Damage assessment of human hair
by electrophoreetical analysis of hair proteins

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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CBB G 250</td>
<td>Commassie Brilliant Blue G 250</td>
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<tr>
<td>CBB R 250</td>
<td>Coomassie Brilliant Blue R 250</td>
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<tr>
<td>CMC</td>
<td>cell membrane complex</td>
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<tr>
<td>C%</td>
<td>percentage by weight of cross-linking agent (gel parameters)</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>HGT</td>
<td>high-glycine/tyrosine proteins</td>
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<td>HPDSC</td>
<td>high pressure differential scanning calorimetry</td>
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<td>h</td>
<td>hour</td>
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<td>HS</td>
<td>high-sulphur proteins</td>
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<td>KAPs</td>
<td>keratin intermediate filament-associated proteins</td>
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<td>KIFs</td>
<td>keratin intermediate filament proteins</td>
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<td>LS</td>
<td>low-sulphur proteins</td>
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<tr>
<td>min</td>
<td>minute</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>Rf</td>
<td>relative mobility of protein bands</td>
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<tr>
<td>SCam</td>
<td>S-carbamidomethylated keratin derivatives</td>
</tr>
<tr>
<td>SCMK</td>
<td>S-carboxymethylated keratin derivatives</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>T%</td>
<td>total concentration of monomer in grams / 100 ml (gel parameters)</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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1. Abstract

The assessment of the damage induced by cosmetic processes on human hair is very important for the cosmetic industry, constituting the first step to prevent hair deterioration and to develop new products. Traditional test methods such as amino acid analysis, tensile and thermal properties, swelling behaviour, spectroscopic and microscopic imaging used in damage evaluation provide only limited information, combinations of a few of them being usually applied for systematic investigations.

In the present study, assessing the status of human hair and its changes imparted by cosmetic processes is realised through gel-electrophoretical fractionation of hair proteins. The basis of the study is the fact that all cosmetic processes influence the chemistry of the morphological components of the hair fibres. Modifications of the hair proteins are specific, a characteristic type and degree of damage being induced by every cosmetic treatment.

The first step of the analysis is the extraction of keratin proteins from hair samples with a dithiothreitol/urea buffer. The resulting thiol groups are protected by alkylation with iodoacetamide and S-carbamidomethylated keratin derivatives are obtained. The soluble proteins are loaded onto polyacrylamide gels in the presence of sodium dodecyl sulphate and their fractionation in the electric field is performed. The gels are then stained with Coomassie Brilliant Blue 250 and subjected to densitometric measurements, resulting densitograms which show the optical density as a function of relative mobility of protein bands. The protein extraction from the hair samples and the gel staining are optimised and standardised, and the adding of internal protein standards with defined molecular weight to the protein solution allows the standardisation of the electropherograms and densitograms. Finally, the densitograms are digitised and the protein profiles are obtained in the form of numerical data, which can be subjected to statistical analysis. This statistical analysis of the protein patterns is performed using cluster analysis, a method of multivariate statistics able to identify the similarities between the digitised protein profiles, and the systematic classification obtained has the form of a dendrogram.

Starting from the analysis of untreated hair samples, the main parameters of the developed method are established. A number of 53 hair samples of individuals are used for the
investigation, the clustering of the samples on the dendrogram depending in this case on the intensity of protein bands. Different classifications are identified for the whole protein profile and for protein areas related to the morphological components, i.e. intermediate filament proteins and keratin intermediate filament-associated proteins. The explanation of these results is the difference in the protein extractability for different hair samples and the unequal variability degrees associated with the protein groups originating from different morphological components.

The cosmetic treatments are applied on a commercial, European mixed hair sample. Beside oxidative and reductive treatments, carried out as single and multiple treatments, at different treatment times, styling procedures such as hot curling are applied. The influence of the contact with swimming pool and sea water is also investigated. Cumulative effects of cosmetic processes on hair proteins are studied applying combined cosmetic treatments. The modifications appearing in the electrophoretical protein patterns are expressed as increasing or decreasing intensity of the protein bands, band broadening, and for extreme cases disappearance of protein bands or appearance of new bands. Cluster analysis performed separately for the intermediate filament proteins and for the keratin intermediate filament-associated proteins allows the identification of changes imparted to the morphological components by a certain cosmetic treatment or by combinations of treatments and the comparative evaluation of the induced damage. The oxidative cosmetic treatments are found to influence to relatively equal extent both morphological components, but systematic changes are identified only for keratin-associated proteins. The modifications induced by reductive cosmetic treatments are evidenced especially through the intensity decrease of intermediate filament protein bands, and the cluster analysis shows a systematic dependence on the treatment number and duration.

A database of characteristic changes of the electrophoretical protein pattern is built by the analysis of hair samples subjected to standard cosmetic treatments, in conformity with the usage instructions. This database is used subsequently for evaluating the effects of non-standard cosmetic treatments on the morphological components of the hair fibre.

UV-irradiation and cosmetic processes have cumulative effects on hair proteins. This aspect is investigated for hair samples subjected to UV-irradiation before and after applying cosmetic treatments. Changes of the electrophoretical protein patterns, expressed mainly as decrease of
protein band intensities are found for relatively short irradiation times and for all types of hair samples investigated.

The enlargement of the application area of the developed method is suggested by the results obtained for the efficacy evaluation of a series of hair sunscreens, subjecting hair samples treated with the corresponding products to UV-irradiation. The changes induced in the electrophoretical protein patterns for treated samples are reduced, compared with non-treated samples; additionally, the classification of the commercial hair sunscreens as a function of their efficacy can be made.

The results obtained using the analysis of the electrophoretical protein patterns for cosmetically treated samples are compared, for selected cases, with the results obtained by traditional test methods, such as amino acid analysis, tensile strength measurements and differential scanning calorimetry. The sets of results obtained using different methods correlate with each other; it can be seen that the results provided by the developed method are more comprehensive than the ones obtained with traditional methods.

The reproducibility of the method is evaluated for triplicate samples and conclusions related to characteristics, advantages/disadvantages and applicability of the method are drawn. The method is found to be very sensitive, stable and reliable, its results corroborating the ones obtained by traditional test methods. Supplementary, the electrophoretical patterns are able to point out differentiated damage at the level of morphological components of the hair cortex.
2. Introduction

Keratin is the generic term applied to the resilient protein-based structures such as hair, horn, nail, feather, and skin which comprise the integument and appendages of the higher vertebrates and whose primary function is to protect the animals from their environment. The strong and durable nature of those keratin materials derives from the synthesis and subsequent cross-linked network of keratin proteins, characterised mainly by their high cystine content. Cystine is an amino acid containing two sulphur atoms in a disulphide bond, providing intramolecular and intermolecular cross-links which largely account for the toughness and resilience of keratin materials. The keratins have been subdivided into two groups, “hard” and “soft”, according to their chemical and physical properties but more particularly according to the amount of cystine they contain. On this basis, skin keratin with a sulphur content of less than 3% is defined as a soft keratin, and the tougher materials as horn, hoof and hair, which contain more than 3% sulphur, are classified as hard keratins [1]. Hard keratin tissues typically express complex patterns of keratin proteins, yet despite obvious phenotypic variation they all appear to express common sets of keratin genes. The proteins of hard keratins were first identified from sheep wool and most of the knowledge about them stems from work on wool [2,3].

2.1. Morphological and chemical structure of human hair

2.1.1. General structure and hair growth

Human hair is a keratin-containing appendage that grows from large cavities or sacs called follicles. Hair follicles extend from the surface of the skin through the stratum corneum and the epidermis into the dermis. The generation of hair is an extremely complex process. At its origin the follicle creates cells which fill themselves with a self-organising and environmentally-resistant protein material. During the process of terminal differentiation, proteins are deposited on the inner surface of the cells and then cross-linked by isodipeptide bonds formed by the action of a transglutaminase, forming the cornified cell envelope. Then the follicle and its subdivisions pigment and extrude their product upon keratinization. [3]. The human hair fibre can be divided into three distinct zones along its axis. The zone of biological synthesis and differentiation resides at and around the bulb of the hair. The next zone outward direction along the hair shaft is the zone of keratinization, where stability is
built into the hair structure via the formation of cystine linkages. The third zone that emerges through the skin surface is the region of the permanent hair fibre, which consists of morphological components such as dehydrated cornified cuticle, cortical and sometimes medullar cells, and intercellular cement.

The morphological hierarchy of the hair components is presented in figure 2.1 [4]. The hair consists of a central core of closely-packed spindle-shaped cortical cells, each filled with macrofibrils, which in turn consist of a microfibril/matrix composite. The long axes of the cells and their fibrous constituents are oriented along the hair fibre. The cortical core is covered by layers of flat scale-like structures which are overlapping from the root toward the tip of the fibre, called the cuticle. The hair may along its central axis contain further cells – the medulla – which are vacuolated and present a series of air-filled spaces. The surfaces of all the cells of the hair shaft are endowed with a thin layer of lipid which is covalently attached to the underlying protein. Between adjacent cuticle cells the lipids are separated by an intercellular cement of relatively constant thickness (18 nm), between adjacent cortical cells the cement is of variable thickness (till 20 nm). The various lipid-cement-lipid structures are known as the “cell membrane complex” (CMC) and can be regarded as the “continuous phase” in the hair’s structure, where all the other components of the hair shaft can be thought of as “discontinuous phase”.

Figure 2.1. Schematic diagram of a human hair fibre showing the morphological hierarchy of the hair components [4].

Human head hair grows in cycles but asynchronously between the various follicles [1,2,3]. The active growing phase is known as anagen and in this the hair grows for 2 to 6 years before entering a resting phase of approximately 100 days, which is known as telogen. In
catagen, which is the precursor of telogen, the follicle shrinks, leaving at its lower end a sac of potential dermal papilla cells and an upper club in which the earlier synthesised hair is only loosely attached.

### 2.1.2. Morphological components of human hair

The cuticle in human hair is generally 5 to 10 scales thick. Each cuticle cell is roughly a square sheet of 60 μm side with round corners and is of relatively constant thickness in the range of 0.3 to 0.5 μm. In a longitudinal direction the sheets are tilted at approximately 5° to the hair’s axis and overlap each other to present a series of scale edges at the fibre surface somewhat irregularly spaced but at an average separation of about 5 μm. At the root-end, approximately ten layers of cuticle are seen in transverse section [5], but the number diminishes in a tip-wise direction [6]. Each cuticle cell shows a well-defined nucleus [7] and is internally multi-laminated, consisting of three major layers: the A layer, a resistant layer with a high cystine content (> 30% as ½ cystine); the exocuticle, also rich in cystine (~15%); and the endocuticle, low in cystine content (~3%) (figure 2.2). The existence of a thin chemically resistant layer, called epicuticle, close to the surface of all undamaged mammalian keratin fibres has been known since 1916. The identification of such a specific structure within the cuticle structure has remained elusive [8-15]; recent microscopical investigations by transmission electron microscopy, sustained by comprehensive examination of human head hair with the atomic force microscope have tentatively identified the epicuticle as being a sharply defined and continuous layer approximately 13 nm thick, covering the entire outwardly facing intracellular surface of every cuticle cell [16,17].

![Figure 2.2. The sublamellar structure of the human hair cuticle, with the cuticle cells separated by the cell membrane complex layers [16].](image)
The cortex constitutes the major part of the fibre mass of human hair and consists of cells and intercellular binding material. The cortical cells are spindle-shaped with longitudinal flutings and with ends which are often separated into smaller finger-like extensions; they are 50 – 100 \( \mu \text{m} \) long and 3 to 6 \( \mu \text{m} \) in diameter. Each cortical cell contains a nuclear remnant, which is spindle shaped longitudinally and stellate in transverse section, pigment granules and spindle-shaped fibrous structures called macrofibrils. Two different types of cortical cells can be distinguished in human hair according to the packing arrangement of the macrofibrils within the cell: paracortical cells, in which the macrofibrils are so closely packed with the intervention of little other material, that is difficult to decide where one macrofibril ends and the adjacent one begins, and orthocortical cells, in which the macrofibrils are seen as discrete near-circular entities separated by small amounts of intermacrofibrillar material. The transverse sections of head hairs contain a ring of orthocortical-type cells, about two or three cells thick, surrounding a core of paracortical-type cells [18]. The spindle-shaped macrofibrils in human hair are approximately 40 to 200 nm in diameter and extend the full length of a cortical cell [19]. Each macrofibril consists of intermediate filaments (originally called microfibrils), highly organised fibrillar units with a diameter of around 7.5 nm, which are embedded in a less organised structure called matrix, as can be seen in figure 2.1 [20,21]. The intermediate filaments contain precise arrays of low-sulphur proteins (keratin intermediate filament proteins KIFs) composed of short sections of \( \alpha \)-helical proteins in coiled-coil formation (1A, 1B, 2A and 2B), showing a hepta-peptide sequence (heptad) repeat unit. The coiled-coils are interrupted at three positions by non-helical fragments (L1, L12 and L2) and are terminated by non-helical N- and C- domains [3,22], as can be seen in figure 2.3:

![Figure 2.3. Schematic diagram of the rod domain of an intermediate filament (adapted from [23])]
Figure 2.4 shows the association of two right-handed $\alpha$-helix chains to form a left-handed coiled-coil structure [24,25,26], realised by the hydrophobic effect due to the apolar residues in position a and d and by electrostatic interactions between the residues in position e and g. The formed dimers then aggregate in an antiparallel arrangement to form structural units of higher order and eventually the intermediate filament [2,14,23].

The cross-section of a dimer with the apolar residues a and d in neighbour positions. The long (from g to e) and short (from g to c) shafts depict the electrostatic interactions at the interhelical, respective intrahelical level (according to [26]).

The matrix comprises the largest structural subunit of the cortex of human hair fibres. It contains the highest concentration of disulphide bonds. The proteins which make up the matrix are called keratin intermediate filament-associated proteins (KIFAPs or KAPs) [19].

The cell membrane complex (CMC) consists of cell membranes and adhesive material that binds the cuticle and cortical cells together. It contains a low proportion of sulphur-containing amino acids, lipids and fatty acids (as, for example, 18-methyleicosanoic acid). Together with the endocuticle the cell membrane complex form the nonkeratinous regions, which are becoming more and more important to cosmetic and wool science because they are believed to be the primary pathway for entry or diffusion into hair and wool fibres [2,3,19, 27].

The medulla is variable in the extent to which it is encountered in normal hair and occupies a minor proportion of the cross-sectional area along the hair’s axis. Medullary cells are loosely packed and during formation they shrivel up, leaving a series of vacuoles along the fibre axis [2,3,19].

The colour of human hair ranges from shades of yellow and red to black, grey and white, and is entirely due to the pigment contained in ellipsoidal particles of about 0,6 $\mu$m length which
are synthesised within specialised cells of the hair follicle, melanocytes. After synthesis, the particles (generally known as melanin granules) are passed on so that they are distributed mainly inside the cortical cells of the fully formed hair shaft. The melanin granules contain small amounts of protein and varying proportions of two types of highly heterogeneous polymeric pigment, eumelanin and phaeomelanin, which are bio-elaborated within the melanocyte from the amino acid tyrosine. Eumelanin is responsible for black and brown hair colours, is insoluble in solvents and chemically intractable to all but powerful oxidising agents such as hydrogen peroxide. Phaeomelanin is responsible for yellow and brownish-red hair colouring, contains significant amounts of sulphur and is soluble in strong alkali [2,28,29]. The melanin granules are found mainly in the keratin macrofibrils and in the macrofibrillar matrix but not in the nuclear remnant region [2].

2.1.3. Chemical composition of human hair

Human hair is predominantly proteinaceous, consisting of (due to the water content) approximately 65% to 95% proteins, the remaining constituents being water, lipids (sterols, free fatty acids, polar lipids), sugars, pigment (melanins), nucleic acids and trace elements [2,3]. The proteins present in hair can be classified in disulphide cross-linked proteins, as are the intermediate filament proteins and keratin-associated proteins, and disulphide and isodipeptide cross-linked mercaptolyse resistant proteins, comprising membrane proteins, cornified envelope proteins and cytoplasmic and nuclear proteins, found in the cuticle layers, cell membrane complex and in the nuclear remnants.

Analysis of the elements contained in hair reveals the presence of carbon, hydrogen, oxygen, nitrogen and sulphur in the average following proportions: carbon 45.2%, hydrogen 6.6%, oxygen 27.9%, nitrogen 15.1% and sulphur 5.2%. In addition, trace elements are detected that may play a role in the composition of pigments. The total ash content of hair ranges from 0.3% to 0.9%. The most frequently trace metals found are Ca, Cd, Cr, Cu, Hg, Zn, Pb, Fe, As and Si. Most of them are incorporated in hair from extraneous sources but are probably integrated in the fibre structure as salt linkages or co-ordination complexes with side-chains of pigment or proteins. Hair trace elements have been studied as an individual unique feature, as an indicator for certain diseases and as a monitor of exposure to environmental pollutants.

Total hydrolysis of the peptide bonds in human hair proteins reveals 24 different amino acids, which are classified in five groups: acidic amino acids, basic amino acids, amino acids with hydroxyl groups, sulphur-containing amino acids, and amino acids with no reactive groups in the side chains [20,30]. Like all proteins, human hair contains both cationic and anionic
groups and is therefore amphoteric. The cationic character is due to the protonated side chains of arginine, lysine, and histidine, and to the small number of free amino groups at the ends of the peptide chains. Anionic groups are present as dissociated side chains of aspartic and glutamic acid and as carboxyl end groups. The individual peptide chains in hair are held together by various types of covalent cross-links and non-covalent interactions, as presented in figure 2.5. In addition to their occurrence between separate polypeptide chains (inter-chain), these bonds can also occur between different parts of the same chain (intra-chain).

Figure 2.5. Schematic representation of covalent and non-covalent interactions between segments of two hypothetical peptide chains [31].

The disulphide cross-link plays an important part in stabilising the hair fibre, leading in particular to its relatively high wet strength, moderate swelling, and insolubility. The cystine content of virgin human hair, expressed in mol % of half cystine, ranges from about 13% to
18% Cystine residues provide stability of the hair as long as it is not exposed to reducing, oxidising and hydrolytic agents or to weathering. A second covalent bridge is provided by the isodipeptide cross-links (especially Nε-(γ-glutamyl)lysine), which provide an additional stabilising effect in the cornified cell envelopes of the cortex and cuticle.

The non-covalent bonds consist of three main groups: hydrogen bonds, ionic bonds (or salt bridges), and the hydrophobic effect. The hydrogen bonds can be realised between –CO and –NH groups in the peptide chains and the amino and carboxyl groups in the side chains, or between suitable donor and acceptor groups in the amino acid side chains, especially in the helical rod domains of the keratin intermediate filaments. The so-called salt bridges are due to the electrostatic interaction between cationic and anionic side chain groups, being responsible for the amphoteric nature of the fibre and its ability to combine with large amounts of acids and bases. The hydrophobic effect results from the approach of two non-polar side groups, with the resultant exclusion of associated water molecules and contributes to the mechanical strength of keratin, particularly at high water content [30,31].

2.2. Methods for damage assessment of hair

The human hair is a reactive substrate, whose structure and physico-chemical properties are of great interest in relation to environmental factors and cosmetic processes applied to it. The surface of hair keratin can be both chemically and physically degraded. Combing, washing and brushing cause physical damage which manifests itself as cuticle degradation. When human hair is exposed to weathering or chemical products, such as permanent waving and bleaching formulations, chemical damage occurs which may be accompanied by physical degradation.

Much of our knowledge about human hair actually derives from wide-ranging research on sheep’s wool in the context of the textile industry. The damage assessment of human hair can be realised by a series of methods, most of them applied initially for wool keratin analysis [2,30,32]. Chemical methods of analysis for human hair consist mainly of amino acid analysis, where special attention is paid to cystine – as an indicator of the hair quality, cysteic acid – as an indicator of bleaching, oxidative dyeing, irradiation, cysteine – for the reduction stage of permanent waving, lanthionine and lysinoalanine – for hair straightening, tryptophan – as an indicator of photo-oxidative damage [32-35], and solubility methods like alkali-solubility, urea-bisulphite solubility and pronase-solubility. Mechanical properties and thermal analysis of human hair are also valuable tools in damage evaluation [20,36-40].
Spectroscopic methods, like Raman and infra-red spectroscopy, X-ray diffraction, X-ray photoelectron spectroscopy, electron spin resonance are employed for quantifying structural and chemical changes in human hair following cosmetic treatments or weathering influences [41-48]. The surface morphology and alterations as a consequence of damaging processes are investigated by means of scanning electron microscopy [49-51] or contact angle determination [52]; furthermore, in the last years a continuously growing use of atomic force microscopy to study surface details and their modification as a result of sample manipulation is registered [16,17,53]. The changes in electrophoretic patterns of cosmetically treated hair contribute also to damage evaluation [54-57].

2.3. Gel electrophoresis of human hair proteins

Proteins occur as components of complex systems in which they interact to a greater or less extent with one another and with other substances. An essential preliminary to the determination of the primary structure of a protein is the cleavage of inter- or intrachain disulfide bonds and, for multichain proteins, the separation of the constituent chains. In the case of the keratins, soluble protein fractions cannot be obtained until almost all the disulfide bonds are broken [1]. The most specific way to achieve solubilization is to break the disulfide bonds by reduction with reagents such as mercaptoethanol, thioglycolic acid or dithiothreitol. Complete reduction usually requires an excess of the reducing agent, a mildly alkaline pH and the presence of reagents such as concentrated urea, guanidine hydrochloride or lithium bromide to swell the keratin and render the disulphides accessible to the reagent [4]. The reduction reaction of keratin with dithiothreitol is presented in scheme 2.1:

\[
\begin{align*}
R-S-S-R + HSCH_2(CHOH)_2CH_2SH & \rightleftharpoons R-S-CH_2(CHOH)_2CH_2SH + R-SH \\
\text{keratin} & \quad \text{dithiothreitol} & \quad \text{mixed disulphide} & \quad \text{reduced keratin} \\
\text{mixed disulphide} & \quad \text{cyclic disulphide of dithiothreitol} & \quad \text{reduced keratin}
\end{align*}
\]

Scheme 2.1. Reduction of keratin with dithiothreitol
For enhancing the stability and the solubility of the reduced keratin, the sulphhydryl groups are generally blocked by reaction with iodoacetic acid, forming S-carboxymethyl keratin derivatives (SCMK), or with iodoacetamide, forming S-carbamoylmethyl keratin derivatives (SCam), as depicted in scheme 2.2:

\[
\begin{align*}
R-SH & + \text{I-C}H_2\text{COOH} \longrightarrow R-S\text{CH}_2\text{COOH} + HI \\
\text{SCMK} & \\
R-SH & + \text{I-C}H_2\text{CONH}_2 \longrightarrow R-S\text{CH}_2\text{CONH}_2 + HI \\
\text{SCam} & \\
\end{align*}
\]

Scheme 2.2. Derivatisation of reduced keratin with iodoacetic acid or iodoacetamide, resulting in S-carboxymethyl keratin derivatives (SCMK), respectively S-carbamoylmethyl keratin derivatives (SCam)

Electrophoresis of proteins is generally carried out in gels made up of cross-linked polyacrylamide. The polyacrylamide gel acts as a molecular sieve, slowing down the migration of proteins approximately in proportion to their charge-to-mass ratio. Migration rate may be affected, on the other hand, by protein shape.

An electrophoretic method commonly employed in protein analysis, for example for purity and molecular weight estimation, makes use of the detergent sodium dodecyl sulphate (SDS), the technique being named Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS binds to most proteins in amounts roughly proportional to the molecular weight of the protein (about 1,4 g SDS / 1 g protein), the bound SDS contributing with a large net negative charge; in this way it renders the intrinsic charge of the protein insignificant and confers on each protein a similar charge-to-mass ratio. In addition, the native conformation of a protein is altered when SDS is bound, and most proteins assume a similar shape. Electrophoresis in the presence of SDS therefore separates proteins almost exclusively on the basis of mass (molecular weight), with smaller polypeptides migrating more rapidly. When compared with the positions to which proteins of known molecular weight migrate in the gel, the position – described by the relative mobility \( R_f \) ( = distance migrated by unknown protein divided by the distance migrated by standard) - of an unidentified protein can provide an excellent measure of its molecular weight [58]. On the other hand, the method is simple and
very fast due to the high electrophoretical mobility of the SDS–protein complexes, and only microgram amounts of sample protein are required [59,60].

The characteristic protein pattern for human hair can be visualised using staining procedures, for example with organic dyes such as Coomassie Brilliant Blue or silver-based staining methods, or radio-labelling of reduced and derivatised proteins. The main classes of hair proteins can be discriminated by their molecular weight [61]. One main family of $\alpha$-keratins constitutes the microfibrillar moiety of the hair cortex with a relatively low sulphur content and molecular weights quoted in the range of 40000–58000 whereas the other major family constitutes the nonfilamentous matrix in which the microfibrils are embedded. This second family includes the high sulphur proteins with molecular weights quoted in the range of 10000 - 35000. Another family of proteins originating from the matrix are the so-called high glycine-tyrosine proteins, with molecular weights under 10000 [4,30], whose detection in human hair is not frequently possible (figure 2.6).

![Human hair protein pattern](image)

**Figure 2.6.** Electrophoretical fractionation of the proteins extracted from human hair samples
LS – low-sulphur proteins
HS – high-sulphur proteins
HGT – high glycine-tyrosine proteins
Molecular weight standards (left to right): 66000, 45000, 36000, 29000, 24000, 20000, 14200 and 6500
SDS – PAGE: T 15%, C 3%
Derivatisation: Iodoacetamide
Detection: Coomassie Brilliant Blue R 250 (CBB R 250)

The stained gels are objected to densitometric measurements and the resulting densitograms constitute the basis for further analysis. The visual comparison is the older applied technique, but the subtle modifications following certain cosmetic treatments cannot be quantified in this way. Special software developments based on statistical methods allow nowadays a reliable interpretation [62,63].
2. Introduction

2.4. Applying chemometrics for evaluation of electrophoretical protein patterns

Chemometrics can be defined as the chemical discipline that uses mathematical, statistical, and other methods employing formal logic to design or select optimal measurement procedures and experiments, and to provide maximum relevant information by analysing chemical data [64]. The development of advanced chemical instruments and processes has led to a need for advanced methods to design experiments, calibrate instruments, and analyse the resulting data.

Progress in the analysis of multicomponent processes and mixtures relies on the combination of sophisticated instrumental techniques and suitable data tools focused on the interpretation of the multivariate responses obtained. In the last 30 years, instruments have evolved in complexity and computational capability has similarly advanced, so that it is possible to develop and employ increasingly complex and computationally intensive methods [65,66].

The relationships of chemometrics to different disciplines are indicated in figure 2.7. On the left are the enabling sciences, mainly quite mathematical. Statistical approaches are based on mathematical theory, so statistics falls between mathematics and chemometrics. Computing is important in as much as chemometrics relies on software. On the right are the main disciplines of chemistry that benefit from chemometrics.

![Chemometrics and its relationships to other disciplines](image)

Figure 2.7. Chemometrics and its relationships to other disciplines [67]

Chemometrics plays a major role and has many of its origins within analytical chemistry, but is also used by environmental chemists, biologists, food chemists as well as geochemists, chemical archaeologists, forensic scientists, which depend on good analytical chemistry measurements and many routinely used multivariate approaches especially for pattern
recognition. The organic chemists need chemometrics primarily in the area of experimental
design and quantitative structure–analysis relationships for drug design. Finally, physical
chemists such as spectroscopists, kineticists and material scientists often come across methods
for signal deconvolution and multivariate data analysis. The most efficient and reliable
approach to the acquisition of experimental data is to introduce statistical considerations at the
planning and design stage. The application of seemingly sophisticated statistical methods to
the analysis of data acquired from poorly-designed experiments may lead to erroneous
conclusions about the results. The use of chemometrics in each phase of an experimental
study requires a clear establishment of the relationships among the desired information and
the way the experiments are to be conducted, the way the data are to be analysed, and the way
the results are to be interpreted [67].

The present study uses as basis material human hair proteins subjected to one-dimensional
SDS-PAGE. The systematic comparison of different protein patterns obtained upon cosmetic
treatments and their classification are possible by applying the statistic evaluation of the
electrophoretical protein patterns, but one should be aware of the fact that the computer
comparison of electropherograms is difficult: electrophoresis conditions, staining, destaining,
densitometry, and sample preparation can modify migration distances from one sample to
another [68]. These aspects can manifest themselves as limitations, as also other investigators
found out [69,70], so that the standardisation of all the intermediate steps is absolutely
necessary. Expressing the one-dimensional protein patterns as densitograms, which describe
the optical density as a function of Rf values, provides an excellent tool for the creation of a
common comparison basis for the electropherograms. The densitograms digitised using the
ScanData software in reproducible Rf intervals provide protein profiles feasible for statistic
analysis.

2.4.1. Uses of cluster analysis

The essential concern of cluster analysis is to find groupings of things such as objects,
experimental units, variables in such a way that the things within groups are more “similar”
(in some sense to be indicated by the measurements on the things) than the things across
groups.

Despite the intuitive appeal of such a goal, however, performing a cluster analysis sensibly
and obtaining meaningful results is far from simple. Questions of what to measure, how to
quantify similarity, what methods to use for performing the clustering and how to assess the results of using clustering algorithms are all critical [71].

In the case of different cosmetically treated hair samples, the classification should be a function of the cosmetic treatment applied to the samples, building a database which allows further classification for new samples.

2.4.2. Measures of distance

The problem that cluster analysis is designed to solve is the following one: given a sample of \( n \) objects, each of which has a score on \( p \) variables, devise a scheme for grouping the objects into classes so that “similar” ones are in the same class.

The data for a cluster analysis usually consist of the values of \( p \) variables \( X_1, X_2, ..., X_p \) for \( K \) objects. The values for object \( k \) can be denoted by \( x_{kl}, x_{k2}, ..., x_{kp} \) and those for object \( l \) by \( x_{ll}, x_{l2}, ..., x_{lp} \). With more than three variables it is not possible to use variable values as the coordinates for physically plotting points, as for two- and three-variable cases, where Pytagoras’ theorem can give the distance between the two objects \( k \) and \( l \). However, the generalised Euclidean distance \( d_{kl} \) presented in equation 2.1 may serve as a satisfactory measure for many purposes [72]:

\[
d_{kl} = \sqrt{\sum_{i=1}^{p} (x_{ki} - x_{li})^2}
\]  

(2.1)

where

- \( d_{kl} \) - Euclidean distance
- \( x_{ki} \) - the value of object \( k \) for variable \( X_k \)
- \( x_{li} \) - the value of object \( l \) for the same variable

2.4.3. Types of cluster analysis

Many algorithms have been proposed for cluster analysis. Here the algorithms of choice are hierarchic techniques, which start with the calculation of the distances of each individual to all other individuals. Groups are then formed by a process of agglomeration or division. With agglomeration all objects start by being alone in groups of one. Close groups are then gradually merged until finally all individuals are in a single group. With division all objects start in a single group. This is then split into two groups, the two groups are then split, and so on until all objects are in groups of their own.
Agglomerative hierarchic methods start with a matrix of “distances” between individuals. All individuals begin alone in groups of size one and groups that are “close” together are merged. There are various ways to define “close”. The simplest is in terms of nearest neighbours (or single-linkage method). Groups are merged at a given level of distance if one of the individuals in one group is that distance or closer to at least one individual in the second group. With furthest neighbour linkage (or complete linkage method) two groups merge only if the most distant members of the two groups are close enough. With group average linkage two groups merge if the average distance between them is small enough.

Ward’s method is sometimes referred to as the minimum variance method since it merges at each stage the pair of objects which minimises the increase in the total within-group squared deviation about the means, i.e., the variance. Since the total variance for a set of points is fixed, the between-cluster variance is maximised as the within-cluster variance is minimised. Ward’s method has several desirable characteristics, including a tendency to produce homogeneous clusters and a symmetric hierarchy, and the incorporation of a definition for the cluster centre which provides a useful cluster summary. Its disadvantages include a sensitivity to outliers and poor recovery of elongated clusters. It has been found to give consistently good results in comparative studies of hierarchic agglomerative clustering methods [72].

The cluster structure resulting from the hierarchical methods is typically shown as a dendrogram, diagrammatic representation summarising the order of pairwise coupling of the objects within the dataset, and the similarity at which each fusion occurred [73].

2.5. Objectives of the study

Human scalp hair provides both protective (thermal insulation, protection against sunburn, light radiation and mechanical abrasion) and cosmetic or adornment functions. Through efforts to improve appearance, hair may be exposed to damaging chemical and mechanical modifications. Chemical methods such as bleaching, dyeing, permanent waving, UV-irradiation induce chemical damage to the cuticle and the cortex, resulting in cleavage of the chemical bonds. Grooming treatments (combing, brushing, shampooing, conditioning) lead to mechanical damage, mainly to the surface of the hair fibre. The assessment of the changes in hair properties and hair composition at the chemical level as well as at the morphological level, following various cosmetic treatments, constitutes the first step to prevent hair deterioration.
Damage evaluation is assessed by traditional methods such as amino acid analysis, tensile and thermal properties, swelling behaviour, spectroscopic or microscopic imaging. In the case of minimal induced changes or complex processes following cosmetic treatments, the results obtained with the above mentioned methods are sometimes combined to obtain comprehensive images of the modifications appeared. For certain circumstances, the results provided by traditional methods do not corroborate or are contradictory, making the damage evaluation of human hair a difficult approach.

The SDS-PAGE provides a reliable and simple technique for separation, visualisation and identification of protein patterns derived from different keratin samples, such as wool and animal hair, as well as for human hair analysis. In the case of wool, for example, where the samples are made up of mixed material, produced by a high number of animals, reproducible protein patterns are obtained. For human hair, a higher variability of the typical protein pattern was observed, sometimes connected with changes of the pattern due to cosmetic treatments or weathering.

The evaluation of electropherograms comprises qualitative and quantitative approaches. While the quantitative aspect is practically of low importance, excepting combined sophisticated techniques, the qualitative interpretation of electrophoretical protein patterns has a capital importance in the majority of the applications. This evaluation can be realised visually or the patterns can be stored in databases and retrieved using special software developments.

In the present work, the one-dimensional electrophoresis shall provide the protein patterns for human hair samples subjected to cosmetic treatments. Their evaluation will be performed using a statistical method, and the comparison with results of traditional methods will serve for verifying the classification obtained. Besides, the electrophoretic analysis shall be able to provide information about the magnitude of the damage at the morphological components level, allowing a higher differentiation for damaging agents and their action. Finally, a database of protein patterns characteristic of certain cosmetic treatments will be built, offering a comparison basis for combined treatments.

The use of statistical analysis for human hair protein patterns obtained using one-dimensional gel electrophoresis in a chemometric approach should offer a reliable tool for assessing the type and the magnitude of the damage induced to human hair after various cosmetic treatments.
3. Results and discussions

3.1. Human hair samples – choosing and characterisation

The human hair samples used during the work consist of Caucasian hair samples from individuals with pigmentation from blond to black and natural “grey” samples, and brown European mixed hair, provided by a commercial source.

The characterisation of the human hair samples is made using amino acid analysis (table 3.1), scanning electron microscopy (figure 3.1) and one-dimensional gel electrophoresis, which gives the characteristical protein pattern of human hair (figure 3.2).

Table 3.1. Typical amino acid composition for human hair samples: hair sample from an individual and commercial, European mixed hair sample (values given in mol %).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Individual’s hair sample (mol %)</th>
<th>Commercial, European mixed hair (mol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cysteic acid</td>
<td>0,2</td>
<td>0,4</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>5,9</td>
<td>6,3</td>
</tr>
<tr>
<td>threonine</td>
<td>7,9</td>
<td>8,2</td>
</tr>
<tr>
<td>serine</td>
<td>11,8</td>
<td>11,0</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>12,6</td>
<td>13,6</td>
</tr>
<tr>
<td>proline</td>
<td>8,1</td>
<td>8,4</td>
</tr>
<tr>
<td>glycine</td>
<td>6,9</td>
<td>6,2</td>
</tr>
<tr>
<td>alanine</td>
<td>5,1</td>
<td>5,2</td>
</tr>
<tr>
<td>valine</td>
<td>6,8</td>
<td>6,8</td>
</tr>
<tr>
<td>cystine</td>
<td>9,0</td>
<td>9,7</td>
</tr>
<tr>
<td>methionine</td>
<td>0,5</td>
<td>0,3</td>
</tr>
<tr>
<td>isoleucine</td>
<td>3,1</td>
<td>3,1</td>
</tr>
<tr>
<td>leucine</td>
<td>7,8</td>
<td>7,4</td>
</tr>
<tr>
<td>tyrosine</td>
<td>1,8</td>
<td>1,2</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>1,8</td>
<td>1,9</td>
</tr>
<tr>
<td>ornithine</td>
<td>0,1</td>
<td>0,2</td>
</tr>
<tr>
<td>lysine</td>
<td>2,9</td>
<td>2,8</td>
</tr>
<tr>
<td>histidine</td>
<td>0,9</td>
<td>0,9</td>
</tr>
<tr>
<td>arginine</td>
<td>6,8</td>
<td>6,4</td>
</tr>
<tr>
<td>lanthionine</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Examination of the literature data suggests a certain variability in the amino acid composition of human hair, particularly with respect to cystine content, reported values ranging from about 7 to 9,9 mol % [74]. The comparison of the amino acid composition obtained for the analysed samples with the amino acid composition of the benchmark of Marshall and
Gillespie [75] showed a pretty good agreement for the untreated hair samples. The cystine content of human hair and its variations were extensively studied, higher amounts of cystine being found in hair from males than from females [3] and various differences being identified to accompany certain dietary and genetic disorders [75]. The others amino acid contents vary in lower limits, excepting hair affected by genetic disorders [15]. An interesting aspect is presented by tryptophan, whose content correlates with pigmentation, age and sex [76]; the present study does not offer information about the tryptophan content, due to its decomposition during acid hydrolysis.

The scanning electron microscope images for the commercial, European mixed hair and for a hair sample of an individual – one fibre of each - are presented in figure 3.1.

![SEM images for a commercial, European mixed hair sample; b hair sample from an individual (bar: 50 µm)](image)

Figure 3.1. SEM images for: a commercial, European mixed hair sample; b hair sample from an individual (bar: 50 µm)

The aspect of the cuticular scales for both samples is characteristic for untreated hair, several centimetres away from the scalp [3,77-79].

Electrophoretic variability among hair samples from different individuals was often reported and used in genetic or forensic studies [54,61,80-83]. A number of eight different phenotypes, characterised by the different number and patterns of major polypeptide bands in the range of 45000 to 60000, were identified on examination of hair from 445 individuals [80]. The electrophoretic method used in the present study does not allow to distinguish between phenotypes but ensure a general view of the entire protein pattern, the differences registered being due mainly to different extractability degrees of the various hair samples.
3. Results and discussions

The hair samples of individuals offer better electrophoretical protein patterns, but the necessity of a systematic study in the case of cosmetic treatments requires a high amount of sample and therefore the use of European mixed hair is recommended. In this way, a common basis for comparisons and interpretations is provided.

The aspect of the electrophoretical patterns for the hair samples analysed during the present work is presented in figure 3.2, where lane a contains the external molecular weight standards, lane b the pattern of a hair sample from an individual and lane c the pattern of the commercial hair.

<table>
<thead>
<tr>
<th>Molecular weight</th>
<th>Chemical classification</th>
<th>Morphological classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>66.000</td>
<td>Low-sulphur proteins (LS)</td>
<td>Microfibrils</td>
</tr>
<tr>
<td>45.000</td>
<td>40.000–58.000</td>
<td>Intermediate filament proteins (KIFs)</td>
</tr>
<tr>
<td>36.000</td>
<td>High-sulphur proteins (HS)</td>
<td>Matrix</td>
</tr>
<tr>
<td>29.000</td>
<td>10.000–30.000</td>
<td>Keratin-associated proteins (KIFAPs or KAPs)</td>
</tr>
<tr>
<td>24.000</td>
<td>High glycine-tyrosine proteins (HGT)</td>
<td></td>
</tr>
<tr>
<td>20.000</td>
<td>MW &lt; 10.000</td>
<td></td>
</tr>
<tr>
<td>14.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.2. Electrophoretical fractionation of the proteins extracted from human hair samples
a. external molecular weight standards
b. hair sample from an individual (S57) (see chapter 4.1, table 4.2 for details)
c. commercial, European mixed hair, untreated

SDS – PAGE: T 15%, C 3%
Derivatisation: Iodoacetamide
Detection: CBB R 250

Traditional methods for hair damage evaluation such as amino acid analysis, tensile parameters measurements, differential scanning calorimetry are used for a number of cosmetically treated hair samples, therefore applied for the case of commercial hair.
3. Results and discussions

3.2. Gel electrophoresis – optimisation and standardisation

For the analysis of the keratin fibres by electrophoretical techniques, the first step to be accomplished is the protein extraction from the hair samples. Common methods include a solubilisation step by cleavage of disulphide bonds using a reagent with thiol groups (see chapter 2.3, scheme 2.1) and a subsequent alkylation step of the reduced proteins usually with monoiodoacetate to prevent reoxidation, the latter step being called S-carboxymethylation. Because S-carboxymethylation drastically modifies the electrophoretic mobility of the extracted proteins [81], the reduced proteins are converted to S-carbamoylmethylated derivatives with nonionisable iodoacetamide (see chapter 2.3, scheme 2.2). The treatment hardly modifies the electrophoretic mobility of proteins, providing better separation of the protein bands and accurate evaluation of their molecular weights [82,84].

In order to perform a systematical analysis of the results obtained for different cosmetic treatments applied on human hair, protein extraction from the hair samples, gel electrophoresis, gel scanning and analysis should be done in identical conditions. All the intermediate steps leading to the final results should be optimised and standardised, obtaining in this way reliable, reproducible results.

3.2.1. Optimisation and standardisation of protein extraction

Due to the higher content of cystine and also probably to certain morphological features, the human hair has a lower extractability than wool fibres. While soluble derivatives of the majority of proteins from wool can be produced relatively easily, human hair is notoriously difficult to dissolve under comparable chemical regimes so that, typically, as little as 50 - 40% and in extreme cases only 5% of the fibre is soluble [2,20,85,86]. Reductive and enzymatic dissolution experiments showed reduced diffusion rates for the reagents into the hair cortex compared to other keratin fibres, but the identification of the morphological component responsible for this behaviour was not possible [87]. The hindered diffusion hypothesis can be also applied to the diffusion of the reduced proteins out of the fibre, explaining cases when almost total reduction of the disulphide bonds in connection with low extraction degrees was registered [88].

The extraction methods for keratins from the literature are used as basis for a systematic investigation of human hair extractability, for both individuals’ and commercial hair samples [81,83,89,90]. The use of protein denaturation agents (as LiBr, 2-propanol or SDS) in the
reducing buffer does not lead to increased amounts of extracted protein [88]. Throughout all the experiments, the same extraction buffer (8M urea, 0.05 Tris, 0.05 DTT) and derivatisation procedure (20% iodoacetamide, 30 min at room temperature) were used. The differences between experiments consist in the temperature and the duration of reduction step, as well as in the occasional applying of ultrasound treatment. The series of attempts can be described as follows:

- 1, 2, 4, 6, 8, 16, 20 and 24 h at 40°C, under constant shaking
- 24 h at room temperature without shaking
- 24 h at room temperature under constant shaking
- 2 h at 45°C under constant shaking, sonication for 1, 5, 10 or 20 min, then another 30 min at 45°C under constant shaking

During the investigations, certain features of the extraction process are observed. First of all, the duration of the extraction step is important, especially in the case of commercial mixed hair. The amount of extracted protein increases visibly with the time until a maximum is attained (8 h of extraction at 40°C); after 8 h no substantial increase in the amount of protein can be observed, as can be seen in figure 3.3. The increase of the extraction temperature associated with long extraction times leads to proteins damage, determining the change of the characteristic protein pattern on the gel. Upon increased extraction times at room temperature no damage of the protein patterns could be observed.

![Image of electrophoretical protein patterns](image-url)

Figure 3.3. Electrophoretical protein patterns for the first two cm of the commercial hair sample after different extraction times (1h, 8 h and 24 h at 40°C)

SDS – PAGE, T 15%, C 3%
Derivatisation: Iodoacetamide
Detection: CBB R 250
On the other hand, the extractions performed without shaking do not provide all the times reproducible results, especially when variable amounts of sample are available (this being mainly the case for hair of individuals). The sonication step does not bring visible improvements for the extraction process, the increase of the protein amount being due probably to increased temperature (45°C compared to room temperature or 40 °C) during the extraction process.

Another observation is that the amount of extracted protein for all hair samples increased for longer extraction times. Furthermore, the differences in the extractability degree between different individual samples observed after 1 h extraction at 40°C (typical extraction method for wool proteins) are much diminished when the same samples are subjected to longer extraction times, even at lower extraction temperatures (figure 3.4).

Figure 3.4. Electrophoretical patterns for the first five cm of hair samples of individuals after different extraction times
a. 1h at 40 °C, sample S55 (see chapter 4.1, table 4.2 for details)
b. 1h at 40 °C, sample S54
c. 24 h at 21 °C, sample S55
d. 24 h at 21 °C, sample S54
SDS – PAGE, 15 % T, 3% C
Derivatisation: Iodoacetamide
Detection: CBB R 250
The amount of extracted protein decreases along the hair shaft, from the root to the tip end, as already known from the literature [77,80,85,87]; this phenomenon is attributed to an effective network of new cross-links of isodipeptide type, formed in the process of weathering of keratin fibres [37,55,91,92]. The variation is observed for easy- and hard-extractable hair samples and also for different extraction methods.

The relative amount of extracted proteins from different samples is visually evaluated from CBB R 250 stained gels. Additionally, the protein extracts for electrophoresis are subjected to a protein assay procedure. The results obtained for a tress of commercial mixed hair at different extraction times and at different distances from the root are presented in table 3.2:

<table>
<thead>
<tr>
<th>Number of cm from the root</th>
<th>1 h extraction at 40°C Protein amount (expressed as mg BSA/ml protein extract)</th>
<th>8 h extraction at 40°C Protein amount (expressed as mg BSA/ml protein extract)</th>
<th>Increase of the extracted protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>1,2 ± 0,08</td>
<td>1,9 ± 0,08</td>
<td>58</td>
</tr>
<tr>
<td>2nd</td>
<td>1,2 ± 0,11</td>
<td>2,0 ± 0,30</td>
<td>67</td>
</tr>
<tr>
<td>3rd</td>
<td>1,2 ± 0,09</td>
<td>1,8 ± 0,08</td>
<td>50</td>
</tr>
<tr>
<td>18th</td>
<td>0,7 ± 0,05</td>
<td>1,4 ± 0,07</td>
<td>100</td>
</tr>
<tr>
<td>19th</td>
<td>0,8 ± 0,08</td>
<td>1,1 ± 0,12</td>
<td>38</td>
</tr>
</tbody>
</table>

The results presented in the table as well as the visual evaluation of the stained gel suggest the necessity to use representative average sample, due to the decrease of the amount of extractable protein along the hair fibre as a result of weathering. For the next studies the whole fibre is used for the protein extraction, and the amount of protein assayed after 8 h extraction at 40°C for the untreated sample is 1,9 ± 0,09 expressed as mg BSA / ml protein extract.

For hair samples from individuals the protein extractability is higher, so that for the majority of the samples 1 h extraction at 40°C is enough to provide clear protein patterns on the stained gels. The protein extraction in mild conditions (room temperature) for a longer time (24 h), applied in the case of hair samples of individuals, provides interesting results:
3. Results and discussions

1 - the amount of protein extracted from the samples is increased around 4-fold compared to the extraction during 1 h at 40°C. The samples had to be diluted before being loaded into the gels, ensuring in this way an optimal staining and subsequent detection of the proteins.

2 - the extractabilities of the samples, very different in the case of 1 h extraction, seem to attain a common value for almost all the samples after 24 h extraction at room temperature. The intensities of the low-sulphur and high-sulphur protein bands are becoming similar for the majority of the samples.

3 - the extraction rate for the different types of proteins is different. The intensity ratio between the bands for the low-sulphur proteins and high-sulphur proteins is frequently lower for the hair samples extracted for 1 h than for the samples extracted for 24 h, suggesting a preferential extraction for the high-sulphur proteins at short extraction times. The preferential extraction of high-sulphur proteins constitutes an aspect encountered not only for human hair [93], but also common to other keratin fibres such as wool [94].

3.2.2. Optimisation and standardisation of gel staining

The methods used for the staining of proteins in polyacrylamide gels are various. Visualisation of individual protein bands or spots is usually performed either in situ within the polyacrylamide gel matrix itself or following “Western” or electroblot transfer of separated proteins onto polymeric membrane support materials such as nitrocellulose, nylon or polyvinylidene fluoride membranes. The proteins could be also stained prior electrophoresis, but the most preferred method is the post-electrophoretic staining and may involve one or more visualisation procedures. These include organic and inorganic metal salt based procedures, fluorescent group tagging, specific protein-ligand binding capacities, enzymatic activity detection, group-specific staining (for glyco-, phospho-, lipoproteins), antibody immunostaining and autoradiography and fluorography for isotopically labelled proteins [95]. A part of these methods is not suitable for keratin staining in polyacrylamide gel, due to the structure and reactivity of this protein class. On the other hand, special requirements for gel evaluation in certain techniques (radiolabelling, fluorescent dye detection) are not adequate for routine analysis or too expensive.

Of the commonly available organic dyes, Coomassie Brilliant Blue R 250 (CBB R 250) and Coomassie Brilliant Blue G 250 (CBB G 250) are the most sensitive and convenient to use [60]. CBB R 250 is a non-polar, sulphated aromatic dye generally used in methanol-acetic acid solutions, where excess dye is removed from the polyacrylamide gel matrix by
destaining. CBB G 250 is a dimethylated derivative of CBR 250, usually used as a colloidal suspension in aqueous trichloroacetic acid (TCA); upon these conditions, the dye does not penetrate the polyacrylamide gel matrix and interacts only with protein thus minimising the background staining [96,97].

Silver staining methods are about 10-100 times more sensitive than various Coomassie Blue staining techniques. Consequently, they are the method of choice when very low amounts of protein have to be detected on electrophoresis gels. A huge number of silver staining protocols have been published, based on the silver nitrate staining technique of Merril et al. [98] and modifications (e.g. Blum et al. [99], Heukeshoven et al. [100]) or the silver diamine procedure of Switzer et al [101], subsequently modified by Oakley et al. [102].

The diamine or ammoniacal silver stains utilise ammonium hydroxide to form soluble diamine complexes. The gels incubated in these solutions are treated then with a solution of citric acid containing formaldehyde for inducing the stain development [102,103]. The non-diamine chemical development stains are using typically silver nitrate and are generally more rapid than the diamine stains. Image development in this case occurs as a result of selective reduction of silver ions to elemental silver by formaldehyde at alkaline pH values [104,105].

Most proteins stain monochromatically with silver yielding brown or black bands, although certain silver stains can produce varying shades of black, blue, brown, red and yellow. Colour formation is highly dependent upon silver-grain size, the refractive index of the gel and the distribution of silver grains in the gel [104,106]. Although silver staining is the most sensitive of all non-radioactive protein visualisation methods currently available it does have a number of drawbacks. For example, high background staining can result, especially if the water used is insufficiently pure or when one of the several steps involved is not performed carefully enough [60]. One of the most serious problems is the fact that certain proteins stain either very poorly or not at all with silver, appearing as negatively stained spots against a darker background. On the other hand, the poor reproducibility is a substantial disadvantage inherent in all modifications of this method described in the literature thus far. This is due to the fact that the process of protein-induced precipitation of metal silver is insufficiently understood to provide effective control of its essential parameters; some literature data suggested that variation of carbon dioxide concentration in air is the main uncontrollable parameter that affects the reproducibility of protein development by silver in polyacrylamide gels [107].
Coomassie Blue staining combined with silver imaging has been used to intensify as well as to differentiate among various classes of proteins. This reduces the protein to protein variability in silver staining considerably and furthermore has higher sensitivity as silver staining alone [108,109]. Such a case is often encountered when a sample to be analysed contains a considerable amount of minor proteins along with certain major proteins. In such circumstances, it is convenient if the gel can be first stained with ordinary dyes such as CBR 250 and then, if necessarily, subjected to the higher sensitive silver stain [110,111]. In another variant, the method of Dzandu et al. [112], the gels are stained first with silver and then with CBR 250.

In contrast to the “positive-staining” procedures described above, alternative less sensitive staining procedures based on the formation of insoluble metal (zinc, copper, potassium etc.) salts have also been described in the literature. These methods, commonly referred to as “negative or reverse staining” are limited to SDS-containing gels and produce a semi-opaque background on the gel surface, while the proteins are detected as transparent bands or spots when viewed against a black background or when properly back-lighted [113,114]. Staining procedures are rapid, display intermediate sensitivity between that of Coomassie staining and silver staining and do not require prior fixation of the proteins within the gel matrix. The unstained proteins are more readily recovered from gels for subsequent characterisation or use. Nevertheless, for quantitation purposes protocols which give positively stained protein bands against a pale background are preferred [60].

The protein patterns for human hair samples stained according to different protocols are presented in figure 3.5 for the commercial, European hair sample and in figure 3.6 for a hair sample from an individual.
3. Results and discussions

Figure 3.5. Electrophoretic protein patterns for the commercial hair sample (1$^{st}$ and 2$^{nd}$ cm from left to right) stained according to different methods:

a. CBB R 250 in acetic acid / methanol [60]
b. CBB G 250 in TCA / methanol [60]
c. CBB R 250 colloidal (in (NH$_4$)$_2$SO$_4$) [97]
d. CBB G 250 colloidal (in H$_3$PO$_4$) [97]
e. Double stain protocol according to Dzandu et al. [112]
f. Double stain protocol according to Irie et al. [111]

Figure 3.6. Electrophoretic protein patterns for an individual’s hair sample (S 57) (see chapter 4.1, table 4.2 for details) (1$^{st}$ and 2$^{nd}$ cm from left to right) stained according to different methods

a. CBB R 250 in acetic acid / methanol [60]
b. CBB R 250 colloidal (in (NH$_4$)$_2$SO$_4$) [97]
c. CBB G 250 colloidal (in H$_3$PO$_4$) [97]
d. Silver stain according to Heukeshoven [100]
e. Simplified Heukeshoven silver stain according to Blum et al [99]
f. Double stain according to Bürk et al. [110]
3. Results and discussions

The silver staining and the double staining methods are more sensitive, but the lack of reproducibility does not recommend their use for a systematic analysis of the gels. The staining with CBB R 250 in methanol/acetic acid, even not so sensitive, is reproducible, easier to quantify and standardise, being therefore the method of choice for systematic investigation.

### 3.3. Chemometric approach of the evaluation of electrophoretical protein patterns

The statistic evaluation of the electrophoretical protein patterns shall allow a systematic characterisation of the different protein patterns obtained from cosmetically treated hair samples. For this purpose the samples are run in similar conditions and the staining protocol is standardised, giving in this way reproducible protein patterns. Densitograms are obtained after subjecting the gels to densitometric measurements in conditions kept as constant as possible.

#### 3.3.1. Standardisation of the electrophoretical protein pattern

The running of the gels proceeds until the tracking dye, Bromophenol Blue, reaches the bottom of the gel, thus providing a similar migration distance of the proteins into the gel. The selection of a representative and reproducible $R_f$ interval for comparison between different samples is ensured by the use of internal standards for the molecular weight, two known proteins added to the protein samples extracts and chosen in such a way that all the bands characteristic for the hair proteins are contained between their bands. As internal standards Phosphorylase b, with high molecular weight (97400), and the B-chain of Insulin, with low molecular weight (3400) are used.

#### 3.3.2. Cluster analysis of densitograms

The densitometric measurements of the gels are performed in a computerised system, using a gel scanner and the software ImageMaster 1D Elite (see chapter 4.2 for details). The densitograms obtained, which give the optical density as a measure of $R_f$, are subjected to cluster analysis in two different approaches: a) using the software Database additional to the gel evaluation software ImageMaster 1D Elite; b) using the software Statistica [115] after manually digitising the densitograms.
3. Results and discussions

3.3.2.1. Cluster analysis in ImageMaster 1D Elite Database

The Database software additional to the gel evaluation software can be seen as “a means of storing useful information in such a way that it can be easily retrieved” [62]. The data retrieving is made by the use of queries, sets of meaningful criteria, which can be used to extract items with desired properties from a database. The database is stored as a hierarchy, each lane being associated with a gel and each gel being associated with an certain experiment. Analysis within the database can be performed:
- through queries – performing a query to find all lanes in the database which contain a particular band may identify relationships between different gels.
- through matching – the lanes are matched against each other in order to establish their similarity. By this method, a sample of newly created lanes can be matched against the lanes of the database to establish which they most closely resemble.
- through dendrogram (or tree diagram) building. A dendrogram depicts the diagram of the total similarity of a set of lanes and may be created in the 1D Database program when a set of lanes has been matched against itself.

The result of each match is a similarity coefficient, which can be calculated by a number of different methods. In the Dice or Jaccard methods, the matching is based on the presence respective absence of the bands on the lanes. When the Pearson method is chosen, a different type of matching occurs. Instead of trying to find identical bands, the Pearson method directly compares the profiles of the two lanes and calculates a Pearson correlation coefficient.

The modifications induced by cosmetic treatments on the protein pattern of human hair are usually connected with intensity modification of different bands; the disappearance of certain bands, respective the appearance of new bands are phenomena associated with very advanced damage of the hair. In these circumstances, the Dice or Jaccard method are not adequate for following small or moderate modifications appearing in electrophoretical patterns. In the Pearson method, the Elite 1D Database program calculates all the Pearson correlation coefficients between pairs of sets of variables, transforms these coefficients into distances and makes a clustering using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm. UPGMA is a simple bottom-up data clustering method used usually in bioinformatics for the creation of phylogenetic trees. The input data is a collection of objects with their pairwise distances and the output is a rooted tree or dendrogram. Initially, each object (in our case, each electrophoretical pattern) is in its own cluster. At each step, the nearest two clusters are combined into a higher-level cluster. The distance between any two
3. Results and discussions

Clusters A and B is taken to be the average of all distances between pairs of objects a in A and b in B [62].

The statistical analysis of the protein patterns using the tools provided by the specialised software program does not lead to satisfactory results, in many cases the classification obtained contradicting the visual evaluation. The reproducibility of the results is also low, different classifications being obtained after applying the analysis procedure for a second time on the same set of gels. The reasons for this behaviour could be as follows:
- the differences between the protein patterns of human hair samples are smaller than the differences induced by a new lane creation procedure in the ImageMaster program, so that the software has difficulties to provide the same classification a second time.
- even the densitograms are standardised by the addition of internal standards for molecular weight to the protein extract, the database functions cannot compensate possible distortions of the lanes due to irregularities in the gel synthesis or current intensity variations during the gel running, as well as to the gel drying process.

However, the results obtained for samples between which clear differences are visible and for gels without distortions possess a high degree of reproducibility. On the other hand, the ImageMaster 1D Elite database could be successfully used for comparison between samples containing different proteins, where the protein patterns differ markedly.

The classification on the basis of the whole protein profiles (described by the densitograms), as our investigations solicits, provides acceptable results just in special cases, being not suitable for interpreting the modifications which appeared in the human hair protein pattern after cosmetic treatments.

3.3.2.3. Cluster analysis using Ward’s method

For this type of analysis the digitisation of the densitograms was previously performed. Using the ScanData software, the Rv values interval between the internal standards for molecular weight was assigned to contain 1000 units, starting from zero at the high molecular internal standard and getting to the value 1000 at the low molecular internal standard, as presented in figure 3.7.
3. Results and discussions

Figure 3.7. Standardised densitogram for the electrophoretical protein pattern of sample S31 (see chapter 4.1, table 4.2 for details). The positions corresponding to the internal molecular standards are marked on the lane selected from the gel and also on the densitogram.

SDS – PAGE, 15 % T, 3% C
Derivatization: Iodoacetamide
Staining: CBB R 250

The manual digitisation of the densitograms allows the elimination of the foreign peaks due to cracks on the gel during drying or to irregular spots of stain on the gel surface. Another advantage of this technique is the possibility to select different protein band areas for comparison, for example the regions of KIFs (low-sulphur proteins) or KAPs (high-sulphur proteins). In this way, the modifications manifested in the protein patterns of human hair following cosmetic treatments can be also associated to the morphological regions of the hair fibre.

Ward’s method is an agglomerative hierarchical method, able to use a set of objects for building groups in which a certain measure of heterogeneity is minimised. As heterogeneity
3. Results and discussions

measure is used the so-called variance criterion $V$ (also called error sum of squares), which is calculated for a given cluster $g$ as $V_g$(equation 3.1):

$$V_g = \sum_{k=1}^{K_g} \sum_{j=1}^{J} (x_{k,jg} - \bar{x}_{jg})^2$$

(3.1)

where $x_{k,jg}$ : value of the variable $j$ ($j = 1, ..., J$) in the case of the individual $k$ (for all the individuals $k = 1, ..., K_g$ in the cluster $g$)

$$\bar{x}_{jg} = \frac{1}{K_g} \sum_{k=1}^{K_g} x_{k,jg}$$

(3.2)

$x_{jg}$ : mean value of the variable $j$ values in the cluster $g$

The distance between a newly built cluster from two clusters $p$ and $q$ and other clusters (for example $r$) can be calculated following Ward’s method as presented in the equation 3.3:

$$d(r, p+q) = \frac{1}{n_r + n_p + n_q} \left[ (n_r + n_p) \cdot d(r, p) + (n_r + n_q) \cdot d(r, q) - n_r \cdot d(p, q) \right]$$

(3.3)

where $d(r, p+q)$ – distance between the cluster $r$ and the cluster $p+q$  
$d(r, p)$ – distance between the cluster $r$ and the cluster $p$  
$d(r, q)$ – distance between the cluster $r$ and the cluster $q$  
$d(p, q)$ – distance between the cluster $p$ and the cluster $q$  
$n_r$ – number of objects in the cluster $r$  
$n_p$ – number of objects in the cluster $p$  
$n_q$ – number of objects in the cluster $q$

The distances calculated with Ward’s method gives the twofold increasing of variance for any new fusion of two clusters [72].

The results obtained using this method are in good agreement with the visual classification of the electrophoretical protein patterns. The densitograms describing the most similar protein patterns occupy neighbour positions in the dendrogram, and the most different are situated at proportional distances with the difference between them.
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3.4. Electrophoretical analysis of untreated human hair

The electrophoretical separation and characterisation of the proteins extracted from keratin fibres is applied since many years. The method was first used for investigations on wool fibres, where the usual analysis utilises a mixture of fibres originating from a high number of individual animals. This aspect, added to the good extractability of the wool proteins, ensures good reproducibility of the data. On the other hand, the analysis of human hair can usually be carried out only on hair samples from individuals, usually in small amounts, or commercial mixed hair samples, made up of a limited number of samples of the same type. In this case, a great variability of the extractability degree of the hair proteins, as well as genetic differences due to the phenotypes are responsible for the variations of the electrophoretical protein pattern. With the electrophoretical method used throughout the study it is not possible to identify phenotypes, so that the genetic differences between the hair samples do not influence the conclusions of statistical analysis. The main differences registered in the case of hair samples from individuals are due to differences in protein extractability between the samples.

The protein pattern is not identical along the hair fibre. Modifications, appearing also for untreated hair samples, are caused by the daily care and the natural weathering process [46]. The human hair is usually exposed to sun and air pollutants and also sometimes to sea and swimming pool water. The consequences are chemical modification of the hair proteins. The irradiation with natural light can be regarded as one of the main factors causing changes at the hair proteins level [116]. The photo-oxidation of the hair proteins proceeds through cleavage of disulphide and peptide bonds, photolytic decomposition of light-sensitive amino acids and building of new cross-links [37,91,117]. These phenomena do not influence obviously the physical properties of the hair, but are clearly expressed in the chemical characteristics [34,74]. The decrease of the protein band intensity in the direction of the tip ends seen on the polyacrylamide gels (figure 3.8) – a fact also observed for wool proteins [118,119] - is connected to the formation of new isodipeptide cross-links, which lower the extractability of the proteins along the hair fibre.
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Figure 3.8. Electrophoretical protein patterns along the hair fibre for the sample S 57 (see chapter 4.1, table 4.2 for details) in cm from the scalp; 1 h extraction at 40°C.
SDS – PAGE, 15 % T, 3% C
Derivatization: Iodoacetamide
Staining: CBB R 250

Starting from the average linear rate of hair growth, evaluated to be around 1cm/month [2], we can presume that the first centimetres from the scalp are only marginally subjected to weathering and grooming practice. It was shown that the cuticle is damaged before any degradation occurs to the cortex [120]. Variation studies of the surface architecture of human hair carried out using scanning electron microscopy [79] have shown that normal hair undergoes gradual deleterious change with time and that mechanical atrophy by brushing, combing and handling contribute considerably to this change. Nevertheless, the first three centimetres from the scalp show the appearance accepted for hair free of externally-promoted defects, with smooth, imbricate cuticle scales, whose free edges are of relatively smooth contour. Limiting the protein analysis to the first two centimetres of the untreated hair samples will ensure thus characteristic protein patterns for undamaged samples. At larger distances from the scalp, mechanical stress can lead to cracking of the endocuticle in the intact hair. As a result of grooming, cuticle fragments can be chipped away at the edges of the scales, letting free way for the action of environmental factors [121].

The electrophoretical patterns of the hair samples from individuals are obtained after protein extraction for 1 h at 40°C. When longer extraction times are used, a higher uniformity of the
protein patterns obtained for different samples is induced. The typical aspect of the electropherograms obtained from samples from individuals is presented in figure 3.9:

![Image of electrophoretic patterns](image)

Figure 3.9. Electrophoretical protein patterns for hair samples of individuals (see chapter 4.1, table 4.2 for details); 1 h extraction at 40°C.
- S30_1 and S30_2 – 1st and 2nd cm of sample 30
- S31_1 and S31_2 – 1st and 2nd cm of sample 31
- S26_1 and S26_2 – 1st and 2nd cm of sample 26
- S25_1 and S25_2 – 1st and 2nd cm of sample 25

SDS – PAGE, 15 % T, 3% C
- Derivatization: Iodoacetamide
- Staining: CBB R 250

As previously discussed, the genetic differences between the samples cannot be distinguished with the electrophoretic method applied, which is more appropriate for the evaluation of all the extractable proteins originating from the intermediate filaments and from the intermicrofibrillar matrix. Protein patterns with different band intensity, related to the individual extractability of the samples, can be seen in figure 3.9, providing the differentiation tool for the hair samples. The corresponding densitograms for two of these samples are superimposed in figure 3.10, depicting the differentiation criterion for hair samples of individuals – differences in protein extractability.
3. Results and discussions

The dendrogram built for the whole protein pattern of untreated hair samples from individuals using cluster analysis is presented in figure 3.11.

Because the densitograms for the first and second cm from the hair root belonging to the same hair sample are not systematically grouped in the same cluster, we can say that the method does not make a differentiation between individuals. That means that the classification is based in fact on the extractability of the protein from the hair samples and not person-specific. A closer analysis of the groups built on the dendrograms reveals that the intensity of the protein bands is the most important classification parameter. The values of the band intensities are direct proportional to the linkage distance between clusters, so that the protein bands with higher intensity – in our case the intermediate filament protein bands - are expected to play a more important role in the samples classification than the protein bands with lower intensity, which are the intermicrofibrillar matrix protein bands.

The contribution of the morphological constituents to the structure of dendrogram presented in figure 3.11 is investigated performing the cluster analysis for the different morphological components as suggested in figure 3.7. The corresponding dendrograms are presented in figure 3.12 for KIFs and in figure 3.13 for KAPs.
Figure 3.11. Dendrogram for the whole protein pattern (KIFs + KAPs) of untreated hair samples of individuals (see chapter 4.1, table 4.2 for details; dendrogram for 106 samples, Ward’s method, parameter Euclidean distance).
The dendrogram of KIFs for the same samples (figure 3.12) is very similar to the one presented in figure 3.11, while the dendrogram of KAPs (figure 3.13) has a different aspect.

The dendrogram obtained for KIFs (figure 3.12) is similar with the dendrogram for the whole protein pattern (figure 3.11). It presents also two main clusters, I and II, and their subgroups Ia, Ib, IIa and IIb contain almost the same samples clustered together for both dendrograms; small differences are due to the transfer of some samples from Ib to IIa when emerging from the KIFs dendrogram to the dendrogram for whole protein patterns.

The aspect of the dendrogram for KIFs looks less complex than the one for KAPs (figure 3.13), suggesting a lower variability degree associated with the microfibrillar protein category. For similar intensities of the protein bands, patterns obtained for the 1st and 2nd cm of the same hair sample are grouped in different clusters, proving the lower specificity of the KIFs region of the protein pattern.

The dendrogram for KAPs (figure 3.13) presents a slightly different structure. In this case we can observe a lower linkage distance between clusters than in the case of KIFs. Moreover, the patterns provided by the 1st and 2nd cm of the same hair sample are more often grouped together, an aspect which shows a higher specificity degree as compared to KIFs area.
Figure 3.12. Dendrogram of KIFs for untreated hair samples of individuals (see chapter 4.1, table 4.2 for details; dendrogram for 106 samples, Ward’s method, parameter Euclidean distance).
Figure 3.13. Dendrogram of KAPs for untreated hair samples of individuals (see chapter 4.1, table 4.2 for details; dendrogram for 106 samples, Ward’s method, parameter Euclidean distance).
The interval length of the protein profile used for comparison is larger for KAPs (800 Rₚ arbitrary units), compared to the interval used for KIFs (200 Rₚ arbitrary units), allowing a higher variability; on the other hand, the intensity values for the protein bands are diminished, giving lower values for the linkage distance between the corresponding clusters. The existence of more differentiated subgroups in the clusters Ia, Ib, IIa and IIb from figure 3.13 is based therefore on differences in protein band intensities, as well as on differences in the protein band form.

In the case of the commercial hair sample the same aspects connected to the weathering process have to be taken into account. Being not possible to establish the position where the hair was cut and its original length, it can be only concluded that the material is untreated but cannot be considered as being undamaged. The decrease of the protein extractability along the hair axis, starting from the hypothetical root, is presented in the figure 3.14:

![Figure 3.14. Electrophoretic protein patterns along the hair fibre for the commercial mixed hair sample](image)

**Figure 3.14. Electrophoretic protein patterns along the hair fibre for the commercial mixed hair sample**

- SDS – PAGE, 15 % T, 3% C
- Derivatisation: Iodoacetamide
- Detection: CBB R 250

The protein pattern of the extract prepared using the whole hair fibre of the commercially available sample, as presented in figure 3.14, contains clear defined bands with a reasonable intensity, providing a good basis for comparison in the case of further cosmetic treatments. In the next electrophoretical investigations the whole fibre extract for cosmetically treated hair samples is to be used, ensuring in this way the reliability of the data.
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3.5. Cosmetic treatments of human hair and their evaluation

The chemical reactivity of the wool fibre has been exploited for a very long time, especially in the cases of bleaching and dyeing processes. Extensive investigations into the chemical nature of treated hair and wool have been described after the introduction of new analytical techniques for protein analysis, as chromatography and electrophoresis, in the middle of the 20th century. Nevertheless, over the last more than 30 years much research has been done also on hair damage from chemical treatments (bleaching, dyeing and permanent waving) and grooming. It has been established that the bleaching and permanent waving processes can result in losses of the mechanical properties [40,122,123], changes in the amino acid composition of hair [33,35,50,56], can damage and wear away the cuticle [124-126], change the IR absorption spectrum of the predominant keratin proteins [126-128] etc.

3.5.1. Oxidative cosmetic treatments

The oxidative treatments of the hair includes the hair bleaching processes, as well as the different dyeing methods which imply a permanent change of the hair colour. All the methods from this group use formulations containing an oxidative agent (usually hydrogen peroxide) and have as a result the modification of the hair colour.

3.5.1.1. Bleaching

The colour of the human hair is due to the inclusion of melanin granules in the hair fibre during keratinisation. The aim of bleaching is to eliminate or tone down the hair colour, this being accomplished by oxidation. During bleaching the melanin pigment undergoes irreversible physicochemical changes and the colour of the hair fibres is changed, but the fact that the pigment granules are distributed within the cortex of the fibre induces simultaneously some oxidation of the keratin matrix [123].

The principal oxidising agent used in bleaching compositions is hydrogen peroxide, and salts of persulphate are often added as “accelerators”. The oxidation systems used have pH values between 9 and 11, and stabilisers are often necessary to reduce the decomposition rate of the hydrogen peroxide. In these conditions the perhydroxyanion ($\text{HO}_2^-$) is the predominant reactive species. The reactivity of melanin with regard to hydrogen peroxide is much higher than that of keratin; however, the melanin pigment represents only a very small fraction of the
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fibre weight - usually about 2% - and thus it is reasonable to expect that some oxidative modification of the fibre matrix will occur [123].

The primary reaction of oxidising agents with the human hair proteins occurs, as Zahn [129] demonstrated, at the amino acid cystine. Robbins and Kelly [35] showed that rather small amounts of degradation occur to the amino acid residues of tyrosine, threonine and methionine during severe bleaching; Chao et al. [50] added to the list of affected amino acid lysine and histidine. Between 15 to 25% of the disulphide bonds in human hair are degraded during “normal” bleaching and up to 45% of the cystine cross-links may be broken during severe “in practice” bleaching [130]. This amount of damage may occur while frosting hair or while bleaching black or dark brown hair to light blond hair [3].

The bleaching agents oxidise disulphide bonds to cystine oxides (intermediate products with intact S-S bonds) and sulphur acids (e.g. cysteic acid, where the S-S bonds is cleaved); under extreme conditions the disulphide bridge is degraded to inorganic sulphate [131] (scheme 3.1).

The oxidative scission of disulphide bonds, as presented in scheme 3.1, may proceed either by path A, where the disulphide cross-links remain intact in the first two oxidation steps or by path B or C, where cleavage of the disulphide cross-links occurs already in the first oxidation step. Indirect evidence for cystine oxides in oxidatively treated wool and hair has been found by a number of workers [132,133]. Because the bleaching of human hair is carried out in an aqueous alkaline oxidising medium, hydrolysis of the cystine oxidation intermediates (I, II, and III from scheme 3.1) should be competitive with their oxidation. In fact, disproportionation of the cystine oxides may also occur, adding to the complexity of the total

\[
\begin{align*}
\text{R-S-S-R} & \rightarrow \text{R-S-S-R} \\
\text{R-S-S-R} & \rightarrow \text{R-SOH} \\
\text{R-S-S-R} & \rightarrow \text{R-SOH} \\
\text{R-S-S-R} & \rightarrow \text{R-SO}_3\text{H} \\
\text{R-S-S-R} & \rightarrow \text{R-SO}_3\text{H} \\
\text{R-S-S-R} & \rightarrow \text{R-SO}_3\text{H} \\
\end{align*}
\]

Scheme 3.1. Schematic representation of disulphide bond oxidation [131].
3. Results and discussions

reaction scheme. The functional groups involved in cystine oxidation are summarised in table 3.3 [74].

Table 3.3. Products of cystine oxidation

<table>
<thead>
<tr>
<th>Cystine oxides (S-S bond intact)</th>
<th>Sulphur acids (S-S bond fission)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-SO-S-R</td>
<td>monoxide</td>
</tr>
<tr>
<td>R-SO₂-S-R</td>
<td>dioxide</td>
</tr>
<tr>
<td>R-SO₂-SO-R</td>
<td>trioxide</td>
</tr>
<tr>
<td>R-SO₂-SO₂-R</td>
<td>tetroxide</td>
</tr>
<tr>
<td>R-SOH</td>
<td>cysteine sulphenic acid</td>
</tr>
<tr>
<td>R-SO₂H</td>
<td>cysteine sulphinic acid</td>
</tr>
<tr>
<td>R-SO₃H</td>
<td>cysteic acid</td>
</tr>
</tbody>
</table>

Cystine oxidation results in decreasing the cross-link density of the protein, providing additional anionic sites in the form of cysteic acid residues and thus an increased alkaline solubility [134]. The isoelectric point of hair is lowered, and the interchain cohesive forces are greatly modified due to disruption of salt and hydrogen links [135]. The water wettability [125,52], the porosity and the moisture adsorption [123,126,135] of the bleached hair increase at the same time with the decrease of the wet tensile properties [123,125,126].

Since the bleaching compositions are usually working at pH values between 9 and 11, side reactions as hydrolysis of the peptide and amide bonds and formation of new cross-links as lanthionine or lysinoalanine are possible. The amide hydrolysis decreases the isoelectric point of the fibres by the hydrolysis of the amide groups of aspartic and glutamic acids; in addition to the cysteic acid formation, this will increase the ratio of acidic to basic residues in the fibres. By the use of high pH values and long reaction times, also the hydrolysis of peptide bonds can occur (scheme 3.2), leading to the decrease of dry tensile properties of the hair fibres [3].

Scheme 3.2. Alkaline hydrolysis of a peptide bond

Under the action of strong bases cysteine residues are decomposed to yield dehydroalanine residues and hydrogen sulphide. Simultaneously, the β-elimination of cystine residues results in dehydroalanine and S-tiocysteine residues. Upon addition of cysteine and lysine residues to
dehydroalanine residues, lanthionine and lysinoalanine residues are formed [74] (scheme 3.3). The formation of new cross-links will decrease the solubility of the hair proteins.

Scheme 3.3. Lanthionine and lysinoalanine residues formation upon keratin treatment in alkaline media

The various changes at the protein level induced by disulphide bond oxidation, peptide bond hydrolysis or new cross-links formation can influence the protein patterns obtained for hair samples subjected to bleaching treatments.

Hair tresses of commercial, European mixed hair are used for bleaching experiments; these include a standard procedure, procedures with prolonged treatment time, and also multiple treatments. The effects of the bleaching treatments on the electrophoretical pattern of the protein extracted from the hair samples are evaluated using the standard protocol previously developed (and used also for the untreated hair samples) and the results obtained from the statistical analysis are discussed.

The influence of the bleaching time on the electrophoretical pattern of human hair proteins

The bleaching experiments are conducted in conformity to the specific instructions for use attached to the commercial product and also at prolonged duration, trying to get closer to the use in practice, when the times recommended are not respected in the hope to obtain “better” results. The bleaching times used for investigating the influence of the bleaching time on the
hair proteins are 30 (standard procedure), 40, 50, 60, 120 and 180 min. The bleaching sequences are followed by a standard washing procedure with 15% laurylethersulphate (LES), pH 5.8, for neutralising the oxidised hair in the alkali medium.

In figure 3.15 the electrophoretical protein patterns of commercial hair sample for different bleaching times are presented.

![Electrophoretical protein patterns](image)

**Figure 3.15.** Electrophoretical protein patterns of commercial, European mixed hair samples for different bleaching times

SDS – PAGE, 15 % T, 3% C
Derivatisation: Iodoacetamide
Detection: CBB R 250

The bleaching treatments applied for 30 or 40 min do not induce significant changes on the electrophoretical pattern of the hair proteins. The intensity of the KIFs is slightly increased, but the densitogram profile does not change much. At longer treatment times a higher solubility of the KIFs is visible, expressed by higher intensity of the low-sulphur protein bands; at the same time, the individual bands start to become indistinct, indicating the presence of proteins with unusual molecular weight produced by peptide bonds cleavage. The changes in the area of low-sulphur proteins are accompanied by a strong decrease of the band intensity for the high-sulphur proteins, for extreme conditions even the degradation of the matrix proteins occurring. An illustrative example of these changes is presented in the figure 3.16, where the densitograms for the untreated sample and the sample bleached for 180 min are superimposed for an easier comparison:
3. Results and discussions

![Figure 3.16. Comparison between the densitograms for untreated and 180 min bleached hair sample (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)](image)

The intensity variation in opposite directions for the protein bands belonging to the low sulphur and high sulphur proteins is due to the concurrent reactions caused in the fibres by the action of the oxidant agents in an alkaline medium, as it is the case for the commercial bleaching products. One of these processes is cysteic acid formation due to the oxidation of the disulphide bridge, producing additional anionic sites especially on the proteins rich in cystine. Another process is the hydrolysis of peptide bonds when proteins with untypical molecular weight are extracted from the hair fibres. On the other hand, the alkaline medium induces the formation of new cross-links such as lanthionine and lysinoalanine.

The traditional physical methods used to detect damage associated with bleaching are satisfactory for measuring the extent of deterioration, but are of little value for elucidating the chemical character of the damage. The latter can best be ascertained by chemical analysis, so that the first correlation uses the content of sulphur-containing amino acids of bleached samples. The comparison of the content in cystine and its degradation products for the bleached samples shows a systematic increase of the cysteic acid content with the bleaching time, which correlates with the systematic decrease of the cystine content. The concurrent process, formation of lanthionine, does not show a systematic dependence on the bleaching duration. The amino acid analysis does not reveal any intermediate oxidation product of
cystine which might be formed during the bleaching process. These compounds are, however, very unstable under alkaline conditions and any remaining would disproportionate to cystine and cysteic acid. The variation of the content of the significant sulphur-containing amino acids with the bleaching time is presented in table 3.4.

Table 3.4. The cystine, cysteic acid and lanthionine content of bleached samples in relation to bleaching time (results in % mol)

<table>
<thead>
<tr>
<th>Bleaching time, in min</th>
<th>Cystine (% mol)</th>
<th>Cysteic acid (% mol)</th>
<th>Lanthionine (% mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9,7</td>
<td>0,5</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>8,6</td>
<td>1,1</td>
<td>0,30</td>
</tr>
<tr>
<td>40</td>
<td>8,2</td>
<td>1,6</td>
<td>0,35</td>
</tr>
<tr>
<td>60</td>
<td>8,0</td>
<td>1,8</td>
<td>0,15</td>
</tr>
<tr>
<td>120</td>
<td>6,7</td>
<td>2,9</td>
<td>0,30</td>
</tr>
<tr>
<td>180</td>
<td>6,5</td>
<td>4,3</td>
<td>0,20</td>
</tr>
</tbody>
</table>

The association of the amino acid analysis data with the electropherograms of the same samples suggests that the main reaction in the case of the bleaching treatments is the oxidation of the disulphide bridge with cysteic acid formation. Small amounts of lanthionine appear also in the bleached samples as a side product, but their variation does not follow any system.

The increase of the protein solubility for the bleached samples, depicted by the higher intensity of the KIF bands on the gels, correlates with the decrease of the cystine content with the bleaching time. This has as a result a lower cross-linking degree of the hair proteins at the intermediate filaments level in the bleached samples and correspondingly a increased solubility.

The increase of the cysteic acid content with the bleaching time leads to an increased number of anionic sites on the protein molecules; this is especially significant for the KAP proteins, due to their higher cystine content. The negatively charged proteins stain fainter with the anionic triphenylmethane dye CBB R 250, the systematic decrease of the band intensity for the high-sulphur proteins with the bleaching time correlating therefore with the increase of the cysteic acid content.

The formation of new cross-links in the form of the amino acid lanthionine cannot correlate with any of the observed variations of the protein band intensity.
The hydrolysis of the peptide bonds with formation of a wide molecular weight protein spectrum is visible at the level of KIFs, expressed especially by the broadening of the protein bands, as well as at the level of KAPs, where some of the protein bands are broader and new protein bands appear. These phenomena are present in particular for long treatment times.

The statistical analysis of the protein patterns, previously applied to the classification of untreated hair samples, has shown that the bands with the higher intensity are the main differentiation factor between the different patterns. The discrepancy between the intensity of KIF and KAP bands is amplified in the case of bleached samples, where the oxidation of the disulphide bridges allows a higher solubility of the proteins contained in the intermediate filaments and at the same time determines a lower staining intensity of the proteins belonging to the matrix of the hair fibres. The low staining intensity of KAP bands is due to the formation of additional anionic sites in the form of cysteic acid. If the cluster analysis is performed using the whole protein pattern, the results will be described probably only by the modification appeared at level of the low-sulphur proteins. For an objective analysis of the bleached hair samples, the digitised densitograms are analysed separately for the areas containing the KIFs and KAPs, as it is presented in the figure 3.7, chapter 3.3.2.2.

The results obtained for KIFs and KAPs are presented in figure 3.17. Both dendrograms show two main clusters containing samples with a different damage degree: Cluster I on both dendrograms displays the samples with a low damage level (the untreated samples and the samples bleached 30, 40 and 50 min), and Cluster II the samples with a higher damage degree (the samples bleached for 60, 120 and 180 min). The clusters II of both dendrograms have highly similar structures, with a shorter linkage distance between the most damaged samples, the ones bleached for more than 2 h. Cluster I on the dendrogram for KIFs does not group the samples as a function of bleaching time, suggesting an unsystematic damage of these proteins for short bleaching times. The damage is expressed in this case mainly as an increased solubility of these proteins. The Cluster I of KAPs presents a different structure, grouping together the bleached samples at linkage distances increasing with bleaching time. This suggests a systematic decrease of the KAP band intensity with treatment duration, in other words, a systematic damage as a function of bleaching time. This result is also supported by previous investigations on bleached wool, which demonstrated a higher susceptibility of intermicrofibrillar proteins to oxidative treatments [136]. Nevertheless, the similarity between
the two dendrograms suggests relatively equal extents to which the morphological components of the hair are affected by bleaching treatments.

Figure 3.17. Dendrograms of KIFs (a) and KAPs (b) for bleached samples (dendrograms for 7 samples, Ward’s method, parameter Euclidean distance)

UN – untreated hair sample
B30 – 30 min bleached
B40 – 40 min bleached
B50 – 50 min bleached
B60 – 60 min bleached
B120 – 120 min bleached
B180 – 180 min bleached
The influence of the multiple bleaching treatments on the electrophoretical pattern of human hair proteins

The continuous growing of the hair makes it necessary to repeat the bleaching treatment every six weeks. In practice it is difficult to avoid repeated treatments of certain hair regions, even when the treatment should be applied only on the hair area next to the root. For this reason, the influence of multiple bleaching treatments with different duration on the hair proteins was studied.

Tresses of commercial, European mixed hair are treated repeatedly with the commercial bleaching product, using different treatment times. The proteins extracted from the hair samples are subjected to SDS-PAGE, and the gels are stained with CBB R 250. The obtained protein patterns are presented in the figure 3.18.

Figure 3.18. Electrophoretical protein patterns for multiple bleached hair samples – commercial, European mixed hair.
SDS – PAGE, 15 % T, 3% C
Derivatisation: Iodoacetamide
Detection: CBB R 250

The changes appearing in the electrophoretical protein patterns are related to the treatment time, as presented before, and also to the number of treatments. It is possible to observe changes in the intensity of the protein bands belonging to the KIFs and KAPs for the
treatments applied. The intensity variation of the KIF bands does not show any systematic dependence on the cosmetic treatment, the increase and decrease of the band intensities suggesting concurrent processes whose effects overlap each other in a complex way. On the other hand, the decrease of the band intensities for KAPs with treatment time and number of treatments underlines once more the favourite reaction path of the bleaching formulation with the hair proteins: the oxidative degradation of the disulphide bridges with formation of cysteic acid, leading to the accumulation of negative charges on the high-sulphur proteins and lower staining intensity with CBB R 250.

After gel scanning and analysis, the densitograms are digitised and subjected to statistical analysis. The result obtained with cluster analysis for KAPs in the case of multiple bleached hair samples is presented in figure 3.19. The samples are distributed in two clusters. Cluster I groups the samples treated one time and the double treated sample for 30 min, the mildest double treatment. In Cluster II are the multiple treated samples, whose protein band intensities decrease systematically.

![Dendrogram of KAPs for multiple bleached samples](image)

**Figure 3.19.** Dendrogram of KAPs for multiple bleached samples (dendrogram for 11 samples, Ward’s method, parameter Euclidean distance)

UN – untreated hair sample
B1x30 – 1x30 min bleached
B2x30 – 2x30 min bleached
B3x30 – 3x30 min bleached
B1x40 – 1x40 min bleached
B2x40 – 2x40 min bleached
B3x40 – 3x40 min bleached
B1x50 – 1x50 min bleached
B2x50 – 2x50 min bleached
B3x50 – 3x50 min bleached
B1x60 – 1x60 min bleached
B2x60 – 2x60 min bleached
The systematic variation of the protein band intensities with treatment time and number of treatments in the case of KAPs can be observed in figure 3.20 for one subgroup of Cluster II from figure 3.19:

![Image of densitograms](image)

**Figure 3.20.** Overlapped densitograms of the KAPs area for multiple bleached samples (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)
- B3x30 – 3x30 min bleached
- B3x40 – 3x40 min bleached
- B2x60 – 2x60 min bleached

The anterior figure depicts the stronger decrease of the protein band intensities for multiple treatments with the duration of the treatment. For multiple bleaching the damage of the hair fibre is more advanced in the case of longer treatment duration, even when the total treatment time is equal, as it is the case for the samples B3x40 and B2x60.

The content of the amino acids of interest, cystine and cysteic acid, prove in the case of multiple bleaching treatments a systematic decrease, respective increase related to the time and number of bleaching treatments, as can be seen from table 3.5. On the other hand, the content of the amino acid lanthionine determined for the multiple bleached samples is again missing a systematic variation with respect to the protein patterns.
Table 3.5. The cystine, cysteic acid and lanthionine content of multiple bleached samples in relation to bleaching time and number of bleaching treatments

<table>
<thead>
<tr>
<th>Bleaching time in min</th>
<th>Cystine (% mol)</th>
<th>Cysteic acid (% mol)</th>
<th>Lanthionine (% mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9,7</td>
<td>0,5</td>
<td>-</td>
</tr>
<tr>
<td>1x30</td>
<td>8,6</td>
<td>1,1</td>
<td>0,30</td>
</tr>
<tr>
<td>2x30</td>
<td>8,3</td>
<td>1,8</td>
<td>0,25</td>
</tr>
<tr>
<td>3x30</td>
<td>7,4</td>
<td>2,3</td>
<td>0,30</td>
</tr>
<tr>
<td>1x40</td>
<td>8,2</td>
<td>1,6</td>
<td>0,35</td>
</tr>
<tr>
<td>2x40</td>
<td>7,5</td>
<td>2,1</td>
<td>0,50</td>
</tr>
<tr>
<td>3x40</td>
<td>7,3</td>
<td>2,9</td>
<td>0,40</td>
</tr>
</tbody>
</table>

The amount of the protein extracted from the hair samples subjected to different bleaching treatments was assayed using an analysis kit able to determine protein in the presence of electrophoresis buffers. The amount of soluble protein in the extracts subjected to electrophoresis increases with the number of treatments applied (table 3.6), suggesting an insignificant role for the cross-linking phenomenon via lanthionine formation in the bleaching processes studied, also evidenced for oxidatively bleached wool [137].

Table 3.6. Extracted protein from the bleached commercial hair samples assayed using the PlusOne 2-D Quant Kit from Amersham Biosciences

<table>
<thead>
<tr>
<th>Bleaching time in min</th>
<th>Protein amount (expressed as mg BSA / ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1,9 ± 0,09</td>
</tr>
<tr>
<td>1x30</td>
<td>2,5 ± 0,05</td>
</tr>
<tr>
<td>2x30</td>
<td>2,8 ± 0,06</td>
</tr>
<tr>
<td>3x30</td>
<td>2,9 ± 0,09</td>
</tr>
</tbody>
</table>

The results show the predominance of the oxidation reaction of the disulphide bridges as effect of the commercial bleaching formulations on the human hair. The increase of cysteic acid content and the decrease of cystine content correlate with the duration and the number of bleaching treatments. The lanthionine formation does not follow a systematic variation, acting as an occasional side reaction.
3. Results and discussions

3.5.1.2. Dyeing

The dyes used for human hair may be described as permanent or oxidation dyes, semi-permanent dyes, temporary dyes or colour rinses and other dyes, including basic dyes, metallic dyes, reactive dyes and vegetable dyes. Oxidation dyeing of human hair has been practised for over 100 years and evolved out of an observation that the colourless \( p \)-phenylendiamine produces a coloured compound when subjected to oxidation, and that this reaction could be used to colour a variety of substrates [138].

The products for temporary dyeing of hair generally comprise water soluble acid dyes and water soluble pigments, which are deposited on the surface of the hair. These colours are removable by a single effective shampooing. The formulations for semi-permanent dyeing of hair contain for the most part simple derivatives of nitroanilines, nitrophenylendiamines and nitroaminophenols. These dyes penetrate into the cuticle and partially into the cortex of the hair. As a result, the colouring effects of these dyes can resist 5-10 shampooings [139].

The most important of the commercial hair dyes are the oxidation dyes, often referred to as permanent hair dyes. The formulation for permanent dye colouring are marketed as two component kits. One component contains the dye precursors, which form active intermediates, and dye couplers, which condense with the active intermediate. The other component is a stabilised solution of an oxidising agent, usually hydrogen peroxide; attempts to minimise the hair and skin damage by the use of air oxidation activated with oxidases were also made [140]. The dye precursors are difunctional ortho- or para-diamines or aminophenols capable of oxidising to diiminium or quinonium ions. These active intermediates condense with electron-rich dye couplers, which can be substituted resorcinols or meta-phenylendiamines, usually containing a vacant position para to the amine or phenolic group. The two components are mixed immediately prior to use. The precursors and peroxide diffuse into the hair shaft, where colour formation takes place after a cascade of chemical reactions. The dye precursors are oxidised by hydrogen peroxide to active intermediates, which undergo rapid reaction with the couplers resulting in dinuclear, trinuclear or polynuclear dyes and pigments (scheme 3.4). These molecules are too large to escape from hair structure. The reactions are usually carried out in alkaline medium, and adjusting the proportions of precursors, couplers and peroxide, as well as the time of contact with the hair, the hair can be made lighter or darker in one process [3]. Oxidative hair dyes are resistant to fading by shampooing, but re-colouring of hair is required every 6 weeks approximately, due to hair growth.
3. Results and discussions

Scheme 3.4. Example of indo dye formed in oxidative hair dyeing from \(\text{p-phenylenediamine} \) and resorcinol.

Last developments in the permanent dyes area made use of the 3,4-dihydroxyphenylalanine, which is converted to the precursor of eumelanins (5,6-dihydroxyindole and its 2-carboxylic acid) and subsequently polymerised \textit{in situ} to dye the hair (scheme 3.5), the chemical imitation of some of the basic steps from the natural biosynthetic pathway to melanin leading to a promising new system for hair dyeing [141].

Scheme 3.5. Simplified scheme of eumelanins synthesis
3. Results and discussions

Comparative studies of oxidative dyeing for wool and human hair [142] showed a higher complexity of the reactions in the case of human hair, suggesting an increase of the dye deposition with dye precursor concentration, dyeing time and solution pH. The alterations produced in hair fibres following oxidative dyeing processes are expected to be similar to those produced by bleaching, although on a smaller scale [126,143].

The oxidation dyes are capable of giving permanent hair colour in an infinite variety of shades and a perfect coverage of white hair. From the very large spectrum available, two different permanent dyes are used for the present study. The first one produces a lighter colour than the initial hair colour and uses an effective hydrogen peroxide concentration of 9%. The second dye used, a so-called tone-on-tone dye, reproduces the initial hair colour and uses an effective hydrogen peroxide concentration of 3%. Beside treatments in conformity to the use instructions, practical modifications regarded as capable to “improve” the results of the dyeing process are applied. These consist of prolonged treatment times with 30 min for both cases and the use of a hair dryer for increased temperature values. As in the case of bleaching, the effects of multiple treatments on hair fibres are investigated.

**The influence of the dyeing time on the electrophoretical pattern of human hair proteins**

The electrophoretical patterns of the proteins extracted from lighter dyed hair samples for different dyeing times are presented in figure 3.21.

![Electrophoretical protein patterns](image)

Figure 3.21. Electrophoretical protein patterns for lighter dyed hair samples

SDS – PAGE, 15 % T, 3% C
Derivatisation: Iodoacetamide
Detection: CBB R 250

The influence of the dye formulations for lighter hair colour at times recommended in the use instructions of the product (50 min), as well as for 30 min prolonged treatment times on the commercial, European mixed hair used for the study are much stronger than for bleaching treatments. This behaviour was repeatedly observed in practice for the material used [88].
The KIFs do not suffer important modifications, although their intensity increased at longer treatment times. On the other hand, the KAPs are strongly affected, their intensity decreasing significantly. Reasons for this contradictory behaviour with regard to the literature data could be the effective hydrogen peroxide concentration (9% for dyeing compared to 6% for bleaching formulations) and the treatment times (50 min for dyeing, 30 min for bleaching) recommended for the commercial cosmetic products. The differences between the protein patterns for the untreated sample and the 50 min lighter dyed sample are presented in figure 3.22 by superimposing the corresponding densitograms.

![Figure 3.22](image)

Figure 3.22. Comparison between the densitograms of extracts from untreated and 50 min lighter dyed hair sample (commercial mixed hair, SDS-PAGE, detection CBB R 250)

The additional heating using the hair dryer does not have any visible influence and the protein pattern remains unchanged.

For dyeing treatments with the *tone-on-tone* dye the effects on the protein pattern are not as strong as for the lighter dye. The aspect of the electropherograms for the recommended treatment time (40 min) and for 30 min prolonged treatment time is presented in figure 3.23, and the corresponding densitograms show a moderate decrease of the intensity of the bands belonging to KAPs, while the band profile for KIFs does not change.
3. Results and discussions

The significant differences ascertained between the effects of the dyeing treatments for the cases investigated seem to be closely related to the effective hydrogen peroxide concentration of the dye formulations. The high concentration of hydrogen peroxide in the lighter colouring dye (9 % H₂O₂) determines a stronger damage of the hair proteins even for the recommended treatment time, while the dye formulation for similar hair colour (3 % H₂O₂) does not much affect the protein pattern at yet longer treatment times.

The influence of the multiple dyeing treatments on the electrophoretical pattern of human hair proteins

In practice it is often necessary to apply repeated dyeing treatments, due to the natural growing of the hair fibres. The narrowing of the treated area to the hair root cannot avoid multiple treatments for neighbouring hair regions.

The influence of multiple treatments in different conditions is investigated. The study includes the influence of the treatment duration (recommended 50 and 40 min for lighter, respective tone-on-tone dye and prolonged with 30 min for every treatment) and of the additional heating with a hair dryer during the treatment. The over-processing of the samples by the use of longer treatment times and additional heating is done intentionally, trying to simulate real life situations, when a better colouring effect is looked for.

The multiple treatments change the electrophoretical protein pattern of the human hair for the lighter colouring dye, as well as for the mild tone-on-tone dye. In the case of lighter colour treatment, the intensity of the KIF bands is mildly modified and the bands present the tendency to spread laterally (figure 3.24) with repeating the treatment. The intensity of KAP bands decreases with every newly applied treatment, the tendency being described by a
3. Results and discussions

continuous decreasing of the intensity of KAP bands compared to the intensity of KIF bands with the number of treatments applied.

Figure 3.24. Comparison between the densitograms for multiple lighter dyed hair samples at 50 min treatment time (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)

With longer treatment time stronger modifications appear in the electrophoretical protein pattern, becoming visible also for the mild dyeing treatment (figure 3.25): the intensity of KIF bands increases and spreading of the protein bands appears; as expected, the intensity decrease for KAP bands is milder than for the case of lighter dyed hair samples.

Figure 3.25. Electrophoretical protein patterns for multiple tone-on-tone dyed hair samples
SDS – PAGE, 15 % T, 3% C
Derivatisation: Iodoacetamide
Detection: CBB R 250

As a conclusion, the general behaviour of the oxidatively dyed samples follows the tendencies already observed in the study of bleached samples. An increased content of cysteic acid and higher alkali solubility were also reported for oxidatively dyed hair [134].
It can be observed that the use of additional heating during the dyeing process produces radical changes in the protein pattern in the case of multiple treatments. These changes are illustrated in figure 3.26, where the densitograms corresponding to the electrophoretical patterns of multiple lighter dyed hair samples with additional heating provided by a hair dryer are superimposed. The densitogram belonging to the sample treated one time does not present modifications of the protein pattern compared to the samples treated without additional heat, as already remarked at the study of the influence of dyeing time on electrophoretical protein patterns of human hair. Dramatic modifications of the protein pattern appear following additional dyeing treatments in similar conditions: the bands belonging to KIFs increase markedly in intensity and broaden very much, while the bands corresponding to KAPs become very faint, denoting an advanced degradation of the initial proteins.

Figure 3.26. Comparison between the densitograms for multiple lighter dyed hair samples with additional heating (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)

A similar evolution is found for multiple tone-on-tone dyed samples with additional heating. Even the tone-on-tone dyeing treatment manifests itself as mild and consequently moderately damaging, the multiple dyeing with additional heating leads to strong changes in the electrophoretical protein pattern. The bands corresponding to KIFs are broadened and diffuse, and the bands corresponding to KAPs either disappeared or have very low intensities. The densitograms for the single treated and three-time treated samples are superimposed in figure 3.27.
Figure 3.27. Comparison between the densitograms of extracts of multiple *tone-on-tone* dyed hair samples with additional heating (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)

The visual analysis of the electrophoretic patterns suggests that, similarly to the bleaching treatments, the easier to quantify and systematic changes take place at the intermicrofibrillar protein level, the band intensities of KAPs varying proportional to the time and/or number of treatments.

The variations associated with KIFs do not follow a recognisable system, being expressed generally through a higher proteins solubility. The severe treatments lead to broadened and diffuse protein bands.

The influence of different dyeing treatments on the electrophoretical pattern of hair proteins can be evaluated using the statistical method previously developed. The densitograms were standardised, digitised and subjected to cluster analysis. The dendrogram of KAPs for dyed samples is presented in figure 3.28.
3. Results and discussions

As can be seen in figure 3.28, the samples are grouped in three main clusters. Cluster I contains the samples whose KAPs suffer only small modifications following dyeing, as are the samples subjected one time to mild tone-on-tone dyeing (1x40 min without and with additional heating and 1x70 min), the low damage induced by tone-on-tone dyeing processes being based on the low effective hydrogen peroxide concentration (3%). Cluster II groups the dyed samples which suffer a stronger damage, these being also divided into two subsequent groups. Cluster IIa comprises the samples dyed once with lighter colour, no matter what conditions were used, the samples dyed two times with lighter colour in conformity with the use instructions and the double tone-in-tone dyed sample, all these samples presenting a
moderate damage degree. In Cluster IIb are grouped the samples whose KAPs have an advanced damage degree due to triple dyeing treatments with lighter colour or with tone-on-tone dyeing with additional heating. Cluster III contains the multiple lighter dyed samples with additional heating, whose bands belonging to KAPs are practically not recognisable. The study of different dyeing treatments in various conditions suggests an extreme damaging effect of the oxidative dyeing formulations associated with additional heat.

3.5.1.3. Comparison of electrophoretical analysis of bleached hair samples with traditional test methods

The damage for bleached hair samples is evaluated by traditional methods such as high-pressure differential scanning calorimetry (HPDSC), tensile properties (stress-strain data), or sulphur-containing amino acids content. These methods are applied for the same bleached samples analysed using the combined electrophoretical and statistical methods previously described for a direct comparison of the obtained results.

Thermal transitions in keratin have been discussed in many papers devoted to the properties of wool and other sources of keratin such as human hair [144,145]. Thermal transitions in keratins are strongly affected by water, depending on the amount of water present. The process characterised by a HPDSC maximum in the temperature range 210-250°C for different dry keratins is referred to as denaturation of the α-helical intermediate filament structures of keratin [146,147]. For human hair, the denaturation peak shifts from approx. 240°C for dry fibres to around 150°C in water, ensuring easier measurements without background effects [148].

The thermoanalytical investigations of bleached hair samples by HPDSC in water lead to the results presented in table 3.6. The values for the denaturation temperature $T_D$ and for the denaturation enthalpy $\Delta H_D$ are in the usual range for α-keratins, but their variation with the applied treatment does not correlate to the literature data. Wortmann et al. [36] described a continuous decrease of the values for denaturation temperature and denaturation enthalpy with the number of cosmetic treatments, which cannot be observed in this study. The explanation for the apparent thermostabilisation of the bleached samples compared to the untreated sample could be an unusual high resistance of the commercial, European mixed hair sample used for the cosmetic treatments to the action of chemical reagents.
Table 3.6. Denaturation temperatures $T_D$ and enthalpies $\Delta H_D$ for multiple bleached hair samples (HPDSC triplicate measurements)

<table>
<thead>
<tr>
<th>Bleaching time in min</th>
<th>Denaturation temperature $T_D$ in °C</th>
<th>Denaturation enthalpy $\Delta H_D$ in J/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>153</td>
<td>16,5</td>
</tr>
<tr>
<td>1x 30</td>
<td>160</td>
<td>19,3</td>
</tr>
<tr>
<td>2x 30</td>
<td>161</td>
<td>16,8</td>
</tr>
<tr>
<td>3x 30</td>
<td>161</td>
<td>18,2</td>
</tr>
</tbody>
</table>

Another type of traditional test methods for oxidative damage assessment is performed by evaluating mechanical properties of treated fibres, such as elastic modulus, stress at break, extension at break etc. The mechanical properties of $\alpha$-keratin fibres are primarily related to the two components of the elongated cortical cells, the highly ordered intermediate filaments which contain the $\alpha$-helices, and the matrix in which the intermediate filaments are embedded [39,126, 149-152].

The presence of the cystine in $\alpha$-keratin fibres is responsible of their high stability to environmental degradation by heat, cold, light, water, biological attack and mechanical distortion. Therefore, the disruption of cystine cross-links during bleaching has a major influence on the wet tensile properties of keratin materials [132]. It is well established that in both hair and wool the disulphide bonds contribute largely to the wet strength, while the dry strength of the fibres remains unaffected unless more than 60% of the cystine cross-links have been broken [123]. Both the dry and wet strength of keratin fibres are greatly influenced by peptide bond breakdown [3,125].

The measurement of the longitudinal mechanical properties of keratin fibres, which are shown to be dependent on temperature, humidity and time-scale of the experiment, is frequently applied to assess the damaging effects of chemical treatments [39]. Several parameters calculated from stress-strain data are reported to be sensitive to modifications of hair fibres as a result of cosmetic treatments or environmental degradation. The most commonly used parameters are the 20% index [126], the yield point (stress) at 15% elongation [122,123,153], the tensile strength or extension to break [123,125] and the hysteresis ratio for the mechanical testing of untreated and treated hair in the wet state [40]. In summary, these papers suggest that “in use” bleaching of hair commonly produces a decrease in tensile properties of as much as 25%, with greater losses occurring when the fibres are frosted or stripped.
The stress-strain measurements in water with an Instron Tensile tester lead to the values of the breaking modulus presented in table 3.7, from which it is possible to correlate the treatment type to the breaking modulus value.

Table 3.7. Breaking modulus of different bleached samples of commercial, European mixed hair, determined in water

<table>
<thead>
<tr>
<th>Bleaching time in min</th>
<th>Breaking modulus in MPa</th>
<th>Standard deviation in MPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>309</td>
<td>30.0</td>
</tr>
<tr>
<td>1x30</td>
<td>292</td>
<td>25.4</td>
</tr>
<tr>
<td>2x30</td>
<td>289</td>
<td>33.2</td>
</tr>
<tr>
<td>3x30</td>
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<td>23.5</td>
</tr>
<tr>
<td>1x40</td>
<td>288</td>
<td>28.4</td>
</tr>
<tr>
<td>2x40</td>
<td>279</td>
<td>32.5</td>
</tr>
<tr>
<td>3x40</td>
<td>274</td>
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<tr>
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<tr>
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<tr>
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<td>17.6</td>
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<tr>
<td>1x120</td>
<td>258</td>
<td>22.2</td>
</tr>
<tr>
<td>1x180</td>
<td>243</td>
<td>16.9</td>
</tr>
</tbody>
</table>

As presented in the previous table, the variation of the breaking modulus is proportional to the time and number of bleaching treatments. The method allows the evaluation of the oxidation degree of disulphide bonds, which contribute largely to the wet strength of the fibres.

The amino acid analysis of the hair samples provides information about the effects of oxidative and alkaline processes on the hair proteins. This analysis takes into account not only the modifications appeared at the level of the cortex proteins, but also oxidation and cross-linking in the high-sulphur proteins of the cuticle, especially in the cystine-rich exocuticle.

The reaction between keratin and hydrogen peroxide, the oxidising agent from bleaching formulations, is confined mainly to cystine residues. Cysteic acid is the major product of cystine oxidation during the bleaching of hair, the decrease in cystine being almost quantitatively matched by a corresponding increase in cysteic acid. On the other hand, the alkaline medium used as basis for bleaching formulations can induce the cross-linking of the protein chains via lanthionine formation. Consequently, the effect of bleaching was investigated determining the content of cystine, cysteic acid and lanthionine in different bleached samples. The content of the mentioned amino acids in hair samples bleached in different conditions is given in table 3.8.
### Table 3.8. The cystine, cysteic acid and lanthionine content of bleached samples in relation to the bleaching time and the number of bleaching treatments

<table>
<thead>
<tr>
<th>Bleaching time in min</th>
<th>Cystine (% mol)</th>
<th>Cysteic acid (% mol)</th>
<th>Lanthionine (% mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9,7</td>
<td>0,5</td>
<td>-</td>
</tr>
<tr>
<td>1x30</td>
<td>8,6</td>
<td>1,1</td>
<td>0,30</td>
</tr>
<tr>
<td>2x30</td>
<td>8,3</td>
<td>1,8</td>
<td>0,25</td>
</tr>
<tr>
<td>3x30</td>
<td>7,4</td>
<td>2,3</td>
<td>0,30</td>
</tr>
<tr>
<td>1x40</td>
<td>8,2</td>
<td>1,6</td>
<td>0,35</td>
</tr>
<tr>
<td>2x40</td>
<td>7,5</td>
<td>2,1</td>
<td>0,50</td>
</tr>
<tr>
<td>3x40</td>
<td>7,3</td>
<td>2,9</td>
<td>0,40</td>
</tr>
<tr>
<td>1x60</td>
<td>8,0</td>
<td>1,8</td>
<td>0,15</td>
</tr>
<tr>
<td>1x120</td>
<td>6,7</td>
<td>2,9</td>
<td>0,30</td>
</tr>
<tr>
<td>1x180</td>
<td>6,5</td>
<td>4,3</td>
<td>0,20</td>
</tr>
</tbody>
</table>

From table 3.8 it is seen that the content of cystine decreases with bleaching time and number of treatments. The cysteic acid content increases systematically with the bleaching gravity, reaching in extreme conditions an almost 9-fold increase, while the lanthionine content variation does not correlate with the treatment applied.

The results of HPDSC measurements and the values of the parameters obtained from stress-strain curves support an observation made during electrophoretical analysis of commercial, European mixed hair. The untreated sample shows a low extractability of the cortical proteins, much lower than for any of the investigated hair samples from individuals, which can be related to an unusually high stability and resistance to chemical reagents of this hair sample.

In spite of this constraint, the electrophoretical analysis is able to detect the modifications induced in the protein pattern by different bleaching processes. These modifications can be correlated with the duration and with the number of treatments and can also offer information about the wrong use of certain cosmetic products. Moreover, the electrophoretical analysis is also able to show the morphological components at which the damage takes place and the local damage degree, providing a more comprehensive view of the modifications induced in the hair fibre than any traditional test method. In contrast to the complex results of the method developed using electrophoretical protein patterns and statistical analysis, the traditional test methods provide only a conditional estimation of the state of hair samples subjected to cosmetic processes.
3.5.2. Permanent waving

The first permanent waves formulations, used almost one hundred years ago, consisting of concentrated solutions of alkali or alkaline sulphites, reacted with hair at elevated temperatures using curling irons or electric heaters. Current permanent waves do not require elevated temperatures, thus the designation “cold waves”. These products are based on mercaptans or sulphites, the most common of these being thioglycolic acid, generally employed at concentrations around 0.6 – 0.8 N and pH values between 9 and 9.5. Sulphite waves employ a pH value near 6 and a hydrogen peroxide neutraliser and are gentle to the hair, which means that they can be used on any type of hair, undamaged or damaged.

In the cold waving are two different phases: a) the reducing action of the waving lotion and b) the action of the fixer (or neutraliser), which is generally oxidising. The first phase is intended to act chemically on the bonds determining the cohesion of the protein structure: covalent disulphide cross-linkages, ionic bonds, or hydrogen bonds. The object is to plasticise the fibre momentarily, so as to render it deformable without elasticity. The hair is wetted with a reducing solution and rolled on curlers so that the imposed deformation is in the shape of curls. In the second phase, the curls are set by restoring the initial chemical structure to the fibre. Rupture of ionic linkages or hydrogen bonds only leads to temporary hair deformations, namely hair set. Cleavage of cystine links and their subsequent reforming in a new position is the process that affords permanent deformation [135].

The cleavage of disulphides in keratin fibres by mercaptans is a bimolecular ionic reaction of the SN2 type, involving two nucleophilic displacement reactions by mercaptide ion, first on the symmetrical disulphide, and then on the mixed disulphide. The reduced fibres are then permanently set by using an oxidising agent (scheme 3.6).

\[
\begin{align*}
R-S-S-R^+ + R*-S^- &\leftrightarrow R-S-S-R^- + R-S^+ \\
R-S-S-R^* + R*-S^- &\leftrightarrow R^*-S-S-R^- + R-S^-
\end{align*}
\]

\[2R-S^- + H_2O_2 \rightarrow R-S-S-R + 2HO^-
\]

**Scheme 3.6. Reduction and reoxidation of keratins in permanent waving processes**

- R-S-S-R – symmetrical disulphide
- R-S-S-R* - mixed disulphide
- R*-S^- - mercaptide ion
- R-S^- – reduced keratin anion
Because the active species in the disulphide scission is the mercaptide ion rather than the unionised mercaptan, pH is a critical factor [3]. The reaction of mercaptans with keratin fibres is a relatively specific reaction in mild acid conditions. However, in alkaline media side reactions as peptide bond hydrolysis (scheme 3.2) and formation of lanthionine (scheme 3.7) can also occur.

\[
\begin{align*}
R-S-S-R^* + R-S^- &\rightarrow R-S-R + R^*-S^- + S
\end{align*}
\]

Scheme 3.7. Reaction between mixed disulphide and reduced keratin with lanthionine cross-link formation  
- R-S-S-R\(^*\) - mixed disulphide  
- R-S\(^-\) - reduced keratin  
- R-S-R – lanthionine cross-link  

By treatment with permanent waving formulations similarly to the commercial ones for 20 min, as often used in practice, degrees of cystine reduction in the range of 15-33 % are realised [154]. The high cleavage of cystine residues and the resultant high concentration of cysteine residues produced from the reaction of thioglycolic acid (respective thioglycolate) or sulphite with hair permit molecular reorientation to occur through a disulphide–mercaptan interchange pathway. The reduction occurs primarily in the high–sulphur regions of the hair fibres, the A layer and the exocuticle of the cuticle and the matrix of the cortex. During the reduction step, a highly reduced zone proceeds into the cuticle and into the outer regions of the cortex, leaving an inner zone of unreduced hair.

The chemical changes produced in hair by permanent waving are expressed by small decreases in cystine and corresponding increases in cysteic acid and cysteine [35,50,155]. Small quantities of mixed disulphide, sorbed thioglycolic and dithiodiglycolic acid, as well as intermediate oxidation products of cystine are also found in cold waved hair [156]. At longer reduction times or an extended rinsing period after the reduction step, certain amounts of protein are dissolved from the hair fibres. The wet tensile properties of hair are decreased upon permanent waving, while the dry tensile properties remain virtually unchanged [126]. The swelling capacity of permanently waved hair is increased proportional to the damage rendered by the waving process, producing a substantial increase in the chemical reactivity of hair toward reactions with diffusion as rate limiting step. Increased swelling is an evidence of cortical damage of hair [3]. The fraction of \(\alpha\)-helix in the microfibrils of human hair decreases during permanent waving treatments, changing to the random-coil structure within a relative short treatment time [36,43].
For the present study the modifications caused by permanent waving treatments on the cortex protein of human hair are investigated. Treatments following the use instructions and possible practical changes varying the reduction and reoxidation time, as well as multiple treatments are applied. In table 3.9 the permanent waving treatments and the codification of the corresponding hair samples are given.

<table>
<thead>
<tr>
<th>Hair sample codification</th>
<th>Reduction time in min</th>
<th>Reoxidation time in min</th>
<th>Number of treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PW311</td>
<td>30</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>PW312</td>
<td>30</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>PW313</td>
<td>30</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>PW321</td>
<td>30</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>PW322</td>
<td>30</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>PW323</td>
<td>30</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>PW331</td>
<td>30</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>PW332</td>
<td>30</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>PW333</td>
<td>30</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>PW411</td>
<td>40</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>PW412</td>
<td>40</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>PW413</td>
<td>40</td>
<td>10</td>
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</tr>
<tr>
<td>PW421</td>
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</tr>
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<tr>
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<td>30</td>
<td>2</td>
</tr>
<tr>
<td>PW433</td>
<td>40</td>
<td>30</td>
<td>3</td>
</tr>
<tr>
<td>PW511</td>
<td>50</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>PW521</td>
<td>50</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>PW531</td>
<td>50</td>
<td>30</td>
<td>1</td>
</tr>
</tbody>
</table>

The conditions chosen for the electrophoretical fractionation of the proteins extracted from hair do not allow the evaluation of the reductive effects of permanent waving formulations on the hair proteins, the extraction of protein from hair samples being based on a reductive method. As established in the anterior study on bleached hair samples, it is possible to evaluate the changes due to oxidation (expressed as increased amounts of cysteic acid) and action of alkaline medium and heating (expressed as additional cross-links) on the hair proteins.
3. Results and discussions

The electrophoretical protein patterns for different permanent waving treatments are presented in figure 3.29, exemplifying the cases of multiple treatments in standard conditions (the conditions recommended for the commercial product: 30 min reduction time, 10 min reoxidation time) and with longer times for reduction and also for reoxidation (50 min reduction, followed by 10, 20 and 30 min reoxidation).

![Electrophoretical protein patterns of commercial, European mixed hair samples for different permanent waving treatments](image)

Figure 3.29. Electrophoretical protein patterns of commercial, European mixed hair samples for different permanent waving treatments

SDS-PAGE, 15% T, 3% C
Derivatisation: Iodoacetamide
Detection: CBB R 250

The visual evaluation of the electrophoretical protein patterns shows an intensity decrease for the bands of KIFs with the treatment duration and the number of treatments. The modifications of the protein patterns in the KAPs area for multiple treated samples in standard conditions cannot be visually evaluated, being extremely low. For treatments using longer reoxidation times (20 or 30 min) a certain decrease of the band intensities for KAPs is visible. These aspects are depicted in figure 3.30, where the densitograms for untreated and multiple treated hair samples at similar reduction times and different reoxidation times are superimposed, and in figure 3.31, where the comparison between protein patterns for different reduction times (30 and 50 min), but identical reoxidation times (10 min) is made.
3. Results and discussions

Figure 3.30. Comparison between densitograms for untreated and multiple permanent waved hair samples; 3 x – three times treated; 30:10 perm. waved: 30 min reduction, 10 min reoxidation; 30:20 perm. waved: 30 min reduction, 20 min reoxidation (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)

Figure 3.31. Comparison between densitograms for different permanent waved hair samples; 1 x – one time treated; 30:10 perm. waved: 30 min reduction, 10 min reoxidation; 50:10 perm. waved: 50 min reduction, 10 min reoxidation (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)
In conformity with the result of the investigation, the band intensity of KIFs is affected, for constant reduction times, by the duration of the reoxidation step. The same situation is observed for different reduction times followed by reoxidation processes with constant duration. On the contrary, the KAPs are apparently affected only by increased reoxidation times. The conditions used for this cosmetic treatment are expected to favour the additional cross-linking of the proteins via lanthionine formation as a side-reaction. During permanent waving processes, relative high amounts of proteins are eluted from the hair fibres [55], so that the extractable protein is diminished. Besides, the decrease of the α-helix content of the hair fibres may imply the decrease of the protein solubility, as found for protein fractions from wool [157].

The cluster analysis for KIFs presents the gradual modifications appearing in the electrophoretical pattern following permanent waving in different conditions, varying the reduction and reoxidation time, as presented in figure 3.32.

![Dendrogram of KIFs for different permanent waved hair samples](image)

**Figure 3.32.** Dendrogram of KIFs for different permanent waved hair samples (dendrogram for 7 samples, Ward’s method, parameter Euclidean distance)

- UN – untreated hair sample
- PW311 – reduction time 30 min, reoxidation time 10 min
- PW321 – reduction time 30 min, reoxidation time 20 min
- PW331 – reduction time 30 min, reoxidation time 30 min
- PW411 – reduction time 40 min, reoxidation time 10 min
- PW421 – reduction time 40 min, reoxidation time 20 min
- PW431 – reduction time 40 min, reoxidation time 30 min
3. Results and discussions

The dendrogram shows a systematic clustering of the permanent waved samples as a function of reduction time, and inside of the clusters as a function of reoxidation time. The sample treated using the standard conditions: 30 min reduction time and 10 min reoxidation time, as recommended in the usage instructions for the commercial product, presents the lowest damage degree and separates from the other ones.

The effects of multiple permanent waving treatments on KIFs gain in importance with the prolongation of the individual steps, reduction and reoxidation. The systematic modifications appearing in the electrophoretical pattern of KIFs due to changes in the treatment times are presented in figure 3.33.

![Dendrogram of KIFs](image)

**Figure 3.33.** Dendrogram of KIFs for different permanent waved hair samples (dendrogram for 7 samples, Ward’s method, parameter Euclidean distance)

- UN – untreated hair sample
- PW311 – reduction time 30 min, reoxidation time 10 min, 1x
- PW312 – reduction time 30 min, reoxidation time 10 min, 2x
- PW313 – reduction time 30 min, reoxidation time 10 min, 3x
- PW411 – reduction time 40 min, reoxidation time 10 min, 1x
- PW412 – reduction time 40 min, reoxidation time 10 min, 2x
- PW413 – reduction time 40 min, reoxidation time 10 min, 3x

The grouping of the samples in figure 3.33 is based on intensity decrease of KIF bands due to multiple permanent waving treatments. The decrease is lower for samples treated following the use instructions, suggesting a limited damage also for repeated applications. Prolongation
of reduction time alone with ten minutes induce an increased degree of hair damage, but only after the third treatment.

As mentioned before, the KAPs are affected by prolonged reoxidation times. The cluster analysis for samples subjected to permanent waving treatments at different reduction and reoxidation times is presented in figure 3.34.

![Dendrogram of KAPs for different permanent waved hair samples](image)

- PW311 – reduction time 30 min, reoxidation time 10 min
- PW331 – reduction time 30 min, reoxidation time 30 min
- PW411 – reduction time 40 min, reoxidation time 10 min
- PW431 – reduction time 40 min, reoxidation time 30 min
- PW511 – reduction time 50 min, reoxidation time 10 min
- PW531 – reduction time 50 min, reoxidation time 30 min

Figure 3.34. Dendrogram of KAPs for different permanent waved hair samples (dendrogram for 6 samples, Ward’s method, parameter Euclidean distance)

The clustering of the samples having as a basis the modifications of KAPs is influenced not only by the reoxidation time, but also by the reduction time. The damaging effects of permanent waving treatments on the electrophoretical protein pattern become stronger with total treatment time, duration of the reduction step having a higher influence than the duration of the reoxidation step.
3.5.3. Influence of hot curling

Changing the styling of human hair can be also be done using the water-set method, what means soaking the hair in water and holding it in a given configuration during drying. The hair is left to dry in the air or an electric dryer can be used [135]. The curls obtained in this way are not very stable against changes to higher humidity [3], but represent a comfortable and widely used technique for temporary styling changes. Water-setting hair provides a reversible set to hair due to the involved hydrogen bonds, which can be easily broken and reformed by changing the humidity of the environment. By contrast, permanent waving involves disulphide bonds, which require the intervention of chemical agents and determine a much longer set stability.

A faster styling of hair without chemical treatments can be made using curling irons or electrical curlers, a method often applied in practice. The use of heated curlers presumes direct contact between hair, either dry or wet, and a hot metallic surface, whose operating temperature ranges from 100°C to more than 160°C [158].

The influence of heat in dry and wet conditions on keratin materials is well known from studies on wool. Chemical changes appearing while heating wool in water are slow liberation of hydrogen sulphide and ammonia and formation of lanthionine and lysinoalanine residues [159,160]. Heating wool causes the formation of isodipeptide cross-links between the ε-amino groups of lysine residues and the side-chain amide groups of asparagine and glutamine residues. The extent of damage produced by heating in dry conditions depends also on whether the material is heated in a closed or in an open system, the presence of oxygen determining oxidation processes [159].

Repeatedly blow-drying, where the hair is exposed to temperatures in the range 50-100°C, was also shown to induce hair damage. Heat drying increases the tendency of hair to acquire a static charge during combing and can produce a short term decrease in fibre stiffness. Moreover, small changes in the wet tensile properties are detected in hair dried at high temperatures [161]. Torsional measurements suggested that even brief exposure to elevated temperatures increases fibre rigidity and that this is not just simply associated with the dehydration of the hair fibre [143].

The heating of hair proteins conducts to isodipeptide links, determining the decrease of the protein extractability (see chapter 2.1.3, figure 2.5). The magnitude of this phenomenon depends on the treatment conditions. The appearance of new cross-links in wool fibres occurs already at 80°C [162]. The higher content of lysine and asparagine and glutamine residues in the intermediate filament proteins compared to the content of the same amino acid residues in
keratin-associated proteins can lead to an increased degree of cross-linking of the low-sulphur proteins via isodipeptide bonds, determining the lower solubility of these proteins. Other possible effects of the heating process, as mentioned in the literature from studies on wool are the formation of hydrogen-bonded aggregates and new lanthionine and dityrosine cross-links beside isodipeptides [160,163].

The practical experiments consist of curling small tresses of commercial, European mixed hair, untreated or previously cosmetically treated, using an electrical curler. The treatment is applied five times for five minutes on dry or wet samples.

The protein patterns of the untreated hair samples, a bleached and a permanent waved hair sample before and after the curling treatment in dry and wet state are presented in figure 3.35. A light decrease of protein pattern intensity for dry curled samples compared with the initial samples is visible; the decrease is much stronger in samples treated in the wet state, and seems to affect especially the KIF intensities.

![Electrophoretical protein patterns of untreated and cosmetic treated hair samples, dry and wet curled (commercial, European mixed hair)](image)

**Figure 3.35.** Electrophoretical protein patterns of untreated and cosmetic treated hair samples, dry and wet curled (commercial, European mixed hair)
SDS-PAGE, 15% T, 3% C
Derivatisation: Iodoacetamide
Detection: CBB R 250

The heating of differently treated hair samples during curling processes conducts to intensity decrease for the electrophoretical patterns obtained for the proteins extracted from these hair samples. The registered decrease is stronger for wet curled samples than for samples subjected to the same treatment without wetting, suggesting a higher influence of the contact
3. Results and discussions

heat on the hair proteins in the presence of water. The comparison of the densitograms of mildly bleached and permanent waved samples, curled in dry or wet state, is presented in figure 3.36.

Figure 3.36. Comparison between densitograms for dry and wet curled hair samples in the cases of mild bleaching (a- 30 min bleaching) and permanent waving (b- 30 min reduction, 10 min reoxidation) (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)

The variations appearing in the electrophoretical protein patterns are expected to be influenced by the non-homogeneous distribution of the hair fibres on the curler’s rod, which
3. Results and discussions

leads to an uneven heating of different parts of the sample. This is for example the case presented in figure 3.37, where the wet curled sample has the same pattern intensity as the starting sample.

![Figure 3.37. Electrophoretical protein patterns for curled hair samples](image)

**SDS-PAGE, 15% T, 3% C**
**Derivatisation: Iodoacetamide**
**Detection: CBB R 250**

The statistical analysis of the samples subjected to heat curling leads to the results presented in figures 3.38 and 3.39 for KIFs and KAPs, respectively.

![Figure 3.38. Dendrogram of KIFs for heat curled hair samples (dendrogram for 9 samples, Ward’s method, parameter Euclidean distance)](image)

UN – untreated sample
UND – untreated sample, dry curled
UNW – untreated sample, wet curled
B – bleached sample
BD – bleached sample, dry curled
BW – bleached sample, wet curled
PW – permanent waved sample
PWW – permanent waved sample, wet curled
PWD – permanent waved sample, dry curled
Cluster I from figure 3.38 comprises the initial samples and the samples whose KIFs are minimally influenced by the curling treatment, such as the dry curled untreated and bleached samples. In Cluster II are included the wet curled and the dry curled permanent waved sample, suggesting a cumulative effect of permanent waving and curling treatments on KIFs.

Cluster I from figure 3.39 comprises the samples whose KAP bands are to a lower degree affected by the applied treatments: the untreated sample, together with the dry and wet curled untreated and the mild bleached sample, showing once more the low influence of heat curling treatments on the matrix proteins. Cluster II groups the previously cosmetically treated samples, respectively bleached and permanent waved, which were subjected to curling. A closer analysis of the classification obtained in figure 3.39 suggests a higher sensitivity of the cosmetically treated samples to heat curling treatments, as it is the case for the bleached hair sample.

Figure 3.39. Dendrogram of KAPs for heat curled hair samples (dendrogram for 9 samples, Ward’s method, parameter Euclidean distance)

- UN – untreated sample
- UND – untreated sample, dry curled
- UNW – untreated sample, wet curled
- B – bleached sample
- BD – bleached sample, dry curled
- BW – bleached sample, wet curled
- PW – permanent waved sample
- PWD – permanent waved sample, dry curled
- PWW – permanent waved sample, wet curled
3.5.4. Influence of swimming pool water and sea water

In practice, even the untreated hair is subjected sometimes to swimming pool water and/or sea water. Swimming is a widely practised sport, so that the influence of water naturally or artificially enriched with salts and another substances on human hair should be taken into account.

The swimming pool water is a complex mixtures of various substances. Typical components for the swimming pool water are the disinfectants, added to destroy vegetative forms of micro-organisms and other contaminants. One of the most frequently used agent is chlorine, whose oxidising effect on the hair keratin is well known. A wide range of other chemicals for ensuring the right pH, flocculents and chelators are also presents in small amounts in the swimming pool water [164].

Sea water is a solution of salts of nearly constant composition, dissolved in variable amounts of water. There are more than 70 elements dissolved in sea water but only six make up > 99 % of all the dissolved salts; all occur as ions, whose weight proportions are: chloride - 55.04 %, sodium - 30.61 %, sulphate - 7.68 %, magnesium - 3.69 %, calcium - 1.16 % and potassium - 1.10 %. Beside the major components there are trace elements in sea water and small amounts of dissolved gases [165].

Allwörden [166] was the first one who observed the formation of bubbles at the surface of keratin fibres treated with chlorine water. This oxidising system diffuses across the epicuticle, degrading the protein beneath and producing smaller, water-soluble species too large to migrate out of the hair. As a result, swelling occurs beneath the epicuticle from osmotic forces, producing the characteristics Allwörden bubbles.

From experiments on wool it is well known that the extent of chemical modifications of proteins during chlorination depends on the pH value of the reaction liquors, where the reactive species are free chlorine, hypochlorous acid and hypochlorite ion. The oxidation of wool by halogens or halogen derivatives is mainly confined to the disulphide bond; in addition tyrosine and tryptophan residues are destroyed during chlorination [167]. Exposure of human hair to chlorine involves much weaker concentrations (0,5 – 6 ppm), but usually much greater liquor-to-fibre ratios and reaction times are implied. In acidic and neutral solutions, cysteic acid is the main oxidation product. In the alkaline region, C-S bond cleavage results in the formation of lanthionine, and at all pH levels, peptide bonds are cleaved [168,169]. The combination of cosmetic treatments with chlorination (10 ppm...
chlorine concentration) showed higher damage in the case of postchlorination treatments, when the fibres were already weakened by prior chlorination [170].

The effects of a concentrated salt solution on hair proteins can be expressed by additional formation of salt linkages between divalent cations such as \( \text{Ca}^{2+} \) or \( \text{Zn}^{2+} \) [94] present in solution and the anionic sites of the protein chains, leading to a lower protein solubility when the hair sample is not subjected to washing prior protein extraction.

The modifications induced by repeated treatments with sea water and swimming pool water – which will be called with regard to the most significant component for our investigation chlorine water - on the electrophoretical protein patterns of human hair samples are presented in figure 3.40 for the case of untreated sample.

![Figure 3.40. Electrophoretical patterns for untreated hair sample (untreated) and hair samples treated with chlorine water (ClW) and sea water (SW) (commercial, European mixed hair) SDS-PAGE, 15% T, 3% C Derivatisation: Iodoacetamide Detection: CBB R 250](image)

As can be seen in figure 3.40, the intensity of KIF and KAP bands decreases for both applied treatments. The same effect appears for previously cosmetically treated hair samples, at a lower magnitude, though.

The intensity decrease of the protein bands for the hair samples treated with chlorine water is due to cysteic acid formation due to cystine oxidation, the increase of the negative charge of the proteins leading to a weaker staining with CBB R 250.

A closer examination of the protein patterns from figure 3.40 shows lower intensity of the bands belonging to KIFs for the sample subjected to sea water, while the bands for KAPs have a comparable intensity with the ones obtained for chlorine water treated samples. This surprising phenomenon can have different explanations. First, the presence of small amounts of divalent metals in the extraction buffer was shown to decrease the extractability of KIFs in the case of wool samples [94]; we can presume that a certain amount of cations remain on the hair surface after the contact with sea water, having a similar influence. On the other hand, the
moderate homogeneity degree of the commercial hair sample can also contribute to uneven behaviour of different hair tresses.

Figure 3.41. Densitograms for untreated hair sample and hair samples treated with chlorine (CIW) and with sea water (SW) (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)

The results of the statistical analysis in the case of treatment with chlorine water and sea water will be presented in the next chapter (3.5.5.)

3.5.5. Combined cosmetic treatments

The application of different cosmetic treatments one after another is a case often met in practice. Therefore, the study of the influence of combined cosmetic treatments on hair proteins is a useful approach.

The cosmetic treatments alone or in combinations are applied on tresses of commercial, European mixed hair, using cosmetic formulations commercially available and respecting the usage instructions. A few samples are also subjected to incubation with chlorine water and sea water. The treatments applied and the samples codification are presented in table 3.10.
### Table 3.10. Combined cosmetic treatments applied on commercial, European mixed hair and their codification

<table>
<thead>
<tr>
<th>Sample codification</th>
<th>1st cosmetic treatment</th>
<th>2nd cosmetic treatment</th>
<th>3rd cosmetic treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTA1</td>
<td>Middle blond dyeing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTA2</td>
<td>Middle blond dyeing</td>
<td>Permanent waving for dyed hair</td>
<td></td>
</tr>
<tr>
<td>CTA3</td>
<td>Middle blond dyeing</td>
<td>Permanent waving for dyed hair</td>
<td>Incubating with chlorine water</td>
</tr>
<tr>
<td>CTA4</td>
<td>Middle blond dyeing</td>
<td>Permanent waving for dyed hair</td>
<td>Incubating with sea water</td>
</tr>
<tr>
<td>CTB1</td>
<td>Permanent waving for hard-to-perm hair</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CTB2</td>
<td>Permanent waving for hard-to-perm hair</td>
<td>Frost bleaching</td>
<td>-</td>
</tr>
<tr>
<td>CTB3</td>
<td>Permanent waving for hard-to-perm hair</td>
<td>Frost bleaching</td>
<td>Incubating with chlorine water</td>
</tr>
<tr>
<td>CTB4</td>
<td>Permanent waving for hard-to-perm hair</td>
<td>Frost bleaching</td>
<td>Incubating with sea water</td>
</tr>
<tr>
<td>CTC</td>
<td>Frost bleaching</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CTD</td>
<td>Permanent waving for dyed hair</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CTF</td>
<td>Incubating with swimming pool water</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CTG</td>
<td>Incubating with sea water</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Following cosmetic treatments, the samples are subjected to protein extraction. The soluble proteins are subjected to electrophoresis and the gels are stained with CBB R 250. The electrophoretical protein patterns obtained for samples subjected to the main combined treatments described in table 3.10 are showed in figure 3.42, from which the cumulative effects of different cosmetic treatments can be easily visually evaluated.
3. Results and discussions

Figure 3.42. Electrophoretical protein patterns of hair samples subjected to combinations of cosmetic treatments
- Untreated – untreated hair sample
- CTA1 - Middle blond dyeing
- CTA2 - Middle blond dyeing + Permanent waving for dyed hair
- CTA3 - Middle blond dyeing + Permanent waving for dyed hair + Chlorine water
- CTA4 - Middle blond dyeing + Permanent waving for dyed hair + Sea water
- CTB1 - Permanent waving for hard-to-perm hair
- CTB2 - Permanent waving for hard-to-perm hair + Frost bleaching
- CTB3 - Permanent waving for hard-to-perm hair + Frost bleaching + Chlorine water
- CTB4 - Permanent waving for hard-to-perm hair + Frost bleaching + Sea water
- CTC - Frost bleaching

SDS-PAGE, 15% T, 3% C  
Derivatisation: Iodoacetamide  
Detection: CBB R 250

The effects of the first combined treatment (CTA), dyeing and permanent waving, on the electrophoretical protein pattern can be described by the decrease of the band intensities, especially in the region of KAPs, as can be also seen by the comparison of the densitograms obtained for the middle blond dyed (CTA1) and middle blond dyed and permanent waved (CTA2) hair samples in figure 3.43.
The second combined cosmetic treatment brings dramatic changes in the protein pattern, as can be seen by superimposing the densitograms for the corresponding samples (CTB1, CTB2) in figure 3.44.

Figure 3.43. Comparison between densitograms for middle blond dyed and middle dyed + permanent waved hair samples (commercial, European mixed hair, SDS-PAGE, detection CBB R 250).

Figure 3.44. Comparison between densitograms for permanent waved and permanent waved + frost bleached hair samples (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)
The KIF bands of the samples subjected to the second combined treatment (figures 3.42 and 3.44) are widened and diffuse, and the KAP bands are severely reduced. The effect of frost bleaching is extremely enhanced by the previous permanent waving treatment, producing advanced modifications of the hair proteins.

The frost bleaching alone produces the characteristic damage for oxidative treatments, the electrophoretical pattern preserving the number and position of protein bands. The oxidative damage is more pronounced as in the case of middle dyeing treatment, but only the performing of frost bleaching after permanent waving treatments leads to an advanced damage of hair proteins. The changes appeared in the electrophoretical pattern after the mentioned combination are compared with the case of frost bleaching alone in figure 3.45.

![Figure 3.45. Comparison between densitograms for frost bleached and permanent waved + frost bleached hair samples (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)](image)

For performing the cluster analysis for the hair samples subjected to combined cosmetic treatments the KAPs area is chosen, systematic variations of protein bands with the applied treatment being identified from anterior analysis. The result obtained is presented in figure 3.46.
Cluster I comprises, beside the untreated sample, the samples which suffer mild modifications following cosmetic treatments, as are the middle blond dyed sample and the samples incubated with chlorine water and sea water. The samples in Cluster II present higher modifications of the electrophoretic pattern, whose different magnitude determines the classification in two subgroups: Cluster IIa, which includes hair samples subjected to a single cosmetic treatment and to the first combined treatment, and Cluster IIb, comprising the samples subjected to the second combined cosmetic treatment, whose protein patterns are completely changed.

The dendrogram of figure 3.46 suggests also differences which appear in the electrophoretical protein pattern after incubating the hair samples with chlorine and sea water. A better view of
the changes after these treatments can be obtained when comparing the corresponding densitograms for the entire protein pattern, as presented in figures 3.47 and 3.48.

![Optical density vs. Rf values](image)

Figure 3.47. Comparison between densitograms for combined cosmetically treated hair sample CTA2 (middle blond dyed + permanent waved) before and after incubation with chlorine water (CTA3) and sea water (CTA4) (commercial, European mixed hair sample, SDS-PAGE, detection CBB R 250)

The final treatment applied in the case of CTA3 and CTA4 hair samples has a certain influence on the electrophoretical protein pattern. As expected, the chlorine contained in the swimming pool water acts as a weak oxidising agent on the hair proteins [170], determining an intensity increase for the bands belonging to KIFs, while the KAPs remain in principle unchanged. The incubation with sea water does not bring consistent changes in the protein pattern, except a very slight decrease of the intensity of KAP bands.

Similar modifications can be observed in figure 3.48 for hair samples subjected to the second combined treatment CTB2 (permanent waving + frost bleaching), followed by incubation in chlorine (CTB3) and sea water (CTB4).
3. Results and discussions

Figure 3.48. Comparison between densitograms for combined cosmetic treated hair sample CTB2 (permanent waving + frost bleaching) before and after incubation with chlorine water (CTB3) and sea water (CTB4) (commercial mixed hair sample, SDS-PAGE, detection CBB R 250).

3.5.6. Classification of cosmetically treated hair samples using statistical evaluation of electrophoretical protein patterns

The effects of different cosmetic treatments on the electrophoretical protein pattern of human hair can be classified and evaluated using the statistical analysis method presented above. As already discussed, all cosmetic treatments (bleaching, oxidative dyeing with a lighter colour, tone-on-tone dyeing, permanent waving) can be classified as a function of the modification induced in the electrophoretical protein pattern.

To evaluate the effects of different cosmetic treatments on the protein pattern of human hair as a function of their damage mechanism and of the morphological level of the hair fibre at which these processes take place, protein patterns obtained after different treatments carried out in conformity with the usage instructions of commercially available products (forming the so-called “standard” treatments) are separately analysed for KIF and KAP areas. The results of statistical analysis are presented in figure 3.49, expressing a possible “basis cluster”.
The classification obtained for the samples corresponding to different cosmetic treatments is related to the type and concentration of active agents included in the cosmetic formulations. The concentration of hydrogen peroxide or reductive agents, as well as the pH value of the mixture applied on hair are playing a key role. The commercially available cosmetic formulations contain also hair care additives, which can influence in one way or another the effect of active agents on hair proteins.

The dendrograms of figure 3.49 show the classification of differently treated hair samples as a function of the modifications appearing in the electrophoretical protein patterns after cosmetic treatments. As expected, the dendrogram for KIFs is different from the dendrogram for KAPs obtained for the same set of samples, demonstrating that the effects of various cosmetic treatments on different protein groups are different. The reasons for these differences are connected with the amino acid composition, the protein structure and the physical distribution and accessibility to chemical agents of the two morphological components of hair cortex in the fibre, the intermediate filaments and the intermicrofibrillar matrix.

Similar cosmetic treatments whose formulations are created for certain special circumstances have, in their standard application, different effects on the hair proteins. This can be seen for three different permanent waving systems (PW311 – standard permanent wave; CTB1 – permanent wave for hard-to-perm hair; CTD – permanent wave for dyed hair), for which the corresponding samples are distributed in different clusters, as shown in figure 3.49.
3. Results and discussions

Figure 3.49. Dendrograms for cosmetically treated samples of commercial hair: (a) KIFs and (b) KAPs (dendrograms for 9 samples, Ward’s method, parameter Euclidean distance)

- **UN** - untreated hair sample
- **B1x30** – 1x30 min bleached (standard)
- **CTA1** – middle blond dyed
- **CTB1** – permanent waved (perm. wave for hard-to-perm hair)
- **CTC** – white dyed
- **CTD** – permanent waved (perm. wave for dyed hair)
- **H1** – 1x50 min lighter dyed (standard)
- **PW311** – permanent waved (standard: 30 min reduction, 10 min reoxidation)
- **T1** – 1x40 min **tone-on-tone** dyed (standard)
As already found out in the previous investigations, cosmetic treatments applied only one time and in conformity with the usage instructions do not bring spectacular changes in the electrophoretical protein patterns. The modifications are usually related to changes in the protein band intensities, the electrophoretical pattern preserving the same general aspect.

The classification of the samples in *Cluster I* and *Cluster II* in both dendrograms of figure 3.49 is related to the intensity of the protein bands. The first clusters are comprising the samples which suffered a lower damage, and the second clusters are comprising the samples which suffered stronger modifications. The intensity of the protein bands decreases from *Cluster I* to *Cluster II*, and inside every cluster the most similar protein profiles are grouped together.

The dendrograms of figure 3.49 can be used as a basis (“basis cluster”) for evaluating modifications produced on hair proteins by non-standard or multiple cosmetic treatments. The changes appearing at the level of KIFs and KAPs are discussed for some examples.

The hair sample PW411 represents a commercial hair sample subjected to permanent waving using a no-standard method, where the reduction time was extended by 10 min compared to the standard procedure (PW411: 40 min reduction, 10 min reoxidation).

The statistical analysis gives different evaluations for intermediate filament proteins and keratin-associated proteins, as expected. The dendrograms of figure 3.50 show the influence of extending the reduction time for a permanent waving procedure by 10 min at different morphological levels of the hair fibre. The KAPs seem to be less affected by the increased reduction time than the KIFs, so that the sample PW411 is placed next to the standard permanent waved sample (PW311). On the contrary, in the dendrogram of KIFs the sample PW411 occupies a place in a sub-cluster different from the one containing the sample PW311, suggesting a more advanced damage for this protein group upon use of the modified permanent waving procedure.
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Figure 3.50. Classification of the permanent waved hair sample PW411 (reduction time 40 min, reoxidation time 10 min) in dendrograms of (a) KIFs and (b) KAPs for “standard” cosmetically treated hair samples.

In figure 3.51 another example for the classification of non-standard lighter dyed hair samples is given. The samples H2 – dyed one time with additional heating and H5 – dyed two times with additional heating are compared with each other and also with the “standard” treated hair samples. As already discussed in chapter 3.5.1.2, the first dyeing with additional heating does not have strong damaging effects on the hair proteins, while the second treatment is changing the electrophoretical protein pattern dramatically (see fig. 3.26).
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Figure 3.51. Classification of the lighter dyed hair samples H2 (1x50 min dyed with additional heating) and H5 (2x50 min dyed with additional heating) in dendrograms of (a) KIFs and (b) KAPs for “standard” cosmetic treated hair samples.

For the case of KIFs (figure 3.51), sample H2 is positioned next to H1, the “standard” lighter dyed sample while the advanced changes induced to the protein pattern determines the classification of sample H5 out of the clusters built by the initial samples.

In the dendrogram for KAPs sample H2 occupies the position next to H1, stressing once more the low influence of additional heating on the effects of first dyeing treatment. The sample H5 is also comprised in Cluster II, where the samples with higher damage level are grouped, but it is not directly related to any of the other samples.
The examples presented show clearly the possibility to build-up, for the case of a known human hair sample (such as the commercial, European mixed hair), a “basis cluster” comprising certain cosmetic treatments, which can be further used to evaluate the degree and morphological location of damage produced by cosmetic processes.
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3.6. UV-irradiation

Hair exposed to sunlight is claimed to become more brittle, stiffer and drier than before irradiation, exhibits a reduced water sorption capacity and a lower tensile strength. The processes which occur during photolysis are photooxidation, C-S and S-S bond cleavage, decarboxylation and deamination; the relative importance of these processes varies in broad ranges and depends on the morphological part of the fibre where it takes place [3].

The essential first event in hair photodamage, as in all photoprocesses, is light absorption by the fibre. Only wavelengths above 290 nm are effective in natural photo-damage, since shorter wavelength UV light is filtered out by the stratosphere. Melanin and keratin compete for the absorption of photons between 254 and 345 nm. From keratin only the cystine and the aromatic amino acid residues tyrosine, tryptophan and phenylalanine absorb light within this wavelength range [116]. The primary reaction of the hair photodegradation occurs at or near the hair surface and involves degradation of cystine residues via C-S mechanism (route C on scheme 3.1, chapter 3.5.1); as already discussed, chemical oxidation of disulphide in hair by alkaline hydrogen peroxide is different and appears to proceed through S-S cleavage [47].

Possible reaction products of photolysis and photooxidation of cystine following formation of thiyl (HOOC-CH(NH$_2$)-S$^•$) and perthiyl (HOOC-CH(NH$_2$)-SS$^•$) radicals are trisulphide, tetrasulphide, lanthionine, alanine, dehydroalanine, cysteine, hydrogen sulphide, S-sulphocysteine, cysteine sulphenic acid and in the final oxidation state cysteic acid [3,74]. Upon wetting, photodamaged hair produces an odour of mercaptan, typical of reduced sulphur; although mercaptans have been proposed as one of the many products that may form during oxidative hair damage [3], there is only a limited number of literature data reporting the formation of mercaptans in hair upon exposure to light [42]. In the last years, an alternative possible mechanism for the formation not only of oxidised sulphur species, but also of thiol groups in keratin following exposure to UV radiation as a function of the energy of incident radiation was proposed by Millington and Church [171]. In conformity with this mechanism, the first stages involved in the photolysis are the formation of the radical anion RSSR$^•$. The proposed reactions are presented in scheme 3.8 a for high energy of the incident radiation, which acts directly on cystine residues, and in scheme 3.8 b for lower energy of the incident radiation, which is absorbed by tyrosine and tryptophan residues and rapidly transferred to cystine residues. For keratin at room temperature, an equilibrium exists between the disulphide radical anion and the thiyl radical and thyl anion, which leads to the rapid formation of thiol groups, as presented in scheme 3.8 c.
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\[
\begin{align*}
\text{(a)} & \quad \text{RSSR} + \text{hv (254 nm)} \rightarrow \text{RSSR}^\cdot + \cdot \cdot \\
\text{RSSR} + \cdot \cdot & \rightarrow \text{RSSR}^\cdot \\
\text{Tyr} + \text{hv (280-320 nm)} & \rightarrow \text{Tyr}^* \\
\text{Trp} + \text{hv (280-320 nm)} & \rightarrow \text{Trp}^* \\
\text{Tyr}^* + \text{RSSR} & \rightarrow \text{Tyr}^\cdot + \text{RSSR}^\cdot \\
\text{Trp}^* + \text{RSSR} & \rightarrow \text{Trp}^\cdot + \text{RSSR}^\cdot \\
\text{(b)} & \quad \text{RSSR}^\cdot \leftrightarrow \text{RS}^- + \text{RS}^\cdot \\
\text{RS}^- + \text{H}^+ & \rightarrow \text{RSH} \\
\end{align*}
\]

Scheme 3.8. Cystine residues degradation by high (a) and lower (b) energy irradiation with thiol formation

RSSR – species containing intact cystine
RSSR^\cdot – species containing cystinyl radical cation
RSSR^- – species containing cystinyl radical anion
Tyr – tyrosine residue; Tyr^* – excited tyrosine residue
Trp – tryptophan residue; Trp^* – excited tryptophan residue
\cdot \cdot – free electron
RS^- – thiol radical
RS^\cdot – thiol anion
RSH – thiol

Hair pigments (eumelanins and pheomelanins) function to provide some photochemical protection to hair proteins, especially at lower wavelengths where both the pigments and the proteins absorb light [116,172,173]. Hair pigments accomplish this protection by absorbing and filtering the impinging radiation and subsequently dissipating this energy as heat, the pigments themselves being degraded or bleached. In absence of melanins, the hair is more strongly affected by environmental factors; it has been found that grey hair undergoes more severe UV damage and needs more UV protection than dark brown hair [174,175].

The decrease of the cystine content along the hair fibre - easy to quantify for long hair fibres by comparing the amino acid composition of the root and of the tip end - due to the photodamage of the hair protein does not necessarily imply a higher solubility of these proteins under usual extraction conditions, since secondary cross-linking of amino acid residues may occur as well [37]. From irradiation experiments on wool it is known that, in addition to extensive damage of the fibre by the breaking of disulphide bridges with formation of cysteic acid and by main chain scission at N-C\alpha bonds (scheme 3.10) [92,159], exposure to UV radiation causes formation of secondary cross-links between protein residues such as
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Lanthionine [176] or dityrosine [177] (scheme 3.11) which limit the extractability of the protein from the fibre.

\[
\begin{align*}
   &\text{-CO-\text{NH-\text{CR-\text{CO-}}} \\
   \text{hv} &\rightarrow \text{-CO-\text{NH-\text{\textbullet CR-\text{CO-}}} + \text{H\textbullet}} \\
   &\text{-CO-\text{NH-\text{CR-\text{CO-}}} [\text{-CO-\text{N=CR-\text{CO-}}}]} \\
   &\text{-CO-\text{NH-\text{CR-\text{CO-}}} \rightarrow \text{OH}} \\
   &\text{-CO-\text{NH-\text{CR-\text{CO-}}} \rightarrow \text{OOH}} \\
   &\text{-CO-\text{NH-\text{CR-\text{CO-}}} \rightarrow \text{amid end group}} \\
   &\text{-CO-\text{NH-\text{CR-\text{CO-}}} \rightarrow \text{keto-acyl end group}} \\
\end{align*}
\]

Scheme 3.10. Cleavage of main peptide chain in UV-irradiated keratin with formation of keto-acyl protein end groups

\[
\begin{align*}
   \text{CO} &\text{C} \text{H} \text{NH} \text{CH}_2 \text{S} \text{CH}_2 \text{C} \text{H} \text{NH} \text{CO} \\
   \text{H-C-CH}_2 \text{S-CH}_2 \text{C-H} &\text{lanthionine} \\
   \text{CO} &\text{H} \text{C-CH}_2 \text{NH} \text{CO} \\
   \text{CH}_2 &\text{dityrosine}
\end{align*}
\]

Scheme 3.11. Lanthionine and dityrosine cross-links in UV-irradiated keratin.

3.6.1. Cosmetic treatments followed by UV-irradiation

Usually, damaging agents as daily care, cosmetic treatments or sunlight act simultaneously on human hair. Their cumulative action on the hair proteins constituted the subject of systematic investigations [117,125,178,179] and was evaluated by changes in hair structure (bulk properties) or by damage of hair surface. Cosmetic history prior to weathering is an important factor. For example, it was shown that dyed hair suffers less of a cystine loss than undyed fibres and shows as good a retention of mechanical properties in the early stages of weathering as the cosmetically unprocessed samples. On the other hand, bleached hair, when exposed to similar conditions, weakens and swells more in water [91].
Commercial, European mixed hair tresses are subjected to various cosmetic treatments (bleaching and permanent waving in the form of complex cosmetic treatments, incubation with swimming-pool water or with sea-water) and subsequently to UV-irradiation. The irradiation of hair samples is performed in a climatised room, using a table top solarium (see chapter 4.2 for details). The electrophoretical patterns for UV-irradiated samples are presented in figure 3.52.

Figure 3.52. Electrophoretical protein patterns for cosmetic treated and UV-irradiated hair samples (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)

The intensity of KIF bands decreases for the irradiated samples compared to non-irradiated samples without depending on the type of cosmetic treatment previously applied to the hair samples. The intensity of KAP bands decreases perceptibly for the untreated and minimum treated hair samples; the decrease for complex cosmetically treated samples is reduced due to the already low intensity of those protein bands.

The decrease of band intensity of KIFs correlates with the formation of new cross-links, induced by photolysis of hair fibre proteins. A similar phenomenon is responsible for the decrease of band intensity of KAPs; besides, cystine oxidation produces cysteic acid, leading to a decrease of CBB R 250 staining intensity. The reason for the lower staining intensity of KAPs is due to the affinity decrease of negatively charged proteins for the anionic dye.
The main modifications appearing in the electrophoretical protein patterns following UV-irradiation are emphasised in figure 3.53 by superimposing the densitograms for minimum treated and cosmetically treated hair samples, before and after UV-irradiation.

Figure 3.53. Densitograms for hair samples subjected to: a) sea water (SW) and sea water followed by UV-irradiation (SW_UV); b) combined cosmetic treatments (CTA3: oxidative dyeing + perm. waving + chlorine water) and additional irradiated (CTA3 + UV)
The statistical analysis of the electrophoretical protein patterns in the region of KAPs leads to the dendrogram presented in figure 3.54. The clustering of the samples is closely related to the damaging potential of the applied cosmetic treatments. The samples more affected by cosmetic treatments such as the CTA and CTB combinations are grouped in Cluster I. The cluster is once more divided into two subclusters, depending on the cosmetic combination type, CTA and CTB. In Cluster II are grouped the untreated sample and the samples less affected by cosmetic treatments, as are the samples incubated with chlorine water and sea water; this cluster comprises also two subgroups, one with the samples before UV-irradiation and the other one with the same samples after UV-irradiation.

Figure 3.54. Dendrogram of KAPs for untreated, minimum treated and combined cosmetically treated hair samples subjected to UV-irradiation (dendrogram for 14 samples, Ward’s method, parameter Euclidean distance)

- UN – untreated hair sample
- UV - UV-irradiation treatment
- ClW – chlorine water treatment
- ClW_UV – chlorine water + UV-irradiation treatment
- SW – sea water treatment
- SW_UV – sea water + UV-irradiation treatment
- CTA3 – oxidative dyeing + perm. waving + chlorine water
- CTA4 – oxidative dyeing + perm. waving + sea water
- CTA5 – CTA3 + UV-irradiation
- CTA6 – CTA4 + UV-irradiation
- CTB3 – perm. waving + bleaching + chlorine water
- CTB4 – perm. waving + bleaching + sea water
- CTB5 – CTB3 + UV-irradiation
- CTB6 – CTB4 + UV-irradiation
3.6.2. UV-irradiation followed by cosmetic treatments

It is known that weathered hair is more susceptible to swelling when compared to unweathered controls, suggesting also a higher susceptibility to oxidation or reduction. It may be that the newly formed cross-links are not easily affected by such treatments but that the cystine residues which remain are those which originally were more shielded and perhaps more important for the organisation of the macromolecular structure [91]. On the other hand, the internal lipids which, together with globular proteins, form the cell membrane complex, are destroyed by the visible range of sunlight. In this way the diffusion of foreign materials into the hair fibres is enhanced for irradiated samples [180]. Investigations made on proteins eluted from hair by various harmful agents have shown a lower solubility of hair proteins following UV-irradiation [55].

Another group of samples was first subjected to UV-irradiation and subsequently cosmetically treated (bleached or permanent waved), for investigating the magnitude of the modifications appearing in practice for hair samples which are exposed to sunlight and then cosmetically treated, as could be the case for long hair samples. The aspect of the electrophoretical protein pattern for hair samples which were first UV-irradiated and then subjected to bleaching and permanent waving treatments is presented in figure 3.55.

Figure 3.55. Electrophoretical protein patterns for UV-irradiated and subsequently cosmetically treated hair samples.
SDS-PAGE, 15% T, 3% C
Derivatisation: Iodoacetamide
Detection: CBB R 250
The protein extractability of the hair samples which are subjected to UV-irradiation and afterwards to cosmetic treatments decreases compared to non-irradiated samples. This is visible especially for the samples subjected to permanent waving, as can be seen from the electrophoretical patterns presented in figure 3.55 and from the comparison of the densitograms, carried out in figure 3.56.

![Graph showing comparison between densitograms of permanent waved and UV-irradiated and permanent waved hair samples](image)

**Figure 3.56.** Comparison between densitograms of permanent waved (PW311) and UV-irradiated and permanent waved (UV_PW311) hair samples (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)

- PW311 – 1x 30 min reduction, 10 min reoxidation
- UV_PW311 – UV-irradiated + 1x 30 min reduction, 10 min reoxidation

The decrease of protein band intensities correlates with the processes taking place at different morphological levels of the hair fibre, as already discussed for hair samples subjected to cosmetic treatments and subsequently to UV radiation. The cosmetic treatments performed after UV-irradiation do not have a substantially different influence on the electrophoretical protein pattern of human hair, resembling the ones encountered for the hair samples subjected only to cosmetic treatments.

The UV-irradiation leads to the formation of new cross-links at the KIF level, which are not as sensitive to the oxidation induced by bleaching treatments as disulphide bonds. Because of this reason, the intensity of KIF bands is reduced for hair samples which were previously UV-irradiated. This effect is even increased by permanent waving processes, suggesting a cumulative action and leading to an extremely low solubility of KIFs for multiple treatments.
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The intensity decrease is also visible for the bands belonging to KAPs, the different applied treatments (bleaching, permanent waving, UV-irradiation) manifesting an additive effect at the matrix proteins level. Nevertheless, the whole densitogram profile preserves the same general aspect after performing multiple treatments on the hair samples, suggesting a lower damage degree as, for example, for combined cosmetic treatments as CTB (see chapter 3.5.5) or multiple dyeing processes with additional heating (see chapter 3.5.1.2).

The irradiation of untreated samples for the double time period leads to an advanced decrease of the protein band intensities for the entire pattern, as can be seen in figure 3.57; the magnitude of this phenomenon is higher for KIFs.

![Figure 3.57](image)

Figure 3.57. Comparison of dendrograms of untreated and UV-irradiated hair samples (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)

Moderate magnitude of the changes appearing in the electrophoretical protein patterns of UV-irradiated hair samples which were subsequently bleached or permanent waved suggests a minimum of sensitisation of hair samples following UV-irradiation. The reduced effect of the irradiation on hair behaviour can be attributed to the short exposure time, which may not lead to a sufficient increase of the hair porosity and does not lead to an advanced damage of internal lipids taking part in the cell membrane complex building.

The result of the statistical analysis for samples subjected to different cosmetic treatments after UV-irradiation is presented in figure 3.58.
3. Results and discussions

Figure 3.58. Dendrogram of KAPs for UV-irradiated hair samples subsequently subjected to different cosmetic treatments (dendrogram for 15 samples, Ward’s method, parameter Euclidean distance).

Cluster I of figure 3.58 comprises the less damaged samples due to different applied treatments, as are the samples subjected to a single cosmetic treatment B1x30 and PW311, as well as the UV-irradiated samples for different time periods. The internal classification of the samples in Cluster II, which contains the samples subjected to UV-irradiation and multiple cosmetic treatments, suggests a non-specific damage of KAPs in the case of standard cosmetic treatments associated with UV-irradiation.

UN- untreated hair sample
UV – UV-irradiated
UVx2 – 2x UV-irradiated
B1x30 (1x30 min bleached)
B2x30 (2x30 min bleached)
B3x30 (3x30 min bleached)
UV_B1x30 (UV + 1x30 min bleached)
UV_B2x30 (UV + 2x30 min bleached)
UV_B3x30 (UV + 3x30 min bleached)
PW311 (1x30:10 perm. waved)
PW312 (2x30:10 perm. waved)
PW313 (3x30:10 perm. waved)
UV_PW311 (UV + 1x30:10 perm. waved)
UV_PW312 (UV + 2x30:10 perm. waved)
UV_PW313 (UV + 3x30:10 perm. waved)
3. Results and discussions

3.6.3. Sunscreen formulations for hair care

UV damage at hair surface level, expressed as dryness, dullness and increase of fibre friction may be repaired or remedied by the use of conditioners, humectants and moisturisers. At the cortex level, the UV damage may be prevented or at least retarded by the use of UV-absorbers, the so-called sunscreens.

The sunscreens are substances able to filter the luminous radiation. The most used filtering agents are benzophenone derivatives, esters of aminobenzoic and salicylic acid, dibenzoyl methane derivatives, phenylbenzoxazole and –imidazole, as well as esters of cinnamic acid [181]. Surprisingly, the presence of some of them in certain cosmetic composition have been found to accentuate the deterioration in mechanical properties induced by sun radiation on human hair [182].

A test series is performed on untreated tresses of commercial, European mixed hair to determine the efficiency of a number of commercially available sunscreen products for UV-protection of hair. Five sun-protection formulations, available as lotions, sprays or oils and containing UV filtering agents such as phenylimidazole sulphonic acid, 4-aminobenzoic acid and butylmethoxydibenzoyl methane (scheme 3.12) in different combinations and in presence of various conditioners (see chapter 4.3.5. for details) are applied on wet hair tresses. Subsequently, the hair samples are subjected to UV-irradiation. During the UV-irradiation time, the samples are periodically rinsed with water and treated again with the sunscreen formulations. For two of the sunscreen products, randomly chosen, the procedure is repeated in order to evaluate the damage progress with irradiation time.

![Scheme 3.12. Structures of a few UV filtering agents](image-url)

The aspect of the electrophoretical protein patterns of hair samples UV-irradiated after sunscreen application is presented in figure 3.59.
3. Results and discussions

As can be seen from the electrophoretical patterns from figure 3.59, as well as from the comparison of the densitograms of the untreated sample and the sunscreen treated and UV-irradiated samples in figure 3.60, the intensity of the protein bands decreases for the UV-irradiated samples compared to the untreated sample, especially for KIFs. However, the intensity decrease of the protein bands for UV-irradiated samples is lower in the presence of the sunscreen, as can be seen by comparing figure 3.57 with figure 3.60.

Figure 3.59. Electrophoretical protein patterns for sunscreen treated and UV-irradiated hair samples (commercial, European mixed hair)
SDS-PAGE, 15% T, 3% C
Derivatisation: Iodoacetamide
Detection: CBB R 250

Figure 3.60. Comparison of densitograms of untreated hair sample and sunscreen treated hair samples subjected to UV-irradiation (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)
The evaluation of the protective action of sun screen products is more accurately made using the statistical approach developed for protein profile analysis. The results of statistical analysis for KAPs is presented in figure 3.61.

![Dendrogram of KAPs for extracts of UV-irradiated hair samples, without and with sunscreen application (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)](image)

All the hair samples, with or without sunscreen, are affected by the UV-irradiation. The magnitude of the damaging effect is higher for UV-irradiated hair samples in the absence of UV-absorbers, the corresponding samples building their own sub-cluster (UV, UVx2). The other UV-treated samples are further divided into two groups. One group consists of hair samples treated with the sunscreens 2, 3, 4 and sunscreen 1 after double exposure, whose close neighbourhood with no-protected samples suggests a higher damage. The other group comprises the samples treated with sunscreen 1 and sunscreen 5 even after double exposure, which manifest a lower decrease of protein bands and respectively a lower damage degree,
suggesting a higher efficacy in maintaining the integrity of hair proteins for these two sunscreen formulations.

The anterior study demonstrates the feasibility of the method in the comparative evaluation of efficacy of different cosmetic products, suggesting a new possible application area for the statistical analysis of electrophoretical protein patterns.
3. Results and discussions

3.7. Reproducibility of the method

The reproducibility of the electrophoreetical/statistical analysis method is evaluated using triplicate determinations. Three equivalent portions of hair samples whose electrophoretical patterns are not strongly changed following cosmetic treatments are subjected to protein extraction, the extracts are run on gels and the patterns obtained stained with CBB R 250. The densitograms obtained after densitometric measurements are separately digitised for KIFs and KAPs and subjected to cluster analysis. All the individual steps are performed in conformity with the standard procedure used throughout the work.

The samples used for reproducibility investigations consist of untreated, single and double bleached samples, as well as hair samples subjected to two simple combinations of standard treatments. The degree of change for the characteristic electrophoretical pattern of hair proteins for these samples ranges from low until medium. Higher differences in the protein profile aspect implies a clear differentiation of the samples and does not bring an objective evaluation of the reproducibility of the method. The differences between the protein patterns are suggested by the comparison of the densitogram for the untreated sample with the densitograms for bleached and permanent waved and permanent waved and bleached samples, presented in figure 3.62.

![Densitogram comparison of samples used for investigating the method’s reproducibility: untreated and combined treated hair samples, using standard cosmetic treatments (commercial, European mixed hair, SDS-PAGE, detection CBB R 250)](image-url)
The dendrogram obtained for KIFs is presented in figure 3.63:

Figure 3.63. Dendrogram of KIFs - triplicate determinations for differently treated hair samples (dendrogram for 18 samples, Ward’s method, parameter Euclidean distance)

- A, B, C - endings for triplicate hair samples
- UN - untreated
- B1x30 - bleached 1x30 min
- B2x30 - bleached 2x30 min
- B2x40 - bleached 2x40 min
- B1x30+PW311 - bleached 1x30 min and perm. waved 1x 30 min reduction:10 min reoxidation
- PW311+B1x30 - perm. waved 1x 30 min reduction:10 min reoxidation and bleached 1x30 min

Clearly defined groups, made up of three elements for each type of treatment, are visible on the dendrogram of figure 3.63, with linkage distances of maximum 0.3 units. The linkage distance increases for every new connection with the neighbour clusters, the final value reaching 2.7; nevertheless, the higher similarity of the KIF bands for the first four groups in Cluster I determines a lower linkage distance for their common cluster (around 1.02 units) than for Cluster II, which comprises only two groups, but at a higher linkage distance (1.3 units). The low capability of the method in the case of using the KIFs to distinguish between single and multiple treated hair samples, especially when processes influencing the intensity of the protein bands in different directions are present, is depicted once again in the previous figure.
3. Results and discussions

The dendrogram for KAPs obtained for the reproducibility investigation of the method for different cosmetic treated hair samples is presented in figure 3.64:

![Dendrogram of KAPs - triplicate determinations for different treated hair samples](image)

Figure 3.64. Dendrogram of KAPs - triplicate determinations for different treated hair samples (dendrogram for 18 samples, Ward’s method, parameter Euclidean distance)

- A, B, C - endings for triplicate samples
- UN - untreated hair sample
- B1x30 - bleached 1x30 min
- B2x30 - bleached 2x30 min
- B2x40 - bleached 2x40 min
- B1x30+PW311 - bleached 1x30 min and perm. waved 1x 30 min reduction:10 min reoxidation
- PW311+B1x30 - perm. waved 1x 30 min reduction:10 min reoxidation and bleached 1x30 min

The maximum linkage distance for the groups made up of three elements, which characterise every hair sample, is in the case of KAPs lower than 0.13 units. As previously discussed in chapter 3.4, this small value is given by the low intensity of the protein bands. The increase of the linkage distance with the further joining of the neighbour clusters is closely connected with the differences between the protein patterns. Thus, even Cluster I contains only two groups, the linkage distance is slightly higher than the global linkage distance for Cluster II, which contains four groups. This behaviour is also observed for the distribution of the clusters in figure 3.63.

On the other hand, we can observe again that the statistical analysis of the protein patterns for the area of KAPs is able to differentiate between multiple cosmetic treatments. Moreover, the
clustering of the samples in Cluster II of figure 3.64 suggests different damage degrees induced at the matrix protein level by cosmetic treatments which does not respect the usage instructions (see chapter 3.5.1.1) or by combinations of cosmetic treatments applied in different successions (see chapter 3.5.5).

The reproducibility of the classification presented before can be also verified for arbitrarily chosen samples from the previous analysis. For example, statistical analysis of duplicate samples using the bands of KAPs leads to the dendrogram presented in figure 3.65, which possesses a high similarity with the dendrogram of triplicate samples of figure 3.64. The difference between the above mentioned dendrograms consists, as expected, of lower linkage distances for the clusters of the dendrogram for duplicate samples, leading to a global linkage distance of around 0.57 units, while the global linkage distance for the triplicate samples reaches 0.71 units.

![Dendrogram of KAPs - duplicate determinations for different treated hair samples](image)

Figure 3.65. Dendrogram of KAPs - duplicate determinations for different treated hair samples (dendrogram for 12 samples, Ward’s method, parameter Euclidean distance)

- A, B, C - endings for initial triplicate samples
- UN - untreated hair sample
- B1x30 - bleached 1x30 min
- B2x30 - bleached 2x30 min
- B2x40 - bleached 2x40 min
- B1x30+PW311 - bleached 1x30 min and perm. waved 1x30 min reduction:10 min reoxidation
- PW311+B1x30 - perm. waved 1x30 min reduction:10 min reoxidation and bleached 1x30 min
The aspects previously presented show a satisfactory reproducibility of the standardised electrophoretical/statistical analysis method and allow some concluding remarks:

- The method is able to classify in a reproducible way the highly similar protein profiles, as it is the case for triplicate and duplicate determinations.

- The protein profiles changing in a characteristic way following cosmetic treatments are clustered as a function of the specific modifications induced by cosmetic processes.

- For oxidative and/or multiple cosmetic treatments the analysis using KAP bands is recommendable, providing reliable and reproducible results; nevertheless, additional information can be obtained applying the same method in the case of KIFs.
3. Results and discussions

3.8. Summary and conclusions

The objective of the present study is the development of a comprehensive tool, able to identify and classify changes induced to the human hair cortex proteins by various cosmetic treatments. Systematic comparison and evaluation are performed using statistical analysis.

The starting point is represented by one-dimensional protein patterns obtained using sodium dodecyl sulphate polyacrylamide gel electrophoresis. Standardisation of all intermediate steps is absolutely necessary for the reproducible and reliable analysis of protein patterns. Protein extraction from hair fibres, electrophoresis conditions, staining and destaining are performed in a standardised manner for all investigated samples. Besides, the migration distance of the protein bands in the gel is also standardised by adding internal standards of molecular weight. Subsequently, the electropherograms stained with Coomassie Brilliant Blue R 250 (CBB R 250) are subjected to densitometric measurements. The densitograms are then digitised and classified using cluster analysis, a statistical method able to identify the similarities between the protein profiles, which gives the results in the form of a dendrogram. Comparing the dendrograms obtained with the results of traditional test methods for hair damage assessment, an excellent correlation is found. Furthermore, the method can be applied separately for keratin intermediate filaments (KIFs) and keratin intermediate filament-associated proteins (KAPs), allowing a more detailed analysis of the effects of cosmetic processes on the morphological components of the hair fibre and their constitutive proteins.

The method provides good results not only in the systematic study of protein pattern changes for various cosmetic treatments, but can also be used for the classification of untreated hair samples. The analysis in the chosen conditions does not evidence different phenotypes, but the strong differentiation of hair samples of individuals as a function of protein extractability emphasises the necessity of a common starting material for the systematic study of changes imparted by cosmetic treatments on human hair proteins.

For this reason, a large number of cosmetic procedures is applied on commercial, European mixed hair, as single, multiple or combined treatments in standard conditions – in conformity with the usage instructions of the commercial product - or non-standard conditions – at treatment duration or temperature different from the standard ones, simulating possible in practice circumstances. The evaluation of the changes appearing in the electrophoretical
protein patterns for KIFs and KAPs by the use of cluster analysis leads to the finding that different cosmetic treatments act in a different way on the hair cortex.

Oxidative processes such as bleaching and dyeing change the intensity of KIF as well as KAP bands, but in different directions. The intensity increase of KIF bands is connected to increased solubility of proteins originating from intermediate filaments due to disulphide bridges cleavage and corresponding lower crosslinking degree of the protein chains; simultaneously, on the proteins rich in sulphur the cleavage of disulphide bridges with cysteic acid formation leads to supplementary anionic sites, which determine a lower staining intensity with the anionic dye CBB R 250 and a corresponding intensity decrease of KAP bands.

Applying reductive processes such as permanent waving results mainly in the decrease of intensity for KIF bands, accompanied by a quite small decrease of KAP band intensities. The predominant effect in this case is the appearance of new crosslinks, which decreases the extractability of the protein from fibres.

For various treatment times and multiple treatments, the changes of the electrophoretical patterns evolve systematically for KAPs in the case of oxidative treatments and for KIFs in the case of reductive treatments. These systematic changes are expressed as decrease of the protein band intensities.

Combined cosmetic treatments induce usually advanced changes of KIFs and KAPs. For a systematic comparison, more appropriate are the changes of KAP bands. Nevertheless, applying cluster analysis for KIF bands provides additional information.

The investigations show pattern changes, expressed as decrease of band intensities for KIFs as well as for KAPs, produced by the contact with hot surfaces – for styling procedures – and by incubation with swimming pool water and sea water, depicting the sensitivity of the method and its potential applicability for sensing minimal pattern changes.

Characteristic changes induced by cosmetic treatments on the electrophoretical pattern of human hair proteins are centralised in a database of standard cosmetic processes. The database comprises two basis dendrograms, built for KIFs and for KAPs, and is a very useful reference for interpreting changes induced at the level of KIFs or KAPs by non-standard treatments, when the treatment time or conditions are changed.

For example, prolonging the reduction time for a permanent waved hair sample by 10 min determines its classification next to the standard permanent waved sample on the KAPs
dendrogram, but in a more damaged cluster on the KIFs dendrogram. This shows higher changes induced by the reduction step in the KIFs than in the KAPs.

Another example is given for oxidatively dyed hair samples, treated one and two times under a hair dryer. While the single treated sample occupies the position next to the standard dyed sample on both dendrograms, the double treated sample shows a higher damage level than the other samples on the KAPs dendrogram and even builds a separate cluster on the KIFs dendrogram. Starting from these observations, the high damaging potential of multiple dyeing treatments with additional heat is emphasised.

This feature of the database suggests its capability to be used in implementation studies of new products in the cosmetic industry.

UV-irradiation is also able to induce modifications of the electrophoretical pattern of human hair proteins. The intensity decrease for KIF bands for all investigated samples suggests as predominant phenomenon the formation of new cross-links, which lead to lower solubility of KIFs. The intensity of KAP bands decreases due to the same reason, more visible for untreated samples and at a lower extent for cosmetically treated hair samples. Moreover, it is possible that the cleavage of disulphide bridges with cysteic acid formation induced by UV-irradiation decreases the band intensities of high-sulphur proteins by inducing a lower staining intensity with CBB R 250.

By the use of sunscreen products during UV-irradiation experiments, the changes of electrophoretical protein pattern are reduced; small differences are also identified between samples treated with different products. In this way, the statistical classification provides information about the efficacy of different sunscreen formulations, opening new application areas for the method.

As final conclusion, it can be said that standardisation and optimisation of protein extraction and staining constitute the first step in the development of a sensitive, stable and reproducible analytical method for the hair proteins, based on SDS-PAGE. Electrophoretical protein patterns for cosmetically treated hair samples show progressive and consistent changes of cortex proteins, so that it is possible, by applying statistical tools, to classify the electropherograms and to evaluate the changes imparted by cosmetic processes on human hair proteins. Furthermore, the method can be applied for development and implementation studies of new cosmetic products.
4. Experimental part

4.1. Materials

The hair samples used for the study were hair samples collected from individuals and commercial, European mixed hair. The samples from individuals were cut at the scalp level. The commercial hair was provided by Kerlin International Haarfabrik GmbH, and consists of brown tresses of 21 cm long (together with the glue band on which the hair fibres are fixed). In this case the level at which the hair was cut is not exactly known.

The quality characteristics for the commercial hair are presented in table 4.1.

Table 4.1. Quality parameters of commercial, European mixed hair

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<th>Colour type 4</th>
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<td><strong>Microscopic characterisation of fibres diameter</strong></td>
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<tr>
<td>Major fibre axis in µm</td>
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<tr>
<td>Minor fibre axis in µm</td>
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<td>Ellipticity factor</td>
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<td>Equivalent circular diameter in µm</td>
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<td><strong>Chemical parameters</strong></td>
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<td>Cystine in mol%</td>
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<td>Cysteic acid in mol%</td>
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Hair tresses of 1 cm width from the commercial hair were used for cosmetic treatments. The hair samples from individuals were available in small amounts, allowing usually just the electrophoretical analysis, where a few mg of sample are enough for the routine analysis. A number of samples (codified from Z1 to Z4) were available as thin tresses.

The hair samples from individuals, together with the available data as sample colour, sample length and the age of the person who provided the sample are presented in the table 4.2.
Table 4.2. Hair samples from individuals and their characteristics

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<td>27</td>
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</table>

4.2. Apparatus

The laboratory devices used are:

Gel electrophoresis: Mini PROTEAN 3 Electrophoresis, BioRad
Power supply: Power Pac 300, BioRad
Gel drier: Gel Air Dryer, BioRad
Shaker: KS 501, Ika Labortechnik
Amino acid analyser: Alpha Plus, Pharmacia Biotech
Differential scanning calorimeter 7, Perkin Elmer
Scanning electron microscope S360, Leica
Instron 1122, Instron Wolpert
UV – Visible spectrophotometer Cary 100 Bio, Varian
Scanner Sharp JX-330
HI 9321 Microprocessor pH-meter, Hanna Instruments
Rotary evaporator IKA Werk, Janke & Kunkel
Thermomixer 5436, Eppendorf
Hot plate IKAMAG RET - G
Centrifuge 2-15, Sigma
Table top solarium 406 B, Philips
Curling device: Circurl 200, Petra Electronic
Software products used:
- ImageMaster 1D Elite, Amersham Biosciences
- Image Master 1D Elite Database, Amersham Biosciences
- QuatroPro
- Statistica, StatSoft Inc. 2000
- ScanData
- Peakfit Jandel Scientific

### 4.3. Cosmetic treatments

#### 4.3.1. Bleaching

The standard procedure implies the treatment of 1g hair sample with a mixture made up of 2.5g bleaching powder and 7.5ml of bleaching lotion containing 6% $\text{H}_2\text{O}_2$. The mixture was applied on the dry hair using a brush. In the standard procedure the bleaching formulation was let to act on the hair for 30 min, then the sample was rinsed with tap water (at $\sim$35 °C). The sample was subsequently washed with a solution 15% sodium lauryl sulphate with pH 5.6 (ratio solution : hair = 1:4) for 1 min, let to stay another 1 min and rinsed with tap water for 5 min. The wet sample was then dried using a hair-dryer.

Variations of the bleaching time were applied for simulating possible practical use of the bleaching products.

#### 4.3.2. Dyeing

The colour product was applied on hair tresses and let to act in the standard procedure for 40 or 50 min, in conformity with the use instructions. Then the hair was washed using the method described above and dried.

The dyeing formulations used were:

- Middle-blond dye, with a $\text{H}_2\text{O}_2$ concentration of 3%
- Light-blond dye, with a $\text{H}_2\text{O}_2$ concentration of 9%
- Tone-on-Ton dye, with a $\text{H}_2\text{O}_2$ concentration of 3%
4.3.3. Permanent waving

In the standard procedure the hair tresses were wetted with water and treated with the reducing solution (sodium thioglycolate, pH 9.5), which was let to act for 30 min. Then the hair was rinsed with tap water (5 min) and treated with the oxidation solution (3% H₂O₂) for another 10 min. The hair was rinsed again with water and lauryl sulphate solution and dried using the hair-dryer.

Variations of the reducing and oxidation step were applied for simulating possible practical wrong use of the permanent waving formulations.

4.3.4. Hot curling

Small tresses of hair untreated and subjected to various cosmetic treatments were hot curled using a commercial curling device. The hair tresses were curled around the rod and treated 5 times for 5 min. Between subsequent treatments the hair samples were allowed to cool down for 5 min.

4.3.5. Swimming pool and sea water incubation

Hair tresses were incubated for 2 hr at room temperature in swimming pool and respectively sea water, at a weight ration of liquid : hair of 50:1 and dried with a hair-dryer, without previous washing.

4.4. UV-irradiation

Untreated and cosmetically treated hair tresses, in a dry or wet state, were irradiated in a climated room (20°C, 55% RH) using a table top solarium HB 406 (Philips). The irradiation conditions were chosen to represent a medium exposure of the human skin to sun light in the temperate regions of the earth during three months.

Similar conditions were used in the case of samples treated with sunscreen formulations.

Sunscreen products ordered as used for experiments: 1) sun-protection spray; 2) sun-protection splash; 3) sunshine cream spray; 4) sunshine lotion; 5) sunshine oil.
4. Experimental part

4.5. Analytical methods

4.5.1. Protein extraction

The extraction of proteins from the hair samples was made using a method adapted from Marshall [81]. The hair samples were cut in small pieces (around 1-2 mm long) and were treated with a reducing buffer containing 8M urea, 0.05M Tris-(hydroxymethyl)-aminomethan and 0.05M dithiothreitol (DTT). For every 5 mg of sample 500 µl reducing buffer were used. The extractions were conducted in nitrogen atmosphere on a thermomixer (40°C, continuous shaking) for different times, depending on the sample type: 1 h for hair samples from individuals and 8 h for commercial, European mixed hair samples.

4.5.2. Derivatisation of reduced proteins

For avoiding the reoxidation and improving the solubility of the reduced proteins, alkylation of the thiol groups was performed. Corresponding volumes of 20% iodoacetamide (50 µl for every 500 µl reducing buffer) were added and the samples were allowed to react 30 min at room temperature [84].

The separation of the soluble proteins from the hair residues was made by 15 min centrifugation at 10000 RPM. The protein solutions as well as the residues were stored in the freezer for further investigations.

4.5.3. Internal standards

The internal standards used were phosphorylase b (M=97400) and insulin B-chain (M=3700), prepared as stock solutions (1mg/ml) in Laemmli buffer (0.0625M Tris / HCl, pH 6.8, 2% SDS, 10% glycerine, 5% DTT and 0.002% Bromophenol Blue) [183]. The working solution consisted of a mixture of 50 µl phosphorylase stock solution, 50 µl insulin B-chain stock solution and 100 µl SDS 2% solution, heated up to 80°C for 5 min. From the latter solution 10 µl were added to 90 µl protein extract before running the gels.
4. Experimental part

4.5.4. Sodium dodecyl-sulphate polyacrylamide electrophoresis (SDS-PAGE)

The gel electrophoresis of the proteins extracted from the hair samples was performed after the discontinuous method described by Schaegger and Jagow [184].

*Gel solutions*

Resolving gel: 15% T, 3% C

- 1M Tris / HCl pH 8,45
- 0,1% SDS
- 13,3% glycerine
- 0,05% ammonium persulphate
- 0,05% N,N,N’N’-tetramethylethylendiamine (TEMED)

Stacking gel: 4% T, 3% C

- 0,75M Tris / HCl pH 8,45
- 0,075% SDS
- 0,05% Ammonium persulphate
- 0,10% N,N,N’N’-tetramethylethylendiamine (TEMED)

Electrode buffers

Cathodic buffer: 0,1M Tris

- 0,1M Tricine
- 0,1% SDS

Anodic buffer: 0,2M Tris / HCl pH 8,9

The resolving gel solution, after deaerating and adding ammonium persulphate and TEMED, was pipetted into the slab gel sandwich to a level about 1,5 cm from the top. After polymerisation (around 1 h), the stacking gel solution was poured on the top of the resolving gel and a Teflon comb of 0,75 mm was inserted between the glass plates forming the gel sandwich. After another 1 h allocated for the polymerisation of the stacking gel, the comb was removed and the wells formed used for loading the samples. Usually, a volume of 5 µl for the protein samples was used. The buffer chambers were filled with the appropriate buffers and the electrophoresis cell was connected to the power supply. The gel was run at 60 mA until
the tracking dye reached the bottom of the gel (mainly 2 h), then the sandwich was disassembled and the gel carefully placed in a tray of staining solution.

4.5.5. Staining methods

4.5.5.1. Coomassie Brilliant Blue R 250 and Coomassie Brilliant G 250

The detection of proteins subjected to polyacrylamide gel electrophoresis with Coomassie Brilliant Blue R 250 was made after a method adapted from Weber and Osborn [185]. The solutions used have the following composition:

Staining solution: 0.1% Coomassie Brilliant Blue R 250
45% methanol
10% acetic acid

Destaining solution: 5% methanol
10% acetic acid

After electrophoresis, the gels were stained for 1 h under constant shaking. The destaining was made overnight also under constant shaking.

The colloidal staining method with Coomassie Brilliant G 250 consisted of constant shaking of the gels in 2% H₃PO₄, 6% (NH₄)₂SO₄ for more than 48 h [97].

4.5.5.2. Silver staining

The silver staining method of Heukeshoven [100] uses silver nitrate in an acidic medium for the staining step and a basic medium for the developing step.

The method described by Morrisey [105] uses also silver nitrate. The main difference between this method and the anterior one consists in a supplementary reduction step with DTT.

Another important group of silver staining methods uses in the staining step a basic solution, the silver being in the diamine form. The developing step uses a slightly acidic solution of formaldehyde [102].
4.5.5.3. Double staining

The method described by Bürk [110] consists of two steps, in the first one the gels being stained with Coomassie Brilliant Blue R 250 in ethanol/formaldehyde solution. After complete destaining of the background, the gels are stained with silver diamine in alkaline solution and the colour development is made in a slightly acidic solution of formaldehyde.

The double stain of Irie et al. [111] uses as the first step a Coomassie R 250 staining, followed by a complex silver staining, using silver nitrate and also silver diamine.

The double staining method after Dzandu [112] has as the first method a silver nitrate staining. Subsequently the gels are stained with Coomassie R 250 from acetic acid/methanol solution.

4.5.6. Protein assay

The amount of protein in the protein extract prepared for electrophoresis was assayed using the PlusOne 2-D Quant Kit from Amersham Biosciences [186]. The proteins were precipitated with a solution based on trichloroacetic acid and the pellet re-suspended in a copper-containing solution. The unbound copper was measured with a colorimetric reagent, the colour density being inversely related to the protein concentration. The protein used for standard curves was bovine serum albumin (BSA).

4.5.7. Amino acid analysis

Hair samples of around 5 – 10 mg together with 5 ml HCl 6N were introduced in hydrolysis tubes and a vacuum atmosphere was created using a water pump. After 24 h hydrolysis at 110°C, the acid is eliminated by distillation under vacuum on a water bath (50°C) and the hydrolysis product is washed a few times with distilled water. The dry amino acid mixture is subjected to amino acid analysis after the method of Spackman et al. [187].
4.5.8. Tensile measurements

The stress-strain measurements (load elongation measurements) were made analogously to the Standard IWTO-32 in water, at 20°C. From every hair sample 90 fibres were taken, which were fixed on a special clamp with double-sided tape in subgroups of 10 fibres. The individual force to break and extension to break for every fibre from each subgroup were registered, and the samples were climatised and weighted for determining the medium value of the transversal section area in normal conditions (20°C, 65% RH). The test conditions were as follows:

Device: Instron 1122
Sample length: 10 mm
Extension length: 10 mm
Extension rate: 10 mm / min
Applied force: 10 N
Medium: Water, 20 °C
Specific hair density 1,31 g / cm³
Fibres number: 9 x 10

4.5.9. High-pressure Differential Scanning Calorimetry (HPDSC)

The thermoanalytical investigations were done with a DSC 7 apparatus from Perkin Elmer. Amounts of samples in the range 5 – 10 mg were weighted in climatised conditions in stainless steel crucibles and then 50 µl water added with a Eppendorf pipette. The crucibles were tightly closed using the appropriate top and rubber fittings and then subjected to a heating program with 10°C / min until 200°C were reached. The thermal energy necessary for this process was recorded parallel to a reference, and the resulted differential calorimetric curve was used to calculate the temperature – given by the peak maximum - and the enthalpy variation - given by the normalised peak area - of the melting process of the α-fibrils.

4.5.10. Scanning electron microscopy

Hair segments from the middle of the hair tresses were stuck down to double-sided adhesive tape on a scanning electron microscope mounting stub. The fibre specimens were vacuum-coated with gold and examined by secondary electron emission in a Leica S360 scanning electron microscope.
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