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Reproducibility of high-performance thin-layer chromatography (HPTLC) in textile dye analysis

Ethan Groves *, Skip Palenik, Christopher S. Palenik

Microtrace LLC, 790 Fletcher Dr., Suite 106, Elgin, IL 60123, United States

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ABSTRACT

In the forensic comparison of dyed fibers, thin-layer chromatography (TLC) is one of the few analytical techniques that have proven sensitive enough to detect and separate the minute quantities of dyestuffs present in single fibers. The method has become well-established by trace evidence examiners as a means of further distinguishing fibers whose colors appear to be the same by both comparison microscopy and microspectrophotometry. As practiced at present, the forensic analysis of dyes is limited to a comparison of the separated dye bands from known and questioned fibers performed on the same TLC plate. It is recognized, however, that retardation factor (R_f) alone is not sufficient proof of identify. The limited use of investigative TLC analysis in forensic fiber examinations is due in part to the range of uncontrolled or poorly defined variables that affect the reproducibility of the developed plate. Through the study of a six component test dye mixture developed on over 50 high-performance thin-layer chromatography plates, the effects of several critical variables that affect reproducibility and resolution, including: plate selection, pre-elution, tank saturation, developing distance, and eluent stability have been evaluated.

When considered collectively, the results of this research provide a means for acquiring and archiving repeatable data, both from casework and known reference samples collected on different plates and at different times. These provide a pathway for the development and utilization of reference databases for the identification of dyes. The empirical uncertainty established for the generalized separation procedure used in this research provides objective guidance for evaluating the significance of associations (or eliminations) made on the basis R_f. Ultimately, this research also opens a pathway to the use of forensic dye analysis as an investigative tool, rather than one exclusively restricted to comparative analyses.

1. Introduction

Fiber comparisons represent a significant proportion of all forensic trace evidence analyses [1]. Color is perhaps the most important optical property exhibited by evidential fibers, since along with thickness and shape these are the easiest characteristics to recognize when searching for target fibers through a stereomicroscope. In addition to polymer identification by polarized light microscopy (PLM) and Fourier-transform infrared microspectroscopy (micro-FTIR), the principal property relied upon in most fiber comparison is color, which is evaluated most commonly by comparison microscopy and microspectrophotometry (MSP). It may come as a surprise to some that the colors of most fibers are only rarely, if ever, due to absorptions from a single dyestuff. Since colors are commonly matched to shades specified by their customers, most dye houses are experts at color matching. They characterize these colors using the same spectrophotometric techniques as those employed by forensic scientists. From a spectrum obtained from an exemplar fabric, a dye house will formulate a dye mixture, which can change over time due to changes in dye lots, dyestuff availability (as a function of price and/or quality), and a master dyer's preferences for specific dyestuffs. One consequence of this method of coloring fibers, by accurately matching a spectrophotometrically specified color produced from different dyes, is an additional level of discrimination in forensic fiber comparisons that is achieved by dye identification.

Planar chromatography is a cost effective, routine analytical tool that can be conducted in almost any forensic laboratory. When properly performed, the results provide a direct visual comparison of the dyes extracted from both known (K) and questioned (Q) fibers. The thin-layer chromatography (TLC) plates, or high-quality color images of the developed plate, make easy-to-understand exhibits that can be displayed to the court and jury when providing testimony regarding the significance of a fiber comparison. If a reproducible technique is employed in performing

* Corresponding author.
E-mail address: egroves@microtracellc.com (E. Groves).

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this analysis, not only do the developed plates demonstrate the correspondence in the dye bands with respect to both color and retardation factor (R_f), they also permit the semi-quantitative estimation of the relative proportions of the individual dyestuffs. Furthermore, the development of a database of the chromatographic, physical, and chemical properties of the most commonly used textile fiber dyestuffs make it possible for a laboratory using the database to identify the dyes by their Colour Index (C.I.) designations [2]. Neumann et al. (2011) reports on search algorithms that can be used to screen standardized HPTLC data of the Secret Service Digital Ink Library allowing unknown samples to be compared and identified against a database of known inks [3].

There is a wealth of information in the literature concerning the characterization and identification of a wide range of compounds using TLC [4–7], including a standard guide for the analysis of textile dyestuffs [8]. The development of high-performance thin-layer chromatography (HPTLC) plates, with higher resolution and smaller sample requirements than conventional plates [9], makes this approach more amenable to the limitations of sample size imposed by typical forensic casework samples. Despite the versatility of this method, HPTLC (and TLC, in general) has typically been limited to a strictly comparative role, when utilized at all, in forensic casework. However, HPTLC need not be limited to a strictly comparative role. Investigative HPTLC in forensic casework can provide detailed information about a sample, which cannot be obtained through alternative analytical methods (*e.g.,* MSP, FTIR, Raman*, etc.*) alone. For example, Palenik et al. (2015) show a pair of fibers in which different combinations of dyes have resulted in fiber colors that are indistinguishable by microscopy and MSP alone [10]. In addition, the actual identification of the dyes present in an unknown sample may yield additional investigative information, such as application, timescale, or location constraints, depending on the dye, or dye combinations identified.

This work explores several analytical variables inherent in basic HPTLC technique that are anticipated to have the largest potential for impact on the quality of the resulting data. Specifically, these topics include: plate selection, pre-elution, tank saturation, developing distance, and eluent stability. Based on the results of these studies, an optimized experimental path has been developed for the separation and analysis of dyes extracted from textile fibers. This controlled approach has been developed to provide an optimized balance between the chromatographic separation of components as well as time considerations while taking into account the ever-present size limitations associated with typical forensic samples. By fixing experimental constraints, the data collected in this work provides a means by which to objectively evaluate the uncertainty associated with this approach. The ability to obtain reproducible HPTLC separations provides a basis from which a) dyes on separate plates, collected at different times can be evaluated for quality, b) tolerances (uncertainty) that can be established for the forensic comparison of questioned and known HPTLC data, and c) a database of HPTLC data from a collection of reference dyes can be developed and evaluated for quality [11]. This, in turn provides a means by which the identification of dyes in casework samples can be more reliably and more practically accomplished [12].

2. Experimental variables and evaluations

The following sections address several aspects of HPTLC analysis, which impact repeatability and resolution. Each sub-section: plate selection, pre-elution of plates, tank saturation, developing distance, and eluent stability, was explored both through a survey of the literature on the topic and actual experimentation to maximize the repeatability and define the anticipated variation of the method when configured for forensic dye separations.

2.1. Plate selection

Chromatography plates are available in a variety of configurations, which include variations in plate types (preparatory, analytical, *etc.*), sizes, stationary phase thicknesses, adsorbent chemistries, support substrates, with and without visualization additives (among others). Therefore, plate selection is a critical first variable to consider when developing a planar chromatography application. The plates which had been used reliably for forensic TLC dye analyses in the authors' laboratory for decades were the Whatman HPTLC plate (Cat. #: 05-713-255); however, production was discontinued in approximately 2011. The manufacturer's specifications for these plates are provided in Table 1.

Analogous products from four suppliers (Analtech, EMD Millipore, Machery-Nagel, and Sorbtech) were sourced, and a survey of plate performance of these products was conducted; manufacturers' specifications for these plates are provided in Table 1. Analtech, at the time, did not have a stock of HPTLC plates without a fluorescent brightener, and could only supply plates containing a fluorescent dye which is typically added for locating colorless UV absorbing compounds. Communications with chromatography suppliers indicated that the majority of chromatography plates are produced with a fluorescent indicator. The presence of such an indicator is unnecessary for the examination of dyestuffs, which are themselves are highly colored. Furthermore, the presence of a fluorescent indicator presents an added complication to the subsequent evaluation and analysis of developed plate.

Each plate was spotted with an equivalent amount of a reference dye mixture (Analtech, test dye I), consisting of four dyes: Sudan IV, Bismarck Brown Y, Rhodamine B, and Fast Green FCF. This dye mixture was used to compare the developing characteristics of the different HPTLC plates. The plates were then dried and developed using an eluent proposed by Wiggins [13]:n-Butanol, acetone, water, and ammonia (5:5:1:2), which was selected for evaluation because it was reported by Wiggins [13] to be applicable to the widest range of dye application classes. The evaluation was completed in triplicate, developing a single plate from each manufacturer on separate days using a fresh batch of eluent. In each evaluation the plates show similar separations of the dye mixture, which also show variations in R_f and band densities. Fig. 1 shows a comparison of the developed HPTLC plates from one of the evaluations. The images have been cropped to show only the developed lanes (no post processing of the images was conducted).

Table 1
Specifications of the HPTLC plates examined in this study.

Manufacturer	Catalog #	Adsorbent			Fluor. indicator	Pre-conc. Zone size	Plate size	Plate substrate
		Thickness	Particle size	Chemistry				
Analtech	61077	150 μm	8–10 μm	Unmodified Silica	Yes	1.5 cm	10 × 10 cm	Glass
EMD Millipore	13748	150–200 μm	5–7 μm		No	2.5 cm		
Machery-Nagel	811032	200 μm	2–10 μm		No	2.9 cm		
Sorbtech	4214056	200 μm	2–10 μm		No	2.8 cm		
Whatman	05-713-255	200 μm	4.5 μm		No	2.0 cm		