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Identification of Dyes on Single Textile Fibres by HPLC-DAD-MS

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Abstract An HPLC-DAD-MS method is described to analyse textile dyes in different dye classes (reactive, basic, acid, direct, disperse). The described method is sensitive enough to analyse single fibres with a length of a few mm or less, which makes it suitable for forensic analyses. The current paper describes the information content of the acquired data and as well as the results of a validation study, in which the repeatability, specificity and limit of detection of the method were assessed by repeated measurements of nine different dyes in the mentioned dye classes. The mass accuracy (deviation generally < 2 ppm) and absorbance spectra were found to be highly stable in several easurements over a period of 8 weeks. Deviation in retention times were observed and attributed to small experimental effects and a pre-column blockage. The results show that dye analysis is possible for most fibres with a minimum length of one or a few mm.

Keywords: Forensic science, Dyes, Fibres, HPLC-DAD-MS.

Introduction

Many textiles tend to shed fibres. Only a mild contact with such a textile suffices to cause transfer of textile material to a receptor. If transferred material ('traces') can be attributed to a certain donor, it may establish a relation between a suspect and a crime scene or a victim. The easy transfer of material thereby makes investigation of transferred fibres a powerful forensic tool.

However, textiles are generally mass produced. A match found between a reference material of known origin and questioned textile traces does not imply that the reference material is the source of the investigated fibre trace: there will most likely be other possible sources that also could have transferred matching fibre traces. The evidential value of a match is the strength of the indication that the reference material is indeed the source of the textile trace. The evidential value is determined by the extent to which fibres from different sources can be discriminated and is thus directly related to the analytical methods used in their analysis. A very discriminative method will lead to a high evidential value, while the evidential achievable by a less-discriminative method will be poor. Useful reviews of studies on the discrimination of different textile fibres using routine techniques (microscopy, optical spectrometry[1]) are provided by Siegel[2] and Grieve[3]. Newer studies are reviewed by Palmer[4].

The various studies show that the evidential value of a match involving synthetic fibres is generally much higher than the evidential value of a match involving cotton. This is due to the fact that cotton is used in many textiles and, being a natural product, is rather inhomogeneous. The morphology of cotton fibres from different sources cannot generally be distinguished and discrimination by routine techniques is mainly based on colour.

Obviously, optimisation of the evidential value is very beneficial, especially for cotton fibres. It is expected that the elucidation of the chemical nature of the fibre dyes that induce the textile colour improves the achievable evidential value for different reasons. Firstly, it enables the discrimination of dyes that have an equal or very similar colour, but are chemically different. A similarity of colour of different dyes can occur by chance, but also by subtle tuning of dye chemicals by dye manufacturers to make a specific chromophore suitable for alternative application methods and fibre types or to circumvent patent issues. Secondly, it would be the preferred method for fibres that are dyed unevenly as an uneven dyeing often hinders microscopic and spectrometric colour comparison. A chemical identification would identify the same (mixture of) dyes in various fibres, albeit in different amounts or ratios and

thereby provides a more robust and objective answer to the question whether a specific fibre trace may have originated from a questioned textile.

There are many studies that aim to improve identification of textile dyes. Published studies focus on

- Absorbance microspectrometry (UV/Vis), which provides a rough classification of dyes, but is not specific enough for a detailed chemical identification[8];

- Raman spectrometry, which has been introduced in several laboratories in the past decade. Raman spectrometry has been used to study the chemical nature of dye molecules[9–11]. It has several advantages, such as a general high sensitivity and chemical specificity for dye molecules and an easy, quick and non-destructive analysis procedure. However, Raman spectrometric analyses are often hindered by fluorescence and may be limited in the discrimination of a mixture of dyes;

- Direct mass spectrometry, as proposed by Tuinman et al.[12]

- Chromatographic techniques. Thin layer chromatography (TLC), as used routinely in a number of forensic fibre laboratories, enables separation of dyes, but only yields limited chemical identification. HPLC[5,13], CE[14–17], and UPLC[18] have been proposed for their higher resolution and sensitivity. Moreover, these techniques can be connected to a mass spectrometer[19,20] to provide a high chemical specificity.

Nevertheless, none of the proposed techniques has proven powerful enough to routinely identify fibre dyes. In our opinion, this is due to two main challenges:

The first main challenge is the small sample size: often, only one or a few fibre traces are available for analysis. To be relevant for forensic analyses, it should be possible to analyse samples as small as 5 mm[5] or 10 mm[6]. Such small samples contain between 2 and 200 ng of dye[5,7]. In addition, dyes are often present as a mixture.

A second challenge is the wide chemical variety between different classes of dyes. This is especially challenging for chromatographic approaches, where dyes need to be isolated from the fibre material before analysis. Isolation procedures are complex and may vary for different classes of dyes. In his standard text on TLC, Wiggins mentions 15 different solvent mixtures that are needed for the classification and separation of different dye types[21]. The analyst has to follow a classification scheme to decide which method is most likely to lead to a good separation. Later studies, summarised by Griffin and Speers[13], mostly report on the analysis of dyes from a single class. A more versatile method proposed by Speers et al.[22] enabled analysis of dyes in three separate classes (basic, acid, disperse), thereby improving the versatility of the technique. However, dyes for cotton are excluded. This is a serious omission, as cotton is the textile most frequently encountered[23-24] and a relatively low evidential value is currently attributed to matching cotton fibres accordingly. The preference for synthetic fibres in previous work is probably based on the easy extraction of dyes from these fibres. Isolation of dyes from cotton, especially reactive dyes, is more laborious.

Experimental

Samples and materials

Samples of textiles with nine different dyes as well as the corresponding powder dye references were used. Powder dye references and dyed textiles were kindly provided by Chemische Fabriek Triade BV (Naaldwijk, The Netherlands). The Colour Index (CI) and commercial names of the used dyes are presented in table 1; their formulae are presented in figure 1.

Solvents and solutions

During extraction, digestion, and analysis, several materials were used:

Dimethyl sulphoxide (DMSO, z.a.), methanol (reag), formic acid (98-100%), acetonitrile (lichrosolv), ammonium acetate (98%, z.a.), acetic acid (glacial 100%) and sodium hydroxide (Emsure) were obtained from Merck (Amsterdam, The Netherlands). Water was purified using Millipore equipment. Methanol (HPLC grade) was obtained from Rathburn (Rathburn Chemicals Ltd, Walkerburn, Scotland). Cellulase (Trichoderma Viride, 1,1 U/mg) was obtained from Brunschwig Chemie.

Acetic acid solution (0,5 M) was obtained by dissolving 1 mL of acetic acid in 32 mL distilled water. Sodium hydroxide (NaOH) solution (3 M) was prepared by dissolving 2 g of NaOH into 16,7 mL distilled water. Cellulase solution was prepared by adding 10 mL of an acetic acid solution in water (pH5) to 0.01 g of cellulase. This solution was allowed to settle at least 4 hours before use.

Extraction and digestion

Three procedures, namely the formic acid, DMSO, and cellulase digestion procedures, are used to isolate the dye molecules from the fibres. These three methods are detailed below. The choice of the suitable procedure depends on the class of the dye. In the current study, each procedure is used for three of the nine textile samples, as shown in table 1. All analyses reported below are preceded by a separate isolation. In this way, the validation is not limited to the analysis by HPLC-DADMS, but also covers the dye isolation procedures.

In the current study, the selection of the suitable extraction procedure is based on the (known) composition of the reference textiles. Unknown samples need to be analysed by optical and/or infrared microscopy to select the suitable procedure. Acrylic fibres are identified based on their morphology and low birefringence,

or on their infrared spectrum. They are mostly dyed using basic dyes and are subjected to the formic acid procedure. Other fibres types (e.g. cotton, regenerated cellulose, polyester, polyamide, wool often contain direct, disperse, or acid dyes and are subjected to the DMSO procedure. If dyes in cotton or regenerated cellulose are not extracted using DMSO, the cellulase digestion procedure is applied as the fibre may contain reactive dyes.

Formic acid procedure

The formic acid method is used for basic dyes, which are mostly used on acrylic fibres. 10 mm of fibre (unless stated otherwise) is submerged in 20 KL of formic acid in a closed vial and heated to 60 ºC until the fibre is discoloured or to a maximum of 20 minutes. The formic acid (without the fibre) is then transferred to a new, open vial and left at 60 ºC. After complete evaporation of the formic acid, 20 KL of DMSO is added.

DMSO procedure

The DMSO procedure can be used to extract direct, disperse and acid dyes, which are used on several types of fibres, including cotton, regenerated cellulose, wool, polyamide, and polyester. 10 mm of fibre (unless stated otherwise) is submerged in 20 KL DMSO and heated to 100 °C until the fibre is discoloured or to a maximum of 2 hours.

Cellulase digestion procedure

The cellulase digestion procedure can be used to hydrolyse cellulose polymer, e.g. cotton, so that reactive dyes covalently bound to cotton dissolve and become available for analysis by chromatographic techniques. In our experience, extraction using a 2% NaOH solution, as proposed by Home and Dudley[26], and used later by Xu et al.[17] and Dockery et al.[6] failed to provide reproducible results. Therefore, we used the procedure proposed by Wiggins[21]. The method was altered, as the cellulase proposed by Wiggins could not be obtained commercially.

10 mm of fibre (unless stated otherwise) is submerged in 10 KL NaOH solution and cooled (4 ºC for 4 hours). Trapped air bubbles, if present, are removed before cooling by gentle tapping. The NaOH solution is removed after cooling and the fibre rinsed in acetic acid solution and twice in cellulase solution. The fibre is then submerged in 10 KL cellulase solution and mixed in a thermo mixer (Eppendorf Comfort, 50 ºC, 550 RPM) for 20 hours. Afterwards, the samples are centrifuged (5000 RPM, 5 minutes) and 10 KL methanol is added.

Reference dye samples

10 mg of a dye reference is dissolved in 10 mL MeOH (1 Kg/KL) as a stock solution. This solution

Table 1. Dyes used in the current study. Commercial names are provided by Chemische Fabriek Triade, supplier of the used samples. (CI: Colour index name)

Formic acid		DMSO		Cellulase	
Commercial Name	**C.I. Name**	**Commercial Name**	**C.I. Name**	**Commercial Name**	**C.I. Name**
Triacryl red GTL	Basic Red 18	Triamin Blue 3RL	Direct Blue 67	Triactive Yellow S3R 150%	Reactive Yellow 145
Triacryl red GRL	Basic Red 46	Trianyl Light Blue ANB	Acid Blue 78	Triacion Red SE-3B	Reactive Red 120
Triacryl blue 5G	Basic Blue 3	Triasperse Blue BGL-S	Disperse Blue 73	Triactive Orange 3R	Reactive Orange 16

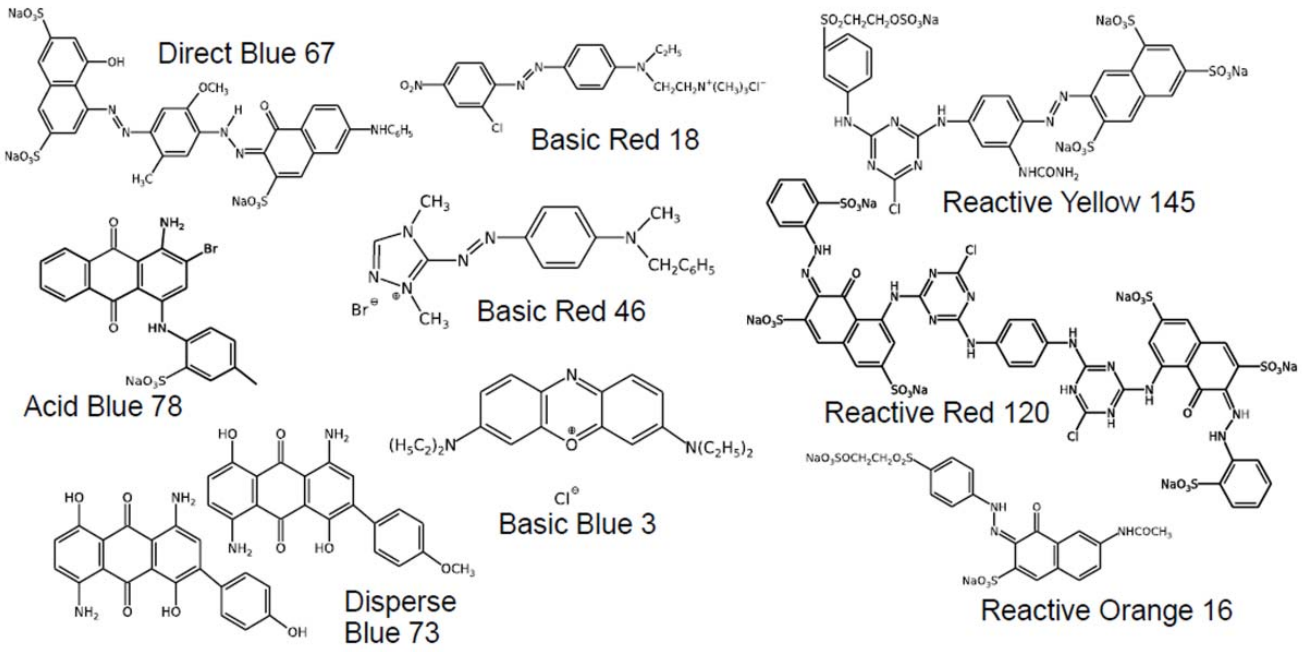

Fig. 1 Structures of the dyes used in the current studies. Information extracted from the Colour Index (www.colour-index.com; Society of Dyers and Colourists).

is diluted with MeOH 10 times (100 ng/KL) as working solution. The injection sample is made by adding 1 KL of the working solution to 19 KL DMSO solvent (5 ng/KL).

HPLC Instrumentation

Analyses were carried out on an HPLC system, consisting of an auto sampler (Thermo Scientific Finnigan Surveyor Auto sampler Plus), a pump (Thermo Scientific Finnigan Surveyor MS Pump Plus), a pre-column (AJO-4286 and Guard cartridge holder KJ10-4282), a column (Grom-sil 120 ODS-5 ST 150×2.0 mm i.d., 3 µm, Grace Davison Discovery Sciences, Deerfield, USA). The column is kept at 22 ºC by a column oven Spark Holland Mistral). The column oven is equipped with both a heating and a cooling system to accurately maintain the set temperature.

The injection volume amounted to 10 KL. The analysis time of the analysis was 67 minutes. During the first 53 minutes of a run, two mobile phases were used as a linear gradient, namely ammonium acetate 10 mM in water/ methanol (95:5) and ammonium acetate 25 mM in acetonitrile/ methanol (50:50). From 53-67 minutes, the acetonitrile containing mobile phase was used.

Eluents were analysed by diode array detection (DAD, Thermo Scientific Finnigan Surveyor PDA Plus Detector, spectral range 200-800 nm) and mass spectrometry (Thermo Scientific LTQ Orbitrap, scan range

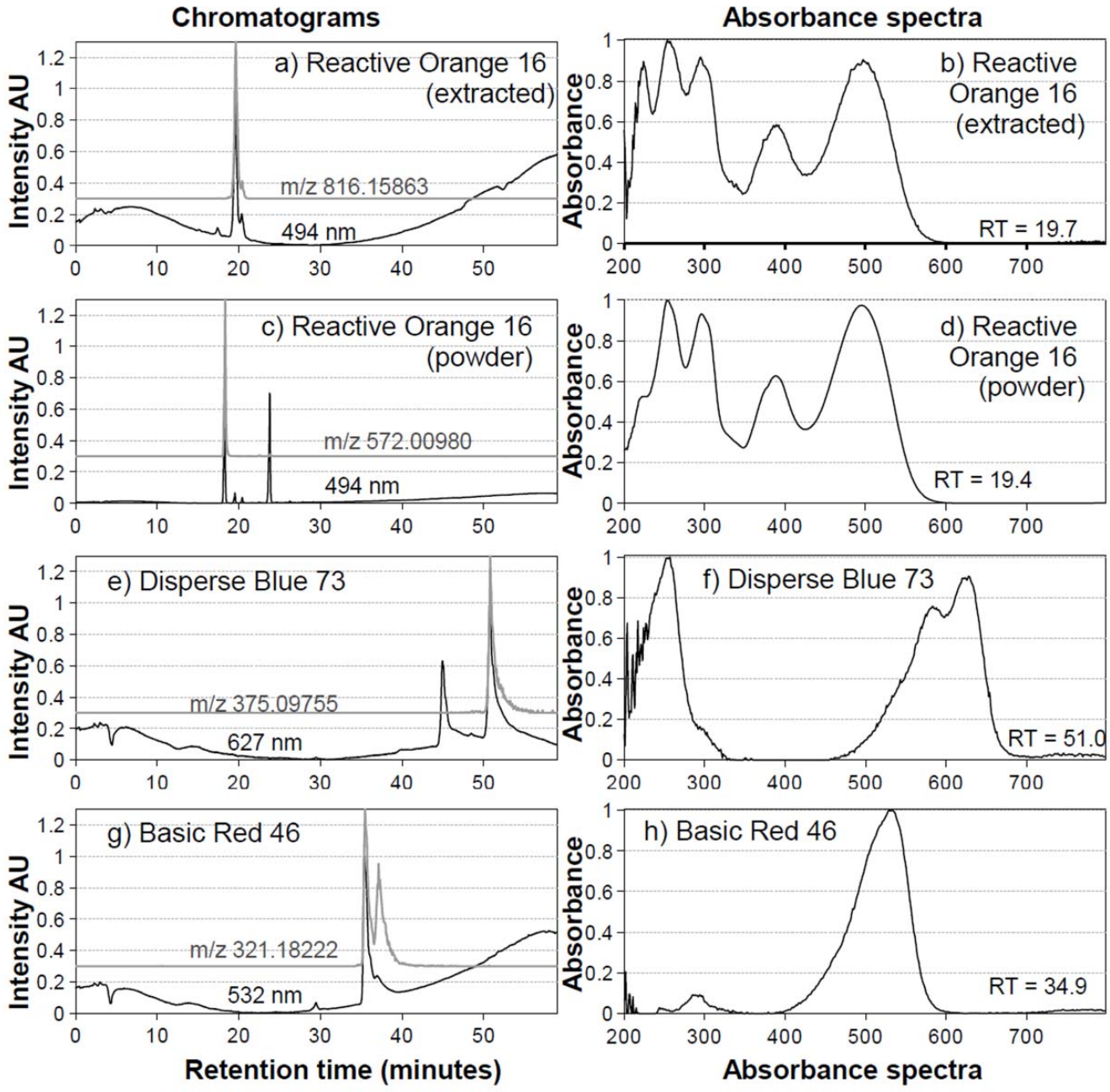

Fig. 2 Chromatograms and absorbance spectra of a, b) Extracted Reactive Orange 16; c, d) Reactive Orange 16 powder dye reference; e, f) Disperse Blue 73 and g, h) Basic Red 46. Plots a, c, e, and g present absorbance chromatograms, extracted by monitoring the light absorption at a single wavelength (black curves) and mass chromatograms, extracted by monitoring the response at a single m/z value (grey curves). Also see table 2 for the selected wavelengths and masses. Plots b, d, f, and h present absorbance spectra at selected retention times (RT, indicated in minutes). All curves, except those in plots c and d are acquired from dyes isolated from fibres. All curves are normalised and, where appropriate for clarity, given an offset.

150 – 2000 m/z). The obtained mass accuracy of the Orbitrap system is better than 2 ppm with the use of a lock mass.

Entrance of the eluents into the mass spectrometer was enabled by an ESI source at 4 kV, a discharge current of 20 µA, and a capillary temperature of 300 °C. A nitrogen flow was used as sheath gas.

Data were acquired using standard instrument software (Thermo Scientific). The spectral search engine of NIST MS search 2.0 was used to compare the acquired spectra. The NIST software was developed for mass spectra, but achieves excellent results when used with absorbance spectra and has an easy integration with the Thermo Scientific software. The mass spectral databases provided by NIST are not used for this application. Instead, user databases based on known reference samples need to be composed. A match factor calculated by the NIST software is, according to the manual, based on a normalised dot product. The same manual describes that a match factor of 800 or higher is a 'good match', while a match factor higher than 900 is an 'excellent match'.

Results and Discussion

During the HPLC analyses, both detectors (DAD, MS) acquire spectra continuously. In this way, ample data are recorded. In the next section, a number of cross-sections of data obtained from Reactive Orange 16 will be shown in detail. Afterwards, summaries will be provided for the other dyes analysed.

Reactive Orange 16

Chromatograms of extracted Reactive Orange 16 are shown in figures 2a. The black curve in this figure represents the absorbance chromatogram, acquired after hydrolysis of dyed fibres using the cellulase method. It shows the absorbance of the effluent at a wavelength of 494 nm during the HPLC run. The value of 494 nm was chosen, as the absorption at this wavelength is high while the interference with other compound is limited. The mass chromatogram (signal at m/z 816.15863) acquired of the same effluent (grey curve) in figure 2a is related to the molecular ion. Detection of the molecular ion is possible as electron spray ionisation (ESI) is a relatively 'soft' ionisation method.

Comparison of the grey and black curves shows that the mass chromatogram has a flat baseline and little or no signals other than the band at retention time 19.7 minutes. The absorbance chromatogram (black line) shows a variable baseline. This is attributed to the lower specificity of the absorbance spectra compared to mass spectra. Figure 1b shows the absorbance spectrum at 19.7 minutes. The spectrum shows several broader bands. The band at 494 nm is visible as the highest band in the visible region (> ~400 nm). However, the spectrum is non-zero from 200 to around 600 nm. It can be anticipated that other materials leaving the column also cause absorbance at 494 nm, thereby inducing a variable baseline.

Figure 3 shows the mass spectrum at an RT of 19.7 minutes. The main peak in this spectrum, (m/z 816.15863) is attributed to the structure drawn in figure 3. The inset in figure 3 shows that the relevant peak is narrow due to the high mass resolution of the used mass spectrometer. Consequently, the chance that other species disturb the chromatogram is small. This explains the almost featureless baseline of the mass chromatogram in figure 2a.

Figure 2c shows chromatograms obtained from powder references. The absorbance chromatogram (black curve) shows two main bands, which are attributed to the original dye molecules and the dye molecule

Table 2. Retention times (RT), calculated mass and optimal wavelength of detection (absorbance spectra) of the studied dyes under the proposed condition. Properties are mentioned for extracted and for dissolved powder dye references. A number of dyes lead reproducibly to the presence of a mixture of compounds. These are shown as separated entries in the table.

Dye	Source	Rt (minutes)	λmax (nm)	Calculated mass (m/z)
Direct blue 67	Pure, fibre	30.8	600	395.53282
Acid blue 78	Pure, fibre	41.3	586	486.97808
Disperse blue 73	Pure, fibre	45.6	627	361.08190
		51.0	627	375.09755
Basic red 46	Pure, fibre	35.0	532	321.18222
Basic bue 3	Pure, fibre	38.0	649	324.20704
Basic red 18	Pure, fibre	44.6	478	390.16913
		45.0	478	410.13783
Reactive yellow 145	Pure	15.9	417	458.49050
		18.4	417	409.50681
		19.9	417	467.47256
	Fibre	14.5	417	823.64415
		14.8	417	519.64296
		16.0	417	580.56492
		15.7	417	830.65198
		17.2	417	742.61774
Reactive orange 16	Pure	19.4	494	572.00980
		24.8	494	474.04242
	Fibre	17.6	494	774.14806
		19.7	494	816.15863
Reactive red 120	Pure	22.8	540	444.30740
	Fibre	15.5	540	756.43563
		15.8	540	760.79802
		17.0	540	594.34948
		16.9	540	892.07812
		17.5	540	599.05469
		17.5	540	899.08595
		18.7	540	540.36520
		18.5	540	811.05172

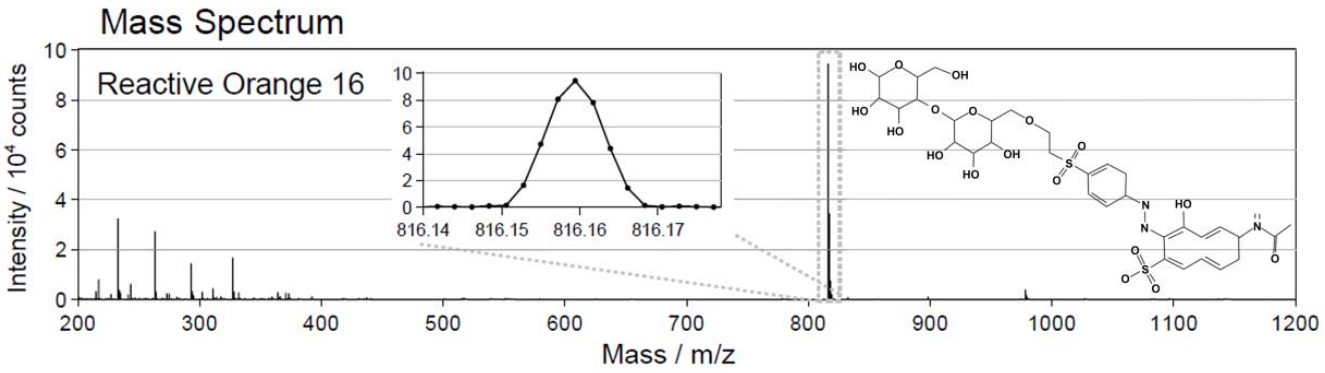

Fig. 3 A mass spectrum of Reactive Orange 16 isolated from cotton by cellulase digestion. The shown mass spectrum was acquired at a retention time (RT) of 19.7 minutes. The inserts show a detail of the spectrum at m/z 816.15863 and the structure attributed to this signal.

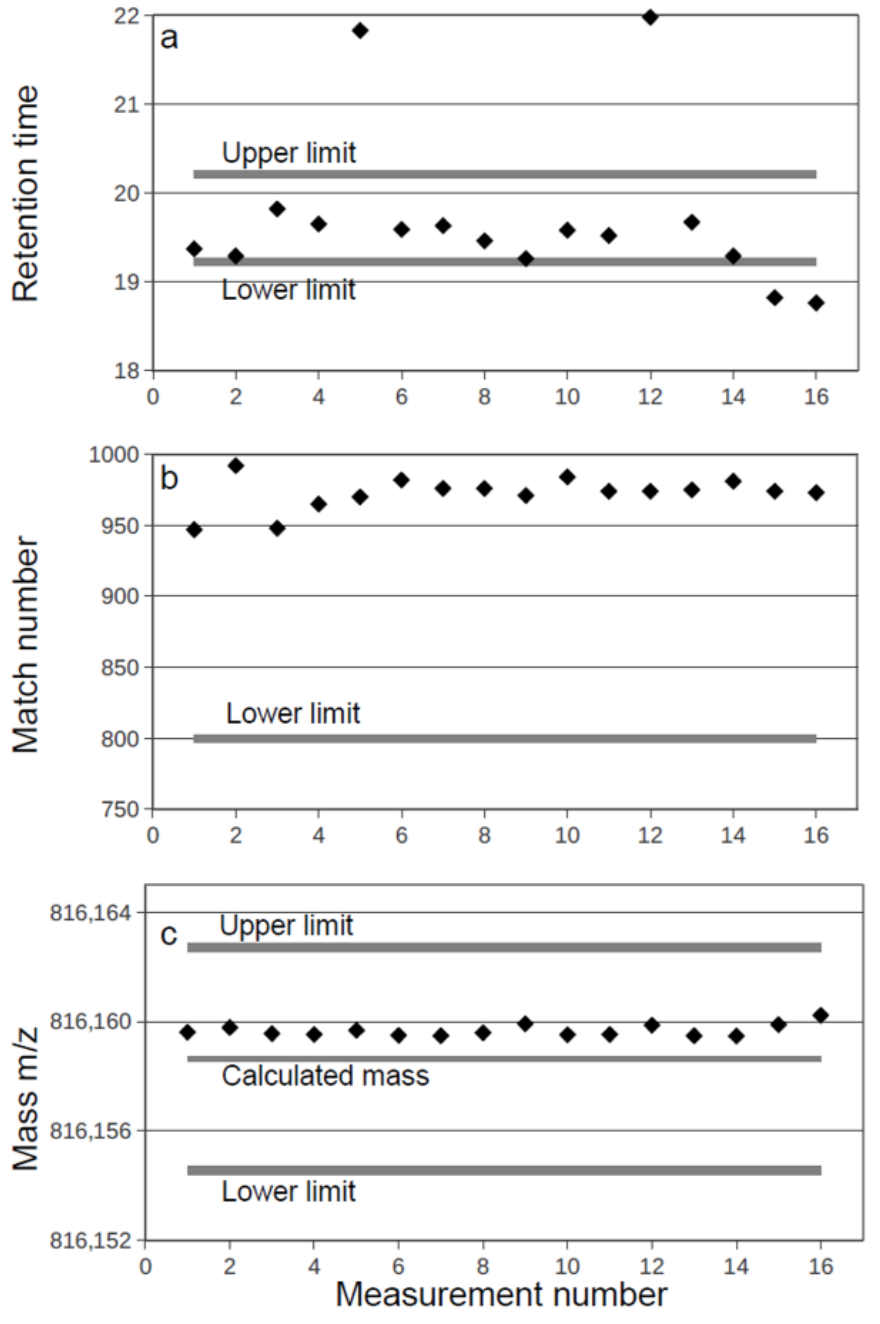

Fig. 4 Results of the validation study for Reactive Orange 16, presenting the repeatability of a) the retention time (RT, limits set at a deviation of 2.5% from the mean value) b) the similarity of absorbance spectra, expressed as a match factor (see text, lower limit set at 800) and c) the mass accuracy. (limits set at a deviation of 5 ppm from the calculated molecular mass).

after hydrolysis of the reactive site. The molecular mass of original and hydrolysed dyes is different, and the mass chromatogram (grey curve) consequently shows a single peak.

The retention times (RTs) observed for the powder dye references (figure 2c) and extracted dyes (figure 2a) shown for Reactive Orange 16 differ (also see table 2). This is explained by the properties of reactive dyes and cellulase. On application, reactive dyes form a covalent bond with the cellulose units of cotton. This bond cannot easily be broken. Instead, the pre-processing step with cellulase hydrolyses the bond between different cellulose units rather than the bond between the dye and the attached cellulose unit. This implies that the recovered dye is still linked to one or more cellulose units and thus differs from the chemical structure of unreacted dye. This alters the RT and the observed molecular mass. The signal at m/z 816.15863 is attributed to the structure drawn in figure 3, which contains the dye molecule connected to two cellulose units.

Other dyes

Figure 2 also shows extracts from Disperse Blue 73 (figure 2e-f) and Basic Red 46 (figure 2g-h). These figures are prepared in a similar to those in figures 2a-d, though the used wavelengths and m/z values are optimised for the specific dyes. The variables used to extract the different curves from the data sets are

presented in figure 2 and in table 2. For disperse, basic, acid and direct dyes, no relevant differences were found between powder dye references and dyes extracted from fibres. Therefore, the results of analyses of powder dye references are not included in figures 2e-h.

The chromatograms of Disperse Blue 73 (figure 2e) shows two bands in the absorbance chromatogram (black line, 45.6 and 51.0 minutes) while the mass chromatogram (grey curve) only shows a single peak (51.0 minutes). This mass chromatogram was extracted at m/z 375.0976. The mass spectrum acquired at an RT of 45.6 minutes shows the presence of a compound with m/z 361.082. These results imply that Disperse Blue 73 consists of two different structures having different masses, but both absorb light at 627 nm. Indeed, figure 1 shows that Disperse Blue 73 contains a mixture of two different structures. One of these contains a methoxy group; the other contains hydroxy group. The structures shown have comparable absorbance spectra, but their masses were found to differ by 14 amu (i.e. the difference between H and CH3, see table 2).

The mass chromatogram of Basic Red 46 (grey curve in figure 2g) contains a double band (35 and 37 minutes). The light absorption of the latter of these bands (black line) is relatively small and seen as a shoulder. This effect is assigned to the presence of two isomers with different properties.

A number of characteristics can be deduced from Figure 2 and table 2:

- The RTs reported for different dyes vary considerably. This facilitates a good separation of different dyes. In our experience (no further data shown), the combination of RT, mass range, and charge state of a dye provides a first indication on the class of the dye;

- The absorbance spectra show large differences between dyes and thus can aid the identification and discrimination of different dyes.

- The obtained mass spectra have a very high accuracy and can thereby assist in the elucidation of the molecular formula of the dye structure under investigation.

Validation study

Based on the first results obtained with the HPLC-DAD-MS system, the method was considered a very powerful addition in forensic fibre investigation. To facilitate the introduction into routine case work, we set up a validation study. This validation is divided in two parts. The first part, reported here, focuses on the robustness of the method and the accuracy of the used procedures and instrumentation. The second part will focus on the evidential values that can be obtained and will be based on a much larger set of samples. The second part is currently in progress and planned as a future publication.

The validation of the procedures and instrumentation is based on several analyses on a set of nine dyes. This set, shown in table 1, was chosen to cover all three isolation techniques (formic acid, DMSO, and cellulase). For every measurement in the validation, three different fibres were put together and their dyes isolated simultaneously. The resulting solution thus contains a mixture of dyes. Initial results showed that these could be separated well by HPLC under the chosen conditions. Therefore, the different dyes are analysed independently. This approach was chosen to test the possibility to separate dyes and to reduce the required instrument time.

The current section presents the experiments carried out to validate the repeatability, limit of detection, and specificity of the proposed method.

Reproducibility

As stated above, the HPLC-DAD-MS system can yield indications on the identity of a compound by three properties, namely via the retention time RT, the absorbance spectrum and the mass spectrum. The reproducibility of these properties was studied by a series of replicate measurements over a period of 16 weeks. During this period, dyes were isolated from the fibres before every analysis to include the isolation procedure in the reproducibility study. In addition, solutions of pure dyes were analysed over the same period. Figures 4a-c respectively show the main results of the robustness study for the RT, the absorbance spectra and the mass spectrometric results of Reactive Orange 16. The data for other dyes is summarised in table 3.

In figures 4a-c, the x-axis shows the measurement number, corresponding to the 16 analyses that were carried out during 16 weeks. Figure 4a shows the results of the RT. The limits shown in this figure amount to a difference of 2.5% of the average value of the determined RT (19.7 minutes, see table 2). It appears that the RT is within these limits for most measurements. In some cases, RTs outside the set limits were obtained. These deviations have been attributed to the replacement of one of the solvents used in the analyses and to a partial blockage of the HPLC pre-column. Similar deviations have also been observed for other dyes. Later adjustments improved the repeatability, but it is realised that the RT is relatively sensitive for small changes in the analysis and are not fully repeatable.

The rather large deviations found for the RT did not affect the separation of the different dyes analysed in one run, as shown in table 1. Instead, the RTs of all dyes in a single run were all affected in a similar fashion and did not compromise the observed separation or band widths. Results for the other dyes are presented in table 3.

In order to assess the repeatability of the absorbance spectra, we calculated the similarity of the absorbance spectrum and a previously acquired spectrum. The similarity of these spectra, calculated by NIST software, is expressed as the match

factor. Based on initial tests, we decided that a match factor of 800, described by NIST as a 'good match' is useful for identification purposes. Match factors found for Reactive Orange analyses over a period of 16 weeks are provided in figure 4b. The robustness of absorbance detection appears high: the match factors presented in figure 4b are generally above 900. Also the match factors of other dyes, provided in table 3, are generally at or above 900.

Figure 4c shows the m/z values obtained for Reactive Orange 16 over the 16 week period. As the formula of Reactive Orange 16 is known, and the formula of the dye bound to a few cellulose units can be derived, the expected masses can be calculated. The mass is indicated in figure 4c along with the upper and lower limits defined as a deviation of 5 ppm of the calculated mass. The mass accuracy of the mass spectrometer is shown to be very high. The deviation is well within the 5 ppm range. The m/z values shown have a very high robustness, albeit with a slight offset. Similar robustness was observed during the analyses of the other dyes (see table 3).

The mass resolution is calculated by the instrument software and was found to be higher than 120,000 at m/z 400 in all reported experiments.

Limit of detection

The limit of detection (LOD) has been obtained using dissolved powder dye references. These solutions can be prepared at known concentrations and provide an accurate value for the LOD. However, the derived values may not be relevant for practical use, as the dye content, thickness, and weight of a fibre are normally unknown. Therefore, we also used extracted dye to analyse the limit of detection. In this case, the limit of detection is expressed as the length of the fibre needed for identification of the dye. This expression of the LOD is closer to the considerations of the forensic scientist. However, an inhomogeneous colouration of the fibre, or a variable diameter of the fibres will affect the accuracy of these analyses. Results on the limit of detection of Reactive Orange 16 are presented in figure 5. Numeric data on the different dyes included in the current study are provided in table 3.

Figure 5a shows the signal intensities obtained in mass spectra from dissolved Reactive Orange 16 powder at different concentrations. The noise level indicated in this figure is the highest noise level encountered during the series. The limit of detection was set at the concentration where the signal is three times this noise level. Figure 5b shows the signal intensities obtained for fibres at three different lengths.

The plots in figure 5 show that the system response is roughly proportional to the introduced amount of dye: reduction of the dye concentration by a factor of 100 (10^4 to 10^2 ng·mL^{-1}) reduces the obtained signal by two orders of magnitude. In a similar fashion, reduction of the fibre length from 10 to 1 mm decreases the obtained signal by one

Table 3. Summary of results of the validation study. The values for the HPLC retention times (RT), the match factor, and the mass accuracy are based on analyses of dyes extracted from fibres. Other values are provided for both the extracted dyes and for powder dye references. The values for the LOD represent the minimum concentration dissolved powder dye references (Kg/L) and on the minimum length of fibre needed for identification of the dye. A number of dyes lead reproducibly to the presence of a mixture of compounds. These are shown as separated entries in the table.

Dye name	HPLC				DAD				MS			
	Rt fibre minutes	(sd)	match index Arb.	(sd)	LOD fibre mm	(sd)	LOD powder µg/L	(sd)	Acc ppm	(sd)	LOD fibre mm	(sd)	LOD powder µg/L	(sd)
Direct blue 67	30.7	(0.99)	996	(5)	0.04	(0.03)	20.5	(39.2)	1.7	(0.1)	0.017	(0.007)	7.3	(11.1)
Acid blue 78	41.2	(0.67)	998	(2)	0.01	(0.00)	5.4	(3.8)	1.9	(0.2)	0.003	(0.002)	1.9	(0.9)
Disperse blue 73	45.5	(0.47)	922	(46)	0.23	(0.12)	72.0	(41.4)	2.5	(0.4)	0.003	(0.002)	1.1	(0.9)
	51.0	(0.25)	939	(18)	0.14	(0.08)	50.7	(23.1)	2.5	(0.2)	0.044	(0.022)	20.6	(20.4)
Basic red 46	34.9	(0.75)	902	(47)	0.17	(0.12)	13.9	(5.5)	1.0	(0.2)	0.014	(0.011)	2.2	(2.5)
Basic blue 3	37.9	(0.70)	911	(72)	0.13	(0.15)	4.7	(2.6)	0.8	(0.1)	0.002	(0.002)	0.1	(0.0)
Basic red 18	44.5	(0.38)	894	(51)	0.32	(0.16)	60.8	(27.8)	0.4	(0.2)	0.268	(0.352)	0.5	(0.2)
	45.0	(0.42)	894	(51)	0.32	(0.16)	-		0.2	(0.2)	0.003	(0.002)	-	
Reactive yellow 145	14.5	(0.94)	897	(44)	0.41	(0.20)	47.6	(15.0)	1.3	(0.5)	0.242	(0.165)	29.4	(15.4)
	14.8	(0.98)	897	(44)	0.41	(0.20)	-		1.5	(0.2)	0.153	(0.093)	-	
	16.0	(0.95)	922	(46)	0.37	(0.25)	132.0	(43.3)	1.5	(0.2)	0.130	(0.119)	61.2	(32.4)
	15.7	(0.38)	932	(40)	0.37	(0.25)	-		1.0	(0.4)	0.352	(0.291)	-	
	17.2	(0.90)	933	(39)	0.35	(0.26)	41.6	(13.6)	1.2	(0.3)	0.096	(0.105)	53.5	(43.3)
Reactive orange 16	17.6	(0.95)	911	(39)	0.46	(0.29)	25.8	(7.7)	1.6	(0.3)	0.178	(0.166)	8.2	(2.8)
	19.7	(0.90)	973	(12)	0.06	(0.05)	37.3	(11.2)	1.3	(0.3)	0.011	(0.009)	4.2	(1.3)
Reactive red 120	15.5	(0.93)	952	(40)	0.24	(0.26)	107.0	(81.2)	1.2	(0.7)	0.418	(0.320)	153.3	(79.5)
	15.8	(0.79)	959	(26)	0.24	(0.26)	-		1.2	(0.7)	0.741	(0.709)	-	
	17.0	(0.85)	944	(47)	0.32	(0.33)	-		0.3	(0.2)	0.248	(0.238)	-	
	16.9	(0.75)	955	(28)	0.32	(0.33)	-		0.9	(0.4)	0.615	(0.408)	-	
	17.5	(0.15)	930	(26)	0.21	(0.10)	-		1.2	(0.9)	0.473	(0.340)	-	
	17.5	(0.19)	932	(29)	0.21	(0.11)	-		1.1	(0.9)	1.080	(0.708)	-	
	18.7	(0.70)	916	(37)	0.44	(0.29)	-		1.1	(0.5)	0.687	(0.454)	-	
	18.5	(0.14)	916	(37)	0.44	(0.29)	-		0.9	(0.4)	0.889	(0.430)	-	

order of magnitude. We are aware that the quantitation of the mass signal is not perfect. We did not strive to optimise quantitation, as the described method is proposed as a qualitative method.

The limit of detection can be calculated by interpolation of the curves in figure 5. However, if proportionality between introduced amount of dye and the obtained signal is assumed, the limit of detection can also be assessed by a single measurement. This approach was followed for the other dyes. The results are presented in table 3. The limit of detection based on absorbance spectra was calculated in a similar way. Also these results are presented in table 3.

Specificity

The specificity of the described method is based on three basically independent parameters, namely the RT, the absorbance spectrum, and the mass spectrum. The combined specificity is therefore considered promising. The most straightforward way to assess the specificity is the analysis of a large number of fibre dyes. This is the subject of current research and will be reported in due time. Nevertheless, the data presented in the current study provides a number of indications that the specificity of the proposed method is remarkably high. A number of results will be summarised below:

- The described validation procedure was based on mixtures of three dyes. In all cases, these could be discriminated based on RT, absorbance spectrum, and mass spectrum.

- The insert in figure 3 shows that the mass peak has a high resolution. The combination with a high mass accuracy makes a dye identification based on mass spectra highly specific.

- The presence of a mixture of compounds was shown, even for reference textiles that were, according to the supplier, dyed with a single dye. Most of the mixed compounds could be attributed to different reaction products of expected dye on the basis of their molecular mass (results not shown). The presence and relative amounts of these mixtures were found to be relatively stable and the LOD has been calculated for each of these compounds. An overview is provided in tables 2 and 3. Specificity is thus improved, as the identification is based on a number of compounds.

- Disperse Blue 73 is, according the colour index, a mixture of two related compounds. Both compounds were identified.

A further improvement in specificity may be achieved by the acquisition of higher order mass spectrometry (MS^n). These were not taken into account in the current study.

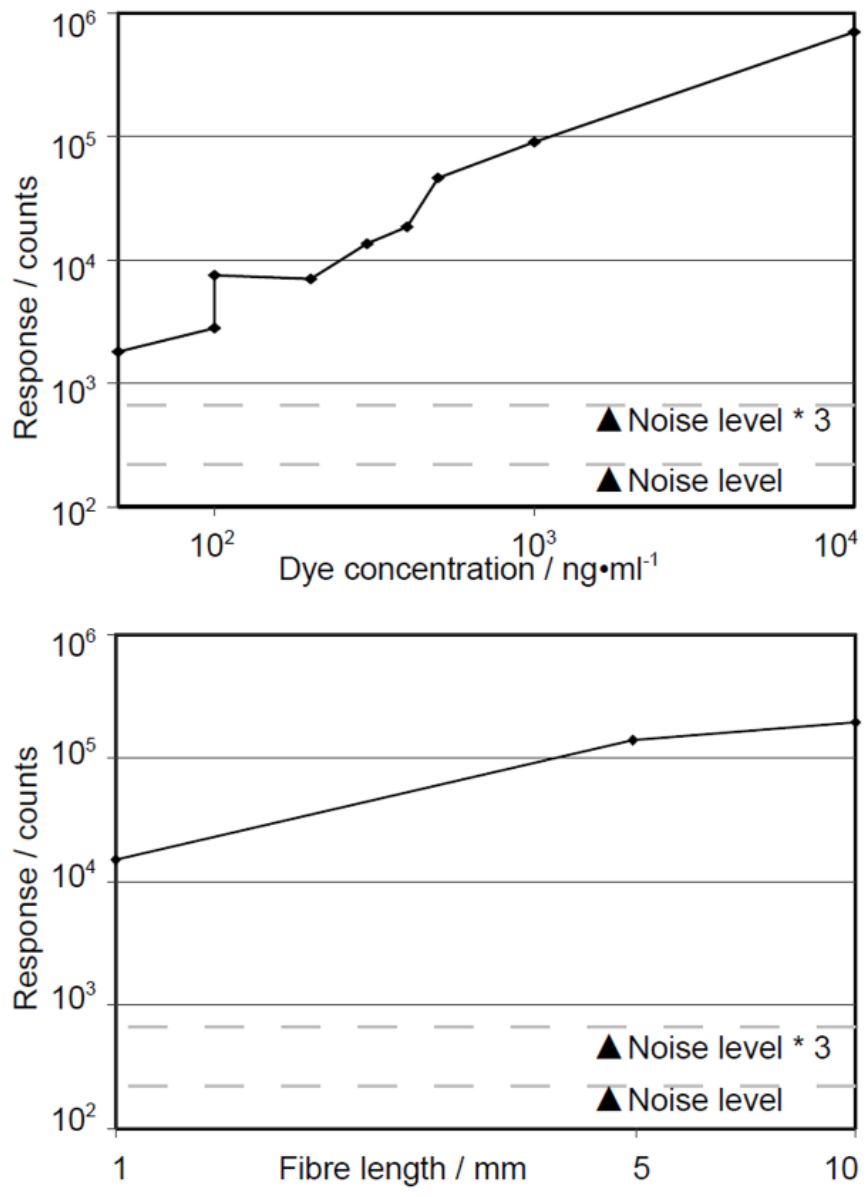

Fig. 5 The limit of detection of Reactive Orange 16. a) Response of the mass spectrometer as a function of the dye concentration (dissolved powder dye reference). b) Response of the mass spectrometer as a function of the length of the fibre from which the dyes were extracted.

Conclusion

ConclusionThe HPLC-DAD-MS method described was shown to be suitable for the forensic analysis of textile dyes in different classes (acid, basic, reactive, direct, disperse). In addition, the limit of detection was found to be around 1 mm or less for the analysed fibres.

To our knowledge, this is the first study in which reactive dyes on

cotton are analysed using an HPLC approach. In addition, it is the first study since the work of Speers[22] and Dockery[6] that describes an HPLC system that is suitable for the analysis of different classes of dyes.

The repeatability of the retention time RT was lower than expected. Recent work (not reported here) indicates that robustness can be improved by a more frequent replacement of the pre-column. However, it is realised that the RTs are relatively sensitive for small changes in the analysis and are not fully repeatable. Repeatability of the analyses was found to be very high. The accuracy of the determined mass was generally found to be better than 2 ppm over a period of several weeks. Mass resolution was found to be higher than 120,000 at m/z 400 in all reported experiments. The reproducibility of the acquired absorbance spectra (as analysed by NIST MS search 2.0 software) was found to be excellent.

Analyses on reference dye samples indicate a limit of detection of less than 100 Kg/L for most dyes. This suffices to analyse single fibres with a length of a few mm or less. For most fibres, except those with a very pale colour, this implies that dye analysis is possible whenever physical handling of the fibre using tweezers is possible.

The provided results indicate that the specificity of the proposed method is very high, as closely related compounds can be discriminated based on RT, absorbance spectrum and/or mass spectrum. Current research aims at a more thorough evaluation of the specificity by the compilation of data bases of reference materials and street samples.

The analysis time, around one hour excluding sample preparation, is relatively long, but analysis can run unattended by use of an auto sampler. The described features make the technique reliable, versatile, and very specific. Therefore, we are currently implementing this methodology into routine case work in our laboratory.

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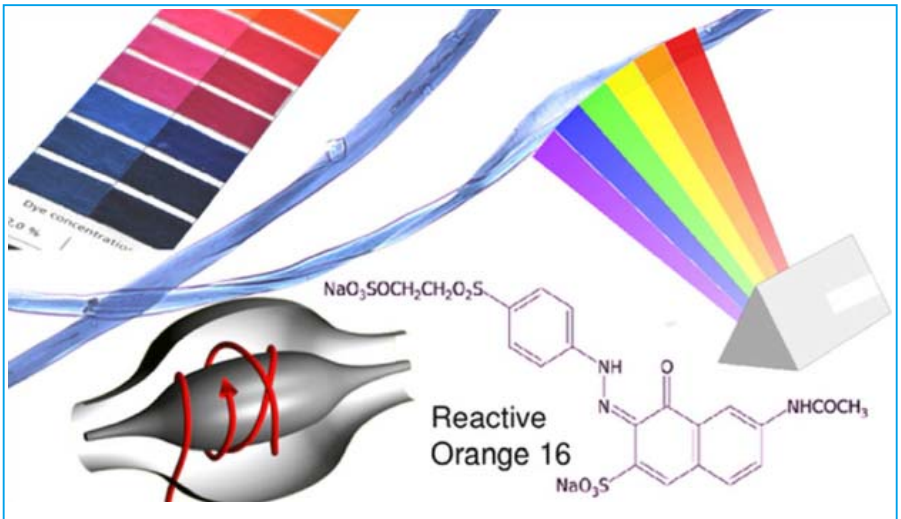

Fig. 6 TOC Infographic