Applied Chemistry Project

Project Title: Determination of α-casein protein in pasteurised and UHT nonfat bovine milk using RP-HPLC

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Program: Bachelor of Science in Applied Chemistry
Academic Year: 2020

Faculty of Science, Chulalongkorn University

Determination of α-casein protein in pasteurised and UHT nonfat bovine milk using RP-HPLC

by

Chayanitasa Punyathanarwat

In Partial Fulfilment for the Degree of Bachelor of Science

Program in Applied Chemistry (International Program)

Department of Chemistry, Faculty of Science

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ABSTRACT

In this study, α-casein proteins in pasteurised and UHT nonfat bovine milk available in the Bangkok supermarket are detected and quantified by RP-HPLC. Two brands of pasteurised milk and one brand of UHT nonfat milk were studied. The gradient elution was carried out using a mixture of two eluents at 1 mL/min flow rate, and column temperature of 25°C; Solvent A was water, acetonitrile, and TFA in a ratio of 900:100:1 (v/v/v); Solvent B was water, acetonitrile, and TFA in a ratio of 100:900:1 (v/v/v). The signal was monitored by a UV detector at a wavelength of 220 nm. The gradient elution program was set from 20-80% (B) for 0-23 min and hold at 80% (B) for 2 min. Thus, the total time of a single run was 25 min. As a result, pasteurised milk contain more α-casein content than UHT milk. The concentration of α-casein in pasteurised milk were 33.7, 30.1 g/L and in UHT milk was 28.1 g/L. The aim of this study is to provide nutrition information for customers.

Keywords: α-casein, RP-HPLC, Pasteurised Milk, UHT milk

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LIST OF ABBREVIATIONS

CN	casein
α-CN	alpha-casein
α_{S1}-CN	alpha-S_1-casein
α_{S2}-CN	alpha-S_2-casein
β-CN	beta-casein
k-CN	kappa-casein
RP-HPLC	Reversed-phase High Performance Liquid Chromatography
UHT	Ultra High Temperature
H-Bond	Hydrogen bond
2D	2-Dimentional
Tris	Tris (hydroxymethyl) aminomethane
TFA	Trifluoroacetic acid
β-ME	β-mercaptoethanol
ACN	Acetonitrile
USDA	The United States Department of Agriculture
RSD	Relative Standard Deviation

CHAPTER 1: INTRODUCTION

1.1 Introduction to research problem and significance

Milk products are popular worldwide. They were used as the main ingredient in many different kinds of food and beverage including yoghurt, ice-cream, cheese, butter, etc. According to Food and Agriculture Organisation of the United Nations, there are over 6 billion people consume milk and milk products in 2018 and in places as North America and Europe, where dairy consumption per capita is more than 150 kg/capita/year.[1] Although, at present, the dairy consumption in China and South East Asia countries is low (less than 30 kg/capita/year), it is expected to grow faster in the next decade leading to an increase in dairy import.[1] Aside from the delicious taste, milk products are one of the good sources of proteins and calcium for human since all essential amino acids required by humans can be found in milk.[2] However, it has been studied that dairy consumption can increase the proliferation of cancer cells.[3]

The experimental results conducted by Research Institute of Biomedical Science and Technology, Pusan National University show that the most abundant milk protein, casein protein, can significantly increase proliferation of the prostate cancer cells in the human body. The α-casein and other casein proteins increase proliferation by 228% and 166%, respectively.[3] For this reason, people should aware of the concentration of casein protein in different types of milk products. In accordance with Amy Shapiro, the founder and director of Real Nutrition in New York, although adult needs protein at 0.8 g/kg of body weight on daily basis,[4] casein protein consumption should be less than 30 g each day.[5]

Casein protein is only present in the animal,[6] such as bovine milk, which is the most produced milk worldwide.[7] Additionally, skim milk products are gaining their popularity among customers and the trend of consumption is expected to increase.[1] This inspires our project to investigate the concentration of α-casein protein in pasteurised and UHT nonfat bovine milk using Reversed-phase High-Performance Liquid Chromatography (RP-HPLC) to provide the nutrition fact that may be beneficial to people.

1.2 Research Objectives

There are two objectives for this project. (1) Apply Reversed-phase High Performance Liquid Chromatography (RP-HPLC) method for determination of α-casein proteins in milk samples. (2) Quantify α-casein protein content in selected pasteurised and UHT nonfat bovine milk brands available in Bangkok supermarket to provide nutrition information for customers.

1.3 Literature search

1.3.1 Milk Protein

There are more than 100 different proteins in milk, which they are classified into three major groups based on nitrogen fraction: casein protein (CN), whey protein (serum protein), and non-protein nitrogenous compound (NPN).[8] On average, the abundance of caseins, whey proteins, and non-protein nitrogenous compound of total milk protein are 80%, 18%, and 2%, respectively.[9]

According to the USDA nutrient database, bovine milk contains 3.15%[10] of protein, where the total milk protein content fluctuates with individual animal genetics, breed, health, and stage of lactation.[9] The nutrient content of bovine milk is illustrated in Table 1.[10]

Table 1: Nutrient content of bovine milk by 100 g reference amount

Overall Composition	Bovine Milk	Unit
Water	88.13	g
Energy	61	kcal
Protein	3.15	g
Total lipid (Fat)	3.25	g
Carbohydrate	4.8	g

1.3.2 Casein Protein

Casein protein is the most abundant milk protein that 80% of the total milk protein is caseins.[8] There are four fractions of casein protein: alpha-S_1-casein (α_{S1}-CN), alpha-S_2-casein (α_{S2}-CN), beta-casein (β-CN), and kappa-casein (k-CN).[9] The abundance of αS_1-,αS_2-,β-, and k-CN are as follow: 38%, 10%, 35%, and 12%.[9] Each casein fraction has its amino acid composition, genetic variation, and functional properties. Caseins are usually precipitate at pH 4.6.[11] They are rich in essential amino acids and have an average molecular weight of 2062 g/mol.[12] The molecular

formula of casein protein is $C_{81}H_{125}N_{22}O_{39}P$ and the 2D structure of the molecule is displayed in Figure 1.[12]

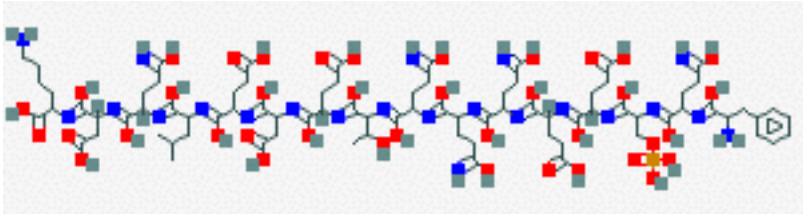

Figure 1: *Chemical structure of Casein Protein*[12]

Casein protein is conjugated protein that has porous micellar structure, acquiring about 6-12% of the total volume fraction of milk.[13] It is mostly conjugated with the phosphate group, which helps to stabilise the micelle through calcium-binding. The calcium binding by the individual casein is proportional to the phosphate content.[13] It has been investigated that more than 90% of calcium content in skim milk is involved with the casein micelle.[13] Therefore, the complex structure of casein is difficult to quantify straightforwardly.[8]

1.3.3 Protein Denaturation

Due to protein aggregating nature, denaturation is required when working on protein analysis to avoid errors.[9] Protein denaturation is the process by which proteins lose their folded structure and cease to function.[14] Protein denaturation can be brought by various external stress, for instance: exposure to heat, acid or alkaline, oxidising or reducing agents, urea, and organic solvents. These exposures do not have an impact on the primary structure but they affect the secondary and tertiary structure by weakening and breaking the bonds.[15]

- Heat can be used to denature proteins via interrupting hydrogen bonds and non-polar hydrophobic interactions. Since heat increase kinetic energy, it causes the molecule to move and vibrate violently which could lead to bond breaking.[16] Milk proteins are denatured above 65°C.[17]

- Addition of acid or alkaline denatures the proteins by disrupting the salt-bridge,[16] the bonds between oppositely charged residues that contribute to protein structure[18] arise from the neutralisation of acids and amines on the side chain.[16] The positive and negative ions in the salt-bridges would change partners with the positive and negative ions introduced from the addition of acid or alkaline.[16] Proteins would completely be denatured if the pH is below 2.5 or above 11.5.[19]

- Exposure to oxidising or reducing agent involves disturbing the disulphide bonds. The reducing agents break the disulphide bonds in the protein, while the oxidising agents can generate new disulphide bonds to where they don't belong.[16] This causes a loss of structure.

- Urea denatures proteins through direct interaction that disrupt the H-bonding at the surface of the proteins. The hydrogen bonds from urea polarise charged area, such as peptide groups, which enervate the intermolecular bonds and interactions. Consequently, the secondary and tertiary structure are all debilitated and the protein structure is unfolded.[20] The chemical structure of urea is shown in Figure 2.

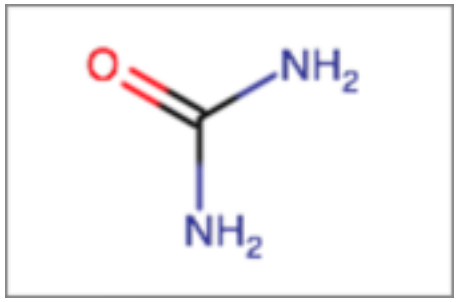

Figure 2: *Chemical structure of urea*

- The addition of organic solvent especially ethers and alcohol can disrupt the intermolecular H-bonds within the protein molecules by forming new bonds with the protein side chains. Moreover, this allows them to break through the bacteria cell wall and denature both proteins and enzymes inside of the cell.[16]

1.3.4 Pasteurisation and Ultra High Temperature process

Pasteurisation and Ultra High Temperature (UHT) process are the most popular milk treatment methods used for expanding the shelf life and eliminating bacteria. Pasteurisation involves heating milk to 72-75°C for 15-20 seconds and immediately cool it to less than 3°C.[21] UHT process requires more step. It begins with preheating stage that can be proceeded either with or without a holding time, where the temperature would rise to around 90°C. Next, the milk is heated to sterilisation temperature (137-145°C) and hold about 5 seconds before rapidly cooled.[22] After pasteurisation and packaging, a small group of bacteria may remain and can slowly grow during storage at low temperature. On the other hand, the UHT process heats the milk to a higher temperature to inactivate spore-forming bacterias from growing during storage and causing

spoilage. Therefore, UHT milk has a longer shelf life than pasteurised milk and can be stored at room temperature.[22]

In comparison to raw milk, pasteurisation and UHT process alter some of the nutritional value. For example, it decreases a minimal level of iodine, vitamin B12, and digestive enzyme.[23] In addition, as stated in section 1.2.3 that heat is one factor involving in denaturing milk proteins through weakening and breaking bonds, it has been proved that heat treatment processes irreversibly destroy and coagulate whey proteins in milk. However, casein proteins are heat-stable proteins and hardly coagulated when exposed to high temperature.[24] Nevertheless, it is claimed by one article in the International Dairy Journal that pasteurisation and UHT process do not affect the quality of proteins.[25]

1.3.5 Analysis Techniques

1.3.5.1 RP-HPLC

Reverse-phase High Performance Liquid Chromatography (RP-HPLC) is one of the chromatographic methods that is commonly used to separate low polarity compounds.[8] This separation technique is based on the partition of hydrophobic interaction between the static non-polar stationary phase (C4, 8, or 18) and solute molecules from the polar mobile phase.[26] Generally, the solutes are eluted by the addition of eluent (organic solvent) to the mobile phase. The increment of organic solvent increase eluent strength and solubility of solutes in the mobile phase than in stationary phase. Elution can be proceeded by either isocratic or gradient condition. Isocratic elution is when the concentration of a single solvent or solvent mixture remains constant. While gradient elution involves two solvent systems that differ in polarity and the concentration of the solvents increases overtime. In this system, the solutes are eluted in the order of increasing molecular hydrophobicity.[26] Compared to other liquid chromatographic techniques, HPLC advantages in time efficiency, separation capacity, preciseness of the result, and highly reproducible because it is largely automated.[27]

There are seven major components in HPLC system: (1) mobile phase, (2) degasser, (3) pump, (4) sample injector, (5) column, (6) detector, and (7) computer.[28] The schematic diagram of the HPLC instrument is demonstrated in Figure 3.

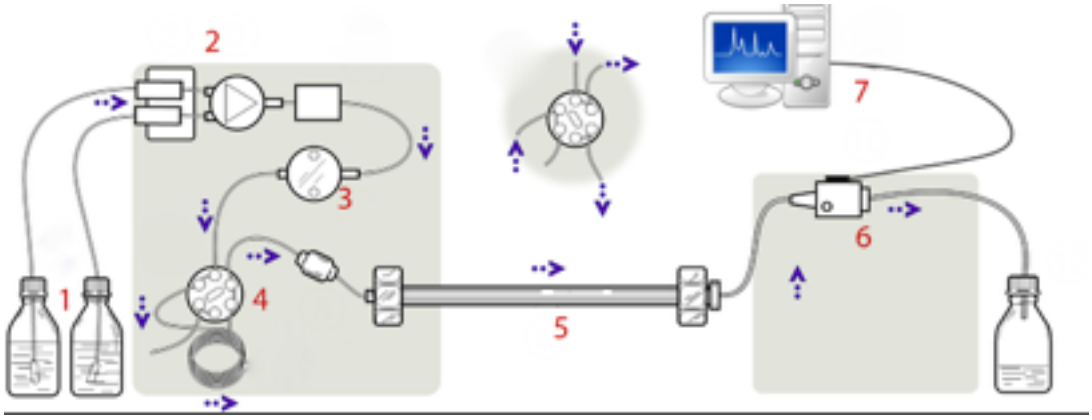

Figure 3: *Schematic diagram of HPLC instrument*[28]

Role of each component[28]:

(1) Mobile Phase: Transport the sample through the HPLC system.

(2) Degasser: The degassing system removes gases from the solvent. This step is important because air bubbles that are generated from the solution could alter the flow of mobile phase and affect the accuracy of the data.

(3) Pump: Mix the mobile phase and deliver them to the system at the desired flow rate.

(4) Injector: Introduce liquid samples to the system.

(5) Column: Separate sample component.

(6) Detector: Detect individual molecules released from the column.

(7) Computer: Convert the signal from the detector and display data in the chromatogram.

1.3.5.2 DATA INTERPRETATION

Retention Time

The retention time (t_R) is the time taken for a sample to travel through the column from the injection point to the detector. On the chromatogram, the retention time of a certain molecular species is taken at the apex of the peak.[28] Retention time is often used for qualitative analysis. It plays a role in peak identification of the unknown sample by comparing it with the referenced data. If the retention time of the unknown sample matches with the existing data, then a confident identification can be made. However, some factors can cause shifts in retention time. For instance, flow rate, column temperature, characteristic of both stationary phase and mobile phase, leaks, etc.

[29] Therefore, the HPLC condition needs to be carefully controlled and should be as similar to the referenced data as possible.

Retention Factor

The retention factor (k) is a ratio of the duration of an analyte spends in the stationary phase to the mobile phase. The stronger the interaction between the analyte and the stationary phase, the longer the retention and the higher the retention factor.[30] It can be used to describe the chromatographic behaviour of a sample component in the column. The retention factor advantages over retention time in the analysis because it is independent of the column length and the flow rate of the mobile phase. The formula used for calculating retention factor for solute 'A' is demonstrated in Figure 4. The variable 't_R' and 't_M' stands for *retention time* and *dead time* (the retention time of a non-retained species).[31]

$$k_A = \frac{t_R - t_M}{t_M} = \frac{t_S}{t_M}$$

Figure 4: *Retention factor calculating formula*[32]

Sample Spiking

Sample spiking is a technique used to confirm the identity or peak of an analyte among the rest of sample component peaks by adding a known purified reference material to sample matrix.[32] A positive identification can be achieved if any of the peak within the chromatogram grow and retain symmetry. On the other hand, if the new peak is appeared or any of the peak develops a shoulder. Then, the analyte might be absence in the sample.[32] By the way, to ensure the accuracy of an analytical method, the recovery percentage is usually calculated from the spiked data by comparing the nearness of expected value (added amount) and resulted value, where, 100% recovery is desired. The calculating formula is shown in Figure 5.

$$\%\text{Recovery} = \frac{C_{\text{Spiked sample}} - C_{\text{non-spiked sample}}}{C_{\text{analyte}}}$$

Figure 5: *Recovery percentage calculating formula*

Calibration Curve and External Standard

A calibration curve (standard curve) is the graphical representation of the response signal to concentration.[32] It is one of the most powerful methods in the quantitative analysis since the curve is used to predict the concentration of the unknown analyte in a sample. A calibration curve is obtained by preparing a set of standards containing known amounts of the interested analyte, measuring the instrument response for each standard and constructing the relationship between the instrument response and analyte concentration. The linear relationship or the line of best fit is preferred.[33]

External standard quantification is one of the most common calibration methods. In this technique, both standards and unknown samples are separately analysed under the same process and condition.[32] There are two types of external standard quantification: single-point calibration and multi-level calibration. These two methods differ in response factor (ratio between signal and concentration of the analyte) determination. Single-point calibration method uses the origin point of the graph to determine the second point of the regression line. On the other hand, multi-level calibration uses at least two data points to determine the regression line as it involves preparing a wide range of standard solution concentrations.[32] For this reason, multi-level calibration of the external standard takes advantage over single-point calibration in terms of accuracy since the linearity is experimentally proved. The calibration curves of both single point and multi-level calibration are exhibited in Figure 6 and 7.

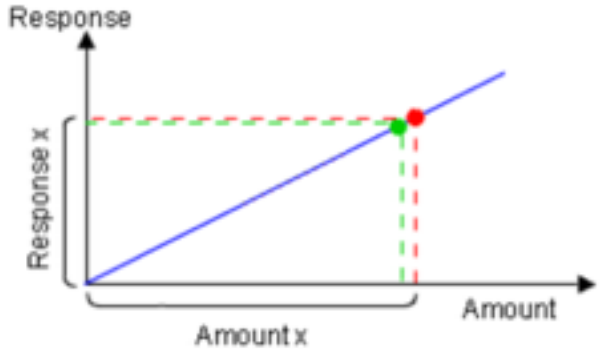

Figure 6: *Single point calibration of external standard*[30]

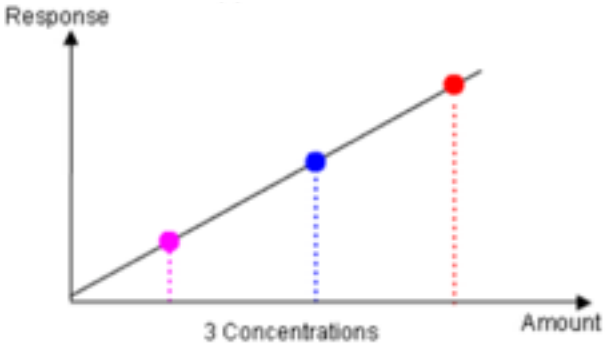

Figure 7: *Multi-level calibration of external standard*[30]

A linear relationship between the instrument response and analyte concentration from the calibration curve is usually express through equation $y=mx+c$. The variable 'y' represents instrument response, 'm' is the gradient (slope) or response factor, 'x' is analyte concentration, and lastly 'c' is the intercept on the y-axis. This linear equation will be used to quantify the unknown concentration of an analyte in the sample.

1.3.5.3 PREVIOUS STUDY AND INSPIRATION

Many published scientific papers study about casein protein in raw bovine milk. The techniques that have commonly been used for protein analysis include Bicinchoninic Acid assay (BCA), RP-HPLC, and variations of HPLC. For example, coupling HPLC with evaporative light scattering detector (ELSD), Electrospray Ionisation Mass Spectrometry (ESI-MS), etc. Our project decided to minimise sample preparation steps suggested by one research article published on the 'Journal of Chromatography A'[27] with RP-HPLC for both time and cost-efficiency. The distinctive point of our project is that we primarily focus on pasteurised and UHT nonfat bovine milk in different brands available in Thailand. The information about protein content in pasteurised and UHT nonfat bovine milk has not yet been explored.

CHAPTER 2: EXPERIMENTAL

2.1 List of equipment and instrument

HPLC Instrument

The Agilent 1200 series HPLC system is displayed in Figure 8. It consists of a degasser (G1322A), quaternary pump (G1311A), an automatic sampler (G1329A), a column oven (G1316A) and a various wavelength UV-detector (G1314E). The chromatographic separation was performed on Zorbax Eclipse XDB-C18 column (4.6 ✕ 150mm, 5-µm, USA). The equipment was controlled by Agilent ChemStation software.

Figure 8: *The Agilent 1200 series HPLC system*

Laboratory Instruments

The laboratory equipment used for mobile phase filtration (1-6) and preparing both standard solutions and sample solutions (7-18) are listed below. The mobile phase filtration apparatus is shown in Figure 9.

1. Filtering cup
2. Filtering head
3. Filtering membrane
4. Clamp
5. Funnel base

6. Conical flask
7. Beaker
8. Pipette
9. Graduated cylinder
10. Volumetric flask
11. Dropper
12. Stirring rod
13. Balance
14. Spatula
15. Syringe
16. Syringe filter
17. HPLC Vial
18. Bottle

Figure 9: *Mobile phase filtration apparatus*

2.2 List of chemicals and materials

The chemicals used in this experiment include purified α-casein (Sigma-Aldrich, St. Louis, MO, USA), Urea (Sigma-Aldrich, St. Louis, MO, USA), Tris (Carlo-Erba Reagents, SAS, France), Trisodium citrate (Fisher-Chemicals, Leicestershire, UK), β-mercaptoethanol (Acros Organics, New Jersey, USA), HPLC-grade water, acetonitrile (RCI Labscan, Bangkok, Thailand), and 99% TFA (Acros Organics, Geel, Belgium).

The pasteurised and UHT nonfat bovine milk were sampled from both Tops supermarket and gourmet market in Bangkok, Thailand. There were two brands of nonfat pasteurised bovine milk (Meiji and DutchMill) and one brand of nonfat UHT bovine milk (Anlene) available.

2.3 Experimental Procedure

2.3.1 Preparing standard solutions

A 25 g/L of α-casein stock solution was prepared by dissolving 250 mg of purified α-casein in 10 mL of denaturing solution (8 M urea, 165 mM Tris, 44 mM trisodium citrate and 0.3% (v/v) β-mercaptoethanol). The role of each chemical in the denaturing solution is presented in Appendix

A. Then, a set of four standard solutions were obtained by mixing the α-casein stock solution with the denaturing solution following the dilution scheme displayed in Table 2. The standard solutions were filtered through a syringe filter (13 mm, Nylon, 0.22-µm, WhatMan) and collected in the 2 mL amber glass HPLC vial as shown in Figure 10.

Table 2: Dilution scheme of standard solutions

Standard Level	Dilution Scheme		Concentration (g/L)
	Stock sol. (mL)	Denaturing sol. (mL)	
1	0.4	1.6	5
2	0.8	1.2	10
3	1.2	0.8	15
4	1.6	0.4	20

Figure 10: *Standard solutions*

2.3.2 Sample preparation

All skim milk samples were stored in the refrigerator at 8°C until analysis. Then, a volume of 0.4 mL of each milk sample was diluted with 1.6 mL of denaturing solution described in section 2.2.1 which makes the dilution factor equals to 5. The diluted samples were filtered through a syringe filter (13 mm, nylon, 0.45-µm, VertiClean) and directly analysed.

Figure 11: *Skim milk before and after adding denaturing solution*

2.3.3 RP-HPLC separation conditions

Chromatographic conditions were as follow; Solvent A: Water, Acetonitrile, and TFA in a ratio of 900:100:1 (v/v/v). Solvent B: Water, Acetonitrile, and TFA in a ratio of 100:900:1 (v/v/v). The gradient elution was set from 20-80% (B) for 0-23 min and hold at 80% (B) for 2 min. Thus, the total time of a single run was 25 min. The column temperature was 25°C. The flow rate was 1.0 mL/min. The detection wavelength was 220 nm. An injection volume of sample solution was 20µL.

2.3.4 Calibration curve

Each standard level of standard solutions was analysed twice. The chromatograms are displayed in Appendix B. The obtained peak areas (Appendix C) were statistically treated by linear regression against concentration to obtain four levels calibration curve of α-casein. The resulting linear equation was used to quantitate samples as the mean of 3 repeats.

2.4 Analytical Procedure

2.4.1 Qualitative Data Processing

The analysis of qualitative data in chromatography deals with peak identification. In this experiment, the peak of α-casein in milk samples is determined by injecting both standard solutions and sample solutions under identical analytical conditions. The retention factor (k) was obtained to confirm the identity of analyte and identify the chromatographic behaviour of α-caseins in the column. The formula used for calculating retention factor is shown in Figure 4, and the raw data table of retention time and dead time for both standard solutions and sample solutions are appeared in Appendix D and E, respectively. Moreover, spiking sample method was also conducted to validate the method and confirm the identity of α-casein peak among the rest that appear on the chromatogram of the milk sample that has matrix effect. A volume of 0.4 mL of each milk samples was spiked with 0.2 mL of α-casein stock solution before diluted with 1.2 mL of denaturing solution. The peak areas and recovery percentages of spiked milk samples are reported in Appendix F and the chromatogram of both spiked and non-spiked milk samples are shown in Appendix G. The spiked chromatogram grows and retains symmetry providing positive identification.

2.4.2 Quantitative Data Processing

To quantify the concentration α-casein in milk samples, the sum of the peak area (α_{S2}-CN and α_{S1}-CN) obtained from each trial of sample solutions (Appendix H) was replaced in the variable 'y' of the linear equation acquired from the calibration curve mentioned in section 2.2.4 ($y = 10074x - 33626$), and solve for variable 'x' which represents the diluted concentration. (Appendix I) Then, the initial concentration of α-casein in milk samples were calculated by multiplying diluted concentration with the dilution factor. The raw and processing data of initial concentration is reported in (Appendix J).

CHAPTER 3: RESULTS & DISCUSSION

3.1 Separation of purified α-casein

The chromatogram of one standard solution in Figure 12 and the rest in Appendix B show a separation of two α-casein fractions (α_{S2}-CN, α_{S1}-CN). The peaks of both α_{S2}-CN, α_{S1}-CN grow as the concentration increased which established a linear relationship between peak area and concentration. The calibration curve is displayed in Figure 13. The strong linearity between these two variables is affirmed by the linear correlation coefficient (R^2) of the calibration curve, 0.9908.

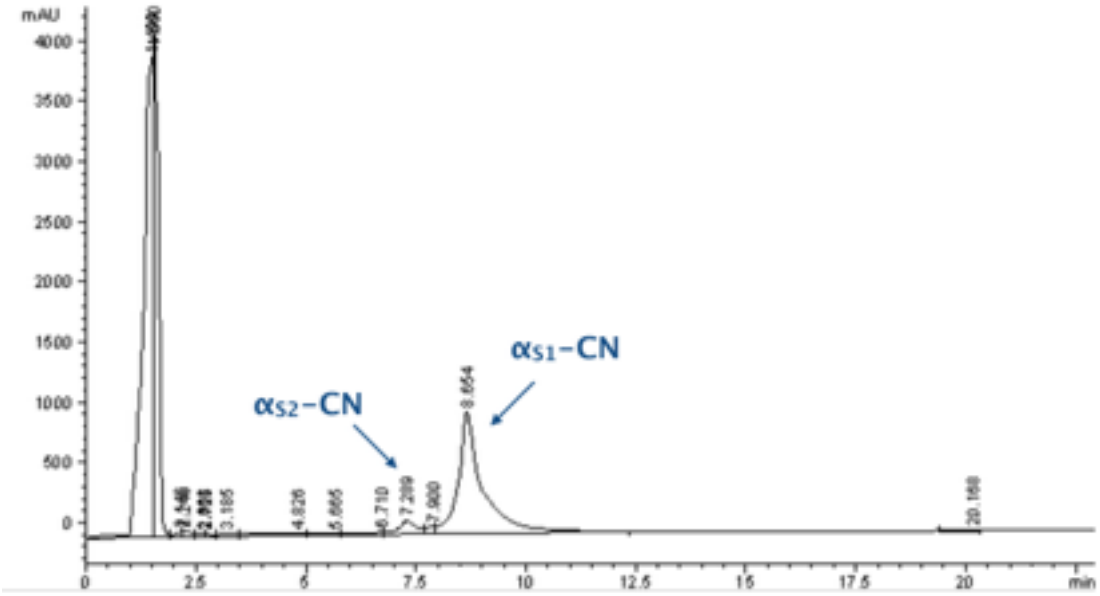

Figure 12: *Chromatogram of standard solution containing α_{S2},α_{S1}-CN fractions*

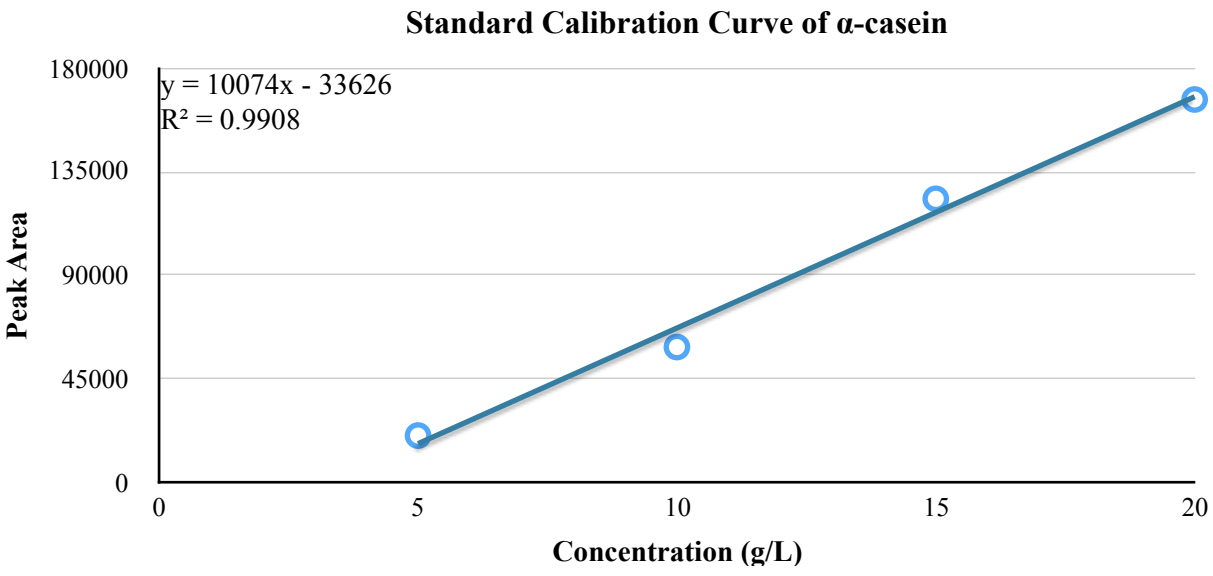

Figure 13: *Standard Calibration Curve of α-casein*

Referring to the data in Appendix C, the RSD of the peak area of standard solutions results are 2.2% or lower, signifying a satisfactory level of precision since according to the Horwitz equation,[34] the acceptable RSD value in analytical chemistry is 2.55% for 20% analyte, and 2% for 100% analyte. Nevertheless, the accuracy and precision of the result could be improved by increasing the repeatability. Although the total analysis time of single run was 25 minutes, the separation of purified α-CN fractions was achieved within 9 minutes, which approximately three times faster than the previous studies that yield the separation in 30 minutes.[27] Therefore, the modified analytical condition used in this experiment is regarded as successful in both time and result efficiency.

3.2 α-casein in milk sample

The results of the qualitative data confirmed the identity of α-casein in milk samples. Firstly, the peak of the spiked samples grow and retain symmetry at the same retention time as the non-spiked sample. The shape of the peaks in both chromatograms are identical. (Appendix G) As shown in Table 3, the recovery percentage of spiked Meiji, DutchMill, and Anlene samples are 89.4%, 98.9%, and 96.9%, respectively, which, all lie within the acceptable range of the mean recoveries of 70-120%.[35] The smaller recovery percentage of spiked Meiji sample might due to stronger matrix effects, inferences and random errors.[36]

Table 3: Recovery percentage of spiked and non-spiked milk samples

	Pasteurised Milk				UHT Milk	
Brand:	**Meiji**	**Meiji Spiked**	**DutchMill**	**DutchMill Spiked**	**Anlene**	**Anlene Spiked**
Concentration (g/L)	6.75	8.99	6.01	8.48	5.58	8.00
%Recovery		89.4%		98.9%		96.9%

Secondly, the retention factor of α_{S2}-CN and α_{S1}-CN in standard solutions and milk samples reported Table 4 are close to each other. However, the differences of α_{S2}-CN retention factor between standard solution and sample solutions are greater than α_{S1}-CN. The maximum range differences of the retention factor between standard solution and sample solutions in α_{S2}-CN is 0.14 while α_{S1}-CN is 0.02. This might be due to the low concentration of α_{S2}-CN that affect the sensitivity of the instrument since α_{S2}-CN account only 10% abundance among the rest of other casein fractions.[9] Nevertheless, according to Codex Alimentarius International Food Standards, the acceptable range of differences retention factor in liquid chromatography between the referenced

value and an analyte in the sample should be ±0.1. Hence, comparing to the retention factor of standard solutions, the differences in retention factor for all sample solutions attained this acceptable range except for α_{S2}-CN in Meiji sample. The reason behind this might due to the sample component that yields a different degree of interaction with the stationary phase.[37] Overall, the results obtained from qualitative data (recovery of spiked sample and retention factor) are adequate to confirm the identity of α-casein in milk samples.

Table 4: Retention Factor of αS2, αS1-CN in standard solutions and sample solutions

	Standard Solutions	Pasteurised Milk		UHT Milk
		Meiji	Dutchmill	ANLENE
α_{S2}-CN	3.84	3.70	3.76	3.77
α_{S1}-CN	4.70	4.68	4.72	4.72

Each milk sample solutions were analysed 3 times under the same analytical condition as standard solutions. The quantitative results yield good precision of data as the RSD for all types of the sample shown in Table 5 are below 1.5% which is lower than the Horwitz's RSD value of 2.55%.[35] The mean result of the initial concentration of α-casein in Meiji, Dutch Mill, and Anlene is 33.7 g/L, 30.1 g/L, and 28.1 g/L, respectively. According to the data provided by one pharmaceutical research,[38] there are 35.8 g/L of protein in bovine milk, and 29.5 g/L is casein protein. Thus, based on the calculation referring to the information that α-casein is 38% of the total protein,[9] the concentration of α-casein in milk should approximately be 17.2 g/L. This indicates that the experimental results of α-casein content in the bovine milk samples are greater than the referenced value.

Table 5: The initial concentration of α-casein in milk samples

Brand:	Pasteurised Milk		UHT Milk
	Meiji (g/L)	DutchMill (g/L)	Anlene (g/L)
Mean	33.7	30.1	28.1
RSD	1.49%	1.33%	0.98%

There are many possible reasons behind the outcome. Firstly, the peaks of α_{S1}-CN and β-CN are not clearly separated. As shown in Figure 14, the peaks of α_{S1}-CN and β-CN in milk samples are overlapping each other, which lead to an error in integrating peak area since they could mix with each other and cause the area of the α_{S1}-CN peak to be greater than it should. This analytical

condition might only be suitable for purified casein protein. The overlapping peak resulted from the fast elution so the analytical condition needs to be adjusted. Secondly, a systematic error of HPLC instrument could affect the accuracy of the result. According to an article in the Journal of Chromatography A[39], the systematic error of HPCL instrument is very hard to detect, the problem could arise from the balance, incorrect integration setting, flow rate error from non-ideal mobile phase behaviour, and instrumental imperfections. Thirdly, the sample could be contaminated with unexpected impurities. This could result from the removal of vial cap during analysis, human error in sample preparation, and repeat injection of a single vial. These three possible errors may lead to a positive deviation of the concentration of α-casein in milk samples.

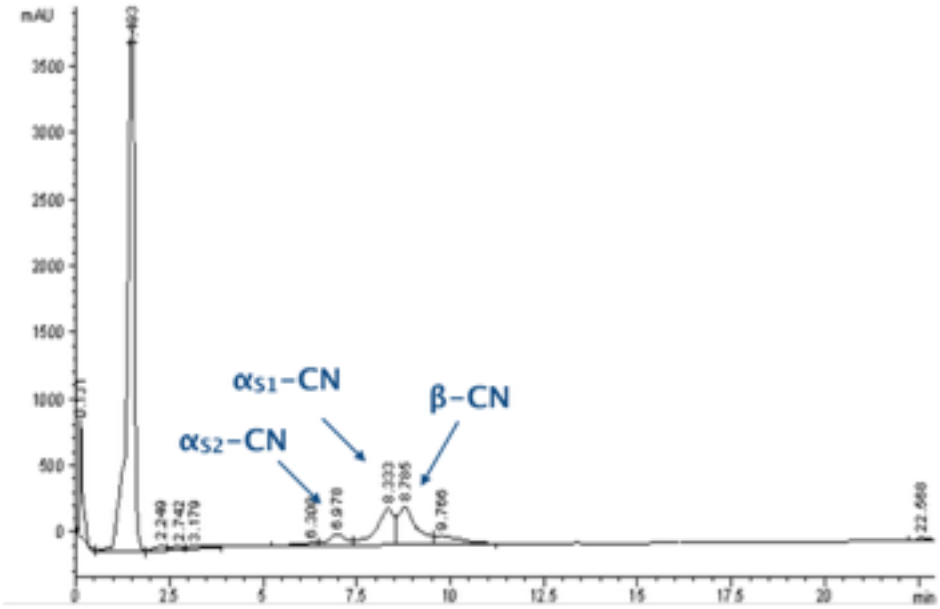

Figure 14: *Chromatogram of Meiji sample*

3.3 Pasteurised milk vs UHT milk

Despite the deviation to the referenced data, the experimental results indicate that pasteurised milk contains more α-casein content than UHT milk. Meiji has the highest α-casein concentration among the three brands, followed by Dutch Mill and Anlene, respectively. (Table 5) Comparing to Anlene, Meiji and Dutch Mill has 18.1% and 6.87% greater α-casein content. This might due to the fact that Meiji contains 7 g of protein, Dutch Mill contains 6 g of protein and Anlene holds 5 g of protein. The nutrition fact attached to the milk package of each milk sample is provided in Appendix K. Moreover, the higher concentration of α-casein in pasteurised milk might be related to the treatment process mentioned in section 1.2.4 that pasteurised milk has been heated

at lower temperature and shorter time comparing to the UHT milk. Thus, there are less denatured proteins and result in higher protein content. Although casein proteins are considered as heat-stable proteins, yet heating to the temperature that is about two times higher than milk protein denature temperature of 65°C, there might be some loss in protein content.

Based on the experimental results, UHT milk is preferred for consumption over pasteurised milk regarding the lower α-casein content that plays a significant role in increase the proliferation of cancer cells in the human body. Since casein protein consumption should be less than 30 g each day,[5] it is recommended to consume both pasteurised and UHT milk less than 1 L per day. Nevertheless, the sign of danger of α-casein in the human body does not reveal immediately after consumption because it takes some time for a cancer cell to build up, which the effect of casein is varied among individual.[40] Therefore, humans should not heavily rely on milk as a major source of protein.

CHAPTER 4: CONCLUSION

In conclusion, the two objectives of this project are achieved. The application of RP-HPLC method for determination and quantification of α-casein protein in pasteurised and UHT nonfat bovine milk is successful. However, the analytical condition needs to be adjusted for better peak resolution in milk samples. Based on experimental results, there is a greater α-casein protein content in pasteurised milk than UHT milk. Therefore, in regards to the health effect of α-casein protein, UHT milk is preferable.

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APPENDICES

Appendix A: Chemicals in denaturing solution

Urea: As mentioned in section 1.3.3, Urea disrupt the H-bonding at the surface of the protein. The urea hydrogen bonds polarised areas of charge, such as peptide groups which enervate the intermolecular bonds and interactions.[20]

Tris: Tris is a buffer used to maintain pH of the solution.

Trisodium Citrate: The addition of citrate alters the chemical and functional properties of milk by decreasing the activity or concentration of ionic calcium and increasing solubilisation of colloidal calcium phosphate.[41]

β-mercaptoethanol: β-ME is a reducing agent that irreversibly denature RNases by reducing disulphide bonds and destroying the native conformation required for enzyme functionality.[42]

Appendix B: Chromatogram of standard solutions

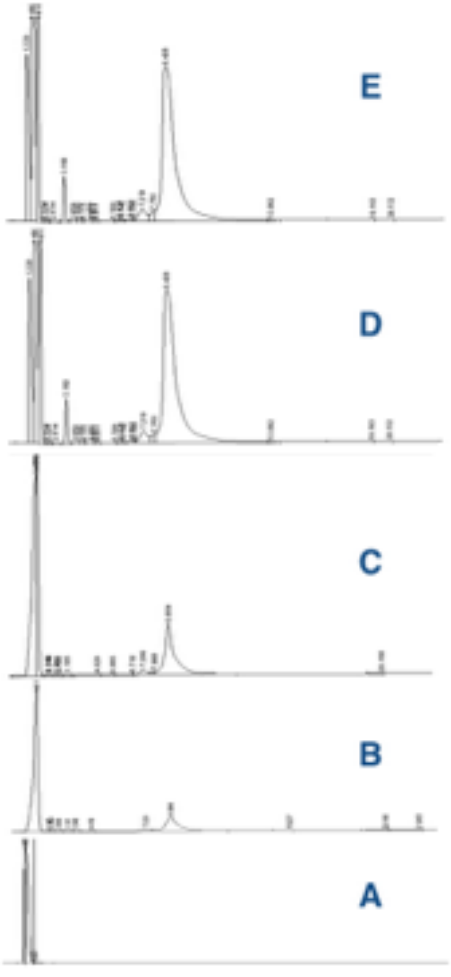
A: Denature solution (blank)

B: Standard level 1 (α-CN 0.4:1.6 DS)

C: Standard level 2 (α-CN 0.8:1.2 DS)

D: Standard level 3 (α-CN 1.2:0.8 DS)

E: Standard level 4 (α-CN 1.6:0.4 DS)



Appendix C: Peak Area of Standard Solutions

Table 6: Peak area of standard solutions

Standard Level	1	2	3	4
Trial 1	19,972.6	59,693.4	121,784.8	164,586.8
Trial 2	20,603.8	57,989.3	125,011.1	168,752.8
Mean	**20,288.2**	**58,841.4**	**123,398.0**	**166,669.8**
SD	**446.3**	**1,205.0**	**2,281.3**	**2,945.8**
RSD	**2.2%**	**2.0%**	**1.8%**	**1.8%**

Appendix D: Data table for retention factor calculation of standard solutions

Table 7: Retention time and dead time of standard solutions

	Retention Time (min)							
Standard Level	**1**		**2**		**3**		**4**	
	α_{S2}-CN	**α_{S1}-CN**	**α_{S2}-CN**	**α_{S1}-CN**	**α_{S2}-CN**	**α_{S1}-CN**	**α_{S2}-CN**	**α_{S1}-CN**
Trial 1	7.280	8.634	7.221	8.656	7.223	8.487	7.220	8.438
Trial 2	7.313	8.618	7.298	8.647	7.266	8.482	7.219	8.436
Trial 3	7.328	8.666	7.289	8.654	7.222	8.486	7.217	8.435
	Dead Time (min)							
Standard Level	**1**		**2**		**3**		**4**	
Trial 1	1.511		1.499		1.493		1.490	
Trial 2	1.499		1.512		1.498		1.492	
Trial 3	1.500		1.510		1.499		1.497	
Mean: α_{S2}-CN	**7.258**							
Mean: α_{S1}-CN	**8.553**							
Mean: Dead Time	**1.500**							

Appendix E: Data table for retention factor calculation of sample solutions

Table 8: Retention time and dead time of milk samples

	Retention Time (min)					
	Pasteurised Milk				UHT Milk	
Brand:	**Meiji**		**DutchMill**		**Anlene**	
	α_{S2}-CN	**α_{S1}-CN**	**α_{S2}-CN**	**α_{S1}-CN**	**α_{S2}-CN**	**α_{S1}-CN**
Trial 1	6.938	8.464	6.936	8.360	6.995	8.391
Trial 2	6.978	8.333	6.935	8.358	6.987	8.382
Trial 3	6.934	8.399	6.998	8.375	6.990	8.386
Mean	**6.950**	**8.399**	**6.956**	**8.364**	**6.991**	**8.386**
	Dead Time (min)					
Brand:	**Meiji**		**DutchMill**		**Anlene**	
Trial 1	1.455		1.489		1.452	
Trial 2	1.490		1.420		1.474	
Trial 3	1.493		1.481		1.469	
Mean	**1.479**		**1.463**		**1.465**	

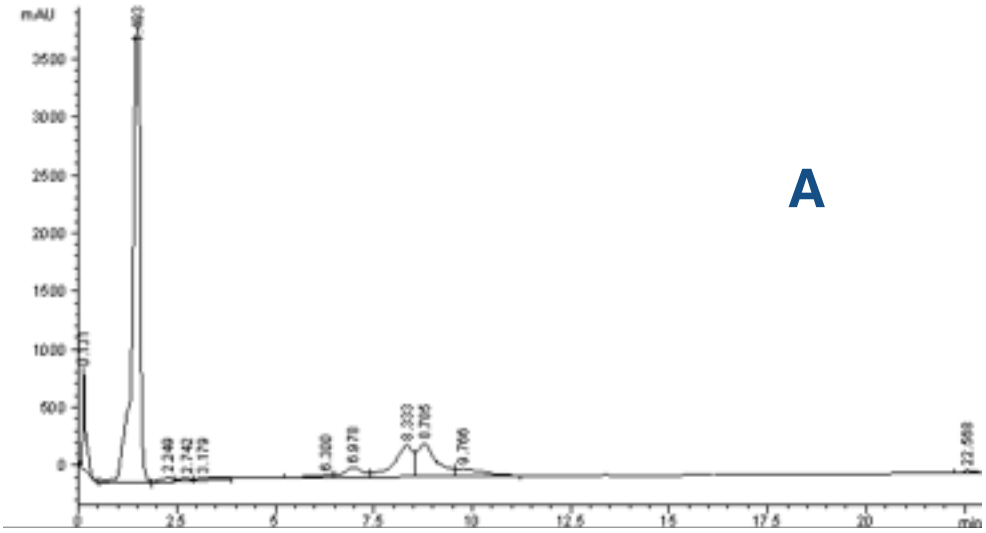
Appendix F: Data table of spiked and non-spike milk samples

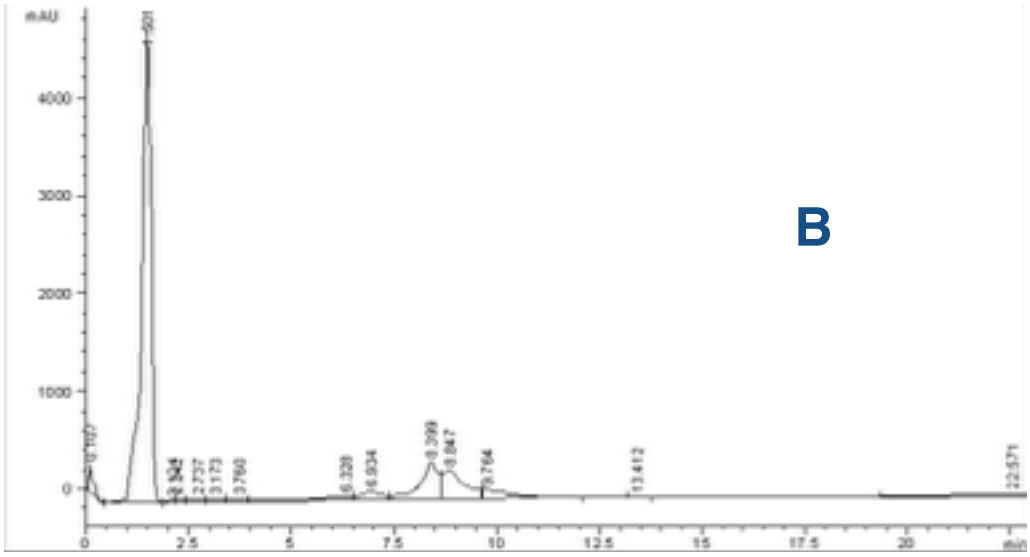
Table 9: Peak area and recovery percentage of spiked and non-spike milk samples

	Pasteurised Milk			UHT Milk		
Brand:	**Meiji**	**Meiji Spiked**	**DutchMill**	**DutchMill Spiked**	**Anlene**	**Anlene Spiked**
Peak Area Trial 1	35,679.0	57,056.5	26,878.0	51,783.1	22,571.3	46,984.9
Peak Area Trail 2	33,141.0	56,788.3	26,289.4	49,522.1	23,387.9	47,612.0
Mean	**34,410.0**	**56,922.4**	**26,583.7**	**50,652.6**	**22,979.6**	**47,298.5**
Concentration (g/L)	6.75	8.99	6.01	8.48	5.58	8.00
%Recovery		89.4%		98.9%		96.9%

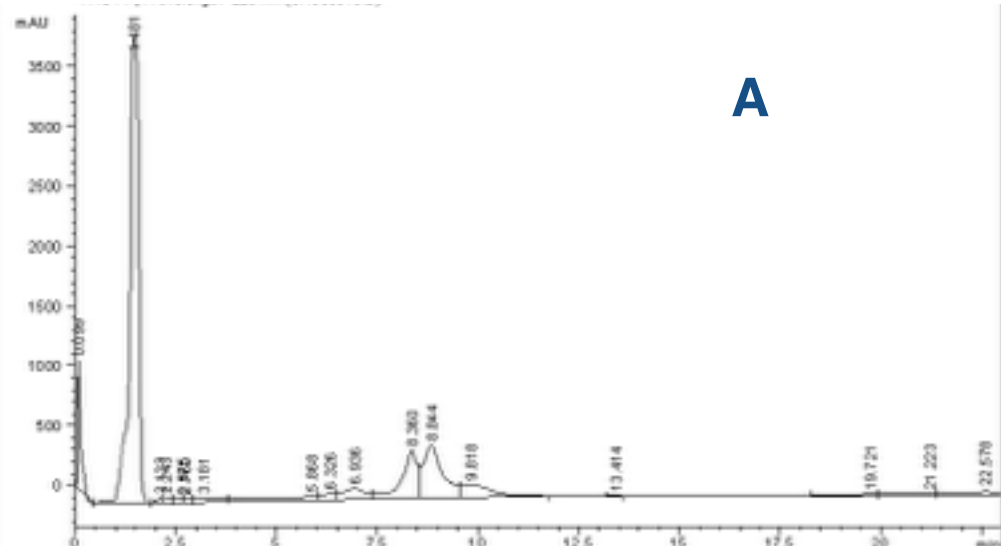
Appendix G: Chromatogram of spiked and non-spike milk samples

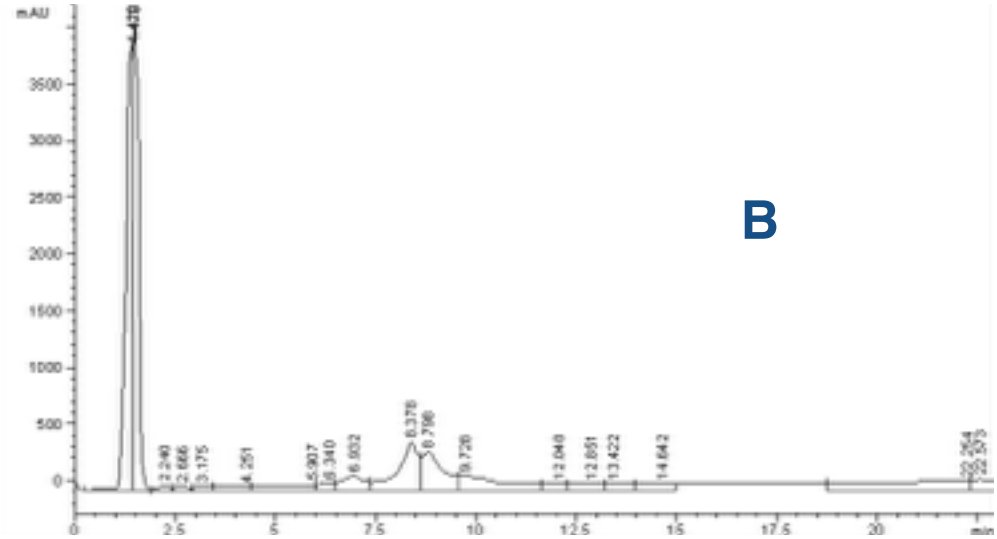
1. Meiji (A = non-spike, B = spiked)



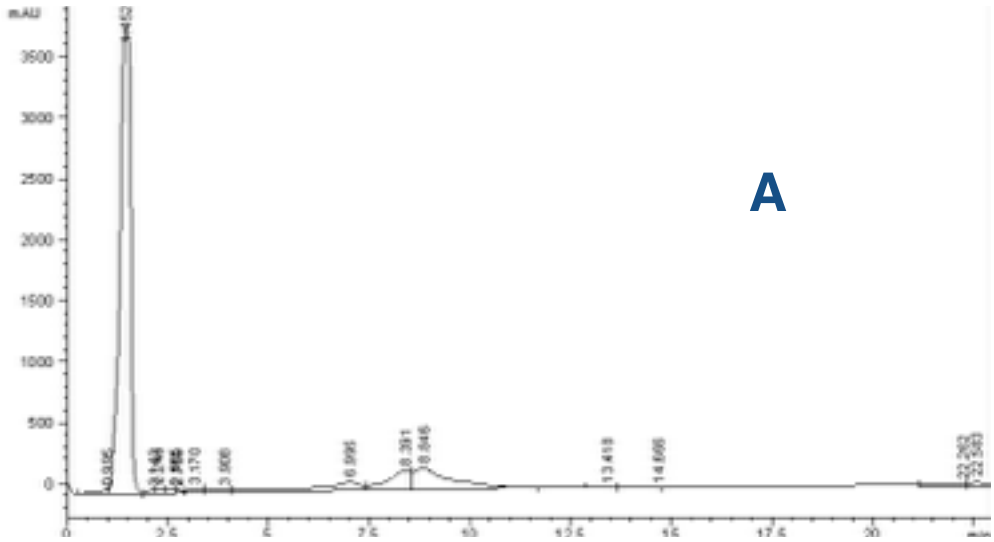


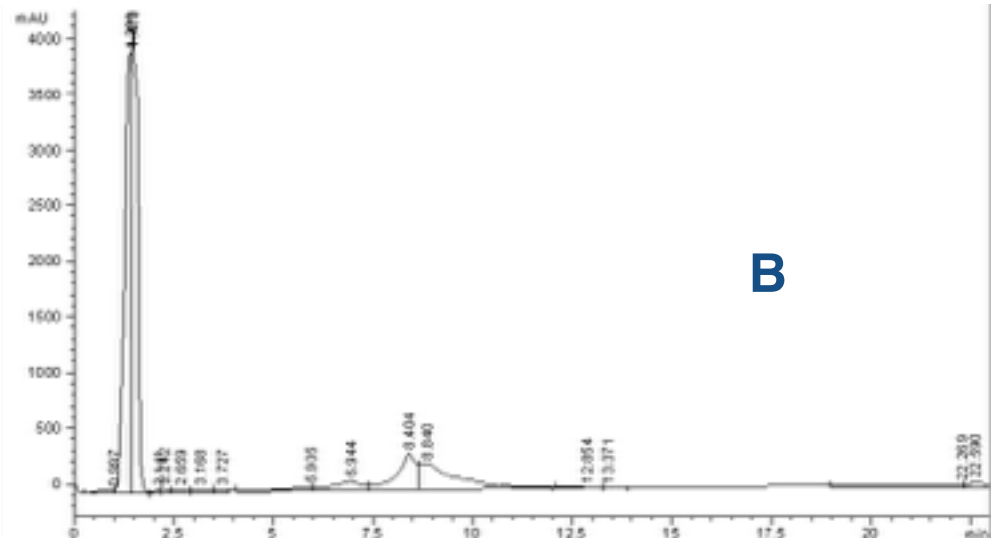
2. Dutch Mill (A = non-spike, B = spiked)





3. Anlene (A = non-spike, B = spiked)





Appendix H: Peak area of sample solutions

Table 10: Peak area of milk samples

Brand:	Pasteurised Milk		UHT Milk
	Meiji	DutchMill	Anlene
Trial 1	33,100.0	26,154.1	22,526.7
Trial 2	34,931.6	27,771	23,640.5
Trial 3	34,552.1	26,957.6	22,988.4
Mean	**34,194.6**	**26,960.9**	**23,051.9**

Appendix I: Data table of diluted concentration of a-CN in milk samples

Table 11: The diluted concentration of a-CN in milk samples

Brand:	Pasteurised Milk		UHT Milk
	Meiji	DutchMill	Anlene
Trial 1	6.62	5.93	5.57
Trial 2	6.81	6.09	5.68
Trial 3	6.77	6.01	5.62
Mean	**6.73**	**6.01**	**5.63**
SD	**0.10**	**0.08**	**0.06**
RSD	**1.43%**	**1.33%**	**0.99%**

Appendix J: Data table of initial concentration of a-CN in milk samples

Table 12: The initial concentration of a-CN in milk samples

Brand:	Pasteurised Milk		UHT Milk
	Meiji (g/L)	DutchMill (g/L)	Anlene (g/L)
Trial 1	33.10	29.65	27.85
Trial 2	34.05	30.45	28.40
Trial 3	33.85	30.05	28.10
Mean	**33.7**	**30.1**	**28.1**
SD	**0.50**	**0.40**	**0.28**
RSD	**1.49%**	**1.33%**	**0.98%**

Appendix K: Nutrition fact of milk samples

1. Meiji

2. Dutch Mill

3. Anlene

ข้อมูลโภชนาการ

หนึ่งหน่วยบริโภค : 1 กล่อง (180 มิลลิลิตร)
จำนวนหน่วยบริโภคต่อกล่อง : 1

คุณค่าทางโภชนาการต่อหนึ่งหน่วยบริโภค
พลังงานทั้งหมด 70 กิโลแคลอรี (พลังงานจากไขมัน 0 กิโลแคลอรี)

	ร้อยละของปริมาณที่แนะนำต่อวัน *
ไขมันทั้งหมด 0 ก.	0 %
ไขมันอิ่มตัว 0 ก.	0 %
โคเลสเตอรอล น้อยกว่า 5 มก.	1 %
โปรตีน 5 ก.	
คาร์โบไฮเดรตทั้งหมด 11	4 %
ใยอาหาร 0 ก.	0 %
น้ำตาล 9 ก.	
โซเดียม 85 มก.	4 %

ร้อยละของปริมาณที่แนะนำต่อวัน *

วิตามินเอ	0 %	วิตามินบี 1	0 %
วิตามินบี 2	25 %	แคลเซียม	60 %
เหล็ก	0 %	วิตามินดี	70 %
วิตามินบี 6	45 %	วิตามินซี	40 %
ฟอสฟอรัส	25 %	วิตามินบี 12	20 %
วิตามินอี	20 %	แมกนีเซียม	15 %
สังกะสี	15 %		

*ร้อยละของปริมาณสารอาหารที่แนะนำให้บริโภคต่อวัน สำหรับคนไทยอายุตั้งแต่ 6 ปีขึ้นไป (Thai RDI) โดยคิดจากความต้องการพลังงานวันละ 2,000 กิโลแคลอรี

Biography

I, Chayanitasa Punyathanarwat, grew up as an only child in the family. I was born in December 1998 in Bangkok, Thailand. I studied at Saint Joseph Convent School from Grade 1 to Grade 3. Then, I moved to KIS International School and stayed until I finished Grade 12, which I graduated International Baccalaureate (IB) Diploma program. Each student was required to select three higher level subjects and three standard level subjects based on personal interest during the last two years of high school. My higher level subjects are (1) Chemistry (2) Language and Literature (3) Business and Management. My standard level subjects include (1) Mathematics (2) Mandarin (3) Environmental Systems and Societies. These 6 subjects lead me to study Bachelor degree at BSAC program of Chulalongkorn University. I believed that '*Industrial Chemistry and Management Program*' fit my previous knowledge of chemistry and business. Plus, it would allow me to expand my knowledge in Applied Chemistry.

Nonetheless, my interest has changed after I got a chance to touched on a few subjects from the Faculty of Commerce and Accountancy during my third year. Thus, I planned to continue my master degree in the UK either in Finance and Management or Data Science as soon as I graduated from BSAC. For further information, please contact chayanitasa@gmail.com.