2] covers the determination of the degree of biodegradability of alkylbenzene sulfonates. It serves as an index of the suitability of the sulfonate for general use as a surfactant.

1.2 In general, this test method distinguishes between sulfonates in which the alkyl side chains are linear and those in which they are branched, since the former are more readily biodegradable. If the alkylbenzene sulfonate in fully formulated products is to be examined, it must be extracted using the method noted in Annex A1. (See Appendix X1 for data.)

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Material Safety Data Sheets are available for reagents and materials. Review them for hazards prior to usage.

2. Referenced Documents

2.1 *ASTM Standards:*[3]

E1298 Guide for Determination of Purity, Impurities, and Contaminants in Biological Drug Products

D2330 Test Method for Methylene Blue Active Substances (Withdrawn 2011)[4]

E1625 Test Method for Determining Biodegradability of Organic Chemicals in Semi-Continuous Activated Sludge (SCAS)

3. Summary of Test Method

3.1 The sample is first subjected to a presumptive test based on shake culture. When necessary, the sample may be subjected to a confirming test based on semicontinuous treatment with activated sludge.

3.2 In the presumptive test, microorganisms are inoculated into a flask that contains a chemically defined microbial growth medium (basal medium) and the surfactant to be tested. Aeration is accomplished by continuous shaking of the flask. Following two adaptive transfers, biodegradation is determined by measuring the reduction in surfactant content during the test period.

3.3 In the confirming test, activated sludge obtained from a sewage treatment plant is used. The sludge, the surfactant to be tested, and a synthetic sewage used as an energy source for the sludge microorganisms are all placed in a specially designed aeration chamber. The mixture is aerated for 23 h, allowed to settle, and the supernatant material removed. The sludge remaining in the aeration chamber is then brought back to volume with fresh surfactant and synthetic sewage and the cycle repeated. Biodegradation is determined by the reduction in surfactant content during each cycle.

4. Significance and Use

4.1 This test method is designed to determine whether the sulfonate tested will be removed sufficiently by usual methods of sewage treatment for the effluent to be safely discharged to the environment without further treatment.

4.2 If the surfactant reduction in the presumptive test equals or exceeds 90 %, the material is considered to be adequately biodegradable without further testing.

4.3 If the surfactant reduction in the presumptive test is between 80 and 90 %, the material should be subjected to the confirming test.

4.4 If the surfactant reduction in the presumptive test is below 80 %, the material is considered inadequately biodegradable.

[1] This test method is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fateand is the direct responsibility of Subcommittee E47.04 on Environmental Fate and Transport of Biologicals and Chemicals.

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[2] This test method is based on "A Procedure and Standards for the Determination of the Biodegradability of Alkyl Benzene Sulfonate and Linear Alkylate Sulfonate" by the Committee on Biodegradation Test Methods of the Soap and the Detergent Association, *Journal of the Americal Oil Chemists' Society,* Vol 42, 1965, p. 986.

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[4] The last approved version of this historical standard is referenced on www.astm.org.

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4.5 If it is necessary to run the confirming test, the surfactant reduction in this test must be at least 90 % for the material to be considered adequately biodegradable.

4.6 An example of data from both the presumptive and confirming test can be found in Appendix X4.

PRESUMPTIVE TEST (SHAKE CULTURE)

5. Apparatus

5.1 *Shaking Machine*—A reciprocating shaker operating at about 128 strokes of 51 to 101.6 mm (2 to 4 in.)/min or a gyrator shaker operating at 225 to 250 r/min with an amplitude of 25 to 51 mm (1 to 2 in.) should be used. (Other shakers may be used if equivalent aeration can be demonstrated.)

6. Reagents and Materials

6.1 *Purity of Water*—Either distilled or deionized water may be used in this test. It must be free of bacteriostatic materials. Water derived from steam condensate in many cases will contain amines which are inhibitory to microbial growth.

6.2 *Basal Medium:*

6.2.1 The composition of the basal medium shall be as follows:

NH_4Cl	3.0 g
K_2HPO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.25 g
KCl	0.25 g
$FeSO_4 \cdot 7H_2O$	0.002 g
Yeast extract	0.30 g
Water	1.0 L

6.2.2 The basal medium may be prepared by sequentially dissolving the NH_4Cl, K_2HPO_4, KCl, and $FeSO_4$ in approximately 800 mL water and adjusting the pH to 7.2 ± 0.2 with a dilute solution of hydrochloric acid or sodium hydroxide. The yeast extract and $MgSO_4$ dissolved in 200 mL water are then added with stirring to the former solution. Alternatively, the medium may be prepared using suitable stock solutions of the salts, but the pH must be adjusted before the $MgSO_4$ is added. In either case, the yeast extract must be added in dry form immediately before use. It is important to use the basal medium immediately after preparation to avoid bacterial growth. The basal medium shall be dispensed into one of the following Erlenmeyer flasks: 500 mL in a 1-L flask, 1000 mL in a 2-L flask, and 1500 mL in a 4-L flask.

NOTE 1—The 1-L and 2-L flasks are best suited for a gyratory shaker and the 4-L flask for a reciprocating shaker.

NOTE 2—The pH of the medium should be checked before use and adjusted to pH 6.8 to 7.2 if necessary.

6.2.3 The flasks shall be stoppered with cotton plugs or the equivalent to reduce contamination and evaporation.

6.3 *Microbial Culture:*

6.3.1 *Source*—The microbial inoculum may be obtained from any of the following sources:

6.3.1.1 Natural sources (soil, river/lake water, sewage, activated sludge, secondary effluent, and so forth).

6.3.1.2 Laboratory cultures (activated sludge, river die-away, and so forth).

6.3.2 *Maintenance of Culture*—If desired, the culture may be maintained as a shake flask culture by weekly transfers in the basal medium plus 10 mg/L linear alkyl sulfonate (LAS). For each weekly transfer use 1 mL of 7-day culture for each 100 mL of fresh medium.

7. Standardization

7.1 As a control on the culture and test conditions used, the total run is invalid if the result with a suitable reference sample of linear alkyl sulfonate is less than 90 % removal as measured by methylene blue active substance (MBAS) loss.

8. Procedure

8.1 *Addition of Surfactant to Basal Medium:*

8.1.1 Add 10 mg/L of surfactant (active basis) to the flasks containing basal medium. If surfactant stock solutions are used, stability during storage must be confirmed.

8.1.2 Use one flask for each surfactant being tested, plus one control flask for LAS, additional controls if desired (see Note 3), and one blank flask containing all basal medium components but with no surfactant.

NOTE 3—A reference LAS sample that meets the standards of biodegradability of both the presumptive and confirming tests is available through the Environmental Protection Agency (EPA). This sample is a composite of several commercially available products, believed to be typical (from a biodegradability standpoint) of LAS surfactants in commercial use. It is suggested that a control test should be conducted using this material, whenever surfactant biodegradability determinations are undertaken. Biodegradation values for the EPA LAS standard are noted in the accompanying data from the EPA. A more complete and recent analysis may be found in Appendix X2.

NOTE 4—Failure to repeatedly attain prescribed biodegradation values for the surfactant control (LAS) indicates that conditions are unfavorable for normal microbial activity or that an analytical problem exists. Such problems should be investigated by an experienced microbiologist or an analytical chemist.

8.2 *Inoculation*—Using the culture described in 6.3, inoculate the flasks. Use the same culture for all flasks including control and blank. Use 1 mL of inoculum for each 100 mL of basal medium in the flask.

8.3 *Incubation*—Place flasks containing basal medium, surfactant, and inoculum on a shaking machine that will produce acceptable aeration and mixing for biodegradation. Maintain the temperature of the flask contents at 25 ± 3°C, and measure, and, if necessary, adjust the pH of the media at the start of each incubation period to pH 6 to 8.

8.4 *Adaptation* (acclimation)—Prior to beginning the biodegradation test, make two 72-h acclimation transfers from the flasks from 8.3 according to the following illustrative drawing describing the sequence comprising 8.2, 8.3, and 8.4.

8.5 *Analysis* (see Test Method D2330):

NOTE 5—It is important to follow Test Method D2330 exactly since it is known to eliminate the effects of interfering ions that might be present.

8.5.1 To follow the course of biodegradation, remove samples from the shake flasks for analysis. Samples must be taken during the 8-day test at zero time (immediately after inoculation and mixing of the flask contents) and on the seventh and eighth days. Samples at zero time of the two adaptive transfers are desirable to ensure proper initial concentration. Unless analyses are run immediately, the addition of 1 mL of formaldehyde/100 mL of sample should be used for

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preservation for any sample (0 time or 7 or 8 days). When preservative is used, add to all samples including blank, and store the samples at 4°C.

8.5.2 Since the analytical result from the blank sample is used to correct the results from the other flasks, use the same sample size (or dilution factor) for the blank as is used for the other samples.

9. Calculation

9.1 Calculate the net surfactant concentration by subtracting the analyzed blank values from the analyzed values for the other flasks.

9.2 Calculate the percentage removal from the reduction in surfactant concentration as follows:

$$\text{Percentage removal (Day } x) = \left[\left((S_0 - B_0) - (S_x - B_x)\right)/(S_0 - B_0)\right] \times 100 \quad (1)$$

where:

S_0 and S_x = analyses of test surfactant cultures, and
B_0 and B_x = analyses of blank cultures, on Days 0 and x, expressed as concentrations of MBAS, mg/L.

9.3 The result of the test shall be calculated as the average of the seventh and eighth day percentage removals.

10. Precision and Bias

10.1 *Summary*—Statistical analyses were employed to determine the reproducibility of the methods and the best estimate of the true percentage removal. Using these statistics for each surfactant, confidence limits around the true percentage removal and lower tolerance limits for individual results were calculated.

10.2 *Statistical Approach Used:*

10.2.1 Three cooperative experiments were conducted during a 15-month period. Each experiment was designed to provide for replicate units within each run and replicate runs for each laboratory. Additionally, in the first experiment, replicate analyses for each unit were obtained, Thus, four levels or sources of variability were investigated: laboratory-to-laboratory, run-to-run within laboratories, unit-to-unit within runs, and analysis-to-analysis within units.

10.2.2 Since all the participating laboratories did not have the facilities to conduct the entire testing scheme, the statistical analysis was performed recognizing the varying number of degrees of freedom in the experimental design. Test results at each level of variability were averaged to yield the average for the next higher level; for example, the grand mean is the average of laboratory means rather than the average of individual runs or unit means. It is believed that any slight loss in precision of the confidence limits is of less importance than unduly biasing the results when a few laboratories submit a larger proportion of the determinations.

10.2.3 It was observed from the first set of data that variaility increased as the percentage removal decreased, and that the distribution of results was skewed toward the lower percentage removal values. As a variance stabilizing step, the square root transformation attributed to Yates and discussed by Bartlett[5] was applied to the data prior to analysis. The transformation used was:

$$X = (100 - Y + Z) \quad (2)$$

where:

Y = observed percentage removal value, and
Z = small value.

As all calculations were done by computer, a range of Z values from 0 to 2.0 was explored. It was found that $Z = 0.1$ successfully stabilized the variance. In the transformed state the population was found to approach normality.

10.2.4 After transformation, means were determined and an analysis of variance performed to estimate the components of variance for the sources listed above. Using these statistics, confidence limits around the true percentage removal and lower tolerance limits for individual results were calculated.

10.3 *Results:*

10.3.1 *Components of Variance*—During the early work, analyses of the components of variance indicated no need for duplicate analyses and only single analyses were run for the remainder of the study. Considering the other sources of variability, laboratory-to-laboratory variations were significantly greater than variation between runs in the same laboratory. Table 1 summarizes the relative importance of the sources of variability. These data are pooled variances from five LAS materials.

10.3.2 *Confidence and Tolerance Limits*—Table 2 presents the means and limits obtained. The lower tolerance limit is that value above which 95.0 % of the results of single determinations are expected to fall (with 95 % confidence).

TABLE 1 Sources of Variability

Source of Variation	Shake Flask		Semicontinuous	
	Variance	Degrees of Freedom	Variance	Degrees of Freedom
Laboratory-to-laboratory	0.1928	14	0.2045	10
Run-to-run	0.0585	66	0.0425	39
Unit-to-unit	0.0120	97	0.0033	36
Total for single determination	0.2633	31[A]	0.2503	20[A]

[A] Harmonic mean.

CONFIRMING TEST (SEMICONTINUOUS ACTIVATED SLUDGE)

11. Apparatus

11.1 *Aeration Chambers* (see Fig. 1):

11.1.1 *Construction*—Use methyl methacrylate tubing 83 mm (3¼ in.) in inside diameter. Taper the lower end 30° from the vertical to a 13-mm (½-in.) hemisphere at the bottom. Locate the bottom of a 25.4-mm (1-in.) diameter opening for insertion of the air delivery tube 25.4 mm above the joint of the vertical and tapered wall. The total length of the aeration chamber should be at least 600 mm (24 in.). An optional draining hole may be located at the 500-mL level to facilitate sampling. Units are left open to the atmosphere. Glass can be used as an alternative to methyl methacrylate.

[5] Barlett, M. S., "The Use of Transformation," *Biometrics*, Vol 3, No. 1, March 1947, pp. 39–52.

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