Chemical analysis and potential application of corn bio-ethanol co-products

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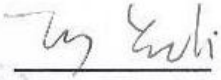
Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously."



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Summary

Due to global warming and concerns of depletion of fossil fuel, bioethanol, a type of renewable energy, is highly sought after. In 2013, bioethanol production exceeded 50 billion liters in USA. Of that production, 82% of ethanol facilities employed dry grind processing technology from corn. The increased production of bioethanol is accompanied by a significant increase in the quantity of its major co-product, distillers dried grains with solubles (DDGS). Conventional usage of DDGS is livestock feed, but due to its high unsaturated fatty acids content and lack of lysine, tryptophan, and carbohydrates, DDGS can only be fed less than 30% of total mix ration (TMR) an thus its disposal or utilization is a challenge for bioethanol industry.

Aiming at maximizing the value of the huge amount of surplus ethanol co-products, this thesis proposed a process from which ingredients for potential dietary supplements, coating materials, and seasoning can be obtained. Extraction with ethyl acetate and absolute alcohol from seven type of bioethanol plant feeds (1, Liquefied corn mash; 2, Beer; 3, Whole stillage; 4, Syrup; 5, Corn oil; 6, Wet distillers' grain with solubles (WDGS); 7, DDGS) were analyzed for phenolic acids, α-tocopherol, and carotenoids and their changes during the producing process of DDGS was found. The combined effect of depletion of starch, fermentation and heat treatment result in fluctuant content of these compounds. Among the seven samples, whole stillage has the second highest content of phenolic acids (14.9±1.78 mg/100 g)

and highest contents of carotenoids (14.4±0.87 mg/100 g) and α-tocopherol (6.49±0.36 mg/100 g) on dry weight basis, becoming best raw material for extraction of lipophilic compounds.

The residues from above extraction were further extracted with aqueous alcohol (70%) to obtain zein. Both zeins isolated from whole stillage and DDGS showed close molecular profiles with commercial zein in SDS-PAGE analysis, while the content of zein in the extracted sample from whole stillage was higher than that from DDGS. The resulting residues after removal of lipophilic compounds and zein, was further extracted for the nucleotides from yeast and water-soluble proteins. The nucleic acid were hydrolyzed to give 5′-mononucleotides by Nuclease P1 to yield 5′-GMP (0.66±0.06 mg/g), 5′-AMP (0.64±0.03 mg/g) in dry DDGS, and 5′-GMP (0.94±0.07 mg/g), 5′-AMP (0.96±0.04 mg/g) in dry whole stillage. Furthermore, the proteins were hydrolyzed into amino acids and peptides by the combination of Protamex and Flavourzyme and obtained 2.81±0.16 mg/g glutamic acid in dry DDGS or 3.60±0.27 mg/g glutamic acid in dry whole stillage after 32 hours' hydrolysis. Therefore, the aqueous fraction may have potential to be developed into seasoning with flavour enhancers including 5′-GMP, 5′-AMP (can be deaminized to 5′-IMP), and glutamic acid (MSG).

Overall, our findings suggest bio-ethanol coproducts, typically whole stillage and DDGS, can be utilized in the above novel and efficient process, which is promising and practical to significantly improve the commercial value of co-products of biofuel.

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List of Abbreviations

WDGS	Wet distillers' grains with solubles
DDGS	Dried distillers grains with solubles
HPLC	High-performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis
GMP	Guanosine monophosphate
AMP	Adenosine monophosphate
UMP	Uridine monophosphate
CMP	Cytidine monophosphate
MSG	Monosodium glutamate
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
CGM	Corn gluten meal
DH	Degree of hydrolysis

Chapter 1 Introduction

1.1 Corn fermentation co-products

Due to the concern of global warming and increased price of fossil fuels, the biofuel industry, as a type of renewable energy source, witnessed rapid growth over the past decades and the increasing trend is expected to continue for the foreseeable future (*1, 2*). In 2013, bioethanol production exceeded 50 billion liters in USA, 82% of which was from the corn dry-grind process for ethanol production (*3*). Another type of process is wet-milling, in which corn is steeped in water to soften the kernel fraction and separate it from other components. The co-products in wet-milling process include corn gluten meal (CGM) and corn oil. The main difference between the two processes is in the initial treatment of corn.

In dry-grind process for ethanol production, also called dry milling (**Figure 1.1**), the whole grain is first ground into flour, which is with a mean particle diameter of approximately 1 mm (*4*). The flour is referred as "meal" in the industry and will be processed without any separation from other components of the grain. The meal is then with water and form a liquefied mash. In the presence of enzymes, starch of the corn is converted to dextrose, a simple sugar that can be fermented by yeast. After high-temperature sterilization, the saccharified mash is cooled and fermented in fermenters with the addition of brewer's yeast, from which the conversion of sugar to ethanol and carbon dioxide (CO_2) begins. After 40 to 50-hour of fermentation, the obtained "beer" is distilled to separate ethanol from the remaining "whole stillage" (*5*),

an aqueous slurry of yeast cells and corn kernel residues after grinding (*4*).The whole stillage is centrifuged to separate the coarse grain from the solubles, producing “thin stillage” (the liquid fraction) and “wet distillers’ grains” (the solids fraction). The thin stillage are then concentrated to about 30% solids by evaporation, resulting in condensed distillers solubles (CDS) or "syrup" (*6*). The combination of coarse grain and the syrup is called wet distillers’ grains with solubles, WDGS. WDGS can only be sold in the local place due to short shelf-life, and thus it is dried to produce dried distillers grains with solubles (DDGS).

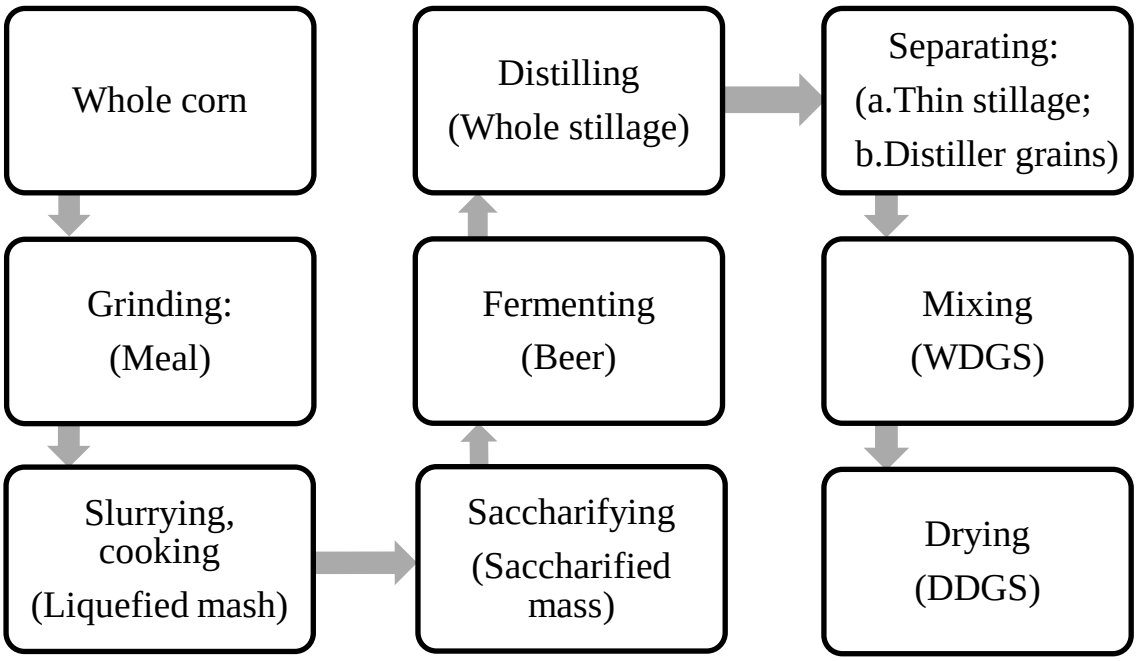

Figure 1.1 General flow diagram of a dry grind ethanol process from corn

In dry grind process of corn, each bushel (25.4 kg) of ground corn can be fermented to produce 2.8 gallons (10.6 L) of ethanol in optimal process (*5*). Since the starch portion accounts for only 65-70% of the kernel, the remaining 25-30% is recovered and sold as DDGS at the price of around $100 per ton, fluctuating based on

its various nutrient quality (*7*). The increased production of bioethanol is accompanied by a significant increase in the quantity of its major and low-value co-product, DDGS. In 2009, with over 10 billion gallons of bioethanol production from corn, there were 25 million tons of DDGS generated in the United States (*8*).

Conventional usage of DDGS is livestock or poultry feed. However, it is not an ideal animal feed because its high unsaturated fatty acids contents leads to rancidity problem. It is not nutritionally balance because it lacks essential amino acids such as lysine and tryptophan. Yet the presence of ruminally indigestible proteins make harder to digest. Therefore, DDGS can only be fed less than 30% of total mix ration (TMR) for most livestock (*9, 10*). It was found that egg yolk from hens fed with a diet with DDGS tend to have lower protein contents (*10*). Therefore, the generally accepted percentage of DDGS in layer's diet is only 10% (**Table 1.1**) (*11*). Another limitation of DDGS utilization in animal feed industry is the high variation in nutritional quality and nutrient concentration among DDGS from various sources, resulting inconsistency in nutritional value and difficulty in making regulated feed formations (*12*).

Other growing new energy sources such as shale gas, solar, and wind energy are strong competitors for bioethanol. The price of bioethanol could be constrained and the DDGS oversupply as animal feed would make the bioethanol industry less profitable while creates environmental problem if the DDGS were to be disposed as it is. It is, therefore, a grand challenge for bioethanol industry to find alternative and value-added usage of DDGS and other co-products.

Table 1.1 Generally accepted DDGS diet inclusion levels (*11*)

Species		Maximum % of total ration (DM)
Cattle	Lactating dairy cows	20%
	Beef feeders	30-40%
Swine	Weaned pigs	25%
	Grow-Finish	20%
	Gestation	50%
	Lactation	20%
Poultry	Broilers	15%
	Layers	10%

1.2 Composition of corn dry-grind process co-products

Understanding the chemical compositions of dry grind ethanol by products is essential to find new use of DDGS. Since DDGS can be considered as the residue of corn after removal of starch, other chemical components in DDGS concentrate approximately 3-fold compared with in ground corn (*8*). Although most of studies on DDGS composition aimed at evaluating it as feed ingredient, their results are essential references for us to explore valued-added compounds. Spiehs el al. built a database of the composition of 118 DDGS samples which were collected from 10 facilities in United States. On average, major components in DDGS are crude protein (30.2%), crude fat (10.9%), crude fibre (8.8%), acid detergent fibre (16.2%), ash (5.8%) and

starch (5.1%) on dry weight basis (*13*). Han and Liu have found that during dry grind ethanol processing protein was concentrated by 3.6 times and oil was concentrated by 3.4 times upon conversion from ground corn to DDGS (*3*). Kim et al. reported the forage/feed nutritional analysis, in which glutamic acid comprised 5.5%, as the highest amino acid in WDGS on dry basis (*14*). Therefore, the potential application of DDGS can be considered from two aspects as bellows:

(a) Lipophilic fraction

Crude oil comprised around 10% in DDGS (*14*, *15*). With corn as the raw material, DDGS has been known to be rich in phenolic acids (*16*), xanthophyll carotenoids (*15*), phytosterols and tocopherol (*17*), and zein (*18*). Furthermore, corn oil extracted from DDGS is expected to have 2-3 times higher content of these bioactive compounds as the result of removal of starch during fermentation (*19*). In the crude oil fraction, Winkler-Moser et al. compared the fatty acid composition, acid value in the oil extracted from thin stillage and DDGS with corn germ oil and found that post-fermentation corn oils are with higher content of functional lipids (tocotrienols, phytosterols, stearyl ferulates, and carotenoids) and antioxidant capacity than corn germ oil (*15, 17*).

However, current studies focus mostly on the content of a single or specific class of lipophilic compounds. For instance, zein (soluble in ethanol) is neglected in previous study even though it can be co-extracted with other lipophilic compounds, Reclaiming zein from DDGS would significantly enhance its value proposition because zein has found increasing usage as edible biopolymers for food packaging

and nano-encapsulation (*20*-*23*). Despite the previous research on potential value of DDGS and other ethanol co-products, a systematic and comprehensive study is lacking.

Therefore, to increase the commercial value of DDGS, it is necessary to provide a comprehensive picture of the changes of the valued lipophilic compounds during dry-grind processing of corn. These compounds include phenolic acids, tocopherols, phytosterols, carotenoids, and zein.

(b) Hydrophilic fraction

Few studies have investigated the hydrophilic fraction in DDGS. After extraction of lipophilic compounds, the residues consist of yeast cells, corn proteins and fibre.

Yeast is known to be abundant in nucleotides, B-Complex vitamins and proteins, and thus it can significantly increase the nutritive value of DDGS compared to corn (*24*). Based on an amino acid composition multiple regression model, Han and Liu estimated that in DDGS, about 20% of the protein is contributed from yeast (*3*). Yeast extract has already been well-studied and commercialized as a dietary supplement for its nutrients and natural origin (*25, 26*). With high content of nucleotides and glutamic acid in yeast, a seasoning that contained 5′-GMP, 5′-IMP has been developed from yeast in industrial scale since 1960s (*27*). Despite of such mature technology, recycling yeast nucleotides and glutamic acid from bio ethanol co-products, especially residues after lipophilic compounds extracted, remains unexplored.

In addition to the four basic tastes sweet, bitter, salty and sour, a fifth taste, “umami” (flavour), has been found in traditional seasonings of Japanese cuisine.

Flavour enhancers refer to those compounds with no intrinsic flavour of their own, but when they are added in low concentrations to appropriate foods, the palatability of the food can be improved distinctly (*28*). Glutamic acid is often used as a food additive and flavour enhancer in the form of its salt, known as monosodium glutamate (MSG). The substance was discovered and identified by Ritthausen in 1866 (*29*), and the crystal of MSG was obtained in 1908 by Ikeda after the evaporation of Kombu as glutamic acid (*30*). The flavour of MSG was then termed as umami, which is a Japanese word for pleasant savory taste. Glutamic acid is present in every protein-containing food, but it can only be tasted when it is present in an unbound form or glutamic acid containing low-molecular-weight peptides, such as "delicious peptide" (Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala) from beef soup (*31*). Therefore, hydrolysis of protein into free amino acids is one of the approaches to MSG. Glutamic acid accounts for the highest percentage in DDGS (3%) among all the amino acids, indicating DDGS is an ideal source for glutamic acid via protein hydrolysis.

1.3 Objectives

The broad objective of this research is to develop a novel process to significantly increase the commercial value of bioethanol co-products. This approach is supposed to be practical and economical for industrial application, and cost-effective method is ideal. The specific aims of this project include:

1) Identify and quantify the contents of bioactive small molecular compounds in DDGS and study their changes during dry-grind process;
2) Explore the feasibility of reclaim nucleotides from the yeast RNA from the

co-products.

3) Isolate and characterize zein from the co-products in terms of extractability and quality.

4) Study the feasibility of preparing flavour active components from DDGS by applying proteases.

Chapter 2 Functional lipophilic compounds in corn fermentation co-products

2.1 Introduction

(1)Phenolic acids

The intake of whole-grain maize has been revealed to reduce the risk of chronic disease, including type II diabetes, cardiovascular disease, obesity and cancer (*15*). The health benefits are contributed partly by phenolic compounds for their antioxidant capacity (*19*). Phenolic acids are found mainly in plants while human and animals are lack of the pathway to synthesize them (*5*). DDGS has phenolic acids contents 3.4-fold higher than that in the ground corn (*26*). Phenolic acids contents in nine fractions were analyzed for dry-grind process co-products including ground corn; cooked slurry; liquefied slurry; fermented mash; whole stillage; thin stillage; condensed distillers soluble (CDS); distillers wet grains (DWG); and distillers dried grains with solubles (DDGS)). Among the nine materials, distillers wet grains (DWG, also referred as WDGS) were found to have highest total concentration of phenolic acids (*16*), which composed of mainly vanillic acid, caffeic acid, coumaric acid, and ferulic acid **(Figure 2.1**).

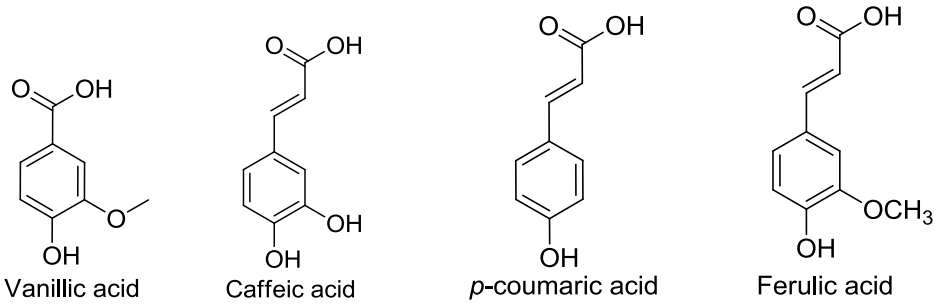

Figure 2.1 Chemical structures of different phenolic acids

(2)Tocopherols

Tocopherols (**Figure 2.2**) are widely spread in vegetable oil as a well-known antioxidant, among which α-tocopherol has the highest antioxidant capacity (*19*). In crude oil extracted from DDGS and thin stillage, tocopherols and carotenoids were found to be higher than that in corn germ oils, and hexane-extracted oil from DDGS was with the highest oxidative stability as evaluated by OSI and storage test at 40 ℃, followed by centrifugally-extracted thin stillage oil (*32*). Extracting solvents of tocopherols included hexane, ethanol and supercritical carbon dioxide ($SCCO_2$). Among them, $SCCO_2$ turned out to be efficient solvents to extract corn DDG oil with high level of tocopherols, tocotrienols and phytosterols, however it is costly comparing to conventional solvent extraction (*17*).

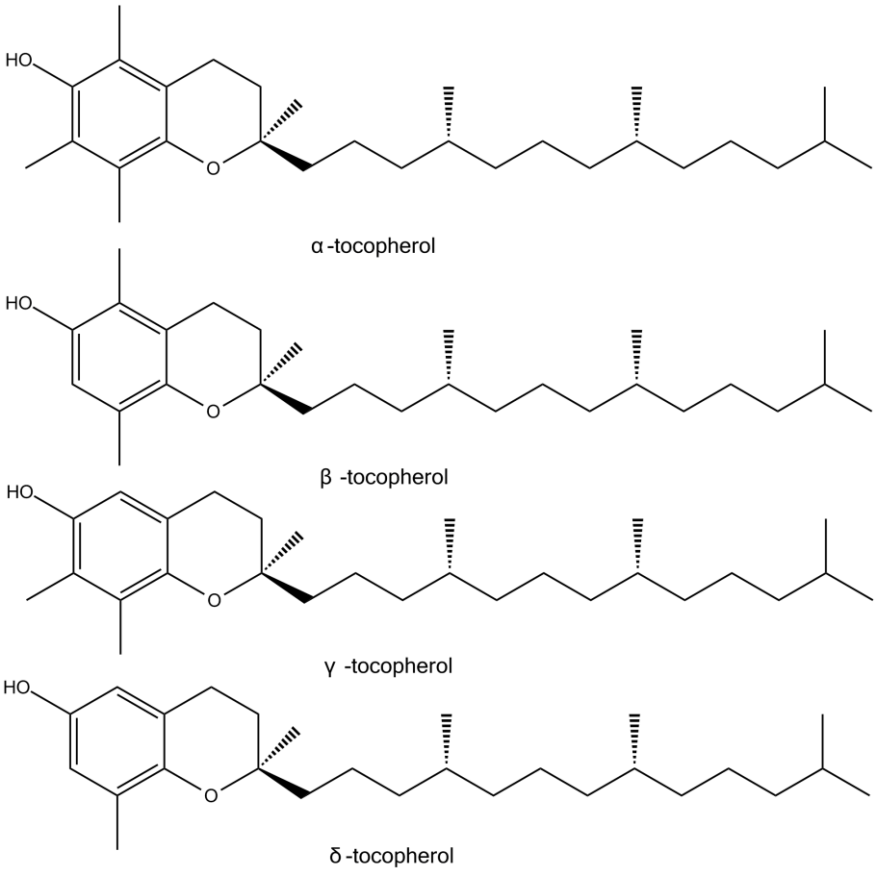

Figure 2.2 Chemical structures of α, β, γ, and δ-tocopherol

(3) Carotenoids

Carotenoids are a variety of natural pigments that result in the yellow, orange and red colors of grains, vegetables and fruits. Carotenoids in corn consist mainly of zeaxanthin, lutein, β-carotene and cryptoxanthin (**Figure 2.3**). Zeaxanthin and lutein, a pair of geometric isomers, are examples of xanthophyll carotenoids that are recognized to have antioxidant properties and act as radical scavengers and singlet oxygen quenchers (*33*). Carotenoid-rich foods have been found to maintain skin health and reduce the risk of degenerative diseases, such as cancer, cardiovascular diseases, and age-related macular degeneration (*34*, *35*).

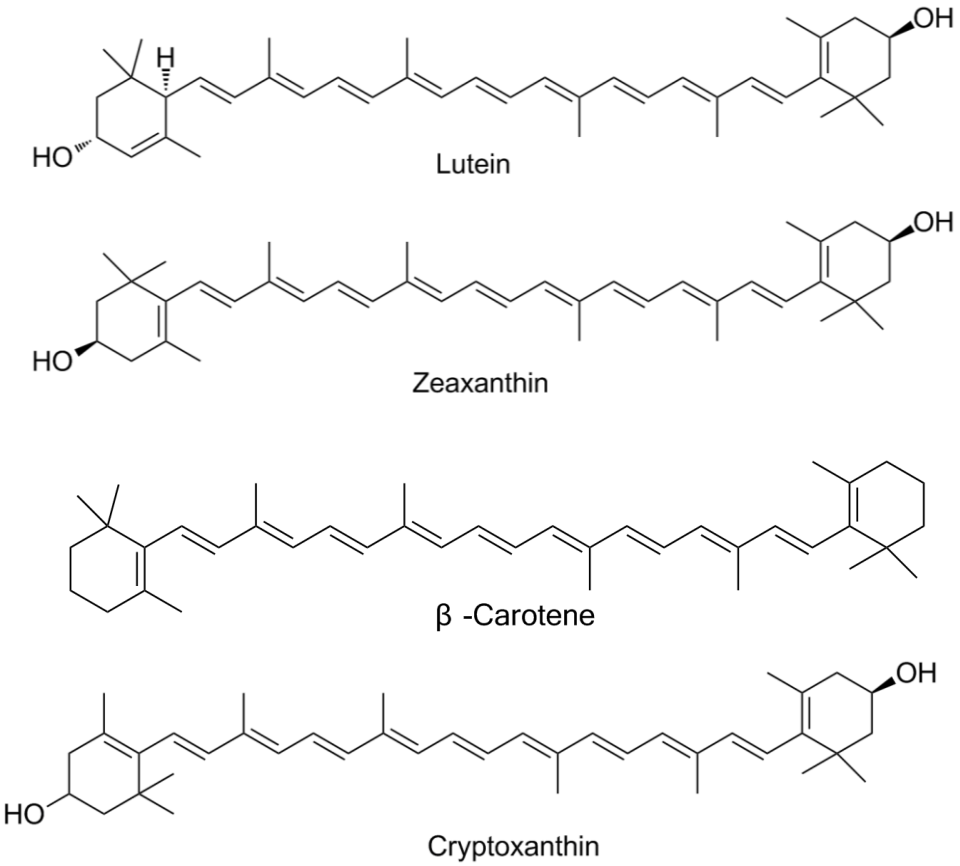

Figure 2.3 Chemical structures of major carotenoids in corn

In corn, the total carotenoids content in whole corn is 11-30 mg/kg , most of which are found in the horny endosperm (*36*). Commercial corn gluten meal, a co-product in bio-ethanol wet milling process, had seven times higher concentration of xanthophyll carotenoids (145 μg/g) than that of yellow dent corn (21.97 μg/g) and eight times higher than that of de-oiled corn (18 μg/g), which indicated xanthophyll carotenoids mostly exist in corn oil (*37*).

(4) Zein

Proteins are mainly located in endosperm and germ of corns. Among the various proteins, albumins and globulins concentrated in the germ, while prolamin-type proteins were found mainly in the endosperm (*38*). Zein is the main and unique prolamin in corn which is not ideal as food ingredients for human because of its

notable absence of tryptophan and lysine and poor solubility in water. However, the property of zein in terms of insolubility in water, resistance to grease, glossy appearance, resistance to microbial attack and large amount of source make it an ideal biopolymer in coating, plastics, textiles and adhesives (*18*). However, the lack of cost-effective zein manufacturing method is the main reason that limits broad commercial application of zein (*18, 39*). There is only 500 tons of zein produced per year dominantly from corn gluten meal with high price of US$10–40 per kilogram (*38*).

Corn zein consists of α，β，γ and δ according to their different solubility in 2-propanol (*40*). Among them, α and δ-zein are true prolamins, while others are glutelins on the basis of Osborne's solubility principles (*41*). In SDS-PAGE, α-zein showed electrophoretic bands at 40, 22 and 19 kDa, corresponding to α-zein dimer, α_1-zein and α_2-zein respectively; δ-zein showed electrophoretic bands at 10 kDa. The α-zein extracted from DDGS was found to retain its film forming capability (*39*). The characteristic property of α-zein recovered by aqueous ethanol from DDG is not affected by hydrolysis and fermentation (*17*).

2.2 Materials and methods

2.2.1 Samples preparation

Seven fractions of bioethanol co-products (1, Liquefied mash; 2, Beer; 3, Whole stillage; 4, Syrup; 5, WDGS; 6, DDGS; 7, Corn oil) were collected at different stages of dry-grinding process from Lakeview Energy LLC, (Chicago, USA). Corn oil was

obtained from high-speed centrifugation of whole stillage and the processing of other six samples can be referred to the flow diagram in **Figure 1.1**. All the samples other than DDGS were frozen at -20 ℃ during transportation and storage.

Two grams of each sample, except for corn oil, was extracted three times, successively with ethyl acetate (100 mL), absolute ethanol (100 mL), and 70% ethanol (100 mL). Each extraction was operated by shaking 5 h and followed by centrifugation and filtration to obtain the solutions. The solutions were combined and the solvent was removed by rotary evaporator under reduced pressure. The recovered oil mixture was washed by ethyl acetate (EA, 10 mL) for three times to separate other lipids from zein. The EA-insoluble residues (mainly zein), were subsequently dissolved in 70% ethanol (30 mL). The solvents were removed under reduced pressure again to afford oil and zein respectively. The yields of oil and zein are stated in **Table 2.3**. The oil was dissolved in ethanol with concentration of 50 mg/mL for HPLC analysis. The zein was dissolved in aqueous ethanol with concentration of 50 mg/mL for SDS-PAGE analysis.

2.2.2 HPLC analysis

Phenolic acid standards (vanillic, caffeic, *trans-p*-coumaric, ferulic acid) and α-tocopherol bought from Sigma-Aldrich (St. Louis, Missouri, USA). Lutein (40 mg/capsule) was purchased from GNC (Pittsburgh, Pennsylvania, USA) and was used as a reference standard for lutein.

The HPLC of oil solutions were carried out on Waters 2695 HPLC system coupled with a photodiode array detector (PDA) (Waters 2996), an auto-sampler (Waters 717 plus). The HPLC column was a 250×4.6 mm, 5μm RP C18 column (Waters, Atlantis T3). The mobile phase consisted of A (0.04% acetic acid in deionized water) and B (0.04% acetic acid in methanol). The gradient procedure for HPLC separation is listed in **Table 2.1**.

Identification of phenolic acid (vanillic, caffeic, p-coumaric, and ferulic acid), lutein, and α-tocopherol were based on comparing the retention time and UV absorbance of the respective compounds.

Table 2.1 Gradient procedure for chromatographic separation

Time/min	Flow rate mL/ min	Phase composition	
		A/%	B%
0	1	100	0
1	1	100	0
8	1	90	10
24	1	75	25
34	1	55	45
45	1	45	55
60	1	0	100
90	1	0	100
95	1	100	0
105	1	100	0

2.2.3 SDS-PAGE

Running gel for SDS-PAGE was prepared in the following procedures. Bromophenol blue, acrylamide/bis-acrylamide solution (19:1, 40%), acrylamide/bis-acrylamide solution (29:1, 30%) were bought from Bio-Rad (Hercules, California, USA). Ammonium persulfate (98%) for electrophoresis was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Comassie brilliant blue G-250 was purchased from AppliChem (Darmstadt, Germany). The separating gel, stacking gel and running buffer were prepared as **Table 2.2** listed.

Table 2.2 Composition of gel and buffer for zein electrophoresis

	Stock solution/100 mL		Radio of stocking solution
Separating gel	A	1 M HCl (48.0 mL) Tris (36.6 g) TEMED (0.23 mL)	A: B: C: H_2O = 1: 3.5: 4: 1
	B	30% (Acr:Bis=29:1)	
	C	Ammonium persulphate (0.14 g)	
Stacking gel	D	1 M HCl (48.0 mL) Tris (5.98 g) TEMED (0.46 mL)	C: D: E: F=0.67: 1: 1: 4
	E	40% (Acr:Bis = 19:1)	
	F	Sucrose (40.0 g)	
Running buffer	Tris- Glycine-SDS Buffer Concentrate for Electrophoresis Reagent from Sigma-Aldrich (St. Louis, Missouri, USA)		

The electrophoresis of zein was operated with electrophoresis apparatus from Bio-rad Company (Hercules, California, USA). The molecular weight profile of extracted zein from DDGS and whole stillage with a 4% stacking gel and 12%

separating gel in an SDS-Tris-Glycine buffer system, following SDS-PAGE method for zein by Paraman (*42*). Briefly, the zein solutions were diluted to 10 g/L by a sample buffer: 125 mM Tris-HCl at pH 7.0, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.05% bromophenol blue. The protein solutions were centrifuged to remove the precipitation, and 15 μL of the solution was loaded on to the gel. Electrophoresis was performed at 200 V for 60 min. The gel was stained by 0.1% Coomassie brilliant blue solution. Bio-rad molecular weight marker ranging from 10 to 200kDa (Hercules, California, USA) was used.

2.3 Results and discussion

2.3.1 Changes of bioactives upon biomass processing

Extraction yields. The recyclable fractions in DDGS include lipophilic compounds, zein and hydrophilic compounds. After being extracted by the combination of ethyl ester, absolute ethanol and aqueous ethanol, crude oil and zein can be obtained (**Table 2.3**). Liquefied mash has the lowest yield of oil and zein among the six samples, which can be explained as that starch in corn was removed in other samples so the total mass of liquefied mash was the highest. The moisture content of "Beer" included the content of ethanol so the yield from this sample remains close with that from whole stillage. With the removal of certain corn oil via centrifugation from whole stillage, WDGS and DDGS reasonably had lower yield of oil in comparison with whole stillage; on the other hand, they had slightly higher yield of zein with reduced total mass. Syrup is the concentrated product from thin stillage

and the thin stillage is the liquid fraction separated from whole stillage, so the oil and zein content of syrup were the lowest among the post-fermented products.

Table 2.3 Yields of oil and zein from bio-ethanol co-products

	Liquefied mash	Beer	Whole stillage	Syrup	WDGS	DDGS
Moisture content, %	67.14	94.10	87.12±0.18	67.51	64.88	12.07±0.37
Oil	2.4	12.5	12.3±1.43	3.2	7.6	7.2±0.89
Zein	0.75	5.2	6.1±0.72	N.A.	6.5	6.7±1.08
Hydrophilics fraction	-	-	17.5±0.97	-	-	18.9±1.20

The extracted oil samples from the seven fractions were analyzed with HPLC to examine the changes of four phenolic acids, lutein and α-tocopherol (**Table 2.4**) as representatives of bioactive compounds. The effects of fermentation and heat treatment could be demonstrated by comparing the contents of these compounds in different samples.

However, among the four phenolic acids tested, only the peaks of *p*-coumaric acid and ferulic acid can be found and integrated clearly in all the seven fractions, due to the close retention time (**Figure 2.4**) and similar UV-vis absorption band of various phenolic acids (**Figure 2.5**).

Table 2.4 Yield of lipophilic compounds in extracted oil samples (mg/100g oil)

	Vanillic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Lutein	α-Tocopherol
Retention time/min	36.37	37.29	41.82	42.68	75.56	84.74
Liquefied mash oil	-	-	11.93	8.78	101.08	89.68
Beer oil	-	7.08	1.91	2.52	62.95	11.68
Whole stillage oil	5.49 ±0.36	-	8.61 ±0.28	5.11 ±0.17	62.97 ±3.34	52.80 ±2.26
Corn oil	1.34	-	0.11	3.99	38.19	61.19
Syrup oil	-	6.34	6.41	6.79	24.98	11.68
WDGS oil	-	5.72	13.21	12.25	76.37	54.42
DDGS oil	8.74 ±0.52	8.68 ±0.47	14.49 ±1.96	16.38 ±0.87	31.62 ±2.03	40.12 ±1.23

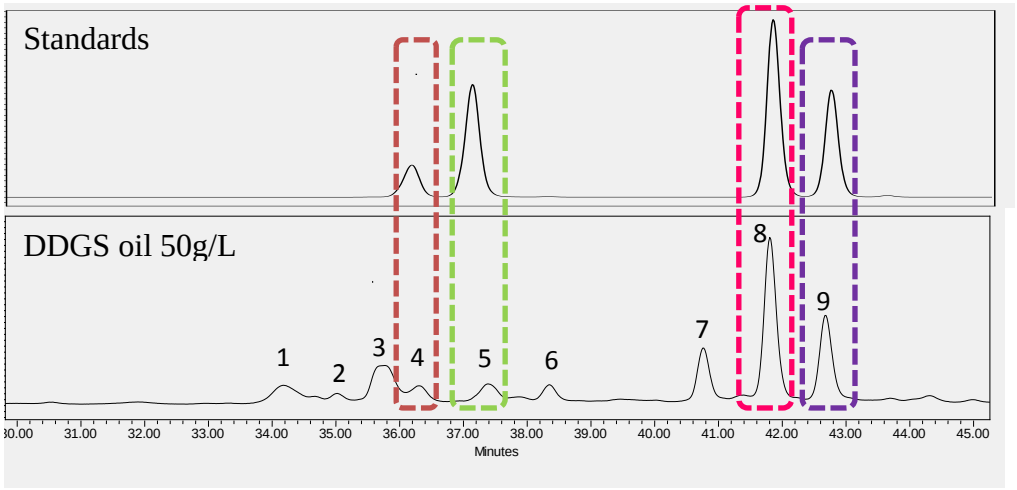

Figure 2.4 HPLC spectra of phenolic acid standards and DDGS oil (λ=300 nm)

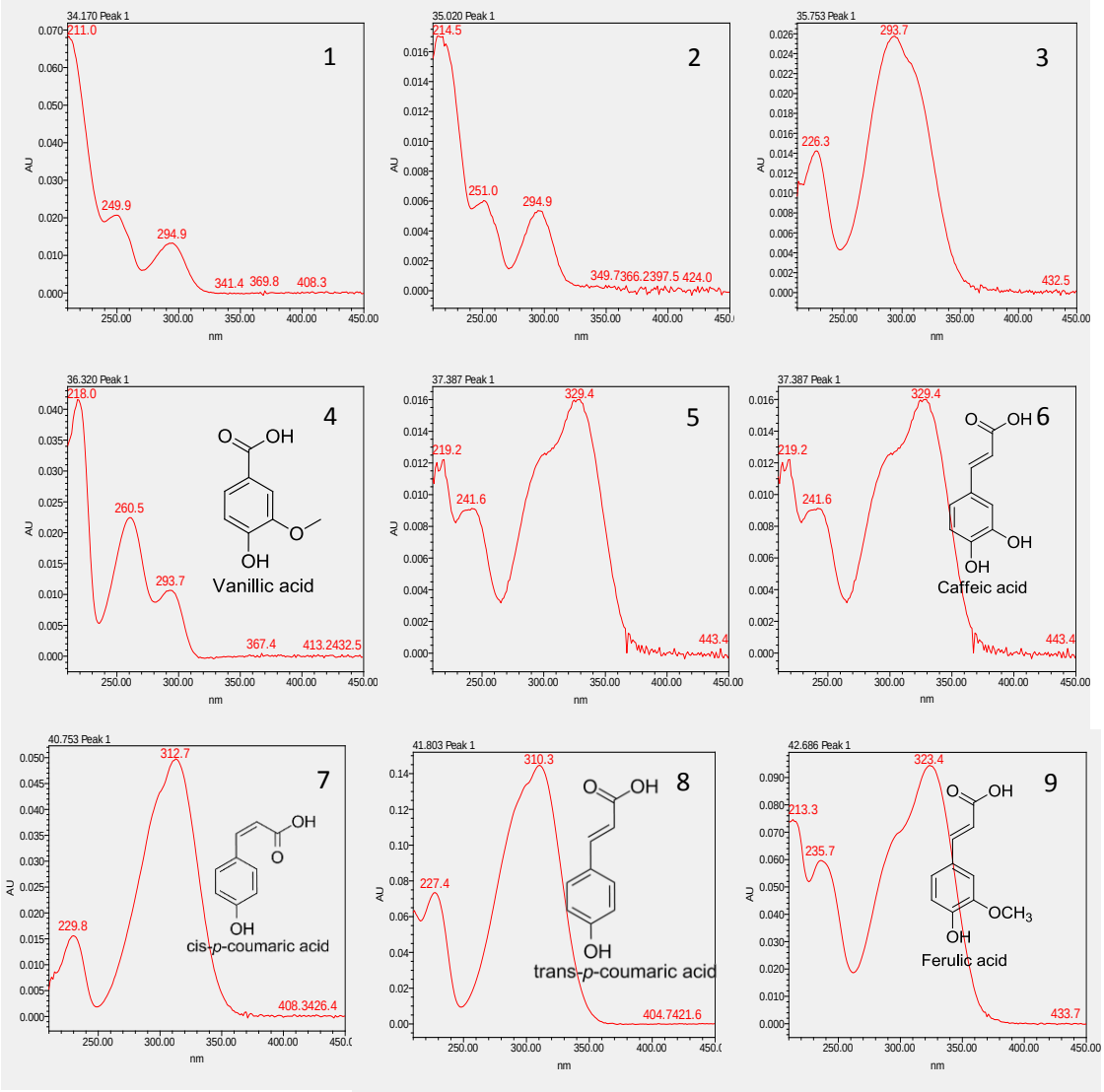

Figure 2.5 UV-vis absorbance of phenolic acids in DDGS oil

As the result of the variability in stability and reactivity of these phenolic acids, the changes trends of different phenolic compounds were different. For instance, caffeic acid content increased from below the limitation of detection in Liquefied mash oil to 7.08 mg/100 g Beer oil, whereas *p*-coumaric acid decreased by six times from Liquefied mash oil to Beer oil. This indicated that comparing an individual phenolic acid is not adequate to summarize the changes of total phenolic acids content; on the contrary, it could be misleading. From **Figure 2.4** and **Figure 2.5**, it is

noticeable that although only four compounds (Compound 4, 5, 8, 9) were identified by comparison with standards, other five compounds (Compound 1, 2, 3, 6, and 7) found between 34-44 min at 300 nm had similar characteristic peaks in the absorbance spectra. In addition to similar polarity, these compounds were probably phenolic acids, concentrations of which have similar response to UV-vis absorbance. Besides, with the carbon-carbon double bond in the structure, caffeic acid, p-coumaric acid and ferulic acid have cis-trans isomers in grains including corn (*43*, *44*). Compound 7 and compound 8, as well as compound 5 and compound 6 have completely identical UV-vis absorbance structure, indicating that they are probably cis-trans isomers. Therefore, in **Figure 2.8**, the total phenolic acids content in oil samples were calculated as ferulic acid equivalent and compared.

Similar treatment for carotenoids was applied for easily comparison (**Figure 2.6**, **Figure 2.7**); the compounds from 73 to 78 min at 450 nm were calculated as lutein equivalent and compared. Compound 10 can be identified as lutein by comparison with lutein dietary supplement. Compound 11, 12 and 13 were identified with LC-MS for zeaxanthin, cathaxanthin, and β-carotene with $[M-H]^-$=567.91, 563.61, 536.66 respectively.

Taking the oil yield into consideration, the content of lipophilic fractions in 100 g dried matter of each raw samples except for corn oil were compared in **Figure 2.9**.

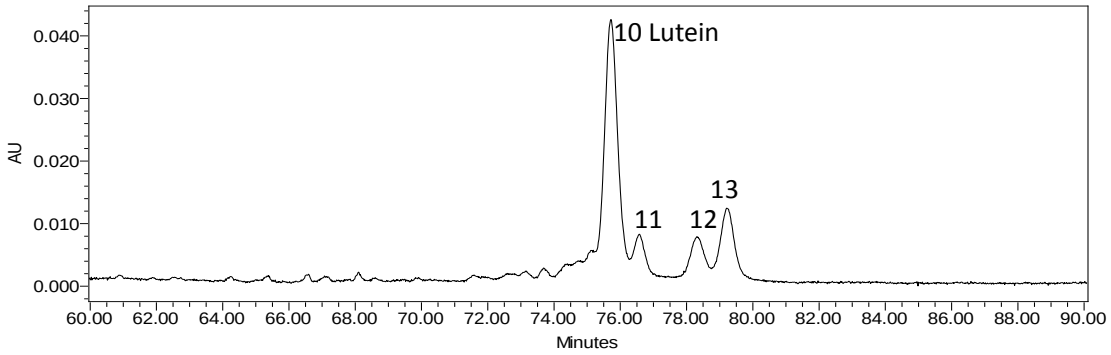

Figure 2.6 HPLC spectra of lipophilic compounds extracted from DDGS (λ = 450 nm)

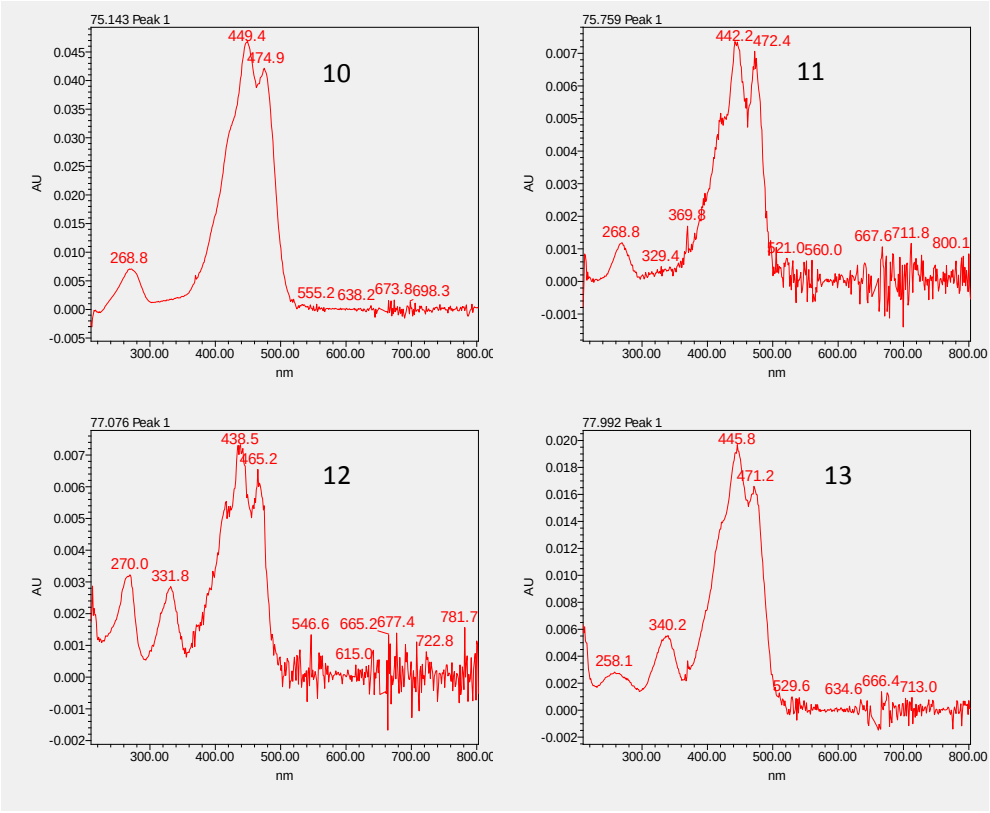

Figure 2.7 UV-vis absorbance of carotenoids in DDGS oil

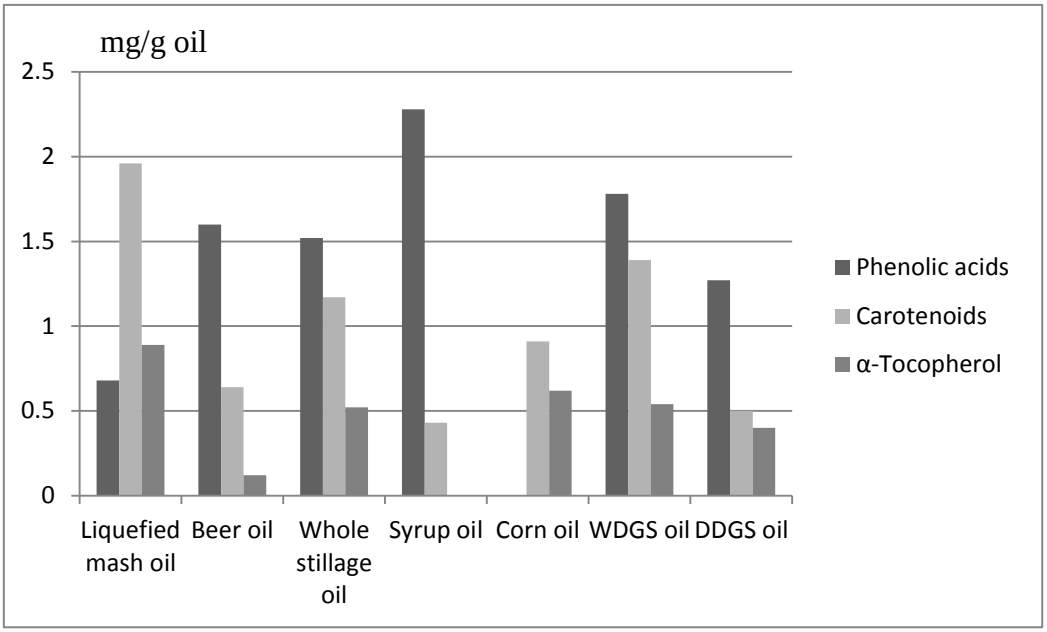

Figure 2.8 Changes of bioactive compounds content (mg/g oil) in extracted oil samples from bio-ethanol products during dry-grind-process

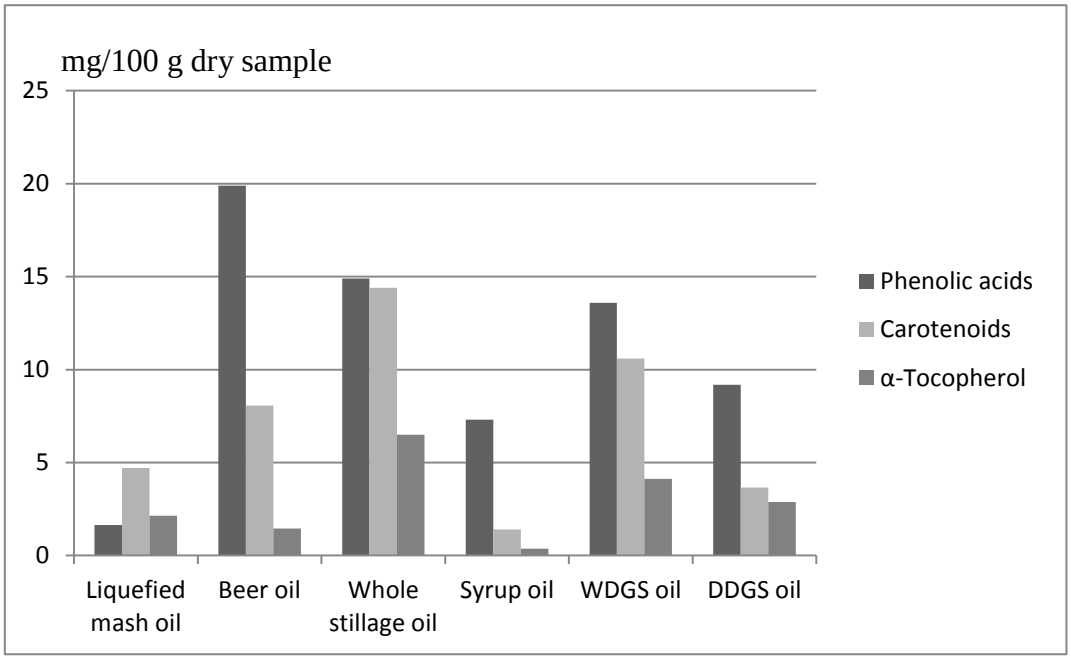

Figure 2.9 Changes of contents (mg/100 g dry sample) of bioactive compounds in bio-ethanol products during dry-grind-process.

The factors resulted in the changes of the three groups of bioactive compounds mainly consist of fermentation and heat treatment. From Liquefied mash to Beer, the product underwent fermentation and 60℃ of heat treatment, while from Beer to

whole stillage, as well as from WDGS to DDGS, 110 ℃ heating was treated to the corn product (**Figure 2.10**).

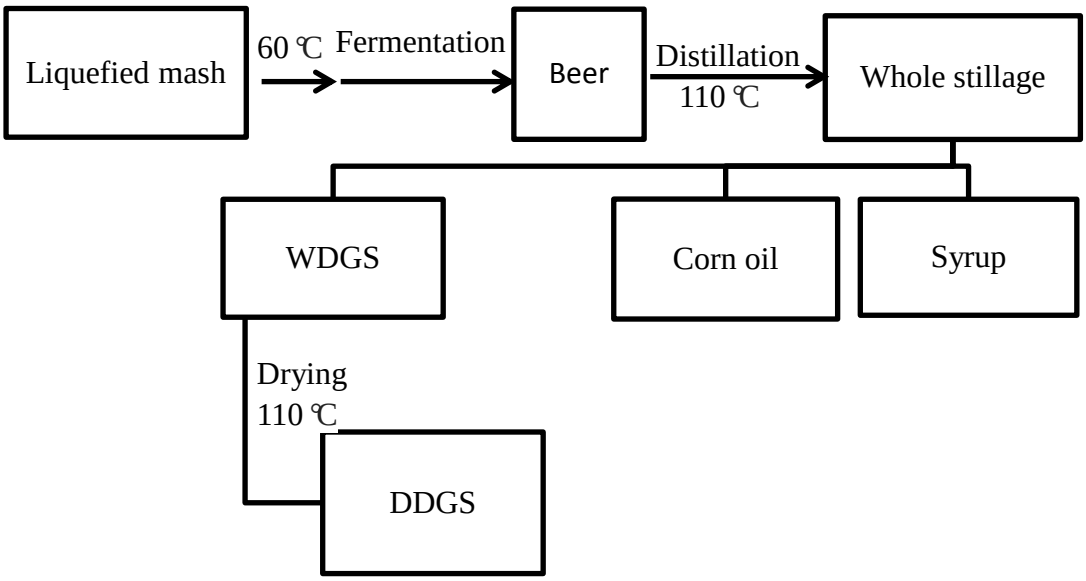

Figure 2.10 Fermentation and thermal treatment steps in dry- grinding procedure.

Based on the data in **Figure 2.8** and **Figure 2.9**, the effects of Fermentation and heating treatment are analyzed as below:

Fermentation When liquefied mash is fermented to Beer, the phenolic acids content increased significantly, reaching more than twice of original content. This may be explained as that the esterase from yeast catalyzed the hydrolysis of phenolic ester. Take ferulic acid for instance, although ferulic acid is abundant in grains, it is generally esterified with polysaccharides that comprise the hemicellulose of plant cell walls and ferulic acid in free form is rarely found (*45, 46*). However, the significant increase of ferulic acid release during Chinese Rice Wine brewing and fermentation by various yeasts was observed; despite of the difference in the increase, wheat Qu (also known as wheat koji in Japanese, 麦曲 in Chinese) and other commercial yeasts

were proved capable in releasing ferulic acid from its conjugates on polymeric cellular materials such as fibres (*47*).

Heat treatment The effects of heat treatment are multiple. Comparing liquefied mash to Beer, phenolic acids content was higher in Beer but carotenoids and α-tocopherol were reduced to around 30% and 20% respectively. The decrease of carotenoids and α-tocopherol was probably the result of degradation of xanthopylls caroternoids and tocopherol when liquefied mash was preheated at 60 ℃ before fermentation. This explanation is in accordance with the study of thermostability of carotenoids and phenolic acids in sweet potato, which demonstrated that heat treatments like boiling (95 ℃ for one hour) and processing of flour (57 ℃ for 12 hours) could result significant decrease in these compounds (*32*). The phenolic compounds were more stable than carotenoids towards heat processing. However, potential release of free phenolic acids in the presence of lipase would make it hard to conclude their thermal stability.

Heat treatment was found to improve the extractability of tocopherols and carotenoids from plant extract due to their release of binding sites (*7*). This can explain that the concentrations of carotenoids and α-tocopherol have increased by 3-4 times from Beer oil to whole stillage oil, which had undergone ethanol distillation at 110 ℃, in despite of their thermo-sensitivity. From WDGS to DDGS, the biomass underwent another heat treatment of 110 ℃, in which reduced the contents of the bioactive compounds due to their thermo-sensitivity.

Other factors The extracted oil from syrup has the highest phenolic acids content but

lowest carotenoids α-tocopherol contents. This could be due to the different polarity of these three classes of compounds. Syrup can be considered mainly as the concentrated product of aqueous fraction of whole stillage that was treated by centrifugation to remove lipids; phenolic acids are more hydrophilic so they tend to have some insolubility in water. Logically, the corn oil has the lowest phenolic acid contents.

Based on the above results, whole stillage has the second highest yield of phenolic acids and highest yields of carotenoids and α-tocopherol. The Lipophilic products extracted from whole stillage have higher content of functional lipid compounds than corn oil, making it potential to be used as health supplements.

2.3.2 Analysis of Zein

Most zein extractions are accomplished by 70% aqueous ethanol (*38*) or aqueous 2-propanol (88%) (*39*). Reducing reagent (e.g. dithiothreitol) and surfactant (sodium dodecyl sulfate) were used in aqueous alcohol to improve the quality and yield of zein extracted from DDGS with the yield of 1.5-6.6%. However, the purity was not ideal at 37-57% (*17, 19, 32*). Both basic (*45*) and acidic (*7*) conditions in the presence of reducing agent have been used to optimize the extracting method. Xu et al. reported an acidic alcohol extraction method that improved the yield of zein improved to 10% from DDGS and extracted zein had better properties than commercial zein: the intrinsic viscosity was increased from 25.1 to 31.6 and the CIE Yellowness Index was reduced from 95 to 54 (*48*). Anderson et al. used ethanol (70%) with sodium bisulfite

(0.5%) and NaOH (0.25%) to extract zein and increased the solvent concentration to 95% to precipitate β and γ-zeins to obtain α-zein-rich solution. The α-zein solid was then precipitated at -18 ℃, with purity of 73%. The film formed by the obtained α-zein was highly transparent and have mostly uniform even surface, but its tensile strength (22.88 mPa) and Young's modulus film (2147.45 mPa) were lower than that of commercial Kobayashi zein (28.72 mPa and 2519.95 mPa, respectively). The effect of enzyme cellulose and pectinase pretreatment was also examined but the results showed that the enzyme-assisted extraction did not improve the extracting yields (*39*). The reported methods of extracting zein suffer from low yields and purity and are not economical for commercial scale production of zein from DDGS.

In my experiment, whole stillage and DDGS were extracted with ethyl acetate, anhydrous ethanol, and aqueous ethanol successively, followed by combination of the extracted solutions. After the solvents were evaporated to dry, the mixture was washed with ethyl acetate and absolute ethanol to remove the oil fraction. The resulting residue mainly contains zein. The simultaneous extraction of lipophilic compounds and zein from biomass not only maximized the recyclable products from bioethanol co-products, but also improved the purity of extracted zein by eliminating other hydrophobic compounds.

The total yield of zein was 6.1% from whole stillage and 6.7% from DDGS. SDS-PAGE protein profiles (**Figure 2.11**) showed zein extracted with 70% ethanol from whole stillage (lane 2) had bands at molecular weight of 15, 22, 25, 37 and 60 kDa. According to the nomenclature of Esen (*40*), the band at around 15 kDa

accounted for γ_2-zein, while the 20 and 25 kDa bands corresponded to α-zein (α_2-zein Z19 and α_1-zein Z22 respectively), and the 37 kDa bands were α-zein dimers. The band at around 60 kDa may be due to impurity. Similar bands were shown in 70% EtOH extracted sample from DDGS (lane 3), except for lack of the band of γ_2-zein.

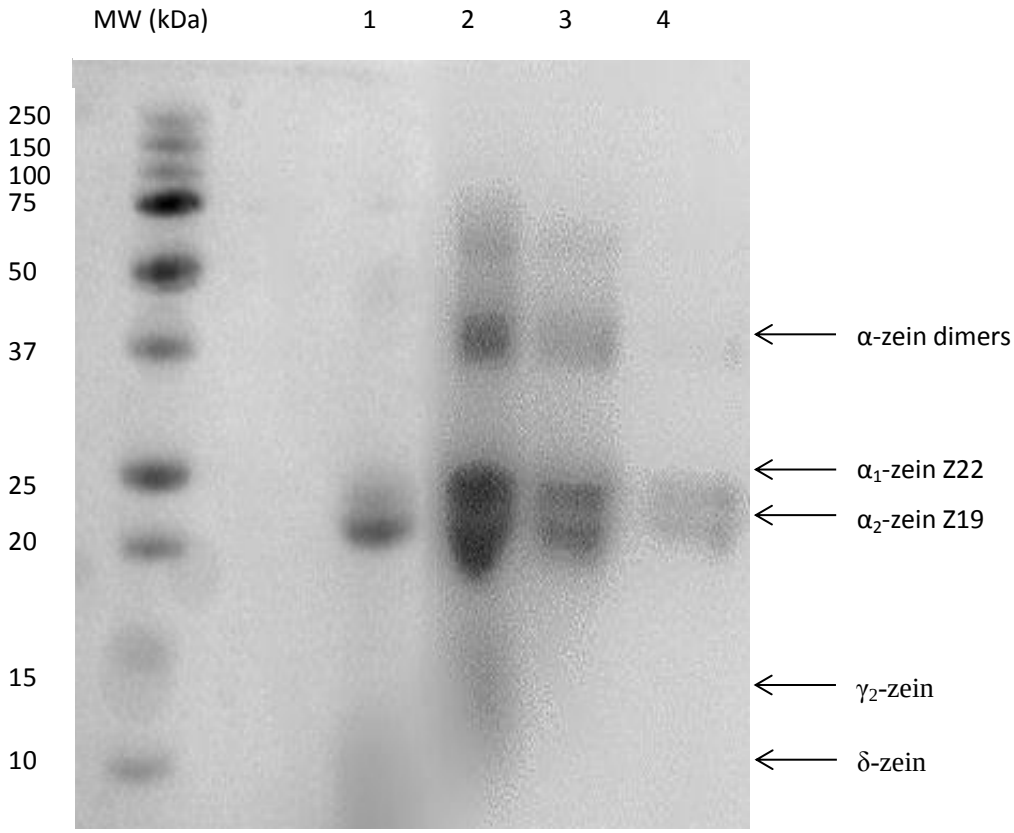

Figure 2.11 SDS-PAGE protein profile of commercial zein (lane 1) and bioethanol coproducts: 70% ethanol extracted zein protein from whole stillage (lane 2); 70% ethanol extracted zein from DDGS (lane 3); 95% ethanol extracted zein protein from DDGS (lane 4). All the samples were dissolved in 70% ethanol with concentration of 50 mg/mL before diluted in sample buffer, except for commercial zein (20mg/mL).

Since α-zein was reported to be relatively more hydrophobic than other zeins, to improve the purity of the zein extraction, 95% ethanol extracted sample was included in SDS-PAGE analysis (lane 4). The purity of zein was improved while the yield decreased to 4.2%, which may be the result of removal of impurity and some loss of α-zein dimers. The properties of purified zein and the crude products need to be further evaluated according to their applications to assess if such purification is needed.

Table 2.5 UV-vis absorbance (650nm) of α-zein bands in SDS-PAGE

	Concentration (mg/mL)	*Abs	Equivalent of commercial zein
Whole stillage	50	0.284	1.378
DDGS (extracted with 70% ethanol)	50	0.165	0.752
DDGS (extracted with 95% ethanol)	50	0.099	0.404
Standard curve (with commercial zein): Abs=0.0038Con.+0.0221 (R^2=0.9899)			

*The total absorbance of α_1-zein Z22 α_2-zein Z19 bands in SDS-PAGE was measured with microplate reader (Bio-Tek, USA).

In the three tested samples, α_1-zein Z22 and α_2-zein Z19 made up the bulk of the extraction products, making them potential to be commercialized. Further comparison was made by measuring UV-vis absorbance of α-zein monomer bands in SDS-PAGE (**Table 2.5**). As the gel was stained with Coomassie Brilliant Blue, the absorbance at 650 nm of α_1-zein Z22 and α_2-zein Z19 bands of the four samples was compared. Zein extracted from whole stillage unexpectedly had 1.378 times of α-zein monomer than

that in commercial zein, indicating higher purity of the extracted product. The purity of zein extracted from DDGS with 70% ethanol was only 0.752 equivalent of commercial zein according to UV-vis absorbance, but that with 95% ethanol was even lower.

Chapter 3 Seasoning developed from corn fermentation co-products

3.1 Introduction

The flavour-enhancing properties of 5′-nucleotides when in combination with MSG have been well-known since 1960s (*49*). They are obtained from two methods:

(1) Hydrolysis from ribonucleic acids to 5′-mononucleotides

Most natural RNase hydrolyzed RNA into 3′-mononucleotides, which have no flavour enhancing effect (*50*). One method of industrial production of guanosine 5'-monophosphate as flavour enhancer is by bioconversion of 5'- amino - 4 -imidazole carboxamide riboside (AICA-R), which is accumulated from fermentation of D-glucose with a non-extracting purineless mutant derived from *Bacillus* megasterium IAM 1245 by x-ray irradiation (*51*). A more common method is hydrolysis of ribonucleic acids. Nuclease P1 is a 5'-phosphodiesterase from *Penicillium citrinum*, which can catalyze the 3'-phosphodiester linkage between 3′-hydroxyl carbon and phosphate group, and specifically split 5'-phosphodiester linkage in RNA, giving rise to 5'-mononucleotides (uridine 5'-monophosphate, inosine 5'-monophosphate, adenosine 5'-monophosphate, guanosine 5'-monophosphate, and cytidine 5'-monophosphate) (*49*). 5'-GMP and 5'-IMP (by deamination of 5'-AMP) can enhance food flavour significantly through synergy effect of glutamic acid. In Japan, as early as in 1980s, more than 1,200 tons of 5'-GMP and 5'-IMP are produced each year by enzymatic hydrolysis of RNA (*27*). However, IMP and GMP are less

thermo-stable than MSG (*52*). The rate of thermal degradation of 5′-ribonucleotides was further increased by lowering the pH (*53*). Thermal degradation of IMP and GMP was the hydrolysis of the phosphoric ester bond in the nucleotides (*54*) and the cleavage of N-glucosidic bonds of IMP and GMP (*55*).

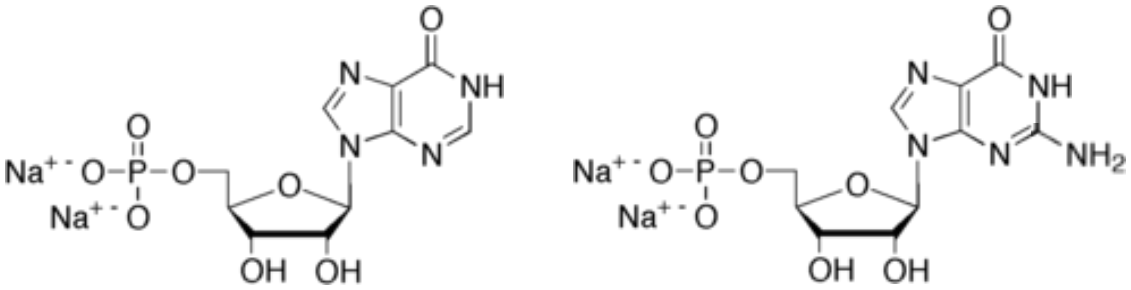

Figure 3.1 Structures of disodium inosinate (left) and disodium guanylate (right)

(2) Hydrolysis from proteins to amino acids

Corn proteins consist of glutelin (68%), albumin (28%) and a little globin (3%) (*56*). These proteins lack essential amino acids such as lysine (0.96%) and tryptophan (0.2%), while are rich in hydrophobic amino acids like leucine (8.24%) and alanine (4.81%). Thus, the low nutritional value, the insolubility and indigestibility of corn protein become the main reasons that limit its application in food industry (*57*).

The hydrolysis of corn proteins can degrade protein into peptides or amino acids, which are easily absorbed. Among the amino acids from corn proteins, glutamic acid accounts for as much as 12%, so that the hydrolysate is potential to be used as a flavour enhancer.

Natural corn protein molecules have dense three-dimensional structures which are hard to be broken by enzymes. Therefore, pretreatment is needed to destroy the disulfide, hydrogen and hydrophobic bonds, so that the tertiary and quartenary structure can be broken. After that, those reactive sites were initially covered inside

protein molecules are exposed due to the loosen structure and the rate of hydrolysis can be improved significantly (*58*). In enzymatic hydrolysis of food proteins, Alcalase, an alkaline protease, was mostly used (*8, 56, 58*). In this project, we used Protemax and Flavourzyme, two neutral proteases, to avoid introducing large amount of salts while maximizing the recovery of glutamic acid. With both endoprotease and exopeptidase activities, Protemax and Flavourzyme have been reported to be more efficient than neutral Neutrase endopeptidase from *Bacillus amyloliquefaciens* and alkaline Alcalase endopeptidase from *Bacillus licheniformis*, etc. (*59, 60*) (**Table 3.1**). The degree of hydrolysis (DH) of soybean protein isolate in the presence of Protemax and Flavourzyme were 19.56% and 10.02% respectively (*61*). Besides, the combinative effect of two proteases can hydrolyze more peptide bonds and degrade more proteins into amino acids. For instance, Protemax has been used in conjunction with Flavourzyme hydrolysis of proteins from brewers' yeast to produce smaller non-bitter peptides with degree of hydrolysis (DH) of 44.8% (*62*).

Table 3.1 Optimal hydrolysis conditions and activity of common proteases

Enzyme	pH	Temperature (℃)	Activity (*59, 60*)
Neutrase	7.1	55	Endopeptidase
Alcalase	8.0	55	Endopeptidase
Papain	6.0	60	Endopeptidase
Protemax	7.5	55	High endoprotease and low exopeptidase activity
Flavourzyme	7.1	55	High exopeptidase and low endoprotease activity (*62*)

3.2 Materials and methods

3.2.1 Samples and chemicals

Whole stillage and DDGS residues after oil extraction were collected. 5′-GMP, 5′-AMP, 5′-UMP and glutamic acid standards were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).and used for the identification and quantification of mononucleotides and glutamic acid. Nuclease P1 was purchased from Hongrunbaoshun (Beijing, China). Protamex and Flavourzyme were purchased from Novozyme (Copenhagen, Denmark).

3.2.2 Extraction and hydrolysis of RNA and protein in whole stillage and DDGS

To confirm the nucleotides were not destroyed during dry-grind process, RNA extraction was conducted as **Figure 3.2** showed. The extracted nucleotides from DDGS had strong absorbance band at 260 nm, which was even stronger in extracted nucleotides from bread yeast as positive control (**Figure 3.3**).

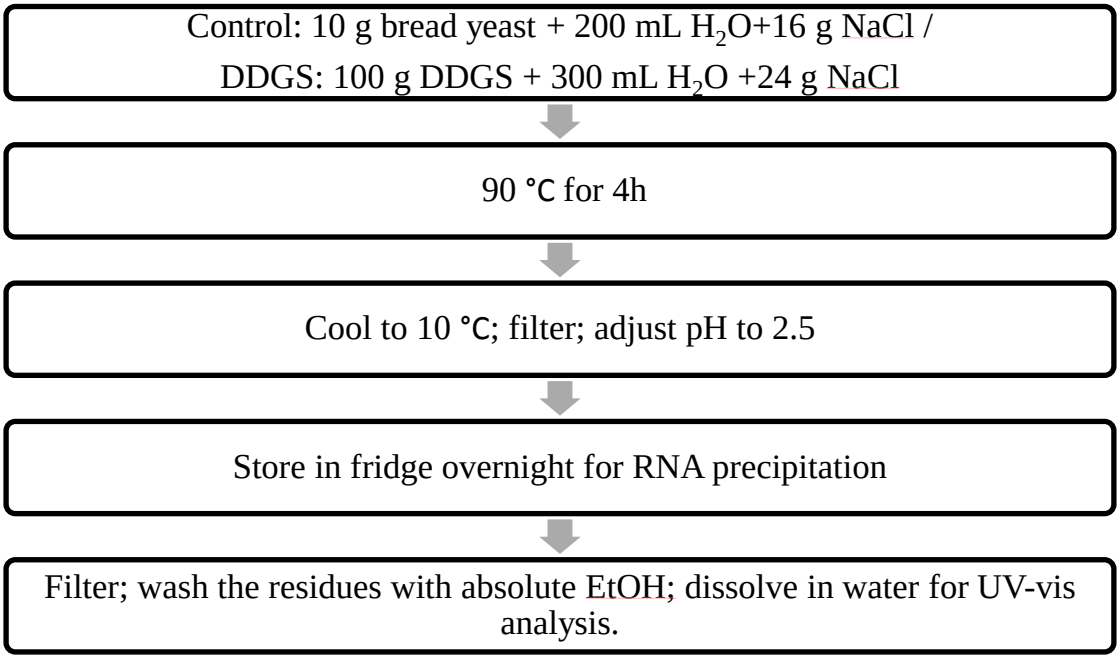

Figure 3.2 RNA extracting process from baker's yeast and DDGS

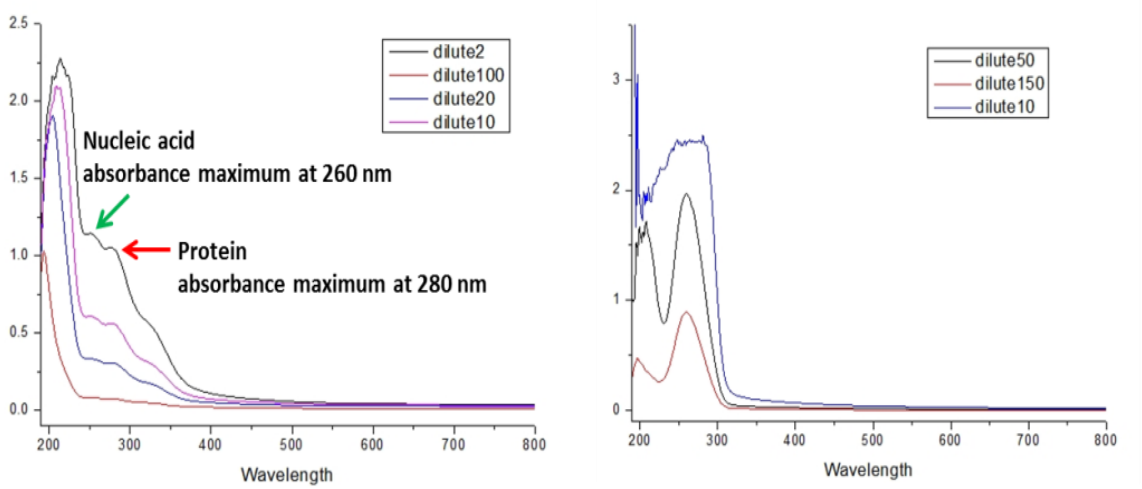

Figure 3.3 UV-VIS spectra of salt water extracts of DDGS (left) and baker's yeast as a positive control (right)

WDGS, Whole stillage and "Beer" were extracted with the same process and the estimated RNA contents were calculated (**Table 3.2**). The results indicated certain amount of nucleotides survived in the production of DDGS and other intermediates from which RNA could be recovered and hydrolyzed into mononucleotides. However, since RNA is known to be unstable once exposed from yeast cells and tend to be degraded into 3′-mononucleotides by RNase, the RNA extraction process may lead to loss of 5′-mononucleotides. It is likely that, during ethanol distillation process at high temperature, the yeast cells might have already been disrupted and the nucleic acids shall be exposed for hydrolysis. Preliminary experiment shown that DDGS with nuclease without any pretreatment gave rise to nucleotides; this result supports our hypothesis that the yeast cells in the DDGS have already been ruptured. Furthermore, when the solids were separated from the whole stillage and only the liquid fraction was hydrolyzed, the amount of 5′-mononucleotides was comparable to that of DDGS hydrolysis. This result further indicated that yeast nucleic acids were released from the yeast cells to the solution phase.

Table 3.2 Estimated nucleic acid contents of distiller biomass on dry weight basis

Sample	Extraction yields* (‰)
Yeast	26.7
DDGS	0.151
WDGS	0.112
Whole stillage	0.126
Beer	0.118

*Calculated based on the absorbance value at 260 nm (One absorbance unit equals to 40 μg/mL.)

With the above RNA recovery results, we developed a simplified method for a two-step hydrolysis of nucleotides and protein from whole stillage and DDGS. The substrate concentration was 10% on dry weight basis. Each sample was hydrolyzed by 2% of Nuclease P1 in water at pH 5.0, 60 ℃ for 6 h. The mixture was then centrifuged to separate the solution and solids. The solution was filter by 0.45 μm micro-filter and analyzed by HPLC and HPLC-MS/MS. The solids were heated in 10mL of boiling water for 12 h, followed by adding 2% of Protamex and hydrolyzing at pH 7.5, 55 ℃ for 8h. After that, 2% of Flavouryme was added for further hydrolysis for 48 h. The hydrolysate was filtered and prepared for amino acids HPLC analysis. The combination of two hydrolysates contained 5′-mononucleotides and glutamic acid.

To evaluate the efficiency of the enzymatic hydrolysis, conventional acidic hydrolysis of protein was performed to get the glutamic acid yield from completely hydrolyzed whole stillage and DDGS. The procedures generally followed that from AOAC (1995) official Method 45.3.05, 982.30 E (*63*). Briefly, whole stillage (4 g) and DDGS (1 g) was added into 40 mL 6 M HCl solution respectively. Freeze the

mixture in dry ice-acetone bath; draw and hold vacuum of ≤ 50 mm for 1 min and seal the tube under vacuum. The mixture was heated at 110℃ for 24 h before neutralization and HPLC analysis. According to duplicate acidic hydrolysis results, the mean of glutamic acid yield on dry weight basis from whole stillage was 18.4 mg/g while that from DDGS was 21.3 mg/g. The yield of glutamic acid from DDGS is comparable with 30 mg/g analyzed by Liu et al.(*14*).

3.2.3 HPLC and HPLC-MS/MS analysis

(1) Nucleotides

The HPLC analysis were carried out on a Waters 2695 HPLC system coupled with a photodiode array detector (PDA) (Waters 2996) and auto sampler (Waters 717 plus). The stationery phase was a HPLC column was a 250×4.6 mm, 5 μm C18 column (Atlantis, Waters). The mobile phase A (K_2HPO_4, 0.1M, pH=5.6) was made by dissolving 13.6 g K_2HPO_4 in 1000 mL of de-ionized water and adjusting the pH to 5.6 with 2 M KOH solution. Mobile phase B was 100% of methanol. The solvent gradient sequence was shown in **Table 3.3**.

Table 3.3 Gradient procedure for nucleotides HPLC analysis

Time (min)	Flow rate (mL/min)	Phase composition	
		%A	%B
0	0.5	100	0
5	0.5	100	0
14	0.5	90	10
15	0.5	80	20
35	0.5	80	20

36	0.5	100	0
50	0.5	100	0

(2) Glutamic acid

The HPLC analysis of glutamic acid was followed the standard method of Waters: AccQ·Tag. The AccQ·Tag Derivitization Kit and AccQ·Tag Eluent A were bought from Waters (Milford, Massachusetts, USA). The mobile phase A consisted of 50 mL of AccQ·Tag Eluent A concentrate and 500 mL DI water and the mobile phase B was acetonitrile, and the mobile phase C was di-ionized water. The hydrolysate was filtered by a 0.45 μm micro-filter and derived. The derivatization procedures were followed Waters: 70 μL buffer and 20 μL derivatization reagent were added to 10 μL of hydrolysate. The mixture was shaken for 15 seconds before putting in a block heater for 10 min at 55 ℃.

Table 3.4 Gradient procedure for amino acids HPLC analysis

Time (min)	Flow rate (mL/min)	Phase composition		
		%A	%B	%C
0	1.0	100	0	0
0.5	1.0	99	1	0
18	1.0	95	5	0
19	1.0	91	9	0
29.5	1.0	83	17	0
33	1.0	0	60	40
36	1.0	100	0	0
45	1.0	100	0	0

3.3 Results and discussion

3.3.1 Nucleotides hydrolysis

The effects of various hydrolysis conditions including amount of enzyme, hydrolyzing time, pH, and pre-treatment were examined first. As 5′-AMP (which can be deaminized to 5′-IMP) and 5′-GMP are the effective flavour enhancers, we used the total yield of the two 5′-mononucleotides as the indicator for hydrolysis. With 2% Nuclease P1 at 50℃, for whole stillage hydrolysis, the concentration of 5′-mononucleotides increased rapidly and reached the highest value at around 4 h, after which 5′-mononucleotides became to decrease due to presumable their instability. With lower concentration of Nuclease P1 (0.2%), it took 20 h to reach the highest amount of mononucleotides; and 0.5%, it took 12 h to complete the hydrolysis.

Extension of hydrolysis time resulted in gradual decrease in 5′-mononucleotides due to their thermo-sensitivity which has already demonstrated by Kuchiba et al (*52*). Therefore, heat-treatment should be avoided after the hydrolysis of RNA, and in our research, the hydrolysate of ribonucleic acids was removed before protein hydrolysis.

Since the original pH of the mixture of whole stillage and water was 4.0 while the optimal pH of Nuclease P1 is 5.0, it is possible that adjustment of pH could help increase the enzyme activity and thus shorten the time of hydrolysis. However, my results shown that the concentrations of 5′-AMP and 5′-GMP in samples hydrolyzed under the two pH have no significant difference, indicating that Nuclease P1 at both pH are comparable (**Figure 3.4**).

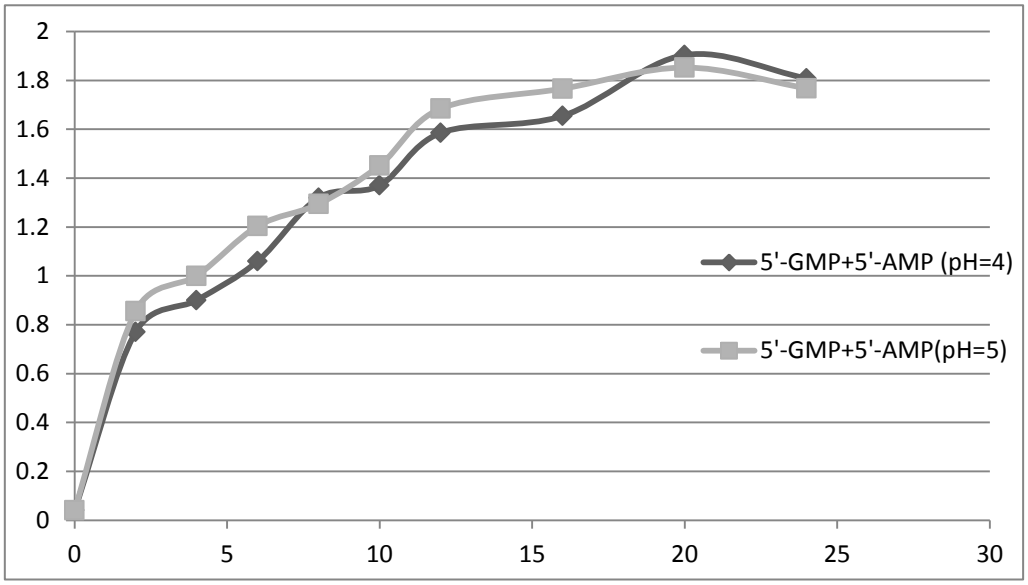

Figure 3.4 GMP and AMP yield (mg/g) from whole stillage hydrolysis (0.2% nuclease) under pH = 4 and pH = 5 (adjusted with NaOH).

For DDGS, complete hydrolysis took 6 h in the presence of 2% enzyme, which can be explained as the solids need extra time to dissolve in water while in Whole stillage they have already dissolved. In order to improve the rate of reaction, pre-heating was done to accelerate the extraction of RNA by heating DDGS suspension in water at 90℃ water for one hour before the addition of Nuclease. However, pre-heating contributed little to shorten the hydrolysis time (**Figure 3.5**). During the first 2 hours, the preheated DDGS was hydrolyzed faster than the non-preheated sample, whereas this difference narrowed down from 2 to 6 hours and both samples took 6 hours to obtain the highest content of mononucleotides. Considering the extra one hour spent in pre-heating, the DDGS sample with pre-treatment was more time-consuming.

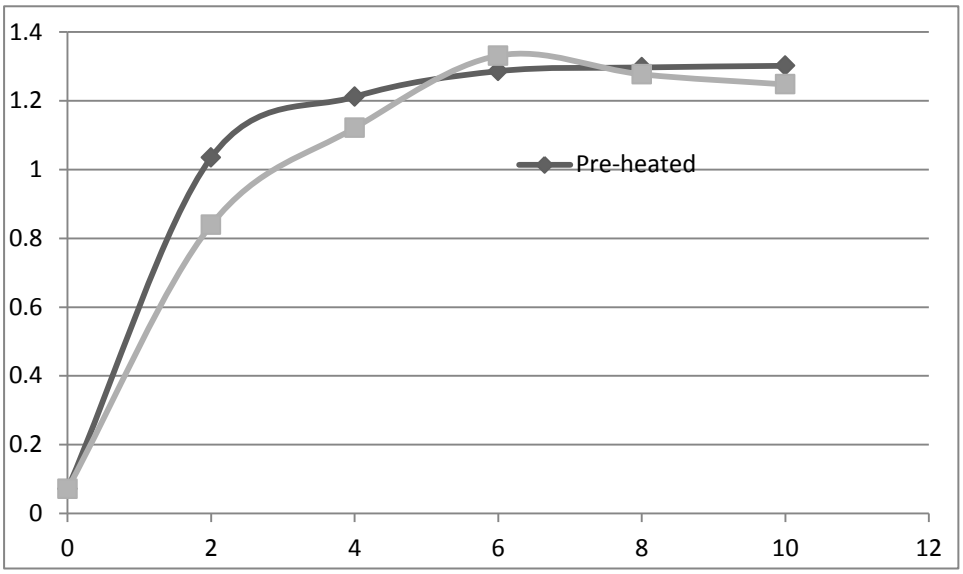

Figure 3.5 Time-course study of GMP and AMP yields (mg/g) from DDGS hydrolysis (2% nuclease).

HPLC spectra of whole stillage hydrolysate under optimal conditions (**Figure 3.6**) show the rapid increase of four 5′-mononucleotides over time. Peaks of 5′-AMP, 5′-UMP, 5′-GMP were identified by comparing the retention time with respective standards and further confirmed by LC-MS/MS (**Table 3.5**). After 6 h-hydrolysis of one gram whole stillage (dried weight), 0.96 mg 5′-AMP, 0.94 mg 5′-GMP and 0.88 mg 5′-UMP were obtained. The yields were much lower from hydrolysis of one g of dry DDGS. The amounts of nucleotides are 0.64 mg (5′-AMP), 0.66 mg (5′-GMP), and 0.78 mg (5′-UMP). The difference in yields may due to RNA degradation during the several additional processing steps from whole stillage and DDGS, which includes high-speed centrifugation, concentration by evaporation and drying under high-temperature, which could be detrimental to RNA.

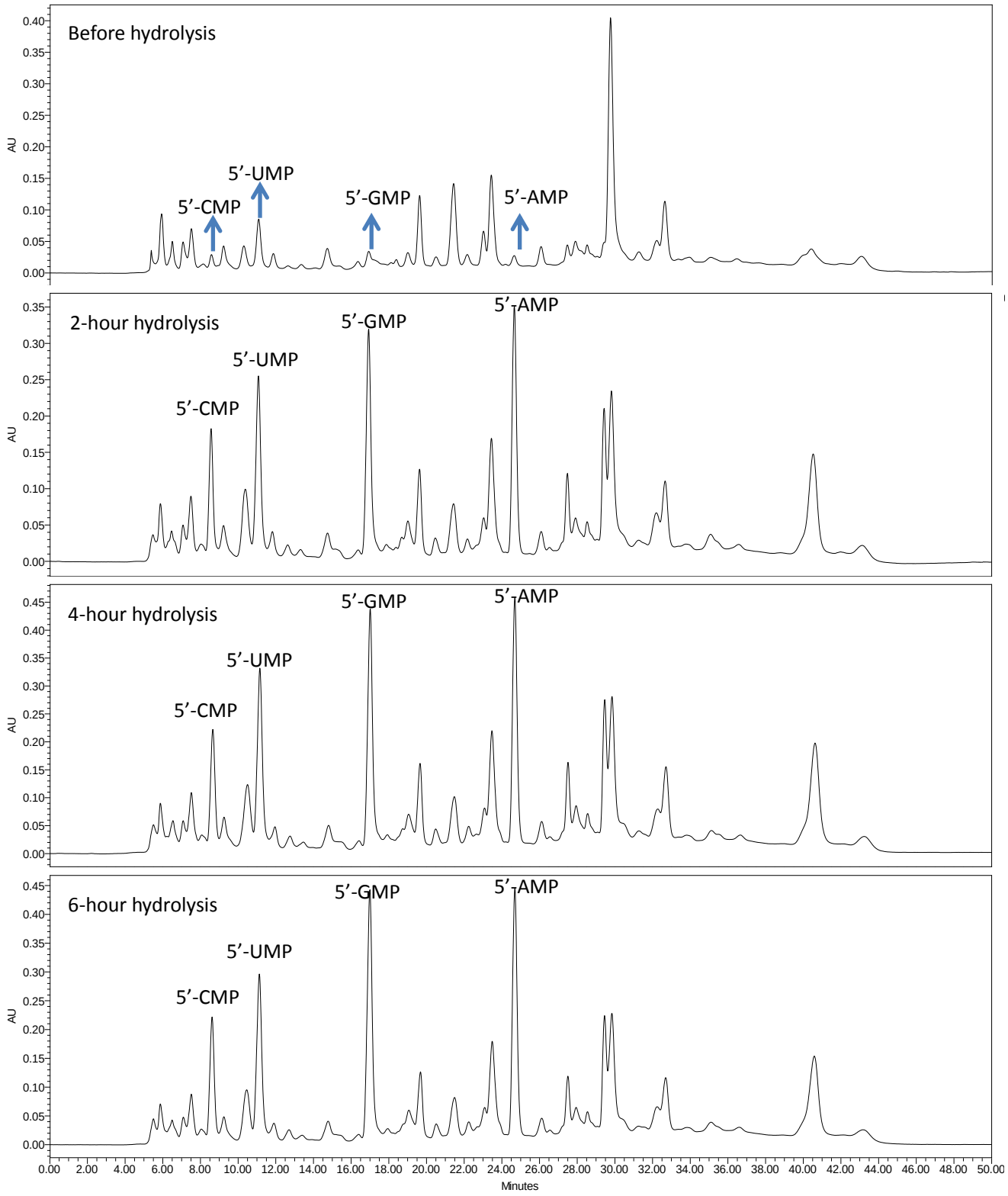

Figure 3.6 HPLC chromatogram of nucleotides hydrolysate (Hydrolysis conditions: 3 g whole stillage and 2% Nuclease P1 in 7 mL water at 50 ℃ without pH adjustment)

Table 3.5 LC-MS/MS of nucleotides from whole stillage hydrolysate (Hydrolysis conditions: 3 g whole stillage and 2% Nuclease P1 in 7 mL water at 50 ℃ for 6 hours without pH adjustment)

Retention time/min	Compound	Molecular weight (m/z)	Precursor ion (m/z)
12.5	5′-CMP	323.20	323.83 (+)
13.1	5′-UMP	324.18	324.95 (+)
26.9	5′-AMP	347.23	348.87 (+)
27.7	5′-GMP	363.22	361.77 (-)

3.3.2 Protein hydrolysis

After the removal of RNA hydrolysate, the proteins in solid residues were further hydrolyzed by Protamex and Flavourzyme in succession. Since different protease can catalyze the hydrolysis of various sites of proteins or peptides, utilizing two proteases with different selectivity can in principle significantly increase the hydrolyzing degree by generating more free amino acids. The increase in the amount of water-soluble peptides was investigated by monitoring the changes of pH of the hydrolysate: the original pH of the hydrolysis reaction is 7.5, and the acidity of carboxyl group is slightly stronger than the basicity of amino group, so the decreasing pH value could be utilized to indicate the hydrolysis degree of protein towards peptides.

Since glutamic acid is the target compound as flavour enhancer, in this experiment, the yield of glutamic acid was defined as the indicator of hydrolyzing degree as follows:

$$\text{Degree of hydrolysis} = \frac{\text{yield of glutamic acid from enzyme hydrolysis}}{\text{yield of glutamic acid from acid hydrolysis}}$$

With the addition of Protamex, the pH of hydrolysate decreased rapidly within 8 hours from 7.5 to 6.2, and extending hydrolyzing time did not lead to further reduction of pH and more degraded peptides. Thus, Flavourzyme was added after 8-hour hydrolysis with Protamex (**Figure 3.9**). The glutamic acid yield did not show noticeable growth until the addition of Flavourzyme after 20 h, indicating with Protamex alone cannot hydrolyze the proteins completely into amino acids. Therefore, the combination of different proteases is necessary. The degree of hydrolysis increased rapidly during the first day after addition of Flavourzyme, from 5% to 21%, indicating the activity of the enzymes can last for about 24 hours. In the second day after addition of Flavourzyme, the growth of DH, from 21% to 22%, was insignificant. Therefore, the end of hydrolysis came around 24 hours after adding Flavourzyme in the hydrolysate.

Optimal conditions were also used in DDGS protein hydrolysis (**Figure 3.9**). In comparison with whole stillage, less free glutamic acids were obtained, which might be caused by the high temperature used in drying of DDGS leading to denaturalization and insolubility of the proteins. The final amount of glutamic acid from DDGS by enzymatic hydrolysis was 13% of that by acid hydrolysis and that from whole stillage and DDGS on dry weight basis were comparable at (3.60 ± 0.27 mg/g) and (2.81 ± 0.16) mg/g respectively.

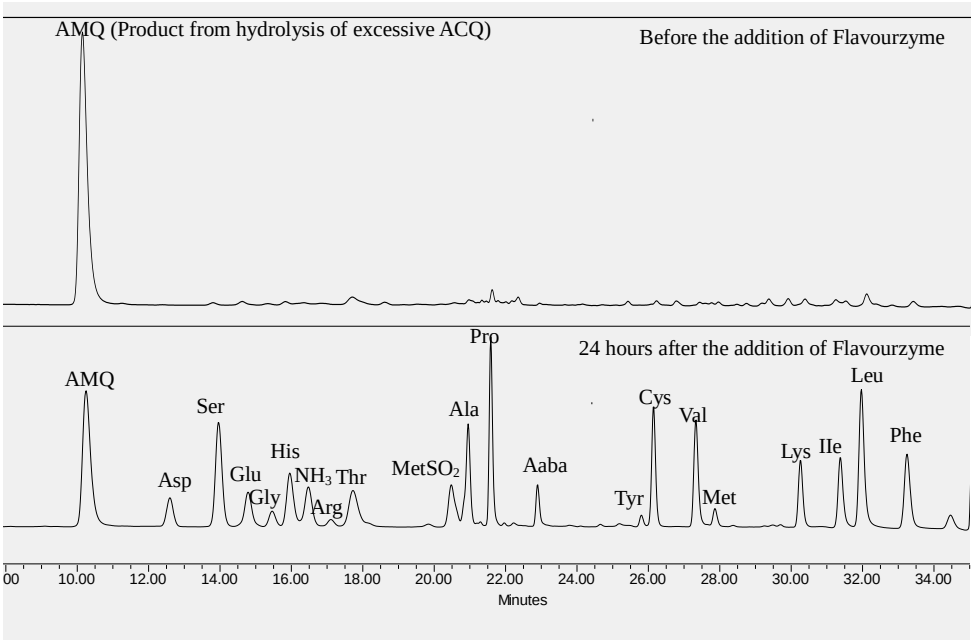

Figure 3.7 HPLC chromatograms of whole stillage proteins hydrolysate

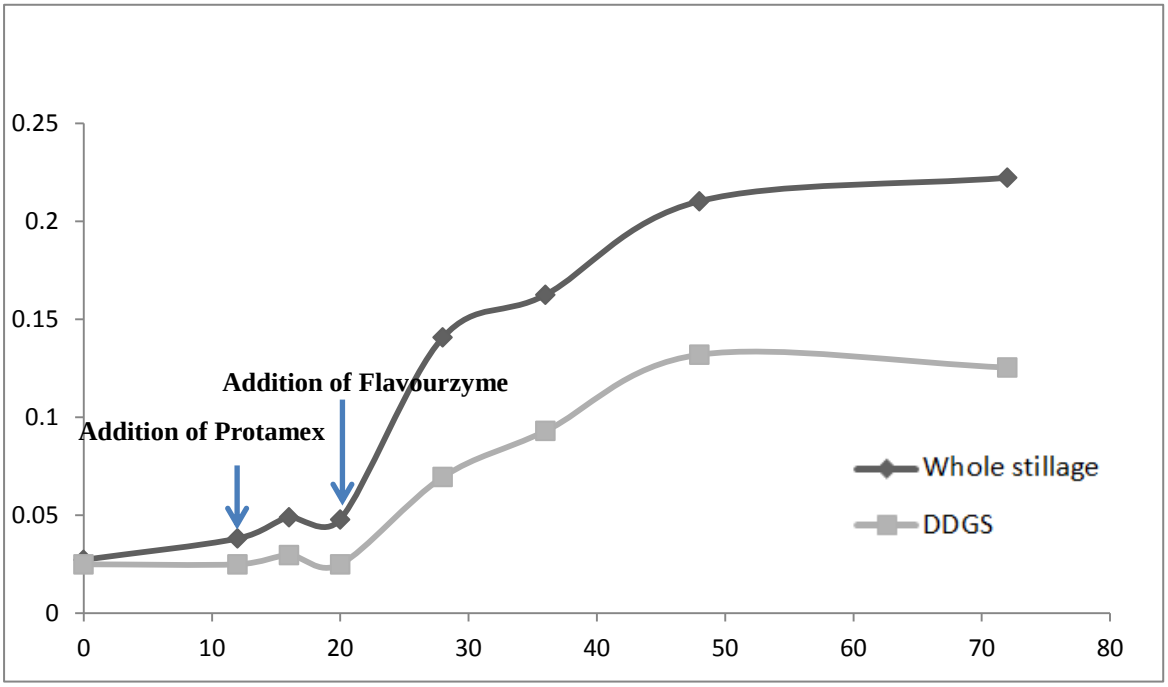

Figure 3.8 Hydrolysis of whole stillage and DDGS (0-12 h: the sample was cooked in boiling water; 12-20 h: the sample was hydrolyzed with Protamex at 55 ℃; 20-72 h: the sample was hydrolysed with Flavourzyme at 55 ℃)

Chapter 4 Conclusions and future work

Based on the results I obtained, a novel and practical valorization of DDGS and other biomass formed in dry-grind bioethanol process could be proposed as shown in **Figure 4.1**. Three fractions were recovered by extraction or hydrolysis in the proposed procedures: the EA/EtOH fraction is abundant in phenolic acids, caroternoids, and tocopherols, which can be developed into dietary supplements. The aqueous EtOH fraction contain high purity of zein proteins, a valuable food coating material that currently has great market demand; and the aqueous fraction extracted from Whole stillage and DDGS have nucleotides and proteins that can be hydrolyzed into flavour enhancer: 5′-GMP and MSG. Considering the fact that zein accounts for about 60% of the total proteins in corn and the glutamic acid content in zein is as high as 7%, it would be ideal that directly hydrolyze the DDGS or whole stillage without depletion of zein to recover more glutamic acid in developing seasoning products.

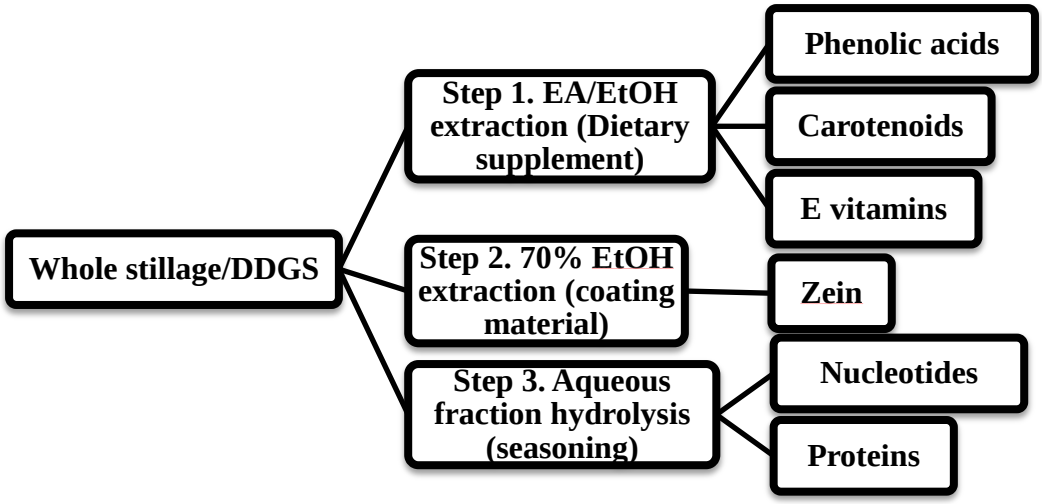

Figure 4.1 Products from Whole stillage and DDGS

Producing DDGS is an energy intensive process. Whole stillage was proven to

be better starting materials (**Table 4.1**). It is noticeable that all products have higher or comparable yields from whole stillage than that from DDGS.

The processing of DDGS is used to extend the shelf-life and improve transportation convenience of the biomass as a livestock feed. However, in our proposed application of corn bio-ethanol product, the effect of these time-consuming and costly procedures is merely lowering the yield of valued products, so the procedures after whole stillage can be omitted to reduce cost yet increase the yields and quality. Whole stillage is a better starting material than DDGS in increasing the commercial value of bio-ethanol coproducts.

Table 4.1 Products from Whole stillage and DDGS

*Products	Whole stillage	DDGS
Yield of oil/%	12.3 ±1.43	7.2 ±0.89
Phenolic acids/mg`100g^{-1}	14.9 ±1.78	9.18 ±1.21
Carotenoids/mg`100g^{-1}	14.4 ±0.87	3.65 ±0.85
α-tocopherol/mg`100g^{-1}	6.49 ±0.36	2.89 ±0.53
Yield of zein/%	6.1 ±0.72	6.7 ±1.08
α- zein content/equivalent of commercial zein	1.378	0.752
Aqueous fraction/%	17.5 ±0.97	18.9 ±1.20
5′-GMP /mg`g^{-1}	0.94 ±0.07	0.66 ±0.06
5′-AMP / mg`g^{-1}	0.96 ±0.04	0.64 ±0.03
5′-UMP / mg`g^{-1}	0.88 ±0.05	0.78 ±0.05
Glutamic acid / mg`g^{-1}	3.60 ±0.27	2.81 ±0.16

*The concentrations were calculated on dry weight basis.

In future study, further effort can be made in the following aspects:

(1) Development of potential dietary supplement based on the corn bioactives extracted from the biomass. Since phenolic acids, carotenoids and tocopherols function and provide health protection in human body mainly as antioxidants, the *in vitro* and *in vivo* antioxidant capacity of the extracted products from whole stillage and DDGS needs to be evaluated. Animal trials and human clinical trials are necessary to verify the product's effects in reducing the risk of chronic disease, including type II diabetes, cancer and cardiovascular diseases before commercialization.

(2) Investigation on zein films characteristics. In order to evaluate and commercialized the extracted zein in our method, the zein film characteristics need to be further examined. Film properties including Tensile strength, Young's modulus, Water vapor permeability and Elongation to break (ETB) are supposed to be tested and compared with current commercial zein. If the properties were unsatisfying, further purification would be necessary to operate.

(3) Savory seasoning from enzyme hydrolysates of whole stillage. Sensory evaluation should be conducted to decide the sufficient concentration of glutamic acid to generate unami and 5′-GMP to enhance the flavour. Besides, bitterness might generate during protein hydrolysis due to small bitter peptides. As savory seasoning, thermos stability of glutamic acid and 5′-GMP should be taken in to consideration since thermal treatment is common in cooking and the loss of glutamic acid and total amount of 5′-IMP and 5′-GMP are 2.2% and 7.59% respectively at 100 ℃, pH= 7, for 15 min (*55*).

Nutritious sauce brewing is another promising research area with high protein content and 5% starch in DDGS. With enzymatic hydrolyzed 5′-GMP and MSG, the flavour of brewed sauce may be enhanced significantly. In addition, the functional lipid compounds may also be attractive to customers with their antioxidant capability and other health benefits. To achieve this, further optimization on hydrolysis conditions for seasoning development is necessary. Unlike seasoning development, the concentration of the flavour enhancer is not necessary to be the highest. Therefore, the hydrolysis procedures can be simplified and the manufacturing cost can be lowered according to the market requirement for umami seasoning by shortening hydrolysis time or reducing the amount of enzyme.

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Appendices

Table A1 Regression equation and detection limits (LODs) of standards (n=5) (Injection volume: 20 μL)

Compound	Wavelength/nm	[a]Regression equation	R^2	[b]LOD/(mg/L)
Vanillic acid	300	Y=33188X-9968	0.9993	1.53
Caffeic acid	300	Y=94488X-169696	0.9998	2.28
p-Coumaric acid	300	Y=152092X+19226	0.9999	0.08
Ferulic acid	300	Y=85649X+25299	0.999	0.12
Lutein	450	Y=144638X-926795	0.9998	9.63
α-tocopherol	300	Y=5000X-29197	1	13.04
5′-AMP	254	Y=60647X+48022	0.9998	4.77
5′-GMP	254	Y=59822X+100541	1	3.21
5′-UMP	254	Y=37787X-185431	0.9998	5.54
Glutamic acid	254	Y=13610X-98200	0.9989	9.86

[a] Y: Peak area; X: mass concentration, mg/L
[b] S/N=3

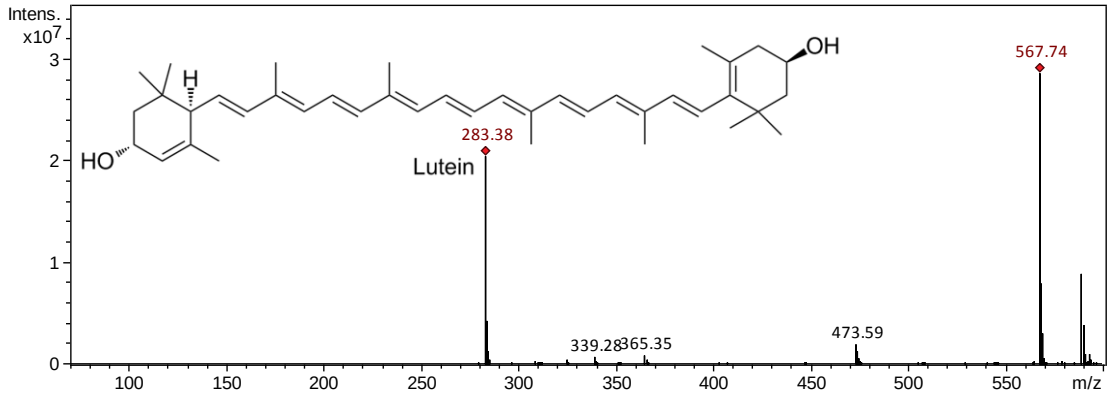

Figure A1 HPLC-ESI-MS spectrum of lutein (negative mode $[M-H]^-$567.74)

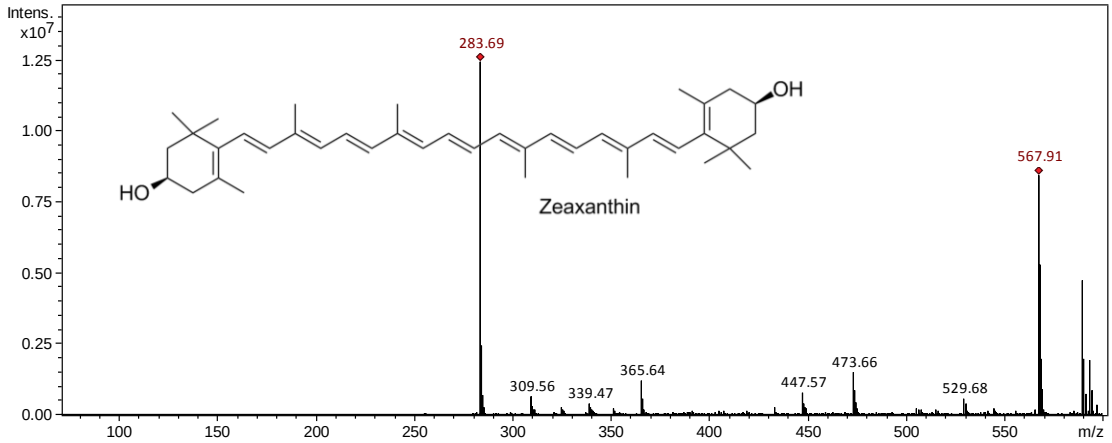

Figure A2 HPLC-ESI-MS spectrum of zeaxanthin (negative mode $[M-H]^-$567.91)

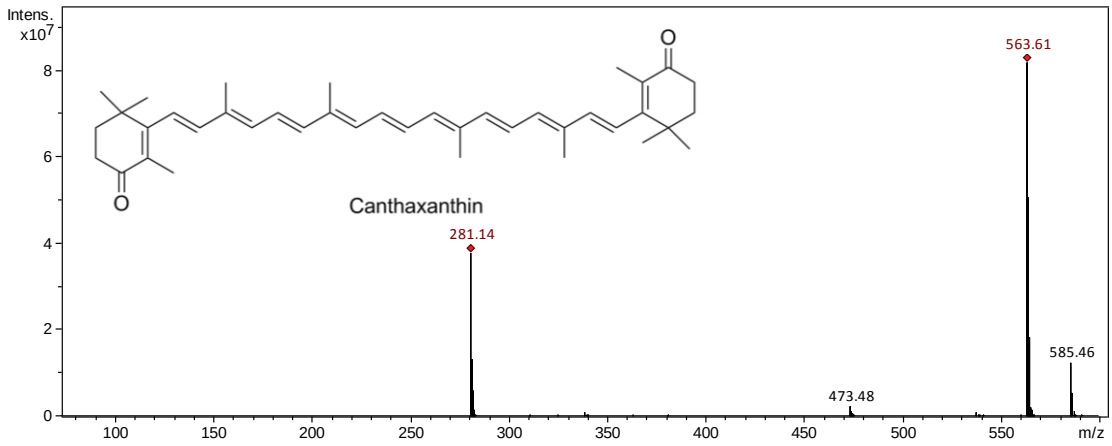

Figure A3 HPLC-ESI-MS spectrum of canthaxanthin (negative mode $[M-H]^-$563.61)

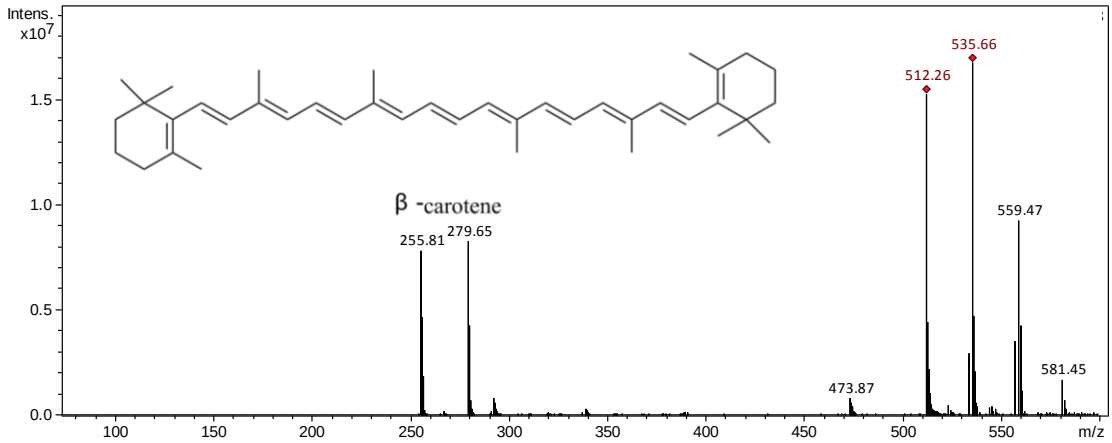

Figure A4 HPLC-ESI-MS spectrum of β-carotene (negative mode $[M-H]^-$535.66)