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Research Article

Biological Coloration of Flax Fabrics with Flavonoids using Laccase from *Trametes hirsuta*

Biological environmentally friendly concepts are emerging to replace chemical treatments of fabrics. In this work, a new process for the coloration of flax fabrics via enzymatic oxidation of natural flavonoids (morin, quercetin) has been developed. Laccase from *Trametes hirsuta* is able to react with flavonoids and polymerize them, resulting in a strongly colored polymeric solution which can be applied to the coloration of flax fabrics. Two methods were investigated: *(i)* the simultaneous enzymatic polymerization and coloration of fabrics and *(ii)* the polymerization of flavonoids with laccase, followed by a further coloration of the flax fabrics. Factors such as temperature, reaction time, presence of NaCl or the use of bleached or unbleached fabrics were evaluated in order to increase the color of the fabrics and the color fastness. The increase of temperature, the presence of salt and the use of unbleached fabrics allowed the final color to be improved. Colorized flax fabrics with oxidized quercetin solution showed a color fixation two times higher than the fabrics colorized with oxidized morin. Finally, the polymerization of flavonoids and their binding to fibers were verified using Fourier transform infrared spectroscopy (FT-IR). The results confirmed this environmentally friendly process as useful for the coloration of flax fabrics. A similar technique could also be extended to the treatment of other types of fabrics in textile processes.

Keywords: Enzymatic polymerization, Flavonoids, Flax, Laccase

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1 Introduction

Flax (*Linum usitatissimum*) is an important source of bio-based natural fibers that recently have renewed interest as textiles (linen), pulp, paper and lightweight high-strength composites [1, 2]. The flax is a ligno-cellulosic fiber composed of cellulose, hemicellulose and lignin (with 2.2 % lignin content) [3]. Cellulose is the main structural component (71 % of the entire components) of flax fibers and is thought to play a predominant role in the mechanical properties of the fiber cell wall [4]. However, other components like pectin, rhamnogalacturonans, homogalacturonans, arbinans and xyloglucans also contribute to the physical and chemical properties of the fiber [5]. Several publications showed the presence of various "phenolic compounds" in flax fibers [6, 7]. Furthermore, these compounds were confirmed by HPLC analysis, ^{13}C NMR Spectroscopy and TEM (Transmission Electron Microscopy) [1, 2] proving the presence of flavonoids. These flavonoids consist of a large group of low-molecular weight polyphenolic substances, naturally occurring in fruits and vegetables and play an integral part of human diet. Basically, flavonoids are present exclusively in the form of β-glucosides and they can be divided based on their basic molecular structure into four main groups: flavones, flavonols, flavonones and isoflavones [8–10]. These phenolic compounds provide color to the natural fibers.

The conventional processes for the treatment of flax fibers involve several steps: the initial step is called "retting" and is accomplished by the breakdown of the materials that bind the fibers into the plant stems [11]. During the scouring process, flax fabric is conventionally treated with alkaline solution (e.g., a high concentration of NaOH) to remove non-cellulosic gummy material in order to make it suitable for further processes [12]. A bleaching process follows with hydrogen peroxide to remove the natural color of flax fabrics, and finally, some fin-

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ishing processes can be performed to improve the characteristics of fabrics.

Aiming to search new “environmentally friendly” alternatives for the treatment of fibers in textile processing, the enzymatic processes are gaining interest as substitutes of chemicals. Their application provides some advantages related to processing conditions as mild temperature, pressure, pH and less water consumption [13]. The replacement of conventional methods by enzymatic textile treatments not only minimizes the COD (chemical oxygen demand) and BOD (biological oxygen demand) in effluents, but also the amount of effluents [14]. Highly specific enzymes affect only non-cellulosic fibers without damaging the cellulose part. The first attempt of enzymatic treatment of flax was applied in the retting process. Moreover, it was also applied in the basic pre-treatments of fabric, like scouring or bleaching, improving the quality process, as well as yarns regularity, dyeability and others.

The functionalization of flax can be obtained by using a variety of enzymes like pectinases, xylanases, cellulases, and semi-cellulases mixed with pectinases or laccases [12, 15–17]. Laccase (EC 1.10.3.2) belongs to the class of multi-copper oxidoreductases that can catalyze the oxidation of various aromatic compounds, especially phenols and anilines by means of oxidative coupling reactions [18, 19]. The synthesis of colorants by laccase has been widely studied with different kinds of substrates such as phenols and polyphenolic compounds [20]. There are several publications focusing on the enzymatic coloration of wool, cotton and human hair by laccase via several reaction substitutes [21, 22–24].

Some of the most interesting natural phenolic compounds are flavonoids. In this research, their interest lies in the color that these compounds add to fabrics. Moreover, these flavonoids can be polymerized by enzymes resulting in dark colored compounds, due to the formation of big conjugate structures along the main chain (polyphenols) [21]. These oxidized compounds contribute to the color of flax fabrics. Morin and quercetin (see Fig. 1) have been selected in this study as they are one of the most common and abundant flavonoids, as well as some of the most extensively studied. Rutin was also studied in previous work, but its use was disregarded due to the low change of color obtained after polymerization, which is the consequence of a very low oxidation rate [24].

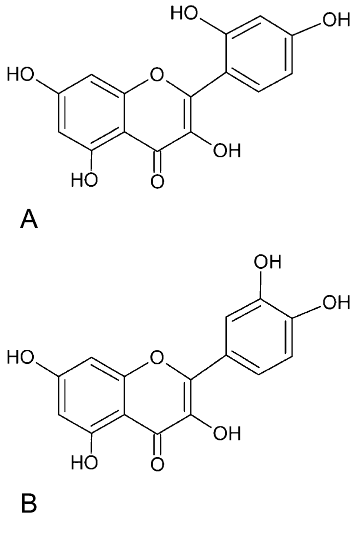

Figure 1. Chemical structure of flavonoids: A) morin; B) quercetin.

In this work, a new type of coloration system for cellulosic fibers was developed. The color of flax fibers was enhanced by enzymatic polymerization of morin and quercetin catalyzed by laccase from *Trametes hirsuta*.

2 Materials and Methods

2.1 Fabrics, Enzyme and Chemicals

100 % raw woven flax fabric having 14/14 yarns (warp/weft) cm^{-1} was used for coloration. Laccase (EC 1.10.3.2) from *Trametes hirsuta* (1000 U/mL, 693 mg/mL) was kindly obtained from Prof. G. Guebitz (Technical University of Graz). Morin and quercetin (high purity) were purchased from Aldrich (St. Louis, USA) and used as phenolic substrates without further purification. Lutensol AT25 was kindly supplied from BASF (Ludwigshafen, Germany). All other chemicals used were also commercially purchased at analytical grade from Aldrich or Sigma and used without further purification.

2.2 Fabric Preparation

The flax fabric samples (each one with 2.5 g) were scoured with a 1 % NaOH and 0.2 % Lutensol AT 25 (non-ionic surfactant) solution in a liquor to fabric ratio of 20:1, using an AHIBA Spectradye dyeing apparatus from Datacolor International (Lucerne, Switzerland) at 98 °C and 45 rpm for 1 h. The alkaline treated fabrics were neutralized with a 0.1 % acetic acid solution at 80 °C for 10 minutes. Some of the fabric samples were then bleached using 5 % hydrogen peroxide, 1 % commercial sodium hydroxide, 3 % sodium silicate and 2 % Lutensol AT 25 (w/w of fabric) in a liquor to fabric ratio of 20:1, at 90 °C for 1 h using the AHIBA apparatus.

2.3 Flax Fabrics Coloration

The colorations were fundamentally processed using two different approaches: a one-step method and a two-step method.

The one-step method included the simultaneous performance of polymerization and coloration. The reaction solutions were prepared with 10 mM of flavonoids (morin or quercetin), 10 % methanol and 2 U/mL laccase in 0.1 M Na-acetate buffer (pH 5.0). Flax fabric (just scoured or scoured and bleached) samples were immersed in the reaction pots before starting the incubation, in a liquor to fabric ratio of 20:1. All assays were performed at 50 °C (optimum temperature of laccase activity) with an agitation of 40 rpm for 24 h using a Rota Wash MK II machine (SDL International Ltd., Stockport, UK). The laccase activity was measured after 1 h, 2 h, 3 h, 5 h, and 24 h.

In the two-step method, the polymerization and coloration were performed separately under different conditions. The polymerization consisted of an incubation of the flavonoid compounds with laccase at 50 °C and an agitation of 80 rpm for 24 h in a shaker water bath using 10 mM flavonoid solutions with 10 % methanol and 2 U/mL laccase in 0.1 M Na-acetate buffer (pH 5.0). The new polymers obtained by the incubation of the flavonoids with the enzyme were used to perform the coloration of the flax fabrics (just scoured and scoured and bleached fabrics). The flax fabrics were immersed in the 24-h incubated solutions with a liquor to fabric ratio of 20:1, at 50 °C, 80 °C, and 90 °C for 2 h using an Ahiba spectradye-Datacolor dyeing machine at 40 rpm. In order to optimize the reaction conditions, the experiments were also performed at 90 °C using different coloration times (10 min, 30 min, 1 h, and 2 h). Finally, the coloration was carried out in parallel in the presence of salt (5 % NaCl), in order to improve the polymer absorption into the flax samples.

After completing the one– or two-step coloration process, fabric samples were thoroughly washed in 0.1 % Lutensol AT 25 solution at 98 °C for 10 min using an Ahiba spectradye-Datacolor dyeing machine in order to eliminate any residual protein or non-reacted products as well as any unfixed dyestuff [25]. The color strength of the fabrics was analyzed by the measurement of color before and after the coloration and washing treatment. As a control, the flavonoid reaction solutions were incubated under the same conditions in the absence of laccase.

2.4 Measurements of Laccase Activity

Standard assays of laccase activity were performed spectrophotometrically by measuring the enzymatic oxidation of 0.5 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) at 420 nm [22]. The molar extinction coefficient for the oxidation product was 3.6×10^4 cm^{-1} M^{-1} [26]. One unit of laccase (U) was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute.

2.5 Evaluation of Color Strength

The color strength of colorized flax fabrics was evaluated using the Color Spectraflash 600 Spectrophotometer (Datacolor, Lucerne, Switzerland) with the standard illuminant D 65. The data was expressed as K/S values calculated by the Kubelka-Munk equation (Eq. 1).

$$K/S = (1 - R)^2/2R \qquad \text{(Eq. 1)}$$

where K is the absorbance coefficient, S is the scattering coefficient and R is the reflectance ratio. $(K/S)_0$ is the color of untreated flax fabrics; $(K/S)_1$ represents the color of the colorized flax fabrics before the washing process with Lutensol; $(K/S)_2$ is the color of the fabrics after the washing process. Before measuring K/S, the samples were dried overnight at room temperature.

2.6 Color Resistance Test

The color resistance test consisted of three sequential washing steps that followed the Standard test method for color fastness to domestic and commercial laundering [27]. Each of the steps was performed at 60 °C for 30 min by using two different detergents: a standard detergent ISO 105-$C0_6$ and a commercial detergent (Skip, Unilevel) containing a bleaching agent based on oxidation-active compounds. In the pots, 25 standard steel balls were included in order to increase the mechanical stress. After each step, the fabric samples were rinsed with tap water and dried overnight at 40 °C. Finally, K/S measurement was performed after each washing process.

2.7 Fourier Transformed Infrared Spectroscopy (FT-IR)

An Avatar System 360 FT-IR spectrophotometer (Nicolet, Waltham, MA, USA) using an attenuated total reflectance accessory (ATR) was employed to record the FTIR spectra of control and treated flax fabrics. The HATR-FTIR consists of a composite ZnSe-diamond crystal that allows a collection of the FTIR spectra directly from the sample without any special preparation [28]. The IR spectra were collected at a spectrum resolution of 8 cm^{-1}, with 60 times of scanning, over the range of 800–4000 cm^{-1} at room temperature. A background scan with no samples and no pressure was acquired before collecting the spectra of the samples.

3 Results and Discussion

3.1 Optimization of Coloration Conditions

Flavonoids require the presence of an organic solvent to improve their solubility in aqueous medium. The resulting mixture, however, could cause the inactivation of the enzyme. Before starting the coloration experiments, it was necessary to investigate the stability of the laccase in buffer solution (sodium acetate 0.1 M, pH 5) in the presence of 10 % methanol. Under these conditions, the activity of laccase was two-fold lower than that obtained in a single buffer solution (sodium acetate 0.1 M, pH 5), indicating that the organic solvent influences the activity of the enzyme negatively. Furthermore, a slight decrease in activity was observed after 24 h of incubation in the solution with methanol (data not shown). In previous studies it was shown that despite the decrease of laccase activity in the presence of methanol, the color depth of fabrics was not excessively affected, showing K/S values similar to the ones obtained in the absence of methanol [24]. Therefore, in order to obtain homogeneous reaction solutions with laccase, flavonoid compounds were diluted in 10 % of methanol and used for flax fabrics coloration.

The optimum reaction conditions for flax fabrics coloration were determined in subsequent experiments by means of comparing the initial K/S values to those obtained after the coloration and washing process (K/S_2). When the one-step method was used to colorize the samples using morin, the measured K/S values were higher than those obtained when the two-step method was applied at the same temperature (see Fig. 2). In the case of quercetin, the behavior of coloration was similar for both methods. An increase of temperature probably improves the absorbance of colored compounds, although it resulted in the inactivation of laccase. Therefore, only the non-enzymatic step of the two-step method was performed at increasing temperatures of 80 and 90 °C. The highest *K/S* values were observed when the coloration was performed at 90 °C (see Fig. 2). The color strength showed a tendency to increase when the reaction temperature rises. High temperatures promote the fibers swelling, increasing the ability of the surface area to absorb the color compounds. Moreover, higher temperatures enhance the molecular vibrations both in fabrics and in the components of flavonoid solution, which induces the sorption kinetics favorably [10].

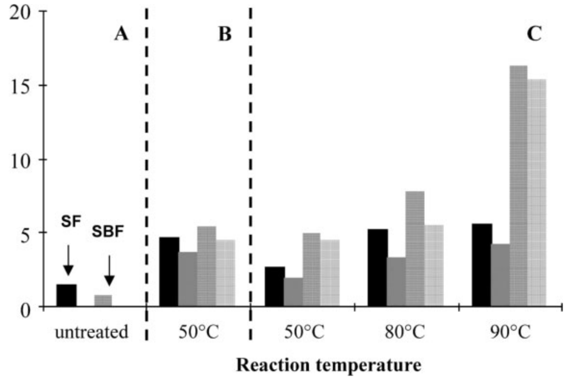

Figure 2. Color of initial flax fabrics (K/S_0) (A) and color strength of fabrics colorized by enzymatic polymerization of flavonoids (K/S_2): (B) one-step method (simultaneous polymerization and coloration) and (C) two-step method, consisting of a 24-h polymerization followed by a 2-h-coloration at different reaction temperatures. (SF) scoured fabric; (SBF) scoured and bleached fabric; (■) scoured fabric treated with morin; (▩) scoured and bleached fabric treated with morin; (▒) scoured fabric treated with quercetin; (░) scoured and bleached fabric treated with quercetin.

In conventional treatments of flax fibers, a bleaching process is performed in order to completely remove the natural color component of fabrics, causing an improvement in whiteness. However, previous work of our group showed that a scoured cotton having flavonoids on surface presented higher color absorption and better color fastness than the scoured and bleached cotton, and these results were proof that the bleaching process could be eliminated [24]. In Fig. 2, the scoured fabrics were observed to present a higher color strength when compared with the scoured and bleached ones. This may be due to the fact that the bleaching process eliminates any residual phenolic compound of fabrics which would mask the real color. Besides, when the reaction is performed in one step, i.e., when the enzyme and the fabric are in contact, the color strength is higher than the one obtained in the two-step method at the same temperature. This suggests that the flavonoids existing on the surface of flax fabrics react with the ones in the reaction solution positively to improve the coupling ability between the flavonoids on flax and in solution.

Comparing both oxidized flavonoid solutions for the two periods of incubation, the oxidized morin has less affinity towards flax fabrics than quercetin, displaying similar *K/S* values under any reaction conditions. On the other hand, the K/S values obtained for flax colorized fabrics via oxidized quercetin tend to increase notably when treated at high temperatures (90 °C). Both flavonoid compounds used in this work have a similar chemical structure differing only in the position of the hydroxyl groups in B-ring (see Fig. 1). In morin, the hydroxyl groups are placed in the ortho– and para-positions (3, 5, 8, 2', 4'), whereas in quercetin molecules, the hydroxyl groups are placed in the meta– and para-positions (3, 5, 8, 3', 4'). Taking this fact and the different *K/S* values obtained using morin and quercetin into consideration, it can be supposed that the steric hindrance phenomena resulting from the different positions of the functional groups influence the capacity of flavonoids to colorize flax fabrics. The substitution pattern can determine the reaction pathway of flavonoids [29], which explains the increase in color strength when the polymer (quercetin) with the functional groups in the meta– and para-positions was applied.

In order to study the effect of the reaction time on the coloration process in more detail, coloration times from 10 to 120 min were applied to the process at 90 °C (optimum reaction temperature). Fig. 3 shows that, by increasing the reaction time, the *K/S* values increased; after 1 h, and the color absorption reached a saturation point. With these results, the best conditions for the coloration of flax fabrics with oxidized flavonoids were decided to be 90 °C and 1 h (see Fig. 3).

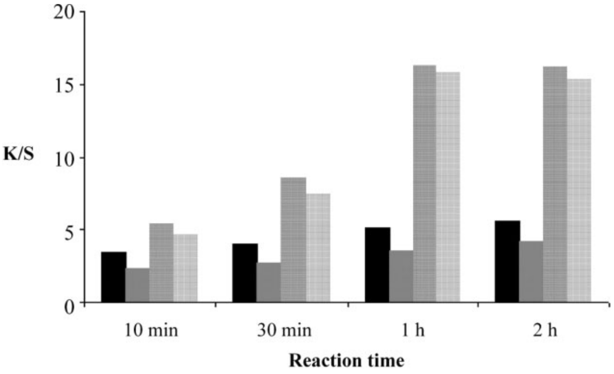

Figure 3. Color strength of fabrics (K/S_2) colorized by a two-step method consisting of a 24-h-polymerization followed by a coloration at 90 °C and different reaction times. (■) scoured fabric treated with morin; (▩) scoured and bleached fabric treated with morin; (▒) scoured fabric treated with quercetin; (░) scoured and bleached fabric treated with quercetin.

As mentioned in the experimental part, some coloration was performed in the presence of salt, in order to increase the polymer absorption onto the flax samples. The color strength of samples, treated in the presence of salt, was evaluated. The data are represented as a dye fixation ratio obtained using Eq. 2 and are shown in Tab. 1.

$$T_{K/S}[\%] = \frac{(K/S)_2}{(K/S)_1} \cdot 100 \qquad \text{(Eq. 2)}$$

where $(K/S)_1$ and $(K/S)_2$ are the color strength values of colorized samples before and after extraction (washing at boiling temperature, for 10 minutes using the dyeing apparatus), respectively [30].

The presence of NaCl in the coloration medium increased the values of $(K/S)_1$ and $(K/S)_2$ for both morin and quercetin. Moreover, the flax fibers colorized with flavonoids in the presence of NaCl showed a higher fixation ratio, with the exception of the scoured fabrics colorized with morin, which resulted in a similar fixation. After washing and rinsing processes, the morin colorized fabrics showed lower values of dye fixation when compared with the quercetin ones. Actually, for morin, more than 50 % of the colorizing compounds vanished from the fabric surface when the washing was performed. Nevertheless, the samples colorized using quercetin presented a great dye fixation ratio (> 80 %). Comparing the scoured and the scoured and bleached fabrics for both polymers it can be observed that the scoured fabrics present a higher color resistance after washing and rinsing $(K/S)_2$.

The enzymatic polymerized phenolic compounds tend to have a characteristic dark brown color due to the formation of conjugates along the main chain. In this work, the incubated flavonoid solutions also showed a color change from yellow or light brown to dark brown produced by the oxidative polymerization reaction catalyzed by laccase. In the control experiments, the solutions of flavonoids were prepared without laccase and incubated and applied to dye flax fabrics. Only a very light natural color (brown for morin; yellow for quercetin) was observed during the incubation period of the control samples, and even flavonoid solutions did not present any color change (data not shown).

3.2 Color Resistance

In the enzymatic coloration of cellulosic fabrics, the dyestuffs produced by polymerization with laccase usually have a low affinity towards cellulose, showing no covalent fixation on the fabrics [25]. In this work, the samples with most coloration (i.e. those treated in the presence of NaCl) were washed at boiling temperature and then treated with detergents three sequential times (see Fig. 4), in order to analyze the resistance of the binding between the colored compounds and fabrics. Some steel balls were added to the pots to increase the mechanical stress (beating effects) on fabrics. The mechanical stress would improve the formation of microfibrils at the surface of flax fabrics reducing the colorized area by morin and quercetin [24]. The disappearance of the color through the washing process was remarkable, especially after the first washing step. This was because the flavonoid compounds did not deeply penetrate through the fabrics. The flavonoid molecules that were not covalently bound to the cellulose structures of the flax fabrics disappeared into the washing solution by mechanical stress.

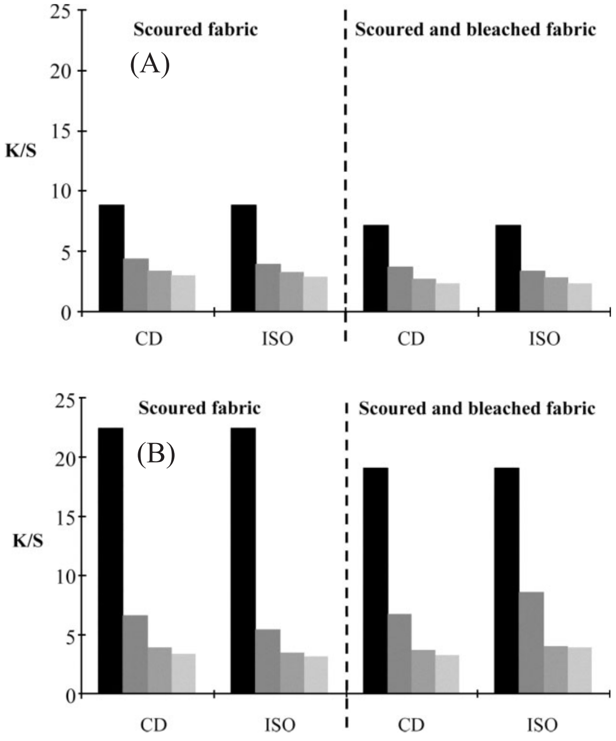

Figure 4. Color strength (K/S_2) of flax fabrics colorized in the presence of 5 % NaCl by enzymatic polymerization of flavonoids: (A) morin; (B) quercetin; before and after washing processes with a (CD) commercial detergent; (ISO) standard detergent. (■) *K/S* after coloration and extraction; (▓) *K/S* after the first washing process; (▒) *K/S* after the second washing process; (░) *K/S* after the third washing process.

Table 1. Dye fixation ratio $(T_{K/S})$ of colorized flax fabrics at 90 °C and 2 h, treated with or without 5 % NaCl: (S) scoured fabric; (SB) scoured and bleached fabric; $(K/S)_1$ color strength before the washing process; $(K/S)_2$ color strength after washing process.

	Morin				Quercetin			
	S		SB		S		SB	
	–	NaCl	–	NaCl	–	NaCl	–	NaCl
$(K/S)_1$	12.5	20.7	14.9	17.5	19.9	24.3	19.0	21.4
$(K/S)_2$	5.6	8.8	4.2	7.1	16.2	22.4	15.3	19.0
$T_{K/S}$ [%]	45	43	28	41	82	92	81	89

The influence of both detergents, commercial and standard, on color removal is similar. A commercial detergent with active compounds was used expecting an improvement of the color strength due to the oxidation of the fixed flavonoid compounds on the surface of flax. However, the effect of the mechanical stress induced in the experiments was stronger than the effect of these oxidative compounds, producing the removal of the colorized compounds from the treated fabrics.

3.3 FT-IR Analysis

All the fabric samples treated with the new polymeric compounds, as well as raw samples and controls were analyzed by FT-IR (Fourier Transformed Infrared) spectroscopy, in order to characterize the new phenolic polymeric structures. Comparing the results with the controls, several new bands appeared in the samples colorized with oxidized flavonoids (in Fig. 5 only the range between 1200–1800 cm^{-1} is shown). It can be assumed that the bonds observed at wavenumbers higher than 3750 cm^{-1} are attributed to the aromatic rings in phenolic compounds existing in flavonoids [31, 32]. The several absorption bands observed at wavenumbers higher than 3200 cm^{-1} (3300, 3690, 3750, 3890 cm^{-1}) are due to the vibration of the O-H linkage of phenolic and hydroxyl groups [28, 31, 32]. Among them, the band obtained at 3300 cm^{-1} is also found in the untreated flax fabric, confirming that flax fabrics naturally include phenolic compounds in their structure. The band at 2360 cm^{-1} observed in the samples after the treatment with flavonoids belongs to the stretching asymmetric and symmetric vibration of CO_2 and is attributed to the benzene ring [33]. The 1735 cm^{-1} band is attributed to the C = O ester absorption and can be originated by the oxidation reaction of flavonoids. The flavonoids have the capacity of antioxidants by free radical scavenging and the OH group of the B-ring, possessing electron donating properties and being a radical target, formed phenolic radicals (FIO’) [34]. The formed radical may react with a second radical to form a quinone, which may be further spontaneously polymerized [34].

The shift of the band from 1640 to 1650 cm^{-1} was also commonly discovered in flavonoids colorized samples.

4 Conclusions

In this study, flavonoids were easily polymerized by laccases, forming intermediate quinones, which spontaneously react to give colored compounds. These new compounds were very useful for fabric dyeing processes, as they provide a strong color and high absorbance to flax fibers. The best results were obtained in two sequential steps, at high temperature and in the presence of salt, which improves the polymer absorption onto the flax fibers. A high coloration and resistance against the washing process was obtained when the fabrics were only scoured, rather than the results obtained after scouring and bleaching processes, demonstrating that the natural flavonoids in flax have a positive effect, and may function like grafting points to which the quinones formed by laccase oxidation could bind. Therefore, the use of the biological synthesized flavonoids allows eliminating the bleaching stage from the traditional textile process, and consequently, to improve the fabric quality, as bleaching processes usually cause damage to the fibers via radical reactions. This study results in a more economical and environmentally friendly process, as the flax coloration using laccase can be performed under milder conditions such as neutral pH, short periods of incubation and less water and chemical consumption.

To obtain dark brown colors, flax fabrics coloration can be performed using quercetin flavonoid. Compared to morin, the quercetin flavonoid is a proper colorant to apply to flax fabrics, as these compound demonstrated acceptable values of color strength. However, resistance tests indicated that no covalent binding occurs between the fiber and the oxidized compounds, resulting in a poor affinity and a high removal of color during the washing processes, especially when the fibers are subjected to high mechanical stress. Future work should be focused on the application of a crosslinker agent between the cellulose structures and oxidized flavonoids, with the aim to

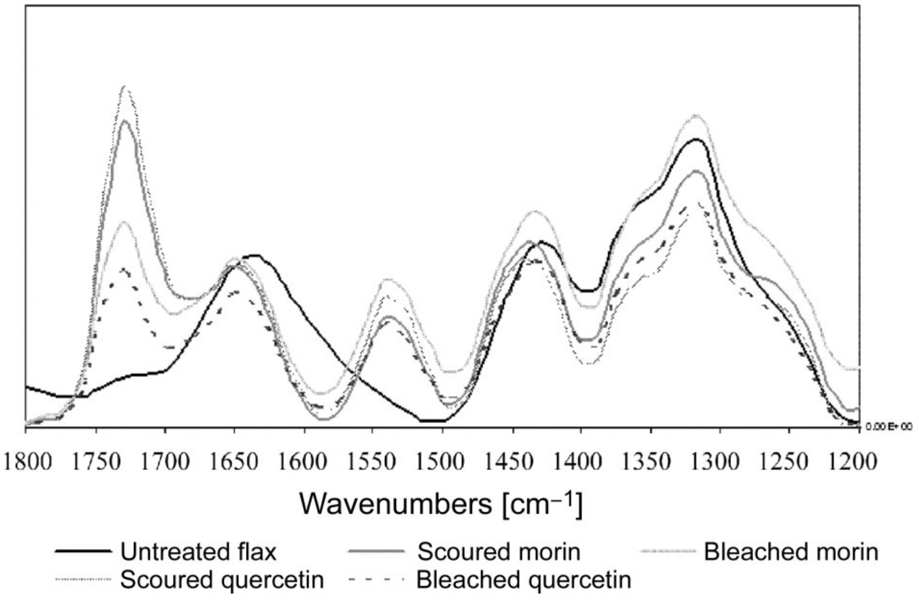

Figure 5. FT-IR analysis of flax fabrics colorized with morin and quercetin: 1200–1800 cm^{-1}.

attain higher amounts of oxidized flavonoids on the fabrics and the desirable washing fastness values.

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