



Study of the interaction between collagen and naturalized and commercial dyes by Fourier transform infrared spectroscopy and thermogravimetric analysis



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ARTICLE INFO

Article history:

Received 25 September 2014

Received in revised form

10 January 2015

Accepted 12 January 2015

Available online 21 January 2015

Keywords:

Dyes

FT-IR

TGA

Cross-linking

Collagen

Secondary structure

ABSTRACT

The naturalized dyes (ND) and the traditional acid dyes (ADs) were compared by studying the different behavior during the leather dyeing process. NDs are glyconjugated compounds synthesized by the covalent union of a dye species with a natural sugar (e.g. lactose) able to confer water-soluble properties to the dye molecule as a whole. The interactions between the dyes and the leather proteins were studied by FT-IR spectroscopy and thermogravimetric (TG) analyses. The protein cross-linking of the dyed leather samples was investigated by studying the 1654/1690 cm^{-1} peak height ratio and a deconvolution procedure of the amide I peak. The helix secondary structure was the predominant component of the leather proteins of the samples dyed with low concentrations of NDs (2%), while the β -sheets prevailed when leather samples were dyed with the traditional ADs and high concentrations of NDs (>5%). The data were discussed with respect to TG results.

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1. Introduction

The transformation of animal skin into leather is a complex process encompassing several steps, among which leather dyeing adds value to the material for market purposes [1]. Routinely, the dyeing is carried out with sulfonated colorants at acidic pH, in order to establish electrostatic interactions between the residual amino groups of collagen and the sulfonic groups of the dyes [2]. This process takes place in water and in the presence of a wide variety of auxiliary chemicals: from salts to adjust the pH of dyeing baths, to surfactants and mordants to aid dye penetration into leather and enhance the overall fastness properties. Therefore, effluents from a tannery include a broad spectrum of contaminants, impacting heavily on the environment [3].

Recently, we synthesized a new class of environmentally friendly naturalized dyes (NDs) [4]. Briefly, a naturalized dye is a glyconjugated chemical, which is the covalent union of a dye species (e.g., azo, anthraquinone, aniline type chromophore) with a natural sugar, privileging lactose, able to impart remarkable water-soluble properties to the dye molecule as a whole. Moreover, dyeing processes involving NDs do not require the addition of chemical auxiliaries [5] and the resulting fastness properties of the dyed materials (e.g., polyester and nylon) are competitive with those observed from commercial colorants [6]. On the basis of these findings we prepared a second generation of NDs, in view of replacing traditional acid dyes (ADs) to advance to a more eco-sustainable leather industrial process. In particular, we achieved a new chemical bridge linking the chromophore and lactose [5], introducing a more stable amide moiety in place of an ester (Fig. 1) [4].

This modification to the first reported structures generated NDs with an amphoteric character, which is essential for leather dyeing process [5]. Furthermore, we observed that chemically different glyconjugated dyes of this new class were able to dye leather when combined among them, giving a finished material with

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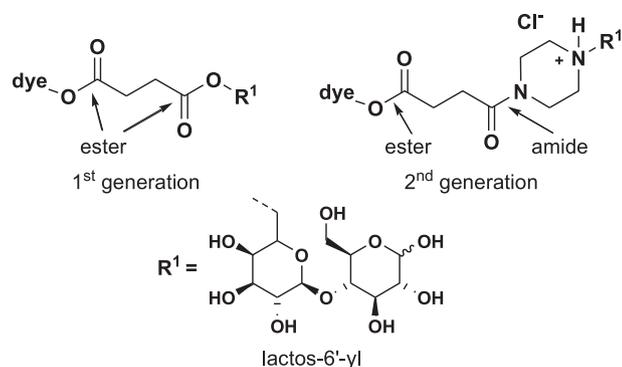


Fig. 1. Naturalized dyes.

homogeneous hue and fastness properties [6]. Elasticity, flexibility and longitudinal/tensile strength characterize collagen-based materials depending on the degree of intermolecular cross-links between the collagen triple helical units: lengthwise strength is enhanced by a parallel alignment of the fibrils (tendons), compliance is obtained through random layered arrangements (skin), flexibility is gained with laminated sheets, tensile strength is improved by means of concentric fibrillar layers [7]. These properties have been studied experimentally but also through successful computational materiomics studies [8–12]. While amino acid side chains play an important role in self-association of collagen helices and lateral packing of collagen helices, they are also crucial in the binding of a wide variety of molecular species which influence the microstructure and physical performance of the collagen matrix [13,14].

In this work, we investigated the interaction of second generation NDs **7–9** and **12** with leather at molecular level by Fourier transform infrared spectroscopy (FT-IR) and thermogravimetric analyses (TGA). IR spectroscopy is a well-established technique to analyze the secondary structure of polypeptides and proteins [15–19]. The IR spectral data of polypeptides and proteins are usually interpreted in terms of the vibrations of a structural repeat unit, which give rise to nine characteristic IR absorption bands, namely amide A, B and I–VII [17–19]. Among these, amide I and II bands are the two most prominent absorptions of the protein backbone, with amide I ($1700\text{--}1600\text{ cm}^{-1}$) being the most sensitive one of the spectral region. The amide I band is due almost entirely to the carbonyl stretch vibrations of the peptide linkages and the frequencies arising from each component of the complex absorption correlate closely to each secondary structural element of the proteins [20]. Thus, second derivative spectra allow the identification of the various secondary structures present in the protein [18] and the curve fitting can be applied to calculate the contribution of each component of the absorption band to the secondary structure [18,21–24].

The results were compared to those obtained from a group of commercial ADs. An interaction model between the dyes and the leather proteins was described, indicating that NDs interact better than ADs with the polypeptide matrix.

2. Experimental section

2.1. Materials and solutions

Chromium tanned leather samples and the commercial traditional ADs Acid Orange 37 (AO37), Acid Yellow 49 (AY49), Acid Red 249 (AR249) and Acid Blue 113 (AB113) were provided by BIO-KIMICA. The NDs **7–9** and **12** were synthesized according to

patented experimental procedures, elaborating commercial Disperse Orange 30 (DO30), Disperse Red 202 (DR202), Disperse Blue 27 (DB27) and Disperse Yellow 42 (DY42), respectively [5]. Formic acid was purchased from Sigma-Aldrich-Fluka (F0507, purity > 95%). All solutions were prepared with deionized water (18.2 MΩ cm) using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Synthesis of second generation ND: the (piperazin-6'-yl)lactose moiety

The selective protection of the hydroxyl groups of lactose **1** [25] generated the protected diol **2**, whose 6' and 2' positions were available for further elaboration. Regioselective tosylation of the primary alcohol [26] was followed by an $\text{S}_{\text{N}}2$ process using an excess of piperazine to obtain **4** in 62% overall yield for three steps (Scheme 1).

Next, the piperazine derivative **4** was coupled to the dye **5**, which had been prepared conveniently from chromophore DO30 [5]. The free carboxylic acid moiety was activated with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) in mild conditions [27], recovering compound **6** in good yield. Final deprotection of the acetonide moieties was carried out in acid conditions, to restore the original structure of the lactose portion within **7** (Scheme 2) [5].

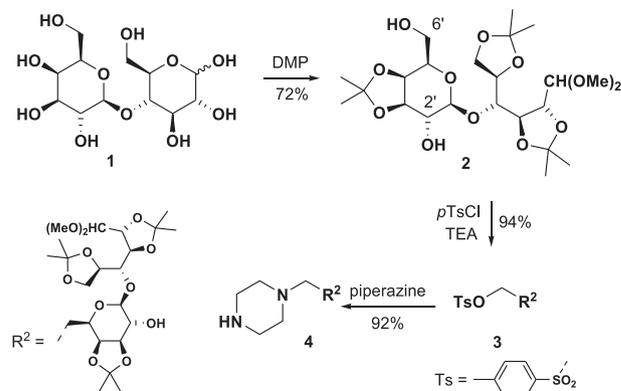
Adduct **7** showed a significant solubility in water, even though the piperazinil unit was present as a hydrochloride salt. A similar strategy was followed to synthesize compound **8** and **9** (Fig. 2) [5].

A slightly different approach was adopted for compound **12**. In this case, DY42 **10** was made to react with ethyl chloroacetate in the presence of a catalytic amount of potassium iodide [28]. The resulting ester derivative was hydrolyzed to acid **11**, which was converted to **12** following the route described previously (Scheme 3).

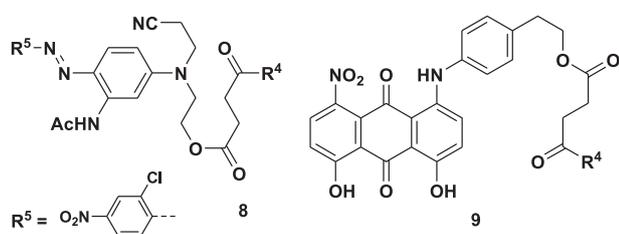
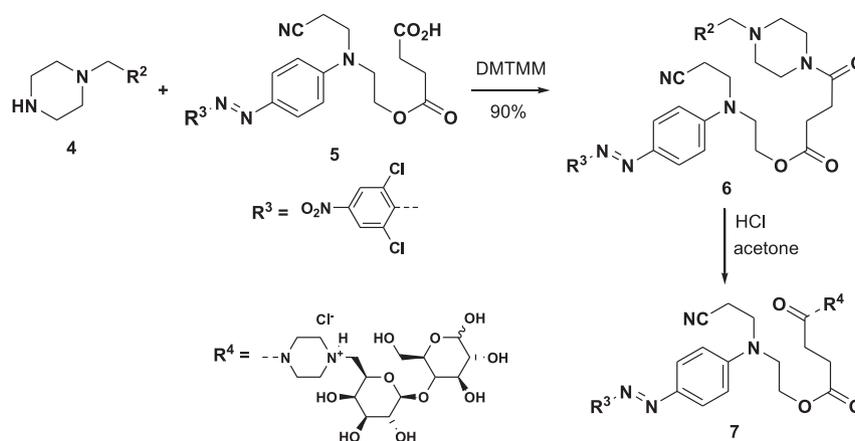
Thanks to this easy procedure to achieve the second generation NDs, it is possible to process chemically different chromophores in a reproducible manner, whenever a free carboxylic acid is available for amide coupling [5].

2.3. Leather dyeing

The operational procedure for leather dyeing was an adaptation to laboratory scale of the daily large scale dyeing of a tannery. Briefly, 0.2 g of chrome tanned leather specimen were put in a plastic tube with a 2 mL aqueous solution of dye at C1 concentration (4 mg, 2% w/w) and stirred (magnetically) for one hour at 20 °C. The dyeing bath was heated at 55 °C and 0.4 mL of water and



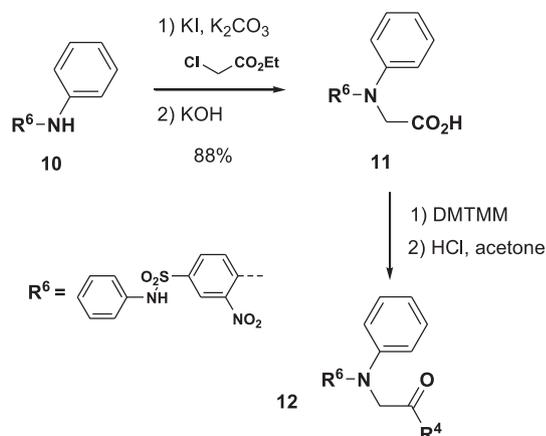
Scheme 1. Synthesis of protected (piperazin-6'-yl)lactose.



4 mg of formic acid were added. The sample was stirred for 30 min and then rinsed with 4 mL of water for three times and dried at room temperature. In the case of NDs we performed also another set of experiments increasing the amount of dye up to C2 (5% w/w), C3 (10% w/w) and C4 (15% w/w) concentrations. It has to be highlighted that the w/w percentage of an ADs chromophore is approximate, due to the presence of additives in commercial products [2]. Instead, NDs are chemically pure products and the percentage refers to the actual weighted amount of dye.

2.4. Colorimetry

The final color of the leather samples were characterized by colorimetry [29]. The three coordinates of the CIELAB color space were measured by using a CM2600d KONICA MINOLTA portable



spectrophotometer. The illuminant was D65 and the light source wavelengths were 360–740 nm. The diameter of the spot was 5 mm and the light incident angle was 10°. The specular component included (SCI) and specular component excluded (SCE) coordinates were acquired simultaneously.

2.5. FT-IR spectroscopy

Infrared spectra were recorded on a Perkin–Elmer Spectrum One FTIR spectrophotometer, equipped with a universal ATR accessory and a TGS detector. After recording the background spectrum, sample spectra were obtained on different area of blank and leather samples. For each sample, 128 interferograms were recorded (in order to obtain a suitable S/N ratio), averaged and Fourier-transformed to produce a spectrum with a nominal resolution of 4 cm⁻¹. The spectra were run and processed by means of the Perkin–Elmer spectrum software and a written-in house LabVIEW program for peak fitting, respectively. Control analyses were performed on leather specimens before entering into the dyeing

Table 1

L*a*b* coordinate values for both SCI and SCE configurations of all dyed leather specimens.

Dye	%(w/w)	SCI			SCE		
		L*	a*	b*	L*	a*	b*
7	C1	62.92	25.73	39.27	62.9	25.65	39.22
	C2	57.97	31.73	44.06	57.91	31.7	44.02
	C3	53.6	36.09	45.14	53.59	36.03	45.08
	C4	53.28	36.39	44.83	53.21	36.37	44.9
8	C1	48.11	39.65	13.66	48.08	39.59	13.64
	C2	41.18	38.98	14.73	41.06	39	14.74
	C3	39.59	37.27	14.39	39.45	37.35	14.43
	C4	37.25	36.1	14.53	37.11	36.16	14.57
9	C1	53.56	-13.29	-22.35	53.4	-13.29	-22.39
	C2	49.7	-10.88	-25.83	49.59	-10.86	-25.87
	C3	44.54	-9.46	-25.34	44.49	-9.45	-25.37
	C4	40.93	-7.58	-24.93	40.8	-7.58	-25.01
12	C1	81.47	-7.6	25.79	81.35	-7.56	25.75
	C2	80.16	-7.25	40.23	80.04	-7.23	40.19
	C3	78.81	-6	49.71	78.7	-5.98	49.68
	C4	77.84	-4.62	54	77.7	-4.61	54.02
AO37	C1	55.68	32.81	40.07	55.56	32.87	40.19
AR249		52.46	48.56	11.48	52.33	48.62	11.56
AB113		32.12	3.76	-22.11	31.96	3.79	22.21
AY49		78.02	-10.69	57.24	77.85	-10.62	57.18
blank-1	/	82.38	-4.39	1.34	82.21	-4.36	1.37
blank-2	/	85.85	-4.27	0.39	82.68	-4.25	0.43

process (blank-1) and subjected to dye conditions in the absence of the colorants (blank-2).

2.6. Thermogravimetric (TG) analyses

TG analyses were carried out on an EXSTAR series TGA/DTA7200 apparatus, with nitrogen as purging gas (200 mL/min). Each sample (5–10 mg) was placed in an alumina sample pan and the run was carried out at a standard rate of 10 °C/min from 30 to 700 °C in the presence of nitrogen.

3. Results and discussion

3.1. Dyeing efficiency

The leather dyeing efficiency of the NDs was compared to that of the traditional ADs. The dyeing baths at the end of the dyeing procedure were almost colorless (the absorbance decreased of 95% after the first rinsing step) when ADs were used, indicating that the leather samples had retained the dyes very efficiently. In the case of NDs the dyeing baths and the three rinsing solutions were colored, with a decrease of the absorbance of about 60% after the first rinsing step (data not shown for brevity). The final colors of the leather samples dyed with NDs and ADs were characterized by the $L^*a^*b^*$ coordinate values for both SCI and SCE configurations (Table 1).

Leather samples dyed with NDs showed a decrease in the lightness and an increase in the colour saturation as the amount of the dye increased from C1 to C4. Furthermore, the sections of the leather samples dyed with NDs were completely colored, indicating their ability to penetrate across the samples. On the contrary the ADs did not penetrate the leather, as shown by the absence of colour in the central region of the sample sections (Fig. 3).

The increasing concentration of ADs from C1 to C2 did not improve dye penetration. This result was likely due to the poor mechanical stir of the leather samples in the plastic tube, compared to the rolling and tumbling effects on whole box calf type leather specimens (4–6 m² wide) during the large scale operations in tannery drums (50–60 m³ volume, running at 20% capacity) [30]. However, it suggests that NDs penetrate across the leather sample better than ADs and also in mild stirring conditions.

3.2. FTIR spectroscopic analysis of leather samples dyed with NDs and ADs

Leather is composed by structural proteins (88% collagen, 6% keratin, 0.9% elastin) and non-structural proteins (3% albumins and globulins, 2.1% mucins and mucoids). Collagen is the most abundant protein of leather and therefore, it is likely to assume that FTIR spectra of leather mainly depend on collagen absorptions.

Fig. 4 shows the comparison of representative FT-IR spectra of blank-1, blank-2, ND 7 and AO37 in the 1800–900 cm⁻¹ region.

The strong absorption bands at 1650 and 1550 cm⁻¹ were attributed to the amide I (C=O stretching) and amide II (CN

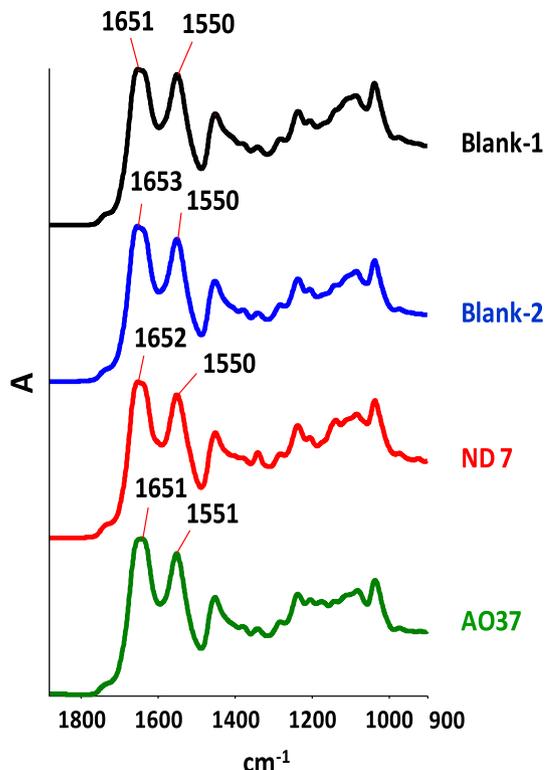


Fig. 4. Comparison of representative FT-IR spectra of blank-1, blank-2, ND 7 and AO37 in the 1800–900 cm⁻¹ region.

stretching and NH bending) of the leather proteins. Absorption features in the fingerprint region from 1451 to 1000 cm⁻¹ were attributed to CH₂ wagging, CH₃ deformation, C–N stretching and C–OH stretching of leather proteins [31] and the contribution of dye absorptions, which is small because of the low dye concentration. The amide region shows significant differences.

A careful investigation of specific absorption bands is fundamental to obtain useful information about the interaction of dyes with leather proteins. In particular, FT-IR spectroscopy has been widely employed to study the collagen cross-linking in bone and cartilage samples through the analysis of the 1660/1690 cm⁻¹ absorbance (or peak area) ratio, which increases as the collagen cross-linking increases [32–34]. Intermolecular cross-linking is responsible for the mechanical strength of collagen fibrils. *In vivo* intermolecular cross-links are due to the formation of covalent bonds from aldehydes generated enzymatically from lysyl and hydroxylisyl sidechains by lysyl oxidase [35]. Cross-linking process is also due to the reaction of reducing sugars (*e.g.* glucose) which is strictly related *in vivo* to aging processes [36]. The nonenzymatic glycosylation of certain lysine and hydroxylysine residues is the first step in a series of reactions known as the Maillard reaction [37], and it involves the nonenzymatic condensation of a free amino group on a sugar aldehyde or ketone. The resulting Schiff base is

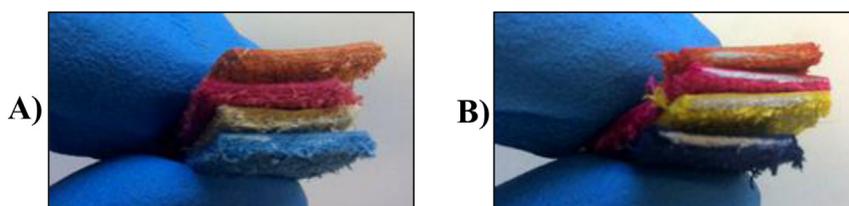


Fig. 3. Sections of the leather samples dyed with NDs (A) and ADs (B).

likely to undergo a rearrangement to form a fairly stable ketoamine called the Amadori product [38].

Barth et al. in a study on bone collagen reported that the suppression of plasticity from fibrillar sliding gave an increase in the 1660/1690 ratio and it has been primarily attributed to an increase in the concentration of the nonenzymatic (AGE) cross-links. The decrease in the 1660/1690 ratio is more explicitly related to changes in enzymatic cross-links [39].

It is quite clear that the collagen structural changes and the eventual cross-linking responsible for the spectral changes observed in dyed leather samples are not due to enzymatic processes, but to the interaction/reaction with dyes and/or with formic acid employed in the dyeing procedure.

Fig. 5 shows the values of the 1654/1690 cm^{-1} absorbance ratio calculated from the spectra of the dyed leather samples investigated.

The 1654/1690 cm^{-1} absorbance ratio was found significantly higher for the leather samples dyed with NDs at C1 and for the blank-2 sample. The leather samples dyed with the ADs at the same concentration gave an absorbance ratio similar to that of the chrome tanned leather (blank-1).

By increasing the concentration of NDs, the ratio value decreased, reaching the value calculated from the FT-IR spectrum of the blank-1 (weak chrome-tanned leather) and the leather samples dyed with ADs (Fig. 6).

These spectral changes may be the result of significant structural changes of collagen, better discussed in the next paragraph.

3.3. Amide I peak fitting

The interaction of NDs and ADs with leather proteins and the spectral differences observed in the 1654/1690 cm^{-1} ratio of Amide I band of FT-IR spectrum, were studied more in detail applying a peak fitting analysis to the vibrational C=O stretching frequencies associated to the amide I (Fig. 7).

As mentioned above, different secondary structures of proteins contribute to the overall stretching of the carbonyl double bond and a second derivative spectrum analysis provides information about the number and frequencies of the components. Secondary structure motifs were estimated by expressing the amplitude of the bands, assigned to each structure as a fraction of the total sum of the amplitudes of the amide I components [40]. The leather

samples treated with ADs were explored at C1, while the samples treated with NDs were explored at C1 and C4. Each single component of amide I was assigned according to the literature [7,20,39,41,42], namely *ca.* 1680 cm^{-1} to turns, 1690 cm^{-1} to antiparallel β -sheets, 1656 cm^{-1} to helix, 1630 cm^{-1} to intramolecular β -sheets, 1610 cm^{-1} to intermolecular β -sheets. The peak fitting analysis showed that all the samples had almost the same secondary structure components, but the different percentages reflected the different type of interaction between the dyes and the leather proteins (Table 2).

The helix is the predominant component of native collagen secondary structure. Modifications observed in the component percentages of leather samples suggested that major changes of collagen in leather samples treated with NDs at C1 were the increase of helix structure and the decrease of β -sheets. Helix percentage in NDs at C1 dyed samples was indeed $48 \pm 4\%$, not statistically different from the blank-2 (47%). In samples dyed with traditional ADs or NDs at C4 the helix percentage was instead $38 \pm 4\%$ and $31 \pm 8\%$, respectively, comparable with the helix percentage found in blank-1 (39%). Thus, the structure of the leather proteins (basically collagen) in samples dyed with NDs at C1 was richer in helices, while in those treated with ADs at C1 and with NDs at C4 had a higher percentage of β sheets.

Fig. 8 shows, for each dyed leather sample, the ratio of the component percentage at 1660 cm^{-1} assigned to helix and at 1690 cm^{-1} assigned to turns and antiparallel β sheets, analogous to the ratio of the absorbance values at 1654 and 1690 cm^{-1} .

The results calculated on the basis of the Gaussian component percentages confirmed the previous results calculated on the basis of the ratio of absorbance values of the amide I band. The single component analysis evidenced significant conformational changes and significant variations of the 1660/1690 cm^{-1} absorbance ratio.

It is interesting to note that blank-1 is a weak-chrome tanned leather specimen before entering into the dyeing process; blank-2 is a weak-chrome tanned leather specimen processed with the dyeing procedure in the absence of colorants. The blank-2 leather sample showed higher helix percentages and 1660/1690 cm^{-1} percentage components ratio values comparable with those obtained in leather samples dyed with NDs at C1. These structural changes can be likely due to the treatment at 55 °C with 3.63×10^{-2} M (1.67 g/L, i.e. 0.17% w/w) formic acid, conditions commonly employed to fix the color without compromising the

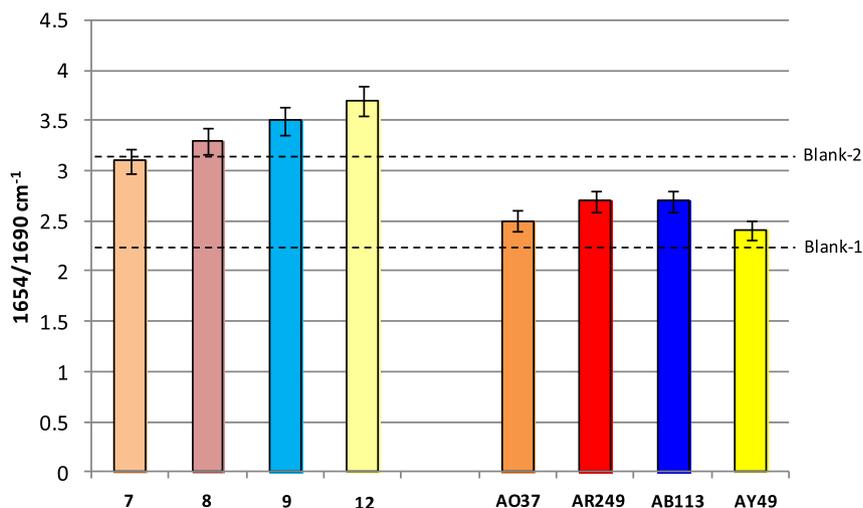


Fig. 5. Absorbance ratio (1654/1690 cm^{-1}) from the FT-IR spectra of leather samples dyed with NDs and Ads at C1 concentration. The dotted lines represent the ratio values of the blank-1 and blank-2 samples.

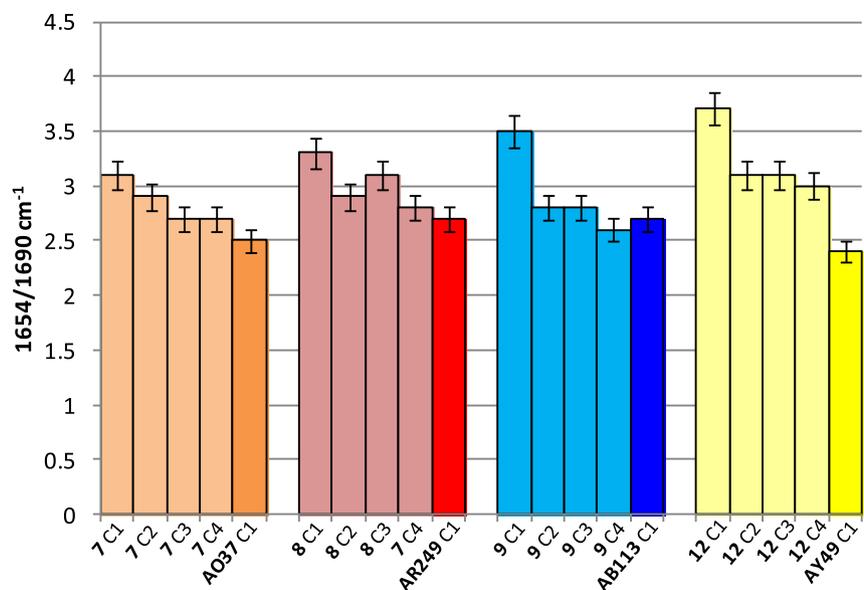


Fig. 6. Comparison of the absorbance ratio values ($1654/1690\text{ cm}^{-1}$) between leather samples dyed with NDs at C1–C4 and ADs at C1.

collagen structural integrity. The weak acid conditions of leather ($\text{pH} < 3.5$, isoelectric point of collagen) are mandatory in order to favor the interaction of sulphonic groups of ADs with protonated amino groups of collagen [43]. We can hypothesize that in these conditions formic acid may act as a cross-linker for collagen, as well as succinic acid [44]. In the case of succinic acid the crosslinking is due to the ion interaction between the carboxylate groups and the protonated amine group of the collagen. The crosslinking between succinic acid and collagen is also due to multiple intermolecular hydrogen bonding between the acid and the lysine residue of the

collagen. The results obtained analyzing the blank-2 sample can be explained assuming the reactions of formic acid analogous to those reported for succinic acid.

Thus, all the conformational changes observed may be induced by the primary interaction with colorants and/or by the formation of cross-links among collagen fibers [45] induced by treatment with formic acid and/or by colorants.

The samples dyed with NDs at C1 had $1660/1690\text{ cm}^{-1}$ absorbance ratio higher than those dyed with ADs at the same concentration and with NDs at C4. The $1660/1690\text{ cm}^{-1}$ absorbance ratio of

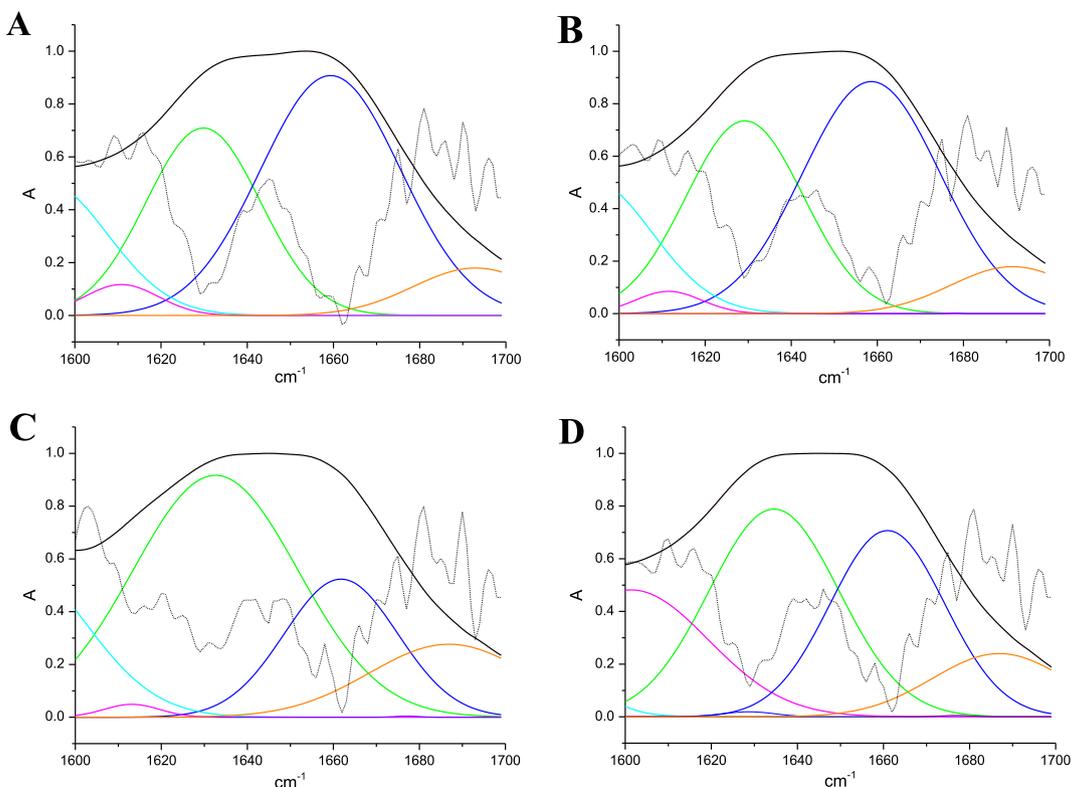


Fig. 7. Representative FT-IR spectra in the amid I region of (A) blank-2, (B) C1 12, (C) C4 12, (D) C1 AY49, compared with the predicted spectra (bold dotted line). The second derivative spectra (dashed line) and the single Gaussian components are also shown.

Table 2
Results of the deconvolution procedure applied to the amide I region of FT-IR spectra of leather samples dyed with NDs and ADs.

Frequency/cm ⁻¹ (bandwidth/cm ⁻¹)	Assignment	%
Blank-1		
1630 (34)	intramolecular β -sheets	42
1658 (34)	Helix	39
1610 (19)	intermolecular β -sheets	5
1682 (43)	turns	13
Blank-2		
1630 (31)	intramolecular β -sheets	37
1659 (38)	Helix	47
1611 (20)	intermolecular β -sheets	6
1693 (34)	Antiparallel β -sheets	9
7^a		
1632 (30)	intramolecular β -sheets	36
1659 (37)	Helix	54
1692 (34)	Antiparallel β -sheets	11
8^a		
1629 (33)	intramolecular β -sheets	40
1659 (37)	Helix	46
1610 (18)	intermolecular β -sheets	4
1691 (34)	Antiparallel β -sheets	10
9^a		
1628 (31)	intramolecular β -sheets	36
1609 (22)	intermolecular β -sheets	10
1657 (40)	Helix	45
1692 (35)	Antiparallel β -sheets	8
12^a		
1629 (33)	intramolecular β -sheets	39
1659 (37)	Helix	46
1611 (18)	intermolecular β -sheets	5
1691 (34)	Antiparallel β -sheets	9
AO37^a		
1630 (38)	intramolecular β -sheets	45
1660 (34)	Helix	39
1609 (17)	intermolecular β -sheets	4
1689 (35)	Antiparallel β -sheets	11
AR249^a		
1629 (34)	intramolecular β -sheets	34
1658 (38)	Helix	38
1603 (26)	intermolecular β -sheets	16
1684 (24)	turns	6
1698 (22)	Antiparallel β -sheets	5
AB113^a		
1629 (35)	intramolecular β -sheets	42
1659 (36)	Helix	43
1610 (18)	intermolecular β -sheets	4
1690 (35)	Antiparallel β -sheets	10
AY49^a		
1635 (36)	intramolecular β -sheets	36
1601 (42)	intermolecular β -sheets	21
1661 (31)	Helix	32
1687 (37)	Antiparallel β -sheets and turns	11
7^b		
1631 (32)	intramolecular β -sheets	34
1658 (35)	helix	38
1601 (35)	intermolecular β -sheets	18
1686 (38)	turns	10
8^b		
1633 (32)	intramolecular β -sheets	30
1659 (33)	Helix	35
1609 (37)	intermolecular β -sheets	21
1679 (31)	turns	9
1697 (27)	Antiparallel β -sheets	5
9^b		
1631 (50)	intramolecular β -sheets	55
1662 (29)	Helix	19
1609 (15)	intermolecular β -sheets	4
1681 (58)	turns	21
12^b		
1633 (45)	intramolecular β -sheets	52
1662 (31)	Helix	30
1613 (15)	intermolecular β -sheets	3
1687 (44)	Antiparallel β -sheets and turns	16

^a C1.

^b C4.

the leather samples dyed with **8**, **9** and **12** at C4 was even lower than that observed for traditional ADs.

Two phenomena may contribute to high cross-linking and higher helix percentage observed in leather samples dyed with NDs at C1: (i) the treatment with formic acid, (ii) the presence of the lactose unit in the dye structure. The protonated structure of the piperazine bridge of NDs (Scheme 2) and the contemporary presence of the polyhydroxylated structure of lactose may favor the hydrogen bonding within the amino acid residues of collagen, contributing to the stabilization of helix structure and to the increase of cross-linking.

However, leather samples dyed with higher dye concentrations up to C4 had lower helix and higher β -sheet percentages. This can be likely due to the stacking interactions between chromophore rings in excess [46] that surround collagen fibers, thus avoiding the intermolecular covalent crosslinking and stretching the protein structure because of their steric hindrance.

3.4. Thermogravimetric (TG) analyses

The thermal stability of the leather samples dyed with NDs and ADs was investigated by TGA. Fig. 9 shows a representative thermogram of a leather sample dyed with **12** at C1.

The trends of the TG% and DTG were found to be not significantly different for all the samples analyzed. The first weight loss corresponded to the loss of water, while the second weight loss corresponds to the polypeptide chain thermal decomposition. Table 3 summarizes the TGA parameters found for all the samples analyzed.

Fig. 10 shows the trend of the T_0^2 values for all the samples analyzed.

The thermal stability of the leather samples was found to be dependent on the class and the percentage amount of dyes used in the dyeing procedure. The leather samples dyed with NDs at C1 had higher T_0^2 values (338 ± 1 °C) which corresponded to higher thermal stability and were not statistically different from the blank-2 (338 °C). These results suggest that the treatment with formic acid at 55 °C increases the thermal stability of collagen fibers by itself and low concentrations of NDs (C1) do not interfere with this stabilization. The higher thermal stability of collagen fibers due to treatment with formic acid found in blank-2 with respect to the blank-1 sample is compatible with the hypothesis that formic acid favors the cross linking process [44].

The correlation between the thermal stability of the samples analyzed and the higher values of the 1660/1690 cm⁻¹ ratio found in the FTIR study seems to support the correlation of this ratio with crosslinked structures, in agreement with the literature data [32–34].

The leather samples dyed with ADs at C1 and NDs at C4 had lower values of T_0^2 (335 ± 1 °C and 332 ± 2 °C, respectively), with values comparable (ADs) or even lower (**8**, **9** and **12** NDs showed the lowest T_0^2 value) than the T_0^2 found in blank-1 (334 °C). In all these cases it seems that the presence of the dyes interferes with the mechanism of formic acid-induced cross linking. This interference could be due to a "shielding effect" of stacked dye chromophores around collagen fibers. These TG results agreed, indeed, with data obtained from FT-IR analysis. Higher values of the 1660/1690 cm⁻¹ ratio evidenced in the leather samples dyed with NDs at C1 samples suggested a more cross-linked structure of the samples, compatible with higher thermal stability. Covalent bonds between collagen helices stabilize, indeed, the material. Leather samples dyed with ADs and NDs at C2–C4 showed lower thermal stability and corresponding lower values of the 1660/1690 cm⁻¹ ratio, a decrease in helices and higher percentages of β -sheets. Stacking interactions between dye chromophores and β -sheets structures of

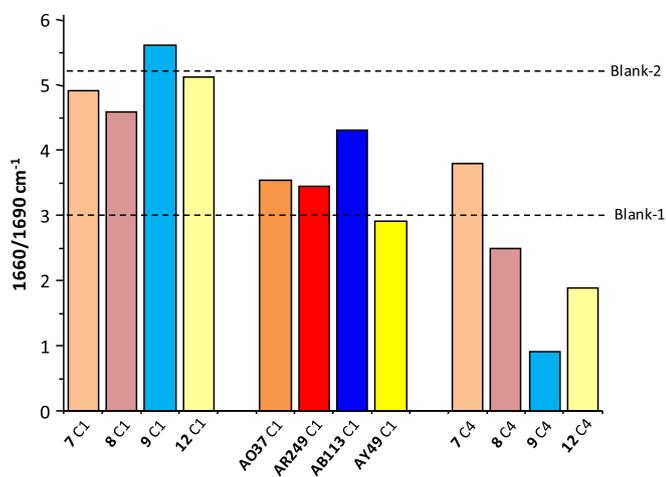


Fig. 8. 1660/1690 cm^{-1} percentage components ratio calculated from FT-IR amide I peak fitting of all leather samples dyed NDs at C1 and C4 and with ADs at C1. The dashed lines correspond to the ratio values for the blank-1 and blank-2 samples.

collagen likely avoid the intermolecular covalent crosslinking and may be responsible for the lower stability of these samples.

4. Conclusions

FT-IR and TG analysis were useful to investigate the interaction of NDs, a new and eco-friendly class of dyes, and traditional ADs with the proteins of chrome tanned leather at molecular level. The NDs were found to exhibit a higher penetrating capacity into the leather compared to that of traditional ADs, since they were capable to impregnate the leather sample in mild agitation conditions. The FT-IR analysis of amide I band, both in terms of the absorbance ratios at two different wavelengths (the 1654/1690 cm^{-1} absorbance ratio) and the analysis of the single components found by peak fitting evidenced that the leather proteins in samples dyed with NDs at C1 (2%) were characterized by higher percentage of helices and higher values of the 1660/1690 cm^{-1} ratio, analogous to the values found in blank-2 leather sample. We hypothesized that all these conformational changes observed may be induced by the primary interaction with colorants and/or by the formation of cross-links among collagen fibers induced by the treatment with formic acid and/or by colorants.

We hypothesized that the lactose unit in the NDs structure favors, by one hand, the nonenzymatic condensation of the sugar

Table 3
Experimental temperatures and weight loss percent of thermal degradation steps of leather samples dyed with NDs and ADs.

Sample	T_0^1	T_{ONSET}	T_0^2	% Final weight
Blank-1	77.4	302.2	334.3	22.3
Blank-2	72.7	299.9	338.0	20.3
7^a	76.6	298.2	337.0	21.6
8^a	74.1	299.1	339.0	21.6
9^a	70.6	300.6	339.5	20.9
12^a	74.0	297.7	337.6	20.3
AO37 ^a	75.4	302.0	335.2	22.1
AR249 ^a	73.0	299.1	336.0	21.4
AB113 ^a	76.1	299.2	335.4	22.4
AY49 ^a	71.9	300.6	333.5	20.4
7^b	77.7	297.4	334.6	22.5
8^b	79.0	299.9	330.6	22.1
9^b	72.7	299.3	330.9	22.7
12^b	76.5	299.5	331.5	22.6

^a C1.

^b C4.

aldehyde or ketone [37,38] with free amino groups, responsible for the cross-linking. On the other hand, the hydroxyl groups of lactose may interact by H-bonding with specific amino acid residues of collagen, contributing to the stabilization of helix structure.

In samples dyed with traditional ADs at C1 and NDs at higher concentrations (particularly at C4, 15%), β -sheet structures were predominant and the spectra were characterized by lower values of the 1660/1690 cm^{-1} ratio.

Traditional ADs, instead, interact with collagen primarily by electrostatic forces between chromophore sulphonated groups and the protonated amino groups of amino acid side chains [2]. Furthermore, the additional π -stacking [47] occurring across the AD molecules, exclude the hydrogen bonding cross-linkage, essential for the stabilization of helix secondary structures. The excess of NDs (up to C4) causes the inhibition of cross-linking, as well, lower helix and higher β -sheets percentages. This can be likely due to the stacking interactions between the excess of chromophore rings that surround collagen fibers, (i) stabilizing the β -sheet structures and (ii) avoiding the intermolecular covalent crosslinking.

The thermoanalytical study confirmed these results and matched with this hypothesis, revealing a higher thermal stability for the leather samples dyed with NDs at C1 concentration and lower thermal stability in leather samples dyed with ADs and NDs at C4 concentration.

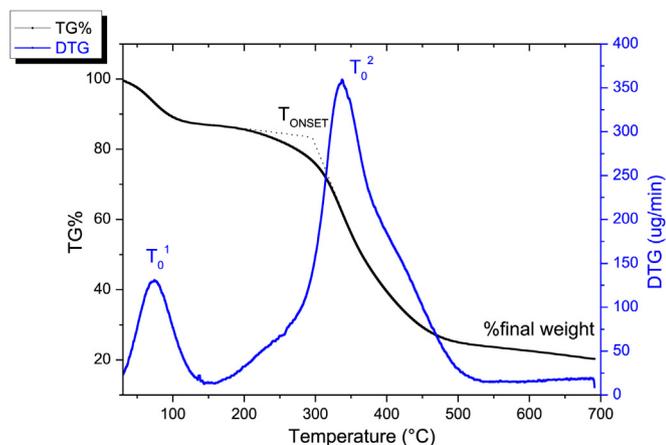


Fig. 9. Thermogravimetric curve (left axis) and its derivative (right axis) of leather sample dyed with **12** at C1, performed under nitrogen flow at 10 $^{\circ}\text{C}/\text{min}$ heating rate.

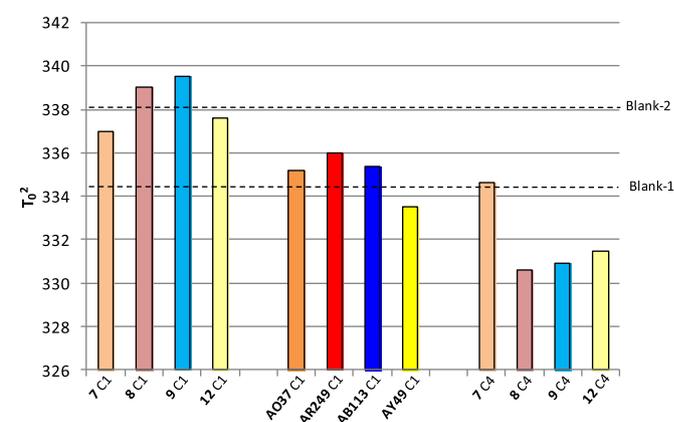


Fig. 10. Trend of the T_0^2 values for the leather samples dyed with NDs at C1 and C4 and with ADs at C1. The dashed lines correspond to the T_0^2 values for the blank-1 and blank-2 samples.

In principle, NDs may be competitive with the traditional AD because of their eco-friendly properties, because of their better penetrating capacity and because their employment at low concentrations (about 2%) give more cross-linked structures. This last characteristic may provide better quality features to the leather in terms of resistance and flexibility.

Currently, studies for scale-up dyeing at industrial level with NDs are in progress [48], taking into account the recently displayed biodegradability properties of this new class of colorants in the presence of common bacteria strains (e.g., *Escherichia coli*). This would offer the opportunity to treat dyeing effluents in an eco-friendly manner and re-use the water for further dyeing cycles, cutting the costs associated to water management [49].

Acknowledgments

This work has been financially supported by the European Project Life+ 2012 ENV/IT/352-“BIONAD”. The authors would like to thank Dr. Stefano Legnaioli, Dr Giulia Lorenzetti and Dr. Emanuela Grifoni for the colorimetric analysis.

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