

DIFFERENTIATION OF L-CYSTEINE SOURCES USING
SPECTRAL ANALYSIS AND AMINO ACIDS
ANALYSIS

BY

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ABSTRACT

L-cysteine is a food additive that is used in bakery ingredients. It is used as a stabilizer to soften the texture of bakery dough. However, L-cysteine's primary sources could be derived from animal and human parts, which lead to non-halal food sources. Five samples of pig bristle, human hair, duck feather, chicken feather and cow horn, were extracted with 6M HCl and freeze-dried into a powder form. One gram of L-cysteine powder form from five different samples was analyzed using spectral fingerprinting profile and chromatography separation analysis. Spectral fingerprinting profile of L-cysteine sources was obtained by using combination of Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) and Raman spectroscopy. Meanwhile, the amino acid content of L-cysteine sources was analyzed through amino acid analysis (AAA) by ultra-high performance liquid chromatography (UHPLC). The result found that the ATR-FTIR is preferable as a fingerprinting tool rather than Raman spectroscopy in differentiating L-cysteine's primary sources. This is because the infrared ray can capture and compare the spectral fingerprinting profile of L-cysteine sources compare to Raman spectroscopy. Precisely, ATR-FTIR was able to differentiate the samples by the presence of dominant Amide I band at the wavenumber region between 1800 cm^{-1} - 1250 cm^{-1} . Data pre-treatment by using KMO test, Barlett test and eigen value were carried out to determine data reliability. Five distinct groups were successfully differentiated in PCA. Accordingly, the amino acid content of L-cysteine sources was analyzed through AAA. The result showed that 17 amino acids concentration were successfully separated and identified in all five samples. The data also proved that human hair had the highest L-cysteine concentration compared to other samples. The separation of 17 amino acids by AAA lead to further identification of specific biomarker compounds. By using AAA data, all samples were successfully separated into different clusters with PCA aid. Based on the variable maximum rotation diagram, lysine to L-cystine ratio (LYS/CYS) was the only ratio located at the pig bristles cluster. In conclusion, ATR-FTIR and UHPLC have successfully differentiated L-cysteine sources through spectral fingerprinting profile and AAA, respectively. The initial screening through ATR-FTIR can interpret and differentiate L-cysteine origin sources through spectral fingerprinting profile. In fact, the implementation of diagnostic ratio as sample differentiation is another promising analytical approach for biomarker compounds. Therefore, this study could be used on an industrial scale to create a database and detect L-cysteine origin sources in commercial products.

خلاصة البحث

السيستين (L-cysteine) هو مادة مضافة غذائية تستخدم في مكونات المخبوزات ويستخدم كعامل استقرار لتليين قوام عجينة المخبوزات. ومع ذلك، قد تكون المصادر الأولية لـ L-cysteine مثيرة للجدل لأنها قد تكون مشتقة من أجزاء حيوانية وبشرية. ولذلك تهدف هذه الدراسة إلى التمييز بين مصادر L-cysteine باستخدام تقنية التحليل الطيفي. تم استخلاص خمس عينات من شعيرات الخنزير، وشعر الإنسان، وريش البط، وريش الدجاج، وقرن البقر باستخدام حمض الكلور HCl بتركيز 6 مولاري، وتجفيفها بالتجميد إلى شكل مسحوق. تم تحليل جرام واحد من مسحوق L-cysteine من خمس عينات مختلفة باستخدام ملف البصمة الطيفية وتحليل الفصل الكروماتوجرافي. وتم الحصول على ملف البصمات الطيفية لمصادر L-cysteine باستخدام دمج كل من الانعكاس الكلي المنخفض لتحويل فوربييه للأشعة تحت الحمراء (ATR-FTIR) والتحليل الطيفي لرامان. وفي الوقت نفسه، تم تحليل محتوى الأحماض الأمينية لمصادر L-cysteine من خلال تحليل الأحماض الأمينية (AAA) عن طريق تحليل كروماتوغرافيا السائل عالية الدقة (UHPLC). وأظهرت النتائج تفضيل ATR-FTIR كأداة للبصمات الطيفية بدلاً من التحليل الطيفي لرامان في التمييز بين مصادر L-cysteine الأولية. وذلك لأن الأشعة تحت الحمراء يمكنها التقاط ومقارنة ملف البصمات الطيفية لمصادر L-cysteine مقارنة بمطياف رامان. وعلى وجه التحديد، فقد كان ATR-FTIR قادرًا على التمييز بين العينات من خلال وجود نطاق Amide I المهيمن في المنطقة بين 1800 سم⁻¹ - 1250 سم⁻¹. تم إجراء المعالجة المسبقة للبيانات باستخدام اختبار KMO واختبار Barlett وقيمة Eigen لتحديد موثوقية البيانات. تم تمييز خمس مجموعات متميزة بنجاح في PCA. ووفقًا لذلك، تم تحليل محتوى الأحماض الأمينية لمصادر L-cysteine من خلال AAA. وأظهرت النتائج أن 17 حمض أميني تم فصله وتحديد بنجاح في جميع العينات الخمس. كما أثبتت البيانات أن شعر الإنسان يحتوي على أعلى تركيز من L-cysteine مقارنة بالعينات الأخرى. وأدى فصل 17 من الأحماض الأمينية بواسطة AAA إلى تحديد مركبات محددة للمعلم الحيوي. وباستخدام بيانات AAA، تم فصل جميع العينات بنجاح إلى مجموعات مختلفة بمساعدة PCA. ومن خلال مخطط الدوران الأقصى للمتغير، كانت نسبة اليسين إلى السيستين (LYS / CYS) هي النسبة الوحيدة الواقعة ضمن مجموعة شعيرات الخنزير. وكاستنتاج لما تم التوصل إليه، تم تحديد مصادر L-cysteine بنجاح بواسطة ATR-FTIR و UHPLC من خلال ملف تعريف البصمات الطيفي و AAA، على التوالي. وأثبت الفحص الأولي من خلال ATR-FTIR أن التفسير المباشر للتمييز بين مصادر أصل L-cysteine يمكن أن يحدد من خلال ملف تعريف البصمات الطيفية. وفي الواقع، فإن تنفيذ نسبة التشخيص لتمايز العينة هو نهج تحليلي واعد آخر لمركبات الملعقات الحيوية. ولذلك، يمكن استخدام هذه الدراسة على نطاق صناعي لإنشاء قاعدة بيانات واكتشاف مصادر لأصل L-cysteine في المنتجات التجارية.

APPROVAL PAGE

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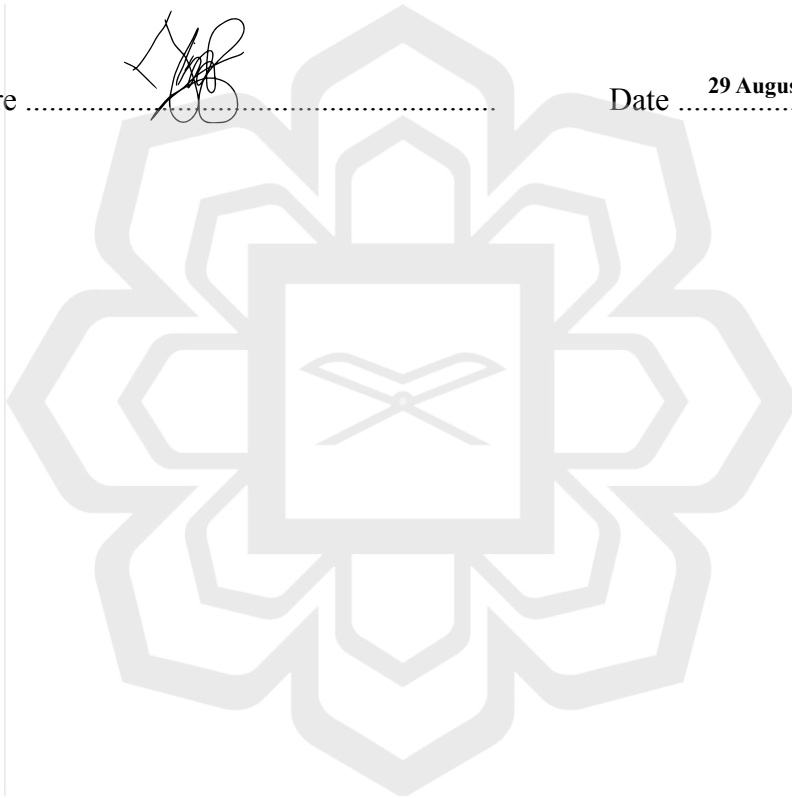
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DECLARATION

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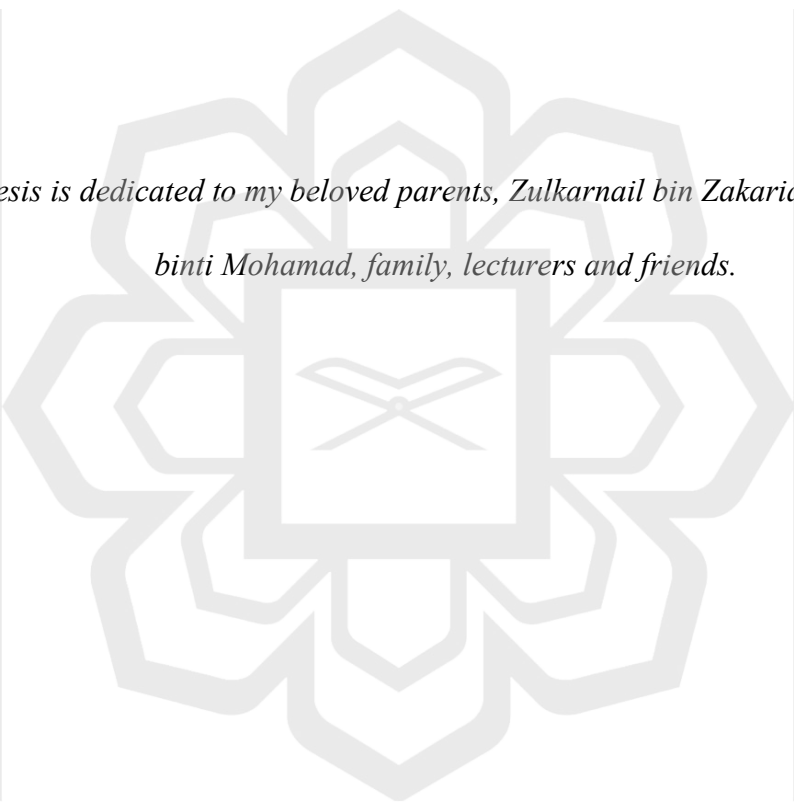
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*This thesis is dedicated to my beloved parents, Zulkarnail bin Zakaria and Khadijah
binti Mohamad, family, lecturers and friends.*

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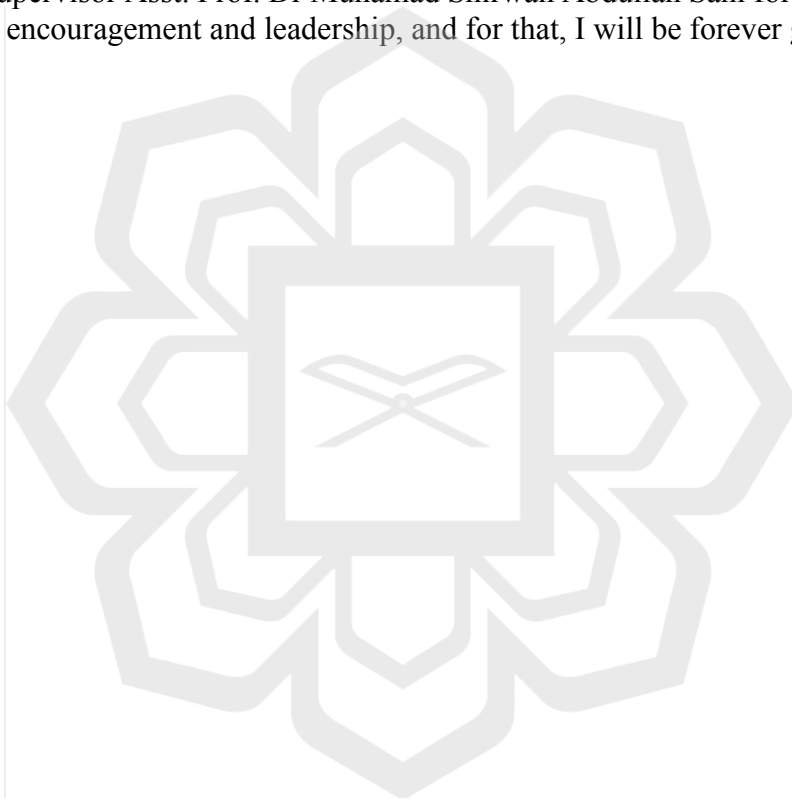


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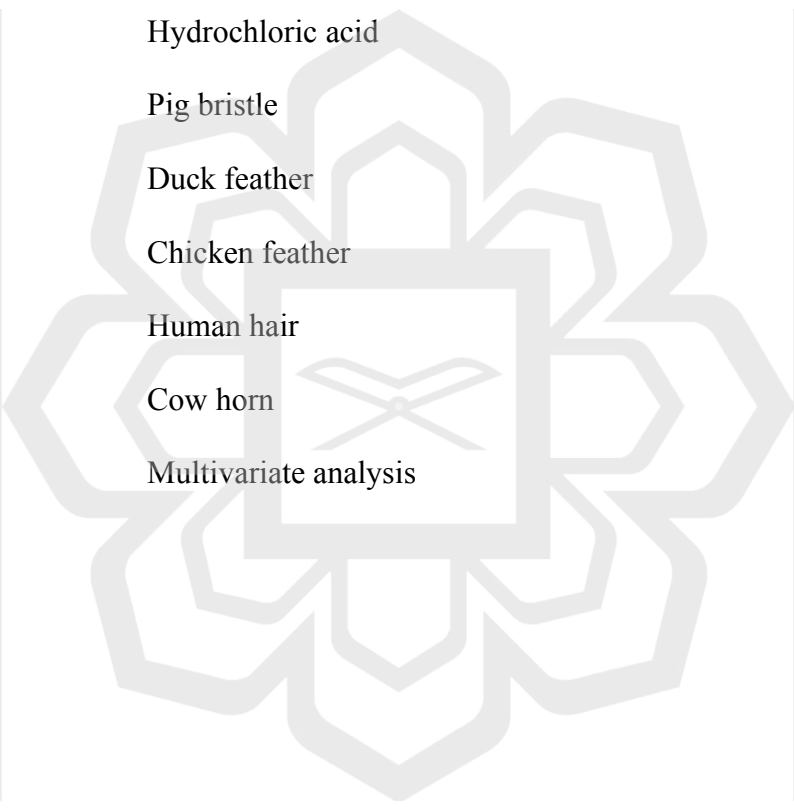
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LIST OF ABBREVIATIONS

ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared
AAA	Amino Acid Analysis
UHPLC	Ultra High-Performance Liquid Chromatography
DR	Diagnostic ratio
PCA	Principal component analysis
HCl	Hydrochloric acid
PB	Pig bristle
DF	Duck feather
CF	Chicken feather
HH	Human hair
CH	Cow horn
MVA	Multivariate analysis



CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

L-cysteine is an amino acid common to many proteins and enzymes. It is of interest due to the presence of a reactive thiol group as a side chain (Demirkol et al., 2004; Helmutgmunder et al., 1990; Hunt, 1985). Thiol side-chain involves wide applications such as detoxification of heavy metals in living organisms, antioxidant capabilities of tissues and mitochondria, blood coagulation in mammals, transport across cell membranes and electrochemical sensing (Borase et al., 2015; Cai et al., 2014; Cebi et al., 2017; Ensafi et al., 2009). L-cysteine is usually found in a relatively low dietary protein concentration, which does not exceed 5% of total amino acids (Demirkol et al., 2004; Ismail et al., 2014). It can be found in whole foods such as meat, grains, nuts, fruits, and vegetables. Worldwide L-cysteine production can reach up to 400 tons, where L-cysteine is mostly used as a food additive (Berehoiu et al., 2013; Jahangir et al., 2013). It acts as a stabilizer in bakery ingredients by softening the texture of the yeast and preventing further oxidation. Meanwhile, in animal food production, L-cysteine has been used as an artificial flavor by mimicking meat flavor (Ismail et al., 2014; Wada & Takagi, 2006; Wu, 2013). Production of L-cysteine additives derived from human hair, cow horn, pig bristles, and duck feather as starting raw materials extraction (Cai et al., 2014; Ismail et al., 2014). Therefore, this study aims to develop a method for L-cysteine source detection (Cuadros-Rodríguez et al., 2016).

Spectral fingerprinting profiles from attenuated Fourier transform infrared (ATR-FTIR) and Raman spectroscopy advanced approaches due to robust and simple

analytical measures (Hashim, 2013; Nemecek et al., 2013; Ramin Jorfi, 2012). Tremendously, they can determine origin sources from meat samples without targeting any specific molecule compounds (Cebi et al., 2017; Ramin Jorfi, 2012). Theoretically, the polar functional group is better detected in ATR-FTIR, while the aromatic carbon compound is better detected in Raman spectroscopy. However, spectral analyses only reveal chemical fingerprinting profile based on functional group and carbon compound detection. It is proposed that spectral analyses can be further identified using protein content analysis carried out by UHPLC. In fact, chromatogram data of amino acid analysis (AAA) can sort varied amounts of amino acid content based on the injected sample concentration quantitatively. Hence, there is a need to combine spectral analyses using both ATR-FTIR and Raman spectroscopy with AAA using UHPLC identification to develop an origin-based spectral fingerprinting profile for L-cysteine.

A study done by Lamp et al. (2018) proved that AAA application was able to differentiate amino acids in unknown plant sample materials. This can be supported by a study done by Azilawati et al. et al. (2015), where porcine, bovine, and fish gelatin amino acid concentration were differentiated through AAA by UHPLC. In addition, AAA can be utilized to develop a specific biomarker for species identification tools. The difference of amino acid concentration on each sample helps develop a specific biomarker for species compound identification. Thus, AAA has a great advantage in developing a specific biomarker for source identification in this study. Hence, combining a biomarker compound with chemometric analysis is advantageous for L-cysteine source determination in food samples.

Combining the chemometrics test with spectral or chromatogram data is advantageous to determine the underlying relationship between variables. The spectral fingerprint between each sample could be described in the simplest way with the aid of

PCA. Every sample could be discriminated with respect to their spectral fingerprint and chromatography profile based on the data obtained. Hence, this study aims to provide a comprehensive chemical composition of L-cysteine source through a combination of ATR-FTIR, Raman spectroscopy, and UHPLC data. In addition, PCA will be performed to monitor the classification as a graphical display to determine the relationship between each source.

1.2 STATEMENT OF THE PROBLEM

The utilization of L-cysteine ingredients in a wide variety of products, especially bakery products, has led to the huge concern of various groups of consumers, especially those who are restricted to religion-based dietary. Moreover, the most controversial issue is that L-cysteine's major production could have come from animal hair or human hair. Therefore, there is a necessity to develop a reliable method for the differentiation of L-cysteine sources in foods.

1.3 PURPOSE OF THE STUDY

The proposed method of differentiating L-cysteine sources would benefit the enforcement authorities for ingredient verification and offer a new technique in detecting L-cysteine amino acids that can contribute to food authentication.

1.4 RESEARCH OBJECTIVES

1.4.1 General Objective

This study's general objective is to differentiate L-cysteine sources (cow horns, pig bristles, human hair, duck feathers, and chicken feathers) using a spectroscopy and chromatography analysis combined with PCA.

1.4.2 Specific Objectives

1. To differentiate the L-cysteine sources in cow horns, pig bristle, human hair, duck feather, and chicken feathers, using Raman spectroscopy.
2. To differentiate L-cysteine sources in cow horns, pig bristle, human hair, duck feathers, and chicken feathers using ATR-FTIR and PCA.
3. To validate the differentiation of L-cysteine sources in cow horns, pig bristle, human hair, duck feather, and chicken feathers using AAA and PCA.

1.5 RESEARCH SCOPE

Five L-cysteine sources (cow horns, pig bristles, human hair, duck feathers, and chicken feathers) were analyzed using spectroscopy techniques (ATR-FTIR and Raman). The results obtained were validated by using AAA.

1.6 HYPOTHESIS

A combination of spectroscopy technique and PCA could differentiate L-cysteine sources from cow horn, pig bristles, human hair, duck feathers, and chicken feathers. In addition, AAA can identify the protein content of the spectral data from cow horns, pig bristles, human hair, duck feathers, and chicken feathers through UHPLC.

CHAPTER TWO

LITERATURE REVIEW

2.1 HALAL CONCEPT

The number of Halal products that existed in the market is increased due to consumer demand. The term Halal is defined as permissible. Technically, anything related to Halal products should be free from any *najs* and safe to consume. The context of Halal is restricted not only to food industries, but it also covers all aspects, including pharmaceuticals and cosmetics. Al-Baqarah, verse 168 mentioned that:

“O mankind, eat from whatever is on earth [that is] lawful and good and do not follow the footsteps of Satan. Indeed, he is to you a clear enemy.”

It has clearly been mentioned in the Quran that it is an obligation for people to consume lawful and good things to protect themselves from evil deeds. From the Islamic perspective, all kinds of food are permitted to be consumed except those Allah clearly prohibited (Ali et al., 2014). According to these criteria, Islamic scholars have shown a clear guideline for human needs, especially Muslims, to prevent prohibited things.

The Halal concept is based on producing goods that include services (processing system) in line with Islamic law or *Shari'ah* (Manaf Bohari et al., 2013). The Halal industry is promising to supply Muslim consumers' needs. The term Halal is crucial for Muslims worldwide as it must provide consumer demand and the items that are either consumable or non-consumable that comply with *Shari'ah* (Manaf Bohari et al., 2013). In fact, Halal industries also compromise hygiene practice, which is one of Islamic Law requirements. Thus, Halal industries offer services that meet consumer demands in religious aspects and product satisfaction. The Halal concept can be a standpoint for

the consumer toward changes into a better life and their ideas and manifestation of quality, health, safety, and environment (Arrifin et al., 2010).

2.2 L-CYSTEINE

2.2.1 L-Cysteine Amino Acid and Its Religious Issue

Amino acids (AAs) are essential for growth and development. They act as precursors for building up proteins and other biologically important substances such as peptides, hormones, and enzymes (Baker, 2009; Kimura, 2014; Kodera et al., 2017; Poinso et al., 2016). AAs are categorized into two groups: essential AAs that can be obtained from the diet and non-essential AAs that are synthesized directly in the human body. Accordingly, L-cysteine is a non-essential AA as it can be directly synthesized in the body. However, the essential AA classification should comprise the organism's ability to synthesize carbon skeleton or just nitrogen compounds (Wu, 2009). Since L-cysteine carbon skeleton cannot be synthesized directly, it is counted under nutritionally essential AAs (Wu, 2009).

L-cysteine is an uncharged polar AA containing thiols – SH side-chain (Demirkol et al., 2004) (Figure 2.1). It takes part in protein synthesis, cellular metabolism, stabilizer, and detoxification (Borase et al., 2015; Cebi et al., 2017) and important for stabilizing tertiary and quaternary protein conformation through disulfide (Wirtz & Droux, 2005). In addition, protein-associated and free thiols are responsible for binding metals and react with nucleophilic drugs and reactive oxygen (Plaza et al., 2018; Yin, 2015). The conversion of free thiol groups to disulfide bridges and vice versa constitutes a dynamic reactive system that is the basis for redox switches in protein (Wirtz & Droux, 2005).

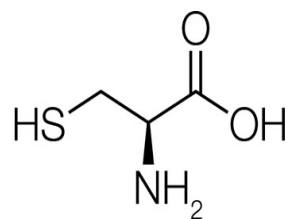


Figure 2.1: Chemical structure of L-cysteine. L-cysteine consist of amino group and thiol side chain (SH).

2.2.1.1 L-Cysteine as Food Additives

According to the food additives database European Commission, L-cysteine is labeled under the E numbers of E910, E920, and E921 (EC, 2008), which are L-cysteine, L-cysteine hydrochloride, and L-cysteine hydrochloride monohydrate, respectively (Cebi et al., 2017). It falls under the antioxidant category that the added L-cysteine proportions shall not be in a greater amount than the maximum permitted proportions according to Food Regulations 1985 (FoSIM, 1985). According to Food Drugs and Administration (FDA), the maximum concentration of L-cysteine in the dough is up to 0.009 in 100 parts and flour bakery products.

On the other hand, L-cysteine has been used in animal food as additives to mimic an artificial meat flavor (Berehoiu et al., 2013). In bakery industries, L-cysteine is widely used as a stabilizer to prevent further oxidation from occurring and create a fluffy texture of cakes by reducing the gluten network of dough (Cebi et al., 2017; Ismail et al., 2014). Theoretically, it can break the disulfide bond in the protein, which will increase the elasticity of bakery dough (Cebi et al., 2017). Thus, the addition of L-cysteine will increase the flow properties of the flour, reduce mixing time and lessen the stretch of the dough.

2.2.2 Production of L-Cysteine

Mass production of L-cysteine take place in China. Keratin hydrolysis is a production method for L-cysteine additive. Keratin can come from four different sources: pig bristles, human hair, duck feather, and cow horn, and it is considered the major process production of L-cysteine in China (Ismail et al., 2014). The importance of L-cysteine in bakery ingredients required a cost-effective production to meet supply demand. Therefore, acid hydrolysis of keratin has been used by the manufacturers to produce L-cysteine (Berehoiu et al., 2013; Ryu et al., 1997; Xu et al., 2013). To produce of one kilogram of L-cysteine in keratin hydrolysis, at least 27 kg of HCl is needed, and the temperature must be maintained at 100°C for 6 hours (Berehoiu et al., 2013). Hence, human hair is chosen as one of the raw materials due to its abundantly for mass production. However, animal sources like feathers, bristles, or hooves are also used as L-cysteine sources.

There are three types for synthesizing L-cysteine keratin hydrolysis, enzymatic bioconversion, and fermentation (Wada & Takagi, 2006). Figure 2.3 shows the summary of the keratin hydrolysis process.

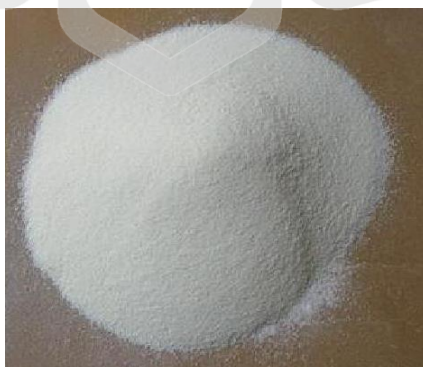


Figure 2.2: Industry scale L-cysteine powder.