Enzymatic extraction of dyes for differentiation of red cotton fibres by TLC coupled with VSC

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ABSTRACT

The purpose of this work was to assess the usefulness of thin layer chromatography (TLC) for discriminating single cotton fibres dyed with red reactive dyes. An effective enzymatic extraction procedure with the use of cellulase for the red reactively-dyed cotton fibres was developed and used for the discrimination of fibres derived from 21 garments purchased commercially. Discrimination of the fibres relied on the separation of the extracted dyes by thin layer chromatography (TLC). Four eluents were used to develop the plates with the extracted dyes, and the obtained results were analysed using, among others, video spectral comparator (VSC). Observation of TLC plates in visible, ultraviolet and infrared light allowed unambiguous discrimination of 5 and probable discrimination of 6 of the 21 fibres tested. The remaining fibres were divided into several groups. Comparison of the acquired results with those obtained for the same examination material by standard non-destructive methods used in forensic fibres examinations (transmitted light microscopy, fluorescence microscopy, UV–Vis micro-spectrophotometry and Raman spectroscopy) has shown that efficiency in fibres differentiation is similar for all methods. TLC coupled with VSC was even found to be more effective in differentiation of red cotton fibres. The chemometric analysis was helpful to discriminate dyed cotton fibres, characterized by very similar colour.

1. Introduction

Fibres and textiles are part of the human environment since ancient times. Fibres come in a variety of forms and have multiple functions. They are characterized by different chemical composition, structure, thickness, length, shape of cross section, etc. An additional very important feature introducing significant diversity between fibres is their colour, obtained by the use of various dyes or mixtures thereof, and by various dyeing techniques [1].

Identification and comparative studies of the single fibres, and more specifically their fragments of approximately 0.5–2 mm length and 5–25 μm diameter, are the subjects of forensic science. Due to their considerable abundance, single fibres constitute a very important group of traces (mainly microtraces) occurring at the site of the event. According to the Locard's principle [2] during the commission of a criminal act, the material from which the objects are made may be transferred. Identification and differentiation of the fibres collected at the scene of the crime can make it easier to connect people and places [3]. Forensic examination of fibres consists of determining their physical and chemical characteristics and then identifying and differentiating them [4].

For the analysis of fragments of the single fibres and dyes mainly techniques such as optical microscopy, UV–Vis micro-spectrophotometry (MSP), infrared spectrometry (IR) and Raman spectrometry are used [3,5–10]. Sometimes, the information obtained with the use of these techniques is not sufficient for discrimination of fibres. In such cases, with the consent of the court and the appropriate amount of material, destructive methods may be used. These are mainly chromatographic methods: liquid chromatography and thin-layer chromatography.

Thin-layer chromatography (TLC) is the simplest of all commonly used chromatographic methods. Optimization of techniques and materials allows for efficient separation and precise determination of the components of the analysed samples [11]. TLC was once one of the most popular analytical techniques found in many fields of chemistry. At present, it is rarely applied in forensic science despite its great advantages such as simplicity and low cost of analysis. Instrumentation and automation of thin-layer chromatography creates new perspectives on the application of this method in modern laboratories, including forensics [11]. Despite the lower popularity of the TLC method in textile dye analysis, the procedures described and examples of its use can be found in the literature [12,13].

The use of chromatographic techniques requires the extraction of dyes from fibres. Depending on the dye, different extraction mixtures are used. In the case of reactive dyes, enzymatic digestion of cotton fibres with cellulase solution is an effective method [14]. Cellulases belong to the class of enzymes that convert cellulose into glucose. During the hydrolysis process, β-1,4-glycosidic bonds are removed [15]. Cellulase is not a single enzyme but a mixture of three different types of cellulases which act synergistically [16]. These are en-doglucanases, exoglucanases (including cellobiohydrolase) and β-glu-cosidases [15,17] that act in various parts of the cellulose chain. For the process of hydrolysis, the reaction environment, in particular pH and temperature, is of great importance. Cellulase activity depends on these parameters. For most enzymes used in industry, the optimum temperature is in the range of 40–60 °C, while the optimum pH is 4.5–5.5 [18], but for each type of cellulose they should be optimized. In order to improve the hydrolysis of cellulose, the fibres are subjected to a solution of sodium hydroxide prior to enzymatic digestion. It is used to break hydrogen bonds between cellulose chains. This results in so-called "swelling" of the fibre, which makes the cellulose chains more accessible for the enzyme [14]. In nature, cellulase is produced by various organisms such as bacteria and fungi [15]. In the industry for the chemical processing of cellulose, including cotton fibres, mainly fungal cellulase is used. Most suitable for cellulase production are fungal strains such as *Trichoderma* and *Aspergillus* which are capable of producing large amounts of extracellular proteins [16].

The purpose of the study was to assess the effectiveness of differentiation of red cotton fibres, and to compare the effectiveness of this method with other methods used in comparative studies of the samples of coloured fibres retrieved from the same source. Discrimination of the different red fibres relies on the extraction and separation of the components of the fibres such as dyes. The following paper presents an efficient procedure for the extraction of the reactive dyes derived from red cotton fibres, which has been developed based on the procedures described in the available literature sources. The developed extraction procedure was applied to fibres from consumer products.

2. Material and methods

2.1. Chemicals and material

The test material was composed of 21 pieces of red clothing, of a similar shade of colour, made solely of cotton (Co) or with addition of other type of fibres (polyesters – PES, modal – Mod., elastane – EL) (Table 1). These items were purchased using current statistics on clothing sales in Poland and availability of the consumer product, and they consisted of blouses, trousers, shirts, sweaters, a dress and a hat [19]. The preliminary research focused on textile identification and confirmation of its composition in relation to the information contained on textile labelling was conducted with the use of transmitted light microscopy. Then, 21 samples of red cotton fibres were selected to the further examinations.

NaOH, cellulase from *Aspergillus niger* fungus (≥0.3 u/mg) and cellulase from *Trichoderma reesei* ATCC 26921 fungus (≥1 u/mg) were obtained from Sigma Aldrich (Saint Louis, Missouri, USA). Acetic acid (glacial, 99.5% cz. d. a.), sodium acetate (anhydrous, cz. d. a.), ethanol (99.8% cz. d. a.) and pyridine (cz. d. a.) were purchased from ChemPur (Piekary Śląskie, Poland). Methanol (reag. ph. eur.) was obtained from Merck (Darmstad, Germany). Ammonia (25% cz. d. a.) and isoamyl alcohol (cz. d. a.) were obtained from POCH (Gliwice, Poland). Acetic acid and sodium acetate were used to prepare an acetate buffer of 0.1 mol/l and pH = 5.

2.2. Samples and extraction procedure

A strip of material (2 × 1 cm) was cut from each piece of clothing. Samples were retrieved the back side of each object, 5 cm away from

Table 1
Details of the examined red clothing according to its labelling.

Sample	Trademark	Type of clothing	Fibre composition	Origin
1	Adidas	T-shirt	100% Co	Honduras
2	Big Star	T-shirt	100% Co	Bangladesh
3	C&A	Sweater	95% Co 5% EL	no details
4	C&A	T-shirt	100% Co	no details
5	Cropp	Dress	95% Co 5% EL	Bangladesh
6	Cropp	T-shirt	100% Co	Bangladesh
7	Esprit	T-shirt	60% Co 40% Mod.	Bangladesh
8	H&M	Sweater	83% Co 17% PES	China
9	H&M	Hat	100% Co	China
10	House	Blouse	100% Co	Bangladesh
11	Lee	T-shirt	100% Co	Macedonia
12	Nike	Trousers	79% Co 21% PES	Cambodia
13	Puma	T-shirt	100% Co	Georgia
14	Reebok	T-shirt	100% Co	Indonesia
15	Reserved	T-shirt	100% Co	Bangladesh
16	Tom Tailor	Sweater	100% Co	Bangladesh
17	Vero Moda	Trousers	70% Co 28% PES 2% EL	China
18	Vistula	Sweater	100% Co	Poland
19	Wolczanka	Shirt	75% Co 25% PES	Poland
20	Zara	T-shirt	100% Co	Portugal
21	Zara	T-shirt	100% Co	Portugal

the seam. From each stripe 4 threads were extracted using metal tweezers and they were cut with a scalpel to a length of 1 cm. 4 threads from each garment were transferred to Eppendorf tube with a capacity of 1.5 ml and provided with a corresponding number from 1 to 21. Such prepared samples were covered with 50 μl NaOH solution. The closed Eppendorf tubes were put in a bag filled with ice and placed in a refrigerator for 4 h. After this time the solution was removed. The fibres were washed first in 50 μl of 0.5 M acetic acid solution and then twice in 150 μl of a 1.6 g/dm^3 cellulase solution in acetate buffer (pH = 5). After completion of the rinsing procedure, the fibres were again covered with 150 μl of a cellulose solution. Eppendorf tubes were placed in a thermomixer (Eppendorf Thermomixer C) for 20 h at 45 °C. The speed of shaking was 500 rpm. After that, the Eppendorf tubes contents were centrifuged (Eppendorf Centrifuge 5415D). The centrifugation time was 5 min, while the centrifugation speed was 7000 rpm. The above procedure was taken from the literature [12]. The cellulase solution digests only cellulose fibres, so the separation of cotton fibres from other types of fibres was not necessary.

The procedure described in literature was subjected to some modifications. Using the *Aspergillus niger* cellulase solution, the extraction of dyes was carried out at temperatures of 50 °C, 55 °C and 60 °C, and solutions of a concentration of three and ten times greater than described were applied. Instead of a thermomixer, a water bath was also used, and the ultrasounds (Bandelin Sonorex Super) were applied. The procedure was repeated with the use of *Trichoderma reesei* cellulase solution and subjected to the same modifications. While using *Trichoderma reesei*, the extraction was also carried out for 8 threads taken from each textile product. In this case, a double volume of reagents was used, and after centrifugation the samples were evaporated to dryness. The residues were dissolved in 150 μl of acetate buffer (pH = 5). Each version of procedure was performed at least 3 times.

2.3. Preparation of eluents

To prepare eluent E1, the following reagents were mixed: n-butanol, ethanol, ammonia, pyridine and distilled water, respectively 6:3:2:6:6 (V/V). The following reagents: n-butanol, ethanol, ammonia, pyridine and distilled water, respectively 8:3:4:4:6 (V/V) were mixed in order to prepare eluent E2. To prepare eluent E3: n-butanol, ethanol, ammonia, pyridine and distilled water, respectively 8:3:4:4:3 (V/V) were mixed. Eluent E4 was prepared by mixing: methanol, isoamyl alcohol and distilled water, respectively: 5:5:2 (V/V).

2.4. TLC

Chromatography was performed on TLC silica gel 60 F254 glass plates, 10 × 10 cm obtained from Merck. From each extract, 50 μl of liquid was withdrawn and 50 μl of methanol was added. The sample solutions were spotted on the TLC plates and developed in a mobile phase for about 90 min. A horizontal elution chamber was used. Four eluents were used to develop the plates with the extracts: E1, E2, E3 and E4. After development, the TLC plates were removed from the chamber followed by air drying. This step was repeated 3 times for every pair of sample solution with each eluent.

The procedure was subjected to some modifications. Additional separation of dyes was carried out, changing the volume of methanol added to the extracts. 50 μl of the dye solution was mixed sequentially with 40 μl, 30 μl, 20 μl and 10 μl of methanol. The development of the plates on which only extracts were applied was also performed. This step was repeated 3 times for every pair of sample solution with each eluent.

2.5. Video spectral comparator (VSC)

Developed plates were analysed using a Foster + Freeman VSC-6000 video spectral comparator equipped with a FireWire 5 CCD camera. Each plate was observed successively in visible light, ultraviolet light at wavelengths of 254 nm, 312 nm and 365 nm, and infrared light. The integration value was modified in order to improve the clarity of the image. Detailed measurement parameters are presented (Table 2). During the analysis, photographs were obtained, independently processed to improve readability and saved using the dedicated VSC Suite software. Based on them, the separated dye components were labelled and the Rf retention factors were calculated for them, both digitally and by hand.

3. Results and discussion

3.1. Extraction

The use of cellulase from *Aspergillus niger* did not result in the extraction of dyes. Modification of any of the parameters under consideration did not alter this extraction outcome. The too low hydrolytic activity of *Aspergillus niger* cellulase against cellulose was the most likely cause for the above results. The activity of cellulases produced by *Aspergillus niger*, is relatively low when compared to cellulases produced by other fungi [20,21]. Because of its "mildness", *Aspergillus niger* cellulase does not cause the effective depolymerisation of cellulose, which was confirmed experimentally in presented study.

The use of *Trichoderma reesei* cellulase resulted in obtaining solutions containing extracted fibre dyes of all analysed samples. The hydrolytic activity of this enzyme was sufficient enough to digest cotton fibres. The extraction process performed at all tested temperatures (50 °C, 55 °C and 60 °C), while maintaining the value of the other process parameters, resulted in complete digestion of cotton fibres. The optimum process temperature was assessed as 50 °C, as the application of higher temperature was not necessary. Application of a *Trichoderma reesei* cellulase solution of a concentration of three and ten times greater than described was also not needed, as the use of the solution of original

Table 2
VSC measurement parameters.

Wavelength	Magnification	Integration [ms]	Aperture [%]	Brightness
Visible light	2.13	45	50	60
254 nm	2.13	1000	90	60
312 nm	2.13	500	90	60
365 nm	2.13	500	60	60
Infrared light	2.13	500	90	60

concentration (1.6 g/dm^3) resulted in complete digestion of cotton fibres. Moreover, it was noted that optimal results are gained by application of the developed procedure for 8 threads, as the obtained spots on the TLC plates were significantly more visible. In this case an Eppendorf Thermomixer C was used, because in the water bath enzymatic digestion of fibres did not occur completely. This probably resulted from the fact that such a number of threads occupied too much volume in the Eppendorf tube and hence the enzyme did not have access to the fibres. This significantly affected the efficiency of the extraction process. After eliminating this problem by applying a thermomixer, optimal concentration of dyes was obtained. In addition, it has been found that the use of ultrasound can significantly shorten the time needed for complete digestion of cotton fibres. Due to the preliminary character of that part of the experiment, no further conclusions could be drawn, but this part of research is worth pursuing.

3.2. TLC and VSC

The use of thin-layer chromatography allowed the successful separation of the dyes from 21 fibre samples into individual components. It was proven that the use of different eluents influences the efficiency of this separation.

Plates were observed in visible light and in ultraviolet light with three different wavelengths. Images of the plates that were obtained in light of wavelengths of 254 nm and 365 nm were considered not readable enough so they were not used during further analysis. The best images were achieved by illuminating the plates with ultraviolet light at a wavelength of 312 nm. The images obtained in ultraviolet light are presented for one of the plates (Fig. 1). It was considered that photographs obtained in ultraviolet light were not sufficient basis for the correct calculation of the Rf coefficients of the separated components. For this reason, plates were observed in infrared light. The obtained images considered in further analysis are presented for one of the plates (Fig. 2).

Based on images obtained in ultraviolet and infrared light compared to images obtained in visible light, the retention factors for the separated components were calculated (Tables 3 and 4). It has been observed that the use of E4 eluent leads to the least informative results. The dyes developed with this eluent were separated into fewer components than with the other three eluents. In addition, the values of the retention factors for the components of all samples were very similar, and the resulting spots from these components were placed at one level. This fact significantly impedes or even prevents the differentiation of the fibres in which the tested dyes were contained. Observation of plates in infrared and ultraviolet light did not eliminate this problem. In this case, the only basis for fibre discrimination was the comparison of colour of the resulting spots. Since the assessment of colour is subjective and highly dependent on the observer, fibre differentiation after use of only the eluent E4 was not possible. For the largest sample group, the eluent E2 was considered to be the best mobile phase. However, on this basis it cannot be inferred that it will be most suitable for the majority of the population of red dyes extracted from cotton fibres. In addition, it was concluded that observation of TLC plates in visible light was not sufficient to properly differentiate the fibres. As a result of observation of TLC plates in UV and IR light, components that were invisible or difficult to observe in previous conditions were labelled. This proves the extraordinary usefulness of such observation in the analysis of TLC plates.

The optimal volume ratio of extract to methanol was assessed as 50:50 μl. It was found that application of different ratios gives the same separation results but the concentration of dyes in these cases was too great and caused the phenomenon of "tailing" the spots of individual components. They became "blurry", which prevented an unambiguous determination of the points at which the components were on the plates. Further analysis of such plates was not possible.
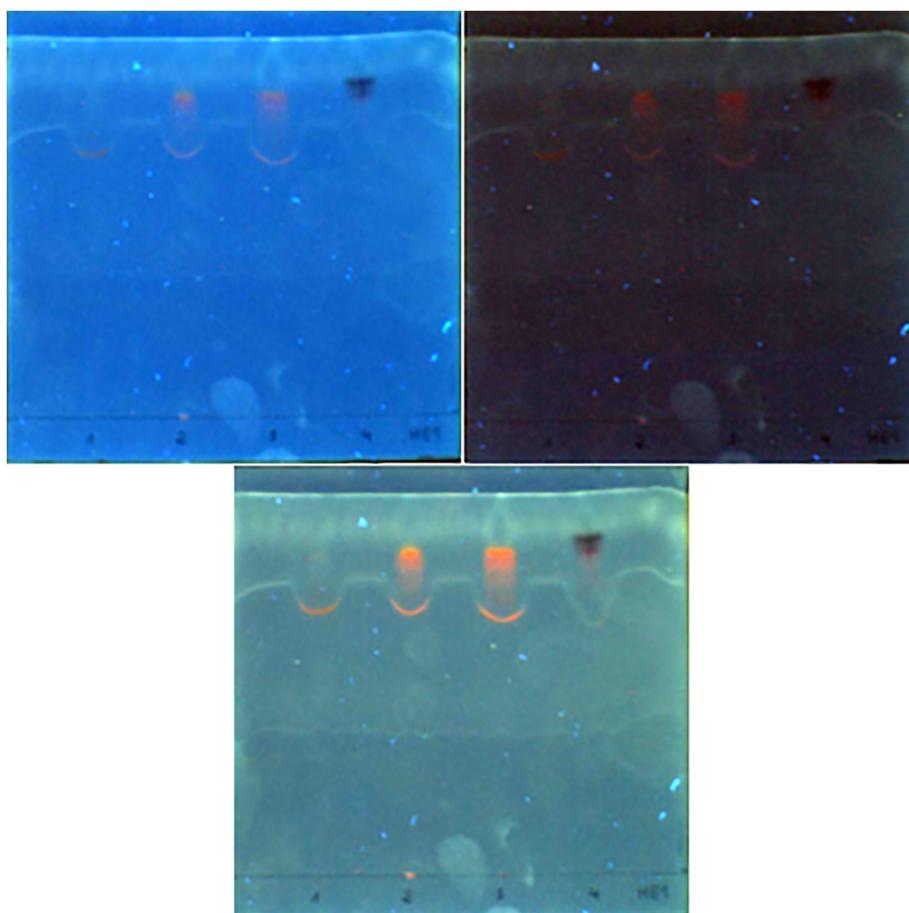
Fig. 1. Pictures of the same plate with the dyes extracted from samples 1-4 developed with eluent E1, observed in UV light with a wavelength of 254 nm (top, left), 312 nm (bottom) and 365 nm (top, right).

3.3. Differentiation of fibres

Based on the comparison of the number of labelled components of each extract, the retention factors calculated for the components of each extract and the visual evaluation of the obtained plate images, an attempt to evaluate the similarity of the dyes extracted from the examined fibres was made (Fig. 3). Among them, 5 dyes were distinguished (from samples no. 1, 18, 19, 20 and 21), as they show major differences from each other and from other dyes. Differences between the results obtained for them are so significant that they allow their unambiguous discrimination. Other dyes were divided into several groups.

Obtained results were compared with those obtained by other methods for the same red reactively-dyed cotton fibres [19]. Standard techniques used in forensic examination of fibres such as transmitted light microscopy (OM) and fluorescence microscopy (FM), UV-Vis microspectrophotometry (MSP) and Raman microspectroscopy (μ-Raman) were considered. For standard methods [19] and TLC, the value of discriminating power (DP) was calculated (Table 5) according to Smollan and Moffat's procedure [22] as a ratio of distinguishable pairs to all possible pairs of tested samples.

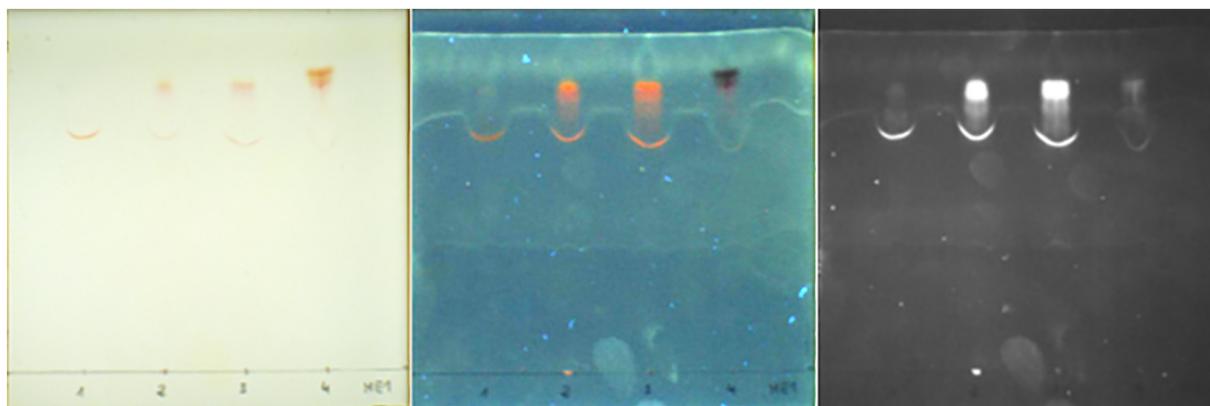The effectiveness of TLC and non-destructive methods in fibre

Fig. 2. Pictures of the same plate with the dyes extracted from samples 1-4 developed with eluent E1 observed respectively in: visible light, in UV light at wavelength of 312 nm and in the infrared light.

Table 3
R_f factors calculated for plates observed under visible light (for each sample developed with each eluent).

No. of sample	E1		E2		E3		E4	
	Number of components	R_f	Number of components	R_f	Number of components	R_f	Number of components	R_f
1	1	$R_f = 0.70$	1	$R_f = 0.53$	2	$R_{f1} = 0.37$ $R_{f2} = 0.38$	2	$R_{f1} = 0.55$ $R_{f2} = 0.60$
2	2	$R_{f1} = 0.69$ $R_{f2} = 0.84$	2	$R_{f1} = 0.53$ $R_{f2} = 0.57$	1	$R_f = 0.42$	1	$R_f = 0.60$
3	2	$R_{f1} = 0.67$ $R_{f2} = 0.85$	2	$R_{f1} = 0.53$ $R_{f2} = 0.57$	1	$R_f = 0.41$	1	$R_f = 0.59$
4	3	$R_{f1} = 0.66$ $R_{f2} = 0.86$ $R_{f3} = 0.87$	5	$R_{f1} = 0.52$ $R_{f2} = 0.57$ $R_{f3} = 0.59$ $R_{f4} = 0.76$ $R_{f5} = 0.85$	5	$R_{f1} = 0.41$ $R_{f2} = 0.47$ $R_{f3} = 0.54$ $R_{f4} = 0.71$ $R_{f5} = 0.85$	3	$R_{f1} = 0.58$ $R_{f2} = 0.69$ $R_{f3} = 0.76$
5	2	$R_{f1} = 0.68$ $R_{f2} = 0.86$	2	$R_{f1} = 0.54$ $R_{f2} = 0.57$	1	$R_f = 0.44$	2	$R_{f1} = 0.57$ $R_{f2} = 0.62$
6	2	$R_{f1} = 0.66$ $R_{f1} = 0.86$	2	$R_{f1} = 0.53$ $R_{f2} = 0.57$	1	$R_f = 0.43$	1	$R_f = 0.60$
7	3	$R_{f1} = 0.65$ $R_{f2} = 0.84$ $R_{f3} = 0.89$	2	$R_{f1} = 0.54$ $R_{f2} = 0.82$	2	$R_{f1} = 0.43$ $R_{f2} = 0.44$	3	$R_{f1} = 0.55$ $R_{f2} = 0.57$ $R_{f3} = 0.60$
8	2	$R_{f1} = 0.86$ $R_{f2} = 0.88$	4	$R_{f1} = 0.54$ $R_{f2} = 0.78$ $R_{f3} = 0.82$ $R_{f4} = 0.85$	4	$R_{f1} = 0.45$ $R_{f2} = 0.51$ $R_{f3} = 0.75$ $R_{f4} = 0.81$	2	$R_{f1} = 0.60$ $R_{f2} = 0.77$
9	2	$R_{f1} = 0.63$ $R_{f2} = 0.83$	3	$R_{f1} = 0.53$ $R_{f2} = 0.57$ $R_{f3} = 0.80$	1	$R_f = 0.42$	2	$R_{f1} = 0.58$ $R_{f2} = 0.60$
10	2	$R_{f1} = 0.65$ $R_{f2} = 0.84$	2	$R_{f1} = 0.53$ $R_{f2} = 0.56$	1	$R_f = 0.42$	2	$R_{f1} = 0.58$ $R_{f2} = 0.61$
11	2	$R_{f1} = 0.64$ $R_{f2} = 0.84$	4	$R_{f1} = 0.52$ $R_{f2} = 0.56$ $R_{f3} = 0.76$ $R_{f4} = 0.80$	3	$R_{f1} = 0.43$ $R_{f2} = 0.47$ $R_{f3} = 0.79$	3	$R_{f1} = 0.61$ $R_{f2} = 0.74$ $R_{f3} = 0.76$
12	2	$R_{f1} = 0.64$ $R_{f2} = 0.85$	1	$R_f = 0.53$	1	$R_f = 0.43$	1	$R_f = 0.60$
13	2	$R_{f1} = 0.62$ $R_{f2} = 0.86$	3	$R_{f1} = 0.53$ $R_{f2} = 0.57$ $R_{f3} = 0.81$	2	$R_{f1} = 0.42$ $R_{f2} = 0.81$	1	$R_f = 0.61$
14	3	$R_{f1} = 0.61$ $R_{f2} = 0.85$ $R_{f3} = 0.87$	3	$R_{f1} = 0.52$ $R_{f2} = 0.57$ $R_{f3} = 0.81$	2	$R_{f1} = 0.41$ $R_{f2} = 0.83$	1	$R_f = 0.60$
15	3	$R_{f1} = 0.62$ $R_{f2} = 0.88$ $R_{f3} = 0.90$	3	$R_{f1} = 0.53$ $R_{f2} = 0.56$ $R_{f3} = 0.83$	1	$R_f = 0.40$	1	$R_f = 0.60$
16	2	$R_{f1} = 0.62$ $R_{f2} = 0.88$	2	$R_{f1} = 0.53$ $R_{f2} = 0.56$	1	$R_f = 0.40$	2	$R_{f1} = 0.58$ $R_{f2} = 0.59$
17	2	$R_{f1} = 0.59$ $R_{f2} = 0.86$	2	$R_{f1} = 0.49$ $R_{f2} = 0.54$	1	$R_f = 0.41$	2	$R_{f1} = 0.58$ $R_{f2} = 0.60$
18	2	$R_{f1} = 0.63$ $R_{f2} = 0.86$	4	$R_{f1} = 0.51$ $R_{f2} = 0.56$ $R_{f3} = 0.77$ $R_{f4} = 0.81$	4	$R_{f1} = 0.41$ $R_{f2} = 0.43$ $R_{f3} = 0.76$ $R_{f4} = 0.81$	2	$R_{f1} = 0.61$ $R_{f2} = 0.75$
19	2	$R_{f1} = 0.86$ $R_{f2} = 0.90$	4	$R_{f1} = 0.72$ $R_{f2} = 0.77$ $R_{f3} = 0.80$ $R_{f4} = 0.88$	3	$R_{f1} = 0.42$ $R_{f2} = 0.81$ $R_{f3} = 0.83$	2	$R_{f1} = 0.63$ $R_{f2} = 0.79$
20	2	$R_{f1} = 0.65$ $R_{f2} = 0.84$	1	$R_f = 0.52$	2	$R_{f1} = 0.45$ $R_{f2} = 0.75$	3	$R_{f1} = 0.59$ $R_{f2} = 0.60$ $R_{f3} = 0.73$
21	3	$R_{f1} = 0.61$ $R_{f2} = 0.84$ $R_{f3} = 0.87$	2	$R_{f1} = 0.53$ $R_{f2} = 0.56$	2	$R_{f1} = 0.44$ $R_{f2} = 0.80$	1	$R_f = 0.60$

differentiation was similar. The discriminative force was the highest for TLC. TLC can also be considered as a complementary method in cases where the application of non-destructive methods does not allow discrimination of the fibres or their differentiation is problematic. The use of non-destructive methods and TLC in the appropriate sequence allowed for discrimination of all tested red cotton fibres. The obtained results are presented in the form of a dendrogram (Fig. 4).

In the similar study [13], where 12 samples of red reactively-dyed cotton fibres were tested, commercial dye names, colour index names and combination of dyes applied to colour the material were known in advance. The use of high-performance thin layer chromatography (HPTLC) in combination with enzymatic digestion also led to a discrimination of some extracted dyes. Moreover, the calculated value of the DP for the TLC was 0.98, both in cited research and this paper, even though there was no detailed information about dyes tested in this research. This indicates a high reproducibility of the applied method, and

Table 4

R_f factors calculated for plates observed under UV and IR light (for each sample developed with each eluent).

No. of sample	E1 Number of components	E1 R_f	E2 Number of components	E2 R_f	E3 Number of components	E3 R_f	E4 Number of components	E4 R_f
1	1	$R_f = 0.70$	1	$R_f = 0.53$	3	$R_{f1} = 0.37$ $R_{f2} = 0.38$ $R_{f3} = 0.40$	2	$R_{f1} = 0.55$ $R_{f2} = 0.60$
2	3	$R_{f1} = 0.69$ $R_{f2} = 0.81$ $R_{f3} = 0.84$	3	$R_{f1} = 0.53$ $R_{f2} = 0.58$ $R_{f3} = 0.60$	2	$R_{f1} = 0.40$ $R_{f2} = 0.43$	3	$R_{f1} = 0.57$ $R_{f2} = 0.59$ $R_{f3} = 0.60$
3	3	$R_{f1} = 0.67$ $R_{f2} = 0.82$ $R_{f3} = 0.84$	3	$R_{f1} = 0.53$ $R_{f2} = 0.58$ $R_{f3} = 0.60$	3	$R_{f1} = 0.40$ $R_{f2} = 0.43$ $R_{f3} = 0.47$	3	$R_{f1} = 0.56$ $R_{f2} = 0.58$ $R_{f3} = 0.60$
4	4	$R_{f1} = 0.66$ $R_{f2} = 0.83$ $R_{f3} = 0.85$ $R_{f4} = 0.87$	5	$R_{f1} = 0.52$ $R_{f2} = 0.57$ $R_{f3} = 0.59$ $R_{f4} = 0.76$ $R_{f5} = 0.85$	6	$R_{f1} = 0.40$ $R_{f1} = 0.41$ $R_{f2} = 0.47$ $R_{f3} = 0.54$ $R_{f4} = 0.71$ $R_{f5} = 0.85$	5	$R_{f1} = 0.54$ $R_{f2} = 0.56$ $R_{f3} = 0.58$ $R_{f4} = 0.69$ $R_{f5} = 0.76$
5	3	$R_{f1} = 0.68$ $R_{f2} = 0.84$ $R_{f3} = 0.86$	3	$R_{f1} = 0.40$ $R_{f2} = 0.54$ $R_{f3} = 0.57$	3	$R_{f1} = 0.42$ $R_{f2} = 0.44$ $R_{f3} = 0.50$	3	$R_{f1} = 0.57$ $R_{f2} = 0.59$ $R_{f3} = 0.62$
6	3	$R_{f1} = 0.66$ $R_{f1} = 0.84$ $R_{f3} = 0.86$	5	$R_{f1} = 0.40$ $R_{f2} = 0.53$ $R_{f3} = 0.57$ $R_{f4} = 0.72$ $R_{f5} = 0.78$ $R_{f2} = 0.53$	3	$R_{f1} = 0.42$ $R_{f2} = 0.44$ $R_{f3} = 0.50$	3	$R_{f1} = 0.55$ $R_{f2} = 0.57$ $R_{f3} = 0.60$
7	4	$R_{f1} = 0.65$ $R_{f2} = 0.84$ $R_{f3} = 0.85$ $R_{f4} = 0.89$	5	$R_{f1} = 0.54$ $R_{f2} = 0.56$ $R_{f3} = 0.73$ $R_{f4} = 0.80$ $R_{f5} = 0.82$	5	$R_{f1} = 0.43$ $R_{f2} = 0.44$ $R_{f3} = 0.50$ $R_{f4} = 0.56$ $R_{f5} = 0.67$	4	$R_{f1} = 0.55$ $R_{f2} = 0.57$ $R_{f3} = 0.60$ $R_{f4} = 0.68$
8	3	$R_{f1} = 0.65$ $R_{f2} = 0.86$ $R_{f3} = 0.88$	4	$R_{f1} = 0.54$ $R_{f2} = 0.78$ $R_{f3} = 0.82$ $R_{f4} = 0.85$	4	$R_{f1} = 0.45$ $R_{f2} = 0.51$ $R_{f3} = 0.75$ $R_{f4} = 0.81$	2	$R_{f1} = 0.60$ $R_{f2} = 0.77$
9	3	$R_{f1} = 0.63$ $R_{f2} = 0.83$ $R_{f3} = 0.85$	5	$R_{f1} = 0.41$ $R_{f2} = 0.53$ $R_{f3} = 0.57$ $R_{f4} = 0.74$ $R_{f5} = 0.80$	3	$R_{f1} = 0.42$ $R_{f2} = 0.45$ $R_{f3} = 0.77$	3	$R_{f1} = 0.58$ $R_{f2} = 0.60$ $R_{f3} = 0.73$
10	3	$R_{f1} = 0.65$ $R_{f2} = 0.82$ $R_{f3} = 0.84$	4	$R_{f1} = 0.41$ $R_{f2} = 0.54$ $R_{f3} = 0.58$ $R_{f4} = 0.74$	2	$R_{f1} = 0.42$ $R_{f2} = 0.45$	2	$R_{f1} = 0.58$ $R_{f2} = 0.61$
11	2	$R_{f1} = 0.64$ $R_{f2} = 0.84$	4	$R_{f1} = 0.52$ $R_{f2} = 0.56$ $R_{f3} = 0.76$ $R_{f4} = 0.80$	3	$R_{f1} = 0.43$ $R_{f2} = 0.47$ $R_{f3} = 0.79$	3	$R_{f1} = 0.61$ $R_{f2} = 0.74$ $R_{f3} = 0.76$
12	3	$R_{f1} = 0.64$ $R_{f2} = 0.83$ $R_{f3} = 0.85$	4	$R_{f1} = 0.41$ $R_{f2} = 0.53$ $R_{f3} = 0.56$ $R_{f4} = 0.75$	1	$R_f = 0.43$	2	$R_{f1} = 0.59$ $R_{f2} = 0.60$
13	3	$R_{f1} = 0.62$ $R_{f2} = 0.85$ $R_{f3} = 0.86$	4	$R_{f1} = 0.40$ $R_{f2} = 0.53$ $R_{f3} = 0.57$ $R_{f4} = 0.81$ $R_{f2} = 0.53$	3	$R_{f1} = 0.42$ $R_{f2} = 0.46$ $R_{f3} = 0.81$	2	$R_{f1} = 0.59$ $R_{f2} = 0.61$
14	3	$R_{f1} = 0.61$ $R_{f2} = 0.85$ $R_{f3} = 0.87$	4	$R_{f1} = 0.40$ $R_{f2} = 0.53$ $R_{f3} = 0.57$ $R_{f4} = 0.81$	3	$R_{f1} = 0.41$ $R_{f2} = 0.45$ $R_{f3} = 0.83$	2	$R_{f1} = 0.59$ $R_{f2} = 0.60$
15	3	$R_{f1} = 0.62$ $R_{f2} = 0.88$ $R_{f3} = 0.90$	5	$R_{f1} = 0.41$ $R_{f2} = 0.53$ $R_{f3} = 0.57$ $R_{f4} = 0.82$ $R_{f5} = 0.84$	3	$R_{f1} = 0.41$ $R_{f2} = 0.48$ $R_{f3} = 0.83$	2	$R_{f1} = 0.58$ $R_{f2} = 0.61$
16	2	$R_{f1} = 0.62$ $R_{f2} = 0.88$	2	$R_{f1} = 0.53$ $R_{f2} = 0.56$	1	$R_f = 0.40$	2	$R_{f1} = 0.58$ $R_{f2} = 0.59$
17	3	$R_{f1} = 0.59$ $R_{f2} = 0.83$ $R_{f3} = 0.86$	3	$R_{f1} = 0.49$ $R_{f2} = 0.54$ $R_{f3} = 0.58$	3	$R_{f1} = 0.41$ $R_{f2} = 0.47$ $R_{f3} = 0.76$	2	$R_{f1} = 0.58$ $R_{f2} = 0.60$

Table 4 (*continued*)

No. of sample	E1		E2		E3		E4	
	Number of components	R_f	Number of components	R_f	Number of components	R_f	Number of components	R_f
18	3	$R_{f1} = 0.63$ $R_{f2} = 0.84$ $R_{f3} = 0.86$	5	$R_{f1} = 0.51$ $R_{f2} = 0.56$ $R_{f3} = 0.72$ $R_{f4} = 0.76$ $R_{f5} = 0.81$	4	$R_{f1} = 0.41$ $R_{f2} = 0.43$ $R_{f3} = 0.76$ $R_{f4} = 0.81$	4	$R_{f1} = 0.61$ $R_{f2} = 0.64$ $R_{f3} = 0.75$ $R_{f4} = 0.81$
19	3	$R_{f1} = 0.63$ $R_{f1} = 0.86$ $R_{f2} = 0.90$	7	$R_{f1} = 0.51$ $R_{f2} = 0.57$ $R_{f3} = 0.72$ $R_{f4} = 0.77$ $R_{f5} = 0.80$ $R_{f6} = 0.83$ $R_{f7} = 0.88$	4	$R_{f1} = 0.42$ $R_{f2} = 0.78$ $R_{f3} = 0.81$ $R_{f4} = 0.83$	3	$R_{f1} = 0.63$ $R_{f2} = 0.79$ $R_{f3} = 0.81$
20	2	$R_{f1} = 0.65$ $R_{f2} = 0.84$	1	$R_f = 0.52$	3	$R_{f1} = 0.45$ $R_{f2} = 0.75$ $R_{f3} = 0.81$	3	$R_{f2} = 0.59$ $R_{f3} = 0.60$ $R_{f4} = 0.73$
21	4	$R_{f1} = 0.61$ $R_{f2} = 0.79$ $R_{f3} = 0.84$ $R_{f4} = 0.87$	2	$R_{f1} = 0.53$ $R_{f2} = 0.56$	3	$R_{f1} = 0.44$ $R_{f2} = 0.72$ $R_{f3} = 0.80$	1	$R_f = 0.60$

thus shows that the TLC can be useful for distinguishing dyes, although it is a destructive and time-consuming technique.

4. Conclusions

Discrimination of coloured fibres based on distinction of dyes present in the single fibres was found to be a difficult and complex problem. Enzymatic digestion was an effective technique of extracting reactive dyes from cotton fibres. Choosing an enzyme with sufficiently high hydrolytic activity and determining the proper parameters of the procedure were essential steps. The use of *Trichoderma reesei* cellulase with a concentration of 1.6 g/dm^3 has resulted in complete digestion of fibres. The procedure performed at a temperature of 50° C for 8 threads retrieved from each product was considered the best. However, the use of 8 threads is not necessary, as the procedure can also be applied to a smaller or larger number of threads, depending on the amount of material available. Then, proportionally smaller or larger volumes of reagents should be used.

Table 5
Values of discriminating power for techniques used in fibre examination.

Technique	OM	FM	μ-Raman	MSP	TLC
DP	0.76	0.79	0.83	0.94	0.98

Obtained results confirmed that TLC may be a useful technique in forensic fibre examination. Comparing the results obtained with the use of standard, non-destructive, forensic fibres examination methods, TLC was found to be even more effective. This means that TLC coupled with plate observation using VSC in light of different wavelengths, is able to give satisfactory results, especially in a case of the absence of more advanced methods. However, further studies concerning greater number of samples conducted in this direction are advised. In addition, TLC was found a simple, inexpensive and low-cost reagent method, compatible with the principles of green chemistry, worth developing and optimizing.

The use of the sequence of four analytical techniques: transmitted
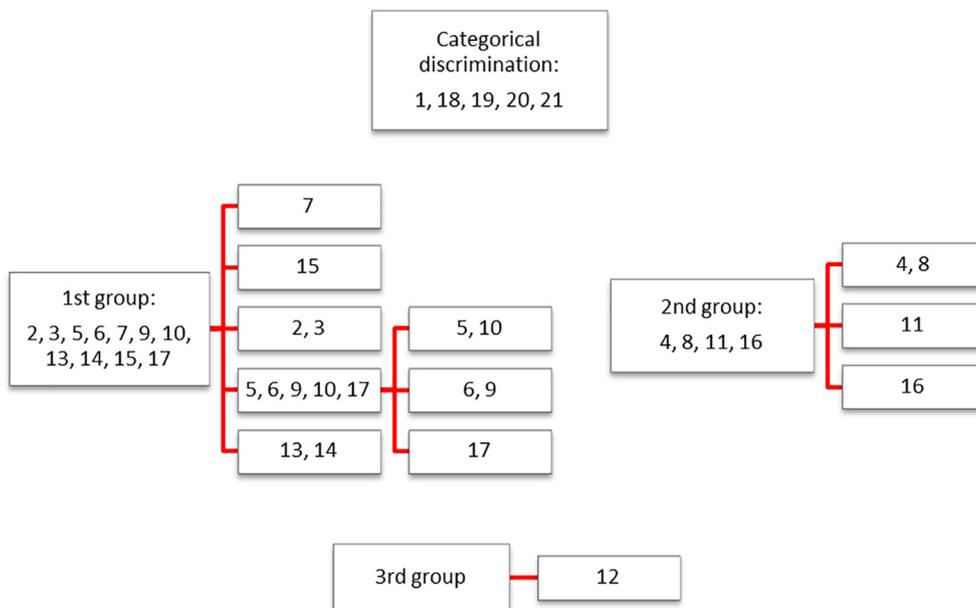

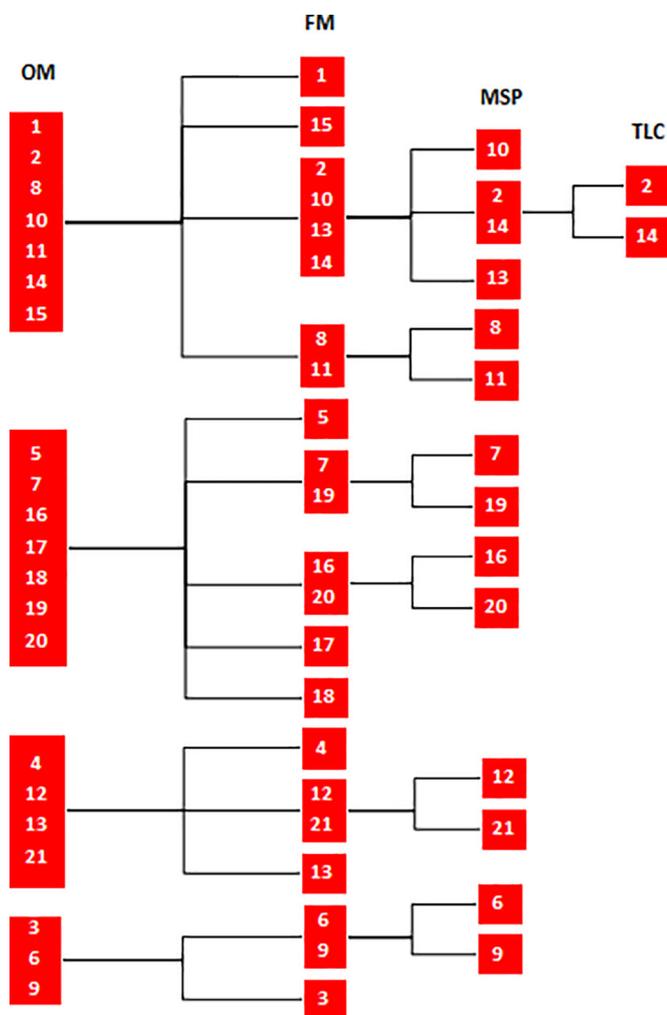**Fig. 3.** Results of differentiation of red cotton fibers using TLC and VSC.

Fig. 4. Results of differentiation of red cotton fibers using transmitted light microscopy (OM), fluorescence microscopy (FM), UV-Vis microspectrophotometry (MSP) and TLC.

light microscopy, fluorescence microscopy, UV–Vis microspectrophotometry and TLC allowed complete discrimination of the analysed red cotton fibres, originating from 21 clothing coming from the textile market. From the forensic science point of view, such a result demonstrates the capabilities of the distinction of the popular microtraces in the form of red cotton fibre with the use of methods, which are present in almost every forensic laboratory.

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