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DETERMINATION OF CALCIUM AND MAGNESIUM BY FAAS IN MILK AND HELLIM CHEESE PRODUCED IN TRNC

MASTERS THESIS

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NEAR EAST UNIVERSITY

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Approval.

We certify that we have read the thesis submitted by EDMUND AGBOR AGBORAYOR titled **"DETERMINATION OF CALCIUM AND MAGNESIUM BY FLAME ATOMIC ABSORPTION SPECTROMETRY IN MILK AND TRADITIONAL HELLIM CHEESE PRODUCED IN TRNC"** and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Educational Sciences.

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DECLARATION

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

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Abstract

Determination Of Calcium and Magnesium by Flame Atomic Absorption Spectrometry in Milk and Traditional Hellim Cheese Produced In TRNC.

Edmund Agbor Agborayor.
Master of Science Thesis, Department of Analytical Chemistry,
June 2023, 34 pages

Milk and dairy products such as the traditional Hellim cheese, a white, hard and salty cheese are largely produced locally and industrially in TRNC as a source of livelihood and for exportation respectively. The content of macro minerals such calcium and magnesium in some of these milk and cheese products purchased from supermarkets was analysed in thesis. The analysis of 6 samples was done using flame atomic absorption spectrometry (FAAS) and aqueous calibration method after sample was solubilized by HNO_3/H_2O_2 open wet-acid digestion at room temperature and pressure. The concentration of calcium and magnesium in the samples recorded varied quiet significantly compared to what was written on some with company nutrition data labels. The highest average concentration after 4 replicate analysis, 18.64mgg^{-1} was recorded for calcium in cheese sample 1 followed by an average of 14.19mgg^{-1} in cheese sample 2 and sample 3 brands. An average of 0.79mgg^{-1} was observed for magnesium in cheese sample 1 and 0.69mgg^{-1} for cheese sample 2 and sample 3 brands. Concentrations between 0.89-1.03mgg^{-1} in calcium and 0.097-0.10mgg^{-1} in magnesium was also recorded for milk samples. From the observed values, we can conclude that Hellim cheese and milk from TRNC are reliable sources of calcium and magnesium in health based on recommended daily average (RDA) but much needs to be done in standardising the observed variations due to regional production conditions such as by selective breeding in improving the quality rather than nutritional manipulations. This work should open room for research work into the Hellim cheese sector because it is still very much under researched in TRNC.

Keywords: Hellim cheese, milk, calcium, magnesium, determination, FAAS.

ÖZET

Sütte Alev Atomik Absorpsiyon Spektrometresi ve KKTC'de Üretilen Geleneksel Hellim Peyniri ile Kalsiyum ve Magnezyum Tayini.

Edmund Agbor Agborayor.
Bilim Tezi Yüksek Lisansı, Analitik Kimya Bölümü,
Haziran 2023. 34 sayfa

Geleneksel Hellim peyniri, beyaz, sert ve tuzlu peynir gibi süt ve süt ürünleri, geçim kaynağı olarak ve ihracat için büyük ölçüde yerel ve endüstriyel olarak TRNC'de üretilmektedir. Süpermarketlerden satın alınan bu süt ve peynir ürünlerinin bazılarında kalsiyum ve magnezyum gibi makro minerallerin içeriği tezde analiz edilmiştir. 6 numunenin analizi, alev atomik absorpsiyon spektrometresi (FAAS) ve numune oda sıcaklığı ve basıncında HNO_3/H_2O_2 açık ıslak asit sindirimi ile çözündürüldükten sonra sulu kalibrasyon yöntemi kullanılarak yapıldı. Kaydedilen numunelerdeki kalsiyum ve magnezyum konsantrasyonu, şirket beslenme veri etiketleri ile bazılarında yazılanlara kıyasla önemli ölçüde sessiz değişmiştir. 4 replikat analizinden sonra en yüksek ortalama konsantrasyon olan $18.64 mgg^{-1}$, peynir örneği 1'de kalsiyum için kaydedildi, ardından peynir örneği 2 ve örnek 3 markalarında ortalama $14.19 mgg^{-1}$ kaydedildi. Peynir örneği 1'de magnezyum için ortalama $0.79 mgg^{-1}$ ve peynir örneği 2 ve örnek 3 markaları için $0.69 mgg^{-1}$ gözlenmiştir. Süt numuneleri için kalsiyum içinde $0.89\text{-}1.03 mgg^{-1}$ ile magnezyumda $0.097\text{-}0.10 mgg^{-1}$ arasındaki konsantrasyonlar da kaydedildi. Gözlenen değerlerden, TRNC'den gelen Hellim peyniri ve sütünün, önerilen günlük ortalama (RDA) 'e göre sağlıkta güvenilir kalsiyum ve magnezyum kaynakları olduğu sonucuna varabiliriz, ancak besinsel manipülasyonlardan ziyade kalitenin iyileştirilmesinde seçici üreme gibi bölgesel üretim koşullarından dolayı gözlenen varyasyonlar. Bu çalışma Hellim peynir sektörüne yönelik araştırma çalışmaları için yer açmalıdır, çünkü hala KKTC'de araştırılmamaktadır.

Anahtar Kelimeler: Hellim peyniri, süt, kalsiyum, magnezyum, belirleme, FAAS.

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List of Abbreviations

TRNC	Turkish Republic of Northern Cyprus
FAAS	Flame Atomic Absorption Spectrometry
NIH	National Institute of Health
RDA	Recommended Daily Average
RDI	Recommended Daily Intake
LOD	Limit of Detection
LOQ	Limit of Quantitation
LDR	Linear Dynamic Range
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
RSD	Relative Standard Deviation
SD	Standard Deviation
AOAC	Association of Official Analytical Chemists
RCT	Rennet Coagulation Time
ICP-OES	Inductively Coupled Plasma- Optical Emission Spectrometry
ICP-MS	Inductively Coupled Plasma- Mass Spectrometry
IR	Infra-red
UV	Ultraviolet
D_2	Deuterium
EDTA	Ethylene Diamine Tetraacetic Acid
CKD	Coronary Kidney Diseases
MCP	Milk Coagulation Properties

CHAPTER 1

This chapter covers general information on thesis topic which includes literature review, research objectives, limitations. Theory on spectroscopy, atomic absorption spectrometry was also summarised in this chapter.

Introduction

1.1 Calcium and Magnesium in Health.

Calcium and Magnesium are some of the most important macro minerals in the human body as their salts, especially calcium are minerals that constitute the human skeletal system and deficiencies in adults and children can lead to health complications such as osteoporosis, rickets paralysis and even poor blood clotting. (Antonio & Gustavo, 2017, p 720; Burrow et al., 2018; Vannucci et al., 2018). In as much as calcium plays a vital role in maintaining the quality of human anatomy, scientific studies have revealed that excessive or uncontrolled in take of calcium might increase the risk of kidney stones, cardiovascular diseases, osteoporotic or hip fracture especially in females and gastro intestinal disorders (Li et al., 2018).

Calcium recommended intake varies throughout life and most health agencies suggest for age groups 9yrs and higher a recommended daily average (RDA) between 1000-1300mg per day of calcium in order to meet the requirements of 97-98% healthy individuals (Cano et al., 2018).

Magnesium also acts as a cofactor or activator of several enzymes in humans, maintains cell stability and mitigates the effects of oxidative stress caused by its deficiency especially in obese people (Morais et al., 2017). Low values have also been reported in patients suffering from Fibromyalgia, a common chronic pain syndrome that causes fatigue, depression and sleep disturbances (Shin et al., 2020). Magnesium balance in the human body is highly regulated by a continuous balance between intestinal absorption, storage in bones and kidney excretion for eliminating excess. However, adverse effects of excessive magnesium intake or toxicity especially in patients with coronary kidney diseases (CKD) have been reported for serum magnesium concentrations exceeding 1.74-2.61mmol/L, with symptoms such as nausea, face flushing, retention in urine (Mg overload), irregular heartbeat, difficulty breathing, cardiac arrest (Pickering et al., 2020).

The National Institute of Health (NIH) office of dietary supplements suggests a recommended daily intake (RDI) of 320mg magnesium for adult females and 420mg for adult males (Rojo –Gutierrez et al., 2022).

1.2 Milk and Dairy Products Produced in the Turkish Republic of Northern Cyprus (TRNC).

Milk and milk derivatives such as cheese are the richest natural sources of these minerals (Antonio & Gustavo, 2017). Some of such derivatives produced locally and consumed in TRNC are the hellim (Turkish) or halloumi (Greek), a traditional Cyprus cheese originally made from a blend of rennet (for coagulation), pasteurized cow, goat and sheep milk and it is widely manufactured at household levels as a source of livelihood and in about ten modern factories for mainly exports and local consumption (Wilson, 2017).

1.3 Chemistry of Milk Cheese Making.

Since cheese is made from milk, it is important to understand the basic chemistry of milk.

Milk contains four major constituents: water, lipids or fats, proteins (casein and whey), lactose or sugars, vitamins and minerals, the most significant of which from cheese making point of view is calcium phosphate which binds protein casein. The quality of cheese depends on one of its protein groups, the caseins (Fox et al., 2017, p. 71), reason why a brief discussion of casein only was done in this thesis.

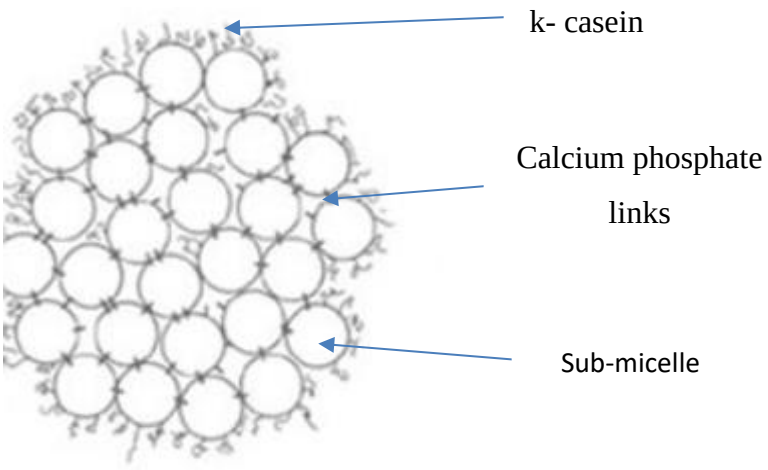
1.3.1 Milk Proteins.

Milk proteins are categorized into two with respect to their solubilities at pH 4.6 and temperature at 20℃ as insoluble **casein micelles** and soluble **whey proteins**.

Casein micelle consist of four sub proteins with different properties namely, alpha s-1 casein (α_{s1}-casein), alpha s-2 (α_{s2}), beta casein (β-casein) and kapa casein (k-casein). The casein micelle is surrounded by hydrophobic negatively charged k-casein heads projecting outwards which prevents casein micelles from sticking together as in liquid state milk. This is the reason k-casein is of most importance during cheese processing because they have to be eliminated ormfor casein micelles to stick together and form cheese curd (Fox et al., 2017, p. 71; Polowsky, 2023).

Figure 1

Walstra and Jenness Sub-Micelle Model Of Casein Micelle (Fox et al., 2017, p. 97)



1.3.2 Converting Milk To Cheese.

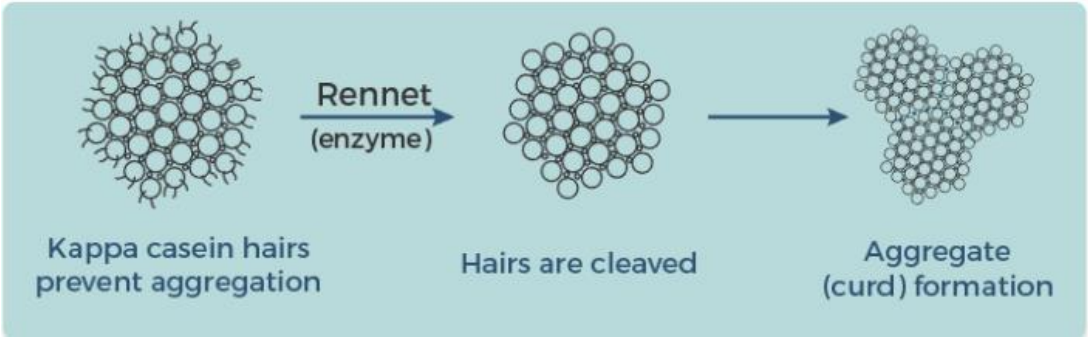
Cheese making basically is the controlled decomposition and dehydration of milk. It generally involves the following stages (Fox et al., 2017, p. 13-24):

- Milk selection.
- Standardization of milk composition.
- Heat treatment.
- Coloring.
- Acidification or Rennet addition as milk coagulant.
- Salting and Ripening.

Acidification can be Direct that is addition of HCl or Microbiological that is fermentation of milk lactose to lactic acid by addition of starter culture bacteria such as Lactobacillus. The objectives of these additions are to introduce protons to neutralize negatively charged k-casein heads or cut them off completely as with rennet coagulant in order to promote aggregation of milk casein micelles (curd formation). Most companies make use of rennet because direct acidification most at times dissolves calcium phosphate bonds within sub-micelles leading to poorly formed and soft cheese curds.

Figure 2

Aggregation Of Casein Micelles Caused By Rennet Addition (Polowsky, 2023)



1.4 Theory of Spectroscopy Based On Absorption.

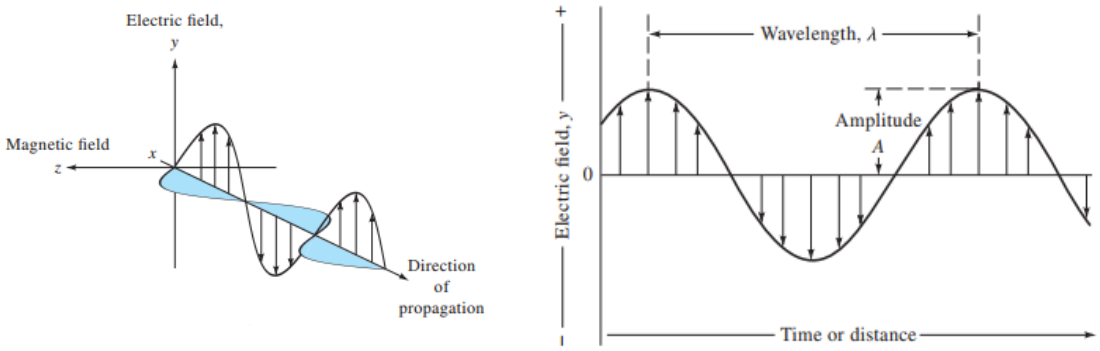
Spectroscopy is a science discipline that studies the interaction of electromagnetic radiation and matter or substances.

Much of our present day understanding of the electronic structure of atoms, measurement of analyte concentration and structure determination has come from the instrumental analysis of light or electromagnetic radiation either emitted or absorbed by substances (Harris & Lucy, 2020).

Electromagnetic radiation is a form of energy propagated through space as waves of electric (xy-plane) and magnetic (yz-plane) fields at right angles to each other and in the direction of propagation as shown below.

Figure 3

Plane Polarized Wave Propagating along the x-axis (left image) and only Electric Field Oscillations (right image)



In a vacuum, electromagnetic radiation moves at a constant speed **c =** 2.998×10^8m/s, which is related to its frequency **(ν)** and wavelength **(λ)** by **equation 1** below;

$$c = \nu \lambda \qquad \textbf{Equation 1.}$$

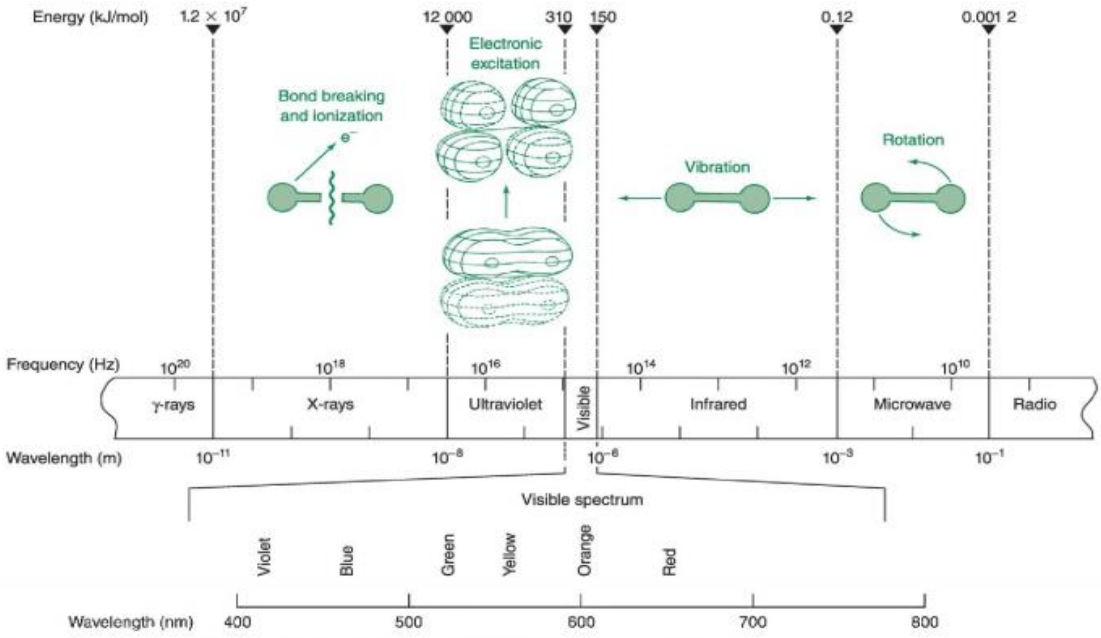
Visible light for example is one form of electromagnetic radiation we can see. The electromagnetic radiation is a whole spectrum (Skoog et al., 2018).

1.4.1 The electromagnetic spectrum.

The electromagnetic spectrum, showing representative molecular processes that occur when light in each region is absorbed is shown below

Figure 4

The Electromagnetic Spectrum



1.4.2 Interaction of Radiation with Matter

When samples absorb electromagnetic radiation, they undergo a change in energy because of a permanent transfer of energy from the radiation to the sample. These observations were made by Max Planck (1900) and Albert Einstein (1905) through

their quantum theories of Black-body radiation and photoelectric effect of light respectively (Skoog et al., 2018).

These models concluded that the energy involved in this process exist as a stream of discrete tiny particles in these radiations called **photons** and that this energy is related to the frequency and wavelength of the radiation as shown in the equation below (Daniel & Harris, 2020).

$$\mathbf{E = h\,\nu = h\frac{c}{\lambda}} \qquad \textbf{Equation 2}$$

Where **E** is the energy of the particles in joules, **h** is the Planck's constant (6.626 x 10^{-34}J.s), **ν** is the frequency of the radiation expressed as **c/ λ**.

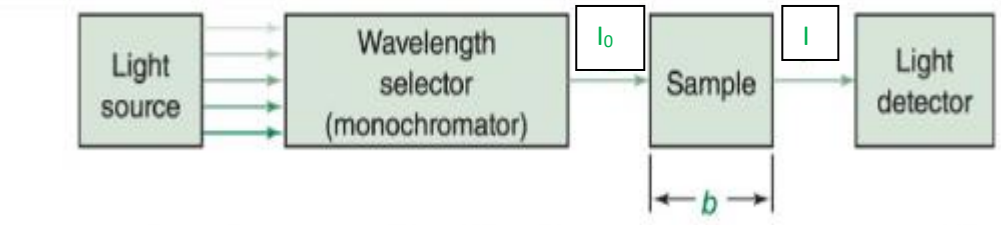
This interaction is used to obtain useful qualitative or quantitative information about analyte in a sample.

1.4.3 Measurement of Transmittance and Absorbance (Beer's Law)

When a sample absorbs radiation, the intensity (**I**) of the radiation defined as the energy flux per second per unit area of the radiation beam (W/m^2) is decreased. The block diagram below shows a typical spectrophotometric experiment where I_0 is the intensity of the radiation before absorption or intensity of blank and I the intensity after absorption or intensity after absorption by analyte in sample (Harris & Lucy, 2020).

Figure 5

Block Diagram of a Typical Spectrophotometer



The fraction of original light that passes through the sample is called the transmittance (T) of the radiation and it is expressed as;

$$\mathbf{T = I/\ I_0} \qquad \textbf{Equation 3}$$

And the Absorbance (**A**) of the medium is defined as;

$$\mathbf{A = -\ Log\ (T)} \qquad \textbf{Equation 4}$$

The absorbance is very significant because it is directly proportional to the concentration of analyte absorbing light in the sample as defined by the **Beer-Lambert** equation;

$$\mathbf{A = Ɛbc} \qquad \textbf{Equation 5}$$

Where **b** is the path length (cm) containing analyte which radiation travels through, **Ɛ** is the molar absorptivity ($M^{-1}cm^{-1}$) of the medium and **c** the concentration of the sample (M).

Equation 5 is the core principle behind the use of spectrophotometers in analytical chemistry.

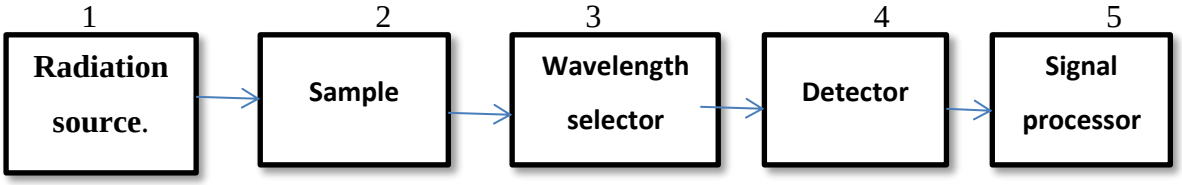
1.4.4 Components of Optical Instruments used in Spectroscopy.

All optical spectroscopic instruments have been designed for measurements in the ultraviolet (UV), visible and infrared (IR) regions of the electromagnetic spectrum. The methods are often based on six phenomena: absorption, emission, scattering, chemiluminescence, fluorescence and phosphorescence (Skoog et al., 2018).

The instruments may somewhat differ in structure, but they all have the five basic components present in all spectroscopic instruments shown below;

Figure 6

Five Basic Components Present in all Spectroscopic Instruments



- **Radiation** comes from a **line source** such as element specific hollow cathode lamps, lasers, etc. OR from **continuum source** such as deuterium lamp, argon, xenon and tungsten. Line sources emit limited number of lines or narrow bands of wavelengths whereas continuum sources provide a band distribution of wavelength within a particular spectral range.
- **Wavelength selectors (monochromators)** such as glass prisms, gratings and filters have been designed to allow only one wavelength or a range of wavelengths pass through to the detector. They also have exit slits to control

bandwidth of selected wavelength which affects signal-to-noise and hence precision of the detector.

- **Detectors** present in all modern instruments consist of sensitive transducers to convert radiation signals containing photons from monochromators into easily measured electrical signals. Some examples include phototubes, photomultiplier tubes, photovoltaic cells, thermocouples etc.
- **Signal processor** such as a computer equipped with software mathematically transforms signals from the detector.

1.4.5 Atomic Absorption Spectrometry.

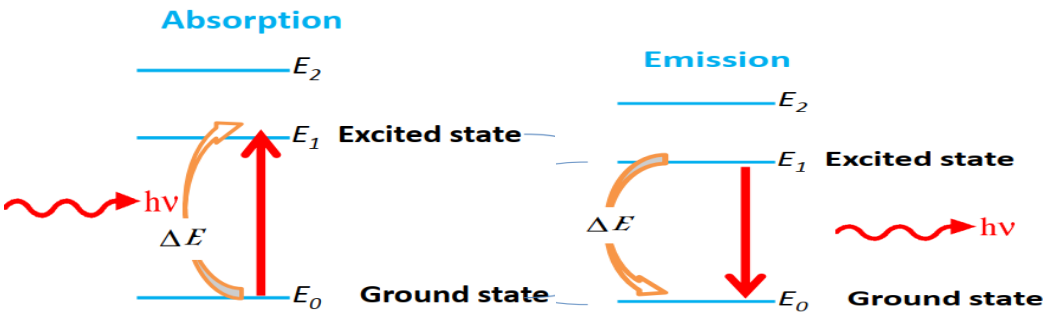
Atomic absorption spectrometry measures the amount of radiation absorbed by ground-state gaseous atoms of analyte for quantitation **unlike atomic emission** spectrophotometry which measures the amount of excess radiation emitted by excited-state particles of analyte as they return to ground-state.

A decrease in the amount of radiation reaching the detector is measured as absorbance which is directly proportional to the concentration of the element in the original sample.

When the analyte is a molecular species, it is referred to as **molecular absorption** or emission spectrometry.

Figure 7

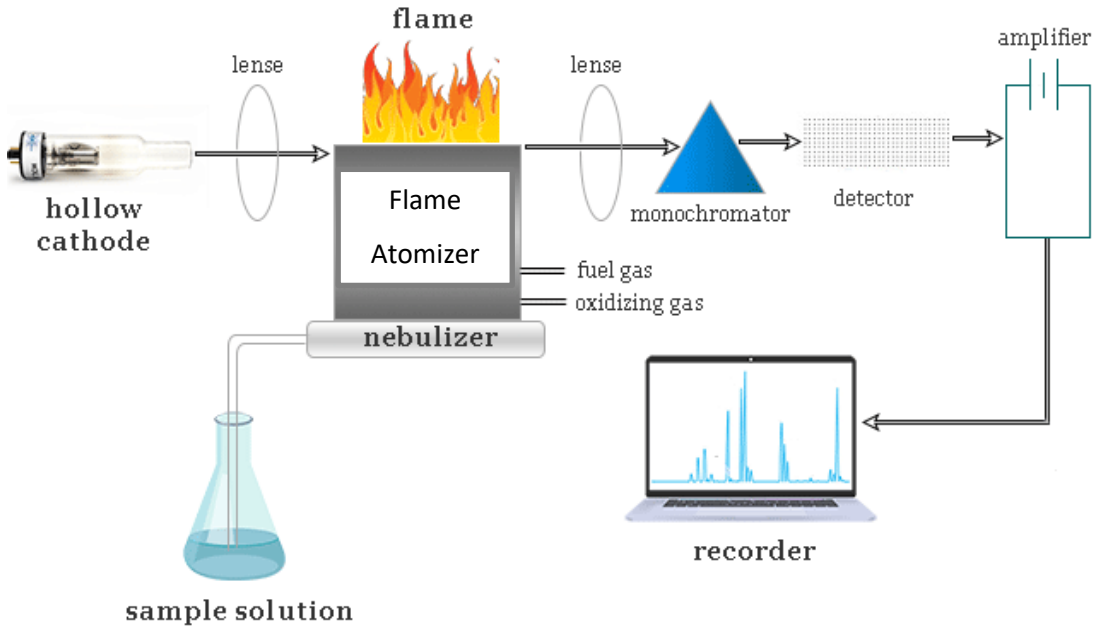
Absorption and Emission Process Illustration.



The working principles of a typical atomic absorption spectrophotometer are summarized in the figure below:

Figure 8

Flame Atomic Absorption Spectrophotometer Setup



1.4.5.1 Atomizer

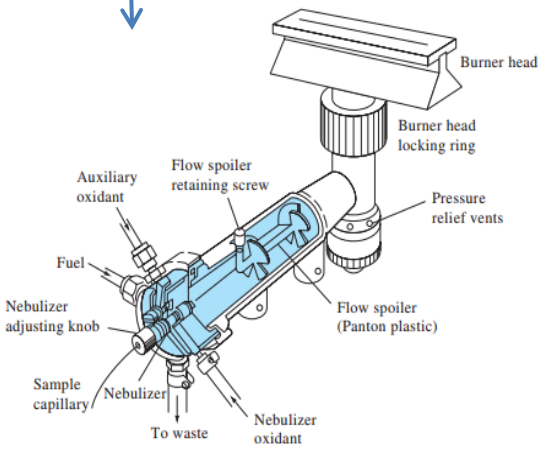
The role of the atomizer is to convert elements in a nebulized sample solution to gaseous atoms.

With respect to atomizer used, atomic absorption spectrophotometry can be classified as **flame atomic absorption spectrophotometry (FAAS)** if atomizer is flame as illustrated in figure 8 above OR **graphite furnace (electro thermal) atomic absorption spectrophotometry (GFAAS**) if atomizer is a graphite furnace. The flame is a gaseous mixture of an oxidant (such as air, oxygen, or nitrous oxide) and a gaseous fuel (such acetylene, hydrogen or natural gas).

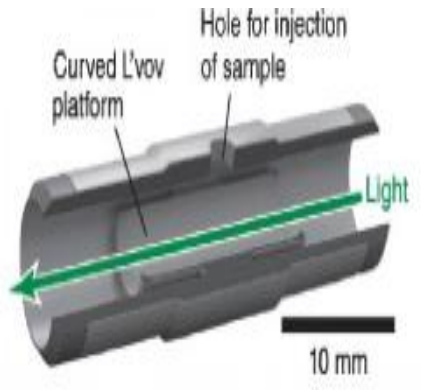
The photo below shows ***iCE 3000 series model atomic absorption spectrophotometer*** with laminar flow burner flame (left) and graphite furnace (right) compartments and their respective cross-sections (Skoog et al., 2018).

Figure 9

iCE 3000 Series Model Atomic Absorption Spectrophotometer. (Courtesy of Instrumental Analysis Laboratory in the Department of Analytical Chemistry, Near East University TRNC).



Flame atomizer (cross view)



Graphite furnace atomizer (cross view)

Table 1

A Summary of the Types of Atomizers and their Typical Temperature Ranges used in Atomic Spectroscopy

Type of atomizer	**Typical atomization temperature, ^{o}C**
Flame	1700-3150
Graphite furnace	1200-3000
Inductively coupled argon plasma (ICP)	4000-6000
Direct current argon plasma (DCP)	4000-6000
Microwave induced argon plasma (MIP)	2000-3000
Laser-induced plasma (LIP)	8000-15000
Glow-discharge (GD) plasma	Non thermal

The **inductively coupled argon (Ar) plasma (ICP)** atomizer listed in table 1 above is very popular and it is mostly employed in **atomic emission spectrophotometry (ICP-AES)** were population of excited state atoms affects the sensitivity and hence detection limit of the instrument. This is because the ICP flame is twice as hot as flame from combustion atomizers, flame stability and relatively inert Ar environment which eliminates most of the interferences encountered in flames. The ICP atomizer is also used regularly with a mass spectrometer **(ICP-MS)** for multi elemental analysis and with detection limits as low as parts per trillions. ICP atomizers are however more expensive to purchase and operate than combustion flames.

1.4.5.2 Types and properties of flame.

Table 2

Types and Properties of Oxidant-Fuel Flame Mixtures

Oxidant	**Fuel**	**Temperature, ^{o}C**	**Max. burning velocity, cm s^{-1}**
Air	Acetylene HC≡CH	2100-2400	158-266
Nitrous oxide N_2O	Acetylene	2600-2800	285
Oxygen	Acetylene	3050-3150	1100-2480
Air	Hydrogen	2000-2100	300-440
oxygen	Hydrogen	2550-2700	900-1400
Air	Natural gas	1700-1900	39-43
Oxygen	Natural gas	2700-2800	370-390

Of all the flames listed in table 2, air-acetylene is the most commonly used for safety concerns. Nitrous oxide- acetylene is also used because of its higher temperature which can easily break strong bonds between refractory compounds and analyte but the explosive nature of nitrous oxide –acetylene mixture makes it a rare choice for most analysts.

The burning velocities also listed are very significant because they affect flame stability, atomization efficiency and laboratory safety in the following ways;

- If gas flow rate is less than burning velocity, flame propagates back into the burner, giving pop sounds or flashback.
- If the gas flow rate goes far above the burning velocity, the flame blows off the burner.
- Flame is only stable when gas flow rate equals burning velocity. The process to stability is gradual, reason why it is advisable to allow the flame flow for at least 10 minutes after switching it ON before sample injection.

The pros and cons of flame atomic absorption spectrophotometers are summarized in **table 3** below;

Table 3

The Pros and Cons of Flame Atomic Absorption Spectrophotometers

Pros	**Cons**
High reproducibility	Poor atomization
Fast and easy to use	Moderate detection limits
Lowest capital cost	Large sample size
Very compact instrument	1-10 elements per determination
Relatively few interferences	No screening ability

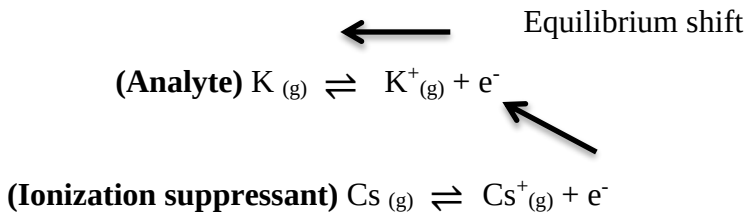
1.4.6 Interferences in Atomic Absorption Spectrophotometry

Interference is any effect that changes the intensity of analyte signal (either positive or negative) while analyte concentration remains unchanged. They are four common types encountered in atomic absorption spectrophotometry namely: spectral, matrix, chemical and ionization interferences (Harris & Lucy, 2020).

- **Spectral interferences** occur when the signal of analyte overlap with signals from other elements or molecules in the sample such as molecular emissions from oxides of other elements in the sample. This overlap can be corrected by choosing another wavelength for analysis or by using high-resolution spectrometers which better resolves closely spaced lines. Other source of spectral interference is overlap of signals from flame or furnace with analyte signal. This can be corrected by using deuterium (D_2) or Zeeman background correction to subtract flame signals.
- **Matrix interference** is caused by any component of the sample that alters the sample movement or nebulization such as differences in viscosity or density of the samples due to their matrix or the reagents used for digestion, preservation and salt deposits may cause sample to nebulize differently. Peristaltic pumps ensure steadier solution flow to flame or plasma.
- **Chemical interference** is caused by any component of the sample that affects any of the chemical processes in atomization of the analyte such as bond breaking. For example, SO_4^{2-} and PO_4^{3-} hinder the atomization of Ca^{2+} because they form non-volatile compounds with Ca^{2+}. Chemicals called ***releasing agents*** that preferentially reacts with these compounds to free Ca^{2+}

are usually added in excess to sample solutions to overcome chemical interferences. Lanthanum and strontium are commonly used. EDTA and 8-hydroxyquinoline can also be used as ***capping agents*** as they readily form volatile complexes with Ca^{2+}. A fuel-rich flame or higher temperature flame such as nitrous oxide- acetylene also eliminates chemical interference because they provide higher dissociation energy to break free analyte from thermally stable compounds.

- **Ionization interference** is common with the analysis of easily ionizable metals such alkali metals at relatively low temperatures and in the analysis of other elements at high temperatures. Most ions and their metal atoms don't absorb at the same wavelength leading to an overall decrease in analyte signal. Ionization suppressors such as potassium (K), cesium (Cs) and rubidium (Rb) are used to decrease extent of ionization by Le Chatelier's principle illustrated below. For example, it is recommended to add a solution that contains 1000ppm of CsCl (Cesium chloride) in the analysis of potassium.



1.5 Sample Preparation for Metal Analysis in Atomic Absorption Spectrophotometry

Samples for atomic absorption spectrometry are usually dissolved or solubilize through processes that destroys organic matter and free inorganic elements in forms suitable for analysis. Organic matter may be destroyed by combustion (**dry ashing**) with aid of a muffle furnace or by oxidation with reagents (**wet acid digestion**) such as with a strong acid or combination of acids depending on the nature of sample matrix. Wet ashing is aided with the use of a hot plate or more sophisticated and faster microwave assisted decomposition (Cruijsen et al., 2019).

Nitric acid (HNO_3) is commonly used reagent because it is readily available in high purity and does not form insoluble salts as might happen with hydrochloric acid (HCl) or sulfuric acid (H_2SO_4). Hydrogen peroxide (H_2O_2) may be added to increase the oxidizing power of the digestion solution and also because it reduces nitrogen oxides, NO (seen as brown fumes during digestion) generated by digestion of organic matter (Harris & Lucy, 2020).

1.6 Statement of Problem (s)

- Lack of correct nutrition data on labels of most of the local Hellim cheese products is a call for concern. Majority labels show just fats and protein contents while very few give details of Calcium present. This shows lack of state regulation, laboratory sophistication, cooperation between local producers and research laboratories in higher institutions, financing for local producers to get their final products analyzed and controlled so that they can meet the standards of a bigger international market.
- Variations in quantities of these macro minerals and even other trace elements in these dairy products comes from the milk used in the production stage and whose quality is affected by several factors such as breed, feeding system, animal's nutritional and health status, stage of lactation, season and to a lesser extent, additives such $Cacl_2$ added to manipulate mineral contents or reduce rennet coagulation time (RCT) which is very common in cheese curd formation in cheese processing (Guggisberg et al.,2022; Masotti et al., 2020)

1.7 Research Objectives

Analytical data on nutrition composition with respect to macro minerals in these products is necessary for regulation of milk sources, quality improvement through standardization and hence the general health and safety of the consumers. This should also create invitation for more in-depth research into the entire livestock sector of Turkish Republic of Northern Cyprus in future. Also, since the overall mineral content including heavy metals of Hellim and milk produced here in Turkish republic of Northern Cyprus is very much under reported or completely absent, this work is aimed at calling for more research into this sector of the country.

This exercise will employ the use of **Flame Atomic Absorption Spectrometry (FAAS) for analysis.**

In the absence of commonly used modern microwave digester for sample digestion, an optimized and low cost open wet acid digestion method with nitric acid and hydrogen peroxide mixture (HNO_3/H_2O_2) as digestion reagents was used to solubilize the samples before analysis. All guides plus safety for sample preparation, analysis and method validation was taken from AOAC official method 2011.14 (Cruijsen et al., 2019).

1.8 Limitation

- Open wet acid digestion for sample preparation though less cheap and easier to operate than recommended closed microwave system, it is time consuming and liable to contamination from unclean laboratory environment because digestions are carried at atmospheric pressure and room temperature on just a hot plate.
- I worked with open windows due to extreme heat in the instrumental analysis lab 4 because the AC system was faulty. Slight wind from the windows led to flame instability during analysis.
- Fewer glassware.

CHAPTER 2

The objective of this chapter is to highlight articles with similar research objectives to this thesis irrespective of analysis method used, sample digestion method and number of analytes.

Literature Review

Articles with related objectives from different countries have been published with most utilizing a more sophisticated and faster microwave assisted decomposition of sample and an inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled mass spectrometry (ICP-MS) for analysis.

Diniz et al. analyzed Ca, Mg, K, and Na in Brazilian cream cheese with Flame Atomic Absorption Spectrometry (FAAS) and Microwave Induced Plasma Optical Emission Spectrometry (MIP OES) after acid decomposition with HNO_3/H_2SO_4 in reflux system in 2017. Their objective was to compare the two spectrometric methods and as well as to evaluate the concentrations of these minerals Brazilian cream cheese. The concentration values of the elements evaluated were not in accordance with the values indicated on the labels of all products showing the importance of monitoring these products.

Snirc et al.,2019, analyzed the content mineral of 19 samples of traditional Oštiepok cheese with inductively coupled plasma optical emission spectrometry (ICP-OES) and got an average of 685mg/100g Calcium and 38.1mg/100g magnesium. Sample digestion was done with HNO_3/H_2O_2 with the aid of pressure microwave. Though the traditional cheese was considered a suitable source of macro and microminerals for human health but overall concentrations vary from sample to sample and values were low based on recommended daily average.

Karasakal, 2019, also analyzed trace and major elements in 81 vegan milk samples and oil samples from Turkey covering three different brands by

inductively coupled plasma optical emission spectrometry (ICP-OES) after HNO_3/H_2O_2 and microwave assisted digestion.

Lee et al. analyzed macro and micromineral content of 20 whole milk, 12 skimmed milk and 18 organic milk samples from top 10 milk manufacturing companies in South Korea with ICP-OES and ICP-MS. Higher concentrations of calcium and magnesium were found in whole milk samples. An average of 106.8mg/100g calcium and 10.7mg/100g magnesium were found.

CHAPTER 3

This chapter covers instrumentation and optimum conditions used, reagents used, apparatus and overall sample preparation procedure.

Experimental

3.1 Instrumentation

The analysis of Calcium and Magnesium in the various samples purchased locally was carried out using ***Thermo scientific iCE 3000 series*** atomic absorption spectrometer with element specific hollow cathode lamps as radiation source. Instrument's operating conditions are summarized in table 4 below:

Table 4

Thermo Scientific Ice 3000 Series Operating Conditions

Parameters	**Calcium**	**Magnesium**
Wavelength (nm)	422.5	285.1
Lamp current (%)	100	75
Measurement time (s)	4	4
Number of resample	3	3
Band pass (nm)	0.5	0.5
Nebulizer flow (L/min)	0.8	0.8
Background correction	D_2 Quadline	D_2 Quadline
Flame	Air-acetylene	Air-acetylene
Burner height (mm)	50	50
Fame height (mm)	8	7

3.2 Reagents and solutions

Analytical grade reagents from Sigma-Aldrich Germany were used throughout this exercise. Nitric acid, HNO_3 (65%w/v) and hydrogen peroxide, H_2O_2 (35%w/v) were used as digestion reagents. Magnesium nitrate hexahydrate $Mg(NO_3)_2.6H_2O$ and calcium nitrate tetrahydrate, $Ca(NO_3)_2.4H_2O$ were used to prepare standards for respective metals. 1000mg/L of each metal was prepared

from stoichiometric mass values of each respective metal salts in 1% nitric acid. Strontium nitrate (releasing agent) and deionized water (diluent).

3.3 Apparatus

Ceramic mortar and pestle (from ISO LAB, Germany), micropipettes from ISO LAB Germany, plastic knives, hot plate, vortex machine from Heidolph Reax, binder oven (USA), 15ml falcon tubes from Sigma-Aldrich (USA), Whatman filter papers (110mm, ashless), 100ml and 50ml volumetric flask from ISO LAB Germany.

All glassware were soaked in 10% nitric acid overnight, rinsed with deionized water and oven dried before used.

3.4 Samples and Sample Preparation.

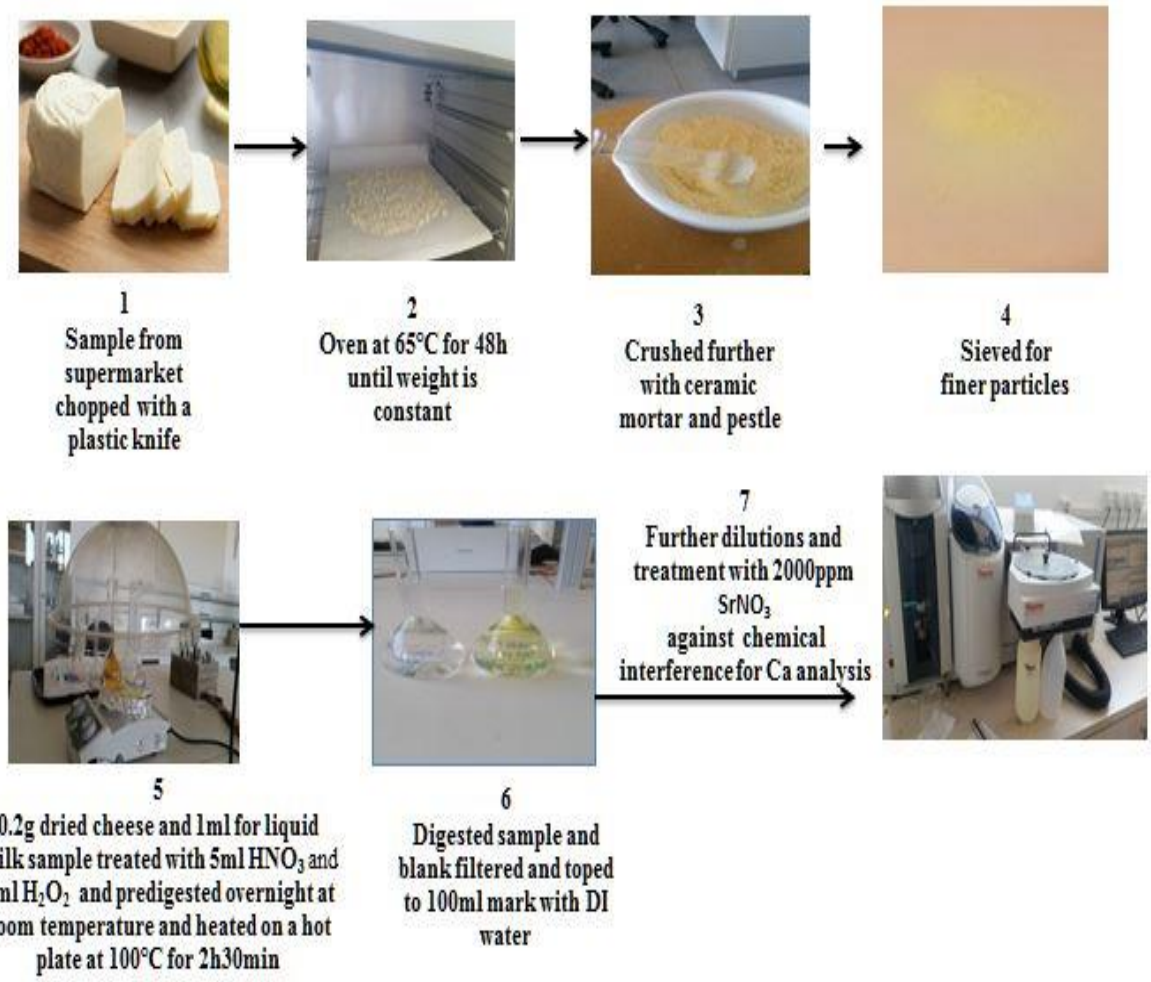
Cheese and whole milk samples were bought from supermarkets in Lefkosa and Gazimagusa municipalities. Cheese samples were cut into small pieces with a plastic knife and dried at $65^{O}C$ for 48hours in an oven until constant weight was obtained. The dried samples were further crushed into finer particles by means of a ceramic mortar and pestle. 0.2g portions from each cheese sample and 1ml portions from each milk sample was dissolved in a mixture of 5ml HNO_3 (65%w/v) and 2ml H_2O_2 (35%w/v) in a 100ml volume flask and pre-digested overnight at room temperature. The pre-digested sample was then placed on a hot plate the following morning and digested further at $100^{O}C$ for 2h30mins under a fume hood. The digests were allowed to cool and then filtered using a Whatman filter paper into another acid washed 100ml volumetric flask and toped to the 100ml mark with deionized water. Further dilutions were made into separate falcon tubes for each sample so that concentrations fit appropriately into the linear dynamic range of the calibration curves plotted. Analysis was done on the same day of sample preparation.

Falcon tubes with samples for calcium analysis only were treated with 2000mg/L Sr $(NO_3)_2$ as releasing agent against phosphate interferences. Reagent blank as control for contamination was prepared under same conditions as sample.

Calibration curves of corresponding pure metal nitrate salts were prepared in concentrations ranging from 2 to 14mg/L for Ca and 0.1 to 0.7mg/L for Mg.

Figure 10

Summary of Sample Preparation Steps



CHAPTER 4

Results and Discussion

Optimization of some variables such as digestion reagent will be discussed here. Calibration data, figures of merit and data on concentration of elements will also be discussed in this chapter.

4.1 Optimization of Results

Optimum instrument's conditions for each element were used as stated in the element's cookbook or manufacturer's recommendation. Digestion reagents for samples was of the variables optimized throughout this exercise

4.1.1 Optimization of Digestion Reagent.

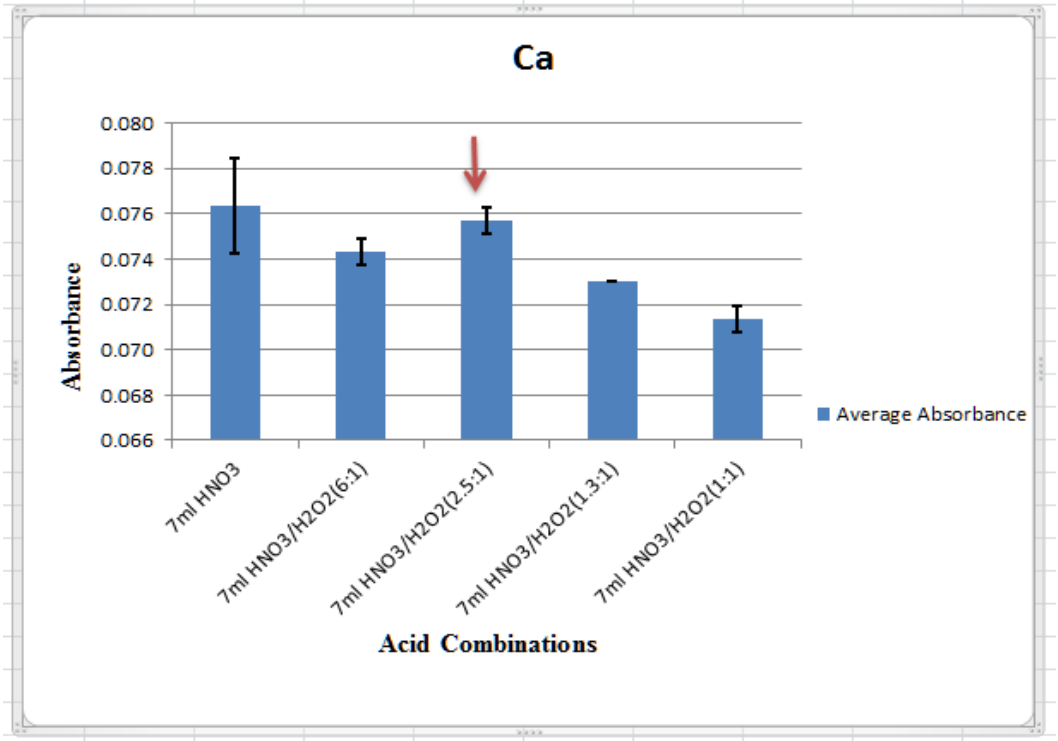
With respect to sample matrix and metals being analyzed, nitric acid, hydrochloric acid, sulfuric acid and hydrogen fluoride are reported to be the most effective acids used in the digestion of inorganic and organic samples for metal analysis (Mohammed et al.,2017). This is because of their powerful oxidizing abilities when used in combination with other reagents such as hydrogen peroxide, permanganate and chlorate.

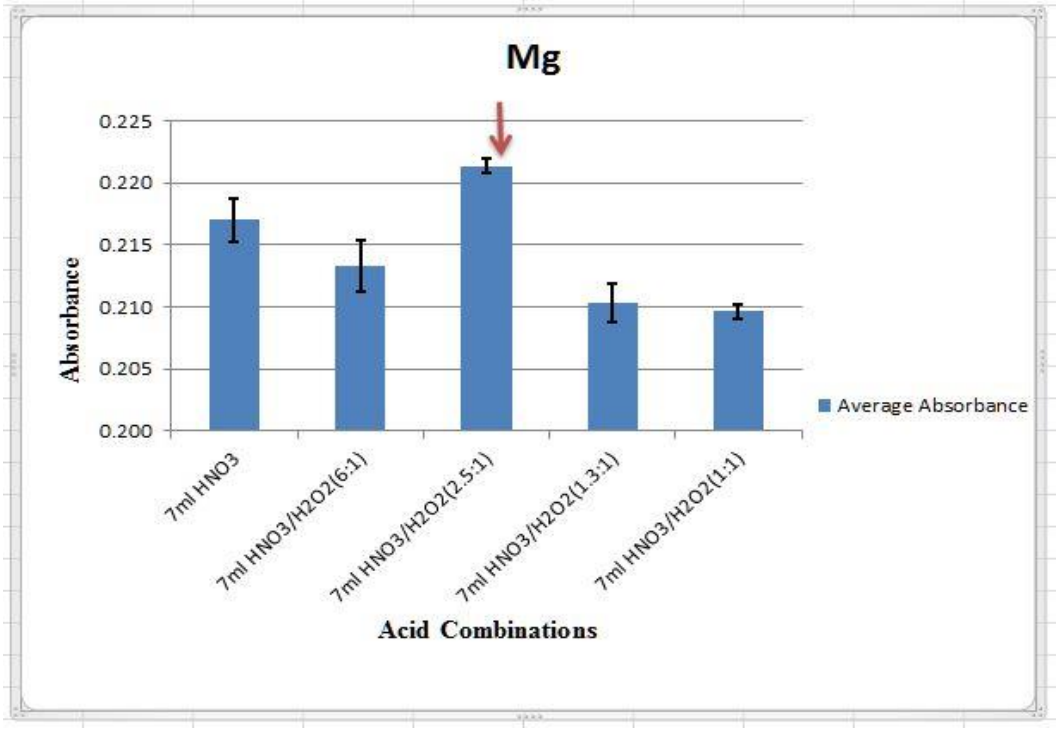
Nitric acid was used alone and in combination with hydrogen peroxide and the efficiency or oxidizing strength of each digestion media in the breakdown of sample matrix was assessed through absorbance signal after three repeats of both metals. All combinations were subjected to the same heating time and temperature conditions and sample cheese 2 was used because of its high fat content. Signals observed were not too different but 5ml HNO_3 and 2ml H_2O_2 (2.5:1) gave best signal for both metals as shown in figure 11

- 7ml HNO_3 and 0ml H_2O_2
- 6ml HNO_3 and 1ml H_2O_2 (6:1)
- 5ml HNO_3 and 2ml H_2O_2 (2.5:1)
- 4ml HNO_3 and 3ml H_2O_2 (1.3:1)
- 3.5ml HNO_3 and 3.5ml H_2O_2 (1:1)

Figure 11

Optimization of Digestion Reagent.





4.1.2 Addition of Excess Strontium Nitrate Concentration as a Releasing agent against Chemical Interferences.

Cheese and milk samples were treated with excess strontium nitrate as releasing agent against chemical interferences. The change in signal for both analytes was observed and accuracy was checked with recovery of spiked samples. A better signal and recovery were gotten for calcium when at least 2000mg/L strontium nitrate was used. The releasing agent had a slightly negative but insignificant effect on the overall signal of magnesium, reason why releasing agent was not used for analysis of magnesium because better signals were obtained for untreated samples. Analytical recoveries were gotten when strontium nitrate concentration from at least 2000mg/L was used. The increase in the signal of calcium when treated with releasing agent showed calcium was greatly affected by refractory compounds. Calcium standards also treated with 2000mg/L strontium nitrate didn't show any effect on the signal.

4.2 Figures of Merit and Quantitation.

The instrument's limit of detection (LOD) was calculated as 3 times the standard deviation of 8 replicate reagent blank aspirations divided by the slope of the linear square fit equation of the calibration curve for both elements. Same calculation was done for limit of quantitation (LOQ) but 10 times the standard deviation of 8 replicate reagent blank aspirations was used. The LOD and LOQ for the method were 0.46mg/L and 1.34mg/L respectively for calcium and 0.01mg/L and 0.03mg/L respectively for magnesium as summarized with other parameters in **table 5 below.**

In the absence of a certified reference material, only recovery test was used to check accuracy of the method. Cheese and milk samples were all spiked with known analyte concentrations at two levels for cheese and milk samples and percent recovery calculated after subjecting spiked samples through the same digestion procedure and analysis. The analytes were successfully recovered between 80% and 114% as shown in **table 6 below.**

The concentration of calcium and magnesium in the samples were finally determined from aqueous linear calibration curves **(Figure 12 and 13 below)** of

respective analytes standards and the average of 4 replicate measurements reported in **table 7**

Figure 12

Calcium Aqueous Calibration Curve.

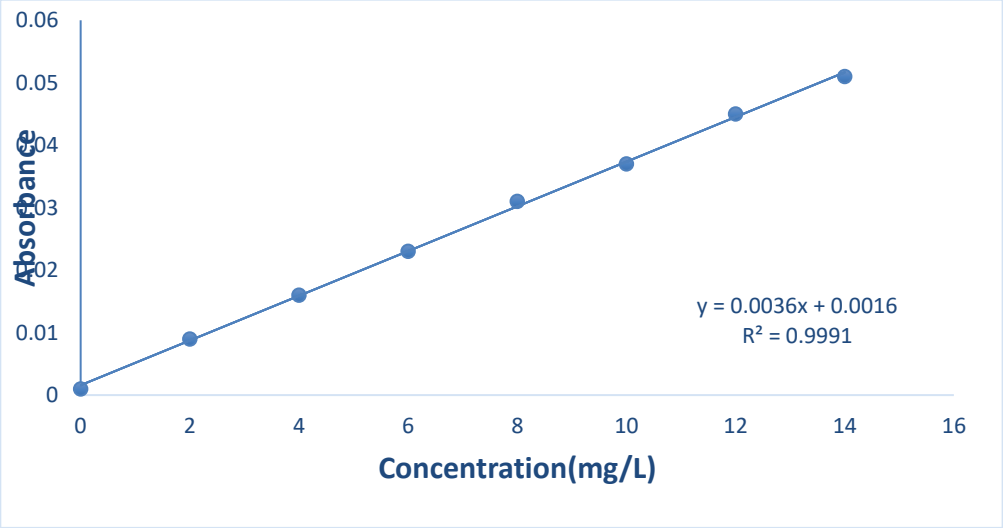


Figure 13

Magnesium Aqueous Calibration Curve

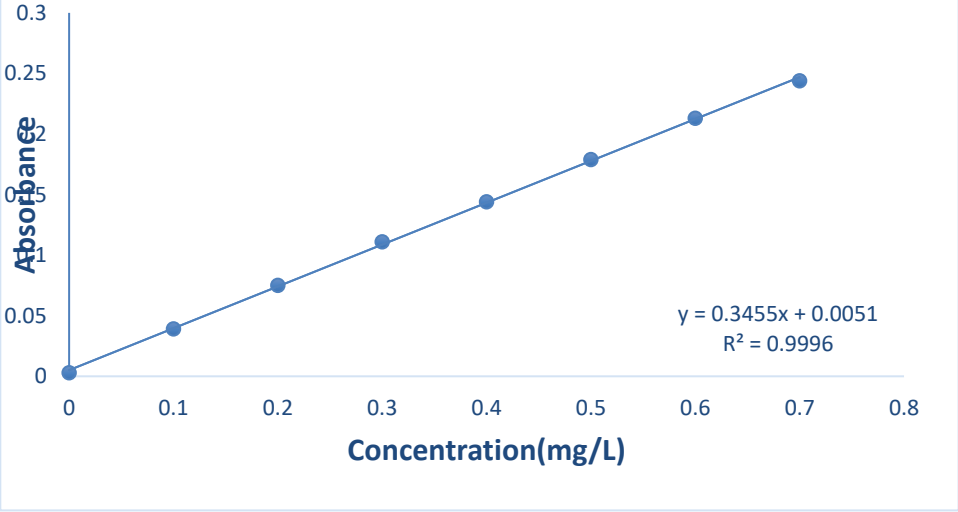


Table 5

Analytical Figures of Merit

Elements	Linear equation[a]	R^2	LOD[b]mg/L	LOQ[C]mg/L	LDR[d]mg/L
Ca	Y=0.0036x+0.016(±0.0005)	0.9991	0.46	1.34	2-14
Mg	Y=0.3455x+0.0051(±0.001)	0.9996	0.01	0.03	0.1-0.7

[a]Signal =slope x concentration (mg/L) + blank (±σ), n=8

[b]Limit of detection = 3σ/slope

[c]Limit of quantitation = 10σ/slope

[d]Linear dynamic range

R^2 (Linear correlation coefficient).

σ Standard deviation

Table 6

Relative Recoveries of Spiked Hellim Cheese and Milk Samples.

Sample	Analyte	Added(mg/g)	Found(mg/g)	Recovery (%)
Cheese sample 1[(a)]	Ca	0.00	20.18	-
		2.5	22.28	84
		3.75	24.12	104.9
	Mg	0.00	0.79	-
		0.25	1.03	96
		0.38	1.19	105
Cheese Sample 2[(b)]	Ca	0.00	13.58	-
		2.5	15.68	84
		3.75	17.53	105
	Mg	0.00	0.69	-
		0.25	0.93	96
		0.38	1.04	92
Cheese sample 3[(c)]	Ca	0.00	14.28	-
		2.5	17.08	112
		3.75	17.75	92
	Mg	0.00	0.69	-
		0.25	0.94	99
		0.38	1.08	103
Milk sample 1[(d)]	Ca	0.00	0.99	-
		0.49	1.48	100
		0.73	1.66	92
	Mg	0.00	0.10	-
		0.05	0.15	100
		0.07	0.18	114
Milk sample 2[(e)]	Ca	0.00	1.03	-
		0.49	1.53	102
		0.73	1.70	92
	Mg	0.00	0.10	-
		0.05	0.146	92
		0.07	0.171	101
Milk sample 3[(f)]	Ca	0.00	0.89	-
		0.49	1.28	80
		0.73	1.58	95
	Mg	0.00	0.1	-
		0.05	0.15	107
		0.07	0.17	92

Table 7

Average (n=4) Concentration (mgg^{-1}) and (mg/100g) of Ca and Mg in the Different Hellim Cheese and Milk Brands Obtained by FAAS against Company Labelled Concentration (mg/100g)

Sample	Analyte	Concentration (mgg^{-1}) ±SD[g]	Concentration (mg/100g)	RSD%[h]	Company label
Cheese sample 1[a]	Ca	18.64±1.20	1864	6.4	NA
	Mg	0.788±0.017	78.8	2.17	NA
Cheese sample 2[b]	Ca	14.19±0.85	1419	5.95	NA
	Mg	0.695±0.029	69.5	4.15	NA
Cheese sample 3[c]	Ca	14.27±0.00	1427	0	700
	Mg	0.690±0.016	69	2.37	NA
Milk sample 1[d]	Ca	1.03±0.1	103	10.20	114
	Mg	0.100±0.05	10	4.89	NA
Milk sample 2[e]	Ca	0.99±0.18	99	15.33	114
	Mg	0.100±0.008	10	8.08	NA
Milk sample 3[f]	Ca	0.89±0.05	89	5.33	110
	Mg	0.097±0.0005	9.7	0.51	NA

[a] White salty high fat hellim 1

[b] White salty high fat hellim 2

[c] White reduced salt high fat Hellim 3

[d] semi skimmed liquid milk1

[e] Semi skimmed liquid milk 2

[f] Whole fat liquid milk 3

[g] SD (Standard deviation, n=4)

[h] %RSD (Relative standard deviation)

Table 8

Calcium and Magnesium content of some cheese brands and milk produced in other countries, mg per 100g.

Cheese type	**Ca**	**Mg**	**Reference**
Normal Cheddar	720	25	O'Brien&O'Connor.2017
Reduced fat Cheddar	840	39	O'Brien&O'Connor.2017
Mozzarella	590	27	O'Brien&O'Connor.2017
Parmesan	1200	45	O'Brien&O'Connor.2017
Cream cheese	98	10	O'Brien&O'Connor.2017
Traditional Oštiepok cheese	685	38.1	Šnirc et al., 2019
Hellim Cheese	1427	69.5	This thesis
Milk			
South Korean whole milk	106.8	10.7	Lee et al., 2022
TRNC whole milk	99	10	This thesis

Concentration of calcium in the above three cheese and three milk samples produced in TRNC was found between 1419 – 1864mg/100g and 89 – 103mg/100g respectively. Magnesium in cheese and milk samples was found between 69 – 78.8mg/100g and 9.7 – 10mg/100g respectively. The values given on some the labels don't match what was gotten in this analysis with milk sample values below company label and cheese values far above company labels too.

The differences observed must have come from regional and production conditions of milk.

A study conducted with milk from 1860 primiparous Dutch Holstein-Friesian cows from 388 herds in the Netherland reported that genetics had greater effect on the overall mineral quality of milk than on environmental and management factors or herds effects (Oh & Deeth, 2017; Stocco et al., 2017). This means that the best ways of altering the levels of these minerals in milk is by selective breeding rather than nutritional manipulations as in the case of cheese and milk industrial production. Selective breeding improves milk quality and hence milk coagulation properties (MCP) for quality cheese production (Stocco et al., 2017).

CHAPTER 5

Conclusion and Future Work.

This study provides useful information on the concentration of calcium and magnesium in the traditional Hellim cheese and milk produced in the Turkish Republic of Northern Cyprus (TRNC). This will open room for more research into this sector because this is the first study comparing the mineral content of traditional Hellim cheese produced in TRNC.

Calcium concentration in cheese was found between 1419 – 1864mg/100g for the three cheese samples analyzed and between 69 -79mg/100g was recorded for magnesium.
Calcium was also in the range 89 – 103mg/100g for milk samples analyzed and between 9.7 – 10mg/100g for magnesium. Although these values vary and very much don't reflect the company labels, we can conclude that Hellim cheese and milk from TRNC compared to values shown in table 8 is a reliable source of calcium and magnesium in health based on recommended daily average (RDA).

Much needs to be done in standardizing the observed variations due to regional production conditions such as by selective breeding in improving cattle milk quality rather than nutritional manipulations such as the addition $CaCl_2$ to decrease rennet coagulation time in cheese processing (Oh & Deeth, 2017; Stocco et al., 2017; Guggisberg et al., 2022; Masotti et al.,2020).

This is necessary to prevent excessive intake of these minerals which also come with serious health complications. Ideally, the minerals our body needs to function properly comes from so many other food sources and the water we drink daily. So consuming dairy products with uncontrolled mineral quality puts the population at risk to mineral toxicity. Therefore, Government through food safety regulatory bodies needs to get actively involved and monitor or control the dairy sector of TRNC from farms to industrial production centers.

Future work will aim at extending the number of minerals to be investigated to others such as sodium, potassium, phosphorus, sulfur etc. and to trace elements or heavy metals. Also, investigations to determine the various factors affecting livestock milk mineral composition such as feeding system of cattle, health status, lactation stage and breed of livestock production in TRNC.

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