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Comparative Examination of Inks by Using Instrumental Thin-Layer Chromatography and Microspectrophotometry

ABSTRACT: It is well-known that in thin-layer chromatographic (TLC) densitometry and reflectance microspectrophotometry (MSP) the relationship between signal output and content of substance(s) absorbing light is neither a directly, nor reciprocally proportional one. For this reason, the discriminating power of the widespread signal-to-signal ratio technique is not high in relation to similarly colored materials, especially to those having subtle differences of same colored components.

In this paper the modified signal-to-signal ratio approach applicable for both, TLC and MSP methods, is proposed. The approach includes transforming (by using the recommended equations and iterative procedure) the values of the signals obtained for compared materials, in such a manner that the ratios of these transformed signals become less mass-dependent in compared with the ratios obtained by using the signal-to-signal ratio technique. As a result of applying this approach, the discriminating power of densitometric TLC and MSP methods has been enhanced significantly. The value and limitations of the proposed techniques are considered

KEYWORDS: questioned documents microspectrophotometry, thin-layer chromatography, densitometry, signal, content, relationship, signal-to-signal ratio, iterative procedure, comparison, dyes, inks

Thin-layer chromatography (TLC) and microspectrophotometry (MSP) are often used for forensic comparative examination of similarly colored inks and other materials [1–6].

These methods assist the analyst in assessing the similarity or lack of similarity between the materials in question in cases when these materials have different qualitative compositions or significant differences in relative proportions of their same components. Besides qualitative visual assessment of relative proportions of the samples components separated by TLC or of the shape of reflectance spectra recorded for the samples that are to be compared, the following techniques of objective evaluation of data obtained with the help of TLC and MSP are applied in forensic analysis.

Densitometric Thin-Layer Chromatography

This method is more frequently used when comparing similarly colored forensic materials (mainly inks and fibers) by means of evaluating the relative proportions of the various dye components of such materials using the peak ratioing technique [1,2,7]. The following equation for calculating these ratios is·used in this approach:

$$Ratio_i = (S_1)_i/(S_2)_i,$$ (1)

where S is the signal output (in the form of peak height or peak area) corresponding to chosen sample's components, 1 and 2, separated by TLC; i is the index of the sample taken from the material analyzed.

However, some serious problems interfere with successful application of this procedure. These problems originated from the "native" character of a relationship between the signal and the amount of substance per spot. This relationship for most cases of measurements in reflectance mode with a slit-type scanner is sufficiently nonlinear [8,9]. Furthermore, even in cases when such relationships can be satisfactorily approximated by straight lines, these lines, as a rule, do not pass through the origin of the graph "signal-content" (see for example, [8,10]) as it will be shown in the following. This is the reason why the signal-to-signal ratio technique often produces statistically poor data and therefore cannot be considered a reliable technique for discriminating samples having subtle differences in their components' proportions.

In this paper the modified version of the signal-to-signal ratio technique (introduced partly in the work [11]) that can cope with the above mentioned problems, is described.

Reflectance Microspectrophotometry

For comparative examination of apparently similar materials by using MSP some kind of computerized techniques [4,5] that are based on "cross-correlation" procedures [12] have been developed.

Such techniques are effective in situations when: 1) it is necessary to select (for further examination) from a large collection of similarly colored samples (or from the library of previously recorded spectra) those that are the most similar to a questioned or reference sample (spectrum); 2) samples compared are sufficiently different either in their spectral profiles (for example, when metameric ink samples of different formulations are compared) or spectral intensity (when samples being compared are far from being equal with respect to the concentration of their colored components: samples to be compared are obviously different in lightness).

However, these techniques—taking compared spectral curves as whole—do not readily detect subtle differences in those spectra. For example, such kind of differences occur when samples compared consist of the same components that are in similar but not equal proportions, or when there are minor differences in qualitative dye composition of compared samples.

The technique ("2λ-method with correction of reflectance values") discussed in this paper allows one to find and evaluate such differences in spectra to be compared. It is based on a "reflectivity ratioing method" [13]—a kind of the signal-to-signal ratio technique—that enables the ratios of the values of reflectance (R) measured at two wavelengths, λ_1 and λ_2, to be calculated and compared for different measuring sites (i) of the material analyzed

$$Ratio_i = (R_{\lambda 1})_i/(R_{\lambda 2})_i$$ (2)

Being applied to discriminate between samples having similar reflectance spectra, the method [13] shows much less efficiency than the proposed technique. The cause of this is discussed in the section "Theory."

Theory

If, suppose, there is a directly or reciprocally proportional relationship between the content of light absorbing substance (in a chromatographic zone, for TLC, or in a measuring field, for MSP[2]) and the corresponding reflectance signal

$$Signal = Constant \times Content \tag{3}$$

or

$$Signal = Constant / Content \tag{4}$$

then the signal-to-signal ratio technique would allow one to determine RATIO values that are directly proportional to the masses' ratios of components in samples compared.

Figure 1 illustrates a case described by Eq. 3 when the relation between signal output (S) and content (C) of any light absorbing component (for example, components A and B) is not simply linear (see Eqs. 7, 8, and 11 as follows) but is a directly proportional one (as it is taken place, for example in absorption spectrophotometry, provided Lamberth:Beer's law holds [6].

If we assume that the contents of both components, A and B, in a material analyzed are equal, then one can easily see in Fig. 1 that for samples 1, 2 . . . i in which the contents of both components, A and B, increase from C_1 to C_i, ratioing of corresponding signal outputs

$$S_{A1}/S_{B1} = S_{A2}/S_{B2} = \ldots = S_{Ai}/S_{Bi} = Ratio \tag{5}$$

produces one and the same (mass independent!) value, Ratio: in the case of $C_A = C_B$ this RATIO value shows how much the detector used is more sensible to component A as compared with component B.

Therefore, it can be expected that if a situation like that, occurs in a real analysis, the mean value of such ratio values calculated for different samples of analyzed material could be characterized by a very small statistical error (of course, provided that instrumental and other systematic and statistical errors are not large).

However, it is well known [8,9,14] that in densitometric TLC (except when fluorescence or absorbance of nanogram quantities of a substance are measured—the situation that does not often occur in most of ink analyses) and in reflectance MSP a relationship between signal output and content of absorbing substance is not directly proportional.

In TLC such a relationship is often satisfactorily described by the nonlinear Michaelis-Menten function [9] (see Fig. 2)

$$S = S_{max} \frac{C}{K + C}, \tag{6}$$

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[2]In MSP the situation usually is more complex because the reflectance signal taken at a given wavelength represents nonadditive sum of the signals of sample's components. But for simplicity of description (to avoid complex mathematical expressions), in further consideration it is assumed that only one colored component of an analyzed sample absorbs at a wavelength chosen as the analytical one.
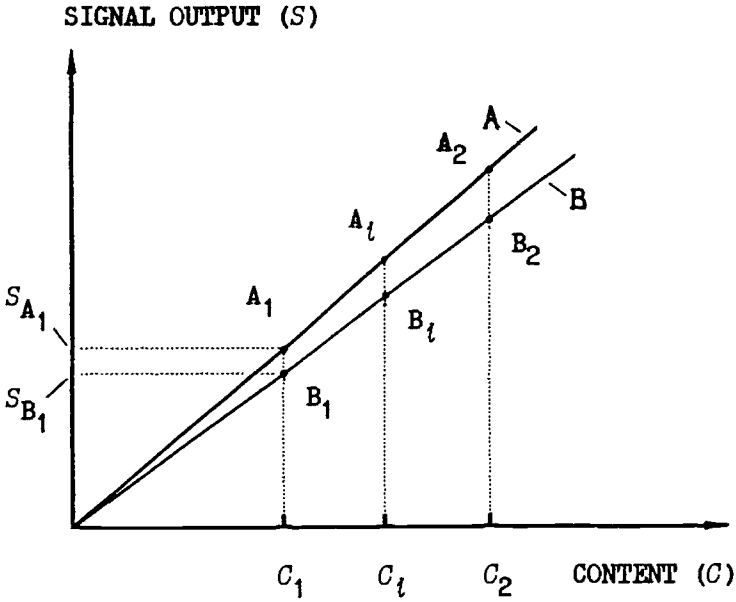
FIG. 1—*Theoretical Beer type relation between signal output* (S) *and content* (C) *of light absorbing substances A and B.*
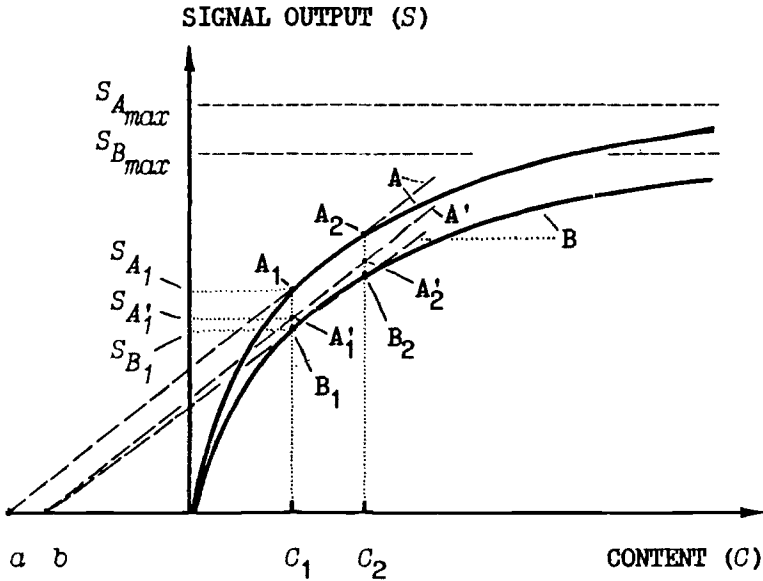
FIG. 2—*Theoretical Michaelis-Menten type curves relating signal output* (S) *and content* (C) *of substances A and B in the thin layer of sorbent.*

where S is the peak signal, S_{max} is the limit of the peak signal, K is a constant, and C is the content of the separated zone.

Linearization of this function results in Eq. 7

$$\frac{1}{S} = \frac{K}{S_{max}} \frac{1}{C} + \frac{1}{S_{max}}, \tag{7}$$

that graphically can be depicted as a line with the slope of K/S_{max} and the intercept of $1/S_{max}$ (see lines A and B in Fig. 3).

It should be pointed out that within short intervals of contents, functions $S = f(C)$ can be satisfactorily approximated by straight lines (as it is shown in Fig. 2 for the interval of contents $C_1 - C_2$: near linear parts, $A_1 - A_2$ and $B_1 - B_2$, of curves A and B, respectively). But contrary to the case shown in Fig. 1, these lines practically never converge at the origin of the graphics "signal–content." Otherwise, they are described not by Eq. 1, but by Eq. 8:

$$S = a C + b, \tag{8}$$

where a and b are coefficients (slope and intercept, resp.)

For this reason, the applications of the signal-to-signal ratio technique using Eq. 1 frequently leads to producing error results of the analysis.

In reflectance MSP "signal–content" relationships have also nonlinear shapes which, in general, can be satisfactorily approximated by the Kubelka-Munk function relating the diffuse reflectance (R) for an infinitely thick, opaque layer of absorbing material and
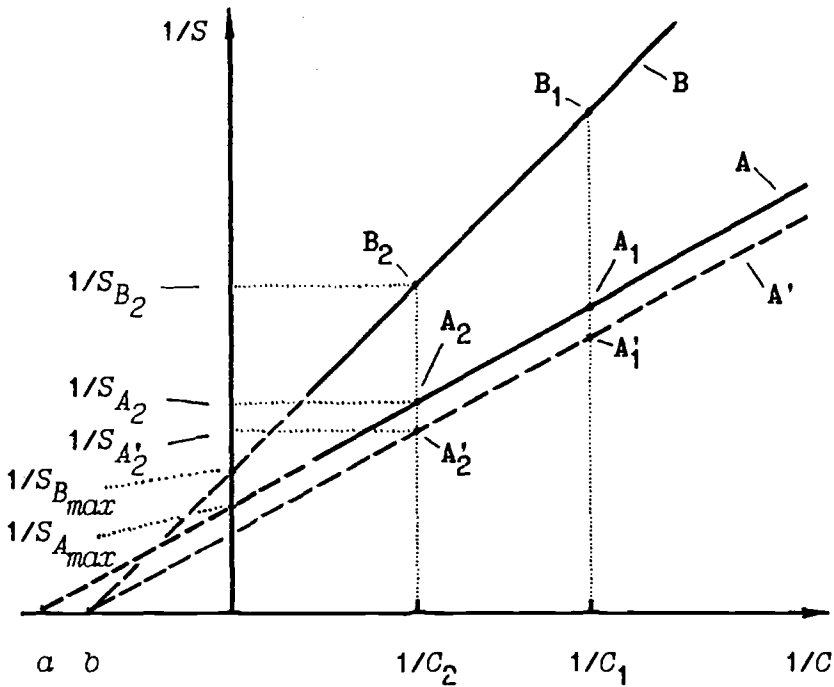

FIG. 3—*Linearization of the Michaelis-Menten type curves.*

the ratio of the absorption and scattering coefficients (k/s) [9,14]

$$F(R) = \frac{k}{s} = \frac{(1 - R)^2}{2R} = \frac{2.303 \; \varepsilon \; C}{s}, \tag{9}$$

where ε and C are extinction coefficient and content of the absórbing substance, respectively.

In accordance to the nonlinear function Eq. 9 typical "signal (R)—content (C)" curves in reflectance MSP have hyperbolic shape (see Fig. 4).

Combination of Eqs. 6 and 9 results in Eq. 10

$$F(R) = F(R)_{max} \frac{C}{K + C}, \tag{10}$$

where $F(R)_{max}$ is the limit of the Kubelka-Munk function, K a constant, and C the amount of the light absorbing component in the measuring field of the sample analyzed.

It has been shown by Pollak [14] that the reciprocal value of reflectance R is a linear function of absorption k with the coefficient of scatter s as a constant parameter. According to this, substitution of $F(R)$ and $F(R)_{max}$ by $1/R$ and $1/R_{min}$, respectively, and simple transformation of Eq. 10 results in Eq. 11

$$R = R_{min} + R_{min} K \frac{1}{C} \tag{11}$$
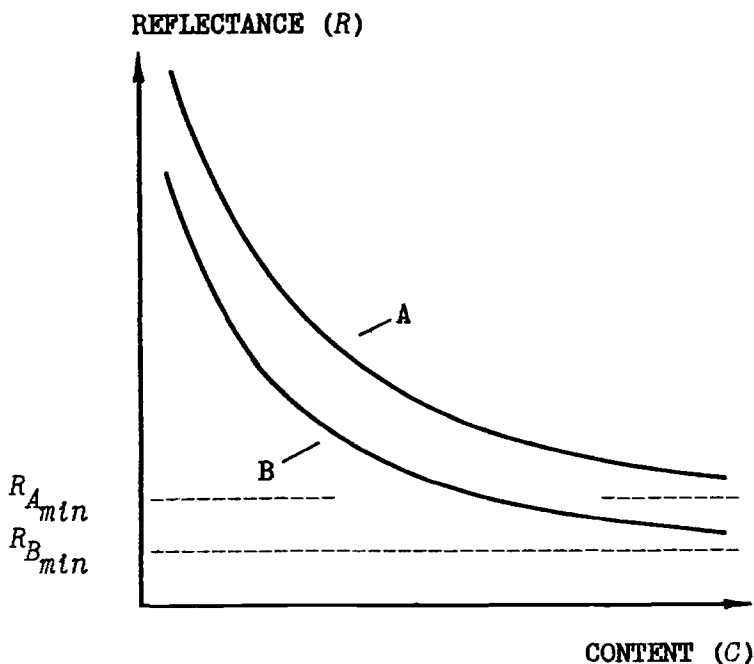
REFLECTANCE (R)

CONTENT (C)

FIG. 4—*Theoretical Kubelka-Munk type curves relating reflectance* (R) *and content* (C) *of light absorbing substances in the sample analyzed: measurements at two wavelengths* λ_1 *(curve A) and* λ_2 *(curve B).*
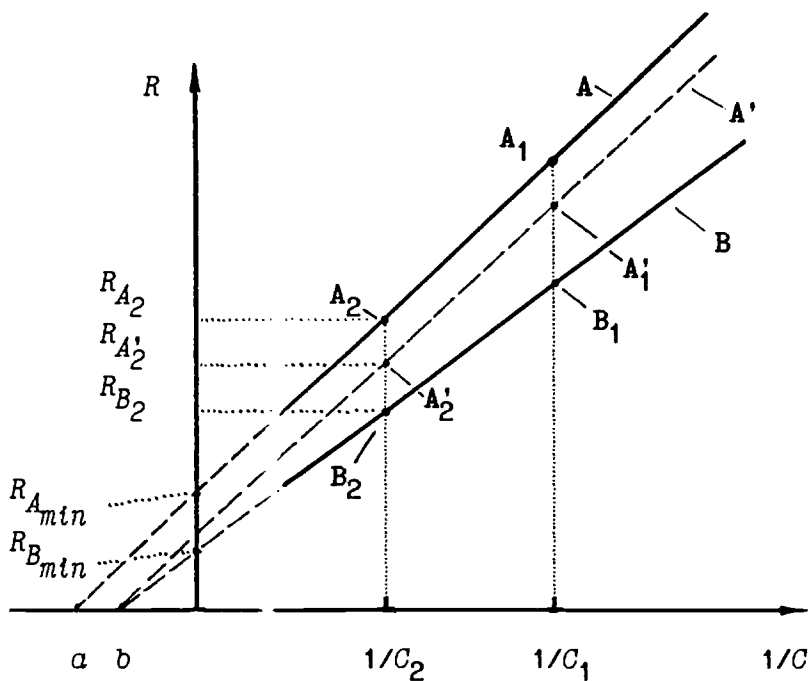
FIG. 5—*Linearization of the Kubelka-Munk type curves.*

So, Eq. 11 shows that reflectance (R) is a linear function of the reciprocal value of content (C) that graphically can be depicted as a line with the slope of $R_{min}{\cdot}K$ and the intercept of R_{min} (see lines A and B in Fig. 5).

Figures 2, 3, and 5 clearly demonstrate why the signal-to-signal ratio technique cannot be considered as a reliable tool for comparison of similar materials by using densitometric TLC and reflectance MSP. Contrary to the situation illustrated by Fig. 1, in these cases the relationships "signal–content" are not directly proportional: they are approximated by linear functions like those given previously which all contain the values of the intercepts unequal to zero (like $1/S_{A_{max}}$ and $1/S_{B_{max}}$ in Fig. 3, or $R_{A_{min}}$ and $R_{B_{min}}$ in Fig. 5) and unknown to an examiner.[3]

For this reason, ratioing signals gives the ratio values that are mass dependent:

$$S_{A_1}/S_{B_1} \text{ is not equal to } S_{A_2}/S_{B_2} \tag{12}$$

(see Fig. 2), and

$$(1/S_{A_1})/(1/S_{B_1}) \text{ is not equal to } (1/S_{A_2})/(1/S_{B_2}) \tag{13}$$

[3]Of course, such information can easily be determined by the calibration procedure. But in forensic practice inks and many other samples are compared without using preliminary calibration procedures (there are some reasons explaining why calibrations are not in demand in forensic comparative examinations but, of course, the main one being, as a rule, that an examiner does not have full information about all components of samples to be compared).

(see Fig. 3), and

$$R_{A_1}/R_{B_1} \text{ is not equal to } R_{A_2}/R_{B_2} \qquad (14) \text{ (see Fig. 5)}$$

and so the application of the previously mentioned ratio technique for comparison of similar materials always produces statistically incorrect results including an unpredictable "systematic" error. The rate of this error can vary within a wide range depending on the casework values of slopes and intercepts of the approximizing linear functions, from small (in such cases the ratios values look quite good as regards the calculated statistical parameters [7,10]) to very big one [7,11].

A new approach developed to solve the considered problem is discussed in this paper. This approach includes transforming signals' values (by using the proposed equations and iterative procedure) in such a manner that their ratios become less mass-dependent in comparison with the signal-to-signal ratio technique. Graphically it can be demonstrated just as it is in Figs. 2, 3, and 5: one of the lines, A or B, moves up or down until, being extrapolated, both lines (lines A' and B in these figures) intersect at a point belonging to the X-axis of the corresponding graph (points "b" in the figures). It leads to the situations when

$$S_{A_1'}/S_{B_1} = S_{A_2'}/S_{B_2} = \ldots = S_{A_i'}/S_{B_i} \qquad (15)$$

(see Fig. 2), and

$$(1/S_{A_1'})/(1/S_{B_1}) = (1/S_{A_2'})/1/S_{B_2}) = \ldots = (1/S_{A_i'})/(1/S_{B_i}) \qquad (16)$$

(see Fig. 3), and

$$R_{A_1'}/R_{B_1} = R_{A_2'}/R_{B_2} = \ldots = R_{A_i'}/R_{B_i} \qquad (17) \text{ (see Fig. 5)},$$

where i relates to any content (C_i, it is not shown in the figures) of components A and B for which Eqs. 15 to 17 hold (at least, to any C_i that is between C_1 and C_2 in Figures 2, 3, and 5). In the proposed approach such a "moving"—line A . . line A'—is carried out by using the iterative procedure described below.

To use this approach the following equations have been derived.

Thin-Layer Chromatography

Suppose several samples were taken from materials A and B that are to be compared. Every sample was analyzed by TLC and then evaluated by using scanning densitometry. If, for a given densitogram, C_1 and C_2 are the contents of substances 1 and 2 in chromatographic zones, respectively, then using Eqs. 7 and 8 their ratio is given by Eqs. 18 and 19, respectively:

$$\frac{C_2}{C_1} = \frac{\dfrac{1}{S_1} - \dfrac{1}{S_{1max}}}{\dfrac{1}{S_2} - \dfrac{1}{S_{2max}}} \frac{k_2 \, S_{1max}}{k_1 \, S_{2max}} \qquad (18)$$

$$\frac{C_1}{C_2} = \frac{a_2}{a_1} \frac{S_1 - b_1}{S_2 - b_2} \qquad (19)$$

For a given analysis, $S_{1_{max}}$, $S_{2_{max}}$, k_1, and k_2, or a_1, a_2, b_1, and b_2 are empirical constants. So substitution of S_1 by PH_1 and S_2 by PH_2 in Eqs. 18 and 19 results in Eqs. 20 and 21, respectively[4]:

$$Ratio_i = K[1/(PH_1)_i - K_1]/[1/(PH_2)_i - K_2] \qquad (20)$$

$$Ratio_i = K[(PH_1)_i - K_1]/[(PH_2)_i - K_2] \qquad (21)$$

where i is the sample index, PH_1 and PH_2 are peak heights values obtained for two separated analytes whose relative proportion in the materials compared is evaluated, K is a constant[5], and K_1 and K_2 are coefficients (determined by using an iterative procedure considered below) which change the peak height values, $(PH_1)_i$ and $(PH_2)_i$ so that the sum of the $Ratio_i$ values' dispersion measures, calculated for each material compared, becomes minimum. (See example that follows.)

Microspectrophotometry

Suppose several reflectance spectra have been recorded for materials X and Y containing two ingredients, A and B, which absorb at wavelengths λ_1 and λ_2, respectively (see footnote 1 and Figs. 4 and 5). If for a given spectrum, C_1 and C_2 are the contents of the ingredients A and B, respectively, then using Eq. 11 their ratio can be written in the form

$$\frac{C_B}{C_A} = \frac{R_{B_{min}} \cdot K_B \cdot (R_A - R_{A_{min}})}{R_{A_{min}} \cdot K_A \cdot (R_B - R_{B_{min}})} \qquad (22)$$

Since $R_{A_{min}}$, $R_{B_{min}}$, K_A, and K_B are empirical constants for a material analyzed under certain measuring conditions (see Fig. 5), Eq. 22 after substitution of subscripts A and B by λ_1 and λ_2, respectively, can be transformed into Eq. 23 for calculating the ratios of the aperture reflectance values taken at two wavelengths λ_1 and λ_2 for the materials compared:

$$Ratio_i = K[(R_{\lambda_1})_i - K_{\lambda_1}]/[(R_{\lambda_2})_i - K_{\lambda_2}] \qquad (23)$$

where i is the spectrum index, K is a constant (see footnote 5) K_{λ_1} and K_{λ_2} are coefficients (determined by using an iterative technique considered below) which change the reflectance values, $(R_{\lambda_1})_i$ and $(R_{\lambda_2})_i$, so that the sum of the $ratio_i$ values' dispersion measures, calculated for each material compared, becomes minimum. (See example that follows.)

The Eqs. 20, 21, and 23 are used in two techniques for comparison of similarly colored materials described in this paper.

Experimental Procedure

Samples

Acid Brilliant Blue Z (dye 1) and Acid Anthraquinone Brilliant Blue (dye 2) were used for preparation of two solutions in 50% aqueous methanol: solution A (0.2% of

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[4]Eq. 20 is the basic one for this technique. But Eq. 21 can also give good results when "signal–content" relationships are satisfactorily approximated by straight lines (like it is depicted in Fig. 2). So in every analysis, both these equations should be tested in order to choose that one that gives better statistical results for the materials compared (see as follows).

[5]In comparative analysis it is not necessary to know the actual value of the empirical constant K. So in all examples given in this paper, $K=1$.

dye 1; 0.6% of dye 2) and solution B (0.2% of dye 1; 0.65% of dye 2). These solutions, A and B (with the masses' ratio of dye 2 and dye 1 equal to 3, for solution A, and 3.25, for solution B), were diluted to prepare solutions A1-A5 and B1-B5 containing 0.5, 0.75, 1.0, 1.5, and 2.0 mg/mL of dye 1, respectively. The solutions A1-A5 and B1-B5 were studied by TLC.

Acid Brilliant Blue Z (dye 1) and Acid Orange Fast (dye 2) were used for preparation of two aqueous solutions: solution X (0.9% of dye 1; 1.0% of dye 2) and solution Y (0.9% of dye 1; 1.1% of dye 2). With the aid of a pen these solutions were applied on a sheet of paper as lines of approximately equal width and thickness (lines X and Y). These lines were studied by reflectance MSP.

The previously mentioned and some other dyes (all obtained from NPO "NIOPIK," Moscow, Russia) were used for preparation of several indistinguishable to the eye colored pairs (duplicates) of ballpoint inks, fountain pen inks, and artists' paints with slightly different relative proportions of the same colored ingredients. These pairs of materials were applied onto a paper as lines of approximately equal width and thickness, and then studied by TLC and MSP.

Thin-Layer Chromatography

Samples (1 μL of solutions A1-A5 and B1-B5) were spotted on a precoated 10 × 10 cm silica gel Merck 60-F_{254} HPTLC plate. The plate was developed to a distance of about 3.5 cm by the linear ascending mode in a Camag (Switzerland) twin-through chamber, without saturation. The eluent was ethyl acetate—isopropanol—water—acetic acid = 30:15:10:1 (v/v). On the resulting chromatograms two compact colored zones, with R_f values of approximately 0.2 for dye 1 and 0.4 for dye 2 were observed.

Plates were scanned by absorbance (at preliminary chosen wavelength, λ = 585 nm) with a Gamag TLC/HPTLC Scanner connected to an SP4100 Integrator (Spectra-Physics). All ten tracks were measured with positioning at each run in order to minimize positioning error [11]. An HP 86B computer (Hewlett-Packard) and software developed by the author were used for calculating differences between the mixtures A (solutions A1-A5) and B (solutions B1-B5) compared. The results obtained for the mixtures compared are considered below (see Table 1).

For comparative TLC analysis of inks that were prepared as described above, the ink samples (15 samples for every duplicate) were cut out from the paper. Every sample was extracted with 3 to 5 μL of chloroform (ballpoint inks), dimethylformamide (paints and some ballpoint inks), or 50% aqueous ethanol (fountain pen inks) in Camag micro vials (N-11 polypropylene) with cone shaped interiors. The extracts (1 μL) were spotted onto Merck (Germany) 20 × 20 cm silica gel 60 TLC plates (without fluorescent indicator) by means of a Camag Micro-Applicator.

Microspectrophotometry

Reflectance spectra were taken directly from the ink lines X and Y (see the previous by using the following equipment and instrumental conditions.

Leitz (Germany) MPV-SP spectral photometer with current-stabilized light source (tungsten-halogen lamp, 12 V/100 W), measurement grating monochromator (1200/mm), Hamamatsu-type R928 photomultiplier, dark-field "Ultropak" condenser and objective "U0-6.5 × 0.12"; wavelength range, 400 to 800 nm; monochromator half bandwidth, 3 nm; lamp current, 7A; photomultiplier output, 500 V.

The HP 86B computer (Hewlett-Packard) and software developed by the author were used for calculating differences between the ink samples compared.

The ink lines X and Y were analyzed according to the "two wavelength method with

TABLE 1—*Results of comparative evaluation of two mixtures.*

	Mixture A					Mixture B				
	Integrator reading		$Ratio_i^a$			Integrator reading		$Ratio_i^a$		
	PH_1 1	PH_2 2	Using Eq. 1 3	Using Eq. 20 4	Using Eq. 21 5	PH_1 6	PH_2 7	Using Eq. 1 8	Using Eq. 20 9	Using Eq. 21 10
	5952	5076	1.173	0.986	1.275	5847	5163	1.132	0.949	1.233
	7634	6614	1.154	1.011	1.286	7763	7092	1.095	0.961	1.228
	8893	7612	1.168	1.044	1.321	8691	8097	1.073	0.956	1.223
	10593	9524	1.112	1.013	1.294	10689	10101	1.058	0.964	1.242
	12315	11299	1.090	1.006	1.302	12016	12065	0.996	0.917	1.203
Mean value (MEAN):			1.139	1.012	1.296			1.071	0.950	1.226
Standard deviation (STD):			0.037	0.021	0.018			0.050	0.019	0.015
Relative standard deviation (REL STD):			0.032	0.021	0.013			0.047	0.020	0.012
Sum of the relative standard deviations:								0.079	0.041	0.025
"Resolution", $R_{\alpha,\beta}$ [b]:								0.79	1.57	2.17

[a]See text for the explanation of the K_1 and K_2 values used in Eqs. 20 and 21.
[b]See Eq. 24.

TABLE 2—Results of comparative evaluation of two green ink writings.

	Writing X				Writing Y			
	Reflectance value		Ratio$_i$		Reflectance value		Ratio$_i$	
Measuring site	440 nm	512 nm	Using Eq. 2 Set X	Using Eq. 23 Set X'	440 nm	512 nm	Using Eq. 2 Set Y	Using Eq. 23 Set Y'
1	2	3	4	5	6	7	8	9
1	0.2772	0.2118	1.309	1.054	0.2909	0.2338	1.244	1.014
2	0.3047	0.2393	1.273	1.048	0.3386	0.2805	1.207	1.015
3	0.2571	0.1912	1.345	1.063	0.2432	0.1853	1.312	1.021
4	0.2964	0.2280	1.300	1.064	0.2550	0.1985	1.285	1.013
5	0.2711	0.2014	1.346	1.078	0.2992	0.2435	1.229	1.007
6	0.2684	0.2014	1.333	1.065	0.2937	0.2350	1.250	1.020
7	0.2767	0.2058	1.345	1.083	0.2716	0.2160	1.257	1.008
8	0.2909	0.2220	1.310	1.067	0.2964	0.2391	1.240	1.014
9	0.3249	0.2601	1.249	1.042	0.2494	0.1954	1.276	1.000
10	0.2826	0.2148	1.316	1.065	0.2909	0.2335	1.246	1.015
Mean value (MEAN):			1.313	1.063			1.255	1.013
Standard deviation (STD):			0.032	0.012			0.030	0.006
Relative standard deviation (REL STD):			0.025	0.012			0.024	0.006
"Resolution," $R_{\alpha,\beta}^{a}$:							0.93	2.68

^{a}See comments to Table 1.

correction" for comparative examination of similarly colored materials introduced in this paper.

In regard to comparing similarly colored inks this method is used as follows.

For every ink writing that is to be compared, several (usually about 10) sites that have apparently similar thickness (depth of ink), surface characteristics (morphology, gloss, and so on) and homogeneity (homogeneous distribution of the ink in the measuring fields), are located under the microscope.

The reflectance spectra of these sites are recorded in visual range of electromagnetic spectrum (it should be stressed that this method can also be used in ultraviolet and near infrared regions).

The sets of the recorded spectra are investigated to eliminate outliers (the spectra that seem to be very probably wrong: ones located far away from the others or ones having obvious distortions of their shape, etc.)

The remaining spectra (those that seem OK) are compared by using the following procedure (with the help of an appropriate program developed in this work as a specific routine applicable in forensic analysis for comparative evaluation of similarly colored materials).

Two analytical wavelengths, λ_1 and λ_2, are determined (alternatively, they can be chosen by the examiner) as those at which the distances (Euclidean distances [15]) between the spectra recorded for the compared materials, X and Y, have maximum ($\text{dist}_{\max}^{X,Y}$) and minimum ($\text{dist}_{\min}^{X,Y}$) values, respectively.

For every spectrum (i) the ratio (*ratio_i*) of the aperture reflectance values taken at these two wavelengths is determined by using Eq. 23. Finally, the sets of the *ratio_i* values obtained for the inks analyzed are compared by using statistical methods (see the following).

The *ratio_i* values obtained for the ink lines X and Y are given below (see Table 2).

Results and Discussion

Densitometric Thin-Layer Chromatography

The densitogram was recorded for each sample of the mixture A (solutions A1-A5) and mixture B (B1-B5). Peak height values calculated by the integrator for separated analytes, PH_1 (for dye 1) and PH_2 (for dye 2) were used for computing their ratios by using Eqs. 1, 20, and 21 (see Table 1).

The values of *Ratio_i* listed in columns 4, 5, 9, and 10 of Table 1 were calculated by using the following coefficients[6]: $K_1 = 9.472$ and $K_2 = 0$ in Eq. 20; $K_1 = -0.00172$ and $K_2 = 0$ in Eq. 21.

These (optimal) values of coefficient K_1 were determined by using an iterative procedure as follows.

The value of K_1 was changed successively (with a chosen increment) within a certain interval of values. At every step of this procedure the REL STD of the *Ratio_i* values were computed for each mixture compared (A1-A5 and B1-B5) and the sum of these REL STD was calculated. The optimal value of K_1 was chosen as one at which this sum became minimum.

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[6]In this paper, the simplified way of calculating the ratios of signal outputs by using Eqs. 20, 21, and 23 is considered, that is the way when $K_2 = 0$. In the examples described in the paper, such approach demonstrated quite satisfying discriminating power. However, the maximum of the discriminating power of the proposed techniques can be achieved only when optimal values of both coefficients, K_1 and K_2, determined by using an iterative procedure, are used simultaneously (capabilities of this approach are being studied).

The portion of the table of the results, received when the procedure using Eq. 21 was executed in the example considered follows.

K_1-value	(REL STD)$_A$	(REL STD)$_B$	Sum of REL STD
−0.00223	0.020	0.011	0.031
−0.00206	0.017	0.010 (min)	0.027
−0.00189	0.015	0.011	0.026
−0.00172	0.013	0.012	0.025 (min)
−0.00155	0.012	0.014	0.026
−0.00138	0.011 (min)	0.017	0.028
−0.00121	0.012	0.023	0.035
. . .	. . .	. . .	. . .
0	0.032	0.047	0.079
0.0017	0.036	0.052	0.088

These results show that for every set, A and B, there is a value of K_1 at which the REL STD of the $Ratio_i$ values is minimum. (It corresponds to the situation shown in Fig. 3 when lines A′ and B cross at a point belonging to the X-axis). The use of the minimum sum of (REL STD)$_A$ and (REL STD)$_B$ is a compromise allowing one and the same value of K_1 to be used for calculating the $Ratio_i$ values for all samples of the mixtures compared.

The parameter of $R_{\alpha,\beta}$ (it is similar to a "Peak Resolution" parameter that is widely used in chromatography for evaluating the extent of separation of two adjacent peaks on the chromatogram [16]) was calculated as

$$R_{\alpha,\beta} = \frac{|(MEAN)_\alpha - (MEAN)_\beta|}{(STD)_\alpha + (STD)_\beta} \quad (24)$$

where α and β relates to materials compared (A and B, in the example considered).

With the aid of this parameter[7] the discriminating power of the techniques can easily be evaluated. Thus, it is clearly seen in Table 1 that "resolution" between $Ratio_i$ values obtained for the mixtures compared is quite satisfying in cases when Eq. 20 and especially Eq. 21 were applied (in these both cases $R_{\alpha,\beta} > 1$: it means that the two compared sets, A and B, of $Ratio_i$ values do not overlap). At the same time, the signal-to-signal ratio technique produced bad "resolution" of the $Ratio_i$ values calculated for the mixtures A and B: the value of $R_{\alpha,\beta} = 0.79 < 1$ shows significant overlapping of the sets of $Ratio_i$ values (hence, these sets cannot be considered as discriminated ones).

The values of the peak heights represented in Table 1 (columns 1, 2, 6, and 7, respectively) and the corresponding values of the content of dyes 1 and 2 in the separated zones (see above) are well approximated by the Michaelis-Menten function (6) (correlation coefficients were 0.9991, 0.9978, 0.9984, and 0.9979, respectively) and not so satisfactorily—by the linear function (8) (correlation coefficients obtained for the same sets of the peak heights' values, were 0.9897, 0.9954, 0.9839, and 0.9921, respectively). In consequence, the ratioing of the peak heights by using Eq. (21) gives statistically better results (the values of REL STD are 0.013 and 0.012 for the mixtures A and B, respectively) than using Eq. 20 (the values of REL STD are 0.021 and 0.020, respectively).

At the same time, the example considered shows obvious advantages of both techniques over the signal-to-signal ratio technique (the corresponding values of REL STD are 0.032 and 0.047, respectively).

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[7]The sets of $Ratio_i$ values can also be compared by using nonparametric tests such as, for example, that described the following.

All above mentioned can be illustrated with the help of the following equations:

$$(\text{MEAN})_A \pm \Delta_A = K_A \text{ (masses' ratio)}_A \tag{25}$$

$$(\text{MEAN})_B \pm \Delta_B = K_B \text{ (masses' ratio)}_B \tag{26}$$

where A and B relate to the mixture A (solutions A1-A5) and mixture B (solutions B1-B5), respectively; MEAN is the arithmetic mean of the $Ratio_i$ values, Δ is an interval taken as STD or some other precision quantity, K is an experimental constant, masses' ratio is the actual value of the ratio of the masses of dye 1 and dye 2 in solutions A1-A5 ($\frac{1}{3}$, μg/μg) and B1-B5 ($\frac{1}{3.25}$, μg/μg), respectively (see the previous).

In this example and many other similar cases tested by the author (for example, see [11]) the obtained values of Δ are small when peak heights' ratios are calculated by using Eqs. 20 and 21, and are sufficiently higher when the technique using Eq. 1 is applied.

It means that correlation between "masses' ratios" and corresponding "$Ratio_i$ values" is usually much better for the introduced technique using Eqs. 20 and 21 than that for the signal-to-signal ratio technique. Otherwise, the proposed technique is less mass-dependent than the signal-to-signal ratio technique.[8]

The steps of the proposed "modified signal-to-signal ratio procedure" are illustrated by the flow diagram shown in Fig. 6. The procedure includes the described above operations of sampling and extracting dye components of materials that are to be compared, applicating the extracts on a TLC plate, developing and scanning the TLC plate in reflectance mode, and comparing the corresponding sets of ratios obtained for the analyzed materials, by using Eqs. 20 and 21 that include the iterative procedure described above.

The proposed technique was tested to discriminate between similarly colored pairs of inks and paints prepared for this work. The technique proved its high discriminating power with regard to these pairs of materials whereas the usual signal-to-signal ratio technique failed to distinguish those "duplicates" in all cases considered. The results obtained were similar to those that had already been published [11] and so, for the sake of saving place, they were not included in this paper.

In addition to the description given above it is also important to stress that the proposed technique has its maximum efficiency (see also footnote 6) in cases when the ranges of contents of analytes per spots are rather similar. So ink samples taken from materials which are to be compared should be of approximately equal mass.

Microspectrophotometry

The $ratio_i$ values obtained for two indistinguishable to the eye green lines that have been written by the inks X and Y prepared as color duplicates with slightly different proportions of their two colored components (see the previous) are shown in columns 5 and 9 of Table 2 together with the values of two analytical wavelengths determined as recommended above.

Also, Table 2 (columns 4 and 8) contains the values obtained by direct ratioing the corresponding reflectance values measured, that is, by using Eq. 2.

---

[8]In the work [7] it is claimed that "chromatographic variation is a major contributor to the variation in the ratio value obtained." However, basing on the results of this study described previously, it is possible to state that it is mass-dependence inherent to the signal-to-signal ratio technique, that is the main cause of the variations in the $Ratio_i$ values obtained (provided, of course, relevant quality of the chromatograms obtained). To decrease these variations, the proposed technique could be used instead of the usual peak ratioing technique.
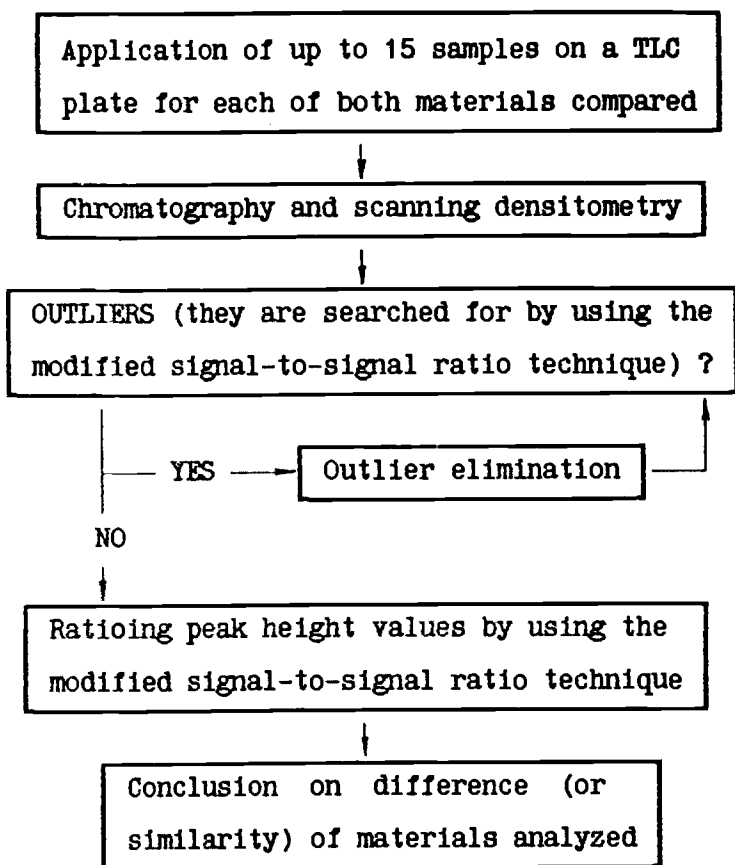
Application of up to 15 samples on a TLC plate for each of both materials compared

↓

Chromatography and scanning densitometry

↓

OUTLIERS (they are searched for by using the modified signal-to-signal ratio technique) ?

— YES ——→ Outlier elimination ——┘

NO

↓

Ratioing peak height values by using the modified signal-to-signal ratio technique

↓

Conclusion on difference (or similarity) of materials analyzed

FIG. 6—*Flow diagram of the functional sequence of operations in comparison of similarly colored materials by using thin-layer chromatographic densitometry coupled with the introduced modified signal-to-signal ratio technique.*

Comparison of the sets of the *ratio*$_i$ values determined for ink writings X and Y were executed by using the parameter $R_{\alpha,\beta}$ considered previously. Here again, the proposed technique shows much better "resolution" ($R_{\alpha,\beta}$ = 2.68) between the *ratio*$_i$ values calculated for the ink writings X and Y than that ($R_{\alpha,\beta}$ = 0.93) obtained when the signal-to-signal ratio technique was applied.

Similar results were obtained with the help of the nonparametric test[9] described in [18] (see also [19]).

According to this test the data from the sets, X' and Y' (or X and Y), were arranged in ascending order. The data from set X' (and set X) were marked by a "+" sign and the data from set Y' (and set Y) by a "−" sign. As a result of this arrangement, the

---

[9]The nonparametric statistics was used in this work for comparing the sets of the obtained *Ratio*$_i$ values because these values were not normally distributed (it is due to nonlinear transformation taking place when ratioing signals is being carried out [17]). At the same time, the use of the $R_{\alpha,\beta}$ or similar parameters assuming a Gaussian distribution, is also possible for evaluating, at least in a first approximation, the results of comparative examination of similar materials, as it has been demonstrated in the examples described. (This "statistical problem" needs special consideration that is beyond the scope of the present paper).

successions of the figures were obtained for the data from the sets X' and Y' (see Table 2, columns 5 and 9)

$$
\begin{array}{cccc|cccc}
1.000\ 1.007\ \ldots\ 1.020\ 1.021 & 1.042\ 1.048\ \ldots\ 1.078\ 1.083 \\
-\qquad\qquad -\qquad\quad -\qquad\ - & +\qquad\ +\qquad\qquad\ +\qquad\ + \\
\hline
\text{Series 1} & \text{Series 2}
\end{array}
$$

and for the data from the sets X and Y (see Table 2, columns 4 and 8)

$$
\begin{array}{ccccc}
1.207\ \ldots\ 1.246 & 1.249\ 1.250\ 1.257 & 1.273 & 1.276\ 1.285 \\
- & + \qquad - & + & - \\
\hline
1 & 2 \qquad 3 & 4 & 5
\end{array}
$$

$$
\begin{array}{ccc}
1.300\ \ldots\ 1.310 & 1.312 & 1.316\ \ldots\ 1.346 \\
+ & - & + \\
\hline
6 & 7 & 8
\end{array}
$$

that formed two and eight " + " and " − " series, respectively.

It is known [18] that if the data sets (for example, the sets X' and Y') come from the same population, then the probability of getting m-series ($m = 2, \ldots, d$, where d is a number of series obtained in a real case of analysis) in this succession is expressed via a function $h(m)$ that, for equal sets ($N_1 = N_2 = N$, where N is the number of data in each set) is calculated using Eqs 27 and 28

$$
\text{For } m = 2i \qquad h(m) = 2(i - 1_{C_{N-1}})^2/N_{C_{2N}} \tag{27}
$$

$$
\text{For } m = 2i + 1 \qquad h(m) = 2(^iC_{N-1})(^{i-1}C_{N-1})/^NC_{2N} \tag{28}
$$

where ^iC_j is the number of combinations of i items from a total of j items, that is

$$
^iC_j = \frac{j!}{(j - i)! \times i!} \tag{29}
$$

The probability that the sum, m, of series will be less than or equal to the value of d, is calculated by Eq 30

$$
P(m \Leftarrow d) = \Sigma\, h(m) \tag{30}
$$

If $P(m \Leftarrow d) < \beta$ (β is a significance level: in this case, in order to be more certain that we made a correct decision, a high level of significance, 0.005, was used), then the difference between the sets compared is significant.

In this example the following results were obtained:

For the proposed technique using Eq 23 the data from the sets X' and Y' (see Table 2, columns 5 and 9) formed 2 " + " and " − " series as it was shown above (that is, $d = 2$): $P(m = 2) = h(2) = 1.1 \times 0^{-5} \ll \beta$, that is, using this technique it was possible to show that there was a significant difference between the two inks analyzed.

For the "reflectance values ratio" method [13] the data from the sets X and Y (see Table 2, columns 4 and 8) formed 8 " + " and " − " series as it was shown above (that is, $d = 8$): $P(m \Leftarrow 8) = h(8) + h(7) + \ldots + h(2) = 0.35 \gg \beta$, so this method did not enable discrimination between inks X and Y which, in fact, contained dyes in different proportions (see section "Experimental Procedure").

Recording reflectance spectra for two samples compared

↑

Obvious differences between spectra ?   — YES →   Conclusion on discrimination between samples

NO

↓

Recording about ¡0 spectra for every sample

↓

OUTLIERS (they are searched for in each set of spectra by using the "2λ-method with correction of spectral values") ?

— YES →   Outlier elimination

NO

↓

Calculating $Q = DIS_{max} / DIS_{min}$ (see in the text)

0 < Q < 2 ? — YES

NO

↓

Comparing samples by using "2λ-method with correction of spectral values"

Calculating Euclidean distances between sets of spectra compared

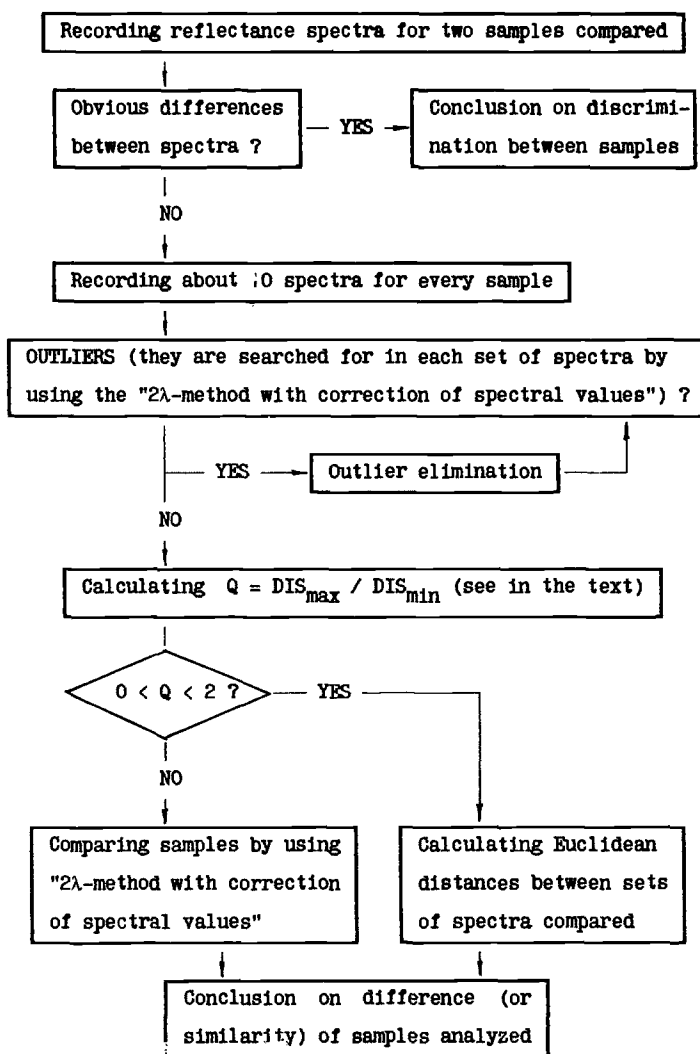Conclusion on difference (or similarity) of samples analyzed

FIG. 7—*Flow diagram of the functional sequence of operations in comparison of similarly colored materials by using reflectance microspectrophotometric method coupled with the introduced "2λ-technique with correction."*

By using the considered technique several pairs of inks and paints, prepared with small differences in proportions of their colored ingredients, were discriminated whereas both, the "2λ-method" [13] and the method of determining distances between spectra [4], failed to discriminate between those "duplicates."

Position of the Modified Two Wavelength Technique in the General Procedure

The proposed technique seems an effective tool for discriminating samples having similar spectral curves in ultraviolet, visible and near infrared ranges. At the same time there is a condition that should be taken into consideration to use this technique with maximum efficiency.

The point is that this technique is not effective for discriminating samples that are sufficiently different in lightness, that is, when one of the samples which are to be compared looks obviously lighter (or darker) than the other one. For such pairs of samples the "Euclidean distance" method [4,15] should be applied.

In order to determine which method should be used first the following calculating operation has been incorporated into the procedure described previously.

Before ratioing the measured reflectance values by using Eq. 23 it is necessary to calculate the quotient (Q) of the obtained values of maximum and minimum distances between the groups of spectra recorded for compared samples X and Y:

$$Q = (\text{dist}_{max}^{X,Y})/(\text{dist}_{min}^{X,Y}) \tag{31}$$

It was found in this work that the proposed technique is most effective in cases when either Q is a negative number (this means that mean spectra calculated for samples compared have at least one point of intersection) or Q is more than 2. And, on the contrary, in cases when $O < Q < 2$ the application of the method of determining distances between the spectra of samples compared [4] is preferable.

Thus, with this addition considered, the whole procedure of using the MSP method for comparative examination of similarly colored materials can be illustrated by the following flow diagram (Fig. 7).

Conclusion

New techniques are proposed for comparison of similarly colored inks and other materials of forensic interest by using scanning thin-layer chromatographic densitomety and reflectance microspectrophotometry. The techniques demonstrate high discriminating power with regard to the indistinguishable to the eye colored materials having subtle differences in relative proportions of their dye components.

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