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Characterization of Dye Extracts from Historical Cultural-Heritage Objects Using State-of-the-Art Comprehensive Two-Dimensional Liquid Chromatography and Mass Spectrometry with Active Modulation and Optimized Shifting Gradients

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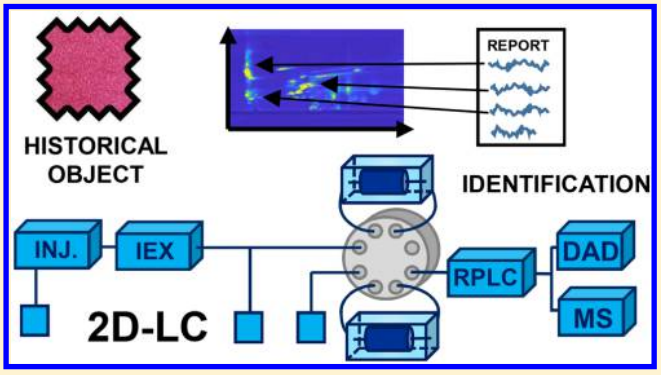
Supporting Information

ABSTRACT: Unbiased characterization of dyes and their degradation products in cultural-heritage objects requires an analytical method which provides universal separation power regardless of dye classes. Dyes are small molecules that vary widely in chemical structure and properties, which renders their characterization by a single method challenging. We have developed a comprehensive two-dimensional liquid chromatography method hyphenated with mass spectrometry and UV−vis detection. We use stationary-phase-assisted modulation to enhance the method in terms of detection limits and solvent compatibility and to reduce the analysis time. The PIOTR program was used to optimize an assembly of shifting second-dimension gradients, which resulted in a high degree of orthogonality (80% in terms of the asterisk concept). The resulting method is universally applicable to all classes of dyes extracted from cultural-heritage objects. Thanks to the high peak capacity and orthogonality, dye components can be separated from chemically similar impurities and degradation products, providing a detailed fingerprint of the dyes mixture in a specific sample. The method was applied to a number of challenging dye extracts from 17th- and 19th-century cultural-heritage objects.



Throughout the history of humanity, dyes and pigments have been widely used.[1,2] Pigments have been used at least since the Paleolithic period, as is evident from the findings of red-ocher-dyed corpses dating back to the Upper Paleolithic age, around 30 000 B.C.[3] The oldest dyed cloth ever found dates back to around 4000 B.C. and was discovered in the Indus Valley in South Asia.[1] Natural dyes have been employed predominantly throughout time until the first synthetic dye, mauveine, was discovered in 1856.[4] In the following years, the dyes industry developed dramatically, and synthetic dyes gained popularity due to their ease of production and overall better lightfastness.[5]

Dyes and dyestuff samples pose significant challenges when it comes to their analysis, due to a number of reasons. Historically, dyes were typically prepared with the goal to obtain, often brilliant, colors without paying attention to the purity of the compounds. Dyestuffs may thus contain a large number of side products. In objects dyed with natural dyes, several components are usually present, and the combination of these components is used to identify the biological source.[6,7] Also when synthetic dyes were used, complex mixtures of very similar components are often found, all contributing to the color.[8]

Dyes and their side products are also prone to degradation in time or by exposure to air, light, and/or humidity. Furthermore, all the dye components and degradation products may interact with each other and/or with the material to which they are applied. Considering that one object may be colored with a number of dyes, a single art object can give rise to 10−20 different samples, each one containing 10−20 dye-related compounds.[9]

Dyes can be classified in a large number of categories, including mordant, direct, acidic, basic, and vat dyes.[1] Typical LC methods for dye characterization allow analysis of a limited

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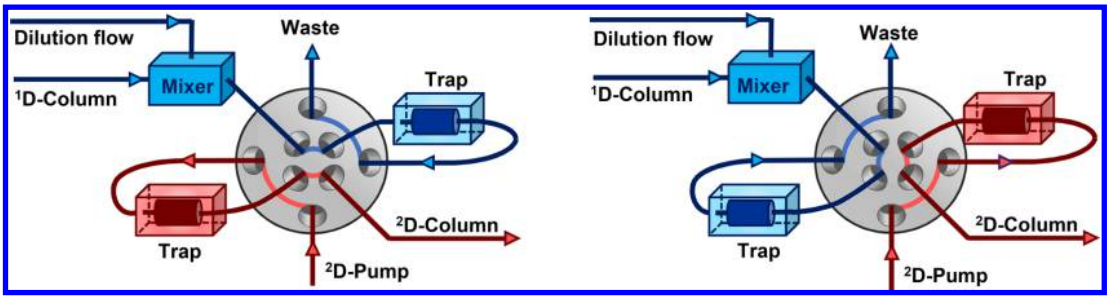

Figure 1. Schematic of the two positions of a SPAM interface. Rather than using large storage loops, analytes are effectively filtered out of the ^{1}D effluent using low-volume trapping columns. Reprinted with permission from ref 13. Copyright 2018 American Chemical Society.

subset of these classes.[8,10] In addition, while consultation of historical documents may provide conservation scientists with clues on possibly applied dyestuffs in a given object, unequivocal historical analysis is usually not possible, and a universal analytical method is required.

Ideally, an analytical technique is (i) unbiased with regard to pre-existing knowledge on a given cultural-heritage object; (ii) universal for as many classes of dyes extracted from cultural-heritage objects as possible; (iii) offers sufficient selectivity to analyze degradation products that can be chemically similar to the main compound, regardless of dye class; and (iv) provides a high separation power (chromatographic efficiency in both dimensions) and a high precision (repeatability), to ensure reliable identification of dyes present. The above premises demand a high selectivity and separation power of the analytical technique. A conventional (one-dimensional) LC-MS method may suffice to identify the dyes used. However, our goal is to also study the nature and (approximate) concentrations of dye-degradation products, so as to determine the condition of cultural-heritage objects and to support the development of conservation strategies. Because a single dye compound may give rise to 30−40 degradation products[11] that occur in vastly different concentrations, we need a separation technique with a very much higher separation power, such as LC × LC.

In this context, we recently developed a separation method based on comprehensive two-dimensional liquid chromatography (LC × LC) for the simultaneous analysis of synthetic acidic and basic dyes.[9] In LC × LC, the first-dimension (^{1}D) effluent is divided in many fractions by a modulation interface, and each fraction is injected into a second-dimension (^{2}D) separation. The total peak capacity is approximately equal to the product of the individual peak capacities of the respective separation dimensions. If two sufficiently different (so-called "orthogonal") separation mechanisms are used, this total peak capacity can be fully exploited. Our method therefore utilized a strong anion-exchange mechanism in the ^{1}D separation (separation based on charge) and a fast ion-pair reversed-phase LC separation in the ^{2}D separation (separation based on hydrophobicity).

While successful at the separation of dyes, acidic and basic dyes, the previous method showed insufficient separation for other dye classes. In the present work, we aimed to extend the method to other dye classes, including the natural dyes. Moreover, the ion-pair agent in the ^{2}D separation, tetramethylammonium hydroxide, proved to be incompatible with mass spectrometry. Other drawbacks of the previous LC × LC method were (i) high detection limits, due to the additional dilution of the sample mixture by the ^{2}D separation; (ii) the occurrence of "breakthrough" (premature elution of sample components from the ^{2}D column), due to incompatibility of the ^{1}D-effluent and the ^{2}D phase system; and (iii) a rather long analysis time of 4 h. All of these issues may be addressed using active-modulation techniques.[12]

In this work, we present a method for the characterization of natural and synthetic dyes by LC × LC with UV/vis and mass-spectrometric (MS) detection. The method is universally applicable to all types of dyes extracted from cultural-heritage objects. We have developed a stationary-phase assisted modulation (SPAM) interface to overcome incompatibility issues between the two dimensions, to enhance detection sensitivity and to reduce the analysis time. Shifted-gradient assemblies are explored to maximize the orthogonality of the two separation dimensions. The target application of the universal method is the detailed characterization of dyes, contaminants, and degradation products found in cultural-heritage. To demonstrate the potential of the technique, dye analysis is performed on a selection of 17th- and 19th-century historical objects.

EXPERIMENTAL SECTION

Hardware. *Instruments.* For all experiments, an Agilent (Waldbronn, Germany) 1290 series Infinity 2D-LC system was used for liquid chromatography and UV−vis detection. The system included two binary pumps (G4220A); one 1200 series isocratic pump (G1310A); a 1290 series autosampler (G4226A) equipped with a 20-μL injection loop; two thermostated column compartments (G1316C), in one of which an 8-port, 2-position 2D-LC valve was installed (G4236A); and one diode-array detector (DAD, G4212A) equipped with an Agilent Max-Light Cartridge Cell (G4212-60008, 10 mm path length, V_{det} = 1.0 μL). The injector needle drew and ejected at a speed of 10 μL·min^{-1}, with a two second equilibration time. All tubing was fabricated of stainless steel (SS). The chromatographic and UV−vis systems were controlled using Agilent OpenLAB CDS Chemstation Edition (Rev. C.01.04 [35]) software. For MS detection, a Bruker (Bremen, Germany) MicroQ-ToF mass spectrometer was used. The mass spectrometer was controlled by Bruker Daltonics micrOTOF control Compass 1.3 for micrOTOF-sr1 version 2.0 on a separate computer. Data were extracted using Bruker Daltonics Data Analysis, version 4.0 SP4 build 281.

Columns. For the first dimension, an Agilent PL-SAX 1000 Å (PL1951-3802, 150 × 2.1 mm i.d., d_p = 8 μm, max ΔP = 150 bar) strong anion-exchange column was used. To protect the ^{1}D column, an Agilent 1290 Infinity In-Line Filter (G5067-4638) was used. For the second dimension, an Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HD 95 Å

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(959757-902, 50 × 2.1 mm i.d., d_p = 1.8 μm, max ΔP = 1200 bar) column was used.

Trapping: SPAM. For trapping, two Phenomenex SecurityGuard ULTRA (P/N: AJ0-9000) guard-column holders were used in conjunction with two UHPLC C18 2.1 mm i.d. SecurityGuard ULTRA cartridges (P/N: AJ0-8782). Each combination of a holder and a cartridge is henceforth referred to as a trap. For each modulation loop, one trap was directly installed into one of the ports of the valve with the male end, and the female end was connected to another port using an 140 mm long SS tubing with 0.12 mm i.d.

For all actively modulated LC × LC experiments, the end of the ^{1}D column was connected through a connection-tee (P/N: U-428, IDEX, Illinois, US) with a dilution flow of 5 mM ammonium formate/formic acid buffer (pH = 3.2) in water. The combined flow-stream (at a ratio of 20:1, v/v, [formate buffer]/[^{1}D column effluent]) was directly connected to the 2D-LC valve using a Waters Zirconia 50 μL Y-mixer, P/N: 700002911, internal volume 50 μL. Figure 1 clarifies the connections and operating principle of this active-modulation technique.[13]

Chemicals. Acetonitrile (LC-MS grade) was obtained from Biosolve (Valkenswaard, The Netherlands), and deionized water (MS-grade) was procured from Merck (Darmstadt, Germany). Acetonitrile (AR grade) was obtained from VWR International (Fontenay-sous-Bois, France). Triethylamine (≥99.5%), tris(hydroxymethyl)aminomethane hydrochloride (≥99.0%) and tris(hydroxymethyl)aminomethane (≥99.9%), ammonium sulfate (BioXtra, ≥ 99%), ammonium formate (BioUltra, ≥ 99%), sodium chloride (BioXtra, ≥ 99.5%), dimethyl sulfoxide (DMSO, ≥ 99%), and formic acid (≥96%) were obtained from Sigma-Aldrich (Darmstadt, Germany). Eighty different dyestuffs and the extracts of the samples from historical objects were obtained from the Cultural Heritage Agency of The Netherlands (RCE, Amsterdam, The Netherlands).

Sample 1 was an extract from a textile found in a 17th-century shipwreck located in the Waddenzee in The Netherlands. The textile was remarkably well preserved for a 400-year-old object, rendering it very useful for studies as to their creation. The other three samples were extracts from embroideries designed by Emile Bernard. Sample 2 was from *Plant and flowers on gray fond* and dates from 1892 to 1904, and samples 3 and 4 were extracts from different sections of *Plant and flowers on yellow fond*, dating from 1891 to 1892. Readers seeking more information on these historical samples and the applied extraction procedure are referred to in the Supporting Information, section S-1.

Analytical Conditions. *Preparation Methods.* The original dyestuffs provided by the RCE were supplied as powdered pigments, which contained the dyestuff, but also degradation products, impurities, mordants, and other materials. Each sample was diluted into a stock solution, in concentrations of 5000 ppm by weight, in a mixture of ACN and DMSO 1:1 (v/v). For the analyses, these were further diluted to a concentration of 50−100 ppm, in the same solvent mixture.

A mixture of all dyestuffs in the set was also created by mixing all the 5000-ppm stock solutions in equal ratio. The result is a solution which contains all the 80 dyes, their contaminants, and degradation products, in a concentration of approximately 60 ppm for each dye.

Methods. This section describes the general method with standard parameters used in this study unless otherwise specified in the text.

For the ^{1}D strong-anion-exchange separation, the flow rate was set at 60 μL·min^{-1}. Both mobile phases consisted of 5 mM TRIS buffer, pH = 7.5, in water/acetonitrile (6:4, v/v) without (mobile phase A) or with (mobile phase B) 100 mM ammonium sulfate. The gradient program was 100% A from 0.0 to 7.5 min, a linear gradient from 100% A to 100% B from 7.5 to 45.0 min, maintained at 100% B from 45.0 to 55.0 min, and a linear gradient from 100% B to 100% A from 55.0 to 60.0 min.

The dilution flow was set at 1.14 mL·min^{-1} to achieve a ratio of 20:1 (v/v) relative to the ^{1}D effluent.

For the ^{2}D ion-pair reversed-phase LC separation, the run time (equal to the modulation time) was 0.75 min, and the flow rate was 1.2 mL·min^{-1}. The mobile phase consisted of a 5 mM triethylamine (TEA) buffer, brought to pH = 3.0 with formic acid, and acetonitrile (ACN) in a ratio of 95:5 TEA buffer/ACN (v/v, mobile phase A) and 5:95 TEA buffer/ACN (v/v, mobile phase B). A shifting gradient assembly for the second dimension was optimized using PIOTR.[14,15] In a shifting gradient, the initial modifier concentration (ϕ_{init}) and final modifier concentration (ϕ_{final}) were varied across ^{2}D modulations as a function of the (^{1}D) analysis time (^1t). The functions of $\phi_{init}(^1t)$ and $\phi_{final}(^1t)$ can be represented as boundaries in between which the ^{2}D gradients are operated. For every modulation, the ^{2}D gradient program was a linear gradient from $\phi_{init}(^1t)$ at ^2t = 0 min to $\phi_{final}(^1t)$ at ^2t = 0.4 min, maintained at $\phi_{final}(^1t)$ from ^2t = 0.4 to 0.55 min, a linear gradient from $\phi_{final}(^1t)$ at ^2t = 0.55 min to $\phi_{init}(^1t)$ at ^2t = 0.6 min maintaining at $\phi_{init}(^1t)$ until 0.75 min. The optimized lower boundary function $\phi_{init}(^1t)$ started at 30% B from 0 until 10.0 min, then decreased linearly from 30% B to 20% B from 10.0 to 40.0 min, and maintained at 20% B from 40.0 to 60.0 min. The optimized upper boundary function $\phi_{final}(^1t)$ started at 100% B from 0.0 to 10.0 min, then decreased linearly from 100% B to 65% B from 10 to 50 min, and then decreased linearly from 65 to 55% from 50 to 60 min.

Data Analysis. All data and displayed images were processed and created using the in-house written PIOTR program.[14]

RESULTS AND DISCUSSION

Development and Optimization of Analytical Method. While the previously developed method featured a good identification power for synthetic dyes, a number of fundamental and practical complications limited its applicability. These limitations included (i) concerns about quantification, due to the additional dilution by the second-dimension separation, which reduced the detection limits of the method; (ii) the occurrence of breakthrough in the ^{2}D separation; (iii) the rather long analysis time; (iv) the limited surface coverage, rendering a fraction of the theoretical peak capacity useless; (v) the incompatibility with the MS, due to the ion-pairing agent in the second-dimension mobile phase; and (vi) the lack of universality of the method (not covering natural dyes and neutral synthetic dyes).

Improvement of Detection Limits, Phase-System Compatibility, and Speed of Analysis. While the added second-separation dimension yields additional selectivity and separation power, it also further dilutes the analytes. Active-modulation techniques, such as stationary-phase-assisted

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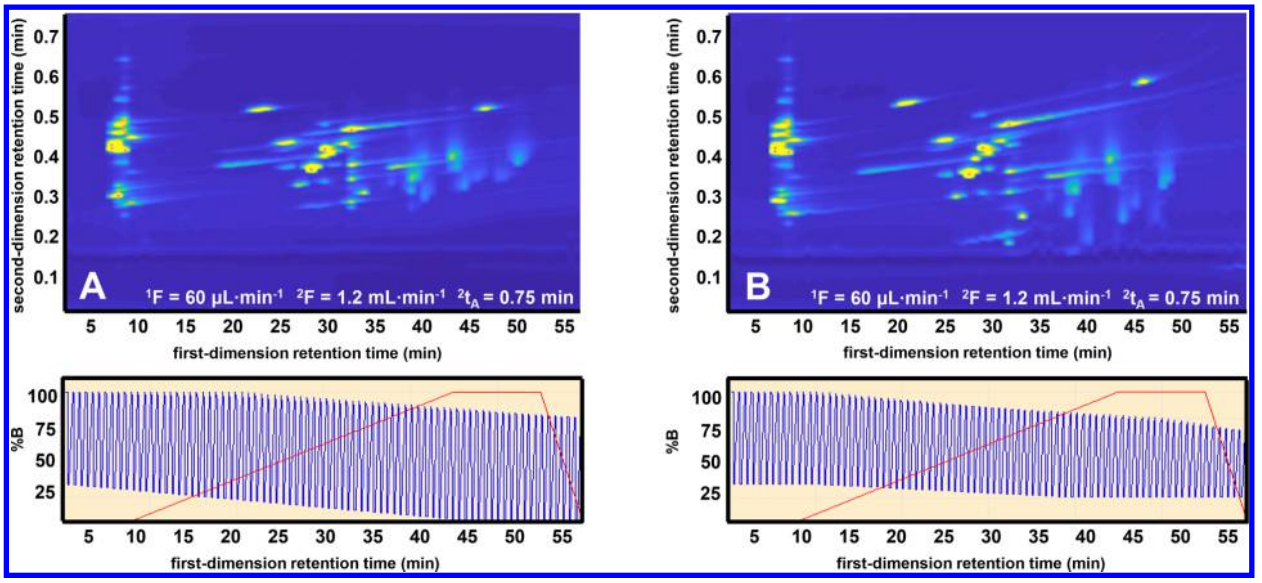

Figure 2. LC × LC chromatograms of a mixture of 80 synthetic and natural dyes using a modulation time of 0.75 min, and two different shifting gradient assemblies shown at the bottom. Detection wavelength shown, 254 nm.

modulation, can potentially alleviate this problem. In this case, each sampling loop of the ^{2}D-LC valve is replaced by a small trapping unit ("trap"), typically a guard column. As the ^{1}D effluent passes through the trap, analytes must be retained by the stationary phase. Often an additional dilution flow is utilized to establish conditions under which the analytes can be sufficiently retained. Once the modulation cycle is complete (i.e., the ^{2}D system is ready for the next injection), the valve switches, and the trap is brought in-line before the ^{2}D column, while the other trap is brought in-line after the ^{1}D column. The ^{2}D mobile phase (composition−gradient program) is then used to elute the retained analytes into the ^{2}D column.

For this study, a ^{1}D flow rate of 60 μL·min^{-1} was used in conjunction with a dilution flow of 5 mM ammonium formate/formic acid buffer (pH = 3.2) in water at 1.140 mL·min^{-1} to achieve an effective dilution ratio of 1:20 (v/v). Other dilution ratios were also investigated, and the results are shown in the Supporting Information, section S-2. A low pH was used to neutralize any organic acids. One of the diode-array detectors was used to monitor the waste stream from the ^{2}D-LC valve to verify the absence of premature elution from the traps.

The dead volume of active-modulation traps typically is in the order of a few microliters, and the little tubing required merely serves to connect the trap to the valves. Consequently, most of the ^{1}D mobile phase is discarded, which effectively increases the concentration of the analyte bands in the trap, eventually improving the detection limits. Moreover, the absence of ^{1}D mobile phase during the release of analytes from the trap diminishes the risk of breakthrough resulting from solvent incompatibility. Moreover, as the full volume of the fractioned ^{1}D effluent must no longer be transferred to the second dimension, the ^{1}D flow rate can be increased, which may result in a shorter analysis time, provided that undersampling[16] of the first dimension is avoided. Finally, a smaller ^{2}D injection volume may result in a better separation efficiency and a higher peak capacity.[16]

Improvement of MS Compatibility and Separation-Space Coverage. As a result of the reduced volume of the collected ^{1}D fraction, the ^{2}D injection volume is significantly decreased. Consequently, shorter and narrower columns can be used, leading to a lower ^{2}D flow rate and allowing more (or all) of the ^{2}D effluent to be directed to, for example, a mass spectrometer with a flow-rate restriction (e.g., micro- or nanoelectrospray). To exploit this fully, the previously employed ion-pairing agent, tetramethylammonium hydroxide, was replaced by the reasonably volatile triethylamine in the ^{2}D mobile phase, while the pH was kept at 3.0.

By increasing the ^{1}D and ^{2}D flow rates, the modulation and analysis times of the method could be reduced from 1.0 and 90 min (Supporting Information, section S-3, Figure S3A) to 0.5 and 45 min (Supporting Information, section S-3, Figure S3B), respectively. However, the narrow peaks observed in the ^{2}D separation proved challenging for the specific MS instrument used. To sample each chromatographic peak sufficiently, while maintaining sufficient sensitivity, a sampling rate of 4 Hz was selected for the MS. This led to a minimum modulation time of 0.75 min.

To improve the surface coverage, an optimal shifting gradient assembly was predicted using the PIOTR program according to the procedure described elsewhere.[14] The improvement resulting from the application of this program becomes apparent from the two chromatograms and corresponding gradient assemblies shown in Figure 2A,B. The coverage of the separation space is improved in Figure 2A (asterisk orthogonality of 0.700 to 0.804), thus increasing the effective peak capacity. Readers seeking more information on general interpretation or a clarification on the appearance of LC × LC chromatograms are referred to the Supporting Information, section S-4.

Improvement of the Mixed-Mode ^{1}D Separation. Originally, anion-exchange was selected as the ^{1}D separation mechanism, because a large fraction of synthetic dyes contains anionic moieties. In anion-exchange chromatography, negatively charged analytes are retained, whereas cationic or neutral species elute early (i.e., effectively unretained). All of the neutral and synthetic dyes which coelute around the dead-volume from the ^{1}D separation then must be separated by a fast and efficient ^{2}D reversed-phase separation. This is not a realistic expectation. The total number of neutral analytes exceeds the local peak capacity of this part of the separation

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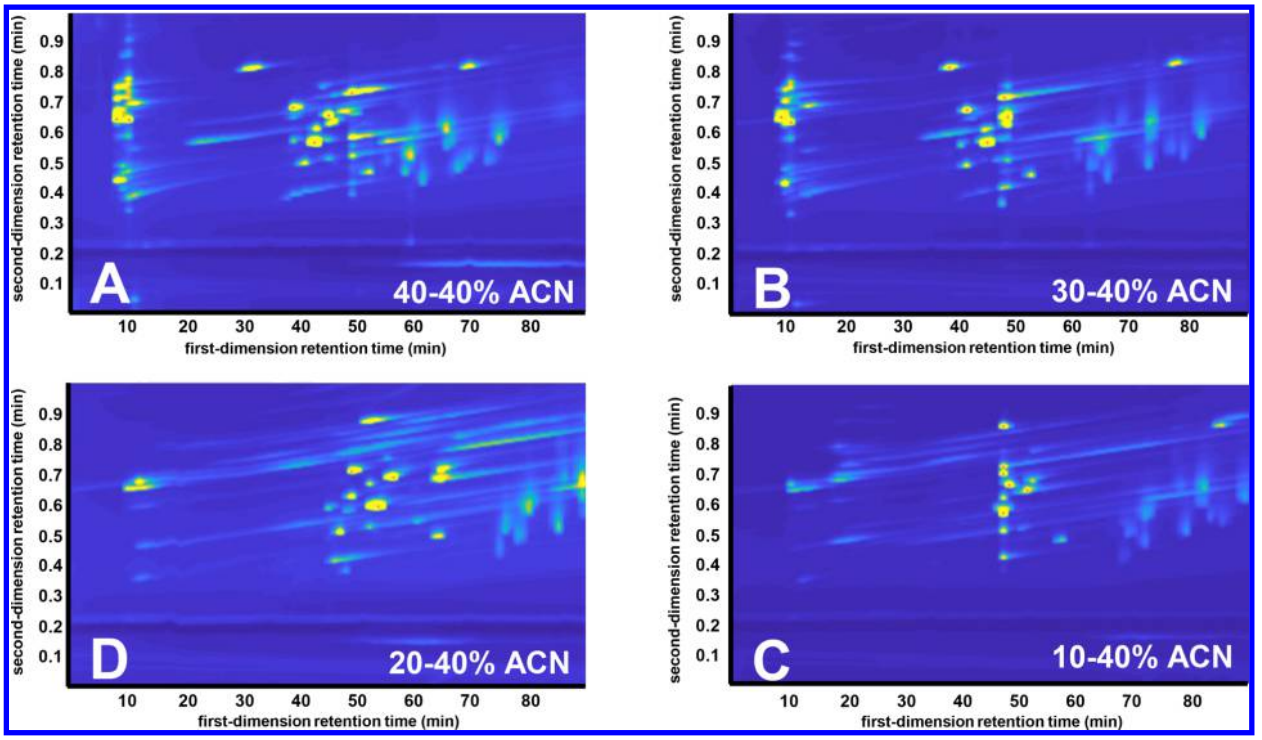

Figure 3. Separation of 80 natural and synthetic dyes with a ^{1}D ionic-strength gradient (see the Methods section) and (A) a constant concentration of 40% ACN, or a gradient from (B) 30 to 40%, (C) 20 to 40%, or (D) 10 to 40% of ACN. Detection wavelength shown, 254 nm.

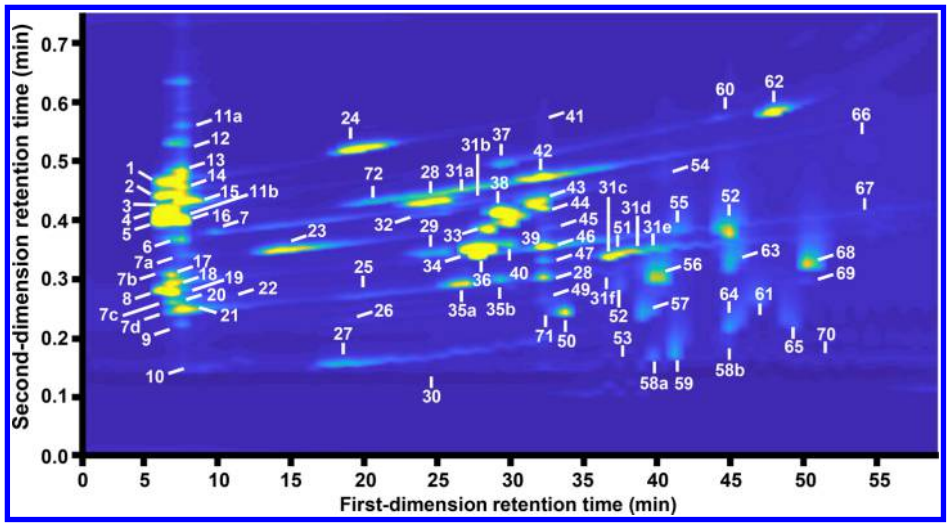

Figure 4. LC × LC separation of a mixture of reference standards using mixed-mode strong anion-exchange in the ^{1}D and ion-pair reversed-phase LC in the ^{2}D. Tentative identification using MS and UV−vis spectra, (1) victoria blue B, (2) victoria blue R, (3) methyl violet (= crystal violet), (4) rhodamine B, (5) methyl violet−CH_3, (6) diamond green B, (7) fuchsin (multiple components), (8) berberin, (9) isatin, (10) (+) epi-catechin and (+) catechin hydrate, (11) vesuvine BA, (12) auramine A, (13) azo flavine, (14) diamond green G, (15) turmuric, (16) rhodamine 6G, (17) safranine T, (18) auramine B, (19) orcein, (20) chrysoidin, (21) methylene blue, (22) Negrosin, (23) Kaempferol, (24) Emodin, (25) Fisetin, (26) Brazilin, (27) Rutin, (28) Alizarin Yellow, (29) Picric acid, (30) Murexide, (31) Water blue IN, (32) Azo flavine 3R, (33) Flavazin L, (34) crocein orange G, (35) quinoline yellow, (36) metanil yellow, (37) wool-cloth scarlet, (38) martius yellow, (39) orange IV, (40) patent blue V, 4(1) erythrosin, (42) alizarin, (43) fast red AV, (44) uranin A, (45) purpurin, (46) quercetin, (47) sulfuretin, (48) orange I, (49) silk scarlet, (50) chrysoin, (51) morin, (52) cotton scarlet, (53) carminic acid, (54) wool red B, (55) amido black 10B, (56) ponceau RR, (57) amido naphtol red G, (58) indigo carmine, (59) azo fuchsine 6B, (60) eosin degradant (one bromine atom lost), (61) orange GG, (62) eosin A, (63) fast red B, (64) naphtol yellow, (65) fast acid magenta B, (66) congo red, (67) brilliant yellow, (68) crystal ponceau, (69) yellowish light green SF, (70) amaranth, (71) tartrazin, and (72) rhamnetin. Chromatogram shown reflects absorption at 254 nm.

space. We must remedy this if we seek extension of the method to include neutral, hydrophobic natural dyes.

Fortunately, some ion-exchange columns also provide retention based on hydrophobicity under RPLC conditions. This so-called mixed-mode (ion-exchange/RPLC) behavior offers opportunities for creating additional retention (and increased peak capacity) for the neutral dyes. Various ^{1}D dual-gradient programs were explored, as illustrated in Figure 3. During the ion-exchange counterion gradient, the initial acetonitrile concentration was also varied from 40 (Figure

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Table 1. Repeatability of ^{1}D Retention Times for Five Components in Five Different Experiments Conducted on Different Days across 2 Weeks

	$t_{R,1}$ (min)	$t_{R,2}$ (min)	$t_{R,3}$ (min)	$t_{R,4}$ (min)	$t_{R,5}$ (min)	$t_{R,avg}$ (min)	σ (min)
picric acid	22.787	22.785	22.775	22.775	22.766	22.778	0.009
quinoline yellow	23.488	23.484	23.488	23.486	23.488	23.487	0.002
orange II	25.041	25.043	25.041	25.044	25.046	25.043	0.002
martius yellow	26.590	26.589	26.590	26.588	26.593	26.590	0.002
naphthol yellow S	42.216	42.213	42.219	42.225	42.185	42.212	0.016

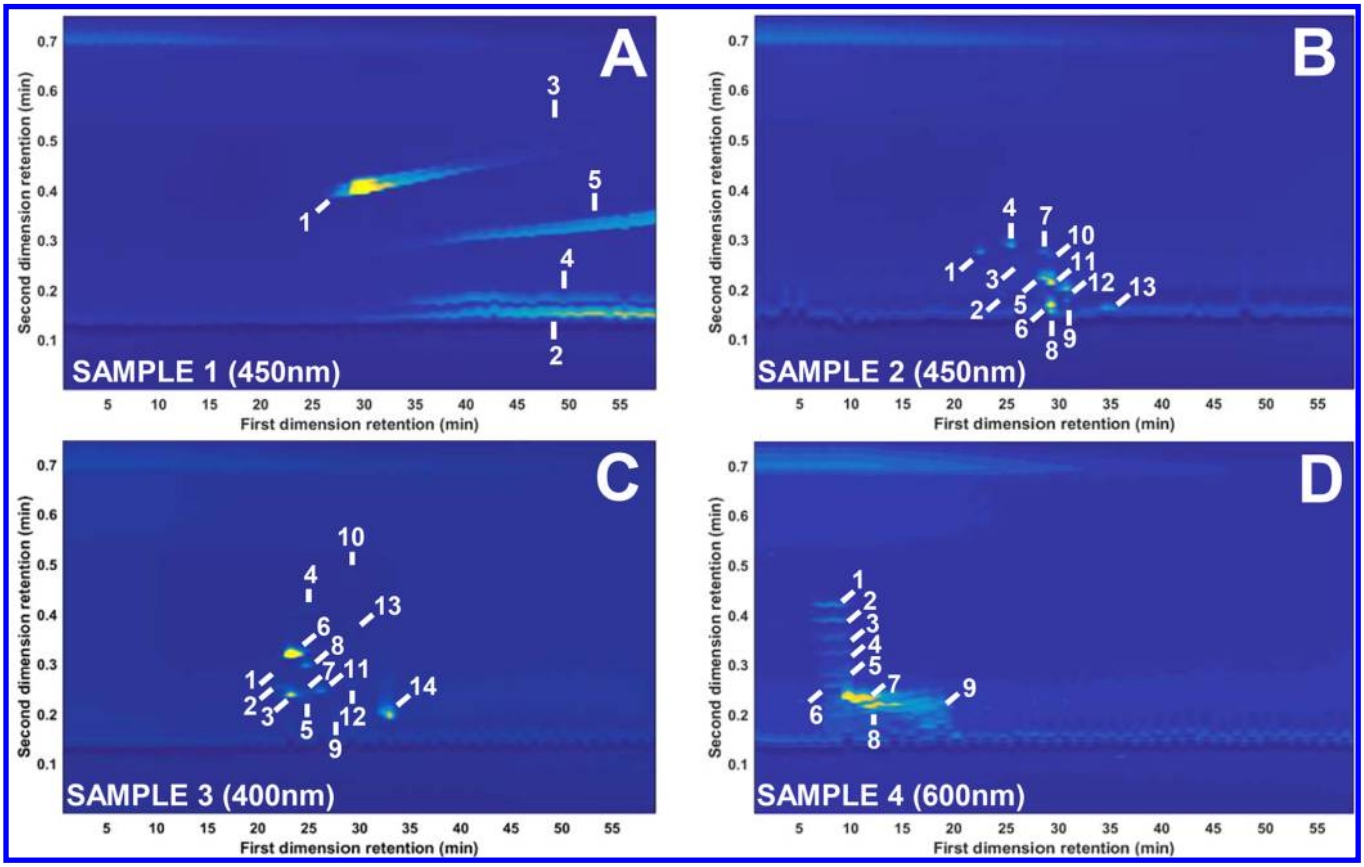

Figure 5. LC × LC separations for samples 1−4 (A−D, respectively). Peak assignments, (A) (1) alizarin, (2) carminic acid, (3) purpurin, (4) unknown, and (5) unknown; (B) (1) picric acid, (2, 3) trace unknown, (4) patent blue V, (5) patent blue V isomer with two C_2H_4 groups lost, (6) water blue IN, (7) patent blue V isomer with one C_2H_4 group lost, (8) water blue IN, (9) patent blue V isomer with three C_2H_4 groups lost, (10) patent blue V isomer with one C_2H_4 group lost, (11) patent blue V isomer with two C_2H_4 groups lost, (12) patent blue V isomer with three C_2H_4 groups lost, and (13) unknown; (C) (1, 2) trace unknown, (3) quinoline yellow component 1, (4, 5) trace unknown, (6) flavazine L, (7) yellowish light green SF, (8) orange II, (9, 10) trace unknown, (11) quinoline yellow component 2, (12, 13) unidentified trace components, and (14) tartrazine; (D) (1−5) crystal violet components, (6) unknown, and (7−9) degradation products of patent blue V. Data from DAD is shown at optimal wavelengths for clarity. Identification based on MS and UV−vis spectra.

3A), 30 (Figure 3B), 20 (Figure 3C), and 10% (Figure 3D) ACN in mobile phase A to 40% ACN in mobile phase B. In essence, the two gradients were concurrent. Unfortunately, the increase in retention, as the initial ACN concentration is lowered, is accompanied by a deterioration of the peak shapes. This occurred especially for weak anions, which were found to coelute with the strongly charged anions.

One possible solution was thought to have the ACN gradient preceding the salt gradients, instead of having them run concurrently. While ^{1}D-LC experiments with two successive gradients were promising (see the Supporting Information, section S-5 for chromatograms), proceeding with this approach would, however, require a quaternary pump with a low-dwell volume, which was not available. Further attempts to increase the retention of neutral compounds were abandoned.

Figure 4 illustrates the optimized LC × LC separation with almost all of the components that give rise to significant peaks identified using the information provided by both the MS and UV−vis detectors for each data point (see the Supporting Information, section S-8 for a full reference database of all dyes). Both synthetic and natural dyes are well separated, and the surface space is well covered. In terms of the asterisk equation, the orthogonality is 0.80 (Z_-, 0.94; Z_1, 0.97; Z_+, 0.85; and Z_2, 0.82), which is very high.[17] See the Supporting Information, section S-6 for a full description of the used data and a detailed calculation. The good separation and high orthogonality contribute to a universal applicability of the

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method to all dyes extracted from cultural-heritage objects regardless of class.

The final attribute for the method to be successful for this purpose is a good repeatability. Retention times in both dimensions were found to be highly repeatable. Some representative values for standard deviations in measured retention times for the standard mixture are listed in Table 1. When an unknown sample is injected using the universal method, any known dyes present can be rapidly identified with a high degree of confidence, even without the availability of MS information.[18] Moreover, LC × LC chromatograms give an instant impression of additional dyes, related impurities, and degradation products present in the sample.

Application. The developed universal method was applied to the historical samples 1−4. For sample 1, the resulting separation is shown in Figure 5A. The main components found were alizarin, carminic acid, and purpurin. In addition, two unknown compounds were found, which have not yet been identified. Compounds that are highly and variably charged at the ^{1}D mobile-phase pH of 7.5 elute as very broad peaks from the mixed-mode ion-exchange separation. A case in point is carminic acid (analyte #2), with pK_a values of 3.39, 5.78, 8.35, 10.27, and 11.51,[19] and a resulting charge close to −3 at the ^{1}D conditions. In the vicinity of a pK_a value, different analyte ions will be differently charged. The pK_a may also vary locally, especially in the vicinity of the stationary phase, where the solvent composition is different. All of this contributes to very broad and poorly shaped ^{1}D peaks. However, for identification purposes, oddly shaped peaks are very useful, adding yet another factor to reduce the uncertainty of peak identification. For compound #4, which could not directly be identified, its retention times and peak shapes suggest that it is quite similar to carminic acid.

The separation of sample 2 is shown in Figure 5B. Overall, the peak assignments clearly show the presence of the dyestuff patent blue V as the main component. However, thanks to the high local peak capacity of the method, compounds that are quite similar are also separated. Multiple isomers and degradation products of patent blue V can be discerned. This fingerprint may provide insight in the extent of dye degradation. In addition, traces of picric acid, water blue IN, and a number of yet unknown components were found. The separation of sample 3, shown in Figures 5C, also reveals the presence of a large number of unknown compounds and degradation products next to several major dye components flavazine L, yellowish light green SF, orange II, quinoline yellow components, and tartrazine. Sample 4 (Figure 5D) is an example of a challenging case. The extract appears to consist of several crystal violet and patent blue V components. However, the retention patterns of components #7−9 suggest that all of the patent blue V components have lost their charge due to conditions encountered during the history of the cultural-heritage object.

The universal method developed in this study opens new avenues for the characterization of dyes in cultural-heritage objects. Individual samples never contain the vast numbers of dyes that may potentially be separated (Figure 5). Instead, a typical sample contains a small number of dyes, the most common of which can be readily identified with a good deal of certainty. However, prior to analysis, it is not known what dyes to expect. A universal method, as presented here, allows for the identification of a wide variety of components from different dye classes without changing the chromatographic system as done in the past.[7,8] Dyes that are revealed using the present method but cannot currently be identified present challenges to the art-science community and, as such, may spur progress in the field. The new knowledge created may provide crucial information from a historical perspective.

Typical samples also contain minor peaks, which may provide information on the origin of the material (side products, contaminants) or on the state of degradation (degradation products). Figure 6B provides a case in point.

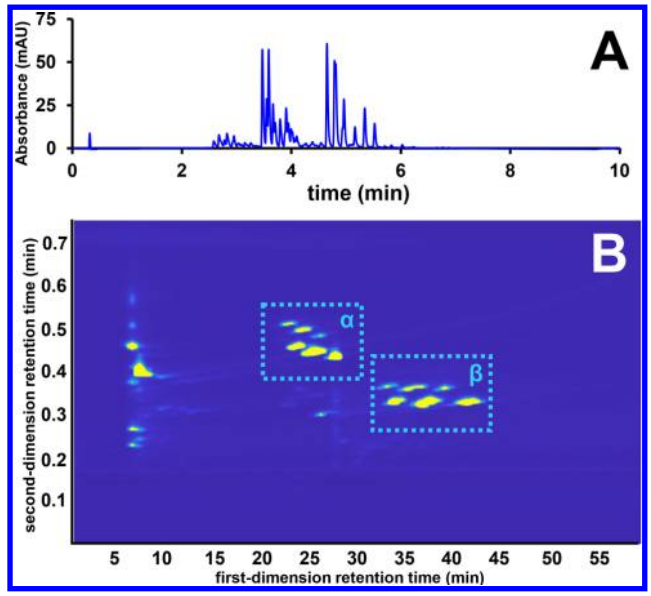

Figure 6. (A) RPLC-DAD chromatogram of a water blue IN reference mixture. (B) LC × LC separation of a mixture containing water blue IN. The two highlighted ensembles of peaks illustrate how the orthogonal separation power of ^{2}D-LC can improve the information provided. Detection wavelength shown, 600 nm.

The LC × LC chromatogram shows a number of neutral or positively charged dyes that elute around t_0 in the first dimension and two highly informative sextets of peaks which belong to differently methylated forms of the dye water blue IN. The difference between the two sextets is one charged (sulfonate) group. Within a sextet, a horizontal step to the left reflects the presence of an additional polar group, and the upward shift reflects isomers. The LC × LC chromatogram provides a great deal of instant information on the variations encountered around the basic structure of the dye (Supporting Information, section S-7). When performing high-resolution ^{1}D separations (Figure 6A), a good separation can be obtained, but the interpretation of the chromatogram is not straightforward and requires careful interpretation of UV/vis and MS spectra. Also, trace compounds are revealed much more clearly in LC × LC chromatograms.

CONCLUSION

The analysis of dye extracts from cultural-heritage objects presents a major challenge for analytical chemists. A large variety of dye classes exist, which can feature almost all types of molecular properties that are typically encountered in small-molecule separations. This puts very high demands on LC separations. To fill the need for a universal tool for the separation of dyes and their degradation products, we have developed a highly orthogonal method based on actively modulated comprehensive LC × LC, which provides similar

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separation power regardless of dye class. We have demonstrated the method on extracts taken from 17th- and 19th-century historical objects. The representative separations shown indicate that the dye components in a real sample will rarely span the entire separation space. However, the separations also underline the benefits of high overall and local peak capacities. Real samples typically exhibit a number of highly similar dyes, side products, contaminants, and degradation products.

The proposed method allows for hyphenation of LC × LC with MS, uses stationary-phase-assisted modulation to improve detection sensitivity and mobile-phase compatibility, and uses a computer-optimized shifting gradient assembly to obtain a high degree of orthogonality. Optimization of the ^{1}D mobile-phase composition was also attempted with an additional organic-modifier gradient preceding the ion-exchange salt gradient. However, this was not fruitful. Good peak shapes could not be obtained for all classes of peaks, and the mixed retention mechanisms complicated computer-aided modeling.

Due to the separation power offered by the method, many additional analytes have been clearly separated and characterized by MS and UV−vis spectra, most of which are not identified at this stage. We envisage that the proposed method opens new possibilities to perform detailed online dye-degradation research.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b05469.

Photographs and the extraction procedure for the historical objects, a number of sections concerning method development, and an extensive database of the investigated dyes with reference chromatograms and UV and MS spectra (PDF)

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Notes

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