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Michele Di Foggia,[1] Carla Boga,[2] Gabriele Micheletti,[2] Benedetta Nocentini,[2] Paola Taddei[1*]

[1] Department of Biomedical and Neuromotor Sciences, Alma Mater Studiorum – Università di Bologna, Via Belmeloro 8/2, 40126 Bologna, Italy

[2] Department of Industrial Chemistry 'Toso Montanari', Alma Mater Studiorum – Università di Bologna, Viale Del Risorgimento 4, 40136 Bologna, Italy

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* Corresponding author:

Prof. Paola Taddei

Department of Biomedical and Neuromotor Sciences

Alma Mater Studiorum – Università di Bologna

Via Belmeloro 8/2

40126 Bologna

Italy

e-mail: paola.taddei@unibo.it

phone: +39 051 2094280

Abstract

Many restoring formulations for damaged hair keratin have been developed. Some patents claim that the hair repair occurs through the reconstruction of disulfide bridges of keratin, through α,β-unsaturated Michael acceptors, such as shikimic acid and bis-aminopropyl diglycol dimaleate. To gain more insights into the possible repairing mechanism, this study is aimed at assessing, by IR and Raman spectroscopies coupled to scanning electron microscopy (SEM), the structural changes induced in keratin from bleached hair by the treatment with commercial reconstructive agents as well as shikimic acid and dimethyl maleate, chosen as model compounds. Vibrational spectroscopy revealed that shikimic acid- and maleate-based restoring agents interacted with hair fibers modifying both their cortex and cuticle regions. None of the investigated treatments induced an increase in the S-S disulfide bridges content of the hair cortex, although it cannot be excluded that this phenomenon could have occurred in the cuticle. S-S rearrangements were found to occur. None of our results can be interpreted as direct evidence of the sulfa-Michael reaction/cross-linking. From a morphological point of view, beneficial effects of the restoring agents were observed by SEM analyses, in terms of a more regular hair surface and more imbricated scales.

Keywords: keratin; Raman spectroscopy; IR spectroscopy; disulfide bridges.

1. INTRODUCTION

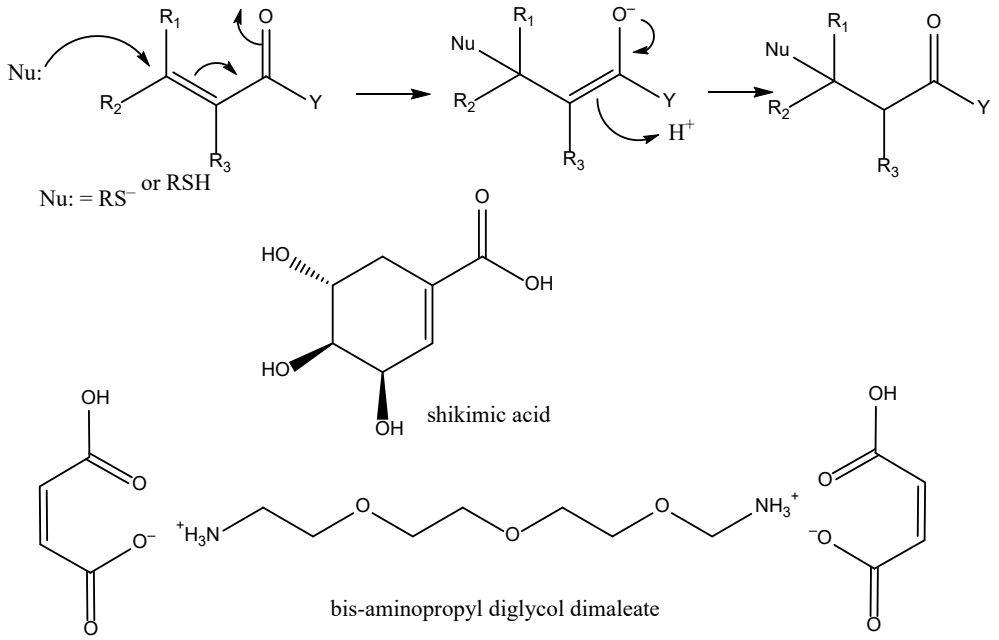
Depending on its moisture content (up to 32% by weight), a human hair is made up of approximately 65% to 95% keratin, as well as water, lipids, minerals and pigments. The two primary morphological tissues of hair are the cuticle and the cortex. The former is composed of imbricated plate-like cells at the outermost layer of hair (with unordered conformations and β-sheet as the prevailing structures), whereas the cortex is composed of aggregates of spindle-shaped cortical cells (with prevailing α-helix conformation) and contains melanin pigments [1-3].

The reaction of oxidative decolorization of melanin pigments, called "bleaching", is usually the first step in hair coloring, a technique widely used in the cosmetic industry and in hairdressers' salons.

In the first step of the process, a bleaching base is commonly used to provide a source of bleach and to obtain a lighter blonde shade. In general, bleaching creams contain persulfates as sodium (or potassium) and ammonium salts. The latter are very active salts because, once dissolved in an alkaline solution, they produce ammonia, which is easily absorbed by the hair, allowing it to swell. Among other components of the bleaching base, there are sodium metasilicate, heavy metal sequestrants, anti-caking agents (*e.g.,* silica), thickening agents (*e.g.,* long-chain alcohols), self-emulsifying waxes, fatty acids, cationic surfactants and quaternary polymers. Immediately before use, to destroy the melanin pigments, the bleaching cream must be mixed with an oxidizing emulsion, usually an aqueous solution of hydrogen peroxide in a concentration-dependent on the desired level of hair color removal.

Due to the use of the above-cited compounds, bleaching is considered one of the hardest treatments on hair bonds, besides over-curling and straightening [4]. It may cause physical changes of fibers [5,6] and deterioration of mechanical properties, due to the cleavage of disulfide bonds of cystines and, in case of severe bleaching, degradation of amino acid residues until to the breaking of bonds of peptide chains [6]. Moreover, during the bleaching treatment, the formation of by-products such as sulfenic, sulfonic, and cysteic acids can also occur [7,8]. Differently from the skin, which regenerates to recover from minor damage, hair, unfortunately, is not able to repair itself. For this reason, many restoring formulations for damaged hair have been developed and patented by cosmetic

manufacturers. Some patents claim that the hair repair occurs through the reconstruction of disulfide bridges, owing to the presence in the formulation of molecules reactive towards thiols [9-13]. In many formulations, great importance is devoted to the presence of α,β-unsaturated systems able to act as Michael acceptors, mainly α,β-unsaturated carbonyl- or carboxyl- compounds. Actually, the thia-Michael addition reaction (Scheme 1) between thiols or thiolate groups, derived from the residual presence of cysteine SH groups after the bleaching (or straightening, curling, or other hair treatments) is an easily occurring reaction [14] that, owing to the nucleophilicity of the sulfide group, is also classified as a click-chemistry reaction [15]. A plethora of Michael acceptors has been claimed in the patent literature as suitable to bind thiol residues in restructuring formulations [9-13]. Among them, many maleic acid derivatives have been proposed. In particular, bis-aminopropyl diglycol dimaleate (Scheme 1) is a component of a commercial hair restoring formulation [11] and shikimic acid (Scheme 1) is claimed as an active ingredient of the hair restoring formulation of a second commercial formulation [13].



Scheme 1. Top: Thia-Michael addition mechanism. Bottom: currently considered examples of active ingredients in formulations for the restoration of damaged hair.

According to the patent literature [9-13], the formulations provide long-lasting moisturized feel and smooth feel without leaving the hair greasy, improved appearance (e.g., sheen), increased dry strength (tensile strength), ease of combing the hair when wet or dried, less hair breakage, and decreased frizz. To gain more insights into this subject and to investigate the possible repairing mechanism of damaged human hair, the present study is aimed at assessing the structural changes induced in keratin from bleached hair by the treatment with commercial reconstructive agents, as well as with shikimic acid and dimethyl maleate, chosen as model compounds to avoid matrix interferences. To this purpose, hair samples were characterized by vibrational Attenuated Total Reflectance (ATR)/Fourier Transform (FT)-IR and FT-Raman spectroscopies coupled to scanning electron microscopy (SEM). The advantage of the chosen vibrational techniques is that they are non-destructive and do not require any sample manipulation. They are complementary since they provide different pieces of information: ATR/IR spectroscopy was used to gain insights into the hair cuticle, while FT-Raman spectroscopy is more sensitive to the sample bulk (i.e., the hair cortex). Both techniques were used to assess the possible changes in secondary structure induced by the treatments in the different sample areas. Besides, Raman spectroscopy appeared particularly useful since it provides information on the S-S disulfide bridges content and their conformation, through the Raman active S-S stretching mode.
To investigate the influence of the bleaching degree, the restoring agents and the model compounds were applied to hair bleached three and four times. The bleached fibers were analyzed as starting control samples.

2. EXPERIMENTAL

2.1. Materials

Lunex system Ultra Cream, Lunex system Restore (hereinafter indicated as BCC), Uni.Color OXI 40, and Actyva Colore Brillante Shampoo were produced by Kemøn S.p.A (Perugia, Italy). OLAPLEX® Hair Perfector™ N°3 (Santa Barbara, CA, USA), hereinafter indicated as Olaplex, and brown human hair were kindly provided by B. Nocentini. The ingredients of the above commercial

products are reported in Supplementary Material. Shikimic acid, dimethyl maleate, and methanol were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Preparation of the samples for vibrational and SEM analyses

Each sample of human hair used for Raman, ATR/FT-IR and SEM analyses was a lock of hair of about 12 cm length and 0.3 cm diameter.

A lock of a brown human hair (10 g) was immersed for 45 min at 35 °C in a 1:1 mixture of Lunex Ultra Cream and Uni.Color Oxi, then washed with Actyva Colore Brillante Shampoo, rinsed with water and dried with a hairdryer. This bleaching treatment was repeated for three consecutive times. The use, as starting material, of a hair sample that has undergone three consecutive bleaching treatments is in line with the approach used in hairdresser saloons and with the treatment described in patent literature [12]. Actually, the three-time bleaching assures the obtainment of a homogeneous decoloration grade, independently of the nuance of the starting lock. An image of the lock before and after the three bleaching treatments has been included in Supplementary material (Fig. S1).

This lock, after the above-cited three bleaching treatments, was divided into samples of ~0.35 g. Two of them were treated at 25 °C for 45 min with the restoring agents (BCC or Olaplex), whereas the other two were immersed at 25 °C for 45 min in 0.05 M methanolic solution of the model compound (shikimic acid or dimethyl maleate). The use of methanol was chosen to favor the solubility of the model compounds since they are poorly soluble in water.

Five locks bleached three times (~0.35 g each) were subjected to a further bleaching treatment for 15 min as above described, then treated with the same compounds (BCC, or Olaplex at 25 °C for 45 min, methanolic solution 0.05 M of shikimic acid or dimethyl maleate at 25 °C for 45 min) except one that was used as control and compared with the lock that underwent three bleaching treatments. The choice of reducing the time of the fourth bleaching to 15 min was due to the finding that, when it was carried out for 45 min, the fibers were not homogeneous and showed the presence of a high amount of hair deprived of cuticle and cortex (Fig. S2, Supplementary Material). Probably, this advanced deterioration could derive from a too long time of application of the bleaching mixture on samples

that have already previously undergone three bleaching treatments. For this reason, all the data reported below refer to a fourth bleaching treatment that lasted 15 min.

2.3. Vibrational spectra

Raman spectra were recorded in triplicate by using a Bruker MultiRam FT-Raman spectrometer equipped with a cooled Ge-diode detector. The excitation source was a Nd^{3+}-YAG laser (1064 nm) in the backscattering (180°) configuration. The focused laser beam diameter was about 100 μm, the spectral resolution 4 cm^{-1}, the laser power at the sample about 80 mW. The number of scans was 5000 for each spectrum.

The relative contents of disulfide bridges and cysteic acid (as sulfonate salt, $R\text{-}SO_3^-$) were evaluated through the $A_{s\text{-}s}/A_{1450}$ and A_{1040}/A_{1450}, where $A_{s\text{-}s}$, A_{1040} and A_{1450} were the areas of the bands assignable to disulfide bridges (calculated drawing a baseline between 482 and 585 cm^{-1}), to cysteic acid at about 1040 cm^{-1} [16,17] (calculated drawing a baseline between 1070 and 1020 cm^{-1}) and to CH_2 bending at 1450 cm^{-1} (calculated drawing a baseline between 1500 and 1375 cm^{-1}), chosen as an internal standard [18,19].

The 580-470 cm^{-1} spectral range was analyzed by a curve-fitting procedure to evaluate the conformation of the $C_\alpha\text{-}C_\beta\text{-}S\text{-}S\text{-}C_\beta\text{-}C_\alpha$ linkage in cystine disulfide bridges [20-22]. A linear correction in the above mentioned spectral range brought the baseline of the Raman spectra to approximately zero intensity. The frequencies of the band centers found in the fourth-derivative spectra (obtained with 13-point smoothing) were used as starting parameters for the curve-fitting procedure. The curve-fitting analysis was performed using the OPUS version 6.5 program, which uses the Levenberg–Marquardt algorithm. The Raman component profiles were described as a linear combination of Lorentzian and Gaussian functions. The contents of strained, *gauche-gauche-gauche*, *gauche-gauche-trans* and *trans-gauche-trans* $C_\alpha\text{-}C_\beta\text{-}S\text{-}S\text{-}C_\beta\text{-}C_\alpha$ conformations were determined from the areas of the bands at about 495, 505, 520, and 540 cm^{-1}, respectively [20-22]. The content of each conformation was calculated from the area of the individually assigned bands and expressed as a fraction of the total area of the above-mentioned bands.

IR spectra were recorded in triplicate on a Bruker Alpha Fourier Transform FTIR spectrometer, equipped with a Platinum Attenuated Total Reflectance (ATR) single reflection diamond module (penetration depth 2 μm) and a Deuterated Lanthanum α-Alanine doped TriGlycine Sulfate (DLaTGS) detector; the spectral resolution was 4 cm^{-1}, and the number of scans was 64 for each spectrum.

The relative content of cysteic acid (as sulfonate salt) was evaluated through the $I_{1040}/I_{Amide\ I}$ and $I_{1175}/I_{Amide\ I}$ ratios, where $I_{1040,}$ $I_{1175,}$ and $I_{Amide\ I}$ were the absorbances (measured as peak heights) of the cysteic acid bands at about 1040 and 1175 cm^{-1} [19,22,23] (calculated drawing a baseline between 1330 and 946 cm^{-1}) and Amide I, used as an internal standard [24,25] (calculated drawing a baseline between 1724 and 1348 cm^{-1}), respectively.

Due to their intrinsic orientation, the Raman and IR spectra were recorded by positioning the fibers along one specific direction. Average spectra were reported.

The Raman and ATR-IR spectroscopies were applied to the study of hair locks bleached three and four times before and after treatment with restructuring formulations (BCC or Olaplex) as well as with solutions of pure dimethyl maleate or shikimic acid, chosen as model compounds. The bleached samples were characterized as control samples; a more complete set of data, including comparison with starting brown hair, is reported elsewhere [26].

The Raman and ATR-IR techniques were used to gain complementary information on the composition of the fibres. In fact, the former is sensitive to the sample bulk (and thus to the cortical region of hair), the latter to the surface skin (and thus to the cuticle region). Actually, according to Kim et al. [27], the cuticle and whole diameter of hair are genetically different and vary in the 2.7-3.6 μm and 65-91μm ranges, respectively, with an average contribution of the cuticle to the overall hair of 2.1-2.2%. According to the results reported by Greve et al. [28], the NIR laser used in Raman spectroscopy penetrates several hundreds of microns, i.e. more than a single hair fiber. These data support the negligible contribution of the cuticle to the overall FT-Raman spectrum.

Statistical analysis on Raman and IR data was performed with R statistical software (version 3.5.3;

GNU GPL license). The data have a non-Gaussian distribution, so a non-parametric Kruskal–Wallis test was used for the statistical significance (set at $P < 0.05$), and a Dunn–Bonferroni post-hoc analysis has been performed for any dependent variable for which the Kruskal–Wallis test was significant. The Kruskal–Wallis test does not compare means but is based on ranks and was used to verify if the rank means are different. Nevertheless, we reported the data as average values with their associated standard deviation (SD) for better readability.

2.4. Scanning electron microscope (SEM) evaluation

The scanning electron microscopic evaluation of the surface morphology of samples of human hair was performed longitudinally with a Zeiss Evo 50-EP (Carl-Zeiss, Oberkochen, Germany). To minimize artefacts, sputtering was avoided, and the samples were observed in variable pressure (VP) mode. All measures were carried out at an accelerating voltage of 20 kV and 100 Pa in the chamber pressure. The signal revealed secondary electrons. For each sample, SEM images were recorded on the central region of the fiber belonging to the same lock; at least two analyses on two different and near regions along the same fiber were made.

3. RESULTS AND DISCUSSION

3.1. Vibrational analyses

3.1.1. Restoring agents and model compounds

Fig. 1 and 2 show the Raman (A) and IR (B) spectra of BCC and Olaplex together with their corresponding model compounds (i.e., shikimic acid and dimethyl maleate), respectively.

As can be easily seen, the IR spectra of the commercial formulations appeared broadened according to their character of aqueous solutions; this effect is obviously more pronounced in the BCC *versus* shikimic acid comparison because of the solid-state of the latter.

The spectra of the BCC formulation (Fig. 1) appeared more complex than those of the corresponding model compound, according to its character of mixture. Some bands appeared shifted in their

wavenumber positions compared to shikimic acid due to the interaction with water; in the spectra of BCC, the shikimic band at about 1680 cm^{-1}, assignable to the COOH stretching mode in both Raman and IR spectra, was substituted by bands at about 1600 and 1400 cm^{-1}, ascribable to the COO^- group [29,30] of shikimate, besides other carboxylate salts present in the formulation. Actually, at the pH value of BCC (i.e., 6.25), most shikimic acid (pK_a about 4.5) should be deprotonated. Several bands in both Raman and IR spectra of the BCC commercial formulation were ascribable to the model compounds, although some bands (indicated with a circle) should be assigned to other constituents. In particular, the IR band at about 1725 cm^{-1} of BCC could be assigned to the C=O stretching ester group of the esterified fatty acids present in the formulation. The strong Raman band at about 1650 cm^{-1} is due to the C=C stretching mode of shikimic acid, besides other unsaturated compounds present in the formulation.

With regards to the Olaplex formulation, its active ingredient is bis-aminopropyl diglycol dimaleate. Several bands present in the spectra of Olaplex may be assigned accordingly and are consistent with the presence of this compound. In particular, as detailed in Fig. 2, bands attributable to both the COOH and COO^- groups were detected together with modes assignable to NH_3^+, C-O, and C-C bonds [31,32]. The spectra of dimethyl maleate are consistent with those reported in the literature for maleate esters [33,34].

3.1.2. Treatment with shikimic acid

Fig. 3 and 4 show the Raman and IR spectra of the hair locks bleached three times and treated with BCC, respectively; the spectra of the corresponding restoring agents are reported for comparison. Bands assignments for keratin are reported in the literature [35]. Fig. S3 and S4, Supplementary Material, show the Raman and IR spectra of the hair locks bleached three times and treated with 0.05 M shikimic acid, respectively; the spectra of shikimic acid are reported for comparison.

More detailed data on the spectrum of the hair lock bleached three times are reported elsewhere [26]. Raman spectroscopy showed that upon treatment with BCC (Fig. 3), the bands at about 1395 and

1160 cm^{-1} strengthened. The former was above assigned to the COO^- group, the latter to BCC components other than shikimic acid. It is interesting to note that significant variations occurred in the S-S conformational distribution (Fig. 5A), although the $A_{s\text{-}s}/A_{1450}$ and A_{1040}/A_{1450} ratios did not change (Fig. S5A, Supplementary Material). In other words, the disulfide bridges and cysteic acid contents did not significantly vary upon BCC treatment, but substantial S-S rearrangements occurred. In fact, the fitting data (Fig. 5A) indicated that the *gauche-gauche-gauche* conformation significantly increased at the expenses of the strained and *gauche-gauche-trans* conformations.

Interestingly, also the Amide I range underwent significant changes, with a shift of its maximum from 1656 cm^{-1} (wavenumber position typical of α-helix [35] from the cortical region of hair [20,21]) to 1653 cm^{-1} and a weakening of the component at 1670 cm^{-1} (assignable to β-sheet [20,21]). However, the former change may be due to the incorporation of an unsaturated component belonging to BCC. The treatment with 0.05 M shikimic acid did not produce any significant changes in the Raman spectra (Fig. S3, Supplementary Material), and the same result was obtained by increasing the concentration of shikimic acid to 0.1 M (data not shown). The Raman $A_{s\text{-}s}/A_{1450}$ and A_{1040}/A_{1450} ratios did not significantly vary upon treatment with the model compound (Fig. S5A, Supplementary Material), and only slight changes in S-S conformational distribution were observed (Fig. 5A). These results show that the hair cortex (to which Raman spectroscopy is sensitive) is affected by the treatment with BCC, but negligibly by that with the model compound.

IR spectroscopy shows that the BCC restoring agent was also incorporated in the cuticle. In fact, the spectrum recorded on the hair lock bleached three times and treated with BCC (Fig. 4) showed some bands ascribable to BCC (indicated with an asterisk); some of them were definitely due to the BCC constituents other than shikimic acid (such as the 1725 cm^{-1} component assigned to the ester group), others (those in the 1150-1000 cm^{-1} range) may be ascribed to shikimic acid. It is interesting to note that the 1043 and 1175 cm^{-1} bands, both attributed to cysteic acid, and thus also the corresponding IR $I_{1040}/I_{Amide\ I}$ and $I_{1175}/I_{Amide\ I}$ ratios (Fig. S5B, Supplementary Material), had a significantly different trend upon treatment with BCC: the former remained nearly constant, while the latter significantly

decreased. This apparently strange behavior may be explained by considering that the 1043 cm^{-1} band had a contribution from BCC, while the 1175 cm^{-1} component did not. In confirmation, it may be observed that the correlation between the IR $I_{1040}/I_{Amide\ I}$ and $I_{1175}/I_{Amide\ I}$ ratios significantly improved by excluding the sample bleached three times and treated with BCC (Fig. S6, Supplementary Material). Therefore, for the latter sample, the $I_{1175}/I_{Amide\ I}$ ratio was the most reliable to evaluate the cysteic acid content of the cuticle; the obtained results showed that this component appeared to decrease upon the BCC treatment. The weakening of this band may be due to the loss of salt bridges involving the SO_3^- groups consequent to rearrangements in the fiber, revealed by the significant changes in the $I_{Amide\ I}/I_{Amide\ II}$ IR ratio ($I_{Amide\ I}/I_{Amide\ II}$ = 1.13 ± 0.01 and 1.20 ± 0.04 in the sample bleached three times and after treatment with BCC, respectively), as well as in the spectral profile and wavenumber position of Amide II and weakening of Amide III. It cannot be excluded that some groups belonging to the constituents of BCC (for example, the OH groups of shikimic acid) could have interacted via hydrogen bonds with the C=O, OH, and NH groups present in the fiber. Actually, it must be observed that also the 3500-3000 cm^{-1} range (where OH stretching, Amide A and B modes fall) was affected by the treatment with BCC (Fig. 4): a more prominent component at 3400 cm^{-1} appeared and Amide B, i.e., NH_3^+ stretching, shifted from 3066 to 3078 cm^{-1}, probably due to the loss of interactions with the SO_3^- groups.

Actually, due to the low pK_a value reported for cysteic acid (comprised between 0 and 1.3 [36,37]), several authors have reported that at pH noticeably lower than our treatments [38,39], sulfonic acid groups are ionized (i.e., SO_3^-) and internally compensated by the ionized basic groups. Evidently, in the presence of BCC, some interactions between these groups are lost.

The IR spectra of the sample treated with 0.05 M shikimic acid (Fig. S4, Supplementary Material) showed a spectral profile generally different if compared with the BCC treatment, as previously observed for the Raman spectra; a more defined band at 1022 cm^{-1} was detected, which would suggest the incorporation of the model compound. However, the expected strengthening in the 1050-1100 cm^{-1} range, above reported for the BCC treatment, was not observed. The bands at 1040 and 1175

cm^{-1} weakened, and the IR $I_{1040}/I_{Amide\ I}$ and $I_{1175}/I_{Amide\ I}$ ratios (Fig. S5B, Supplementary Material) decreased accordingly. The apparent decrease of the cysteic acid content, already observed for the BCC treatment, might be explained analogously as a loss of salt bridges involving the SO_3^- groups consequent to rearrangements in the fiber. Actually, some variations in the Amide I and II profiles and a weakening of Amide III (Fig. S4, Supplementary Material) were detected upon treatment with the model compound. In the 3500-3000 cm^{-1} range, similar changes were previously observed for the BCC treatment.

The possible occurrence of a sulfa-Michael reaction between free SH groups and shikimic acid, according to scheme 1, has been taken into account.

If the reaction had occurred, a weakening in the C=C stretching band would have been observed together with a strengthening in the 740-570 cm^{-1} C-S-C stretching range [30,31]. These changes are better detectable in the Raman spectra since, in the IR ones, these modes are weaker (in particular, the latter is hardly detectable [23]). Such behaviors were not clearly detected in our spectra, and only a slight strengthening near 670 cm^{-1} was observed (Fig. S7, Supplementary Material); on the other hand, the trend of the C=C stretching is not easily disclosable since this mode is overlapped to the Amide I maximum at about 1650 cm^{-1}, which was found to strengthen (Fig. 3).

Analogous BCC and shikimic acid treatments were also performed on the hair fibers bleached four times; the corresponding Raman and IR spectra are reported in Fig. S8-S9 and S10-S11, Supplementary Material, respectively. The spectral changes were less significant than for the samples bleached three times. The Raman spectrum of the hair lock bleached four times and treated with BCC (Fig. S8, Supplementary Material) showed minor variations in the Amide I range. The cysteic acid 1040 cm^{-1} band appeared to weaken, and the A_{1040}/A_{1450} ratio decreased accordingly (Fig. S12A, Supplementary Material). The $A_{S\text{-}S}/A_{1450}$ ratio did not change, while S-S rearrangements occurred; the fitting data (Fig. 5B) indicated that the *trans-gauche-trans* conformation increased at the expenses of the *gauche-gauche-trans* one. No significant changes were observed in the IR spectra (Fig. S10, Supplementary Material) as well as in the calculated $I_{1040}/I_{Amide\ I}$ and $I_{1175}/I_{Amide\ I}$ ratios (Fig. S12B,

Supplementary Material). The treatment with 0.05 M shikimic acid did not induce any significant variations either in the Raman (Fig. S9, Supplementary Material and Fig. 5B) or IR spectra (Fig. S11, Supplementary Material) or the corresponding calculated ratios (Fig. S12, Supplementary Material).

3.1.3. Treatment with maleate compounds

Fig. 6 and 7 show the Raman and IR spectra of the hair locks bleached three times and treated with Olaplex, respectively; the spectra of the restoring agent are reported for comparison. Fig. S13 and S14, Supplementary Material, show the Raman and IR spectra of the hair locks bleached three times and treated with 0.05 M dimethyl maleate, respectively; the spectra of dimethyl maleate are reported for comparison.

Olaplex as well revealed to be incorporated into both the cortex and cuticle; actually, both Raman (Fig. 6) and IR spectra (Fig. 7) revealed several bands ascribable to the restoring agent. The main Raman detected modes were C=O(OH), COO^- stretching, and NH_3^+ bending, besides CC and =CH wagging (Fig. 6), i.e., consistent with bis-aminopropyl diglycol dimaleate. The disulfide bridges and cysteic acid contents did not significantly change upon the treatment as well as the Raman $A_{s\text{-}s}/A_{1450}$ and A_{1040}/A_{1450} ratios (Fig. S5A, Supplementary Material). With regards to the disulfide bridges, the Raman spectral profile around 500 cm^{-1} showed that they underwent conformational rearrangements, with changes slightly different from those observed for the BCC treatment. The fitting data (Fig. 5A) indicated that the *gauche-gauche-gauche* conformation significantly increased at the expenses of the *trans-gauche-trans* conformation. Also in this case, the possible occurrence of a sulfa-Michael reaction between free SH groups and the active ingredient has been taken into account, according to Scheme 1.

The mechanism is analogous to that above reported for shikimic acid; the only difference lies in the possibility of bis-aminopropyl diglycol dimaleate to act as a cross-linking agent, being a difunctional reagent (whilst shikimic acid is monofunctional).

As above observed for shikimic acid, the trend of the Raman C=C stretching band is not easily disclosable due to the overlapping with the Amide I band; with regards to the 740-570 cm^{-1} C-S-C thioether stretching range, a slight strengthening was observed (Fig. S15, Supplementary Material). Some additional information may be inferred from the IR spectra (Fig. 7). Also in this case, the main IR detected bands are assignable to modes involving COO^-, $NH_3^{+,}$ and =CH groups, possibly belonging to bis-aminopropyl diglycol dimaleate. In particular, the Olaplex band at 866 cm^{-1}, assignable to =CH wagging, was still detected in the spectrum of the hair lock treated with the restoring agent, although significantly broadened; if all the active ingredient had reacted with the fiber through both the double bonds, this band should have disappeared. Therefore, this result may be interpreted in different ways. It could indicate (i) the only partial occurrence of the above-reported reaction; (ii) the lack of cross-linking. In other words, this finding could suggest that some molecules of the reagent acted as crosslinkers while others not, and a certain amount of the active reagent remained unreacted and incorporated into the fiber through interactions other than covalent bonds. Alternatively, the detection of the =CH wagging band could be interpreted by considering that bis-aminopropyl diglycol dimaleate behaved as a monofunctional reagent rather than a difunctional. As a third possible explanation, it must be stressed that the 866 cm^{-1} band could be due to other unsaturated compounds present in the formulation. IR spectroscopy showed that upon treatment with Olaplex, no significant changes in the cysteic acid content of the cuticle occurred, as revealed by the constancy of the $I_{1040}/I_{Amide\ I}$ and $I_{1175}/I_{Amide\ I}$ ratios (Fig. S5B, Supplementary Material).

The treatment with dimethyl maleate 0.05 M did not produce any significant changes either in the Raman (Fig. S13, Supplementary Material) or IR spectra (Fig. S14, Supplementary Material). The Raman $A_{s\text{-}s}/A_{1450}$ and A_{1040}/A_{1450} ratios (Fig. S9A, Supplementary Material), and the IR $I_{1040}/I_{Amide\ I}$ and $I_{1175}/I_{Amide\ I}$ ratios (Fig. S5B, Supplementary Material) did not significantly vary; only slight changes in S-S conformational distribution were observed (Fig. 5A). These results showed that the hair cortex and cuticle were negligibly affected by the treatment with the model compound. Therefore, the chosen model compound appeared less active towards hair fibers than Olaplex,

suggesting that the presence of components other than the active ingredient in the commercial formulation favors their penetration into the hair fibers.

Analogous treatments were carried out also on hair locks bleached four times; the corresponding Raman and IR spectra are reported in Fig. S16-S17 and S18-19, Supplementary Material, respectively. The spectral changes were negligible if compared with those observed for the samples bleached three times. Accordingly, no changes were observed either in the Raman $A_{s\text{-}s}/A_{1450}$ and A_{1040}/A_{1450}, and IR $I_{1040}/I_{Amide\ I}$ and $I_{1175}/I_{Amide\ I}$ ratios (Fig. S12, Supplementary Material) or S-S conformations (Fig. 5B).

3.2. SEM analyses

The SEM images of the hair fibers subjected to three bleaching treatments (Fig. 8, A1 and A2) show a damaged cuticle with the presence of opened hair scales. A similar situation was obtained after further bleaching for 15 min (Fig. S20A, Supplementary Material), confirming the findings of the vibrational study that, when the hair has undergone three bleaching treatments, the main effect of further bleaching occurs in the cortical region rather than in the cuticle [26]. The cuticular deterioration joined a stretching level when the fourth bleaching treatment was carried out for 45 min, as evidenced by Fig. S2, Supplementary Material, which shows noticeable abrasion effects, in agreement with literature data [40,41].

Fig. 8B shows SEM images of hair bleached three times and then treated with shikimic acid 0.05 M in methanol. The hair shows a homogeneous and relatively smooth surface, with the cuticles quite adherent to the hair shaft. In the case of Fig. 8C, the sample of bleached hair was treated with the commercial product BCC. Although a fair comparison should be performed on the same fiber in exactly the same region before and after the treatment, at first glance, the SEM images of the surface of the hair treated with BCC appears more regular compared to the untreated sample, with more imbricated scales.

Also, in the case of hair bleached three times and then treated with a methanolic solution of dimethylmaleate (Fig. 8D) or with Olaplex (Fig. 8E), the surface appears more regular compared to that of bleached hair not subjected to restoring treatment. It has to be noted that the restoring effect is showed even in knotted hair (Fig. 8F), which shows a satisfactory closure of the cuticle despite the tension to which it is subjected.

SEM images of hair bleached four times and treated with the commercial restoring agents and model compounds are reported in Fig. S20, Supplementary Material. In general, the hair surface after all the treatments shows more imbricated scales, even if in some cases the presence of knots, made to make the opening of the scales more evident, has caused, in a few isolated areas, an anomalous enlargement of the scales. Given that the treatment with the single component (shikimic acid or dimethyl maleate) produces a restoring effect similar to that of the whole commercial formulation, one can propose an indirect evidence of the beneficial effect on the hair surface of these components.

4. CONCLUSIONS

Vibrational spectroscopy revealed that shikimic acid- and maleate-based restoring agents interacted with hair fibers modifying both their cortex and cuticle regions. Their effects were found to depend on the bleaching degree of the hair since samples bleached three times appeared more modified than those bleached four times, which showed less significant/negligible changes.

At a molecular level, the commercial restoring agents appeared to be more active towards hair fibers than the chosen model compounds, and the incorporation of components other than the active shikimic acid compound was revealed for BCC. SEM analyses showed that restoring agents and model compounds have similar beneficial effects in terms of a more regular hair surface and more imbricated scales. Given that the treatment with the single component (shikimic acid or dimethyl maleate) produces a restoring effect similar to that of the whole commercial formulation, indirect evidence of the beneficial effect on the hair surface can be argued.

None of the investigated treatments induced an increase in the S-S disulfide bridges content, as revealed by Raman spectroscopy, although S-S rearrangements were found to occur; at this purpose, it must be stressed that this technique is sensitive to the hair cortex. Therefore, it cannot be excluded that this phenomenon could have occurred in the cuticle.

The decrease in the cysteic acid content observed in some samples (mainly upon treatment with BCC and shikimic acid) was explained as a sign of the loss of salt bridges involving the SO_3^- and NH_3^+ groups consequent to rearrangements in the fiber, which can also be due to weak interactions with the constituents of the restoring agents/model compound. However, it must be stressed that only slight structural changes were observed in keratin, whose main conformation remained α-helix.

None of our results can be interpreted as direct evidence of sulfa-Michael reaction/cross-linking: only a slight strengthening in the C-S-C stretching Raman range was detected. The broadening of the =CH wagging IR mode observed for the treatment with Olaplex cannot be univocally interpreted. Actually, it must be stressed that the different treatments were deliberately applied to bleached hair fibers, reproducing the conditions used by hairstylists. Raman and IR spectroscopy showed that under these conditions, the S-S disulfide bridges underwent oxidation to cysteic acid, and free SH thiol groups were not detected. Further studies are in plan to investigate the occurrence of the reaction under reducing conditions, to maximize the concentration of free SH thiol groups [42].

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FIGURES

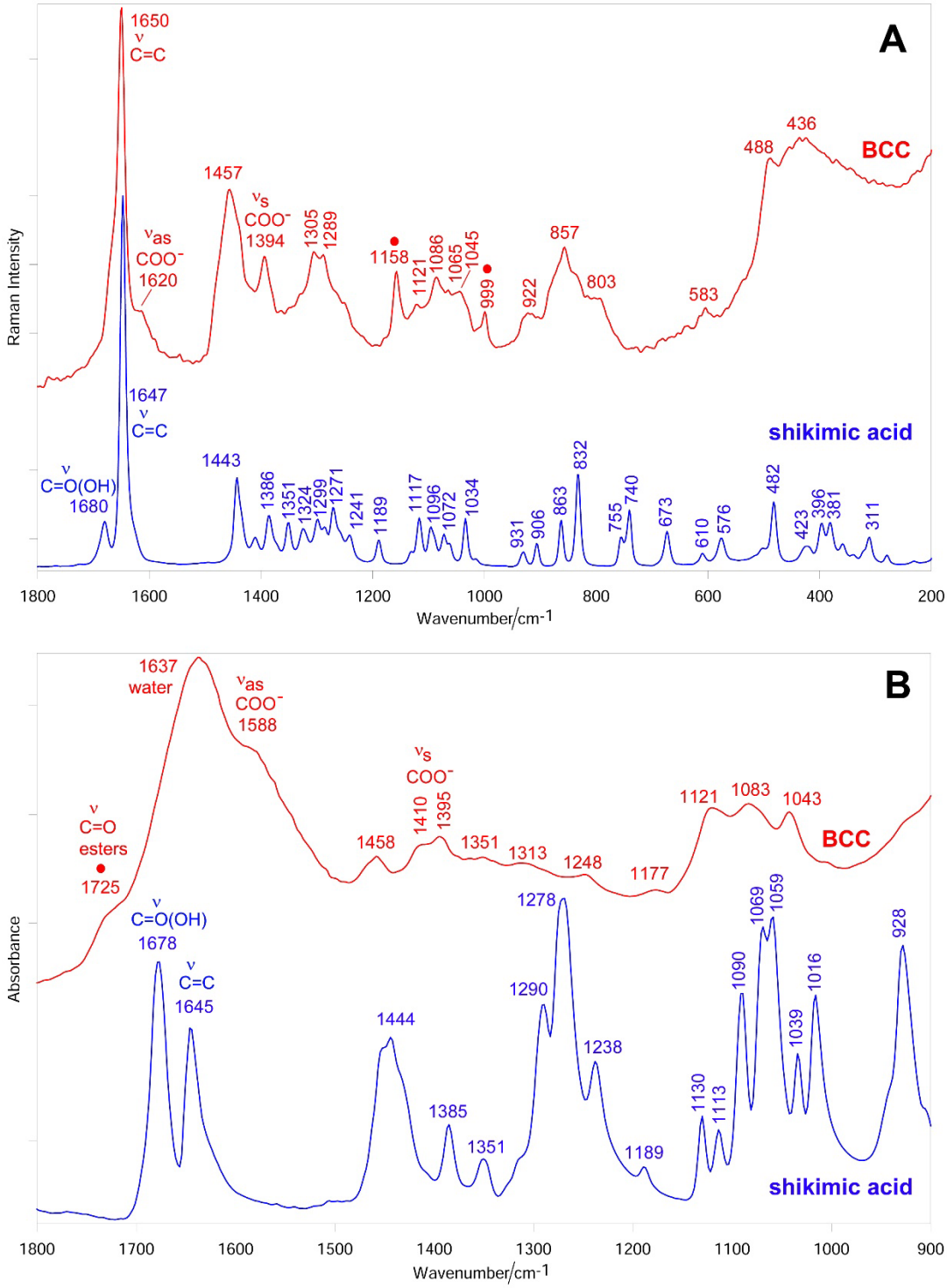

Figure 1. Raman (A) and IR (B) spectra of BCC and shikimic acid. The assignments of the main bands [29,30] are indicated (as = asymmetric; s = symmetric; ν = stretching) together with the components of BCC not detected in the spectrum of shikimic acid (circle symbol). IR spectra of BCC and shikimic acid. The IR band at 1637 cm^{-1} has a contribution from water.

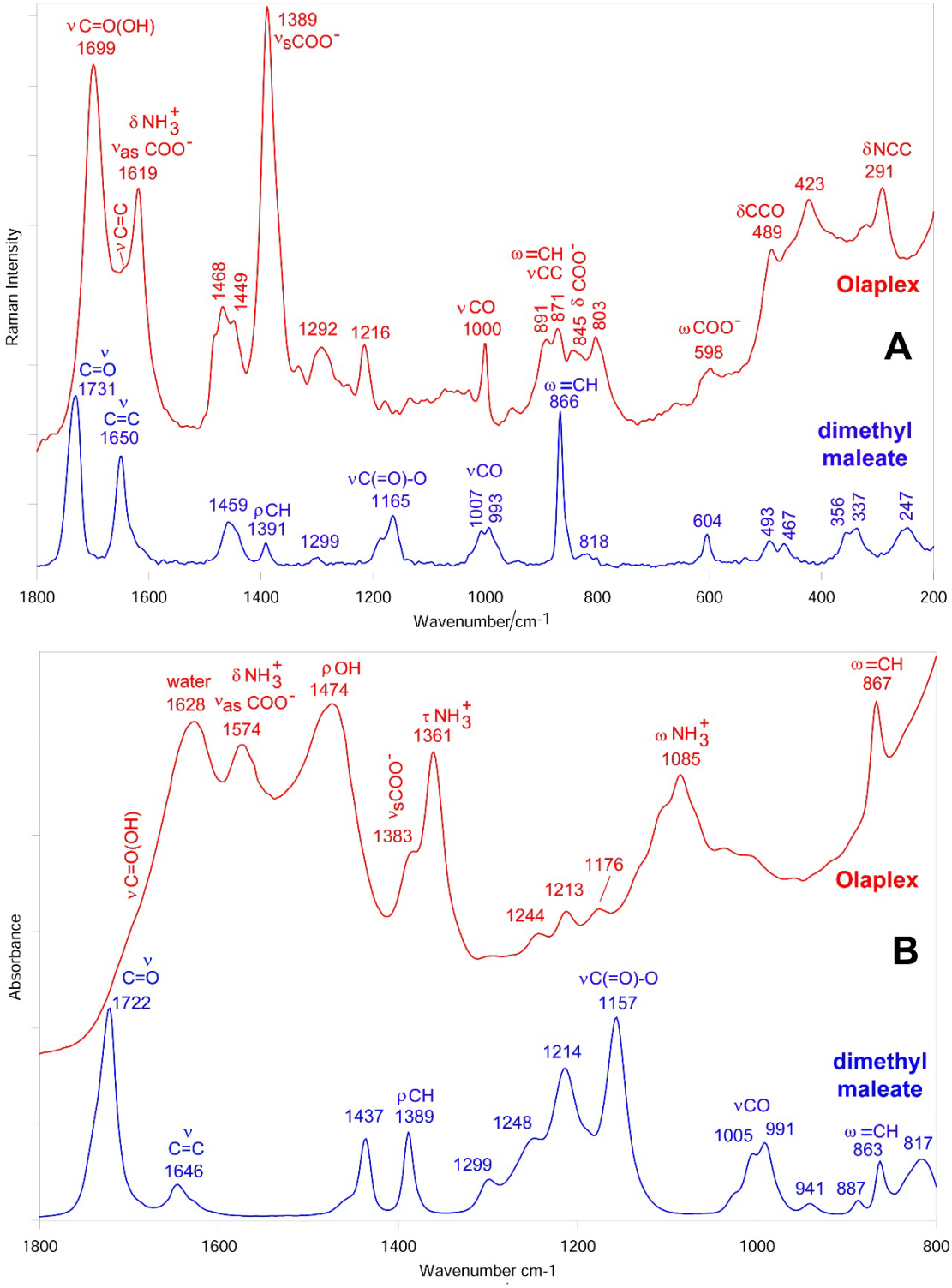

Figure 2. Raman (A) and IR (B) spectra of Olaplex and dimethyl maleate. The assignments of the main bands are indicated (as = asymmetric; s = symmetric; ν = stretching; δ = bending; ω = wagging; ρ = rocking; τ = twisting) according to the literature [29-34]. The IR band at 1628 cm^{-1} has a contribution from water.

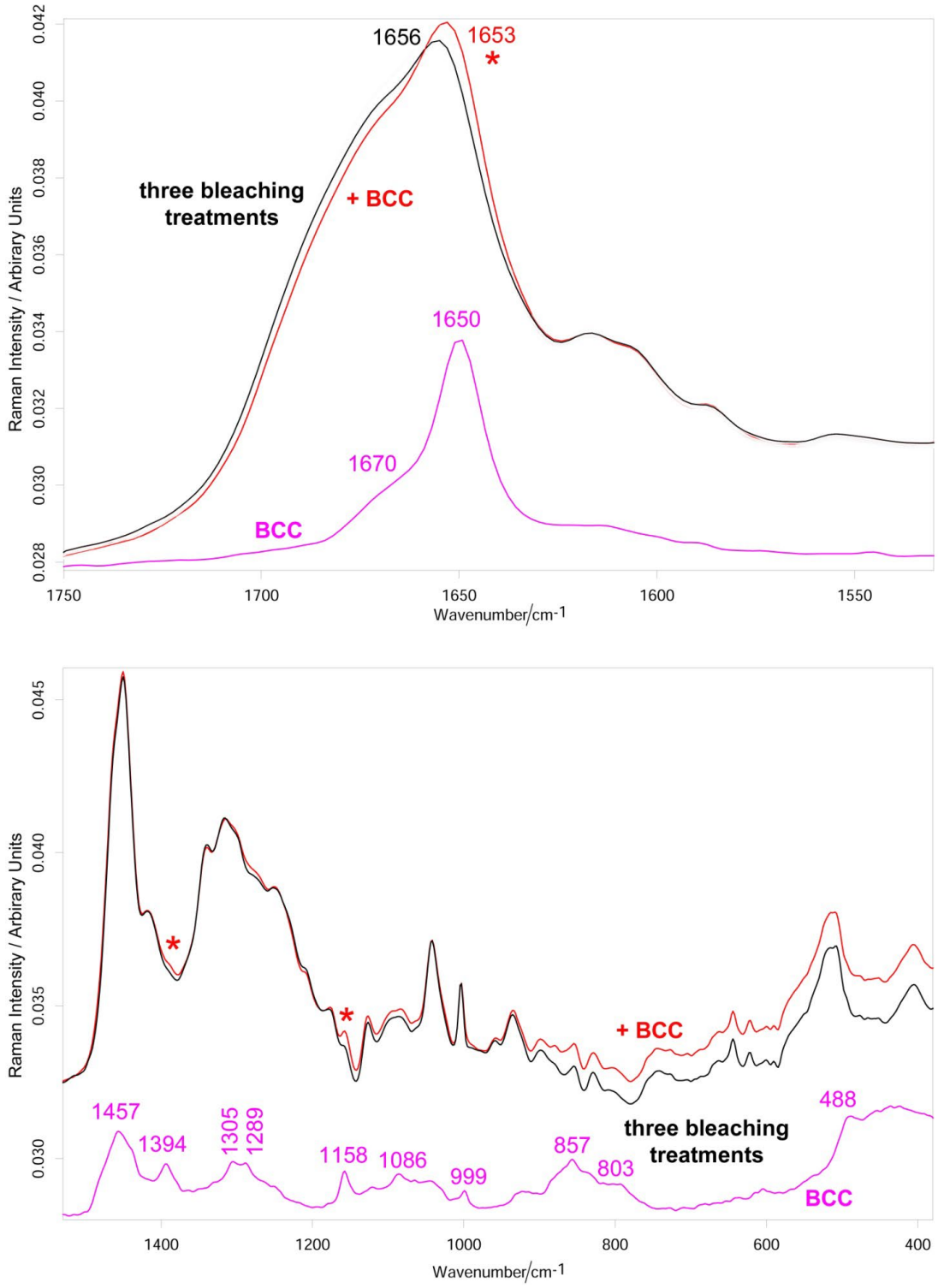

Fig. 3. Average Raman spectra of the hair lock bleached three times and after treatment with BCC. The spectrum of BCC is reported for comparison. The spectra are normalized to the intensity of the band at 1450 cm^{-1} (CH_2 bending). The bands indicated with an asterisk have a contribution from BCC.

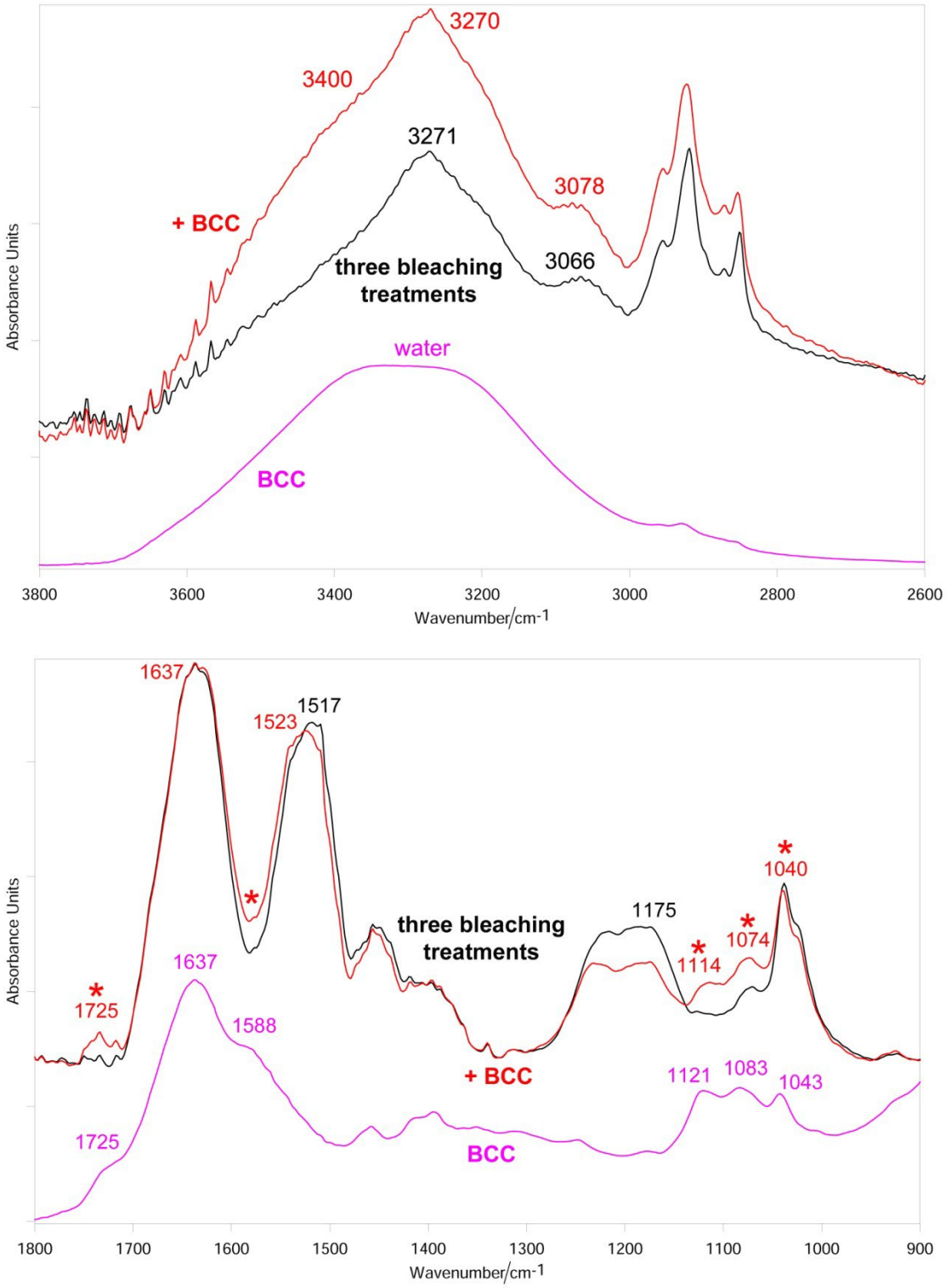

Fig. 4. Average ATR-IR spectra of the hair lock bleached three times and after treatment with BCC. The spectrum of BCC is reported for comparison. The spectra are normalized to the absorbance of the Amide I band. The bands indicated with an asterisk have a contribution from BCC.

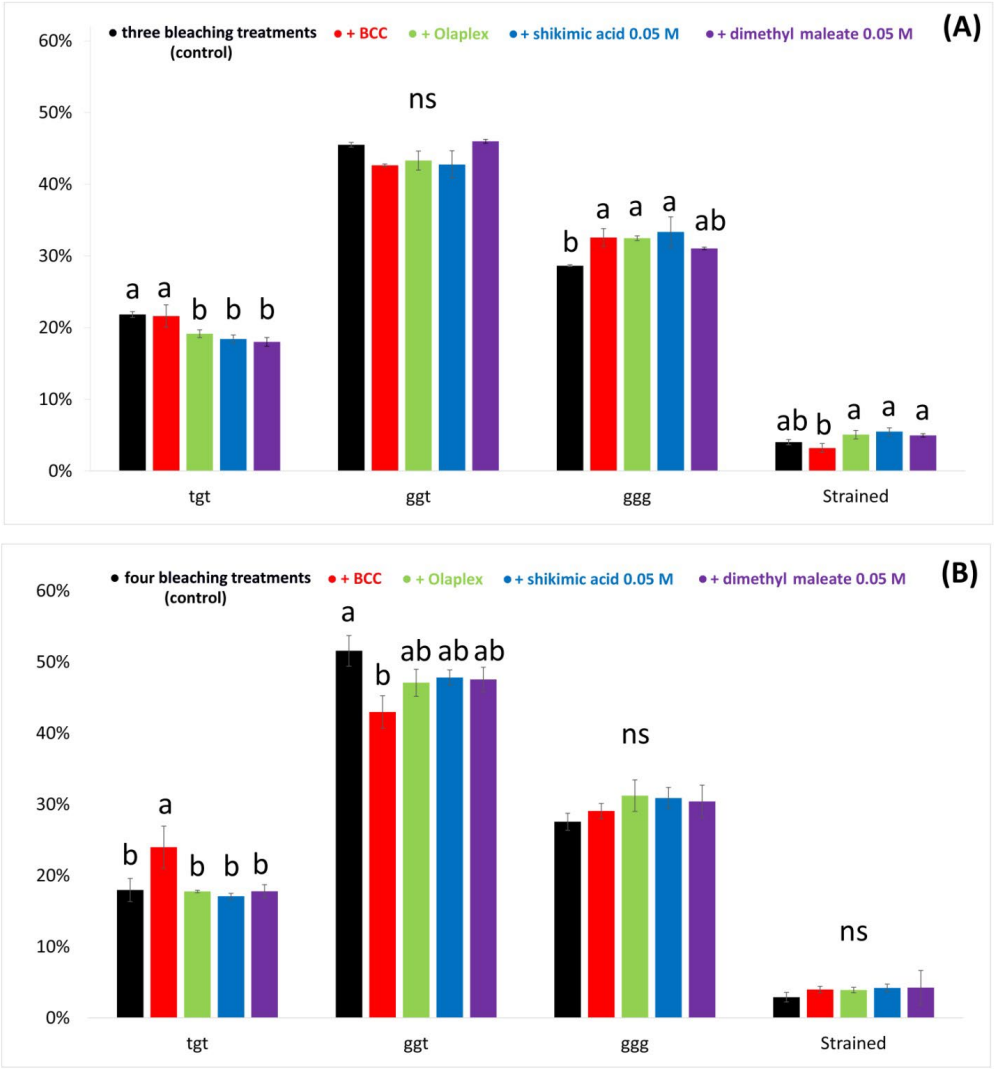

Fig. 5. (A) Percentages (average ± standard deviation) of strained, *gauche-gauche-gauche* (ggg), *gauche-gauche-trans* (ggt) and *trans-gauche-trans* (tgt) C_{α}-C_{β}-S-S-C_{β}-C_{α} conformations as obtained by the curve fitting of the Raman S-S stretching range of: (A) the hair locks bleached three times and after treatment with BCC, Olaplex, shikimic acid 0.05 and dimethyl maleate 0.05 M; (B) the hair locks bleached four times, and after treatment with BCC, Olaplex, shikimic acid 0.05 and dimethyl maleate 0.05 M. For each conformation, different letters on histogram bars represent statistically significant differences; ns = not significant.

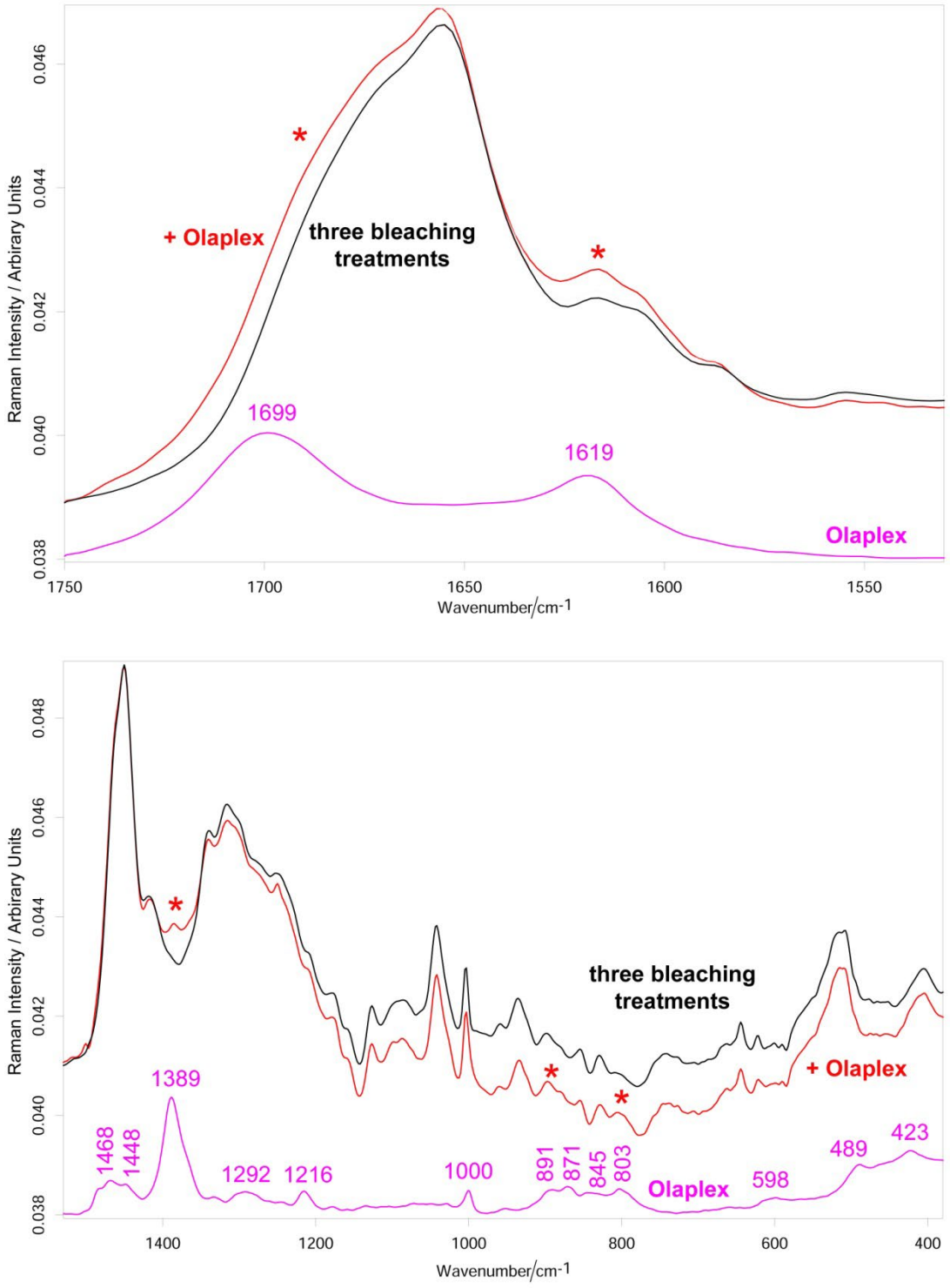

Fig. 6. Average Raman spectra of the hair lock bleached three times and after treatment with Olaplex. The spectrum of Olaplex is reported for comparison. The spectra are normalized to the intensity of the band at 1450 cm^{-1} (CH_2 bending). The bands indicated with an asterisk have a contribution from Olaplex.

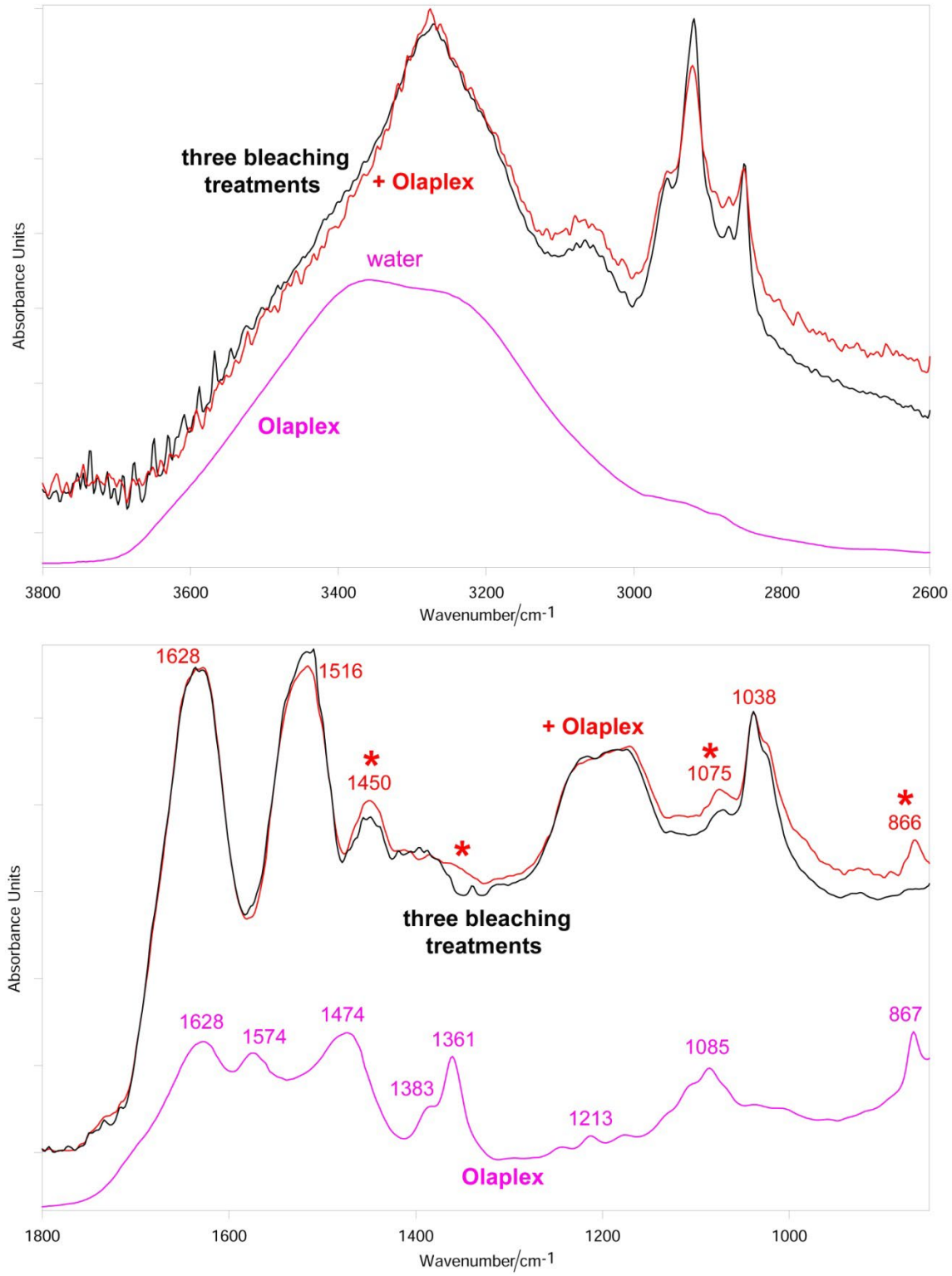

Fig. 7. Average ATR-IR spectra of the hair lock bleached three times and after treatment with Olaplex. The spectrum of Olaplex is reported for comparison. The spectra are normalized to the absorbance of the Amide I band. The bands indicated with an asterisk have a contribution from Olaplex.

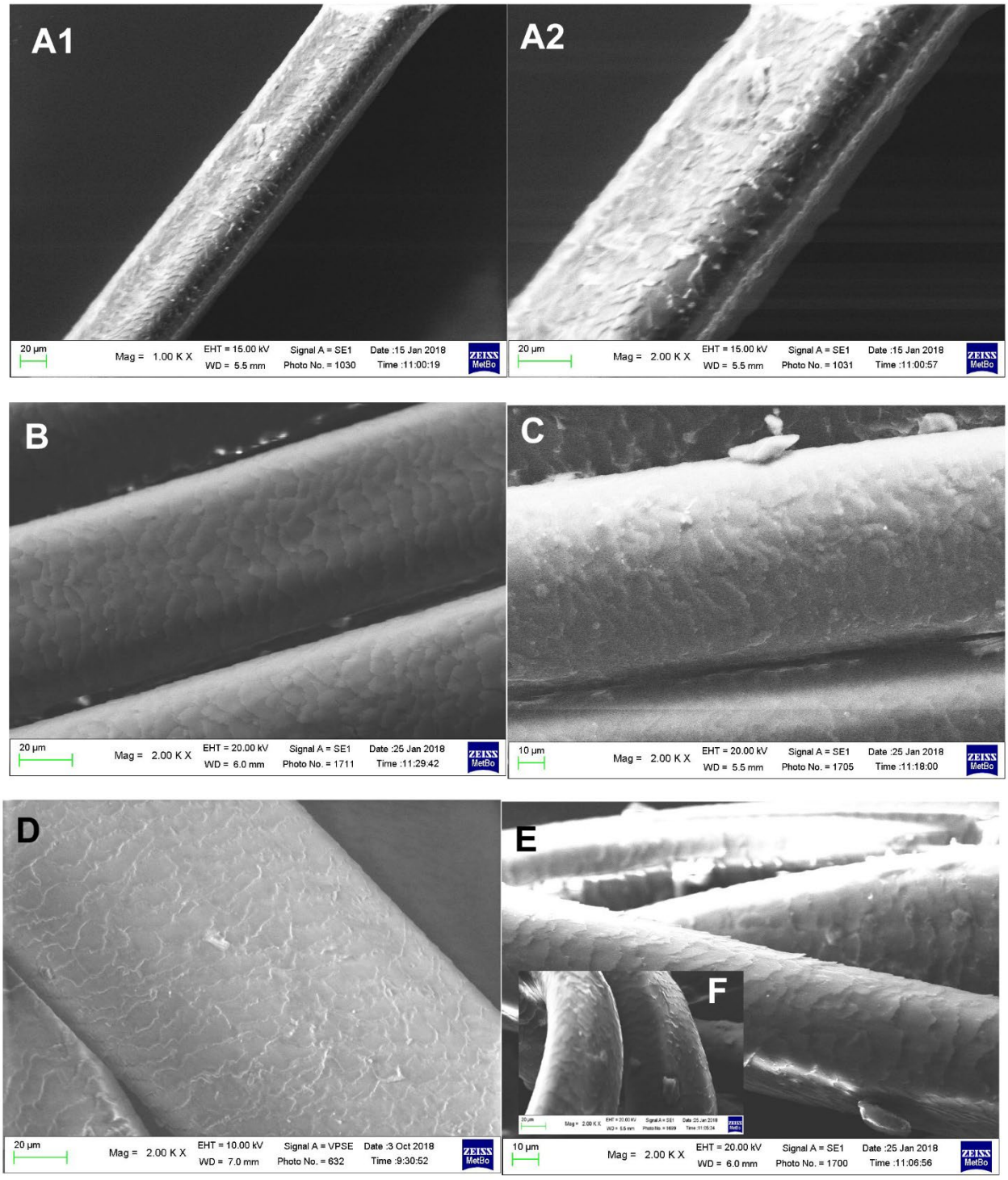

Fig. 8. Image of human hair fiber (A1 and A2) subjected to three consecutive bleaching treatments (right: magnified image) and then treated with: (B) shikimic acid 0.05 M in methanol, (C) BCC (small solid residues on the surface are probably due to dust contamination), (D) dimethyl maleate 0.05 M in methanol, (E) Olaplex, (F) Olaplex, image on knotted hair.