KTH ROYAL INSTITUTE OF TECHNOLOGY

Degree Project in Chemical Science and Engineering
Second cycle, 30 credits

The effect of temperature on the chemical stability of Vitamin C in a cosmetic product

- Development of an HPLC-method for quantitative analysis

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Sammanfattning

Vitamin C är en populär ingrediens i hudvårdsprodukter, bland annat på grund av dess positiva effekter på kollagen-produktionen i huden och dess antioxidativa egenskaper. Dessvärre är vitamin C en känslig ingrediens som lätt bryts ned av ljus, syre, hög temperatur eller fel pH-värde. I dagsläget finns inga regleringar för kontroll av nedbrytning av aktiva ingredienser i kosmetiska produkter.

I den här studien har en metod utvecklats med omvänd-fas högupplösande vätskekromatografi (RP-HPLC) och detektor med ultraviolett (UV) ljus för att analysera halten C-vitamin i en kosmetisk testprodukt, hädanefter kallad OTP och en modifierad variant av denna, kallad MTP. En stabilitetstudie gjordes vid olika temperaturer och ljusförhållanden på dessa produkter. En jämförelse gjordes även mellan en högre och lägre syretillgång.

I metoden som valdes för stabilitetsanalys använde UV-detektor (254 nm). Den kromatografiska kolonnen innehöll C-18-stationärfasoch hade en längd om - 250x4.6 mm, med 5 µm partikelstorlek, från Chrompack. Mobilfasen bestod av 20 mM lösning av ättiksyra i Milli Q-vatten och metanol i förhållandet 95:5 (v/v) och mobilfashastigheten 2 ml/min, vilket gav en retentionstid på ca 2.4 min för vitamin C.

I MTP bröts vitamin C ned snabbare än i OTP. Den lägsta nedbrytningshastigheten kunde ses vid den lägsta temperaturen (4℃) och den lägre syretillgången av dem som studerades. Ett linjärt samband kunde ses mellan förvaringstiden och koncentrationen av vitamin C som fanns kvar i produkten vid detta förvaringsförhållande.

Nyckelord: *Vitamin C, Kosmetisk produkt, HPLC, Stabilitet, Nedbrytning, Antioxidant*

Abstract

Vitamin C is a popular ingredient in skin care products, due to its positive effect on the collagen production and its antioxidative characteristics. Unfortunately, vitamin C is a sensitive ingredient that is easily degraded by light, oxygen, elevated temperatures, or the wrong pH-value. To today's date, there are no regulations controlling the degradation of active ingredients in cosmetic products.

In this study, a method has been developed for Reversed Phase-Liquid Chromatography (RP-HPLC) and Ultra Violet (UV)-light detector to analyze the vitamin C content in a cosmetic test product, in this study called OTP and a modified variant of the same product, called MTP. A stability study was made at different temperatures and light conditions. A comparison was also made between higher or lower levels of oxygen present.

The method chosen was performed at a wavelength of 254 nm. The column used contained a C-18 stationary phase (250x4.6 mm, 5 µm particle size) from Crompack. The mobile phase consisted of 20mM solution of acetic acid in Milli Q water and methanol 95:5 (v/v) and the flow rate was set to 2 ml/min, which gave a retention time of approximately 2.4 min for vitamin C.

Vitamin C was degraded faster in MTP than in OTP. The lowest degradation rate was found at the lowest temperature (4℃) and the lower oxygen content among the ones studied. A linear relationship was found between storage time and the vitamin C concentration left in the product at this storage condition.

Keywords: *Vitamin C, Cosmetic product, HPLC, Stability, Degradation, Antioxidant*

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Abbreviations

ANOVA	Analysis of Variance
HPLC	High-Pressure Liquid Chromatography
MeOH	Methanol
MS	Mean of Squares
MTP	Modified version of Test Product
O/W	Oil-in-Water Emulsion
OTP	Original Test Product
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
RT	Retention Time
SS	Square of Sums
STD	Standard Deviation
W/O	Water-in-Oil Emulsion
ZnA	Zinc Ascorbate

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2 Introduction

2.1 Background

2.1.1 The history of Vitamin C

Vitamin C is best known as an essential vitamin and antioxidant that humans must eat in sufficient amounts not to get deficiency disease, more specifically, scurvy. The word “Ascorbic” means “no scurvy.” Most plants and animals are able to make their own vitamin c from glucose. However, for humans, it is essential to get vitamin c from food intake. Humans lack a certain enzyme called L-glucono-gamma lactone oxidase needed for *in vivo* synthetization. Vitamin C can be found in citrus fruits, leafy vegetables and broccoli for example (*1*).

2.1.2 Vitamin C and our body

In the human body, vitamin C has a number of functions. It is involved in important metabolic functions as well as being crucial for the growth and maintenance of healthy bones, gums, ligaments, teeth and blood vessels. It is necessary for wound healing and immune responses to infections. The body also uses it for absorption of iron and for utilizing folic acid (*2*).

The least amount found necessary to avoid scurvy is 6.5 mg per day. This is barely enough to prevent the levels to drop below 50% in our body. The last organ that receives nutrients from dietary intake though, is the skin (*3*). Therefore, topical application is a given alternative for boosting skin levels of vitamin C. The levels from topical application can be as much as 20-40 times higher than from oral intake (*4*).

2.1.3 Vitamin C and our skin

Our skin is subject to everyday stress by UV-light exposure, air pollutants, ozone and other stimuli which cause cumulative damage and photoaging (*5*).

Vitamin C is also found in our skin, where it has multiple functions. It helps in the biosynthesis of collagen, act as a scavenger of reactive oxygen species (ROS) and acts as a treatment of hyperpigmented spots (*6*). It reduces both nitrogen- and oxygen based free radicals, which can slow down the aging process (*2*). It is now recognized that sagging skin, wrinkles and age spots are primarily caused by oxyradical damage (*7*).

UV-light from the sun causes ROS to form in our skin when exposed to sun light. When the production of ROS overwhelms the cellular antioxidant capacity, it results in a condition called oxidative stress. Oxidative stress is thought to be involved in the development of a wide variety of diseases, including atherosclerosis, diabetes, neurodegenerative diseases, chronic inflammatory diseases, cancer and in aging (*8*).

Ascorbic acid neutralizes ROS by donating one electron in a single-electron oxidation reaction. This oxidates the ascorbic acid to an ascorbyl radical, a less reactive species than ROS, which does not cause damage. The ascorbyl radical disproportionates to ascorbate and dehydroascorbate. (*9*)

2.1.4 Chemical description of Vitamin C

In nature, ascorbic acid comes in two different variants that are mirror images of each other, L-Ascorbic Acid (LAA) and D-Ascorbic Acid (DAA). It is only the former that is bioactive. In Figure 1, the structure can be seen. It contains a 5-carbon ring, similar to that of glucose (*1*).

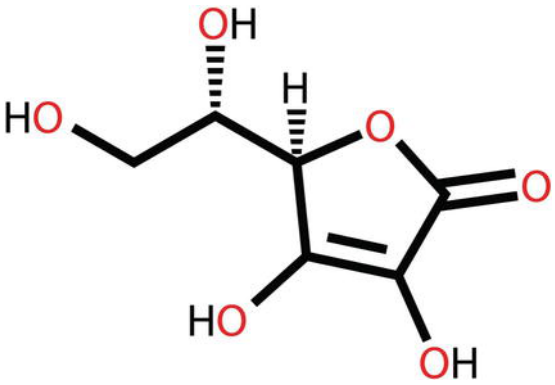

FIGURE 1. THE STRUCTURE OF ASCORBIC ACID (*9*)

2.2 Vitamin C in cosmetic products

In cosmetic products, vitamin C can act complimentary to UV-filters. UV-filters absorbs and reflects the UV-light from the sun, and thereby reduce the formation of free radicals in the skin. However, UV-filters and sunscreens do not reduce the free radicals formed completely. UVA radiation has a wavelength of 315-400 nm and UVB has a wavelength of 280-315 nm (*10*). Vitamin C does not primarily act as a sunscreen since it does not absorb UV-light of wavelengths above 295 nm (*11*). Instead, Vitamin C acts as an antioxidant and reacts with and neutralizes the free radicals formed.

For the best penetration through the outer layer of the skin (stratum corneum), the vehicle used for transportation is preferably a lipid based. Hydrophilic compounds have more trouble penetrating stratum corneum than lipophilic compounds (*12*). However, vitamin C has been shown to penetrate through the skin despite its hydrophilic nature (*13*).

2.2.1 Stability of Vitamin C

Vitamin C is unfortunately known to be sensitive to light, pH, high temperatures and exposure to oxygen, which cause degradation (*14*).

2.2.2 Derivatization improves the stability of vitamin C

To reduce the problem of degradation of vitamin C, a number of different chemically stable ascorbyl derivatives has been used in cosmetic products. However, the pharmacological effect can be reduced. Therefore, it is of great interest to stabilize vitamin C in other ways (*2*).

Zinc ascorbate is a salt, which means that it consists of two ions bonded together with an ion-ion bond (see Figure 2). In a water solution, they exist as separate units. The ascorbate ion is easily protonated to form ascorbic acid in acidic conditions and can be used as an alternative to ascorbic acid in cosmetic products.

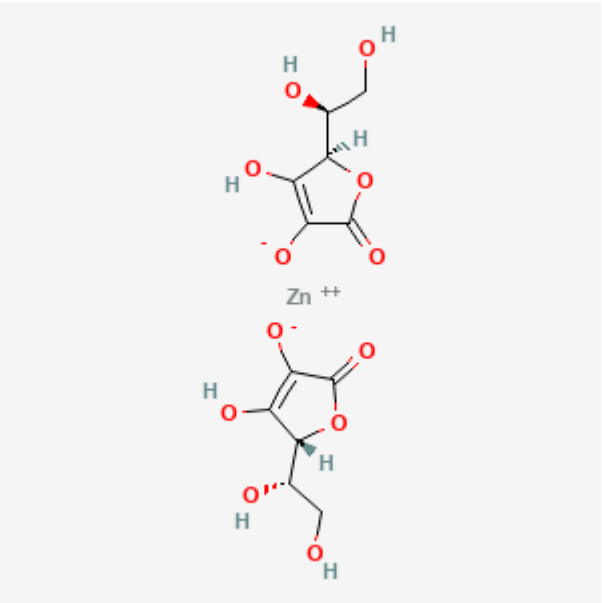

FIGURE 2. ZINC ASCORBATE. TWO ASCORBATE IONS IONICALLY BONDED TO Zn^{2+}. (*15*)

2.2.3 Regulations of cosmetic products

For cosmetic products, there is no requirement in the cosmetic regulation in the European Union (EC) No 1223/2009 demanding declaration of the concentration of the active ingredients. It is not demanded to control the degradation of the ingredients either. This means that a product claiming to contain a certain ingredient is not necessarily doing so in any effective amount, and might not have desired effect on the skin. Especially not after a few months of storage. For the consumer, it also means a great deal of uncertainty to know neither the content from the beginning nor what is left in the product.

2.3 The aim of the study

The aim of the study was to develop a method for quantitative analysis of vitamin C, in the form of zinc ascorbate (ZnA), in a cosmetic product and to measure the degradation over time in the product. Four different storage conditions were used for studying the effect of different temperatures and lighting conditions:

1. Room temperature (20-25 ℃), light
2. Room temperature (20-25 ℃), dark
3. 4℃, dark
4. 40℃, dark

2.4 Quantitative methods for determination of Vitamin C

Zinc ascorbate can be analyzed in the same way as ascorbic acid itself. Upon the use of an acidic mobile phase the ascorbate ion gets protonated, turning it into ascorbic acid.

Analysis of vitamin C in a cosmetic product involves two main steps:

1. Extraction of vitamin C from the test product (sample preparation)
2. Analysis of vitamin C concentration in the extract

The oil content in a cosmetic product must be removed since it could otherwise be retained in the column and accumulate over time, or in worst case scenario, block the column entirely.

3 Method

3.1 Instrumentation

3.1.1 High Pressure Liquid Chromatography (HPLC)

HPLC is an analytical method for separation, identification, quantification and separation. A liquid, containing the sample is pumped under high pressure to a column containing a stationary phase. The stationary phase interacts with each of the components in the sample to different extents based upon polarity. This means that some of the components are retained in the column longer and some exit the column faster. This makes it possible to separate and identify each component when compared to the retention time of a known reference standard under the same conditions. The sample can also be quantified when compared to the reference standard concentration. After separation by the column, the components are sent to a detector measuring the concentration of the sample and sends a signal to a computer. A chromatogram is shown on the computer where each component in the sample corresponds to a peak. The size of the peak corresponds to the concentration of the component.

In normal phase chromatography, the stationary phase is polar and the liquid phase is non-polar. In reverse phase chromatography, the polarity of the phases are the opposite with a polar liquid phase and a non-polar stationary phase.

3.1.2 Instrumentation used

The method development as well as the final stability analysis was performed with Reverse Phase High Pressure Liquid Chromatography (RP-HPLC). HPLC air actuated 4 port valve manual injector (Valco Instruments Co.) and pump (Perkin Elmer Series 200) equipped with UV-detector (Spectra 100 variable wavelength detector, Spectra-Physics). The software used was EZChrom Chromatography Data System Version 6.8 (Scientific Software, Inc.)

3.2 Method development

3.2.1 HPLC-method

A Milli Q-water solution of vitamin C was injected into the HPLC-system and different combinations of columns, mobile phases and flow rates were evaluated to find a combination giving a satisfying peak from vitamin C (Table 1 and Table 2). All runs were isocratic.

TABLE 1. THE DIFFERENT COLUMNS STUDIED

Column number	Column
1	Spherisorb ODS-2 100x 4.6 mm
2	Dionex Acclaim PolarAdvantage II C18 3 um 120 Å 3x 150 mm
3	Purospher STAR RP-18 endcapped (150 x 4.6 mm, 5µm particle size)
4	Dionex Acclaim Mixed-MTPe HILIC-1 3um 120 Å 3.0 x 150 mm
5	Chrompack SS 250 x 4.6 mm ODS-3

TABLE 2. DIFFERENT COMBINATIONS OF PARAMETERS USED IN HPLC ANALYSIS.

Experiment number:	Column number:	Mobile phase Acetic acid water solution/methanol (v/v)	Flow rate [ml/min]	Wavelength [nm]
1	1	90:10	1	254
2	2	98:2	1	254
3	2	95:5	1	254
4	3	90:10	1	254
5	3	95:5	1	254
6	3	99:1	1	254
7	4	70:30	1	254
8	4	50:50	1	254
9	4	30:70	1	254
10	4	10:90	1	254
11	3	99:1	1	254
12	3	99:1	1	254
13	5	95:5	1	254
14	5	95:5	2	254

3.2.2 Sample preparation (extraction)

Different combinations of mixing techniques and temperatures were evaluated to find a satisfying method for extracting vitamin C from the serum with as accurate yield as possible.

Milli Q water was added to the serum before the start of mixing. Three different mixing methods were studied: shaking by hand, vortex and sonication. A spiked sample of serum was used, where 0.0197 g of ZnA was weighed and stirred into 6.009 g of serum and divided into three different 50 ml Falcon tubes. The exact amounts in each tube can be seen in Table 3. The remaining serum was discarded. 25 ml of Milli Q water was then added to each Falcon tube and they were mixed for 1 min in three different ways, filtered to avoid large particles that can block the column, and evaluated by the earlier developed HPLC-method.

The spiked sample was used since the first samples of the product were not freshly made and the concentration of vitamin C was uncertain. A spiked sample made sure that a certain amount of vitamin C was present in the sample before extraction.

TABLE 3. MIXING TECHNIQUES STUDIED.

Number	Mixing	Amount of serum [g]	Time [min]
1	Shaking by hand	1.505	1
2	Vortex	1.702	1
3	Sonication	1.513	1

After choosing the most suitable mixing method, trials were made with heating up the water for extraction and also to shake the Falcon tube after separation of the oil and the water phase before filtration.

3.3 Products for testing

3.3.1 Original Test Product (OTP)

The serums for testing were formulated at Skinome Research AB's laboratory. The test product analyzed in this study consists of a continuous oil phase and an aqueous phase dispersed within the oil phase, stabilized with emulsifiers, better known as a water-in-oil emulsion (W/O). The ingredients in the product are described in more detail in Table 4. Zinc ascorbate has a high water solubility, but a very poor solubility in oils and is therefore solubilized in the water droplets of the medium. The concentration of ZnA added is 0.5% (w/w).

The relatively low water content contributes to a stabilizing effect on vitamin C since the continuous oil phase acts as a physical barrier for oxygen from the ambient air that it needs to pass through (*16*).

TABLE 4. THE INGREDIENTS OF OTP AND THEIR STATED FUNCTION IN COSMETIC PRODUCTS ACCORDING TO THE EUROPEAN DATABASE FOR INFORMATION ON COSMETIC SUBSTANCES AND INGREDIENTS, COSING (*17*).

INCI	Function
Squalane	Hair conditioning Refatting Skin conditioning Skin conditioning – emollient
Avena sativa (Oat) Kernel Oil	Skin conditioning
Jojoba Esters	Moisturizing Skin conditioning Skin conditioning – emollient Soothing
Hydrogenated Vegetable Oil	Skin conditioning Skin conditioning – emollient
Caprylyl Glycol	Deodorant Hair conditioning Skin conditioning Skin conditioning – emollient
Hydrogenated Lecithin	Skin conditioning Surfactant – emulsifying
Aqua	Solvent
Niacinamide	Smoothing
Alpha-Glucan Oligosaccharide	Cleansing Skin conditioning Smoothing
Acetyl glucosamine vegetal	Skin conditioning
Zinc ascorbate	Antioxidant Oral care Skin protecting

3.3.2 Modified Test Product (MTP)

In the modified version of the serum, the pH of the water phase was lowered to about 3-4 before it was added to the oil phase compared to pH 5 in OTP. In the oil phase, Avena sativa (Oat) Kernel Oil was not added as in OTP, but erythritol was added instead.

3.4 Calibration curve

3.4.1 Stock solution preparation for calibration- and standard solutions

6 mg of ZnA was weighed directly into a 1.5 ml Eppendorf tube. 1 ml of Milli Q water was added with a micropipette followed by 3 min sonication to yield a stock solution with a concentration of 6 mg ZnA/ml Milli Q water. From the stock solution, a calibration- or standard solution was prepared immediately. The calibration- or standard solution was either used immediately or put into storage at -18℃ for later use, to avoid degradation of ZnA.

3.4.2 Construction of the calibration curve

From the stock solution, five different calibration solutions were made in the concentrations 0.06, 0.15, 0.3, 0.45 and 0.6 mg ZnA/ml Milli Q. From the stock solution, an aliquot was taken and diluted with Milli Q water in a 1.5 ml Eppendorf tube (see Table 14 in the Appendix for dilution scheme). All five solutions were prepared at once, just before the first injection and injected in triplicate. The first injection was made in a randomized order. The second and third injection followed the order of the first injections. All calibration solutions were kept in aluminum foil during the analysis to avoid degradation from light.

The equation of the trend line obtained from the mean values of the areas and the corresponding concentrations of the calibration standards were later used to calculate the yields of the extraction of vitamin C from the serums.

3.5 Stability testing of serum

3.5.1 Storage conditions

Both OTP and MTP were formulated at Skinome Research AB. Out of practical reasons, OTP was kept refrigerated at Skinome Research AB for 1 day and MTP for 9 days before they were put into respective storage conditions. They were analyzed after 4 weeks (28 and 29 days respectively) and after 6 weeks (42 and 43 days respectively) of storage at the chosen conditions.

TABLE 5. DIFFERENT STORAGE CONDITIONS OF SAMPLES FOR STABILITY TESTING.

Storage condition	OTP (number of jars)	MTP (number of jars)
Room temperature (daylight)	4	4
Room temperature (dark)	4	4
Refrigerated	4	4
40℃	4	4

3.5.2 Additional testing of older samples of OTP

Older samples of OTP, stored at 4℃ for 9 and 11 months respectively, were also analyzed to get additional time points for this condition. Samples stored at other conditions for a longer period of time were not available for this study.

3.5.3 Sample preparation

In the final method for sample preparation used in the stability study of the serum, 1.5 g of serum was weighed directly into a 50 ml Falcon tube. 25 ml of room temperature water was added. The tube was shaken by hand for approximately 10 seconds for the serum and water to blend, followed by 5 min vortex mixing by Vortex-Genie 2 G560/E 50 Hz, at the highest speed. The tube was then left in an upright position for 2 min for the oil and water phases to separate. A syringe with a needle attached was used to collect 1 ml of the water phase. The needle was detached, and a 0.45 mm syringe filter was screwed on to the syringe. The filtrate was collected into a 1.5 ml Eppendorf tube and injected in triplicate.

3.5.4 Standard solutions

Standard solutions were injected prior to analysis of the samples in the stability study to confirm the validity of the calibration curve. The standard solutions were prepared from the stock solution immediately after it was prepared to avoid degradation. The standard solutions were prepared to match the highest and the lowest concentration on the calibration curve and were stored at -18℃. 20 min before injection, the standard solutions were taken into room temperature to thaw. If the high or low standard injections did not match the set criteria (i.e., differed too much from the calibration curve), a new standard, matching the mid-point in the calibration curve would be made from a new stock solution. If the freshly made standard solution would not match the calibration curve, the calibration curve should no longer be seen as accurate and a new calibration curve would have been made.

3.5.5 Analysis

Before injection of the samples, the injection needle was washed with MeOH three times, then the injection loop was washed with MeOH three times. The needle was then washed with the sample. 60 µl of sample was collected, whereas the first 10 µl and the last 10 µl was discarded due to the risk of inserting air bubbles to the loop. Thus, 40 µl of sample was injected. The chromatograms would run for 10 min.

For the 4-week stability analysis, an aliquot from one jar of each storage condition was collected from OTP and MTP. For the 6-week analysis, from OTP, in the storage conditions room temperature (light), room temperature (dark) and refrigerated, an aliquot was taken both from the jar analyzed at the 4-week time point as well as from another, not yet analyzed jar. For OTP, stored at 40℃ as well as for MTP, only the already analyzed jars were analyzed at the 6-week time point.

A starting point value for OTP was later obtained by formulating a fresh batch of serum, and analyzing it the same day. This was due to that the method for extraction was not yet developed completely at the start of the stability study. The starting point value shows the maximum yield the chosen method

can obtain. The measurements made in the stability study was adjusted according to this value when calculating the concentration.

3.5.6 Materials and instrumentation

UV-detector (Spectra 100 variable wavelength detector from Spectra-Physics), 254 nm. C-18 column (250x4.6 mm, 5 µm particle size) from Crompack (column no 5). The mobile phase consisted of 20mM solution of acetic acid in Milli Q water and methanol 95:5 (v/v), and the flow rate was set to 2 ml/min, which gave a retention time of approximately 2.4 min.

TABLE 6. ANALYSIS SCHEME FOR SERUM STABILITY TESTING.

Serum type	Storage condition	4-week analysis (jar number analyzed)	6-week analysis (jar number analyzed)
OTP	Room temperature (daylight)	1	1 & 2
	Room temperature (dark)	1	1 & 2
	Refrigerated	1	1 & 2
	40℃	1	1
MTP	Room temperature (daylight)	1	1
	Room temperature (dark)	1	1
	Refrigerated	1	1
	40℃	1	1

3.6 Stability testing of standard solutions

A fresh standard solution of 0.3 mg/ml ZnA in Milli Q water was prepared by pipetting 5 x 950 ml of Milli Q water and 5 x 50 ml of a stock solution of 6 mg/ml in an Erlenmeyer flask. After mixing, 1 ml was pipetted into 3 different Eppendorf tubes. The three Eppendorf tubes were stored in different storage conditions (room temperature in aluminum foil, 4℃ or -18℃) for 4 hours and then analyzed in duplicate. The frozen Eppendorf tube had to be thawed at room temperature for 20 min before analysis. One of the solutions was analyzed prior to storage to get a starting point value.

3.7 Validation of the method

3.7.1 Repeatability – intra-day precision

Three identical extractions were performed and analyzed in triplicate within the same day to a total of 9 injections. Standard deviation (STD) and relative standard deviation (RSD) were calculated.

3.7.2 Repeatability – inter-day precision

When using a method for stability testing, the analysis will be performed over several different days to evaluate different time points. In order to estimate the uncertainties related to this variation, an inter-day precision test was made to see if the different conditions at different days affect the results significantly. The conditions can be different humidity or temperature at different days at the lab, for example.

The statistical test associated with this is called Analysis of Variance (ANOVA). This technique can separate various sources of errors. The hypothesis is that all the measurements are taken from the same population (i.e., that there is no significant difference between results obtained on different days). On the basis of this assumption, the variance of the population can be calculated either from the within-sample variation or from the between-sample variation. To evaluate if the hypothesis is true, a F-test was performed. A F-test is a test where two variances are compared to see if one variance differs significantly from the other.

3.7.3 Recovery (trueness)

The recovery of extracted vitamin C (i.e., the maximum yield obtainable with the chosen method) was calculated from the analysis of freshly formulated serum analyzed the same day as it was manufactured, and the measurements in the stability study was adjusted according to this value that was seen as being corresponding to the concentration of ZnA added to the serum at the manufacturing stage.

3.7.4 Linearity

From the stock solution, five different calibration solutions were made in the concentrations 0.06, 0.15, 0.3, 0.45 and 0.6 mg ZnA/ml. From the stock solution, an aliquot was taken and diluted with Milli Q water in a 1.5 ml Eppendorf tube (see Appendix for dilution scheme). All five solutions were prepared at once, just before the first injection and injected in triplicate. The first injection was made in a randomized order. The second and third injection followed the order of the first injections. All calibration solutions were kept in aluminum foil during the analysis.

The equation of the trend line obtained from the mean values of the areas and the corresponding concentrations of the calibration standards were later used to calculate the yields of the extraction of vitamin C from the serums.

3.7.5 Specificity

The specificity was tested using spiked samples (i.e., with added ZnA) of product analyzing degraded samples of ZnA in Milli Q water, looking for interfering peaks of degradation of ZnA.

A blank sample was also analyzed by analyzing a sample of serum with the same ingredients added except for ZnA.

4 Results and discussion

4.1 Method development

4.1.1 HPLC-method

The first part of the study consisted of varying different parameters for the HPLC-analysis and determining the optimal analysis method. The column, mobile phase composition and flow rate were varied to obtain the best parameters for the analysis when injecting a water solution of vitamin C.

The first attempts showed peaks with a short RT (~ 1 min), suspected to be close to, or even overlapping the retention time of the void peak (see Appendix, Figure 13). This means that the analyte has not had any interaction with the stationary phase, at the silica particles inside the column. This cannot be accepted since any unretained molecules merge together to one large peak with no separation of the analytes, and quantification is impossible in this case. There might be several compounds hiding behind one peak.

There was also a problem with peak splitting (see Appendix, Figure 14). The flow at the inlet of the column can be disturbed by the presence of void causing a fraction of the analyte to enter the column earlier than the other fraction. This would look like two peaks, or split peaks in a chromatogram. A partially blocked frit of the column could also cause peak splitting since a fraction of the sample is delayed by the blockage at the frit. (*18*)

It was also tested to inject vitamin C from another manufacturer, but the split peaks remained the same. This reduces the possibility of degradation or contamination of the product being the cause of the split peak.

The final choice of a 25 cm long C18 column gave a symmetric peak, well separated from the injection peak (see Appendix, Figure 15). With a flow rate of 1 ml/min, the RT was ~4.6 min, but to shorten the time required for analysis, the flow rate was increased to 2 ml/min and the RT was shortened to ~2.4 min in the final method.

TABLE 7. DIFFERENT COMBINATIONS OF PARAMETERS TESTED FOR DEVELOPMENT OF HPLC-METHOD

Experiment number:	Column number:	Mobile phase composition. Acetic acid water solution/methanol (v/v)	Flow rate [ml/min]	Wavelength [nm]	Chromatogram	Split peak (Y/N)	RT > 1.5 min (Y/N)
1	1	90:10	1	254	Figure 13	Y	N
2	2	98:2	1	254	-	Y	N
3	2	95:5	1	254	-	N (but broad shape)	N
4	3	90:10	1	254	-	Y	Y
5	3	95:5	1	254	-	Y	Y
6	3	99:1	1	254	Figure 14	Y	Y
7	4	70:30	1	254	-	N	N
8	4	50:50	1	254	-	N (but flat top)	N
9	4	30:70	1	254	-	N	N
10	4	10:90	1	254	-	Y	N
11	3	99:1	1	254	-	Y	Y
12	3	99:1	1	254	-	Y	Y
13	5	95:5	1	254	-	N	Y
14	5	95:5	2	254	Figure 15	N	Y

4.1.2 Extraction method

In order to get access to the water content in the serum, the two phases, oil and water, had to be separated. Thus, the emulsion had to be broken. One way can be to break the emulsion physically by mixing in a solvent, which was evaluated in this study. The solvent added, can dissolve vitamin C and also be injected into the HPLC-system.

Vitamin C shows a much higher solubility in water as compared to many other common solvents such as methanol, ethanol, propan-2-ol, acetone, acetonitrile, ethyl acetate and tetrahydrofuran (*19*). Therefore, water was chosen as the extraction medium.

TABLE 8.THE SOLUBILITY IN MOLE FRACTIONS OF VITAMIN C IN DIFFERENT SOLVENTS AT 20℃ (*19*).

	Water	Methanol	Ethanol	Propan-2-ol	Acetone	Acetonitrile	Ethyl Acetate	Tetrahydrofuran
Solubility at 20℃ [mole fraction]	28.96+/- 0.02	10.06 +/- 0.05	2.29 +/- 0.03	0.51 +/- 0.01	0.26 +/- 0.01	0.20 +/- 0.01	0.10 +/- 0.01	0.53 +/- 0.01

TABLE 9. DIFFERENT WAYS OF MIXING SERUM WITH WATER, THE RESULTING MEAN YIELD AND THE HIGHEST YIELD FROM THREE INJECTIONS.

	Mean yield (%)	Highest yield (%)	Peak area 1	Peak area 2	Peak area 3	RSD (%)
Shaking by hand	73	88	345753	472907	351324	18
Vortex	67	110	678150	398910	142802	66
Sonication	35	47	228563	243907	65949	55

EQUATION 1

$$RSD = \sqrt{\frac{\sum_i (x_i - \bar{x})^2}{(n-1)}}$$

Equation 1 shows the calculation of RSD, where $\bar{x}$ is the arithmetic mean and x_i is each individual value of peak area, RT or peak height obtained from each chromatogram and n is the number of injections.

When evaluating the different mixing techniques, it could be seen that the highest mean yield was obtained by shaking the Falcon tube by hand. The yield was calculated from Equation 8 and Equation 9 (See Appendix). However, the RSD value (calculated from Equation 1) of the peak areas was remarkably high. One possible explanation is that the third injection was made later than the first two, and the filtrate was stored at 4℃ for a few hours in between, making degradation possible.

All three extractions were made in parallel until the filtration step where one Falcon tube at the time was filtered and the first injections were made. This resulted in that the sample from the first mixing technique was injected in closer conjunction to the mixing and might have a lesser degree of degradation in the filtrate. This cannot explain the very much larger peak area for the first injection of vortex compared to the second injection for the vortex technique. Despite of the inconclusive result,

vortex was chosen as the technique to proceed with since it had the largest yield overall and also for the ability to control the extraction procedure more precisely compared to shaking by hand.

It was also studied to melt the serum with hot water during the extraction, varying the time for vortex mixing and also mixing the oil and water phase again before taking the final sample to filtrate. This was studied in different combinations, shown in Table 10.

The results were spread and no conclusion could be drawn other than that there was no possibility of degradation causing the variation since the serum tested had been formulated almost the same number of days before analysis in all cases. One combination that stood out was the melted serum that was shaken before filtration and vortexed for 5 min.

TABLE 10. DIFFERENT COMBINATIONS OF EXPERIMENTAL CONDITIONS OF EXTRACTION OF VITAMIN C FROM SERUM.

Vortex [min]	Melted serum	Shaken before filtration	Serum age (days)	Yield (%)
10	x	x	7	27
5	-	x	8	29
5	-	-	6	49
1	x	x	7	49
5	x	-	7	57
1	x	-	6	59
10	-	-	7	68
5	x	x	7	81
5	x	x	6	87

The results obtained with melted, shaken and 5 min vortexed serum would be shown hard to repeat, though. Three repeated extractions in the same day of the chosen method analyzed in triplicate yielded 66%, 40% and 37% respectively. This led to an investigation of all the possible variations that could occur during the analysis.

It was found that the shaking of the tube after separation of the phases might lead to a variation in the composition of oil and water of the sample taken since the separation of the phases starts very quickly and it is hard to get a homogenous sample. If the composition of the oil (in which vitamin C is practically insoluble) and the water (in which vitamin C dissolves very easily) is varying, the concentration of vitamin C will vary a lot. It also increases the risk of injecting oil into the column since it might not be separated from the extract during filtration. Instead, a certain time (2 min) was set for the two phases to separate before collecting an aliquot from the water phase.

The higher yield In the first extraction might be partially due to water from washing the holder of the filter paper used. Because of the relatively large volume, it diluted the next sample filtered. This was eliminated by changing to a small syringe filter with a smaller volume in the final extraction method.

The same procedure was then studied again. Three repeated extractions in the same day now yielded 43%, 45% and 45% with Milli Q water at room temperature and 44%, 48% and 48% with Milli Q water

at 50℃. Because of the very small difference in yields and the higher risk of degradation with high temperature, water at room temperature was chosen for the final method.

4.2 Validation

4.2.1 Repeatability–- intra-day precision

The intra-day precision study was conducted by extracting vitamin C in accordance with the sample preparation method chosen for stability testing and analyzing the filtrate in triplicate. This was repeated three times to a total of 9 injections. RSD values were calculated using Equation 1.

TABLE 11. RSD VALUES FOR PEAK AREA, RT AND PEAK HEIGHT OF THREE IDENTICAL EXTRACTIONS OF VITAMIN C FROM SERUM PERFORMED WITHIN THE SAME DAY.

Extraction, number:	RSD, peak area (%)	RSD, RT (%)	RSD, peak height (%)
1	3.8	1.0	7.4
2	4.3	0.4	7.5
3	3.0	1.0	4.2

4.2.2 Repeatability–- inter-day precision

Table 15, Table 16 and Table 17 (See Appendix) show data from analysis of 0.06 mg/ml ZnA in Milli Q water. This corresponds to the lowest concentration of the calibration curve and also the lowest concentration of the standards injected prior to the analysis of the stability samples. Table 18, Table 19 and Table 20 (See Appendix) show data from analysis of 0.6 mg/ml ZnA in Milli Q water. This corresponds to the highest concentration of the calibration curve and also the highest concentration of the standards injected prior to the analysis of the stability samples. The F-test shows a value well above the tabulated value (*20*), for both concentrations. This indicates a larger variation between the results obtained on different days compared to results obtained the same day.

In the stability testing for example, this means that the results at the same time-point between different storage conditions tested are exposed to the same bias coming from variations in the laboratory setting. The mean values might all be too high or too low (accuracy), but the STD (precision) is not affected. Between different time-points of the same storage conditions tested, the results have different bias affecting it, making the results spread more when the analysis is performed on different days. One day the results may be too high and the other day too low, making the difference look larger than it truly is.

4.2.3 Linearity

The calibration curve ranging from 0.06 to 0.6 mg/ml with a R^2-value of 0.997 indicates linearity within this range (see Figure 3 and Table 12).

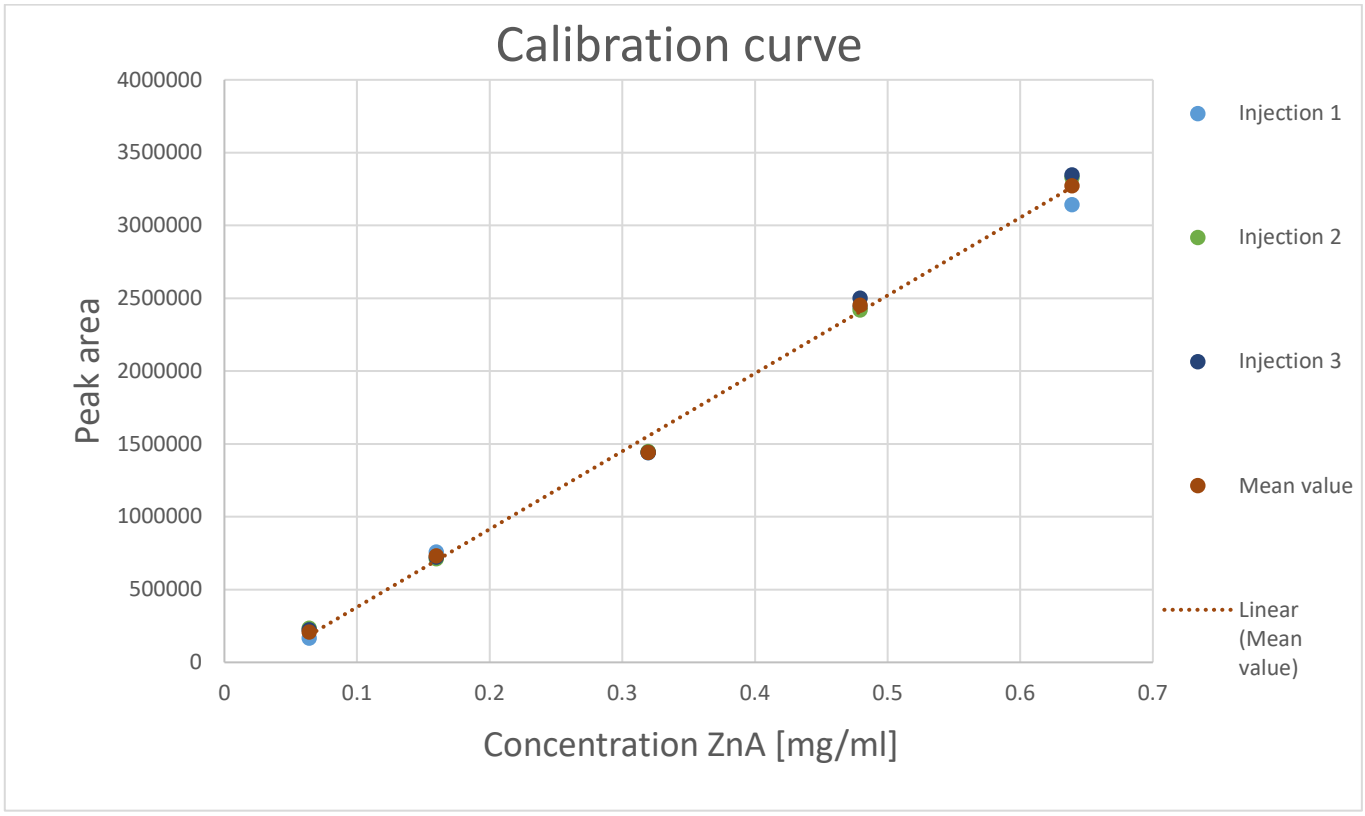

FIGURE 3. CALIBRATION CURVE OF ZNA IN MILLI Q WATER.

R^2-VALUE	SLOPE	INTERCEPT	EQUATION
0.997	**5350000**	**-155000**	**Y=5350000X-155000**

TABLE 12. CALIBRATION CURVE. NUMERICAL VALUES

A residual plot (see Figure 4) was made by plotting the obtained difference between the calculated value from the calibration curve equation and the obtained value from the injection peak for each injection at every concentration level to see the pattern. This can separate random errors from systematic errors. Random errors should be evenly spread out (i.e., have equal numbers of data points below, as well as above the calculated value.

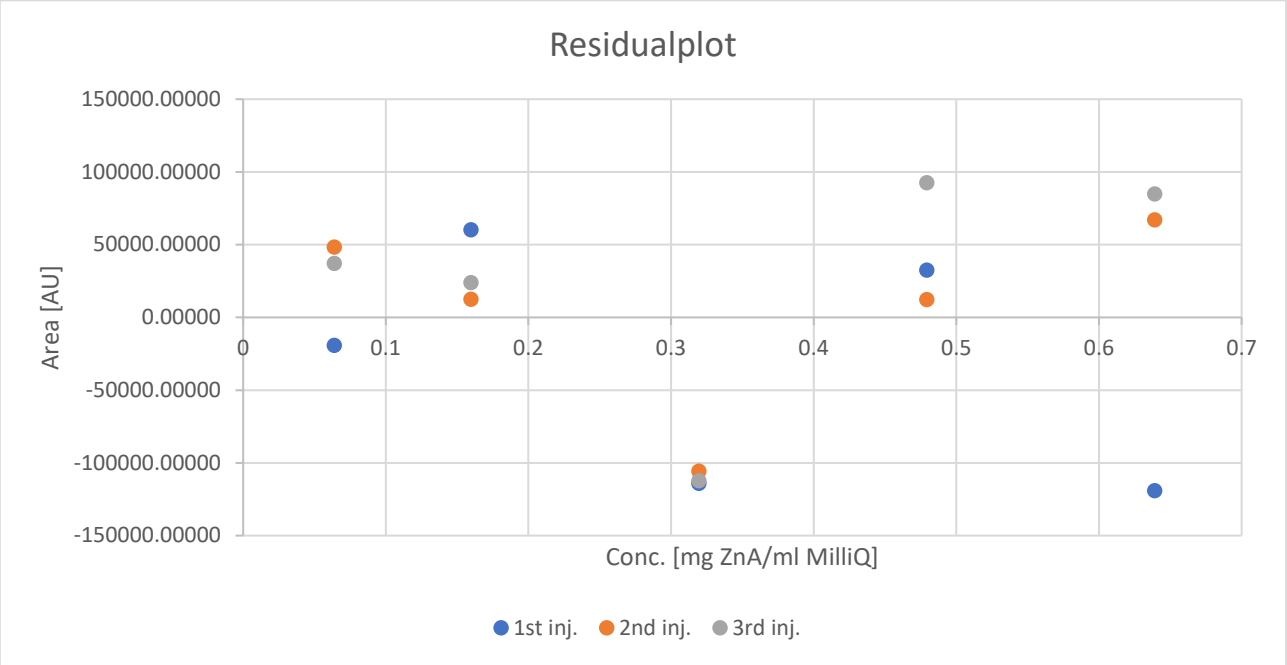

FIGURE 4. RESIDUAL PLOT FROM THE CALIBRATION CURVE. THE VERTICAL AXIS SHOWS THE AREA AND THE HORIZONTAL AXIS SHOWS THE CONCENTRATION OF THE CALIBRATION SOLUTIONS IN MG ZNA/ML MILLI Q.

The residual plot showed a suspicious pattern at the mid-point on the curve. The mid-point which would correspond to a 100% yield from an extraction lays lower than the calibration curve. If this point is accurate it results in a systematic error in all of the calculations of the yield, giving a lower result than the true value. That means that the true value of vitamin C in the serum might be slightly higher than what the results show in the stability study.

4.2.4 Specificity

No interfering peaks were shown when analyzing serum without ZnA. Neither did interfering peaks show up when analyzing ZnA in Milli Q water where a substantial amount had been degraded. This shows that the method is specific for ZnA.

4.3 Stability testing of serum

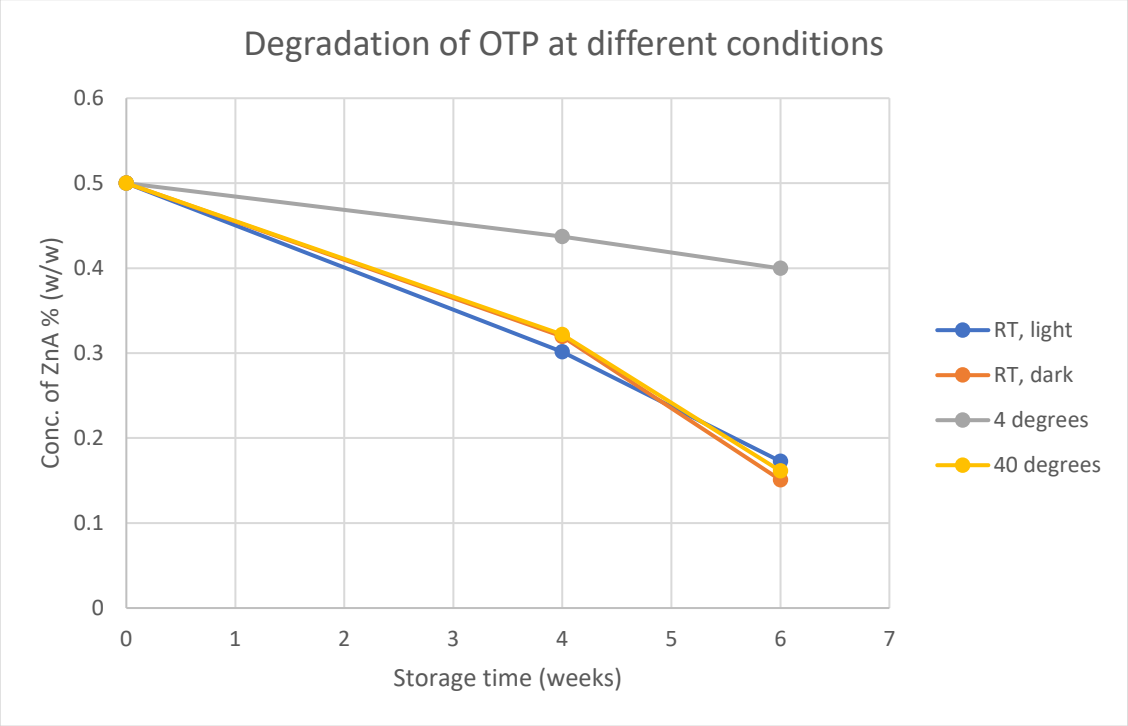

FIGURE 5. DEGRADATION OF OTP AT DIFFERENT STORAGE CONDITIONS OVER TIME.

The results show clearly that the lowest temperature in the study (kept at 4℃) is more favorable for the stability of vitamin C in OTP than the higher temperatures (see Figure 5). This was expected since several studies show that degradation of vitamin C is enhanced at higher temperatures.

The serum, both OTP and MTP, analyzed at 40℃ had severely decomposed already at the first measuring-point (4 weeks). It had a brown color and consisted of a liquid with aggregated material (see Appendix, Figure 11 and Figure 12). The inhomogeneity of the serum made the analysis of it less likely to obtain a reliable result. It would be expected for the higher temperature for vitamin C to degrade faster than in room temperature, unlike what the results are showing. The change in color would also indicate a degradation of light sensitive ingredients, including vitamin C.

Another study showed however that after storage at 45℃, a W/O emulsion containing 0.1% (w/w) from the beginning, showed no vitamin C content after 20 days. The water content in this emulsion was a lot higher though, 71 % (w/w). (*16*) This would suggest that a lower water content might have protected vitamin C from degradation.

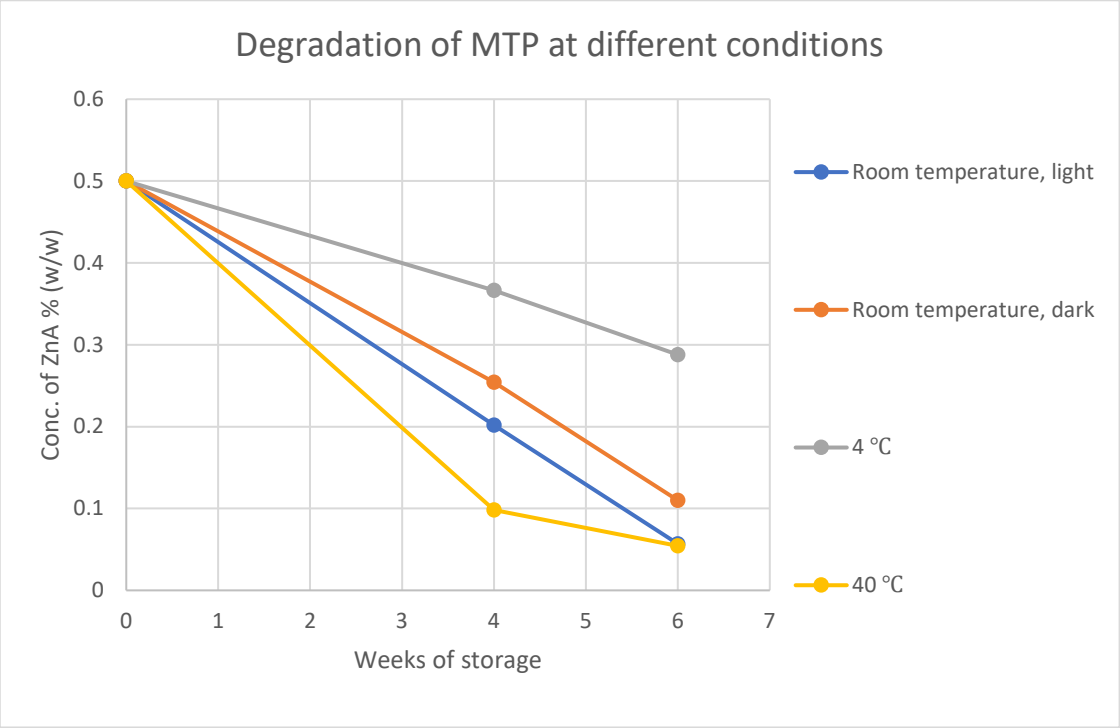

FIGURE 6. DEGRADATION OF MTP AT DIFFERENT STORAGE CONDITIONS OVER TIME.

The results for MTP at the different storage conditions showed a similar pattern to that of OTP. It did however degrade at a higher speed (see Figure 6).

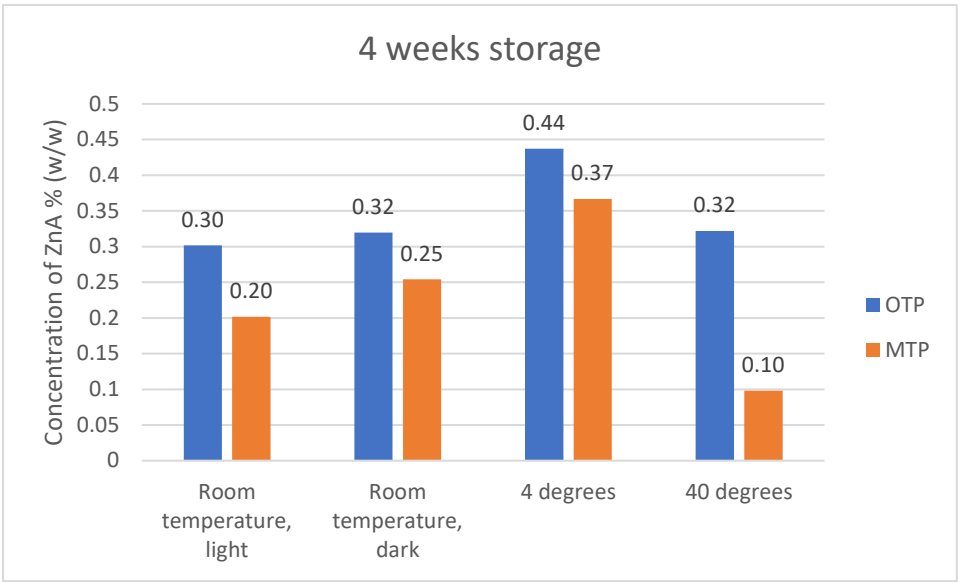

FIGURE 7. COMPARISON BETWEEN OTP AND MTP AT 4 WEEKS OF STORAGE

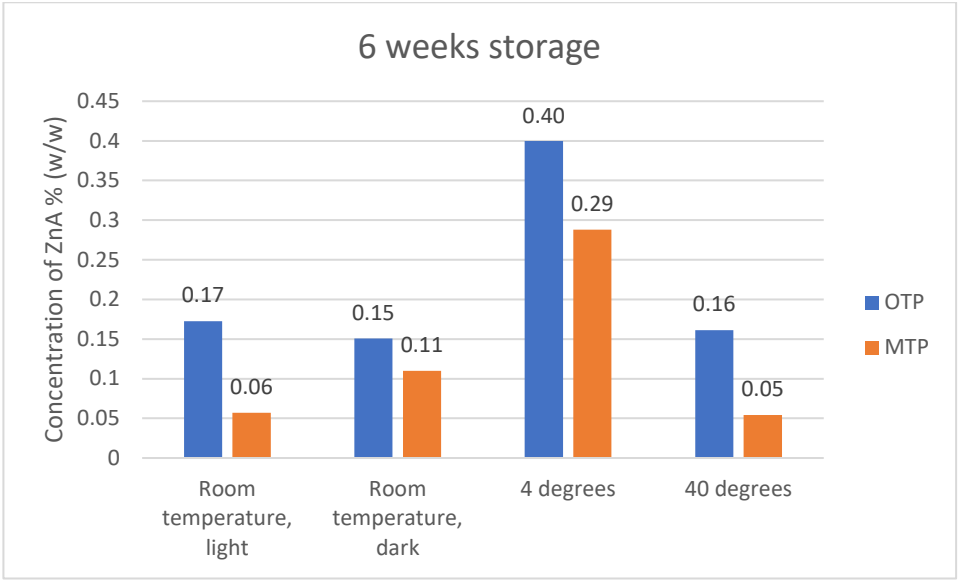

FIGURE 8. COMPARISON BETWEEN OTP AND MTP AT 6 WEEKS OF STORAGE

MTP showed throughout a lower concentration than OTP at both 4 and 6 weeks of storage in different storage conditions (see Figure 7 and Figure 8). This was unexpected since a lower pH, as in MTP has shown to stabilize vitamin C (*16*). The result might be influenced by MTP being formulated 9 days earlier than OTP.

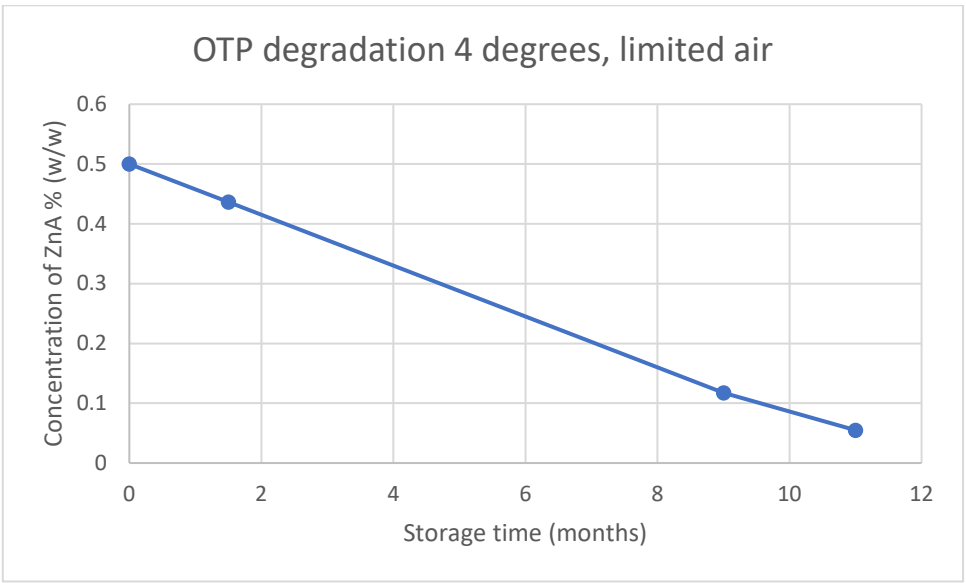

FIGURE 9. DEGRADATION OF OTP UNDER OPTIMAL STORAGE CONDITIONS.

It has been strongly suggested that the presence of oxygen has a great impact on the rate of degradation of vitamin C. One study show that at an excess of oxygen, the degradation follows pseudo-first order kinetics, thus only depends on the concentration of vitamin C, and is independent on the concentration of oxygen. When the oxygen in the headspace of the jar has been consumed to a

certain level, the degradation no longer show this pattern but is slowed down due to the limited access to oxygen (*16*). The opening and closing, and also stirring in a jar with serum introduces new oxygen and can severely accelerate the degradation compared to a jar kept closed. Figure 9 shows the degradation of OTP stored in refrigerator in closed containers.

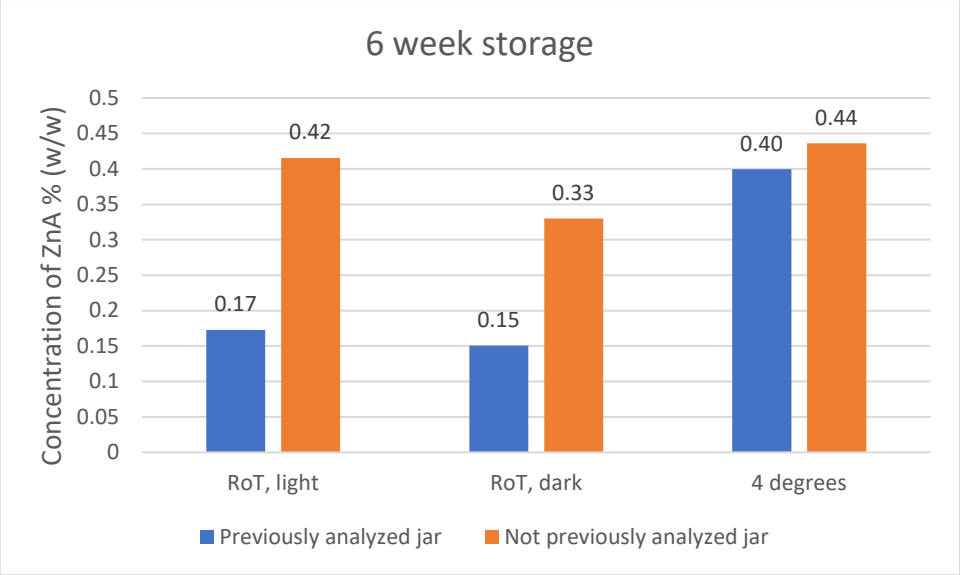

FIGURE 10. COMPARISON BETWEEN PREVIOUSLY OPENED AND ANALYZED JARS (AT THE 4 WEEK TIME-POINT) AND NOT PREVIOUSLY OPENED AND ANALYZED JARS OF OTP.

The serum for stability testing was not weighed and the jars were not filled to the top to eliminate the effect of oxygen present before the start of the stability study. At the 6-week time-point in the study, a new jar, not previously analyzed was chosen by visually selecting a jar with less air inside than the previously analyzed jar. The results show a large effect, especially on the serums kept in room temperature (see Figure 10). A new jar stored in 40℃ was not analyzed after 6 weeks due to the inhomogeneity of the serum.

4.4 Stability testing of calibration standards

TABLE 13. THE PERCENTAGE LEFT OF THE KNOWN VALUE AFTER STORAGE OF ZNA IN MILLI Q WATER (0.3 MG/ML) UNDER DIFFERENT CONDITIONS.

Time	4 ℃	Room temperature	-18 ℃
0	-	95	-
3 h	71	33	86

The stability testing of the calibration standard shows a rapid degradation, largely depending on the temperature of the solution. This shows the importance of a fast handling of the calibration standards and also of the filtrates from the extractions of vitamin C from serums, which mostly consists of water and vitamin C and perhaps other water-soluble components of the serum. Another study shows results of 93.4%, 91.6% and 86.4% non-degraded ascorbic acid (1.0% w/w) in water solution after 1, 2

and 4 hours respectively under aerobic conditions, pH 7.0 at 45℃ (*16*), which is contradictive to the results in this study. One difference was the concentration, which was roughly 0.0003% (w/w) in this study. One possibility is that there is a concentration dependence on the degradation rate.

5 Conclusions

The HPLC-method developed in this project showed linearity of ascorbic acid from 0.06 mg/ml to 0.6 mg/ml in a water solution. This corresponds to a content of 1-10 % w/w of vitamin C in the test product when extracting vitamin C according to the developed extraction method, making it suitable for analysis of products with a vitamin C content in this range.

No interfering peaks were showing from a degraded sample of a water solution containing vitamin C which means that the method is suitable for stability studies of vitamin C. No interfering peaks were showing from OTP without ZnA either, meaning that for OTP and possibly other similar products, the HPLC- and extraction method is specific for analysis of vitamin C.

The mean recovery of vitamin C from freshly made OTP (0 days old) from 3 extractions was 92.4% of the theoretical value with RSD 3,4%. This shows that the method is accurate and repeatable on a satisfying level.

The degradation rate of vitamin C was faster in MTP than in OTP. The slowest degradation rate was found at the lowest temperature (4℃) and the lower oxygen content among the ones studied. A linear relationship was found between storage time and the vitamin C concentration left in the product at this storage condition. However, only 10% of the initial concentration of vitamin C was left in the product after almost 12 months storage, even at this favorable storage condition. This suggests that a limitation in contact with oxygen as well as a cold storage is preferred. To prolong the life of vitamin C further, more studies need to be performed. A product with a lower water content, addition of antioxidants or another formulation such as a W/O/W emulsion might help to protect vitamin C from degradation.

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7 Appendix

TABLE 14. THE DILUTION OF THE CALIBRATION SOLUTIONS

Calibration number	Stock solution [µl]	MilliQ [µl]	Dilution	Conc. [mg/ml]
1	10	990	1:100	0.06
2	25	975	1:40	0.15
3	50	950	1:20	0.3
4	75	925	1:13.3	0.45
5	100	900	1:10	0.6

FIGURE 11. OTP, AFTER 4 WEEKS OF STORAGE AT 40℃, BEFORE STIRRING (TO THE LEFT) AND AFTER STIRRING (TO THE RIGHT).

FIGURE 12. MTP, AFTER 4 WEEKS OF STORAGE AT 40℃, BEFORE STIRRING (TO THE LEFT) AND AFTER STIRRING (TO THE RIGHT).

TABLE 15. THE DIFFERENT PEAK AREAS, THEIR MEAN VALUES, STD AND s^2 OBTAINED FROM THREE INJECTIONS MADE AT FIVE DIFFERENT DAYS FOR INTER-DAY PRECISION ANALYSIS OF 0.06 MG/ML ZNA IN MILLI Q WATER.

Date of injection	Area (1st injection)	Area (2nd injection)	Area (3rd injection)	Area (mean value)	STD	s^2
2022-05-11	52313	84669	68082	68355	16180	261783444
2022-05-12	101361	117508	114202	111024	8530	72757754
2022-05-18	95601	127133	124670	115801	17537	307556692
2022-05-19	118962	128347	138423	128577	9733	94722420
2022-05-31	74450	88308	84492	82417	7158	51241297

TABLE 16. MS, SS AND DEGREES OF FREEDOM CALCULATED FOR INTER-DAY PRECISION ANALYSIS OF 0.06 MG/ML ZNA IN MILLI Q WATER.

	Mean square (MS):	Degrees of freedom:	Sum of squares (SS):
Within-sample	262687203	10	2626872029
Between-sample	3736267865	2	7472535730

EQUATION 2.

$$SS\ within-sample \sum_i \sum_j \left(x_{ij} - \bar{x}_i\right)^2 / h(n-1)$$

EQUATION 3.

$$SS\ between-sample = n \sum_i (\overline{x_i} - \overline{x})^2 / (h-1)$$

EQUATION 4.

$$Degrees\ of\ freedom\ between-sample = h-1$$

EQUATION 5.

$$Degrees\ of\ freedom\ within-sample = h(n-1)$$

Equation 2, 3, 4 and 5 describes the calculations made in Table 16 and Table 19, where i is the injection number, j is the sample number, h is the total number of samples and n is the total number of injections. MS is calculated by dividing SS with the degrees of freedom.

TABLE 17. THE OVERALL MEAN OF THE AREAS, THE VALUE CALCULATED FROM THE F-TEST AND THE TABULATED VALUE FOR COMPARISON, FOR 0,06 MG/ML ZNA IN MILLI Q WATER.

Overall mean of peak areas:	F-test (calculated value)	P=0.05 (tabulated value)
101235	14.223	4.103

EQUATION 6.

$$F-value = \frac{MS\ between-sample}{MS\ within-sample}$$

Equation 1 and Equation 6 describes the calculation of the F-value in Table 17 and Table 20. Note that the F-value is defined so that F is always ≥ 1.

TABLE 18. THE DIFFERENT PEAK AREAS, THEIR MEAN VALUES, STD AND S^2 OBTAINED FROM THREE INJECTIONS MADE AT FIVE DIFFERENT DAYS FOR INTER-DAY PRECISION ANALYSIS OF 0.6 MG/ML ZNA IN MILLI Q WATER.

Date of injection	Area (1st injection)	Area (2nd injection)	Area (3rd injection)	Area (mean value)	STD	s^2
2022-05-11	2710331	2728751	2708385	2715822	11239	126309545
2022-05-12	2904121	3059528	2827070	2930240	118410	14020819002
2022-05-18	3005336	2975088	2906686	2962370	50540	2554266268
2022-05-19	3024800	3095127	3051490	3057139	35502	1260405133
2022-05-31	2840894	2822442	2760459	2807932	42135	1775359636

TABLE 19. MS, SS AND DEGREES OF FREEDOM CALCULATED FOR INTER-DAY PRECISION ANALYSIS OF 0.6 MG/ML ZNA IN MILLI Q WATER.

	Mean square (MS):	Degrees of freedom:	Sum of squares (SS):
Within-sample	6579053195	10	65790531950
Between-sample	107632033242	2	215264066484

TABLE 20. THE OVERALL MEAN OF THE AREAS, THE VALUE CALCULATED FROM THE F-TEST AND THE TABULATED VALUE FOR COMPARISON, FOR 0.6 MG/ML ZNA IN MILLI Q WATER.

Overall mean of peak areas:	F-test (calculated value)	P=0.05 (tabulated value)
2894701	16.360	4.103

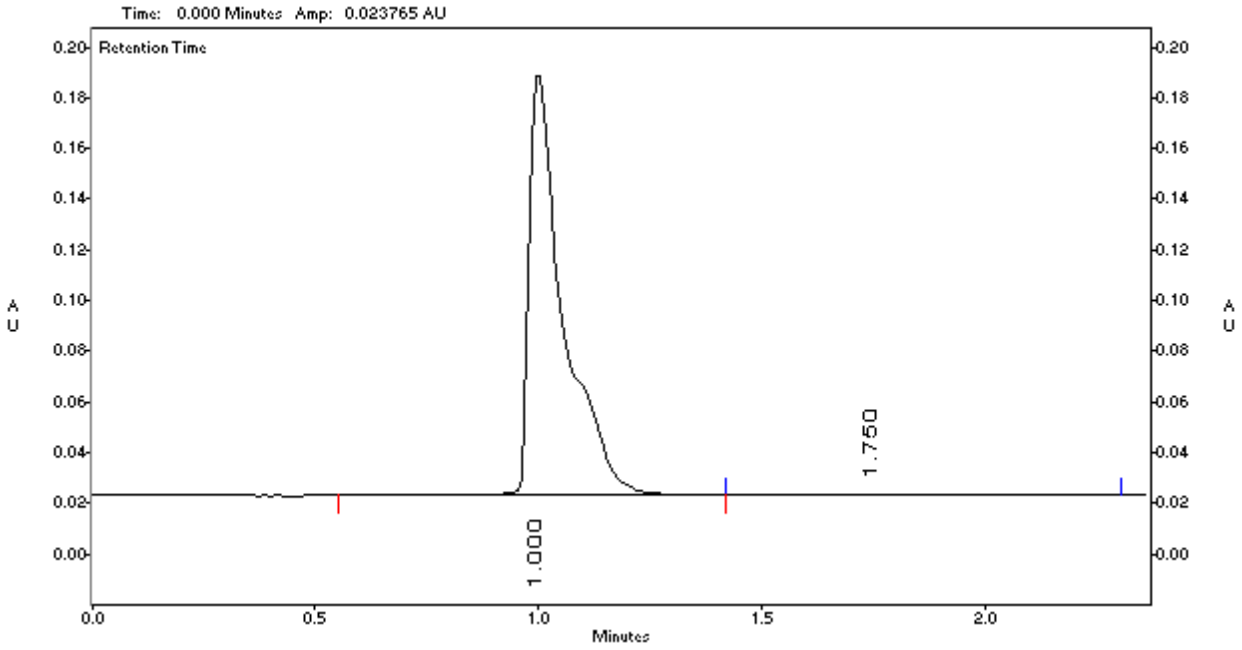

FIGURE 13. CHROMATOGRAM WITH COLUMN 1, SHOWING SIGNIFICANT TAILING AND SHORT RT.

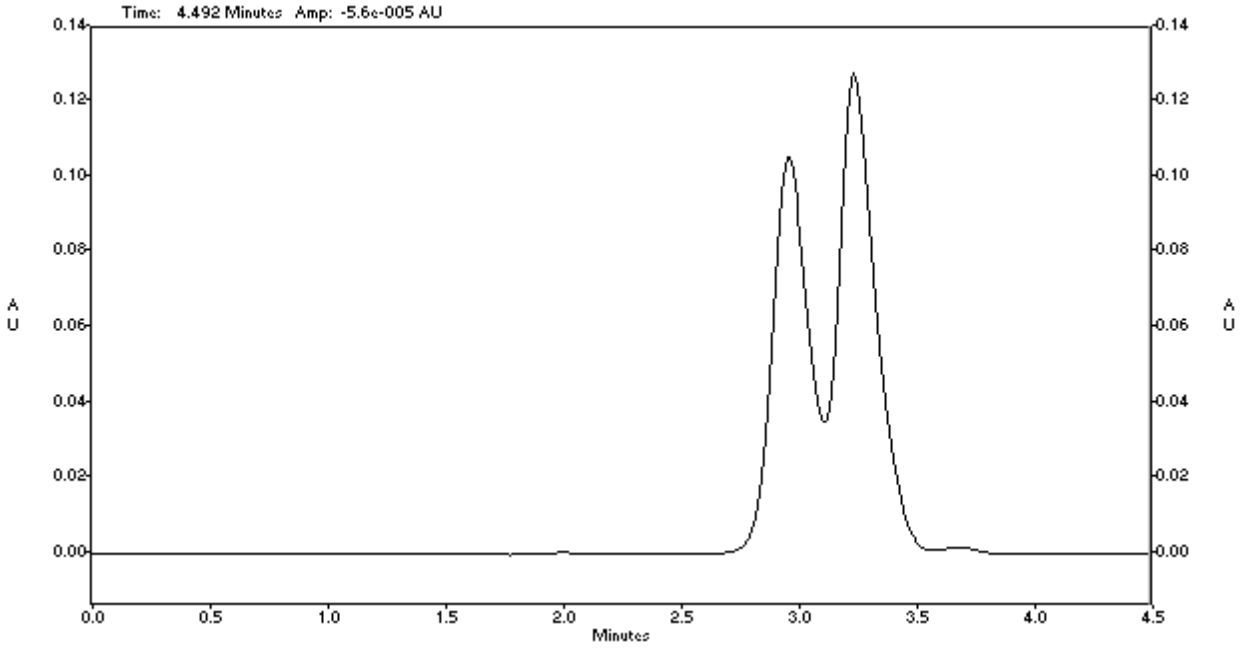

FIGURE 14. CHROMATOGRAM FROM COLUMN 3 SHOWING SIGNIFICANT PEAK SPLITTING.

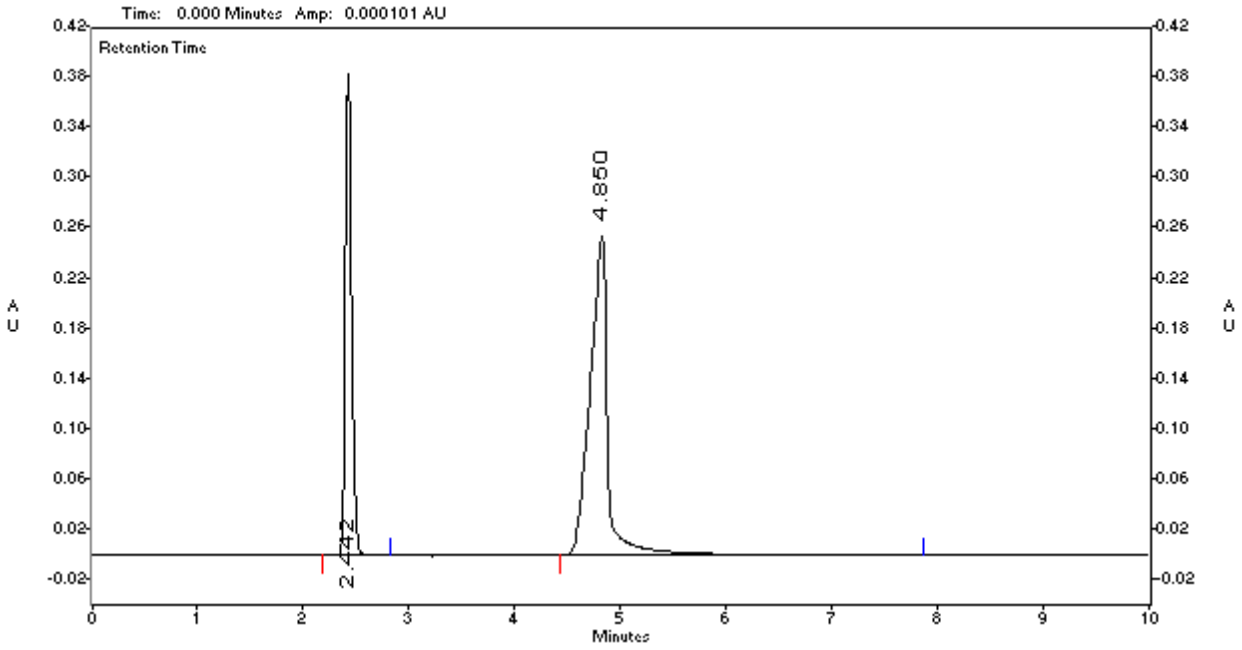

FIGURE 15. CHROMATOGRAM FROM STABILITY TESTING SHOWING ZNA AT 2.442 MIN AND LATER ANOTHER PEAK FROM ANOTHER WATER SOLUBLE COMPONENT OF THE SERUM, ALWAYS PRESENT IN CHROMATOGRAMS FROM EXTRACTIONS OF SERUM IN THIS STUDY.

EQUATION 7.

$$Calculated\ concentration\ of\ ZnA\ in\ Milli\ Q\ water\ \left[\frac{mg}{ml}\right] = \frac{\bar{x}_{area} + intercept}{slope}$$

Where the intercept and slope is taken from the equation of the calibration curve.

EQUATION 8.

$$Theoretical\ concentration\ of\ ZnA\ in\ Milli\ Q\ water\ \left[\frac{mg}{ml}\right] = \frac{pm \cdot 10}{v}$$

Where p is the percentage (w/w) of ZnA in the serum, m is the mass of the serum weighed for analysis in grams, and v is the volume of water added, in milliliter.

EQUATION 9.

$$Yield\ (\%) = \frac{Calculated\ concentration\ of\ ZnA\ in\ Milli\ Q\ water}{Theoretical\ concentration\ of\ ZnA\ in\ Milli\ Q\ water}$$