CHARACTERIZATION OF RECOMBINANT GROWTH FACTOR BASED COSMECEUTICALS

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ABSTRACT

Skin integrity is something that matters to a lot of people since it makes up a protective barrier but also contributes to a beautiful appearance. To maintain skin integrity there is a wide range of skin care products on the market to choose from. However, lately one particular product group called "cosmeceuticals" have increased. Cosmeceuticals contain biologically active substances among which recombinant growth factors are commonly seen. Recombinant proteins have already been used for a long time in pharmaceuticals where they are under much stricter regulations than in cosmetics. This means that neither the purity or the effectiveness, nor the concentration of the growth factors in cosmeceuticals are meeting the requirements of cosmetics. Thus, there is a risk that consumers will be misled or, in even worse cases, have adverse effects.

In this master's thesis five different cosmeceutical products containing recombinant growth factors were studied with the aim of detecting and characterizing protein. Different methods of sample preparation were performed to detect protein with electrophoresis followed by characterization with chromatography and two-dimensional fluorescence difference gel electrophoresis.

The obtained results showed that only one out of five products contained protein. This could mean that no protein was included in a majority of the products, although in future experiments additional analysis with higher sensitivity should be carried out to confirm this. Considering the protein that was found, it was not only the growth factor FGF, claimed to be there, but mainly another one that shared a lot of properties with bovine serum albumin. Hence, there seems to be reason to question the quality of cosmeceuticals and it would be advisable to establish stricter regulations.

SAMMANFATTNING

Att ha välbevarad, vacker hy är något som många människor eftersträvar. Frågan är vilka medel folk är beredda att ta till för att uppnå detta och hur långt tillverkarna vågar gå i sina försök att tillfredsställa konsumenterna.

På senaste tiden har en ny typ av skönhetsprodukter dykt upp på marknaden. Produkter som vid första anblick ser ut som vilka ansiktskrämer och serum som helst. Tar man sig en närmare titt ser man dock att de påstås innehålla biologiskt aktiva ingredienser såsom mänskliga tillväxtfaktorer. Tillväxtfaktorer är proteiner som normalt finns i huden hos alla människor och hjälper till att kontrollera bland annat celldelning och celltillväxt. Dessa egenskaper har man utnyttjat under en längre tid inom läkemedelsbranschen där tillväxtfaktorer har inkluderats med goda resultat. På senare tid däremot, har forskare inte bara sett botande egenskaper hos dessa protein utan även en skönhetsfrämjande effekt som skulle kunna ge föryngrad och vackrare hud om de inkluderades i ansiktskrämer eller dylikt.

Det må låta lovande, men läkemedelsindustrin och kosmetikaindustrin har några väsentliga skillnader som inte bör förbises. Nya läkemedel som kommer ut på marknaden har genomgått en strikt process och regleras av lagar och regler som ska se till så att alla produkter är säkra och saknar allvarliga biverkningar. När det kommer till kosmetika däremot, är regelverket betydligt mildare och det saknas liknande krav på produktkvaliteten. Det är där skon klämmer. Det finns alltså nya kosmetiska produkter på marknaden med samma ingredienser som i läkemedel men som inte regleras till närmelsevis lika hårt.

I det här examensarbetet har fem olika hudvårdsprodukter som sägs innehålla en utav de två tillväxtfaktorerna, EGF och FGF, undersökts. Detta gjordes i syfte att komma fram till huruvida dessa protein verkligen har inkluderats, hur stor koncentrationen i så fall är, samt för att få en bild utav produkternas renhet. Flertalet experiment utfördes för att kunna detektera protein vilket resulterade i oväntade svårigheter. Efter mängder av olika försök med varierande metoder kunde nämligen protein endast upptäckas i *en* utav produkterna. Slutsatsen kunde dras att det antingen saknades protein eller att det förekom i extremt låg dos. Detta borde bekräftas med känsligare analysmetoder i framtida experiment för att säkerhetsställa att de biologiskt aktiva hudvårdsprodukterna inte är en marknadsföringsbluff. Hursomhelst, den enda produkten som påvisade närvaro av en tillväxtfaktor kunde vidare analyseras. Olika egenskaper såsom storlek, koncentration, laddning och hydrofobicitet undersöktes och dokumenterades. Det visade sig att FGF som hävdats ingå i produkten fanns där, men desto mer protein utgjordes av en helt annan sort, nämligen serum albuminer - blodproteiner.

Vidare studier borde göras utav användandet av tillväxtfaktorer i skönhetsprodukter. För att det som konsument ska vara värt att lägga stora summor pengar på hudvårdsprodukter med påstådda effekter, ska det gå att säkerhetsställa vad som faktiskt ingår i produkterna och huruvida några risker tillkommer vid användandet.

PREFACE

This master's thesis project was conducted during the spring semester 2019 at Boku University in Vienna. With the help of an established network between professors at LTH and Boku, the final course of the education abroad was made possible. The subject "cosmeceuticals", which is concerned in the thesis, belongs to an upcoming group of skin care products on the market. Those products contain recombinant proteins, which there is good knowledge about in the biotechnology department at Boku.

After spending several months in the lab I got to learn different analytical techniques and methods. I also got the opportunity to work in a new environment and gained insight in how research is conducted in another country.

I would like to thank Leif Bülow and Alois Jungbauer who enabled my studies at Boku. I would also like to thank my supervisor, Nico Lingg, for guiding me, introducing the analytical techniques to me and for discussing the project and answering all my questions. I want to thank Beate Beyer who patiently helped me in the lab, especially with the 2D-DIGE. I am also very thankful to my friend Ronja who I went together with to Vienna. Thank you for all the time we spent together in the lab, it was very encouraging! Further I would also like to thank Johan, my supervisor at LTH for answering my questions when something was unclear. Finally I would like to thank my family and friends for constantly encouraging me, and not to forget A.G.

ABBREVIATIONS

2D-DIGE	- Two-dimensional fluorescence difference gel electrophoresis
BSA	- Bovine serum albumin
DNA	- Deoxyribonucleic acid
EGF	- Epidermal growth factor
FGF	- Fibroblast growth factor
GF	- Growth factor
GFP	- Green fluorescent protein
HCP	- Host cell protein
HPLC	- High-performance liquid chromatography
HAS	- Human serum albumin
IEF	- Isoelectric focusing
IEX	- Ion-exchange chromatography
IPG	- Immobilized pH gradient
MS	- Mass spectrometry
pI	- Isoelectric point
RP-HPLC	- Reversed phase high-performance liquid chromatography
SDS-PAGE	- Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SPR	- Surface plasmon resonance

CONTENTS

1

INTRODUCTION

People of all times have been interested in beauty and how to improve their look. Something that many associate with unwanted changes in appearance is aging, especially in the skin. Skin integrity is of great importance not only because it works as a protective barrier but also because it makes us look younger and more beautiful, in many people's opinion. Naturally a lot of companies have seen the potential in creating solutions for this problem, which has resulted in a wide range of skin care products on the market. Lately, a new product group called "cosmeceuticals" have appeared on the shelves. These products contain biologically active substances such as recombinant proteins, among which human growth factors have increased. Recombinant proteins have already been used for a long time in pharmaceutical products, however those products are under much stricter regulations than cosmetic products (Hadmed and Castillo, 2016). Although cosmeceutical products are used with a cosmetic purpose, they do contain the same substances as pharmaceuticals and should be regulated in the same way. However, the fact is that neither the purity, the effectiveness nor the concentration of the active substances are usually meeting the requirements of pharmaceuticals. This means that there is a risk that impurities, such as host cell protein and DNA, can have unknown effects when they are being used in topical products. In addition, this might lead to misfolded proteins that are incapable of binding to their receptors which might lead to consequences like making them ineffective or even adverse reactions (DePalma, 2018).

The purpose of this master's thesis is to detect protein, investigate the impurity profile, protein variant pattern and the concentration in five different cosmeceutical products containing epidermal and fibroblast growth factors. The initial part of the report is describing the background of skin aging, the concept of cosmeceuticals as well as the properties and functionalities of growth factors. Also the techniques that are used in this project are described and in what way they are being used. Then, the methods of this master's thesis are explained, including lab experiments with different analytical tools and how they are applied to detect and characterize protein. Finally, the results are analyzed and discussed and ending with drawing a conclusion.

2

THEORETICAL BACKGROUND

This chapter is divided into two parts. The first part, 2.1, explains what the skin products investigated in this master's thesis are used for, how skin aging occurs and the role of growth factors. The second part, 2.2, explains the techniques used for the protein analysis of the growth factors claimed to be in the cosmeceutical products.

2.1 Skin aging, cosmeceuticals and growth factors

2.1.1 Skin aging

Skin integrity is important, not only because the skin acts as a protective barrier against external effectors but also because undamaged and intact skin makes us look younger and more beautiful, in many people's opinion. During aging the skin is affected by natural, intrinsic factors as well as extrinsic factors like exposure to sunlight, pollution and cigarette smoke (Hadmed and Castillo, 2016). Consequently the mechanical, protective and compliance properties deteriorate with time due to breakdown of collagen and elastin network in the dermis which can visibly be seen as wrinkles, laxness and exaggerated expression lines as seen in Figure 2.1 (Fabi and Sundaram, 2014).

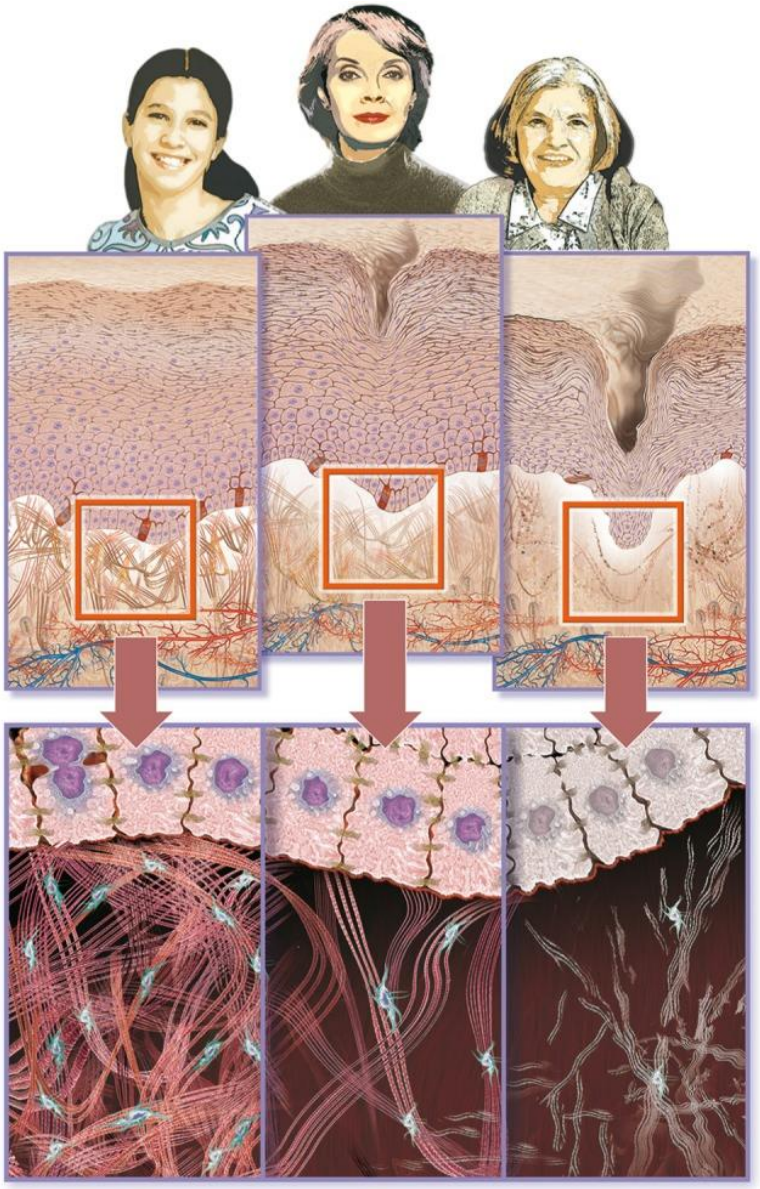

Figure 2.1. Changes in the skin over time. In the epidermis, so called corneocytes accumulate causing a rough and dull surface of the skin. In the dermis there is a decline in collagen content. Also, collagen and elastin fibers rearranges and become fragmented. Consequently, the structure loses its strength resulting in wrinkles (Aldag *et al*., 2016, Figure 2, Illustration: © Kleinhans RED).

Research have been performed on acute and chronic wound healing which has resulted in a deeper insight in the process of skin aging (Martin, 1997) (Hensley and Floyd, 2002). There are several pathways that are similar between the mechanisms of wound healing and regeneration of aging skin. Furthermore, knowledge about these mechanisms have been used in the development of a quite new product group called “cosmeceuticals” (Fabi and Sundaram, 2014).

2.1.2 Cosmeceuticals

Cosmeceuticals can be described as products which are combinations of pharmaceuticals and cosmetics. They contain biologically active substances but are applied with a cosmetic purpose, primarily with the aim of getting younger looking skin. These biologically active substances are made up of peptides and proteins, among which growth factors are commonly used. Since cosmeceuticals are not defined as pharmaceuticals they have not undergone the same procedure of proving efficiency as would have been necessary for a pharmaceutical product (Hadmed and Castillo, 2016).
Furthermore, a lot of biopharmaceutical products have recently been developed in which proteins are included with a therapeutic purpose. When these proteins are produced there is a risk of also including host cell proteins (HCPs), which are inevitable byproducts of cell culture - or fermentation-based biomanufacturing. The biggest risk of not removing the HCPs is their potential of inducing severe immune responses in patients (DePalma, 2018).
Apart from getting undesired HCPs in the product there is also a risk of introducing misfolded proteins or aggregates, and molecules with undesired post-translational modifications. Misfolding can be accelerated by different factors like heating during the manufacturing process, for instance. The impurities that might be present during biopharmaceutical production can be quantified and controlled to be kept at an allowed limit. However, although the same problem applies to the cosmeceutical manufacturers who also use recombinant proteins, they do not work under regulations as stringent as those for the pharmaceutical producers. Consequently, the purity might be disregarded by some (DePalma, 2018) (Ghorbani Aghdam *et al*., 2019).
In this master's thesis focus will be on human fibroblast growth factor (FGF) and human epidermal growth factor (EGF) present in a selection of cosmeceutical products.

2.1.3 Growth factors

Growth factors (GFs) are usually proteins with the capability of stimulating cell growth. By binding to specific receptors they can control pathways inter as well as intracellularly. In this way they monitor several mechanisms such as cell growth, differentiation and proliferation including collagen and elastin production and distribution (Fabi and Sundaram, 2014) (Żerańska *et al.*, 2016).
In this master's thesis cosmeceuticals containing epidermal growth factor (EGF) and fibroblast growth factor (FGF) are examined. EGF is a 53 amino acids long growth factor with a molecular mass of 6200 Da which has the effects of promoting mesenchymal (multipotent stem cell with potential of differentiating into different cell types (Nature, 2019)) and epidermal cell proliferation and differentiation (National Center for Biotechnology Information, n.d) (Uniprot, 2019). It participates in wound healing and promotes dermal recovery (Bodnar, 2013). FGF on the other hand stimulates the proliferation of fibroblasts, angiogenesis and thus wound healing by sending out signals created when binding to fibroblast growth factor receptors (Yun *et al*., 2010). FGF has a mass of 17000-18000 Da and around 155 amino acid residues (R&D Systems, a Bio-Techne brand, n.d.).

Furthermore, the interest in GFs lays in the fact that it is thought to be the same mechanisms occurring during skin aging as during wound appearance. When these events occur, the inherent repair system of the

skin is initiated which includes the mechanisms of GFs. Since the levels of GFs in the skin peak in youth and decline with age, these mechanisms will weaken with time. Therefore, the purpose of applying topical GFs when using cosmeceuticals is to refill the skin's own decreasing levels and upregulate the activity of skin rebuilding cells. This could slow down the aging process and possibly even have a reverse effect (Fabi and Sundaram, 2014).

Used in topically applied cosmeceuticals, GFs are thought to increase collagen production and decrease the collagen degradation in the skin. Although the mechanisms are not fully clear there is a hypothesis that if GFs such as KGF (FGF7), TGF-β and EGF successfully go through the stratum corneum, the outermost layer of the skin, they can induce a signaling cascade by binding to keratinocytes (Fabi and Sundaram, 2014) (Mehta and Fitzpatrick, 2007). Keratinocytes have receptors for these GFs, some of which are included in cosmeceutical products. Next after binding to the receptors, the keratinocytes start to secrete GFs themselves which subsequently affects fibroblasts which then start to produce GFs that exert their effects in the dermis. These fibroblast produced GFs also stimulate the proliferation of keratinocytes which leads to an extension of the starting signaling loop which can be seen in Figure 2.2 below (Fabi and Sundaram, 2014).

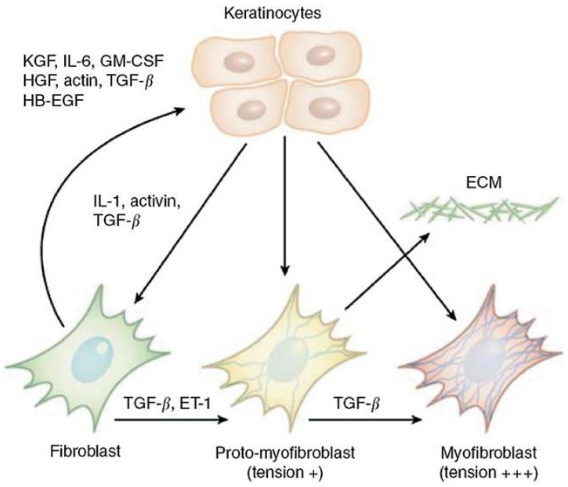

Figure 2.2. The proposed signaling cascade for growth factors and cytokines. ECM, extracellular matrix: EGF, epidermal growth factor: ET-1, endothelin 1: HGF, hepatocyte growth factor: IL-1, interleukin 1: IL-6, interleukin 6: KGF, keratinocyte growth factor: TGF, transforming growth factor. Interleukins are a group of cytokines which are signaling molecules in biochemical reactions (Fabi and Sundaram, 2014, Figure 3).

What is questionable about the effects of cosmeceuticals is that the included GFs, such as EGF and FGF, struggle to penetrate the skin. They are two big hydrophilic molecules with molecular masses of 6200 Da respectively 17000-18000 Da, and since studies have shown that molecules larger than 500 Da have difficulties penetrating the outermost skin layer, this means that a sheer mass of EGF and FGF cause problems (Hadmed and Castillo, 2016). On the other hand, there are studies investigating the effect of cosmeceuticals containing GFs which show good results despite the big size of the molecules (Gold, Goldman and Biron, 2007) (Żerańska *et al*., 2016). An explanation to this could be that GFs are capable of transporting themselves via sweat glands and hair follicles. In this way they can reach the keratinocytes and then interact and release chemical signals for fibroblasts. Another explanation is that it was older

people testing the products, who usually have drier skin which is more sensitive and facilitates larger molecules to enter (Hadmed and Castillo, 2016). Nevertheless, whether the presence of growth factors in cosmeceuticals have any effect and whether they are harmless is a controversial issue. The mechanism of how such large molecules can penetrate the skin has not been elucidated yet (Ranaweera, 2019).

2.2 Protein analysis methods

To be able to analyze and characterize the protein in the cosmeceutical products it is necessary to start by doing sample preparation. Thereafter different analytical tools can be used to analyze properties such as mass, isoelectric point and hydrophobicity. As a result an overview of the product content, concentration and purity will be obtained. Below, the different analytical methods used in this master thesis are explained.

2.2.1 Sample preparation

Samples that are too dilute, acidic, viscous or have a lot of interfering components can cause problems during some protein analysis. Thus, it is of great importance to prepare the samples with a suitable pre-treatment to be able to get good results. Several methods can be used during sample preparation. To find the best one for a sample that lacks information and experience, different alternatives can be carried out and compared. For instance, using detergents to dissolve proteins and to obtain a lower viscosity can be done. Another option is to use ultrafiltration to obtain a higher concentration and a third method is to remove interfering substances from the sample (Grabski and Burgess, 2001).

2.2.2 Sample preparation - ultracentrifugation

Another technique that can be used for sample preparation to concentrate and purify protein, is centrifugation. Zonal separation is a centrifugation method where a sample is placed on the surface of a density gradient or, like in the case of this project, a cushion made up by a liquid media. By applying sufficient gravitational force the particles of interest (in this case the proteins) will sediment and separate from contaminants in the sample when they migrate through the density gradient or cushion. Due to Stoke's law, Equation 1, it is possible to both concentrate and purify the particles simultaneously. The equation describes the physics of a sinking particle.

Stoke's law:

$$v = [d^2(p_p - p_m)g]/18\mu \quad (1)$$

v= sedimental velocity (m s^{-1})
d= diameter
p_p = the density of the particle (kg m^{-3})
p_m = the density of the liquid medium
g= gravitational acceleration (m s^{-2})
μ= the viscosity of the liquid medium (Pa s)

Under the conditions that arise during ultracentrifugation, centrifugal forces decrease and replace gravitational forces. Thus, the velocity of a particle under ultracentrifugation can be described with the relationship seen in Equation 2 below.

$$v = [d^2(p_p - p_m)\omega^2 r]/18\mu \quad (2)$$

ω = the angular velocity (radians,s^{-1})
r= the distance from the particle to the axis of rotation

In Equation 2, the "modified" version of Stoke's law, it is noticeable that the particle is affected by the two dominating forces: buoyancy ($p_p - p_m$) and sedimenting force $\omega^2 r$ and thereby the viscosity of the liquid medium and the size of the particle have an impact. The balance of these forces determines the sedimentation behavior of the particle in a centrifugal field.

In zonal separation the centrifugal force makes the sample components migrate through the gradient or cushion according to their s, sedimentation coefficient, which can be described by the relationship described in Equation 3. The migration rate is different for different particles which creates bigger distance between them over time depending on their s value.

$$s = v/\omega^2 r \quad (3)$$

To complete the centrifugation process a suitable rotor and density media need to be chosen. For the experiment in this master's thesis a swinging-bucket rotor was used, which creates long path lengths and causes the sedimenting particles to have minimal interaction with the sides of the tube. Sucrose solution was the media that was used as a cushion in the bottom of the centrifugal tubes, on top of which the sample was placed. Sucrose solution is a non-ionic medium with the advantages of being stable, biologically inert and cheap (Lawrence and Steward, 2010).

2.2.3 SDS-PAGE

One of the easiest and most straightforward characterization methods is sodium dodecyl sulfate–polyacrylamide gel electrophoresis, or SDS-PAGE as it is usually called. SDS-PAGE is a technique where the number and mass of polypeptides within a sample can be analyzed. Samples are prepared and injected into a gel (Nowakowski *et al*., 2014). To start, the polypeptides will be denatured by an anionic detergent which will subsequently bind to them and give them a negative charge which will correspond to their molecular mass. This step is followed by the application of an electric field over the gel where the charged molecules are separated according to their sizes (Nowakowski *et al*., 2014). Furthermore, to be able to see what has bound to the gel and where, it is stained. Coomassie Brilliant Blue is a commonly used dye which stains protein bands blue via electrostatic interaction with protonated amino acids and via hydrophobic association with aromatic residues. However, it does only bind to the acrylamide with low affinity and can easily be removed by a destaining step (Steinberg, 2009). Finally, the bands which have appeared on the gel can be compared with a marker which shows bands with known molecular weights. Thus, SDS-PAGE can detect if any protein is present in a sample and by looking at the mobility of the

polypeptides their molecular weight can be determined (Garfin, 2009). Considering the limitations of SDS-PAGE, this technique is incapable of separating small peptides and has difficulties to resolve proteins with similar molecular masses (Tomky-Cassiday, 2007).

2.2.4 Matrix Spiking

Matrix spiking is a technique which is used to evaluate the capacity of an analytical method when a specific sample matrix is being investigated. By doing this, it is possible to get information about the quality of the results and figure out whether they are valid or not. A successful result of matrix spiking increases the confidence in the validity of the sample test result (Bowen and Volchok, 1980).
Matrix spiking is carried out by adding a known amount (a spike) of analyte to the sample. The sample is then tested with the analytical method and the recovery of the analyte is determined. A spiked sample with known amount of analyte can then be compared with a "normal" sample. If the analytical method is not working well, the matrix spike result will be much higher or lower than expected (Bowen and Volchok, 1980).

2.2.5 High-Performance Liquid Chromatography with Cation Exchange Column

High-performance liquid chromatography (HPLC) is an analytical technique with the ability to separate, identify and quantify different components in a mixture and is commonly used for analyzing peptides and proteins. The principle of the method is to pump the liquid sample under pressure through a column where the sample components will travel with different speed depending on their affinity for the immobilized stationary phase of the column. If a component has high affinity for a molecule in the immobilized phase, it will retain longer than one with lower affinity. Moreover, the binding and thereby the separation can occur in different ways. For instance ion-exchange (IEX) chromatography, which is commonly used for separation of biomolecules like peptides and proteins, separates components by differences in their charge. The charged groups on the peptide molecules will bind to molecules of opposite charge present in the column while non-bound components will be washed out. An anion-exchanger has positively charged gel matrix and will therefore bind and exchange negatively charged counter ions while a cation-exchanger has a negatively charged matrix and will bind and exchange positively charged counter-ions. Peptides and proteins are ionized in solution to an extent depending on the pH. Every peptide and protein has an isoelectric point (pI), where all its negative charges are the same amount as its positive charges. Thus, the net charge is zero when pH=pI. When the pH<pI the net charge is positive which means the protein should bind to a cation-exchange column. When instead the pH>pI the net charge is negative and the protein should bind to an anion-exchange column. Usually proteins with a pI<6 are analyzed with an anion-exchange column and proteins with a pI>8 with a cation-exchange column (Aguilar, 2004).

There are different elution modes to elute the bound components from the ion-exchange column. One option is to increasing the salt concentration of the eluent with the purpose of introducing competing counter ions. Another one is to change the buffer pH with the purpose of decreasing the net charge (Aguilar, 2004). Using a salt gradient is an uncomplicated and commonly used method whereas a pH

gradient is easier to control and reproduce, however it is a more complex method. Nevertheless, the use of pH gradients has shown to give high resolution separation of charge variants as described by Lingg *et al*. (2014). Since one part of the aim of this master's thesis was to investigate the protein variant pattern of the growth factors the natural choice was to use a pH gradient with high resolution.

Furthermore, what also should be considered when using IEX is the choice of buffers. Since binding of the proteins to the column are depending on the charge of the proteins there should preferably not be any competition from other similarly charged ions. Therefore, the buffer ions should have the same charge as the ion-exchanger which means that anionic buffers are used for cation-exchangers and cationic buffers are used for anion-exchangers. An advantage with the buffer components used in IEX is that they do not denature the proteins which can then be further analyzed with functional assays for instance (Aguilar, 2004).

2.2.6 Reversed-Phase High Performance Liquid Chromatography

Reversed phase high performance liquid chromatography (RP-HPLC) is another kind of chromatography which separates proteins and peptides based on their hydrophobicity. Instead of having a hydrophilic stationary phase (sorbent) like "normal-phase" chromatography, RP-HPLC has covalently bound hydrophobic ligands to the stationary phase, which creates a hydrophobic sorbent. Thus, the affinity for hydrophobic molecules is higher (Aguilar, 2000).

The solute mixture, containing the sample with the proteins to be separated, is initially introduced to the stationary phase together with aqueous buffers. Hydrophobic molecules will adsorb to the hydrophobic surface of the stationary phase while hydrophilic molecules in the mobile phase will pass through the column and be eluted first. The adsorbed molecules are then eluted by addition of an organic solvent, such as acetonitrile, which reduces the hydrophobic interactions and releases them. Elution can be carried out either by a constant concentration of organic solvent or by a gradient, where the amount of organic solvent is increased over time. The elution order of the solutes will be based on increasing molecular hydrophobicity, the more hydrophobic the longer the retention time (Aguilar, 2000).

In RP-HPLC the conditions are quite rough and not suitable if it is desirable to maintain the 3D-structure of the protein. It might be that the proteins are irreversibly denatured and consequently the recovery of biologically active forms is lost. Therefore it is preferable to use IEX when isolating protein whose functionality is to be investigated as well (Aguilar, 2000).

2.2.7 Two-Dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE)

Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) is a method where proteins are separated based on two characteristics: charge and size. The first dimension includes isoelectric focusing (IEF), a technique which separates proteins based on their pI. Since the proteins are denatured in the 2D-DIGE procedure the separation is based on all their charges, not only those on the surface which is the

case in IEX. Thus, separation based on pI values can look slightly different when comparing the two methods. The second dimension, SDS-PAGE gel electrophoresis, separates proteins based on their size as previously described (Beckett, 2012).

An essential part of 2D-DIGE is the minimal labeling. Fluorescent dyes are used to label each protein by binding to a restricted number of lysine residues. The fluorescent cyanine dyes, CyDye DIGE Fluors, are spectrally resolvable and includes Cy2, Cy3 and Cy5. The minimal dyes are matched for molecular weight and charge and each of them carry a positive charge which replaces the one of the lysine, and thus does not affect the pI value. The labeling is dye limiting and assures that only 1-2 % of the lysine residues are labelled which means there should only be one dye label per protein. This will later on be seen as one protein spot on the gel. To avoid gel-to-gel variation if several attempts are carried out, one of the dyes is used to label a pooled internal standard (Beckett, 2012). The labelled protein is pooled into one sample and transferred to an IPG (immobilized pH gradient) gel strip having a pH range from acidic to basic. In the IEF technique an electric field is applied which creates an electric force that causes proteins to move towards the acidic or basic end of the gel strip depending on the charge they have. When the proteins reach their respectively isoelectric point, they stop moving (Diez *et al.*, 2010) (GE Healthcare Life Sciences, 2012). The IEF strip is then placed on top of the SDS-PAGE gel where the electric forces helps to transfer the proteins from the IEF gel into the SDS-PAGE gel (Diez *et al.*, 2010).

The individually labelled proteins are separated and the gel is scanned at different wavelengths resulting in fluorescence associated with each CyDye. Image analysis can finally be carried out where the colored spots represent protein. If a spot appears at the same place in two images it means they contain the same kind of protein. An overview of the 2D-DIGE technique can be seen below in Figure 2.3 (Beckett, 2012).

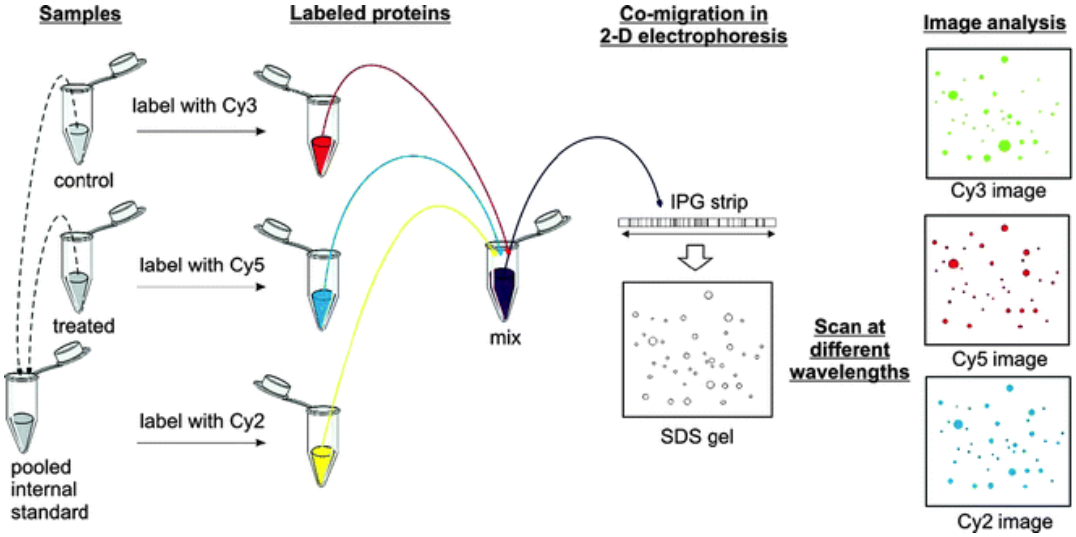

Figure 2.3. An overview of the 2D-DIGE procedure (Beckett, 2012, Figure 1).

2D-DIGE is a technique with the capability of identifying biological variability like protein post-translational modifications. With dyes having comparable sensitivity to silver staining and with the ability to label several proteins and separate them on the same gel, avoiding gel-to-gel variability makes this technique a research tool of great importance. However, it is a denaturing technique which excludes the possibility of further analyzing the proteins with functional assays (Diez *et al*., 2010).

3

MATERIAL, EQUIPMENT AND METHOD

Several experiments were carried out with the purpose of detecting and characterizing the recombinant protein in the cosmeceutical products. Here the methods of the experiments are described including the material and equipment.

3.1 Cosmeceutical products

Purified FGF sample which had been prepared in-house was used as an internal standard for comparison in the experiments. The FGF concentration of the internal standard was measured to 0.322 mg/mL.
The cosmeceutical products that were studied in this master's thesis are stated below.

FGF products:

- Norelift, anti-wrinkle cream, produced in France. Claimed effect of stimulating the skin cells' anti-aging processes and to have similar effect to botox by erasing wrinkles and fine lines.
- Cell shock, eye cream, produced in Switzerland. Claimed effect of improving the feeling and touch of the skin's inner "architecture" and to restore its luminosity and uniformity.
- rh-bFGF Gaifu (further referred to as "Gaifu"), produced in China. Claimed effect of promoting wound healing and can be used for chronic wounds. Contains 70000 IU/vial.

EGF products:

- Bioeffect, serum, produced in Iceland. Claimed effect of stimulating the skin's ability to renew itself.
- Terproline, face cream, produced in Italy. Claimed effect of improving the aesthetic appearance of facial skin that is in need of regeneration and improve the condition of ageing skin. EGF present in encapsulated form.

3.2 Sample preparation

Chemicals

The chemicals used to prepare samples for SDS-PAGE were PBS (Invitrogen), 2-propanol (isopropanol) and Triton X-100 (Sigma-Aldrich), acetonitrile (J.T Baker), 2-D Clean-Up Kit (GE Healthcare). As reference proteins bovine serum albumin (BSA) and skim milk powder (Sigma-Aldrich) were used together with green fluorescent protein (GFP) (produced in-house).

Method

Several attempts were carried out in order to come up with a way to extract and detect the protein in the products for further analysis. To see if the preparation process had been successful, samples were subsequently run in SDS-PAGE experiments.

3.2.1 SDS-PAGE

Chemicals

The chemicals used in the sample preparation were NuPAGE® LDS Sample Buffer (4X) (Invitrogen) and DL-dithiothreitol (DTT) (Sigma-Aldrich). The gel used in the experiments was NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm (Invitrogen) and a 20x MES SDS Running Buffer stock solution was used*. Furthermore, the molecular weight marked was SeeBlue Plus2 (Invitrogen) and the Coomassie-Brilliant Blue R250 (Sigma-Aldrich). The chemicals used in the fixing and destaining solution came from (Merck)*. The silver staining included 4 different solutions for the staining procedure: fixing solution, incubation solution, silver solution, developing solution and a stop solution*.
*All details for the buffer solutions can be found in Appendix 7.1.1.

Method

In the SDS-PAGE experiments the presence/absence of protein was examined as well as the molecular weight of the protein. This was compared between the product samples but also with a marker, an internal standard and other reference proteins.
Several experiments were carried out with the initial aim of just comparing the products in their normal states. This was followed by several attempts of extracting the proteins by using different solvents and precipitation methods. The sample volume was 100 mg each time and the basic approach was the same in all the experiments with the only varying parameter being the dissolving/precipitating method.

Samples were prepared by mixing 15 µL product solution, 5 µL NuPAGE LDS Sample Buffer (4X), NP0007 and 5 µL DTT (2M). The samples were heated for 10 minutes at 95 ℃and then loaded into a NuPAGE Novtex 4-12% Bris-Tris Gel (1.0 mm) filled with 20x MES SDS Running Buffer stock solution. In addition to the product samples, a SeeBlue Plus2 Marker (10 µL) and an internal FGF standard were loaded for comparison. The gel was run for approximately 50 minutes at 200 mA followed

by a procedure of being put into a fixing solution (10 minutes), a Coomassie staining solution (10 minutes) and finally a destaining solution. Lastly, the presence or absence of bands could be investigated. In the experiment with silver staining the gel was instead put in fixing solution and incubation solution for 30 minutes respectively followed by a washing step in RO-water 6 times for 5 minutes. Afterwards the gel was incubated in silver solutions for 20 minutes, shortly rinsed and then put into developing solution until bands appeared on the gel. Finally the gel was placed into stop solution for 10 minutes to stop the reaction.

The SDS-PAGE experiments were performed with the following product samples (all Coomassie stained as well as one additional silver stained gel for the ultracentrifugation experiment):

- Product samples in 1:5 dilution with phosphate-buffered saline (PBS).
- Direct product samples.
- Product samples diluted with isopropanol (70%) → centrifuged → supernatants ultrafiltrated and used.
- Product samples diluted with acetonitrile (70%) → centrifuged → supernatants vacuum centrifuged, - mixed with 50 µL water and used.
- Product samples acid precipitated with 2-D Clean-Up Kit.
- Product samples diluted with 350 µL 1:2 mixture of PBS and Triton.
- Product sample microwaved and diluted with 50 µL PBS (only Terproline).
- Product samples diluted with 5 mL paraffin oil and ultracentrifuged. Samples taken from the arisen separated phases and used in SDS-PAGE experiments with Coomassie staining as well as silver staining (for more details about the ultracentrifugation experiment see section 3.2.2 below).
- Gaifu serum sample compared to FGF standard and BSA. All samples loaded on the gel in three different concentrations of 0.5 g/L, 0.25 g/L and 0.125 g/L.

3.2.2 Ultracentrifugation

Chemicals

In the ultracentrifugation experiment the chemicals used were sucrose (Acrose Organics) and paraffin oil (Merck).

Equipment

In the ultracentrifugation the equipment used was Beckmann Ultra Clear 344053 13.2 mL centrifuge tubes, Beckmann SW 41 Ti swinging-bucket rotor and Beckmann 2 Nano Beckmann L-100XP ultracentrifuge.

Method

Ultracentrifugation was performed in another attempt to extract the protein in the creams. A volume of 700 µL 30 % sucrose solution was pipetted into centrifuge tubes and then put in the freezer overnight. The product samples were mixed with 5 mL paraffin oil for easier handling and introduced on top of the

frozen sucrose solution, which was then topped up with pure paraffin oil. The centrifugation tubes were balanced and placed in a swinging-bucket rotor. Thereafter the rotor was placed into the centrifuge which was set to 38 000 rpm for 2 hours. The sample phases in the bottom of the centrifuge tubes were used in SDS-PAGE experiments with Coomassie staining as well as silver staining.

3.3 Protein Characterization

Further protein characterization was carried out with Gaifu, which was the only product in which protein could be detected.

3.3.1 Ion-exchange Chromatography

Gaifu was the only product that could be run in the IEX chromatography. Gaifu contained FGF2 which has a pI value above 8 (PhosphoSitePlus, 2019), hence a cation-exchange column was chosen for the experiments.

Chemicals

When the buffers for the ion-exchange chromatography were prepared the following chemicals were included*: 4-(2-hydroxyethyl)piperazine-1-ethansulfonic acid (HEPES), N,N-bis(2-hydroxyethyl)glycine (bicine), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 2-(N-morpholino)ethanesulfonic acid (MES), formic acid ~98% all from (Sigma-Aldrich) and natrium chloride and natriumacetat from (Merck). The reference proteins used here as well were BSA, skim milk powder protein and green fluorescent protein (GFP, produced in-house).
*All details for the buffer solutions can be found in Appendix 7.2.

Equipment

Instrument: Agilent Technologies 1220 Infinity LC.
Cation-exchange column: ProPac™ WCX-10 LC Column, 054993, Dionex.

- Diameter: 4 mm.
- Length: 250 mm.
- Particle size: 10 µm.

Method

In the ion-exchange chromatography the proteins were separated according to their pI, analyzed and compared to the internal standard and some reference proteins.
The experiments were carried out as follows:

- Buffer protocol were chosen in order to get a pH gradient with good separation capacity of the protein. The buffers were mixed, filtered and degassed before being connected to the chromatography device.

- The samples together with a blank were prepared by diluting with either PBS or Buffer A, or they were kept undiluted.
- The samples and the blank were filtered into chromatography vials to get rid of undesired particulate matter. The final sample volume was 200 µL to have marginal for the injection volume of 100 µL while the blank volume usually was a bit bigger, around 500 µL, to be able to be reused.
- The sample vials were placed into the chromatograph and a method was chosen on the software before the run was started.

Experiment details

1. The **first** ion-exchange chromatography analysis was performed only with Gaifu. All details are stated below.

- Sample preparation: Gaifu, 1:5 dilution with PBS.
- Buffer A: pH 8.
- Buffer B: pH 10.5.
- Buffer components: HEPES, Bicine, CAPSO, CAPS and NaCl (see protocols in Table 7.17 and 7.18 in Appendix 7.2).

2. The **second** ion-exchange chromatography analysis included several samples of Gaifu of different dilutions together with the FGF standard for comparison. All details are stated below.

- FGF in 1:2 dilution with buffer A.
- Gaifu in 1:5 dilution with buffer A.
- Gaifu in 1:10 dilution with buffer A.
- Blank: PBS in 1:2 dilution with buffer A.
- Buffer A: pH 4.
- Buffer B: pH 11.
- Buffer components: CAPS, CAPSO, Bicine, HEPES, MES, Na-acetate, formic acid and NaCl (see protocols in Table 7.19 and 7.20 in Appendix 7.2).

3. The **third** ion-exchange chromatography analysis included new dilutions of Gaifu and also reference proteins for new comparison. The running conditions were the same as in the second chromatography anlysis. All details are stated below.

- Gaifu diluted 1:2 with buffer A.
- Gaifu non-diluted.
- Green fluorescent protein (GFP) 1 g/L → 1:10 dilution with buffer A.
- Skim milk powder 10 g/L in PBS → 1:10 dilution with buffer A.
- Bovine serum albumin (BSA) 10 g/L in PBS → 1:10 dilution with buffer A.
- Blank: PBS in 1:2 dilution with buffer A.
- Buffer A: pH 4.
- Buffer B: pH 11.
- Buffer components: CAPS, CAPSO, Bicine, HEPES, MES, Na-acetate, formic acid and NaCl (see protocols in Table 7.19 and 7.20 in Appendix 7.2).

The samples were prepared to be 1g/L, comparable with the FGF standard which had been injected with that concentration earlier.

4. The **fourth** ion-exchange chromatography experiment also compared with reference proteins
A new experiment was prepared to analyze FGF standard, BSA and Gaifu in the same run. This would give a more credible result than when comparing curves from different runs which was done in the third experiment. All details are stated below.

- Gaifu 0.5 g/L undiluted.
- FGF, which at this point had been measured to 2.2 g/L, were prepared in a 1:3.38 dilution with buffer A to obtain 0.5 g/L as the concentration of Gaifu.
- BSA stock solution (10 g/L) was also diluted with Buffer A, 1:19, to obtain a concentration of 0.5 g/L.
- Blank: 1:2 mixture of PBS and Buffer A.
- Buffer A: pH 4.
- Buffer B: pH 10.5.
- Buffer components: CAPS, CAPSO, Bicine, HEPES, MES, Na-acetate, formic acid and NaCl (see protocols in Table 7.21 and 7.22 in Appendix 7.2).

3.3.2 Reversed Phase-HPLC

Chemicals

Acetonitrile (J.T Baker).

Equipment

Instrument: Waters e2695 HPLC.
Column: Tosoh TSKgel Protein C4-300, L × I.D. 5 cm × 4.6 mm, 3 μm column with a guard column.

Method

In the RP-HPLC the proteins were separated according to their hydrophobicity, analyzed and compared to the internal standard. The experiments were carried out as follows:

- The samples together with a blank were prepared by diluting with either PBS or they were kept undiluted.
- The samples and the blank were filtered into chromatography vials to get rid of undesired particulate matter. The final sample volume was 200 μL to have marginal for the injection volume of 100 μL while the blank volume usually was a bit bigger, around 500 μL, to be able to be reused.
- The sample vials were placed into the chromatograph and a method was chosen on the software before the run was started.
- Acetonitrile was used as organic solvent.

Experiment details

1. The **first** RP-HPLC run contained the following samples:

- Gaifu undiluted.
- BSA, diluted with PBS to a concentration of 0.5 g/L.
- FGF standard diluted with PBS to a concentration of 0.5 g/L.

2. The **second** RP-HPLC run contained different dilutions of FGF standard with PBS with the aim of creating a calibration curve with known concentrations to see where Gaifu could fit in and thus figure out its FGF concentration. The dilutions prepared were:

- FGF standard 1:2.
- FGF standard 1:4.
- FGF standard 1:8.
- FGF standard 1:16.
- FGF standard 1:32.
- FGF standard 1:64.
- FGF standard 1:128.

200 µl of each sample was filtered and injected into chromatography vials which were placed into the chromatography device and injected into the column for analysis.

3.3.3 Two-Dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE)

Equipment

For the first dimension of the 2D-DIGE an 18 cm Immobiline DryStrip Gel pH 3-11 NL (GE Healthcare) and the Ettan IPGphor3 Isoelectric focusing unit (GE Healthcare) were used. When performing the second dimension an Ettan DALTsix instrument (GE Healthcare) was used.

Chemicals

A minimal dye labeling kit (2 nmol), containing a CyDye DIGI Fluor, obtained from GE Healthcare was used. In addition Tris pH 8.8 (Merck), 99.8% anhydrous N,N-dimethylformamide (Sigma–Aldrich), 10 mM L-lysine (Fluka, Bucharest, Romania), 100 mM dithiothreitol (DTT) (Sigma-Aldrich), 8 M urea (Merck), 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma–Aldrich), 2% immobilized pH gradient (IPG) buffer 3-11 (GE Healthcare), DeStreak reagent (GE Healthcare), agarose (Sigma-Aldrich) and paraffin oil (Merck) were used.

Method

Labelling

Three samples including FGF standard, Gaifu and BSA were prepared by diluting with water to obtain concentrations of 1 mg/mL. A volume of 30 µL of each sample was labeled by adding 3 µL of 200 mM

Tris, pH 8.8 for pH adjustment and 0.6 µL of 400 pmol/µL CyDye working solution in 99.8% anhydrous N,N-dimethylformamide. Three different CyDye solutions were used: Cy2, Cy3 and Cy5 which were added to one sample each. This was followed by incubation on ice under light-protection for 30 minutes. Thereafter 0.6 µL of 10 mM L-lysine was added in order to stop the reaction. The samples were incubated once more on ice under light-protection for 10 minutes. The samples labeled with Cy2, Cy3 and Cy5, respectively, were pooled and mixed. A 5 µL of 100 mM DTT was added to the pooled sample which was then incubated 10 minutes at room temperature for denaturation. Then, the pooled sample was mixed with 400 µL of rehydration solution* containing 8 M urea, 2% CHAPS, 2% IPG Buffer 3-11. Finally, 6 µL DeStreak reagent was added and the samples were applied to the isoelectric focusing strip by rehydration loading. For this procedure the sample mixed with rehydration solution* (8 M urea, 2% CHAPS, 2% IPG buffer, RO-water) was pipetted into a narrow well in a plastic container. Afterwards, the IPG strip was placed into the liquid with the gel-side facing downwards, covered with paraffin oil and incubated for 20 h in the dark.

First dimension

The gel strip was placed into the IPGphor3 instrument according to the manufacturer's instructions and the IEF program suitable for the length and pH range of the gel was started.

Second dimension

For the second dimension the gel strip was denatured and reduced in equilibration buffer 1* and then alkylated with equilibration buffer 2*. The incubation time was 15 minutes for each buffer and took place on a shaker. Thereafter the gel strip was placed in a one well self-cast acrylamide gel and closed with melted agarose solution (containing bromphenolblue) in Tris/glycine running buffer. After preparation of the gel cassette, which included filling of the lower chamber with 400 mL 1x SDS running buffer* and adding of 4 liters of RO-water the gel was inserted in Ettan-Dalt-six and the upper chamber was fixed on top of the gel. The upper chamber was filled with 200 mL 10x SDS running buffer and 800 mL RO-water. Finally, the gel was run 1 h at 10 mA, 80 V, 1 W and 5 h at 40 mA, 500 V and 13 W.
*See buffer protocols in Appendix 7.4.

Fluorescence scanning

After running the gels the bands were detected with Typhoon FLA 9500 Fluorescence scanner (GE healthcare).

3.3.4 Spiking samples

Method

After all the struggle with the sample preparation and attempts to discover protein in the cosmeceutical products it was decided to try a different approach. New samples were weighed (100 mg) and this time spiked with a 0.322 mg/mL FGF standard (100 µL). The idea was to carry out the same sample preparation methods exactly as described in section 3.2.1 however this time with samples containing a

known amount of FGF. The aim of this experiment was to get a clue if protein actually was detectable in the matrixes of the cosmeceuticals and if so, which sample preparation that would be the best one.

4

RESULTS AND DISCUSSION

4.1 Sample preparation

4.1.1 Sample preparation and SDS-PAGE

Several attempts were carried out in order to prepare samples from which the EGF and FGF, claimed to be in the cosmeceutical products, could be detected and analyzed. For this purpose a proper sample preparation is essential to be able to perform SDS-PAGE with clear and accurate resolution of protein bands. If the samples are very dilute, viscous or contain a lot of interfering substances pre-treatment is needed to make the protein visible on the gel (Grabski and Burgess, n.d.). Since the cosmeceutical products were both dilute, viscous (except for the serums) and contained a lot of interfering ingredients, sample preparation was necessary.
The solubility of a protein is depending on the charges and hydrophobic residues at its surface. The amino acid sequences of proteins differ in charge, hydrophobicity and polarity and hence they display different solubility under particular conditions. A common characteristic for most proteins is the way of folding to have hydrophobic residues on the inside and hydrophilic ones on the surface. By changing the solving properties one can change the solubility of a certain protein and cause precipitation which can be done in order to concentrate the protein of interest. Protein precipitated from a large volume can then be dissolved into a smaller one and thus, a more concentrated solution can be obtained. Ways of causing precipitation are for instance addition of detergents, changing of pH by adding bases or acids and by applying heat. Precipitation is initially caused by the creation of an unstable solution which leads to protein aggregation and flocculation which further separates the protein from the rest of the solution (Hatti-Kaul and Mattiasson, 2003).

During the sample preparation different solvents were used and in some cases in combination with centrifugation and ultrafiltration to concentrate the sample and obtain a higher protein concentration. The solvents that were used were Triton-X 100, acetonitrile and isopropanol. Non-ionic detergents, such as Triton-X 100, are usually preferred since they cause the least amount of damage to proteins and is usually

considered as an option in protein analysis (Islam *et al*., 2017). Acetonitrile is a nitrile which is often being used as an organic solvent that causes precipitation. Using acetonitrile also have the advantage that the dilution caused by its addition can be reduced by evaporation (vacuum centrifugation was used here) (Alshammari *et al*., 2015) (Abian and Carrascal, 2003), while isopropanol is a polar organic solvent which also has the capability of precipitating protein (Marney *et al*., 2008).

Gaifu was the only product in which protein could easily be detected when it was directly applied on the gel without any sample preparation. The result can be seen in Figure 4.1 where three bands are visible indicating the presence of proteins with molecular masses of approximately 17, 49-62 and 98 kDa respectively. Since 17 kDa is the molecular mass of FGF, this band is showing the presence of the growth factor in the cosmeceutical while the other bands indicate presence of two other proteins as well. Apparently the matrix of Gaifu was of such kind that no sample preparation was necessary for protein detection in contrast to the other products. Therefore Gaifu was not included in the other electrophoresis experiments where different sample preparation methods were tried.

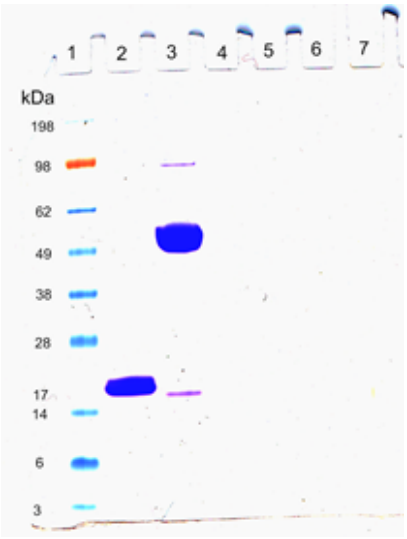

Figure 4.1. The first SDS page gel with directly applied samples. FGF standard in well 2 and Gaifu in well 3.

Moreover, the protein detection and molecular mass determination of the other products gave no results after diluting the samples in PBS. Neither did dissolving in the organic solvent isopropanol give any clear results nor did the attempt with Triton™ X-100 or the use of the 2-D Clean-Up Kit (GE Healthcare) with the purpose of causing acid-precipitation. Microwaving and ultracentrifugation did just result in white smears and acetonitrile followed by vacuum centrifugation gave blue smears instead of the desired blue bands (all the gels can be seen in Appendix 7.1.2).

Since all the manufacturers had claimed the presence of FGF or EGF in their products there had to be an explanation for the lack of protein on the SDS-PAGE. It could either be that the products contained a large amount of interfering substances making the protein very difficult to detect, or it could be that the protein was added in such low concentration that it was undetectable with SDS-PAGE. However, Coomassie brilliant blue staining has a sensitivity of 8-10 ng per band for some proteins and 25 ng for most proteins (Thermofisher Scientific). According to the Canadian drug bank, which is a bioinformatics and cheminformatics resource with an extensive amount of detailed drug data and information, the amount of FGF2 in several cosmeceutical products is 2 µg/g (Drugbank, 2019). Using this as a reliable reference it is remarkable that no protein could be detected in 4 of 5 products from which 0.1 g was used in each sample preparation. In 0.1 g of product 200 ng protein should be present, which is 8 times more than the detection limit of SDS-PAGE. Based on these numbers it seems like there was no protein in the products. Even though it has to be considered as unlikely, a third explanation is that that no protein was included and it was all false advertisement.

4.1.2 Matrix spiking

By looking at the properties of the product matrices it was possible to see whether the presence of interfering substances would be a reasonable explanation to the absence of protein on the SDS-PAGE. Matrix spiking was the chosen method and was carried out with the four different cosmeceutical products with undetectable protein content: Bioeffect, Cellshock, Norelift and Terproline. FGF was injected into each product sample which was then mixed with the purpose of incorporating it into the creams/serum. All the sample preparation methods that were initially carried out were repeated with the spiked samples. If the FGF standard would be detected by any of these methods, this would confirm a successful sample preparation. Thus, such a method should be able to detect protein in the original products.
Spiking the samples with FGF standard, gave another picture of the functionality of the sample preparation methods. In all the experiments the products contained 32.2 µg FGF standard. Such an amount appeared to be detectable in Bioeffect and Cellshock when the samples were diluted with only PBS (Figure 4.2). Acetonitrile followed by vacuum centrifugation was successful for all the spiked products except for Cellshock (Figure 4.3) while using the 2D Clean-Up Kit and ultracentrifugation protein was detectable in all the different product matrices which can be seen in Figure 4.4 and 4.5. The summarized results can be seen in Table 4.1 where the successful methods for each product are marked: ✓.

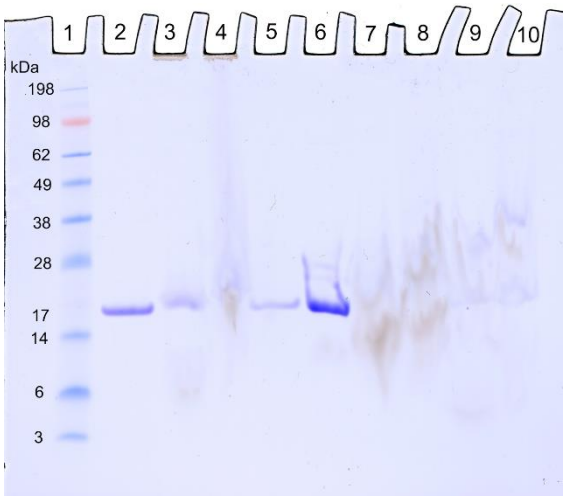

Figure 4.2 Samples spiked with FGF. Some applied directly and some diluted with PBS. From 1-10: 1. Marker, 2. FGF standard, 3. Cellshock (dil.1:5), 4. Cellshock dir., 5. Bioeffect (dil. 1:5), 6. Bioeffect dir., 7. Terproline (dil. 1:5), 8. Terproline dir., 9. Norelift (dil. 1:5), 10. Norelift dir.

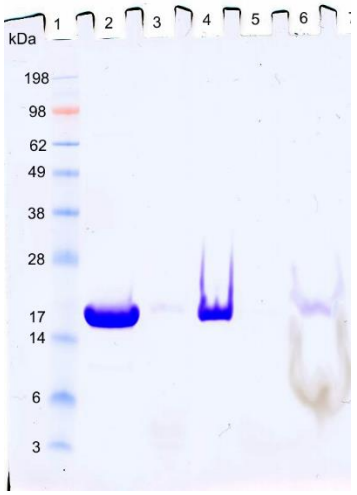

Figure 4.3. Samples spiked and diluted in acetonitrile followed by vacuum centrifugation. From 1-6: 1. Marker, 2. FGF std, 3. Norelift, 4. Bioeffect, 5. Cellshock, 6. Terproline

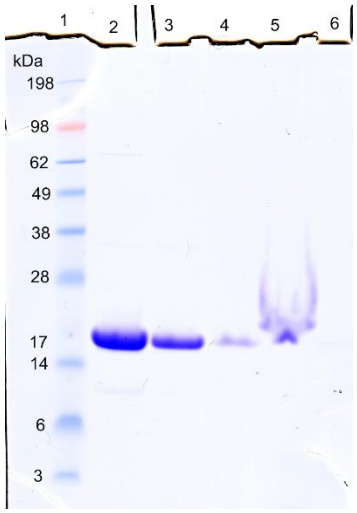

Figure 4.4. Samples spiked and treated with 2D Clean-Up Kit.
From 1-6: 1. Marker, 2. FGF standard, 3. Bioeffect, 4. Cellshock, 5. Norelift, 6. Terproline

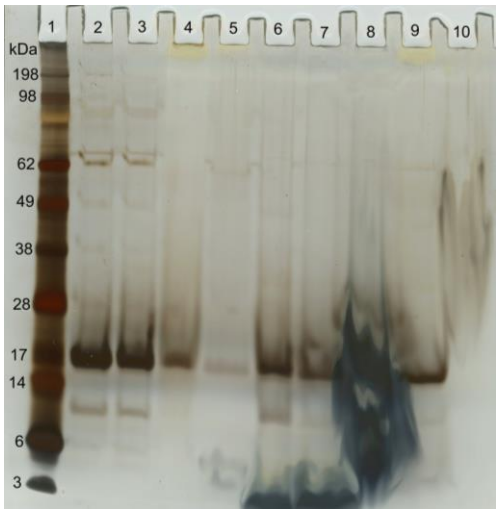

Figure 4.5. Samples spiked and ultracentrifuged.
From 1-10: 1. Marker, 2. FGF standard, 3. Bioeffect, 4. Cellshock 1, 5. Cellshock 3, 6. Norelift 1, 7. Norelift 2, 8. Norelift 3, 9. Terproline 1, 10. Terproline 2.
(The different numbers of each sample comes from the different layers created during the ultracentrifugation).

Table 4.1. Listing the results of each sample preparation method with the spiked samples. Successful result are marked ✓.

Product	**Diluted in PBS**	**Acetonitrile+vacuum centrifugation**	**Isopropanol +UF**	**2D Clean-Up Kit**	**Triton-X 100/PBS**	**Triton-X 100/PBS +UF**	**Ultracentrifugation**
Bioeffect	✓	✓	-	✓	-	-	✓
Cellshock	✓	-	-	✓	-	-	✓
Norelift	-	✓	-	✓	-	-	✓
Terproline	-	✓	-	✓	-	-	✓

The conclusion of the matrix spiking is that if FGF had been present in the cosmeceutical products it should have been detected after treatment with at least the 2D Clean-Up Kit and ultracentrifugation. Probably it would be the same for the EGF. Therefore, the explanation of the samples having too much interfering substances can be excluded.

4.1.3 Sample preparation with bigger sample sizes

Moreover, since sample preparation with 2D Clean-Up Kit and acetonitrile followed by vacuum centrifugation gave detectable protein in the spiked samples, those methods were repeated with 10 times bigger samples sizes, without spiking. Ultracentrifugation was not performed again due to lack of time. The purpose of these experiments was to confirm the validity of the methods.

Nevertheless, no protein could be detected which can be seen in Figure 4.6 and 4.7 which both lack blue protein bands. This means the successful results obtained from the spiking experiment cannot be confirmed and the question still remains whether Bioeffect, Cellshock, Norelift and Terproline contain too small amounts of GF to be detected or nothing at all.
Further protein characterization was performed on Gaifu since none of the other products showed the presence of protein.

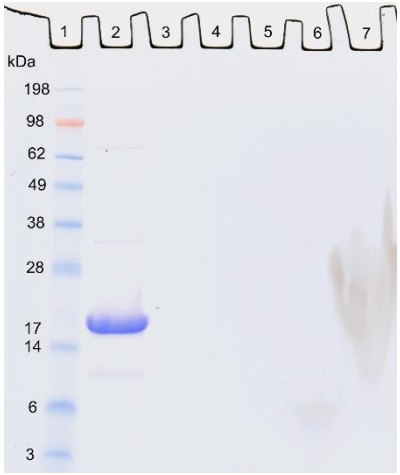

Figure 4.6. Samples of 1 g treated with acetonitrile followed by vacuum centrifugation. From 1-7: 1. Marker, 2. FGF standard,3. Bioeffect 1, 4. Bioeffect 2, 5. Cellshock, 6. Norelift, 7. Terproline

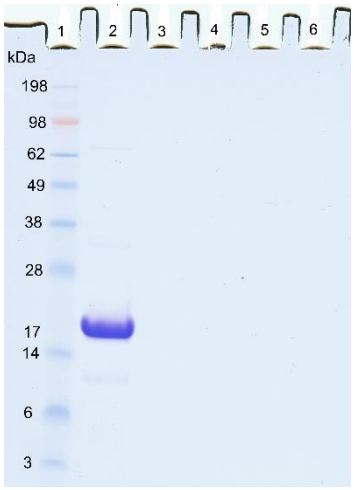

Figure 4.7. Samples of 1 g treated with 2D Clean-Up Kit. From 1-6: 1. Marker, 2. FGF standard, 3. Bioeffect, 4. Cellshock, 5. Norelift, 6. Terproline

4.2 Characterization of Gaifu

4.2.1 Comparison with reference protein

When Gaifu was run on the SDS-PAGE three protein bands appeared which could be seen in Figure 4.1. Those bands show that there was a chance that other protein than FGF was present in the serum. For comparison, reference proteins were run together with Gaifu to try to identify all of its content.
BSA, which is a protein with stabilizing properties, was chosen as one of the reference proteins. BSA is used in numerous applications due to its lack of effect in many biochemical reactions and its low cost (Chakraborty, 2017). There might be a chance that the manufacturers have used BSA as a stabilizer which is the reason why it was chosen as a reference protein.

4.2.2 Comparison of molecular mass

Interesting observations were seen when comparing Gaifu and BSA, which turned out to share several properties. Looking at the gel with Gaifu, FGF standard and BSA of different concentrations, which can be seen in Figure 4.8, Gaifu shows a vague band next to the FGF standard whereas the most intense band around 62 kDa has the same molecular mass as BSA. In addition, several bands of higher molecular

masses then follow. Globular proteins, among which BSA is a well-studied one, have a tendency to aggregate under certain conditions (Babcock and Brancaleon, 2013). Hence, the lined up bands could be multimers; protein aggregates consisting of multiple monomers. No variants of FGF can be seen in Gaifu, only multimers of potential BSA.

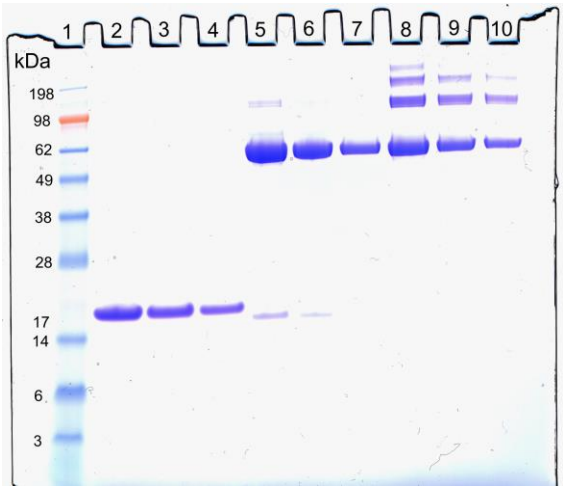

Figure 4.8. The result of SDS-PAGE with following contents of the wells: Marker (1), FGF standard (well 2-4), Gaifu (well 5-7) and BSA (well 8-10).

4.2.3 Comparison of surface charge

Furthermore, separating proteins by surface charge distribution with IEX is a sensitive method with high resolution and the ability of separating molecules with only small charge differences (Aguilar, 2004). Cation-exchange chromatography is used when the molecules to separate have a positive charge which they have when the pH is lower than their pI. Positively charged groups of the proteins will then bind to negatively charged molecules in the column.
In the IEX, Gaifu and BSA are showing almost identical peaks (A) with the same retention time whereas the FGF standard is eluting much later at higher pH value (B), which can be seen in Figure 4.9. The higher the affinity is for the molecules in the immobilized stationary phase, the longer a certain component will remain in the column. This means that Gaifu and BSA, which showed a much shorter retention time, must have lower affinity for the column compared to the FGF standard which retained much longer. The surface charge profile of Gaifu and BSA are similar due to the fact that they bind and elute from the column almost identically whereas the FGF standard differs a lot from the others and seem to consist of several variants with small conformational differences.
The advantage with IEX is that it is non-denaturing and the separated protein could be further analyzed if wanted (Aguilar, 2004).

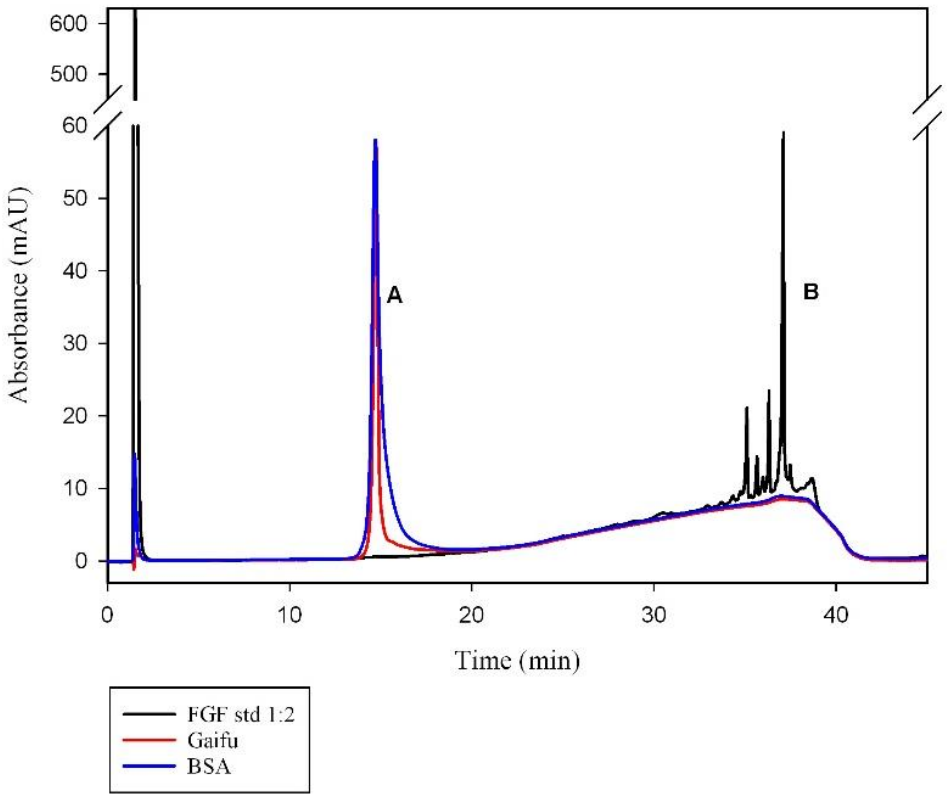

Figure 4.9. Showing the obtained chromatogram from the run with FGF standard, Gaifu and BSA in the ion-exchange chromatography.

4.2.4 Comparison of hydrophobicity

RP-HPLC has the capacity of separating proteins that are nearly identical and the chromatographic behavior in RP-HPLC of a protein can be correlated to its amino acid composition. Since the resolving power is so high and the detection sensitivity is very good, RP-HPLC can obtain important information about proteins. When looking at the chromatograms of the RP-HPLC which can be seen in Figure 4.10 and Figure 4.11, they show that FGF (peak A) elutes earlier with lower hydrophobicity while Gaifu and BSA (peak B) are eluting later. This means that the hydrophobicity of these molecules are higher and they adsorb stronger to the hydrophobic stationary phase and therefore it takes longer to leave the column. In addition, Gaifu shows a small, almost undetectable peak at 18.7 minutes which must be the FGF that could be seen in the SDS-PAGE. In Figure 4.11 an in-zoom of peak A, B and C in Figure 4.10 can be seen. Gaifu has a hydrophobicity which is similar to the one of BSA which means they have about the same amino acid sequences which is rather different from the FGF standard. The double peak of Gaifu can be represented by multimers of BSA, described previously in section 4.2.2.

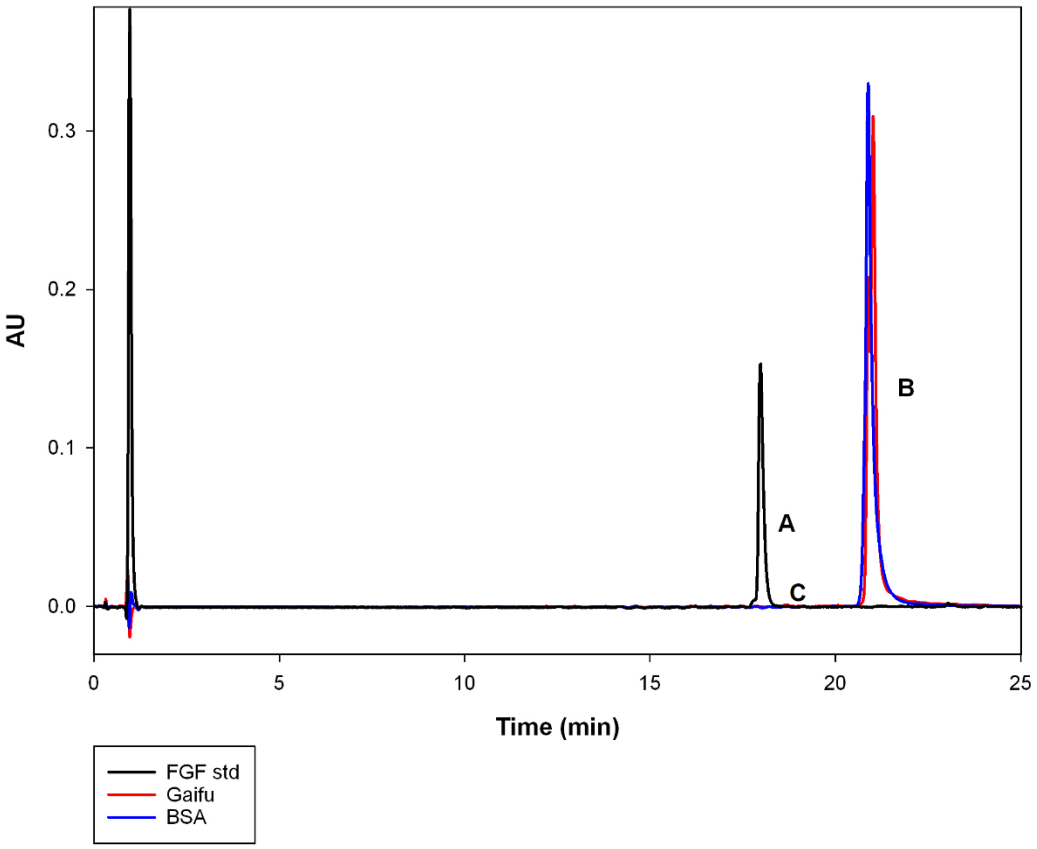

Figure 4.10. Showing the chromatogram obtained from the RP-HPLC.

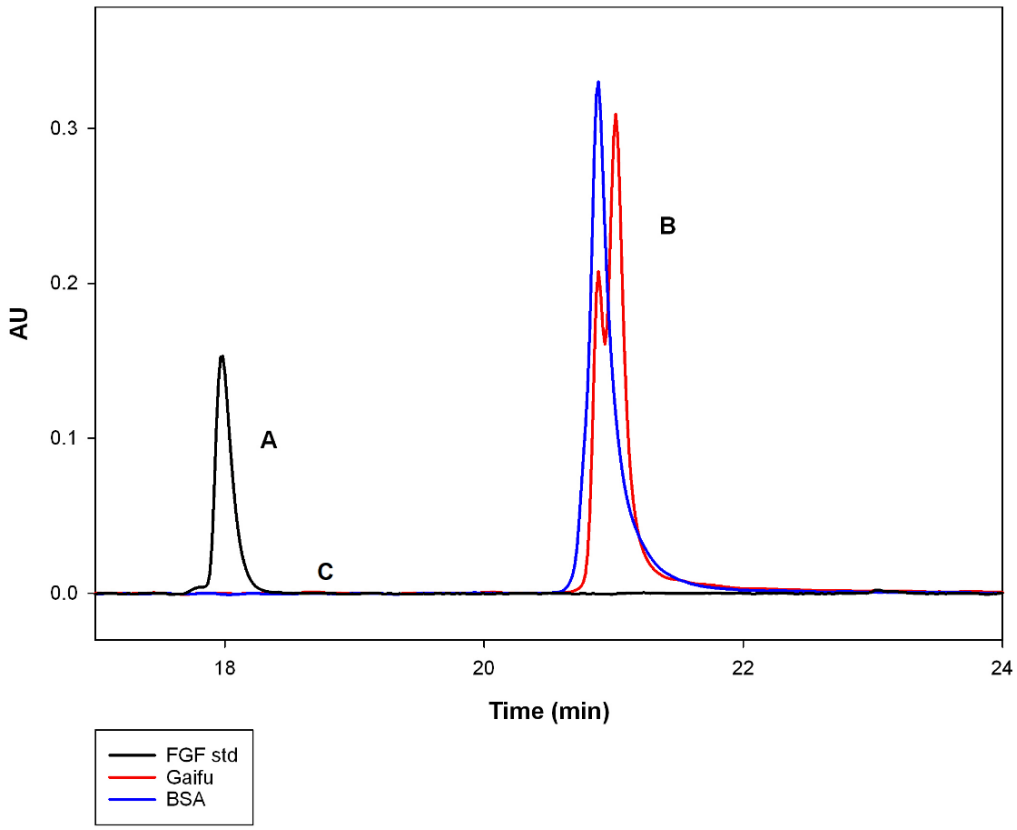

Figure 4.11. Showing an in-zoomed picture of peak A, B and C in Figure 4.10.

4.2.5 Concentration determination

Since it seemed like Gaifu contained at least some FGF it was of interest to determine the concentration of the growth factor. A calibration curve was prepared from different dilutions of the FGF standard which was run in the RP-HPLC (see chromatogram in Figure 4.12). Seven dilutions ranging from 1:2 to 1:128 (see Table 4.1 for corresponding concentrations) was included and compared to Gaifu. This resulted in a calibration curve (Figure 4.13) within which Gaifu did not fit but appeared outside the lower limit. Thus, the FGF concentration in Gaifu was lower than a 1:128 dilution of FGF standard which is less than 2.52 µg/mL. The negative peak of Gaifu is noise and the blank subtraction. For better accuracy, more dilutions should be included in a future experiment.

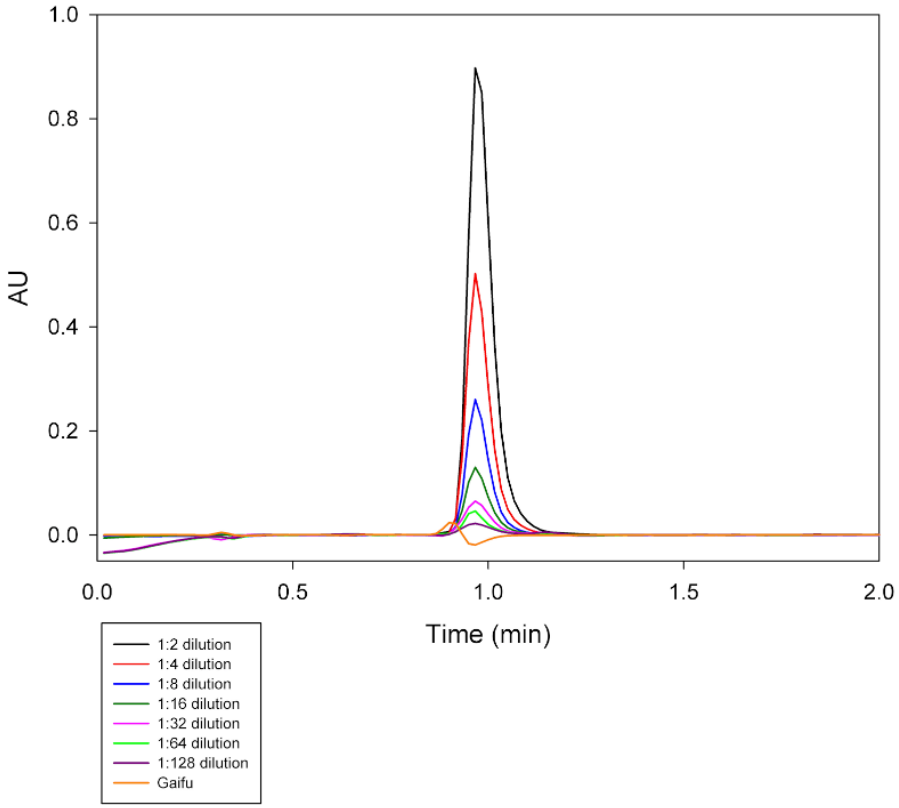

Figure 4.12. The chromatogram obtained from the RP-HPLC comparing different dilutions of FGF standard with Gaifu.

Table 4.1. The concentrations corresponding to each dilution of the FGF samples which can be seen in Figure 4.12 above.

Dilution	**Concentration** (mg/mL)
Undiluted	0.322
1:2	0.161
1:4	0.081
1:8	0.040
1:16	0.020
1:32	0.010
1:64	0.005
1:128	0.003

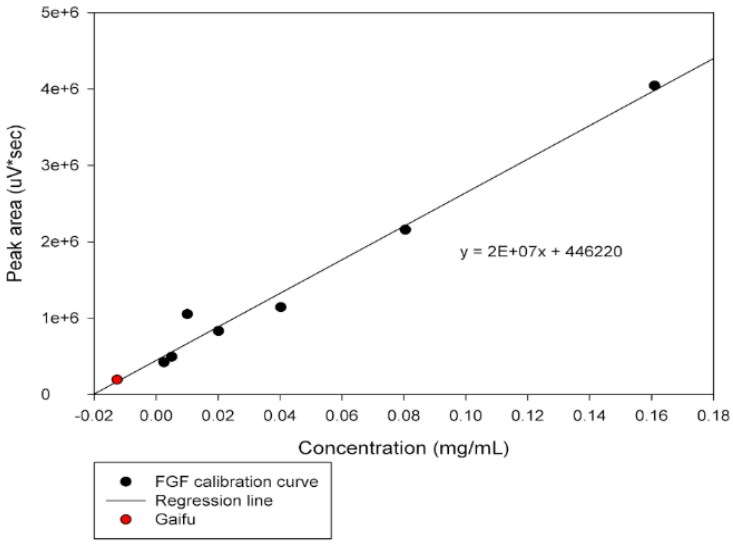

Figure 4.13. The calibration curve made up of several FGF standard-dilutions in comparison with Gaifu.

4.2.6 Characterization with 2D-DIGE

With the 2D-DIGE technique three samples labelled with different fluorescent dyes Cy2, Cy3 and Cy5 could be compared on one gel. Here FGF standard was labelled with Cy2, Gaifu with Cy3 and BSA with Cy5. By comparing overlaid pictures of the gel it is possible to see similarities and differences in molar masses and charges between the samples. Compared to cation-exchange chromatography, which also separates based on charge, the 2D-DIGE separates proteins based on all their charges while in the chromatographic method the separation is only based on the surface charges. In Figure 4.14 all the obtained pictures from the 2D-DIGE can be seen.

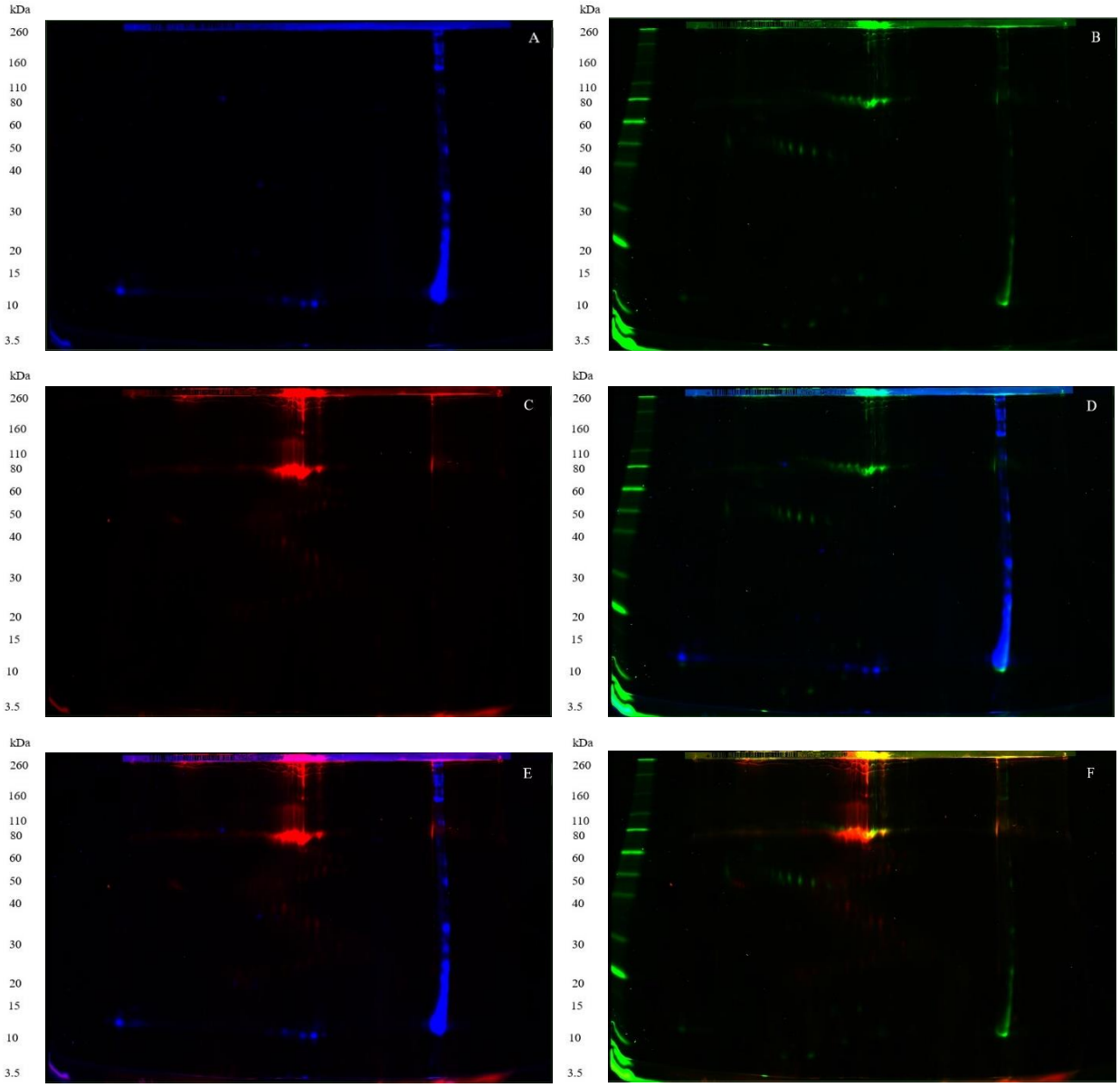

Figure 4.14. Qualitative comparison of FGF standard, BSA and Gaifu. A) Gel with FGF standard labelled with Cy2 shown in blue. B) Gel with Gaifu labelled with Cy3 in green. C) Gel with BSA labelled with Cy5 in red. D) Overlay of A and B (FGF standard and Gaifu). Spots that are present in both samples appear in a blue-green color. E) Overlay of A and C (FGF standard and BSA). No spots are present in both samples. F) Overlay of B and C (Gaifu and BSA). Spots that are present in both samples appear in a yellow color.

If a spot is present in more than one sample, it should appear in roughly the mixture of the sample colors. In gel F, an overlay of Gaifu and BSA, it is clear that they share several spots which appear in a yellow color. Also in gel D, the overlay of Gaifu and FGF standard there seems to be a mixture of the colors in some spots, however the blue is much more intense. Once again it has been shown that Gaifu contains FGF in addition to BSA which is present in higher concentration.

4.3 Limitations

The procedure of the electrophoresis was performed according to protocols from the manufacturers. There might be parameters that could be adjusted to give better results such as the heating temperature and heating time of the samples, the addition of reducing DTT and the amount of samples used. However, since different sample preparation methods were tried no other parameters were varied in order to keep consistency and knowing the reasons for the results.
Furthermore, it might be that small adjustments of the buffer gradients used for the HPLC could be made, however several experiments were carried out, all with clear results, so it should not be making much difference or improvement. Regarding the ultracentrifugation, the settings were based on earlier experiments described in literature and should be applicable on those of this project as well.
In addition, there is always a risk for human errors in all the experiments such as mistakes during pipetting, buffer preparation, handling and adjustment of the instruments etc. To exclude any such risks the experiments could be repeated additional times if any future research would be carried out.

4.4 Future Prospects

There was not enough time to repeat all the methods that were used successfully during the matrix spiking. Ultracentrifugation with bigger sample sizes would have been performed if the project had gone further. Mass spectrometry (MS) is a technique that would have been suitable to use as well. MS is an indispensable analytical tool in biochemistry identifying compounds from the molecular masses of their constituents (Gross, 2017). Naturally, economic aspects had to be taken into account, which unfortunately led to the exclusion of MS. What else could be done in future experiments with Gaifu is using functional assays to detect/quantify the binding or activity of the FGF to its receptor. For instance, surface plasmon resonance (SPR) is a technique that can be used for this purpose. SPR is commonly used when measuring binding affinity of proteins with high sensitivity and without the need of labels. The technique is based on the fact that SPR occurs when polarized light hits a metal surface (usually silver or gold) at the interface of dielectric media with different refractive index. When SPR hits a prism the angle will change with the refractive index of the dielectric media. This can give information about mass and density changes in the media which, for instance, can be noticeable when molecules bind to their receptors (Douzi, 2017) (Tang *et al*., 2010). By doing this it would be possible to see if the FGF is functioning by checking if it is binding to its receptor or not.

5

CONCLUSION

In the project of this master's thesis five different cosmeceutical products, claimed to contain either epidermal growth factor (EGF) or fibroblast growth factor (FGF), were investigated. The aim was to detect growth factors, investigate the impurity profile, protein variant pattern and the concentration in five different cosmeceutical products. All parts were included although successful results could not be reached in all cases. Four out of five products could not be included in the characterization experiments.
Only one product, Gaifu, had detectable FGF while all the other products failed to show the claimed protein. Several sample preparation methods were tried including organic solvents, surfactants and ultracentrifugation among others. Nothing could be detected with any of the methods although the sensitivity of the SDS-PAGE should have been enough to detect the amount that was thought to be present. This could not be explained by interfering substances in the product matrices since matrix spiking gave successful results where the injected protein was detectable. The only explanation for these results is that four out of five cosmeceutical products did not contain any protein at all or extremely small amounts. There is very vague information about how much GF cosmeceutical products are containing and how much that is necessary to improve skin integrity. When 1 gram of sample was analyzed with preparation methods that had been successful in the spiking attempts, no protein could be seen. Since the detection limit in SDS-PAGE is 25 ng per band for most proteins, it means that the products contained less than 25 ng/g when a cosmeceutical product should contain around 200 ng/g, according to the Canadian Drug Bank. Either if the manufacturers did not include any GFs or if they used a concentration as low as < 25 ng/g, both cases are questionable. More experiments such as mass spectrometry should be carried out to confirm this.

Gaifu could be further characterized by using SDS-PAGE, ion exchange-chromatography, RH-HPLC and 2D-DIGE which made it possible to show that it contained at least two different proteins. These proteins had molecular masses of around 69 kDa and 17 kDa which correspond to the molecular masses of BSA and FGF. When separating the proteins based on their charge with isoelectric focusing, a bigger part of the content in Gaifu shared properties with those of BSA. This was the case also when the protein was separated based on surface charge with ion exchange chromatography. Even the hydrophobicity of Gaifu, correlated to its amino acid sequence, was more similar to BSA than to FGF. Although what is questionable is that even though Gaifu showed clear presence of FGF in the SDS-PAGE, it was almost not detectable in the RP-HPLC. The similarities between Gaifu and BSA can probably be explained by the fact that BSA is commonly used as a stabilizer and might have been included to stabilize FGF in the serum. Alternatively, this could be an impurity. No comparison with human serum albumin (HAS) was done, perhaps this would have shown even more similarities. Regarding the protein variant pattern did

Gaifu show clearer evidence of containing different proteins rather than variants of FGF. More sensitive methods could be used to confirm this. Since there was no time to study the functionality of the FGF in Gaifu no conclusion can be reached regarding this.

Cosmeceuticals might be an up-coming product group although more information about their content and verification of working should preferably be available to create trust among customers. Stricter regulations, similar to those for pharmaceuticals, would be good in order to keep the quality and safety as high as possible.

6

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7

APPENDIX

7.1 SDS-PAGE

7.1.1 SDS-PAGE buffer protocols

20x MES SDS Running Buffer stock solution
Including 2-(N-Morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich), Tris(hydroxymethyl) aminomethan (Tris) (Merck), sodium dodecyl sulfate (SDS) solution (Merck) and EDTA (Merck).

Table 7.1. Protocol for 20x MES SDS Running buffer stock solution.

20x MES SDS Running buffer stock solution	
MES	195 g
Tris	121 g
SDS	20.0 g
EDTA	6.00 g
RO-water	Fill up to 1000

Buffers (ST=silver staining, CB=Coomassie Blue):

Fixing solution (ST, CB)
Including ethanol (96.0%) (Merck), acetic acid (100%) (Merck) and RO-water.

Table 7.2. Protocol for fixing solution.

Chemical	**One gel (50 mL)**
Ethanol (96.0%)	25.0 mL
Acetic acid (100%)	5.00 mL
RO-water	Fill to 50.0 mL

Coomassie staining solution (CB)

Including Coomassie R250 (Sigma-Aldrich), ethanol (Merck), acetic acid (Merck) and RO-water.

Table 7.3. Protocol for Comassie staining solution.

Chemical	**1000 mL**
Coomassie R250	1.16 g
Ethanol	250 mL
Acetic acid	80.0 mL
RO-water	Fill to 1000 mL

Destaining solution (CB)

Including ethanol (Merck), acetic acid (Merck) and RO-water.

Table 7.4. Protocol for destaining solution.

Chemical	**One gel (50 mL)**
Ethanol	12.5 mL
Acetic acid	4.00 mL
RO-water	Fill to 50.0 mL

Incubation solution (ST)

Including ethanol (96.0 %) (Merck), Na-acetate (waterfree) (Merck), Thiosulfate-pentahydrate (Merck), RO-water and glutaraldehyde (50.0 % in water, 5.60 M) (Fluka).

Table 7.5. Protocol for incubation solution.

Chemical	**One gel (50 mL)**
Ethanol (96%)	15.0 mL
Na-acetate (water free)	3.40 g
Thiosulfate-pentahydrate	0.100 g
RO-water	Fill to 50.0 mL
Add before use:	
Glutaraldehyde (50.0 % in water (5.60 M))	125 µL

Silver solution (ST)

Silver nitrate (Merck), formaldehyde 35.0 % (Sigma-Aldrich) and RO-water.

Table 7.6. Protocol for silver solution.

Chemical	**One gel (50 mL)**
Silver nitrate	50.0 mg
RO-water	Fill to 50.0 mL
Add before use:	
Formaldehyde (35.0 %)	10.0 µL

Developing solution (ST)
Including sodium carbonate (Merck), formaldehyde 35 % (Sigma Aldrich) and RO-water.

Table 7.7. Protocol for developing solution.

Chemical	**One gel (50 mL)**
Sodium carbonate	3.75 g
RO-water	Fill to 150 mL
Add before use:	
Formaldehyde 35.0 %	15.0 µL

Stop solution (ST)
Including EDTA (Merck) and RO-water.

Table 7.8. Protocol for stop solution.

Chemical	**One gel (50 mL)**
EDTA	0.730 g
RO-water	Fill to 50.0 mL

7.1.2 SDS-PAGE gels sample preparation

Below the gels from the different SDS-PAGE experiments can be seen in Figure 7.2-7.9 together with Table 7.1-7.8 which describe what is in each well. Figure 7.1 shows the marker which indicates the molecular weights of the proteins that has been used in the experiments. It is obtained from Thermofisher.

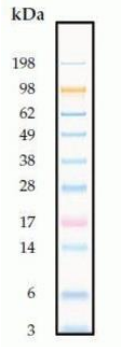

Figure 7.1. Showing the marker used in the SDS-PAGE experiments (ThermoFisher Scientific).

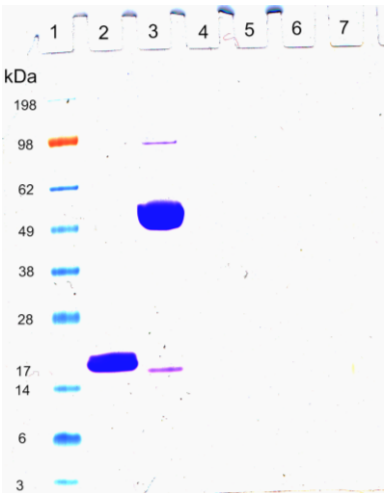

Figure 7.2.Showing the first SDS page gel with direct EGF and FGF samples.

Figure 7.3. (Right) Showing the SDS page gel with EGF and FGF samples extracted in isopropanol.

Table 7.1. Showing the contents of the wells in Figure 7.2.

Nb.	**Well**
1	Marker
2	FGF standard
3	Gaifu
4	Empty well (Cell shock could not be transfered)
5	Bioeffect
6	Empty well (Terproline could not be transfered)
7	Norelift

Table 7.2. Showing the contents of the wells in Figure 7.3.

Nb.	**Well**
1	Marker
2	FGF standard
3	Norelift 1
4	Norelift beginning
5	Norelift later
6	Cellshock beginning
7	Cellshock later
8	Terproline beginning
9	Terproline later
10	Bioeffect

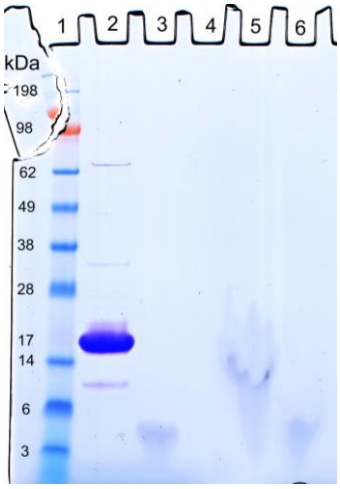

Figure 7.4. Showing the SDS page gel with EGF and FGF samples extracted in acetonitrile.

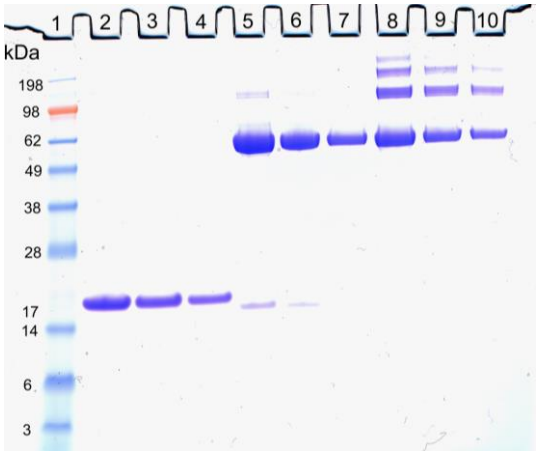

Figure 7.5. Showing the SDS page gel with samples of FGF standard, Gaifu and BSA of different concentrations.

Table 7.3. Showing the contents of the wells in Figure 7.4.

Nb.	Well
1	Marker
2	FGF standard
3	Cellshock
4	Bioeffect
5	Terproline
6	Norelift

Table 7.4. Showing the contents of the wells in Figure 7.5.

Nb.	Well
1	Marker
2	FGF standard, 0.5 g/L
3	FGF standard, 0.25 g/L
4	FGF standard, 0.125 g/L
5	Gaifu, 0.5 g/L
6	Gaifu, 0.25 g/L
7	Gaifu, 0.125 g/L
8	BSA, 0.5 g/L
9	BSA, 0.25 g/L
10	BSA, 0.125 g/L

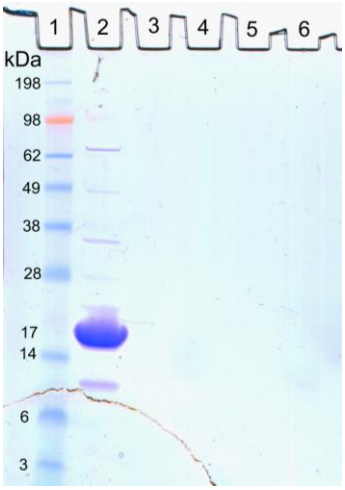

Figure 7.6. Showing the SDS page gel with EGF and FGF samples treated with 2D Clean-Up Kit to cause acid precipitation.

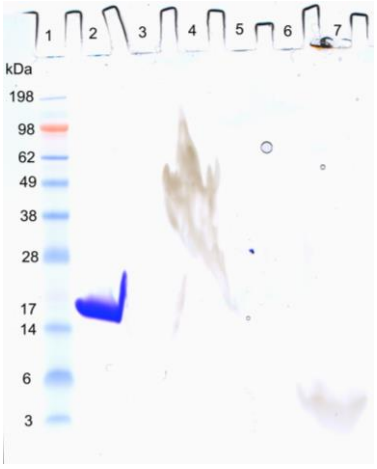

Figure 7.7. Showing the SDS page gel with EGF and FGF samples treated with Triton and PBS together with one microwaved sample.

Table 7.5. Showing the contents of the wells in Figure 7.6.

Nb.	Well
1	Marker
2	FGF standard
3	Terproline
4	Bioeffect
5	Norelift
6	Cellshock

Table 7.6. Showing the contents of the wells in Figure 7.7.

Nb.	Well
1	Marker
2	Triton + FGF standard
3	Triton + Terproline
4	Triton + Norelift
5	Triton + Bioeffect
6	Triton + Cellshock
7	Microwaved terproline

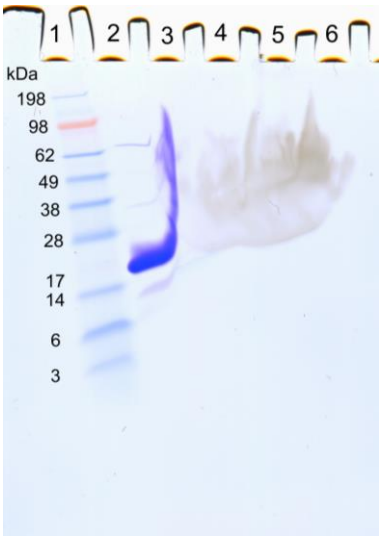

Figure 7.8. Showing the SDS page gel with EGF and FGF samples treated with Triton and PBS followed by ultrafiltration.

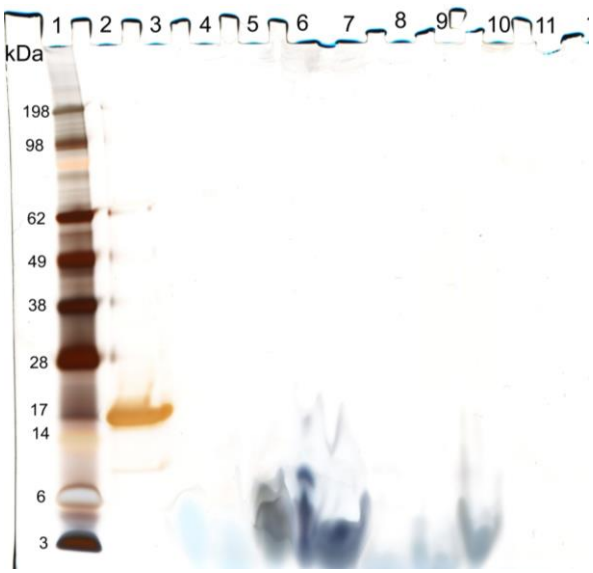

Figure 7.9. Showing the silver stained SDS page gel with EGF and FGF samples treated with ultracentrifugation.

Table 7.7. Showing the contents of the wells in Figure 7.8.

Nb	Well
1	Marker
2	FGF standard
3	Bioeffect
4	Norelift
5	Terproline
6	Cellshock

Table 7.8. Showing the contents of the wells in Figure 7.9.

Nb.	Well
1	Marker
2	FGF standard
3	Terproline middle phase
4	Terproline bottom phase
5	Norelift 1st middle phase
6	Norelift 2nd middle phase
7	Norelift bottom phase
8	Cellshock 2nd middle phase
9	Cellshock bottom phase
10	Cellshock mixed phases
11	Bioeffect

7.1.3 SDS-PAGE gels with spiked samples

The gels with spiked samples can be seen below in Figure 7.10-7.17 together with Table 7.9-7.16 which describe what is in each well.

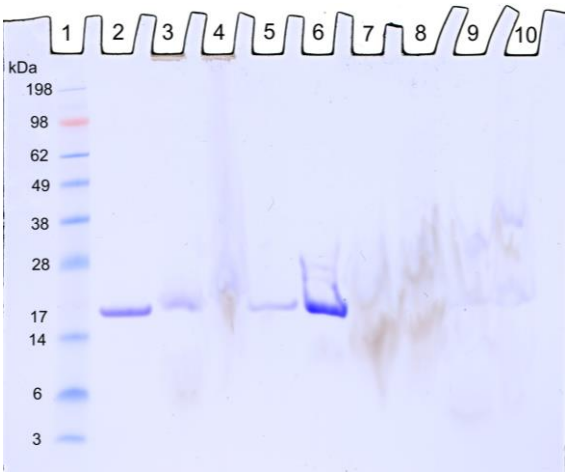

Figure 7.10. Showing the SDS page gel with FGF standard spiked into the products. One of each sample has been loaded directly and one is diluted 1:5 with PBS.

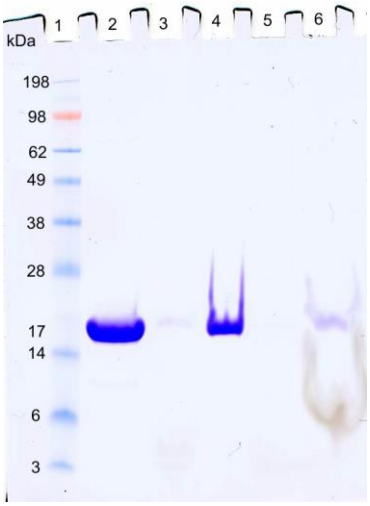

Figure 7.11. Showing the SDS page gel with FGF standard spiked into the products and dissolved in acetonitrile.

Table 7.9. Showing the contents of the wells in Figure 7.10.

Nb.	Well
1	Marker
2	FGF standard
3	Cellshock 1:5 dilution with PBS
4	Cellshock direct
5	Bioeffect 1:5 dilution with PBS
6	Bioeffect direct
7	Terproline 1:5 dilution with PBS
8	Terproline direct
9	Norelift 1:5 dilution with PBS
10	Norelift direct

Table 7.10. Showing the contents of the wells in Figure 7.11.

Nb.	Well
1	Marker
2	FGF standard
3	Norelift
4	Bioeffect
5	Cellshock
6	Terproline

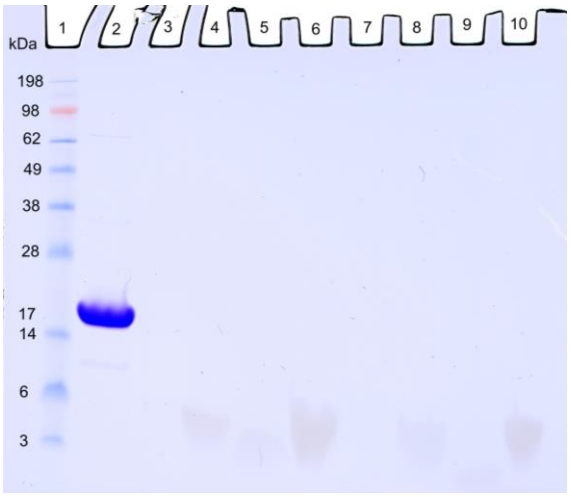

Figure 7.12. Showing the SDS page gel with FGF standard spiked into the products and diluted in isopropanol.

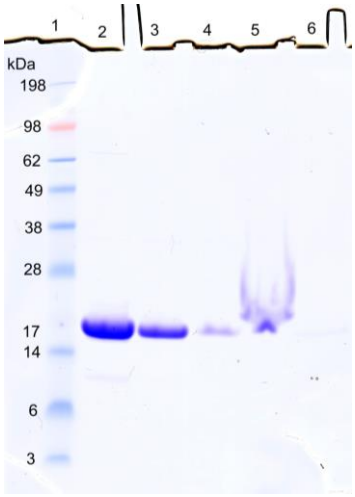

Figure 7.13. Showing the SDS page gel with FGF standard spiked into the products and samples treated with 2 Clean-Up Kit.

Table 7.11. Showing the contents of the wells in Figure 7.12.

Nb.	Well
1	Marker
2	FGF standard
3	Bioeffect
4	Cellshock
5	Norelift
6	Terproline
7	Bioeffect waste
8	Cellshock waste
9	Norelift waste
10	Terproline waste

Table 7.12. Showing the contents of the wells in Figure 7.13.

Nb.	Well
1	Marker
2	FGF standard
3	Bioeffect
4	Cellshock
5	Norelift
6	Terproline

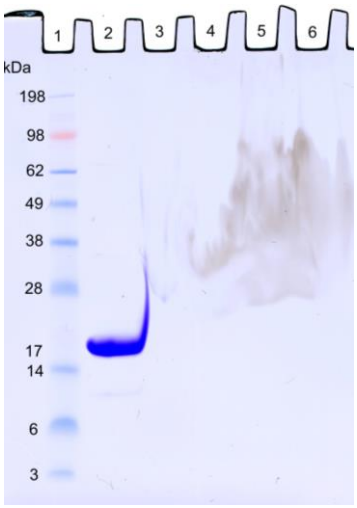

Figure 7.14. Showing the SDS page gel with FGF standard spiked into the products and mixed with Triton/PBS.

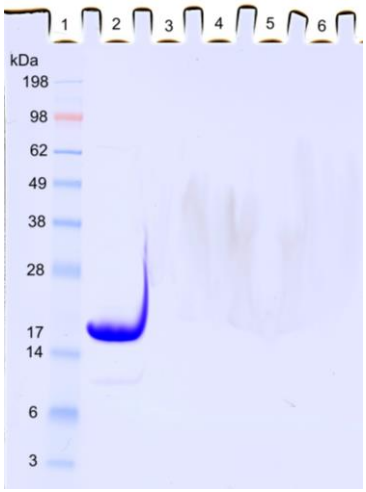

Figure 7.15. Showing the SDS page gel with FGF standard spiked into the products and mixed with Triton/PBS followed by ultrafiltration.

Table 7.13. Showing the contents of the wells in Figure 7.14.

Nb.	Well
1	Marker
2	FGF standard
3	Bioeffect
4	Cellshock
5	Norelift
6	Terproline

Table 7.14. Showing the contents of the wells in Figure 7.15.

Nb.	Well
1	Marker
2	FGF standard
3	Bioeffect
4	Cellshock
5	Norelift
6	Terproline

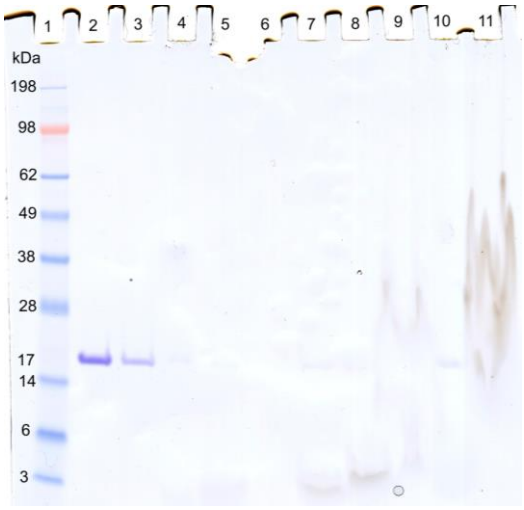

Figure 7.16. Showing the SDS page gel with FGF standard spiked into the products followed by ultracentrifugation.

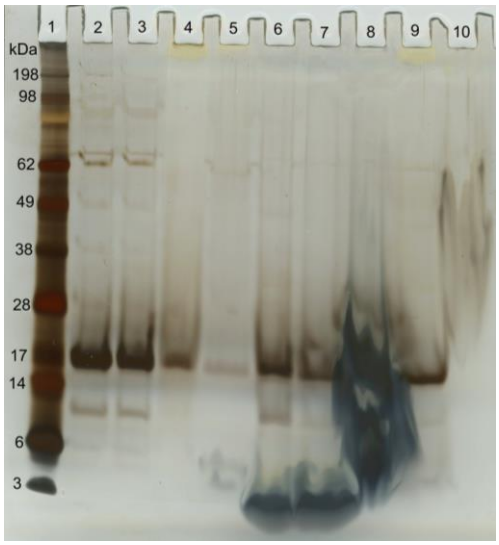

Figure 7.17. Showing the SDS page gel with FGF standard spiked into the products followed by ultracentrifugation and silver staining.

Table 7.15. Showing the contents of the wells in Figure 7.16.

Nb.	Well
1	Marker
2	FGF standard
3	Bioeffect
4	Cellshock 1
5	Cellshock 2
6	Cellshock 3
7	Norelift 1
8	Norelift 2
9	Norelift 3
10	Terproline 1
11	Terproline 2

Table 7.16. Showing the contents of the wells in Figure 7.17.

Nb.	Well
1	Marker
2	FGF standard
3	Bioeffect
4	Cellshock 1
5	Cellshock 3
6	Norelift 1
7	Norelift 2
8	Norelift 3
9	Terproline 1
10	Terproline 2

7.2 Ion-Exchange Chromatography

Below the buffers for the HPLC and the obtained results from the experiments can be seen.

1st run of the ion-exchange chromatography

For the first ion-exchange chromatography experiment the protocol used can be seen in Table 7.17 and 7.18.

Table 7.17. Below the molar of each chemical to obtain the desired pH is presented.

	HEPES	**Bicine**	**CAPSO**	**CAPS**	**NaCl**	**pH**
Buffer A (mM)	5.50	4.20	9.50	0.80	6.30	8.00
Buffer B (mM)	0.00	10.5	2.50	7.00	0.00	10.5

Table 7.18. Below the mass of each chemical to obtain the desired pH is presented.

1 liter	**HEPES**	**Bicine**	**CAPSO**	**CAPS**	**NaCl**	**pH**
Buffer A (g)	1.31	0.685	2.26	0.177	0.370	8.00
Buffer B (g)	0.000	1.71	0.593	1.55	0.000	10.5

2nd and 3d run of the ion-exchange chromatography

For the second ion-exchange chromatography experiment the protocol used can be seen in Table 7.19 and 7.20.

Table 7.19. Below the molar of each chemical to obtain the desired pH is presented.

	CAPS	**CAPSO**	**Bicine**	**HEPES**	**MES**	**Na acetate**	**Formic acid**	**NaCl**	**pH**
Buffer A (mM)	0.000	25.7	0.000	10.2	1.00	2.90	7.70	37.0	4.00
Buffer B (mM)	7.60	0.00	8.80	0.00	17.8	30.0	6.00	0.000	11.0

Table 7.20. Below the mass or volum of each chemical to obtain the desired pH is presented.

1 liter	**CAPS**	**CAPSO**	**Bicine**	**Hepes**	**MES**	**Na acetate**	**Formic acid**	**NaCl**	**pH**
Buffer A (g)	0.000	6.10	0.000	2.43	0.195	0.238	296 (µL)	2.16	4.00
Buffer B (g)	1.68	0.000	1.44	0.000	3.48	2.46	231 (µL)	0.000	11.0

4th run of the ion-exchange chromatography

For the third ion-exchange chromatography experiment the protocol used can be seen in Table 7.21 and 7.22.

Table 7.21. Below the molar of each chemical to obtain the desired pH is presented.

	CAPS	**CAPSO**	**Bicine**	**HEPES**	**MES**	**Na acetate**	**Formic acid**	**NaCl**	**pH**
Buffer A (mM)	0.000	30.0	0.000	11.3	1.20	2.20	8.10	20.0	4.00
Buffer B (mM)	6.80	1.40	8.00	0.000	15.8	30.0	13.3	50.0	10.5

Table 7.22. Below the mass of each chemical to obtain the desired pH is presented.

1 liter	**CAPS**	**CAPSO**	**Bicine**	**Hepes**	**MES**	**Na acetate**	**Formic acid**	**NaCl**	**pH**
Buffer A (g)	0.000	7.12	0.000	2.69	0.195	0.227	312 (µL)	1.17	4.00
Buffer B (g)	1.50	0.331	1.30	0.00	3.48	3.09	512 (µL)	2.92	10.5

7.3 2D-DIGE

Below the buffer protocols used in the 2D-DIGE procedure can be seen in Table 7.23 and 7.24.

Table 7.23. Rehydration solution

Chemical	**Amount**
8M Urea (Merck)	15.0 g
2% CHAPS (Fluka)	0.625 g
2% IPG buffer (GE Healthcare)	156 µL
RO-H2O	To 25.0 mL

Table 7.24. Running buffer 1 and 2

	Buffer 1	**Buffer 2**
Urea (Merck)	7.20 g	7.20 g
SDS (10%) (Merck)	4.00 mL	4.00 mL
Glycerin (Merck)	6.00 mL	6.00 mL
Tris/HCL (50 mM pH 8.8) (Merck)	2.00 mL	2.00 mL
DTE (Sigma Aldrich)	0.400 g	-
Jodacetamid (Sigma Aldrich)	-	0.500 g
Bromphenolblue (Sigma Aldrich)	-	400.0 µL of 0.100 % Bromphenolblue solution.
RO-H2O	Fill to 25.0 mL	Fill to 25.0 mL

Table 7.25. 10 x SDS running buffer stock solution

Chemical	**Amount**
Tris (Merck)	60.5 g
Glycine (Sigma Aldrich)	288 g
SDS (Merck)	20.0 g
RO-H2O	to 2.00 L