Abstract

This project examines an optimization of a HPLC method for detecting two common UV-filters in different sunscreen products. The UV filters, Octocrylene and Padimate O, are organic compounds that will block UV radiation from the sun that mainly causes skin cancer. The analyses were performed by a HPLC with a reversed phased C18 column. An optimization of the HPLC method was made by changing different parameters to get the most precise and optimal method to achieve best peak separation. Due to the fat content in sunscreens, several extraction methods, based on Ultrasonication, were made and investigated for the sample preparations. Additionally, statistic has been used as a tool to investigate the UV-filters in the sunscreens. Herein, an ANOVA test has been made to investigate which factors had the most influence on the concentration hence a reproducibility test was made. Furthermore this study examines the difference between a new and an old sunscreen by doing a two sample t-test to test whether storage of a sunscreen has an effect on the UV –filters. Finally a validation on the final method is performed. Calibrations curves have been made for the two standards in order to quantify the total amount of UV-filters in the sunscreens.

The method that was validated was found to be partly good. The two-sample t-test showed that statistically there was no significance between the new and old sunscreen. The samples were dissolved in methanol and sulfuric acid and treated with heat and ultrasonic. The retention time for the optimization of the HPLC method was not reduced compared to the method from the scientific article, but the amount of mobile phase was reduced.

Abstrakt

Dette projekt omhandler en optimering af en HPLC metode hvor to UV filtre fra forskellige solcremer ønskes bestemt. UV filtre, Octocrylene og Padimate O, er organiske forbindelser der blokere UV stråling fra solen, som primært forårsager hudkræft. Til at analyserer på prøverne bruges et HPLC instrument med en omvendt faset C18 kolonne. En optimering af HPLC metoden blev udført ved at ændre på de forskellige parametre for at opnå den bedste metode. På grund af det høje fedtindhold i solcreme er der lavet flere ekstraktionsmetoder til prøveforberedelserne. Udover dette, blev statistik anvendt som et redskab til at undersøge UV filtrene i Solcreme. Heri er der blevet lavet en ANOVA test hvor forskellige faktorers indflydelse undersøges; til dette formål blev der lavet en reproducerbarheds test. Derudover blev der også fortaget en two – sample t-test hvor en gammel og en ny solcreme blev testet med henblik på deres opbevaring. Til sidst blev der valideret på den sidste metode. To kalibreringskurver blev ligeledes lavet for de to standarder med henblik på kvantitative at kunne bestemme koncentration af UV filtre i solcremerne.

Den optimerede metode blev valideret og fundet til at være nogenlunde god. På baggrund af two-sample t-test blev det fundet at der statistisk ikke var nogen signifikans forskel på den gamle og nye solcreme. Prøverne blev opløst i methanol og svovlsyre og behandlet med varme og ultralyd. Retentions tiden på den optimerede HPLC metode blev ikke reduceret i forhold, til den videnskabelige artikel. Mængden af den mobile fase blev derimod reduceret.

Preword

This fourth semester project was performed by Elisabeth Højkær Villumsen Nielsen, Borja de Diego Mateo Maja Niemann Jensen and Mariam Khaled Abdel Rahman during the period February - June 2011.

It was carried out in the Chemistry Engineering Department of the *University of Southern Denmark* (Odense) under the supervision of Kathrine Bisgaard Christiensen and Lene Pedersen.

The aim of the present project is to optimize the determination of UV filters in Sunscreens using a Liquid Chromatographic technique and is divided into two parts: Report and appendix.

It should be noted that all the references are written in sharp parentheses. Author and page number refer to books, capital letters “A+number” refers to articles and “W+number” refers to web sites that will be found on the reference index.

The entire group would like to thank all the support shown by the instructors, without whom none of this would have been possible.

In Odense, June 3rd 2011

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1.0 Introduction

Although sunlight is necessary for life and for most living organisms on earth, too much sunlight is harmful and can damage the skin. Damaged skin can result in skin cancer, which is one of the most common types of cancer. More than 1 million people are diagnosed with skin cancer in the US every year. As prevention is better than cure, we found out sunscreens as a good subject for this project.

The aim of this project is to optimize a HPLC method and sample preparations for detecting common organic UV-filters in 3 different sunscreens. For this purpose, different sunscreens with the same Sun Protection Factor (SPF) were investigated. The pure standards; Octocrylene and Padimate O, are used for a quantitative and qualitative analysis. The optimization is based on a method found in a scientific article and is going to be developed so it can determine and quantify the UV-filters in 3 different sunscreens. It is also want to test the known myth that the sunscreens should be through away if is the bottle from last season because of the concentration drop of the UV-filters. It is also wanted to make a method validation to investigate have well the method is.

2.0 Theory

2.1 Sunscreens

Sunscreens are products that usually come in a form of lotions or creams that are applied to the human skin. Sunscreens contain UV-filters that can block UV radiation from the sun that are damaging to the human skin and can lead to skin cancer. The UV radiation is ultraviolet light that is invisible to the eye and is usually divided in 3 categories:

- UVA Rays (320-400nm): long waved rays, which are not absorbed by the ozone layer. They are able to penetrate deep in the skin and produce premature aging caused by sun exposure.
- UVB (280-320nm): powerful and short waved rays partially absorbed by the ozone layer and they are the primary cause of sunburns. As the ozone layer is decreasing, they can become more and more dangerous.
- UVC (190-280nm): do not reach earth because of the filtration in the atmosphere, although is the most dangerous one.

Figure 1 illustrates how UVA and UVB range in comparison to each other and to the visible light.

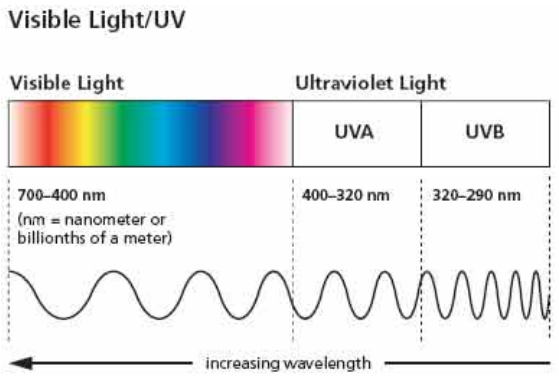


Figure 1 UVA and UVB radiation

To prevent UVA - and UVB radiations from damaging the skin UV – filters are used. [W8]

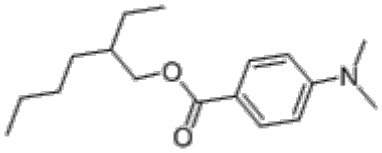
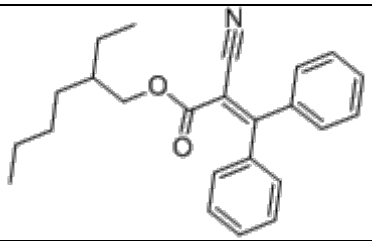
2.1.1 UV filters

UV-filters are compounds that will prevent damages on the skin and will either block or reflect UV radiation. UV filters are classified into two types, organic and inorganic UV filters. Most of the UV filters are organic compounds also called chemical sunscreens it will absorb the UV radiation and makes it difficult for the UV to penetrating though the skin. The sunscreens are absorbing solar radiation and transforming it into another form of energy, which is not as dangerous. They usually have a sun protection factor (SPF), which is a way to measure the effectiveness of a sunscreen. The inorganic filters also called physical compounds will block the UV radiation [W6]. The requirements for a good sunscreen are as following:

- Good ability to absorb ultraviolet radiation
- Resistance to external agents: sweating and water
- Skin care, so that it doesn´t cause any irritation
- Stable in the sun
- Compatible with other substances of the formula

The main ingredients in sunscreens are usually aromatic molecules conjugated with carbonyl groups. This general structure allows the molecule to absorb high-energy ultraviolet rays and converts them into lower energy, so that skin is protected from radiation. It´s important that most of the ingredients do not suffer any significant chemical change, so they maintain the capacity to absorb UV rays intact.

As in everything else there are rules for the concentration and compound that most contain in the product. In the United States the US food and drug administration controls most of the ingredients and the permitted limits. In table 1, it is possible to see the minimum and maximum of the content of different compounds in the USA. Europe and Japan make other restrictions [Salvador, p. 87].

Compound	**Structure**	**Density [g/mL] (at 25 °C)**	**Boiling point [°C]**
Padimate O		0.995	325
Octocrylene		1.051	218

There are many UV filters that are used in cosmetic and are usually classified into UVA, UVB or UVA and UVB filters. Two common compounds are chosen in this study that is listed in the following table [W5] [A2, p. 2]:

2.1.2. Side effects

Even though sunscreens are meant to protect the skin from sun damage a sunscreen itself can also be damaging to the skin. This is one of the most discussed issues today. Some UV – filters is said to cause changes on the skin and many studies are still researching for how different UV –filters may affect the human body. Some side effects that may occur by the two chosen UV-filters that are used in this project can cause health problems. The side effects of the UV-filters are listed below.

Octocrylene is a compound that absorbs both UVA and UVB rays. The maximum amount by the FDA (The US food and drug administration) is only 10w%. As a UV- filter this compound is stable comparing it to other UV-filters. However a study states that when Octocrylene penetrates to the skin it can increase the production of free radicals and can furthermore cause indirect damages to the DNA. [W4]

Padimate O is a compound that falls in the types of chemical sunscreens and blocks UVA radiations. A study in Oxford University has shown that the derives of Padimate O damages the DNA. The study showed that instead of absorbing the UVA rays the compound set of a chemical reaction that released high active agents that damages the DNA and will certainly cause cancer. [W3]

Allowable ingredients and concentrations should be taken in consideration because in rare cases, these chemical ingredients may cause skin irritation, redness, burning or any other reaction. Table 1 permitted UV filters and maximum concentrations.

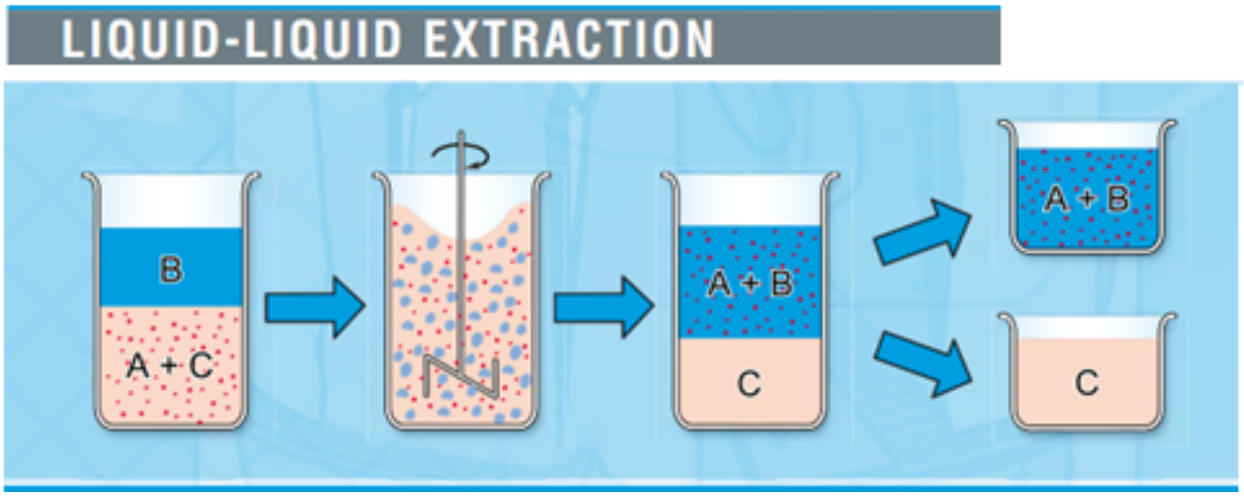
Drug Name	Concentration, %	Absorbance
Aminobenzoic acid	Up to 15	UV-B
Avobenzone	2-3	UV-A I
Cinoxate	Up to 3	UV-B
Dioxybenzone	Up to 3	UV-B, UV-A II
Ecamsule*	2	UV-A II
Ensulizole	Up to 4	UV-B
Homosalate	Up to 15	UV-B
Meradimate	Up to 5	UV-A II
Octocrylene	Up to 10	UV-B
Octinoxate	Up to 7.5	UV-B
Octisalate	Up to 5	UV-B
Oxybenzone	Up to 6	UV-B, UV-A II
Padimate O	Up to 8	UV-B
Sulisobenzone	Up to 10	UV-B, UV-A II
Titanium dioxide	2 to 25	Physical
Trolamine salicylate	Up to 12	UV-B
Zinc oxide	2 to 20	Physical
*Only available in United States in patented products.		

Tabel 1; permitted UV filters and maximum concentrations [A2, p. 2]

2.2 Extraction

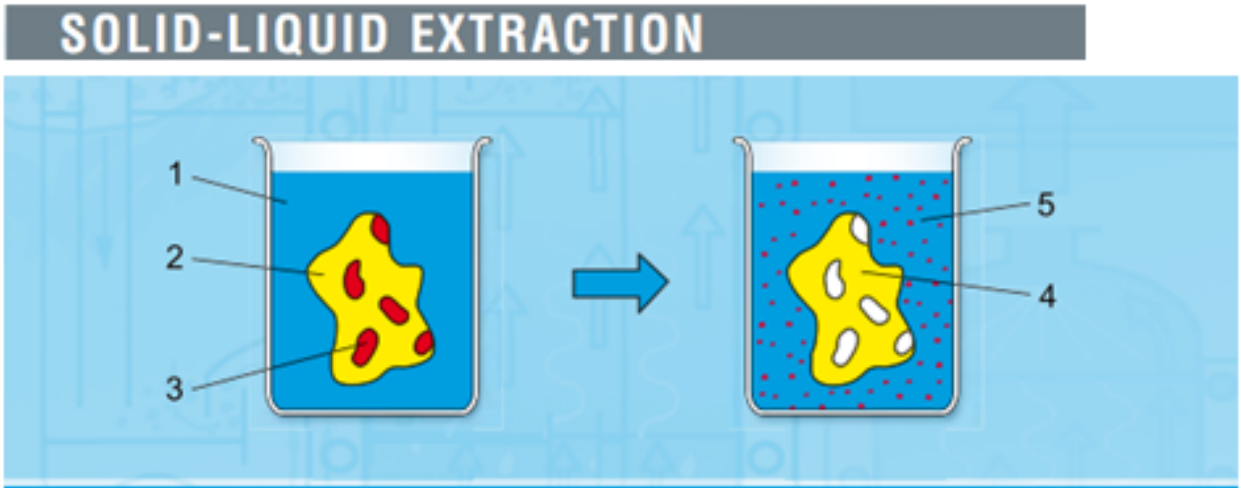
Extraction is a technique or a process that is often used to separate and isolate a desired substance from a complex mixture that can either be a liquid - or a solid mixture. In chemistry, extraction is a method used to separate a substance that can be dissolved in two immiscible solvents together with varying degrees of solubility and are in contact through an interface.

There are many types of extraction methods that are widely used depending on the substance that has to be isolated. Some of the most common extraction methods are a liquid –liquid extraction and a solid – liquid extraction. A liquid-liquid extraction is a method that is based on immiscible organic solvents to separate a substance from a mixture. The substance can either be nonpolar, some uncharged substance or a polar, ionized substance. The separation can in most cases be done successfully as a formation of phases will be created. The phases that will be created are a result of the difference polarity of the certain substances in the mixture where the electrostatic interactions and intermolecular forces between the molecules play an important role. Technically, the liquid-liquid extraction method can be done where the desired solute, in an aqueous solution, is extracted by a nonpolar or a polar organic solvent depending on the solute's polarity. The solute will be in the phase that it is most attracted to and a separation can be made [D. Kealey p. 109-114]. Following illustration [W9] shows the principle behind the liquid-liquid extraction method.



Figur 2 Ideal liquid-liquid Extraction; When the initial mixture (A+C) and the solvent (B) are mixed, the transition component (A) is transferred into the solvent. After setting, two phases are obtained; the extract (A+B) and the carrier liquid (C)

In the project, a solid-liquid extraction method was made. A solid-liquid extraction is a technique that allows substances from a solid to be dissolved. Likewise the solvent extraction, this extraction method also uses organic solvents to dissolve the substances from the solid. However the solvent must be chosen carefully based on the polarity for the certain substance that is wished to be extracted from the solid. This following illustration [W9] shows the principle behind the solid-liquid extraction:

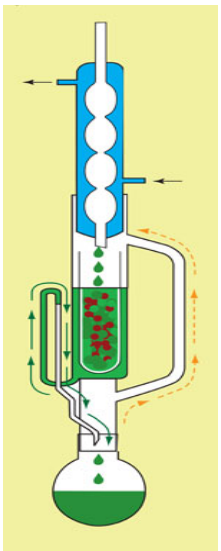


Figur 3. Solid Liquid Extraction; Schematic extraction - before extraction (left) and after extraction (right): 1 Solvent, 2 extraction material (solid carrier phase with transition component), 3 transition component, 4 depleted solid carrier phase, 5 solvent with dissolved transition component

Solid –liquid extraction is basically an extraction of a solid by a liquid. There are many methods that cover up a solid-liquid extraction. The following sections show 3 common methods of a solid-liquid extraction.

2.2.1 Soxhlet extraction

A soxhlet extraction is a method where a soxhlet apparatus is used to extract a solid. The principle behind this extraction is extracting a solid by recycling a solvent through it. The figure shows a soxhlet apparatus where the solvent phase lies in the bottom, a condenser at the top and a glass reservoir in the middle where a thimble shaped filter sits. The theory behind this method is to heat up the solvent in the bottom flask that will start to evaporate where the vapor is condensed by the condenser at the top. The vapor that is condensed will start dripping into the thimble that contains the solid matrix and the extraction will start.



Figur 4 soxhlet apparatus

The condensed solvent will eventually return to the bottom flask allowing new fresh condensed solvent to pass through the thimble. [W10][R. Kellner p. 405-406]

2.2.2 Microwave-Assisted Method

This method uses microwaves to heat up the extraction by providing the necessary energy to increase the temperature. The extraction container that is heated must however be microwave transparent. Furthermore the solvents polarity must be taken into consideration, as only solvents that absorb microwaves are suitable. Using the microwaves will convert the absorbed electromagnetic energy to heat energy making an extraction possible. [W11][R. Kellner p. 409]

2.2.3 Ultrasonic Extraction

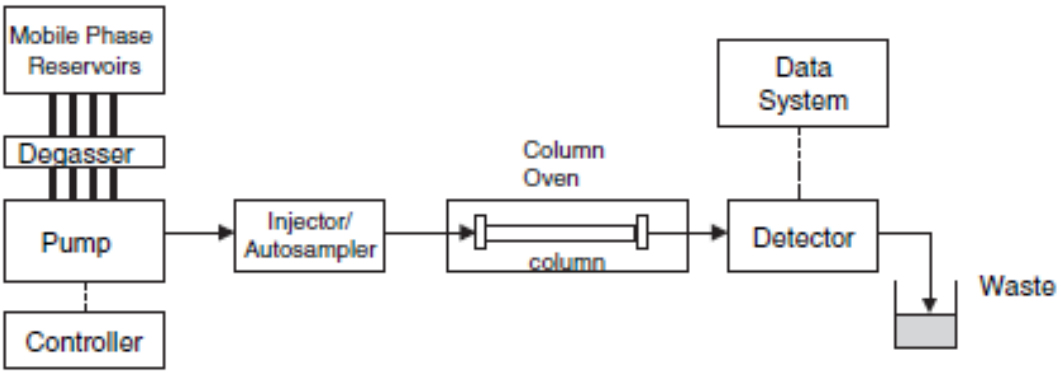
The Ultrasonication extraction is one of the most common extraction methods as it can be used with many solvents and the sample preparation is usually simple. The solid will be dissolved in a liquid and treated in Ultrasonication for a given time and temperature.

2.2.3.1 Ultrasonication

Ultrasonic is a sound with an inaudible frequency to the human ear and a high magnitude. It is a cheap, simple and effective procedure to dilute solid samples. Sound waves are pressure waves, which are transmitted through a medium, without this medium, the transmission is impossible. The reason why the sound waves cause contraction and subsequent expansion is due to the medium in which they propagate. When this medium is a solvent, it may form bubbles or cavities in the liquid, which exploited in a process known as cavitation. These bubbles burst violently causing a local increase in pressure and temperature. It is very remarkable that it is not noticeable in the system because the bubbles are very small. In addition, these local energy increases, resulting in the formation of radicals in the solvent. Due to increased processing temperature, it helps the solubility Ultrasonication which produces the bubble burst, in sum, increased pressure results in better penetration of solvent into the solid and the formation of a reactive medium to attack the sample. The bath temperature and the desired frequency can easily be regulated. [W12]

2.3 Theory HPLC

In this project a HPLC instrument was used to make the analysis. The HPLC, High Pressure Liquid Chromatography is a physical separation technique conducted in the liquid phase, where components are distributed between a stationary and mobile phase by using high pressure and small-particle stationary phases. The HPLC works by separating a sample into its constituent components or analytes by distributing between the mobile phases, which is the flowing liquid, and a stationary phase, which is the sorbents packed inside a column. The analytes are separated as they move through the column. For example there is two compounds A and B, and the A molecule has a stronger affinity for the stationary phase than the B molecule, the B will run through the column and the signal from A will come out later than B.



Figur 5, HPLC [Dong, p.79]

The mobile phase reservoirs or eluent reservoirs contains two bluecap bottles with two different eluents. A degasser that removes the gasses in polar eluents to avoid air running through the system and make noise on the detector. The pump regulates the composition of the two eluents and pumps the eluent with the sample through the system. The injector is connected with an auto sampler that automatically injects the sample into the system. The column is the most important part of the HPLC, it is where the separation takes place and the type of column depends on the compounds that are going to be separated. In the used HPLC the detector is an UV-detector the purpose for the detector is by selecting the optimal wavelength to be more sensitive for the wanted compound and less for the unwanted. The HPLC system is controlled by a computer where the adjustments are made on eluent composition, injection volume, flow rate, temperature of the column and the computer is also loading and saving the data on the method, sequent and chromatography data. [Dong, p. 2-4]

2.3.1 Mobile phase

The mobile phase is, as mentioned earlier, the solvent that moves the analyte through the system. In HPLC the mobile phase makes interactions with both, the analyte and the stationary phase. Therefore it has a powerful influence on the retention time and separation; see further description in *Method optimization*.

In reversed phase chromatography, RPC, the mobile phase is often methanol and water or acetonitrile and water. Water is a weak solvent where methanol and acetonitrile are the strong solvents, where acetonitrile is the strongest. The strength of the mobile phase is the ability to elute the analyte from the column and the strength is under normal phase conditions often characterized by Hildebrand's elution strength scale (E^o), see the table below. The strength of the solvent is related to the polarity, in a normal phase chromatography is the nonpolar compound hexane the weakest solvent and water is the strongest, see the table below. In the RFC where the stationary phase is hydrophobic the strength of the solvents is in reversed order compared to the Hildebrand scale.

Solvent	Solvent strength (E°)	bp (°C)	Viscosity (cP) at 20°C	UV cut-off (nm)	Refractive index
n-Hexane	0.01	69	0.31	190	1.37
Toluene	0.29	78	0.59	285	1.49
Methylene chloride	0.42	40	0.44	233	1.42
Tetrahydro-furan	0.45	66	0.55	212	1.41
Acetonitrile	0.55–0.65	82	0.37	190	1.34
2-Propanol	0.82	82	2.30	205	1.38
Methanol	0.95	65	0.54	205	1.33
Water	Large	100	1.00	<190	1.33

E° (solvent elution strength as defined by Hildebrand on alumina). Data extracted from reference 2 and other sources.

Figur 6, Common solvents and their properties, [Dong, p. 27]

Besides having an appropriate solvent strength, the mobile phase needs to be cheap, high purity, UV transparent, and having a low viscosity and toxicity, non flammability and have a high solubility for the sample components and of course be non corrosive to the HPLC system. [Dong, p. 27-31]

2.3.2 Stationary phase

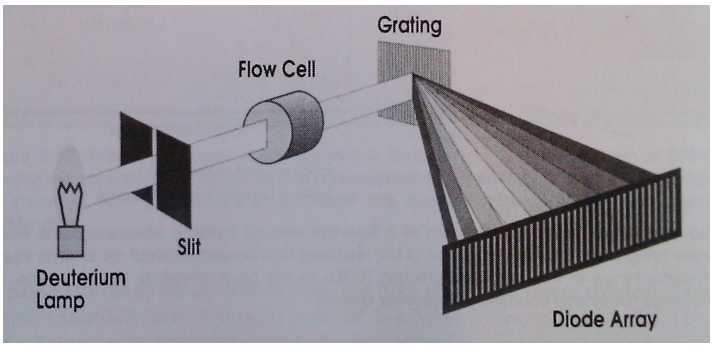
The stationary phase or the column is, as mentioned earlier, the heart of the HPLC instrument. The characteristics of column packing are the support type, bonded groups, particle size, pore size and surface area. A typical column will be packed with unbounded silica, because it is the most widely used support material and is often used for analytical purposes due to the strong adsorptive characteristics. The quality of the stationary phase and determines of efficiency is related to the particle size and size distribution and also affects the backpressure of the column. A large surface area gives a better resolution and to ensure that the stationary phase should be porous.

The stationary phase can be categorized based on the mode of the HPLC and the length and inner diameter (i.d.) of the column; the range of the length is typically 50-250 mm.

The first stationary phases that were developed for RPC had solid particles that were coated with nonpolar liquids. These were quickly replaced by more permanently bonding hydrophobic groups, such as octadecyl (C18) bonded groups, on silica support. The numbers of carbon contained in the hydrophobic group gives the name to the column so that such as octadecyl as the hydrophobic group will be a C18 column [Dong, p. 49-56]

2.3.3 Detector

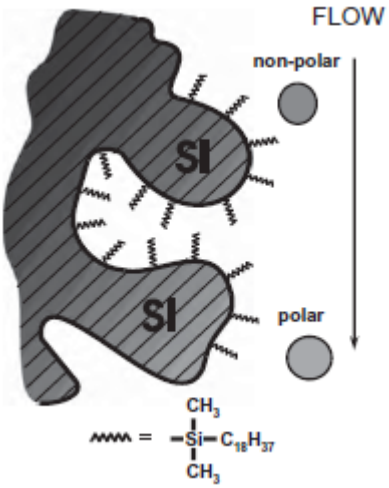
The detector purpose is to measure the concentration of the analytes by monitoring one of their inherent properties such as the UV absorbance. The table of different detectors is very long, and can be classified either as universal to all analytes or specific to particular classes of analytes. As the table is long and there are many different detectors there will only be focused on the UV/Vis absorbance detector, as this is the used detector in this project. The principle for this detector is based on the Lambert Beer's law; $A = \varepsilon * l * c$, where the absorbance is equal to the molar absorptivity times path length times concentration. This detector is the most used detector because most of the analytes have UV absorbance. The operating principles behind this detector are that the detector consists of a deuterium lamp, a monochromator and a small flow cell. See the figure below.



Figur 7, A schematic of a UV-Vis absorbance detector,[Dong, p. 89]

The monochromator consists of a movable grating or prism that allows the selection of a specific wavelength to pass through the exit slit. The light source is split into a sample and a reference beam, and the intensity of each beam is monitored by a separation photodiode. Only the sample beam passes through the sample flow cell. [Dong, p. 87-90]

2.3.4 Reversed phase chromatography (RPC)



Figur 8, reversed-phase chromatography, [Dong, p. 6]

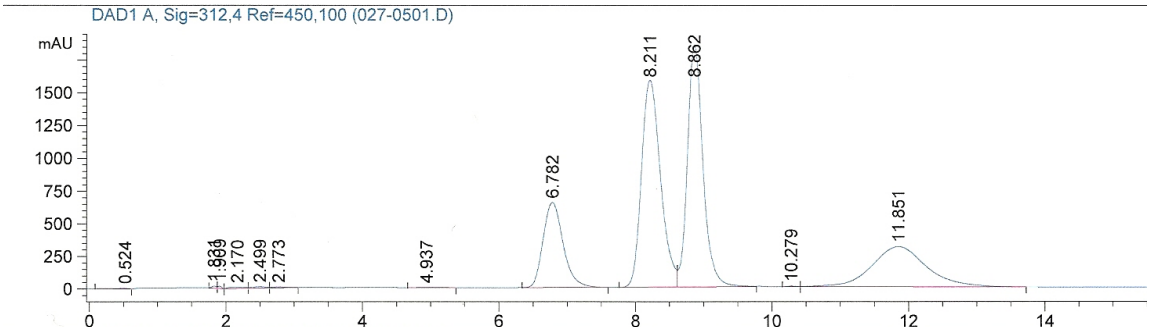
RPC is the most used HPLC mode and is used in more than 70 % of all HPLC analyses. It is so popular because the method is suitable for the analysis of polar, medium-polarity and some non-polar analytes. RPC have opposite normal-phase chromatography (NPC), a non-polar (hydrophobic) stationary phase and a polar mobile phase. The elution order is also reversed compared to NPC where first the polar elutes and last the nonpolar compounds, as the nonpolar compound is better bonding to the stationary phase. The hydrophobic interactions occur as a result of the repulsive forces between a polar eluent, a medium-polar analytes and a nonpolar stationary phase. The most used types of stationary phase for the RPC are C8 or C18 column. [Dong, p. 5-7]

2.3.5 Isocratic vs. Gradient

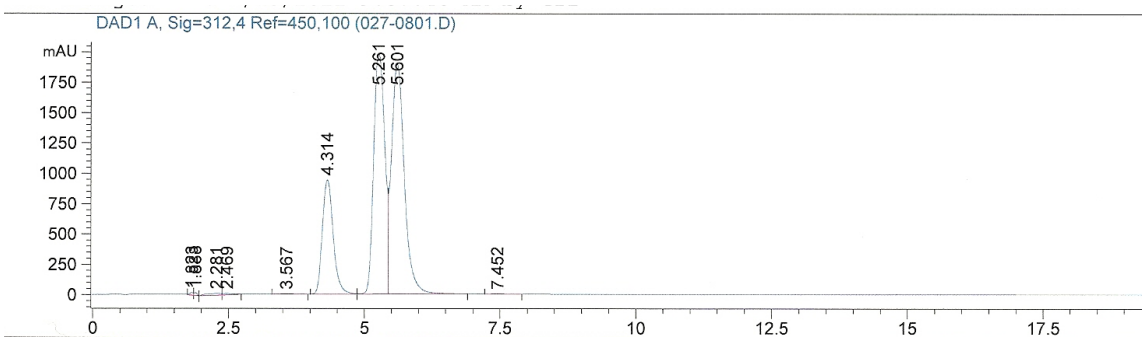
In most HPLC separations the isocratic conditions are used in which the same mobile phase is used throughout sample analysis time. The method is very useful for simple samples with uniform polarity and where peaks are broadened by increasing elution time. The gradient analysis where the strength of the mobile phase is increased with the time of the analysis is often used for samples that are of varying or unknown polarity. The formulas for column efficiency using the peak width and standard formulas for retention factor cannot be used when the analysis is with gradient conditions. The reason is that the solvent strength increases through a gradient method and therefore the peak width decreases compared to the isocratic. In the gradient analysis the resolution of early and late eluting peaks will be better as well as the sensitivity of late eluting peaks. [Dong, p. 39-41]

2.3.6 The chromatogram

As seen below, the chromatogram with the time in minutes on the x-scale and the absorbance in mAU on the y-scale. Gives a good idea of what have been accomplished in this project.



Figur 9, a good chromatogram with gradient D3 and all 3 standards



Figur 10, a bad chromatogram with gradient E and all 3 standards

The conclusion whether a chromatogram is useful depends on how good the peak that is interesting have been separated. The idea is to get good separation so that the area of the peak and hereby the concentration can be determine with accuracy and preciseness.

2.3.7 Retention time

The time from the injection to the maximum of the first peak is called the retention time, t_R. The retention time of an unretained compound or the first baseline disturbance by the sample solvent is called the void time, t_M, which is the time that the solute spend in the mobile phase. The adjusted retention time, t_R', is the time that the solute spend in the stationary phase.

Thus, $t_R = t'_R + t_M$

In other words is the retention time the total time that the solute, spend in the stationary and in the mobile phase, the faster the compound runs through the column the shorter the retention time will be. See the figure below. [Dong, p. 17]

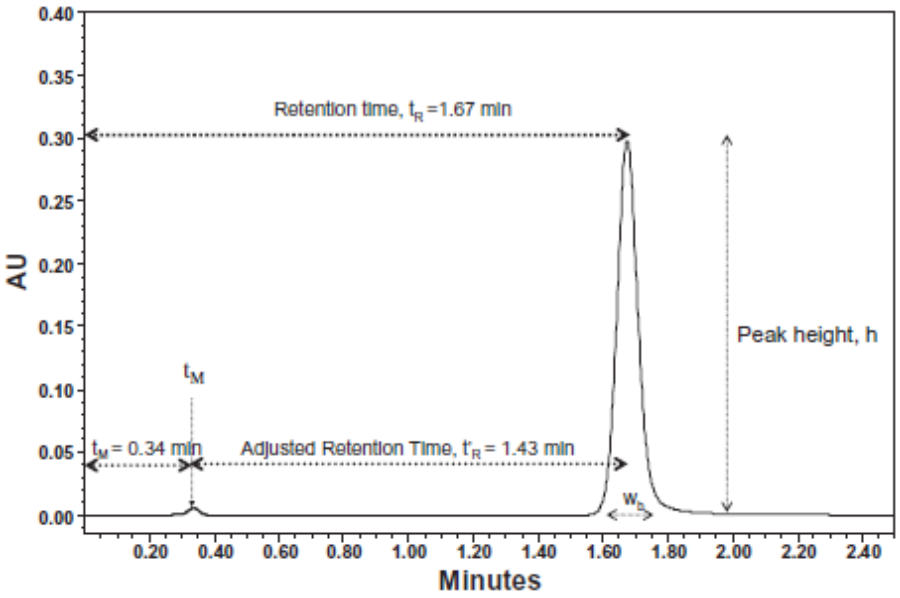


Figure 11, Chromatogram showing retention time, void time, peak width and peak height, [Dong, p. 17]

2.3.8 Retention factor

The retention factor, k, is as the name indicates the degree of retention of the sample component in the column. The definition of the retention factor is seen below in the figure how the adjusted time is related to the void time.

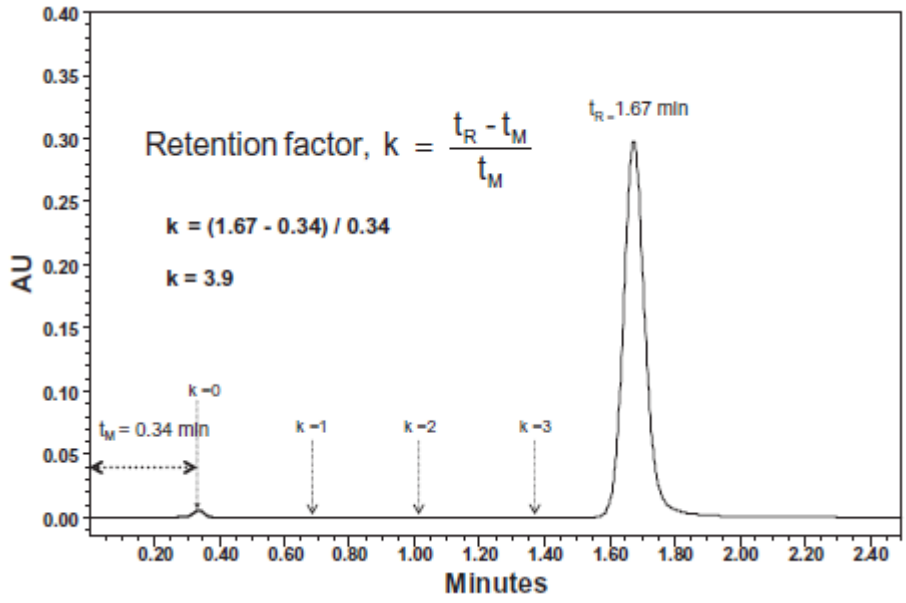


Figure 12. A chromatogram showing the calculation for retention factor, k, which is equal to t_R'/t_M. [Dong, p. 19]

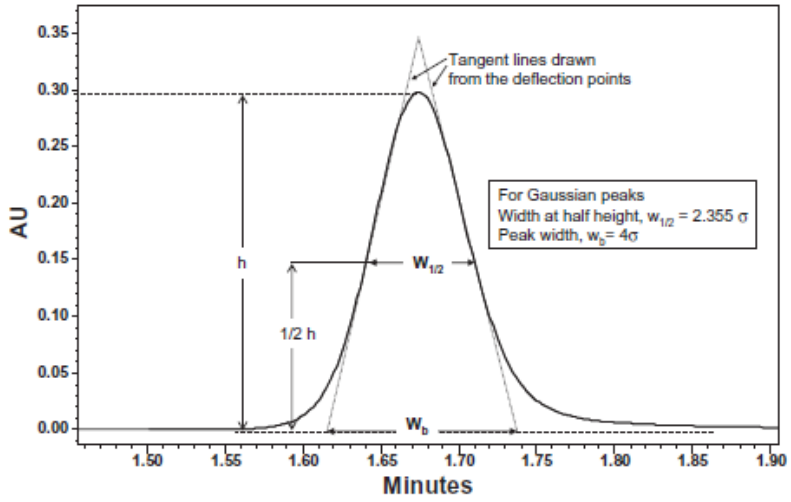
The retention factor is an important parameter defining the retention of the analyte. Desirable k values for isocratic analyses are 1 to 20, but the preferable range is 1 < k < 5-10. The factors that determine the value of k are the strength of the mobile phase, the type of stationary phase and the temperature. As the retention time decreases the faster the compound runs through the column and the smaller will the retention factor be. [Dong, p. 19-20]

2.3.9 Peak area

Every solute peak has both a peak width, and a peak height, h. The peak width is measured at the base, w_b, or at the peak half-height, $w_{½}$. To measure the exact w_b two tangent lines are drawn for the steepest inflection points of the peak the distance between the intercept with the baseline is w_b.

Peak area is roughly equal to ½(w_b*h). For Gaussian peaks, w_b is approximately equal to four times standard deviation, σ, but w_b and σ are usually difficult to measure hence, so the width at half height, $w_{½}$ is usually used to calculate column efficiency, the $w_{½}$ is equal to 2.355 σ.

The height or the area of a peak is proportional to the amount of analyte component and the peak area is commonly used to perform quantitative calculations. In the real world the area of the peak is calculated with integral by the computer. [Dong, p. 17-18]



Figur 13, diamgram illustrating how peak width and peak width at half height are measured.[Dong, p.18]

2.3.10 Selectivity

The separation factor or selectivity, α, is related to the retention factor to calculate the relative retention between two samples components by k_2/k_1 . The values for separation of two components must be above 1.0. The separation factor is an important factor of the method optimization and the desirable range is $2 > \alpha > 1.05$. The factor depend on many factors that affect k such as the nature of the stationary phase, the mobile phase composition and properties of the solutes. [Dong, p. 20-21]

2.3.11 Column efficiency

The column efficiency and plate number, N, need to have an efficient column because it produces sharp peaks and can separate many components from the sample in a relatively short time. In most chromatograms, peaks tend to be Gaussian, a clear sharp peak, in shape and broaden with time.

The number of theoretical plates or plate number, N, is the measure of the efficiency of the column and is defined as:

$$N = \left(\frac{4 * t_R}{w_b}\right)^2 = 16\left(\frac{t_R}{w_b}\right)^2$$

[Dong, p. 21-22]

2.3.12 Resolution

The resolution, R_S, is a measure of the degree of separation between two adjacent analytes and R_S is defined as:

$$R_S = \frac{t_{R2} - t_{R1}}{\left(\frac{w_{b1} + w_{b2}}{2}\right)}$$

The values of R_S that needs to be noted is; R_S= 0 indicates complete co-elution or no separation. A value of R_S= 0.6 indicates that a shoulder is discernible or a slight partial separation, R_S= 1 indicates that a partial separation and is the minimum separation required for quantification and R_S= 1.5 indicates baseline separation. All analytes should ideally have R_S values in the range R_S= 1.5 – 2.0. [Dong, p. 23-24]

2.3.13 The resolution equation

The resolution equation have an influence on three parameters;

$$R_S = \left(\frac{k}{k+1}\right)\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{\sqrt{N}}{4}\right)$$

The three independent factors are retention, selectivity and efficiency. The retention, k, and the selectivity, α, is depending on both thermodynamic factors and the peak width and column efficiency, N, is a kinetic factor, this equation gives an idea of what parameters affect the separation of peak. [Dong, p. 34-35]

2.3 Method optimization

The purpose of the method optimization is to make a short and precise method with good separation of the UV-filter that is going to be tested. It is wanted to develop a short method because the time of analysing the UV-filters will be shorter and the amount of mobile phase will be reduced. To develop the best suitable method for the analysis, that gives the best chromatogram with the lowest cost, there are different parameters that can be changed during the optimization. Below is listed the parameters that have influence on the chromatogram and in the following text there will be further explanation of the parameters that have been changed during optimization of this project.

Mobile phase parameters:

- Solvent strength and type
- Buffer type and concentration
- pH

Operating parameters:

- Flow rate
- Temperature
- Gradient range and time

Column parameters:

- Type of stationary phase
- Length
- Diameter
- Particle size

Detector setting:

- Wavelength
- Sample amount

The list of parameters that has impacted on the chromatogram is long, but in this project the focus has been on the operating parameters, the solvent strength and type.

The mobile phase is a parameter that is easy to change by changing the composition or type of the mobile phase this is much easier than to change the type of the column. The composition and the type of the mobile phase have a huge influence on the separation and retention time of the chromatography. As described in the section *Mobile phase;* the strength of the mobile phase is the ability to elute the analyte from the column and is related to the polarity. The stronger the mobile phase is the faster it will run though the column and the shorter the retention time will be. If the analytes on the other hand runs too fast through the column it will result in a bad separation.

The Gradient range and time is defined as the gradient slope and is well related to the mobile phase. With increasing the time it often increases overall resolution of complex samples and by changing both the slope and time it is possible to fine-tuning separation of complex samples.

The flow rate does not affect isocratic retention or selectivity, because the flow has the same effect on the retention time of each solution, but in a gradient elution it is an important factor affecting both average retention and selectivity. With a higher flow rate it reduces the retention time and analysis time but it is also increases the column backpressure. As a guideline the flow rate should be proportional to the square of the column inner diameter.

If the temperature increases it can reduce the retention for RPC and can have some effects in the selectivity in general. As a higher temperature gives a lower viscosity of the mobile phase it gives a better flow and a lower column backpressure. [Bisgaard, S1, HPLC4], [Dong, p. 33-34,204-207]

2.4 Method validation

The aim of method validation is to investigate if a method of analyzing is suitable for a certain analyte.

To which extent a method validation has to be used on an analyte depends on the purpose of the desired experiment. It depends on the purpose of the method and the reason for the validation.

A method validation should be made [Lund, p. 7];

- If a new method is developed from the beginning or with background in some relevant theories
- If an old method is optimized
- If the method is changed in the laboratory
- If a standard method or an already validated method is modified
- If a validated method is used on a new sample matrix
- If new instruments are used or old ones have been moved
- If an already validated method is to be used by a new analyst

For analyzing methods using HPLC or GC a couple of parameters must be taking into consideration. The following parameters will typically be relevant for the method validation [Simonsen, p. 265] and [S5, p. 3];

- Specificity
- Linearity and range
- Sensitivity
- Accuracy/bias
- Limit of detection (LOD)
- Limit of Quantification (LOQ)
- Precision
- Robustness

These parameters are generally used when working with a HPLC and will be explained in the following sections and what relevance it has on this project.

2.4.1 Specificity

According to statistics, the definition of specificity is how accurately and specifically it is possible to measure an amount of a certain compound in a sample matrix where a lot of other compounds are present. Specificity measures the degree of interference from other compounds [S3, p. 4]. In general the specificity

is determined by spiking a sample with a known amount of a certain compound and then calculates the percentage retrieval [Simonsen, p. 264].

In this project the specificity will be measured by spiking the compounds, which are known to be in the different sunscreens of a sample and then calculate the percentage retrieval. The percentage retrieval has to be used theoretically to calculate whether there will be a discrimination of related compounds to the compounds that are measured during the analyses.

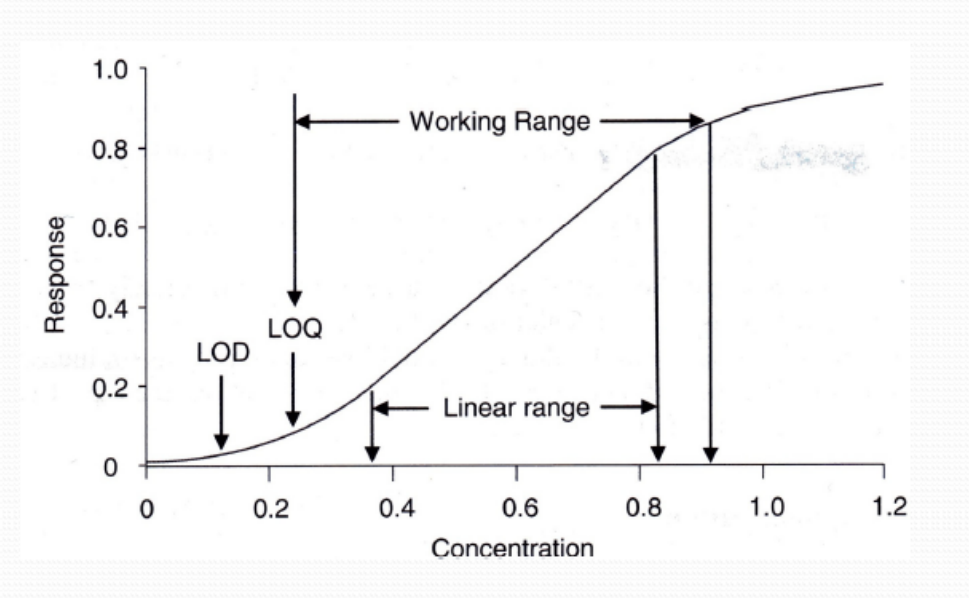
2.4.2 Linearity and range

The range is the concentration area where the current substance can be determined with a given precision and accuracy. The measuring range does typically cover a larger area than the linearity. The reason is that there is no need of a linear correlation between the response and the concentration to give a good method. Although the correlation does not need to be linear, the accuracy and precision must be acceptable in the whole measuring range[A9, p. 15-16]

When the measuring range is determined, the precision and accuracy have to be detected in the ends of the measuring range. Because it is only interesting to cover the area where the method is being used and therefore it is not necessary to cover the whole theoretical range.

In this project the range will only be explained in the used interval. Precision and accuracy will be determined not only in the ends of the range but it will be detected in the whole measuring range that is used. An accurate and precise range is important in concentrations from $2.18 \cdot 10^{-4} \frac{g}{mL}$ *to* $1.20 \cdot 10^{-4} \frac{g}{mL}$.

Linearity is defined by a linear regression, a response vs. concentration [simonsen, p. 34]. This is done by analyzing a series of standards with different concentrations covering the area that is believed to be linear. The linearity is investigated to the minimum where a double determination of 5 concentrations has to be done. For illustration see figure 13



Figur 14; illustration of the different terms do to linearity and working range [S5, p. 9]

The investigation of linearity can be done at the same time, as the measuring range is determined. The purpose of linearity is to demonstrate in which area there is a linear correlation. If it is not possible to find a linear correlation in the area of the measuring range, the range can be split into two areas, each with their own linearity.

To make a linear regression their have to be reasonable variance homogeneity through the measuring range. This means that all points should contribute equally to the location on the line. In this project the variance homogeneity will be determined by using a hypothesis test with a 95% confidence interval.

If there is variance homogeneity the linearity can be made by using a simple linear regression. In case of no variance homogeneity, the linear calculation should be weighted [Lund, p. 18-19].

After making the regression the interception of the y-axis is investigated to find out if the regression crosses the point [0.0]. The testing is done by using a hypothesis test with a confidence interval of 95% where [Montgomery, p. 406];

$$H_0 = \beta_{0.0}$$
$$H_1 \neq \beta_{0.0}$$

In theory the interception should be in [0.0] because the two compounds investigated will be the only compounds in the standards to absorb light at a wavelength of respectively 312nm and 358nm.

When the regression is made the uncertainties for all the concentrations will be calculated by making a 95% confidence interval on all of the points in the regression by using the following equation [Montgomery, p. 412];

$$\overline{\mu}_{Y|x_0} - t_{\alpha/2,n-2}\sqrt{\overline{\sigma}^2\left[\frac{1}{n}+\frac{\left(x_0-\overline{x}\right)^2}{S_{xx}}\right]} \le \mu_{Y|x_0} \le \overline{\mu}_{Y|x_0} - t_{\alpha/2,n-2}\sqrt{\overline{\sigma}^2\left[\frac{1}{n}+\frac{\left(x_0-\overline{x}\right)^2}{S_{xx}}\right]}$$

where $\overline{\mu}_{Y|x_0} = \overline{\beta}_0 + \overline{\beta}_1 \cdot x_0$ is from the regression model.

$t_{\alpha/2,n-2}$ is the *t* distribution value by a significance divided by 2. It is divided by 2 because it is a two-sided test. *n* is the number of measurements in the regression.

$\overline{\sigma}^2$ is an estimated of standard deviation σ^2.

S_{xx} is equal to $\sum\left(x_0-\overline{x}\right)^2$.

$\overline{x}$ is the mean value of all of the points in the regression.

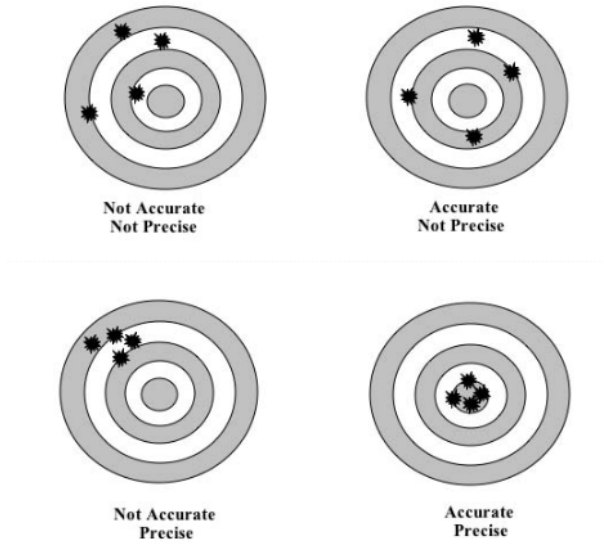
$\mu_{Y|x_0}$ is the mean value for a given x.

2.4.3 Sensitivity

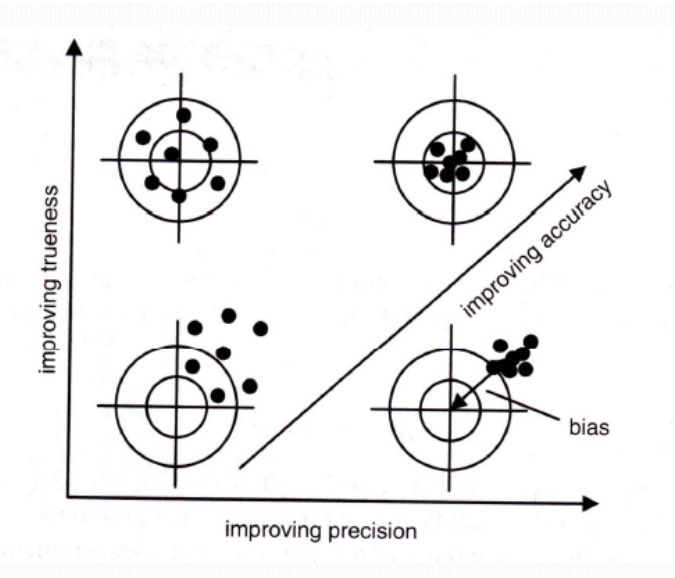
The methods sensitivity is the smallest difference in concentration, which can be determined. The sensitivity is detected by using the slope from the calibration curve [Lund, p. 11].

2.4.4 Accuracy/bias

Accuracy is defined by the exactness of an analytical method or how close a single measured value is to the accepted reference value [S3, p. 3]. For illustration see figure 14 and figure 15.



Figur 16; Illustartion of accuracy and precision [S5, p. 6]



Figur 15; Illustartion of accuracy and precision [S5, p. 7]

The most common way to measure the accuracy is by making a recovery test. In this project this is done by spiking a sample with a known amount of a compound. The recovery test reveals interference with impurities or discriminating compounds. For determination of the recovery a minimum of 10 spiked samples is used.

A method's accuracy is expressed by bias. Bias is defined as [S5, p. 4];

$$Bias = measured\ \ value - reference/"true"\ value\ \ (\bar{x} - \mu_0)$$

2.4.5 Limit of Detection (LOD)

The limit of detection is the lowest concentration of an analyte that can be determined (not quantified). Limit of detection is detected by calculation of standard deviation on a minimum of 6 blank samples or samples with a concentration close to the expected limit of detection. The concentration determination must be made under repeatability. The conditions for repeatability are as follows [Simonsen, p. 33];

- Same measure method and procedure
- Same person must make the measurements
- Same instrument for the measurements must be used
- The measurements must be repeated within a short time

Limit of detection (LOD) can be calculated by using the t-distribution and the following equation can be taking under consideration [Lund, p.22];

$$LOD = t_{0.995}(f) \cdot s_{blank} \cdot \sqrt{1 + \frac{1}{n}}$$

where s_{blank} is the standard deviation calculated for a minimum of 6 blank samples. n is the number of samples. $t_{0.995}$ is to be found in the table of quintiles in the t-distribution and f is the degree of freedom found by the following equation; $f = n - 1$.

2.4.6 Limit of Quantification (LOQ)

Limit of quantification is defined as being the lowest concentration of analyte which can be quantified with acceptable precision and accuracy [S3, p. 5]. Limit of quantification is determined by measuring blank samples or samples with a very low concentration [Simonsen, p. 264]. After measuring samples, the standard deviation SD can be calculated and the limit of quantification (LOQ) can be detected by using the following equation;

$$LOQ = 10 \cdot SD_r$$

2.4.7 Precision

Precision is defined by random errors [Lund, p. 5]. Precision indicates how close several measured points lie to the same population. There are two ways to measure precision; repeatability and reproducibility.

Repeatability is the standard deviation obtained by running the samples under repeatability conditions. Repeatability conditions are as earlier mentioned; same method, same instruments, same analyst and is repeated within a short time.

Reproducibility is the standard deviation measured under a maximum of varying conditions; same method, different equipment, different laboratories, different analyst and the samples are measured within a long period of time.

In this project reproducibility will be determined. Reproducibility will be investigated by changing the analyst and running the samples in different days.

2.4.8 Robustness

Robustness is defined by the ability of a method to remain unaffected by small changes. In other words it is the reliability of the method that is measured. The small changes can be; temperature, pH, variation of glass etc [Lund, p. 5].

In this project the robustness are determined by changing the temperature, flow, solvent combination, the analyst and time.

2.5 Statistical terms and tests

In this section different statistical terms and tests that are used in this project will be described. The statistical calculations are made in the program SAS JMP version 8.

2.5.1 Standard deviation

Standard deviation is a measure of variability [Simonsen, p. 760]. It is an expression used to determine how close the measurements are to the mean value. The standard deviation for a sample can be calculated by the following equation[Bøye, p. 17] and has the same units as the measurements;

$$s = \sqrt{\frac{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)^2}{n-1}}$$

n is the number of samples, x_i is the observations, $\bar{x}$ is the mean value. The mean value is calculated by taking the sum of all the observations and divided it with the number of observations. *n-1* is the degree of freedom. If the sample variance, s^2 is known, then the standard deviation can be calculated by taking the numeric square root of s^2.

2.5.2 Relative standard deviation

The relative standard deviation (RSD) also called coefficient of variance (CV%) can be calculated by the following equation;

$$RSD = \frac{s}{\bar{x}} \cdot 100\%$$

The relative standard deviation is typically given in percentage. It is therefore possible to compare with the relative standard deviation calculated for other data [Simonsen, p. 25].

2.5.3 Variance

The variance s^2 is a measure of how large the distance is between the different measurements. The bigger the variance is, the longer the distance between the measurements are. [Montgomery, p. 761]

2.5.4 Confidence interval

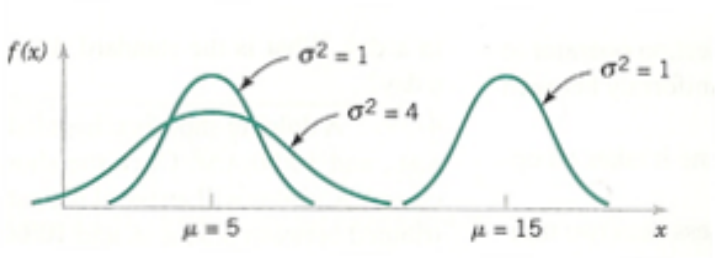
Confidence interval (CI) is constructed on the mean or variance. The limits for the confidence interval indicates the area where it is relatively likely to find the measured value with a probability of 100(1-α) % [Montgomery, p.752] . A confidence interval of 95%, 99% or 99, 9% is typically used. However, a confidence interval of 95% is the most commonly used.

2.5.5 Normal distribution

The normal distribution, also called the Gaussian distribution, is a model that is used widely for the distribution of a random variable and is a continuously probability distribution. The normal distribution can basically contain all real numbers and be determined by using the mean value μ and the variance σ^2 [Montgomery, s. 121-122];

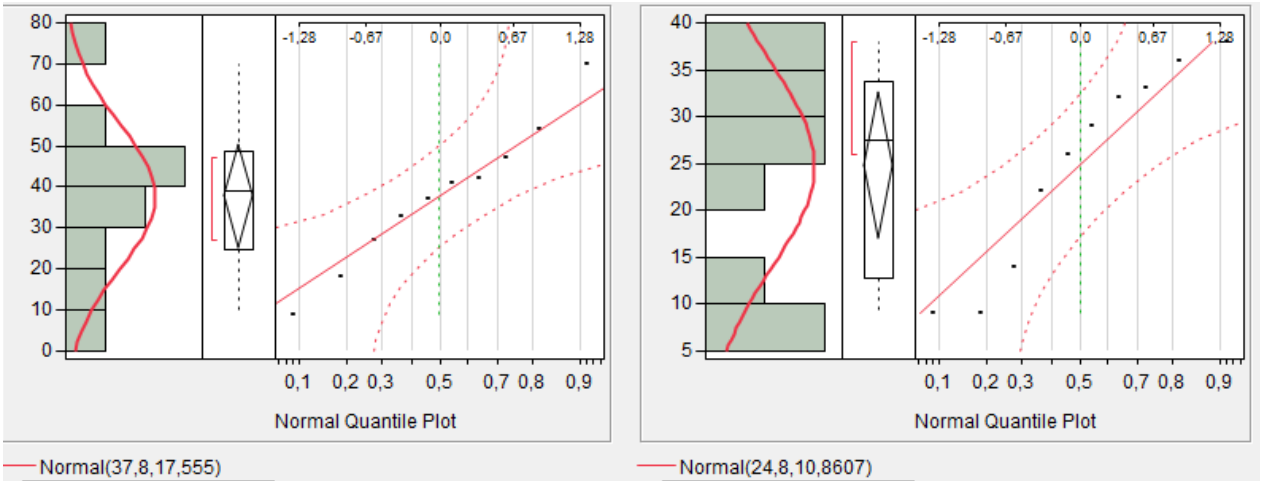
$$f(x) = \frac{1}{\sqrt{2\pi\sigma}} e^{\frac{-(x-\mu)^2}{2\sigma^2}}, \quad -\infty < x < \infty$$

The normal distribution is a symmetrical plot as shown in the figure below;



Figur 17, Normal probability density functions for selected values of the parameters μ and σ^2, [Montgomery, p. 122]

The Gaussian distribution can be investigated by using a normal quantile plot in SAS JMP, see figure below

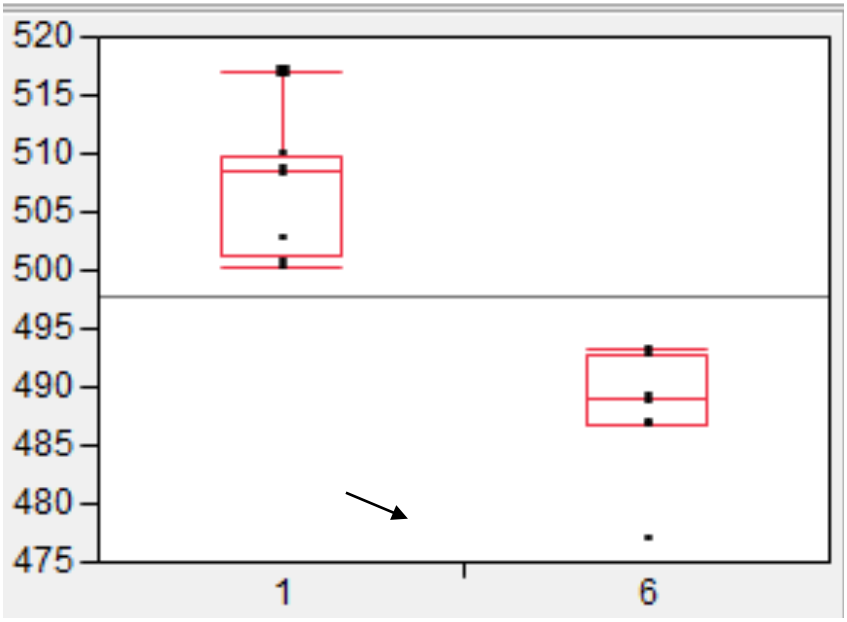


Figur 18; Normal quantile plot for the two different set of data where both datasets are normal distributed

The figure shows that both dataset is normal distributed because the data lies approximately on a straight line however the two datasets consist only of 10 observations. The conclusion for the normal distribution can be made, but it will be a stronger conclusion if there were more measurements.

2.5.6 Quartiles - outliers

Before making an analysis and fitting the data into a model it is important to investigate if there are any outliers present. Outliers can be investigated by using a box plot. The box plot can be made in SAS JMP and uses quartiles. 25% quartiles is called the lower quartile, it indicates a number *i* where 25% of the data is less than or equal to the number *i*. The 50% quartile is called the median while the 75% quartile is called the upper quartile and is a number *j* where 75% of the data is less than or equal to the number *j* - below is shown an example of a box plot. The box plot on the left side has no outliers while the plot on the right side has one potential outlier.



Figur 19; The box plot in the left side have no outliers while the box at the right side have one possible outlier

As shown in the figure there is one line in both ends of the box. Those are respectively the maximum and minimum. The maximum is calculated by taking the biggest point within the 75% and adding 1.5*interquartiles width. The minimum is determined by taking the smallest point within the 25% and minus 1.5*interquartiles width. The interquartiles' width is defined by the distance between the 25% quartile and the 75% quartile. If a point as shown in the figure 18 is placed outside this interval it is necessary to consider if it is an outlier and should be removed from the dataset [Bøye, p.15].

2.5.7 Hypothesis testing

Hypothesis testing can be defined as a statement on a relevant parameter of one or more samples [S4, s. 3]. There are different kinds of statistical hypotheses, the one-sided and the two-sided [S4, s.3]. The two-sided will be used in this project.

A general procedure of hypothesis testing is as follows [S4, s. 9];

- Identify the parameter
- Form a null hypothesis, H_0
- State an alternative hypothesis H_1
- Choose a level of significance, α

- Determine a proper statistical test
- Define the region for the statistics
- Compute any necessary values for rejecting/falling to reject the null hypothesis
- Decide wherever to rejecting/falling to reject the null hypothesis.

For hypothesis testing there are two different kinds of error; type 1 and type 2. An error of type 1 occurs when the null hypothesis is true and rejected. A type 2 error occurs when the null hypothesis is false but is failed to reject.

2.5.8 Two sample t-test

A two sample t-test or a two sample comparison is a test where two means are compared to each other. This test examines the difference in the means of two normal distributed populations where the variance is unknown.

The two sample test mainly investigates the difference in the means of two samples or two measurements where the variances σ_1^2 and σ_2^2 are unknown and is assumed to be unequal $\sigma_1^2 \neq \sigma_2^2$. However before making the t-test, the normal distribution has to be checked, as well as if there is any outliers, to confirm if the basic conditions are fulfilled. The variance homogeneity can be tested using the *Bartless test* and is described in the following section, *Assumptions for making an ANOVA*.

A hypothesis test is made to find whether the Null hypothesis is true or not thus $\mu_1 - \mu_2$ must be found. Therefore two hypothesis tests are usually established; the Null hypothesis and the alternative hypothesis where the mean is different from zero:

$$H_0 = \mu_1 - \mu_2 = 0$$
$$H_1 = \mu_1 - \mu_2 \neq 0$$

The populations are represented as X_1 and X_2. When the variances, $\sigma^2_1$and σ^2_2, of the two populations are unequal, an equation is established to test whether the Null hypothesis can be rejected or not at a certain significant level. However there is not a precise method for testing $H_0 = \mu_1 - \mu_2 = \Delta_0$ as the variances are unequal. Therefore assuming that the Null hypothesis is true a test statistic is calculated to see whether the Null hypothesis has to be rejected or not.

Test statistics:

$$T_0^* = \frac{\overline{X_1} - \overline{X_2} - \Delta_0}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

In addition to this, determining the degree of freedom can be calculated using the test statistics value:

Degree of freedom:

$$v = \frac{(\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2})^2}{\frac{(S_1^2/n_1)^2}{n_1 - 1} - \frac{(S_2^2/n_2)^2}{n_2 - 1}}$$

Using these 2 equations above can determine whether the Null hypothesis is true or no. If the Null hypothesis is true then the test statistic has to be distributed approximately as the degree of freedom for the test. [Montgomery, p. 346]

2.5.9 ANOVA

ANOVA is an **an**alysis **o**f **va**riance and it is said to be a robust way of testing the variability between results from experiments with one or more factors involved. In the following sections the assumptions for making an ANOVA test will be described. A short explanation of the 1-, 2- and 3-factor ANOVA is described as well. For a two- and three-sided analysis of variance there can be an interaction between the factors which is shortly explained.

2.5.9.1 Assumptions for making an ANOVA

For making an analysis of variance it is necessary that the data is normal distributed. The normal distribution will typically be shown by a graph that is symmetrical about the mean, or it can be investigated by using a normal quantile plot as earlier mentioned. If there is a small amount of samples it will be necessary to make an assumption.

The measurements should be independent of each other and variance homogeneity is important. Variance homogeneity is defined by the variances should be equal for each experiment and can easily be tested by using the following equation;

$$F_{obs} = \frac{s_{\max}^2}{s_{\min}^2}$$

s_{max}^2 is the maximum variance while s_{min}^2 is the smallest variance. A more precise way to investigate the variance homogeneity is the *Bartlett's test* but it can only be used for a one factor ANOVA. The *Bartlett's test* is as follows;

$$x_{obs(r-1)}^{\ 2} = \frac{(n-r)\cdot \ln MSE - \sum_{i=1}^{r}(n_i - 1)\cdot \ln\left(s_i^{\ 2}\right)}{c}$$

$$Where,\quad c = 1 + \frac{\sum_{i=1}^{r}\frac{1}{n_i - 1} - \frac{1}{n-r}}{3\cdot(r-1)}$$

The *Bartlett's test* is a one-sided x^2 test with a r-1 degrees of freedom. In the equation; MSE is the mean square error, s_i^2 is the variance for the calculated mean of each "different" type of test, n is the number of observations while r is the number of samples [Bøye, p. 101].

2.5.9.2 1-, 2- and 3-factor ANOVA

An analysis of variance can be calculated with different factors. In the following section a 1-, 2- and 3-factor will be shortly explained.

In ANOVA the factors can either be fixed or random. If the factors are fixed the levels are selected and controlled and the conclusion that will be drawn from the analysis will only involve some parts of the population.

If the factors are random the levels have been selected unsystematically from the population of levels. The conclusions that can be made on the basis of the random-effects model applies to the entire population.

In a single factor ANOVA as the name says only one factor varies. The total sum of square SST describes the variability of the data and can be calculated by the following equation (fixed-effect model) [Montgomery, p. 505];

$$SS_T = SS_{Treatments} + SS_E$$

$SS_{Treatments}$ is the symbol for treatment sum of square. SS_E stands for error sum of square. If the model were random the equation would be the same for a single factor analysis of variance.

In a two-sided analysis of variance 2 factors varies and the total sum of square SS_T can be calculated for a fixed model, by using this equation;

$$SS_T = SS_A + SS_B + SS_{AB} + SS_E$$

SS_A is the sum of square treatment for the factor A while SS_B is the sum of square treatment for factor B. SS_{AB} is the sum of square for the interactions between the two factors A and B. SS_E is the sum of square error.

In a 3-factor ANOVA, 3 factors (A, B, C) varies and the interactions will not only happen between A and B but also between AC and BC. The total sum of square SST can be calculated by using the following equation [Montgomery, p. 547];

$$SS_T = SS_A + SS_B + SS_C + SS_{AB} + SS_{AC} + SS_{BC} + SS_{ABC} + SS_E$$

It is assumed that the factors A, B and C are fixed. In words the SS_A is the square deviation of response, measured by factor A and the total average while for example SS_{AB} is the square deviation of the interactions between factor A and B. SS_E can be explained as the variance in samples that occur by random mistakes. See table 2 below for a better overview [Montgomery, p. 556].

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_0
A	SS_A	a-1	MS_A	MS_A/MS_E
B	SS_B	b-1	MS_B	MS_B/MS_E
C	SS_C	c-1	MS_C	MS_C/MS_E
AB	SS_{AB}	(a-1)(b-1)	MS_{AB}	MS_{AB}/MS_E
AC	SS_{AC}	(a-1)(c-1)	MS_{AC}	MS_{AC}/MS_E
BC	SS_{BC}	(b-1)(c-1)	MS_{BC}	MS_{BC}/MS_E
ABC	SS_{ABC}	(a-1)(b-1)(c-1)	MS_{ABC}	MS_{ABC}/MS_E
Error	SS_E	abc(n-1)	MS_E	
Total	SS_T	abcn-1		

Tabel 2 [Montgomery, p. 556]

The mean square can be calculated by dividing the sum of square with the degrees of freedom. See below for an example for factor C;

$$MS_C = \frac{SS_C}{(c-1)}$$

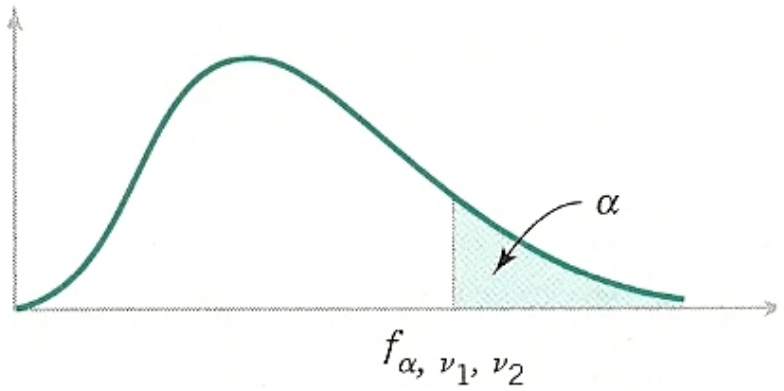
Mean square C is the variance for the effect of factor C.

The equation for a three factor ANOVA can also be described by the following [Montgomery, p. 556] ;

$$Y_{ijkl} = \mu + \tau_i + \beta_j + \gamma_k + (\tau\beta)_{ij} + (\tau\gamma)_{ik} + (\beta\gamma)_{jk} + (\tau\beta\gamma)_{ijk} + \varepsilon_{ijkl} \begin{cases} i = 1, 2...., a \\ j = 1, 2...., b \\ k = 1, 2...., c \\ l = 1, 2...., n \end{cases}$$

where τ_i, β_j *and* γ_k represents the three factors while $(\tau\beta)_{ij}$, $(\tau\gamma)_{ik}$ *and* $(\beta\gamma)_{jk}$ is the interactions between factors. $(\tau\beta\gamma)_{ijk}$ stands for the interactions between all three factors while ε_{ijkl} is the random error. i, j and k describes the level of each factor and l is the replicates. μ is the overall mean effect.

The purpose of making analyses of variance is to investigate if there is significance between the factors. The calculation of the significance can be done by using a F-test. First a critical F-value should be decided for example $f_{0.05}$ and afterwards the f_0 value could be calculated by dividing the mean square for a certain factor with the mean square of error. If the calculated value for f_0, based on the variance, is higher than the $f_{critical}$ then the null hypothesis can be rejected if not then there is significance. See figure 20 for illustration



Figur 20 illustartion of f-test

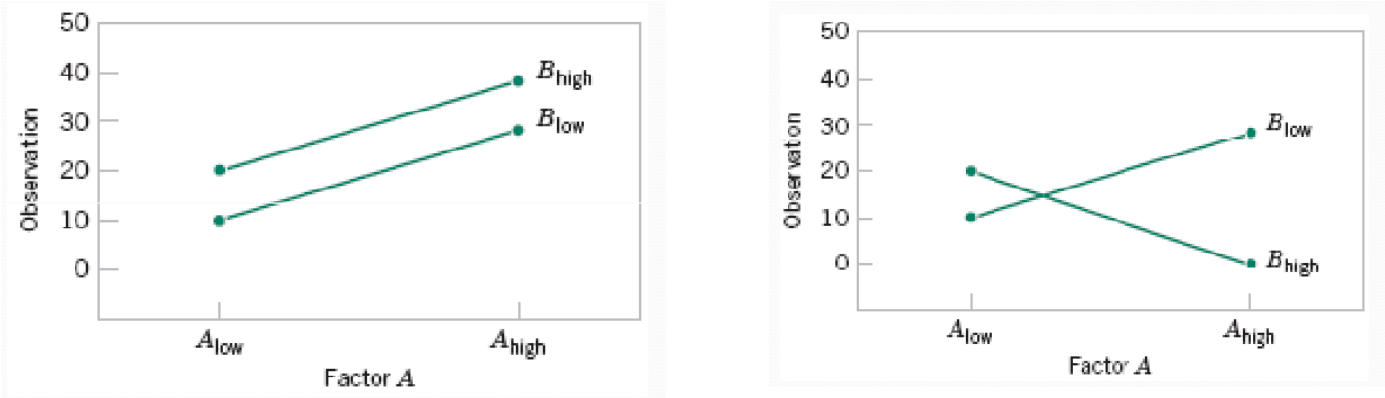
There are three different levels of significance; level *; 0,05, level **; 0,01 and level ***; 0,001.

In this project the significance for each of the listed parameters in the table will be calculated by SAS JMP where the p-value will be compared with the level of significance. If the p-value lies between level * and **

it will have a significance level of *or if the p value is larger than the level of significance there will be no significance.

2.5.9.3 2- and 3-factor ANOVA - interactions

An interaction between factors is when the response between different levels of one factor is not equal to the response from all levels of the other factors [Montgomery, p. 542]. As seen in figure 21 it is possible to see a two-factor interaction plot, the high and low values of A and B only indicate that it is different values;



Figur 21; At the left side the plot shows no interactions while the graph at the right side shows interactions [S2, p. 5-6]

3.0 Materials and methods

3.1 Standards

The standards; *Padimate O and Octocrylene* were purchased from the company Sigma – Aldrich, the compounds are very complex and have many synonyms, see some of the synonyms below.

Padimate O: Benzoic acid, 4-(dimethylamino)-, 2-ethylhexyl ester.

Octocrylene: 2-Propenoic acid, 2-cyano-3,3-diphenyl-, 2-ethylhexyl ester.

3.2 Solvents

All solvents and reagents were of analytical purity grade or of HPLC grade and purchased from Sigma - Aldrich. The solution of sulfuric acid 2M was prepared from 98% concentrated acid. The used solvents were methanol, acetonitrile and water. The used water that was ultrapure water, UWP, which was prepared from a SG water –Siemens system.

3.3 Sunscreen products

Four sunscreen products with the same sun protection factor (SPF), from 3 cosmetic manufacturers in Europe were analyzed in this project, the sunscreens were;

- *L'Oréal;* Solar expertise, active anti wrinkle and brown spot, Sun Cream, SPF 30, volume; 75mL Price; 89.95DKK.
- *Derma;* Baby solcreme, SPF 30, volume; 200mL, price; 48.99DKK
- *Garnier;* Ambre solaire, moisturing sun protection milk, SPF 30, volume; 200mL, price; 103.55DKK.

The *Derma* product was special formulated for application on children´s skin. The products were collected from Danish retail outlets in the period February-March 2011, excepting the old *Garnier* product, which was from the previous year.

3.4 Equipment

The HPLC-2 system place on the room B5 of the *Faculty of Engineering of the University of Southern Denmark* in Odense was used. It consisted of an *Agilent 1100 Series HPLC by Hewlett Packard* with binary pump. The instrument was equipped with a UV detector in the range 200-600nm and data acquisition was by ChemStation for LC 3D Systems software and a personal computer.

The column used for the method was a Reversed phase column of *Gemini C18 110A* (100 mm x 3 mm i.d. 3µm particle size).

3.5 Experiment planning

The laboratory work was planned carefully as the HPLC was not available all the time, because the HPLC was shared between two groups. Therefore the HPLC was only available in 3 weeks. Research has been made in order to have an idea of how the laboratory work has to be done to reach the purpose of the project. For that reason relevant articles were found that deals with this subject.

The following table shows the laboratory plan roughly:

Date:	Experiment:	Comments on the lab process:
Week 13 28/03-11	**Testing extraction methods.** Derma and L'Oreal was diluted in methanol and treated with Ultrasonic.	This extraction method did not give good results.
Week 13 29/ 03-11	**Testing standards on the HPLC.** Several solutions of the standards have been made to find roughly the same peak height as the samples.	
Week 13 30/03-11 1/04-11	**Testing extractions methods**. Testing different extractions on the sunscreens.	
Week 14 06/04-11 07/04-11	**Testing extractions methods**. Experimenting on additional extraction methods.	An extraction method was chosen – using methanol, sulfuric acid (2M) and treated

		with heat and ultrasonic.
Week 15 11/04-11	**Upgrading of the chosen extraction method** by decreasing the conc. **Standard preparation** with the new extraction method.	
Week 15 12/04-11	**HPLC method optimization.** Running the standards and L'Oreal with the new HPLC methods.	Several methods have been made to find the best method for optimization.
Week 15 13/04-11	**New Developed HPLC methods. A mixture of all standards** was made and analyzed on the new methods.	An optimized was chosen considering the best resolution and retention time.
Week 15 14/04-11	**Change of mobile phase.** The mobile phase changed from water/methanol to water/ACN	ACN gave a bad resolution therefore the mobile phase was changed back to water/methanol.
Week 15 15/04-11	**Statistics – Reproducibility.** Sample preparation was made by 2 group members separately in order to examine the reproducibility.	The samples were extracted with the final extraction method and the optimized HPLC method as well.
Week 18 02/05-11	**Method Validation using the final optimized method.** Standard curves were made.	

Week 18 03/05-11	**Statistics – A two sample test.** A test was made to compare the new and old Garnier. **Spiking – 3 factor ANOVA.** A recovery test was made on the three sunscreens	Error source: The **spiking** results showed that the UV-filter Padimate O was not present in the Derma sunscreen.

For more details see the Laboratory Journal in appendix 3a

3.6 Sample preparation

Many types of different extractions were developed to find the preferred method. Nine sample preparations were made where each of them presented a different method, see appendix 3b.

The sample preparation 9 was the final method that had been chosen, and is described below.

Sample preparation 9

As we still had a high concentration, new samples of sunscreens were prepared with a less amount of sunscreen than earlier.

Around 150 mg of sunscreen were diluted in 12,5mL of methanol and 0,062mL of 2M H_2SO_4. The samples were heated for 60min and treated with ultrasonic for 60min.

3.7 HPLC optimization during laboratory

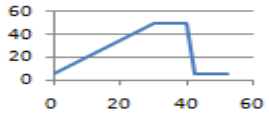
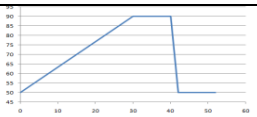
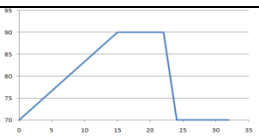
The method development is based on the method from the scientific [A3, p. 48], where they used a 5 µm Symmetry Shield C18 column, 150 mm length, 4.6 mm i.d. The column temperature was 40 ^{o}C and had a flow rate of 1.2 mL/min. The detectors wavelengths were 312 nm and 358 nm. The mobile phase was 85 % ethanol and 15 % water.

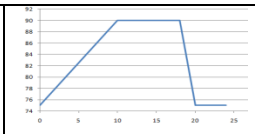
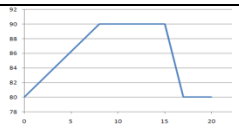
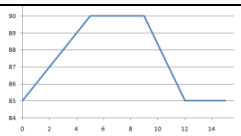
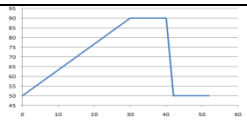
For all the methods that have been developed, during this project, the samples have been analyzed on the HPLC machine, with the column 3 µm Garmini C18, 100 mm length, 3.00 mm i.d..The flow rate, column temperature, gradient time, gradient range, solvent strength and type are changed during the method optimization. Below there is tables over the different methods. All the methods below are with a mobile phase of water and methanol if nothing else is mentioned. There are also illustrations of the gradient time and range in the tables for %B, where % B is the amount of methanol in percentage.

Isocratic A

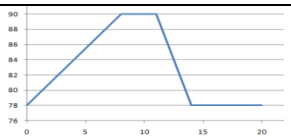
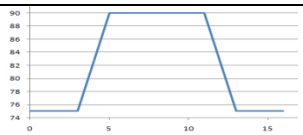
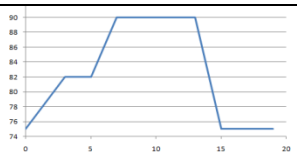
Flow rate (mL/min)	Temperature (^{O}C)	Wavelength (nm)	% B	Time (min)
0.3	40	312	85	45

Gradient

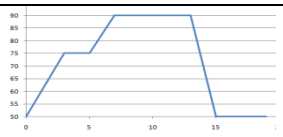
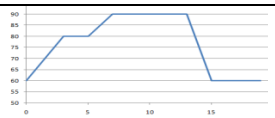
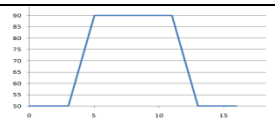
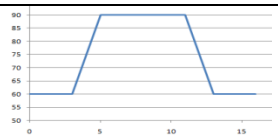
Method	Flow rate (mL/min)	Temp. (^{O}C)	Wavelength (nm)	% B min/max	Gradient range and time	Time (min)
Gradient A	0.3	40	312, 350	5/50		52
Gradient B	0.3	40	312, 350	50/90		52
Gradient C	0.2	40	312, 350	70/90		32

Gradient D	0.2	40	312, 350	75/90		24
Gradient E	0.2	40	312, 350	80/90		20
Gradient F	0.2	40	312, 350	85/90		17
Gradient G	0.3	40	312, 350	70/90		32

Methods gradient H, I, J and K is with same values as gradient C except from the temperature it is respectively 45, 50, 60 and 30^{O}C in the different methods.

Method	Flow rate (mL/min)	Temp. (^{O}C)	Wavelength (nm)	% B min/max	Gradient range and time	Time (min)
Gradient D2	0.3	45	312, 350	50/90		20
Gradient D3	0.3	45	312, 350	50/90		16
Gradient D4	0.3	4	312, 350	50/90		19

In the methods below the mobile phase is water and acetonitrile now % B is indicating the percentage of acetonitrile.

Method	Flow rate (mL/min)	Temp. (^{O}C)	Wavelength (nm)	% B min/max	Gradient range and time	Time (min)
Gradient ACN1	0.3	45	312, 350	50/90		19
Gradient ACN2	0.3	45	312, 350	60/90		19
Gradient ACN3	0.3	45	312, 350	50/90		16
Gradient ACN4	0.3	45	312, 350	60/90		16

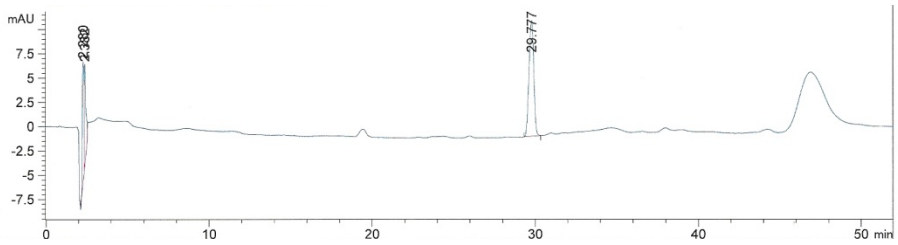
All the chromatograms that will be show in the report will be the chromatogram with the wavelength at 312 nm if nothing else is noted.

4.0 Results

4.1 Method optimization

The purpose of the method optimization was to develop a method with a short retention time and still good separation of the standards. During the method optimization there have been made a lot of chromatograms and methods. In the treatment of the results there have only been focusing in some of the chromatograms and methods, all the details about the methods can be found under HPLC *optimization during laboratory*. All the chromatograms below is from a *L'Oréal* sample, which active UV-filter is *Octocrylene*, where nothing else is mentioned.

Below is seen the chromatograms from the method that have been developed based on the article.

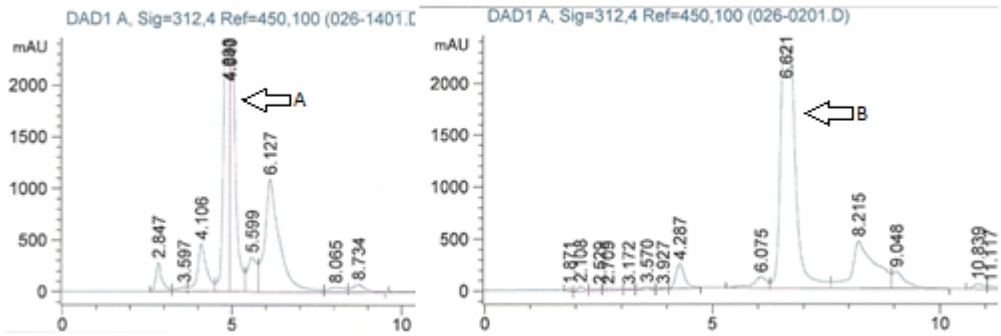


Figur 22, Method isocratic

It is a chromatogram run with the isocratic method, the intensity of the peak is very low and the length of the method is very long.

4.1.1 Different gradient slope

Beneath there is two different chromatograms the one to the right is with method gradient D and the one to the left is with gradient F.

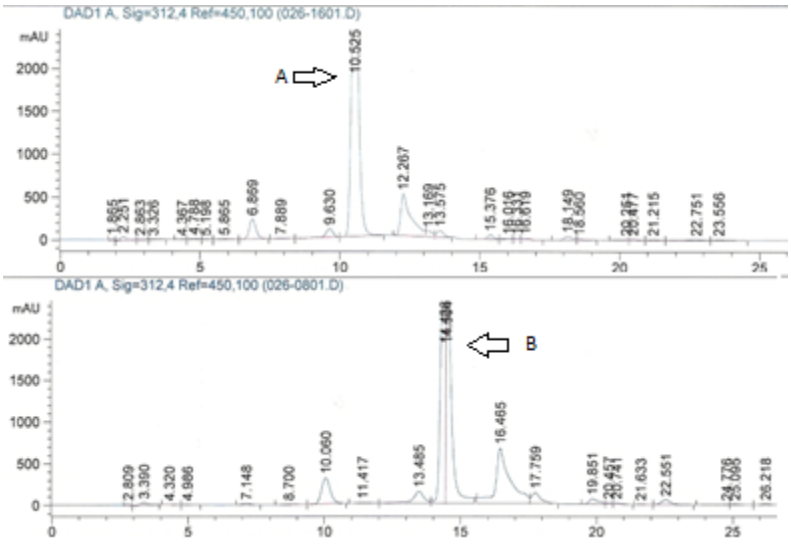


Figur 23, Method gradient F and gradient D

Both method gives short retention time but in the chromatogram with method gradient F there is interference with some of the small impurities. However, there is still interference with impurities in the chromatogram made with method gradient D, but not compared to the other.

4.1.2 Flow rate

Below, there are two chromatograms with different flow rate, the first chromatogram is with method gradient G and the second is with gradient C. The flow rate for the first chromatogram is 0.3 mL/min and the second it is reduced to 0.2 mL/min.

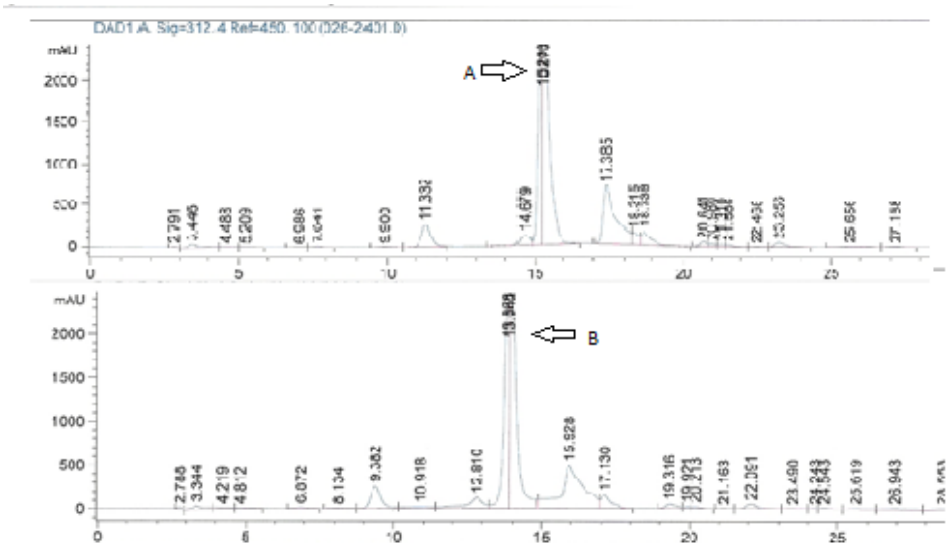


Figur 24, Method gradient G and gradient C

It is easy to see that the change in flow rate results in a much smaller retention time the time is reduced with 4 min. only by changing the flow rate comparing peak A with B.

4.1.3 Temperature

Underneath there is chromatograms with two different temperatures the first chromatogram is with method gradient J with temperature 30°C, the second is with gradient H with temperature 45°C. The only difference between the two methods is the temperature.

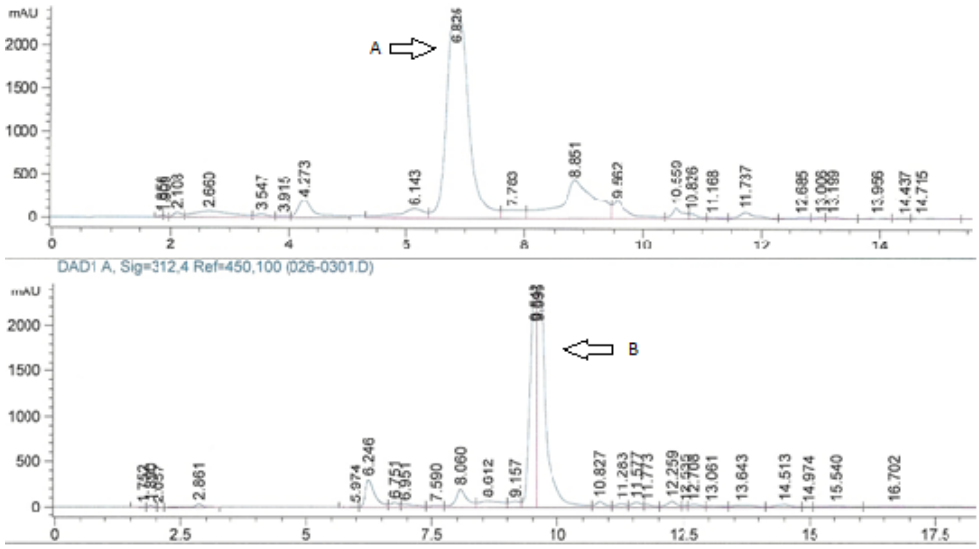


Figur 25, Method gradient J and gradient H

As the temperature is increasing the retention time for the *Octocrylene* peak get smaller and by comparing A with B the retention time is reduced with more than 1 min.

4.1.4 Mobile phase

On the chromatograms below there are two chromatograms with different mobile phase, the upper one is with methanol and water and the other is with acetonitrile and water. The method with methanol is gradient D3 and the method with acetonitrile is gradient ACN1.

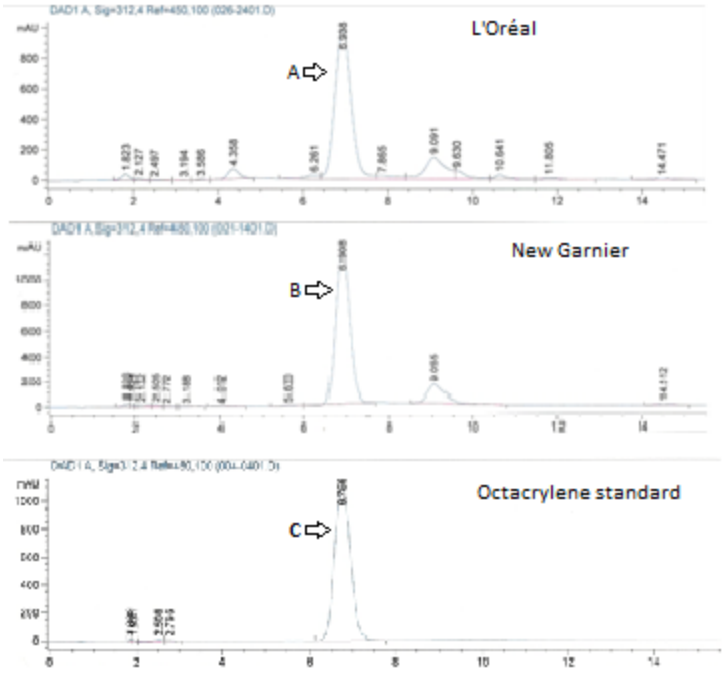


Figur 26, Method gradient D3 and gradient ACN1

On the following blank sample for gradient ACN1 appears two big ghost peaks. The retention time for gradient ACN1 had also been more than 3 min. longer compared B with A.

4.1.5 Plate numbers

Below the chromatogram for *L'Oréal*, *New Garnier* and *Octocrylene* standard with method Gradient D3 is show in the table below the retention time is noted and the plate number is calculated on the equation from the section *Theory HPLC.*



Figur 27, Method gradient D3, L'Oréal, New Garnier and Octocrylene

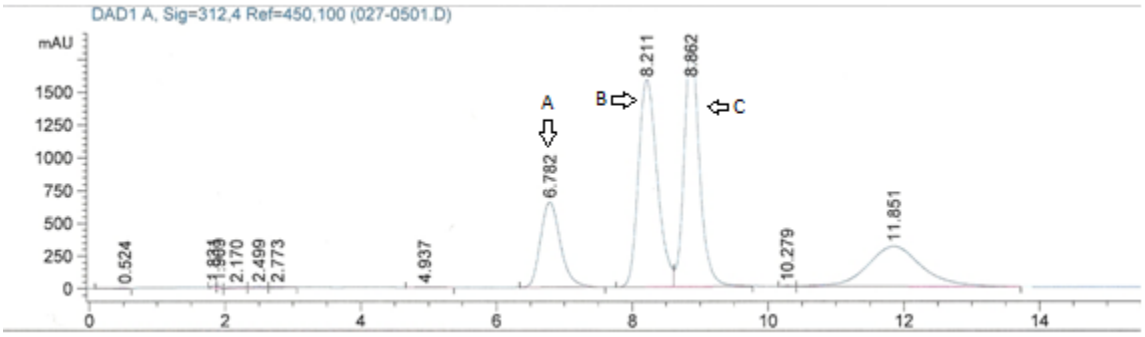
Top nr	Retention time, t_R (min)	Plate number, N
A	6.938	1240,68
B	6.908	1257,43
C	6.756	1088,23

Tabel 3, Retention time and plate number

The plate number or efficiency has been calculated for a *L'Oréal* and a *New Garnier* chromatogram that have been performed regarding the reproducibility experiment. Also a standard chromatogram for *Octocrylene*, which have been performed two weeks later. The calculated plate number for A and B are almost equal. The plate number calculated for C is smaller than A and B, which have to do with the shorter retention time for C. The efficiency is therefore unchanged during the analysis.

4.1.6 Peak separation

To make sure that the standards are nicely separated there have been calculated selectivity and resolution for this. Below a chromatogram is showing for all the three standards with method Gradient D3.



Figur 28, Method gradient D3 standards

Top no.	Retention time, t_R (min)	Between no.	Selectivity, α	Resolution, R_S
A	6.782	A-B	1.29	2.33
B	8.211	B-C	1.10	1.18
C	8.862			

Tabel 4, Separation

For the calculation see appendix 2a.

The resolution and selectivity are good for the three tops, it is wanted that the selectivity is in the range of $2 > \alpha > 1.05$ and both values are in this area. For the resolution it is wanted for ideally analytical that the R_S is in the range 1.5-2.0, the resolution between A and B is above and the resolution between B and C is below. Both values are above 1, which is not ideally analytical but indicates a baseline separation. There was not spend more time on the analytical correct separation method, because the samples only contain one compound and therefore there will be no separation.

4.2 3-factor ANOVA

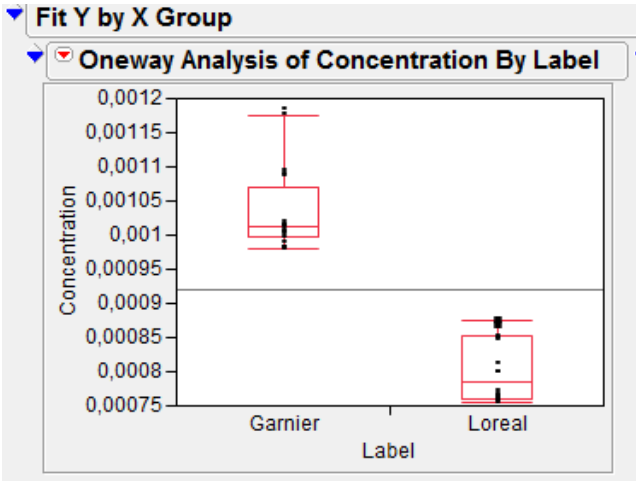
It is wanted to determine the amount of UV-filters in three different sunscreens; L'Oréal, Garnier and Derma, all with a SPF 30. All the sunscreens have been treated with extraction method 9. There are two persons, test person 1 and test person 2 who each have preparing one extraction of each sunscreen and from each extraction there was made two samples that had been run with 3 injections on the HPLC. All of these samples have been running in two different days with 14 days in between and are respectively called time 1 and time 2. The results are depending on the following factors; labels, persons, samples, injections (HPLC) and the time. There are four factors in total but it have been chosen to exclude the injection factor, because it is assumed that the HPLC is a very precise analytical instrument. A selected part of the chromatograms can be seen in appendix 1d and the calculated concentrations can be seen in appendix 2b.

By using a 3 factor ANOVA, three factors have been used to this purpose where the factors are as following: Person, time and label. It is tested if there is a significant different on the measurement of the persons, the time and also if there is a significant different in the amount of UV-filter in the different sunscreens. There have also been made a 95% confidence interval where the true mean value should be.

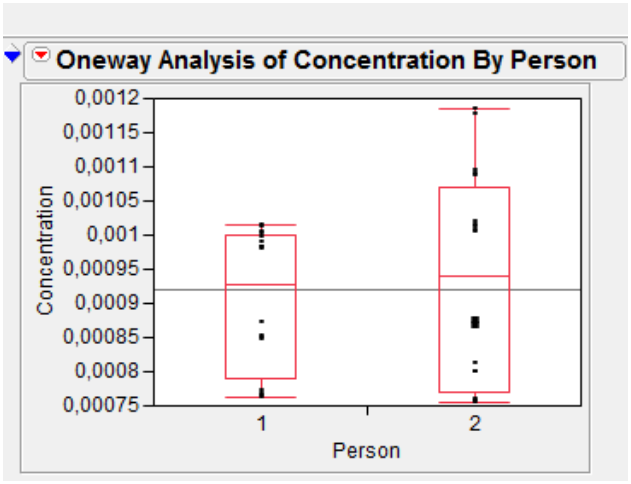
Before making an analysis of variance, a box plot has been made to investigate if there are any present of potential outliers in the data. A normal quantile plot has been made as well for checking the normal distribution. It is assumed that the data are independent of each other. Variance homogeneity is being investigated by plotting the residuals.

4.2.1 Outliers

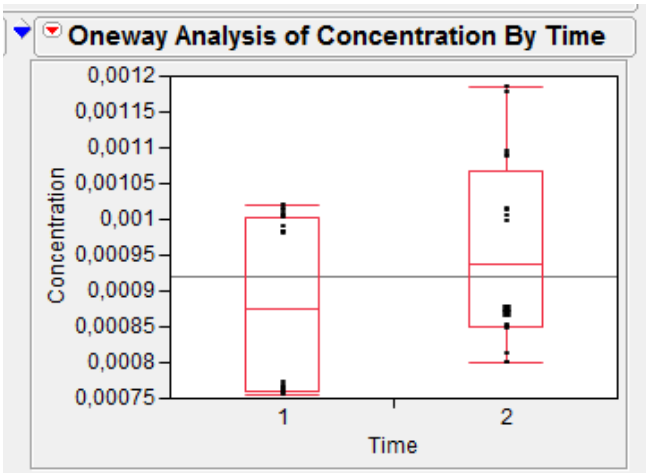
The three figures below shows box plots of respectively concentration vs. label, concentration vs. person and concentration vs. time.



Figur 30; shows a box plot of oncentration vs. label



Figur 29 Shows a box plot of concentration vs. person



Figur 31; shows a box plot of concentration vs. time

The box plot in figure 30 shows that there is two potential outlier but because the outliers lies pretty close to the rest of the data it is not assumed to be an error in the dataset and it has been decided to be kept. In figure 30 the box plot for Garnier stretches longer on top than the bottom and the points lies more concentrated in the bottom of the box plot.

In figure 29 there are no potential outliers but the measurements of test person 2 have a greater spread than the measurements made of test person 1.

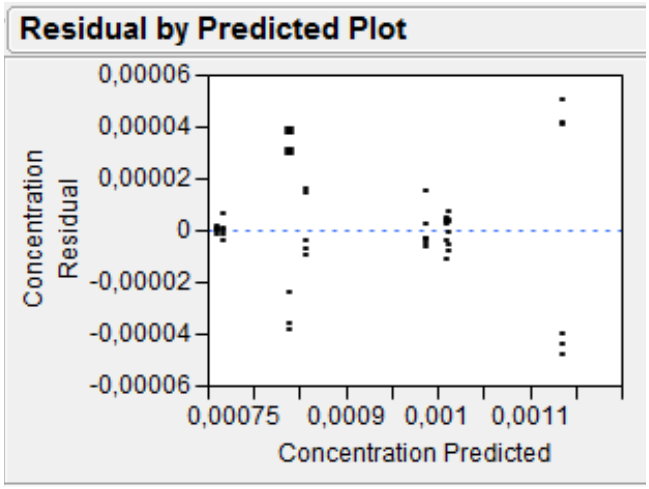
The box plot in figure 31 shows no outliers. However it can clearly be seen that the box plot for time 2 has a greater spread between the measurements than the box plot for time 1.

4.2.2 Normal distribution

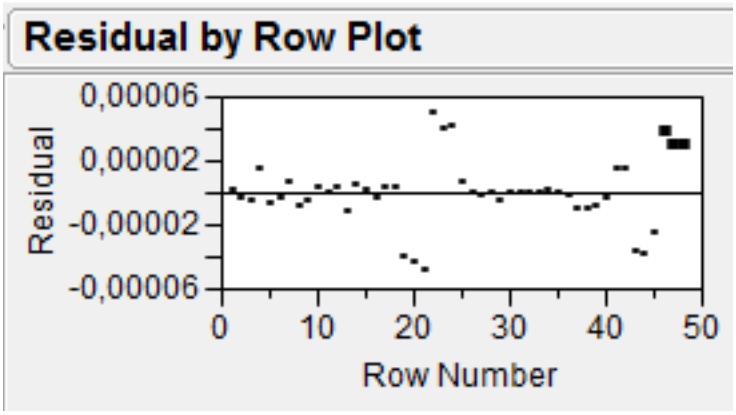
The data is assumed to be normal distributed. It has not been possible to investigate this because of the many factors and the few data for each of the factors.

4.2.3 Variance homogeneity

The variance homogeneity is investigated by plotting the residuals in SAS JMP version 8. The figure 32 and 33 below shows two different plots of residuals. A residual is equal to the difference between the measured and predicted.



Figur 32; shows the residual vs. predicted concentration



Figur 33; shows the residual vs. row number

The residual plots show that the measurements fluctuate randomly around zero. However there are a couple of points that varies and lies pretty high or low. The points that lies higher than the norm is respectively; test person 2 – Garnier – time 2 and test person 2 – Loreal – time 2. It is the same two measurements but from another HPLC sample that lies as the lowest points. The points are randomly spread and there is no tendency to be seen, so that is possible to conclude that there is variance homogeneity.

4.2.4 3-factor ANOVA

Before making the ANOVA the following hypothesis has been made;

1. $H_0; \tau_G = \tau_L = 0 \ (no\ main\ effect\ of\ the\ label\ factor)$
   $H_1; \tau_G \neq \tau_L \neq 0$
2. $H_0; \beta_1 = \beta_2 = 0 \ (no\ main\ effect\ of\ the\ person\ factor)$
   $H_1; \beta_1 \neq \beta_2 \neq 0$
3. $H_0; \gamma_1 = \gamma_2 = 0 \ (no\ main\ effect\ of\ the\ time\ factor)$
   $H_1; \gamma_1 \neq \gamma_2 \neq 0$
4. $H_0; (\tau\beta)_{G1} = (\tau\beta)_{G2} = (\tau\beta)_{L1} = (\tau\beta)_{L2} = 0$
   $(no\ \text{int}eraction\ between\ the\ label\ factor\ and\ the\ person\ factor)$
   $H_1;\ At\ least\ one\ (\tau\beta)_{ij} \neq 0$
5. $H_0; (\tau\gamma)_{G1} = (\tau\gamma)_{G2} = (\tau\gamma)_{L1} = (\tau\gamma)_{L2} = 0$
   $(no\ \text{int}eraction\ between\ the\ label\ factor\ and\ the\ time\ factor)$
   $H_1; At\ least\ one\ (\tau\gamma)_{ik} \neq 0$
6. $H_0; (\beta\gamma)_{11} = (\beta\gamma)_{12} = (\beta\gamma)_{22} = (\beta\gamma)_{21} = 0$
   $(no\ \text{int}eraction\ between\ the\ person\ factor\ and\ the\ time\ factor)$
   $H_1;\ At\ least\ one\ (\beta\gamma)_{jk} \neq 0$
7. $H_0; (\tau\beta\gamma)_{G11} = (\tau\beta\gamma)_{G12} = (\tau\beta\gamma)_{G12} = (\tau\beta\gamma)_{G22} = (\tau\beta\gamma)_{L11} = (\tau\beta\gamma)_{L12} = (\tau\beta\gamma)_{L12} = (\tau\beta\gamma)_{L22} = 0$
   $(no\ \text{int}eraction\ between\ the\ three\ \ factors)$
   $H_1;\ At\ least\ one\ (\tau\beta\gamma)_{ijk} \neq 0$

The 3-factor ANOVA has been made in SAS JMP version 8. The data has been plotted in the following way; Concentration is the response while the factors are; label, person and time factors has been crossed as follows; label*time, label*person, time*person and label*person*time.

The first model made of the 3-factor ANOVA showed a high p-value of 0.34 for the interactions between label and time. Because of the high p-value there is no significance between the two factors and therefore this none existing interaction has been exclude and calculated as a random error instead.

The two tables below show the result of the analysis of variance and the effect of the test.

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	6	7,66876e-7	1,2781e-7	256,7046
Error	41	2,04138e-8	4,979e-10	Prob > F
C. Total	47	7,8729e-7		<,0001*

Tabel 5; Shows the results from the analysis of variance

Effect Tests

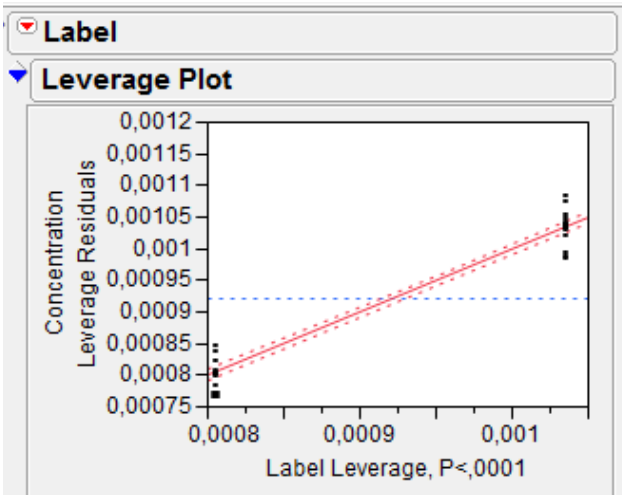
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Label	1	1	6,41489e-7	1288,395	<,0001*
Person	1	1	1,15674e-8	23,2324	<,0001*
Time	1	1	7,44496e-8	149,5279	<,0001*
Label*Person	1	1	2,40483e-8	48,2996	<,0001*
Person*Time	1	1	5,9728e-9	11,9960	0,0013*
Label*Person*Time	1	1	9,34912e-9	18,7772	<,0001*

Tabel 6; Shows the effect on the concentration from the factors and the interactions between the factors

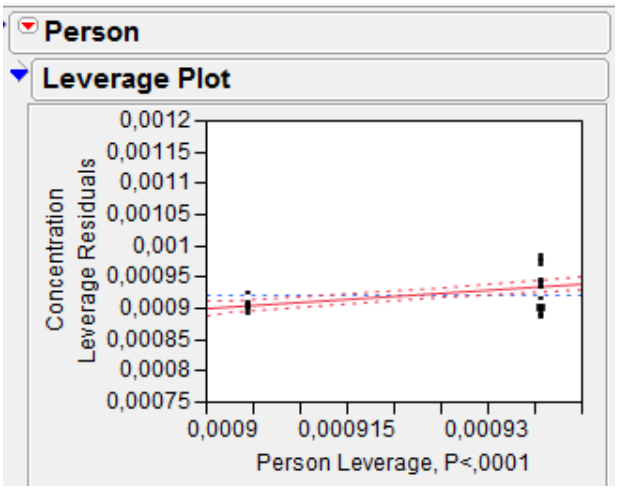
In table 6 it can be seen that the label does have the greatest effect on the concentration because it have a F ratio of 1288.395 and that the time has a pretty high effect on the concentration as well with a F ratio of 149.5279. However, the effect of persons only has an F ratio of 23.2324, which means that the effect on the concentration is smaller compared to the two other factors.
The interactions between the different factors do not have as great effect on the concentration as the factor; label and time has.

The p-value for the three factors and the interactions between label*person and label*person*time is below 0.0001, while the interaction between person*time is 0.0013 and therefore all the factors and the mentioned interactions have a significance level of ***.

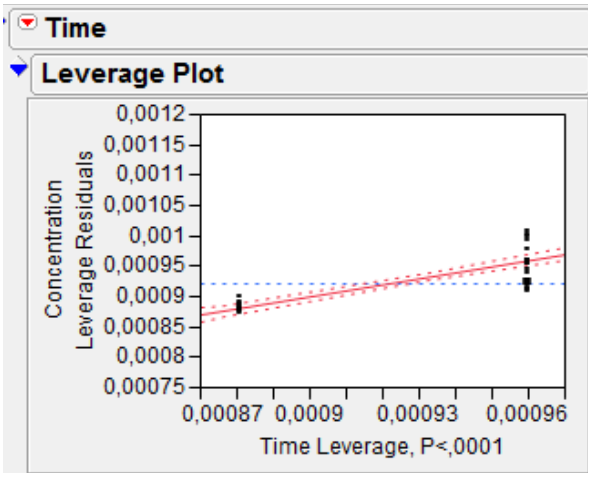
To illustrate the effect on the concentration from the different factors see the three figures below;



Figur 35; Shows the effect of label on the concentration



Figur 34, Shows the effect of person on the concentration



Figur 36; shows the effect of time on the concentration

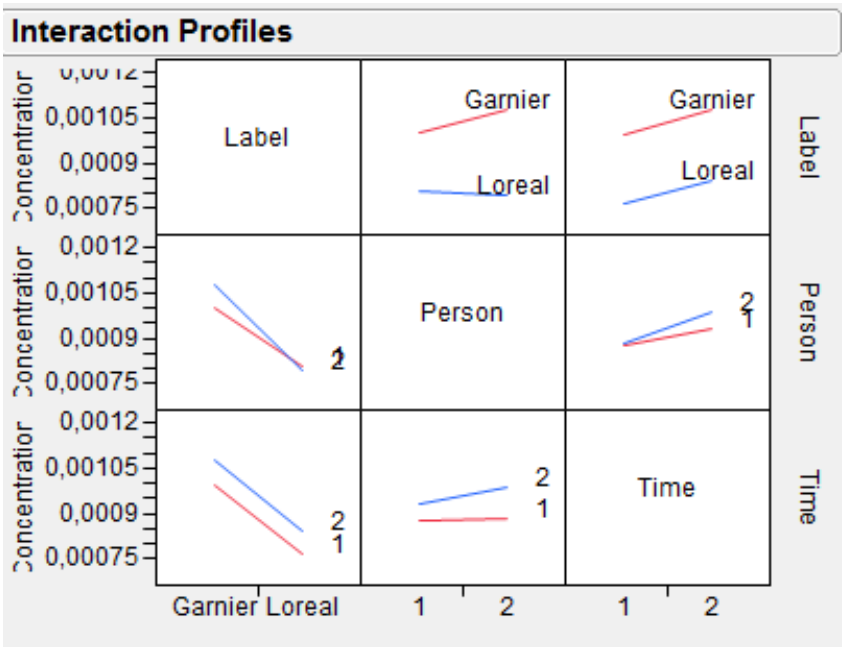
The figures illustrate clearly that the label as mentioned earlier has the greatest effect on the concentration compared with the two other factors. Figure 34 shows that the person has the smallest effect on the concentrations.

The table 7 compares the mean concentrations for each factor and level.

Level	Mean
Label; Garnier	10.36*10^-4
Label; Loreal	8.04*10^-4
Person; 1	9.04*10^-4
Person; 2	9.36*10^-4
Time; 1	8.81*10^-4
Time; 2	9.59*10^-4

Tabel 7; Shows the mean of the concentration depended on the different factors and levels

Figure 37 below shows a plot of the different interactions between the factors. On the interaction plot it can be seen that there is no interaction between the ones where the slops are parallel while the ones that are crossing have interactions with each other. For example there are no interactions between the two labels and time while there is interaction between the persons and labels



Figur 37; illustrate the interactions between factors

Because of the interactions between the different factors the three null hypothesis describing the interaction between factor; label*person, person*time and label*person*time can be rejected, while rejecting the null hypothesis describing the interaction between label*time have failed.

The 3-factor analysis of variance shows that all the three factors; label, person and time has a main effect on the concentration and therefore it is possible to reject the three null hypothesis describing these factors.

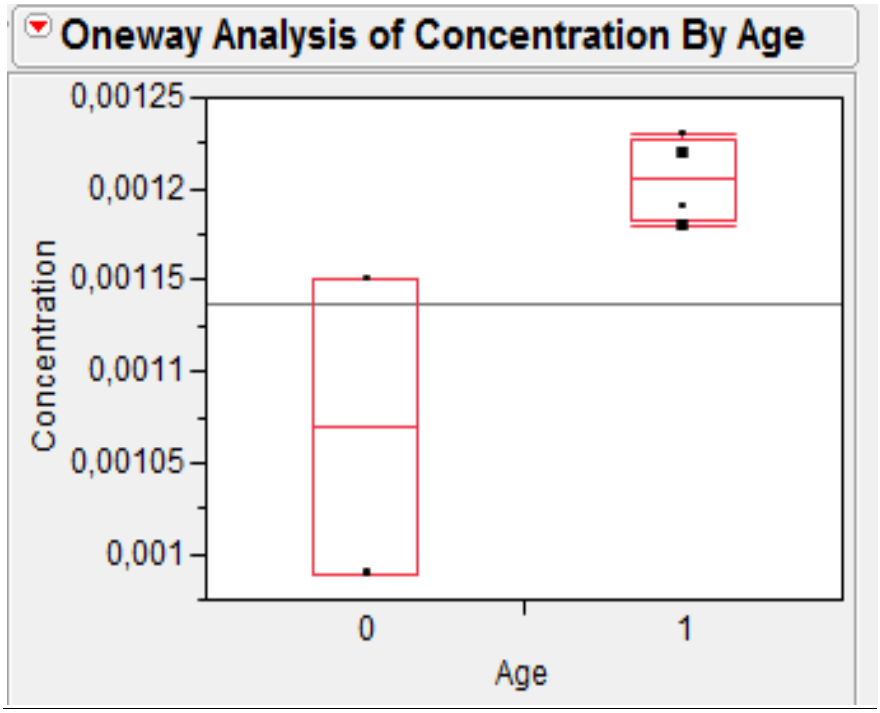
4.3 Two sample t-test

A two sample t-test was made to examine the difference of the means for the old - and new Garnier with an unknown variance. The purpose of this test is to compare the concentrations of the UV-filter in the two sunscreens and investigate if time has an influence.

Before making the actual t-test a box-plot is made to investigate if there are any outliers present. A normal quantile plot is made as well to see if the data is normal distributed. Furthermore, Bartlett´s test is used to check if there is variance homogeneity.

4.3.1 Outliers

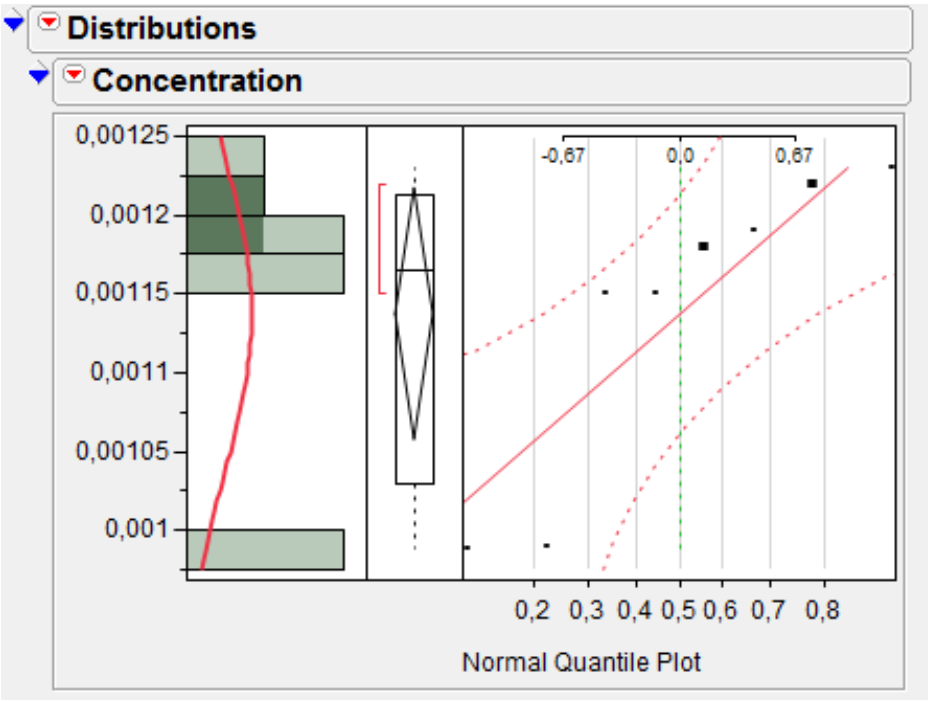
A box plot was made to check the presence of potential outliers. Outliers are observations that can indicate an error, bad data or some random variance and usually appears differently from the rest of the observations. This plot depicts the concentration versus the age of the two sunscreens – The old and new Garnier, age 1 and 0. The figure 38 below shows no presence of outliers as all the points on the plot is within a box plot.



Figur 38; Shows no outliers

4.3.2 Normal distribution

The normal distribution was investigated by making a normal quantile plot as seen in the figure39 below:

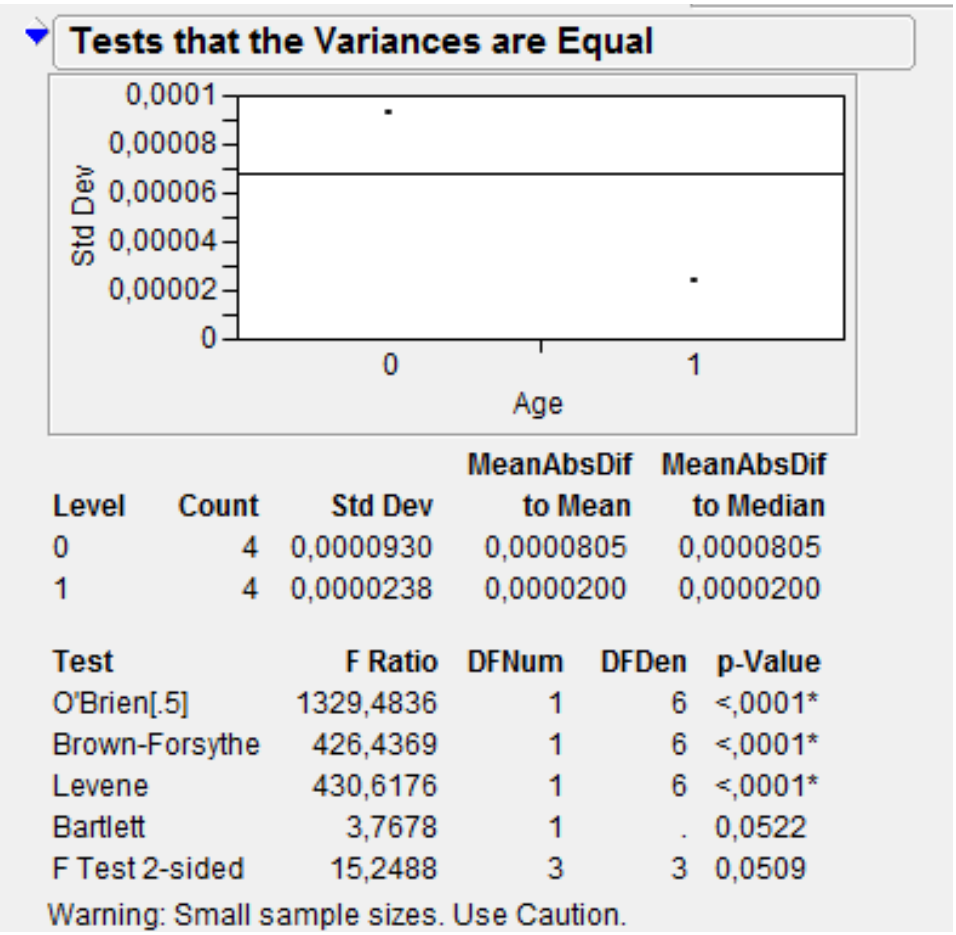


Figur 39; shows a normal quantile plot made on the concentration of the new and old sunscreen

The figure 39 shows a tendency of normal distribution made on the 8 concentrations of the new and old Garnier. A conclusion of normal distribution can be made. However, the conclusion would be stronger if there were more measurements.

4.3.3 Variance homogeneity

Bartless test have been used for investigating if there is variance homogeneity. See figure 40 below



Level	Count	Std Dev	MeanAbsDif to Mean	MeanAbsDif to Median
0	4	0,0000930	0,0000805	0,0000805
1	4	0,0000238	0,0000200	0,0000200

Test	F Ratio	DFNum	DFDen	p-Value
O'Brien[.5]	1329,4836	1	6	<,0001*
Brown-Forsythe	426,4369	1	6	<,0001*
Levene	430,6176	1	6	<,0001*
Bartlett	3,7678	1	.	0,0522
F Test 2-sided	15,2488	3	3	0,0509

Warning: Small sample sizes. Use Caution.

Figur 40; shows the results for investigating homogeneity by using Bartlett's test

The Bartlett test shows that the variances statistical have variance homogeneity. However, the p-value is 0.0522 and lies on the limit to be significance because of this it is hard to conclude that the data actually is homogeny. Because of the low p-value there have been made both a two sampled t-test for equal and unequal variance, the difference between the two tests is minimum and therefore it has been chosen to calculate the t-test for unequal variance.

4.3.4 Two sample t-test

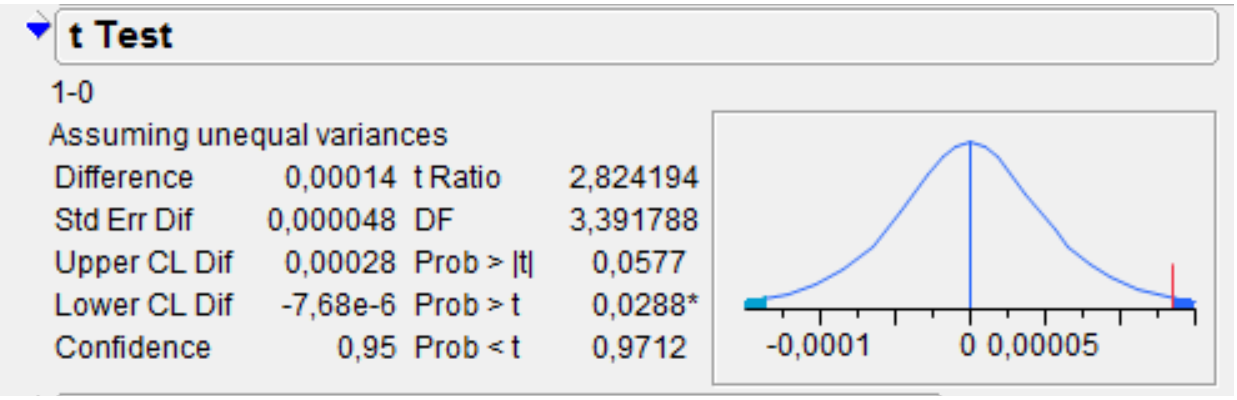
As shown above the presence of outliers were excluded and the data showed that the measurements were normal distributed. The two sample t-test were calculated for unequal variance because of the low p-value is on the limit to be significance. After confirming these conditions a two sample t-test can be made. The test examines the mean differences between two sunscreens – A one-year-old Garnier versus a new

Garnier. 4 samples have been measured and the concentrations were calculated on each sunscreen making it 8 measurements in total. The areas and concentration can be found in appendix 3c.

A hypothesis test was made where the Null hypothesis and the alternative hypothesis was tested subsequently tested. The μ_N represents the **N**ew Garnier and the μ_O represents the **O**ld Garnier.

Hypothesis test: $H_0 = \mu_N - \mu_O = 0$ *and* $H_1 = \mu_N - \mu_O \neq 0$

The calculations for the two sample t-test were made in the Statistical program SAS JMP version 8. The calculated values are shown in the figure below:



Figur 41; Shows the two sample t-test for the new and old *Garnier*

The two sample t-test for comparing the new and old Garnier shows for unequal variance that statistical seen there is no significance between the two samples. However, the p-value is only 0.0577 and therefore lies on the limit for being significant. That means that the null hypothesis is fail to reject. However the difference between the samples is 0.00014 which is a value that is too high to conclude that the two samples are similar although there is no significance.

4.4 Method validation

The project considers an optimization of detecting UV-filters in sunscreens and therefore it is important to validate on the developed method.

The method that is validated considers the determination of the UV-filter Octocrylene that is in the sunscreens from *Garnier* and *L'Oréal*. The method is used for controlling whether the content of UV filters comply with the European legislation and if there is any different between old and new sunscreens.

4.4.1 Specificity

The specificity has been investigated to see if there is any interference from other compounds in the sample that interferes in relation to rediscover the specified UV filter. To investigate the recovery some samples have been spiked with a known amount of the *Octocrylene*. The experiments have been listed in the following way;

Garnier	Injection	*Loreal*	Injection
standard 0,882*10^-3 g/ml	1	Standard 0,54*10^-3g/ml	1
***Garnier* without spiking**	1	*Loreal* without spiking	1
***Garnier* with spiking 1**	3	*Loreal* with spiking 1	3
***Garnier* with spiking 2**	3	*Loreal* with spiking 2	3

Tabel 8; shows how the experiments has been setup

The spiking of the samples gave the following results;

Garnier	Area 1	Area 2	Area 3	Average area	Expected peak
standard 0,882*10^-3 g/ml	31053,7				
Garnier without spiking	33524,3				
Garnier with spiking 1	63285,8	63167,5	63699,9	63384,4	
Garnier with spiking 2	60071,7	59753,6	59703,9	59843,1	64578
Garnier with spiking 1 in %	98,0	97,8	98,6	98,2	Expected peak %
Garnier with spiking 2 in %	93,0	92,5	92,5	92,7	100

Tabel 9; shows areas and expected peaks for Garnier

Loreal	Area 1	Area 2	Area 3	Average area	Expected peak
Standard 0,54*10^-3g/ml	19413,8				
Loreal without spiking	23990,2				
Loreal with spiking 1	43694	43872,9	43845,2	43804,0	
Loreal with spiking 2	42925,5	42701	42777,6	42801,4	43404
Loreal with spiking 1 in %	100,7	101,1	101,0	100,9	Expected peak %
Loreal with spiking 2 in %	98,9	98,4	98,6	98,6	100

Tabel 10; Shows the areas and expected peaks for Loreal

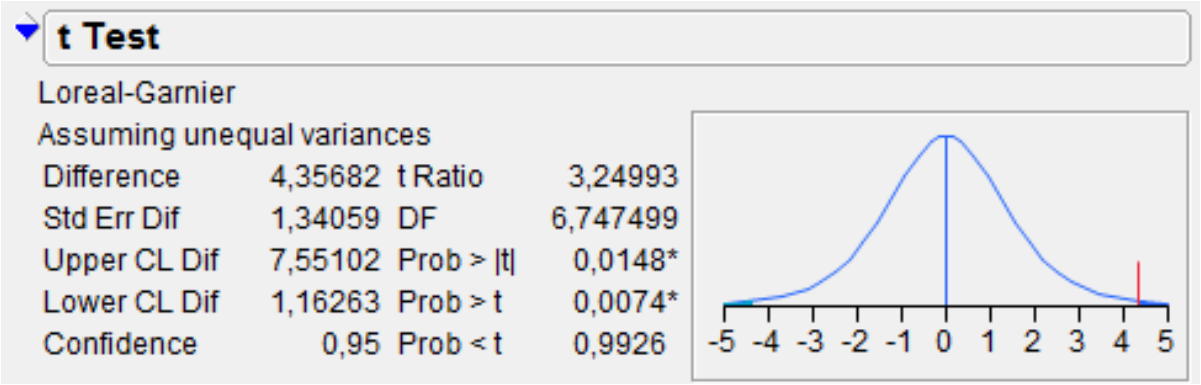
The table 9 shows that the retrieval in percentage for the *Garnier* ranging from 92.45% to 98.16% which gives an average retrieval of 95.41% for all the 6 injections.

The table 10 shows retrieval in percentage for the *L'Oréal* in a range from 98.38% as the lowest to 101.08% as the highest retrieval. The average retrieval for the *L'Oréal* is calculated to be 99.77% for all the 6 injections.

The two dataset has been compared by using a two sample t-test. In appendix 2c the investigation of normal distribution, outliers and variance homogeneity has been made. It can be concluded that the data has no outliers and are normal distributed, while there is no variance homogeneity, and the t-test has therefore been made for unequal variance.

A hypothesis test was made where the Null hypothesis and the alternative hypothesis was tested subsequently tested. The μ_G represents the Garnier and the μ_L represents the Loreal.

Hypothesis test: $H_0 : \mu_G - \mu_L = 0$ *and* $H_1 : \mu_G - \mu_L \neq 0$



Figur 42; shows the t-test for unequal variance

The t-test shows that there is significance different between the retrieval of the compound for each of the two sunscreens. Because of the significance different the null hypothesis is rejected.

The method is specific, because it has been possible to detect the UV-filter in the two sunscreens. However, as the t-test showed, there is significance different between determined the compound in the two sunscreens.

4.4.2 Sensitivity

The sensitivity has been calculated on the same time as the standard curve. The slope from the standard curve describes the sensitivity of the method.

The sensitivity of the method based on Octocrylene is $3,0 \cdot 10^7 \frac{unit\,area}{g/ml}$.

4.4.3 Accuracy/Bias

The methods accuracy has been investigated by spiking the samples for *Garnier* and *L'Oréal*. For explanation of how the experiment has been done see under *specificity* in the *method validation*. The retrieval experiment gave the following results;

Samples	**Injection 1**	**Injection 2**	**Injection 3**	**Average**
Garnier with spiking 1 in %	98,0	97,8	98,6	98,2
Garnier with spiking 2 in %	93,0	92,5	92,5	92,7
Loreal with spiking 1 in %	100,7	101,1	101,0	100,9
Loreal with spiking 2 in %	98,9	98,48	98,6	98,6

Tabel 11; results from the recovery test

The accuracy can be calculated in bias. In this case the accuracy is determined by calculating the relative bias based on the areas under the peaks. The bias is only calculated for the average of the 4 samples. The true value is the sum of the area under the peak for samples without spiking and the area under the peak for a standard peak with the same concentration as the spiked sample. See appendix 2d for the calculations

The result is as follows;

Sample	Relative bias (%)
Loreal with spiking 1	0.92
Loreal with spiking 2	1.4
Garnier with spiking 1	1.8
Garnier with spiking 2	7.3

Tabel 12; showing the relative bias

The relative bias cannot be above 5% if the method has to be accurate because the confidence interval is not accepted if it is below 95%. The three first samples in table 12 have a pretty low relative bias and can be accepted. However, the last Garnier has a high value of 7.3%, which is 2.3% more than the accepted value of 5%. The high value will in this case be ignored and the method is considered to be accurate.

4.4.4 Linearity and range

The linearity of a method defines its ability to achieve proportional results to the concentration of analyte. The linear range defines the analyte concentration in which the method gives linear related results to the concentration.

At the lowest range of concentrations, the limiting factors are the values of *limit of detection* and the *limit of quantification*. At the highest, the limitations depend on the instrument response. Although it does not affect this project because the samples is not close to the limit concentrations.

In our case, a "stock solution" with a concentration of $9*10^{-4}$ mg/l was made. Then 75, 50, 25 and 20% dilutions were made from the stock solution. Since the concentration of the sunscreens lied on the upper end of the standard curve, new solutions with higher concentrations were made. The working range lies in the range of the linearity.

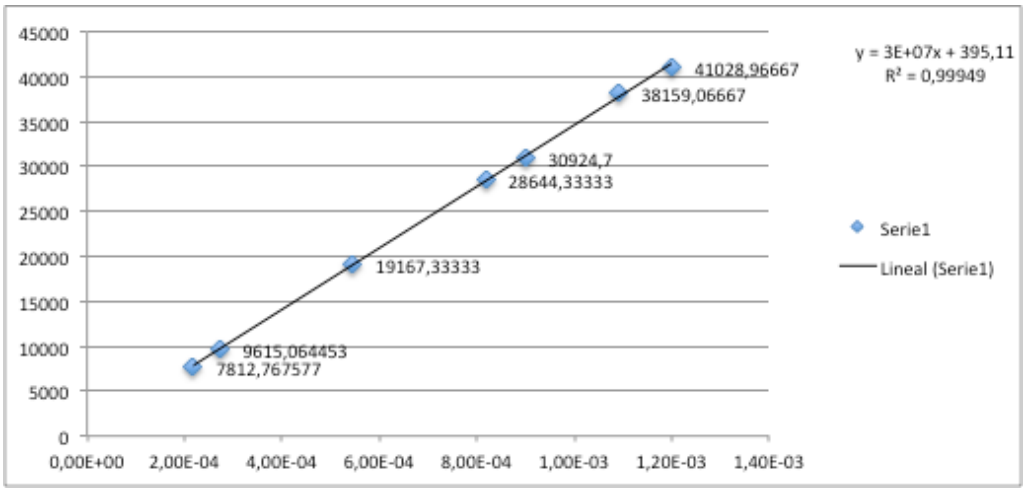
With the data from the concentrations and the areas, the standard derivation from the points, the slope and the intercept are calculated as shown in table 13, 14 and figure 43. So that, it is possible to argue the linearity of the curve:

Calibration standards				
X	**Y**			
(Conc.)	(Area)	X^2	Y^2	XY
0,000218	7812,8	4,8E-08	61039337,3	1,7
0,000273	9615,1	7,5E-08	92449464,4	2,6
0,000546	19167,3	3,0E-07	367386665,8	10,5
0,000818	28644,3	6,7E-07	820497830,2	23,4
0,0009	30924,7	0,00000081	956337070,1	27,8
0,00109	38159,1	1,9E-06	1456114371	41,6
0,0012	41029,0	0,00000144	1683376108	49,3
0,0050450	175352,2	0,000004527393	5437200847,6	156,9

Tabel 13; Shows the concentration and relted areas, its accumulated and the square

Calibration Inform				
Number of samples	7			
Sxx	8,9E-07			
Syy	1,0E+09			
Sxy	3,1E+01			
Slope	3,4E+07			
Intercept	3,9E+02			
Sy	3,4E+02			
s(slope)	3,6+05			
s(intercept)	2,9E+02	Superior Limit	Inferior Limit	Probabillity
t(95%)*s(slope)	9,1E+05	3,5E+07	3,3E+07	2,3E-09
t(95%)*s(intercept)	7,4E+02	1,1E+03	-3,5E+02	2,4E-01
R	9,997E-01			
r2	0,99946			

Tabel 14; Shows the correlation factor calculated manually as well as the confidence errors and the confidence intervals of the slope and the intercept



Figur 43; Callibration curve with the excel formula and correlation factor

For further information about calculation see appendix 2e.

As the correlation factor is very close to one and the confidence errors and confidence intervals of the slope and the intercept are almost irrelevant with a 95% confidence, it is possible to conclude that the calibration curve is linear in the studied range. [Montgomery, p. 420]

4.4.5 Limit of detection and limit of quantification

In this method there will be no point to determine the limit of detection and limit of quantification, because none of the samples contain so low concentration of the UV-filters that it is approaching the limit of quantification and therefore it is pointless to detect the limit of detection. The small peak that contains in some of the chromatograms is irrelevant for this project and has therefore been skipped.

4.4.6 Precision

The precision was examined through reproducibility testing. The reproducibility test was investigated by 2 person's influence on the 3 different sunscreens and a 3 factor ANOVA test was made. The 3 factor ANOVA test is thoroughly reviewed in the section: "A 3 factor ANOVA test". Based on the 3 factor ANOVA it was concluded that the factors that had most influence on the method were the label and time since they had the biggest effect on the concentration. The persons on the other hand had the smallest effect on the concentrations compared to the other factors. However, the differences of the persons play a huge role in the reproducibility test and can clearly be seen in the 3 factor ANOVA where person 1 and person 2 got varied results compared to each other.

The precision can be calculated by calculating the standard deviation where the overall mean of response is divided by the mean square error as the following equation indicates [Simonsen, p.25]:

$$RSD = \frac{SD}{\bar{x}} \cdot 100\%$$

$$RSD = \frac{\sqrt{1.15674 \cdot 10^{-8} + 7.44496 \cdot 10^{-8}}}{9 \cdot 10^{-4}} \cdot 100\%$$

$$= 32.5874 \approx 32.6\%$$

The relative standard deviation for the overall reproducibility model is 32,6 %, which is very high compared to a confidence interval of 95%.

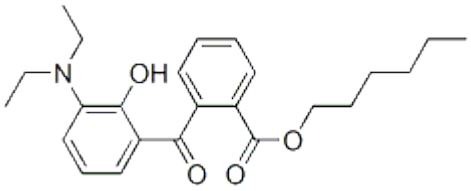
4.4.7 Robustness

If others want to use the method that have been developed in this project it is needed to know how robust the method is and which parameter that could have an influence on the results. During the method optimization the temperature, flow and solvent combination have been changed. Different persons have been doing the sample preparations which have been tested at different times.

The physical parameters as flow, solvent combination and temperature are mostly affecting the retention time but also the concentrations. The temperature is in the range of 30-60 ^{o}C affecting the retention time with 3 min., the higher temperature the shorter retention time, but if the temperature is change on the range of $\pm$2 ^{o}C from 45 ^{o}C it will have no significant different in the result. The flow has a bigger impact on the retention time, when the flow is reduced with 0.1 mL/min from 0.3 mL/min the retention time is reduced with 4 min. The solvent combination is the parameter that has the biggest influence on the retention time and separation and small changes in the mobile phase will change the retention time. When there is only one UV-filter in each sample to detect there is no problem with peaks switching space or impact on the separation, so the impact of the change in retention time is relative. Person and time showed to have a significance influence on the concentration.

4.5 Padimate O

A big error source has been made in the project where the UV-filter has been mistaken for another UV – filter in the Derma sunscreen. The IUPAC name for the UV-filter in Derma is Diethylamino hydroxyl hexyl benzoate and has .the following chemical structure:



The cause of this mistake is due to their similar chemical name. The mistake was eventually been found out by looking at the results of a spiking test where the standard (Padimate O) had another retention time than the UV-filter in the sunscreen. Apparently the UV-filter in the Derma sunscreen has also a different maximal absorption than the Padimate O has.

4.6 Legal amounts of UV-filters in sunscreens

According to the EU legislation, the maximum amount of *Octocrylene* and *Padimate* O on sunscreens is 10% and 8% respectively. To ensure that the amount is respected there has been made some calculations.

As an example the calculations were based on the samples made by person 1. See table 15

Sunscreen	**Person 1 (g)**	**Concentration (g/ml)**
L'Oreal	0,15853	0,012619806
Derma	0,15807	0,012583187
New Garnier	0,14971	0,011917688
Old Garnier	0,1559	0,012410444

Tabel 15; Shows the amount of the four samples and the calculated concentration

According to the chromatograms, *Octocrylene* is present in *L'Oréal* with a value in average of around 0,000804 g/ml, which means in the range of 5-6,5 % of the total content.

It is also present in the *Garnier* samples with an average concentration of 0,001036 g/ml, which means around 8-9%. Higher amount than in the *L'Oréal* samples but still below the maximum 10% [*Chisvert*, p. 87]. All the calculations can be found on appendix 2f.

All the chromatograms showed more or less the same concentration of *Octocrylene*. As expected, they were below the maximum permitted amount so that, any other calculation was made concluding that the manufacturers comply with the European law.

5.0 Discussion

5.1 Extraction method

In this project, one of the difficulties has been to find the right extraction method because the sample is very complex like cosmetic products.

The cosmetic products usually contain a large number of different compounds. Although, sunscreens in general do not require complex sample preparation, since the common creams are usually easily soluble if they are mixed with the appropriate solvent.

Sunscreens contain many different compounds such as water, fatty compounds, parabens and UV-filters. Due to the different properties of the UV filters, it is sometimes very difficult to propose an analytical method for the simultaneous determination of these chemicals. Furthermore it is difficult to find a single solvent for the water-soluble UV filters and the fat soluble that are also compatible with the developed technique. In our project, this has been perhaps one of our initial mistakes as we tried to find simultaneous extractions for 2 or 3 UV filters in different sunscreens - although they have more or less the same properties.

Sometimes, it is not possible to find the completed solubilization of a sample. Lightly cloudy solutions are obtained due to the presence of a few insoluble substances that must be removed by filtration before being analyzed. This fact does not mean that the extraction was not effective.

The target UV-filters on the sunscreens have been the following:

Octocrylene

Padimate O

Octinoxate

As they all are organic compounds, the extraction was carried out mainly with methanol, while they used ethanol in the scientific article. However, methanol and ethanol are almost the same solvents. After investigating the sunscreens, it was found that none of the sunscreens contained the UV-filter; *Octinoxate*.

Because of the similar names of the UV-filter, *Padimate O* and the UV-filter in the Derma, it was assumed that Derma actually contained *Padimate O*. After making the retrieval experiment for the method validation it was discovered that they were two different compounds.

Because of the mentioned problems we only have this two sunscreens; *Loreal* and *Garnier*, where only the UV-filter, *Octocrylene*, was detected.

During the optimization of the extraction method different kinds of solvents were used. Methanol and a mixture of methanol and acetic acid (low concentration) were used, but were not strong enough to dissolve the sunscreens. Even though there had not been any big different between the used solvents, one of the major factors of extraction was the heating and the ultrasonic treatment related to dissolve the fatty compounds. However, it was found that the best solvents for the UV- filters separation in sunscreens were methanol and sulfuric acid. Methanol and sulfuric acid gave the best chromatograms where the peaks had the highest absorbance.

5.2 Method optimization

The method optimization is based on the method described in the article; *Validation of HPLC method for the simultaneous and quantitative determination of 12 UV-filters in cosmetics*[A3] also described in the *HPLC optimization during laboratory*. In our case we have chosen a column with a shorter length, a smaller inner diameter and volume. By choosing another column we expect a shorter retention time and a reducing amount of eluent. In the article they detect Octocrylene after 10 minutes during isocratic method. When we reconstructed the method with another column and analyzed the standards Padimate O and Octocrylene were both detected between 2 and 4 min and the different sunscreens have some peaks in that area but it was difficult to identify whether it was the one standard or the other. We also saw some peak at the end of the chromatogram with very little intensity. Based on the results, it was obvious that we need to find another method to the HPLC and another method for the extraction to get better chromatograms. It was never further investigated if it was the bad extraction method that resulted in a different chromatogram compared to the chromatogram from the article. However it was still the same extraction method as they used in the article. Another problem could be the fact that the method optimization of the HPLC and extraction was developed in the same period of time.

5.2.1Gradient

Based on the results from the first chromatograms with the isocratic method, a lot of different gradient methods were developing see, the total list in *HPLC optimization during laboratory*. In this discussion part there will only be focus on some of the methods.

The first chromatogram that showed some useful peaks was Gradient B where the retention time for *Octocrylene* was 29 min and *Padimate O* was 30 min; all the peaks were nice and sharp. Compared to the method from the article that we want to optimize this method will not be useful. By changing the percentage of mobile phase and gradient, the retention time was reduced to 14.5 min for *Octocrylene* and 15.7 min for *Padimate O* by using method Gradient C. With this method the sunscreen samples also gave a better chromatogram. Gradient D showed a shorter method and retention time for *Octocrylene* compared to gradient C, which was expected as the concentration of methanol at the beginning was higher. The most intensive peak on the chromatogram for *L'Oréal* was previously identified as *Octocrylene* and the time is 10.4 min compared to 14.5 min with the gradient C method. For gradient E and F the time for *Octocrylene* was respectively at 6.9 min and 4.9 min, both chromatograms had so intensive peak that it looks like it was overlapping with some other compounds, the chromatogram with gradient E was still better then gradient F. As the HPLC only was available in a limit period of time it was chosen to change the slope of the gradient in the method Gradient D because this method shows the best separation of our three standards. In these three methods, Gradient D2, D3 and D4, Gradient D3 was the best-developed method. It can be discussed how much the method from the scientific article have been optimized when the time is 10.4 min compared to 10 min, but with the column the amount of costly mobile phase is reduced.

5.2.2 Flow rate

During the optimization we looked at the flow rate because it is one of the parameters that have a huge influence on the retention time, when the compounds run through the column with a higher flow. As we saw in the Results for *Methods optimization*, the time was reduced with 4 minutes when the flow rate was reduced from 0.3 mL/min to 0.2 mL/min. Previously, there had been some problems with too high pressure on the system when the flow was at 0.3 mL/min but after the optimization on the extraction method the problem had been minimized.

5.2.3 Temperature

Another parameter that was investigated during the optimization was the influence of the temperature. As the temperature was increasing the retention time for the *Octocrylene* was decreasing. This was expected as it previously was noted that the viscosity has influence on the retention time and it is related to the temperature. The peaks and separation was best at 45°C.

5.2.4 Mobile phase

In the optimization, we tested if a stronger mobile phase would get the small peaks that appear, to elute faster through the column. It was decided to try the stronger mobile phase; acetonitrile instead of methanol. The trend was overall the same in all the chromatograms for both standards and sunscreen samples, only one peak appears in the standard sample that contains three different standards, on the following blank sample the two missing peaks appears. That indicates that the method of the analysis had been too short to detect all the compounds. There is no point changing the mobile phase to acetonitrile instead of methanol, when the focus of this optimization is to validate a short method.

5.3 Statistics

The three factor ANOVA showed that all the three factors, label, person and time had a great influence on the concentration. However, it was the label factor that had the greatest effect on the determination of the concentration, which was also expected, because it comes from two different producers. However, it was expected that the person had a bigger influence on the concentration than the time. The ANOVA showed the exact opposite. Even though the person showed to have a big significant influence, the f-ratio was only 23.23 compared to a f-ratio of 149.53 for time. The big effect of the time maybe indicates that the methanol and sulfuric acid evaporates or that our samples decomposed during time. Furthermore the 3 factor ANOVA also showed that the concentration rose during the 2 weeks.

This brings us to the discussion whether the UV filters are stabile or not since the time had a huge influence on the concentration. However, this is not investigated further, but the extraction method might have had an influence on the decomposing of the UV-filter and the decomposing might act differently than if it were transferred to the human skin.

The interactions between label*person, person*time and label*person*time show to have a significance influence on the concentration, while the interaction between label*time shows no significance different. This might be due to that the two sample matrixes decompose in the same degree or methanol and sulfuric acid evaporates in the same degree. Therefore it might be the two persons that in some way make the preparation of the samples differently and therefore create the interactions between the factors.

The data for the two sample t-test showed no outliers and indicated normal distribution. However, *Bartlett's* test showed that statistically there were variance homogeneity but the p-value was only 0.0522 and therefore on the limit to be significance, therefore it was chosen to make the t-test for unequal variance. The two sample t-test for unequal variance showed that there was no significance different between the new and the old *Garnier*. However, the p-value was 0.0577, and the difference between the means showed a value of 0.00014, therefore statistically there is no significance different between the new and old, but because of the high difference between the means it cannot in praxis be said to be equal. The two sample t-test actually indicates a greater amount of the UV-filter in the old *Garnier* than in the new one which was not expected. The small different in concentration might due to that the fatty compounds in the old *Garnier* or other compounds that could interfere with the UV-filter, have decomposed and therefore it have been "easier" to extract the UV-filter in the old *Garnier* compared to the new one. However it was very clear that the old sunscreen had changed during time, the cream had a lower viscosity than the new *Garnier* and it looked very different when it was dissolved.

5.4 Method validation

The method validation has been made with the purpose of creating an overview of how good the developed method actually is. However, the extent of the method validation has been limited because of the time in the laboratory and the equipment, which has been available. It has not been possible to run our method and analysis on another HPLC machine for investigating the reproducibility. The determination of linearity by analyzing the standards should have been done at least two times but was only done once. Another thing that we have not investigated during the laboratory for the method validation is the temperature change from day to day and perhaps the pressure, but this change are assumed to be so small that we do not think of it as a source of error.

The specificity was investigated by making a recovery test. The recovery test showed that the method is specific and it is possible to detect the *Octocrylene* compound. The accuracy was investigated as well by making the recovery test and it showed that in average for *Garnier* it was possible to retrieval 95.4% while the *Loreal* could be detected with 99.8% in average. However the calculation of the accuracy was only made on two samples from each label while there should be a minimum of 10 samples. The method is accurate enough for making it quantitative but if a better extraction method was developed, then the accuracy could be improved.

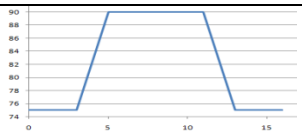
The precision was determined by making a reproducibility test. The 3 factor ANOVA was based on the data from the reproducibility. The relative standard deviation was calculated to 32.6%, which is way over the limit of 5% and can therefore not be accepted. The method is very imprecise because of the huge effect of the person and time that is very difficult to do anything about if the method has to be used in general. However, by investigating the time effects, it is clear that the sample preparation and analysis should be made on the same day to get the best possible results.

The linearity has a correlation coefficient of 0.99946 for the *Octocrylene* and can therefore be accepted. At the beginning of the project, it was wanted to detect the linearity of the two other standards but as they did not show in our sunscreens it was not necessary to determine.

The robustness was investigated for time, person, temperature, solvent combination and flow. The method was found not to be robust for making a quantitative determination. This has to do with the big significance change in concentration during time, therefore the samples for making an analyze have to be made on the same day as the analysis should be run on the HPLC. The big significance different due to time might change as earlier mentioned if another and better extraction method was found.
It also have a huge significance influence which analyst that prepare the samples and therefore it is important that during this analysis. It has to be the same analyst making the sample preparation, which is impossible to do if the method should be used in general. The temperature, solvent combination and flow makes big change at what time the peaks for the UV-filter comes out. It is therefore important to keep these three parameters more or less the same for recreating this analyze.

6.0 Conclusion

It can be concluded that the optimized HPLC method is gradient D3, with the solvents methanol (B) and water. The method is shown below;

Method	Flow rate (mL/min)	Temp. (^{O}C)	Wavelength (nm)	% B min/max	Gradient range and time	Time (min)
Gradient D3	0.3	45	312, 350	50/90		16

It can be concluded, that the peaks for *Octocrylene* had the highest absorbance at a wavelength of 312nm.

We can conclude, that during the optimization of the extraction method it was found that the best solvents for extracting UV- filters from sunscreens were methanol and sulfuric acid. The sample mixtures were treated with heat at 60^{o}C for 1 hour and afterwards treated with ultrasonic at 40^{o}C for 1 hour.

The amount of the UV-filter *Octocrylene* is under the legal permitted amount.

It can also be concluded, that the 3 factor ANOVA, based on the data from the reproducibility experiment, indicate high significant different for all the three factors on the concentration, with a significance level of ***. We can conclude, that there are interactions between the factors; label*person, person*time and label*person*time, while there is no interaction between label*time.

We can conclude, that there is no statistically significance different between the new and the old *Garnier*. However, the difference between the means of these dataset showed a high value of 0.00014, which means that we in praxis cannot conclude, that there is no significance difference.

It can be concluded, that the method is partly good. The relative standard deviation for reproducibility is 32,5%, which is way higher than the wanted value of 5%. We can also conclude, that the method is specific because we are able to detect the UV-filter. The method is also accurate because we are able to determine the UV-filter with a relative bias under 5%, except for one of the samples made from *Garnier* with a relative bias of 7,3%. It can be concluded that the sensitivity of the method is $3.0 \cdot 10^{7} \frac{unit\,area}{g/mL}$.

We can conclude, that the working range is inside the area of the linearity range with a correlation coefficient of 0.99946. It can be concluded that the method is not robust for making a quantitative determination.

It can be concluded that *Padimate O* is not present in any of the sunscreens; this was discovered during the recovery test.

7.0 Perspectives

Nowadays sunscreens are used to prevent radiation from the sun, which causes skin cancer. But, do they really work? There are many lines of investigation that are searching for the stability and the carcinogen nature of the UV-filters. With more time and the proper equipment this fact could be investigated. In any case, UV filters block or reflect the sun radiation and are generally used as a sun protection.

On the other hand, this report investigates the presence of UV-filters in different sunscreens using a HPLC instrument. With more time, we could have done a better and more representative paired t-test. This would lead into a more precise study of the differences between old and new sunscreens; several sample preparations should have been made and kept for a year and compared to a newer sample from the same sunscreen. Or if our samples could be analyzed every six months in a long period of time, a better study of the presence of the UV-filters during the time could be made. It would give more accurate results, as the matrix of the sample would be 100% the same.

The study was based only on two standards, as time was not on our side. Running more standards and other sunscreens would confirm if our method is valid for analyzing other UV-filter. Also working with other HPLC machine or even change the laboratory, our method could be better validated.

A 4-factor ANOVA could also be included in the project, where the precision of the HPLC machine is included in the project.

If someone investigates the precision and accuracy of the machine, our assumption could be examined and validated; otherwise it would be interesting to join the different injections to the analysis of variance.

In order to have the most optimal analysis of the UV filters in sunscreens, a more improved extraction method should be carefully investigated too, as the extraction methods that were examined did not give the desired results.

8.0 Index of reference

8.1 Books

- Bøye , Erik M., *Statistik hjælperen*, Swismark.
- Dong W, Michael, *Modern HPLC for practicing scientists,* Wiley, 2006
- Montgomery & Runger, *Applied Statistics and Probability for Engineers,* 4th Ed.
- Lund, U. Andersen, K. Sørensen, P.S, *Håndbog i methodevalidering for miljølaboratorier,* VKI sag nr. 404444/910, 29. december.
- Salvador, Amparo & Chisvert, Alberto, *Analysis of Cosmetic Products,* Copyright © 2007 Elsevier.
- Simonsen, Flemming, Jeppesen,Helle, Norsker, Merete, *Analyse teknik*, Ny Teknisk Forlag.
- *Problemas de química análitica* by Carlos E. Rubio Navarro, ISBN 8495475006, Departamento Química analítica ed.
- D. Kealey & P. J Haines*, Analytical Chemistry*, Forlag: Bios.
- R. Kellner, J-M Mermet, M. Otto, M. Valcarcel, H.M Widmer, *Analytical Chemistry*, Forlag: Wiley VCH, April 2004

8.2 Articles

A1 *Identification of UV filters in sunscreen products by high-performance liquid chromatography – diode – array detection* by S.C. Rastogi*, G.H. Jensen.

A2 *Sunscreens and Photoprotection* by Stanley B Levy, MD; Chief Editor: Dirk M Elston.

A3 *Validation of HPLC method for the simultaneous and quantitative determination of 12 UV-filters in cosmetics* by M. Nyeborg, M. Pissavini, Y. Lemasson and O. Doucet.

A4 *In vitro evaluation of the cutaneous penetration of sprayable sunscreen emulsions with high concentrations of UV filters* by L. Durand*, N. Habran and K. Amighi.

A5 *Simple Extraction and HPLC determination of UV-A and UV-B filters in sunscreen products* by D. De Orsi, G. Giannini, R.Porra. S. Berri and others.

A6 *The chemistry and HPLC analysis of chemical sunscreen filtyers in sunscreens and cosmetics* by K. Lee Granger and P. R. Brown*.

A7 *Determination of UV-filters in sunscreen by HPLC* by Amparo Salvador, M. C. Pascual - Martí and Alberto Chisvert.

A8 *The Burning Facts* by the US Environmental Protection Agency, EPA 430-F-06-13, September 2006

A9 *Metodevalidering I kemisk analytiske laboratorier, Kvantitative analysemetoder* by DANAK, 2003-08-18.

8.3 Web Sites

W1 https://scifinder.cas.org/scifinder/

W2 http://ec.europa.eu/consumers/sectors/cosmetics/

W3 http://www.skinbiology.com/toxicsunscreens.html

W4 http://www.zimbio.com/Best+Sunscreens/articles/93/Octinoxate+plus+other+sunscreen+ingredients

W5 http://www.chemicalbook.com/ProductChemicalPropertiesCB3218114_EN.htm

W6 http://www.skincancer.org/shining-light-on-ultraviolet-radiation.html

W7 http://www.uv.es/gammmm/Subsitio%20Operaciones/6%20Calibracion.htm

W8 http://www.skincancer.org/understanding-uva-and-uvb.html

W9 http://www.gunt.de/download/extraction_english.pdf

W10 http://www.rsc.org/chemistryworld/Issues/2007/September/ClassicKitSoxhletExtractor.asp

W11 http://www.aidic.it/icheap9/webpapers/14Alupului.pdf

W12 http://www.javeriana.edu.co/Facultades/Ciencias/neurobioquimica/libros/celular/fraccionamiento.htm

8.4 Other references

S1 Bisgaard, Kathrine, Slides from HPLC lectures, 2011

S2 Pedersen, Lene & Broe, Jørgen, Slides from Statistic lectures, 2011

S3 Pedersen, Lene, *A Guide to Analytical Method Validation,* Notes from Statistics, 2011

S4 Pedersen, Lene, *Test of Hypotheses for a Single Sample*, Slides from Statistic lectures, 2011

S5 Pedersen, Lene, *Method Validation,* Slides from Statistics, 2011

9.0 Appendix list

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