Designation: E578 – 07 (Reapproved 2021)

Standard Test Method for Linearity of Fluorescence Measuring Systems[1]

This standard is issued under the fixed designation E578; 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.

1. Scope

1.1 This test method covers a procedure for evaluating the limits of the linearity of response with fluorescence intensity of fluorescence-measuring systems under operating conditions. Particular attention is given to slit widths, filters, and sample containers. This test method can be used to test the overall linearity under a wide variety of instrumental and sampling conditions. The results obtained apply only to the tested combination of slit width and filters, and the size, type and illumination of the sample cuvette, all of which must be stated in the report. The sources of nonlinearity may be the measuring electronics, excessive absorption of either the exciting or emitted radiation, or both, and the sample handling technique, particularly at low concentrations.

1.2 This test method has been applied to fluorescence-measuring systems utilizing continuous and low-energy excitation sources (for example, an excitation source of 450 W electrical input or less). There is no assurance that extremely intense illumination will not cause photodecomposition of the compounds suggested in this test method.[2] For this reason it is recommended that this test method not be indiscriminately employed with high-intensity light sources. It is not a test method to determine the linearity of response of other materials. If this test method is extended to employ other chemical substances, the principles within can be applied, but new material parameters, such as the concentration range of linearity, must be established. The user should be aware of the possibility that these other substances may undergo decomposition, or adsorption onto containers.

1.3 This test method has been applied to fluorescence-measuring systems utilizing a single detector, that is, a photomultiplier tube or a single photodiode. It has not been demonstrated if this method is effective for photo-array instruments such as those using a CCD or a diode array detector.

1.4 This test method is applicable to 10 mm pathlength cuvette formats and instruments covering a wavelength range within 190 nm to 900 nm. The use of other sample formats has not been established with this test method.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Summary of Test Method

2.1 This procedure is used for testing the linearity of fluorescence-measuring systems by using solutions of quinine sulfate dihydrate in sulfuric acid as standard test solutions. Other stable solutions which may be more suitable to the user can be employed (Note 1). The standard used to determine linearity should be stated in the report. The fluorescence of the test solution is measured in the measuring system with the cuvettes, slits, or filters that are to be employed in projected use.

NOTE 1—A substitute standard should have the following properties: (*1*) It should have a large quantum yield at very high dilution; (*2*) it should be stable to the exciting radiation during spectral measurements; (*3*) its fluorescence and its absorption spectra overlap should be small; (*4*) its quantum yield should not be strongly concentration dependent; and (*5*) it should have a broad emission spectrum, so that little error is introduced when wide slits are used.[3]

2.2 *Upper Limit of Linearity*—The fluorescence intensity of a series of standard solutions is measured, the resultant instrument readings are plotted against concentration on a log-log graph, and a smooth curve is drawn through the data points. The point (concentration) at which the upper end of the

[1] This test method is under the jurisdiction of ASTM Committee E13 on Molecular Spectroscopy and Separation Science and is the direct responsibility of Subcommittee E13.01 on Ultra-Violet, Visible, and Luminescence Spectroscopy.

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[2] Lukasiewicz, R. J., and Fitzgerald, J. M., *Analytical Chemistry*, ANCHA, Vol 45, 1973, p. 511.

[3] Gill, J. E., *Photochemistry and Photobiology*, PHCBA, Vol 9, 1969, p. 313.

curve deviates by more than 5 % of the signal from the straight line (defined by the center region of the curve) is taken as the upper limit of linearity. The limit is expressed in micrograms per millilitre of quinine sulfate dihydrate.

NOTE 2—Absorption of the exciting radiation at high solute concentrations is dependent on instrument geometry and pathlength, and can result in fluorescence signal nonlinearity.

2.3 *Lower Limit of Linearity*—The lower limit of linearity is taken as the point (concentration) at which the lower end of the curve deviates from the straight line defined by the central portion of the curve by more than twice the average percent deviation of the points that determine the straight line.

3. Significance and Use

3.1 The range of concentration of a fluorescing substance in solution over which the fluorescence varies linearly with the concentration is the range most useful for quantitative analysis. This range is affected by properties of the solution under analysis and by features of the measuring system. This test method provides a means of testing the performance of a fluorescence measuring system and of determining the concentration range over which the system is suitable for making a given quantitative analysis.

3.2 This test method is not meant for comparing the performance of different fluorescence measuring instruments.

4. Apparatus

4.1 *Fluorescence-Measuring System,* fully equipped for projected use with a suitable UV source to cover the excitation wavelengths of quinine sulfate and a photodetector sensitive at 450 nm.

5. Standard Solutions

5.1 Prepare a stock solution of quinine sulfate dihydrate by transferring 0.100 g of crystalline dihydrate of quinine sulfate, $(C_{20}H_{24}O_2N_2)_2{\cdot}H_2SO_4{\cdot}2H_2O$, National Institute of Standards and Technology SRM 936 (or equivalent), into a 100 mL volumetric flask and fill the flask to volume with 0.1 *N* sulfuric acid. This solution contains 10^3 µg/mL of quinine sulfate dihydrate.

5.2 Make serial dilutions by diluting successive aliquots of this stock solution to ten times their volume with 0.1 *N* sulfuric acid. Prepare, by step-wise dilution, solutions with concentrations of 10^2, 10, 10^0, 10^{-1}, 10^{-2}, and 10^{-3} µg/mL.

6. Procedure

6.1 Select the combination of slit widths or apertures, filters, and the size, type, and illumination of cuvette for which the test is desired.

6.2 Set the wavelength of the exciting radiation to 350 nm by means of filters or an excitation monochromator, whichever is provided with the fluorescence measuring system.

NOTE 3—Instruments equipped with a mercury vapor lamp should be set to isolate the 365 nm mercury line.

6.3 Set the central wavelength of the band pass of the fluorescence-radiation measuring system at approximately 450 nm, using filters or an emission monochromator.

6.4 Rinse the cuvette at least three times and fill with the reagent blank (0.1 *N* sulfuric acid) and record the reading using the appropriate range setting of the instrument.

NOTE 4—When it is necessary to change the measurement settings of the instrument, the reading of the reagent blank should also be determined using the new setting.

6.5 Discard the blank solution used in 6.4, rinse the cuvette at least three times with the most dilute of the solutions described in Section 4, fill the cuvette with this solution, and record the fluorescence intensity reading.

6.6 Discard the more dilute solution, rinse the cuvette at least three times with the next most concentrated standard solution, fill the cuvette with this solution, and record the fluorescence intensity reading. Proceed similarly with the other standard solutions, ending with the 10^2 µg/mL solution.

NOTE 5—The 10^3 µg/mL stock solution is not a recommended test solution due to its large absorbance, A>10, for a 1 cm pathlength at λ = 450 nm, which causes extreme inner filter effects and ineffective corrections (see Note 7).

7. Calculation of Results and Data Presentation

7.1 The fluorescence intensity reading minus the reading of the blank solution is equal to the signal, *S* (using the appropriate multiplication factors corresponding to the amplification ranges). Plot these values of *S* against concentration on a log-log graph and draw a smooth curve through the points.

7.2 Using only the points that fall on the linear portion of the curve, this will include the points at concentrations of 10^0, 10^{-1}, and 10^{-2} µg/mL for most instruments, determine the average percent deviation of the points from the line.

NOTE 6—The data that falls on the linear portion of the curve should be treated by linear regression analysis, which will yield the slope of the line, the standard deviation of the slope, and the standard deviation of the points about the line. To determine which points fall in the linear range, a line connecting the points at 10^0, 10^{-1}, and 10^{-2} µg/mL can be drawn on the log-log graph.

7.3 Note the concentration at which the upper end of the curve deviates by more than 5 % of the signal from the straight line defined by the center region of the curve. Report this concentration, in micrograms per millilitre of quinine sulfate dihydrate, as the upper limit of linearity.

NOTE 7—Absorption of the excitation radiation by the sample before reaching the detection region is usually the major inner filter effect observed at higher concentrations. For collimated excitation radiation and 90° detection region geometry, a correction for excitation radiation absorption has been proposed:[4]

$$F_0/F = (2.303\ D_x\ (X\ 2\ -\ X\ 1))/(10^{-Dx\ X1} - 10^{-Dx\ X2}) \qquad (1)$$

where:

F_0	=	the corrected fluorescence intensity
F	=	the observed fluorescence intensity
D_x	=	the optical density per centimetre of the sample at the excitation wavelength, and
X1 and X2	=	the distances (in centimetres) that the detection region boundaries are from the incident face of the sample cell.

[4] Parker, C. A., and Barnes, W. J., *Analyst*, Vol 82, 1957, p. 606.