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# Standard Guide for Forensic Examination of Non-Reactive Dyes in Textile Fibers by Thin-Layer Chromatography[1]

This standard is issued under the fixed designation E2227; 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 Metameric coloration of fibers can be detected using UV/visible spectrophotometry. If spectrophotometry is restricted to the visible spectral range only, differences in dye components may remain undetected. One method of detecting additional components is to use thin-layer chromatography (TLC). TLC is an inexpensive, simple, well-documented technique that, under certain conditions, can be used to complement the use of visible spectroscopy in comparisons of fiber colorants. The principle of the method is that the dye components are separated by their differential migration caused by a mobile phase flowing through a porous, adsorptive medium.

1.2 This standard does not replace knowledge, skill, ability, experience, education, or training and should be used in conjunction with professional judgment.

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

1.4 *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.*

## 2. Referenced Documents

2.1 *ASTM Standards:*[2]

E1459 Guide for Physical Evidence Labeling and Related Documentation

E1492 Practice for Receiving, Documenting, Storing, and Retrieving Evidence in a Forensic Science Laboratory

E2224 Guide for Forensic Analysis of Fibers by Infrared Spectroscopy

E2228 Guide for Microscopical Examination of Textile Fibers

## 3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *activation*—the heating of the adsorbent layer on a plate to dry out the moisture and maximize its adsorptive power.

3.1.2 *adsorbent*—the stationary phase for adsorption TLC.

3.1.3 *adsorption*—the attraction between the surface atoms of a solid and an external molecule by intermolecular forces.

3.1.4 *chamber*—a glass chamber in which TLC development is carried out.

3.1.5 *chromatography*—a method of analysis in which substances are separated by their differential migration in a mobile phase flowing through or past a stationary phase.

3.1.6 *development*—the movement of the mobile phase through the adsorbent layer to form a chromatogram.

3.1.7 *dye extraction*—the removal of the dye from a fiber by incubating it in an appropriate solvent.

3.1.8 *eluent*—the solvent mixture that acts as the mobile phase in TLC.

3.1.9 *metameric pair*—two colors that appear the same under one illumination, but different under other illumination.

3.1.10 *mobile phase*—the moving liquid phase used for development.

3.1.11 *normal-phase chromatogram*—adsorption in which the stationary phase is polar in relation to the mobile phase.

3.1.12 *origin*—the location of the applied sample or the starting point for the chromatographic development of the applied sample.

3.1.13 *resolution*—the ability to visually separate two spots.

3.1.14 *retardation factor (Rf)*—the ratio of the distance traveled by the solute spot's center divided by the distance traveled by the solvent front, both measured from the origin.

3.1.15 *saturation chamber*—equilibration with mobile phase solvent vapor prior to chromatography.

3.1.16 *solute*—in TLC, a mixture of components to be separated.

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[2] 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.

3.1.17 *solvent front*—the final point reached by the mobile phase as it flows up or across the TLC plate during development of the chromatogram.

3.1.18 *spot*—a round zone of sample application at the origin; or in a chromatogram, a round zone caused by migration of a separated component of the solute where the sharpness of the spot relates to the efficiency of the chromatographic band.

3.1.19 *spotting*—applying a solute sample at the origin of the TLC plate.

3.1.20 *stationary phase*—the solid adsorbent coating layer of a TLC plate.

3.1.21 *tailing*—a spot distorted during development into an elongated streak.

3.1.22 *thin-layer chromatogram*—the series of spots visible on the adsorbent layer after development.

3.1.23 *thin-layer chromatography (TLC)*—a separation technique in which the flow of solvent causes the components of a mixture to migrate differentially from a narrow initial zone over a planar, thinly-applied porous adsorptive medium.

## 4. Summary of Guide

4.1 This guide is intended to advise and to assist individuals and laboratories that conduct forensic fiber examinations and comparisons in their effective application of TLC to the analysis of fiber evidence.

4.2 The guide is concerned with the extraction of dyes from single fibers and from bulk material, classification of the dye or colorant, application and development of the extractants on TLC plates using an optimal elution system, and evaluation and interpretation of the resulting chromatograms. The protocols and equipment mentioned in this document are not meant to be totally inclusive or exclusive.

4.3 Not all fiber type/dye class combinations are covered in this guide.

## 5. Significance and Use

5.1 Forensic analysis of fiber colorants using TLC should be considered for single fiber comparisons only when it is not possible to discriminate between the fibers of interest using other techniques, such as comparison microscopy (brightfield and fluorescence) and microspectrophotometry in the visible range.

5.2 The extraction procedures carried out prior to TLC analysis can provide useful information about dye classification. TLC can provide useful qualitative information about dye components. Similar colors made up of different dye components can be differentiated using this technique. The application of TLC may serve to discriminate between fibers, or it may confirm their similarity.

5.3 TLC may be prohibitively difficult or undesirable in some circumstances. Short lengths of fibers or pale colored fibers may not have an adequate concentration of colorant present to be examined. Dye extraction from some fibers may be impossible. The desire to preserve evidence for possible analysis by another examiner may preclude removing the color for analysis.

5.4 Dye from the known material should first be characterized and eluent systems evaluated to achieve optimum separation of the extract. Dye is then extracted from single known and questioned fibers, using an equivalent amount of material.

5.5 The development of each individual TLC plate will show some variability as a result of the coating and conditioning of the plate, solvent condition, and temperature. It is important to evaluate the performance of each TLC plate by spotting known materials along with the questioned samples. See Ref (**1**).[3]

5.6 Examples for the preparation of Standard dye mixtures are given in Appendix X1.

## 6. Sample Handling

6.1 The general handling and tracking of the samples should meet or exceed the requirements of Practice E1492 and Guide E1459.

6.2 Pre-treatment (mounting medium, washing solvent, etc.) and sample preparation shall be identical for all known and questioned fibers being compared on one TLC plate. For removing single fibers from slide preparations the following procedure is recommended.

6.2.1 Any traces of marker pen ink should be cleaned from the coverslip using an appropriate solvent, for example, acetone.

6.2.2 The coverslip should be cracked all around the fiber and an appropriate solvent, that will dissolve the mountant, but not affect the fiber or the colorant, should be used.

6.2.3 The fiber is removed and extracted in an appropriate solvent. Appropriate solvent selection will depend on the mountant and the sample.

## 7. Analysis

7.1 The ease of dye extraction and the particular extractant required will depend on the generic class of the fiber and the type of dye present. The generic class of the known and questioned fibers shall be determined prior to TLC analysis. See Guides E2224 and E2228.

7.2 Dye classes are classified into broad groups based on their chemical properties or method of application. The determination of the dye class of the known fibers can be helpful in establishing the best extractant, as well as to assist in the subsequent selection of the most efficient eluent system.

7.3 Documented extraction schemes (see Appendix X2) can be used to determine the dye class of fibers of known generic classes, and thus the optimum extractant. Dye classification is done on single fibers or tufts of fiber removed from the known item. A new fiber or tuft can be used for each classification stage.

[3] The boldface numbers in parentheses refer to a list of references at the end of this standard.

7.4 *Dye Extraction*—Known and questioned fibers shall be extracted at the same time under the same conditions. Single fibers can be extracted in a short length (about 25 mm) of fine capillary tube (internal diameter of about 1.5 mm), sealed at one end. A fine wire can be useful in pushing the fiber down the tube. The tube shall be appropriately labeled.

7.4.1 About 10 µl of the appropriate extractant (as recommended in Appendix X2) should be introduced into the tube to cover the fiber sample. A fine glass pipette or syringe can be used for this procedure. The tube should be heat sealed to avoid evaporation and incubated for a constant time and temperature (as recommended in Appendix X2), preferably in an oven. Periodic checks for dye extraction should be made every 15 min for up to 1 h.

7.5 *Dye Extraction: Bulk Material*—Larger fiber tufts can be extracted in a Durham tube or other suitable small stoppered glass tube, using about 100 µl of solvent in a sand bath or oven heated to 100°C. Periodic checks should be made every 15 min for up to 1 h.

7.6 *Nonextractable Dyes*—If classification indicates that a nonextractable dye or pigment other than a reactive dye is present, then place one known and one questioned fiber in labeled capillary tubes. Add approximately 10 µl pyridine/water (4:3) and attempt to extract at about 100°C for one hour. If neither fiber extracts, a positive association is noted. If the questioned fiber extracts and the known fiber does not (or vice versa), there is no association. If both questioned and known fibers "bleed" dye into solution, there may be sufficient dye for analysis.

7.7 *Elution*—Aluminum backed silica gel plates, with nominal particle size of 60 microns and incorporating a fluorphore excited at 254 nm, such as silica gel 60F 254, measuring 5 × 7.5 cm are recommended for normal-phase TLC of fiber dyes (**1**). Plates should be stored in a desiccator; if this is not possible, they should be heat activated before use.

7.7.1 Both known dyes and questioned dyes to be compared shall be applied to the same plate. The extract should be spotted onto the plate about 1 cm from the lower edge. This can be done using a double drawn capillary tube or other suitable device. Spots should not be too near to the edge of the plate or to each other. Care should be taken to avoid scratching the adsorbent coating layer during spot application.

7.7.2 Spots should be dried using a hair dryer or hot plate, with repeat spot applications made until the spot is strongly colored. The spot size should be uniform and not exceed about 2 mm in size.

7.7.3 At least two (preferably more) known dye spots should be included on each plate, on both sides of the questioned sample(s). It is advisable to include a standard dye spot. A note shall be made of the sample order on the plate itself in pencil, well below the sample spots. Plates shall be thoroughly dried before developing.

7.8 *Development Chambers*—Chromatograms can be developed vertically in a glass chamber that may be as simple as a covered glass beaker. Commercial tanks are available (**1**). Twin trough tanks allow the eluent to be transferred to the plate side without removing the cover, but extreme care shall be taken when doing this not to contact the side of the TLC plate.

7.8.1 The eluent should be added to the tank and allowed to stand in the closed container for a few minutes before development, so that the chamber will be saturated with the eluent vapor.

7.8.2 The level of the eluent in a vertical tank should be at least 0.5 cm below the origin/application spots on the TLC plate.

7.9 *Eluent:*

7.9.1 Five parameters shall be considered when selecting the optimum eluent:

7.9.1.1 Separation of component dyes,

7.9.1.2 Sharpness of bands,

7.9.1.3 Movement of the eluted spots from the origin,

7.9.1.4 Components traveling at or close to solvent front, and

7.9.1.5 Strength of dye extract from questioned fibers.

7.9.2 Two or more eluent systems should be assessed with the known fibers to determine the optimum eluent system that can be used for comparison with the questioned fibers.

7.9.3 There are numerous published TLC eluent systems that can be applied to the development of particular fiber/dye class combinations (see Appendix X3).

7.10 Equivalent lengths of fiber should be used for pale fibers or short sample lengths. The extract from known material should be applied to the TLC plate and developed in the trial eluents as previously described. The plate should be eluted until good resolution is achieved (normally 2 cm from the origin), but not so far as to allow the spots to become diffuse, making visualization difficult. The plate should be removed and the position of the solvent front marked in pencil. The plate should be dried in a hot air stream. The eluent should be appropriately discarded. If the eluents produce poor separation, others appropriate to the dye class are evaluated. In exceptional circumstances, eluents appropriate to other dye classes can be used.

7.10.1 After a suitable eluent system has been found, comparison of known and questioned fibers can be carried out. Co-chromatography can be carried out for bulk samples. After drying, plates should be examined immediately in visible and in longwave ultraviolet light. Band position(s) and color(s) should be noted.

7.10.2 If the spots do not move from the origin, a more polar eluent system should be chosen. If the spots move with the solvent front, a less polar eluent system should be chosen.

7.11 Determine and record the color/fluorescence and the Rf value of the spots.

7.12 Plates and samples shall be identifiable. Plates shall be documented by color imaging or retained and stored out of direct sunlight in a manner designed to minimize fading, or both.

## 8. Report Documentation

8.1 Different eluent systems or stationary phases may provide additional discriminating power. The spot colors/fluorescence, sequence, and position of the spots obtained from

the dye of the questioned fibers are compared to those from the corresponding known fibers analyzed under the same conditions.

8.2 A positive association occurs when the colors/fluorescence, sequence, and positions of the spots are consistent between questioned and known fibers. A negative (exclusion) association is noted when either the questioned and known patterns show no similarities, or there are a number of coincident bands but one or more bands are missing from the questioned or known pattern. An inconclusive association is noted when there are no bands on the TLC plate because insufficient colorant is present in the extract. In cases where the amount of extract is very small, the distance traveled by the eluent is very small and in some cases the spots may not be well defined, calculation of the Retardation Factor (Rf) values can easily be inaccurate and therefore meaningless.

8.3 The TLC methods applied to the forensic comparison of fiber colorants should be based on methods in peer-reviewed scientific publications and validated by the individual laboratory. Plates shall be identifiable with respect to case number, sample source, examiner, and date. Case documentation on TLC shall include the source of the samples, method of dye classification, details of extractants/eluent systems tested and/or used, and the results. The use of standard dye mixtures as system performance checks is strongly recommended. All TLC data should be recorded with color imaging to properly document the file.

## 9. Keywords

9.1 fibers; forensic science; thin-layer chromatography

# APPENDIXES

**(Nonmandatory Information)**

## X1. SUGGESTED STANDARD DYE MIXTURES

X1.1 Standard dyes should be used to check eluent performance. The list below suggests some suitable mixtures but is not totally inclusive or exclusive. Any (simple) mixture of dyes separating in these eluents can be used. As many dyes are light sensitive, the solutions should be stored in sealed amber glass containers.

X1.2 *Recommendations for Preparation of Standard Dye Mixtures*—Approximately 5 mg of each dye component is made up to a final volume of 25 mL with pyridine/water (4: 3 v/v). Use until the supply is exhausted.

### X1.3 Solution A for Eluents 2, 3, 4, 5, 13, 14, 17

X1.3.1 Solway green G (CI Acid Green 25), Solway blue RNS (CI Acid Blue 47) and Naphthalene fast orange 2GS (CI Acid Orange 10).

### X1.4 Solution B for Eluents 6, 11, 12

X1.4.1 Supracet fast orange G (CI Disperse Orange 3), Supracet fast violet B (CI Disperse Violet 8) and Supracet scarlet 2G (CI Disperse Orange 1).

### X1.5 Solution C for Eluent 8

X1.5.1 Supracet fast orange G (CI Disperse Orange 3) and Supracet fast violet B (CI Disperse Violet 8).

### X1.6 Solution D for Eluent 16

X1.6.1 Solway green G (CI Acid Green 25), Supracet fast orange G (CI Disperse Orange 3) and Supracet fast violet B (CI Disperse violet 8).

X1.7 *Testing of Eluents and Extraction Solutions*—Checks are carried out, just prior to use, on eluent performance and the extractants are tested to ensure that they have not been contaminated. Suggested TLC plates are Merck DC Alufolien Kieselgel 60F254 (7.5 × 5.0 cm). The standard dye and extraction solution are spotted side by side onto a TLC plate that is resting on a hotplate at about 700 C or dried with a heat source such as a blow-drier. They are spotted 1 cm from the base and eluted as described in Section 7. The chromatogram is compared with previous stored tests to ensure that the separation is acceptable, and that there are no visible bands obtained from the extraction solution. If unacceptable, fresh eluent/extractant is made and tested.

## X2. EXTRACTION SCHEMES AND CLASSIFICATION OF DYES

### X2.1 WOOL Fibers (2)

X2.1.1 *Stage 1:*
Pyridine/Water (4:3), 100°C, 10 min
Good extraction ACID dye
Little/ no extraction—Go to Stage 2

X2.1.2 *Stage 2:*
2 % aqueous oxalic acid, 100°C, 20 min then pyridine/water (4:3), 100°C, 10 min
Improved extraction METALIZED Dye
Little/ no extraction REACTIVE Dye

### X2.2 COTTON and VISCOSE Fibers (3)

X2.2.1 *Stage 1:*
Glacial acetic acid, 100°C, 20 min
Good extraction AZOIC Dye
Little/ no extraction—Go to Stage 2

X2.2.2 *Stage 2:*
Pyridine/Water (4:3), 100°C, 20 min
Good extraction DIRECT Dye
Little/ no extraction—Go to Stage 3

X2.2.3 *Stage 3:*
Dithionite/polyvinylpyrrolidone, 100°C, 20 min
Apply extract to TLC plate; check color of spot
Fiber color changed REACTIVE Dye
(No colored spot or spot not original fiber color)
Fiber color unchanged INGRAIN Dye
(No colored spot or spot not original fiber color)
Fiber color changed—Go to Stage 4
(original colored spot)

X2.2.4 *Stage 4:*
10 to 14 % Sodium hydroxide, 100°C, 10 min (new fiber)
Fiber color changed SULFUR Dye
Fiber color unchanged VAT Dye

### X2.3 ACRYLIC Fibers (4)

X2.3.1 *Stage 1:*
Formic acid/water (1:1), 100°C, 20 min
Good extraction—Go to Stage 2

X2.3.2 *Stage 2:*
TLC procedure—methyl acetate eluent
Movement DISPERSE Dye
No movement—Go to Stage 3

X2.3.3 *Stage 3:*
TLC procedure—methanol eluent
Sharp line at solvent front ACID Dye
Little/ no movement/ smearing BASIC Dye

### X2.4 POLYESTER Fibers (5)

X2.4.1 *Stage 1:*
Chlorobenzene, 130°C, 10 min
Good extraction DISPERSE Dye
Little/ no extraction—go to Stage 2

X2.4.2 *Stage 2:*
Dimethylformamide/formic acid (1:1), 100°C, 20 min
Good extraction BASIC Dye

### X2.5 POLYAMIDE Fibers (5)

X2.5.1 *Stage 1:*
Chlorobenzene, 150°C, 15 min
Little/ no extraction—Go to Stage 2
Good extraction DISPERSE Dye

X2.5.2 *Stage 2:*
Pyridine/water (4:3), 100°C, 20 min
Little/ no extraction REACTIVE or DIAZO Dye
Good extraction—Go to Stage 3

X2.5.3 *Stage 3:*
TLC procedure—methanol eluent
Sharp line at solvent front ACID Dye
Little/ no movement/ smearing BASIC Dye

### X2.6 POLYPROPYLENE Fibers (6)

X2.6.1 *Stage 1:*
Methyl acetate/water/acetic acid (5:5:1), 100°C, 20 min
Good extraction DISPERSE Dye
Little/ no extraction—Go to Stage 2

X2.6.2 *Stage 2:*
Pyridine/ water (4:3), 100°C, 20 min
No extraction PIGMENT
Some extraction—Go to Stage 3

X2.6.3 *Stage 3:*
2 % aqueous oxalic acid, 100°C, 20 min then Pyridine/water (4:3), 100°C, 20 min
Improved extraction METALLIC Dye
No improvement ACID Dye

### X2.7 ACETATE/TRIACETATE Fibers (7)

X2.7.1 *Stage 1:*
CA pyridine/water (4:3), room temp., 15 min
CTA pyridine/water (4:3), 100°C, 20 min
Little/no extraction = DIAZO Dye
Good extraction = DISPERSE Dye

NOTE X2.1—Reactive, Sulfur, Vat, Diazo, Ingrain, and pigmented dyes do not extract.

## X3. COMPOSITION OF ELUENTS

X3.1 The following list summarizes eluent systems that have been recommended in the relevant forensic literature. This list is not meant to be totally inclusive or exclusive.

| Eluent | Solvents | Proportions (v/v) | Ref. |
|---|---|---|---|
| 1 | *n*-Butanol, acetone, water, ammonia | 5 : 5: 1 : 2 | **(8,1)** |
| 2 | Pyridine, amyl alcohol, 10 % ammonia | 4 : 3: 3 | **(8,9,1)** |
| 3 | *n*-Butanol, ethanol, ammonia, pyridine, water | 8 : 3 : 4: 4 : 3 | **(8,9,1)** |
| 4 | Methanol, amyl alcohol, water | 5 : 5 : 2 | **(9,1)** |
| 5 | Toluene, pyridine | 4 : 1 | **(8,1)** |
| 6 | Chloroform, ethyl acetate, ethanol | 7 : 2 : 1 | **(9)** |
| 7 | *n*-Hexane, ethyl acetate, acetone | 5 : 4 : 1 | **(8,1)** |
| 8 | Toluene, methanol, acetone | 20 : 2 : 1 | **(8,1)** |
| 9 | *n*-Butanol, acetic acid, water | 2 : 1 : 5 | **(8)** |
| 10 | *n*-Butanol, ethanol, ammonia, pyridine | 4 : 1 : 3 : 2 | **(8,1)** |
| 11 | Chloroform, butanone, acetic acid, formic acid | 8 : 6 : 1 : 1 | **(8)** |
| 12[A] | *n*-Butanol, acetic acid, water | 4 : 1 : 5 | **(8,1)** |

[A] These eluents form an upper and a lower phase. Use the upper phase as the eluent.

NOTE X3.1—The ethanol used is 99 %; the ammonia 0.880 SG unless otherwise stated.

X3.2 *Eluents Recommended for Certain Dye Classes*—Certain fiber type/dye class combinations have been found to give better separation in certain eluents. These are shown in the table below and are recommended as a first choice.

| Fiber Type | Dye Class | Eluent Number |
|---|---|---|
| Wool | Acid or Metallized | 1, 2 |
| Cotton and Viscose | Direct | 1, 4, 3 |
| Acrylic | Basic | 11, 12, 1 |
| Polyester | Disperse | 6, 7, 8, 5 |
| Polyamide | Acid | 9, 10 |
| Polypropylene[A] | | |

[A] Polypropylene rarely contains an extractable dye. If the dye can be extracted an eluent appropriate to the dye class is used.

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