MEAT PROTEIN HYDROLYSATES AND THEIR ADVANCED GLYCATION END-PRODUCTS AS SOURCES OF BITTER TASTE MODIFIERS

By

Chunlei Zhang

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Food and Human Nutritional Sciences
University of Manitoba
Winnipeg, Manitoba, Canada

ABSTRACT

Bitterness is an important issue in various food products because of the negative effects on consumer acceptability. Protein hydrolysates have been shown to be sources of various bioactive peptides but there is scanty information on their ability to act as bitter taste modifiers. Meat was chosen because it has multiple proteins and offers the possibility of generating a wide range of peptides with desirable function as bitter taste modifiers. In this study, beef protein was hydrolyzed with each of six commercial enzymes (Alcalase, Chymotrypsin, Trypsin, Pepsin, Flavourzyme, and Thermoase). Using an electronic tongue showed that Alcalase hydrolysates (AH) had significantly ($P<0.05$) lower bitter scores compared to other enzymatic hydrolysates. Separation of the AH and chymotrypsin hydrolysate (CH) by RP-HPLC yielded peptide fractions with significant ability to inhibit quinine activation of a human bitter taste receptor (T2R4) in HEK 293 cells based on calcium imaging assays. Eight peptides were identified from the T2R4-inhibitory AH and CH fractions, most of which were dominated by leucine and polar amino acids such as glutamic acid, serine, threonine and aspartic acid.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Rotimi Aluko for his excellent guidance, support, patience, and motivation. The door of Dr. Aluko was always open to provide students with guidance and support, and he allowed us to express our opinions, but steered us in the right direction. Besides, I am also deeply grateful to my co-supervisor Dr. Prashen Chelikani for his guidance during cell culture and calcium assay experiments. Furthermore, I would like to thank my committee member Dr. Sijo Joseph for his precious time, insightful feedback and guidance. I also owe my deepest appreciation to Dr. Adeola Alashi and Dr. Nisha Singh for teaching me multiple experiments and their endless patience and passion. It was impossible to accustom myself to a new environment quickly in the beginning and get through my master program without their help and encouragement. I also want to thank my colleagues, Hongyi, Ifeanyi, Tola and Ayo for their help and cooperation. Last but not least, I deeply appreciate the love, support and encouragement from my parents and my friends. Last but not least, I want to thank Alberta Agriculture and Forestry for the financial support.

DEDICATION

I wish to dedicate this thesis to

My great parents, Bin Zhang and Xinling Zheng

ABBREVIATION

Bitter Taste Receptors or Taste type 2 Receptors	BTRs, T2Rs
Defatted Beef	DB
Defatted Beef Hydrolysates	HBP
Beef Protein Alcalase Hydrolysates	AH
Beef Protein Chymotrypsin Hydrolysates	CH
Beef Protein Trypsin Hydrolysates	TH
Beef Protein Pepsin Hydrolysates	PH
Beef Protein Flavourzyme Hydrolysates	FH
Beef Protein Thermoase Hydrolysates	TMH
Hydrophobic Amino Acids	HAA
Positively Charged Amino Acids	PCAA
Negatively Charged Amino Acids	NCAA
Aromatic Amino Acids	AAA
Sulphur-containing Amino Acids	SCAA
Branch Chain Amino Acids	BCAA
N,N-Bis(carboxymethyl)-L-lysine	BCML
Gamma-aminobutyric acid	GABA
L-ornithyl-L-alanine	OA
Phenylalanine	Phe
Ornithine	Orn
Alanine	Ala
Asparagine	Asn
Methionine	Met
Tyrosine	Tyr
Serine	Ser
Aspartic acid	Asp
Glutamine	Gln
leucine	Leu
Proline	Pro
Glycine	Gly
Arginine	Arg
Isoleucine	Ile
Histidine	His
Valine	Val
Taurine	Tau

TABLE OF CONTENTS

CHAPTER ONE-INTRODUCTION

Being averse to bitter taste is a common phenomenon for humans and other animals, which requires the pharmaceutical and food industries to render their products without this taste (Levit et al., 2014; Meyerhof, 2005). Besides, the increasing health awareness of consumers results in reduced addition of sodium salt, fat, and sugar, which accentuates the undesirable taste of some foods (Eckert & Riker, 2007). Moreover, some nutritional supplements fortified in foods also cause severe taste deficiency, leading to a reduction of consumer acceptance of such products (Oszmianski et al., 2007). There are some techniques that have been applied to reduce food bitterness, but these ingredients have to be used at high concentrations in order to be effective, which may increase the health risks for some specific populations (Keast, Breslin, & Beauchamp, 2001).

With the development of molecular biology techniques, the bitter taste receptors (BTRs) and bitter taste transduction pathways have been gradually clarified. Manipulating the bitter taste by using low molecular weight compounds, which can act at the cellular level or receptor level is promising (Ley, 2008; Sai P Pydi et al., 2014). So far, however, there are only a few compounds identified as bitter taste inhibitors through trial and error means. Moreover, scanty information on the structure-function relationship of bitter tastants makes it difficult to explore potential bitter taste modifiers. However, among those identified inhibitors, some are from classes that are usually identified as bitter taste carriers, such as flavanones and peptides (Kenji Maehashi et al., 2008; Sai P Pydi et al., 2014; Roland et al., 2014). Therefore, it can be assumed that small conformation changes or group changes may turn bitter agonists to antagonists.

Given that protein hydrolysates were reported to have extensive novel functions, such as anti-oxidation, antihypertension, and cholesterol reduction (Pripp & Ardö, 2007; Wu & Aluko, 2007), peptides and other types of amino acid derivatives (BCML and GABA) can be considered as efficient bitter taste inhibitors (Sai P Pydi et al., 2014). Therefore, it is reasonable to deduce that the wide range of non-bitter peptides and their advanced glycation end-products could be exploited as potential bitter taste inhibitors. Meat (specifically beef) was chosen for this work because of the potential to generate (through enzymatic hydrolysis) flavor-promoting peptides that could act as bitter taste suppressors or antagonists.

1.1. HYPOTHESES

- Non-bitter tasting peptides can be generated through enzymatic hydrolysis of beef proteins.
- Peptides, which have no bitter taste, are potential bitter taste inhibitors.
- Peptides derivatives such as advanced glycation end-products are potential bitter taste modifiers.

1.2. OBJECTIVES OF STUDY

The objectives of this project were to determine:

- Bitter taste-suppressing ability of different enzymatic beef protein hydrolysates and their advanced glycation end-products (AGEs) using an electronic tongue
- Antagonistic effect of the AGEs, protein hydrolysates and reverse-phase HPLC peptide fractions against a human bitter taste receptor (T2R4) on cell based assays culture
- The amino acid sequence of peptides present in the most peptide fractions

CHAPTER TWO-LITERATURE REVIEW

2.1. BITTER TASTE

The sensation of taste is crucial for animals to survive in nature, because it can provide valuable information about the environment and the quality of food. For example, sweetness signals nourishing and high energy foods, in contrast bitterness suggests foods comprising toxic or life-threatening components. Therefore, animals have evolved to be innately averse to bitter taste for self-protection (Davis et al., 2010; Dong, Jones, & Zhang, 2009; Roper, 2007, 2013). Due to the life environment and food types, the sensitivity of bitter taste varies across different species; for example, compared to carnivores, herbivores generally are more sensitive to bitter taste, because of the more toxic compounds that exist in plants in comparison to meat (Dong et al., 2009; Li & Zhang, 2014). There are many bitter taste substances that exist in our daily life such as caffeine in coffee, humulones in beer, bitter glucosinolates in broccoli, and phenol flavonoids in tea (Barratt-Fornell & Drewnowski, 2002; Meyerhof, 2005). Proper dosage of bitter taste bioactive compounds have beneficial influence on human health, such as bitter peptides that inhibit blood pressure-promoting protein, anti-oxidative polyphenols from apple juice and limonoids from citrus fruit (Oszmianski et al., 2007; Upadhyaya, Pydi et al., 2010). Moreover, bitter taste is a common attribute of several drugs, which constantly is a big obstruction for compliance of drug treatment for patients, especially children who are very sensitive to tastes (Calcagno, Lobatto, Robson, & Millon, 2016; J. A. Mennella, Spector, Reed, & Coldwell, 2013). Therefore, efficiently and safely reducing bitter taste in foods and medicines is desirable.

2.2. BITTER TASTE RECEPTORS

All the gustatory signals are produced by the brain after taste molecules interact with taste receptor-expressing cells (TRCs) that are situated in taste buds of the oral cavity (Chaudhari & Roper, 2010; Stone, Tan, Tam, & Finger, 2002). Each taste bud consists of a 50-100 TRCs and each TRC has dozens of different taste receptors (Stone et al., 2002). Salty and sour taste are sensed by ion channels directly, while sweet, umami and bitter are sensed by G protein-coupled receptors (GPCRs) (Jaggupilli et al., 2016; Pydi et al., 2012).

More than 30 GPCRs have been discovered to be important for human taste perception with 25 of these identified as important for bitter taste sensing alone and are termed type 2 taste receptor (T2Rs), the least studied and understood subfamily of GPCRs (Jaggupilli et al., 2016; Sai Prasad Pydi et al., 2012; Sainz et al., 2007). In general, T2Rs are 290 to 330 amino acids-long proteins that have seven transmembrane helices (TM) and 3 extracellular loops (ECL), 5-19 amino acids long amino-termini and 3 intracellular loops with 7-32 amino acid long carboxyl-termini (Jaggupilli et al., 2016; Pydi et al., 2012). The amino acid sequences in extracellular loops are significantly divergent, among the T2Rs which partly explain why different T2Rs are activated by different bitter compounds (Chandrashekar et al., 2000; Ueda et al., 2001; Upadhyaya et al., 2010b). As mentioned before, there are hundreds of structurally heterologous compounds perceived as bitter by the 25 human T2Rs. The assignment of T2Rs with corresponding bitter taste ligands has been developed for some receptors (shown in Table 1), but some have not been deorphanized, like the hTAS2R42, hTAS2R45, hTAS2R48, and hTAS2R60. Certain receptors like TAS2R14 and TAS2R16 have broader molecular receptive ranges than others such as TAS2R3 and TAS2R7. To date, more than one hundred bitter taste compounds have been matched the 25 T2Rs in human, but there is no obvious common chemical substructures discovered in these compounds (Brockhoff, Behrens, Niv, & Meyerhof, 2010; Ji et al., 2014; Miguet, Zhang, & Grigorov, 2006). This makes it challenging to predict bitter taste of compounds and understand the underlying ligand-binding mechanisms. Previously, it was believed that T2Rs exclusively resides in the tongue to sense bitter taste (Behrens, Foerster, Staehler, Raguse, & Meyerhof, 2007; Sbarbati, Merigo, Benati, Tizzano, & Bernardi, 2004), but recent studies have revealed that T2Rs are also distributed in the gastrointestinal tract, enteroendocrine STC-1 cells, respiratory system, male reproductive system and central nervous system (Maik Behrens & Meyerhof, 2010; Singh et al., 2011; Wu et al., 2002; Xu, Cao, Iguchi, Riethmacher, & Huang, 2013). Recent nCounter sequence analysis of different human tissues shows expression of T2Rs at significant levels in many tissues (Jaggupilli et al., 2017). This suggests that T2Rs may possess more potential important physiological functions other than bitter taste sensation.

2.3. MECHANISMS OF BITTER TASTE SENSATION

2.3.1. T2Rs LIGAND BINDING

The initiation of signal of bitter taste starts from water-soluble bitter taste molecules (ligands or agonists) binding to extracellular and/or transmembrane domains of T2Rs. Some receptors like TAS2R16 and TAS2R43 are broadly tuned to make contact with numerous compounds (Bufe, Hofmann, Krautwurst, Raguse, & Meyerhof, 2002). Amino acid sequence analysis of T2R31, T2R43 and T2R46 suggest 85% homology, but their ligands are notably different (Brockhoff et al., 2010), which indicates that each T2R has its own unique ligand binding pocket.

Furthermore, classic chimeric receptor approach and site-directed mutagenesis were used to identify the receptor regions and amino acid residues critical for ligand binding (Pronin, Tang, Connor, & Keung, 2004; Pydi et al., 2014; Singh et al., 2011). Studies found that TM3, TM5 and TM6 are the binding sites of β-glucopyranosides in T2R16 and the key amino acids residues involved include Glu86, Trp94 and His181(Sakurai et al., 2010). TM1, TM7 and some amino acid residues from ECL1 and ECL2 in T2R1 were found to form one binding site for peptide ligands (Upadhyaya et al., 2010a). Seven amino acid residues located at TM3, TM4, ECL2 and ECL3 in T2R4 were discovered to form a binding pocket for quinine (Sai P Pydi et al., 2014). An amino acid at position 262 of T2R38 was found to play an important role in ligand binding and stabilizing a network of inter-helical hydrogen bonds (H-bond) (Tan, Abrol, Trzaskowski, & Goddard, 2012). Besides, swapping two amino acid residues in TM7 between T2R46 and T2R31, which are activated by strychnine and aristolochic acid respectively, was observed to reverse their agonist selectivity (Brockhoff et al., 2010). Therefore, the amino acid residues existing in extracellular loops and transmembrane domains form different flexible and selective binding pockets to accommodate a large and diverse group of bitter taste ligands.

Table 1 Bitter taste receptors with their identified agonists

Receptor	**Identified agonists**	**Reference**
TAS2R1	dipeptide and tripeptide	(Upadhyaya et al., 2010a)

TAS2R4	6-n-propyl-2-thiouracil, quinine, amarogentin, arborescin, artemorin, campher, colchicine, denatonium benzoate	(Meyerhof et al., 2009; Reed, Bartoshuk, Miller, Duffy, & Lucchina, 1999)
TAS2R7	Strychnine, caffeine, papaverine, quinine	(Bufe et al., 2002; Meyerhof et al., 2009)
TAS2R10	Strychnine, quinine, caffeine	(Brockhoff, Behrens, Massarotti, Appending, & Meyerhof, 2007)
TAS2R14	α-Thujone, picrotoxinin, picrotin, 1-naphthoic acid, benzoate, piperonylic acid, 1-nitronaphthalene, 1,8-naphthalaldehydic acid, (-)-epicatechin gallate, (-)-epigallocatechin gallate	(Maik Behrens et al., 2004; Yamazaki, Narukawa, Mochizuki, Misaka, & Watanabe, 2013)
TAS2R16	Various β-glucopyranosides, including salicin, helicin, arbutin, phenyl-β-D-glucoside, methyl-β-D-glucoside, amygdalin, esculin, 2-nitro-phenyl-β-D-glucoside, naphthyl-β-D-glucoside	(Bufe et al., 2002; Sakurai et al., 2010)
TAS2R38	Various thioamides, including propylthiouracil, phenylthiocarbamide, diphenylthiurea, acetylthiourea, methylthiouraci	(Bufe et al., 2005)
TAS2R39	(-)-epicatechin gallate, (-)-epigallocatechin gallate	(Yamazaki et al., 2013)
TAS2R43	Aristolochic acid, 6-nitrosaccharin, saccharin, acesulfame K, n-isopropyl-2-methyl-5-nitrobenzenesulfonamide	(Kuhn, 2004a; Pronin et al., 2004)
TAS2R44	Aristolochic acid, saccharin, acesulfame K, denatonium, 6-nitrosaccharin	(Kuhn, 2004b; Pronin et al., 2004)
TAS2R46	Sesquiterpene lactones, clerodane, labdane diterpenoids, strychnine, denatonium	(Brockhoff et al., 2007)
TAS2R47	Denatonium, 6-nitrosaccharin	(Pronin et al., 2004)

2.3.2. T2Rs AND G-PROTEIN COUPLING

Activation of T2Rs leads to structural changes in the intracellular loops and triggers activation of the coupled heterotrimeric guanine-nucleotide-binding proteins (G-proteins), which function as the molecular switches to turn on intracellular signaling cascade (Oldham & Hamm,

2008; Schachman, 2000). Heterotrimeric G-proteins are composed of alpha (α), beta (β) and gamma (γ) subunits. Gustducin is a taste receptor cell-specific G-protein and is highly homologous to transducin (the rod and cone photoreceptor G-protein), suggesting that gustducin plays the same role as transducin (McLaughlin, S.K., McKinnon, P.J., and Margolskee, 1992). Upon activation of receptors, subunits of the heterotrimer dissociate and gustducin stimulates phosphodiesterase (PDE) to decrease the concentration of cyclic adenosine monophosphate (cAMP). The decrease of cAMP subsequently weakens the inhibition of a cyclic nucleotide-inhibited cation channel to release calcium. Simultaneously, the Gβγ dimer activates PLC β2 to produce inositol triphosphase (IP3), which stimulates calcium ion to release from the endoplasmic (Gwendolyn, Kimberly, & Robert, 1996; McLaughlin, S.K., McKinnon, P.J., and Margolskee, 1992; Oldham & Hamm, 2008). Therefore, intracellular calcium mobilization has been used to identify agonists and bitter taste modulators as well as potential antagonists (Sai P Pydi et al., 2014). Calcium release subsequently activates TRPM5 ion channel to depolarize the cell and release neurotransmitters (Meyerhof, 2005; Ueda, Ugawa, Yamamura, Imaizumi, & Shimada, 2003). The general process of activation is shown in Fig. 1.

Due to the limited life span of taste receptor cells, the human embryonic kidney (HEK) 293 cell system has been used to express BTRs and transporting parts and/or other segments of the gustatory signaling system (Bufe et al., 2002; Pronin et al., 2004; Ruiz-Avila, Ming, & Margolskee, 2000). Multiple studies used this HEK 293 cell system to express different T2Rs and Ga16/44 to examine the ligand specificities of members of the T2R family (Maik Behrens et al., 2004; Greene et al., 2011; Sai P. Pydi et al., 2015; Sai P Pydi et al., 2014; Roland et al., 2014). The process of developing HEK 293 cells system and calcium assay is shown in Fig. 2.

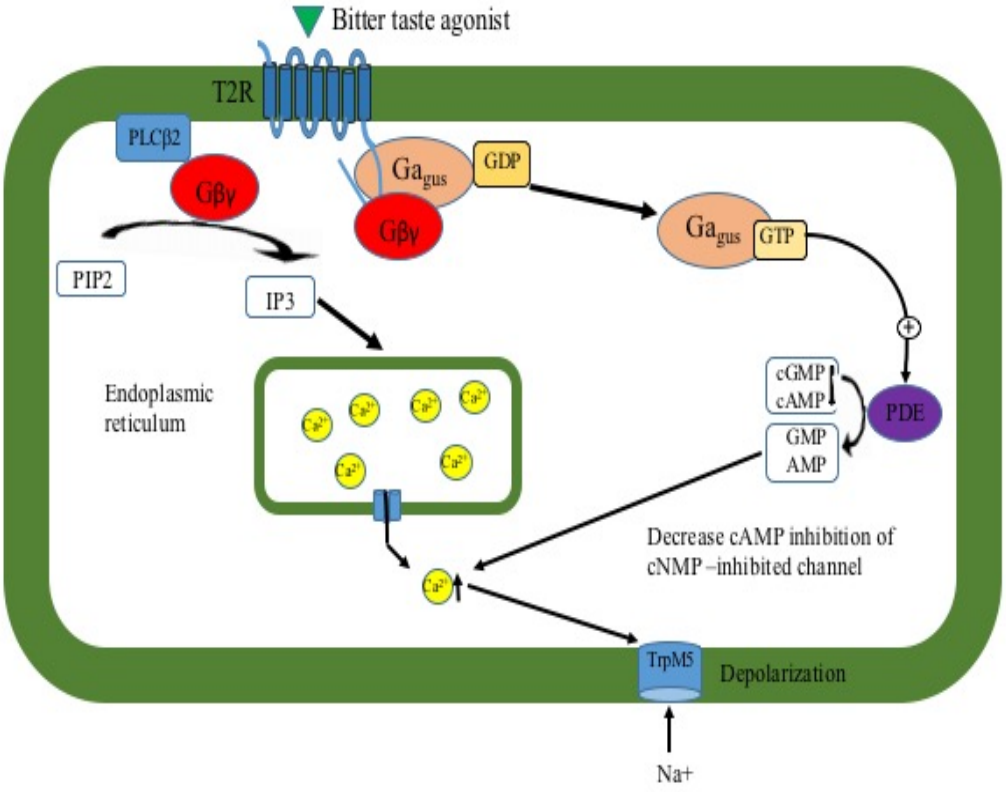


Fig. 1. Bitter taste transduction mechanism

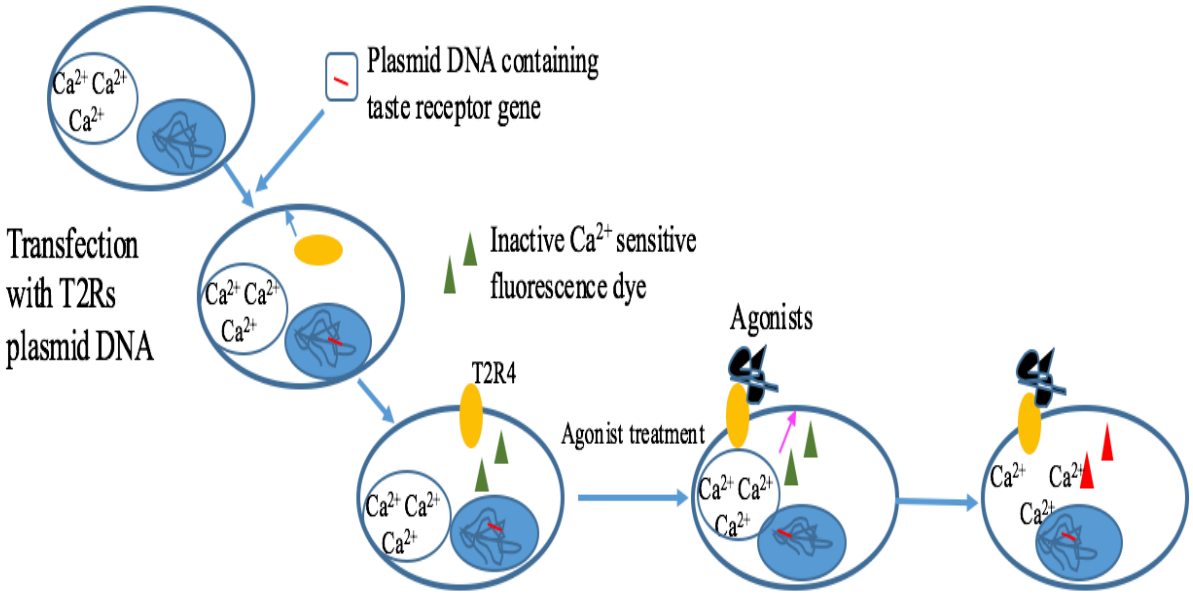


Fig. 2. Schematic of T2R assay in HEK 293 cells

2.4. BITTER TASTE MODIFIERS

For pharmaceuticals, applying physical barriers to a solid formulation is a common method, such as encapsulation, coating, emulsion, and suspensions, but this efficient method is problematic for children liquid drugs (Gupta et al., 2010; Mennella et al., 2013). Adding artificial sweeteners, sucrose, sodium chloride, and potassium chloride to mask bitter taste is an accepted strategy applied in foods and beverages to improve sensory profile (Eckert & Riker, 2007; Harwood, Loquasto, Roberts, Ziegler, & Hayes, 2013; Keast et al., 2001). However, adding artificial sweeteners was found to impart a metallic bitter taste and astringency, which is undesirable as well (Ley, 2008; Montmayeur & Matsunami, 2002). Due to the popularity of health foods, the use of high doses of sugar and salt are limited because of health risks for certain groups (e.g. diabetes, hypertension and pregnant women). Besides, the use of congruent flavor (e.g. chocolate, grapefruit, caffeine) and scavenging molecules (e.g. cyclodextrins and

cyclofructans) are implemented to decrease bitter taste (Allison A.-M.A., 2000; Binello, Cravotto, Nano, & Spagliardi, 2004; Vummaneni & Nagpal, 2012). Unfortunately, these methods cannot be applied generally and some of them could change the texture and flavor release properties of foods.

Many papers reported that amino acids and peptides diminished bitter taste efficiently. For example, L-aspartyl-L-phenylalanine and L-ornithyl-L-alanine (OA) reduced the bitterness of potassium chloride (Fuller & Qurtz, 1997). Further, the simple nucleotides, cytosine monophosphate (CMP) and 2-deoxyadenosine triphosphate (dATP) were demonstrated to cause a 40% and 60% reduction in bitterness of a 10 mM quinine solution, respectively. Besides, a mixture of amino acids (L-asparagine, L-methionine, L- tyrosine, L-serine, L-aspartic acid, L-glutamine, L-alanine, L-leucine, and L-proline) was reported to suppress the bitter taste of high potency sweeteners (Lindqvist, 2011). However, the mechanisms of bitter taste suppression of these amino acid and their derivatives have not been elucidated; information on extracellular receptor activation or interactions with components of intracellular gustatory signal cascade transmission is scanty.

The gradual decoding of the structure-function relationships between T2Rs and agonists enables discovery of potential specific bitter receptor antagonists, which can bind to the same receptors as agonists without evoking the signal transduction process but blocks receptor activation (Jaggupilli et al., 2016). 4-(2,2,3-trimethylcyclopentyl) butanoic acid (GIV3727) was one of the first discovered bitter taste receptor antagonist, which specifically acted as an orthosteric and insurmountable antagonist for hTAS2R31. GIV3727 inhibits hTAS2R31 activation by saccharin and acesulfame K with IC_{50} values of 6.4±2.4 uM and 7.9± 6.1 uM respectively (Slack et al., 2011). P-(dipropylsulfamoyl) benzoic acid (Probenecid), an inhibitor of the Multidrug Resistance Protein 1 (MRP1) transporter and a clinical drug for gout, was another bitter taste receptor antagonist, which inhibited the activation of hTAS2R16, hTAS2R38, and hTAS2R43 (Greene et al., 2011). Moreover, several flavanones including sakuranetin, 6-methoxysakuranetin, 6-methoxyflavanones have been illustrated as antagonist for hTAS2R31 and hTAS2R39 (Fletcher et al., 2011; Roland et al., 2014). Pydi and colleagues utilized molecular modelling guided site-directed mutagenesis to predict the ligand-binding pocket of

T2R4 for agonist quinine and then docked 75 different amino acids derivatives with T2R4 ligand-binding pockets. Based on binding affinity, 19 amino acid derivatives were selected for competition assays to characterize their ligand specificities. Ultimately, GABA and BCML showed antagonist activity in calcium imaging assay (Sai P Pydi et al., 2014). Later in 2015, the structure-function information successfully led to the discovery of another T2R4 antagonist, abscisic acid (ABA), which blocked the quinine activation with an IC_{50} value of 34.4 ± 1.1 μM. Site-directed mutagenesis indicated that the T2R residues responsible for interaction with quinine was also involved in binding to ABA (Sai P. Pydi et al., 2015).

2.5. MEAT PROTEIN HYDROLYSATES

2.5.1. GENERAL FUNCTIONS OF PROTEINS AND PROTEIN HYDROLYSATES

Proteins are regarded as one of the most important food category in food industry not only because of their well-known nutritional value but also because of their various dynamic functional properties, such as emulsification, foaming, gelation, hydration, and textural properties (Liu & Tang, 2014; Phillips, Horn, & Smith, 1995). Protein hydrolysates, especially those obtained from enzyme hydrolysis are showing great advantages in terms of medical diets when compared with intact protein and amino acids because of their high absorption efficiency (Clemente, 2001; Siemensma, Weijer, & Bak, 1993; WHO, 2003). Bioactive peptides (BAPs), generated from enzyme hydrolysis of food protein, are gaining growing focus, because they have been reported to possess remarkable multifunctional health-promoting bioactive properties; for example, chicken breast skin and soy protein peptides were reported to have potent antioxidant activities (Liu et al., 2012; Onuh, Girgih, Aluko, & Aliani, 2014). Furthermore, BAPs from milk protein, meat muscle protein, casein, and egg were demonstrated to possess various bioactivities, such as antihypertensive, anti-inflammatory and anticancer (Kannan, Hettiarachchy, Marshall, Raghavan, & Kristinsson, 2011; Ryu, Qian, & Kim, 2010; Udenigwe & Aluko, 2012). This research area of BAPs is still growing continuously due to the numerous types of proteins and enzymes, and the increasing number of newly discovered disease targets.

2.5.2. FLAVOUR PROPERTIES OF PROTEIN HYDROLYSATES

In addition to their health-enhancing properties, amino acids and peptides also play important roles in taste modification (Maehashi & Huang, 2009a; Ogawa et al., 2004). Most amino acids exhibit many basic tastes but one taste is predominant. Glycine, alanine, threonine, proline, serine, glutamine are recognized as sweet-tasting amino acids, while phenylalanine, tyrosine, arginine, leucine, isoleucine, valine, methionine, and histidine are bitter but glutamate and aspartic acid have umami taste (Akitomi et al., 2013a; Birch; Kemp, 1989; Solms, 1969). Peptides have also been reported to have unusual tastes. For example, l-aspartyl-l-phenylalanine methyl ester is a peptide with sweet taste while Orn-Tau produces a salty taste even though it has no sodium ion (Tada, Shinoda, & Okai, 1984). Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala, which was isolated from beef soup was named "delicious peptide" because of its umami taste (Masahiro Tamura et al., 1989) and multiple peptides from chicken protein hydrolysates such as Glu-Glu, Glu-Val, Ala-Asp-Glu, Ala-Glu-Asp, Asp-Glu-Glu, and Ser-Pro-Glu were reported to enhance umami taste (Maehashi, Matsuzaki, Yamamoto, & Udaka, 1999). Miraculin, a protein extracted from miracle fruits, has the ability to modify sourness into sweetness (Theerasilp et al., 1989). And as mentioned above, many amino acids and amino acid derivatives have been used to reduce bitter taste by masking unpleasant tastes or inhibiting activation of bitter taste receptors.

However, peptides with bitter taste have been shown to be present in aged, fermented, and hydrolyzed food products (Maehashi & Huang, 2009a; Maehashi et al., 1999). So far, more than 200 kinds of bitter-tasting peptides have been found to occur with a great diversity in amino acid sequences (Kohl, Behrens, Dunkel, Hofmann, & Meyerhof, 2012; Maehashi & Huang, 2009b). Furthermore, synthesized peptides were shown to interact with specific bitter taste receptors. It was found that the tripeptide Phe-Phe-Phe activated hTAS2R1 with EC_{50} value in micromolar range (Upadhyaya et al., 2010b), while Phe-Phe-Pro-Arg was reported to activate hTAS2R8 and hTAS2R39 (Ueno, Sakurai, Okada, Abe, & Misaka, 2011). Two peptides isolated from Gouda cheese, Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn-Ser and Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn, were also demonstrated to activate TAS2R1 and TAS2R39 (Kohl et al., 2012). With the discovery of more bitter peptides, some common structural properties have been proposed. Firstly, the sizes are between 0.36 kDa to 2.10 kDa, because peptides that are too bulky cannot

bind to bitter taste receptors while smaller (<0.36 Da) peptides failed to achieve the required configuration required for activation (Kim et al., 1999; Ley, 2008; Maehashi & Huang, 2009a). Secondly, the hydrophobicity of peptides closely correlates with their bitter taste. This notion was firstly proposed by Ney who invented a Q value, the average amount free energy needed to transfer amino acid chains from ethanol to water, and found that all bitter peptides had a Q value greater than 1400kcal/mole (Ney, 1971). Later, many investigators used synthesized peptides to examine this notion and found that hydrophobicity of peptides plays an important role in bitter taste but other factors like positions and modification of amino acid as well as sizes of peptides also had crucial influence on bitter taste (Kim & Li-Chan, 2006; Maehashi & Huang, 2009b; Matoba & Hata, 1972; Toelstede & Hofmann, 2008). Due to the dual flavor properties of peptides, it is hypothesized that peptides can be a good source for bitter taste receptor antagonists

2.5.3. SOURCES OF PROTEIN HYDROLYSATES

Recently, bioactive peptides isolated with antioxidative and anti-hypertension properties, from plant proteins such as soybean, peas, chickpeas, are becoming increasingly important (Han & Baik, 2008; Udenigwe & Aluko, 2012; Yimit, Hoxur, Amat, Uchikawa, & Yamaguchi, 2012). Compared to plant proteins, animal proteins have more complete amino acids profile. Cow's milk is the most important protein source to produce protein hydrolysates for nutritional diets of patients and infant formulations by food-grade proteases (Clemente, 2001; Cordle, Mahmoud, & Moore, 1991; Siemensma et al., 1993). Beef muscle has high protein (20-25g protein/100g) content and contains all the essential amino acids (lysine, threonine, methionine, phenylalanine, tryptophan, leucine, isoleucine, valine in adequate quantities (Williams, 2007). And the umami amino acid, glutamic acid/glutamine are present in the highest amounts (16.5%), followed by the sweet amino acids, aspartic acid and asparagine (9%) (Akitomi et al., 2013a; Nishimura & Kato, 1988). Besides, the protein of beef muscle is highly digestible, with ~ 94% when compared to the 78% protein digestibility of beans and 86% in whole wheat (Robert, 2012). Therefore, in terms of flavor studies, all the characteristics of beef suggest that the protein can be used as a good source to produce desirable peptides, especially those that modify bitter taste.

2.6. ADVANCED GLYCATION END-PRODUCTS (AGEs)

AGEs are an heterogeneous group of compounds generated from non-enzymatic reaction between carbonyl group of the a reducing sugar and an amino group of a protein which is termed as Maillard reaction (Uribarri et al., 2015). The reaction process includes three stages which are initiation, propagation and an advanced stage. The early glycation and oxidation processes form Schiff bases and Amadori products, further glycation of amino group of peptides or proteins results in molecular rearrangements leading to the generation of AGEs. The types and yields of final products are attributed to selected reducing sugar types, pH, temperature, and heating time (Nie et al., 2013; Wu, Huang, Lin, & Yen, 2011). The process of AGE formation produces reactive oxygen species (ROS), which are believed to be deleterious for human health and to contribute to several chronic diseases, such as diabetes, cardiovascular disease, neurodegenerative disease, and chronic kidney disease (CKD) (Chen, Pyzik, Yong, & Striker, 2013; Goldin, Beckman, Schmidt, & Creager, 2006; Uribarri et al., 2015). However, peptides from soy protein and milk protein after Maillard reaction were shown to possess enhanced antioxidant activity (Liu et al., 2012; Oh et al., 2013a)

In addition to the above mentioned effects, AGEs are also undoubtedly important substances for unique aroma and taste of thermally processed foods. For example, 4-hydroxy-2 (or5)-ethyl-5(or2)-methyl-3(2H)-furanone, 2-hy-droxy-3-methyl-2-cyclopenten-1-one, and 3-hydroxyl-4,5-dimethyl-2(5H)-furanone as inner salt were reported to enhance sweetness perception of sugar (Namiki & Nakamura, 1992; Ottinger, Soldo, & Hofmann, 2003). Besides, the bitter taste intensity of casein peptide AGEs decreased after 3 hours heating, compared to heating casein peptides alone for 12 hours (Dong, Wei, Chen, McClements, & Decker, 2011). It was also reported that Maillard reaction products of soy protein hydrolysates exhibited strong caramel-like odor and had notably weaker bitter taste (P. Liu et al., 2012).

Table 2 Summary of some amino acids and amino acids derivatives functioning as bitter taste modifiers and their potential inhibiting mechanisms

Bitter taste modifiers	**Bitter taste substances**	**Masking effect**	**Method**	**Potential mechanism**	**References**
1% of a phosphatidic acid (PA) and β-lactoglobulin (LG) complex	Quinine Solution (5mM)	90%	Human gustatory test	PA-LG blocks bitter substances from interacting to target sites on the taste receptor membranes	(Katsuragi et al., 1995)
L-Aspartyl-L-phenylalanine potassium salt (0.6 g/L)	potassium chloride (20 g/L)	50%	Human gustatory test		(Fuller & Kurtz, 1997)
L-Arginine (0.15% w/v)	L-Ile solutions (100 mM) L-Phe solutions (100 mM)	70% 19%	Human gustatory test	L-Arg not only binds at the receptor site, but also mediates elsewhere in the process of bitterness perception	(Ogawa et al., 2004)
L-Arginine (<10 mM)	quinine solution (0.3mM)	67%	Human gustatory test	L-Arg competitively binds at quinine binding sites	(Ogawa et al., 2005)
L-Ornithin (100 mM)	L-isoleucine (100 mmol/l) L-leucine (150 mmol/l) L-valine (300 mmol/l)	67% 63% 50%	Human gustatory test and Artificial taste sensors	The structure of L-org resembles that of L-Arg, thus the mechanism are assumed to be similar	(Tokuyama et al., 2006)
γ-Amino butyric acid (GABA) (50ppm)	caffeine (500 ppm)	33%	Human gustatory test		(Kindel & De, 2010)

4-(2,2,3-trimethylcyclopentyl) butanoic acid (GIV3727)	Inhibiting the activation of T2R31 from Acesulfame K (2 mM) and saccharin (3 mM)	IC_{50} 6.4 ± 2.4 uM and 7.9 ± 6.1 μM respectively	Measuring changes in intracellular calcium mobilization of HEK293T cells	GIV3727 is disrupting G-protein coupling	(Slack et al., 2010)
GABA Na, Na–bis (carboxymethyl)-L-lysine (BCML)	Inhibiting activation of T2R4 from Quinine Solution (1 mM)	IC_{50} 3.2 ± 0.3 uM and 59 ± 18 nM respectively	Measuring changes in intracellular calcium mobilization of HEK293T cells	GABA and BCML share the same binding sates of quinine to T2R4, thus competitively inhibiting the binding of quinine to activate receptors	(Sai P Pydi et al., 2014)

CHAPTER THREE - MATERIALS AND METHODS

3.1. MATERIALS

Ground beef was bought from local market (Safeway, Manitoba). Chymotrypsin® (from bovine pancreas, EC 3.4.21.1), trypsin® (from porcine pancreas, EC 3.4.21.4), Pepsin® (from porcine gastric mucosa, EC 3.4.23.1), Alcalase® (from fermentation of *Bacillus licheniformis*, EC 3.4.21.62), and Flavourzyme® (from *Aspergillus oryzae*, EC 232.752.2) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Thermoase® (from *Bacillus stearothermophilus*, EC 3.4.24.27) was a product of Amano Enzymes Inc. (Nagoya, Japan).

3.1.1. PREPARATION OF DEFATTED BEEF (DB)

Raw ground beef (approximately 250 g) was packed in aluminum foil plates evenly, frozen at -20°C for 24 h and freeze-dried. Freeze-dried beef was blended thereafter in a Waring blender to fine powder and defatted twice by mixing 100 g with 1 L food grade acetone under fume hood. The defatted beef was placed in aluminum foil plates and air-dried overnight in the fume hood at

room temperature. The dried defatted beef was then milled in the Waring blender into fine powder and stored at -20°C (Hou, Li, & Zhao, 2011).

3.1.2. PREPARATION OF DEFATTED BEEF HYDROLYSATES (DBH)

DB was mixed with water to prepare 5% (w/v) slurries and protein hydrolysis initiated by adding deferent enzymes at 1% (w/w, protein basis). The hydrolysis conditions (temperature and pH) of each enzyme are shown in Table 3, which were based on manufacturers' instructions and literature information (Hou et al., 2011; Lin, Tian, Li, Cao, & Jiang, 2012; Nchienzia, Morawicki, & Gadang, 2010). Each mixture was stirred continuously for 4 h and the reaction terminated by heating the slurries to 95°C for 15 min. The mixtures were thereafter centrifuged (3270 g at 4°C) for 30 min and the resulting supernatants freeze-dried and stored at -20°C until needed for further analyses (Nchienzia et al., 2010).

Table 3 Optimal conditions of Alcalase®, Thermoase®, Trypsin®, Flavourzyme®, Pepsin®, Chymotrypsin®.

Enzyme	Temperature (°C)	pH	Incubation time (h)
Alcalase®	55	8.0	4
Thermoase®	37	8.0	4
Trypsin®	37	8.0	4
Flavourzyme®	50	6.5	4
Pepsin®	37	2.0	4
Chymotrypsin®	37	8.0	4

3.2. DETERMINATION OF PROTEIN CONTENT

The protein content of DB was determined by Lowry method (Melander & Tømmeraas, 2008; Winters & Minchin, 2005), which needs four reagents including reagent A (2% sodium carbonate, 0.4% sodium hydroxide, 0.16% potassium sodium tartrate tetrahydrate, 1% sodium dodecyl sulfate), reagent B (4% copper sulfate), reagent C (a mixture of 100 parts of reagent A and 1 part of reagent B), and reagent D (a mixture of 1 part of Folin Ciocalteu's phenol with 1

part of distilled water). These four reagents were prepared freshly before every use. Bovine serum albumin (BSA) was used to prepare 10 mg/ml standard stock solution and DB was hydrated with distilled water to obtain 10 mg/ml stock solution. The BSA stock solution and DB stock solution were serially diluted to give 20-1000 µg/ml using distilled water. A 3 ml aliquot of reagent C was added to each test tube, mixed and incubated at room temperature for 1 h in the dark. Then 0.3 ml of reagent D was added to each tube and incubated at room temperature for 45 min. Finally, the absorbance was at 660 nm in a spectrophotometer and the protein content calculated using the following relationship:

A $\left(\frac{Abs\ sample}{Abs\ \text{BSA}}\right) * Conc\ BSA = Corresponding\ Conc\ Sample\ Protein$

B $\frac{Conc\ Sample\ Protein}{Conc\ Sample} * 100 = Protein\ content\ \%$

(Abs: absorbance; Conc: concentration)

3.3. **DETERMINATION OF DEGREE OF HYDROLYSIS OF DBH-OPA METHOD**

The degree of hydrolysis (DH) of DBH was determined by the O-phthalic aldehyde (OPA) method, which was based on previous reports (Charoenphun, Cheirsilp, Sirinupong, & Youravong, 2013; Nielsen, Petersen, & Dambmann, 2001). The OPA reagent, which was prepared fresh daily contained 6 mM OPA dissolved in 95% methanol and 5.7 mM DL-dithiothreitol in 0.1 M sodium tetraborate decahydrate with 2% (w/v) SDS. N-Gly-Gly glycine solution was prepared as standard solution in 8 serial concentrations (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4 mg/ml) while DB and DBH was dissolved in distilled water to 0.25 mg/ml. The spectrophotometer was preheated to 37 °C. 10 ul of the standard solutions and 200 ul OPA reagent were added in 96 cell plates and read by spectrophotometer at 340 nm to make a standard curve. Then 10 ul of the DB and DBH and 200 ul OPA reagent were added in 96 cell plates and read by spectrophotometer. The total amino groups in the DB were determined by acid hydrolysis in 6 M HCl at 110°C, for 24 h. The DH was calculated by the following equation:

$$\%DH = [((NH_2)_{DBH}) - (NH_2)_{DB}/((NH_2)_{Total} - (NH_2)_{DB})] * 100$$

$(NH_2)_{DBH}$: Content of free amino groups in DBH

$(NH_2)_{DB}$: Content of free amino groups in DB

$(NH_2)_{Total}$: Content of free amino groups in acid hydrolyzed DB

3.4. **DEGREE OF GLYCATION (DG)**

The OPA method as described above (section 3.3) was used to determine contents of free amino groups (FAG) of the Alcalase hydrolysate, Chymotrypsin hydrolysate and their AGEs (Charoenphun et al., 2013; Nielsen et al., 2001). The degree of glycation (DG) was calculated according the following equation:

$$\%DG = \frac{FAG\ of\ hydrolysates - FAG\ of\ AGEs}{FAG\ of\ hydrolysates} * 100$$

3.5. **ANALYSIS OF AMINO ACID COMPOSITION**

The amino acid profile of the DB and DBH were determined according the method of Bidlingmeyer (Bidlingmeyer, Cohen, & Tarvin, 1984), using an HPLC system to analyze amino acid composition samples were hydrolyzed with 6 M HCl. The contents of cysteine and methionine are measured after finishing oxidation (Gehrke et al., 1985). The tryptophan content is determined after alkaline hydrolysis according to the method of Landry (Landry & Delhaye, 1992).

3.6. **ESTIMATION OF MOLECULAR WEIGHT DISTRIBUTION**

Molecular weight distribution of DBH was determined based on the method of He et al. (2013), using an AKTA FPLC system (GE Healthcare, Montreal, PQ) equipped with a Superdex Peptide12 10/300 GL column 154 (10 x 300 mm), and UV detector (λ = 214 nm). The column was calibrated using the standard proteins and an amino acid: cytochrome C (12,384 Da); aprotinin (6,512 Da); vitamin, 855 Da); and glycine (75 Da). A 100 µl aliquot of the 5 mg/ml DBH samples (dissolved in 50 mM phosphate buffer, pH 7.0 containing 0.15 M NaCl) were loaded onto the column and elution performed at room temperature using the phosphate buffer at a flow rate of 0.5 ml/min. The molecular weights (MW) of peptide in samples were estimated from a linear plot of log MW versus elution volume of standards.

3.7. ESTIMATION OF BITTER SCORES BY ELECTRONIC-TONGUE

3.7.1 PREPARATION OF MATERIALS

Diagnostic solutions including Hydrochloride (0.1 M HCl), sodium chloride (0.1 M NaOH) and monosodium glutamate (0.1 M MSG) as well as the calibration solution (1 M HCl) were purchased from Alpha M.O.S (Toulouse, France). Known bitter score substances such as acetaminophen, caffeine monohydrate, quinine hydrochloride (QHCl), leporamide hydrochloraide and femotidine were purchased from MP Biomedicals (Solon, OH, USA). Each DBH was dissolved in distilled water to give 0.5, 1.0, 2.5, 5.0, and 10.0 mg/ml concentrations followed by filtration first through a 0.45 μm filter disc and then a 0.2 μm filter.

3.7.2 BITTERNESS EVALUATION BY ELECTRONIC TONGUE

Bitter scores of DBH were evaluated using the Astree II e-Tongue system (Alpha M.O.S., Toulouse, France). The Alpha-MOS electronic tongue instrument is shown in Fig. 3. This system is a completely automated taste analyzer equipped with seven sensors, BD, EB, JA, JG, KA, OA, and JE, based on the ChemFET technology (Chemical modified Field Effect Transistor) to analyze liquid samples (Alpha MOS, 2004). Firstly, 0.01 M HCl was used to condition and calibrate sensors and the reference electrode repeatedly until stable signals were obtained for all seven sensors with minimal or no noise and drift. Secondly, diagnostic procedure was performed repeatedly, using 0.1 M HCl, NaOH, and MSG to ensure the sensors can identify distinctive tastes, until the discrimination index achieved at least 0.94 on a principle component analysis (PCA) map. After that, the bitter scores of a serial different concentration of enzymatic hydrolysates (0.5 mg/ml, 1mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/ml) were detected to get the threshold of the e-Tongue sensors to an appropriate range of protein enzymatic hydrolysates concentrations. And the bitter scores projected using the bitterness standard PLS model of the instrument. The PLS bitterness standard model was constructed from several bitter taste compounds with known bitter taste scores (Table 4) as determined from human panelists. 5 mg/ml was selected as the maximum strength to evaluate the bitterness of BPH. Finally, protein hydrolysates at 5 mg/ml were used to evaluate their bitter taste.

Table 4 Bitter taste compounds with known bitter scores tested by sensory analysis panel (SAP), the values provided by Alpha M.O.S.

Reference Compounds	**Used to build bitterness standard model**	**Used to validate bitterness standard model**	**Concentration (mM)**	**Published values**
Caffeine	√		0.24 2.36	2.5 8.5
Quinine	√		0.03 0.12	9 15.5
Prednisolone	√		0.44 0.88	13.5 17
Paracetamol	√		3.31 19.85	4 11
Loperamide		√	0.002 0.01	7.5 14
Famotidine		√	0.06 0.15	4.2 9

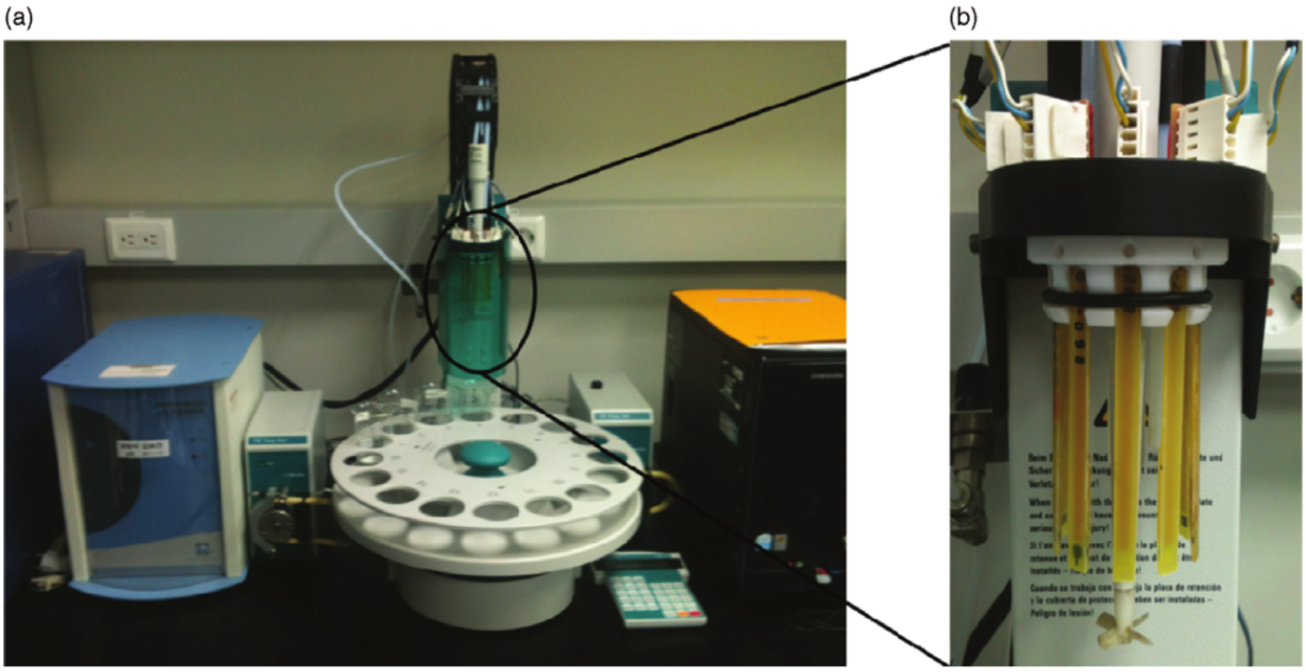


Fig. 3. the overview of Alpha-Mos sensory evaluation system

3.8. DETERMINIATION OF CALCIUM MOBILIZATION

Determination of the activating or potential bitter taste blocking activity of DBH was carried out by measuring intra-cellular Ca^{2+} mobilization using Fluo-4 NW calcium assay kit. Stable transfected HEK293 cells expressing T2R4 and Ga16/44 and HEK293 only expressing G-alpha 16/44 were used as experimental and control group, respectively. A viable cell count was taken after 6–8 h of transfection, and each well in the 96-well clear bottom black-walled plates contained 1×10^5 cells, which were then incubated at 37°C in a CO_2 incubator for 16 h. After that, the culture medium was substituted with Fluo-4 NW dye (lyophilized dye in 10 ml of assay buffer and 100 ul 2.5 mM probenecid added to prevent dye leakage from cytosol) for 40 min at 37°C CO_2 incubator and following 30 mins incubation at room temperature. Calcium mobilization was measured after addition of DBH on T2R4 agonists and DBH in terms of relative fluorescence units (RFUs) using a Flexstation-3 microplate reader (Molecular Devices, CA, USA) at 525 nm, following 494 nm excitation.

3.9. SEPARATION OF DBH BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC)

3.9.1 1st ROUND OF SEPARATION OF AH AND CH BY HPLC

Purification of AH and CH was conducted on an RP-HPLC system (Varian 940-LC) fitted with a Phenomenex C12 preparative column (21 x 250 mm) according to the method of Girgih et al (2015). Briefly, freeze-dried AH and CH was dissolved in double distilled water that contained 0.1% trifluoroacetic acid (TFA) as buffer A to give 200 mg/ml. After sequential filtration through 0.45 μm and 0.2 μm filters, 4 ml of the DBH solution was injected onto the C12 preparative column (Phenomenex Inc., Torrance, CA, USA). Fractions were eluted from the column at a flow rate of 10 ml/min using a linear gradient of 0–100% buffer B (methanol containing 0.1 % TFA) over 60 min. Peptide elution was monitored at 214 nm absorbance**.** Eluted peptides were collected using an automated fraction collector every 1 min and pooled into four fractions according to elution time. In this study, according to the distribution of peaks of chromatograms, 4 fractions were collected from AH and CH. The collecting time for each fraction was shown in Table 5 and Table 6. The solvent in the pooled fractions was evaporated

using a vacuum rotary evaporator maintained at a temperature range between 35 and 45°C and thereafter the aqueous residue was freeze-dried.

Table 5 Collecting time (minutes) of 1st round separation of AH

Fraction	Time	Time duration
1	5.6	9.4
2	14.6	8.4
3	23.6	10
4	33	8.2
	41.3	0ff

Table 6 Collecting time (minutes) of 1st round separation of CH

Fraction	Time	Time duration
1	5.6	7
2	12.6	9.2
3	21.8	9.8
4	31.6	11.4
	43	0ff

3.9.2 2nd ROUND OF SEPARATION OF AH-FRACTION1 (AH-F1) AND CH-FRACTION 4 (CH-F4) BY HPLC

The same RP-HPLC system fitted with a Phenomenex C12 preparative column (21 x 250 mm) as mentioned above (section 3.6.3) was used to conduct the 2nd round purification of AH-fraction 1 (AH-F1) and CH-fraction 4 (CH-F4)**.** Freeze-dried AH-F1 and CH-F4 were dissolved in buffer A (double distilled water with 0.1% trifluoroacetic acid) to 4 mg/ml and filtered through 0.45 μm and 0.2 μm filters. 4 ml of the DBH solution was injected onto the C12 preparative column (Phenomenex Inc., Torrance, CA, USA). Fractions were eluted from the column at a flow rate of 10 ml/min using a nonlinear gradient of 0–100% buffer B (methanol containing 0.1 % TFA) over 49 min (Table 7). Peptide elution was monitored at 214 nm absorbance**.** Eluted peptides were collected using an automated fraction collector every 1 min and pooled into four fractions according to elution time. The collecting time for each fraction was shown in Table 8 and Table 9.

Table 7 Nonlinear gradient of buffer A and buffer B in 2nd round RP-HPLC fractionation

Time (min)	Flow (ml/min)	% A	% B
Pre-run	10	100	0
7	10	95	5
14	10	90	10
21	10	85	15
28	10	80	20
35	10	75	25
42	10	70	30
49	10	65	35

Table 8 Collecting time duration of 2nd round separation of AH

Fraction	Time (min)	Time duration (min)
1	6.8	1.2
2	8.4	0.8
3	12	1.6
4	18	1.8
	19.8	0ff

Table 9 Collecting time duration of 2nd round separation of CH

Fraction	Time (min)	Time duration (min)
1	20.4	1.6
2	22	0.8
3	22.8	0.8
4	23.6	1.6
5	25.2	3.2
6	28.4	4.6
7	33	1.8
8	34.8	40.2
	40.2	0ff

3.10. MAILLARD REACTION

The Maillard reaction was carried out based on the protocol reported by Chen et al. (2014). DBH at concentration of 1% and 3% (w/v) was mixed with 100, 200, 300, 400, and 500 mM D-glucose in phosphate buffer, pH 10. The mixtures were heated at 120^{o}C in an oven for 30 min followed by rapid cooling in ice water and then freeze dried. In order to extract water soluble advanced glycation end-products (AGEs), 2 g of dried AGEs was mixed with 50 ml water and 50 ml ethanol for 2 h at room temperature with continuous stirring. The mixture was then

centrifuged (3270 g at 4°C) for 30 min and the resulting supernatant was collected while the residue was used to repeat the extraction procedure. Finally, the supernatants were pooled and evaporated using the vacuum rotary evaporator maintained at a temperature range between 35 and 45°C. The concentrated supernatant was filtered through a 0.2 μm disc to remove insoluble materials and then freeze dried.

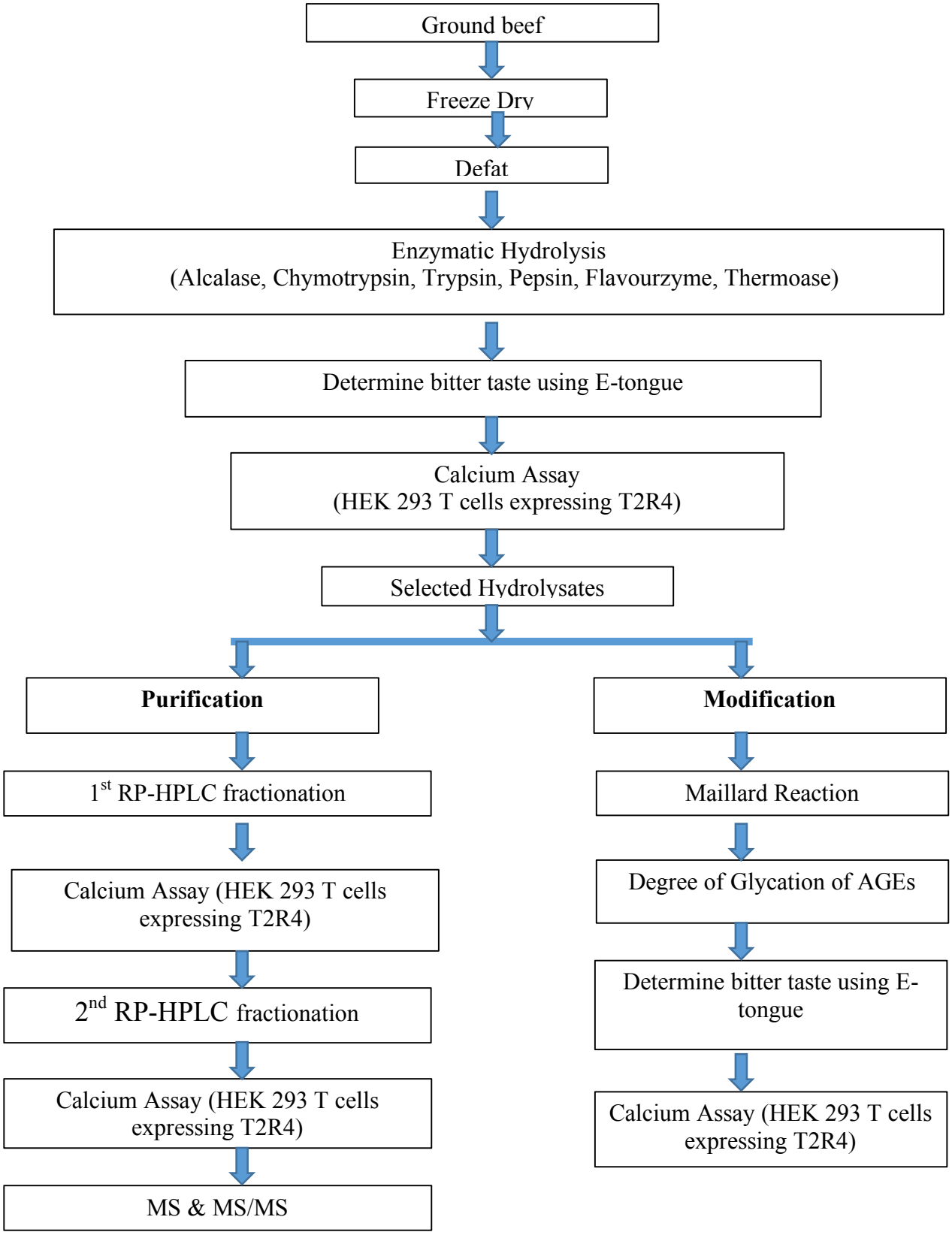
3.11. PEPTIDE IDENTIFICATION AND SEQUENCING

The most active HPLC peptide fractions against T2R activation (from cell culture) were characterized by tandem mass spectrometry. Briefly, a 10 ng/μL aliquot of the sample (dissolved in an aqueous solution of 0.1% formic acid) was infused into an Absciex QTRAP® 6500 mass spectrometer (Absciex Ltd., Foster City, CA, USA) coupled with an electrospray ionization (ESI) source. Operating conditions were 5.5 kV ion spray voltage at 200 ^{o}C, and 30 μL/min flow rate for 2 min in the positive ion mode with 2000 m/z scan maximum. MS/MS spectra were analyzed by the PEAKS Studio software (Bioinformatics Solutions, Waterloo, ON, Canada) to obtain peptide sequence.

3.12. MASS SPECTRA ANALYSIS OF AH, CH, AND THEIR AGEs

Alcalase hydrolysate (AH), Chymotrypsin hydrolysate (CH) and their respective 10 AGEs were characterized by mass spectrometry analysis to determine the major ions present. Briefly, a 10 ng/μL aliquot of the sample (dissolved in an aqueous solution of 0.1% formic acid) was infused into an Absciex QTRAP® 6500 mass spectrometer (Absciex Ltd., Foster City, CA, USA) coupled with an electrospray ionization (ESI) source. Operating conditions were 5.5 kV ion spray voltage at 200 ^{o}C, and 30 μL/min flow rate for 2 min in the positive ion mode with 2000 m/z scan maximum.

Fig. 5. Overview of methods used in this study



CHAPTER FOUR-RESULTS AND DISCUSSION

4.1. DEGREE OF HYDROLYSIS (DH)

The DH of beef protein hydrolysates are shown in Table 10. Raw lean beef contains about 73.1% moisture, 23.2% protein and 2.8% fat, thus after defatting and freeze-drying, the main nutrients remaining are proteins (myofibrillar, sarcoplasmic, stroma and granular) free amino acids and some soluble vitamins (Robert, 2012; Williams, 2007). Based on the same initial protein material, properties of peptides, including flavour have been attributed to enzyme type and DH (Maehashi et al., 1999; Zhao, Schieber, & Gänzle, 2016). As shown in Table 10, the DH for six the protein hydrolysates are all below 50%. The highest DH (47.02 %) was produced by Flavourzyme, which agrees with the fact that Flavourzyme is a complex mixture of endoprotease and exoprotease with at least eight active key enzymes (Del et al., 2007; Merz et al., 2015), suggesting a potent hydrolysis capability. 40% of DH of round scad protein hydrolyzed by flavourzyme was reported by Thiansilakul et al (2007). The lower DH are probably because of the much shorter hydrolyzing time (1 h) contrast to 4 hour hydrolyzing in this study. The second highest DH (35.57 %) is from Alcalase, which also has a broad substrate specificity (Guerard, Dufosse & Broise, 2001). Using Alcalase to hydrolyze protein has been commonly used, but the DH of alcalase varied significantly between them. For example, Thiansilakul et al (2007) reported 40 % of DH of Alcalase but 20 % of DH was showed in the study of Guerard et al (2001), even though their initial protein material both are fish, but the former one is scad and the second one is tuna, which means the DH is significantly influenced by protein substrates. Chymotrypsin and trypsin, both are extracted from the pancreas and have similar tertiary structures, but they have different substrate specificities (Hedstrom, Szilagyi, & Rutter, 1992). Chymotrypsin prefers large hydrophobic residue involving L-isomers of tyrosine, phenylalanine, and tryptophan, while trypsin preferentially cleaves peptides bonds at arginine and lysine residues, thus the DH are largely depend on the amino acid composition of protein substrates. In this study, chymotrypsin (25.83 %) showed slightly higher DH than that of trypsin (24.18 %) and this pattern is consistent with the results of casein and bonito, but opposite with the DH of soybean and chicken, and the DH of chicken and bonito were markedly higher than that of soybean (Maehashi et al., 1999b), suggesting that meat protein is more easier for chymotrypsin and trypsin to hydrolyze than plant protein. It was reported that thermoase was used to form

peptide bonds during a hydrolysis reaction (Trusek-Holownia, 2003), which may be responsible for the lower DH (18.26 ± 0.01%). Pepsin hydrolysate had the lowest DH (8.60 ± 0.02%), which is similar to the DH of soy protein isolates from 30 mins hydrolysis (7.9 %) (Meinlschmidt et al., 2016). The similar results probably from great different time duration are likely attributed to easy digestibility of beef protein (Williams, 2007). Besides, pepsin can be inhibited by phenylalanine-containing peptides, which are released during its activity (Eerlingen & Haesendonck, 1994; Knowles, Sharp, & Greenwell, 1969), which possibly results pepsin having lower DH. In summary, DH is greatly affected by protein substrates, hydrolyzing time duration, and other related conditions. And DH also based on the results, it can be assumed that Flavourzyme and Alcalase hydrolysates may have relatively smaller molecular weight peptides when compared to the other enzymatic four protein hydrolysates.

Table 10 Degree hydrolysis of six commercial enzymes of defatted beef

Enzyme Types	**Degree of Hydrolysis (%)**
Alcalase®	35.57 ± 0.01
Chymotrypsin®	25.83 ± 0.02
Trypsin®	24.18 ± 0.02
Pepsin®	8.60 ± 0.02
Flavourzyme®	47.02 ± 0.02
Thermoase®	18.26 ± 0.01

4.2. DEGREE OF GLYCATION OF AGEs

The glycation degree of AH and CH AGEs are summarized in Table 11-12 respectively. Glycation is a common method used to modify protein, and the properties and characteristics of the resulting AGEs are closely related to the degree of glycation (Li et al., 2013; Song et al., 2013), which is influenced by many factors including temperature, time, water activity and reactants molar ratio (Garcia-Amezquita et al., 2014; Oh et al., 2013b). Previous studies reported that adding 500-1000 Da and 1000-5000 Da fractions of wheat gluten hydrolysates enhanced umami taste and improve the yield of AGEs (Y. Li et al., 2013; Ogasawara,

Katsumata, & Egi, 2006; Song et al., 2013; L. Sun & Zhuang, 2012). But cereals are rich in asparagines (Friedman, 2003), which produced acrylamide, known as a carcinogenic factor, during Maillard reaction (Vattem & Shetty, 2003). Thus, meat protein hydrolysate is a better choice to produce safer AGEs. Given the same saccharide and same reacting conditions, the difference of glycation degree could have been due to different peptides within the enzymatic hydrolysates. In this study, the CH had higher content of molecules in this 1000-5000 Da range, but showed significantly ($P<0.05$) lower glycation degree than that of AH with low molecular weight peptide at all concentrations, suggesting that in addition to peptide size, the types of amino acids of peptides are important in determining degree of glycation. Li et al (2013) reported that the content of lysine and arginine decreased significantly in AGEs, implying that peptides with more lysine and arginine may have higher glycation degree. In this case, AH and CH had no difference in content of these two amino acids. The degree of glycation was improved with the increase of the D-glucose concentration; thus the AGEs of produced with 0.5 M D-glucose had significant higher ($P<0.05$) glycation degree than the other AGEs from lower glucose concentrations. Thus reducing sugar comprised the pharmaceutical activity of therapeutic antibodies and proteins (Zhang et al., 2009), and even non-reducing disaccharide also resulted in Maillard reaction through hydrolysis to glucose and fructose (Townsend & DeLuca, 1988). The relationship between percentage of hydrolysates and glycation degree of the AGEs was not linear. For example, the 1% hydrolysate AGEs did not always have a lower glycation degree than those from 3%. Therefore, degree of glycation was more dependent on glucose concentration rather than protein hydrolysate concentration. Glycation is believed to be an important way to reduce bitter taste and generate aroma taste because attachment of sugars can dramatically reduce the surface hydrophobicity of peptides and increase solubility (Garcia-Amezquita et al., 2014; Li et al., 2016). Thus the AGEs with higher glycation degree may have a better taste and higher potentials to mask bitter taste. However, over decrease of surface hydrophobicity reduced emulsification activity and emulsification stability (Y. Li et al., 2013).

Table 11 Degree of Glycation of AH-AGEs

Alcalase hydrolysates	Con of D-glucose	Glycation Degree
1%	0.1M	46.67[a]

1%	0.2M	47.53[b]
1%	0.3M	54.15[e]
1%	0.4M	54.57[f]
1%	0.5M	56.45[g]
3%	0.1M	48.00[c]
3%	0.2M	48.29[c]
3%	0.3M	48.68[d]
3%	0.4M	54.88[f]
3%	0.5M	54.96[fg]

($P<0.05$)

Table 12 Degree of Glycation of CH-AGEs

Chymotrypsin hydrolysates	Con of D-glucose	Glycation Degree
1%	0.1M	29.53[a]
1%	0.2M	29.11[a]
1%	0.3M	30.13[b]
1%	0.4M	36.67[d]
1%	0.5M	37.14[e]
3%	0.1M	29.49[a]
3%	0.2M	28.86[a]
3%	0.3M	36.63[cd]
3%	0.4M	36.40[c]
3%	0.5M	38.60[f]

($P<0.05$)

4.3. MOLECULAR WEIGHT DISTRIBUTION

Peptide distributions of the six enzymatic hydrolysates were determined by FPLC column according to their molecular size. High molecular weight (MW) peptides elute at shorter volumes, while low MW peptides elute with longer elution volumes. Four known compounds were used as MW standards to calibrate FPLC column and their chromatograms are shown in Fig. 5. The chromatograms for each of the six enzymatic hydrolysates are shown in Fig. 6-11 while a comparative chromatogram is shown in Fig. 12. The Alcalase hydrolysate (AH) chromatogram exhibited a main peak at 16.76 ml elution volume with an estimated molecular weight (CMW) of 445 Da, which indicates tetrapeptides since the mean MW of amino acids has been estimated to be 110 Da (Wu & Aluko, 2007). It was reported the molecular weight distribution of whey protein hydrolysates ranges from 300 to 1400 Da and the most of whey protein hydrolysates was under 1000 Da when the DH was 20.04 %. In this case, the DH of Alcalase is higher than 20.04%

(Li, Chuan, & Zheng, 2008), thus it is reasonable to get peptides with smaller MW. The main peak in the CH was observed at 14.67 ml elution volume, indicating most of the hydrolysates were less than 1800 Da. In addition there were two smaller peaks at 17.05 and 18.54 ml elution volumes with 367 and 136.98 Da estimated MW presenting in CH chromatogram. However, the 136.98 Da cannot be a peptide and is probably a free amino acid (Wu & Aluko, 2007). Most of TH were under 2800 Da, which is significantly higher than the MW of human-like collagen (500 – 1400 Da) (Wang et al., 2014) which is probably due to the higher DH (70.9 %) of collagen. And at the same study, the MW range of the collagen hydrolysates of pepsin was from 4000 – 6500 Da as the DH was 24.5 %. However, in this study, most PH were under 18200, even though the DH was lower than that of collagen, suggesting that beef protein is easier to hydrolyze to generate small peptides than collagen. For Flavourzyme hydrolysates (FH) , due to its extensive capability of hydrolysis, FH generally had a medium and small sized peptide with a broad distribution (Bamdad, Wu, & Chen, 2011; Berends et al., 2014). However, in this case, FH have a wide range of MW distribution from 3.17 KDa to 80.5 Da, which suggests that the hydrolysis of flavourzyme to beef protein was not complete. The TMH peptides were also mainly observed at short elution volumes, showing the MW range was from 132 KDa to 1.18 KDa, which is consistent with its low DH. In summary, the MW distribution results are generally in accord with DH; smaller DH are associated with bigger peptides and vice versa. Extensive studies have demonstrated that bitter taste of peptides is closely related to their molecular weights (Ishibashi, Kouge, & Shinoda, 1972; Kim & Li-Chan, 2006; Maehashi & Huang, 2009b). It was reported that the bitter intensity of peptides increased as amount of amino acids increased, but there was no significant difference in bitterness when the number of amino acids of peptides is more than seven (Otagiri, Nosho, Shinoda, Fukui, & Okai, 1985; M Tamura et al., 1990). Thus, the molecular weight of potential bitter taste peptides is around 110 Da to 800 Da. However, the average molecular weight of peptides isolated from the most bitter taste fractions of soybean proglycinin were lower than 1700 Da (Kim et al., 1999) while peptides with 2 - 12 amino acid residues in the 200 to 1400 Da molecular weight range , were obtained from soybean 11S glycinin (Kim, Kawamura, & Lee, 2003). Therefore, based on size, it can be assumed that Alcalase hydrolysate and chymotrypsin hydrolysate peptides have the most potential to interact with bitter taste receptors either as agonists or antagonists.

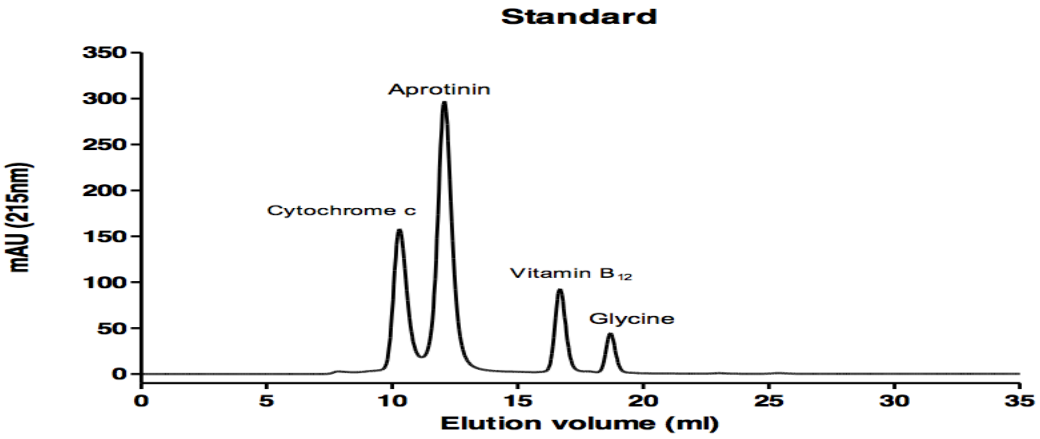


Fig. 5. MW distribution of standard substances

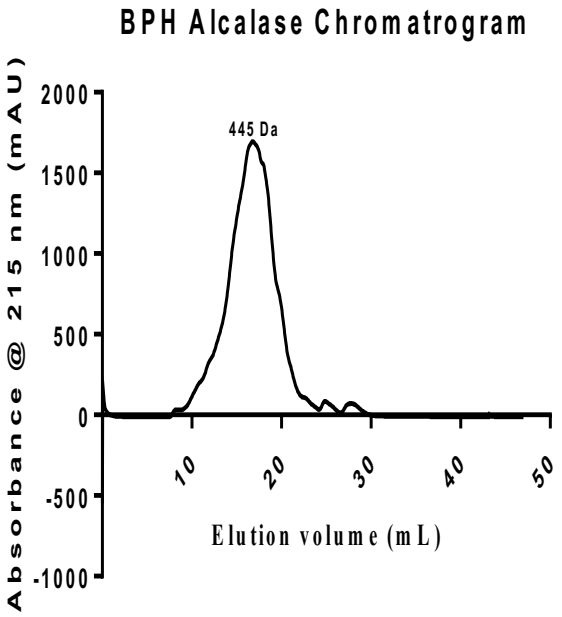


Fig. 6. MW distribution of AH

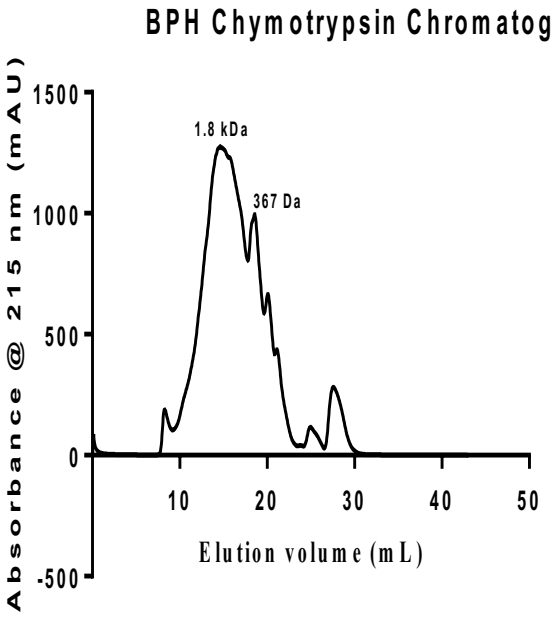


Fig. 7. MW distribution of CH

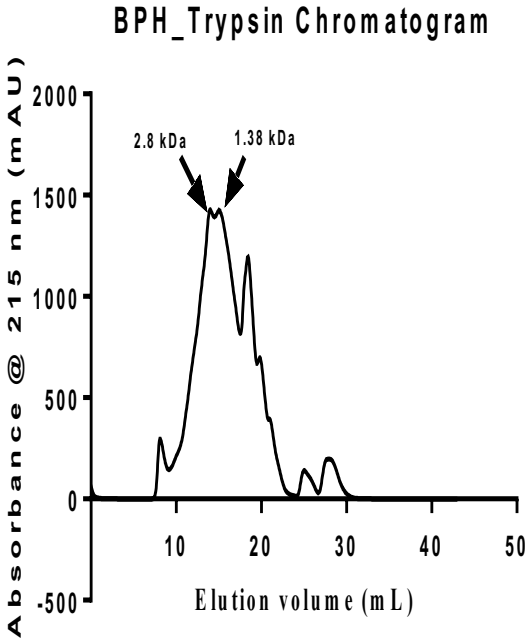


Fig. 8. MW distribution of TH

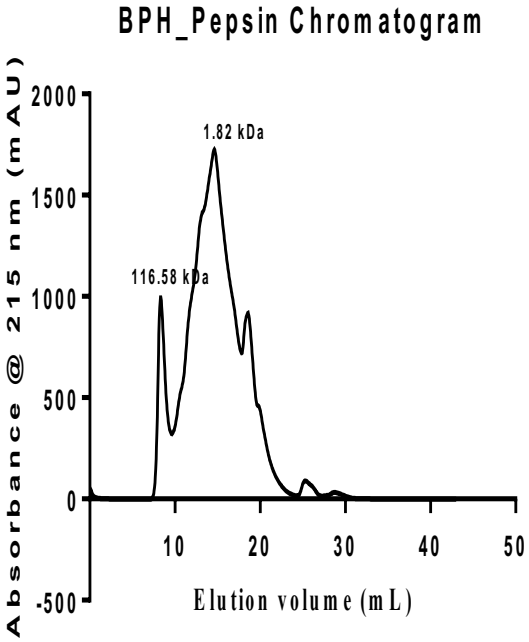


Fig. 9. MW distribution of PH

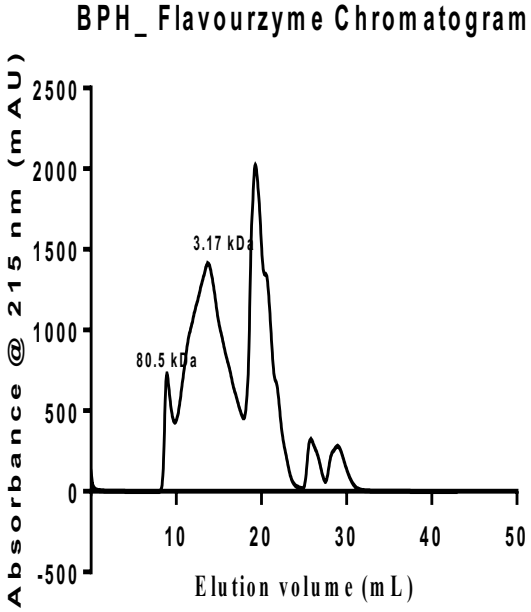


Fig. 10. MW distribution of FH

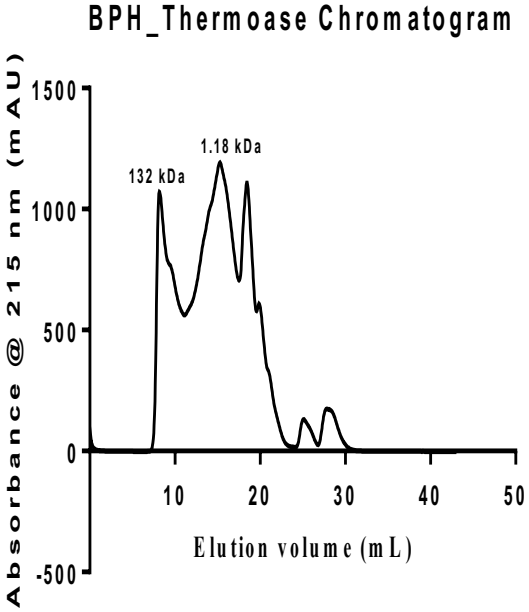


Fig. 11. MW distribution of TMH

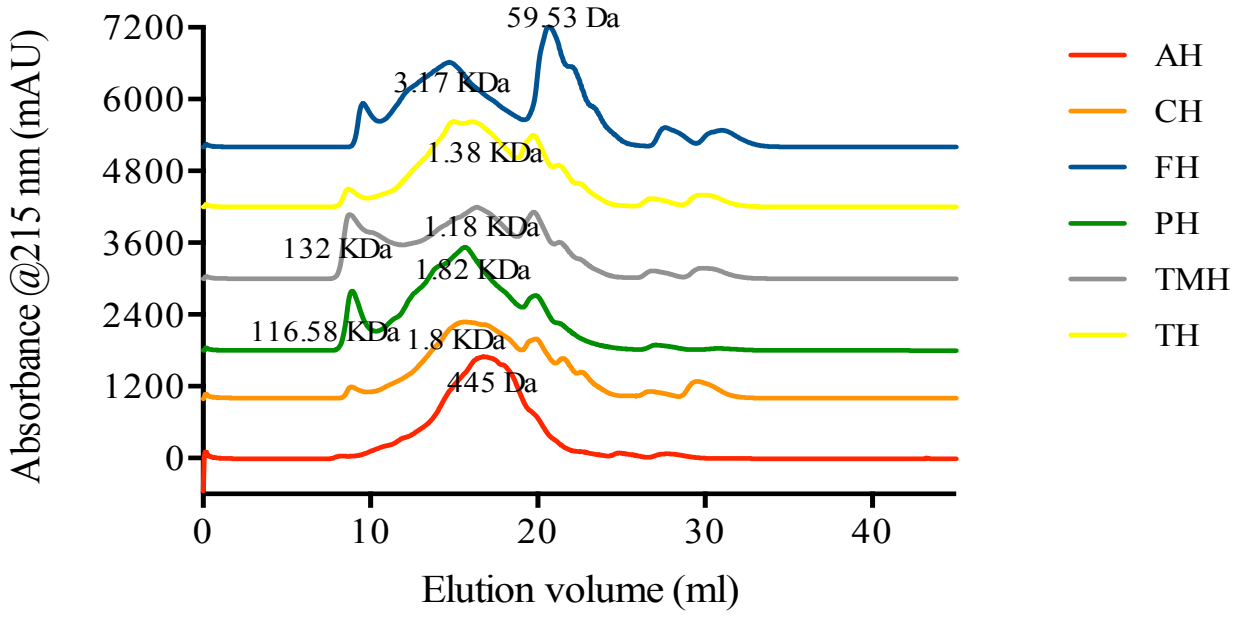


Fig. 12. Overall FPLC chromatogram of six enzymatic BPH

4.4. AMINO ACID COMPOSITION

Amino acid compositions of DB and DBH are shown in Table 13. Properties and functions of peptides are closely correlated with amino acid composition (BirchKemp, 1989; Udenigwe & Aluko, 2011), especially the flavor of hydrolysates (Nishimura & Kato, 1988; Zhao et al., 2016). This is because most amino acids have many basic taste but one primary or dominant taste; for example, glycine, alanine, threonine, proline, serine, glutamine are recognized as sweet taste amino acids, while phenylalanine, tyrosine, arginine, leucine, isoleucine, valine, methionine, and histidine are bitter but glutamate and aspartic acid have umami taste (Birch; Kemp, 1989; Kurihara, 2009; Solms, 1969). In this study, the umami taste amino acids, Glx, are the most abundant amino acids in DB, which is consistent with previous study that Glx (17.28 %) was the major amino acid in beef protein, chicken and ostrich, the content of which were 17.28%, 16.48 % and 15.89 % respectively (Sales & Hayes, 1996). Glutamic acid and glutamine were reported to be the most abundant amino acid in alcalase hydrolysates and flavourzyme hydrolysates of yellow strip trevally, while glycine was the major amino acid in fish flesh (Klompong et al., 2009). However, in this study, the major amino acid remained were Glx and the content was not significantly changed by enzymatic hydrolysis, which is probably because of different protein

sources. Another flavor-enhancing amino acid, Asx, the commonly known sweet amino acids, are the second predominant amino acids in DB and DBH, which also agrees with previous study that the Asx content of beef protein was 9.6 % (Sales & Hayes, 1996). In the case of Asx, their levels were significantly ($P<0.05$) increased in the enzymatic hydrolysates when compared to DB, suggesting the enzymatic hydrolysis possibly increases the sweetness of protein hydrolysates. However, a demonstrated bitter taste amino acid, Arg, was significantly ($P<0.05$) increased in the enzymatic hydrolysates. Besides, there were significant ($P<0.05$) reductions in the levels of some hydrophobic amino acids in the enzymatic hydrolysates ($P<0.05$), which suggests possible reduced bitter taste of peptides based on the Q value theory and multiple previous studies (Maehashi & Huang, 2009a; Ney, 1971; Shigenaga, 1988). Previous research indicated that compositions of protein hydrolysates from protein were based on the types of enzyme used, because different enzymes have different specificity which affected the removal of hydrolyzed debris mainly collagen and stroma with special amino acid profiles (Shahidi & Han, 1995; Wu, Chen, & Shiau, 2003). Thus the supernatant after hydrolysis had slightly different amino acid composition. Actually, in this study, the amino acid composition of these six enzymatic hydrolysates showed no significant difference. Negatively charged amino acid (NCAA) and positively charged amino acid (PCAA) are not closely related with flavor. However, NCAA was proposed to enhance the radical scavenging ability of 2,2-diphenyl-1-picrylhydrazyl (DPPH), while positively charged amino acid (PCAA) had a negative influence on DPPH (Udenigwe & Aluko, 2011). In this study, enzymatic hydrolysis exerted no effect on the content of NCAA, but the level of PCAA was significantly ($P<0.05$) increased after hydrolysis. Aromatic amino acid (AAA) also have been described to have positive influence on radical scavenging activity due to their loose electrons and hydrogen atoms which can neutralize free radicals (Udenigwe & Aluko, 2011); enzymatic hydrolysis also significantly ($P<0.05$) enhanced their level. Sulphur-containing amino acids (SCAA) were proposed to promote ferric reducing but negatively affect superoxide scavenging ability (Udenigwe & Aluko, 2011), and their content was significantly lower in enzymatic hydrolyzed DBH. Branch chain amino acids (BCAA) have been added to sports drinks and even oral medicine at high concentrations, but their intense bitter taste makes them undesirable, while in this study, enzymatic hydrolysis process did not increase BCAA level. Therefore, it is possible that the enzymatic hydrolysates have lower bitter taste property in addition to possessing antioxidant activity.

Table 13 Amino Acid Composition of Defatted Beef Protein (DBF) and DBF Enzymatic-hydrolysates

AA	DBP	AH	CH	PH	TH	TMH	FH	Mean±SD	P-Value
Asx	9.98	10.41	10.25	9.99	10.19	10.48	10.46	10.23 ± 0.22	0.02
Thr	4.63	4.45	4.67	4.26	4.43	4.73	4.36	4.48 ± 0.18	0.11
Ser	4.27	4.21	4.32	4.13	4.07	4.41	4.35	4.25 ± 0.13	0.71
Glx	15.51	15.95	16.18	14.30	16.31	15.89	17.86	15.94 ± 0.90	0.23
Pro	4.79	5.07	4.28	5.58	4.74	4.44	4.18	4.72 ± 0.53	0.75
Gly	5.46	6.50	4.34	7.33	5.56	5.15	4.57	5.58 ± 1.15	0.82
Ala	6.01	6.32	5.87	6.31	6.09	5.98	6.51	6.18 ± 0.24	0.14
Cys	1.03	0.89	0.98	0.89	0.83	0.91	0.82	0.89 ± 0.06	0.002
Val	4.55	4.39	4.57	4.89	4.34	4.64	4.57	4.57 ± 0.20	0.84
Met	2.63	1.81	2.29	1.88	1.85	1.89	2.02	1.96 ± 0.18	0.001
Ile	4.21	3.91	4.24	4.30	3.97	4.35	4.18	4.16 ± 0.18	0.51
Leu	7.86	7.56	7.95	7.12	7.56	7.87	8.07	7.69 ± 0.35	0.28
Tyr	3.35	3.10	3.49	2.93	2.96	3.43	2.72	3.11 ± 0.30	0.10
Phe	4.22	3.92	4.31	4.15	3.77	4.27	3.82	4.04 ± 0.23	0.12
His	4.13	4.03	4.26	4.42	4.40	4.05	5.05	4.37 ± 0.37	0.18
Lys	8.85	8.98	9.30	8.75	9.66	9.20	7.73	8.94 ± 0.67	0.76
Arg	6.29	6.59	6.52	6.80	7.29	6.31	6.88	6.72 ± 0.37	0.04
Trp	1.02	0.67	0.88	0.75	0.66	0.92	0.59	0.75 ± 0.13	0.004
HAA	39.66	37.64	38.86	38.80	36.75	38.70	37.48	38.04 ± 0.87	0.01
PCAA	19.27	19.61	20.08	19.97	21.34	19.45	19.66	20.02 ± 0.69	0.05
NCAA	25.49	26.36	26.43	24.21	26.50	26.37	28.32	26.37 ± 1.30	0.16
AAA	8.59	7.69	8.68	7.83	7.38	8.62	7.14	7.90 ± 0.64	0.04
SCAA	3.66	2.70	3.27	2.76	2.68	*2.80*	2.84	2.84 ± 0.22	0.001
BCAA	16.62	15.86	16.76	16.31	15.87	16.85	16.81	16.41 ± 0.47	0.32

HAA-hydrophobic amino acids-alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine

PCAA- positively charged amino acids- histidine, lysine, and arginine

NCAA- negatively charged amino acids- ASX (asparagine +aspartic acid) and GLX (glutamine + glutamic acid)

AAA- aromatic amino acids- phenylalanine, tryptophan and tyrosine

SCAA- Sulphur-containing amino acids- cysteine and methionine

BCAA- Branch chain amino acids

4.5. PREDICTION OF BITTER SCORE FROM ELECTRONIC-TONGUE

Bitter scores of beef protein hydrolysates and their advanced glycation end-products were estimated by electronic tongue, the results of which are shown in Fig. 13-15. Sensory tests are commonly performed by sensory panelists through physical tasting of samples, however, this method has some disadvantages, such as low objectivity and reproducibility (Akitomi et al., 2013b; Choi, Kim, Nam, Lee, & Jeong, 2014). In order to solve these problems, electronic-tongues have been developed. The overview of this sensory evaluation system is shown in Fig 3. In this system, there is one Ag/AgCl electrode and seven sensors coated lipid/polymer membranes, which govern the sensitivity and selectivity of individual sensor by interacting with tastants and produce electric potential (Choi et al., 2014; Miyanaga et al., 2002; Technology & Field-effect-, n.d.). This system can not only detect bitter taste of samples, but also can determine the suppression ability of bitter taste modifiers, such as high potency sweeteners suppressing bitter taste of quinine hydrochloride, and acesulfame K and citric acid suppressing the bitter taste of epinephrine (Rachid, Simons, Rawas-Qalaji, & Simons, 2010; Wu et al., 2016). For example, E-tongue has been used to detect the bitter taste of BCAA and bitter taste suppression effect of L-arginine on BCAA (Akitomi et al., 2013b) and evaluate the masking efficacy of sweetening and/or flavoring Agents on the bitter taste of epinephrine (Rachid et al., 2010). Example of a successful bitter taste standard model is shown in Fig. 15. The straight line represents an ideal 100% correlation and the colored dots are the predicted electronic-tongue measurements in comparison with the actual in vivo sensory analysis panel measurements for four standards (caffeine, paracetamol, quinine, prednisolone); loperamide and famotidine were used to validate the bitter taste standard model. The correlation coefficient (R^2) is 0.97 in this model, indicating the sensor system can predict bitter taste to a similar extent the human sensory analysis panelists. Kang et al (2014) reported that the correlation coefficient of bitterness is lower than other four basic taste because compared to other taste, such as salty, the mechanism of sensing salty by human which is mediated by sodium ion flux through apical channel resembles the mechanism of E-tongue, however, E-tongue appears to have difficulty in sensing organic bitter taste substances, such as amino acids and peptides (Miyanaga et al., 2002; Rudnitskaya et al., 2010). However, prediction of bitter taste of dairy protein hydrolysates showed 0.94 R^2 value between response of E-tongue and sensory panel (Newman et al., 2014). This study aims to find high efficient bitter taste modifiers which are expected to be able to function at receptors level.

Considering the diversity and complexity of 25 human bitter taste receptors, the T2R4 whose chemical structure and binding sites of its agonist quinine have been extensively studied was selected as future target receptor; quinine and BCML were selected as bitter agonist and antagonist, respectively (Sai P Pydi et al., 2014). Predicted bitter score of beef protein hydrolysates are shown in Fig. 13. Here, all enzymatic hydrolysates (5 mg/ml) were significantly higher than that of BCML (59 nM, the IC_{50} to suppress bitter taste of 1 mM quinine), but significantly lower than that of quinine (1 mM, the EC_{50} of quinine for T2R4) (Sai P Pydi et al., 2014). Among the six beef hydrolysates, AH exhibited the lowest bitter taste score of ~ 8. Based on the acceptability of human for bitter taste, shown in Table 14, bitter score 8 means the sample exhibited slight taste. Besides, consistent with the previous study, BCML (59 nM) can significantly reduce the bitter taste of quinine (Sai P Pydi et al., 2014). The beef hydrolysates also showed suppressing effect on quinine bitter taste, but significantly lower than the capability of BCML. The weaker bitter taste-suppressing effect of the beef hydrolysates may be because they are a complex mixture of different peptides, some of which may not be active suppressors. Bitter scores of AH-AGEs and CH-AGEs were significantly ($P<0.05$) lower than that of quinine and within the acceptable range for human acceptance. For combination of AH-AGEs and quinine, AGEs of 0.3 M, 0.4 M, 0.5 M D-glucose had significantly ($P<0.05$) lower bitter scores than other AGEs, suggesting less bitter taste when AH peptides react with higher concentrations of reducing sugar. CH-AGEs showed significantly ($P<0.05$) stronger bitter taste suppression ability to quinine than AH-AGEs, even though themselves bitter scores resembled. Which is probably because CH had more sulfur containing amino acids, such as methionine and cysteine, which were reported to contribute meaty aromas (Oh et al., 2013c), but theses pleasant taste cannot been detected based on the mechanism of artificial sensor.

Table 14 Bitter Intensity Level with Corresponding Scores Used in Building the Bitterness Standard Model

Bitter Intensity Level	From	To
Taste not Detected	1	4.5
Slight taste	4.5	8.5
Acceptable	8.5	12.5
Limit acceptable	12.5	16.5
Not acceptable	16.5	20

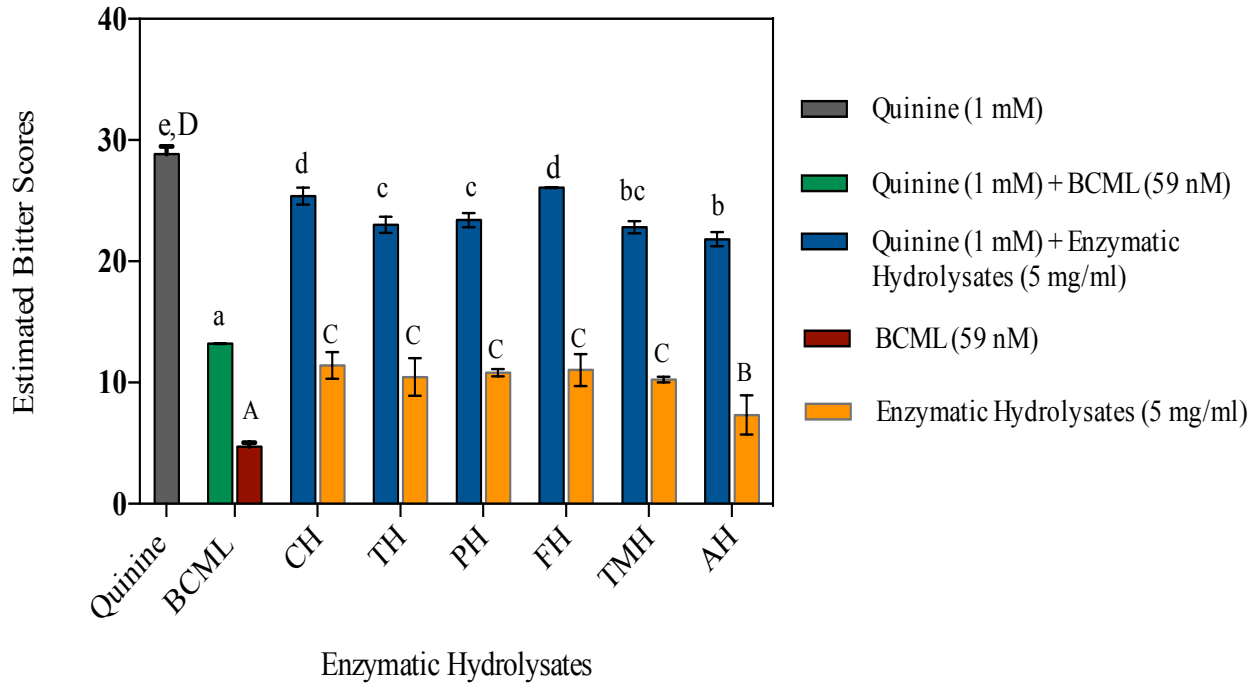
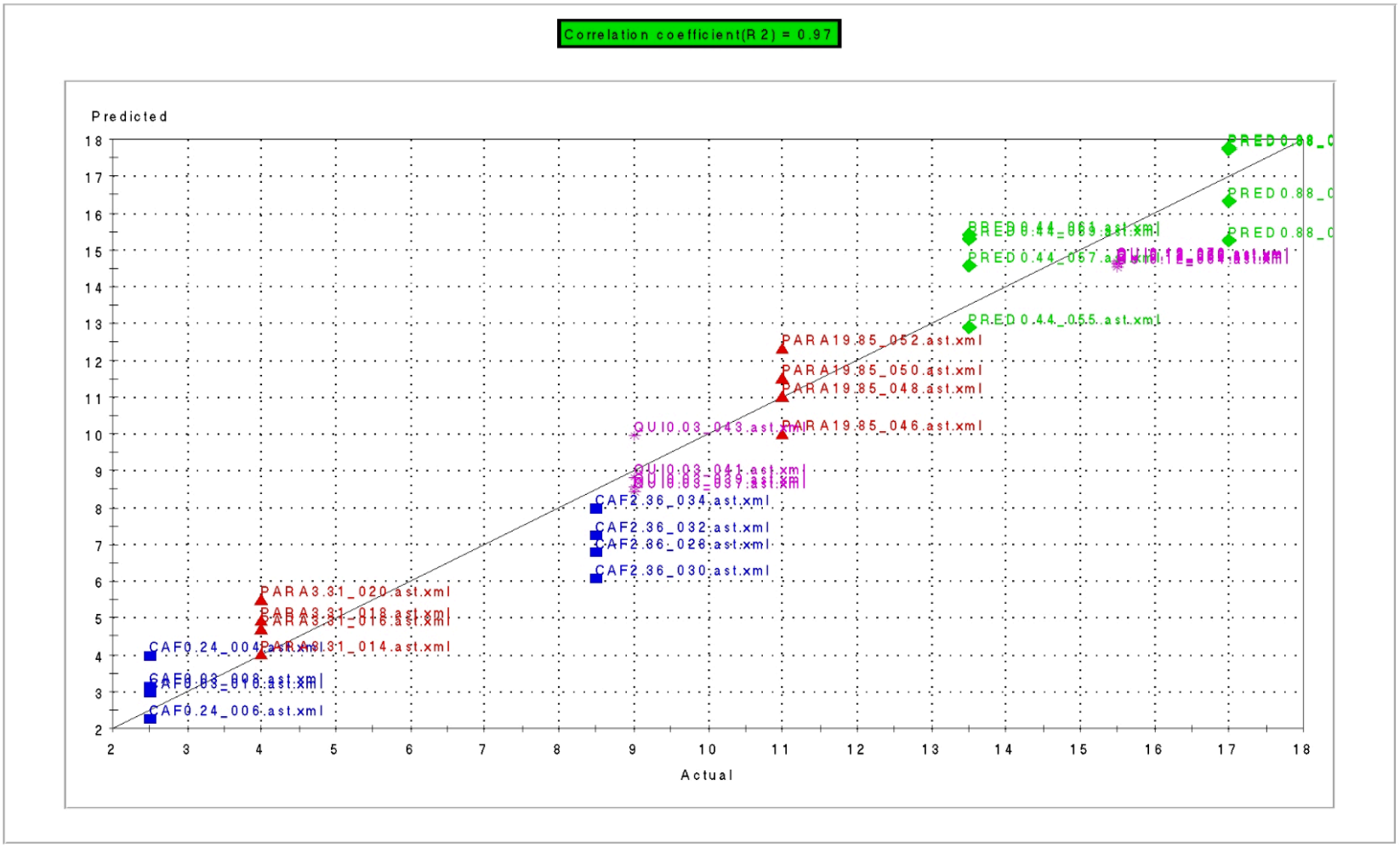


Fig. 13. Estimated bitter scores of AH, CH and their combination with quinine



Selected sensors : ZZ-63424-09008133-67A JE-56904-08007041-60B BB-63701-09008282-67A CA-58704-09007065-63A GA-60705-09007341-64A HA-60906-07007073-54F

Fig.16. Bitter taste standard model

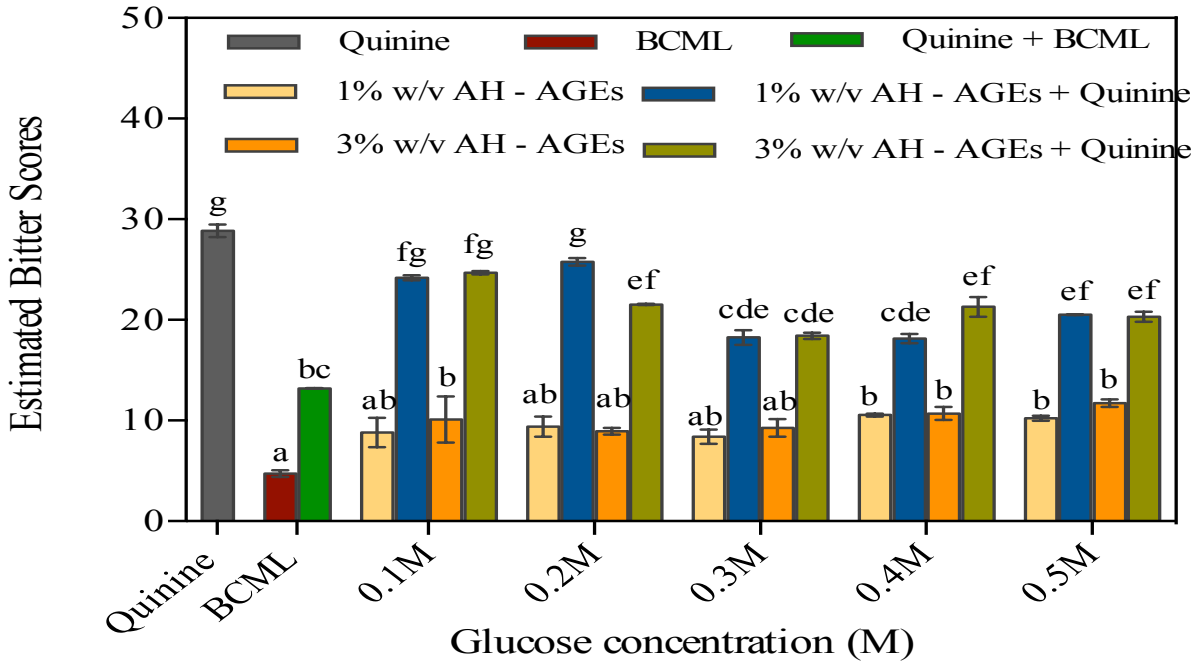


Fig. 14. Estimated bitter scores of AGEs of Alcalase hydrolysates

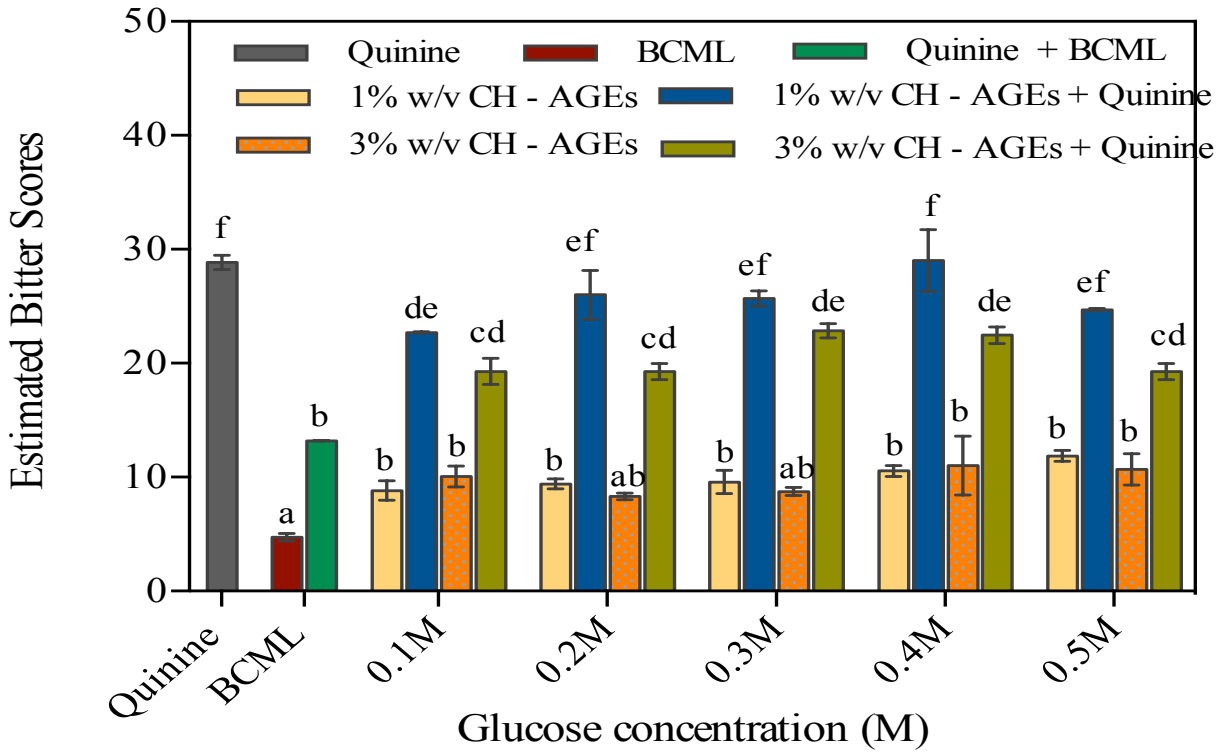


Fig. 15. Estimated bitter scores of AGEs of Chymotrypsin hydrolysates

4.6. DETERMINATION OF INHIBITORYABILITY AGAINST ACTIVITAION OF T2R4 BY QUININE

As mentioned in bitter taste sensation mechanism in chapter 2, the activation of T2Rs will stimulate calcium release from intracellular stores, hence calcium assay is used to identify bitter taste agonists and antagonists or modifiers of T2Rs (Article, 2009; Behrens & Meyerhof, 2006; Pydi et al., 2014). This is a cell based system coupled with intracellular calcium release as a reactant. HEK 293 cell heterologous expression system is a robust assay method to measure GPCR activation and wildly used for T2Rs and others human membrane proteins (Chakrabarti et al., 2015). Most of the T2R inhibitors including GABA, BCML, GIV3727, abscisic acid, probenecid and 6-Methoxyflavanones were discovered by the calcium assay and HEK 293 cell based system method (Greene et al., 2011; Sai P. Pydi et al., 2015; Sai P Pydi et al., 2014; Roland et al., 2014; Slack et al., 2010). In this study, HEK293T cells expressing T2R4-Ga16/44 were used as experiment group, HEK 293 T cells only expressing Ga16/44 were used as control group. Results of the calcium assay of beef protein hydrolysates are shown in Fig. 17. Here, calcium mobilization of quinine, CH and FH were similar, indicating that the two hydrolysates activated T2R4 in the manner as the known quinine agonist. But this result is different with some previous reports that indicated that adding flavourzyme improved the taste and aroma of Chinese sausage and wheat gluten hydrolysates (Feng et al., 2014; Koo et al., 2014), which is probably due to some big molecular weight hydrolysates existing in flavourzyme hydrolysates. TH, PH, TMH and AH had significantly lower calcium mobilization but significantly higher than that of buffer, which means they can also activate T2R4 but the potency was weaker than those of quinine, FH and CH. And Alcalase has been extensively reported to improve food flavour, including Chinese sausage, soy protein isolates, yellow tuna and lean beef (Feng et al., 2014; Gu et al., 2001; Meinlschmidt et al., 2016; O'Meara & Munro, 1985), but all these sensory test were performed by human panelists. The calcium assay results were not totally consistent with predicted bitter scores from electronic-tongue, which may be because the electronic-tongue mimics human tongue with 25 bitter taste receptors (Technology & Field-effect-, n.d.), but the calcium assay focuses only on T2R4. Moreover, the enzymatic hydrolysates are comprised of different peptides, some of which could have interacted with the polymer membranes of sensors of the electronic tongue in a manner different from interactions with T2R4. AH and CH were furtherly selected to be purified by RP-HPLC, because AH showed lower bitter taste from the

electronic-tongue and calcium assay data while CH was selected as a comparison group. Fig. 18 displayed the calcium assay results for fractions from the 1st round of RP-HPLC separation of AH and CH. Surprisingly, CH fractions showed significantly ($P<0.05$) lower calcium mobilization than that of quinine and even lower than some AH fractions. Fraction 1 and fraction 4 from AH and CH exhibited significantly ($P<0.05$) lower calcium mobilization, and their combination of quinine showed significantly ($P<0.05$) reduced calcium mobilization, which means that some compounds in fraction 1 and fraction 4 are potent suppressors of T2R4 activation by quinine. One possible mechanism responsible for this inhibitory function could be that the peptides bind to the receptor ligand-binding site(s), which subsequently prevented quinine binding or peptide-quinine interactions reduced quinine binding ability (Pronin et al., 2004; Sai P Pydi et al., 2014; Roland et al., 2014). It is also possible that peptide interactions with T2R4 at places other than the ligand-binding sites could have reduced binding efficiency of quinine to the sites or post-binding activation effectiveness was reduced. However, Fig. 19 showed that HEK293 T cell only with Ga16/44 had similar trend as HEK293 T cells with T2R4-Ga16/44, suggesting that fractions stimulated calcium release not only through activating T2R4 but also via other ways. Besides, samples also reduced the calcium mobilization of the control group, which supports the proposed mechanism that the peptides combined with quinine and blocked the interaction between quinine and receptors. In order to get more pure compounds to enhance inhibitory efficiency, AH fraction 1 and CH fraction 4 were selected to conduct 2nd RP-HPLC fractionation. For AH fraction 1, calcium mobilization of four fractions significantly ($P<0.05$) declined compared to 1st RP-HPLC fractions, and inhibitory potency to quinine significantly ($P<0.05$) increased. For CH fraction 4, there were eight fractions collected. Fractions 1, 3, 4 had significantly ($P<0.05$) lower calcium mobilization compared to other fractions or quinine and they also significantly ($P<0.05$) suppressed T2R4 activation by quinine. Fig. 20 and Fig. 21 were similar as Fig. 18 and Fig. 19, suggesting that peptides from the 2nd round of RP-HPLC fractions can stimulate calcium release by means other than interaction with receptors and may have functioned as bitter taste modulators by blocking the ability of quinine to interact with T2R4 receptors. Moreover, effects of AH-AGEs and CH-AGEs on T2R4 calcium release were also determined by calcium assay. The results showed that some AGEs such as A1 to A5 had significantly ($P<0.05$) lower released calcium when compared to AH. Among the AGEs, A1, A2 and A4 also significantly ($P<0.05$) reduced quinine-dependent calcium

mobilization. CH AGEs also significantly ($P<0.05$) decreased ability to stimulate calcium release. And C1, C2, C3, C4 significantly ($P<0.05$) suppressed the bitter taste of quinine.

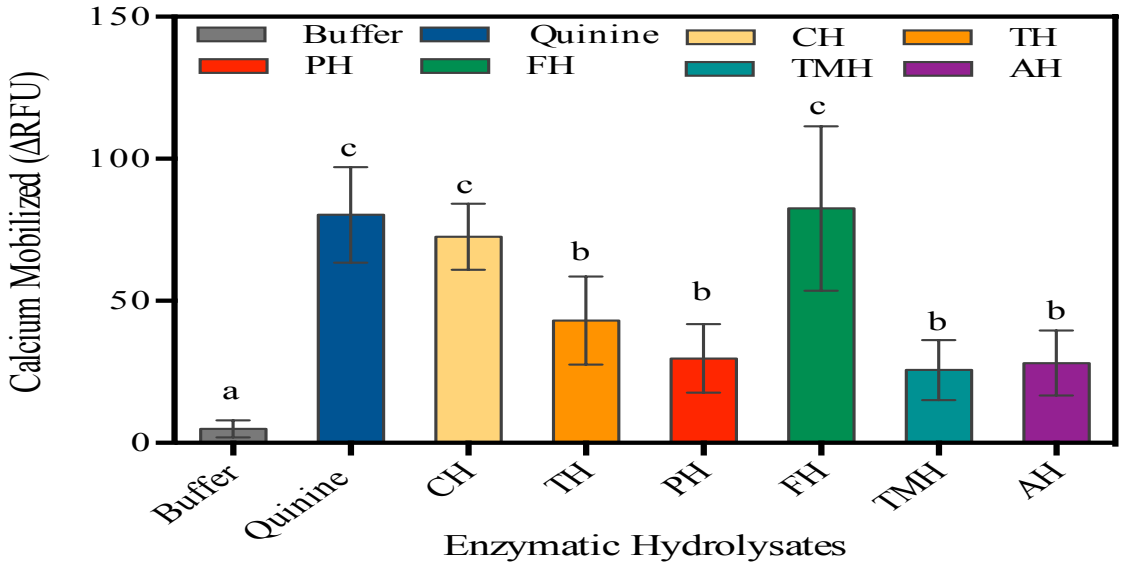


Fig. 17. Calcium mobilization of beef protein hydrolysates

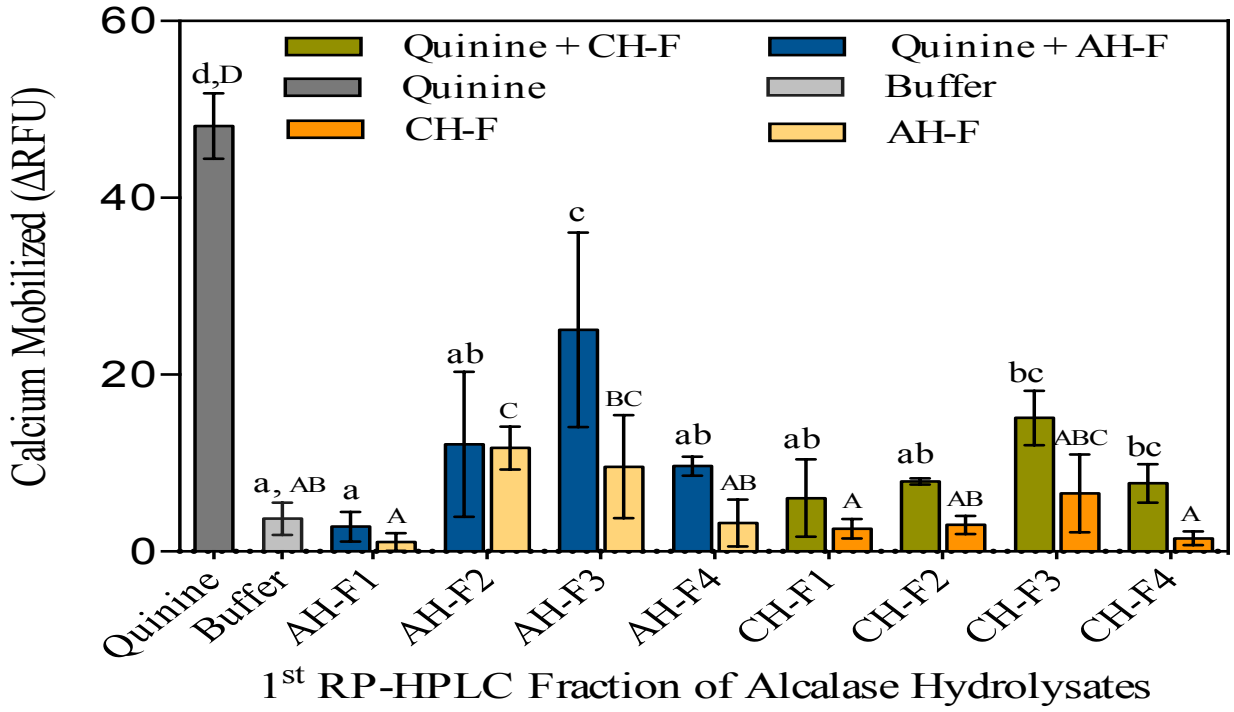


Fig. 18. Calcium mobilization of 1st RP-HPLC fractions of AH and CH

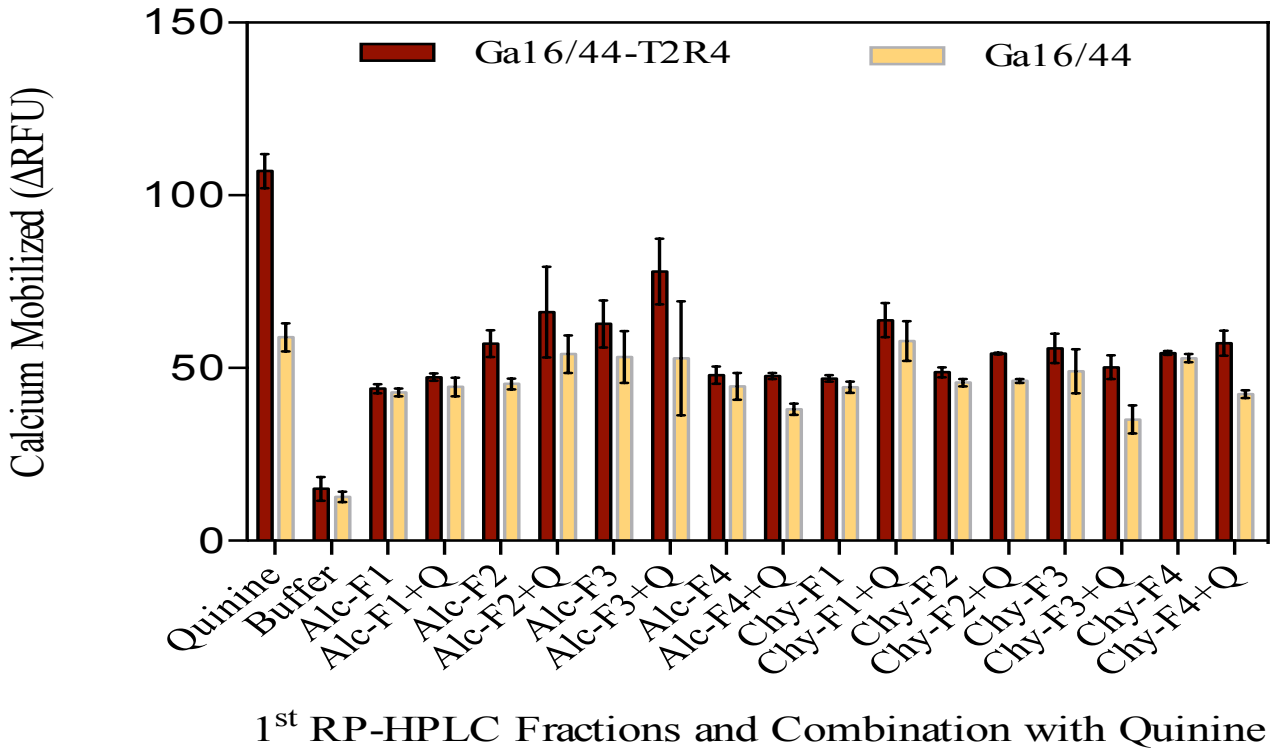


Fig. 19. Calcium mobilization of 1st RP-HPLC fractions of AH and CH on HEK 293 cells expressing T2R4 and Ga16/44 and HEK 293 cells expressing Ga16/44

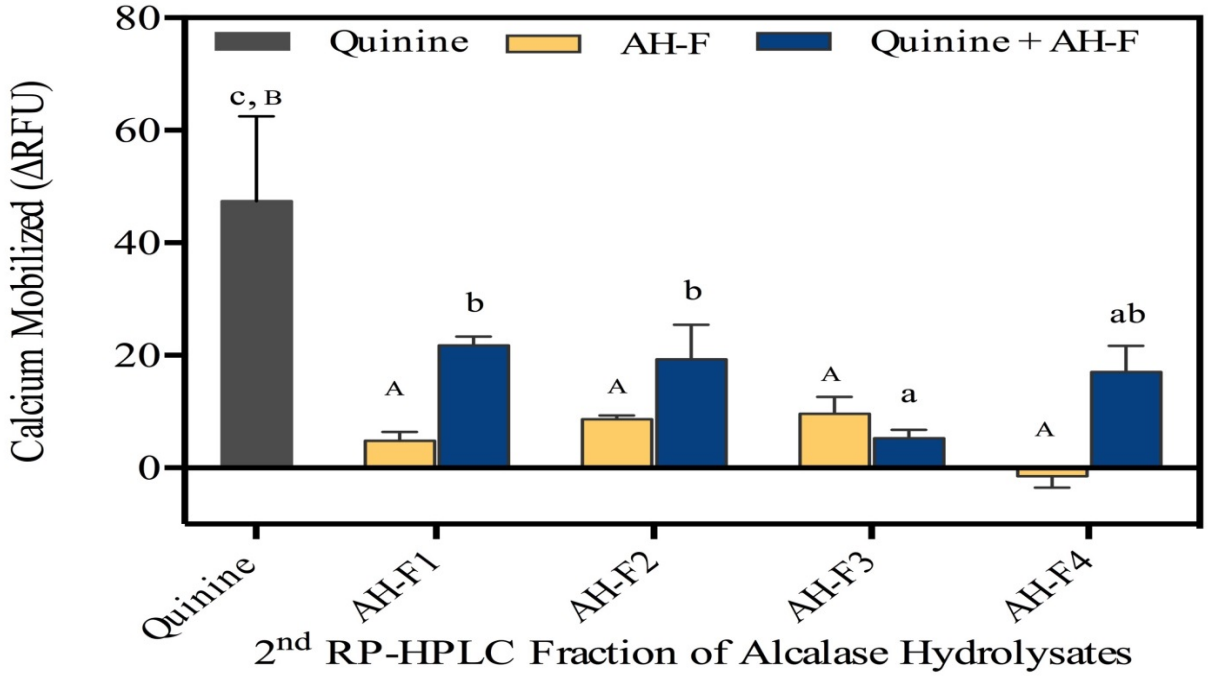


Fig. 20. Calcium mobilization of 2nd RP-HPLC fractions of AH-F1

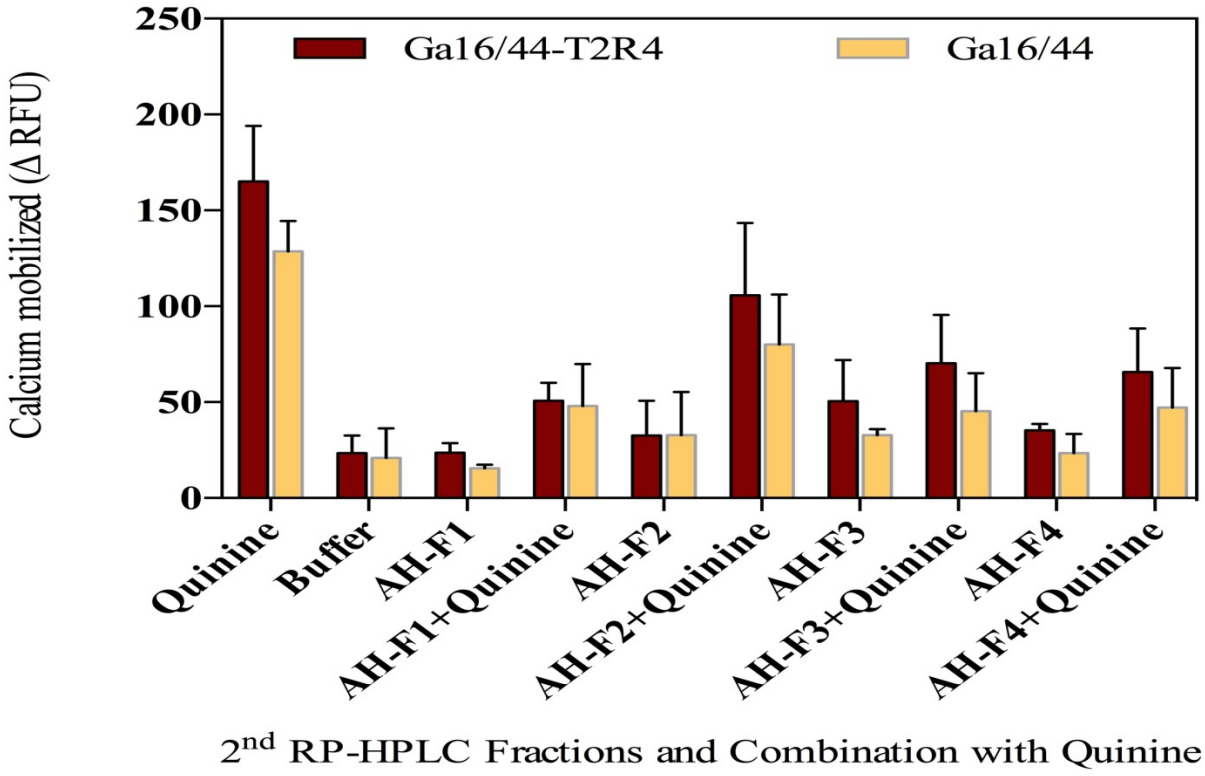


Fig. 21. Calcium mobilization of 2nd RP-HPLC fractions of AH-F1 on HEK 293 cells expressing T2R4 and Ga16/44 and HEK 293 cells expressing Ga16/44

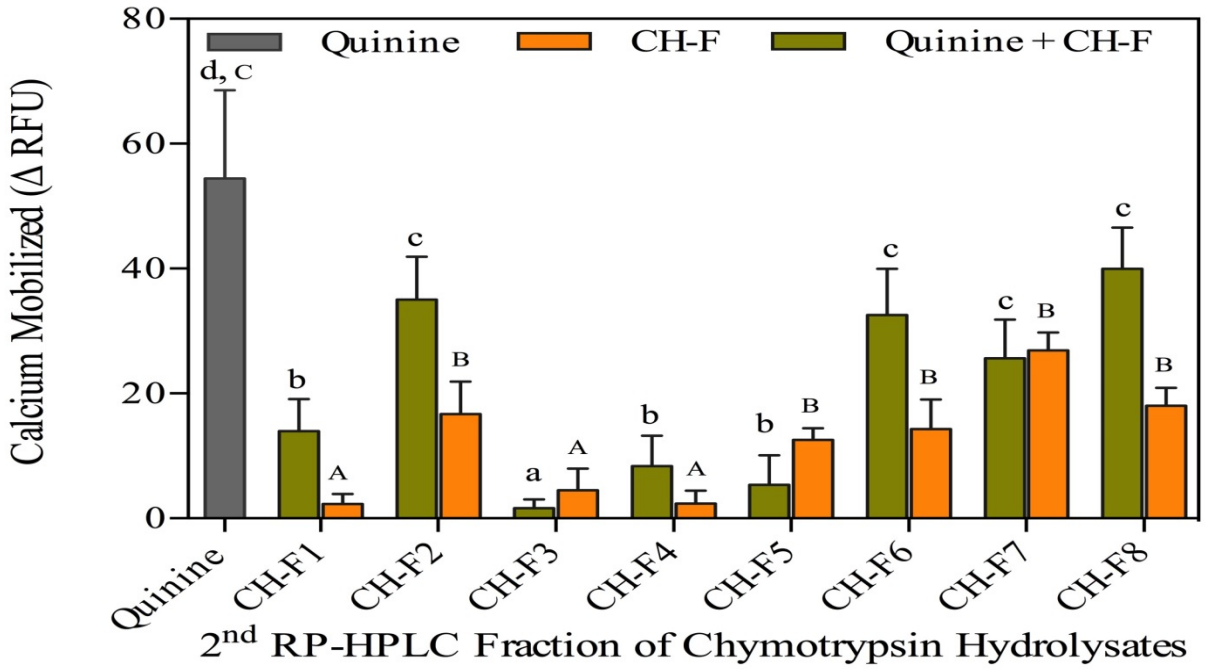


Fig. 22. Calcium mobilization of 2st RP-HPLC fractions of CH-F4

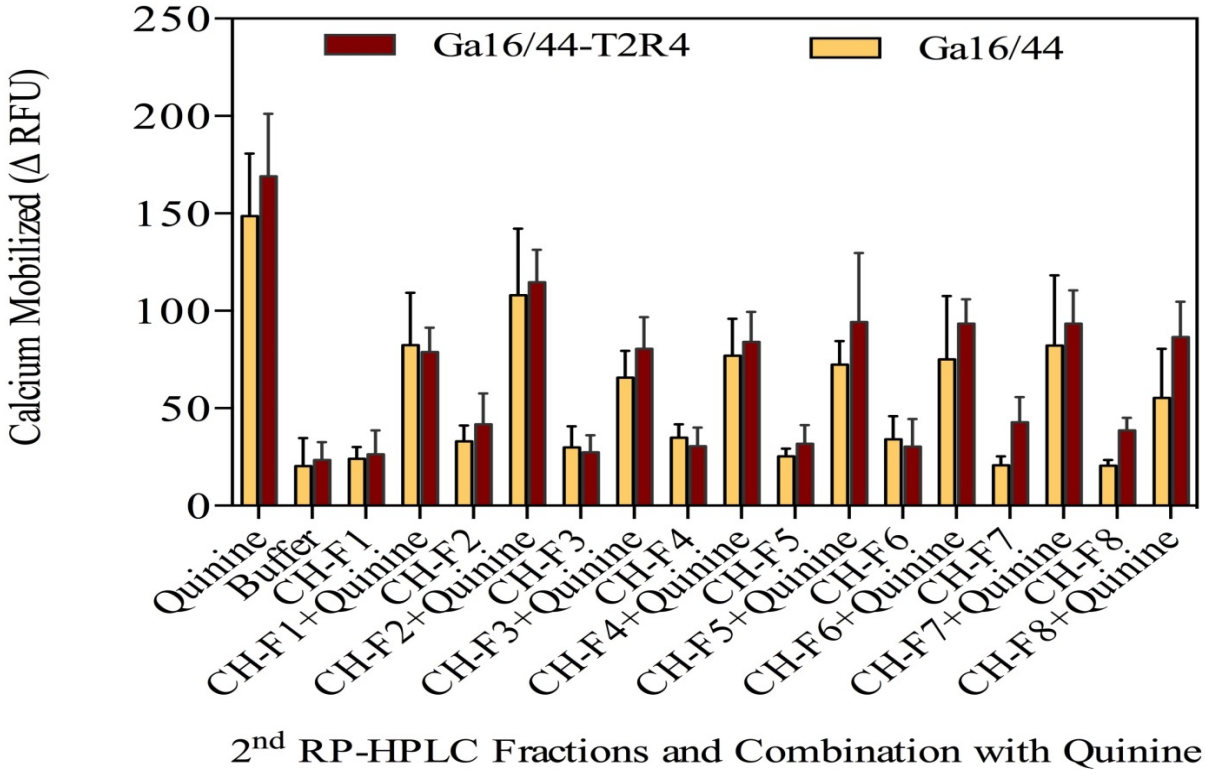


Fig. 23. Calcium mobilization of 1st RP-HPLC fractions of CH-F4 on HEK 293 cells expressing T2R4 and Ga16/44 and HEK 293 cells expressing Ga16/44

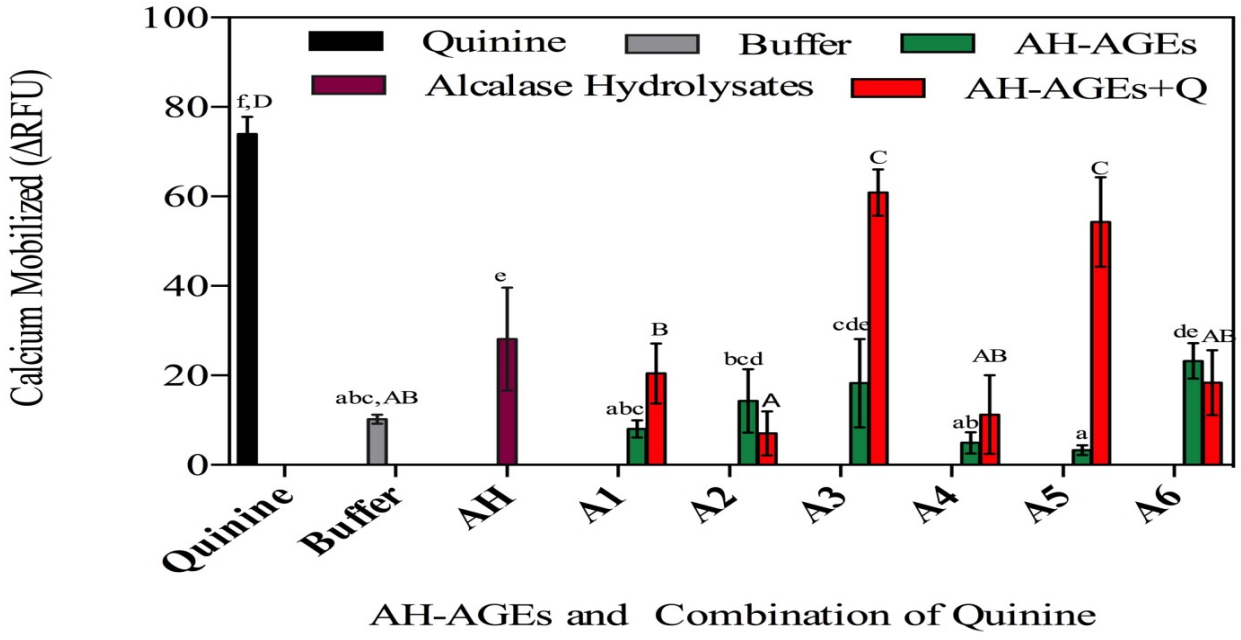


Fig. 24. Calcium mobilization of AH-AGEs

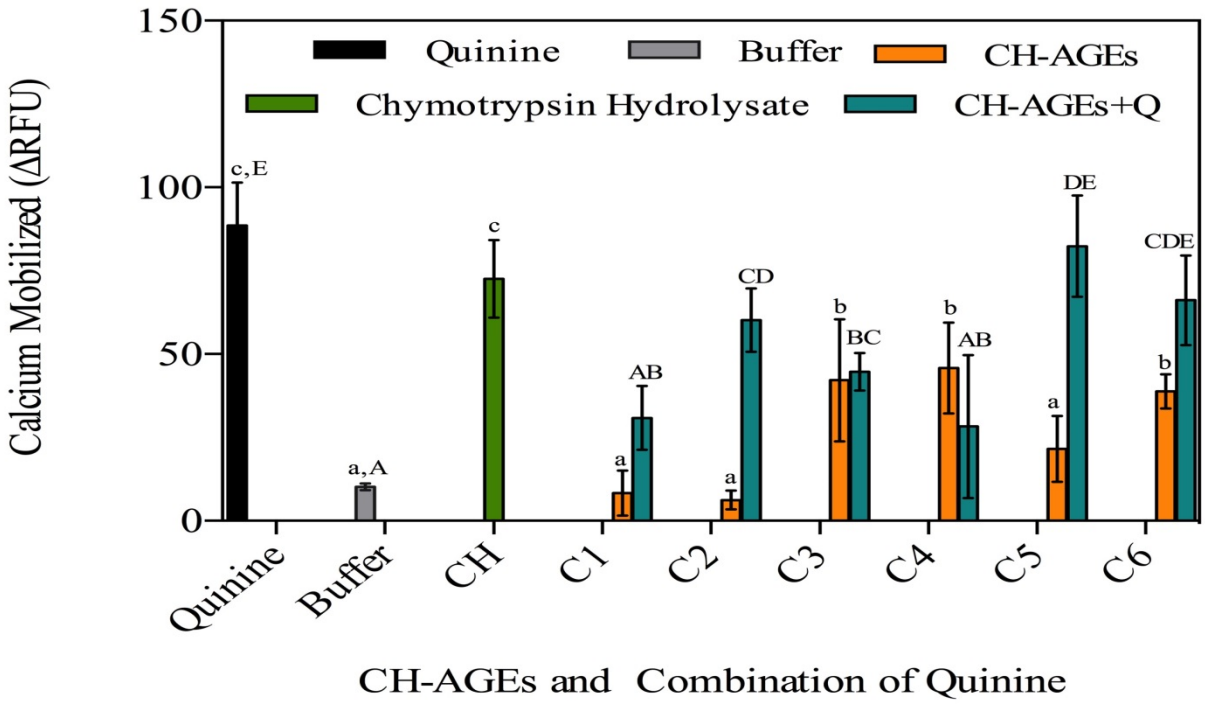


Fig. 25. Calcium mobilization of CH-AGEs

A1: AH-AGEs (1% AH, 0.3M D-glucose); A2: AH-AGEs (3% AH, 0.3M D-glucose)
A3: AH-AGEs (1% AH, 0.4M D-glucose); A4: AH-AGEs (3% AH, 0.4M D-glucose)
A5: AH-AGEs (1% AH, 0.5M D-glucose); A6: AH-AGEs (3% AH, 0.5M D-glucose)
C1: CH-AGEs (1% CH, 0.1M D-glucose); C2: CH-AGEs (3% CH, 0.1M D-glucose)
C3: CH-AGEs (3% CH, 0.2M D-glucose); C4: CH-AGEs (3% CH, 0.3M D-glucose)
C5: CH-AGEs (3% CH, 0.4M D-glucose); C6: CH-AGEs (3% CH, 0.5M D-glucose)

4.7. RP-HPLC PURIFICATION

Peptides are fractionated by RP-HPLC on the basis of their different hydrophobic properties. Peptides with high hydrophobicity were collected at longer elution times compared to more hydrophilic peptides (Aguilar, 2004; Boysen & Hearn, 2001), which implies that F1 is supposed to have the high content of least content of positively charged (PCAA) and negatively charged (NCAA) amino acids and low content of hydrophobic amino acids (HAA) and aromatic amino acids (AAA) (Girgih, Udenigwe, & Aluko, 2013). Thus fractionation increases the

concentration of peptides with similar amino acids. As shown in Fig. 26 and Fig. 27, AH and CH were pooled into four fractions (F1-F4), and F1 for both of them started eluting at 5.6 min, the same elution time was seen in hemp seed protein hydrolysates digested by pepsin and pancreatin (Girgih et al., 2013), suggesting they may have peptides with similar hydrophobicity. Previous study about pea seed and hemp seed showed that F4 and F5 had higher percentage of hydrophobic amino acids (valine, leucine and isoleucine) compared to F1-F3 (Pownall, Udenigwe, & Aluko, 2010). AH had more peptides eluted from 13 mins to 32 mins, while CH eluted more peptides from 32 mins to 43 min, which indicates that CH have more hydrophobic peptides compared to AH, which is consistent with the results of amino acid composition. Based on the Q rule theory mentioned before, hydrophobic peptides are more likely to have bitter taste; therefore, fraction 4 may have the strongest bitter taste among these fractions. However, fraction 1 and fraction 4 showed significantly ($P<0.05$) lower ability to activate T2R4 and significant ($P<0.05$) higher masking ability to reduce the bitter taste of quinine, which suggests that there are more factors affecting the bitterness property of peptides apart from hydrophobicity, such as structure and size of peptides (H.-O. Kim & Li-Chan, 2006; K. Maehashi & Huang, 2009a). Fraction 1 of AH and F4 of CH were selected to perform 2nd RP-HPLC to continually get more purified fractions. As shown in Fig. 28 and Fig. 29, the 2nd RP-HPLC chromatograms of fraction 1 and fraction 4 were like horizontally expanded chromatograms of these fractions from the 1st RP-HPLC separation. Four fractions and eight fractions were selected from AH fraction 1 and CH fraction 4, respectively. And the selected fractions of AH fraction 1 were purer compared to that of fraction 4 of CH because the individual fraction had only one peak while some fractions of CH had more than one peak. The determination of the bitter taste suppression ability of these 12 fractions showed that AH-F3 and CH-F1, CH-F3, CH-F4 and CH-F5 significantly inhibited the activation of T2R4 by quinine (Fig. 21 and Fig. 22), indicating the inhibitory ability is not only dependent on polarity.

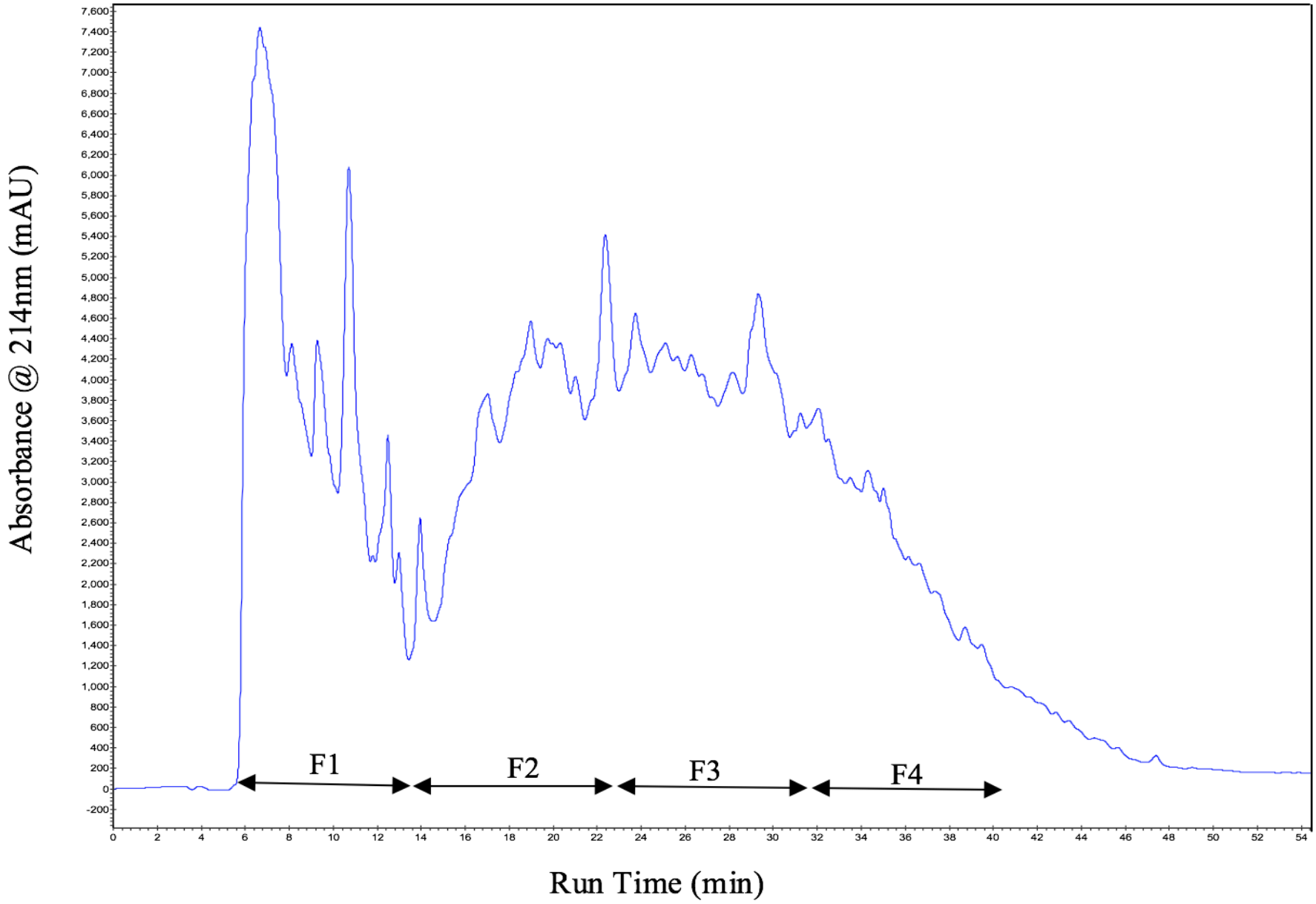


Fig. 26. 1st RP-HPLC Chromatogram of Alcalase hydrolysates

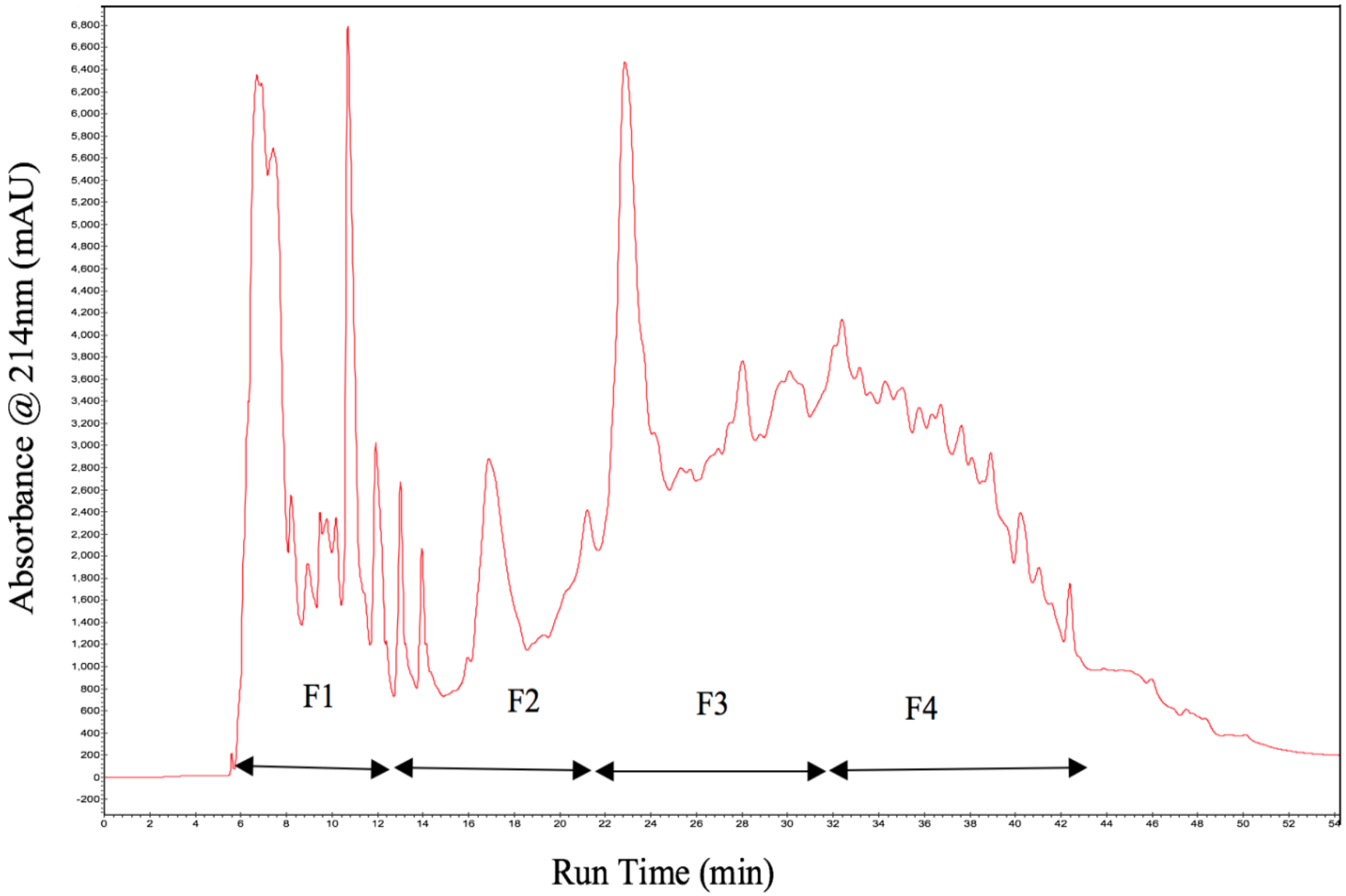


Fig. 27. 1st RP-HPLC chromatogram of Chymotrypsin hydrolysates

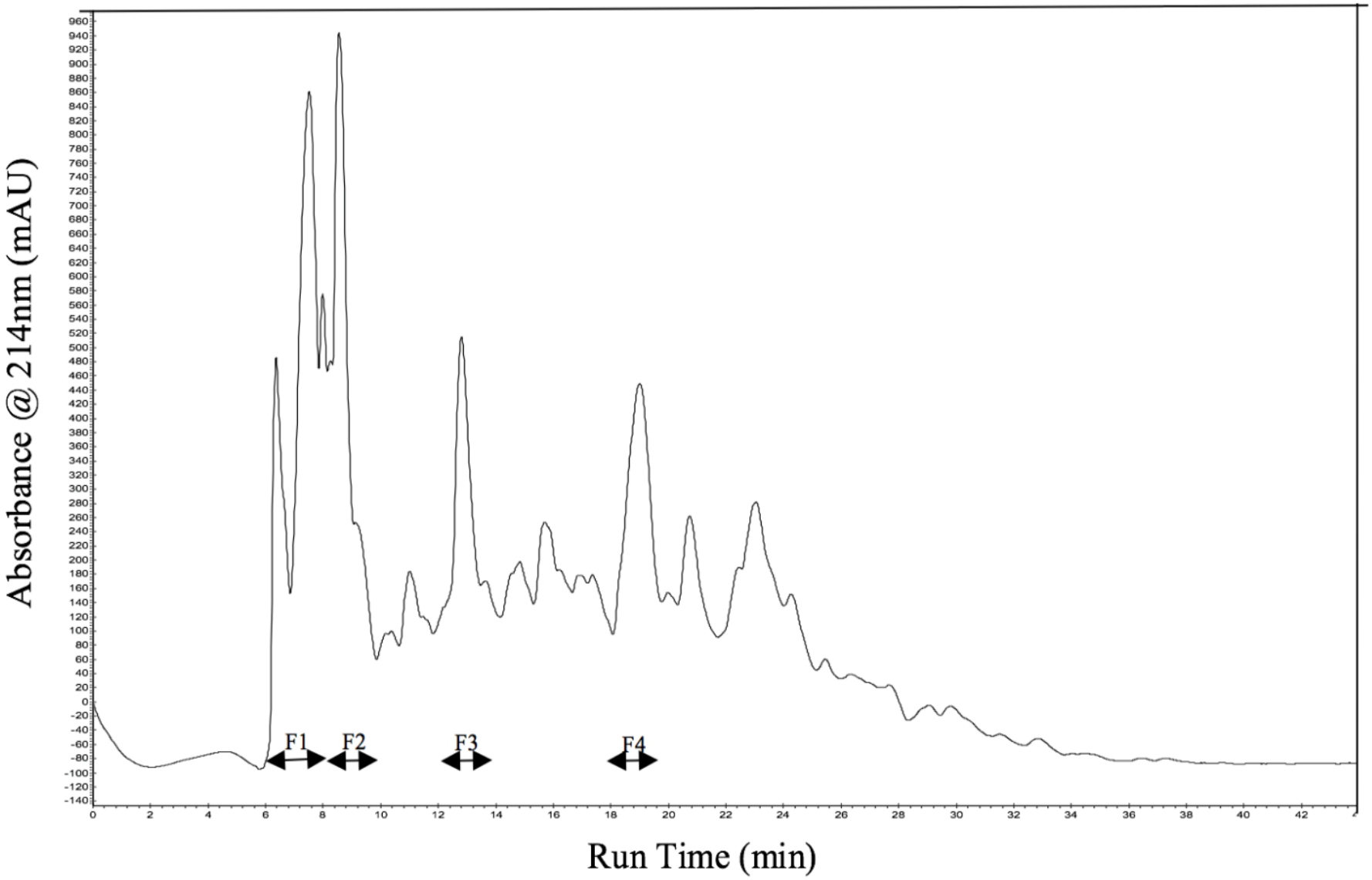


Fig. 28. 2nd RP-HPLC Chromatogram of Alcalase hydrolysates

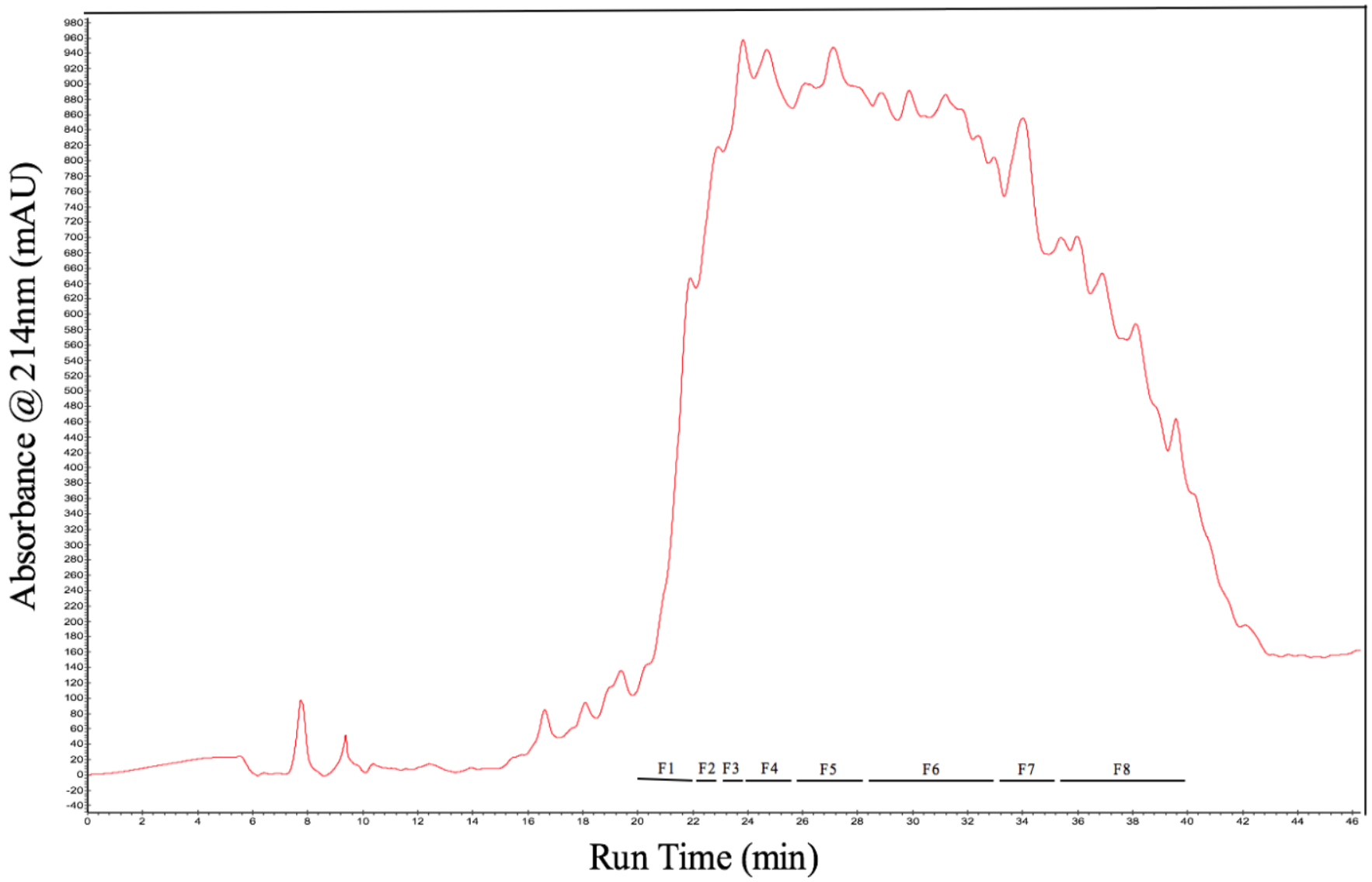


Fig. 29. 2nd RP-HPLC Chromatogram of Alcalase hydrolysates

4.8. IDENTIFICATION OF BITTERNESS INHIBITORY PEPTIDES

Fraction 1 from the 2nd RP-HPLC separation of AH and fraction 1, 3, 4, 5 of CH were selected to identify peptide compositions and their amino acid sequences. The MS scans of these fractions are shown in Fig. 30-34. In contrast to CH fractions, molecular species presented in AH fraction 3 had smaller mass-to-charge ratio (m/z), which is accordance with the results of molecular weight distribution that most AH peptides are intensively distributed around 450 Da. MS scan of four fractions of CH had similar shapes and a great amount of common molecular species besides those from baseline. Molecular species with high intensity were submitted for MS/MS fragmentation and resultant ions were used to analyze de novo amino acid sequence using PEAKS software (Aluko et al., 2015). The amino acid sequence of identified peptides are shown in Fig. 35-42 and summarized in Table 15 which also shows the parent proteins, positions and calculated molecular weight (CMW). Eight peptides were identified and five of them were tetrapeptides (TMTL, ETCL, AAMY, VSSY and AAYM), and their MW were around 450 Da. Besides, there was one hexapeptide (SSMSSL) with MW 592.72Da, one heptapeptide with MW 794.85 Da and one decapeptide whose MW was 1000.08 Da. Tetrapeptides and pentapeptide identified from hemp seed protein efficiently scavenged DPPH and had high efficacy of metal chelation activity (Girgih et al., 2014), implying that maybe four and five amino acid peptides have higher bioactivity. Previous studies have reported that peptides of 0.36-2.10 kDa were primary contributors to bitterness of protein hydrolysates, because highly bulky peptides cannot bind to bitter taste receptors while very small peptides failed to achieve the particular conformation required for binding (Kim, Choi, & Lee, 1999; Maehashi & Huang, 2009a). The results suggest that peptide sizes identified from this work fall within the 0.36-2.10 kDa range and can interact with bitter taste receptors. It has been observed in several peptides from soy protein that leucine residue at C-terminal was responsible for bitterness, and after applying carboxypeptidase, the bitterness was often markedly reduced (Edens et al., 2005; W. Wang & Gonzalez De Mejia, 2005). The results are contrary to data from this work since leucine is located at the C-terminals in four peptides (TMTL, ETCL, SSMSSL, and ETSARHL) identified from bitter taste suppressing fractions. Moreover, except AAMY and AAYM, the ratio of hydrophobic amino acids in each identified peptides were all less than 50%, which may be the

primary reason for low bitter taste. But Sarmadi et al (2010) proposed that hydrophobic properties can enhance peptides entry into target organs through cell membrane lipid bilayer, which is possible to achieve bioactivity inside cells. Besides, some hydrophobic compounds, such as D-Tryptophan benzyl ester and N,N-Dibenzyl-L-serine methyl ester, were reported to have high predicted binding affinity to T2R4 (Sai P Pydi et al., 2014), implying that it is possible for hydrophobic substances to inhibit bitter taste receptors. Thus, peptides with high content of the hydrophobic amino acids also have charge to suppress bitterness. So far, there has been no peptide proposed as specific bitter taste receptor antagonists, however, some peptides have been reported to suppress bitter taste. For example, L-Glu-L-Glu eliminated the bitter taste of brucin and caffeine (Belikov, 1986) and dipeptides containing asparaginic acid was used to reduce bitter taste of potassium salt in a US patent (Ensemble & Cited, 1998), but the mechanisms were not described. Therefore, further determination of bitter taste suppression ability of these identified peptides are necessary.

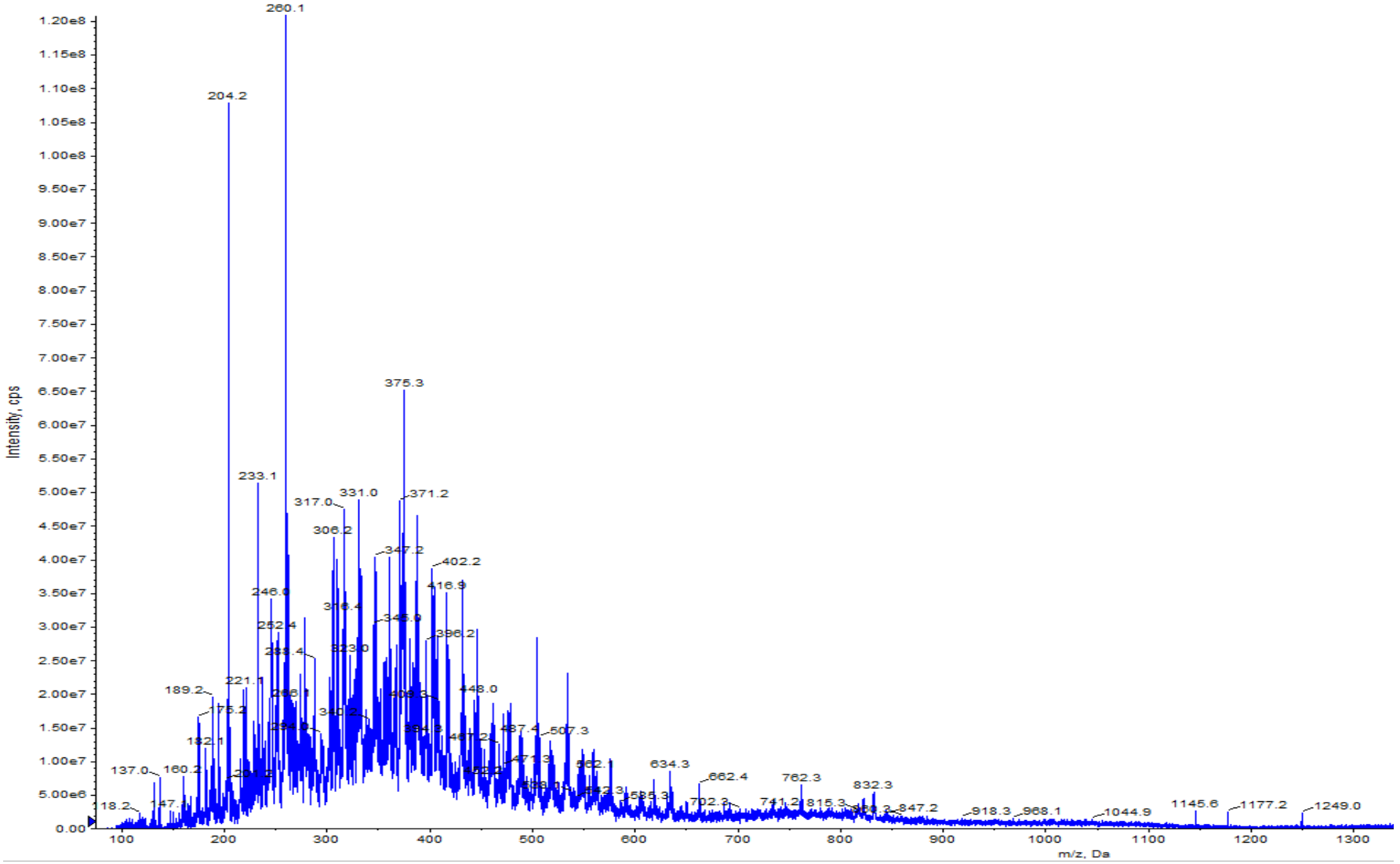


Fig. 30. Liquid chromatography-mass spectrometry chromatogram of 2nd RP-HPLC fraction 1 of Alcalase

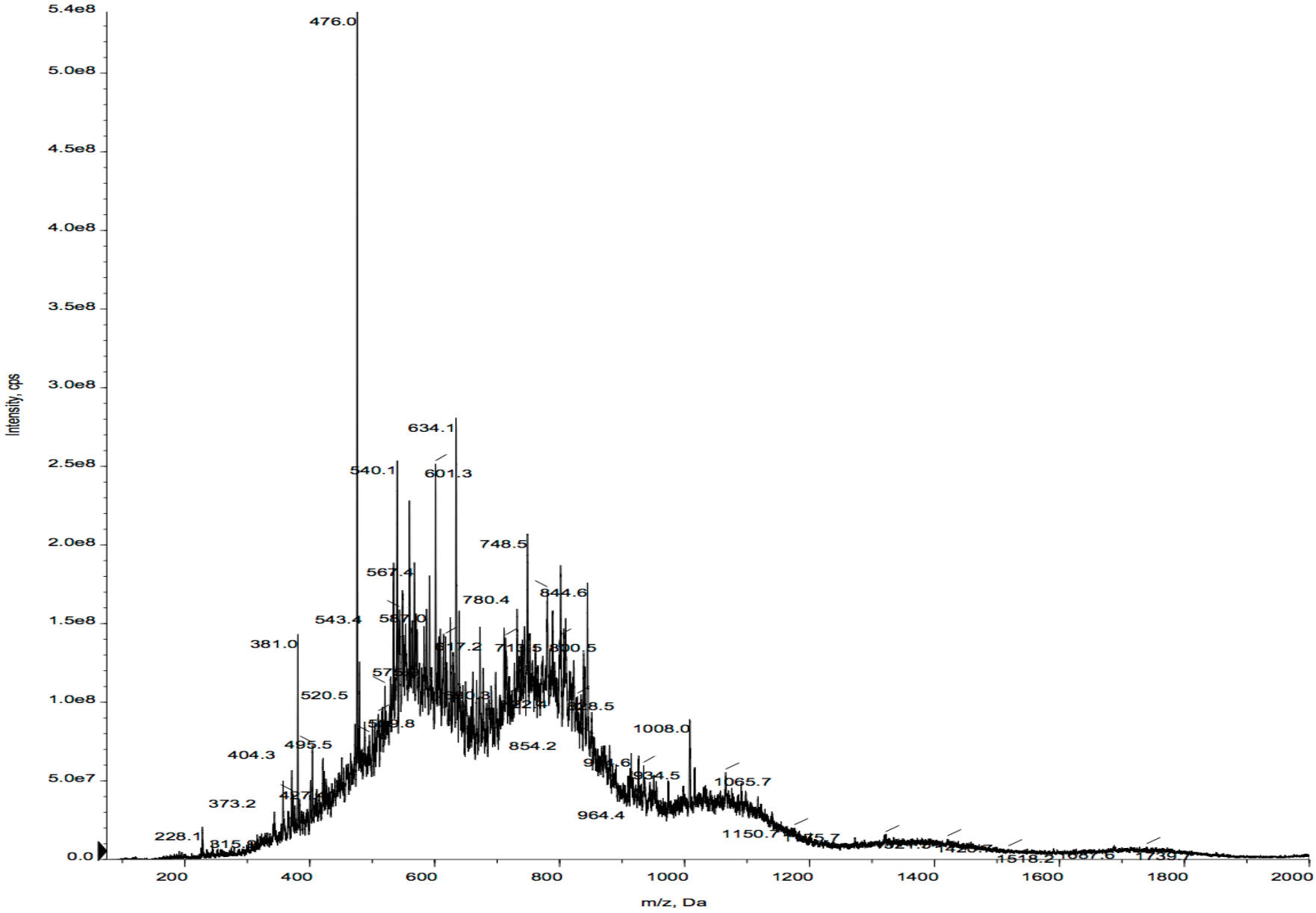


Fig. 31. Liquid chromatography-mass spectrometry chromatogram of 2nd RP-HPLC fraction1 of Chymotrypsin

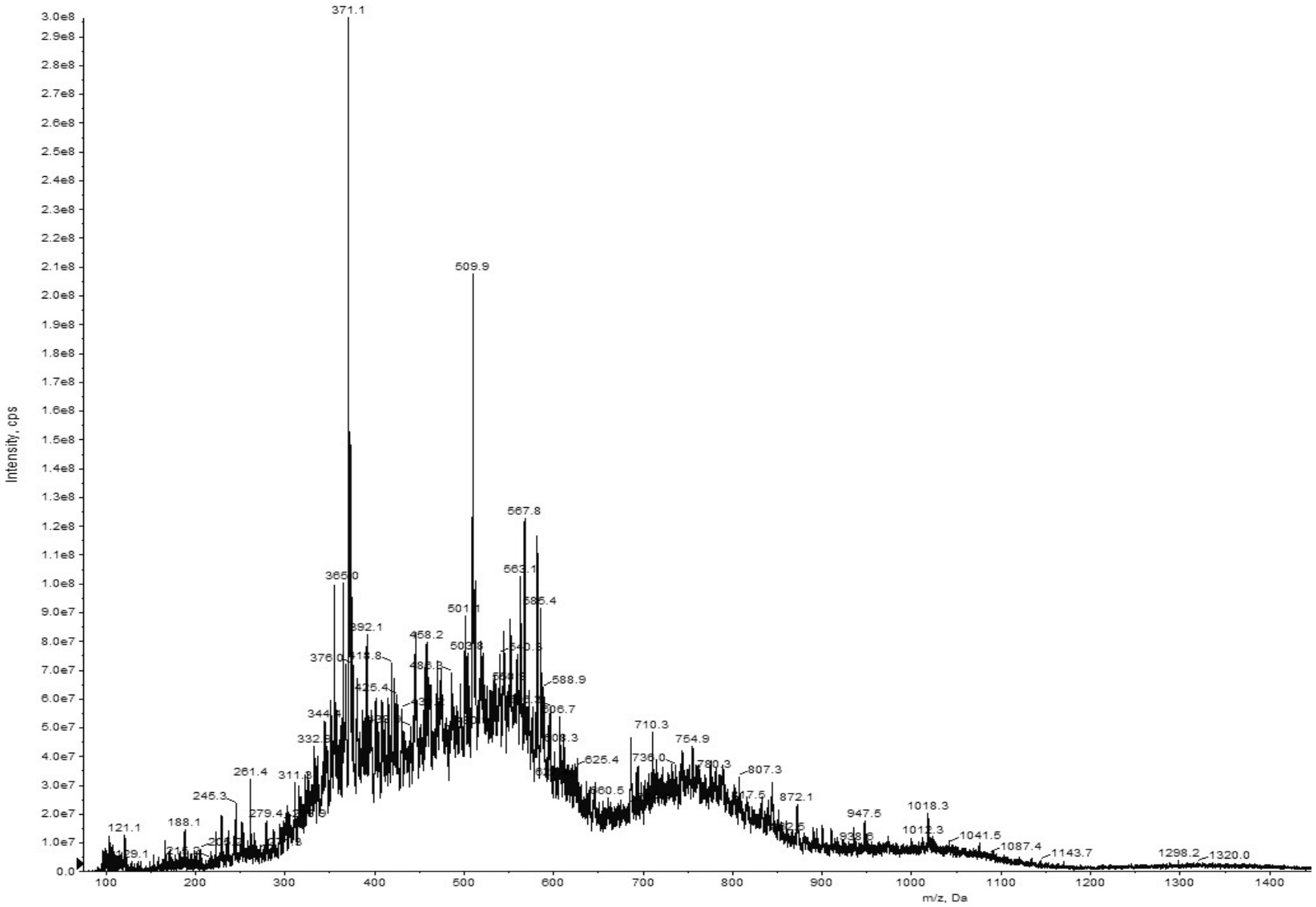


Fig. 32. Liquid chromatography-mass spectrometry chromatogram of 2nd RP-HPLC fraction 3 of Chymotrypsin Hydrolysates

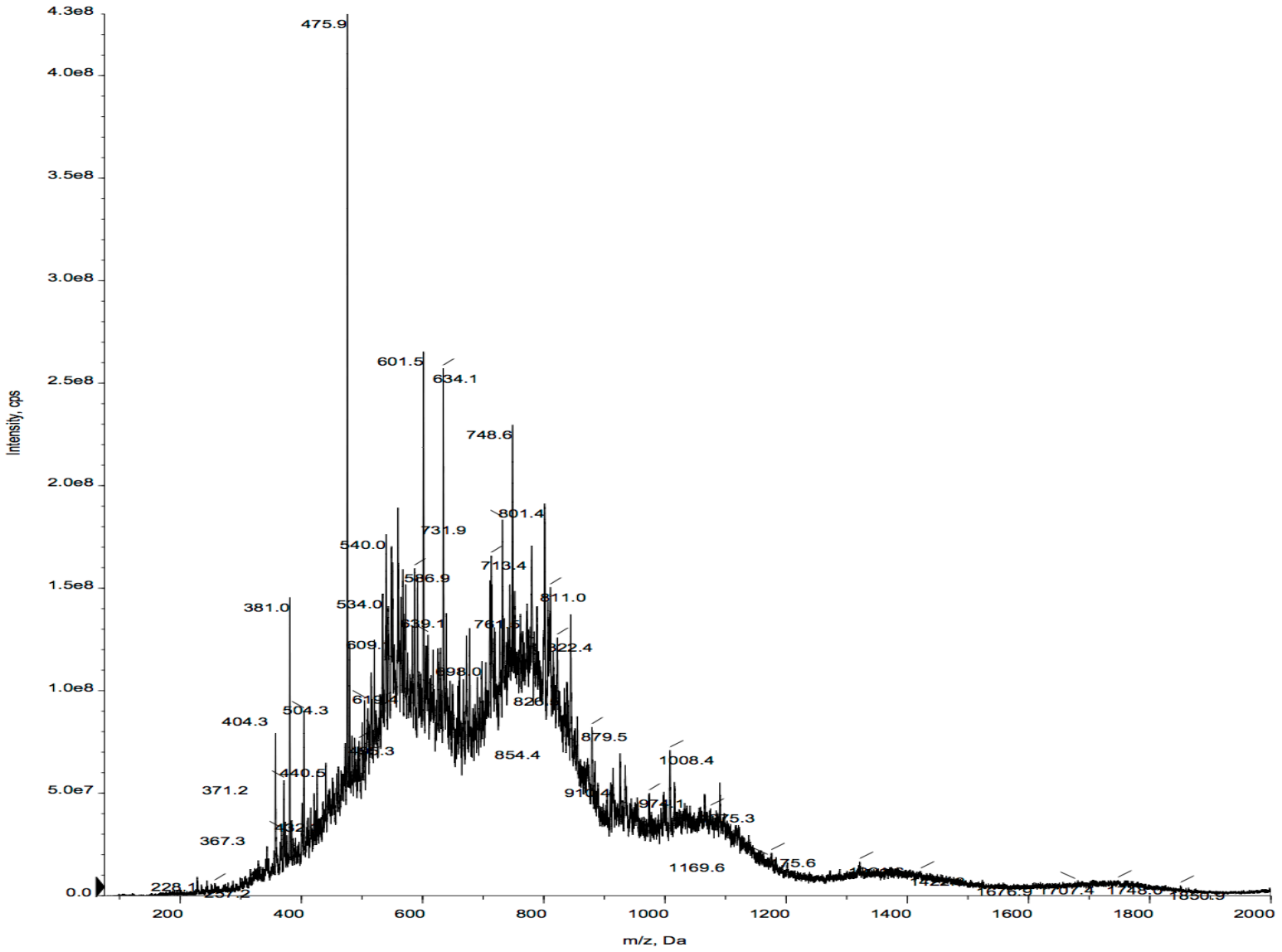


Fig. 33. Liquid chromatography-mass spectrometry chromatogram of 2nd RP-HPLC fraction 4 of Chymotrypsin Hydrolysates

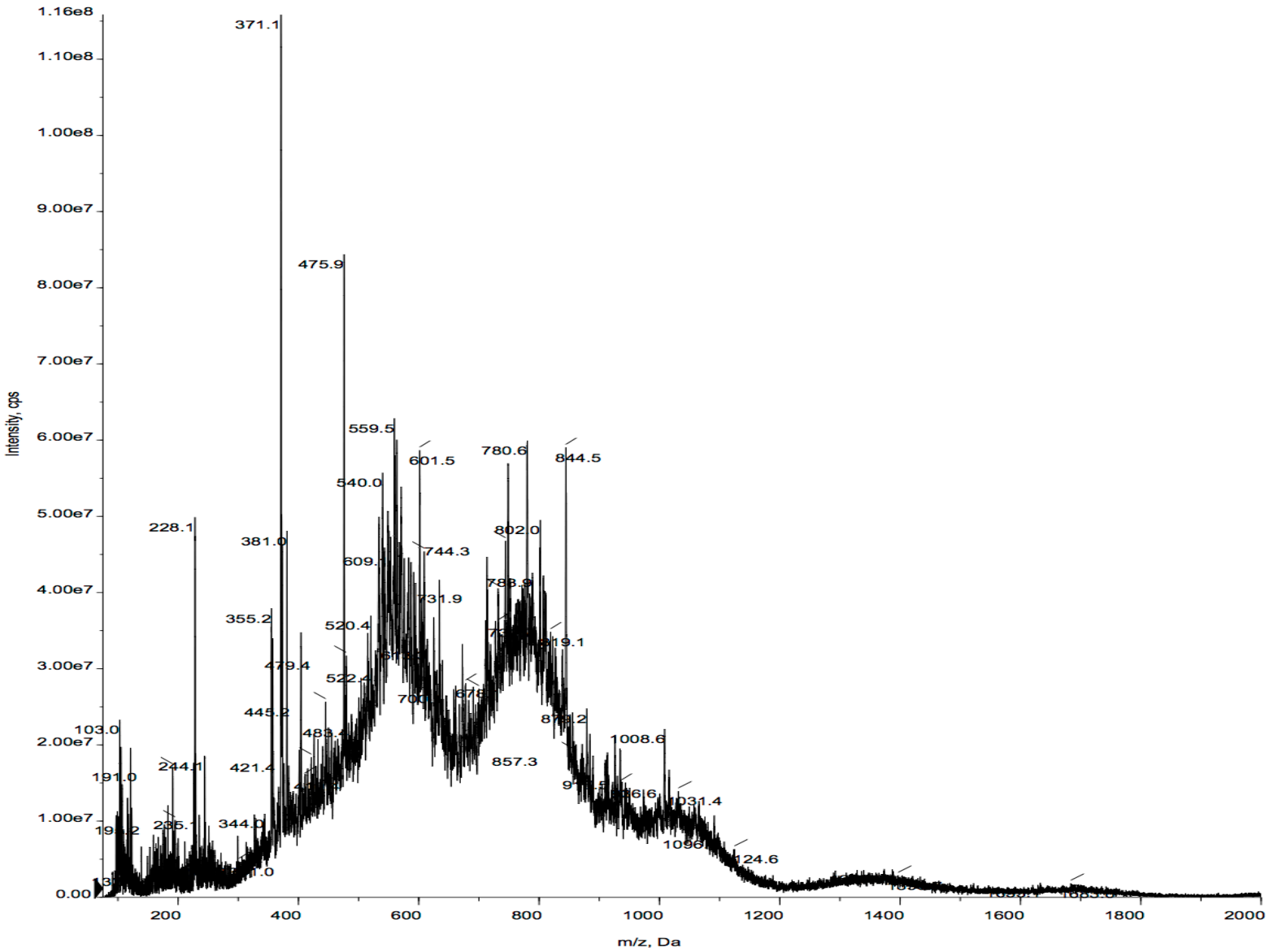
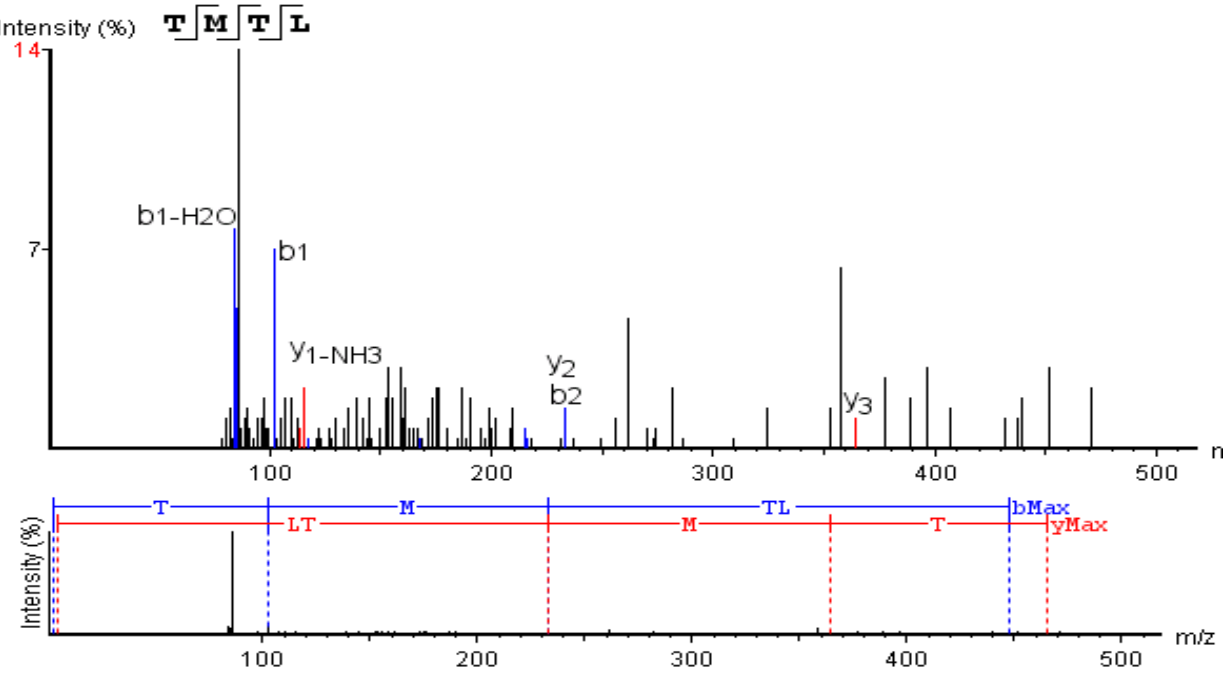
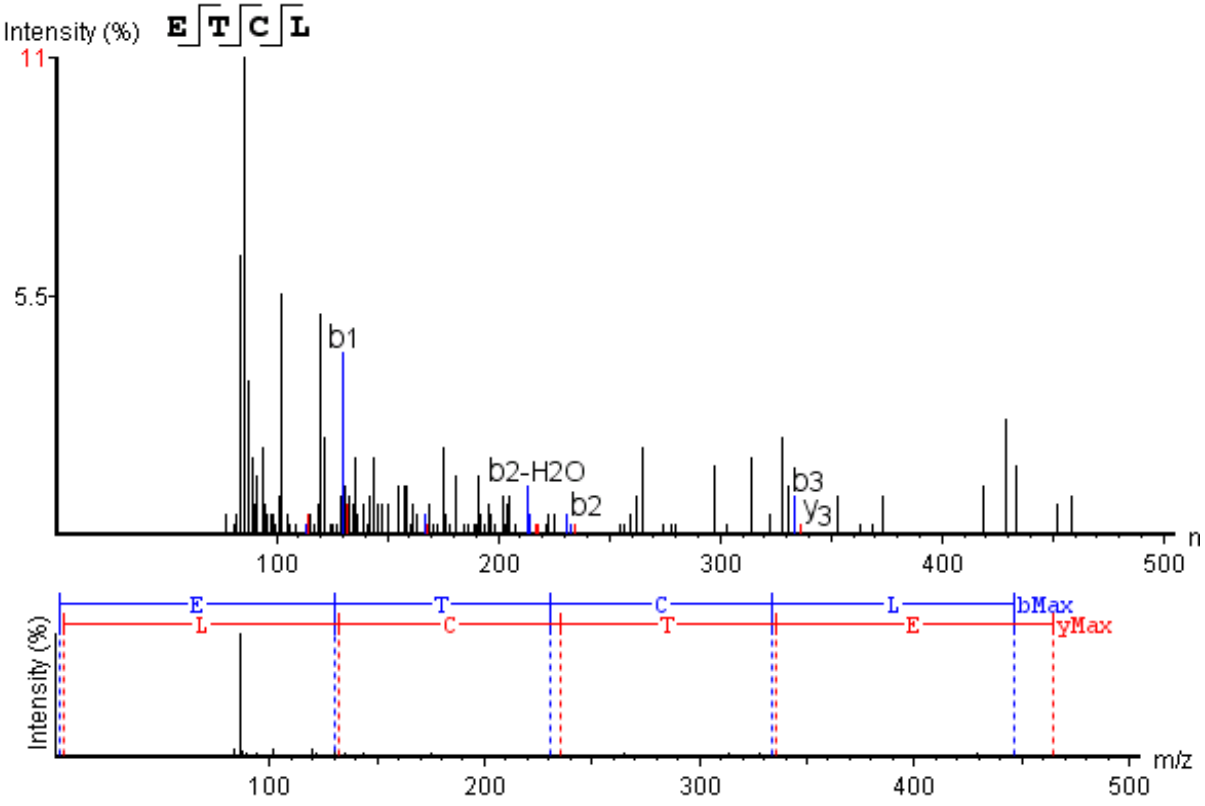


Fig. 34. Liquid chromatography-mass spectrometry chromatogram of 2nd RP-HPLC fraction 5 of Chymotrypsin

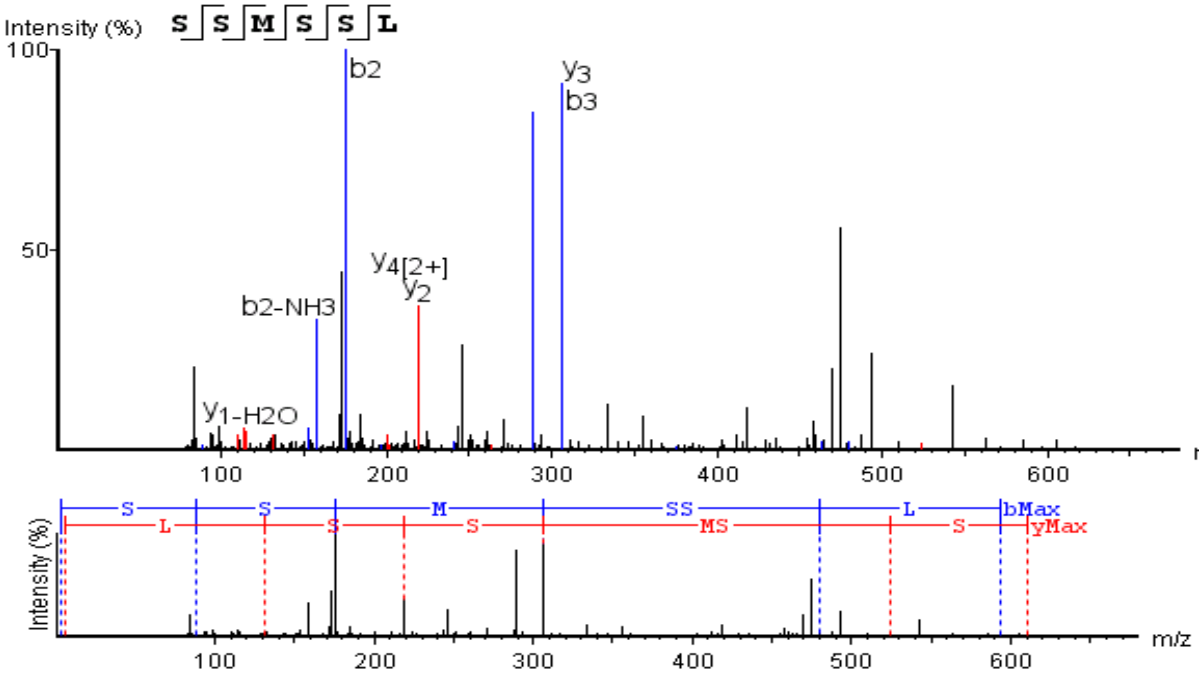
A


Intensity (%)
T M T L
14
7
b1-H2O
b1
y1-NH3
y2
b2
y3
n
100
200
300
400
500
T
M
TL
bMax
LT
M
T
yMax
Intensity (%)
m/z
100
200
300
400
500

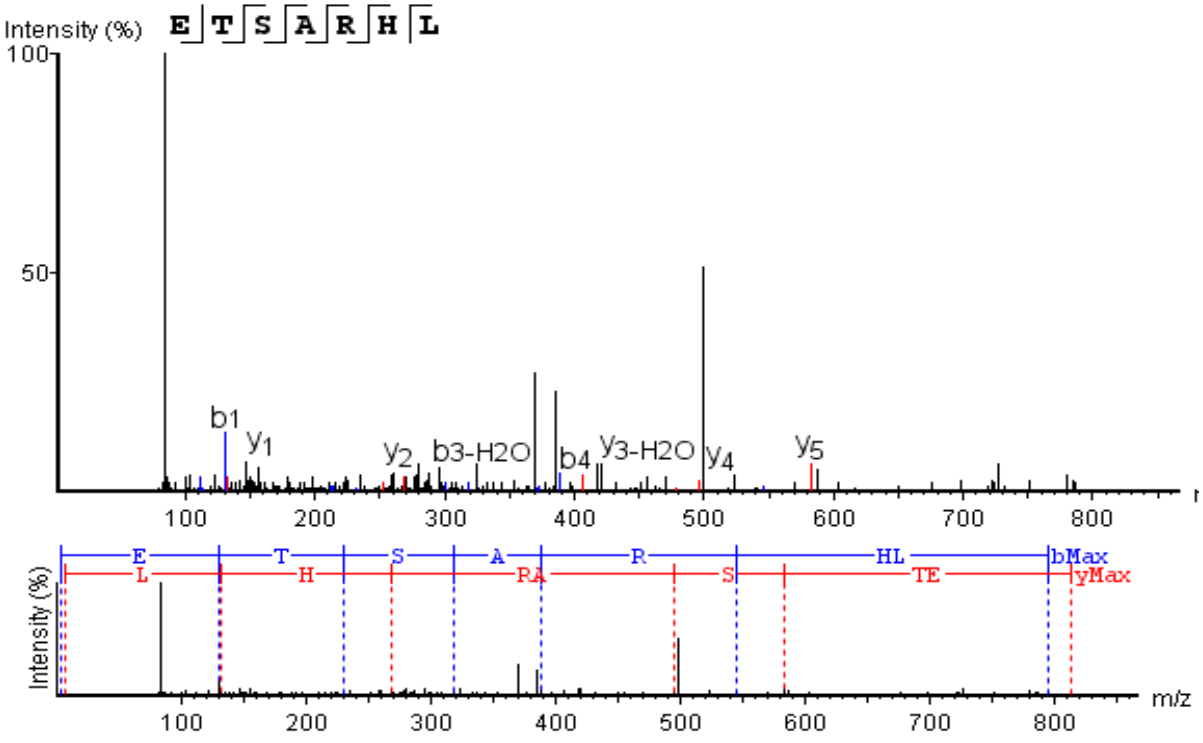
B


Intensity (%)
E T C L
11
5.5
b1
b2-H2O
b2
b3
y3
n
100
200
300
400
500
E
T
C
L
bMax
L
C
T
E
yMax
Intensity (%)
m/z
100
200
300
400
500

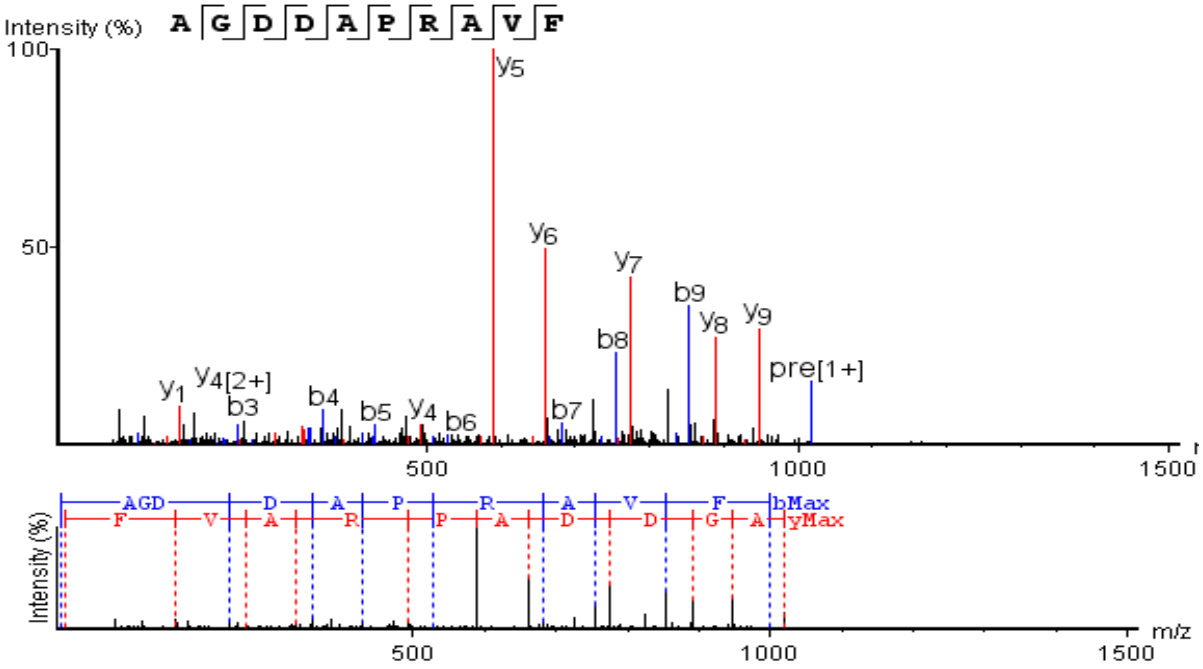
C


Intensity (%)
S S M S S L
100
50
b2
y3
b3
y4[2+]
y2
b2-NH3
y1-H2O
100
200
300
400
500
600
m/z
Intensity (%)
S S M SS L bMax
L S S MS S yMax
100
200
300
400
500
600
m/z

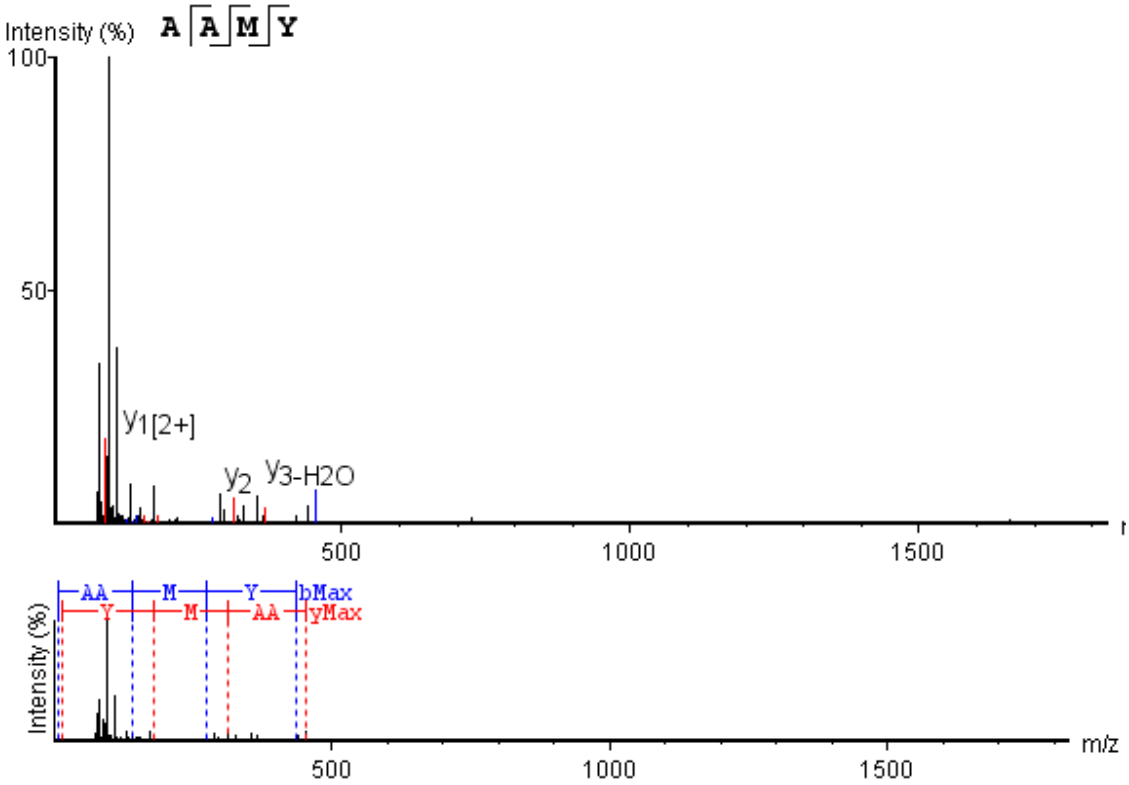
D


Intensity (%)
E T S A R H L
100
50
b1
y1
y2
b3-H2O
b4
y3-H2O
y4
y5
100
200
300
400
500
600
700
800
m/z
Intensity (%)
E T S A R HL bMax
L H RA S TE yMax
100
200
300
400
500
600
700
800
m/z

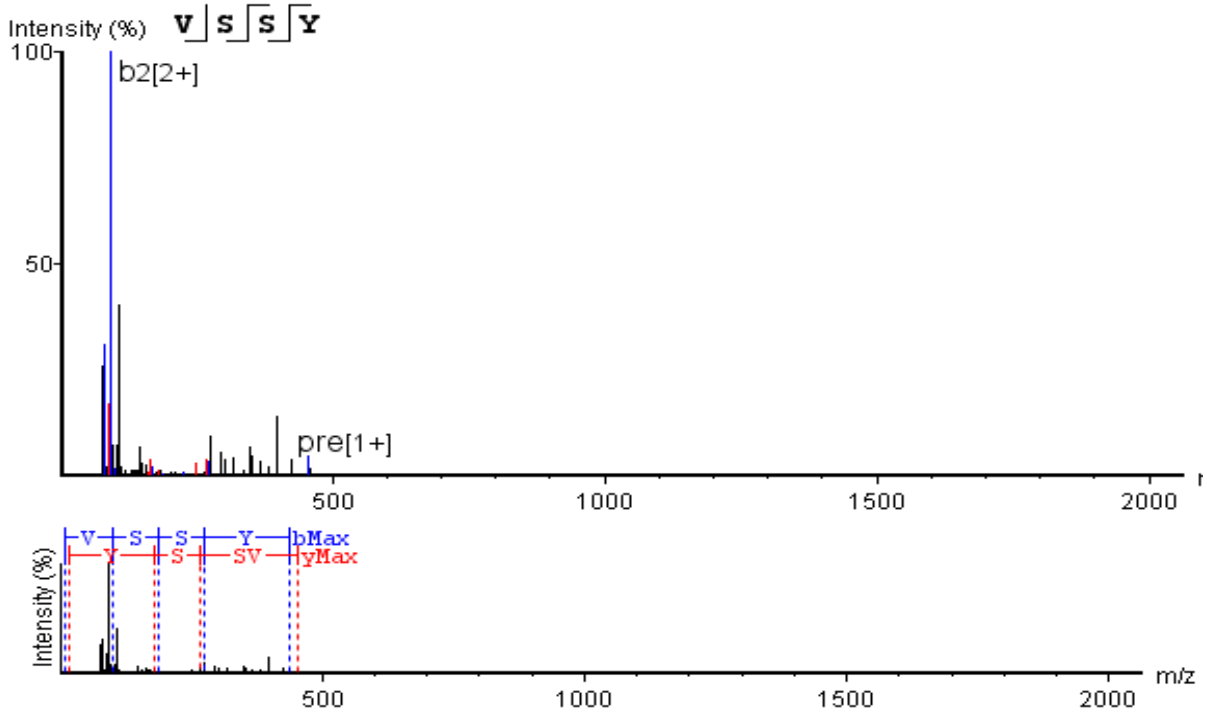
E


Intensity (%)
A G D D A P R A V F
100
50
y5
y6
y7
b9
y8
y9
b8
pre[1+]
y1
y4[2+]
b3
b4
b5
y4
b6
b7
500
1000
1500
AGD
D
A
P
R
A
V
F
bMax
F
V
A
R
P
A
D
D
G
A
yMax
Intensity (%)
m/z
500
1000
1500

F


Intensity (%)
A A M Y
100
50
y1[2+]
y2
y3-H2O
500
1000
1500
AA
M
Y
bMax
Y
M
AA
yMax
Intensity (%)
m/z
500
1000
1500

G



H

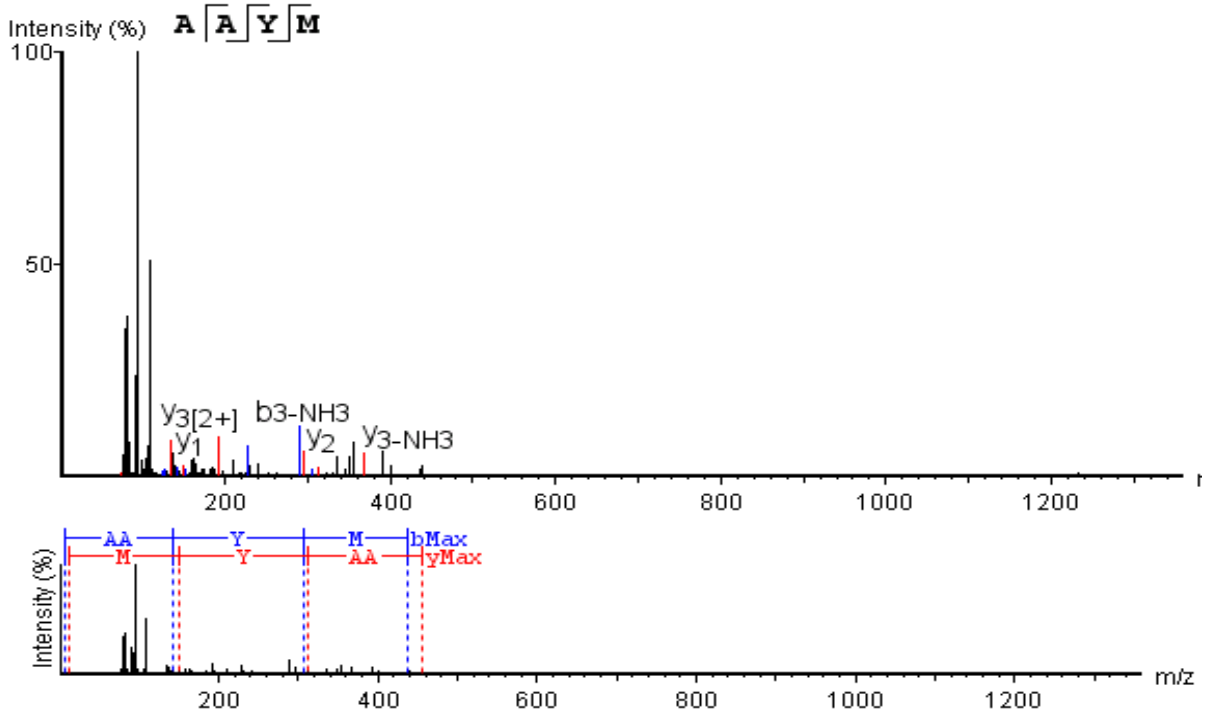


Fig. 35. Tandem mass spectrometry chromatogram and amino acid sequence of peptides (A-H)

Table 15 Identified Peptides from BPH and their Calculated molecular weight and Position

Peptide Source	Obs (m/z)	Z	Suggested Peptide	Parent Protein	Position	CMW (Da)
2nd AH-F3	233.1	2	TMTL	Versican core protein	f529-532	446.6
2nd AH-F3	233.1	2	ETCL	Coagulation factor XIII, B polypeptide	f1540-1545	446.5
2nd AH-F3	306.2	2	SSMSSL	Cardiomyopathy associate protein 1	f1540-1545	592.72
2nd AH-F3	407	2	ETSARHL	Myosin class II heavy chain (MHC)	f23-29	794.85
2nd CH-F3	509	2	AGDDAPRAVF	Alpha-actin-2, Alpha-actin-1, Alpha-cardiac actin	F24-33	1000.08
2nd CH-F5	228.1	2	AAMY	DDR2 protein, FDPS protein	F368-371, f280-283	436.56
2nd CH-F5	228.1	2	VSSY	Desmin, Fibrillin-1, Glucagon	F20-23, f308-311, f107-110	436.43
2nd CH-F5	228.1	2	AAYM	KRT5 protein	F282-285	436.56

CMW: Calculated Molecular Weight

4.9. MASS PROFILE OF AH, CH AND AGEs

In order to confirm that the Maillard reaction led to structural modifications of the peptides, 12 selected AGEs and their initial substrates, AH and CH, were subjected to mass spectrometry analysis. The LC-MS chromatograms are shown in Fig. 36-49. Firstly, there are no common peaks seen in AH chromatogram and CH chromatogram, which suggests there are no common compounds in these two enzymatic hydrolysates. And chromatogram of AH accumulated significant peaks from 200 to 600 m/z, while chromatogram of CH showed an obvious peak from 800 to 1000 m/z, which is compatible with the results of molecular weight in which CH showed more composition with bigger molecular weights than AH. According to previous study that 1000-5000 Da peptides can enhance AGEs yield (Y. Li et al., 2013; Zeng, Zhang, Guan, & Sun, 2012), thus CH-AGEs should have more peaks in MS spectra. Besides, mass spectra for heated different amino acid (lysine, arginine, aspartic acid, tyrosine, serine, cysteine) with sucrose showed that cysteine-sucrose had most peaks observed followed by serine-sucrose, while arginine-sucrose had least peaks (Golon et al., 2014), which also suggests that CH-AGEs should have more peaks observed. It was obvious that all the MS chromatograms of AGEs were different from their corresponding substrates, AH and CH, and there are no common peaks observed, indicating new molecular species were produced during Maillard reaction (Mennella, Visciano, Napolitano, del Castillo, & Fogliano, 2006; Sun, Hayakawa, Ogawa, & Izumori, 2005). Similar species were observed in AGEs chromatograms with all having ions ranging from 100 to 800 Da and two common peaks 202.8 and 383 Da, but which are possibly from contamination. Besides, AGEs produced from same glucose concentration had more common peaks, for example, A1 (1% AH, 3% D-glucose) had more common peaks with A2 (3% AH, 3% D-glucose) rather than with other AH AGEs. Martins et al (2014) studied the kinetics of the glucose/glycine Maillard reaction and found that the initial concentration of glucose and glycine had no effect on estimated rate constants, which could partly explain the similar effect of all AGEs of AH and CH.

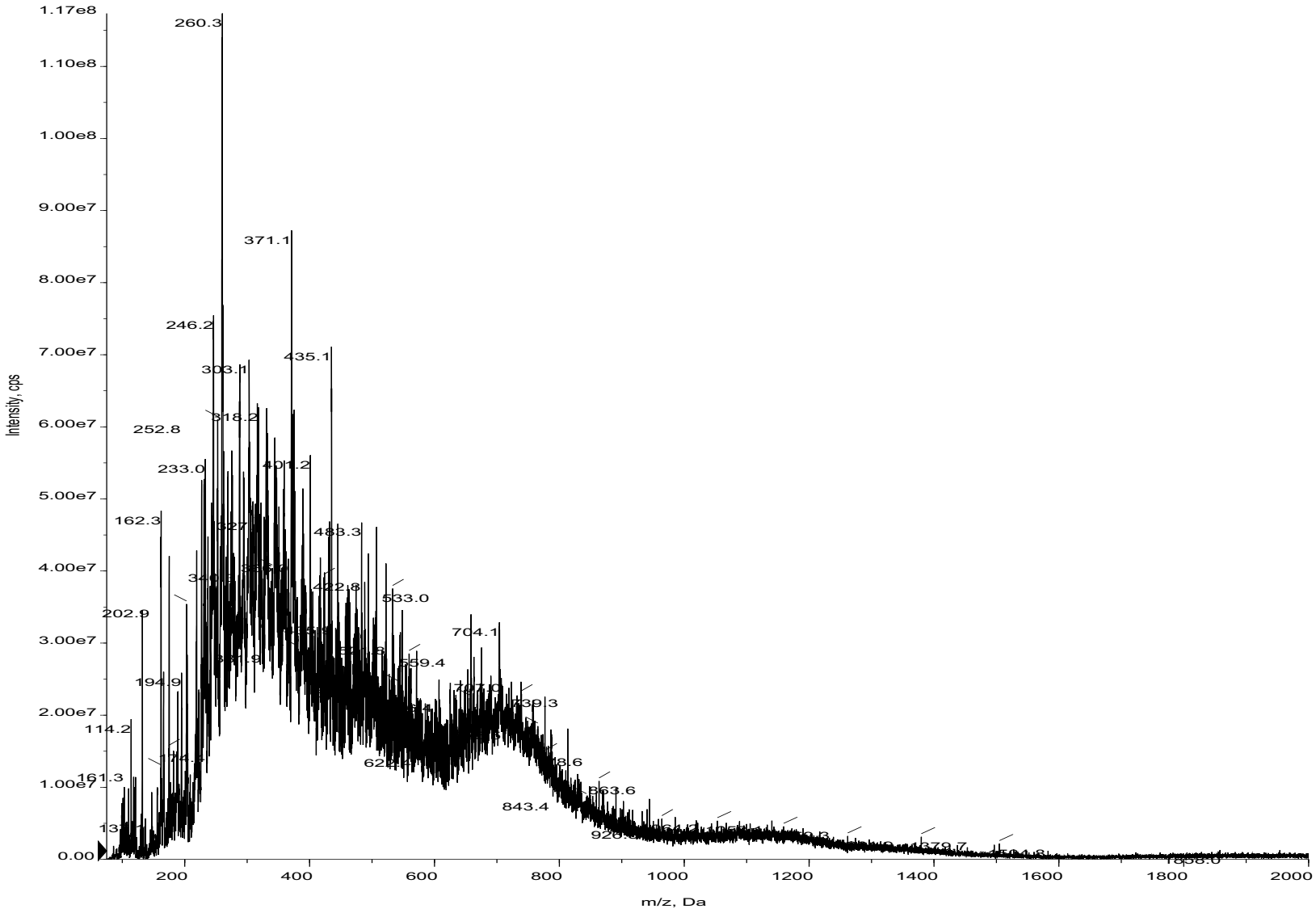


Fig. 36. Liquid chromatography-mass spectrometry chromatogram of Alcalase Hydrolysates

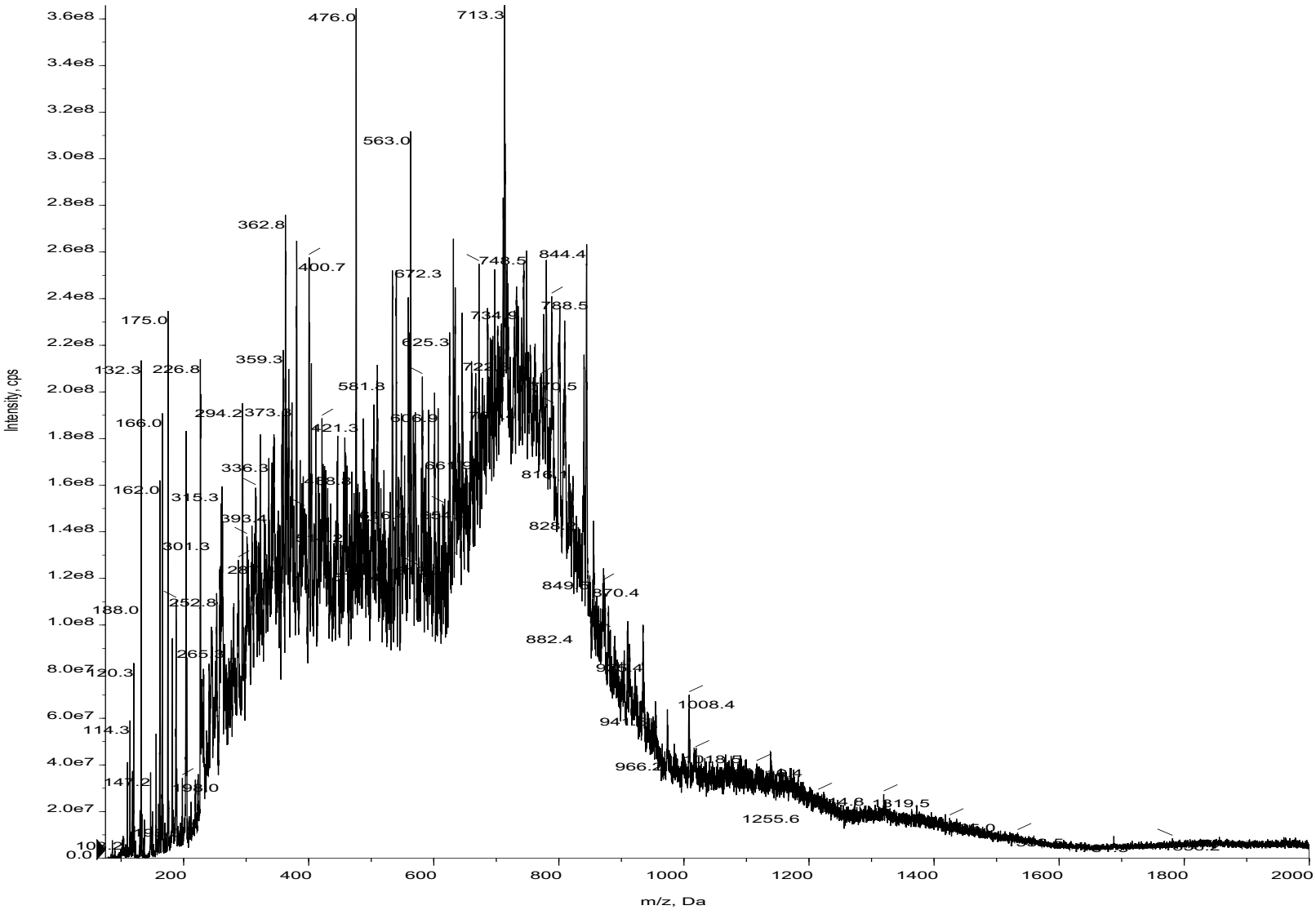


Fig. 37. Liquid chromatography-mass spectrometry chromatogram of Chymotrypsin Hydrolysates

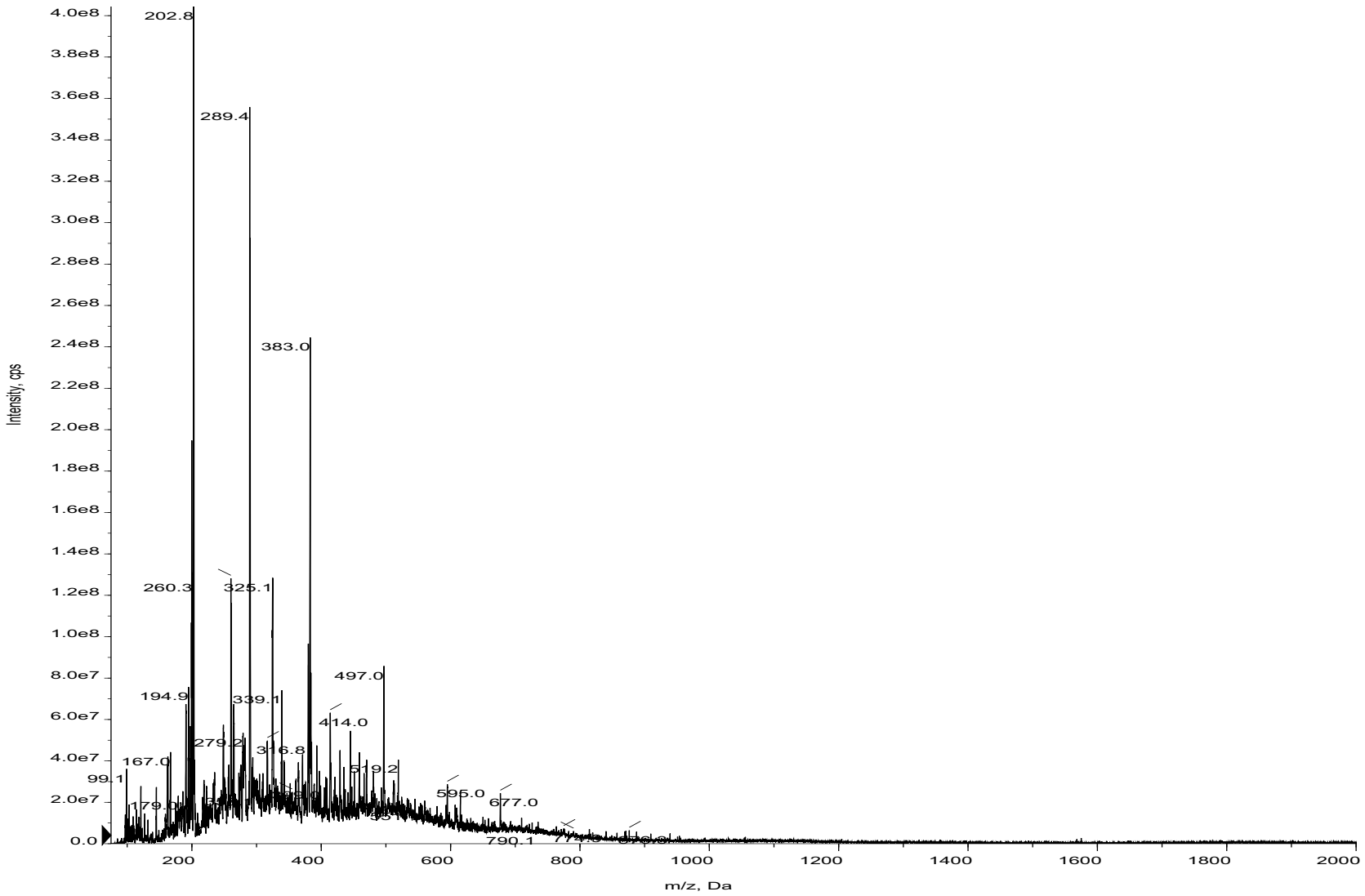


Fig. 38. Liquid chromatography-mass spectrometry chromatogram of Alcalase Hydrolysates AGEs1

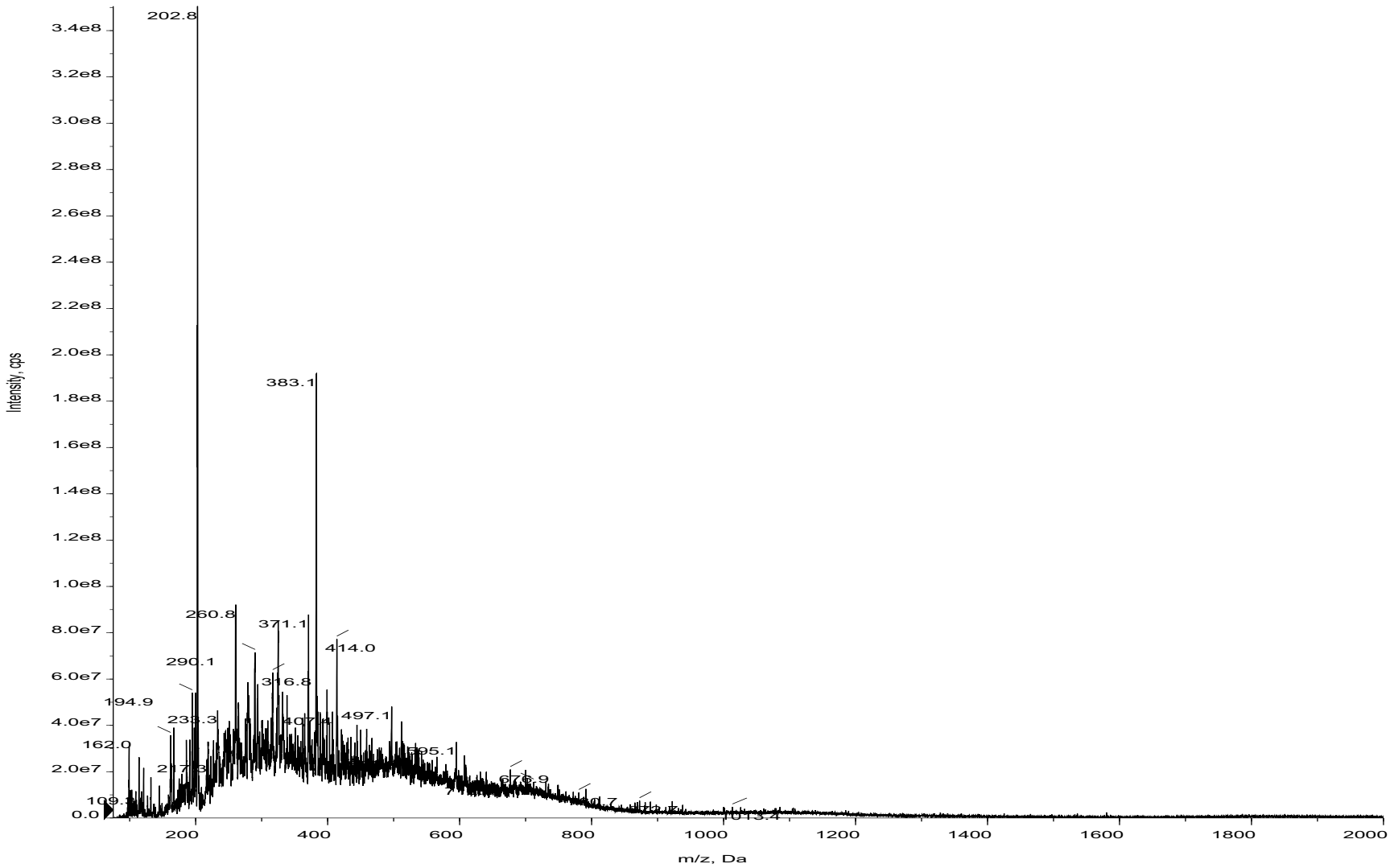


Fig. 39. Liquid chromatography-mass spectrometry chromatogram of Alcalase Hydrolysates AGEs2

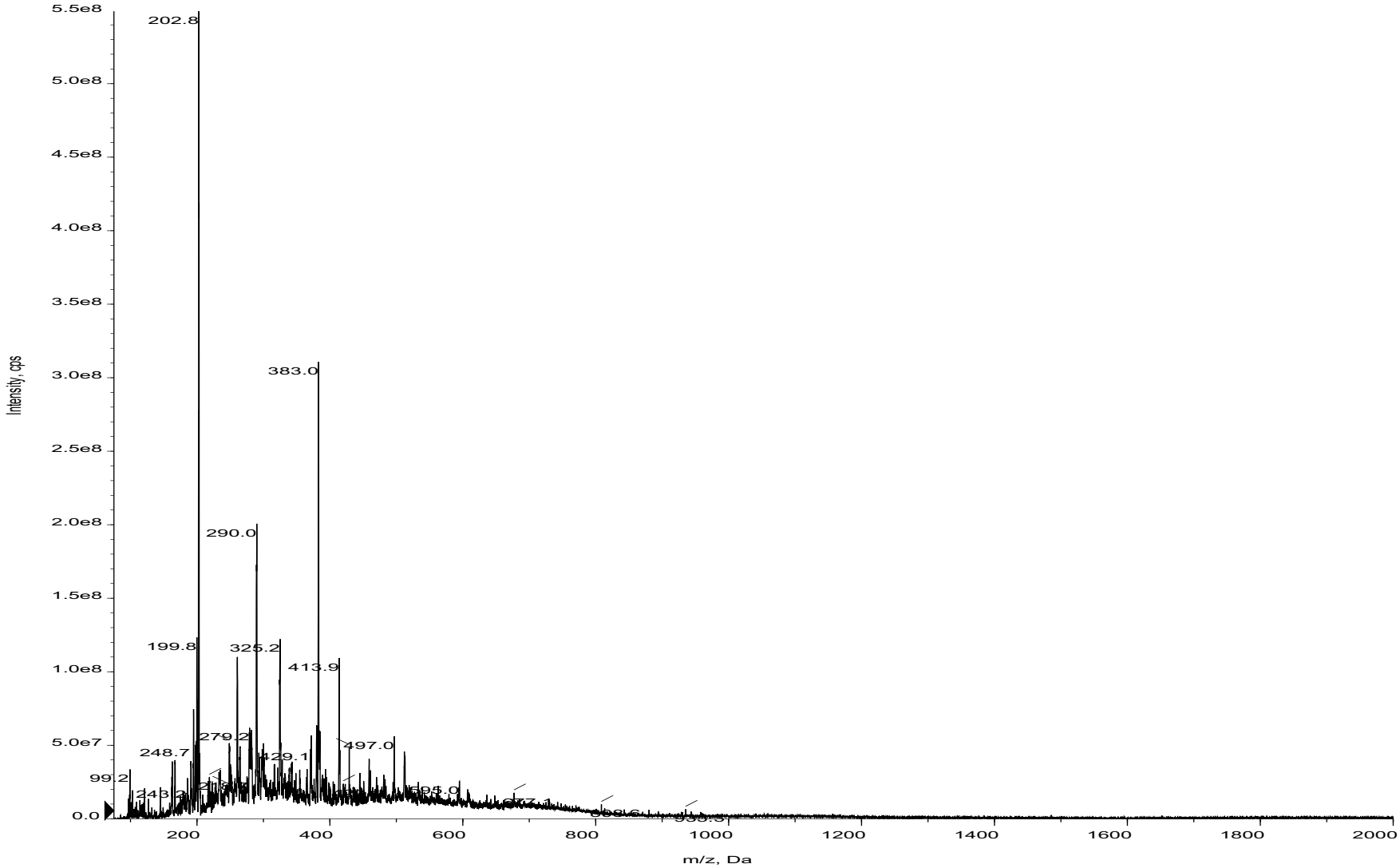


Fig. 40. Liquid chromatography-mass spectrometry chromatogram of Alcalase Hydrolysates AGEs3

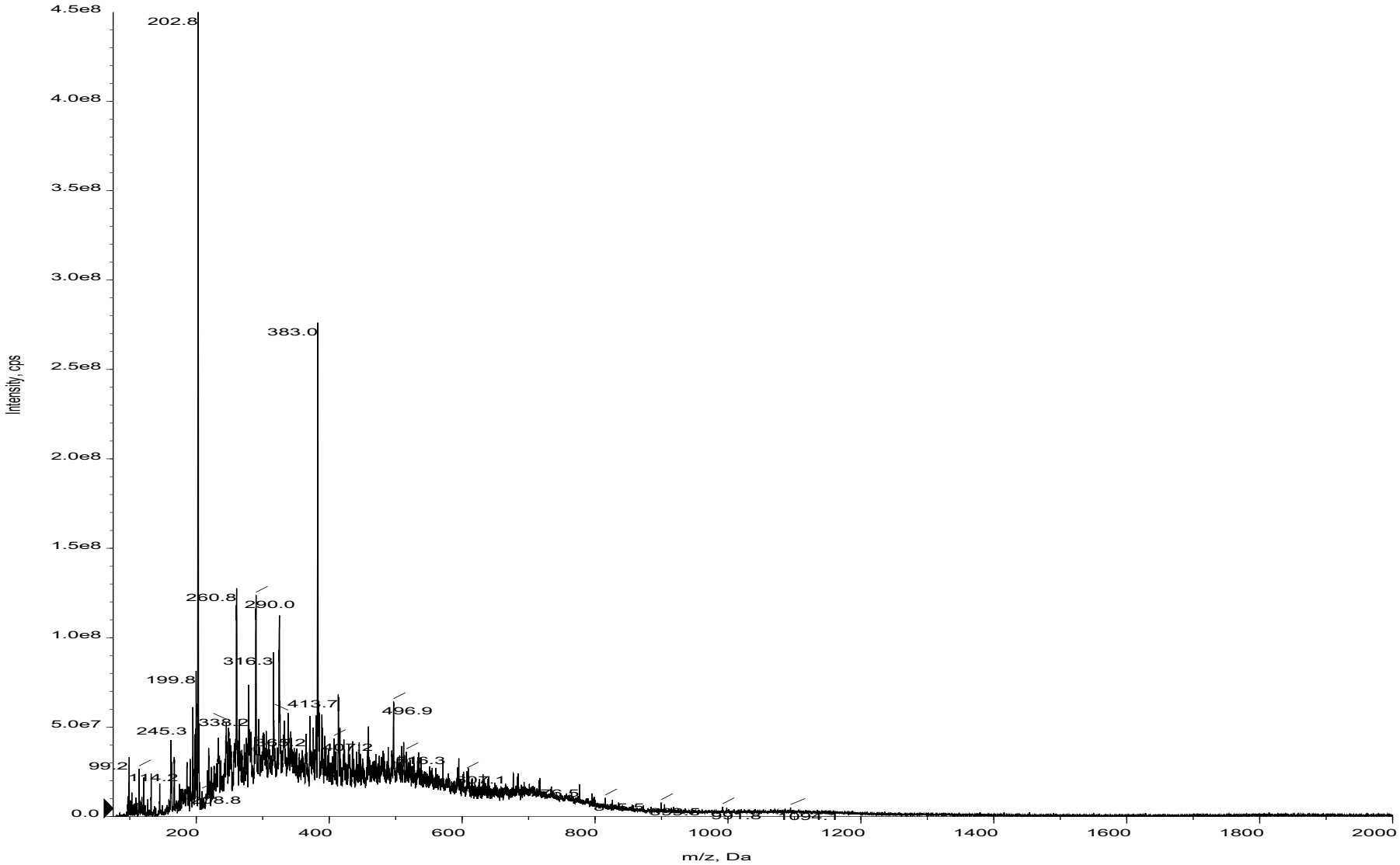


Fig. 41. Liquid chromatography-mass spectrometry chromatogram of Alcalase Hydrolysates AGEs4

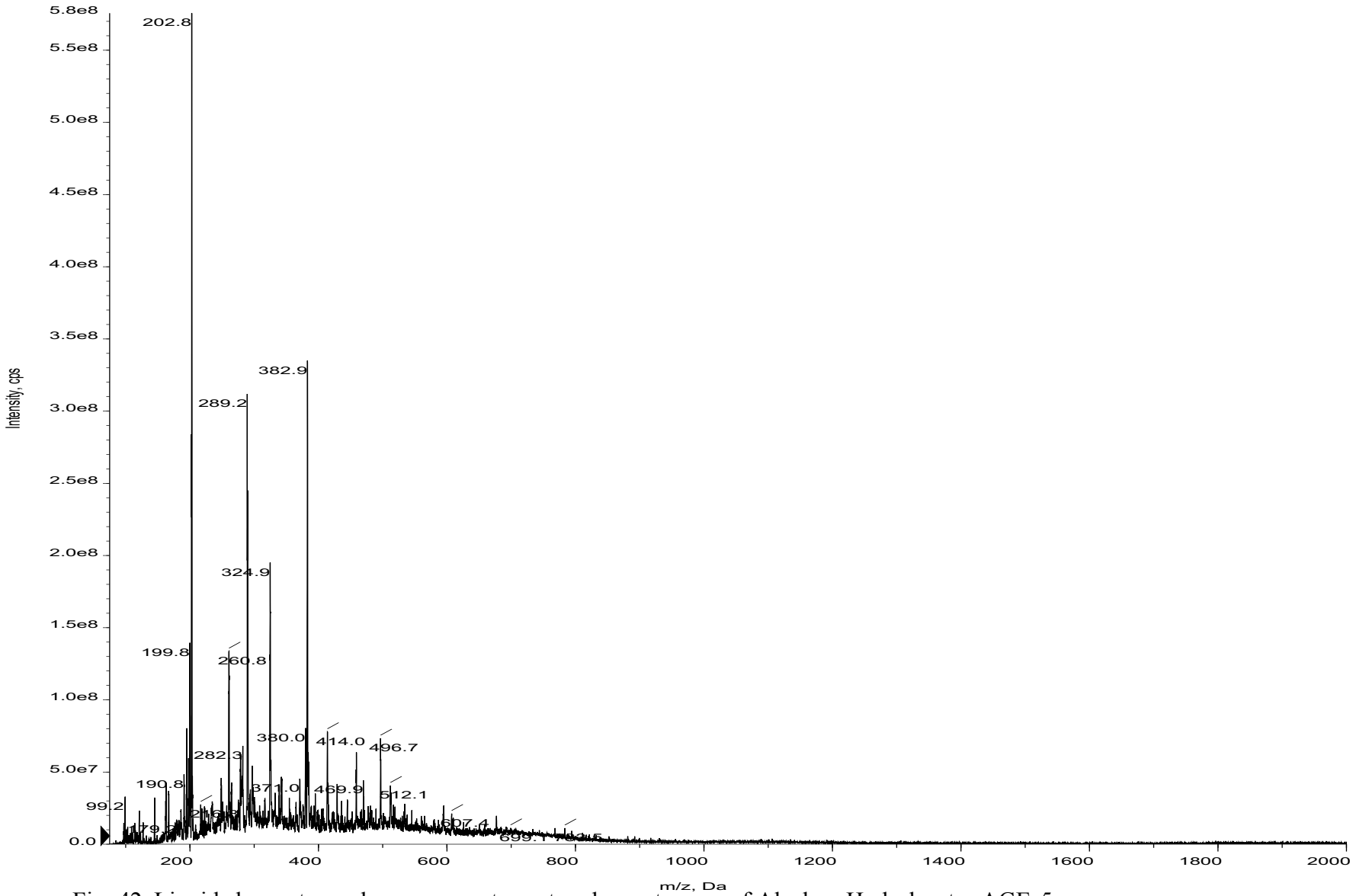


Fig. 42. Liquid chromatography-mass spectrometry chromatogram of Alcalase Hydrolysates AGEs5

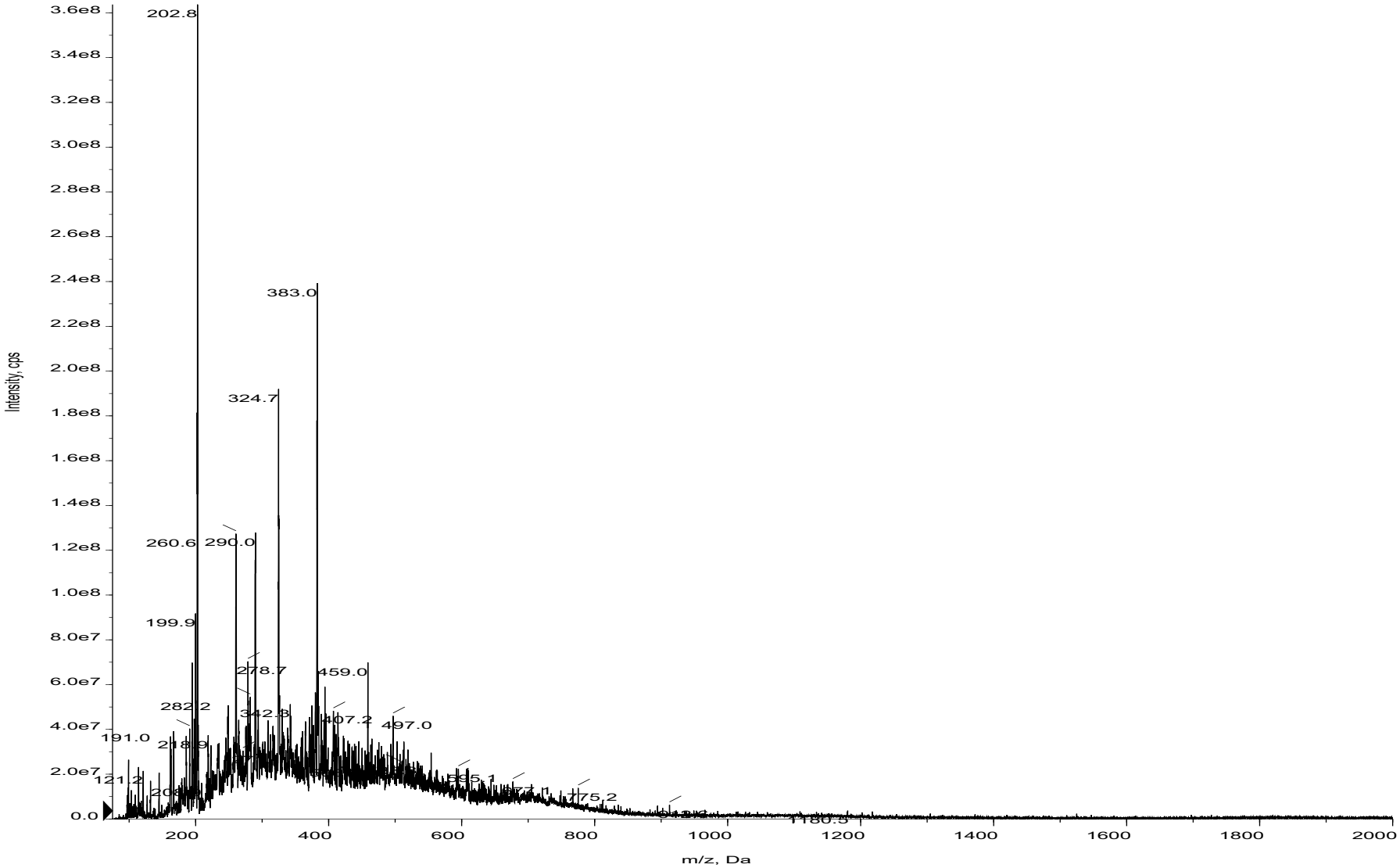


Fig. 43. Liquid chromatography-mass spectrometry chromatogram of Alcalase Hydrolysates AGEs6

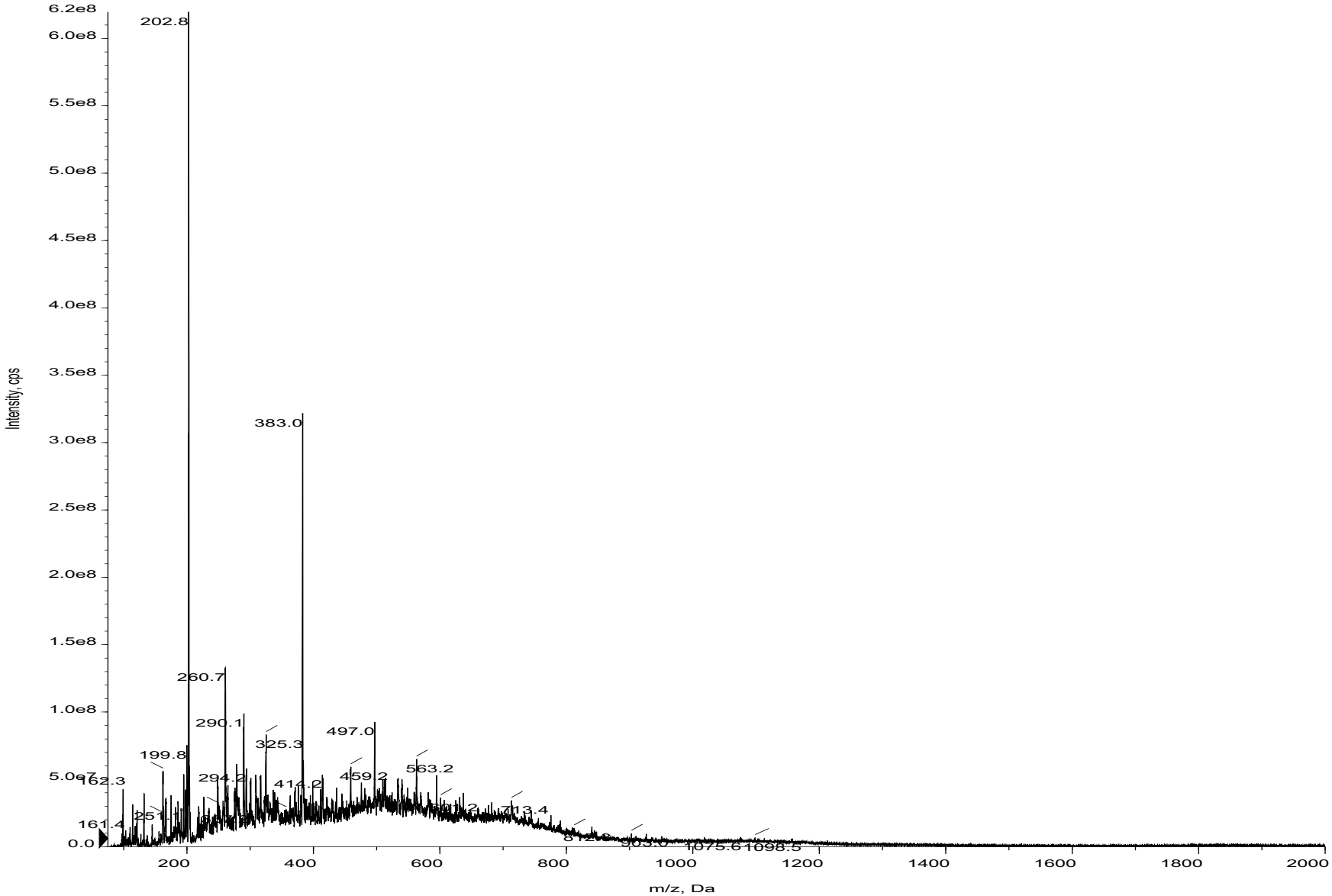


Fig. 44. Liquid chromatography-mass spectrometry chromatogram of Chymotrypsin Hydrolysates AGEs1

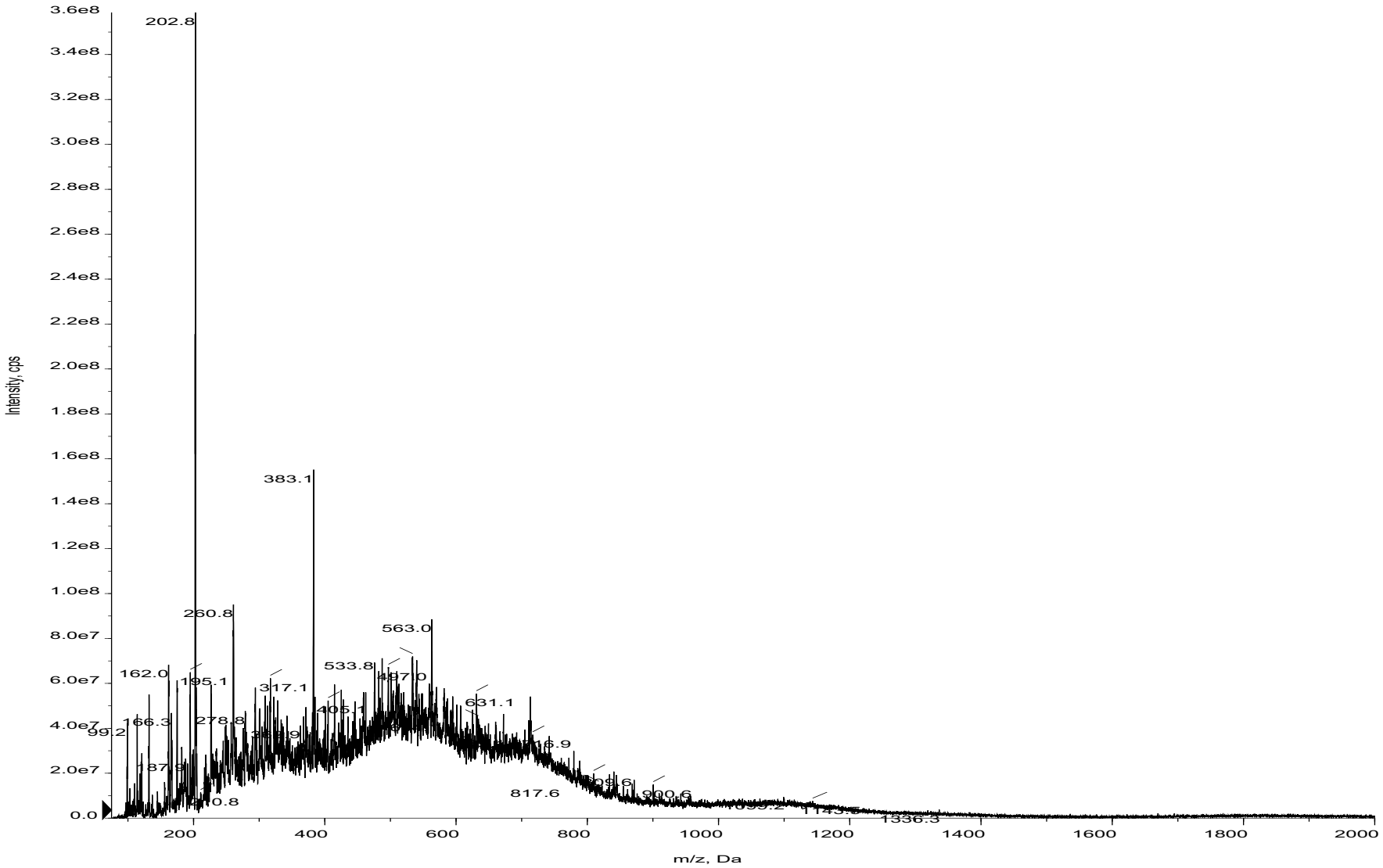


Fig. 45. Liquid chromatography-mass spectrometry chromatogram of Chymotrypsin Hydrolysates AGEs2

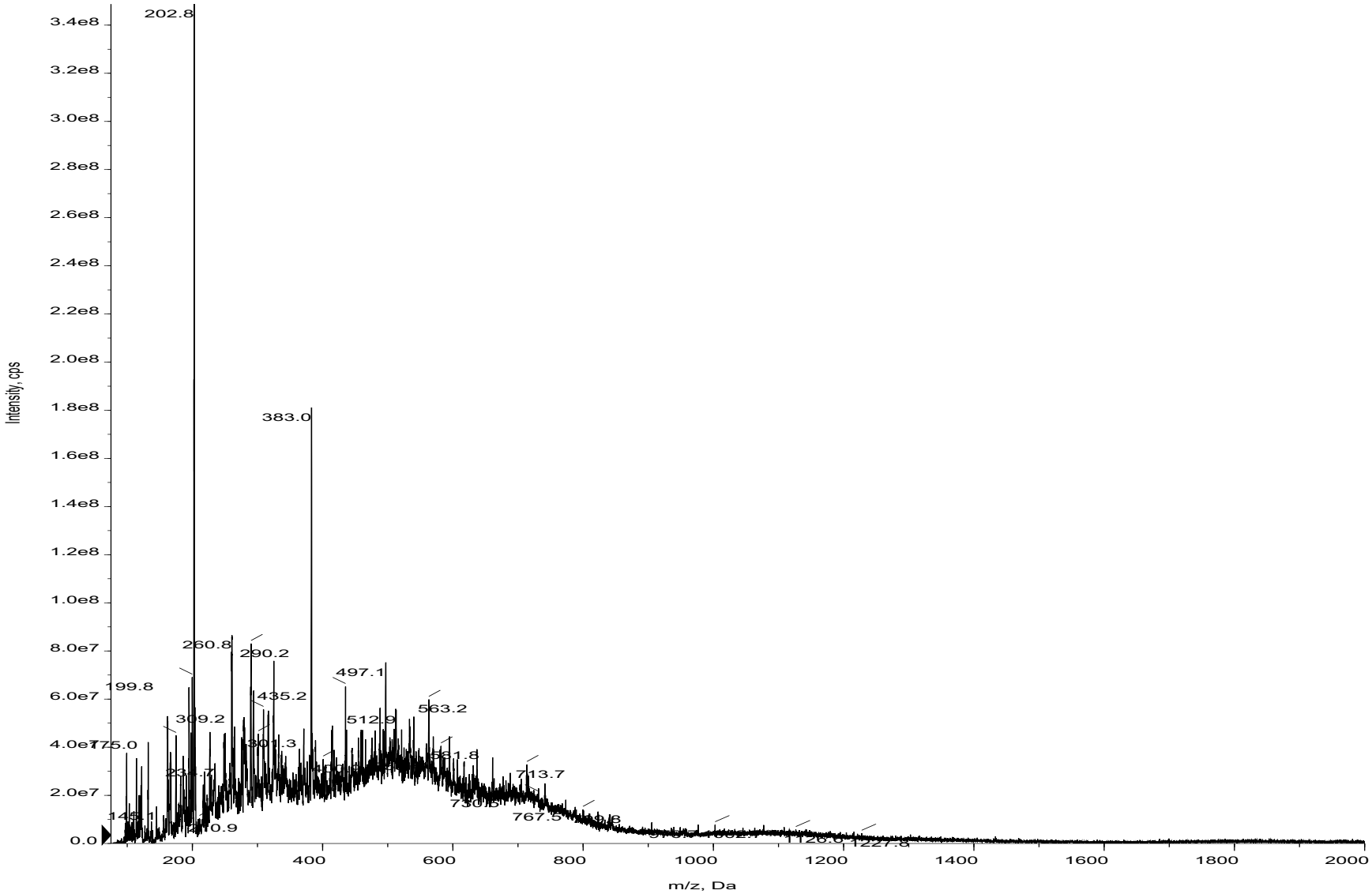


Fig. 46. Liquid chromatography-mass spectrometry chromatogram of Chymotrypsin Hydrolysates AGEs3

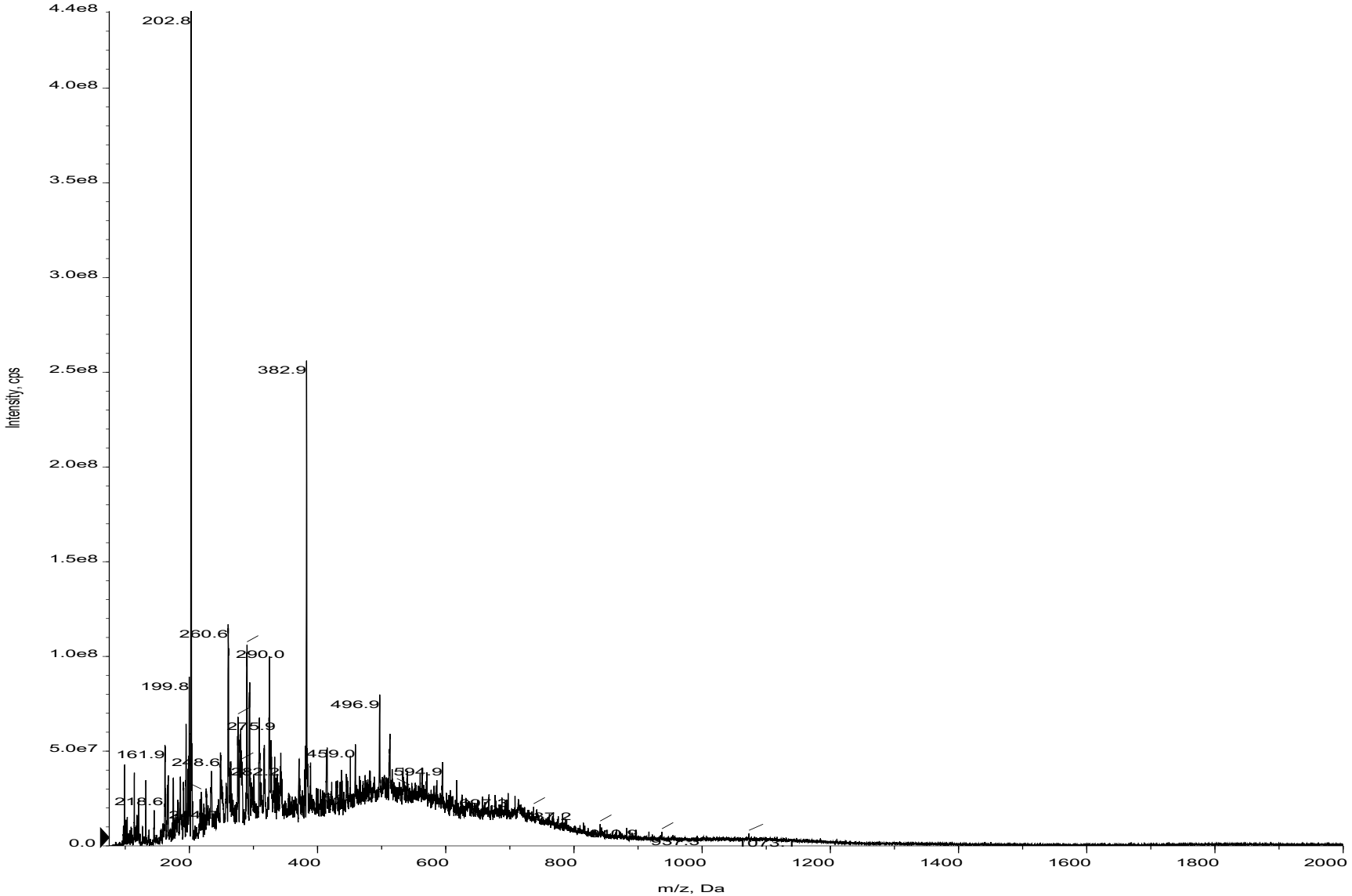


Fig. 47. Liquid chromatography-mass spectrometry chromatogram of Chymotrypsin Hydrolysates AGEs4

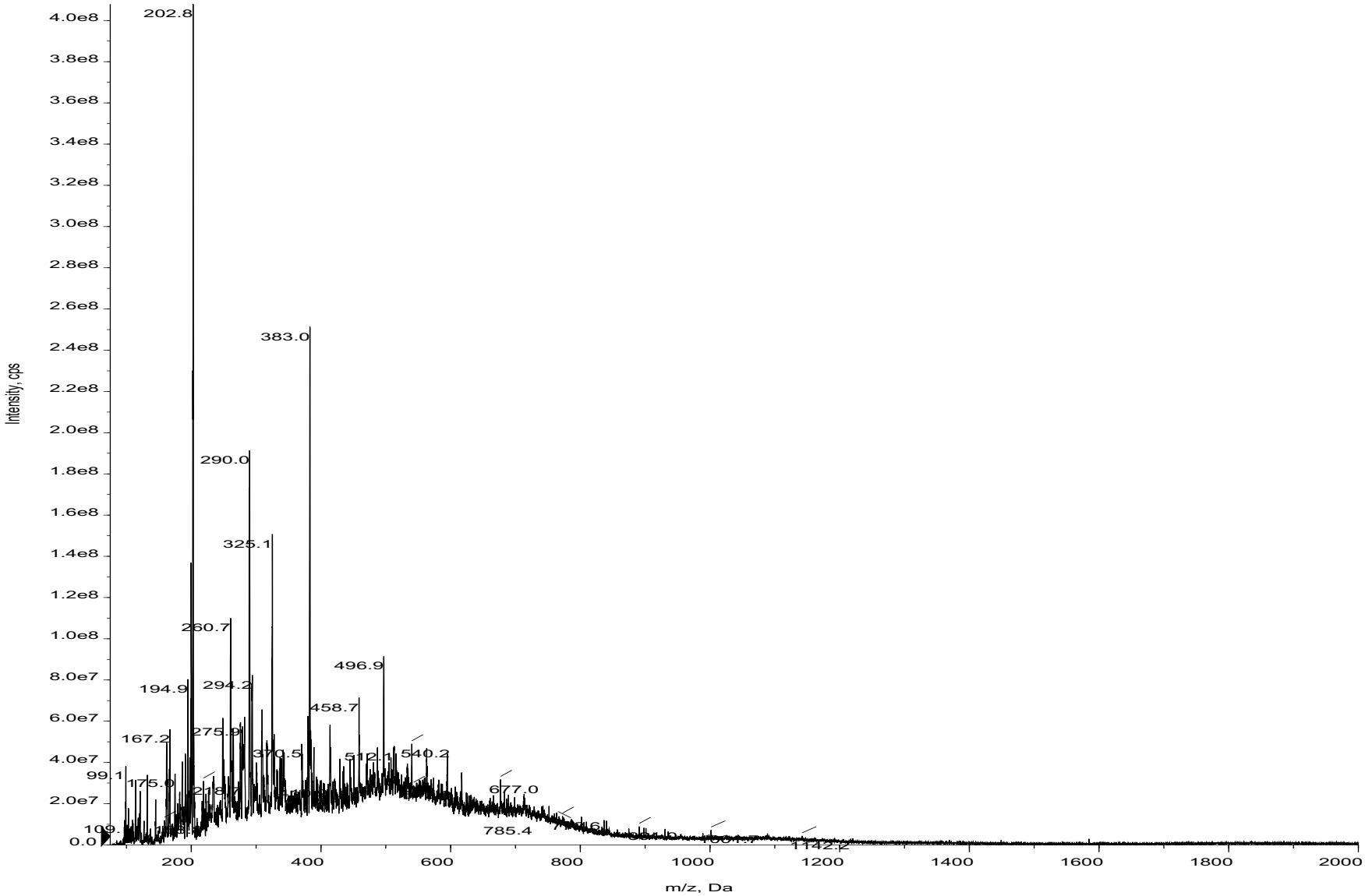


Fig. 48. Liquid chromatography-mass spectrometry chromatogram of Chymotrypsin Hydrolysates AGEs5

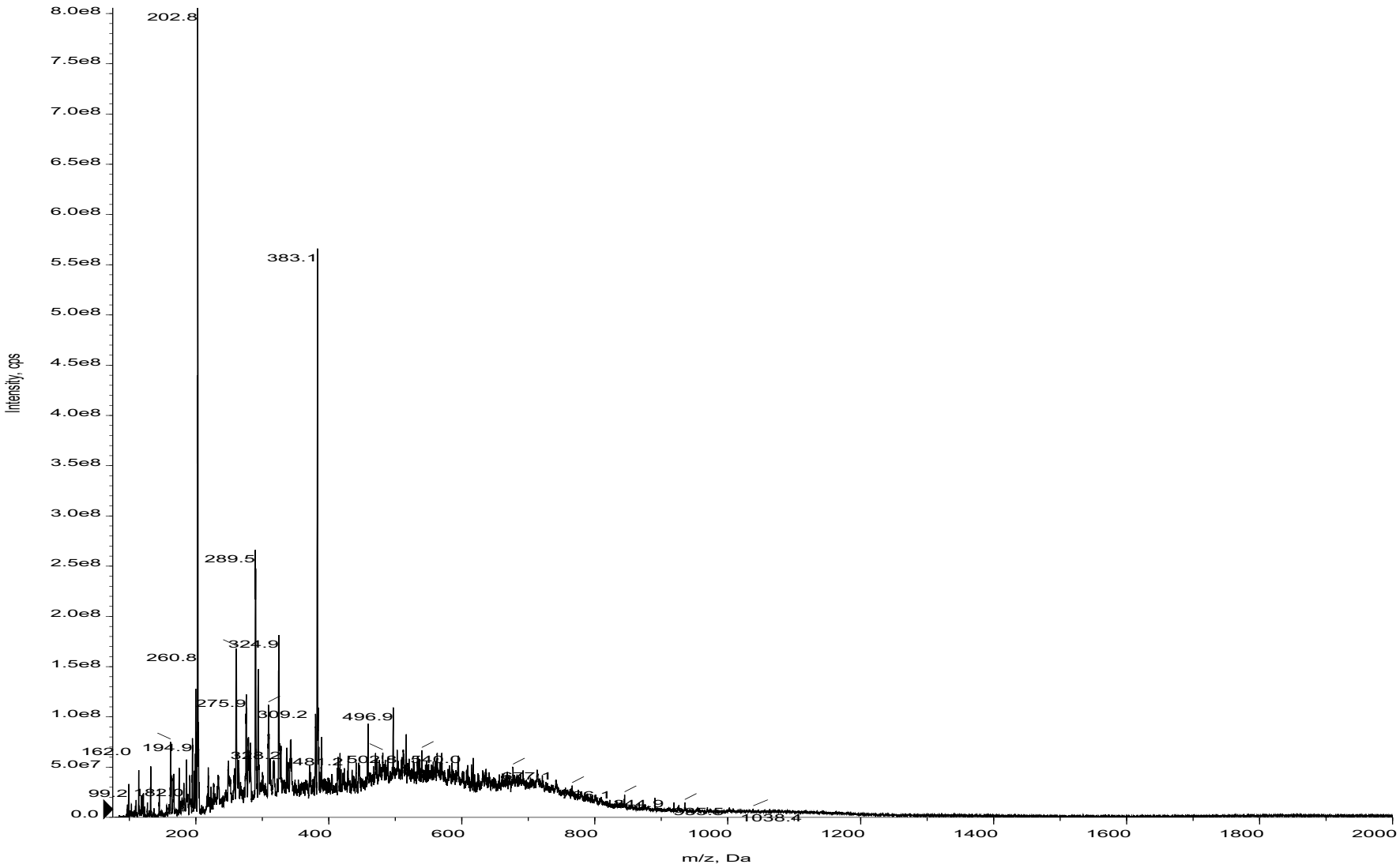


Fig. 49. Liquid chromatography-mass spectrometry chromatogram of Chymotrypsin Hydrolysates AGEs6

CHAPTER FIVE-GENERAL DISCUSSION AND CONCLUSIONS

After the discovery of 25 bitter taste receptors and elucidation of the activation mechanisms of these receptors, the search for bitter taste modulators that can specifically block bitter taste receptors are gaining increasing attention. So far, there are only a few bitter taste inhibitors (GIV 372, GABA, BCML, abscisic acid, probenecid, 6-Methoxylflavanones) reported acting at BTRs levels (Greene et al., 2011; Sai P. Pydi et al., 2015; Sai P Pydi et al., 2014; Roland et al., 2014; Slack et al., 2011). Even though lots of amino acid derivatives and peptides shown bitter taste suppression ability in previous studies, most of them only used human panel or E-tongue to determine bitter taste instead of supplementing vivo experiment to discover mechanisms. This study aims to find some novel peptides and AGEs with low bitter taste and bitter taste inhibitory ability on T2R4. Firstly, beef protein was hydrolyzed by six commercial enzymes with diverse preferential cleavage. Flavourzyme showed the highest DH (47.02%) because it is a mixture of endoprotease and exoprotease (Merz et al., 2015). Alcalase had the second highest DH (35.57%) due to its broad substrate specificity (Guerard & , Dufosse, De La Broise, 2001). Chymotrypsin (25.83%) and trypsin (24.18%) had similar DH. Thermoase (18.26%) and pepsin (8.60%) had the lowest DH. Enzymatic hydrolysis is an important approach to produce novel peptides, and higher DH suggests more peptides are produced, thus more peptides with small molecular weighs was assumed to be generated in the Flavourzyme and Alcalase hydrolysates. However, molecular weight distribution showed that Alcalase hydrolysates had most abundant small peptides with sizes close to 450 Da, while Flavourzyme hydrolysates had big MW. Other enzymatic hydrolysates comprised big molecular weight peptides as well as small molecular peptides. The relationship between molecular weights and bitterness has been extensively studied that 0.36-2.10 KDa peptides was demonstrated suitable to interact with BTRs (Kim et al., 1999; Maehashi & Huang, 2009a). Therefore, Alcalase hydrolysates and chymotrypsin hydrolysate had more peptides that can potentially bind to the bitter taste receptors. Later results of bitter scores of E-tongue supported the theory, that AH had significantly lower bitter score than other hydrolysates and reduced bitter taste of T2R4 agonist quinine. However, there were no significant differences on amino acid compositions of BPH. Calcium assay based on HEK 293 cell system showed that AH had low ability to activate T2R4 while CH extensively activated T2R4, indicating that AH and CH were presenting bitter taste. However, as L-Arginine, a

known bitter taste amino acids, was mixed with NaCl to suppress bitter taste of BCAA (Ogawa et al., 2004; Tokuyama et al., 2006), suggesting that bitter taste compounds also have potential to suppress bitter taste of other substances. Further purified fractions of AH and CH showed increasing deference probably due to the more concentrated effective components. But in terms of the mechanisms, AH and CH fractions unlikely suppressed bitter taste by interacting with T2R4, because the calcium in control cells (HEK 293 cell expressing Ga16/44) was also released obviously. Thus further study about the mechanism is required. Eventually, eight peptides were identified from AH and CH. Every peptide was composed of hydrophobic amino acids and hydrophilic acids but for most of them had more hydrophilic amino acids, which increases solubility of peptides in aqueous solution. The higher solubility makes peptides easier interact with receptors. The same principle has been observed in pharmaceutical industry that in order to improve the efficacy of formulation of protein pharmaceuticals, site-directed mutagenesis have been used to make hydrophobic to hydrophilic mutations on the protein surface (Shaw et al., 2001; Trevino, Scholtz, & Pace, 2007). In this study, some AGEs of AH and CH showed bitter taste suppression ability to quinine and reduced calcium mobilization activated by quinine, the principle of these phenomena partly can be contributed to increased solubility, because Maillard reaction is known to increase surface solubility of protein hydrolysates (J.-S. Kim & Lee, 2009; P. Liu et al., 2012). Besides, multiple pleasant compounds were produced from Maillard reaction (Golon et al., 2014; Liu et al., 2012). Compared to other bitter taste inhibitors, such as GABA and probenecid, one is chief neurotransmitter in mammalian central nerve system (Oláh et al., 2009; Sai P Pydi et al., 2014), and another is an inhibitor of the Multidrug Resistance Protein 1 (MRP1) transporter and also clinically used to treat gout in humans (Greene et al., 2011), identified peptides were extracted from natural food, thus they can be used in daily food with bitter taste such as cheese, yogurt and beer, or even pharmaceuticals. Saccharides were generally used to mask bitter taste of many drugs or added in to drug formula to reduce bitter taste (Ley, 2008; Rachid et al., 2010), however, Maillard reaction may happen between saccharide and some ingredients (Golon et al., 2014). Thus stability of peptides makes them more valuable as bitter taste suppresser in pharmaceutical industry. Moreover, given the proposed kinds of bioactivities of peptides, such as antioxidant, anti-hypertension, immune function and brain function (Je, Lee, Lee, & Ahn, 2009; Onuh et al., 2014; Yimit et al., 2012), therefore, it is possible for these peptides to have more bioactivity apart from suppressing bitterness.

In conclusion, beef protein hydrolysates digested by Alcalase, chymotrypsin, trypsin, pepsin, Flavourzyme, and thermoase as well as their advanced glycation end-products had acceptable slight bitterness. But the RP-HPLC fractions of AH and CH showed remarkable ability to suppress the activation of T2R4 by quinine. Some peptides isolated from these functioning fractions were identified and their parent proteins were all from beef proteins.

CHAPTER SIX-FUTURE RESEARCH

Firstly, from previous studies, many bitter taste masking mechanisms have been reported, such as GABA and BCML because they bind to receptors to disable receptor activation by agonists (Sai P Pydi et al., 2014). Therefore, the identified eight peptides in this study should be synthesized and used for calcium mobilization assay in order to determine their bitter taste inhibitory ability.

Secondly, other bitter taste receptors should be used to detect the suppressing ability of these peptides, because so far found antagonists cannot inhibit activation of all 25 receptor but only a few receptors (Sai P. Pydi et al., 2015; Roland et al., 2014).

Thirdly, quinine is one of T2R4 agonists, suppression its activation of T2R4 does not mean bitter taste of other agonists can be reduced, and thus other T2R4 agonists should be combined with peptides to determine their suppressing ability.

Finally, the AH-AGEs and CH-AGEs showed suppressing ability, but AGEs is a complicated complex comprising abundant compounds; thus, they should be further purified like the protein hydrolysates and used for calcium mobilization assays.

REFERENCES

Aguilar, M. (2004). HPLC of Peptides and Proteins Methods and Protocols. *Methods in Molecular Biology*, *251*, 3–8. https://doi.org/10.1002/0471140864.ps0807s23

Akitomi, H., Tahara, Y., Yasuura, M., Kobayashi, Y., Ikezaki, H., & Toko, K. (2013a). Quantification of tastes of amino acids using taste sensors. *Sensors and Actuators, B: Chemical*, *179*, 276–281.

Akitomi, H., Tahara, Y., Yasuura, M., Kobayashi, Y., Ikezaki, H., & Toko, K. (2013b). Quantification of tastes of amino acids using taste sensors. *Sensors and Actuators, B: Chemical*, *179*, 276–281. https://doi.org/10.1016/j.snb.2012.09.014

Allison A.-M.A., C. I. V. E. (2000). The importance of caffeine as a flavor component in beverages. *Journal of Sensory Studies*, *15*(4), 449–458.

Alpha MOS. (2004). Astree electrochemical sensor technology—Tech- nical note: T-SAS-04.

Aluko, R. E., Girgih, A. T., He, R., Malomo, S., Li, H., Offengenden, M., & Wu, J. (2015). Structural and functional characterization of yellow field pea seed (Pisum sativum L.) protein-derived antihypertensive peptides. *Food Research International*, *77*, 10–16. https://doi.org/10.1016/j.foodres.2015.03.029

Article, R. (2009). Molecular mechanisms of taste transduction in vertebrates, 1–7. https://doi.org/10.1007/s10266-008-0095-y

Bamdad, F., Wu, J., & Chen, L. (2011). Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein. *Journal of Cereal Science*, *54*(1), 20–28. https://doi.org/10.1016/j.jcs.2011.01.006

Barratt-Fornell, A., & Drewnowski, A. (2002). The taste of health: Nature's bitter gifts. *Nutrition Today*, *37*(4). Retrieved from http://journals.lww.com/nutritiontodayonline/Abstract/2002/07000/The_Taste_of_Health__Nature_s_Bitter_Gifts.3.aspx

Behrens, M., Brockhoff, A., Kuhn, C., Bufe, B., Winnig, M., & Meyerhof, W. (2004). The human taste receptor hTAS2R14 responds to a variety of different bitter compounds. *Biochemical and Biophysical Research Communications*, *319*(2), 479–485. https://doi.org/10.1016/j.bbrc.2004.05.019

Behrens, M., Foerster, S., Staehler, F., Raguse, J.-D., & Meyerhof, W. (2007). Gustatory Expression Pattern of the Human TAS2R Bitter Receptor Gene Family Reveals a

Heterogenous Population of Bitter Responsive Taste Receptor Cells. *Journal of Neuroscience*, *27*(46), 12630–12640. https://doi.org/10.1523/JNEUROSCI.1168-07.2007

Behrens, M., & Meyerhof, W. (2006). Bitter taste receptors and human bitter taste perception. *Cell Mol Life Sci*, *63*(13), 1501–1509. https://doi.org/10.1007/s00018-006-6113-8

Behrens, M., & Meyerhof, W. (2010). Oral and extraoral bitter taste receptors. *Results and Problems in Cell Differentiation*, *52*, 87–99. https://doi.org/10.1007/978-3-642-14426-4_8

Belikov VM, G. My. (1986). Plasteins. Their preparation, properties, and use in nutrition. *Nahrung*, *30*(3–4), 281–287.

Berends, P., Appel, D., Eisele, T., Rabe, S., & Fischer, L. (2014). Performance of enzymatic wheat gluten hydrolysis in batch and continuous processes using Flavourzyme. *LWT - Food Science and Technology*, *58*(2), 534–540. https://doi.org/10.1016/j.lwt.2014.03.035

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography B: Biomedical Sciences and Applications*, *336*(1), 93–104. https://doi.org/10.1016/S0378-4347(00)85133-6

Binello, A., Cravotto, G., Nano, G. M., & Spagliardi, P. (2004). Synthesis of chitosan-cyclodextrin adducts and evaluation of their bitter-masking properties. *Flavour and Fragrance Journal*, *19*(5), 394–400. https://doi.org/10.1002/ffj.1434

Boysen, R. I., & Hearn, M. T. W. (2001). HPLC of Peptides and Proteins. *Current Protocols in Protein Science*, *251*, 3–8. https://doi.org/10.1002/0471140864.ps0807s23

Brockhoff, A., Behrens, M., Massarotti, A., Appending, G., & Meyerhof, W. (2007). Broad tuning of the human bitter taste receptor hTAS2R46 to various sesquiterpene lactones, clerodane and labdane diterpenoids, strychnine, and denatonium. *Journal of Agricultural and Food Chemistry*, *55*(15), 6236–6243. https://doi.org/10.1021/jf070503p

Brockhoff, A., Behrens, M., Niv, M. Y., & Meyerhof, W. (2010). Structural requirements of bitter taste receptor activation. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(24), 11110–11115. https://doi.org/10.1073/pnas.0913862107

Bufe, B., Breslin, P. A. S., Kuhn, C., Reed, D. R., Tharp, C. D., Slack, J. P., … Meyerhof, W. (2005). The molecular basis of individual differences in phenylthiocarbamide and propylthiouracil bitterness perception. *Current Biology*, *15*(4), 322–327. https://doi.org/10.1016/j.cub.2005.01.047

Bufe, B., Hofmann, T., Krautwurst, D., Raguse, J.-D., & Meyerhof, W. (2002). The human

TAS2R16 receptor mediates bitter taste in response to β-glucopyranosides. *Nature Genetics*, *32*(3), 397–401. https://doi.org/10.1038/ng1014

Calcagno, C., Lobatto, M. E., Robson, P. M., & Millon, A. (2016). HHS Public Access, *28*(10), 1304–1314. https://doi.org/10.1002/nbm.3369.Three

Chakrabarti, R., Sanyal, S., Ghosh, A., Bhar, K., Das, C., & Siddhanta, A. (2015). Phosphatidylinositol-4-phosphate 5-kinase 1?? modulates ribosomal RNA gene silencing through its interaction with histone H3 lysine 9 trimethylation and heterochromatin protein HP1-?? *Journal of Biological Chemistry*, *290*(34), 20893–20903. https://doi.org/10.1074/jbc.M114.633727

Chandrashekar, J., Mueller, K. L., Hoon, M. A., Adler, E., Feng, L., Guo, W., … Ryba, N. J. . (2000). T2Rs Function as Bitter Taste Receptors. *Cell*, *100*(6), 703–711. https://doi.org/10.1016/S0092-8674(00)80706-0

Charoenphun, N., Cheirsilp, B., Sirinupong, N., & Youravong, W. (2013). Calcium-binding peptides derived from tilapia (Oreochromis niloticus) protein hydrolysate. *European Food Research and Technology*, *236*(1), 57–63. https://doi.org/10.1007/s00217-012-1860-2

Chaudhari, N., & Roper, S. D. (2010). The cell biology of taste. *Journal of Cell Biology*. https://doi.org/10.1083/jcb.201003144

Chen, X. U. E., Pyzik, R., Yong, A., & Striker, G. E. (2013). Advanced Glycation End Products in Foods and a Practical Guide to Their Reduction in the Diet. *J Am Diet Assoc.*, *110*(6), 911–16. https://doi.org/10.1016/j.jada.2010.03.018.Advanced

Choi, D. H., Kim, N. A., Nam, T. S., Lee, S., & Jeong, S. H. (2014). Evaluation of taste-masking effects of pharmaceutical sweeteners with an electronic tongue system. *Drug Development and Industrial Pharmacy*, *40*(3), 308–317. https://doi.org/10.3109/03639045.2012.758636

Clemente, A. (2001). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science and Technology*. https://doi.org/10.1016/S0924-2244(01)00007-3

Cordle, C. T., Mahmoud, M. I., & Moore, V. (1991). Immunogenicity evaluation of protein hydrolysates for hypoallergenic infant formulae. *J Pediatr Gastroenterol Nutr*. https://doi.org/Doi 10.1097/00005176-199110000-00006

Davis, J. K., Lowman, J. J., Thomas, P. J., Hallers, B. F. H. T., Koriabine, M., Huynh, L. Y., … Thomas, J. W. (2010). Evolution of a bitter taste receptor gene cluster in a New World sparrow. *Genome Biology and Evolution*, *2*(1). https://doi.org/10.1093/gbe/evq027

Del Mar Yust, M., Pedroche, J., Alaiz, M., Girón-Calle, J., Vioque, J., Mateo, C., … Fernandez-Lafuente, R. (2007). Partial purification and immobilization/stabilization on highly activated glyoxyl-agarose supports of different proteases from flavourzyme. *Journal of Agricultural and Food Chemistry*, *55*(16), 6503–6508. https://doi.org/10.1021/jf070947l

Dong, D., Jones, G., & Zhang, S. (2009). Dynamic evolution of bitter taste receptor genes in vertebrates. *BMC Evolutionary Biology*, *9*(1), 12. https://doi.org/10.1186/1471-2148-9-12

Dong, S., Wei, B., Chen, B., McClements, D. J., & Decker, E. A. (2011). Chemical and antioxidant properties of casein peptide and its glucose Maillard reaction products in fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *59*(24), 13311–13317. https://doi.org/10.1021/jf203778z

Eckert, M., & Riker, P. (2007). Overcoming challenges in functional beverages. *Food Technology*, *61*(3), 20–26.

Edens, L., Dekker, P., Van Der Hoeven, R., Deen, F., De Roos, A., & Floris, R. (2005). Extracellular prolyl endoprotease from Aspergillus niger and its use in the debittering of protein hydrolysates. *Journal of Agricultural and Food Chemistry*, *53*(20), 7950–7957. https://doi.org/10.1021/jf050652c

Eerlingen, R. C., & Haesendonck, I. Van. (1994). Enzyme-resistant starch. III. The quality of straight-dough bread containing varying levels of enzyme-resistant starch. *Cereal Chem*. Retrieved from http://www.aaccnet.org/publications/cc/backissues/1994/Documents/71_165.pdf

Ensemble, B., & Cited, R. (1998). United States Patent [19], *15*(11), 58–60. https://doi.org/10.1016/j.scriptamat.2005.10.045

Feng, L., Qiao, Y., Zou, Y., Huang, M., Kang, Z., & Zhou, G. (2014). Effect of Flavourzyme on proteolysis, antioxidant capacity and sensory attributes of Chinese sausage. *Meat Science*, *98*(1), 34–40. https://doi.org/10.1016/j.meatsci.2014.04.001

Fletcher, J. N., Kinghorn, A. D., Slack, J. P., McCluskey, T. S., Odley, A., & Jia, Z. (2011). In vitro evaluation of flavonoids from Eriodictyon californicum for antagonist activity against the bitterness receptor hTAS2R31. *Journal of Agricultural and Food Chemistry*, *59*(24), 13117–13121. https://doi.org/10.1021/jf204359q

Friedman, M. (2003). Chemistry, biochemistry, and safety of acrylamide. A review. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/jf030204+

G G. Birch; S E. Kemp. (1989). Apparent specific volumes and tastes of amino acids. *Chem Senses*, *14*(2), 249–258.

Garcia-Amezquita, L. E., Martinez-Alvarenga, M. S., Olivas, G. I., Zamudio-Flores, P. B., Acosta-Mu??iz, C. H., & Sepulveda, D. R. (2014). Effect of Maillard reaction conditions on the degree of glycation and functional properties of whey protein isolate - Maltodextrin conjugates. *Food Hydrocolloids*, *38*, 110–118. https://doi.org/10.1016/j.foodhyd.2013.11.006

Gehrke, C. W., Wall, L. L., Absheer, J. S., Kaiser, F. E., & Zumwalt, R. W. (1985). Sample preparation for chromatography of amino-acids: acid hydrolysis of proteins. *Journal of Association of Official Analytical Chemists*, (68), 811–821.

Girgih, A. T., He, R., Malomo, S., Offengenden, M., Wu, J., & Aluko, R. E. (2014). Structural and functional characterization of hemp seed (Cannabis sativa L.) protein-derived antioxidant and antihypertensive peptides. *Journal of Functional Foods*, *6*(1), 384–394. https://doi.org/10.1016/j.jff.2013.11.005

Girgih, A. T., Udenigwe, C. C., & Aluko, R. E. (2013). Reverse-phase HPLC Separation of Hemp Seed (Cannabis sativa L.) Protein Hydrolysate Produced Peptide Fractions with Enhanced Antioxidant Capacity. *Plant Foods for Human Nutrition*, *68*(1), 39–46. https://doi.org/10.1007/s11130-013-0340-6

Goldin, A., Beckman, J. A., Schmidt, A. M., & Creager, M. A. (2006). Advanced glycation end products: Sparking the development of diabetic vascular injury. *Circulation*, *114*(6), 597–605. https://doi.org/10.1161/CIRCULATIONAHA.106.621854

Golon, A., Kropf, C., Vockenroth, I., & Kuhnert, N. (2014). An Investigation of the Complexity of Maillard Reaction Product Profiles from the Thermal Reaction of Amino Acids with Sucrose Using High Resolution Mass Spectrometry. *Foods*, *3*(3), 461–475. https://doi.org/10.3390/foods3030461

Greene, T. A., Alarcon, S., Thomas, A., Berdougo, E., Doranz, B. J., Breslin, P. A. S., & Rucker, J. B. (2011). Probenecid inhibits the human bitter taste receptor TAS2R16 and suppresses bitter perception of salicin. *PLoS ONE*, *6*(5). https://doi.org/10.1371/journal.pone.0020123

Gu??rard, F., Dufoss??, L., De La Broise, D., & Binet, A. (2001). Enzymatic hydrolysis of proteins from yellowfin tuna (Thunnus albacares) wastes using Alcalase. *Journal of Molecular Catalysis - B Enzymatic*, *11*(4–6), 1051–1059. https://doi.org/10.1016/S1381-

1177(00)00031-X

Guerard, F., & , L. Dufosse, D. De La Broise, A. B. (2001). Enzymatic hydrolysis of proteins from yellowfin tuna (Thunnus albacares) wastes using Alcalase. *Journal of Molecular Catalysis - B Enzymatic*, *11*(4–6), 1051–1059. https://doi.org/10.1016/S1381-1177(00)00031-X

Gupta A K, Madaan S, Dalal M, Kumar, A., Mishra D N, Singh S K, & Verma S. (2010). Practical Approaches for Taste Masking of Bitter Drug: A Review. *International Journal of Drug Delivery Technology*, *2*(2), 56–61.

Gwendolyn, T., Kimberly, S., & Robert, F. (1996). Transduction of bitter and sweet taste by gustducin.

Han, H., & Baik, B. K. (2008). Antioxidant activity and phenolic content of lentils (Lens culinaris), chickpeas (Cicer arietinum L.), peas (Pisum sativum L.) and soybeans (Glycine max), and their quantitative changes during processing. *International Journal of Food Science and Technology*, *43*(11), 1971–1978. https://doi.org/10.1111/j.1365-2621.2008.01800.x

Harwood, M. L., Loquasto, J. R., Roberts, R. F., Ziegler, G. R., & Hayes, J. E. (2013). Explaining tolerance for bitterness in chocolate ice cream using solid chocolate preferences. *Journal of Dairy Science*, *96*(8), 4938–4944. https://doi.org/10.3168/jds.2013-6715

Hedstrom, L., Szilagyi, L., & Rutter, W. J. (1992). Converting trypsin to chymotrypsin: the role of surface loops. *Science*, *255*(5049), 1249–53. https://doi.org/10.1126/science.1546324

Hou, H., Li, B., & Zhao, X. (2011). Enzymatic hydrolysis of defatted mackerel protein with low bitter taste. *Journal of Ocean University of China*, *10*(1), 85–92. https://doi.org/10.1007/s11802-011-1785-6

Ishibashi, N., Kouge, K., & Shinoda, I. (1972). A Mechanismfor BitterTasteSensibility in Peptidest In our previous.

Jaggupilli, A., Howard, R., Upadhyaya, J. D., Bhullar, R. P., & Chelikani, P. (2016). Bitter taste receptors: Novel insights into the biochemistry and pharmacology. *The International Journal of Biochemistry & Cell Biology*, *77*(March), 184–196. https://doi.org/10.1016/j.biocel.2016.03.005

Jaggupilli, A., Singh, N., Upadhyaya, J., Sikarwar, A. S., Arakawa, M., Dakshinamurti, S., … Chelikani, P. (2017). Analysis of the expression of human bitter taste receptors in extraoral

tissues. *Molecular and Cellular Biochemistry*, *426*(1–2), 137–147. https://doi.org/10.1007/s11010-016-2902-z

Je, J. Y., Lee, K. H., Lee, M. H., & Ahn, C. B. (2009). Antioxidant and antihypertensive protein hydrolysates produced from tuna liver by enzymatic hydrolysis. *Food Research International*, *42*(9), 1266–1272. https://doi.org/10.1016/j.foodres.2009.06.013

Ji, M., Su, X., Su, X., Chen, Y., Huang, W., Zhang, J., … Lu, X. (2014). Identification of novel compounds for human bitter taste receptors. *Chemical Biology & Drug Design*, *84*(1), 63–74. https://doi.org/10.1111/cbdd.12293

Kang, B. S., Lee, J. E., & Park, H. J. (2014). Electronic tongue-based discrimination of Korean rice wines (makgeolli) including prediction of sensory evaluation and instrumental measurements. *Food Chemistry*, *151*, 317–323. https://doi.org/10.1016/j.foodchem.2013.11.084

Kannan, A., Hettiarachchy, N. S., Marshall, M., Raghavan, S., & Kristinsson, H. (2011). Shrimp shell peptide hydrolysates inhibit human cancer cell proliferation. *Journal of the Science of Food and Agriculture*, *91*(10), 1920–1924. https://doi.org/10.1002/jsfa.4464

Katsuragi, Y., Sugiura, Y., Lee, C., Otsuji, K., & Kurihara, K. (1995). Selective Inhibition of Bitter Taste of Various Drugs by Lipoprotein. *Pharmaceutical Research: An Official Journal of the American Association of Pharmaceutical Scientists*. https://doi.org/10.1023/A:1016295122461

Keast, R. S. J., Breslin, P. A. S., & Beauchamp, G. K. (2001). Suppression of bitterness using sodium salts. *Chimia*, *55*(5), 441–447.

Kim, H.-O., & Li-Chan, E. C. Y. (2006). Quantitative Structure−Activity Relationship Study of Bitter Peptides. *Journal of Agricultural and Food Chemistry*, *54*(26), 10102–10111. https://doi.org/10.1021/jf062422j

Kim, I. M.-R., Kawamura, Y., & Lee, C.-H. (2003). Isolation and Identification of Bitter Peptides of Tryptic Hydrolysate of Soybean 11S Glycinin by Reverse-phase High-performance Liquid Chromatography. *Journal of Food Science*, *68*(8), 2416–2422. https://doi.org/10.1111/j.1365-2621.2003.tb07039.x

Kim, J.-S., & Lee, Y.-S. (2009). Study of Maillard reaction products derived from aqueous model systems with different peptide chain lengths. *Food Chemistry*, *116*(4), 846–853. https://doi.org/10.1016/j.foodchem.2009.03.033

KIM, M.-R., CHOI, S.-Y., KIM, C.-S., KIM, C.-W., UTSUMI, S., & LEE, C.-H. (1999). Amino Acid Sequence Analysis of Bitter Peptides from a Soybean Proglycinin Subunit Synthesized in *Escherichia coli*. *Bioscience, Biotechnology, and Biochemistry*, *63*(12), 2069–2074. https://doi.org/10.1271/bbb.63.2069

Kim, M. R., Choi, S. Y., & Lee, C. H. (1999). Molecular characterization and bitter taste formation of tryptic hydrolysis of 11S glycinin. *Journal of Microbiology and Biotechnology*, *9*(4), 509–513.

Kindel, G., & De, H. (2010). _ C)_, *1*(19).

Klompong, V., Benjakul, S., Yachai, M., Visessanguan, W., Shahidi, F., & Hayes, K. D. (2009). Amino acid composition and antioxidative peptides from protein hydrolysates of yellow stripe trevally (Selaroides leptolepis). *Journal of Food Science*, *74*(2), 126–133. https://doi.org/10.1111/j.1750-3841.2009.01047.x

Knowles, J. R., Sharp, H., & Greenwell, P. (1969). The pH-dependence of the binding of competitive inhibitors to pepsin. *The Biochemical Journal*, *113*(2), 343–351.

Kohl, S., Behrens, M., Dunkel, A., Hofmann, T., & Meyerhof, W. (2012). Amino Acids and Peptides Activate at Least Five Members of the Human Bitter Taste Receptor Family. *Journal of Agricultural and Food Chemistry*, *61*(1), 53–60. https://doi.org/10.1021/jf303146h

Koo, S. H., Bae, I. Y., Lee, S., Lee, D. H., Hur, B. S., & Lee, H. G. (2014). Evaluation of wheat gluten hydrolysates as taste-active compounds with antioxidant activity. *Journal of Food Science and Technology*, *51*(3), 535–542. https://doi.org/10.1007/s13197-011-0515-9

Kuhn, C. (2004a). Bitter Taste Receptors for Saccharin and Acesulfame K. *Journal of Neuroscience*, *24*(45), 10260–10265. https://doi.org/10.1523/JNEUROSCI.1225-04.2004

Kuhn, C. (2004b). Bitter Taste Receptors for Saccharin and Acesulfame K. *Journal of Neuroscience*, *24*(45), 10260–10265. https://doi.org/10.1523/JNEUROSCI.1225-04.2004

Kurihara, K. (2009). Glutamate: From discovery as a food flavor to role as a basic taste (umami). In *American Journal of Clinical Nutrition* (Vol. 90). https://doi.org/10.3945/ajcn.2009.27462D

Landry, J., & Delhaye, S. (1992). Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *Journal of Agricultural and Food Chemistry*, (40), 776–779.

Levit, A., Nowak, S., Peters, M., Wiener, A., Meyerhof, W., Behrens, M., & Niv, M. Y. (2014). The bitter pill: Clinical drugs that activate the human bitter taste receptor TAS2R14. *FASEB Journal*, *28*(3), 1181–1197. https://doi.org/10.1096/fj.13-242594

Ley, J. P. (2008). Masking bitter taste by Molecules. *Chemosensory Perception*, *1*(1), 58–77. https://doi.org/10.1007/s12078-008-9008-2

Li-jun, L., Chuan-he, Z., & Zheng, Z. (2008). Analyzing molecular weight distribution of whey protein hydrolysates. *Food and Bioproducts Processing*, *86*(1), 1–6. https://doi.org/10.1016/j.fbp.2007.10.007

Li, D., & Zhang, J. (2014). Diet shapes the evolution of the vertebrate bitter taste receptor gene repertoire. *Molecular Biology and Evolution*, *31*(2), 303–309. https://doi.org/10.1093/molbev/mst219

Li, W., Zhao, H., He, Z., Zeng, M., Qin, F., & Chen, J. (2016). Modification of soy protein hydrolysates by Maillard reaction: Effects of carbohydrate chain length on structural and interfacial properties. *Colloids and Surfaces B: Biointerfaces*, *138*, 70–77. https://doi.org/10.1016/j.colsurfb.2015.11.038

Li, Y., Zhong, F., Ji, W., Yokoyama, W., Shoemaker, C. F., Zhu, S., & Xia, W. (2013). Functional properties of Maillard reaction products of rice protein hydrolysates with mono-, oligo- and polysaccharides. *Food Hydrocolloids*, *30*(1), 53–60. https://doi.org/10.1016/j.foodhyd.2012.04.013

Lin, S., Tian, W., Li, H., Cao, J., & Jiang, W. (2012). Improving antioxidant activities of whey protein hydrolysates obtained by thermal preheat treatment of pepsin, trypsin, alcalase and flavourzyme. *International Journal of Food Science and Technology*, *47*(10), 2045–2051. https://doi.org/10.1111/j.1365-2621.2012.03068.x

Lindqvist, M. (2011). Flavour improvement of water solutions comprising bitter amino acids, (277). Retrieved from http://stud.epsilon.slu.se/2232/1/Lindqvist_M_110201.pdf

Liu, F., & Tang, C.-H. (2014). Emulsifying Properties of Soy Protein Nanoparticles: Influence of the Protein Concentration and/or Emulsification Process. *Journal of Agricultural and Food Chemistry*, *62*(12), 2644–2654. https://doi.org/10.1021/jf405348k

Liu, P., Huang, M., Song, S., Hayat, K., Zhang, X., Xia, S., & Jia, C. (2012). Sensory Characteristics and Antioxidant Activities of Maillard Reaction Products from Soy Protein Hydrolysates with Different Molecular Weight Distribution. *Food and Bioprocess*

Technology, *5*(5), 1775–1789. https://doi.org/10.1007/s11947-010-0440-3

Maehashi, K., & Huang, L. (2009a). Bitter peptides and bitter taste receptors. *Cellular and Molecular Life Sciences*, *66*(10), 1661–1671. https://doi.org/10.1007/s00018-009-8755-9

Maehashi, K., & Huang, L. (2009b). Bitter peptides and bitter taste receptors. *Cellular and Molecular Life Sciences*. https://doi.org/10.1007/s00018-009-8755-9

Maehashi, K., Matano, M., Wang, H., Vo, L. A., Yamamoto, Y., & Huang, L. (2008). Bitter peptides activate hTAS2Rs, the human bitter receptors. *Biochemical and Biophysical Research Communications*, *365*(4), 851–855. https://doi.org/10.1016/j.bbrc.2007.11.070

Maehashi, K., Matsuzaki, M., Yamamoto, Y., & Udaka, S. (1999a). Isolation of peptides from an enzymatic hydrolysate of food proteins and characterization of their taste properties. *Bioscience, Biotechnology, and Biochemistry*, *63*(3), 555–559. https://doi.org/10.1271/bbb.63.555

Maehashi, K., Matsuzaki, M., Yamamoto, Y., & Udaka, S. (1999b). Isolation of peptides from an enzymatic hydrolysate of food proteins and characterization of their taste properties. *Bioscience, Biotechnology, and Biochemistry*. https://doi.org/10.1271/bbb.63.555

Matoba, T., & Hata, T. (1972). Relationship between bitterness of peptides and their chemical structures. *Agricultural and Biological Chemistry*, *36*(8), 1423–1431. https://doi.org/10.1271/bbb1961.36.1423

McLaughlin, S.K., McKinnon, P.J., and Margolskee, R. F. (1992). Gustducin is a taste-cell-specific G protein closely related to the transducins .

Meinlschmidt, P., Sussmann, D., Schweiggert-Weisz, U., & Eisner, P. (2016). Enzymatic treatment of soy protein isolates: effects on the potential allergenicity, technofunctionality, and sensory properties. *Food Science and Nutrition*, *4*(1), 11–23. https://doi.org/10.1002/fsn3.253

Melander, C., & Tømmeraas, K. (2008). The influence of sodium hyaluronate molecular weight on protein content according to Lowry and Coomassie blue assays. *Carbohydrate Polymers*, *74*(3), 745–748. https://doi.org/10.1016/j.carbpol.2008.04.001

Mennella, C., Visciano, M., Napolitano, A., del Castillo, M. D., & Fogliano, V. (2006). Glycation of lysine-containing dipeptides. *Journal of Peptide Science*, *12*(4), 291–296. https://doi.org/10.1002/psc.722

Mennella, J. A., Spector, A. C., Reed, D. R., & Coldwell, S. E. (2013). The bad taste of

medicines: Overview of basic research on bitter taste. *Clinical Therapeutics*. https://doi.org/10.1016/j.clinthera.2013.06.007

Merz, M., Eisele, T., Berends, P., Appel, D., Rabe, S., Blank, I., … Fischer, L. (2015). Flavourzyme, an Enzyme Preparation with Industrial Relevance: Automated Nine-Step Purification and Partial Characterization of Eight Enzymes. *Journal of Agricultural and Food Chemistry*, *63*(23), 5682–5693. https://doi.org/10.1021/acs.jafc.5b01665

Meyerhof, W. (2005). Elucidation of mammalian bitter taste. *Reviews of Physiology, Biochemistry and Pharmacology*. https://doi.org/10.1007/s10254-005-0041-0

Meyerhof, W., Batram, C., Kuhn, C., Brockhoff, A., Chudoba, E., Bufe, B., … Behrens, M. (2009). The molecular receptive ranges of human TAS2R bitter taste receptors. *Chemical Senses*, *35*(2), 157–170. https://doi.org/10.1093/chemse/bjp092

Miguet, L., Zhang, Z., & Grigorov, M. G. (2006). Computational Studies of Ligand-Receptor Interactions in Bitter Taste Receptors. *Journal of Receptors and Signal Transduction*, *26*(5–6), 611–630. https://doi.org/10.1080/10799890600928210

Miyanaga, Y., Tanigake, A., Nakamura, T., Kobayashi, Y., Ikezaki, H., Taniguchi, A., … Uchida, T. (2002). Prediction of the bitterness of single, binary- and multiple-component amino acid solutions using a taste sensor. *International Journal of Pharmaceutics*, *248*(1–2), 207–218. https://doi.org/10.1016/S0378-5173(02)00456-8

Montmayeur, J. P., & Matsunami, H. (2002). Receptors for bitter and sweet taste. *Current Opinion in Neurobiology*. https://doi.org/10.1016/S0959-4388(02)00345-8

Namiki, T.; Nakamura, T. (1992). Enhancement of sugar sweetness by furanones and/or cyclotene. Japan: JP 04008264.

Nchienzia, H. A., Morawicki, R. O., & Gadang, V. P. (2010). Enzymatic hydrolysis of poultry meal with endo- and exopeptidases. *Poultry Science*, *89*(10), 2273–2280. https://doi.org/10.3382/ps.2008-00558

Newman, J., Harbourne, N., O'Riordan, D., Jacquier, J. C., & O'Sullivan, M. (2014). Comparison of a trained sensory panel and an electronic tongue in the assessment of bitter dairy protein hydrolysates. *Journal of Food Engineering*, *128*, 127–131. https://doi.org/10.1016/j.jfoodeng.2013.12.019

Ney, K. H. (1971). Prediction of bitterness of peptides from their amino acid composition. *Zeitschrift Für Lebensmittel-Untersuchung Und -Forschung*, *147*(2), 64–68.

Nie, S., Huang, J., Hu, J., Zhang, Y., Wang, S., Li, C., … Xie, M. (2013). Effect of pH, temperature and heating time on the formation of furan in sugar–glycine model systems. *Food Science and Human Wellness*, *2*(2), 87–92. https://doi.org/10.1016/j.fshw.2013.05.001

Nielsen, P. m., Petersen, D., & Dambmann, C. (2001). Improved Method for Determining Food Protein Degree of Hydrolysis. *Journal of Food Science*, *66*(5), 642–646. https://doi.org/10.1111/j.1365-2621.2001.tb04614.x

Nishimura, T., & Kato, H. (1988). Taste of free amino acids and peptides. *Food Reviews International*, *4*(2), 175–194. https://doi.org/10.1080/87559128809540828

O'Meara, G. M., & Munro, P. A. (1985). Hydrolysis of the sarcoplasmic, myofibrillar and connective tissue proteins of lean beef by alcalase and its relationship to whole meat hydrolysis. *Meat Science*, *12*(2), 91–103. https://doi.org/10.1016/0309-1740(85)90018-X

Ogasawara, M., Katsumata, T., & Egi, M. (2006). Taste properties of Maillard-reaction products prepared from 1000 to 5000 Da peptide. *Food Chemistry*, *99*(3), 600–604. https://doi.org/10.1016/j.foodchem.2005.08.040

Ogawa, T., Hoshina, K., Haginaka, J., Honda, C., Tanimoto, T., & Uchida, T. (2005). Screening of bitterness-suppressing agents for quinine: The use of molecularly imprinted polymers. *Journal of Pharmaceutical Sciences*, *94*(2), 353–362. https://doi.org/10.1002/jps.20248

Ogawa, T., Nakamura, T., Tsuji, E., Miyanaga, Y., Nakagawa, H., Hirabayashi, H., & Uchida, T. (2004). The combination effect of L-arginine and NaCl on bitterness suppression of amino acid solutions. *Chemical & Pharmaceutical Bulletin*, *52*(2), 172–7. https://doi.org/10.1248/cpb.59.536

Oh, N. S., Lee, H. A., Lee, J. Y., Joung, J. Y., Lee, K. B., Kim, Y., … Kim, S. H. (2013a). The dual effects of Maillard reaction and enzymatic hydrolysis on the antioxidant activity of milk proteins. *Journal of Dairy Science*, *96*(8), 4899–4911. https://doi.org/10.3168/jds.2013-6613

Oh, N. S., Lee, H. A., Lee, J. Y., Joung, J. Y., Lee, K. B., Kim, Y., … Kim, S. H. (2013b). The dual effects of Maillard reaction and enzymatic hydrolysis on the antioxidant activity of milk proteins. *Journal of Dairy Science*, *96*(8), 4899–4911. https://doi.org/10.3168/jds.2013-6613

Oh, N. S., Lee, H. a, Lee, J. Y., Joung, J. Y., Lee, K. B., Kim, Y., … Kim, S. H. (2013c). The Dual Effects of Maillard Reaction and Enzymatic Hydrolysis on the Antioxidant Activity of

Milk Proteins. *Journal of Dairy Science*, *96*(8), 4899–4911. https://doi.org/10.3168/jds.2013-6613

Oláh, S., Füle, M., Komlósi, G., Varga, C., Báldi, R., Barzó, P., & Tamás, G. (2009). Regulation of cortical microcircuits by unitary GABA-mediated volume transmission. *Nature*, *461*(7268), 1278–1281. https://doi.org/10.1038/nature08503

Oldham, W. M., & Hamm, H. E. (2008). Heterotrimeric G protein activation by G-protein-coupled receptors, *9*(january). https://doi.org/10.1038/nrm2299

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2014). In vitro antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions. *Food Chemistry*, *150*, 366–373. https://doi.org/10.1016/j.foodchem.2013.10.107

Oszmianski, J., Wolniak, M., Wojdylo, A., & Wawer, I. (2007). Comparative study of polyphenolic content and antiradical activity of cloudy and clear apple juices. *Journal of the Science of Food and Agriculture*, *87*(4), 573–579. https://doi.org/10.1002/jsfa.2707

Otagiri, K., Nosho, Y., Shinoda, I., Fukui, H., & Okai, H. (1985). Studies on A Model of Bitter Peptides Including Arginine, Proline and Phenylalanine Residues .1. Bitter Taste of Dipeptides and Tripeptides, and Bitterness Increase of the Model Peptides by Extension of the Peptide-Chain. *Agricultural and Biological Chemistry*, *49*(4), 1019–1026. https://doi.org/10.1271/bbb1961.49.1019

Ottinger, H., Soldo, T., & Hofmann, T. (2003). Discovery and structure determination of a novel Maillard-derived sweetness enhancer by application of the comparative taste dilution analysis (cTDA). *Journal of Agricultural and Food Chemistry*, *51*(4), 1035–1041. https://doi.org/10.1021/jf020977i

Phillips, W. A., Horn, G. W., & Smith, M. E. (1995). Effect of protein supplementation on forage intake and nitrogen balance of lambs fed freshly harvested wheat forage. *Journal of Animal Science*, *73*(9), 2687–2693. https://doi.org//1995.7392687x

Pownall, T. L., Udenigwe, C. C., & Aluko, R. E. (2010). Amino acid composition and antioxidant properties of pea seed (Pisum sativum L.) Enzymatic protein hydrolysate fractions. *Journal of Agricultural and Food Chemistry*, *58*(8), 4712–4718. https://doi.org/10.1021/jf904456r

Pripp, A. H., & Ardö, Y. (2007). Modelling relationship between angiotensin-(I)-converting enzyme inhibition and the bitter taste of peptides. *Food Chemistry*, *102*(3), 880–888.

https://doi.org/10.1016/j.foodchem.2006.06.026

Pronin, A. N., Tang, H., Connor, J., & Keung, W. (2004). Identification of ligands for two human bitter T2R receptors. *Chemical Senses*, *29*(7), 583–593. https://doi.org/10.1093/chemse/bjh064

Pydi, S. P., Bhullar, R. P., & Chelikani, P. (2012). Constitutively active mutant gives novel insights into the mechanism of bitter taste receptor activation. *Journal of Neurochemistry*, *122*(3), 537–544. https://doi.org/10.1111/j.1471-4159.2012.07808.x

Pydi, S. P., Jaggupilli, A., Nelson, K. M., Abrams, S. R., Bhullar, R. P., Loewen, M. C., & Chelikani, P. (2015). Abscisic acid acts as a blocker of the bitter taste G protein-coupled receptor T2R4. *Biochemistry*, *54*(16), 2622–2631. https://doi.org/10.1021/acs.biochem.5b00265

Pydi, S. P., Sobotkiewicz, T., Billakanti, R., Bhullar, R. P., Loewen, M. C., & Chelikani, P. (2014). Amino acid derivatives as bitter taste receptor (T2R) blockers. *The Journal of Biological Chemistry*, *289*(36), 25054–66. https://doi.org/10.1074/jbc.M114.576975

Rachid, O., Simons, F. E. R., Rawas-Qalaji, M., & Simons, K. J. (2010). An Electronic Tongue: Evaluation of the Masking Efficacy of Sweetening and/or Flavoring Agents on the Bitter Taste of Epinephrine. *AAPS PharmSciTech*, *11*(2), 550–557. https://doi.org/10.1208/s12249-010-9402-3

Reed, D. R., Bartoshuk, L. M., Miller, I. J., Duffy, V. B., & Lucchina, L. A. (1999). Localization of a gene for bitter taste perception to human chromosome. *American Journal of Human Genetics*, *64*, 1478–1480. https://doi.org/10.1086/302367

Robert, G. K. (2012). Meat Composition. *Handbook of Meat and Meat Processing, Second Edition*, 45–62. https://doi.org/doi:10.1201/b11479-6\r10.1201/b11479-6

Robert J. Kurtz, ;, & Fuller, W. D. (1997). Umted States Patent, 3–7.

Roland, W. S. U., Gouka, R. J., Gruppen, H., Driesse, M., Van Buren, L., Smit, G., & Vincken, J. P. (2014). 6-Methoxyflavanones as bitter taste receptor blockers for hTAS2R39. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0094451

Roper, S. D. (2007). Signal transduction and information processing in mammalian taste buds. *Pflugers Archiv European Journal of Physiology*. https://doi.org/10.1007/s00424-007-0247-x

Roper, S. D. (2013). Taste buds as peripheral chemosensory processors. *Seminars in Cell and*

Developmental Biology. https://doi.org/10.1016/j.semcdb.2012.12.002

Rudnitskaya, A., Nieuwoudt, H. H., Muller, N., Legin, A., Du Toit, M., & Bauer, F. F. (2010). Instrumental measurement of bitter taste in red wine using an electronic tongue. *Analytical and Bioanalytical Chemistry*, *397*(7), 3051–3060. https://doi.org/10.1007/s00216-010-3885-3

Ruiz-Avila, L., Ming, D., & Margolskee, R. F. (2000). An In vitro assay useful to determine the potency of several bitter compounds. *Chemical Senses*, *25*(4), 361–368. https://doi.org/10.1093/chemse/25.4.361

Ryu, B., Qian, Z. J., & Kim, S. K. (2010). SHP-1, a novel peptide isolated from seahorse inhibits collagen release through the suppression of collagenases 1 and 3, nitric oxide products regulated by NF-??B/p38 kinase. *Peptides*, *31*(1), 79–87. https://doi.org/10.1016/j.peptides.2009.10.019

Sainz, E., Cavenagh, M. M., Gutierrez, J., Battey, J. F., Northup, J. K., & Sullivan, S. L. (2007). Functional characterization of human bitter taste receptors. *The Biochemical Journal*, *403*(3), 537–43. https://doi.org/10.1042/BJ20061744

Sakurai, T., Misaka, T., Ishiguro, M., Masuda, K., Sugawara, T., Ito, K., … Abe, K. (2010). Characterization of the NL - D -Glucopyranoside Binding Site of the Human Bitter Taste Receptor hTAS2R16 * □, *285*(36), 28373–28378. https://doi.org/10.1074/jbc.M110.144444

Sales, J., & Hayes, J. P. (1996). Proximate, amino acid and mineral composition of ostrich meat. *Food Chemistry*, *56*(2), 167–170. https://doi.org/10.1016/0308-8146(95)00201-4

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides*. https://doi.org/10.1016/j.peptides.2010.06.020

Sbarbati, A., Merigo, F., Benati, D., Tizzano, M., & Bernardi, P. (2004). Laryngeal Chemosensory Clusters, *29*(8), 683–692. https://doi.org/10.1093/chemse/bjh071

Schachman, H. K. (2000). CONTENTS, 1–29.

Shahidi, F., & Han, X. (1995). Production and characteristics of protein hydrolysates from capelin (Mallotus villosus). *Food Chemistry*, *53*, 285–293. https://doi.org/10.1016/0308-8146(95)93934-J

Shaw, K. L., Grimsley, G. R., Yakovlev, G. I., Makarov, A. A., & Pace, C. N. (2001). The effect of net charge on the solubility, activity, and stability of ribonuclease Sa. *Protein Science : A Publication of the Protein Society*, *10*(6), 1206–15. https://doi.org/10.1110/ps.440101

Shigenaga, T. (1988). the BitternessAmino of Peptidest.

Siemensma, A. D., Weijer, W. J., & Bak, H. J. (1993). The importance of peptide lengths in hypoallergenic infant formulae. *Trends in Food Science and Technology*, *4*(1), 16–21. https://doi.org/10.1016/S0924-2244(05)80006-8

Singh, N., Pydi, S. P., Upadhyaya, J., & Chelikani, P. (2011). Structural basis of activation of bitter taste receptor T2R1 and comparison with class A G-protein-coupled receptors (GPCRs). *Journal of Biological Chemistry*, *286*(41), 36032–36041. https://doi.org/10.1074/jbc.M111.246983

Singh, N., Vrontakis, M., Parkinson, F., & Chelikani, P. (2011). Functional bitter taste receptors are expressed in brain cells. *Biochemical and Biophysical Research Communications*, *406*(1), 146–151. https://doi.org/S0006-291X(11)00199-9 [pii]\r10.1016/j.bbrc.2011.02.016

Slack, J. P., Brockhoff, A., Batram, C., Menzel, S., Sonnabend, C., Born, S., … Duineveld, K. (2011). Modulation of bitter taste perception by a small molecule hTAS2R antagonist, *20*(12), 1104–1109. https://doi.org/10.1016/j.cub.2010.04.043.Modulation

Slack, J. P., Brockhoff, A., Batram, C., Menzel, S., Sonnabend, C., Born, S., … Meyerhof, W. (2010). Modulation of bitter taste perception by a small molecule hTAS2R antagonist. *Current Biology*, *20*(12), 1104–1109. https://doi.org/10.1016/j.cub.2010.04.043

Solms, J. (1969). The taste of amino acids, peptides and proteins. *Journal of Agricultural and Food Chemistry*, *39*(3), 686–688. https://doi.org/10.1021/jf60164a016

Song, N., Tan, C., Huang, M., Liu, P., Eric, K., Zhang, X., … Jia, C. (2013). Transglutaminase cross-linking effect on sensory characteristics and antioxidant activities of Maillard reaction products from soybean protein hydrolysates. *Food Chemistry*, *136*(1), 144–151. https://doi.org/10.1016/j.foodchem.2012.07.100

Stone, L. M., Tan, S., Tam, P. P. L., & Finger, T. E. (2002). Analysis of cell lineage relationships in taste buds. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(11), 4522–9. https://doi.org/20026432

Sun, L., & Zhuang, Y. (2012). Characterization of the Maillard Reaction of Enzyme-Hydrolyzed Wheat Protein Producing Meaty Aromas. *Food and Bioprocess Technology*, *5*(4), 1287–1294. https://doi.org/10.1007/s11947-010-0406-5

Sun, Y., Hayakawa, S., Ogawa, M., & Izumori, K. (2005). Evaluation of the site specific protein glycation oxidant capacity of rare sugar-protein/peptide conjugatesand antio. *Journal of*

Agricultural and Food Chemistry, *53*(26), 10205–10212. https://doi.org/10.1021/jf051565n

Tada, M., Shinoda, I., & Okai, H. (1984). L-Ornithyltaurine , a New Salty Peptide. *Journal of Agricultural and Food Chemistry*, *32*(1973), 992–996.

Tamura, M., Miyoshi, T., Mori, N., Kinomura, K., Kawaguchi, M., Ishibashi, N., & Okai, H. (1990). Mechanism for the bitter tasting potency of peptides using O-aminoacyl sugars as model compounds. *Agricultural and Biological Chemistry*, *54*(6), 1401–9. https://doi.org/10.1271/bbb1961.54.1401

Tamura, M., Nakatsuka, T., Tada, M., Kawasaki, Y., Kikuchi, E., & Okai, H. (1989). The relationship between taste and primary structure of "delicious peptide" (Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala) from beef soup. *Agricultural and Biological Chemistry*, *53*(2), 319–325. https://doi.org/10.1271/bbb1961.53.319

Tan, J., Abrol, R., Trzaskowski, B., & Goddard, W. A. (2012). 3D Structure Prediction of TAS2R38 Bitter Receptors Bound to Agonists Phenylthiocarbamide (PTC) and 6-n-Propylthiouracil (PROP), *52*, 1875–1885.

Technology, T. C., & Field-effect-, C. M. (n.d.). ASTREE Electrochemical.

Theerasilp, S., Hitotsuya, H., Nakajo, S., Nakaya, K., Nakamura, Y., & Kurihara, Y. (1989). Complete amino acid sequence and structure characterization of the taste-modifying protein, miraculin. *Journal of Biological Chemistry*, *264*(12), 6655–6659.

Thiansilakul, Y., Benjakul, S., & Shahidi, F. (2007). Antioxidative activity of protein hydrolysate from round scad muscle using alcalase and flavourzyme. *Journal of Food Biochemistry*, *31*(2), 266–287. https://doi.org/10.1111/j.1745-4514.2007.00111.x

Toelstede, S., & Hofmann, T. (2008). Sensomics mapping and identification of the key bitter metabolites in Gouda cheese. *Journal of Agricultural and Food Chemistry*, *56*(8), 2795–2804. https://doi.org/10.1021/jf7036533

Tokuyama, E., Shibasaki, T., Kawabe, H., Mukai, J., Okada, S., & Uchida, T. (2006). Bitterness suppression of BCAA solutions by L-ornithine. *Chemical & Pharmaceutical Bulletin*, *54*(9), 1288–92. https://doi.org/10.1248/cpb.54.1288

Townsend, M. W., & DeLuca, P. P. (1988). Use of lyoprotectants in the freeze-drying of a model protein, ribonuclease A. *Journal of Parenteral Science and Technology : A Publication of the Parenteral Drug Association*, *42*(6), 190–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3221279

Trevino, S. R., Scholtz, J. M., & Pace, C. N. (2007). Amino Acid Contribution to Protein Solubility: Asp, Glu, and Ser Contribute more Favorably than the other Hydrophilic Amino Acids in RNase Sa. *Journal of Molecular Biology*, *366*(2), 449–460. https://doi.org/10.1016/j.jmb.2006.10.026

Trusek-Holownia, A. (2003). Synthesis of ZAlaPheOMe, the precursor of bitter dipeptide in the two-phase ethyl acetate–water system catalysed by thermolysin. *Journal of Biotechnology*, *102*(2), 153–163. https://doi.org/10.1016/S0168-1656(03)00024-5

Udenigwe, C. C., & Aluko, R. E. (2011). Chemometric analysis of the amino acid requirements of antioxidant food protein Hydrolysates. *International Journal of Molecular Sciences*, *12*(5), 3148–3161. https://doi.org/10.3390/ijms12053148

Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: Production, processing, and potential health benefits. *Journal of Food Science*. https://doi.org/10.1111/j.1750-3841.2011.02455.x

Ueda, T., Ugawa, S., Ishida, Y., Shibata, Y., Murakami, S., & Shimada, S. (2001). Identification of Coding Single-Nucleotide Polymorphisms in Human Taste Receptor Genes Involving Bitter Tasting, *151*, 147–151. https://doi.org/10.1006/bbrc.2001.5136

Ueda, T., Ugawa, S., Yamamura, H., Imaizumi, Y., & Shimada, S. (2003). Functional interaction between T2R taste receptors and G-protein alpha subunits expressed in taste receptor cells. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *23*(19), 7376–80. https://doi.org/23/19/7376 [pii]

Ueno, Y., Sakurai, T., Okada, S., Abe, K., & Misaka, T. (2011). Human Bitter Taste Receptors hTAS2R8 and hTAS2R39 with Differential Functions to Recognize Bitter Peptides. *Bioscience, Biotechnology, and Biochemistry*, *75*(6), 1188–1190. https://doi.org/10.1271/bbb.100893

Upadhyaya, J., Pydi, S. P., Singh, N., Aluko, R. E., & Chelikani, P. (2010a). Bitter taste receptor T2R1 is activated by dipeptides and tripeptides. *Biochemical and Biophysical Research Communications*, *398*(2), 331–335. https://doi.org/10.1016/j.bbrc.2010.06.097

Upadhyaya, J., Pydi, S. P., Singh, N., Aluko, R. E., & Chelikani, P. (2010b). Bitter taste receptor T2R1 is activated by dipeptides and tripeptides. *Biochemical and Biophysical Research Communications*, *398*(2), 331–335. https://doi.org/10.1016/j.bbrc.2010.06.097

Uribarri, J., Dolores, M., Pía, M., Maza, D., Filip, R., Gugliucci, A., … Wrobel, K. (2015).

Dietary Advanced Glycation End Products and Their Role in Health and Disease 1 , 2, 461–473. https://doi.org/10.3945/an.115.008433.(RAGEs)

Vattem, D. A., & Shetty, K. (2003). Acrylamide in food: A model for mechanism of formation and its reduction. *Innovative Food Science and Emerging Technologies*, *4*(3), 331–338. https://doi.org/10.1016/S1466-8564(03)00033-X

Vummaneni, V., & Nagpal, D. (2012). Taste Masking Technologies : An Overview and Recent Updates. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, *3*(2), 510–524. Retrieved from www.ijrpbsonline.com

W. Fuller; RJ. Qurtz. (1997). Composition comprising bitter- or metallic-tasting eatable and tastand-comprising (hydroxy-, amion- or mercapto)-substituted di:carboxylic acid or derivatives, especially di:peptide containing aspartic acid. US 5,643,955.

Wang, Q., Ma, P., Ma, X., & Fan, D. (2014). Enzymatic hydrolysis of human-like collagen and molecular weight distribution of hydrolysates. *Journal of Chemical and Pharmaceutical Research*, *6*(1), 278–282.

Wang, W., & Gonzalez De Mejia, E. (2005). A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. *Comprehensive Reviews in Food Science and Food Safety*. https://doi.org/10.1111/j.1541-4337.2005.tb00075.x

WHO. (2003). Diet, nutrition and the prevention of chronic diseases. *World Health Organization Technical Report Series*, *916*, i–viii-1-149-backcover. https://doi.org/ISBN 92 4 120916 X ISSN 0512-3054 (NLM classification: QU 145)

Williams, P. G. (2007). Nutritional composition of red meat. *Nutrition & Dietetics*, *64*(Suppl. 4), S113–S119. https://doi.org/10.1111/j.1747-0080.2007.00197.x

Winters, A. L., & Minchin, F. R. (2005). Modification of the Lowry assay to measure proteins and phenols in covalently bound complexes. *Analytical Biochemistry*, *346*(1), 43–58. https://doi.org/10.1016/j.ab.2005.07.041

Wu, C.-H., Huang, S.-M., Lin, J.-A., & Yen, G.-C. (2011). Inhibition of advanced glycation endproduct formation by foodstuffs. *Food & Function*, *2*(5), 224. https://doi.org/10.1039/c1fo10026b

Wu, H.-C., Chen, H.-M., & Shiau, C.-Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomber austriasicus). *Food Research International*, *36*(9–10), 949–957. https://doi.org/10.1016/S0963-9969(03)00104-

2

Wu, J., & Aluko, R. E. (2007). Quantitative structure-activity relationship study of bitter di- and tri-peptides including relationship with angiotensin I-converting enzyme inhibitory activity. *Journal of Peptide Science*, *13*(1), 63–69. https://doi.org/10.1002/psc.800

Wu, X., Onitake, H., Haraguchi, T., Tahara, Y., Yatabe, R., Yoshida, M., … Toko, K. (2016). Quantitative prediction of bitterness masking effect of high-potency sweeteners using taste sensor. *Sensors and Actuators, B: Chemical*, *235*, 11–17. https://doi.org/10.1016/j.snb.2016.05.009

Wu, S. V., Rozengurt, N., Yang, M., Young, S. H., Sinnett-Smith, J., & Rozengurt, E. (2002). Expression of bitter taste receptors of the T2R family in the gastrointestinal tract and enteroendocrine STC-1 cells. *Proceedings of the National Academy of Sciences*, *99*(4), 2392–2397. https://doi.org/10.1073/pnas.042617699

Xu, J., Cao, J., Iguchi, N., Riethmacher, D., & Huang, L. (2013). Functional characterization of bitter-taste receptors expressed in mammalian testis. *Molecular Human Reproduction*, *19*(1), 17–28. https://doi.org/10.1093/molehr/gas040

Yamazaki, T., Narukawa, M., Mochizuki, M., Misaka, T., & Watanabe, T. (2013). Activation of the hTAS2R14 human bitter-taste receptor by (-)-epigallocatechin gallate and (-)-epicatechin gallate. *Bioscience, Biotechnology, and Biochemistry*, *77*(9), 1981–1983. https://doi.org/DN/JST.JSTAGE/bbb/130329 [pii]

Yimit, D., Hoxur, P., Amat, N., Uchikawa, K., & Yamaguchi, N. (2012). Effects of soybean peptide on immune function, brain function, and neurochemistry in healthy volunteers. *Nutrition*, *28*(2), 154–159. https://doi.org/10.1016/j.nut.2011.05.008

Zeng, Y., Zhang, X., Guan, Y., & Sun, Y. (2012). Enzymatic hydrolysates from tuna backbone and the subsequent Maillard reaction with different ketohexoses. *International Journal of Food Science and Technology*, *47*(6), 1293–1301. https://doi.org/10.1111/j.1365-2621.2012.02973.x

Zhang, Q., Ames, J. M., Smith, R. D., Baynes, J. W., Metz, O., & Metz, T. O. (2009). A Perspective on the Maillard Reaction and the Analysis of Protein Glycation by Mass Spectrometry: Probing the Pathogenesis of Chronic Disease. *J Proteome Res.*, *8*(2), 754–769. https://doi.org/10.1021/pr800858h.A

Zhao, C. J., Schieber, A., & Gänzle, M. G. (2016). Formation of taste-active amino acids, amino

acid derivatives and peptides in food fermentations? A review. *Food Research International*. https://doi.org/10.1016/j.foodres.2016.08.042