Determining Monosodium Glutamate in Cafeteria Foods at the Eastern Mediterranean University

Abd Alkarim Khalil Tarda

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Approval of the Institute of Graduate Studies and Research

_____

Prof. Dr. Mustafa Tümer
Director

I certify that this thesis satisfies the requirements as a thesis for the degree of Master of Science in Chemistry.

_____

Assoc. Prof. Dr. İzzet Sakallı
Chair, Department of Chemistry

We certify that we have read this thesis and that in our opinion it is fully adequate in scope and quality as a thesis for the degree of Master of Science in Chemistry.

_____

Asst. Prof. Dr. Mehmet Garip
Supervisor

Examining Committee

1. Prof. Dr. Elvan Yılmaz _____

2. Assoc. Prof. Dr. Mustafa Gazi _____

3. Asst. Prof. Dr. Mehmet Garip _____

ABSTRACT

Monosodium Glutamate (MSG) is a flavor enhancer that is used widely around the world. In this thesis, MSG was extracted from samples of cooked foods purchased from four different cafeterias in EMU; and from packaged foodstuffs such as chicken bouillon and four different powdered soups (Brand II) bought locally. The identification as well as relative concentrations of MSG in these samples were determined by TLC and their actual concentrations were estimated by HPLC using a 50:50 water-acetonitrile mobile phase. Peaks were monitored at 210, 220, 230, 240 and 250 nm but highest sensitivity was obtained with 210 nm radiation. Although good results were obtained with pure MSG standard solutions, there were, however, problems with the food extracts when analyzed with HPLC. There were overlapping unresolved peaks for some of the food extracts that could not be conclusively identified. These peaks, we suspect, are due to water soluble amino acids extracted for the cooked foods. Due to their great solubility, they tend to have low retention times and thus exit the column almost simultaneously. For the samples that could be identified and quantified, relatively high amounts of MSG ranging between 0.94 % and 6.5 % were found. These amounts of MSG may be added or naturally present in the food.

More accurate measurement of the MSG concentration by HPLC can be done by derivatizing the MSG to make it more hydrophobic. This would increase the retention times of the components thereby providing greater degree of separation and resolution.

Keywords: MSG, analysis, food, TLC, HPLC, Derivatization.

ÖZ

Monosodyum Glutamat (MSG), dünyada yaygın olarak kullanılan bir lezzet artırıcıdır. Bu tezde, DAÜ'de dört farklı kafeteryadan alınan pişmiş gıdalardan ve yerel olarak satın alınan tavuk bulyonu ve dört farklı paket çorba (Brand II) de bulunan MSG su ile çıkarıldı. Bu numunelerde MSG'nin tespiti ve nispi konsantrasyonları TLC ile belirlendi ve gerçek konsantrasyonları ise 50:50 su-asetonitril mobil faz kullanılarak HPLC yöntemi ile ölçülmeye çalışıldı. Ölçümler 210, 220, 230, 240 ve 250 nm dalga boyunda çalışıldı ve 210 nm ışın ile en yüksek hassasiyet elde edildi. Saf MSG standart çözeltileri HPLC yöntemi ile iyi sonuçlar vermesine karşın gıda çözeltilerinin analizlerinde sorunlar yaşandı. Bazı gıda çözeltilerinin kromatogramında kesin tanımı yapılamayan ve birbirinden iyice ayrılmamış zirveler gözlendi. Bu zirvelerin pişmiş gıdalarda oluşan ve suda kolay çözülebilen amino asitler olduğunu tahmin ediyoruz. Bu amino asitler sudaki yüksek çözünürlüklerinden ötürü, düşük alıkonma sürelerine sahip olurlar ve kolondan hemen hemen aynı zamanda çıkarlar. Tanımlanan ve konsantrasyonu ölçülebilen örneklerde yüzdelik MSG miktarı % 9 ila % 60 arasında değişen miktarlar olarak bulunmuştur. Ancak bu MSG miktarlarının yiyeceğe yapay olarak mı eklendiği yoksa doğal olarak mı bulunduğu konusunda kesin bir yargıya varılamamıştır.

MSG konsantrasyonunun HPLC ile daha doğru ölçümü, MSG'yi daha hidrofobik hale getirmek için türevlendirmek suretiyle yapılabilir. Bu, bileşenlerin alıkonma sürelerini artırarak daha belirgin ayrışmalarını sağlayacaktır.

Anahtar kelimeler: MSG, analiz, gıda, TLC, HPLC, türevlendirme.

TO MY DEAR FAMILY

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LIST OF ABBREAVIATIONS

ADI	Acceptable Daily Intake
ASA	Acetylsalicylic acid
BBB	Blood Brain Barrier
D-II	Doner-Cafeteria II
BS	Barbecue-Sauce
CASRN	Chemical Abstracts Service Registry Number
CB	Chicken-bouillon
CRS	Chinese Restaurant Syndrome
DAD	Diode Array Detector
DBPC	Double blind placebo controlled
DNFB	1-Fluoro-2,4-dinitrobenzen
D-GLU	Dextrorotatory Glutamic acid
ED_{50}	Effective Dose, 50%
ES	Ezogelin-Soup
FAO	Food Agricultural Organization
FASEB	Federation of American Societies for Experimental Biology
FSANZ	Food Standards Australia New Zealand
F_{254}	Fluorescent indicator at 254 nm
HILC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance/Pressure Liquid Chromatography
IR	Infra-Red
JECFA	Joint Experts Committee of Food Additives
LD_{50}	Lethal Dose, 50%

L-GLU	Levorotatory Glutamic acid
mAU	mille Absorbance Unit
D-I	Doner-Cafeteria I
MS	Mercimek-Soup
MSG	Monosodium Glutamate
NP-HPLC	Normal Phase-HPLC
OPA	O-phthalaldehyde
ppm	part per million
RF	Retention Factor
RP-HPLC	Reversed Phase-HPLC
R_t	Retention time
SCF	Scientific Committee on Food
SS	Sebze-Soup
TLC	Thin Layer Chromatography
UV/VIS	Ultraviolet- Visible
WHO	World Health Organization

Chapter 1

INTRODUCTION

Healthy foods provide our bodies with a lot of essential nutrients such as carbohydrates, fats, proteins, vitamins and minerals. Therefore, selecting the most nutritious food plays a very important role in remaining alive, and having energy and capacity to do any work. On the other hand, unhealthy foods lead to several sicknesses. Diversity of food around the world depends on factors such as religion, culture, climate and habits. In China, for instance, one of the most frequently used food ingredient is monosodium glutamate (MSG) which used as a flavor enhancer (Sanjo, Mamta & Arvind, 2014, pp. 38-41).

Kikunea Ikeda was the first researcher who started a project in 1907 to identify the nature of the compound found in kelp (a type of seaweed used in Fareast cooking) which gives a favored taste for Japanese soups. In 1908 he established conclusively that MSG was the reason behind the fifth taste which he called ***umami***. Furthermore, in 1909 Ikeda and two other co-workers established the industrial process to produce MSG on a large scale (Chiaki, 2009, pp.728S-732S). Currently, the fermentation process - which uses special microorganism such as *Microbacterium ammoniaphlum* in optimal biological system containing carbohydrates and ammonia, and this bacteria produces sufficient amounts of L-glutamic acid which is isolated from the medium and then treated to produce MSG - is the most widely used method around

the world. Its use is much more than the hydrolysis process and the chemical synthesis method (International Food Information Council Foundation, 2014, pp.1-11)

Problems with dietary MSG consumption began to be observed in 1968 when some people claimed that they always suffered with several symptoms after eating Chinese food in restaurants. The symptoms were attributed to the MSG found in all Chinese dishes and soon the term “Chinese restaurant syndrome” (CRS) began to be used to describe such cases (Yoko & Yoichi, 2016, pp.1-7). Consequently, several scientific investigations were carried out to determine if there was any relationship between CRS and MSG. Details and outcomes of these investigations will be discussed further in Chapter 2. In the meantime the use of MSG as a flavor enhancing food additive began to be used all over the world, both in packaged food preparations such as soups, as well as in dishes served in (non-Chinese) restaurants. MSG either in native crystalline salt form or sometimes disguised as Celery Salt (Tuzot in North Cyprus) is widely available in supermarkets everywhere.

In this present study, a few of the popular cafeterias on the university campus were chosen to obtain food samples because of their popularity with staff and students who frequently have their lunch. Because these restaurants are competing for customers with each other, they need to provide good quality tasty dishes but at the same time with low affordable prices in order to attract the students .Therefore, some of these restaurants may resort to using food additives and flavor enhancer, such as MSG in order to make their dishes more tasty and memorable so that they remain popular with the customers. Because of its easy availability and low price we suspect wide use of MSG.

The aims of this study are:

1. Can we determine easily and rapidly the MSG in foods using TLC and HPLC? Can we achieve these determinations qualitatively and/or quantitatively?

2. If yes, can we use these methods to determine how much MSG is present in the foods prepared and sold in cafeterias on the campus?

3. If not, can we identify the problems and suggest ways improve quantitative analysis?

4. Can we distinguish between the MSG naturally present in the foods and that which has been added during the cooking step?

Chapter 2

LITERATURE REVIEW

2.1 Glutamic Acid (GLU), the Precursor of MSG

Glutamic acid (GLU) is one of the 11 non-essential amino acids. The term ***non-essential*** is used because these amino acids are not an important part of our food intake since they are produced within our bodies. Hence there is no need to eat diets that contains them. However, they do play very important role in the metabolism of our bodies and they are needed. (Marie & Sara, 2010, p.172). Glutamic acid itself is one of the most abundant amino acids in foods which is found in two forms; one, as a building block of proteins, therefore chemically bound form, and the other present as the free acid, the unbound form. From stereochemistry view point, GLU has two configurations; one is L-GLU acid which has the flavor enhancing properties when it is in its free form (commonly used as a flavor enhancer, particularly in the form of monosodium salt) and the other is the inactive D-GLU acid. Foodstuffs naturally containing large quantities of free-form L-GLU, such as cheese, mushroom and tomatoes, are frequently used to obtain flavorful dishes (Tiziana et al., 2007, pp. 1712–1717).

2.2. Monosodium Glutamate (MSG)

Monosodium glutamate (MSG) is the sodium salt of the glutamic acid. It is a white crystalline substance as shown in Figure 1, and is freely soluble in water. MSG has a distinctive taste called ***umami*** which is regarded as being different from the other four basic tastes, namely bitter, salty, sour and sweet. Consequently, umami has

acquired broad acceptance among people as the fifth fundamental taste. Thus, glutamate began to be added to foods either as hydrolyzed protein and/or as extracted MSG salt with the purpose of giving umami taste to the final dish. From 1909 until 1965 MSG was obtained by extraction from gluten of wheat and from cakes of defatted soybean, but now it is produced using certain types of microorganisms in a fermentation process utilizing carbohydrates, such as molasses from sugar cane or sugar beet, as well as starch hydrolysates (Mehreen et al., 2012, pp. 39-42).

Figure 1: Crystals of MSG [9]

2.2.1 Characterization of MSG

Several studies have shown that MSG is not a hygroscopic salt, and for this reason it does not absorb water from its surrounding and thus it maintains its function and its appearance throughout the storage duration. Being a strong electrolyte, MSG dissociates completely to give glutamate and sodium ions when dissolved in water, as shown in Figure 2. Furthermore, the thermal stability of MSG means that it does not degrade when it is cooked. It remains unaffected by normal food processing. Some chemical and physical properties of MSG are summarized in Table 1 (Underriner & Hume, 1994, p.29).

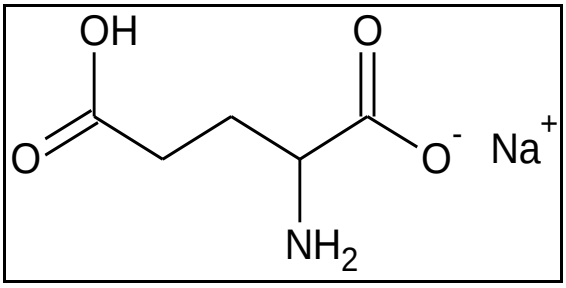

Figure 2: Chemical Structure of MSG

Table 1: Some of Chemical and Physical Properties of MSG

Chemical Properties	
IUPAC Name	sodium;(2S)-2-amino-5-hydroxy-5-oxopentanoate
Molecular Formula	$C_5H_8O_4NNa$
Molecular Mass	169.11
Physical Properties	
Melting Point	232 °C
Color	white crystals
Odor	Odorless
Taste	meaty taste
Solubility	freely soluble in water, sparingly soluble in ethanol , practically insoluble in ether
PH	6.8 Of solution with 5% concentration.

(National Center for Biotechnology Information, 2008), (Food and Agriculture Organization of the United Nations, 2001)

2.2.2 Chinese Restaurant Syndrome (CRS)

The New England Journal of Medicine published a paper in 1968 which examined a syndrome that starts from 15 to 30 minute and continues about two hours after eating some Chinese meals. At the beginning, three symptoms were described as "numbness at the back of the neck, gradually radiating to both arms and the back, general weakness and palpitations" but these symptoms were noticed to be similar to the symptoms of allergy to acetylsalicylic acid (ASA) by the author himself who

also suffered from (ASA) sensitivity. Several probable reasons were proposed, such as MSG, alcohol and salt.

After this study, the syndrome became to be called (CRS) and many reports were written and published about this issue but many of them were anecdotal and were not based on sound scientific principles or were not investigated by proper research methods. Some of these described symptoms analogous to the original 1968 report, while others registered signs dissimilar from those reported originally. Most of these reports mentioned several symptoms such as weakness, sweating, headache, numbness, palpitations, tightness in the chest, etc. In addition, one of these studies claimed a triplet of symptoms of facial pressure, chest pain and burning that appeared in people who ingested large doses of MSG but these signs were different from the three symptoms that were listed in the first report.

Subsequently, several scientific studies such as double blind placebo controlled investigations (DBPC) were carried out in order to examine these symptoms which were classified as severe, impermanent and limited. Consequently, DBPC studies performed many experiments on individuals who claimed sensitivity toward MSG and they showed that the symptoms which were claimed/observed in some cases were not related to MSG ingestion and could also be produced by other sources. Generally speaking, studies that were done on MSG which was added to the foods by suitable amounts (not more than 3 g of MSG) indicated an absence of reverse reactions mostly but when MSG is digested directly without food it will cause some symptoms for individuals who suffering from MSG's allergy. As a result, they could

not establish a clear connection between MSG and previous symptoms (Raif et al., 2000, pp. 1058S-1062S).

2.2.3 Assessment of MSG Hazard

When they tested animals that were given large doses of pure MSG orally, without administering it with food, the concentration of glutamate in plasma became very high and this caused some healthy problems with the animal's brains and these problems were medically characterized as brain lesions and they were the single poisonous effect observed by the MSG researchers. However, when humans used the MSG as a food additive to enhance the taste of their foods, the glutamate levels in blood plasma remained stable and under control, contrary to what happened with laboratory animals. The explanation was that the foodstuff slow down the absorption of glutamate noticeably and reduces the rise of glutamate levels in plasma. Moreover, when the ingested food reaches the small intestine, there will be highly professional cells called enterocytes which are responsible for absorption the nutrients and electrolytes such as glutamate and thereby glutamate levels stay in normal ranges. Furthermore, the threshold limit of acceptability and palatability of MSG with respect to humans is approximately 60 $mg.Kg^{-1}$ bodyweight and based on this value, the human tongue will not accept the food that contains excessive concentration of MSG larger than this value as the food will become unpalatable and be rejected.

Consequently, people are not exposed to the danger of brain lesions when they digest food containing MSG. In conclusion, many studies which included people who suffer from "self-reported sensitivity" to MSG were evaluated and the investigators concluded that there is no clear relation between glutamate digestion

and adverse reactions of MSG. Also, these studies concluded that people who claim their suffering from eating MSG do not have unique characters associated with them.

2.3 Monosodium Glutamate from the Viewpoint of Various Scientific Organizations

There are many global scientific organizations which specialize in food administration and enact rules and laws to ensure the safety of people from risks associated with food additives and other chemicals. In this section, there are four reviews for different international organizations and one university conference.

2.3.1 The Joint Food and Agricultural Organization/ World Health Organization (FAO/WHO) Experts Committee of Food Additives (JECFA) Review

A lot of researches which were performed on animals and human volunteers were reviewed by these joint organizations in order to find any obvious relation between MSG and the list of symptoms that some people claimed they suffered from them when they digested MSG. They found that the amount of MSG which caused the death of 50 percent of a population (referring to mice and rats in this review) under study was between 10-20 g/kg bodyweight and this is called the oral lethal dose (LD_{50}) because it is administered orally. Consequently, the maximum palatability allowed for humans was approximately 60 mg/kg bodyweight.

They also considered 59 different researches which studied the probable toxicity of MSG on brain tissues of hamsters, mice, rabbits, duck, dogs and rats and they observed that mice were the most allergic sample for the brain tissues damage. It was found that when that damage was happening, the glutamate levels in plasma

were “100-300 μmol/dl in neonates, 380 μmol/dl in weanlings and greater than 360 μmol/dl in adults”. These high levels could not be reached in human beings even when they were given bolus dosages with concentration 150 mg/kg bodyweight (nearly 10 grams for adults with an average weight 70 kg).

The concentration of MSG required to generate the brain lesions in half of infant mice was nearly 500 mg/ kg and this was called the ***effective dose*** (ED_{50}). This was given by oral gavage (forced feeding) technique while the maximum acceptable concentration for people is about 60 mg/ kg bodyweight and if the concentration exceeded this value, the individuals will feel nausea. Therefore, the toxic effects related with those of high MSG levels will not appear in mice when the digestion food process is voluntary.

Also several studies were performed on breastfeeding mothers which has shown that when six grams of MSG was admixed with water or liquefied with food and given orally to those mothers, the levels of glutamate in their milk remained in the normal range. Thus, considering the existing evidence, JECFA reached the conclusion that the amount of daily consumption of glutamate, whether from natural sources or from MSG added to food in reasonable amounts to improve the flavor of food, does not form a health risk. For this reason, JECFA decided that there is no need to determine an ADI (Acceptable Daily Intake) for L-GLU acid and its monosodium salt.

2.3.2 The Scientific Committee on Food (SCF) Review

This committee found that there were several reactions that also were noticed with foods which did not contain glutamate. Taking into account the large nutritional consumption of glutamates and data from animal and human studies, the SCF

decided it was not essential to specify an "ADI" for glutamate and that these results also confirmed the JECFA's results.

2.3.3 The Federation of American Societies for Experimental Biology (FASEB) Review

The Federation concluded that there is no effect of the MSG on the nervous system on humans when people take it orally and that there is not enough information to prove the contrary. In addition, they also reported, depending on the review of the related studies, that there was group of healthy people who respond to MSG's effects after one hour from digestion of an amount of MSG greater than three grams. Moreover, when they reviewed the studies that examined a group of people who were suffering from critical unstable asthma, they found that those people may face "bronchospasms" when they were exposed to high dosages of MSG between 1.5 to 25 grams when taken with food.

In another recent review, they reached the conclusion that there is no proof to confirm the relationship between MSG and asthma that happens in people who suffer from chronic asthma and another research used the mice as models to study the relation between MSG and the progress of asthma and they concluded that MSG does not have an effect to cause asthma in mice.

2.3.4 Food Standards Australia New Zealand (FSANZ)

This body also reviewed eight studies to examine the relation between asthma attacks and MSG and they found that the evidence for MSG produced asthma attacks was inconclusive. Moreover, the available data from studies which were recently reviewed show that MSG is not an important cause for generating asthma.

In addition, FSANZ agreed with FASEB if the relation between MSG and its adverse reactions were taken into account, there will be a small group of individuals who may suffer from some of these symptoms when they consume greater than 3 grams of MSG without food. Fortunately, these symptoms are neither severe, nor permanent and are supposed to diminish when MSG is ingested with foodstuff, especially with food containing large amounts of carbohydrates.

2.3.5 The Conference of Hohenheim University

Two scientific conferences were held by Hohenheim University in Germany, once in 1997 to review the data on MSG, and a second time in 2007 as a follow-up of the subject. The scientists came to a number of conclusions, one of which was that "glutamate does not cause asthma or does not have any effect on the lung." Moreover, many studies were performed on individuals who were suffering from asthma with self-reported sensitivity toward MSG and these studies failed to find the relation between MSG and the sensitivity of asthmatics toward it. Scientists also said that there are no obvious standards which can be used to identify the sensitivity of people to MSG.

Additionally, experts did not find any evidence that the intake of glutamate (which is added to food to improve its taste in the form of MSG or naturally present in food) leads to neurological effects in humans and they said even if the food containing added glutamate was used up, the glutamate levels in plasma will not increase since more than 95% of glutamate in diet is consumed as an energy resource by the intestinal absorptive cells. Also, the flow of glutamate to the central nervous system is prevented by the blood brain barrier (abbreviated as BBB) and, moreover, the

hazard of glutamate will not increase even if the BBB is damaged because the diet consumption of glutamate will not raise the glutamate concentrations in plasma.

The probable effects of nutritional glutamate on the unborn babies were considered to be negligible because glutamate is metabolized by the placenta which is considered as an active membrane and that there are many studies which show that glutamate levels in fetuses remained the same even if glutamate levels in mothers became high. So even if glutamate is administered orally, it will be unlikely to affect the fetus's growth.

Finally, all the information which are gathered from different studies confirm that as long as MSG is used as a flavor enhancer at reasonable levels, this will not constitute a health risk or problem to the majority of people. The symptoms that appeared on laboratory animals were because they were artificially given high doses that greatly exceeded allowed levels in food manufacturing. Furthermore, the amount of MSG that can be used in food is restricted and in any case high concentration of MSG yields the food unpalatable and unacceptable, thereby diminishing the ingestion of dangerous levels of MSG. (Kitano, 2014, pp. 465-469).

2.4 The Amount of Glutamate Naturally Present in Foods

Glutamate can be found naturally in various types of foods in the bound form especially in protein rich foods, such as meat, fish and poultry. It is also found in its free form, particularly in foods that are not high in protein, as shown in Table 2. In this table, it can be seen that foodstuffs containing high amounts of protein, such as cheese, have relatively large quantities of glutamate in both bound and free forms while the foods that have low amounts of protein such as tomatoes, can contain large quantities of free glutamate. Because the MSG can be easily formed from free

glutamate, diets which contain large amounts of free glutamate can be used as natural resources for the production MSG (Joseph, 1994, p.389).

Table 2: Examples of Some Foods that Contain Glutamate Naturally

Food Type	**Bound Glutamate (mg/ 100 g)**	**Free Glutamate (mg/100 g)**
Human milk	229	22
Parmesan cheese	9847	1200
Cow's milk	819	2
Pork	2325	23
Beef	2846	33
Duck	3636	69
Chicken	3309	44
Eggs	1583	23
Salmon	2216	20
Mackerel	22382	36
Cod	2101	9
Onion	208	18
Green peppers	120	32
Potatoes	280	180
Tomatoes	238	140
Spinach	289	39
Carrots	218	33
Corn	1765	130
Peas	5583	200

(Kitano, 2014, pp. 465-469), (International Food Information Council Foundation, 2014, pp.1-11).

2.5 The Relationship between MSG and Sodium Intake Reduction

Experts recommended that nutritional sodium consumption must be decreased because when people consume high amounts of salt (NaCl), this leads to an increased hazard of severe illnesses such as hypertension. However, when the salt concentration is decreased, the acceptability or the palatability of several foods decreases. Therefore, in order to improve the taste and palatability of foodstuffs while lowering the salt quantities at the same time, MSG was used for this purpose.

There are many studies that suggest using MSG in low salt diets in order to help people maintain a low salt diet.

When MSG was used in meals and soups to test whether the salt content could be reduced, it was found that the sodium concentration for such diets could be decreased by 40 % while maintaining the acceptability and palatability of these foods. This was found to work when there was an appropriate combination of MSG and salt together. Roininen and others also noted that adding MSG to foods which contain low quantities of salt may enhance their acceptability and palatability, especially “during the period when individuals are becoming accustomed to low salt diets” (David & Fiona, 2007, pp.55-56).

Chapter 3

EXPERIMENTAL

3.1 Chemicals

Reagents with high purity were used for preparing the HPLC mobile phase. These solvents were acetonitrile and methanol (Merck, LiChrosolv®, gradient grade for HPLC) and deionized water. Also, anhydrous glacial acetic acid of 100% purity with CAS number (64-19-7) and methanol of 99.9 % purity with CAS number (67-56-1) were used (both from Merck) for analysis purposes. In addition, pure acetone grade (TEKKIM) with 99.5 % purity and CAS number (67-64-1), pure ethanol from Selim ve Oglu Ltd., crystalline ninhydrin (Merck) with CAS number (485-47-2) and TLC plates of silica gel 60 F_{254} (M105554.0001) and the standard MSG of 99 % purity were used in this study.

3.2 Instruments and Tools

The Agilent 1200 HPLC, magnetic stirrer (VELP. Scientifica), Heidolph 591-00160-00-0 vacuum pump, analytical balance, centrifuge with maximum speed 4000 rpm, sonicator (United Jewelry Company), blender, TLC spotters, TLC chamber , Buchner flask, Buchner funnel, filter paper grade: 391(Sartorius), Erlenmeyer flasks, volumetric flasks (1000 ml ,100 ml, 25 ml), droppers, pipettes (10 ml ,1 ml), filter

membranes, medical syringes with volume of 2.5 ml, vials with a volume of 2 ml (Agilent), plastic spray bottle and aluminum dishes were all used.

3.3 Sampling Process for Cafeteria Foods

The sampling process was done by the researcher himself. He visited seven different university cafeterias, each three times, at lunch times in order to determine which cafeterias will be chosen for sampling. Four restaurants were chosen based on the number of visiting students (customers). It was noted that these four restaurants are the most popular with the students. Similarly, the choice of food selected for analysis was based on the popularity of the various dishes, most popular being selected.

3.4 Raw Material and Description of the Samples

In addition to the sampled dishes from the cafeterias, we also selected some locally available packaged, easy to prepare food preparations from a local supermarket. These products consisted of;

1. Spice mix for barbecue sauce,
2. Chicken bouillon (Brand I),
3. Three different powdered soups (Brand II) Lentil soup, Ezogelin soup and Vegetable soup with cream.

The foods in their packages are shown in Figure 3. The dishes from the selected four cafeterias were purchased all on the same day and each dish was transferred into a separate aluminum dish, and are shown in Figure 4. Each dish was labeled with its name and was stored in the fridge at 4°C until the extraction step.

Figure 3: Photos of Packaged Soups

A) Mercimek soup (MS) B) Ezogelin soup (ES) C) Sebze soup with caramel (SS) D) Spice for barbecue sauce (BS) E) Chicken bouillon cubes (CB).

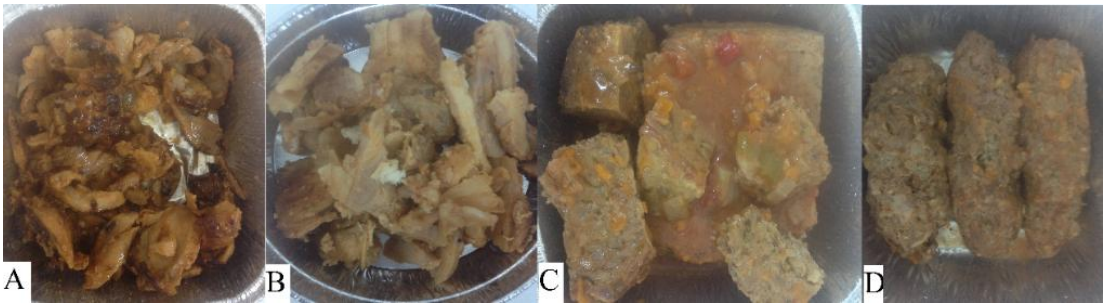

Figure 4: Photos of prepared foods were taken soon after purchase.

A) Chicken shawarma (döner) bought from Cafeteria I (D-I) B) Chicken shawarma bought from Cafeteria II (D-II) C) Beef meatballs (kofta) bought from Cafeteria III (K-III) D) Beef meatballs bought from Cafeteria IV (K-IV).

These samples were two chicken shawarma (in Turkish called döner) bought from Cafeteria I and Cafeteria II respectively, and two meatball (beef) dishes (in Turkish called köfte) bought from Cafeteria III and Cafeteria IV respectively. The sample of meatballs from Cafeteria III had sautéed onions on top, as garnish and were in a broth containing tomatoes and other vegetables. The Cafeteria III meatballs were also in a spicy broth containing onions and tomatoes but no other vegetables. The two shawarma kebabs were spice marinated boneless chicken meat that had been grilled in front of an open gas fire.

3.5 Processing and Preparation of the Laboratory Samples

Careful and equivalent processing of the samples was necessary before carrying out the final analyses because these would invariably affect both accuracy and precision of the results. Although the packaged foods, namely the three soups and the barbeque mix were in the form of powder, they were still sufficiently granular to require better homogenization. Therefore, all the samples to be analyzed were subjected to a homogenization, step, followed by the aqueous extraction step and finally the filtration step for the preparation of the laboratory samples. These processed and filtered solutions were considered to be representative of the raw materials and if these steps were done correctly, then we can be confident about the reliability of the final analytical results.

3.5.1 Replication of Samples

Two replicate laboratory samples for each food specimen were carefully prepared by the exact same procedure so as to improve the quality of our analytical results by enabling us to check precision. Statistically, these replicates will allow us to estimate the precision of the analyses. Table 3 and Table 4 list the details of the replicates.

Table 3: Details of Replicated Samples of Unprepared Foods

Label of the sample	Weight ±0.0001 g	Volume of water ±0.05 ml
BS_1	2.0003	50
BS_2	2.0002	50
ES_1	2.0004	50
ES_2	2.0003	50
MS_1	2.0000	50
MS_2	2.0002	50
SS_1	2.0001	50
SS_2	2.0003	50
CB_1	1.0003	1000

CB_2	1.0002	1000

Table 4: Details of Replicated Samples of Prepared Foods

Label of the sample	**Weight ± 0.0001 g**	**Volume of water ± 0.05 ml**
$K\text{-}IV_1$	10.0003	150
$K\text{-}IV_2$	10.0002	150
$K\text{-}III_1$	10.0003	150
$K\text{-}III_2$	10.0004	150
$D\text{-}II_1$	10.0002	150
$D\text{-}II_2$	10.0004	150
$M\text{-}I_1$	10.0003	150
$M\text{-}I_2$	10.0001	150

3.5.2 Homogenization of Samples

Approximately 100 g was taken from each sample of chicken doner and kofta and it was weighed accurately. Approximately 50 g was taken from each sample of powdered soup, the barbecue sauce spices, and 5 cubes of chicken bouillon (each cube has an average weight of 10 g) were weighed accurately and each sample was blended three times with an electric blender in order to get a homogeneous sample. The two doners and the two koftes were turned in to a creamy paste by the blending process as shown in Figure 5. During the blending process, between the blending of each sample, the blender was carefully cleaned with acetone of 99.5 % purity to remove any remnants of grease; washed with detergent and rinsed with tap water,

and finally washed with small portions of distilled water so as to prevent any contamination between the samples.

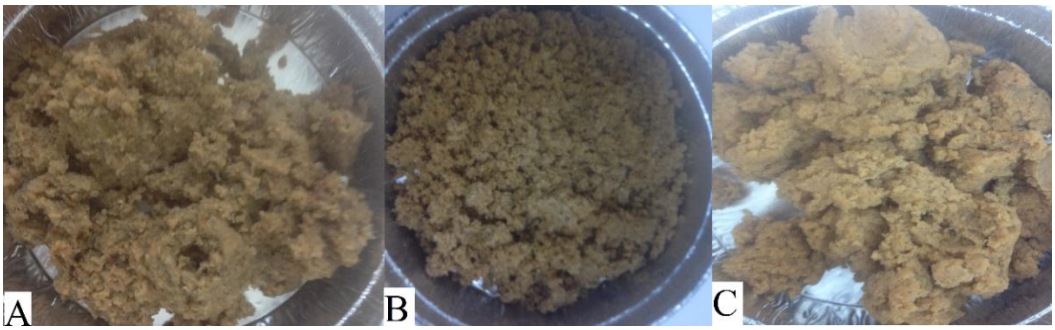

Figure 5: Photos of the Prepared Foods after Homogenization

A) Homogenized meatballs of Cafeteria III B) Homogenized chicken shawarma of Cafeteria I D) Homogenized chicken shawarma of Cafeteria II.

3.5.3 Extraction and Filtration of MSG

All the samples were processed and treated during a two day period; starting on 17.Dec.2016 (for the powdered soups, the chicken bouillon and the barbecue sauce spice) ending on 18.Dec.2016 (for the cafeteria dishes). This was two days before the analysis with HPLC. An accurately weighed amount of approximately 2 g of each of the homogenized packaged foods (soup, bullion or barbeque mix) was transferred into a 100 ml Erlenmeyer flask and 50 ml of distilled water measured with a 50 ml bulb pipette except the sample of chicken bouillon that was prepared by dissolving 1 g of bouillon into 1L of distilled water. Each flask was maintained at 298K and was stirred on a magnetic stirrer hot plate for 10 minutes, until the material in it was completely dispersed and the solution appeared to be homogeneous.

For the prepared foods from the cafeteria, 10 g of each homogenized sample was weighed on an analytical balance and the material transferred into a 200 ml Erlenmeyer flask. To each flask, 150 ml of distilled water was added from a 150 ml volumetric flask. As before, the flasks were maintained at the same temperature of 298 K, and were stirred on the hot plate with a magnetic stirrer. The duration of stirring was 5 minutes longer than the others because these samples were too cold on account of being stored in the fridge.

Vacuum filtration (using a Buchner funnel and Buchner flask) process was chosen instead of gravity filtration because the former is faster than gravity filtration. All the extract solutions were filtered through a grade 391 (Sartorius) filter paper. All the filtrates containing the extracted MSG were collected into individual Erlenmeyer flasks; stoppered; labeled, and stored in the fridge until they were ready to be taken for analysis with the HPLC in Pharmacy Faculty.

3.5.4 Centrifugation of Samples

Once the filtration process for all the samples was finished, most of the samples still appeared turbid and cloudy, so they were not clean enough to be injected into the HPLC. Therefore, 18 test tubes were washed with detergent, tap water, and finally rinsed with distilled water and each labeled with the name of a sample. Each one of these test tubes was filled equally with 5 ml of filtrate with a clean dropper. Then, sets of four test tubes were centrifuged for 15 min at 3000 rpm.

3.6 Preparation the Standard Solutions of MSG

Stock standard solution of MSG was prepared by weighing 0.1001 g of pure MSG which was transferred into 100 ml volumetric flask and mad up to 100 ml with distilled water to produce a solution with concentration of 1001 ppm. This stock

solution was used to prepare the other standard solutions by dilution of the stack. Standard solutions with concentrations of 500, 250, 100, 50 and 25 ppm MSG were prepared. These solutions were subsequently used to construct the calibration curve. Each one of previous solutions was prepared accurately by taking an appropriate volume from the stock solution, which was calculated using the dilution formula shown below.

$$V_{stock} = (M_{diluted} \times V_{diluted}) / M_{stock}$$

All standards were prepared in 25 ml volumetric flasks.

3.7 Filling the Vials with Samples

All the filtered and centrifuged extracts and the MSG standard solutions were filled into 1.5 cm screw capped HPLC auto-injector vials. For the filling step, approximately 2-3 mL of the solution was taken into a hypodermic syringe, then a 0.45µm membrane filter was placed on to it and about 1 o 1.5 mL of the solutions was filtered directly in to the vials. The membrane filter holder used was a stainless steel holder as shown in Figure 6.

Standard solutions from lower to higher concentrations were also transferred into vials by filtration through the 0.45µm membrane filter. The order to transfer from lowest to highest concentration was selected to prevent any probable contamination. For the other samples, the membrane filter was repeatedly washed with deionized water in between samples. All the vials were labeled and stored in the fridge until analysis.

Figure 6: Hypodermic Syringe, Filter Membrane and its Stainless Steel Holder

3.8 The Experiment of Thin Layer Chromatography (TLC)

This experiment was performed to provide a method for identifying MSG and establishing its presence/absence in the extracts.

3.8.1 Making TLC Spotters

A glass tube with two open ends was heated at its middle over a blue Bunsen flame and when it began to glow orange and became soft and flexible the two ends of the tube were pulled smoothly and rapidly away from each other until the middle became a thin capillary tube. The fine tube was broken in the middle to create two spotters for TLC. The spotting edge of the spotters were checked to be level and circular because spotting would be erratic and uncontrollable with uneven edge. In other words, if the edge is uneven many problems will happen such as asymmetry in spot shapes, overloading or scratching the TLC plate itself.

3.8.2 Determination R_f Value of Standard MSG

The retention factor R_f was determined for standard MSG by using two different mobile phases: first one was methanol and water with a ratio of 7 to 3 and the second one was ethanol and water with a ratio of 4 to 1. Three trial runs with each mobile phase was carried out so as to obtain a reliable R_f value for MSG.

3.8.3 Preparing and Spotting TLC Plates

TLC plates with a size of 20 × 12 cm was marked with a thin line 2 cm above the bottom of the plate with a pencil. Medical gloves were worn at all times to avoid the contamination of the plate with amino acids from fingers should they touch it. MSG standard with the 1000 ppm concentration was used as a reference for the other samples and 18 samples of prepared and unprepared foods were spotted onto the plate at marked positions on the line. An effort was made to ensure that equal amounts of each sample was loaded at each spot. Also the first and the final spots were spotted away from the edges of the TLC plate and sufficient space was left between the spots in order to prevent overlapping.

3.8.4 Developing TLC Plate

TLC plates were developed (after the spots became dry) in a closed tank containing the 7 methanol: 3 water mobile phase as shown below in Figure 7. The mobile phase was prepared with 70 ml of 99.9 % methanol and 30 ml of distilled water in an Erlenmeyer flask and once the mixture was completely mixed, it was transferred into the covered TLC tank so as to saturate the atmosphere inside the tank with the vapor of the mobile phase. The level of mobile phase was checked to be under the marked pencil line on the TLC plate, where the samples were spotted, otherwise the spotted samples would dissolve into the bulk mobile phase. The mobile phase traveled up the plate until 1 cm from the top end of the plate. At this point, the plate was taken out and the mobile phase front was marked on the plate before it evaporated, and the TLC plate was dried in the oven at 60 °C.

Figure 7: Development of TLC Chromatogram

3.8.5 Detection of Spots on TLC Plate.

A solution of crystalline ninhydrin (Merck) with a concentration 0.2 % (w/v) in acetone was used as the detection reagent for the amino acid spots. This solution was prepared freshly by dissolving 0.2002 g of the crystals in a 150 ml flask containing 100 ml of 99.5 % purity acetone (TEKKIM) and a few drops of glacial acetic acid. The flask was stirred until all dissolved, and the final solution was transferred into a spray bottle. The dried TLC plates were sprayed with this ninhydrin solution and developed in the oven at 60 $^{\circ}$C for 20 min (Joseph & Bernard, 2003, p.492). After the 20 minute heating, amino acid spots on the TLC plate appeared as purple/violet colored spots as illustrated below in Figure 8.

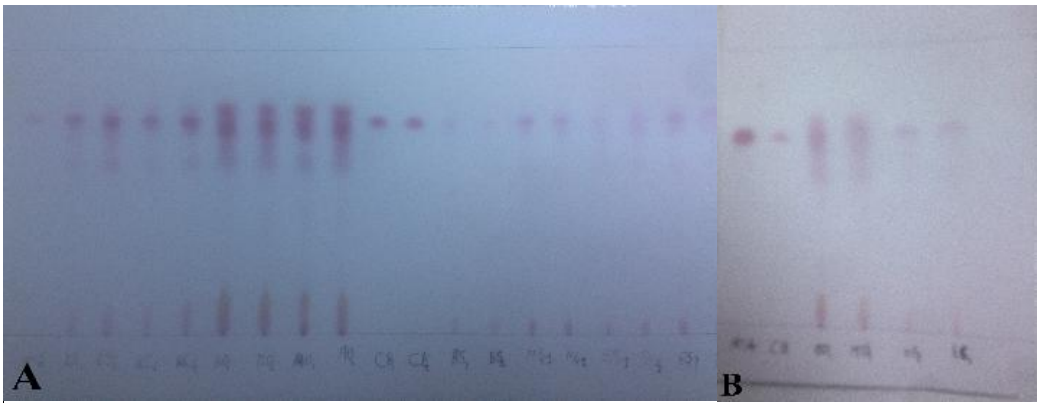

Figure 8: TLC Chromatograms

A) Chromatogram of concentrated prepared foods samples B) chromatogram of diluted prepared food samples.

3.9 Analysis of Samples by HPLC Instrument

3.9.1 Preparation HPLC Mobile Phase

Two different mobile phase systems were tried on the bases of information from the literature for isocratic elution: the first one was 1:1 methanol (HPLC grade Merck) and deionized water and the second was also 1:1 of acetonitrile (HPLC grade Merck) with deionized water. Because of the lower UV cutoff wavelength of acetonitrile (190 nm) as opposed to methanol (210 nm), it was better to us the acetonitrile/water mobile phase rather than methanol because peaks could be monitored more accurately at 210 nm.

3.9.2 Parameters Used in HPLC Method

All samples were analyzed using the Agilent 1200 HPLC instrument with a reversed phase analytical column with brand name of Nucleosil® 120-5C18 (25 cm × 4.6 mm I.D.) at 25°C temperature. The injection volume of each sample was 25 µl which was injected with an automatic injector and the flow rate of mobile phase was 0.5 ml/min. Peaks of the samples were detected at different wavelengths 210, 220, 230, 240 and 250 nm by using a diode array type UV/VIS detector (DAD). For quantitative analysis only the information from 210 and 220 nm were used as the absorbances at the higher wavelengths were negligibly small.

3.9.3 Injection of Samples

The injection order of the standard MSG samples was sequenced to be from lower to higher concentration, and each standard was injected two times. The other 18 extracted samples were injected only once because of time constraint. Deionized

water was injected two times as the blank before injection of any other sample and each run was eluted for 15 minutes.

Chapter 4

RESULTS AND DISCUSSION

4.1 Identification of MSG in Samples Based on R_f Value

The average R_f value for MSG for methanol/water mobile phase was 0.73; and for ethanol/water mobile phase average R_f was 0.57. The average R_f value in the two cases are different because of the differences in the solubility of MSG in the different mobile phases, because their polarity does differ. (Joseph & Bernard, 2003, p.492).

The identification of MSG in samples from TLC chromatogram was achieved by comparing the R_f values of the spots in the samples with the R_f value of standard MSG which was taken as 0.73 in methanol/water mobile phase. As a result, all the samples which were spotted on TLC plate showed that they contained MSG in varying concentrations, as shown in Figure 8 with spots having the same $R_f = 0.73$ value with uncertainty of ± 0.01. Two TLC chromatograms A and B were developed: the first chromatogram (plate A) indicated that the concentrations of MSG in the prepared food extracts were very high compared with the MSG standards highest being 1000 ppm. For this reason, the second chromatogram (plate B) was developed to examine the concentration of MSG in the prepared foods by further diluting the extracts so as to bring their concentrations within range for the MSG standard solutions. However, in both plates on can clearly see the existence of several closely placed spots near the MSG spot such that it was not possible to

scrape the MSG spot and extract the adsorbed MSG for positive identification by IR spectrometry.

Iodine crystals were also tried as a method to visualize the spots of MSG on the TLC plates but it was found to be unsatisfactory because it does not react effectively with unsaturated compounds. Therefore, visualizing the spots by the well-established method of ninhydrin reagent for amino acid detection was used instead of iodine vapor. Ninhydrin is considered to be a specific reagent for amino acids, giving the purple/violet color when it reacts with amino acids.

There are some spots at the beginning of the baseline in TLC chromatograms which appear yellow-purple color after being visualized with ninhydrin reagent. Based on the information in the available literature on the subject, these spots may be one of two amino acids; prolin or hydroxyprolin (Joseph & Bernard, 2003, p.492).

4.2 Detection of Underivatized MSG through Using UV Light

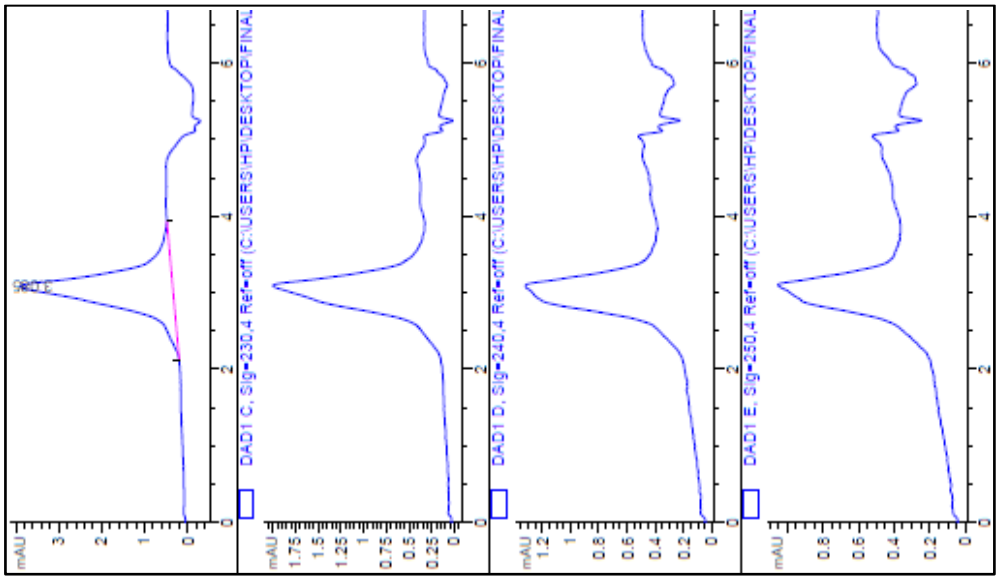

Figure 9: HPLC Chromatograms for MSG Standard with Concentration 25 ppm at 220, 230, 240 and 250 nm

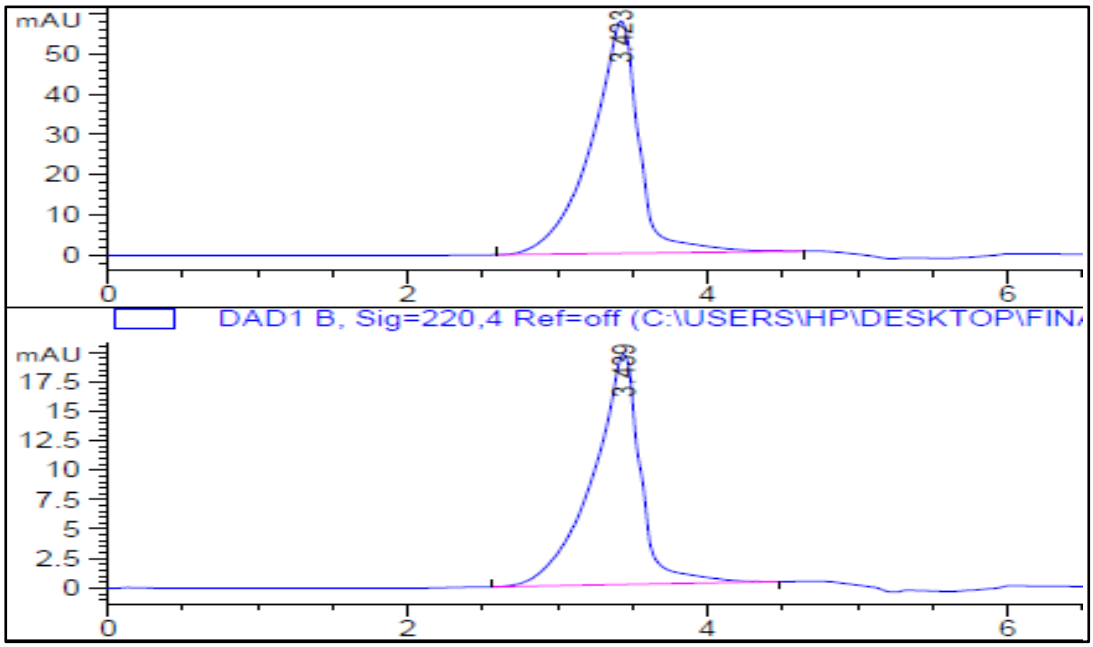

Figure 10: HPLC Chromatograms for Standard MSG with Concentration 500 ppm at 210 and 220 nm

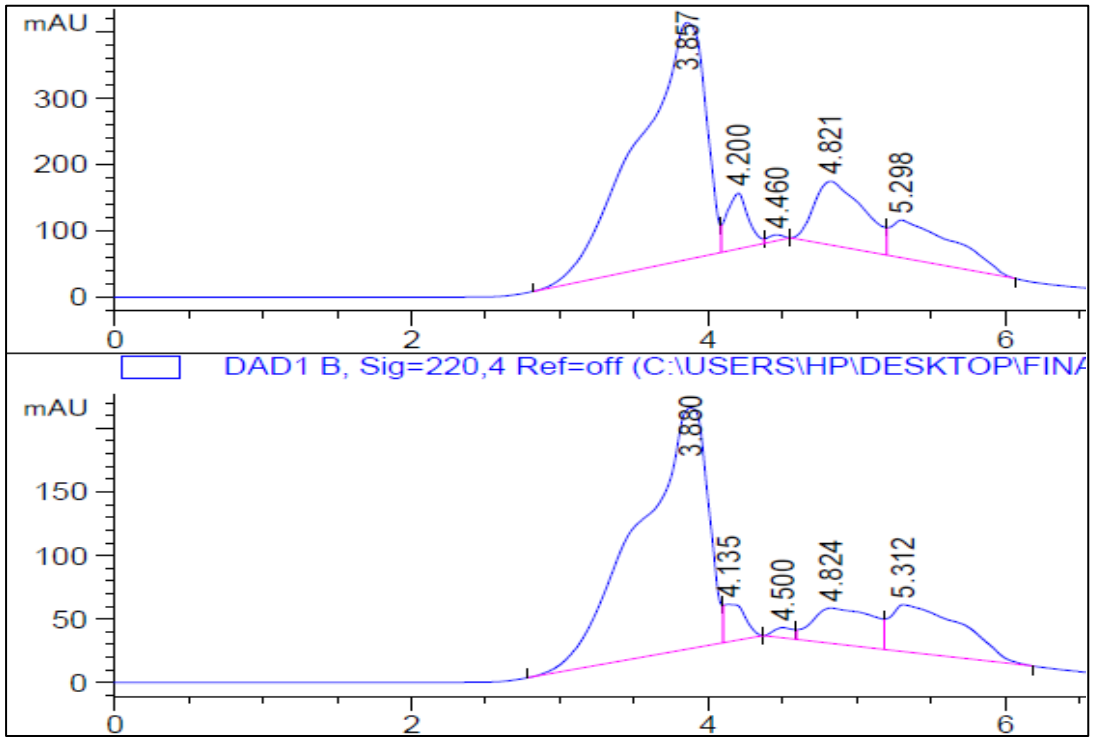

Figure 11: HPLC Chromatograms for One of Prepared Food (K-IV$_1$) at 210 and 220 nm

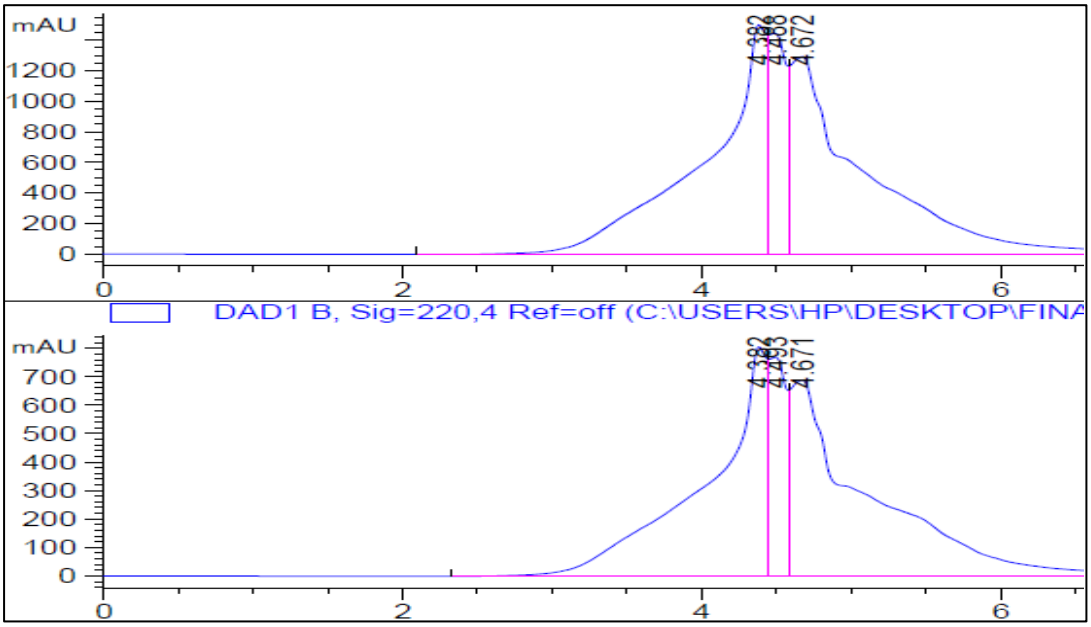

Figure 12: HPLC Chromatograms for One of Soup Powder (SS1) at 210 and 220 nm

All HPLC chromatograms were represented as peak area in (mAU*S) vs. time (minutes). All of them showed that as the detection wavelength increases, the signal intensity and absorbance will decrease and this is also true for the other underivatized aliphatic amino acids, all of which absorb UV light with wavelengths less than 240 nm. [Brain, Valery & Stoyan, 2004, p.125]. Therefore, the peaks detected at the higher wavelengths were much smaller and harder to detect unless the peak detection integration was adjusted from the system to see and integrate them. The retention times of peaks are inconsistent and the reason most probably is because do not use the buffer solution to control the P^H of the mobile phase especially when the compound is ionic or ionizable.

There are many unresolved peaks in cooked and packaged foods samples and the best way to get good resolution for these peaks is using derivatizing process because it i capable of decreasing the hydrophilicity of MSG in order to increase its retention on reversed phase silica gel which has a hydrophobic surface due to the bonded C18

groups. Thus it would be possible to get a good separation and resolution for the derivatized amino acid peaks (including MSG) and also to detect the peaks at wavelengths of 254 nm or more. The increased degree of unsaturation in derivatized MSG and the energy needed for $\prod \rightarrow \prod^*$ transition will decrease due to increase the delocalization of $\prod$ electron system and the probability of observing MSG at longer wavelengths will increase. Also the increased molar absorptivity as a result of the derivatizing groups would make the measurements more sensitive. Unfortunately, because of the unavailability of the necessary reagents for derivatization and time constraints, this could not be done.

Consequently, the chromatographic data at the wavelength of 210 nm was chosen to construct the calibration curve, and calculate the concentrations of the extracted samples.

4.3 The Calibration Curve for MSG Standard Solutions

The concentration of MSG in the samples was calculated based on the calibration curve which was plotted from the data for the standard MSG solutions. The curve is shown in Figure 13. These standards were run before the extracts and each standard was injected twice. The average peak area of the two injections are plotted against standard concentration. The data is shown in Table 5. Peak area was selected as the since it is still the most common option in chromatographic analysis, but there is a possibility to use peak height in calculations as well if the peaks have symmetrical shape. Two calibration curves at different wavelengths 210 nm and 220 nm were drawn to see which one of them will give the best fit line with the best correlation coefficient R^2. This turned out to be the peak areas for measurements made at 210 nm.

Table 5: The Data that were used to build the Calibration Curve

Conc. of Std. MSG in (ppm)	Rt (min)	Average Peak Area at 210 nm in (mAU*S)	Average Peak Area at 220 nm in (mAU*S)
25	3.07	179.5	114.5
50	3.14	324	161.5
100	3.19	444	196
250	3.32	853	363.5
500	3.43	1297	445
1000	3.54	2245	736.5

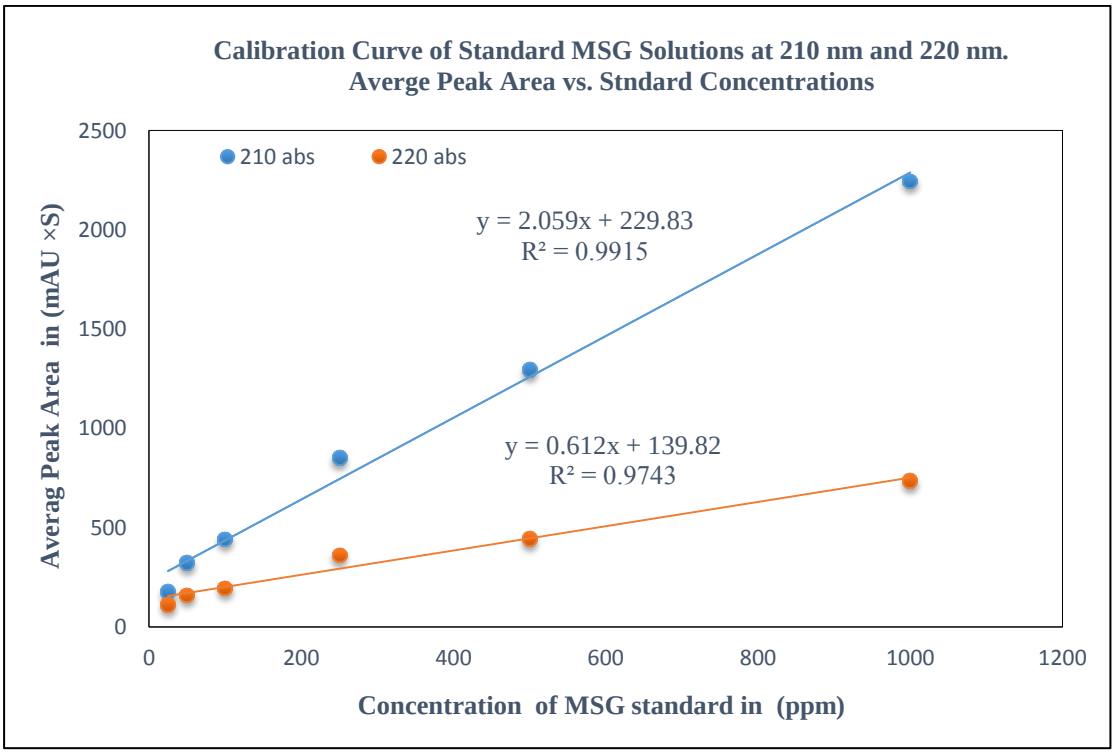

Figure 13: The Calibration Curve of Standard Solutions of MSG

4.4 The Approximate Concentrations of MSG in the Samples

We can see from Table 6 that extracts of the cooked food samples contain higher MSG concentration compared with the extracts of the unprepared food samples. In addition, the concentration of MSG in Kofta samples were also greater than the concentration in Doner samples.

Table 6: The Concentrations of MSG in Samples of Prepared Foods

Symbol of Prepared foods	The Peak Area at 210 nm	The Concentration of MSG in sample at 210 nm × 10^3 ppm	The Peak Area at 220 nm	The Concentration of MSG in sample at 220 nm ×10^3 ppm
D-II_1	5870	2.7	2450	3.8
D-II_2	5080	2.4	2484	3.8
D-I_1	8000	3.8	4190	6.7
D-I_2	7900	3.7	3370	5.3
K-III_1	9200	4.4	4500	7.2
K-III_2	9086	4.3	4710	7.5
K-IV_1	8530	4.0	4350	6.9
K-IV_2	8400	3.9	4487	7.1

Table 7: The Concentrations of MSG in Samples of Unprepared Foods

Symbol of Unprpared foods	The Peak Area at 210 nm	The Concentration of MSG in sample at 210 nm × 10^3 ppm	The Peak Area at 220 nm	The Concentration of MSG in sample at 220 nm ×10^3 ppm
BS1	6578	3.1	3600	5.7
BS2	6600	3.1	3567	5.6
CB1	564	1.6×10^2	208	1.1×10^2
MS1	1000	3.7×10^2	542	6.6×10^2
MS2	1080	4.1×10^2	553	6.8×10^2

All the concentrations of samples were converted from ppm to be in grams of MSG per 100 gram of each sample as shown in Table 8 and Table 9. All of these values appear to be out of the optimal concentration of MSG which is 0.2-0.8 % (w/w). These concentrations do not show any adverse reactions on the majority of people whereas may be cause symptoms similar to MSG adverse reactions in a small group of people according to the reviews of FASEB and FSANZ and they will not have any toxic effects on brain since none of them can raise the level of glutamate in the plasma to the level that may cause brain lesions. Nevertheless, reaction to MSG may appear with some people who suffer from sensitivity toward it, but because the

MSG would be consumed within the food, we suspect that such cases will be very few. Danger is when sensitive people consume 3.6 g or more of MSG without food.

Table 8: The Amount of MSG in each 100 g of each Sample of Prepared Food

Symbol of Sample	Amount of MSG (g/ 100 g food)
D-II_1	4.1
D-II_2	3.5
D-I_1	5.7
D-I_2	5.6
K-III_1	6.5
K-III_2	6.5
K-IV_1	6.0
K-IV_2	5.9

Table 9: The Amount of MSG for 100 g of Unprepared Samples

Symbol of Sample	Amount of MSG (g/ 100 g food)
BS1	7.7
BS2	7.7
CB2	16.2
MS1	0.94
MS2	1.0

The concentration of MSG in the unprepared packaged foods (powdered soups) will be less than these values since more amount of water will be used to prepare an enough amount from soups. With respect to the values of samples BS1 and BS2, it cannot be MSG because on TLC chromatogram they gave spots with very low concentration but on HPLC chromatogram the area for both of them was very large and its concentration exceed the expected value of TLC chromatogram. Therefore, it will be excluded with other samples such as CB1, ES1 and ES2. But we can make an approximation for their concentrations based on the comparison of their spots on TLC chromatogram with the values of other soup powder such as MS1, MS2 and CB2.

Chapter 5

CONCLUSION

In this thesis, qualitative determination of MSG in foods using TLC was done easily and rapidly with ninhydrin reagent as the specific visualizing reagent for detecting amino acids, including MSG. For the quantitative determination of MSG directly by HPLC, a number of problems surfaced which indicates that direct determination with no prior treatment of amino acids on reversed phase HPLC is not very satisfactory. The problem we believe is that other amino acids which are also very water soluble elute form the column at the same time or very close to MSG, thereby producing large unresolved peaks in the chromatogram. One way to overcome this problem is to derivatize the MSG with a suitable derivatizing reagent, such as 1-Fluoro-2,4-dinitrobenzen (DNFB), or o-phthalaldehyde (OPA).

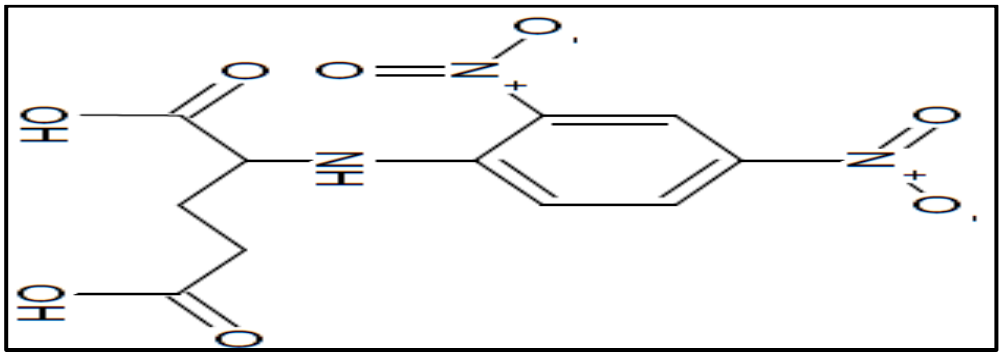

Figure 14: The Chemical Structure of MSG derivative using (DNFB) Reagent.

Although in this study we attempted to analyze MSG without derivatization, we found that this approach was problematic and several troubles occurred after the injection into HPLC column, such as negative peaks, poor peak shape, low retention

time, variation in retention time, poor separation, splitting and broadening. Some of these problems were reduced when we changed from methanol to acetonitrile based mobile phase but this did not solve all the problems. In conclusion, we find that the traditional Reversed Phase -HPLC is not a good option for separation of underivatized amino acids so cannot be used effectively for MSG analysis. However, bearing in mind that normal phase TLC works well with the water soluble amino acids, it would be worth trying a normal phase (NP) HPLC system which is referred to as HILIC (Alpert, 1990).

The results of MSG concentrations in samples were reasonable and acceptable but we are not sure whether the MSG in the foods are present naturally or have been added during processing or cooking? It is well established that MSG is present not only in red-meat and chicken, but is also present in some fruits, vegetables and nuts such as tomatoes, onions, cashew nuts and spices? Further work would be necessary to provide an answer to this issue. Samples without and with added MSG should be extracted and concentrations measured to determine if significant differences exist or that the added MSG can be recovered.

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