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A STUDY OF ANTIMICROBIAL AND SURFACTANT PROPERTIES OF N-LAUROYL AMINO ACIDS AND DEVELOPMENT OF SIMILAR COMPOUNDS FROM CANOLA MEAL PROTEINS

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By

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ABSTRACT

The present study investigated an alternative approach to utilize canola meal proteins. In commercial canola oil production, two different processes are used to extract oil: pre-pressed solvent extraction (PSE) and expeller-pressing. The former is a more widely used process that produces desolventized-toasted (DT) meal; whereas the latter affords cold-pressed (CP) meal. Both DT and CP meals are primarily used in animal feeds. This study investigated the hypothesis that amino acids (AAs) released from meal protein hydrolysis can be chemically modified to generate compounds with useful functions. Pre-treatment of DT and CP meal with ethanol (99%, v/v) and following protein separation from the pre-treated meal were studied as process condition optimizations to obtain protein containing a minimum amount of non-protein components and maximize meal protein recovery. The optimum conditions for ethanol treatment were achieved at 50°C for 30 min at a meal-to-ethanol ratio of 1:4 (w:w), reducing the oil content of the meal to 1%. The protein recovery using aqueous extraction was found optimum at pH 12 with a meal:water ratio of 1:10 (w:v), resulting in 73% and 33% recovery of protein from ethanol pre-treated CP and DT meals, respectively, in a single extraction. Repeated extraction of ethanol pre-treated meal increased protein recoveries to 79% and 38% from CP and DT meal, respectively. Untreated meals and ethanol pre-treated meals were then hydrolyzed with 6 M HCl (protein:acid ratio of 5 mg:2 mL) for 24 h at 110°C. The untreated CP meal released 279 mg AA/g of dbm (dry biomass), and AA recovery was improved to 373 mg AA/g dbm after ethanol pre-treatment. However, untreated DT meal released 400 mg AA/g dbm and no improvement in AA recovery after ethanol pre-treatment. Hydrolysis of separated protein fractions from ethanol pre-treated CP and DT meals yielded 544 mg AA/g dbm and 382 mg AA/g dbm, respectively. H_2SO_4 was examined as an alternative acid. More than 80% of the total AAs of CP proteins were released with 3 M H_2SO_4, while for DT meal proteins, a 5-M concentration was needed to achieve the same level of hydrolysis. H_2SO_4 hydrolysis released less free AAs and more peptides than did HCl at lower acid concentrations of 0.5-1.5 M. Results for the extent of hydrolysis and AA yield indicated that hydrolysis of DT meal protein was less efficient when compared to CP meal proteins.

N-acylated (N-lauroyl) derivatives were prepared from six commercially available reagent grade AAs (glutamic acid, lysine, leucine, proline, valine, glycine) selected based on their abundance in

canola protein, using reaction with lauroyl chloride. The structures of the resulting compounds were confirmed by FTIR and NMR spectroscopy. Aqueous solutions (0.1%, w/v) of N-lauroyl AA derivatives reduced the surface tension of pure water. The critical micelle concentration (CMC) was comparatively lower than sodium dodecyl sulphate (SDS) for all of the tested sodium N-lauroyl AAs except for the proline derivative. Among all the acylated derivatives tested and SDS, the sodium N-lauroyl AA mixture imparted the lowest surface tension (29.5 mN/m), the lowest CMC (1,100 ppm), and the highest foam stability (38% after 180 min). Solutions of the sodium N-lauroyl glutaminate had the lowest foaming capacity and stability. The *in-vitro* growth inhibition of sodium N-acylated AAs was studied against pathogenic and non-pathogenic three Gram-negative (*Escherichia coli* TOP10F, *Pseudomonas fragi*, *Salmonella enteritidis*) and three Gram-positive (*Lactobacillus plantarum*, *Lactococcus lactis*, *Listeria monocytogenes*) bacteria. The sodium N-lauroyl derivatives of glycine, leucine, proline, and valine at 25 ppm inhibited growth (> 90%) of all organisms tested. The N-lauroyl derivatives of lysine and the AA mixture had lower solubilities than the others and exhibited the lowest growth inhibitions.

To confirm the potential of canola meal protein hydrolysate in those functions, N-lauroyl products of the acid hydrolysate of DT meal proteins were prepared and tested. Protein extraction at pH 12 with a meal:water ratio of 1:10 (w:v) followed by ultrafiltration (5 kDa MWCO) gave the highest protein concentration (830 mg/g dbm) and generated the highest free AA yield upon hydrolysis. Prior to acylation, the hydrolysate was separated into three fractions based on charge and polarity, resulting in Fraction 1: containing negatively charged and uncharged polar AAs, Fraction 2: AAs with hydrophobic side chains, and Fraction 3: positively charged AAs. All the separated fractions and the unfractionated hydrolysate were acylated to obtain their N-lauroyl products. The unfractionated protein hydrolysate and the Fraction 1 afforded measurable quantities of N-acylated product that could proceed to further purification and preparation of their sodium salts. Sodium lauroyl products from the unfractionated hydrolysate gave the highest inhibitory activity at 250 ppm exhibiting higher than 87% of growth inhibition against all bacterial strains tested. The sodium salt of acylated Fraction 1 gave complete growth inhibition against *L. lactis* at 125 ppm and 70% growth inhibition against all of the strains except *P. fragi*. This study showed that 1) the differences in the oil extraction process conditions was a significant factor to determine the achievable degrees of hydrolysis and free amino acid content due to acid hydrolysis, 2) AAs

obtained from DT and CP meals can be modified to obtain useful molecules, 3) N-lauroyl AAs were bi-functional molecules having surface-active and anti-microbial activities. These findings lead to uses of canola meal proteins derived AAs. Canola meal proteins can be converted to AAs to generate useful compounds, showing an alternative to using industrially processed oilseed meals.

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LIST OF ABBREVIATIONS

AA	Amino acid
CAGR	Compound annual growth rate
CCC	Central composite design
CFU	Colony foaming units
CMC	Critical micelle concentration
COSY	Correlated spectroscopy
CP	Cold pressed
CV	Column volume
dbm	Dry biomass
DEPT	Distortionless enhancement by polarization transfer
DEPTQ	Distortionless enhancement by polarization transfer with retention of quaternary carbon
dmb	Dry mass basis
DT	Desolventizer-toasted
EAA	Essential amino acids
FAA	Free amino acids
FDA	Food and drug administration
FP	Full pressed
FPLC	Fast protein liquid chromatography
FTIR	Fourier transform-infrared
GRAS	Generally recognized as safe
HPLC	High performance liquid chromatography
MIC	Minimal inhibitory concentration
MWCO	Molecular weight cut off
MSG	Monosodium glutamate
NMR	Nuclear magnetic resonance
PSE	Pre-pressed solvent extraction
RSM	Response surface method
SAE	Sinapic acid equivalents
SDS	Sodium dodecyl sulphates
SLES	Sodium laureth sulphates
SSP	Canola seed storage protein
TEA	Triethyl amine
TLC	Thin layer chromatography
UDEFT	Uniform driven equilibrium Fourier transform
UPLC	Ultra-performance liquid chromatography

1. INTRODUCTION

Crop processing generates a variety of by-products that differ in chemical composition. These by-products can be converted into various chemical compounds and materials depending on the inherent chemical constituents. Utilizing crop industry by-products and generating further industries based on them is crucial in effectively managing agriculture resources. Conversion of agro-industry by-products into useful molecules and materials is the basis of crop biorefining and value-added crop utilization while supporting the sustainability of the crop industry. Transformation of widely available by-products, such as meals, bran, straw, and husk, into building block chemicals for further use plays a vital role in shifting from petroleum-based to renewable bio-based feedstocks. The development of the bio-ethanol industry by utilizing cellulosic fibre-rich crop residues is a good example. Scientific investigation of conversion chemistries and implementation of successful conversion processes strongly support the development of a sustainable crop industry.

Amino acids (AAs) synthesized in plants are incorporated in forming proteins to serve various roles for the plants. In seeds, proteins are in higher concentration than in vegetative tissues. Oil extraction from oilseed like canola generates meal biomass rich in protein after extracting oil. Canola production in Canada was ~19 million tonnes in 2021 (CCC, 2019), of which about 55% was crushed domestically, primarily by pre-press solvent extraction (PSE). This produced ~5.6 million tonnes of canola meal (COPA, 2020). During the PSE oil extraction process, canola meal proteins are modified by their exposure to high temperature and pressure and the hot hexane regimes of the PSE process. Canola meal arising from PSE is desolventized at high temperature (toasting), yielding desolventized-toasted (DT) meal. It is well documented that proteins in DT canola meal are less soluble, lower in available lysine, and have lost native physico-chemical properties required for further applications (Mosenthin *et al.*, 2016; Newkirk *et al.*, 2003). The process-induced changes of the desolventizing-toasting process to canola meal proteins are

primarily at the secondary or higher-order structure level, and no documented evidence of hydrolysis or alteration to the primary structure. Therefore, it can be assumed that the amino acid backbone of the canola meal proteins is not disturbed, and peptide bonds are intact. DT canola meal is currently used in animal feed formulations, particularly in dairy cow rations. Cold pressing, which is expeller pressing under low temperature (60°C), is also employed for canola oil extraction but is limited to organic, non-genetically modified oil markets. The resulting cold-pressed (CP) meal is considered less processed and contains high residual oil level than the DT meal. Canola seed storage proteins (SSPs) are somewhat different in composition from legume seeds such as soy, pea, and other pulses. Canola SSPs are mainly of 12*S* and 2*S* types. The 2*S* canola proteins are napins and have a higher content of sulphur-containing AAs, and disulphide bonds are involved in their structural stabilization than 12*S* canola SSP, cruciferin (Wanasundara, 2011). Although limited in application because of compromised nutritional and functional qualities, several publications report that canola protein derived AAs and peptides have a high potential for use in non-food applications, such as peptides with antihypertensive, antioxidant, and antidiabetic activities, as well as peptides that act as plant growth stimulants (Marczak *et al*., 2003; Wu & Muir, 2008; Zhang *et al*., 2008). The potential use of DT canola meal protein in non-food applications would be advantageous as nutritional and functional quality are compromised.

The AAs industry provides essential AAs for animal feed (*e.g*., lysine, methionine, and threonine as additives), flavour enhancers (*e.g*., monosodium glutamate, serine, aspartic acid), pharmaceutical ingredients, specialty nutrients for various medical diets (*e.g*., branched-chain AAs), and personal care products (*e.g*., glutamic acid-based surfactants), and other applications. These AAs are prepared through fermentation (*e.g*., glutamic acid and lysine) and chemical synthesis (*e.g*., methionine) (Leuchtenberger *et al*., 2005; Tonouchi & Ito, 2017; Zhao *et al*., 2016).

The surfactant industry is moving towards bio-safe products and processes. Surfactants are molecules that stabilize immiscible phases and have applications that go beyond food and feed; personal care, pharmaceutical and industrial applications such as detergents, to name a few. In the "greening" of the surfactant industry, a surfactant having a molecular structure containing at least 50% sustainable, renewable materials are defined as "naturally derived" (Beerling and Gough, 2013). AAs have multiple functional groups and comprise a promising class of bio-molecules to

develop eco-friendly, naturally derived surfactants, mainly due to their bio-degradability attributes. Furthermore, AAs are known to have a wide range of favourable functional attributes (*e.g.*, wetting, foaming, antimicrobial) and applications in consumer products, such as personal care, cosmetics, pharmaceuticals, and household detergents (Bordes & Holmberg, 2015; Morán *et al*., 2004; Pinazo *et al*., 2011; Scott & Jones, 2000). It has also been reported that N-acylated AAs with a fatty acid moiety could have attractive surfactant properties, including mildness and moisturizing, which help minimize surfactant damage to the skin (Ananthapadmanabhan *et al*., 2004). However, finding raw materials and applications of green technologies cost-effectively are major challenges for industries moving toward environment-friendly, green market opportunities. Amino acid extraction from protein-rich underutilized materials requires hydrolysis of proteins that can be achieved via chemical or enzymatic means. Chemical hydrolysis of proteins can be conducted by using alkali or acid. It is a comparatively cheap and easy to control method suitable for large-scale processing to obtain AAs with less peptide production than the enzymatic hydrolysis (Alvarez *et al*., 2012; Fountoulakis & Lahm, 1998). Agri-food processing produces many by-products and wastes that are protein-rich feedstocks and hold potential for reuse or utilization to generate AAs as low-cost raw materials for other industries.

Canola meal processors need to develop alternative methods to provide them with more economic value. The present study was designed based on two considerations: 1) the availability of commercially processed canola meal that contain extensively processed proteins not suited for use in value-added plant proteins, and 2) the opportunities available for AA-based compounds in the bio-based chemical market. The inherent and process-induced interactions of proteins in commercial canola meals complicate downstream processes for protein-specific hydrolysis in obtaining AAs. Therefore, investigations are needed to understand the issues of canola meal proteins in relation to chemical hydrolysis and the nature of the products obtained from such processes. The overall objective was to investigate the generation of AAs from proteins in extensively processed commercial canola meal and then convert them into molecules that possess activities useful for developing bio-based chemicals to enhance the canola bio-economy. The study further focused on improving the value of canola meal for entry into new products, particularly bio-based chemical development from meal protein via converting it into AAs. Understanding the constraints of industrially processed canola meal in generating AAs, ways of mitigating the

constraints, and the potential of converting them into functional molecules were the objectives in this study. Investigations addressed both extensively processed DT meal and cold-pressed (CP) meal that had been subjected to less processing stress. Seven sub-objectives were identified for the investigations needed to achieve the overall objective.

Sub-objective 1: To determine the effects of ethanol pre-treatment conditions (meal-to-ethanol ratio, extraction time and treatment temperature) to reduce oil, free sugars and phenolics (total phenolics) of CP and DT canola meals

Sub-objective 2: To determine the effects of pH and meal-to-solvent ratio for recovering maximum protein content from ethanol-pretreated CP and DT canola meals

Sub-objective 3: To determine suitable H_2SO_4 concentration to release the maximum amount of free AAs from CP and DT canola meal protein

Sub-objective 4: To determine the AA composition of canola protein H_2SO_4 hydrolysates

Sub-objective 5: To generate N-lauroyl AAs from the most abundant AAs (comprising >50% of the total) of canola protein and N-lauroyl product of mixture of those individual AAs and confirm their structures

Sub-objective 6: To determine surfactant properties and antimicrobial activities of N-lauroyl derivatives of the individual AAs and N-lauroyl product of mixture of those individual AAs (from sub-objective 5) compared to the standard surfactant, sodium dodecyl sulphate (SDS)

Sub-objective 7: To study antimicrobial properties of the N-acyl products of canola meal protein hydrolysate

Experiments were designed to test the following hypotheses to achieve the above-mentioned sub-objectives.

1. A pre-treatment for CP meal and DT meal by ethanol extraction will reduce residual oil and small molecules such as free sugars and total phenolics of commercial canola meal.
2. Reducing residual oil and small molecules, such as free sugars and total phenolics, will improve canola meal protein extractability and protein hydrolysis of CP and DT meals.
3. Equal free amino acid yields can be obtained from hydrolysis of proteins separated from CP and DT meals.

4. Sulphuric acid (H_2SO_4) can be used in place of hydrochloric acid (HCl) for acid hydrolysis of canola protein and the release of AAs.
5. N-lauroyl derivatives of individual AAs produced by canola protein hydrolysis possess surfactant and antimicrobial properties.
6. N-lauroyl product of AA mixture has better or similar antimicrobial properties than N-lauroyl derivatives of individual AAs, which are predominant in canola protein.
7. N-lauroyl products of canola protein hydrolysate possess antimicrobial activities.

2. LITERATURE REVIEW

2.1 Amino acids and their chemistry

An amino acid (AA) comprises an amino group, a carboxyl group, a hydrogen atom and a side-chain group around a central carbon atom (Figure 2.1A). AAs are found in free form or amide-linked form in peptides or polypeptide proteins. Twenty AAs are involved in constructing proteins, and they differ in the side-chain structure (Figure 2.1B). Among these AAs, 19 are primary amines while proline is a secondary amine (Bischoff & Schluter, 2012; Lee *et al*., 2013; Pradhan & Vera, 1998); *i.e*., all are α-amino carboxylic acids while in proline, the amino group forms part of a pyrrolidine ring. Pure AAs are non-volatile crystalline solids, insoluble in non-polar solvents and considerably soluble in water depending on side-chain chemical characteristics. AAs have dissociation constants lower than the most carboxylic acids and aliphatic amines. Except for glycine, all AAs contain at least one chiral center and are optically active (Morrison and Boyd, 1987). The carbon atom next to the carboxyl group of the amino acid molecule is the α-carbon (Figure 2.1A).

An AA molecule is an α-amino carboxylic acid, *i.e*., it has two functional groups: an amino group that can yield a positive charge when protonated and a carboxylic group giving a negative charge when deprotonated. When an AA dissolves in water, carboxyl and amino groups ionize, and the AA acts as a zwitterion, *i.e*., at physiological pH, AAs have both negative and positive charges. AAs are amphoteric molecules since they can react as acids or bases. In the acidic medium, amino acid zwitterion is a base that accepts a proton to yield a cation. In the basic medium, it is an acid that loses a proton to form an anion. In an acidic medium, the carboxylic group acts as the basic site and accepts a proton, and it is the ammonium cation that acts as an acidic site and donates a proton in the alkaline medium. The pH at which the AA behaves as neutral, dipolar zwitterion is the isoelectric point (pI), and it depends on the structure, particularly the ionizable side chain of

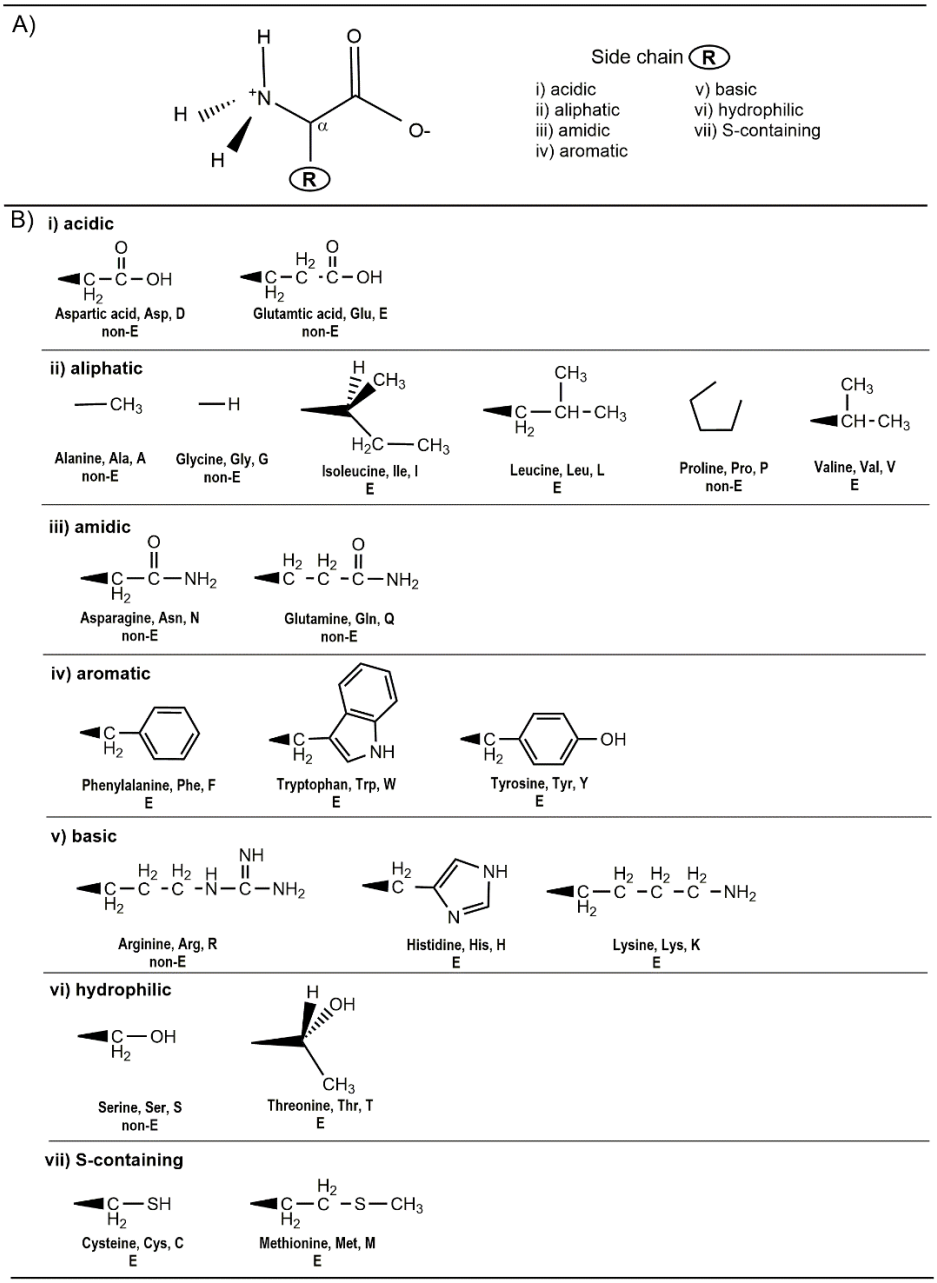


Figure 2.1 Basic structure of an amino acid (L-form) showing essential groups attached to central α-C atom A), and side chain (R) differences of 20 amino acids (B. i to vii).

AA. AAs can have basic or acidic properties depending on the functional side group. Therefore, AAs are further classified as neutral, acidic, and basic (Figure 2.1B), depending on the structure of the side chains (Lee *et al*., 2013; Pradhan and Vera, 2013; McMurray, 2000). In addition to that, the AAs with the side-chain group that is cyclic, non-polar/hydrophobic or hydroxylated also exist. The second amino group of aspartic and glutamic acid could exist in amidated form (Figure 2.1B).

Condensation between the α-amino group of one AA and the α-carboxyl group of another AA forms the peptide or amide bond (Figure 2.2), and it is the most fundamental chemical bond involved in forming a (poly)peptide chain that is the primary structure of a protein. In living systems, peptide bond formation is catalyzed by the large subunit of the ribosome, but the biochemical mechanism is a debated question. The formation of a dipeptide from two AAs is a condensation reaction, as it involves the loss of a water molecule (Figure 2.2). Due to the partial double bond character of the peptide bond, free rotation around the bond is restricted. The steric hindrance between the side chains results in a peptide bond in trans configuration (Rodhina *et al*., 2006; Champe and Harvey, 1994). Electrons are shared by the N and O atoms; therefore, the N-C and C-O bonds exhibit partial double-bond character. The short carbonyl C-N bond length of 1.32 Å compared to the usual C-N single bond length of 1.47 Å is consistent with the partial double-bond character of the peptide linkage.

Peptide bond hydrolysis cleaves polypeptides into AAs by adding a water molecule and resulting in respective AAs (Figure 2.2). Nonenzymatic peptide bond hydrolysis is a slow process (Radzicka & Wolfenden, 1996), except at extreme pH and temperature with catalysis by acids or bases (Smith & Hansen, 1998). Among the hydrolases, proteases and aminopeptidases can catalyze peptide bond hydrolysis and differ in their catalytic ability based on AA specificity and regioselectivity.

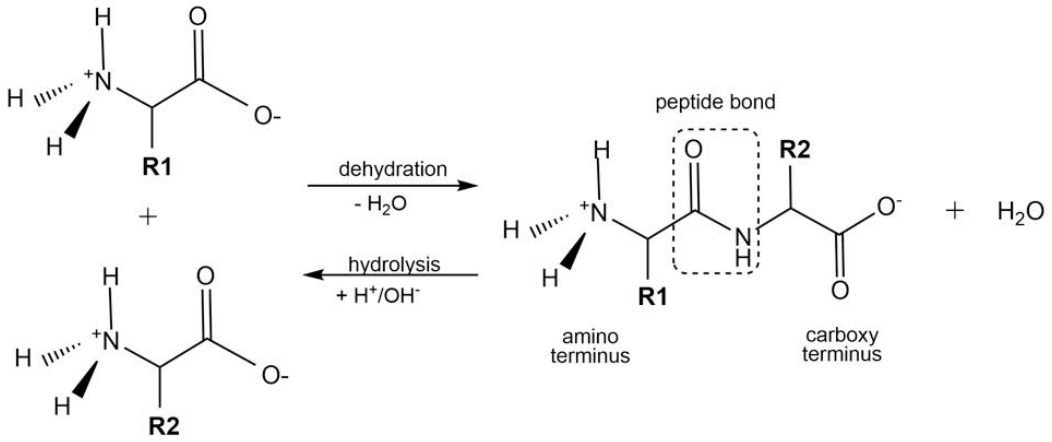


Figure 2.2 Peptide bond formation between two amino acids and hydrolysis of peptide bond to generate constituting amino acids.

2.2 Industrial production of amino acids

The global AA market was ~9.8 million tons in 2020 and is expected to reach 13.1 million tons by 2026, exhibiting a compound annual growth rate (CAGR) of ~5% during 2021-2026 (Imarcgroup, 2021). Similarly, in 2020, the global AA market was estimated at USD 28.6 billion and poised to be at USD 30.2 billion in 2021, at a CAGR of 6.03% to reach USD 40.6 billion by 2026 (Newswire, 2021). Primary markets for AAs are food, feed, health and wellness industries, and their demand rapidly increased with the mass production of individual AA (Scheper, 2003). Glutamic acid is the most demanding AA, followed by L-aspartic acid, L-phenylalanine, L-lysine, and L-threonine. Depending on the raw material(s) used, both animal- and plant-based AAs are available, while plant-origin AAs have a higher demand than animal sources due to rising preference towards vegan and sustainable production systems (Leuchtenberger *et al*., 2005; Scheper, 2003).

Production of AAs can be achieved through microbial methods, chemical synthesis or extraction. Microbial methods are employed through fermentation or enzyme-assisted synthesis. Fermentation is popular in industrial-scale AA production, mostly for producing L-AAs. The enzymatic process is more suitable for producing optically pure AA on a large scale; however, this method uses specific substrates that manufacturing associates with a cost. Production of pure L-isomers by chemical synthesis is difficult, and when racemic forms of AA are produced, an additional optical resolution step is needed to obtain biologically active L-isomers. Production of glycine and methionine via chemical synthesis is economically beneficial since both L-isomer and

D, L racemic mixture of these two AA show similar effects as additives in animal feed. Extraction of AAs from protein-rich plant or animal sources is possible through hydrolysis; however, adequate amounts to fulfil the demand for most AAs are not reachable (D'Este *et al.*, 2018; Leuchtenberger *et al.*, 2005; Scheper, 2003). Therefore, current investigations mainly focus on identifying sources, including non-food non-conventional sources for protein-derived AAs (Lammens *et al.*, 2012; Lasekan *et al.*, 2013).

2.2.1 Feedstocks for amino acid production

One of the significant challenges encountered by the AA industry is ensuring raw material supply for production. In industrial AA production through fermentation, plant-based materials are preferably used as carbon sources, for example, cane molasses, beet molasses and starch hydrolysate from corn (corn syrup) or cassava. The cost of the carbon source is a major consideration in the process. Methanol has become an attractive carbon source in laboratory-scale fermentations than sugars due to its low cost, availability, purity, and water solubility. Inorganic nitrogen sources like ammonia and ammonium salts (*e.g.*, sulphate salts) provide the requirement of a nitrogen source. In addition, cheap, natural, plant- or animal-origin sources supply minerals and vitamins (Scheper, 2003). In the enzymatic synthesis of AA, enzymes are obtained through microbial fermentation with suitable raw materials are employed through chemical synthesis. For instance, L-aspartic acid is one of the first commercialized AAs produced via enzymatic synthesis using fumaric acid and ammonia and immobilized *Escherichia coli* cells with high aspartase activities (Hsiao *et al.*, 1988). For the production of AA via protein hydrolysis, protein-rich feedstocks, primarily the by-products of plant or animal processing industries, are used (Lammens *et al.*, 2012).

2.2.1.1 Animal industry by-products

Animal by-products are a rich source of proteins. By-products of the meat processing industry are used in animal feed and fertilizers. Conversions of proteins in these by-products to protein hydrolysates and their utilizations in the above applications are further beneficial since it reduces final waste streams, which can cause environmental pollution (Lasekan *et al.*, 2013). Useful bio-molecules from animal industry waste include keratin hydrolysate from poultry feathers (Mothé *et al.*, 2017), peptide hydrolysate from poultry processing leftovers (Nikolaev *et al.*, 2016), and AAs

from silkworm industry waste (Ding *et al*., 2017; Wu & Zhang, 2014). Several studies reported on applications of chemical and enzymatic hydrolysis methods to hydrolyze various waste protein sources and their feasibility to achieve expected goals. These studies include acid hydrolysis of hemoglobin from porcine blood (Alvarez *et al*., 2012) and poultry feathers (Bouhamed & Kechaou, 2017) and enzymatic preparation of porcine hemoglobin hydrolysate (Chang *et al*., 2007) and poultry peptide hydrolysate (Nikolaev *et al*., 2016).

2.2.1.2 Plant processing by-products

Utilization of protein-rich plant biomass from main-stream processing, such as dried distiller's grain, vinasse (a by-product from ethanol production from sugar beets or sugar cane), cassava sludge, oilseed meals, have been studied to obtain AAs and protein hydrolysates (Lammens *et al*., 2012). Uses of different plant-based protein sources have been reported in laboratory scales for generating protein hydrolysates, *i.e*., canola/rapeseed meal protein (Alashi *et al*., 2014; Chabanon *et al*., 2007; He *et al*., 2013; Ma & Ooraikul, 1986; Salazar-Villanea *et al*., 2017), chickpea protein (Ertani *et al*., 2019), pea protein (Rondel *et al*., 2011), and cottonseed meal (Zhang *et al*., 2016). In addition, glutamic acid production has been carried out from wheat-dried distiller's grains with solubles after ethanol production using dilute acid HCl (1 M) hydrolysis (Sari *et al*., 2014).

2.2.2 Processes of amino acid production

2.2.2.1 Microbial fermentation

Large-scale fermentation of AAs started in the 1950s after discovering glutamate-producing bacterium, *Corynebacterium glutamicum* in Japan. Today's fermentation process is well developed for large-scale industrial production of major AAs (D'Este *et al*., 2018; Leuchtenberger *et al*., 2005; Zorn & Czermak, 2014). L-glutamic acid is primarily produced by microbial fermentation; in addition, L-lysine, L-threonine, L-tryptophan are also produced on a large scale (Leuchtenberger *et al*., 2005). The fermentation process of AA production generally uses cane molasses, beet molasses, and starch hydrolysates from corn or cassava as carbon sources, and ammonia or ammonium sulphate as nitrogen supplements. The commonly employed bacteria strains in the fermentation process are *Escherichia coli*, *Bacillus subtilis*, and glutamate-producing coryneform bacteria (D'Este *et al*., 2018; Scheper, 2003). Both *C. glutamicum* and *E. coli* produce a range of

AAs, and their genetically modified forms are also employed for increased production yield and obtaining additional types of AAs (D'Este *et al*., 2018).

Fermentation processes comprise three main steps: 1) cultivation of AA producing microbial strains, 2) AA purification from the fermentation broth, and 3) wastewater treatment (Scheper, 2003). Industrial-scale AA production requires purification, which can be achieved via ion exchange followed by crystallization, double crystallization, spray drying or granulation. Food and pharmaceutical industries require AAs of high purity, *i.e*., >99% based on product dry matter (Zorn & Czermak, 2014).

2.2.2.2 Protein hydrolysis

Extraction of AAs from protein hydrolysates is the oldest method of obtaining AA; however, it is not particularly feasible for large-scale production of AAs. Industrial-scale applications of extraction of AAs from protein hydrolysates are limited to the production of a few kinds of AAs, such as L-cysteine, L-leucine, and L-tyrosine. The advantage of this method is the possibility of using animal and plant industry by-products and waste streams as protein sources (Breuer *et al*., 2004; D'Este *et al*., 2018). Hydrolysates can be prepared by chemical or enzymatic means combined or separately (Chabanon *et al*., 2007). Chemical treatments with acid or base rapidly hydrolyze peptide bonds of proteins; however, they associate with the loss of some AAs through chemical reactions beyond hydrolysis. Enzymatic hydrolysis requires milder conditions, a longer reaction time and is substrate-specific and expensive (Alashi *et al*., 2014; Lasekan *et al*., 2013). Studies and reviews published on acid hydrolysis of proteins show that HCl is the most common acid used in the process. In determining amino acid levels of a protein source, the universally accepted method is the Association of Official Analytical Chemists method 994.12b (AOAC International, 2005) which uses acid hydrolysis with 6 M HCl for 20-24 h at 110°C. Under the conditions of this acid hydrolysis, five AAs are modified further: two amide side-chain AAs (asparagine and glutamine) are completely hydrolyzed to their respective acid form, *i.e*., aspartic acid and glutamic acid, tryptophan is completely degraded, cysteine and methionine are oxidized. Base hydrolysis is used to determine tryptophan, which is stable under basic conditions. Base hydrolysis is also applied for samples rich in carbohydrates. Furthermore, there is a possibility of the racemization of AAs during chemical hydrolysis (Fountoulakis & Lahm, 1998).

2.3 Industrial uses of amino acids

History of industrial uses of AAs goes back to 1908, when Prof. Kikunae Ikeda discovered glutamate as a substance providing umami flavor. This discovery resulted in a market for monosodium glutamate, commonly known as MSG for food seasoning since 1909. Among the industries that currently use AAs, pharmaceuticals, food additives, cosmetics, pesticides, and animal feed are the most prominent (Sadovnikova & Belikov, 1978; Tonouchi & Ito, 2017).

2.3.1 Food industry use

As play key roles in human and animal physiology, including the regulation of main metabolic pathways in the body that are responsible for immune responses, enzyme synthesis, cell signalling molecules, gene expression, phosphorylation cascade, synthesis of precursors of hormone synthesis and other nitrogenous substances (Wu, 2009; Wu *et al*., 2014). A balanced dietary intake of AAs, including essential AAs (EAA), is vital for protein nutrition (Wu *et al*., 2014). Protein sources are different in their AA composition and the levels that EAA present in the constituent proteins. Most plant proteins are short for a few EAA. Therefore, blends of different plant proteins or plant and animal proteins can be employed to achieve the requirement of complete protein in the diet (Gorissen *et al*., 2018). Furthermore, L-AAs are considered as a direct food ingredient by US Food and Drug Administration (FDA) since they are recognized to be generally recognized as safe (GRAS) (Burnett *et al*., 2013).

In addition to providing nutrition, AAs are crucial in food taste and flavour. Three AAs occupy a large segment in the AA market; L-glutamic acid (as MSG), and L-aspartic acid and L-phenylalanine as starting materials for peptide sweetener L-aspartyl-phenylalanyl methyl ester or aspartame (Bhalla *et al*., 2007; Leuchtenberger *et al*., 2005). Fermented foods (*e.g*., soy sauce, cheese, fermented meat, wine, and sourdough) are particularly rich in bitter, umami or kokumi taste and flavour generated by taste active AAs and peptides or their AA-derived products (Zhao *et al*., 2016). These flavour compounds in fermented foods are generated by proteolysis of a substrate protein by exogenous proteases secreted from fermenting microorganisms and microbial catabolism of AAs. Lactic acid bacteria are a mainly utilized category of microorganisms in food fermentation (Ardo, 2006; Zhao *et al*., 2016). Although decarboxylation of AAs gives desirable

flavours like savoury and umami, it can also result in off-flavour polyamines, such as cadaverine and putrescine. Branched-chain AAs (*i.e*., leucine, isoleucine, valine) are converted to specific aldehydes, alcohols, and acid with various flavours (*i.e*., malty, fruity, sweaty, rancid, rotten) and catabolized into aromatic AAs that give flavours like rose, bitter almond, putrid. Furthermore, the aroma of boiled cabbage, potatoes, meat, garlic, and eggs are generated by sulphur-containing AAs (Ardo, 2006). Heat processing of food also creates flavour and taste active compounds due to the reaction of AAs with other reactive food components. Maillard reaction between AAs and molecules containing carbonyl groups generates a series of compounds responsible for flavour and taste. Cysteine is used as an additive in meat as it generates meat-flavour-like compounds by Maillard reaction. It can also be an additive in bread dough (Pripis-Nicolau *et al*., 2000).

AAs are nutrition supplements along with protein supplements that have become a lucrative industry worth 14 billion USD which is expected to reach 21. 5 billion USD by 2025 (Huecker *et al*., 2019). Whey proteins as concentrates, isolates or hydrolysates are dominant protein supplements in the market. Since whey proteins are naturally rich in branched-chain AAs, these products are widely used in sports nutrition products, infant food formulations, meat, dairy, bakery, confectionery, *etc*. (Kuesten & Hu, 2020; Shinde *et al*., 2018). In addition, casein and plant proteins (*i.e*., soy, rice, pea) are also popular as protein supplements (Kuesten & Hu, 2020).

2.3.2 Animal feed use

Dietary AAs are needed for animal growth, development, reproduction, lactation, and health while playing a pivotal role in regulating food intake and nutrient metabolism in animals (Li *et al*., 2011; Wu *et al*., 2014). In addition to the grass and forage crops, various crop processing by-products are used as animal feed: *e.g*., wheat feed (flour milling residues, brewers' grains), sugar beet pulp and oilseed meals (soybean, canola/rapeseed, *etc*.) (Wilkinson, 2011). Most of the commonly used feed ingredients do not provide nutritionally balanced protein in animal diets and do not match the animal's physiological demand for proteins and AAs. One main reason for this scenario is the level of available EAAs, such as methionine, lysine, threonine, and tryptophan. They are deficient in cereal and soybean meal-based diets and are the most limiting EAAs for growth and other essential physiological functions (Zorn & Czermak, 2014). This issue is overcome by supplying these limiting EAAs in their free form. Feed-grade L-lysine, D, L-methionine, L-threonine, and L-

tryptophan occupy a large portion of the AA market (Leuchtenberger *et al.*, 2005). Formulation of diets for farm animals, such as laying hens (Dailibard & Paillard, 1995; Rostagno *et al.*, 1995), broiler chicken (Dari *et al.*, 2005), and swine (Parsons, 1996), is practiced based on digestible AAs rather than the total AAs. Feed-grade AAs can satisfy deficiencies of AAs in feeds and reduce additional feed requirements, consequently dropping the demand for resources to generate more feed and the subsequent impact on the environment (Tonouchi & Ito, 2017). These practices prove that supplying feed-grade AAs as an alternative to intact proteins has been reported as advantageous. They are used in grow-finish diets to improve animal performance at optimized cost and reduction in nitrogen excretion. Furthermore, the addition of feed grade lysine to corn-soybean meal-based lactation diets, with supplemental methionine and threonine, had no adverse effect on litter size or growth rate (Greiner *et al.*, 2018).

2.3.3 Non-food or feed use

Today world market for medical-use AAs is more than 30,000 tons per year. Under various medical conditions, medical grade AAs are supplied through gastro-intestinal tract or intravenously. Branched-chain AAs are widely used since they can act on liver cells directly and promote protein synthesis (Tonouchi & Ito, 2017). A large number of natural antibiotics containing D-AAs are isolated mainly from bacteria, and their applications are primarily related to antimicrobial activities (Martnez-Rodrguez *et al.*, 2010). Antimicrobial activities of AA-based surfactants are assessed mainly with the cationic surfactant ability (Clapés & Infante, 2002).

The cosmetics and personal care industry is another market segment that demands AAs. The market for AAs for cosmetics was 14,000 tons per year in 2012. The primary function of using AAs and their salts in cosmetic formulations is to act as hair and skin conditioning agents. In skin-care products, more than half of the “natural” moisturizing agents are AA (especially glutamate) metabolites (AA acid is 40%) since lotions and creams enriched with AAs can improve skin moisture content. AA derivatives, particularly N-acyl glutamate, are known as ultra-mild surfactants for skin and hair (Couteau & Coiffard, 2016; Tonouchi & Ito, 2017). Moreover, there are AAs that provide additional functions, such as oral care agents (arginine), hair waving/straightening agents (cysteine), antioxidants (cysteine and cysteine HCl), fragrances (cystine), buffering (glycine and its calcium and magnesium salts), pH modulators (lysine and its

calcium and magnesium salts), protection against bacteria, and surface activity (Burnett *et al.*, 2013; Sadovnikova & Belikov, 1978). Arginine is the most used AA in the cosmetic industry, and glycine is the second (Burnett *et al.*, 2013).

AAs are commercially available as plant stimulants that can improve plants' growth and development through fertilizer assimilation, increase uptake of nutrients and water, enhance the photosynthetic rate and dry matter partitioning (Sarojnee *et al.*, 2009). The applications of those plant stimulants are shown to suppress diseases and improve plant growth and crop yield, even under adverse conditions, such as salinity and abiotic stress (Abdel-Mawgoud *et al.*, 2011; Murashev *et al.*, 2020; Radkowski *et al.*, 2020; Thomas *et al.*, 2009).

AAs are feedstock molecules for preparing surfactants. Depending on the free functional group/s of the AA, cationic, anionic, non-ionic, or amphoteric surfactants can be obtained. AAs and long aliphatic chains can be combined with linear (single chain), gemini (dimeric) or glycolipid-like structures (Morán *et al.*, 2004) to show surfactant activities.

2.4 Surfactants based on amino acids and their synthesis

Surfactants are used in various ways and applications in large quantities daily and worldwide. Water pollution caused by synthetic detergents is an increasing concern because of the adverse effect on ecosystems. Anionic surfactants are the main component of detergents and cosmetic formulations, and they contribute to the pollutant profile of sewage and wastewaters. After usage, a fraction of surfactants is released to the environment through wastewater discharge and directly into surface waters or sewage systems. These surfactants can accumulate as sediments in soil and eventually leak into water streams (Li *et al.*, 2017). The environmental concerns of surfactants are primarily focused on the slow degradability of petrochemical-based surfactants that pollute water. Therefore, at least partial replacement with surfactants made with natural or renewable sources has become a current need. Such replacement "green surfactants" are expected to be biodegradable, biocompatible, less irritable, less toxic and consumer-friendly (Infante *et al.*, 2004; Lima *et al.*, 2011; Morán *et al.*, 2004; Pinazo *et al.*, 2011). The pressure from legislation and customers toward using environmentally benign chemicals has brought green surfactants into a significant focus in the surfactant industry (Bordes & Holmberg, 2015). Green surfactants are biological or bio-based

amphiphilic molecules synthesized from renewable raw materials using chemical or bio-chemical modifications (Rebello *et al.*, 2014). Developing a green surfactant considers using natural or synthetic starting materials that have a proper rate of biodegradation, inserting weak bonds into the structure to accelerate degradation (cleavable surfactants), and developing more efficient surfactants which require fewer amounts for the task (*i.e.*, gemini surfactant) (Bordes & Holmberg, 2015).

Bio-surfactants are molecules produced by living cells, such as bacteria, yeast, and fungi (*e.g.*, lipo-amino acids, peptides, proteins, phospholipids, fatty acids, glycolipids, *etc.*) and which are having surfactant properties (Clapés & Infante, 2002; Greber, 2017). Bio-based surfactants are in whole or in a significant part composed of biological products or renewable agriculture materials of plant or animal origin (Pinazo *et al.*, 2011). Similarly, these can be defined as synthetic amphipathic molecules which are based on natural biosurfactant structures (Clapés & Infante, 2002). Amino acid-based surfactants can be used as a synthetic equivalent to bio-surfactants with excellent surfactant activity and low environmental impact (Infante *et al.*, 2004; Tripathy *et al.*, 2018). Generally, the hydrophilic moiety of the molecule, which is the AA or AA residue, can be obtained through the hydrolysis of protein or enzymatic synthesis, and the hydrophobic entity is obtained from natural oil (Pinazo *et al.*, 2011; Takehara, 1989). The synthetic lipo-amino acid or lipo-peptides are structurally and functionally analogues for microbial lipo-amino acids and peptides (Clapés & Infante, 2002).

In the synthesis of surfactants, the polyols (*e.g.*, glycerol, simple sugars) or AAs provide the natural building blocks with polar head groups (Bordes & Holmberg, 2015; Holmberg, 2001). Polyols are mostly used for making non-ionic surfactants. Alkyl polyglucosides, such as fatty acid esters of glucose, glycerol, polyglycerol and sorbitan, are non-ionic surfactants that have had an established position in the surfactant market for a long time (Bordes & Holmberg, 2015). Vegetable oils or triglycerides of plant origin are used to introduce hydrophobic groups, such as fatty acyl groups, into surfactants (Chen *et al.*, 2020). AAs are much more adaptable compared to polyols, due to the presence of 20 different AAs, and all are used in the development of different classes of surfactants (Bordes & Holmberg, 2015). Furthermore, an AA possesses two functional groups (carboxylic and amino) in one molecule, which facilitate the transformation of AAs into a single chain surfactant

using modification with a molecule with a hydrophobic group (*i.e.*, fatty acid, fatty ester, fatty amines, fatty alcohols) through ester alkyl or amide linkages (Pinazo *et al.*, 2016).

Synthetic lipo-amino acid/peptides are available in two main types; 1) α-AAs with long aliphatic side chains (lipidic α-amino acid/peptides), and 2) AA/peptide-lipid conjugates having at least one aliphatic chain, which is bound to functional groups of AA or peptide through amide, ester, thioester, and oxime bonds (Clapés & Infante, 2002). AAs and long aliphatic chains can be combined to generate four main structures: linear or single chain, dimeric or gemini (two polar heads/two AAs and two hydrophobic tails per molecule), bola-amphiphiles and glycerolipid-like structures (Chandra & Tyagi, 2013; Clapés & Infante, 2002; Morán *et al.*, 2004). AAs can be converted to surfactants by attaching hydrophobic moiety to either carboxylic or amino group (Bordes & Holmberg, 2015). Combining long aliphatic chains to an AA can be achieved through α-amino, α-carboxyl, or side chain groups of the AA (Morán *et al.*, 2004). Long aliphatic chain is generally provided by fatty acids or alkyl halides that react with an amino group of AA, yielding corresponding N-acyl or N-alkyl derivatives. The carboxyl group of AAs can react with alkyl amines or aliphatic alcohols to generate their N-alkyl amides or esters, respectively. These reactions can be performed under enzyme catalysis or chemical synthesis (Morán *et al.*, 2004). The four main routes of synthesizing surfactants from AA can be summarized as depicted in Figure 2.3: Routes i and ii give surface-active primary amines, acting as cationic surfactants under neutral or acidic conditions. Route iii leads to anionic surfactants under neutral or alkaline conditions. Route iv results in zwitterionic surfactants (Bordes & Holmberg, 2015). Aspartic acid and glutamic acid contain an extra carboxyl group; therefore, dicarboxylic AA-based surfactants are obtained via routes iii and iv. Moreover, AAs, such as lysine, histidine, and tryptophan, contain more than one functional group (an extra amino group) and reactions through routes i and ii give surfactants with two cationic groups at low and neutral pH depending on the amino acid (Bordes & Holmberg, 2015). AAs with reactive side chains, such as lysine or arginine, offer additional opportunities for the molecular design of monocatenary surfactants. From an economic, environmental and preparation point of view, single-chain surfactants with only one AA on the polar head are desirable compounds (Pinazo *et al.*, 2016a).

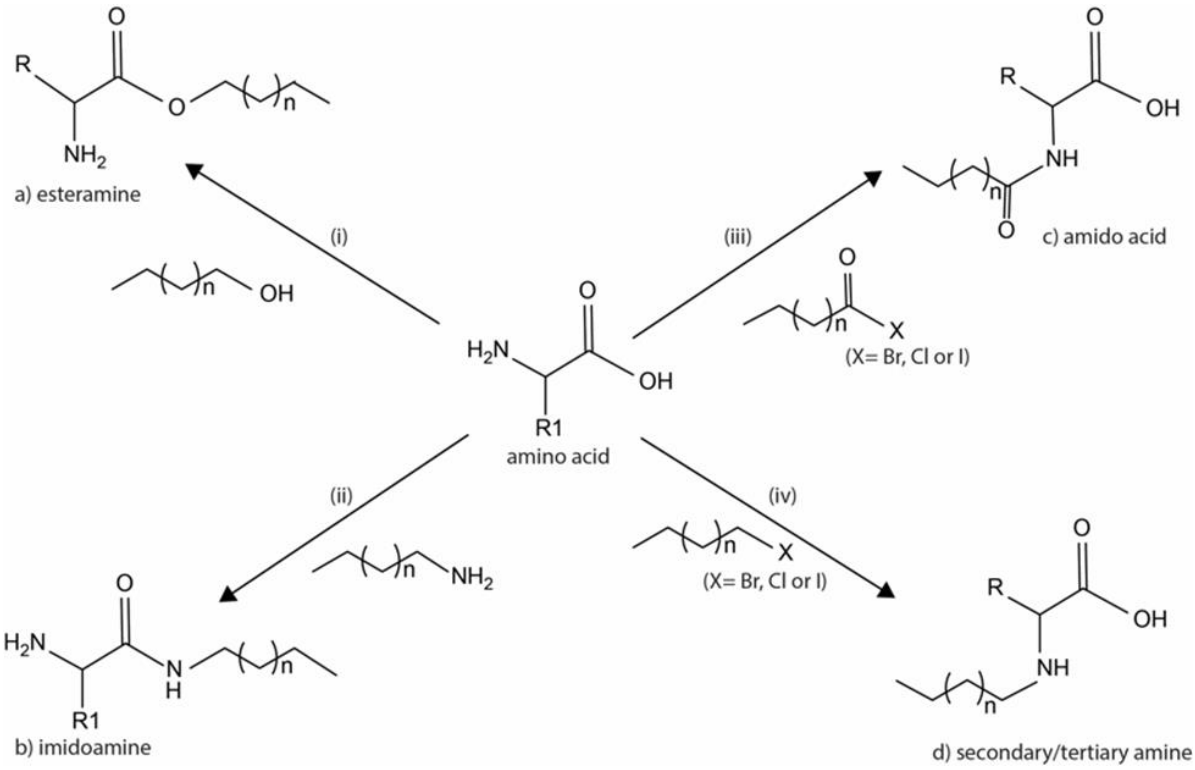


Figure 2.3 Main synthesis pathways of amino acid-based surfactants.

(i) Reacting carboxyl group with fatty alcohol to give a) esteramine; (ii) Reacting carboxyl group with a long chain amine to yield b) amidoamine with α-amino group protected; (iii) Amidation of α-amino group with a derivative of fatty acid giving c) amido acid; (iv) Alkylation of α-amino group with a long chain alkyl halide giving d) secondary/tertiary amine (Sources: Bordes & Holmberg, 2015; Infante *et al*., 1997; Pinazo *et al*., 2016a).

2.4.1 N-acyl amino acid derivatives

N-acyl AAs are anionic amphiphiles, with an amide bond connecting the polar head group and the hydrophobic tail, obtained by route iii, mentioned above. These AA-based surfactants are widely used and extensively investigated for their dermatological properties in skincare products. They are considered milder than sulphate anionic surfactants, such as sodium dodecyl sulphates (SDS) and sodium laureth sulphates (SLES), and lower in skin irritation and sensitization. In addition to

personal care applications, these surfactants are employed as detergents, emulsifiers, wetting agents, food additives and lubricants (Bordes & Holmberg, 2015; Perinelli *et al.*, 2016; Shah *et al.*, 2019). In contrast to fatty acid salts (*i.e.*, sodium laurate soap), the long chain N-acyl fatty acids have excellent water solubility, rapid bio-degradability and good lime resistance (Clapés & Infante, 2002). In addition, they are known to have physiological roles in living systems and some pharmacological properties (Arul Prakash & Kamlekar, 2021).

2.4.2 Properties of amino acid-based surfactants

Surfactants are the class of molecules that exhibit significant properties including surface tension reduction, foaming, antimicrobial, ecotoxicity and biodegradability.

2.4.2.1 Surfactant property

Surfactant monomers possess surface activities in the interface of immiscible phases due to their bi-polar nature and abilities to get adsorbed at the interface (*e.g.*, water-air, water-oil), minimizing the surface or interfacial tension of the bulk solution. With the increase in surfactant concentrations, initially, the interface becomes saturated with monomers, and then, the concentration of the bulk phase increases. At and above a particular concentration, surfactant monomers aggregate into different shapes and sizes (*i.e.*, spheres, ellipsoids, cylinders) to reduce the free energy of the system and establish thermodynamic stability. These aggregates are commonly known as micelles, and the threshold concentrations are defined as the critical micelle concentration (CMC). Micelle formation is an important characteristic of surfactants since many interfacial processes depend on that phenomenon, *i.e.*, interaction with biological membranes, lytic action, solubilization of non-polar materials (Clapés & Infante, 2002; Ivankovic & Hrenovic, 2010; Seweryn, 2018; Shrestha *et al.*, 2007). CMC of AA-based surfactants is lower than conventional surfactants (Chandra & Tyagi, 2013). It has been found that with increasing alkyl chain length, surface activity increases, and CMC decreases. The micelles' morphology depends on the type of hydrophobic moiety and physical conditions of the system, such as temperature and electrolyte content (Chandra & Tyagi, 2013).

Upon studying N,N-dialkyl cystine and N-alkyl cysteine, Yoshimura *et al.* (2007) have found that gemini-type surfactants start to aggregate at a lower concentration compared to monomeric type

surfactants. According to Shrestha *et al*. (2007), N-dodecylglutamic acid in salt-free mixed anionic/cationic surfactant systems forms viscoelastic wormlike micelles. Haldar and Maji (2013) showed that non-covalent interactions play a role in the molecular packing of N-n-hexadecanoyl AA amphiphiles formed with glycine, alanine, valine, and proline.

2.4.2.2 Foaming property

Foam is a two-phase system in that air bubbles are surrounded by a continuous liquid lamella and stabilized by surfactants (Sánchez-Vioque *et al.*, 2001). Foaming ability is determined by the diffusion rate and the adsorption of the surfactant at the air-liquid interface (Zhao *et al.*, 2016). The foaming of AA-based surfactants is influenced by the hydrophobicity of AA moiety and its spatial structure (Sreenu *et al.*, 2014a; Zhao *et al.*, 2019). Better foamability of sodium N-acyl glycinate compared to sodium N-acyl phenylalaninate has been explained by the larger space occupied by the phenylalanine moiety at the interfacial film reducing the foam formation. The structure of an AA has a great influence on foam stability and the above compounds followed the same pattern for the stability of the film as for the foamability. The presence of a branched-chain structure in the hydrophobic portion is known to decrease the film stability. Effect on carbon chain length has also been studied; foaming is difficult for the surfactants with longer hydrocarbon chain (Zhao *et al.*, 2019). Lower foaming ability was observed for sodium-N-oleoyl-isoleucine and -proline compared to SDS showing a decrease of foaming with increasing hydrophobicity (Sreenu *et al.*, 2014a).

2.4.2.3 Antimicrobial property

The antimicrobial activity of AA-based surfactants against pathogenic and non-pathogenic bacteria and fungi species have received considerable research interest. The mechanism of action providing antimicrobial activity is still not completely understood. However, the first interaction of surfactant is on the envelope of the microbial cell. The surfactant molecules interact with the cytoplasmic membrane at different levels, disrupting the cellular membrane and damaging the proton motive force and enzyme transport or enzyme-associated membrane activity. Once the biocide surfactant enters the cytoplasm, variable damage can be expected, depending on the compound or the nature of the bacterial cell (Pinazo *et al*., 2016a). Research attention has been chiefly on cationic surfactants (Fait *et al*., 2015; Greber *et al*., 2014; Perez *et al*., 2020; Pinazo *et*

al., 2016a; Zhou & Wang, 2020). The effect of cationic surfactants on bacteria has been attributed to their readily adsorption to the polyanionic bacterial cell wall, thereby weakening the cell wall integrity (Xia *et al*., 1995). AA-based anionic surfactants have also been studied, and their effectiveness was reported against both Gram-negative and positive bacteria and fungi (McKellar *et al*., 1992; Sreenu *et al*., 2015b; Xia *et al*., 1995).

Furthermore, the relationship between CMC and minimum inhibitory concentration (MIC) of the AA-based surfactants was studied, and no correlation has been observed, concluding that antimicrobial activity is provided by the monomeric form of the surfactants (Greber *et al*., 2014; Xia *et al*., 1995). It has been reviewed that surfactants with lower CMC have higher germicidal activity and increased protein binding ability (Ivankovic & Hrenovic, 2010). Studies have shown a poor effect on Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* than Gram-positive bacteria, such as *Staphylococcus epidermidis* and *Bacillus subtilis* (Greber *et al*., 2014; Perez *et al*., 2020). The structure of AA-based surfactants affects the activity against microbes, so as the length of the hydrocarbon chain (Perinelli *et al*., 2019; Sreenu *et al*., 2015a; Sreenu *et al*., 2014b; Xia *et al*., 1995). In addition, the solubility of the test material plays a significant role in giving sufficient dispersibility to facilitate adsorption to the bacterial cell wall (Xia *et al*., 1995).

2.4.2.4 Biodegradability

Biodegradation is the destruction of a surfactant by the metabolic activity of microorganisms. It depends on the chemical structure and environmental conditions (Ivankovic & Hrenovic, 2010; Scott & Jones, 2000). Biodegradability is considered a major criterion in evaluating the behaviour of surfactants in the living environment (Hosseini *et al*., 2007). Surfactant biodegradation is also influenced by organic contaminants in the medium (ABD-Allahh & Srorr, 1998). Surfactants can undergo primary or ultimate degradation stages. Primary degradation is changing the structure to the extent of losing the surfactant properties of the molecule. Ultimate degradation is when the molecule is broken down to CO_2, CH_4, water, mineral salts, and biomass (Hosseini *et al*., 2007; Lima *et al*., 2011; Scott & Jones, 2000). Long chain Na-acyl arginine methyl ester compounds are cationic surfactants with a satisfactory toxicity profile, high biodegradability and a surface activity compared to conventional long chain quaternary ammonium salts (Morán *et al*., 2004).

2.4.2.5 Ecotoxicity

Surfactants are used in agrochemicals; therefore, they are widely released into the environment. Furthermore, it has been found that the sorption of surfactants on soil or sediments can reduce the toxicity of surfactants in the environment and greatly affect biodegradation. The toxicity of surfactants towards aquatic plants, invertebrates, vertebrates, and terrestrial plants is a significant issue for today's environmental concerns (Ivankovic & Hrenovic, 2010). The toxicity of a single surfactant molecule is highly specific to the targeted organism (Ivankovic & Hrenovic, 2010). Anionic surfactant binds to bioactive macromolecules such as peptides, enzymes, and DNA. The binding of surfactant molecules to proteins can change the folding of the polypeptide chain and their surface charge while modifying the biological activity. The cytoplasmic inner membrane of bacteria is the primary target site of cationic surfactants. Non-ionic surfactants bind to various proteins and phospholipid membranes, increasing membrane permeability (Ivankovic & Hrenovic, 2010). Biological membranes are essentially non-polar interfaces, and the toxic effect of surfactant chemicals is due to their ability to disrupt this biological membrane via hydrophobic/ionic adsorption. The ability of surfactants to change cellular integrity by inducing membrane reorganization and morphological changes leads to cell disruption. Hemolysis occurs by osmotic or solubilization-related mechanisms in a surfactant concentration-dependent manner (Manaargadoo-Catin *et al*., 2016). A study with salts of anionic surfactants of dioctanoyl lysine against planktonic crustacean, *Daphnia magna*, showed their less toxicity than SDS, and no association was found between counter ion size and aquatic toxicity (Sanchez *et al*., 2007).

Biosurfactants are known to have low toxicity. Prepared fatty acid-AA conjugates with valine and phenylalanine showed a lower toxicity profile *in vitro* hemolytic toxicity, cell cytotoxicity and animal toxicity (Katiyar *et al*., 2019). Cytotoxicity of N-decanoyl AA-based surfactants with methionine, leucine, proline, and serine were studied by Perinelli *et al*. (2016) and observed less toxicity than SDS in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay for cell viability when tested in Caco-2, Calu-3 cell lines and A549 cells.

2.4.3 Industrial application of AA-based surfactants

Surfactants play a significant role in the day-to-day activities of humans due to their versatility as an ingredient in washing detergents, food applications, pharmaceuticals, cosmetics, and personal

care products (Pinazo *et al*., 2011). Moreover, their use in bioremediation in the environmental field and the oil industry to remove oil or organic contaminants carries significant ecological importance (Ivankovic & Hrenovic, 2010; Li *et al*., 2017; Menzies *et al*., 2017).

2.4.3.1 Cosmetics and personal care industry

In the personal care industry, demand for milder and greener ingredients is the driving force for selecting raw materials. AAs are used in skincare products like cleansers, toners, face creams, serums, skin moisturizers, hair care, etc. N-acyl AA-based surfactants have been given particular attention due to their foaming ability, antimicrobial properties, antistatic abilities, surface activities and biocompatibility (Ananthapadmanabhan *et al*., 2004). Cosmetic formulations are mostly emulsion-based, and surfactants are required to keep their product stable in terms of durability and viscosity (Burnett *et al*., 2017). Detergency, conditioning, and foaming are properties expected in shampoo formulation, and AA-based dimeric surfactants are widely used in their formulation (Kumar & Tyagi, 2013). AA-based dicationic dimeric surfactants are suitable for providing microbial stability in hair care products. Furthermore, their biocompatibility makes a low impact on the environment (Kumar & Tyagi, 2013).

Dermal irritation and skin sensitization of the ingredients used for personal care products are of significant concern. The initial step to determine the tissue damage is adsorption and interaction of the surfactant molecules, the keratin of the stratum corneum and the hair cuticle. Hydrated micelles cannot penetrate the keratin fibre network due to their larger size. Therefore, only monomeric surfactants can penetrate and attach to keratin, leading to partial denaturation and damaging skin and hair. The CMC of surfactant solutions holds a crucial role in personal care applications. The addition of protein derivatives can alleviate these effects by making a surfactant-protein complex which can produce larger micelles or reduce CMC (Secchi, 2008; Seweryn, 2018). The molecular structure of surfactants is an important factor responsible for causing skin irritation. Ionic surfactants cause irritation due to their electrostatic and hydrophobic interactions with skin proteins. Cationic surfactants are not used as primary surfactants since the skin and hair surface is negatively charged, and therefore, surfactants can bind to them firmly and making removal of dirt more difficult (Seweryn, 2018).

Glycerol AA-based surfactants, particularly with glutamine, glutamic acid, arginine, and tyrosine, were tested in 3T3 fibroblasts and HaCaT keratinocytes. Potential phototoxic effects of these surfactants have not been observed in the neutral red uptake (NRU)-3T3 phototoxicity assay or resazurin assay. Tyrosine-derived surfactants present a lower cytotoxicity and photocytotoxicity in red blood cells and fibroblasts and keratinocytes culture (Benavides *et al*., 2004) than other AA derivatives.

2.4.3.2 Detergent and laundry industry

Since the non-biodegradable petroleum-based surfactants are harmful to aquatic lives, environmental concerns on using eco-friendly substances are the main gearing force that the detergent industry is adapting more bio-based products (Yea *et al*., 2018). AA surfactants are known to have better cleaning ability, foaming ability and fabric softening properties that make them suitable to be used in household detergents (Tripathy *et al*., 2018). Laboratory scale studies have been conducted to identify the suitability of applying AA-based surfactants in detergent formulations. Detergency, dermal and eye irritation, and surface activity were studied for AA-based biosurfactants such as potassium cocoyl glycinate (CGK) and sodium cocoyl glycinate (CGN) that were synthesized from coconut oil (Yea *et al*., 2018). The low toxic and easily degradable L-glutamic acid-, L-arginine-, and L-lysine-based surfactants are the principal ingredients in detergents produced by Ajinomoto company (Tripathy *et al*., 2018).

2.4.3.3 Potential other uses

In addition to the above-discussed applications as a surfactant, AA surfactants have been identified as compounds that provide chelating ability due to their self-assembly, eco-friendly film-forming, antiviral, and emulsifying activity in food processing (Tripathy *et al*., 2018). Vesicular systems are widely used as carriers of many drugs, and they provide the benefit of controlling drug release. Biocompatible cationic vesicles were prepared using diacylglycerol-arginine surfactants and found to be equipped with antimicrobial properties (Tavano *et al*., 2014). Moreover, synthetic acyl AAs/peptides can be employed as drug carriers and for preparing functional liposomes with lipopeptide ligands (Tripathy *et al*., 2018). Lipoamino-based micelles as a delivery vehicle for an antifungal drug, monomeric amphotericin B, were found to be more effective than the solubilizing agent used in commercial formulations of the drug (Serafim *et al*., 2016).

Surfactants are used to bioremediate the hydrocarbons and make them available for the microorganisms to degrade (Karlapudi *et al*., 2018). The removal of aromatic hydrocarbons from contaminated soil was reported with the aid of surface-modified lipopeptide biosurfactant produced using *Bacillus malacitensis* (Christopher *et al*., 2021). Furthermore, Tackie-Otoo and Mohammed (2020) showed that sodium cocoyl alaninate is suitable for enhanced oil recovery techniques.

2.5 Commercial canola meal as a source of protein and amino acids

Canola meal is the co-product of the canola oil extraction process. In Canada, the annual domestic canola seed processing capacity by 2021was about 11 million tonnes, with 14 crushing and refining plants (COPA, 2022). In 2020, nearly 10.3 million tonnes of canola seeds were crushed in Canada, with a meal output of 5.8 million tonnes and 4.5 million tonnes of oil production (COPA, 2020). The domestic production of canola meal in 2021 was 5.7 million tonnes, and 90% of it (or 5.1 million tonnes) was exported, and only 10% of the production was sufficient to fulfil the domestic market requirements. When the average meal protein content of 38% (with 8.5% moisture) is considered, this meal biomass volume equals 2.2 million tonnes of canola protein. The canola oil industry in Canada uses three types of oil extraction processes: pre-pressed solvent extraction, double-pressing (or full-pressing) and cold-pressing. These processes generate canola meals with different compositional and quality characteristics. The widely employed method for commercial-scale oil extraction is pre-pressed solvent extraction (PSE), which includes mechanical pressing and solvent extraction (Adewole *et al*., 2016; Gaber *et al*., 2018; Zago *et al*., 2015).

According to the Canola Council of Canada, almost all of the canola meal production is used in feed formulation for dairy and feed cattle, poultry, swine, small ruminants and cultured fish (CCC, 2019). Statistics are not available for other uses such as for food and non-food, non-feed uses of canola meal, indicating that such applications are either at an experimental level or minor scale that is insignificant for reporting.

2.5.1 Pre-pressed solvent extracted meal

2.5.1.1 Production process

Several processing steps comprise in PSE (Figure 2.4, flow i). After cleaning (feeding to destoner for separating stones, ashes, and any other contaminants), seeds are pre-conditioned (heat treatment to adjust the temperature to 50°-70°C and moisture up to 8% to prevent shattering during flaking) and flaked (physically rapturing seed coat and flatten the seed to increase the surface area and lipid-containing olesomes/oil bodies). Then a cooking step (at 80°-100°C or higher as 120°C, 30-35 min) is applied to reduce flake moisture (which increases pressing efficiency), deactivate myrosinase enzyme and thermally break down oil bodies, and reduce oil viscosity. Mechanical pressing of cooked flakes is the first oil extraction step that extracts up to 80% of seed oil and results in 15% - 20% oil in the pressed cake. Pressed cake goes through solvent extraction, the second oil extraction step; a continuous extraction of pressed cake with boiling n-hexane. Then the solvent extracted meal goes through the desolventizing and toasting step, which heats the meal at a temperature higher than 100°C to reduce the residual hexane level to less than 50 ppm (Adewole *et al*., 2016; Chen *et al*., 2014; Gaber *et al*., 2018; Mustafa *et al*., 2000). The resulting meal of this process is known as desolventized and toasted meal (DT meal). After pressing, the meal contains around 18% - 20% oil, and it has been reduced to less than 1% by solvent extraction. Hexane is the usual solvent for solvent extraction of canola, and the desolventizing and toasting step is necessary to recover the solvent and reduce the solvent level of meal to an acceptable level for animal feed use. This step involves heating the meal by steam injection that can cause meal temperature to rise to around 115°C or higher for about 30 min. At this stage, deactivation of any residual activity of the myrosinase enzyme and glucosinolates and thermal deactivation of microorganisms in the meal, can occur. The majority (nearly 98%) of canola oil production in Canada uses PSE and produces DT canola meal. Expeller pressing is employed as a double-pressing or cold-pressing processes. It is limited to a much lower volume of canola seed processing than PSE.

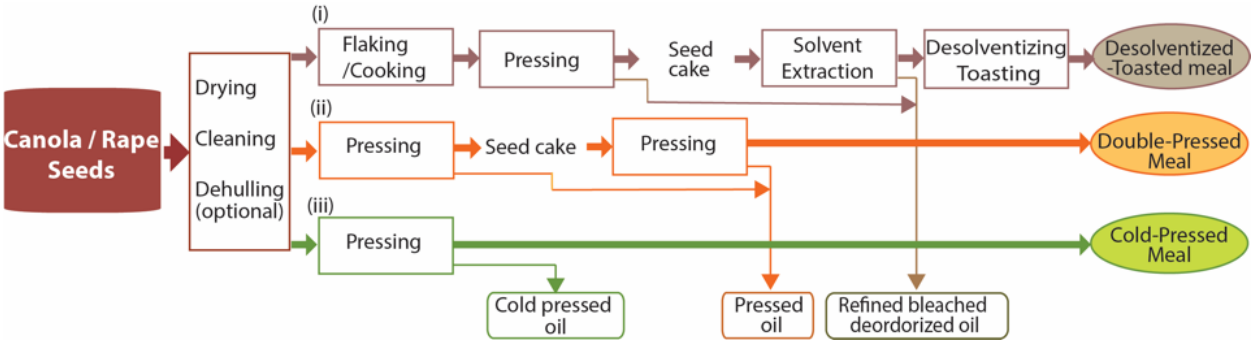


Figure 2.4 Canola meal production processes commonly employed (i) Pre-press solvent extraction process, (ii) double/full pressing, and (iii) cold pressing. Major processing steps are indicated.

2.5.1.2 Meal composition and characteristics

PSE converts up to 60% of canola seed biomass into the meal. Fibre components of seed get concentrated in the meal since the hull portion remains with the biomass left out of oil extraction (Liu *et al*., 2016). Processing conditions employed in PSE affect the composition of meal which has been described in terms of crude protein, neutral detergent fibre, total phosphorus, simple sugars, oligosaccharides, total dietary fibre, glucosinolates, neutral detergent insoluble crude protein, lignin and polyphenols and available lysine (Adewole *et al*., 2016). Canola meal is considered a good source of proteins for animal feed as it is rich in sulphur-containing AAs, methionine, and cysteine (Newkirk *et al*., 2003). However, PSE results in lower AA digestibility compared to soya bean meals. During the toasting step, canola meal undergoes elevated temperatures (up to 130°C), and it severely denatures the meal protein causing poor functionalities (Sun *et al*., 2008). The increase of meal temperature at the desolventizing-toasting step influences protein quality. At elevated temperature and moisture (water activity range), any compound with free amino groups participates in the Maillard reaction with molecules having reducing ends. Free AAs and the epsilon amino group of protein-bound lysine are readily available to react with reducing sugars that are naturally present in canola seeds. As lysine is the AA most vulnerable to heat damage, its availability is a good indicator of the effect of heat treatment. The low solubility of DT meal proteins has been attributed to the Maillard reaction products and protein-protein crosslinking, and the low solubility results in low *in vitro* and *in vivo* digestibility (Adewole *et al*., 2016; Mosenthin *et al*., 2016). As discussed, high temperature at the cooking step negatively

affects the meal quality as animal feed, *e.g*., degradation of tocopherols, development of off-flavours and odours, reduction in available lysine content, protein digestibility, formation of glucosinolate hydrolysis products (Gaber *et al*., 2018). The proteins of DT meal as a source for food and bio-products are limited due to the extensive denaturation and modifications to the molecules (Salazar-Villanea *et al*., 2017).

Glucosinolates, phenolics, phytates, and fibre bring inferior physiochemical properties, such as poor digestibility, off-colour and astringent taste, to the canola meal. Phenolic acid esters, most prominently sinapate esters with sinapolylcholine (sinapine) and sinapoylglucose, negatively affect the digestibility of the meal. During oil processing, sinapine may form complexes with protein through oxidation and decrease the digestibility (Tan *et al*., 2011).

2.5.2 Meals from double-pressing and cold-pressing

2.5.2.1 Production process

Expeller pressing involves oil extraction by applying heat and friction and is considered an environmentally friendly and solvent-free technique (Chen *et al*., 2014). This process follows the same initial seed cleaning steps as the PSE method (Figure 2.4. flow ii and iii). Two types of pressed cake are available as double or full or hot-pressed cake and pre-pressed cake. Full- or double-pressed cake (Figure 2.4. flow ii) is obtained after complete mechanical oil extraction to have 5% - 10% residual oil content in the meal. Pre-pressed cake undergoes mechanical extraction to a lesser extent than the full-pressed meal, and the meal contains 15% - 18% residual oil content. Cold pressing is pre-pressing (Figure 2.4. flow iii) while maintaining the temperature of the meal without exceeding 60°C and resulting in a cold-pressed (CP) meal with 15% - 18% residual oil content. When solvent extraction is applied to pressed cake for further oil removal, the meal has 1% - 2% of oil (Fetzer *et al*., 2018). At present, oil processing of organic and non-genetically modified (non-GM) canola seeds produces CP meals. Canola oil extraction for the biofuel industry uses full-pressing.

2.5.2.2 Meal composition and characteristics

Leming and Lember (2005) studied the composition of canola meal resulted in from different expeller oil extraction methods and reported on a dry matter basis that on average, expeller-pressed

cake contains 36% crude protein, 12% crude fat and 13% crude fibre, and CP cake contains 30.6% protein, 19.4% crude fat and 12% crude fibre. Since the pressed cake is not undergone solvent extraction, a considerable amount of oil remains in the meals, and they increase the energy level of meal in feed ration formulation, particularly a high energy supplement for ruminants (Theodoridou & Yu, 2013). The glucosinolate concentration in pressed cake is at the same level as canola seed. The average aliphatic glucosinolate level in pressed cake is 23.75 µmol/g. The flaking and cooking steps in the pressed cake production inactivate the myrosinase enzyme (Keith & Bell, 1991).

2.6 Research question

When evaluating the current need for bio-based industrial molecules and compounds, it is observed that AA-based surfactants have a wide variety of applications and are required by various product categories that are moving towards an environment-friendly and sustainable bio-economy. Generating AAs from underutilized processing by-products of the agriculture industry is one of the tangible options available to have starting molecules for AA-based surfactant development. Among protein-rich plant sources available in Canada, canola meal is listed at the top with being the sole source of use in animal feeds. The oil recovery maximized industrial oil extraction process makes seed protein undergo several process-induced changes due to the involved high-temperature regimes and hexane, finally producing a meal with an extensively processed and highly stressed protein. Under the current situation, industrially processed PSE meals are not used in protein production for the escalating food-grade plant protein ingredient market. This is because the protein structure changes at or above the secondary structure level resulted in from the PSE process. However, the primary structure of canola seed proteins is intact in the PSE meal, and these proteins could be a source to obtain AAs for further uses. It is envisioned that industrially processed canola meal can be used to generate AAs that can be converted into derivatives including fatty acids having surfactant properties. Reviewing of literature generated, two questions that support the canola industry, as well as the bio-based molecule development industry, have been aroused: Is industrially processed canola meal a suitable source to generate AAs by chemical hydrolysis? Will the AAs obtained by such a process be able to convert into compounds with surfactant properties with additional functions, such as antimicrobial?

The present study was designed to investigate the above questions that previous research has not explored. This work was expected to contribute to the canola industry via value addition of canola meal, bio-based economy, and green chemical development. Outcomes of the study will support:

1) enhancing the value of canola meal to enter new product markets, particularly the meals that are used in animal nutrition only; and

2) bio-based chemical development from underutilized protein-rich crop industry by-products, such as extensively-processed or cannot be used in food or high value applications, to generate functional molecules to the market demand, to address environmental concerns of nitrogen-rich biomass waste and finally to support circularity of agriculture industry.

3. INVESTIGATION OF PRE-TREATMENTS FOR CANOLA MEALS FROM COMMERCIAL PRE-PRESS SOLVENT EXTRACTION AND COLD-PRESSING FOR GENERATING FREE AMINO ACIDS VIA ACID HYDROLYSIS

3.1 Abstract

Canola meals are protein-rich co-product streams of the canola oil processing industry. Non-reversible structural changes in canola seed proteins during commercial oil extraction processes can affect its nutritional and techno-functional properties, thus limiting further value addition. This study presents an alternative approach to utilize extensively modified canola meal proteins. The majority of canola oil produced in North America is obtained by pre-press solvent extraction that yields desolventized-toasted (DT) meal, while a small fraction is processed through pressing processes that produce pressed meal. A portion of the pressed meal is from seed pressed at lower temperatures, and it is called a cold-pressed (CP) meal. Meal co-products are plant protein-rich materials available in large quantities with little or no value in food applications; however, proteins and protein-derived biomolecules generated from these meals could have various uses, including food.

Experiments were conducted to recover free amino acids (AAs) and their mixtures from commercially produced CP (36.4% protein or 340 mg protein/g meal as-is moisture basis) and DT (42.5% protein or 386 mg protein/ g meal on as-is moisture basis) meals. Treatments to reduce the levels of non-protein compounds, namely oil, total phenolics, free sugars and fibre, were applied to improve protein hydrolysis and the purity of final free AA mixtures obtained from these meals. Aqueous ethanol (99% v/v) pre-treatment at 50°C with a meal-to-solvent ratio of 1:4 (w:w) for 30 min was found to be optimal for reducing levels of oil, total phenolics and free sugars (88%, 20%

and 25%, respectively for CP meal and 78%, 23%, and 22%, respectively, for DT meal). Meal protein enrichment was at 16% and 7% for CP and DT meals, respectively. Ethanol pre-treated CP meal had a ~1.9 times higher oil content than DT meal treated the same way, and the values for other components, total phenolics and free sugars, were 1.7 and 1.2 times, respectively higher in CP meal. One-time alkaline extraction at pH 12 with a meal-to-solvent ratio of 1:10 (w:w) produced the highest protein recoveries of 67% and 29% (dry weight basis) from CP and DT meal proteins, respectively. Under the same extraction conditions, two extractions recovered 79% of the protein in CP meal; whereas the protein recovery was 38% for DT meal. These data demonstrate the lower extractability of proteins and the greater extractability of non-protein components from DT meal than from CP meal.

Hydrolysis of meal intact proteins (untreated meal and ethanol pre-treated meal) and separated fractions (extracted protein) with 6 N HCl for 24 h at 110°C yielded a total AAs in the range of 766-889 mg/g protein and 941-833 mg/g protein for CP and DT meals, respectively. The high residual oil level in untreated CP meal interfered with protein hydrolysis resulting in a lower total AA yield (766 mg/g protein) than for the ethanol-pretreated CT meal and the protein fraction from CT meal (884 and 889 mg/g protein, respectively). Glutamic acid, aspartic acid, arginine, lysine, leucine, proline, and valine have the highest abundancy in canola meal protein, and glutamic acid was the predominant AA (20% - 22% of the total AA). These seven AAs comprised 63% and 65% of the total AAs in protein fractions of CP and DT meals, respectively and their hydrolysis with 4 M H_2SO_4 for 24 h at 110°C released 53% and 52% of the total AAs, respectively, compared to the complete hydrolysis achieved with 6 N HCl under the same conditions. A higher concentration of H_2SO_4 was required to release the same amount of free AAs from DT meal compared with CP. It was evident that the yield of free AAs released by the low acid concentration (< 4 M) was related to the partial hydrolysis of proteins to peptides. This study showed that 1 kg of dry CP meal produced 286 g of protein, and hydrolysis of that protein with 4 M H_2SO_4 for 24 h at 110°C produced 209 g of free AAs. These values were 156 g protein and 101 g of free AAs for DT meal. Data from the present study showed that in addition to its lower extractability, the extracted DT meal protein was resistant to hydrolysis with H_2SO_4. This study also showed that the commercially produced canola meals can be used to produce free AA mixtures. However, processing conditions can affect the protein, and free AA yields depend on the processing stresses that the meal has been

exposed to. The mixtures of free AAs are rich in molecules that could be useful for various applications and further product development.

3.2 Introduction

Industrial-scale processing fractionates canola seed into oil and meal with average mass basis recoveries of ~43.5% and ~56.3%, respectively. Oilseed processors in Canada crush 51% of annual global canola production, and it is expected to grow from 9.5 million tonnes in 2019 to 14 million tonnes in 2025, with the volume increase of domestic processing, primarily through pre-press solvent extraction (COPA, 2020). The primary market for canola meals is in animal feeds, where it is a source of protein. Several non-food/feed applications have been investigated for canola meal as proteinaceous materials. Examples include plastics (Manamperi *et al*., 2011; Manamperi *et al*., 2010), adhesives (Wang *et al*., 2014), bio-composites (Li *et al*., 2018), films with barrier properties (Chang & Nickerson, 2014; Shi & Dumont, 2014), surfactants (Sánchez-Vioque *et al*., 2001) and controlled delivery of bio-actives (Akbari & Wu, 2016), all of which are based on canola meal proteins. In addition, hydrolysis of canola meal protein with various proteolytic enzymes has been investigated to generate peptides. Those peptides can exert bioactive properties such as antihypertensive (Wu & Muir, 2008; Yoshie-Stark *et al*., 2008), antioxidative (Zhang et al., 2008), antifungal (Nioi *et al*., 2012), and antiviral (Yust, 2004). All these investigations support expanding utilization of canola meal beyond animal feed applications by adding value to its protein fraction.

Irrespective of its well-balanced amino acid profile and the utilization mentioned above methods, attempts at using canola meal from commercial oil extraction have failed to generate protein products of acceptable quality in the plant protein market. This is mainly due to the changes that occur in meal constituents, including protein, due to the conditions employed in oil extraction processes. The pre-press solvent extraction (PSE) process yields desolventized-toasted meal, and is highly efficient in recovering seed oil. It is the most widely employed process in canola oil production. During PSE, seed and meal are exposed to high heat, *i.e*., 80°-100°C for 30 to 35 min in the seed cooking step, 66°-71°C during the solvent extraction step, and 95°-115°C for ~30 min during the meal desolventizing and toasting step. Components of DT meal go through excessive structural alterations, as the heat generates protein-protein interactions and interactions between

protein and non-protein component interactions, in addition to the consequences of protein surface modifications caused by exposure to the hot, non-polar hexane solvent. These processes significantly alter the native structural characteristics of canola seed proteins and, as a result, their nutritional and native physico-chemical properties (CCC, 2019; Gaber *et al*., 2018). Expeller-pressing at a low temperature is termed cold-pressing and is practiced at a relatively small scale, primarily to obtain organic, non-GMO or virgin canola oil. Expeller-pressing involves only mechanical forces to rupture seeds and express oil. Cold pressing maintains the temperature of the pressed material below 60°C. Cold-pressed (CP) canola meal typically contains a higher residual oil content (13% - 16%) than DT meal. The bio-diesel industry uses repeated expeller pressing to ensure maximum seed oil recovery and therefore produces a full-pressed meal. The frictional forces of mechanical pressing can have the temperature of the pressed material rising as high as 160°C for a short period, which enhances oil recovery and produces a lower residual oil (usually 8% - 11%) in full-pressed meal; however, heat damage to protein is unavoidable because of high process temperature (CCC, 2019; Chen *et al*., 2014; Fetzer *et al*., 2018; Leming & Lember, 2005; Salazar-Villanea *et al*., 2017).

CP and DT canola meals did not show significant differences in crude protein levels and total amino acid (AA) profiles; however, the chemical reactions during processing and their progression among meal matrix components affect the chemical nature of components. This suggests that the DT meal proteins become less soluble and less extractable than CP meal proteins (Mosenthin *et al*., 2016). Furthermore, high process temperature leads to the Maillard reaction and crosslinking between protein and non-protein components, such as reducing sugars in their free form and the fibre fraction (cellulose, hemicellulose), thereby lowering the solubility of meal protein. Poor solubility of proteins results in low protein recovery yields during wet protein extraction processes, especially from the extensively heated DT canola meal. Extracted proteins also exhibit less functionality, such as emulsification and foam formation (Khattab & Arntfield, 2009; Manamperi *et al*., 2012; Mosenthin *et al*., 2016; Sun *et al*., 2008).

Amino acids and peptides derived from canola meal proteins have the potential to be used in non-food applications, even though protein quality is compromised from a nutritional perspective. Amino acid recovery from protein-rich materials generally requires hydrolysis of peptide bonds,

which can be achieved via chemical or enzymatic means. Chemical hydrolysis using alkali or acid is a comparatively economical, easy to control process that can be scaled up to obtain AAs with a lesser amount of peptide production than the limitations of enzyme-assisted hydrolysis (Alvarez *et al*., 2012; Fountoulakis & Lahm, 1998).

In addition to the provision of nutritional requirements, AAs can be feedstocks for several industrial products, *e.g*., surfactants (Clapés & Infante, 2002), pharmaceuticals (Martnez-Rodrguez *et al*., 2010) and plant bio-stimulants (Maini, 2006). Amino acids can be functionalized to attain desirable attributes with promising applications (Pinazo *et al*., 2011). For instance, N-acyl AAs are particularly known to have good skin compatibility, antimicrobial activity, and calcium tolerability, which are required attributes for surfactants in the personal care and detergent industries (Clapés & Infante, 2002; Mhaskar *et al*., 1990; Sreenu *et al*., 2015a). The conventional route of obtaining AAs is microbial fermentation (Scott *et al*., 2007). Studies have reported on using co-products of agricultural material processing as substrates for AA generation. Hydrolysis of cotton seed by acid hydrolysis (Xia *et al*., 1996) and pea flour by enzyme-catalyzed hydrolysis (Rondel *et al*., 2011) were employed to generate AA mixtures that could be converted to surfactant molecules. Zhang *et al*. (2016) reported preparing AA mixtures using cottonseed meal protein using a combination of microbial fermentation and hydrolysis with HCl. Enzymatically prepared rapeseed/canola meal protein hydrolysates rich in short peptides have been studied for potential use in skin care applications (Rivera *et al*., 2015), as a stabilizer in mayonnaise preparation (Aluko & McIntosh, 2005), as a source of bioactive peptides (Wu & Muir, 2008) and as antioxidants (Alashi *et al*., 2014).

The inability of canola meal to penetrate as a source for expanding plant protein markets, the availability of industrially processed meal and the distressed nature of constituent proteins can be addressed through exploring alternative approaches for utilizing commercial canola meal. Utilization of canola-derived AAs in products beyond the usual nutritional and techno-functional applications would support the extension of the canola meal value chain and ensure maximum utilization of processing co-products for a circular crop economy. The utilization of canola meal protein through its conversion to AAs has not been reported in the literature.

Canola meal contains various non-protein components of varying chemical nature, posing side reactions during protein hydrolysis. To mitigate these side reactions and improve AA yield, it is essential to remove non-protein compounds and attain proteins in concentrated forms. Furthermore, alternative acids to HCl to release free AAs from canola proteins need to be investigated. The present work describes suitable meal pre-treatments and the hydrolysis conditions to generate AA mixtures from industrially processed CP and DT meals. The studies that were conducted had the following objectives:

Objective 1: To identify optimum conditions for ethanol extraction (meal-to-solvent ratio, extraction time and treatment temperature) to reduce the levels of oil, free sugars and total phenolics in CP and DT canola meals prior to use as pre-treatment for preparing meal for protein extraction and protein hydrolysis

Objective 2: To identify optimum conditions (pH and meal-to-solvent ratio) for aqueous extraction of ethanol-pretreated CP and DT canola meal, *i.e.*, conditions that maximize protein recovery and protein content

Objective 3: To determine suitable H_2SO_4 concentrations for releasing the maximum amount of free AAs from CP and DT canola meal protein

Objective 4: To determine the composition of AA mixtures obtained from H_2SO_4 hydrolysis of canola protein

In the experiments carried out to achieve the above objectives, the following hypotheses were tested:

Hypothesis 1: Reduction of levels of residual oil and small molecules, such as free sugars and phenolic compounds (total phenolics), in commercial canola meal can be achieved by extraction with 99% ethanol, which can be a suitable pre-treatment for CP meal and DT meal.

Hypothesis 2: Reduction of levels of residual oil and small molecules, such as free sugars and total phenolics, will improve canola meal protein extractability and protein hydrolysis of CP and DT meals.

Hypothesis 3: Equivalent free amino acid yields can be obtained from acid hydrolysis of proteins separated from CP meal and DT meal.

Hypothesis 4: Acid hydrolysis of canola proteins with H_2SO_4 has equal efficiency for releasing AAs as hydrolysis with HCl at the same concentration.

3.3 Materials and methods

3.3.1 Materials

Ethanol (99%, denatured by 1% ethyl acetate) was obtained from Commercial Alcohols, Toronto, ON, Canada). All chemicals were of analytical grade and obtained from Sigma-Aldrich, Canada and Thermo Fisher Scientific, Canada.

Canola meals processed at a commercial scale and by two methods were used in the study. CP meal (Virtex Farm Foods, Saskatoon, SK, Canada and Pleasant Valley Oil Mills, Clive, AB, Canada) and DT meal (Bunge Canada, Nipawin, SK, Canada and ADM Processing, Lloydminster, SK, Canada) were obtained from three random production lots from the same processing plant. For the experiments, meal samples were milled (Restch Ultra Centrifugal Mill ZM 200, Restch GmbH, Haan, Germany) and passed through a 250 µm mesh screen. Table 3.1 provides the particle size distributions of meal samples used in the study.

Table 3.1 Particle size distributions of ground canola meal samples obtained from processors.

Particle size (µm)	Percentage of meal weight (%)	
	DT meal (Untreated)	CP Meal (Untreated)
> 500	0.5 ± 0.1	0.6 ± 0.4
500 - 425	0.9 ± 0.1	1.0 ± 0.1
425 - 250	10.9 ± 0.1	22.7 ± 0.1
250 - 180	22.4 ± 0.3	10.3 ± 0.4
180 - 150	16.7 ± 0.1	13.3 ± 0.6
< 150	55.8 ± 0.5	59.2 ± 0.2

3.3.2 Chemical analysis

3.3.2.1 Moisture and Ash

Moisture and ash contents of all samples were determined according to AOAC 934.01, 2005(a) and AOAC 942.05, 2005(b), respectively. Moisture content was determined using an oven method at 105°C. Pre-weighed amounts of meal were heated to ash in a muffle furnace at 550°C for 6 h to determine ash content.

3.3.2.2 Crude protein

The crude protein content of samples was estimated by determining the total nitrogen content of the materials using the Dumas combustion method (AOAC 990.03, 2005c) and a conversion factor of 6.25.

3.3.2.3 Residual oil

The residual oil content of samples was determined by using the modified Swedish tube method of the AOCS (AOCS, 1997). Briefly, 2 to 3 g of sample was transferred to a Swedish tube, and three ball bearings and 25.0 mL of hexane were added. Tightly closed tubes were shaken for 30 min using an Eberbach shaker at room temperature. Then, the samples were vacuum filtered, and the solvent was transferred quantitatively into a pre-weighed aluminum moisture dish. The solvent was allowed to evaporate overnight, followed by heating in a forced-air oven at 130°C for 20 min to ensure the solvent was completely removed. The residual oil content of the sample was calculated based on the weight of oil recovered by hexane extraction.

3.3.2.4 Total phenolics

The total phenolics content of canola meal was determined spectroscopically using the Folin-Ciocalteu method with reference to the study carried out by Velioglu *et al*. (1998) with modifications. Canola meal (~200 mg) was weighed into a 15-mL plastic centrifuge tube and 6 mL of 70% (v/v) aqueous methanol (1:30, w:v ratio) was added. The mixture was vortexed and shaken for 2 h using an Eberbach shaker at room temperature. Then, the supernatant was recovered by centrifuging at 13,880 × *g* for 20 min. The residue was re-extracted using the same procedure, and the supernatants were combined. The total extract volume was recorded. Aliquots of the extracts were filtered, using 0.45-µm nylon syringe filters, into 1.5-2.0 mL microfuge tubes.

Aliquots of 10 µL were placed in a 96-well microplate. Aqueous methanol (70%, v/v, 10 µL) was used as the blank, and sinapic acid prepared in 70% (v/v) aqueous methanol was used as the standard. Diluted Folin-Ciocalteu phenol reagent (10-fold diluted, 75 µL) was added and mixed by slowly shaking the plate. After 5 min, 75 µL of Na_2CO_3 (6%, w/v) solution was added to each well. Then, the microplate was placed on a shaker to mix the contents for 90 min at ambient temperature. The absorbance values of the generated chromophore were read at 725 nm using a microplate absorbance spectrophotometer (xMark, Bio-Rad Laboratories, Mississauga, ON, Canada), and the total phenolic content was presented as mg of sinapic acid equivalents per 1 g of the meal.

3.3.2.5 Free sugars

Free sugars were extracted by mixing a 150.0 mg of canola meal sample into 1.5 mL of water in microcentrifuge tubes, stirring at 1,400 rpm at 60°C for 1 h using a ThermoMixer C (Eppendorf Canada, Mississauga, ON, Canada). The supernatant was collected in a clean microcentrifuge tube and cleaned up using a Strata ABW cartridge (Phenomenex Inc, Canada) as follows. The cartridge was conditioned with 2 mL of methanol followed by 3 mL of water. Extract (0.8 mL) was added to the cartridge and eluted into a 2-mL volumetric flask. The cartridge was then rinsed with 800 µL of water, and the volume was adjusted with acetonitrile. The solution was centrifuged at 16,200 × *g* for 15 min and filtered through a 0.2-µm PVDF syringe filter. Sugars in the extract were separated and analyzed using ultra performance liquid chromatography (UPLC) method. Sugar separation and elution were achieved in an Acquity UPLC BEH Amide column (1.7 µm, 2.1×100 mm) at 85°C using a gradient solvent system: solvent A (95% acetonitrile, 5% water, 0.1% TEA), and solvent B (30% acetonitrile, 70% water, 0.1% TEA) at a flow rate of 0.3 mL/min and an injection volume of 2-5 µL and detected by an evaporative light scattering detector (ELSD). An external standard prepared, with 800, 600, 400 and 200 mg/mL for each of sucrose, fructose, and glucose, was used for calibration and calculation.

3.3.2.6 Complete (18) amino acid profile

The complete amino acid profiles (18 AAs, aspartic acid and glutamic acid including their respective amide forms) of all samples were determined using three separate hydrolysis methods: a) 6 M HCl hydrolysis, b) 6 M HCl with performic acid pre-treatment, and c) alkaline hydrolysis

followed by high performance liquid chromatography (HPLC) according to the modified method of AOAC 994.12, 2005d (AOAC International, 2005). All protein hydrolysates prepared according to hydrolysis procedures a) and b) were derivatized according to the Waters AccQtag amino acid analysis method, following the Waters AccQFlour Reagent Kit manual (Waters Corp., Mississauga, ON, Canada). Then, samples were run on HPLC (Waters Aliance 2695) with a C18 AccQ-Tag Column (3.9 mm × 150 mm) and a fluorescence detector (Waters 2475, excitation at 250 nm and emission at 395 nm), using Eluent A: Waters AccQTag eluent A buffer, Eluent B: HPLC grade acetonitrile, and Eluent C: water, at a flow rate of 0.75 mL/min for cysteine and methionine and 1.0 mL/min for all other AAs. Hydrolysates prepared by alkaline hydrolysis (procedure c) were analyzed with HPLC directly after cleaning without derivatization.

a) 6 M HCl acid hydrolysis for amino acids (15) except cysteine, methionine, and tryptophan

A 5-mg sample (protein basis) was weighed into a test tube, and 1.9 mL of 6 M HCl acid was added, followed by adding 100 µL of 2% (v/v) phenol in 6 M HCl. The sample was hydrolyzed at 110°C for 24 h in a heating block. Then, the entire hydrolysate was transferred to a 10-mL volumetric flask containing the required volume of NaOH to neutralize the acid, and the volume was adjusted with de-ionized water. Alpha-amino butyric acid was used as the internal standard. Approximately 3 mL of neutralized hydrolysate was passed through a 0.45-µm Phenex RC syringe filter into a 4-mL glass vial. Then, filtered hydrolysate (2.0 mL) was applied to a conditioned Waters Oasis HLB cartridge (Waters Oasis, Waters Corp., Mississauga, ON, Canada) and collected into a 5-mL volumetric flask. The sample was eluted with 2.0 mL of 5% (v/v) acetonitrile in water and collected into the same flask, and the volume was adjusted with water.

b) Performic acid pretreatment followed by 6 M HCl hydrolysis for cysteine and methionine

Performic acid (10.0 mL) was freshly prepared as follows. Phenol (50 mg) was measured into a 30-mL hydrolysis tube, and 1.0 mL of 30% (w/w) hydrogen peroxide was added and gently stirred to dissolve the phenol. Then, 9.0 mL of 88% (v/v) formic acid was added slowly with stirring, followed by letting the covered solution stand at room temperature for 30 min. After 30 min, the performic acid tube was placed in an ice bath for 15 min.

Samples (5.0 mg, protein basis) were weighed into the test tubes, which had been placed in an ice bath for 15 min, and 0.5 mL of performic acid solution was added. The tubes were capped with Teflon-lined caps and vigorously stirred on a magnetic stirrer for 15 min. The tubes were then placed in an ice bath in a cold room (4°C) overnight. Then, the tubes were removed from the ice bath, and solid sodium metabisulfite was added to each tube to decompose the performic acid. Samples were stirred on a magnetic stirrer for 15-20 min until the bubbling stopped. Then, 6 M HCl acid hydrolysis was carried out following a procedure similar to procedure (a). A 100-µL sample was transferred to a clean test tube, 850 µL of 6 M HCl and 50 µL of 2 % (w/v) phenol in 6 M HCl were added, and hydrolysis was performed by incubating in a heating block at 110°C for 24 h. After hydrolysis, the hydrolysate was transferred to a 5-mL flask, neutralized using NaOH, 50 µL of 20 mM 2-aminobutyric acid internal standard was added and brought up to volume with water. The clean-up process for the samples was carried out similar to that described for procedure (a).

c) Alkaline hydrolysis for tryptophan analysis

A 10-mg sample (protein basis) was weighed into a 10-mL hydrolysis tube, added 1.0 mL of 4.2 M NaOH was added, and the contents of the tube were mixed gently using a vortex mixture to disperse the sample. The sample was hydrolysed using a CEM Discover SPD Microwave digester at 205°C for 20 min. The hydrolysate was quantitatively transferred to a 5-mL volumetric flask containing 0.7 mL of 6 M HCl. Two hundred and fifty microlitres of 20 mM 5-methyl tryptophan internal standard was added, and the solution was brought to volume with water. Then, the sample was cleaned up as described below. The neutralized sample (2 mL) was filtered through a 0.45-µm Phenex RC syringe filter into a 2-mL glass vial. The filtered hydrolysate (1.0 mL) was eluted through a Water Oasis HLB cartridge into a 10-mL volumetric flask and then eluted with 1.0 mL of 5% (v/v) methanol: 5% (v/v) acetonitrile: 90% (v/v) water. Next, the column was again eluted with 4.0 mL of 5 % (v/v) acetonitrile in water, the eluate was collected in the same flask, and the volume was brought to 10 mL with water. This sample was analyzed with HPLC directly without derivatization.

3.3.3 Ethanol pre-treatment of meals

Extraction of canola meal with ethanol was studied as a pre-treatment to reduce residual oil, free sugars, and phenolic compounds. Three conditions of ethanol extraction pre-treatment were studied as factors or independent variables-extraction time (X1), temperature (X2) and meal-to-solvent ratio (w:w, X3) in removing residual oil, total phenolics and free sugars and enriching protein content (response variables). Based on the preliminary studies, lower and upper values for the factors: extraction time, temperature and meal-to-solvent ratio, were selected as 30 min to 90 min, 30°C to 55°C, 1:2 to 1:6 (w:w), respectively. The middle and axial point values for each factor were obtained from the calculations according to a central composite design (CCD). Twenty combinations of these three independent variables according to the CCD were employed as experimental points (Table 3.2). At each experiment point, canola meal (~30 g) was stirred with added 99% ethanol according to the pre-determined meal-to-solvent ratio and extracted for the respective time while maintaining the required temperature as indicated in the experimental points of Table 3.2. The resulting meal slurry was filtered under vacuum (Whatman 1 filter paper), and the meal residue was collected, air-dried overnight, and stored in air-tight containers at 4°C for further analysis. Moisture, residual oil, crude protein, total phenolics and free sugars contents of the ethanol-treated meal were determined as described previously and were the responses (dependent variables) used in determining the optimum levels of the three factors or independent variables of ethanol pre-treatment.

Six different CP and DT canola meal samples obtained on different processing days from the respective processing facilities (listed in Section 3.3.1) were used for verifying the applicability of the optimum treatment conditions (meal-to-solvent ratio of 1:4 w/w, extraction time of 30 min, extraction temperature of 50°C). All samples used in the verification study were ground and characterized for their chemical composition before and after ethanol pre-treatment.

Table 3.2 Experimental points and combinations of independent variables (time, temperature, and meal-to-solvent ratio) for the three factor CCD design of ethanol pre-treatment of CP and DT canola meal[a].

Factor / Experiment Number	X_1:Time, min	X_2:Temperature, °C	X_3: Meal-to-solvent ratio, w:w
Axial star point (lower,-α)	10 (-α)	22 (-α)	1:2 (-α)
Factor point (lower,-1)	30 (-1)	30 (-1)	1:3 (-1)
Centre point (0)	60 (0)	42 (0)	1:4 (0)
Factor point (upper, +1)	90 (+1)	55 (+1)	1:5 (+1)
Axial star point (upper,+α)	110 (+α)	63 (+α)	1:6 (+α)
1	30(-1)	30 (-1)	1:3 (-1)
2	30(-1)	30 (-1)	1:5 (+1)
3	30(-1)	55 (+1)	1:3 (-1)
4	30(-1)	55 (+1)	1:5 (+1)
5	90 (+1)	30 (-1)	1:3 (-1)
6	90 (+1)	30 (-1)	1:5 (+1)
7	90 (+1)	55 (+1)	1:3 (-1)
8	90 (+1)	55 (+1)	1:5 (+1)
9	60 (0)	42 (0)	1:2 (-α)
10	60 (0)	42 (0)	1:6 (+α)
11	60 (0)	22 (-α)	1:4 (0)
12	60 (0)	63 (+α)	1:4 (0)
13	10 (-α)	42 (0)	1:4 (0)
14	110 (+α)	42 (0)	1:4 (0)
15	60 (0)	42 (0)	1:4 (0)
16	60 (0)	42 (0)	1:4 (0)
17	60 (0)	42 (0)	1:4 (0)
18	60 (0)	42 (0)	1:4 (0)
19	60 (0)	42 (0)	1:4 (0)
20	60 (0)	42 (0)	1:4 (0)

[a] Coding α= 1.668 for a 3-factor design; $\alpha = (2^k)^{1/4}$, where k = # of different factors in the study.

Calculation of uncoded factor value x_i, $x_i = x_0 \pm X_i(\frac{D}{2})$, where x_0 is uncoded center point value, X_i is coded value for *i*-th factor level and D is the difference between upper and lower points of the uncoded factor points.

Selection of axial point values is based on practical considerations.

Experimental points 1- 8 are factor points, 9-14 are axial points and 15-20 are center points.

3.3.4 Extraction and isolation of the protein fraction from canola meal

It is expected that a concentrated or isolated form of canola protein would contain a lower amount of non-protein components than the canola meal. In turn, the amino acid yield would be higher, with fewer unwanted components expected upon hydrolysis. Cold-pressed and DT meals pre-treated under optimum conditions were used for protein extraction to remove/reduce the meal fibre fraction and associated components. In order to determine a suitable pH and meal-to-solvent ratio that yielded high amounts of protein from CP and DT meals, a two-factor model using CCD with 13 experimental points was employed (Table 3.3). The pH of the extraction solution and meal-to-solvent ratio (w:w) were the independent variables/factors for the experimental points. Selection of lower and upper values for the factors: pH (9 to 13) and meal-to-solvent ratio (1:10 to 1:18, w/v), were selected based on the preliminary studies. Calculations according to the CCD were used to determine the middle and axial point values for each factor. In each experiment, a canola meal (5.0 g) was suspended in water, mixed well and pre-determined levels of NaOH solution (0.1-10 M) were added to reach the required pH. The desired level of meal-to-solvent ratio was reached by adding water, and the pH was readjusted as necessary. The meal slurry was continuously mixed while maintaining the required pH. After 1 h of mixing, the suspension was centrifuged for 20 min at 12,429 × g, and the supernatant was recovered. The recovered supernatant was freeze-dried, and the dry solids were collected. The weight of the collected dry solids was taken as the dry biomass (dbm) recovered from the meal, and the crude protein content of dbm was determined by N analysis. The values for dbm recovered and their protein content were used in calculating the percentage protein recovery (Equation 3.1). The weight of dbm recovered, the weight of protein in the dbm and the percentage protein recovery were the response variables for RSM analysis (Design expert software, Stat-Ease Inc., Minneapolis, MN, USA, version 11) to determine the optimum pH and meal-to-solvent ratio to achieve the maximum level of protein recovered from the meal in a single extraction.

$$\text{Protein recovery, \%} = \frac{\text{Weight of protein in supernatant dbm}}{\text{Weight of protein in starting meal sample}} \times 100 \qquad \text{Equation 3.1}$$

Cold-pressed and DT meals pre-treated under optimum conditions were extracted using the conditions determined to be optimum (pH 12 and meal-to-solvent of 1:10, w:v). After each extraction, the supernatant was recovered as described above and freeze-dried. Then, the values

for dbm recovery and protein content were determined. Six different samples of canola meals (similar to section 3.3.3) were used for verifying the extraction conditions determined to be optimum. In separate experiments with one CP and one DT meal sample, repeated extractions were tested for their impact on protein recovery. Conditions determined as optimum were employed for the first, second and third extractions of the same meal.

Table 3.3 Experimental points and combinations of independent variables (pH and meal-to-solvent ratio) for the two factor CCD design of protein extraction (single run) of CP and DT canola meal[a].

Factor / Experiment Number	X_1: pH of extraction	X_2: Meal-to-solvent ratio, w:v
Axial star point (lower,-α)	8.2 (-α)	1:8.3 (-α)
Factor point (lower,-1)	9 (-1)	1:10 (-1)
Centre point (0)	11 (0)	1:14 (0)
Factor point (upper, +1)	13 (+1)	1:18 (+1)
Axial star point (upper,+α)	13.8 (+α)	1:19.6 (+α)
1	9 (-1)	1:10 (-1)
2	13 (+1)	1:10 (-1)
3	9 (-1)	1:18 (+1)
4	13 (+1)	1:18 (+1)
5	8.2 (-α)	1:14 (0)
6	13.8 (+α)	1:14 (0)
7	11 (0)	1:8.3 (-α)
8	11 (0)	1:19.6 (+α)
9	11 (0)	1:14 (0)
10	11 (0)	1:14 (0)
11	11 (0)	1:14 (0)
12	11 (0)	1:14 (0)
13	11 (0)	1:14 (0)

[a] α= 1.414 for a 2-factor design; $\alpha = (2^k)^{1/4}$, where k = # of different factors in the study.

Calculation of uncoded factor value x_i, $x_i = x_0 \pm X_i(\frac{D}{2})$, where x_0 is uncoded center point value, X_i is coded value for *i*-th factor level and D is the difference between upper and lower points of the uncoded factor points. Axial points are selected based on practical considerations.

3.3.5 Investigation of protein hydrolysis using sulfuric acid

3.3.5.1 Determination of suitable H_2SO_4 acid concentrations for protein hydrolysis

The protein fractions obtained from ethanol pre-treated CP and DT meals were hydrolyzed with a concentration series of H_2SO_4 (1.5-8 M) according to AOAC 994.12, 2005 (AOAC International, 2005) and the resulting hydrolysates were investigated in order to find out the most suitable acid concentration. Briefly, 5.0 mg (protein basis) of the sample was weighed into a test tube with a Teflon lined cap followed by the addition of 2 mL of H_2SO_4. The tube was capped and vortexed to ensure thorough dispersion. The sample tube was placed in a heating block at 110°C and incubated for 24 h. Then, the hydrolysate was quantitatively transferred to a 10-mL volumetric flask containing the required volume of NaOH to neutralize the acid, and the volume was adjusted with de-ionized water. The neutralized hydrolysate was then cleaned by following the same procedure used for HCl hydrolysis in section 3.3.2.6 (a). Finally, the samples were derivatized, and quantification of liberated AAs were performed by the HPLC method described in section 3.3.2.6. The amide forms of AAs, asparagine and glutamine, are further hydrolyzed to form their corresponding acid form; thus, the acid hydrolysis of proteins results in 18 AAs. The content of each of the 18 AAs resulting from hydrolysis of each protein fraction was calculated and taken as the total and compared with the amount of total and individual AAs that were obtained from standard 6 M HCl hydrolysis. It was assumed that 6 M HCl hydrolysis yielded the maximum amount of AAs that could be obtained from each protein source. The AA yield under each hydrolytic condition was calculated according to Equation 3.2.

$$\text{Amino acid (AA) yield, \%} = \frac{\text{Total amount of AAs obtained from (n) M } H_2SO_4 \text{ acid hydrolys, mg/g}}{\text{Total amount of AAs obtained from 6 M HCl acid hydrolysis, mg/g}} \times 100$$

Equation 3.2

where, *n* is the normality of the acid and the total amount of AAs is the sum of the individual AAs obtained from analyzing the hydrolysate.

3.3.5.2 Peptide profile of protein hydrolysates based on peptide size

Since H_2SO_4 hydrolysis produced lower AA yields than complete hydrolysis, an investigation was conducted to see whether partially hydrolyzed peptides were present in the hydrolysates. A protein hydrolysate sample of 200 µL was injected onto a size-exclusion column (Sephadex 30 Increase 10/300) attached to a Fast Protein Liquid Chromatography (FPLC) system (GE AKTA Avant 25,

Cytiva Life Sciences, Montreal, Canada) and eluted with 2× phosphate-buffered sodium (20 mM NaCl, 0.6 mM KCl, pH 7.4) at a flow rate of 0.8 mL/min. Peptides were detected using absorption at 214 nm. Cytochrome C (12,400 Da) 0.2 mg/mL, aprotinin (6,500 Da) 0.2 mg/mL, vitamin B12 (1,355 Da) 0.07 mg/mL, triglycine (189 Da) 0.2 mg/mL and glycine (75 Da) 7 mg/mL were used as standard molecular size markers. The relative abundance of peptides in the hydrolysate was determined using the peak areas. The free AA content of the hydrolysate was determined by the HPLC method after derivatization as described in section 3.3.2.6.

3.3.6 Statistical analysis of data

The experiments described in sections 3.3.3 and 3.3.4 were designed to find the optimum points of the factors affecting the removal of oil, total phenolics, and free sugars and the extraction of the maximum amount of protein from ethanol pre-treated meals respectively. Both of these experiments were conducted as CCDs (described in the respective sections), and data analysis was according to response surface methodology (RSM). Data obtained for response variables (contents of residual oil, total phenolics and free sugars of pre-treated meals in section 3.3.3 and extracted dbm, protein content of dbm and % protein recovery in section 3.3.4) for each experiment were fitted to a second-order polynomial function (Equation 3.3) in order to model by RSM.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \in$$

Equation 3.3

where y is measured/predicted response, β_0 is the intercept, β_i is the linear coefficient of parameter i (i=1, 2, 3….), β_{ii} is the quadratic coefficient of parameter i (i=1,2,3…), $\beta_{ij}$is the coefficient of interaction between variables i and j, and $\in$ is the random error term. Independent variables (experimental factors) are denoted as x_i and x_j.

For the three-factor CCD, the second order polynomial function was:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_1^2 + \beta_3 x_2 + \beta_4 x_2^2 + \beta_5 x_3 + \beta_6 x_3^2 + \beta_7 x_{12} + \beta_8 x_{13} + \beta_9 x_{23} + \beta_{10} x_{123} + \in$$

Equation 3.4

where *y* is contents of oil, protein, total phenolics or free sugars of CP and DT meal after ethanol pre-treatment, x_1 is the time of extraction, x_2 is the temperature of extraction, x_3 is the meal-to-solvent ratio, and β_0 to β_9 are respective regression co-efficients and $\in$ is the error term from data analysis.

For the two-factor CCD, the second order polynomial function was:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_1^2 + \beta_3 x_2 + \beta_4 x_2^2 + \beta_5 x_{12} + \in$$

Equation 3.5

where *y* is pH or meal-to-solvent ratio for protein extraction of ethanol-treated CP and DT meal, x_1 is pH of extraction, x_2 is the meal-to-solvent ratio, β_0 to β_9 are respective regression coefficients and $\in$ is the error term from data analysis.

Data analysis for optimization of independent variables, response surface analysis and response surface development were carried out using Design Experts software (version 11).

All chemical analyses were carried out in triplicate and data are presented as mean ± standard deviation. Further analysis of data was carried out by one-way ANOVA for each response and meal separately. Multiple means comparison was carried out by Tukey's ($p \leq 0.05$). Minitab software was used.

3.4 Results and discussion

The composition of the CP and DT meals used in the study showed that protein and fibre comprised the major fraction of both meals with mean values of 669 mg/g CP meal and 714 mg/g DT meal (Table 3.4). The two meals were different in moisture content, and when the composition was expressed on a dry weight basis, CP meal had ~ 3 times more oil than DT meal, and the protein content was 0.88 times that of DT meal. On a dry weight basis, the free sugar content of CP meal was 69.9 mg/g meal and consisted of glucose, fructose, and sucrose, whereas only sucrose was

detected in the DT meal sugar fraction (75.5 mg/g meal). The absence of free reducing sugars (glucose and fructose) in DT meal may have been a direct result of the conditions of the PSE process, which allowed reducing sugars to be involved in Maillard-type reactions during exposure to high temperature. The values for the contents of ash, total phenolics, fibre and other components of the two meals were similar.

Table 3.4 Characterization of canola meal (dry weight basis)[a]

Parameter	CP meal	DT meal
Ash (%)	6.97 ± 0.02	8.20 ± 0.10
Oil (%)	13.39 ± 0.42	4.33 ± 0.03
Protein (%)	36.38 ± 0.23	42.53 ± 0.40
Total phenolics (mg SAE eq per g of meal)	17.58 ± 0.46	10.90 ± 0.06
Free sugar content[b] (mg per g meal)	69.91[c] ± 5.64	75.48[d] ± 6.88

[a] Data are presented as mean ± standard deviation
[b] Hexane defatted meal was analyzed
[c] Total free sugar content of glucose, fructose and sucrose
[d] Only sucrose content is presented
SAE: sinapic acid equivalent

3.4.1 Ethanol pre-treatment and optimization of treatment parameters

3.4.1.1 Effect on oil, total phenolics and sugar levels

It was expected that ethanol would remove residual oil and small molecules and facilitate meal protein enrichment. The combinations of time, temperature, and meal-to-solvent ratio (three factors, independent variables) for ethanol pre-treatment (Table 3.5) affected the residual oil, total phenolics, total sugars and protein (response variables) contents of the CP and DT meals, resulting in compositionally different meals. Response surface methodology analysis of the data provided information on the treatment combinations that would maximize the removal of oil, phenolics and sugars while enriching the protein content of CP meal (Figures 3.1 A, B, C & D) and DT meal (Figures 3.2 A, B, C & D). This pre-treatment reduced the oil content of CP meal by 78 to 93%. Of the treatment variables, meal-to-solvent ratio was the only parameter that had a significant ($p \leq 0.05$) effect on oil removal. The oil content of DT meal had a 70% - 86% reduction after the pre-treatment with a significant ($p \leq 0.05$) influence from extraction time, meal-to-solvent ratio, and temperature. According to the results, 99% ethanol can be used to extract oil from CP meal as an alternative to hexane.

The ethanol pre-treatment combinations resulted in a reduction in levels of phenolic compounds of 7% - 35% for CP meal and 2% - 27% for DT meal, with temperature having a significant ($p \leq 0.05$) effect (Table 3.5). Most of the phenolic acids in canola cotyledons and seed coats are reported to be in esterified (~80%) and free (~15%) forms, while the remainder is in insoluble-bound forms. Levels of extractable phenolics in defatted canola meal have been reported as 1.59-1.84 g/100 g (15.9-18.4 mg/g) (Naczk *et al*., 1998). The association of canola seed phenolic compounds with proteins has been reported, especially when extracted under alkali conditions, resulting in dark coloration in protein preparations (Matthäus *et al*., 2014; Nadathur *et al*., 2017; Pudel *et al*., 2014; Shahidi & Naczk, 1992; Xu & Diosady, 2000). The present study showed that ~30% of the phenolics in CP meal or DT meal were removed by extraction with 99% ethanol, which would be of limited benefit. The solubility of phenolics depends on their structural chemistry and their interactions with proteins and other seed components via ionic, hydrogen and covalent bonds and hydrophobic interactions (Xu & Diosady, 2000). Therefore, the extraction solvent polarity plays a significant role in the solubility and extractability of phenolic compounds. Aqueous mixtures of methanol (70% - 100%, v/v) (Cai & Arntfield, 2001; Chen *et al*., 2014; Jun *et al*., 2014) or ethanol

(65% - 75%, v/v) (Adem *et al*., 2014; Chabanon *et al*., 2007; Kalaydzhiev *et al*., 2019; Khattab *et al*., 2010), which are more polar than the pure alcohols, have been employed as extraction media for canola meal phenolics for further studies. Therefore, ethanol without water is of limited value in extracting phenolics from canola meals.

The total free sugar content of CP meal was reduced by 0.4% - 49% by ethanol treatment (Table 3.5), with temperature as the only factor affecting the extraction significantly ($p \leq 0.05$). Ethanol treatment reduced the sucrose level of DT meal by 1% - 40% under the conditions tested, with both time and temperature having a significant ($p \leq 0.05$) effect on the final level. Soluble sugars have been reported to represent up to 5.7% of the dry matter in oil-free canola meal and consist of glucose, fructose, and sucrose (Pedroche *et al*., 2004; Wanasundara *et al*., 2016). Aqueous organic solvents effectively lower the levels of soluble sugars in canola meal. Berot and Briffaud (1983) reported washing of defatted rapeseed meal with 80% (v/v) ethanol or methanol, which resulted in final soluble sugar concentrations (expressed as glucose equivalents) of 0.25% and 0.65% of the meal, respectively.

3.4.1.2 Effect on protein level

The difference between untreated CP and DT meal protein contents can be attributed to the level of other non-protein components present, specifically oil, fibre, sugars, etc. The protein level of CP meal increased by 13.7% - 22.8% (414 to 447 mg/g dbm from 364 mg/g of dbm) whereas the change in DT meal was by 4.0% - 8.7% (442 to 462 mg/g dbm from 425 mg/g dbm; Table 3.5). Since CP meal had a higher oil content than DT meal, the most effective protein enrichment was associated with the conditions that resulted in the highest level of oil removal. Both the time and temperature of ethanol treatment showed significant ($p \leq 0.05$) effects on enriching the meal protein content of CP and DT meals; the meal-to-solvent ratio showed a significant ($p \leq 0.05$) effect only for CP meal. A similar observation was reported by (Slawski *et al*., 2012) for a canola meal sample (31% protein and 13% oil) that resulted in a final meal containing 40% protein and 1% residual oil after applying a four-step treatment with 75% (v/v) ethanol. Although ethanol without added water was less effective in removing phenolic compounds and free sugars, a significant amount of oil was removed from both meals, the levels of some other non-protein components were reduced, and the meal protein content was enriched.

It is clear from the above results that ethanol pre-treatment was highly effective in reducing the residual oil level of both meals, but it was less effective in reducing the levels of phenolic compounds and free sugars. The improvement was particularly effective with higher residual oil content of CP meal as further uses would require less interference from oil. Meal protein enrichment was primarily a concentration effect due to removing non-protein components that were soluble in ethanol. Ethanol can be obtained from various bio-processing means (*e.g.*, fermentation of crop industry residues and starch-rich materials), unlike the commonly used hexane, a petroleum industry by-product. Oil and ethanol in the liquid fraction resulting from the canola meal pre-treatment step can be recovered. The recovered oil can be integrated into the vegetable oil supply, and recovered ethanol can be re-used for the ethanol pre-treatment step.

3.4.1.3 Optimum conditions for ethanol pre-treatment

Response surface analysis of experimental results for CP and DT meals identified a 30-min extraction time at 50°C with a meal-to-solvent ratio of 1:4 (w:w) as the optimum pre-treatment conditions to remove non-protein components (oil, free sugars and total phenolics) and to enrich meal protein (Figures 3.1 A, B, C & D; Figures 3.2 A, B, C & D; Table 3.6). There was an excellent agreement with the expected composition of CP and DT meal treated under optimum conditions with the actual values obtained for these meals (Table 3.6). When the pre-treatment was applied to several CP (n=6), and DT (n=6) meal samples obtained from random processing runs at the respective oil extraction facilities, high reproducibility of removing oil, free sugars and total phenolics and enrichment of protein was observed (Table 3.7). On average, upon pre-treatment, a gram of CP meal dbm containing 22.5 mg oil, 426 mg protein, 15 mg total phenolics [as sinapic acid equivalent (SAE)] and 50 mg free sugars, and a gram of DT meal dbm containing 8.5 mg oil, 443 mg protein, 9.4 mg total phenolics (as SAE) and 44.2 mg free sugars, was obtained (Table 3.6).

Table 3.5 Contents of oil, protein, total phenolics and free sugars of CP and DT meals upon various combinations of meal-to-solvent ratio and pre-treatment time and temperature. All values are mg per g dry meal biomass (mg/g dbm).

Exp No.[a]	CP meal				DT meal			
	Oil (mg/g dbm[b])	Protein (mg/g dbm[b])	Total phenolics (mg SAE[c] eq/g dbm)	Free sugars (mg/g dbm[b])	Oil (mg/g dbm[b])	Protein (mg/g dbm[b])	Total phenolic (mg SAE[c]eq/g dbm)	Free sugars (mg/g dbm[b])
1	18.7 ± 0.7	414.1 ± 1.4	16.3 ±1.2	52.8 ± 2.0	13.1 ± 1.6	445.5 ± 2.6	12.7 ± 0.6	68.5± 3.0
2	16.8 ± 0.7	423.6 ± 5.8	15.8 ± 1.3	49.8 ± 0.9	10.7 ± 2.3	448.1 ± 1.1	11.8 ± 0.9	74.2 ± 3.1
3	22.6 ± 0.7	434.2 ± 1.1	13.5 ± 0.7	46.0 ± 1.2	12.9 ± 1.4	449.9 ± 3.3	9.6 ± 0.8	59.5 ± 3.2
4	12.5 ± 4.2	442.1 ± 5.1	14.4± 0.1	45.0 ± 1.6	6.2 ± 0.2	458.1 ± 11.2	9.7 ± 0.4	60.0 ± 1.1
5	17.3 ± 5.9	425.3 ± 2.6	15.7 ± 1.0	54.4 ± 2.2	9.4 ± 0.6	448.4 ± 1.4	11.3 ± 0.3	68.6 ± 2.8
6	11.2 ± 0.2	435.7 ± 2.7	15.6 ± 0.3	52.4 ± 2.0	8.6 ± 1.1	446.9 ± 1.4	10.7 ± 0.3	67.9 ± 0.7
7	19.4 ± 0.8	431.0 ± 3.1	13.5 ± 0.3	49.5 ± 1.8	6.5 ± 0.5	462.0 ± 1.0	8.0 ± 0.1	52.3 ± 3.2
8	13.5 ± 1.0	446.2 ± 10.0	12.5 ± 0.8	35.4 ± 0.4	6.7 ± 0.6	455.6 ± 1.5	9.5 ± 0.0	53.0 ± 3.1
9	29.6 ± 2.6	419.3 ± 2.3	15.5 ± 0.5	44.7 ± 1.5	11.7 ± 0.2	448.7 ± 1.8	10.3 ± 0.3	67.3 ± 2.1
10	9.9 ± 0.8	439.7 ± 11.5	14.8 ± 0.4	38.4 ± 3.6	7.4 ± 0.7	452.5 ± 2.4	10.3 ± 0.0	62.7 ± 3.1
11	19.1 ± 0.7	426.1 ± 3.6	15.9 ± 0.4	69.3 ± 3.2	10.7 ± 1.1	442.4 ± 1.5	11.1 ± 0.2	70.1 ± 2.4
12	21.0 ± 1.0	447.0 ± 13.4	11.5 ± 0.6	57.6 ± 3.8	6.7 ± 1.0	462.4 ± 0.5	8.1 ± 0.2	45.7 ± 3.3
13	12.5 ± 0.1	423.0 ± 4.3	15.7 ± 0.5	55.4 ± 3.1	10.7 ± 0.7	449.5 ± 5.2	11.0 ± 0.9	69.1 ± 0.7
14	14.6 ± 1.0	434.8 ± 5.7	15.6 ± 0.5	59.2 ± 1.1	9.5 ± 0.5	459.2 ± 0.8	10.0 ± 0.4	62.9 ± 3.3
15	16.0 ± 3.5	431.4 ± 7.7	15.4 ± 0.4	53.8 ± 1.7	7.3 ± 0.9	454.5 ± 1.4	9.4 ± 0.5	60.6 ± 5.2
16	13.1 ± 0.6	436.6 ± 4.2	15.0 ± 0.9	64.4 ± 2.4	8.7 ± 0.2	451.6 ± 4.9	9.9 ± 0.5	62.4 ± 2.0
17	16.0 ± 1.0	418.8 ± 2.6	15.2 ± 0.4	55.3 ± 1.7	9.0 ± 1.2	455.7 ± 2.0	10.2 ± 0.7	57.1 ± 2.1
18	14.8 ± 3.2	427.2 ± 1.5	15.6 ± 0.2	46.9 ± 1.6	9.4 ± 0.7	456.3 ± 3.8	9.9 ± 0.5	53.9 ± 3.2
19	15.9 ± 0.4	422.6 ± 0.9	15.4 ± 0.9	56.6 ± 2.1	8.4 ± 0.6	453.8 ± 2.1	9.7 ± 0.5	55.5 ± 2.3
20	12.4 ± 0.8	427.3 ± 2.0	15.2 ± 0.7	54.2 ± 3.1	9.1 ± 1.2	454.4 ± 0.8	9.6 ± 0.6	54.4 ± 3.6

[a] Pre-treatment factor combinations for experimental points refer to Table 3.2; 1- 8 are factor points, 9-14 are axial points and 15-20 are center points

[b] dbm: dry biomass, SAE: Sinapic acid equivalent

[c] As sinapic acid equivalents

Data are presented as mean ± standard deviation

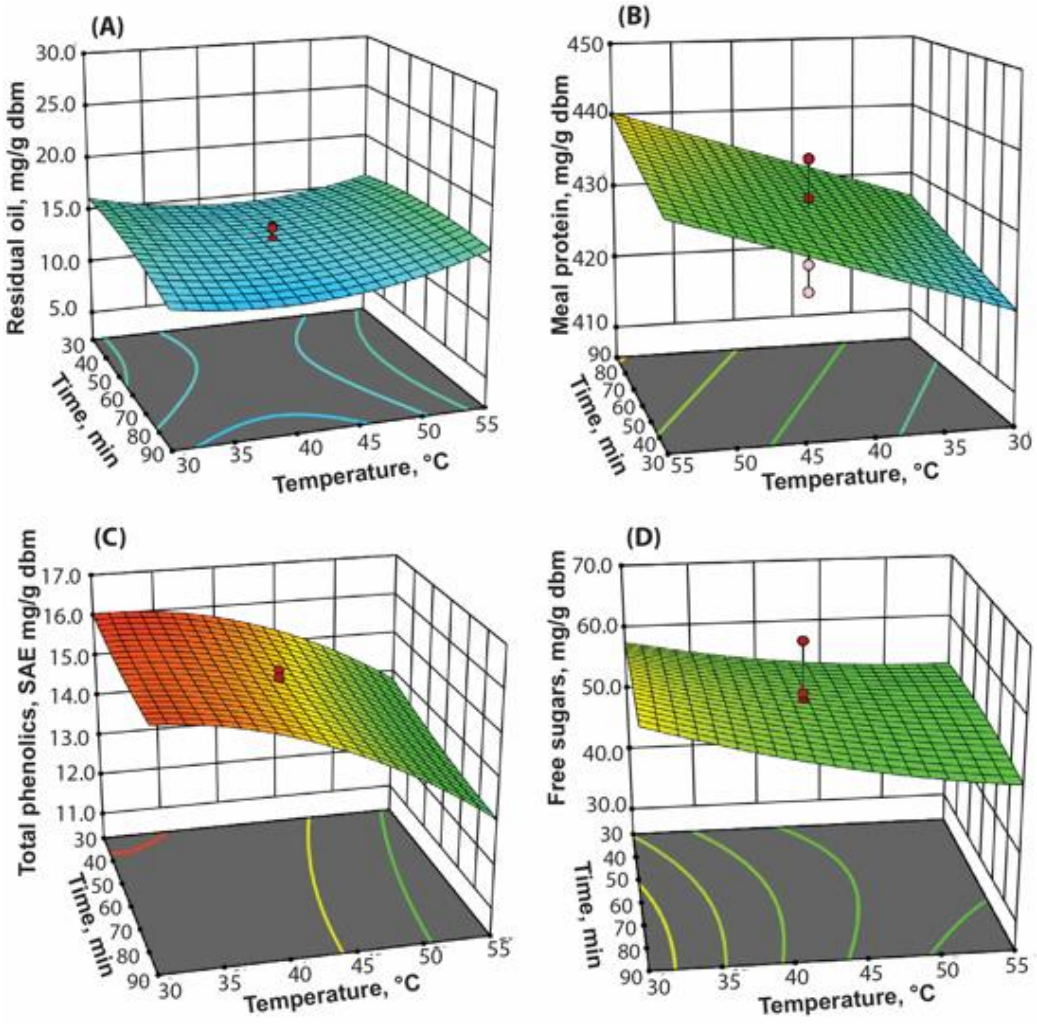


Figure 3.1 (A), (B), (C) & (D). Response surface plots showing the effect of temperature and time of extraction on the contents of: (A) residual oil, (B) protein, (C) total phenolics, and (D) free sugars of CP meal upon ethanol pre-treatment at a meal-to-solvent ratio of 1:4 (w:w).

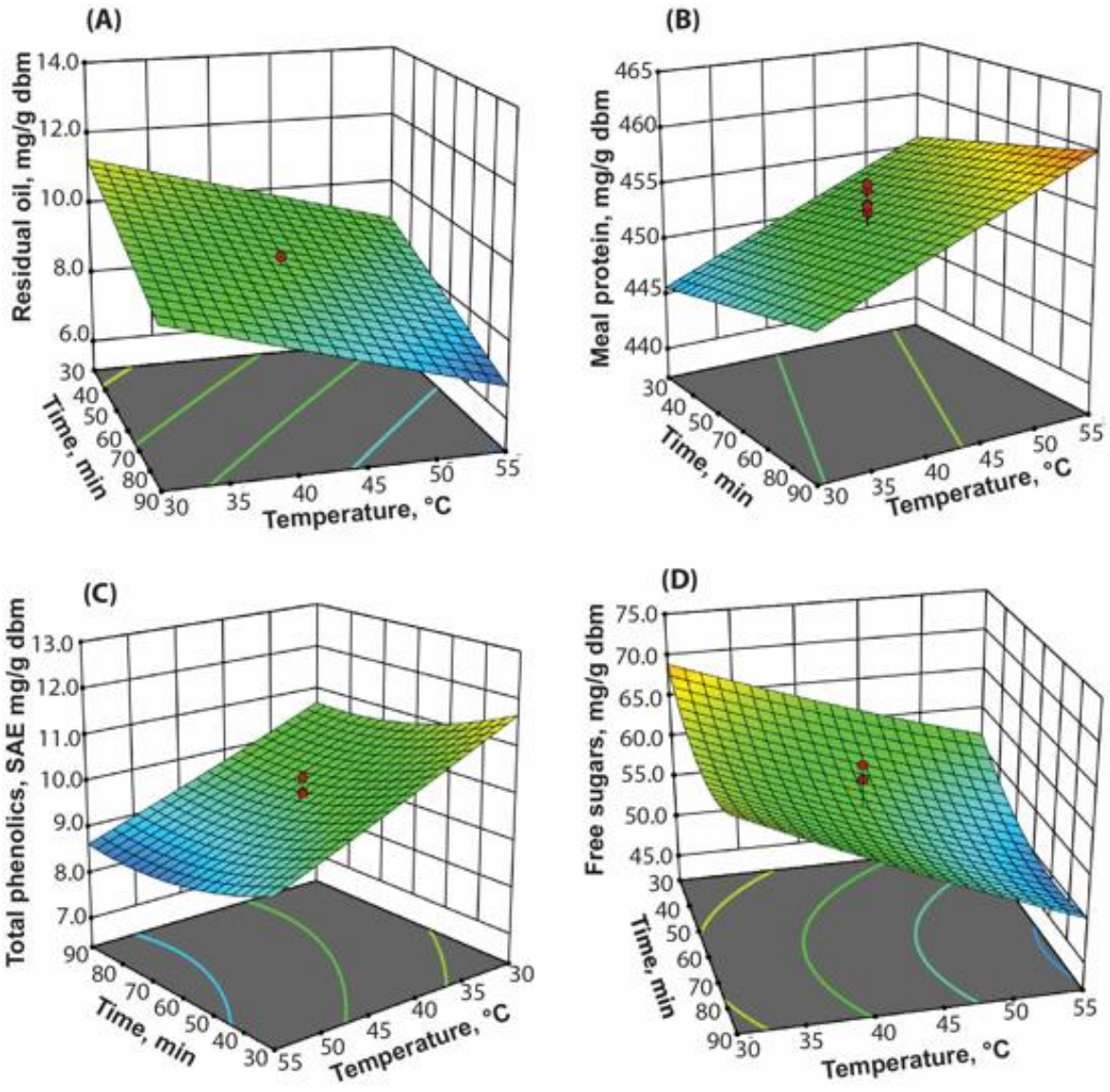


Figure 3.2 (A), (B), (C) and (D). Response surface plots showing the effect of temperature and time of extraction on the contents of: (A) residual oil, (B) protein, (C) total phenolics, and (D) free sugars of DT meal as a result of ethanol pre-treatment at a meal-to-solvent ratio of 1:4 (w:w).

Table 3.6 Expected and observed values for the contents of residual oil, protein, total phenolics and free sugars of ethanol pre-treated CP and DT meals for validating optimum meal-to-solvent ratio (1:4, w:v) and treatment time (30 min) and temperature (50°C)[a]. Expected values were calculated from the equation in the foot note and observed values were obtained from actual pre-treatment experiments.

Parameter	CP meal		DT meal	
	Expected value[b]	Observed value	Expected value[b]	Observed value
Residual oil, mg/g dbm[c]	15.0	16.1 ± 0.2	9.50	8.20 ± 0.3
Protein, mg/g dbm	430.7	422.4 ± 5.6	454.0	456.3 ± 1.7
Total phenolics (mg SAE[c] eq/g dbm)	14.7	14.1 ± 0.4	9.8	8.4 ± 0.4
Free sugars (mg/g dbm)	52.7	53.3 ± 0.8	58.8	46.1 ± 4.4

[a] Oil, protein, total phenolics and free sugars of starting CP meal was 133.9 mg, 363.8mg, 17.6 mg SAE and 69.9 mg per g dry biomass, respectively and for DT meal was 43.3 mg, 425.3 mg, 10.9 mg SAE and 75.5 mg per g dry biomass, respectively. Data are presented as mean ± standard deviation

[b] Equation for expected value calculation was:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_1^2 + \beta_3 x_2 + \beta_4 x_2^2 + \beta_5 x_3 + \beta_6 x_3^2 + \beta_7 x_{12} + \beta_8 x_{13} + \beta_9 x_{23} + \beta_{10} x_{123}$$

where, y is Yield (predicted response), β_0 is the intercept of the model, β_i is parameters related to linear effects (i=1,2,3), β_{ii} is model parameters related to quadratic effects (i=1,2,3), β_{ij} is model parameters related to interaction between variables, x_1 , x_2 , x_3 are the independent factors, and x_{12}, x_{13} , x_{23}, x_{123} are interactions between these factors.

[c] dbm: dry biomass, SAE; Sinapic acid equivalent

Table 3.7 Values of residual oil, protein, total phenolics and free sugars after ethanol pre-treatment of randomly obtained CP and DT meal samples. All meals received ethanol pre-treatment under conditions determined as optimum[a].

Sample	Residual oil (mg/g dbm[b])		Protein (mg/g dbm)		Total phenolics (mg SAE[b] eq/g dbm)		Free sugars (mg/g dbm)	
	Untreated n=3	After treatment n=9	Untreated n=3	After treatment n=9	Untreated n=3	After treatment n=9	Untreated n=3	After treatment n=9
CP meal								
CP 1	137.2 ± 4.0	23.0 ± 5.0^{a}	368.7 ± 5.6	420.0 ± 4.7^{bc}	16.8 ± 0.3	14.4 ± 0.7^{a}	71.2 ± 2.2	48.5 ± 0.57^{ab}
CP 2	140.8 ± 3.1	20.7 ± 2.5^{a}	367.8 ± 7.6	423.6 ± 2.0^{abc}	17.0 ± 0.4	14.1 ± 0.5^{a}	69.6 ± 3.4	51.2 ± 0.56^{a}
CP 3	138.8 ± 2.3	22.0 ± 7.2^{a}	370.2 ± 2.9	431.4 ± 9.4^{ab}	16.7 ± 0.3	14.4 ± 0.7^{a}	68.5 ± 4.1	49.4 ± 0.44^{ab}
CP 4	141.2 ± 4.3	25.6± 5.0^{a}	364.7 ± 3.9	436.8 ± 8.8^{a}	16.9 ± 0.2	14.7 ± 0.5^{a}	70.7 ± 3.0	47.8 ± 1.54^{b}
CP 5	140.9 ± 2.3	21.0 ± 4.2^{a}	363.0 ± 3.8	413.6 ± 8.4^{c}	16.6 ±0.4	14.6 ± 0.5^{a}	72.5 ± 2.3	51.4 ± 0.84^{a}
CP 6	141.4 ± 5.2	22.1 ± 3.6^{a}	361.7 ± 4.2	427.2 ± 4.7^{abc}	17.0 ±0.3	14.7 ± 0.7^{a}	68.4 ± 1.8	49.8 ± 1.69^{ab}
DT meal								
DT1	48.0 ± 5.7	11.1 ± 0.4^{p}	426.5 ± 1.0	443.2 ± 7.4^{p}	11.7 ± 0.2	9.7 ± 1.1^{pq}	78.3 ± 2.7	43.2 ± 1.41^{qr}
DT2	52.6 ± 1.0	11.8 ± 0.7^{p}	424.9 ± 1.4	441.6 ± 4.1^{p}	11.5 ± 0.6	10.0 ± 0.2^{p}	80.2 ± 2.1	45.6 ± 0.91^{pq}
DT3	59.2 ± 1.0	7.7 ± 0.7^{qr}	409.9 ± 2.6	443.7 ± 6.8^{p}	12.1 ± 0.1	9.4 ± 0.4^{pq}	77.2 ± 1.9	43.5 ± 1.23^{qr}
DT4	41.7 ± 5.1	6.6 ± 0.4^{r}	419.7 ± 1.8	443.3 ± 3.2^{p}	11.7 ± 0.3	9.5 ± 0.5^{pq}	78.6 ± 3.1	45.1 ± 0.63^{pqr}
DT5	51.9 ± 1.0	9.7 ± 1.1^{pq}	424.3 ± 1.0	447.3 ± 6.6^{p}	10.9 ± 0.2	9.5 ± 0.9^{pq}	80.3 ± 2.5	46.6 ± 1.42^{p}
DT6	48.9 ± 3.1	10.2 ± 1.0^{p}	422.8 ± 4.9	448.1 ± 3.5^{p}	10.3 ± 0.3	8.3 ± 0.4^{q}	79.8 ± 2.1	42.5 ± 0.66^{r}

[a] Optimum conditions for ethanol pretreatment were: Meal-to-solvent ratio 1:4 w:w, Temperature 50^{o}C, Time 30 min

[b]dbm: dry biomass, SAE: Sinapic acid equivalent

Data were analyzed with one-way ANOVA for each response and meal separately. Values sharing same letter are not significantly different. Multiple means comparison by Tukey's ($p \leq 0.05$)

3.4.2 Protein extraction from ethanol pre-treated meals

3.4.2.1 Optimization of extracted dry matter and protein level of dry matter

Protein extraction was carried out in the alkaline pH range of 8.2-13.8, which was decided upon by a review of the literature and preliminary studies. The protein solubility of DT canola meal is quite low below pH 8, and the same has been observed for laboratory prepared defatted canola meal (Wanasundara, 2011). Increasing the extraction pH to above 8 (Table 3.8) elevated the total dbm (includes both protein and non-protein substances) recovered from both ethanol pre-treated CP and DT meals. The maximum dbm recovered at pH 13.8 was 744 mg/g from CP meal and 539 mg/g from DT meal (Table 3.8). The protein content of the extracted dbm was higher at pH 11 than at pH 13 or 13.8, indicating co-extraction of more non-protein components (*e.g.*, fibre, phytates, sugars, phenolics, *etc*.) occurred at pHs higher than 11. When these values were converted to the amount of protein that could be recovered on a meal weight basis, *i.e.*, protein recovery efficiency, at the same meal-to-solvent ratio (1:14, w:v), values were 65% and ~ 55% for CP meal protein at pH 13.8 and 11, respectively (Table 3.8). Under the same conditions, protein recovery efficiency for DT meal protein was 46% at pH 13.8 and ~20% at pH 11 (Table 3.7), showing that the amount of protein recovered from DT meal was much less than from CP meal with a single extraction. Moreover, the response surfaces provided in Figures 3.3 (A) and 3.3 (B) also showed that the meal-to-solvent ratio had a significant effect on the amount of dbm extracted from both meals; however, pH was the most crucial factor affecting protein recovery. According to statistical optimization using RSM, pH 12 and a meal-to-solvent ratio of 1:10 (w:v) were the most suitable conditions for protein extraction from both meal types. In order to validate the pH and a meal-to-solvent ratio for protein extraction, six random meal samples from each meal type were extracted (Table 3.9). When ethanol pre-treated CP and DT meals were extracted at pH 12 (meal-to-solvent 1:10, w:v), 76.5% - 85.4% of the protein CP meals protein and 26.2% - 36.4% of the protein in DT meals could be recovered. These values show that the difference in protein recovery from the two canola meals was directly related to how the meal was processed. Protein in the highly processed DT meals was sparingly soluble under the conditions suitable for extracting proteins from the lesser processed CP meals.

Table 3.8 Data for protein solubilization and recovery from ethanol pre-treated CP and DT meals conducted for optimizing meal-to-solvent ratio and pH according to a 2-factor CCD.

	CP meal			DT meal		
Experiment No.[a]	**Extractable dry matter (mg/g meal dbm)**	**Protein in extracted dry matter (mg/g dbm)**	**Recovery of meal protein (w/w)%**	**Extractable dry matter (mg/g meal)**	**Protein in extracted dry matter (mg/g dbm)**	**Recovery of meal protein (w/w)%**
1	245.3 ± 22.5	482.8 ± 4.1	28.2 ± 2.3	188.1 ± 5.2	286.3 ± 3.1	12.2 ± 0.2
2	479.9 ± 16.1	545.9 ± 17.4	62.4 ± 2.3	387.3 ± 11.7	425.0 ± 12.5	37.4 ± 0.7
3	274.0 ± 7.1	470.2 ± 12.4	30.7 ± 1.5	212.5 ± 3.2	269.7 ± 5.8	13.0 ± 0.4
4	673.9 ± 2.1	514.4 ± 4.5	67.4 ± 2.1	465.3 ± 6.9	409.6 ± 12.8	44.8 ± 1.6
5	269.3 ± 6.9	453.1 ± 3.5	29.1 ± 0.8	189.4 ± 0.3	242.4 ± 7.2	10.4 ± 0.3
6	744.0 ± 10.2	372.7 ± 20.5	65.1 ± 1.1	539.6 ± 28.8	356.7 ± 25.7	45.6 ± 3.5
7	329.1 ± 20.2	606.5 ± 12.8	49.7 ± 1.1	198.8 ± 5.9	382.8 ± 23.2	17.3 ± 1.5
8	440.8± 5.4	592.1 ± 25.8	63.9 ± 1.1	260.1±1.5	372.3 ± 7.8	22.0 ± 0.5
9	399.2	609.0	57.9	228.3	386.7	20.1
10	384.3	606.4	55.5	226.7	380.7	19.6
11	399.4	608.0	57.8	215.9	381.6	18.7
12	346.8	590.0	48.7	244.8	380.8	21.2
13	389.1	592.8	54.9	238.0	366.7	19.8

[a] Values for experimental points refer to Table 3.2 in section 3.3.3; 1- 8 are factor points, 9-14 are axial points and 15-20 are center points

Data are presented as mean ± standard deviation

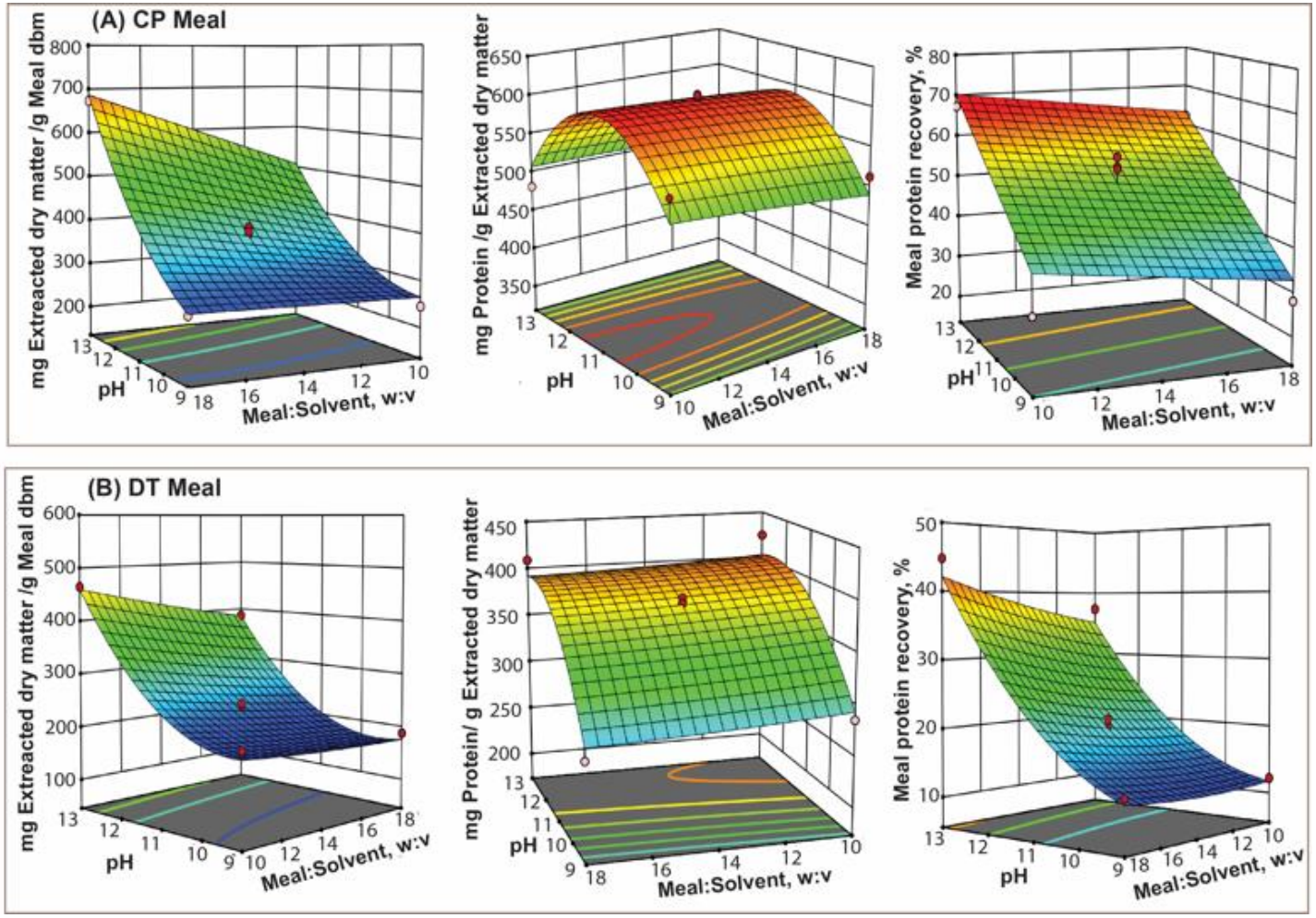


Figure 3.3 (A) & (B). Effect of pH and meal-to-solvent ratio on extracted dry matter (mg/g meal dbm), protein content in extracted dry matter (mg protein/g dbm), and meal protein recovery (%) of ethanol pre-treated CP meal (A) and DT meal (B).

Table 3.9 Verification of applicability of optimum protein extraction conditions for random samples of CP and DT meals (pre-treated with ethanol)[a].

Type of meal	Extractable meal dry matter (mg/g meal dbm)	Protein in extracted dry matter (mg/g dbm)	Protein recovery from meal protein % (w/w)
CP meal			
CP 1	506.3 ± 23.4^{abc}	582.9 ± 0.25^{c}	85.4 ± 2.0^{a}
CP 2	513.1 ± 13.9^{ab}	592.5 ± 8.2^{bc}	84.4 ± 2.0^{a}
CP 3	515.4 ± 4.7^{a}	595.0 ± 0.8^{b}	85.0 ± 1.9^{a}
CP 4	462.8 ± 12.7^{d}	617.1 ± 2.8^{a}	76.5 ± 1.3^{b}
CP 5	468.0 ± 4.7^{cd}	608.4 ± 3.9^{a}	80.9 ± 0.5^{ab}
CP 6	474.6 ± 16.1^{bcd}	612.5 ± 4.6^{a}	79.3 ± 2.6^{b}
DT meal			
DT 1	269.5 ± 16.0^{pq}	496.2 ± 8.7^{p}	30.2 ± 2.0^{pq}
DT 2	251.4 ± 23.5^{q}	463.7 ± 10.6^{q}	26.4 ± 3.1^{q}
DT3	312.3 ± 19.9^{p}	517.8 ± 6.9^{p}	36.4 ± 2.5^{p}
DT 4	304.6 ± 4.8^{p}	510.7 ± 12.8^{p}	35.3 ± 1.6^{pq}
DT 5	252.6 ± 10.7^{q}	466.9 ± 4.7^{q}	26.4 ± 1.4^{q}
DT 6	252.8 ± 16.1^{q}	464.9 ± 6.9^{q}	26.2 ± 1.7^{q}

[a]Optimum conditions were: pH 12, meal-to-solvent 1:10 w:v and two repeated extractions for each sample.

Data were analyzed with one-way ANOVA for each response and meal separately. Values sharing same letter are not significantly different. Multiple means comparison by Tukey's ($p \leq 0.05$).

3.4.2.2 Effect of pre-treatment on protein recovery

When protein extraction was carried out under optimum conditions, values for extracted dbm (solids) and protein from a single extraction of untreated and ethanol pre-treated meal (Figure 3.4 A & B) showed that ethanol pre-treatment slightly improved protein extractability from CP meal. As a percentage of CP meal dry weight, extracted dbm was lower with ethanol pre-treatment but with a higher protein content (Figure 3.4 A). As discussed earlier, this reduction in extracted dry matter weight was mainly due to the lesser amount of non-protein compounds in ethanol pre-treated meal. When CP meal was pre-treated with ethanol, protein extractability improved from 71% (untreated) to 73% (treated) (Figure 3.4 A). In contrast, the values for extracted dry matter and protein were remained lower for DT meal than for CP meal. Ethanol pre-treatment lowered the amount of extractable solids under alkali conditions and the amount of protein (Figure 3.4 A & B). The meal protein recovery from DT meals was between 34% (treated) and 38% (untreated). Calculated data show that 30.6 g of protein can be obtained from 100 g of the ethanol pre-treated CP meal and 25.8 g from untreated CP meal. When DT meal is considered, 100 g of the untreated meal will yield 16.2 g of protein, whereas treated meal will yield 14.8 g, indicating that the ethanol pre-treatment resulted in no improvement in protein extraction from DT meal (Figure 3.4).

According to Kalaydzhiev *et al.* (2019), pre-treatment of canola meal with ethanol can decrease antinutrients such as glucosinolates and phenolic compounds; however, the concomitant increase in fibre content can affect protein recovery from the treated material. The present study showed that ethanol was not efficient in removing significant amounts of small molecule non-protein compounds from industrially processed canola meal. Removal of oil in CP meal may be the primary reason for increased protein extractability and a higher protein level in the extract from ethanol pre-treated meal than untreated. The level of total phenolics in DT meal remained unchanged upon pre-treatment; therefore, co-extraction with protein at alkaline pH is possible (Xu & Diosady, 2000). These reasons may explain the observed poor improvement in the protein level in DT meal after treatment.

3.4.2.3 Effect of repeated extraction on protein recovery

When aqueous protein extraction was repeated for ethanol pre-treated meals, under the optimum conditions, the first, second and third extractions resulted in 67%, 12%, and 2% protein recovery,

respectively, from CP meal (Figure 3.5A) and 29%, 9% and 1%, protein recovery, respectively for DT meal (Figure 3.5 B). Considering the small contribution of the third extraction to the total protein recovery values, preparation of protein-enriched fractions for further use was undertaken with two extractions only. Two extractions of ethanol pre-treated meals with two extractions under the optimum conditions resulted in protein recoveries of 79% for CP meal (Figure 3.5A) and 38% for DT meal (Figure 3.5 B).

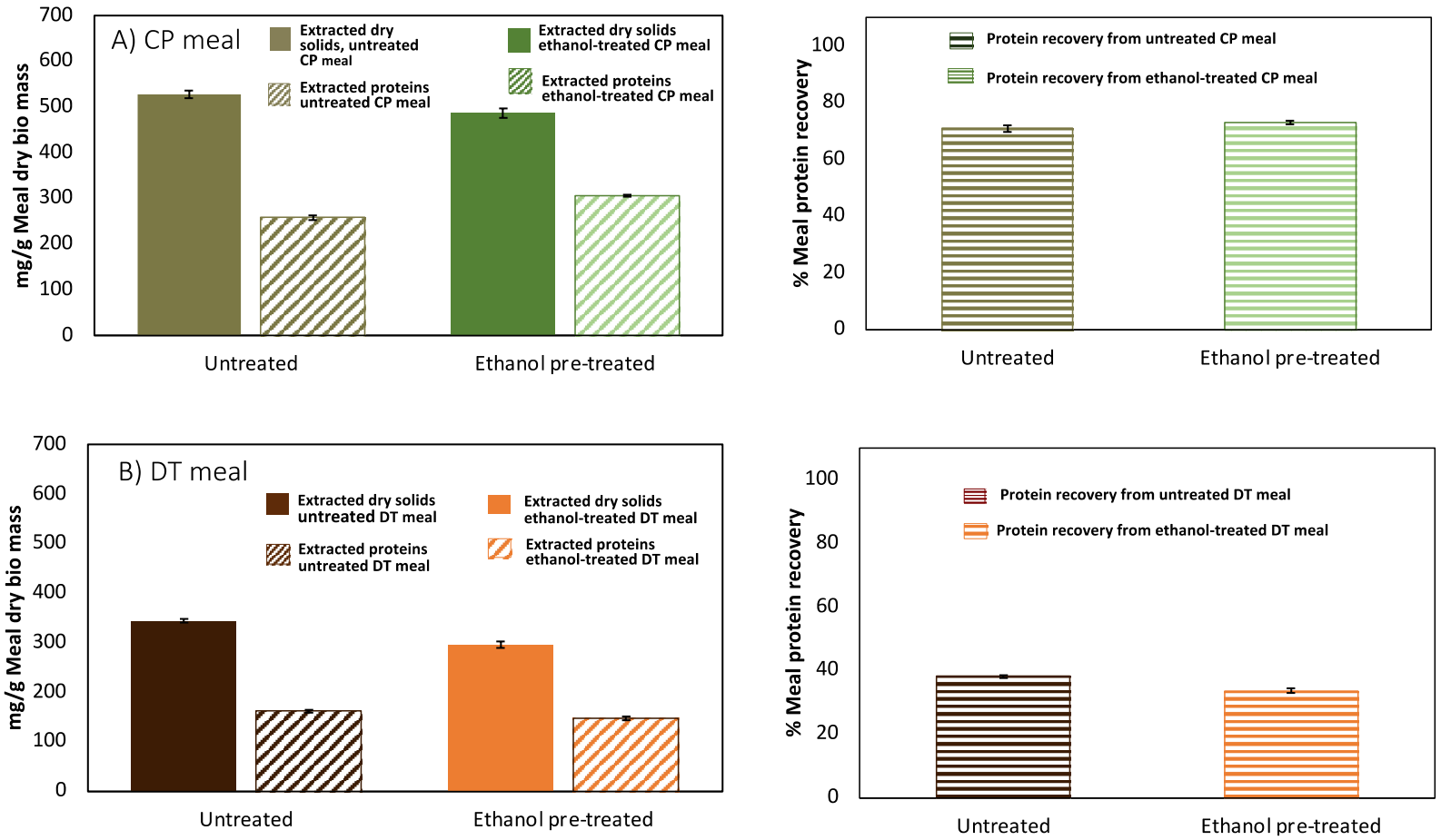


Figure 3.4 (A) & (B). Comparison of untreated and ethanol pre-treated CP (A) and DT (B) canola meal for extracted dry solids, extracted protein, and percentage recovery of meal protein in a single extraction when extracted at pH 12 and the meal-to-solvent ratio of 1:10 (w:v) for 30 min.

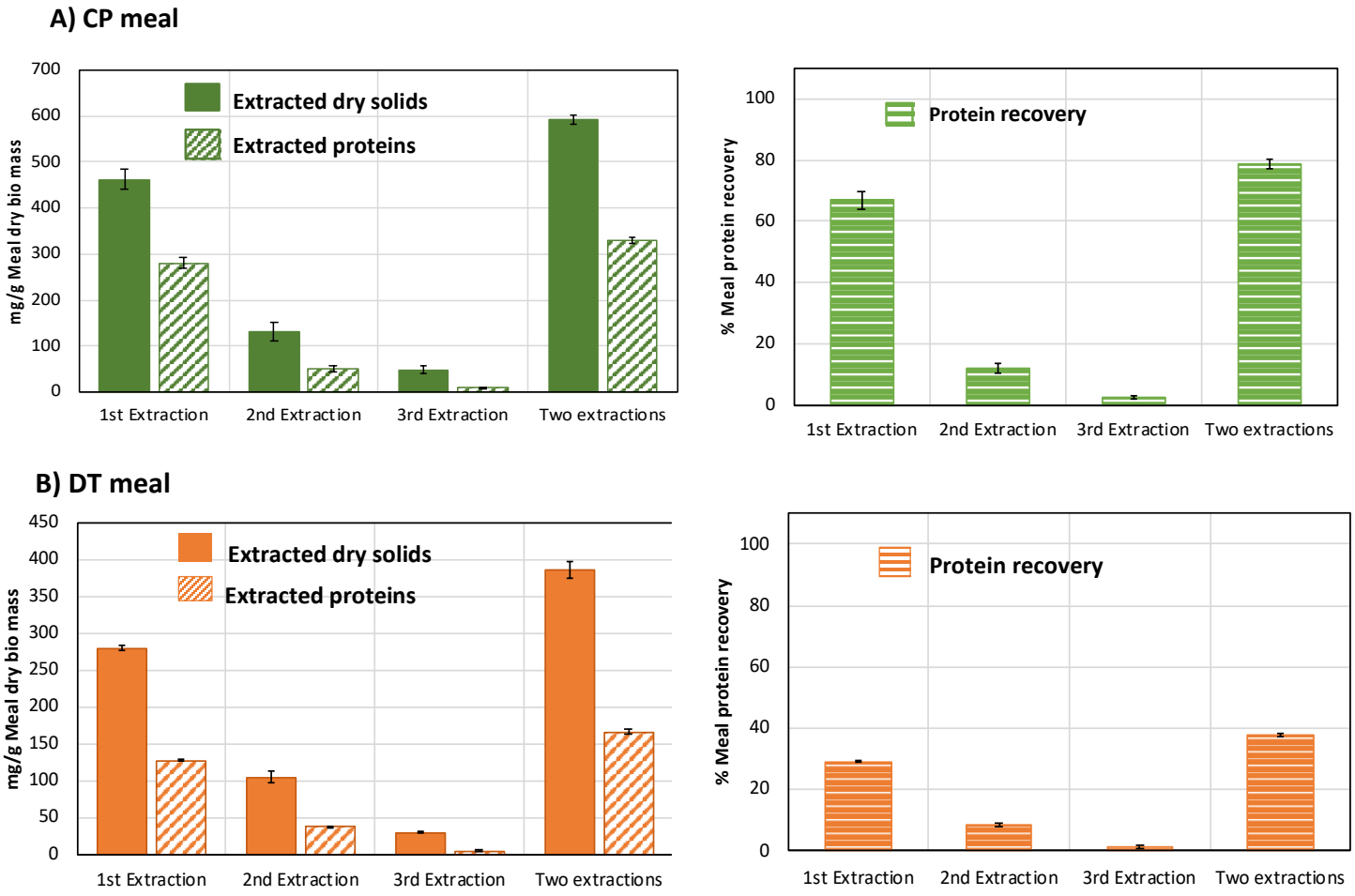


Figure 3.5 (A & B). Comparison of number of repeated extractions under optimum conditions (pH =12, meal-to-solvent 1:10, w:v, 30 min) for the levels of extracted dry solids, extracted protein and meal protein recovery from ethanol pre-treated canola meals. A) CP meal, and B) DT meal. Two-extractions means same meal extracted two times under same conditions and extracts were combined.

Canola meal proteins are primarily seed storage proteins: about 80% of the total protein consists of 12*S* cruciferin and 2*S* napin. Structural proteins associated with oil bodies (2-8%) and cell walls and the minor proteins, such as enzyme inhibitors and lipid transfer proteins comprise the rest (Aider & Barbana, 2011). In terms of solubility behaviour, canola meal proteins exhibit fundamentally different solubility behaviours with changing pH, primarily due to the differences in the protein types in the seed (Wanasundara *et al*., 2016; Wu & Muir, 2008). An alkaline pH medium higher than 11 has been employed to achieve high protein solubility, and consequently to extract more protein from canola meal (Fetzer *et al*., 2018; Ghodsvali *et al*., 2005; Pedroche *et al*., 2004; Tan *et al*., 2011), even for laboratory prepared and ethanol-treated meal (Kalaydzhiev *et al*., 2019). In general, alkaline pH extraction yields 60-90% of protein recovery from canola meal (Alashi *et al*., 2011). In the present study, DT meal proteins showed less solubility in the alkali pH range than CP meal (Figure 3.4 A & B), which may be attributed to the heat- and organic-solvent-induced alterations/interactions in the proteins of PSE meal, rendering them less soluble. Fetzer *et al*. (2018) showed that protein solubility of rapeseed cake from full pressed (FP) or DT meal (exposed to more than 100°C during oil extraction) was lower at alkaline pH than that of CP cake. Thermal treatment during the toasting step of meal de-solventization leads to denaturation, which unfolds protein structure, exposes hydrophobic groups, and facilitates cross-linking by intermolecular disulphide bonding and formation of Maillard reaction products. It also causes a decrease in protein solubility (Mosenthin *et al*., 2016; Mustafa *et al*., 2000; Salazar-Villanea *et al*., 2017). Furthermore, due to the heterogeneous nature of the meal matrix, proteins interact with other meal components, which change the net charge of protein molecules and consequently affect their solubility behaviour (Pedroche *et al*., 2004). An increase in dry biomass extractability of commercial canola meal with increasing pH from 10 to 12 has been observed (Gerzhova *et al*., 2016). Most of the non-protein compounds in alkali-extracted meal dry biomass could be from the alkaline pH soluble components of the seed coat and cotyledon (embryo and endosperm) cell walls. Canola seed coat, a constituent of the meal, is composed of soluble fibre (13%), cellulose (26%) and lignin (31%) (Carré *et al*., 2016) and ~15% N-based protein (Wanasundara *et al*., 2012). When the optimum conditions were applied to random meal samples (Table 3.9), it was clear that 76-86% of CP meal and 26-36% of DT meal protein could be extracted. This confirms the highly processed nature of protein in DT meal, making it less soluble even under highly alkaline conditions at which canola proteins are usually.

3.4.3 Acid hydrolysis of canola proteins

3.4.3.1 Standard HCl acid hydrolysis and 18 amino acid profile

Cold-pressed meal used in the present study (Table 3.10) exhibited slightly lower levels for several of the 18 AAs than did the amino acid profiles for canola meal reported by other researchers (CCC, 2019; Klockeman *et al*., 1997; Shahidi *et al*., 1992) most likely due to the differences in the genetics of the raw materials and the growing conditions (Niemann *et al*., 2018). Complete hydrolysis of peptide bonds of meal protein was expected under the standard acid hydrolysis conditions provided by 6 M HCl. Under these conditions, untreated CP meal, ethanol pre-treated CP meal, and the protein fraction obtained from ethanol pre-treated CP meal yielded 766, 884 and 889 mg of AAs/g protein, respectively (Table 3.10). These meal and protein materials, based on their total N contents, contained 364, 422 and 611 mg protein/g dbm, respectively. The untreated DT meal contained 425 mg protein/g dbm and both ethanol pre-treated DT meal, and its protein-rich fraction contained ~460 mg protein/g dbm. Upon acid hydrolysis, these DT meals and the protein fraction released a total of 941, 884 and 833 mg AAs/g protein, respectively. The amounts of released (or freed) AAs indicated that the ethanol pre-treatment followed by protein separation improved their release and recovery from CP meal due to the removal of oil from the meal. For DT meal, which was low in oil, there was no improvement in AA release due to ethanol pre-treatment or protein separation (Table 3.10). A higher amount of AAs was released from untreated DT meal than from the pre-treated DT meal or protein extracted from DT meal, indicating that either ethanol pre-treatment caused some proteins to become less hydrolyzable, or some of the proteins left behind in the unextracted fraction may have contributed to the total AAs of the untreated meal.

With the assumption that all peptide bonds were broken during HCl acid hydrolysis, the level of AA release from all of the meal types and protein fractions was in the range of 77% - 94% of nitrogen-based protein (Table 3.10). This indicates that there was non-amino acid nitrogen present in the hydrolyzed material, or a complete release of AAs from source protein was not achieved under the conditions employed. In the nitrogen-based protein values, non-protein nitrogen in canola is also included. Primarily, the gluconsinolates (0.4 to 2.0%) and trimethyl amine compounds (*e.g*., betaine, choline and sinapine; 2%) are the main nitrogen-containing non-protein compounds in *Brassica* oilseeds (Wanasundara *et al*., 2012). It has been estimated that for canola-

quality (low in glucosinolates and erucic acid) Brassica oilseeds, 0.8% - 1.3% overestimation of protein content can occur when 6.25 is used as the conversion factor to calculate protein content based on total N values (Wanasundara *et al*., 2012).

3.4.3.2 Hydrolysis with H_2SO_4 acid and effect of acid concentration on amino acid release

The total amount of AAs released from protein fractions of ethanol pre-treated CP and DT canola meals varied depending on the H_2SO_4 acid concentration, from 68.7% to 82.0% for CP meal protein (1.5 to 4 M H_2SO_4 acid, Table 3.11) and 64.4% to 82.8% for DT meal protein (1.5 to 8 M H_2SO_4 acid, Table 3.12). According to standard acid hydrolysis with 6 M HCl, the CP meal protein fraction contained (or released) a total of 89% of protein as AAs based on protein (Table 3.10). Increasing the H_2SO_4 acid concentration up to 4 M released 82% of the total AAs when the total AAs released by 6 N HCl hydrolysis was considered as 100. DT meal protein fraction required comparatively higher H_2SO_4 concentration to reach a similar level of free amino acid release to 6 M HCl. *i.e*., an increase in acid concentration from 4 M to 8 M was needed to achieve 82.8% AA release (Table 3.12).

Direct hydrolysis with H_2SO_4 without any protection (*i.e*., no phenol addition compared to standard 6 N HCl hydrolysis) completely or partially destroyed some of the AAs. Considering the amount of each AA released from each meal protein fraction under varying acid concentration, all the AAs except cystine and methionine showed an increase in their contents (Tables 3.11 & 3.12). In conventional acid hydrolysis, the amide forms of asparagine and glutamine are completely hydrolyzed to aspartic acid and glutamic acid, respectively. Tryptophan is destroyed completely while cysteine, methionine, serine, threonine, and tyrosine are partially lost (Fountoulakis & Lahm, 1998). Although an accurate determination of cysteine, methionine and tryptophan levels is not possible for the hydrolysates of 4 M H_2SO_4 acid, the sums of the concentrations of these AAs in the protein fraction were 0.6% and 0.2% of total AAs for CP and DT meal proteins, respectively (Table 3.11 & 3.12). In the standard acid hydrolysis, the totals of these AAs were 6.9% and 6.8% for protein extracted from CP and DT meal, respectively (Table 3.10). These results show that some degree of AA loss has occurred during hydrolysis with H_2SO_4, which was inevitable.

Table 3.10 Complete amino acid (AA) profile of untreated and ethanol pre-treated meals and protein fraction obtained from ethanol pre-treated meals (n=3, mean values are presented).

Amino acid	Concentration of AAs mg/ g protein[a]					
	CP meal			DT meal		
	Untreated	Ethanol pre-treated	Protein fraction	Untreated	Ethanol pre-treated	Protein fraction
Ala	35.8	42.7	42.7	45.7	41.7	38.3
Asp + Asn	65.6	78.3	79.4	83.8	75.8	73.0
Arg	58.0	63.5	69.2	68.7	68.1	61.7
Cysteic acid[b]	32.7	35.8	28.1	35.2	30.1	27.0
Gly	36.5	40.8	40.9	44.1	42.5	39.7
Glu + Gln	157.2	184.4	194.3	194.6	177.5	214.3
His	18.5	20.3	21.2	21.0	21.3	19.8
Ile	29.1	33.0	33.1	35.5	34.2	28.8
Leu	51.3	58.7	60.0	64.2	60.7	53.9
Lys	51.6	64.4	61.7	61.4	54.4	47.3
Met[b]	20.5	27.6	22.5	25.2	22.3	20.2
Phe	28.9	31.3	31.7	34.5	35.5	28.9
Pro	42.8	49.4	49.3	60.3	56.1	50.6
Ser	32.7	36.7	36.7	40.1	41.1	32.2
Thr	33.1	37.0	35.4	39.2	38.2	27.2
Trp[c]	7.9	8.9	10.9	10.0	9.7	9.3
Tyr	22.8	25.0	25.5	27.0	27.1	19.8
Val	41.0	46.1	46.6	50.7	47.9	40.7
Total AA, mg/g protein	765.9 ± 0.6	884.0 ± 0.5	889.1 ± 1.6	941.1 ± 0.4	884.2 ± 1.4	832.7 ± 0.9
Total AA , mg/g dbm[d]	278.6	373.4	543.7	400.2	403.5	382.2

[a]N-based protein content (% N×6.25). [b]Cysteic acid and methionine were determined from acid hydrolysis performed after performic acid oxidation. [c]Tryptophan was determined from base hydrolysis. [d] Based on percentage protein content of dbm of untreated meal, ethanol treated meal and protein fraction of ethanol-treated meal:36.38, 42.24, 61.15 % for CP meal and 42.53, 45.63, 45.90 % for DT meal, respectively. Only mean values are presented.
For Total AA, mg/g protein, mean ± S.E.M is presented.

Table 3.11 Amino acids released from hydrolysis of protein fraction of ethanol-treated CP meal (n=3, mean values are presented) under different concentrations of H_2SO_4 concentrations.

Amino acid	Released AAs mg/g protein[a]				
	1.5 M	**2.0 M**	**2.5 M**	**3.0 M**	**4.0 M**
Ala	34.8	36.7	39.2	40.5	40.7
Asp + Asn	63.9	63.5	67.1	66.7	66.5
Arg	42.5	46.3	55.0	58.9	61.4
Cysteic acid[b]	11.2	9.1	8.9	6.1	3.3
Gly	41.8	42.9	44.9	46.5	47.0
Glu + Gln	145.2	149.1	159.3	163.3	162.0
His	12.3	13.6	15.9	18.1	19.3
Ile	14.9	16.3	19.1	22.0	24.1
Leu	36.9	41.3	46.5	49.7	51.1
Lys	48.1	44.3	46.1	47.0	46.6
Met[b]	1.4	2.6	0.9	1.5	1.1
Phe	18.6	20.6	22.7	23.7	23.5
Pro	40.7	44.5	48.5	49.7	48.9
Ser	30.9	32.4	35.2	37.3	38.3
Thr	25.3	28.4	32.0	34.3	35.6
Trp[c]	0	0	0	0	0
Tyr	19.6	21.0	23.4	24.3	24.3
Val	23.3	26.2	30.3	33.1	35.3
Total AAs released, mg/g protein	611.4	638.8	695.0	722.8	729.0
	Released amino acids as a % of total amino acids				
Fraction of released AAs from total, %[d]	68.7	71.8	78.1	81.3	82.0

[a]N-based protein content (% N×6.25). [b]Cysteic acid and methionine are reported as it was resulted in from acid hydrolysis.
[c]Tryptophan was not detected.
[d]Calculated based on the content of total amino acids of protein fraction obtained from ethanol-treated CP protein values (889.1 mg/g protein) of Table 3.9. Only mean values are presented.

Table 3.12 Amino acids released from hydrolysis of protein fraction of ethanol pre-treated DT meal (n=3, mean values are reported) under different concentrations of H_2SO_4.

Amino acid	Released AAs mg/g protein[a]								
	1.5 M	**2 M**	**2.5 M**	**3 M**	**4 M**	**5 M**	**6 M**	**7 M**	**8 M**
Ala	29.7	30.4	29.6	32.6	35.4	36.3	37.1	36.7	38.1
Asp + Asn	54.7	53.8	51.2	56.4	60.0	60.6	61.1	59.9	60.8
Arg	33.8	38.8	39.6	46.5	49.3	52.9	52.9	56.5	57.5
Cysteic acid[b]	10.9	7.7	5.9	4.1	3.6	3.9	1.0	0.0	0.9
Gly	40.2	39.9	38.3	42.0	44.0	44.0	44.7	44.4	45.0
Glu + Gln	149.9	154.4	148.8	164.3	172.6	176.8	176.4	174.9	178.8
His	9.9	12.3	12.4	14.9	15.8	17.5	16.1	17.5	17.8
Ile	11.4	13.3	14.1	17.3	20.4	23.3	24.2	25.8	27.4
Leu	31.7	35.5	35.7	40.7	43.7	48.4	45.9	47.2	48.9
Lys	36.5	34.6	32.2	32.7	33.6	34.4	35	34.1	34.5
Met[b]	0.8	0.8	0.5	0.0	0.7	0.0	0.3	0.2	0.4
Phe	15.0	17.0	16.0	17.9	17.6	18.2	16.9	17.6	16.8
Pro	35	39.3	39.9	46.9	46.6	49.4	48.8	47.6	49.3
Ser	26.2	28.1	27.4	30.5	31.7	34.7	30.8	30	30.1
Thr	19.6	21.5	21.9	26.3	26.2	28	27.7	28.2	29.1
Trp[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tyr	13.0	14.3	14.2	16.4	17.0	18.6	15.1	14.8	13.3
Val	18.5	20.7	21.8	26.6	31.3	34.2	36.4	38.4	41.1
Total AA released, mg/g protein	536.8	562.4	549.5	616.1	649.5	681.2	670.4	673.81	689.8
	Released amino acids as a % of total amino acids								
Fraction of released AA from total, %[d]	64.46	67.54	65.99	73.99	78.00	81.81	80.51	80.92	82.84

[a]N-based protein content (% N×6.25). [b]Cysteic acid and methionine are reported as it is obtained from acid hydrolysis. [c]Tryptophan was not detected. [d]Calculated based on the content of total amino acids of protein fraction obtained from ethanol-treated DT protein values (832.7 mg/g protein) of Table 3.9. Only mean values are presented.

Table 3.13 Verification study for AA release due to 4 M H_2SO_4 hydrolysis of protein fraction of ethanol pre-treated CP and DT meal.

Amino acid	Released AAs mg/g protein[a]											
	Ethanol pre-treated CP meal protein						Ethanol pre-treated DT meal protein					
	CP1	CP2	CP3	CP4	CP5	CP6	DT1	DT2	DT3	DT4	DT5	DT6
Ala	38.2	39.1	36.9	38.4	36.7	37.0	32.8	33.8	32.3	35.8	36.9	33.6
Asp + Asn	66.6	65.4	63.7	69.9	64.9	67.4	62.0	64.8	60.9	64.2	66.4	62.1
Arg	57.1	58.5	53.1	49.6	47.7	48.8	50.6	49.3	49.3	53.3	45.4	49.4
Cysteic acid[b]	3.4	7.9	6.0	4.8	4.1	3.9	4.5	3.8	3.2	3.7	4.8	3.8
Gly	46.3	46.4	44.0	48.0	45.2	46.6	43.3	45.7	42.6	46.9	48.1	43.9
Glu + Gln	157.9	155.4	147.9	167.2	157.3	160.6	174.7	165.4	172.9	170.4	164.0	175.6
His	20.2	22.0	20.8	22.8	22.9	22.3	17.6	18.5	17.8	19.8	21.7	18.7
Ile	27.8	30.2	36.1	31.3	28.6	29.4	24.2	26.8	23.6	25.9	27.2	24.2
Leu	51.5	52.7	51.7	55.7	52.4	53.3	46.1	48.4	44.9	49.2	49.4	46.1
Lys	41.4	41.2	41.0	43.3	39.5	41.7	29.7	33.7	29.4	35.6	36.8	30.0
Met[b]	9.2	6.8	8.0	9.1	6.6	7.5	7.4	6.6	6.1	7.8	6.9	6.8
Phe	29.1	27.6	29.4	30.2	29.9	29.7	26.2	24.6	24.5	27.3	28.6	25.5
Pro	53.1	54.7	52.9	56.1	54.3	52.7	54.9	60.8	53.3	57.9	60.4	54.3
Ser	38.3	37.6	36.5	40.0	37.9	38.9	33.0	33.8	33.0	36.9	38.2	33.8
Thr	35.4	36.3	35.5	28.0	35.7	34.6	29.0	29.3	28.0	32.1	33.1	29.1
Trp[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tyr	24.8	25.3	24.5	25.5	25.5	25.2	17.4	18.7	17.9	20.2	20.6	18.4
Val	33.1	38.4	34.5	38.1	33.6	34.3	27.9	33.2	27.6	31.0	32.3	27.7
Total AA released, mg/g protein	733.4	745.5	722.5	758.0	722.8	733.9	681.3	697.2	667.3	718.0	720.8	683.0
	Released amino acids as a % of total amino acids											
Fraction of released AA from total, %[d]	82.5	83.8	81.3	85.3	81.3	82.5	81.8	83.7	80.1	86.2	86.6	82.0

[a] N-based protein content (% N×6.25). [b]Cysteic acid and methionine are reported as it was resulted from acid hydrolysis.
[c]Tryptophan was not detected.
[d]Calculated based on the content of total amino acids of protein fraction obtained from ethanol-treated CP (889.1 mg/g protein) and DT (832.7 mg/g protein) protein values of Table 3.9.
n=3, Only mean values are presented.

During hydrolysis, non-protein components, such as soluble polysaccharides, extracted at alkaline pHs and concentrated along with the meal protein fraction may also consume a portion of the acid and go through hydrolyzed or involved in other reactions. The observed higher acid requirement for DT meal protein for hydrolysis compared to ethanol pre-treated CP meal protein (331mg protein/g meal, Figure 3.8A) could be attributed to the higher level of non-protein constituents in DT protein (166 mg protein/g dbm, Figure 3.8B) which would compete with proteins for acid-induced reactions.

As a diprotic acid, H_2SO_4 provides double the concentration of protons to the hydrolysis medium compared to monoprotic HCl. Conventional acid hydrolysis uses 6 M HCl; therefore, theoretically, a similar extent of peptide bond breakage could be expected when 3 M H_2SO_4 was used. The results for AA yield or % of total liberated AAs in the present study showed that a higher H_2SO_4 concentration than HCl was required to reach high values for AA release/recovery. It has been reported that the H^+ ion generated from the second dissociation of H_2SO_4 is not used in the protein hydrolysis reaction, instead is involved in the degradation of AAs, causing their low yield in the hydrolysate (Flork, 1989). Furthermore, to have satisfactory AA release from hydrolysis with H_2SO_4, Flork (1989) has reported that a minimum of 6 M H_2SO_4, the same molarity as HCl is needed, and the temperature should be maintained at 100°C at least. Several studies have reported the high propensity of H_2SO_4-assisted hydrolysis to generate small peptides. Alvarez *et al*. (2012) have compared hydrolysis of porcine blood hemoglobin with HCl and H_2SO_4 and detected four different fractions: unbroken protein, soluble peptides, non-soluble peptides, and free AAs, in the hydrolysate. Protein soon aggregates with contact with acid. Sufficient hydrolysis time should be allowed to lower the level of the fraction of non-soluble peptides. However, Alvarez *et al*. (2012) showed that the rate of hydrolysis is influenced by the temperature without affecting the average peptide size. An increase in the concentration of H_2SO_4 from 1 M to 3 M produced smaller-sized peptides; however, at higher acid concentrations, more non-soluble peptides also were produced. Therefore, lower concentrations of H_2SO_4 were identified as suitable for soluble peptide production. Furthermore, HCl was identified as a better acid medium for yielding free AAs compared to H_2SO_4. Bouhamed and Kechaou (2017) employed H_2SO_4, HNO_3 and H_3PO_4 to obtain peptides from poultry feather keratin, and H_2SO_4 provided the most efficient acidic medium for peptide production.

In the present study, considering the extent of AA release and AA yield from CP and DT meal protein fractions at different acid concentrations, 4 M H_2SO_4 was identified as suitable to liberate substantial amounts of AAs. Hydrolysis using 4 M H_2SO_4 was verified using six random meal samples of each meal type (Table 3.13) and showed that 81% - 85% of total AAs were liberated from the proteins of CP meal and 80% - 87% from DT meal proteins. Considering the level of protein in the protein fractions separated from CP and DT meals and the hydrolysis efficiency achieved with 4 M H_2SO_4 (Table 3.13), 722-758 mg and 667-721 mg, respectively, of AAs can be obtained from a gram of meal protein fraction dbm.

Safety issues prevent the use of such high acid concentrations, and consequent neutralization generates high salt levels requiring desalination of the resulting hydrolysate. Cold precipitation (Alvarez *et al*., 2012) and the addition of water for termination of hydrolysis (Flork, 1989) have been discussed as techniques for managing this excess salt production. The ratio of solid-to-liquid is also a significant factor in determining the hydrolyzing efficiency and a higher ratio can decrease the yield (Bouhamed & Kechaou, 2017). In the present study, the solid-to-liquid ratio was kept as same as for the standard HCl hydrolysis. In large-scale processing, HCl is extremely corrosive and requires expensive glass-lined reactors, whereas H_2SO_4 hydrolysis can be carried out in stainless steel reactors with a simple protective lining and in a closed vessel to avoid acid fume emission (Flork, 1989). Furthermore, the H_2SO_4-water azeotrope has a boiling point of 338°C at ambient temperature while that of HCl is 108°C. The protein-H_2SO_4 solution boiling point is 120°C, and hydrolysis can be carried out at 110°C without vapour emission (Flork, 1989).

3.4.4 Separation of partially hydrolyzed DT meal protein

In section 3.4.3.2, it was discussed that data obtained for CP and DT meal protein showed that only 81% - 85% of the AAs present in the protein were released when hydrolyzed with H_2SO_4, partly owing to the loss of certain AAs. It has been reported in the literature that with H_2SO_4, protein tends to be hydrolyzed only partially. When CP and DT meal protein was hydrolyzed with 1.5 M H_2SO_4, the AA release was at a level of 64% - 69% (Tables 3.11 & 3.12). Studies on porcine hemoglobin hydrolysis demonstrated that acid hydrolysis (HCl or H_2SO_4) resulted in mixtures of intact proteins, soluble peptides, non-soluble peptides, and free AAs (Alvarez *et al*., 2012). Therefore, an investigation of the hydrolysates obtained with low acid concentrations was carried

out for peptide bond containing (absorbance monitored at 214 nm) molecules (Figures 3.6 & 3.7). Most of the detected components of the hydrolysates eluted between 15 and 25 min (Figure 3.7). According to molecular mass standards, this elution time was indicative for 75-1,355 Da proteins (Figure 3.6). The relative abundances of peptides in the HCl and H_2SO_4 (0.5, 1.0, and 1.5 M) hydrolysates of DT meal protein are presented in Table 3.14. In the hydrolysates obtained from 0.5 to 1.5 M HCl hydrolysis, peptides of sizes up to 189 Da were detected, but peptides larger than that were not observed (Figures 3.7 and Table 3.14). An increase in the concentration of acid for hydrolysis caused a decrease in the relative abundance of the peptides produced, supporting the fact that more AAs were liberated at higher acid concentrations. When 0.5 M H_2SO_4 was used for hydrolysis, larger peptides, up to 12,000 Da, were produced (Table 3.14). In the hydrolysate obtained from hydrolysis with 1.0 M H_2SO_4, 66% of the peptides were in the range of 150-1355 Da indicating that molecules larger than free AAs were present. At the highest H_2SO_4 concentration (1.5 M) tested, all molecules were less than 189 Da. A similar molecular size composition was observed for the hydrolysates obtained with 0.5, 1.0 and 1.5 M HCl. The molecular weight of free AAs is in the range of 75 Da (glycine) to 204 Da (tryptophan). Both peptides and free AA analyses confirmed that lower H_2SO_4 concentrations were not effective in producing free AAs compared to HCl at similar or lower concentrations (Table 3.14 & 3.15). With increasing acid concentration from 0.5 to 1.5 M, released level of AAs based on protein weight increased from 42% to 55% for HCl and 41% to 60% for H_2SO_4 (Table 3.15). When a H_2SO_4 concentration of 1.5 M is considered, it provides double with the H^+ concentration to the medium compared to HCl of the same concentration but resulted in only 5% more free AAs than did HCl hydrolysis at the same concentration (Table 3.15). Considering the other advantages mentioned in section 3.4.3, H_2SO_4 can be an alternative to HCl in generating free AAs from substrates such as canola meal.

Table 3.14 Relative abundance of peptides (absorbance at 214 nm) in DT meal protein hydrolysates produced with different concentrations of HCl and H_2SO_4.

Molecular size range (Da)	Relative abundance of peptides in the hydrolysate %					
	HCl			H_2SO_4		
	0.5 M	1.0 M	1.5 M	0.5 M	1.0 M	1.5 M
<75	36.3 ± 1.4	43.3 ± 3.7	48.0 ± 0.6	32.4 ± 0.5	32.3 ± 9.6	74.6 ± 0.9
150 – 189	63.8 ± 0.8	56.7 ± 3.7	52.0 ± 0.6	34.4 ± 0.9	51.5 ± 9.6	25.4 ± 0.9
189 – 1355	0.0	0.0	0.0	32.6 ± 0.8	16.5 ± 1.8	0.0
1355 – 6500	0.0	0.0	0.0	0.4 ± 0.7	0.0	0.0
6500 – 12400	0.0	0.0	0.0	0.2 ± 0.4	0.0	0.0

Table 3.15 Quantification of free amino acids in the protein hydrolysates produced with different concentrations of HCl and H_2SO_4.

Amino acid	Amino acids (w/w)% on protein basis					
	HCl			H_2SO_4		
	0.5 M	1.0 M	1.5 M	0.5 M	1.0 M	1.5 M
Ala	2.14	2.92	2.78	2.16	2.84	3.02
Asp	5.53	5.66	5.60	6.00	5.93	6.05
Arg	2.48	3.98	4.68	2.46	4.19	5.26
Cysteic acid	0.45	0.51	0.44	0.482	0.44	0.41
Gly	4.33	5.23	5.05	4.63	5.25	5.45
Glu	9.83	11.85	12.73	10.18	12.77	13.65
His	0.74	1.18	1.35	0.77	1.24	1.68
Ile	1.16	1.37	1.01	0.95	1.43	1.58
Leu	2.30	3.16	3.72	2.06	3.22	3.66
Lys	2.40	2.82	2.96	1.84	2.96	3.45
Met	0.45	0.52	0.43	0.45	0.46	0.39
Phe	0.84	0.98	0.87	0.75	0.90	0.76
Pro	3.45	4.93	5.11	2.40	3.83	5.03
Ser	2.42	3.42	3.88	2.50	3.71	4.19
Thr	1.13	1.86	1.49	1.10	2.00	2.38
Trp[c]	ND	ND	ND	ND	ND	ND
Tyr	0.72	0.90	1.04	0.74	1.02	1.13
Val	1.21	1.56	1.84	1.15	1.78	2.06
Total (w/w) %	41.57	52.85	54.99	40.55	54.18	59.93

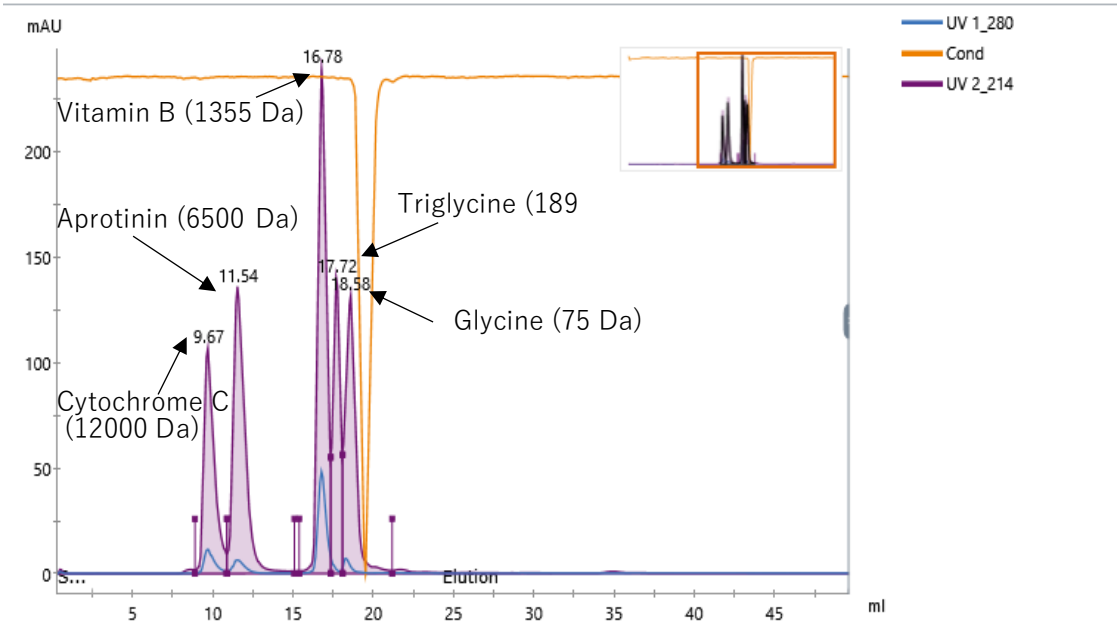


Figure 3.6 Chromatograph for molecular size standards consisted of glycine, triglycine, vitamin B, aprotinin and cytochrome C.

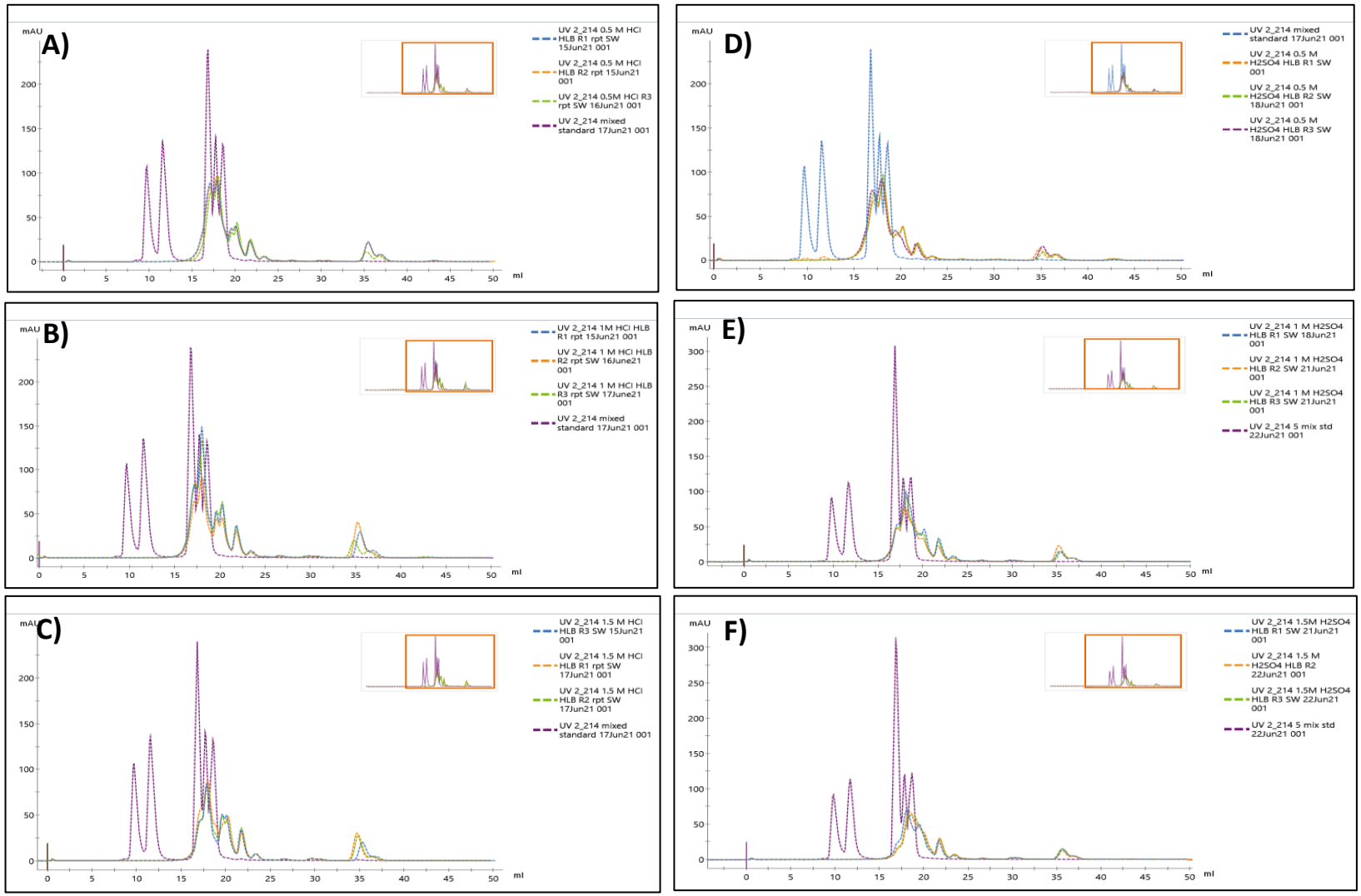


Figure 3.7 A, B, C, D, E, & F. Chromatograph for molecular size standards overlaid with peptide (monitored at 214 nm) profiles of the hydrolysates prepared using: (A) 0.5 M HCl, (B) 1.0 M HCl, (C) 1.5 M HCl, (D) 0.5 M H_2SO_4, (E) 1.0 M H_2SO_4, and (F) 1.5 M H_2SO_4.

3.4.5 Estimation of the amino acid yield from CP meal and DT meal

Experiments of this study showed that ethanol pre-treatment elevated the level of protein that can be extracted from CP meal (Figure 3.4 A). Generation of free AAs by acid hydrolysis was efficient when protein was separated prior to hydrolysis. Figure 3.7 summarized calculated values for the amount of protein and the free AAs obtained from CP and DT meals. These values were calculated in two ways. In Figure 3.8(A), the values are presented based on the product yield from 1 kg of starting materials in each process step. The process steps are ethanol pre-treatment, protein extraction and acid hydrolysis. The values in Figure 3.8(B) are the yield of products of each processing step when the process starts with 1 kg of meal dbm and goes through pre-treatment, protein extraction and hydrolysis. For example, 1 kg of CP or DT meal (no protein separation) yielded 209 g and 101 g of free AAs, respectively, after going through ethanol-pretreatment, protein separation and 4 M H_2SO_4 hydrolysis. Under the same hydrolysis conditions, 1 kg of extracted protein from ethanol pre-treated CP and DT meals yielded 407 g and 280 g, respectively, of free AAs. Considering the differences in the oil extraction processing steps involved in generating CP meal and DT meal, the proteins in CP meal retained their solubility/extractability and ability to break down into monomeric AAs by acid hydrolysis. The proteins of DT meal may have been involved in various other reactions and therefore were structurally modified. Therefore, the DT meal proteins have lost extractability/solubility, and even breaking down peptide bonds is difficult under acid hydrolysis. This study showed that proteins of commercially processed canola meal using PSE have limited utility because of their highly processed nature, and the processing history poses limitations for acid hydrolysis of their peptide bonds as well. However, the free AAs obtained from hydrolysis of either CP or DT meal are the same AA molecular units that can be used directly or in generating compounds for further uses.

The reported non-food/feed uses of canola meal protein primarily focus on utilizing seed storage proteins possessing intact higher-order structure and limited to the inherent nature of the protein. In order to facilitate more uses of these proteins, deliberate structural modifications are required to improve targeted functions, such as adhesive (Wang *et al*., 2014), mechanical (Li *et al*., 2019; Manamperi *et al*., 2010) and rheological (Tan *et al*., 2014) properties. Peptides obtained from partial protein hydrolysis obtained by various proteases (*e.g*., pepsin, papain, Alkalase, Nutrase, *etc*.) have been studied for their biological activities such as antihypertensive (Marczak *et al*.,

2003; Wu & Muir, 2008), antimicrobial (Nioi *et al*., 2012), antioxidative (Zhang *et al*., 2008) and antidiabetic (Mariotti *et al*., 2008). However, the primary AA sequence and enzyme specificity are the main limiting factors to recovering potentially bioactive peptides. This indicates that highly distressed proteins, such as those found in DT canola meal, cannot be a competitive starting material for the above-mentioned routes. Therefore, if they are not used in the feed industry, their fate may be a waste material or incorporation back into the soil. Regenerating monomeric AAs via extensive protein hydrolysis may be a strategic approach to utilizing highly distressed proteins that have a lesser potential for value addition. The free AAs so obtained could be the base molecules for development into tangible products, such as AA mixtures with high nutritional potency, conversion into new molecules and compounds that can function as surface tension reducing (Borders and Holmberg, 2015), antimicrobial (Pinazo *et al*., 2016a) or plant growth stimulating (Ertani *et al*., 2019). Therefore, the generation of free AAs is another approach in the value addition of commercial canola meal.

3.5 Conclusions

From the investigations carried out in this study, the following conclusions can be reached. Commercially produced CP meal has more oil and free sugars than DT meal because of the differences in the processing regimes involved in their production. A pre-treatment with ethanol (99%) can produce a de-oiled CP canola meal by removing ~88% of the residual oil along with ~20% reduction of levels of phenolics and ~25% reduction of the level of free sugars (Table 3.6). The oil content of DT meal can also be lowered to a level of less than 1% of dbm with a limited effect (~20% reduction) on levels of phenolic compounds and free sugars (Table 3.6). Therefore, pre-treatment with 99% ethanol effectively reduces the residual oil content of meals, with partial removal of phenolics and free sugars.

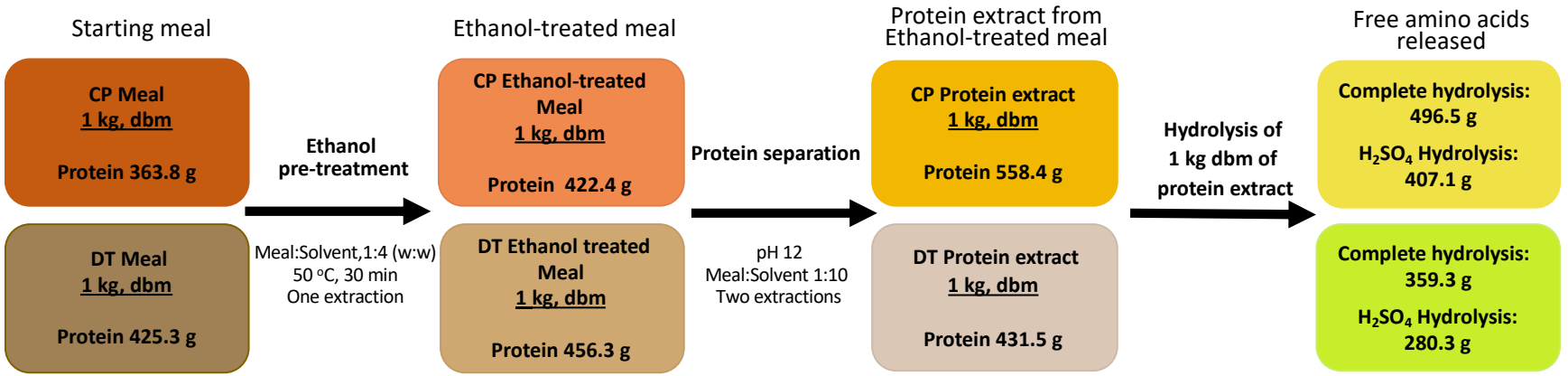
The recovery of proteins from untreated CP meal and DT meals at pH 12 may be affected by the processing conditions of oil extraction more than residual oil content, thus resulting in a lower protein recovery from DT meal (38% of meal protein) than from CP meal (71% of meal protein) (Figure 3.4 A). Ethanol pre-treatment enables an ~2% increment in protein recovery from CP meal and no improvement for DT meal (Figure 3.4 A & B).

Ethanol pre-treatment had no impact on AA liberation from DT meal by standard acid hydrolysis with 6 M HCl, whereas the AA release from CP meal can be slightly improved. The reduction of residual oil in CP meal might lead to this improvement. A unit of protein from CP or DT meal can provide similar amounts of free AAs (830-890 mg/g protein) upon 6 M HCl hydrolysis (Table 3.10). Proteins of CP and DT canola meals can be converted into AA mixtures by H_2SO_4 hydrolysis. Interestingly, DT meal protein yielded a lower amount of free AAs than was obtained from CP meal proteins with the same acid concentration. With 4 M H_2SO_4 hydrolysis, CP and DT meal proteins yielded 729 and 649 mg free AA/g protein, respectively (Tables 3.11 & 3.12).

H_2SO_4 showed less effectiveness in hydrolyzing canola protein than did HCl; the AA yields for CP meal were 729 mg/g *vs*. 889 mg/g of protein, and for DT meal, 649 mg/g *vs*. 832.7 mg/g of protein with 4 M H_2SO_4 and 3 M HCl, respectively. This infers that a higher H_2SO_4 concentration may be required to achieve complete hydrolysis of proteins from both meal types (Tables 3.10, 3.11, 3.12). Using 4 M H_2SO_4, approximately 78% - 82% of protein-bound AAs of these meals can be recovered, with the lesser recovery of sulphur-containing AAs and complete loss of tryptophan (Tables 3.11 & 3.12). There is evidence that partial hydrolysis of canola protein may occur when H_2SO_4 concentration is lower than 1.5 M. This situation results in peptide fragments, consequently reducing free AA yield.

Ethanol pre-treatment and separation of protein by aqueous extraction at pH 12 reduced levels of non-protein compounds and enhanced free AA recovery from both CP and DT meals. The hydrolysis and AA recovery data showed that DT meal protein was less soluble, less extractable, and less hydrolyzable than CP meal proteins. This study showed that the proteins of commercially processed canola meals differ in terms of the solubility required for effective wet extraction. Similarly, their propensity to be hydrolyzed with H_2SO_4 is affected by the process employed for oil extraction. DT meal protein is subjected to more severe processing stresses than CP meal which may impact further uses such as recovering AAs by acid hydrolysis. However, both CP and DT canola meals can be used in generating AAs for further uses.

(A)



(B)

Canola meal type	Ethanol-treated meal	Protein Extract	Free amino acids (FAA)	
			Complete hydrolysis	H_2SO_4 Hydrolysis
CP Meal 1.000 kg dbm 0.364 kg protein	0.865 kg dbm 0.365 kg protein	0.513 kg dbm 0.286 kg protein	0.255 kg FAA	0.209 kg FAA
DT Meal 1.000 kg dbm 0.425 kg protein	0.935 kg dbm 0.426 kg protein	0.361 kg dbm 0.156 kg protein	0.130 kg FAA	0.101 kg FAA

Figure 3.8 (A) & (B). Summary of ethanol pre-treatment, protein extraction and hydrolysis of CP and DT canola meals showing protein and free amino acid (FAA) yields. (A) Amount of product (protein and FAA) that can be obtained from 1 kg dbm of each input (meal or protein hydrolysate) in ethanol pre-treatment, protein extraction and hydrolysis, and B) amount dbm and protein that can be obtained from each meal based on 1 kg dry weight of starting meal. Values obtained from experiments in the study are extrapolated to 1 kg meal material. dbm: dry bio mass.

3.6 Connection to the next study

The next stage of this project was on value addition to the free AAs obtained from canola protein hydrolysates by converting them into functional molecules. Since canola protein hydrolysate is a complex mixture of AAs and non-protein compounds, the application of chemical reactions can be challenging. Therefore, a step-wise approach for understanding the chemical modification of AAs present in the protein hydrolysate was taken. The following study was designed to generate modified AAs. These AAs were selected based on their abundance in canola meal protein and obtained as high purity chemical reagents. An amino acid modification was studied via N-acylation of these reagent grade individual AAs with lauroyl chloride. Furthermore, these N-acylated AAs were used as standard molecules for comparative purposes in evaluating properties of interest, such as those required for surfactant molecules.

4. PREPARATION OF N-ACYL AMINO ACIDS AND THEIR STRUCTURE CONFIRMATION

4.1 Abstract

Six individual amino acids (AAs) of reagent grade, namely, glycine, L-glutamic acid, L-leucine, L-lysine, L-proline, and L-valine, were used in this study. These six AAs comprised 50% of the total AAs of canola meal protein, and they were tested to show their feasibility as the starting materials of new functional molecules. The reagent-grade individual AAs and their mixture were acylated with lauroyl chloride ($C_{12}H_{23}Cl$) following the Schotten Baumann reaction. Final products were evaluated for their structures with Fourier Transform-Infrared (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopic techniques. These structural analyses showed that glycine, L-glutamic acid, L-leucine, L-proline, and L-valine formed N^{α}-lauroyl AA products, whereas the acylated product of L-lysine showed both alpha- and epsilon-N-acylated products. The resulting acylated mixture showed all the characteristic peaks of individual N-acylated AAs in FTIR spectra. The sodium salt of all the acylated derivatives were prepared by reacting with ethanolic NaOH. The success of sodium salt formation was confirmed with their FTIR spectra that showed the disappearance of the characteristic peak for carbonyl bond of the carboxylic group. This study successfully proved the concept for the preparation steps and formation of N-lauroyl derivatives of glycine, L-glutamic acid, L-leucine, L-lysine, L-proline, and L-valine and their mixture.

4.2 Introduction

The use of renewable, low-cost raw materials to design functional molecules has received significant attention along with the increasing demand for biocompatible products. AAs are interesting amphiphilic molecules that can participate in many reactions and can be converted into molecules with useful functions. Among these many reactions of the amino and carboxylic groups

of AAs, reactions with long aliphatic chains, such as fatty acids, fatty esters, fatty amines and fatty alcohols, generate molecules with a wide range of functions. Due to the chemical duality of AAs, these fatty chains can be introduced through ester, alkyl, or amide bonds, and depending on the structure of lipo-amino acid, those synthesized molecules can be anionic, cationic, or amphoteric in ionic nature. The most significant function of lipo-amino acids is the ability to be an alternative to conventional synthetic surfactant molecules. Salts of long chain N-acyl AAs comprise a class of such biocompatible surfactants that have received paramount importance in detergent, cosmetic, pharmaceutical and food industries owing to their structural simplicity, surface and antimicrobial properties, mildness on skin and biodegradability (Bajani *et al*., 2018; Chen *et al*., 2020; Infante *et al*., 1997; Shah *et al*., 2019; Soo *et al*., 2004).

There are three major acylation processes: C-, O- and N-acylation. AAs can go through either O- or N-acylation reactions. In N-acylation, a reacting functional group is an amino group (-NH_2). This reaction is an electrophilic substitution by an acyl group, which forms a final product containing a nitrogen atom between the acyl group and the reactant compound's moiety. In contrast, the reactive group that participates in the O-acylation reaction is the hydroxy group (-OH). It is a nucleophilic acyl substitution on the hydroxy group that yields a compound with the oxygen atom between the acyl group and reaction compound moiety. In the acyl group (R-CO-), R could be an alkyl or aryl group donated from the acylating agent. For N-acylation, the acylation agent is in the form of an acyl chloride or acyl anhydride. The most common N-acyl preparation is N^{α}-acyl AAs reacting the acyl donor group with the α-amino group of an AA. This reaction generates molecules having a general formula of $R^1C(O)NR^2[CH(R^3)CO]_nOH$ (Mikhalkin, 1995).

The N-acyl AAs received chemical industry interest a few decades ago because of the wide range of activities provided by the N-acyl AAs (Mikhalkin, 1995). N-acylation with acetyl chloride and acetic anhydrides is a well-studied reaction (Rajput & Gore, 2011). Formation of an amide by acylation involves nucleophile attack on the carbonyl group, as the rate-determining step, and it forms a tetrahedral intermediate. Subsequently, reformation of the pi-bond on carbonyl group and displacement of leaving group (halide ion) occurs. Proton removal from amide nitrogen is the last step of the reaction. If no other basic molecule species is present, starting amine can act as the base and get protonated. Therefore, having a basic medium during the reaction is crucial as generated

acid (H^+) must be neutralized to avoid the protonation of unreacted amine groups and slow down the further acylation. Hydrochloride acceptor should be a stronger base than the amino group of AA. Another option is to have two equivalents of the base in the medium so that one equivalent is the nucleophile and the other acts as a base for the final proton transfer step. In the cases where acylation depends on the steric effect rather than the components in the acylation mixture, acylation can be facilitated by choosing bases that are not reacting with acyl donor, *i.e*., tertiary amines such as pyridine and triethylamine (Rajput & Gore, 2011; Tolstikov *et al*., 1991).

Acylation of AAs depends on the nucleophilicity of amino groups, steric factors, and the acylating capacity of the carboxylic acid derivative. Types of acylating agents (RCOX) are equivalent to their acidity or the stability of the anion X^-. When the X represents a halogen, it is the most suitable acylating agent among the others as shown in series as: RCO-Halogen > $(RCO)_2O$ ≈ RCOOR' > RCON > RCOOH. It shows that effective acylation needs activation of the carboxyl group in a carboxylic acid. Acid chlorides are the key form of acyl donor in acylation reaction. Thionyl chloride is the most used reagent for preparing acid chlorides (Mikhalkin, 1995; Rajput & Gore, 2011).

Acylation can be carried out either in an aqueous medium by the Schotten Baumann reaction or in anhydride organic solvents (Tolstikov *et al*., 1991). The simplest and the most common way of preparing long chain N acyl AAs is based on the Schotten Baumann reaction, which uses a two-phase solvent system, and introduces a fatty acid acyl residue as acid chloride to an AA in a strongly alkaline aqueous medium for the reaction, where the resulting acids are neutralized to maintain the conditions (Chen *et al*., 2020; Infante *et al*., 1997). Industrial production of N-acylated AAs as surfactant molecules for cosmetics and household cleaning agents uses the Schotten Baumann reaction, an unselective acylation. The use of aminoacylases from *Streptomyces ambofaciens* can selectively catalyze AA acylation reaction by fatty acids in the aqueous medium (Bourkaib *et al*., 2020).

Solubility of fatty acyl donors and AAs have different solvent preferences. All AAs are soluble in an aqueous medium at a suitable pH; however, fatty acyl donor generally needs an organic solvent medium to be soluble. The Schotten Baumann reaction proceeds the acylation of fatty acids and

AAs in an aqueous medium. Furthermore, it has been reported that the reaction that occurs in a water-soluble organic solvent provides a better yield rather than in a completely immiscible solvent, *e.g.*, water- toluene (Mikhalkin, 1995).

Functional properties of these N-acylated derivatives depend on their structure, nature of AA moiety, length, and type of functional groups present in the fatty acid moiety (Mhatre & Singare, 2012). The relationship between their structure and physiochemical and biological activities were reviewed by Mikhalin (1995). Solubility of N-acyl AAs in water or forming colloidal suspensions at low concentrations depends on the acid-base properties of the AA, which include several carboxyl or amino groups, their structural characteristics and polar/non-polar nature of alkyl chains (Mikhalin, 1995).

The hydrophobicity of AA molecules can be improved by grafting a hydrocarbon chain into the AA, which can be achieved via amine or carboxylic function. Of the AAs in both CP and DT canola meal proteins, L-glutamic acid, L-aspartic acid, L-arginine, L-lysine, L-leucine, L-proline, L-valine, glycine constituted ~66% of total AA (Table 3.9). It was hypothesized that the AAs obtained from hydrolysis of canola meal protein can be N-acylated with fatty acids to improve their hydrophobicity and surface-active properties. Furthermore, it was hypothesized that the AAs which are abundantly found in canola meal could be chemically modified with lauroyl chloride providing a C12 fatty chain by N-acylation to make them functionally useful. Moreover, improving the molecule's hydrophobicity may equip these molecules with surface tension changing ability and antimicrobial activity. In order to test these hypotheses, first, the concept of AA modification and conversion to functional compounds needs to be proved through testing with relatively pure molecules. Therefore, the six most abundant AAs of canola protein were selected and converted to N-acyl AAs by introducing a fatty acid (lauroyl) chain via an amine functional group. Generation of N-lauroyl AAs and confirmation of the product via structure characterization are presented in this chapter.

4.3 Material and methods

4.3.1 N-acylation of individual amino acids with lauroyl chloride

Six individual AAs (purity of 98-99%; L-glutamic acid, glycine, L-leucine, L-lysine, L-proline, and L-valine) were purchased from Sigma Aldrich (Oakville, ON, Canada). These AAs were selected based on the AA composition of canola meals obtained in the investigations of Study 3. These six AAs were N-acylated with lauroyl chloride ($C_{12}H_{23}OCl$) using the Schotten Baumann reaction as mentioned in Figure 4.2 A.

Aspartic acid (61-66 mg/g) and arginine (45-53 mg/g) were the other two AAs that also found in abundance in DT meal. Since the preliminary work showed difficulties in obtaining acceptable level of purity for N-lauroyl derivatives of these two AAs, further studies were not carried out with them.

4.3.1.1 Preparation of N- lauroyl products of L-glutamic acid, L-glycine, L-leucine, L-lysine, L-valine

The AAs, glycine, L-leucine, L-lysine, L-valine (1 g, number of moles = n) were dissolved in an equivalent molar amount of aqueous NaOH (number of moles = 2n for all except glutamic acid, which is 3n). Then, lauroyl chloride (equivalent amount is n moles) was added in 10% excess, drop-wise into the AA solution while stirring. The reaction was carried out at ambient temperature for 1 h. A cream-like white compound was formed when the reaction progressed. Then, the pH of the mixture was adjusted to be between 2 and 3 with 6 M HCl solution, and the reaction mixture was stirred for 30 min. The resulting white precipitate was filtered using Whatman No.1 filter paper under vacuum and washed with water several times while checking with 0.1% (w/v) $AgNO_3$ solution for residual chloride ions. The washed precipitate (crude product) was then separated by thin-layer chromatography (TLC; silica gel 60 plates, 20 × 20 cm, 200 µm, Al support) to determine the presence of lauroyl chloride and lauric acid. A solvent mixture of hexane: ethyl acetate 1:1 (v:v) was the mobile phase for TLC, and the plate was sprayed with phosphomolybdic acid (PMA, 5%, v/v) for compound visualization. Unreacted lauroyl chloride and lauric acid formed during the reaction were removed by washing the product with hexane. The final product was recovered by filtering out hexane soluble components. The product was dissolved in hot ethanol or ethyl acetate and filtered to remove any remaining salts, and then the pure product was

recovered by vacuum evaporation using a rotary evaporator. The final product was dried in a vacuum desiccator. The major steps of the process are presented in Figure 4.1 (A).

4.3.1.2 Preparation of N-lauroyl proline

L-Proline 1 g (8.7 mmol) was dissolved in aqueous NaOH (17.4 mmol; 100.0 ml). Lauroyl chloride was added to the proline solution at a 10%-excess mole ratio (9.57 mol) dropwise while the mixture was stirred. The reaction was carried out at room temperature for 2 h. Then, 6 M HCl solution was added to bring the reaction mixture pH between 2-3. A white, viscous product was obtained, and it was extracted into ethyl acetate using a separatory funnel. The ethyl acetate layer was collected and dried using anhydrous Na_2SO_4. After removing ethyl acetate by vacuum evaporation, the reaction product was separated from free fatty acids (formed during the reaction) and unreacted lauroyl chloride using a silica gel column with hexane: ethyl acetate 1:1 (v:v). TLC analysis (hexane: ethyl acetate 1:1, v:v) was used to identify the fractions containing acylated products. The identified fractions were pooled and vacuum evaporated to remove the solvent. The major steps of the process are presented in Figure 4.1(B).

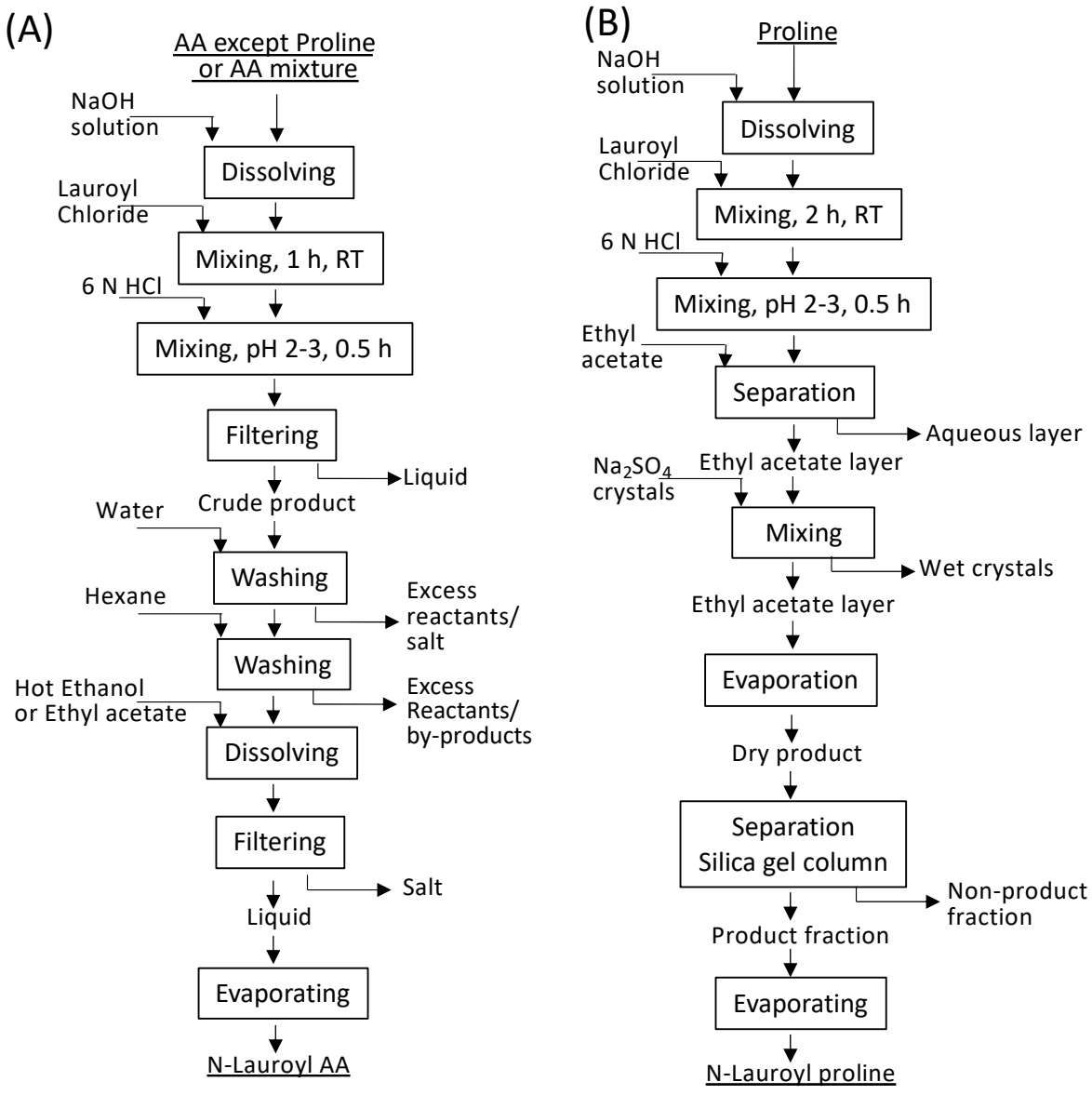


Figure 4.1 Preparation flowchart of N-acyl AA by reacting with lauroyl chloride (A) process for AA except proline or AA mixture, and (B) proline.

4.3.2 Preparation of N-lauroyl product of amino acid mixture

A mixture of AAs (5 g) was prepared to contain glycine, L-leucine, L-lysine, L-proline, L-valine, L-glutamic acid at a ratio of 1.0:1.2:1.2:1.2:1.0:3.6 (w:w) that simulates their amounts in canola meal protein (Refer to Table 3.9). Then, the AA mixture was acylated with lauroyl chloride following the Schotten Baumann reaction. Briefly, the AA mixture was dissolved in an equivalent

amount of NaOH and reacted with lauroyl chloride for 1 h. Then, pH was lowered to 2-3 using 6 M HCl and stirring continued for another 30 min. The reaction products were recovered by filtering with Whatman #1 filter paper and washed first with water and hexane. The completion of reactions was monitored using TLC analysis (hexane: ethyl acetate 1:1, v:v). The major steps of the process were similar to Figure 4.1(A).

4.3.3 Preparation of Na^+ salt of N-lauroyl amino acid products

The prepared N-lauroyl product was dissolved in ethanolic NaOH (20% w/v) at room temperature by stirring for 30 min to obtain the sodium salt of N-acyl AA. Then, the product was recovered by vacuum evaporation of ethanol. The final product, N-acyl AAs' sodium salt, was stored in glass vials at ambient temperature and used for further analyses.

4.3.4 Structure characterization of N-lauroyl amino acid products

4.3.4.1 Fourier transform-infrared (FTIR) spectroscopy

Samples were used in their dry powder form, and ~5 - 10 mg was placed directly on the sample platform of FTIR (Agilent Cary 630 ATR-FIIR spectrometer, Agilent Technologies Inc., Canada). Data acquisition was carried out using Agilent MicroLab software, and transmission mode spectra were used in functional group identification.

4.3.4.2 Nuclear magnetic resonance (NMR) spectroscopy

Samples (5 - 25 mg for ^1H NMR and ~50 mg for ^{13}C NMR) were dissolved in suitable deuterated solvents for each compound and transferred into clean, dry NMR tubes (Norell, Canada, 5 mm). Analysis of ^1H NMR [1D and 2D-correlated spectroscopy (COSY)], ^{13}C NMR [uniform driven equilibrium Fourier transform (UDEFT), distortionless enhancement by polarization transfer (DEPT), distortionless enhancement by polarization transfer with retention of quaternary carbon (DEPTQ) were carried out with Bruker Avance 500 MHz NMR Spectrometer and data were analyzed by TopSpin 3.6 software.

4.3.5 Statistical analysis

All lauroyl derivatives were prepared as three repeated instances. Reaction monitoring and product assessment were conducted for the products of replicated runs. Structure confirmation obtained

was considered the most abundant compound in the reaction mixture. No statistical analysis was performed on the data.

4.4 Results and discussion

4.4.1 N-acylation reaction

Methods for preparing N-acyl AAs are well established (Jursic & Neumann, 2001). Fatty acid or its derivative can be introduced through the amine or carboxylic functional group of AA molecules (Rondel *et al.*, 2009). The acylating agents are carboxylic acids, acid chlorides, anhydrides, or esters. Carboxylic acid chlorides are the most common acylating agent. Preparation of N-acyl AAs can be conducted in an aqueous alkaline medium using the Schotten Baumann method or in an anhydrous organic solvent medium via many synthesis pathways using acid anhydrides, esters, or carboxylic acids (Jursic & Neumann, 2001; Mikhalkin, 1995; Rondel *et al.*, 2009; Tolstikov *et al.*, 1991). The present study carried out the reaction in an aqueous medium following the Schotten Baumann reaction. The reaction process involved the following steps. First, the dissolution of the AA in an equivalent amount of moles of aqueous NaOH was carried out to completely transfer the AA to its salt and made it completely soluble in water. Then, the lauroyl chloride, added in excess, reacted with AA salt. The pH lowering step made the medium acidic (~ pH 3), making carboxyl anion of the AA be protonated, and formed water-insoluble N-acyl AAs, water-soluble NaCl, and water. The acylated product was insoluble in water and made it easy to separate from the rest of the reaction mixture through filtration. However, this separated crude product contained other water-insoluble components, such as fatty acids formed during the reaction, unreacted fatty acid chloride, and some residues of water-soluble components (*e.g.*, unreacted AA and salt). Therefore, further purification steps were required to obtain a pure product. These purification steps were solvent washings, re-crystallization and silica gel chromatography as described by Jursic & Neumann (2001).

In the basic medium, during the acylation reaction, two competitive reactions can occur between an acid chloride and AAs; the main acylation reaction and hydrolysis of acid chloride, as shown in Figure 4.2. The hydrolysis of acid chloride reduces the product yield and necessitates additional steps to remove formed fatty acids from the reaction medium to achieve high product purity. Therefore, maintaining an excess level of acid chloride in the reaction medium ensures a higher

product yield (Jursic & Neumann, 2001; Rondel *et al*., 2009). Since chlorides hydrolyze faster in aqueous solvents, some studies use micellar media (Jursic & Neumann, 2001). Several purification steps were applied to achieve high product purity in the present study. First, the product was washed several times with water to remove unreacted AAs and sodium chloride salt generated during the reaction. Then, a hexane wash was carried out to remove the formed fatty acid molecules and unreacted fatty acid chloride molecules. TLC was performed at each of these steps to monitor the presence of unwanted compounds, *i.e*., lauric acid and lauroyl chloride were removed, and the final product was enriched with N-acylated AA (Figure 4.3). The crude acylated proline derivative formed a slurry that could not be separated from the reaction mixture by using a simple filtration technique. Therefore, extraction of the product with ethyl acetate and separation using silica gel column was performed.

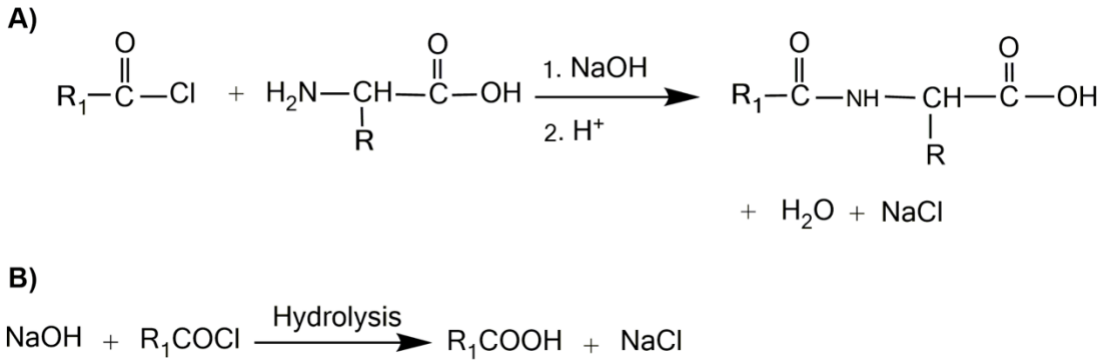


Figure 4.2 Reactions showing A) Schotten Baumann reaction between an AA and a fatty acid chloride, and B) Hydrolysis of a fatty acid chloride in the presence of a base.

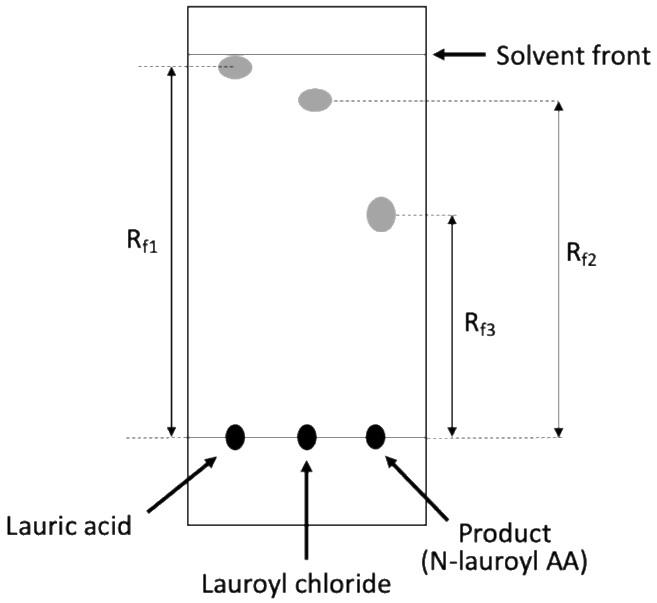


Figure 4.3 A schematic diagram of thin layer chromatography (TLC) separation (normal phase) of N-acylation reaction mixture for identification of lauric acid (Rf_1), lauroyl chloride (Rf_2), and N-lauroyl AA (Rf_3) when hexane:ethyl acetate 1:1 (v:v) as the mobile phase and visualized with phosphomolybdic acid (PMA, 5%, v/v). The Retention factor, R_f was calculated as the distance travelled by the specific compound/distance travelled by the solvent; $Rf_1 > Rf_2 > Rf_3$.

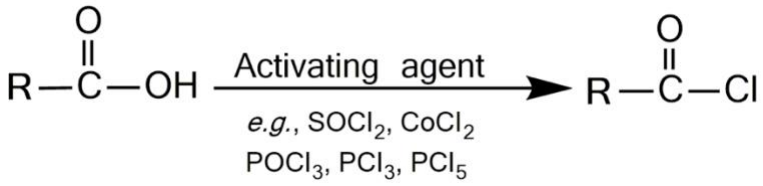


Figure 4.4 Activation of carboxylic acid

4.4.2 Structure characterization

The lauroyl derivatives of AAs obtained from the N-acylation reaction were investigated for their spectral characteristics to confirm the chemical structure of the products. Data obtained from FTIR, ^{1}H NMR and ^{13}C NMR spectra of the compounds were analyzed to see whether N-lauroyl AA derivatives were the dominant molecular species in the formed product.

4.4.2.1 FTIR spectra characteristics

The FTIR spectra of prepared N-lauroyl AA compounds were used in structure characterization by identifying their functional groups (Table 4.1, Figure 4.5 and Figure A1 to A13). For all the synthesised N-acylated AA products, main peaks of the FTIR spectrum were identified as 3,300 cm^{-1} (N-H, amide A), 3,100 cm^{-1} (amide B), 2,850-2,960 cm^{-1} (C-H, sp^3), 1,725 cm^{-1} (C=O, s, carboxylic acid), 1,650 cm^{-1} (C=O, s, Amide I), 1,550 cm^{-1} (O-H or N-H bending, s, Amide II) and 1,250 cm-1 (C-N, amide III). In the spectra of Na^+ salt of the product, the appearance of two bands at 1,600 and 1,450 cm^{-1} was observed with the disappearance of the C=O band at 1,725 cm^{-1}. These observations were in accordance with the spectral data for acids and their salts. Carboxylic group has five characteristic peaks; 2,500-2,700 cm^{-1} (OH stretching), ~1,700 (C=O stretching), ~1,400 cm-1 (C-O stretching or O-H deformation), 1,200-1,300 cm^{-1} (C-O stretching or O-H deformation) and ~900 cm^{-1} (O-H deformation in COOH) and in these compounds their relative intensities were in the ratio of 1:5:2:3:1. All acids gave an intense FTIR band around 1,700 cm^{-1} due to the carbonyl group (C=O), which could also be present in the spectra for aldehydes, ketones, and esters. Salts of carboxylic acid did not show a carboxylic band around 1,700 cm^{-1}; instead, the spectrum showed a characteristic band in 1,510-1,650 cm^{-1} due to asymmetric COO^- stretching. This may be due to the COO^- ion, which has a symmetrical structure where the C atom is equally associated with the two oxygen atoms and the O-C-O bond angle is 100°-130°. Four characteristic bands have been reported for carboxylate ion; 1,510-1,650 cm^{-1} (strong, asymmetric COO- stretching), ~1400 and 1280-1,350 cm^{-1} (broad, symmetric COO^-), and 800-950 cm^{-1} (COO^- deformation vibration) (Filopoulou *et al*., 2021; Hadžija & Špoljar, 1995; Mikhalkin, 1995; Shevchenko, 1963). The N-H and O-H (stretching) bands can overlap in the high-frequency region (around 3300 cm^{-1}), making it difficult to assign to an individual functional group (Mikhalkin, 1995). For potassium cocoyl glycinate and sodium cocoyl glycinate, synthesized from coconut oil, Yea *et al*. (2018) have observed the following specific bands in the FTIR spectra; N-H stretching band (3,300-3,500

cm^{-1}), C-H stretching band (2,800-2,900 cm^{-1}), C=O amide (1,640-1,670 cm^{-1}), C=O carboxyl group (1,700 cm^{-1}) and C-O carboxylic acid (1250 cm^{-1}). According to Katiyar *et al*. (2019), the appearance of characteristic amide -N-H stretching peak (Amide A) and -C=O stretching (Amide I), which cannot be observed in the spectrum of lauric acid but is specific to an amino acid, can be used in identifying the formation of an amide bond. The IR spectrum of acylated products obtained from the mixture of AAs also showed the above-mentioned characteristic peaks of N-acyl products (Figure 4.4).

Table 4.1 Yield, physical form and FTIR wavelengths (cm^{-1}) for main functional groups observed in N-lauroyl amino acids.

Compound	Yield and physical form	FTIR wavelengths (cm^{-1}) for main functional groups
N-lauroyl glutamic acid	Yield: 62% White solid	3300, 3075, 2900, 2850, 1725, 1650, 1500, 1250,
N-lauroyl glycine	Yield: 42% White solid	3300, 3100, 2925, 2850, 1725, 1650, 1550, 1250
N-lauroyl leucine	Yield: 60% White solid	3325, 3075, 2900, 2850, 1700, 1625, 1550, 1250
N-lauroyl lysine	Yield: 71% White solid	3300, 3075, 2925, 2850, 1700, 1625, 1550, 1250
N-lauroyl proline	Yield: 53% White solid	2900, 2850, 1750, 1600, 1450, 1250
N-lauroyl valine	Yield: 55% White solid	3325, 3075, 2900, 2850, 1700, 1625, 1550, 1250
N-lauroyl AA mixture	Yield: 75% White solid	3300, 3075, 2925, 2850, 1700, 1625, 1550, 1250

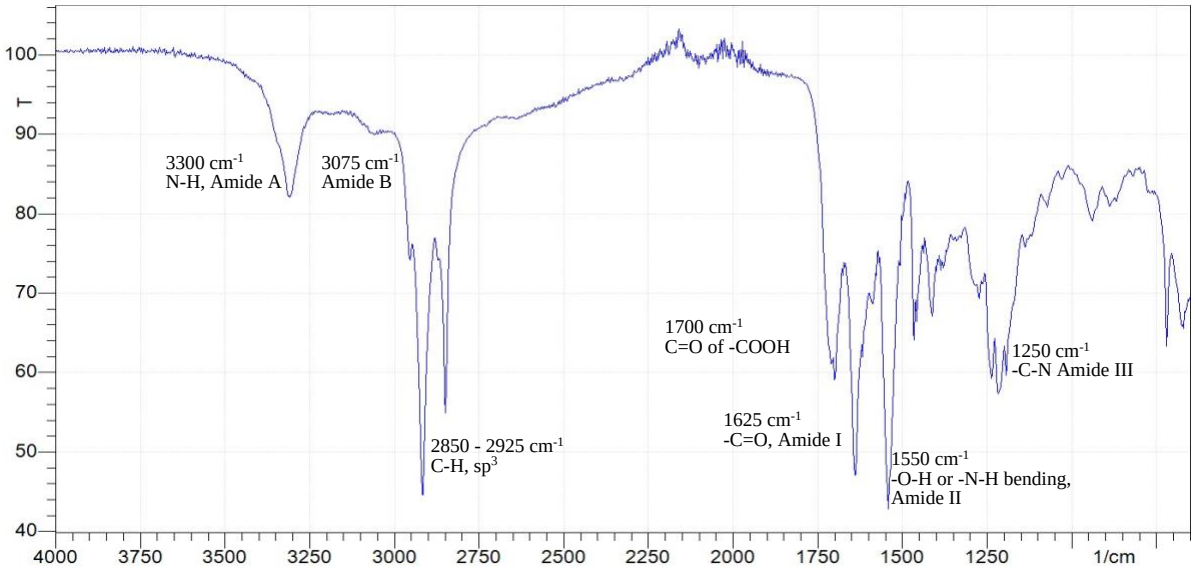


Figure 4.5 Representative FTIR spectrum of N-lauroyl AA mixture (transmission mode).

4.4.2.2 NMR spectral characteristics

The unique and general features of ^{1}H NMR (1D and 2D-COSY), ^{13}C NMR (DEPT 135 and 90, DEPTQ 135 and 90) of N-lauroyl AA products are summarized in Table 4.2. All spectra of these compounds are provided in the appendix (Figures A1 to A28). The lauroyl group was common for all the compounds, and the signals observed in both ^{1}H NMR and ^{13}C NMR spectra for all 6 AAs were similar. Hydrogen atoms of the CH_3 group at ω-C and eight CH_2 groups next to ω-C in the lauroyl group showed peaks at around 0.83 (triplet) and 1.0-1.3 (multiplet), respectively. This was a characteristic of the ^{1}H NMR spectra of all these compounds. Signals for hydrogen atoms of the CH_2 group at β-C and α-C in the lauroyl group showed peaks around 1.4-1.5 (multiplet) and 2.2-2.3 (triplet) ppm, respectively. However, some hydrogen atoms in aliphatic chains of AA side groups can lie in the same range. Also, in ^{13}C NMR spectra, respective C atoms for ω-C, eight C atoms next to ω-C, β-C, and α-C of lauroyl group showed peaks in the ranges of 14-20, 22-29, 30-35, and 35-40 ppm, respectively. Quaternary carbon atoms for the -C=O group of amide and the carboxylic acid group gave their characteristic peak around 170 ppm and was easily identified in DEPTQ spectra. DEPTQ mode of NMR allowed getting information of all C atoms in the structure,

including signals for quaternary C at 180 degrees out of phase with respect to CH and CH_3 carbons and same phase as CH_2 carbons.

The N-glycine molecule has the simplest amino acid moiety. In the ^1H NMR spectrum, a doublet at 3.71 ppm for 2 hydrogen atoms on C-2 and a triplet at 8.07 ppm for H on N atom, which participated in the amide bond, were observed. In the lauroyl group, the 2 H atoms on C-4 (α-C) and C-5 (β-C) were represented by a triplet at 2.10 ppm and a multiplet at 1.47 ppm, respectively. Also, every C atom in the structure gave a peak at ^{13}C NMR UDEFT and DEPTQ spectra. In N-L-valine, CH_3 groups at C-4 and C-4" were not chemically equivalent and distinguished by a multiplet at 0.96-0.98 ppm in ^1H NMR spectrum; further, each C gave two distinct peaks in ^{13}C NMR UDEFT and two positive peaks in ^{13}C NMR DEPT 135 spectrum. Compared to H-3, which gave a multiplet at 2.12-2.21 ppm, the peak for H-2 was more downfield due to the electron withdrawal effect of the carboxylic group and electronegativity of the H atom. Hydrogen atoms on α-C or H-6 were directly influenced by amide -C=O and less shielded (downfield) than the rest of the H atoms in the lauroyl chain. The aliphatic side chain of leucine contains one C atom more than the valine structure. The terminal and branched CH_3 groups of N-L-leucine (at C-5 and C-5") gave similar peaks in both ^1H and ^{13}C NMR spectra. However, hydrogen atoms on C-4, C-3, and C-8 gave a multiplet at 1.55-1.78 ppm. The N-lauroyl proline structure has a chiral centre at C-2. Therefore, the 2 H atoms on each C atom (C-3, C-4, C-5) on the pyrrolidine loop can be diastereotopic and are not chemically equivalent and make those C atoms resonate at different frequencies. The ^1H-NMR spectrum provided the proof as two multiplets at 1.5 ppm and 2.05-2.15 ppm for the 2 H atoms at C-3 in the pyrrolidine loop. Furthermore, a 'triplet × doublet' was given by the 2 H atoms bonded to C-5, and a multiplet was from the 2 H atoms on C-4. The peak at 3.36 ppm found in the spectra could be due to water. The product resulted in N-lauroyl-L-glutamic acid was soluble in hot water, not any other solvent making a limitation to obtaining NMR spectra. Therefore, sodium salt of N-lauroyl-L-glutamic acid was used for structure elucidation with NMR.

Lysine is a dibasic AA of which the ε-amino group has a higher p*Ka* (p*Ka* = 10.5) than the α-amino group (p*Ka* = 8.9). It is categorized as a hydrophilic, basic AA as it is highly polar due to an ε-amino group. Also, lysine has a longer side chain among 20 AAs. The presence of one carboxyl group and two basic amino groups with different p*Ka* or basicity values allows using

lysine molecule for synthesizing surfactant molecules with different ionic characters and tailored functionalities upon grafting a hydrocarbon chain (Colomer *et al.*, 2011; Perez *et al.*, 2014). Lysine-based Gemini surfactant preparation by using spacer molecules, such as 1,4-diaminobutane, spermine or 1,4-bis(3-aminopropyl) piperazine to connect N^{α}- or N^{ε}-acyl lysine molecules has been reported (Perez *et al.*, 2014). The structure elucidation of lysine derivative in the present study showed evidence of the presence of two molecules which can be identified as N^{ε}-lauroyl lysine and N^{α}-lauroyl lysine. This observation has been supported by the work of Leclerc and Benoiton (1968), which mentions that when both amine groups in lysine are unprotected, there is no basis for discriminatory acylation to occur. When acid chloride or anhydride is the acylating agent, acylium ion, $R\text{-}C^{+}=O$, is the nucleophile, and it is highly reactive; therefore, it attacks any amino group without discriminating. Considering these factors, the formation of both N^{ε}-lauroyl lysine and N^{α}-lauroyl lysine is possible in this reaction. The only plausible reason for having a preference for a particular amino group is their relative concentration of the protonated and unprotonated form. However, when the acylation happens by an ester and at a pH higher than 11, the reaction is exclusive with the ε-amino group of lysine. In the study by Paquet (1976), succinimidyl esters have been employed to achieve a selective acylation at an ε-amino group of lysine. It is obtaining N^{α}-acyl lysine as the primary product by protecting $\varepsilon\text{-}NH_2$. Shi *et al.* (2019) have prepared N^{α}-lauroyl lysine using several protections and deprotection steps such as the formation of Cu complex of $\alpha\text{-}NH_2$ and COOH group, protection of $\varepsilon\text{-}NH_2$, acylation of $\alpha\text{-}NH_2$ and deprotection of $\varepsilon\text{-}NH_2$.

In the present study, the AAs that went through acylation were neutral AAs with a non-ionic side chain (*i.e.*, glycine, leucine, valine), acidic AA with a negatively charged side chain (*i.e.*, glutamic acid), basic AA with a positively charged side chain (*i.e.*, lysine) and proline, which has a special structure and the only AA with a secondary amine group. The structural evidence for the reaction products of monoamino carboxylic acids, diamino carboxylic acids (lysine) and amino polycarboxylic acid (glutamic acid) was also studied. Acylation ability of AAs is determined by many factors: nucleophilicity of amino group (p*Ka* = 2.23 - 4.43), steric factors, and acylating capacity of fatty acid derivatives: *i.e.*, RCOX (determined by the stability of the anion X^{-}, where X is a halide). For an effective acylation, activation of the carboxylic acid group is needed, *i.e.*, formation of the acid chloride, anhydride, ester, *etc*., (Figure 4.4). Acylation of AA with a fatty

acid chloride is the most common method (Mikhalkin, 1995). Amino group AA acts as a nucleophile in the basic medium to react with acyl chloride. In the reaction mixture, NaOH neutralizes the protonated amine. Therefore, adding an equivalent base or two equivalents of amine groups is important to have final neutralization by removing a proton from the amide nitrogen. If there is no base, the amines/amine group in the reaction mixture acts as the base and gets protonated and is no longer available to act as a nucleophile to continue with acylation of remaining acid chloride, and the reaction cannot take place (Rajput & Gore, 2011).

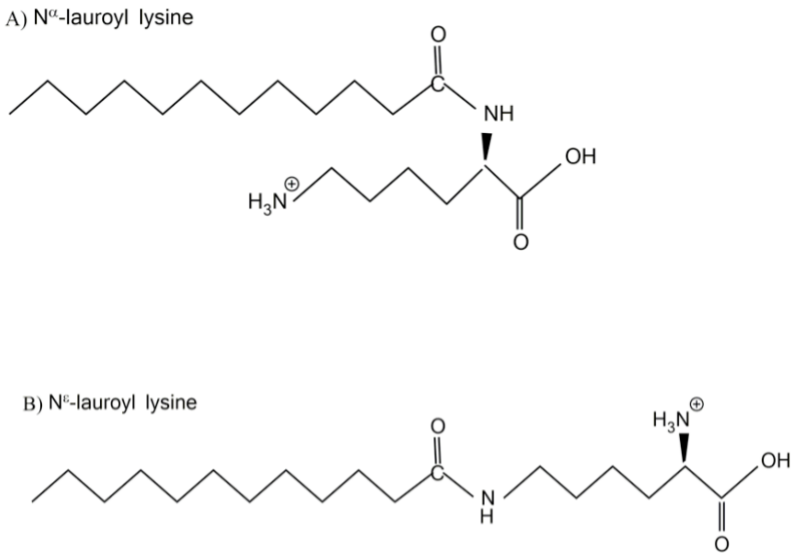


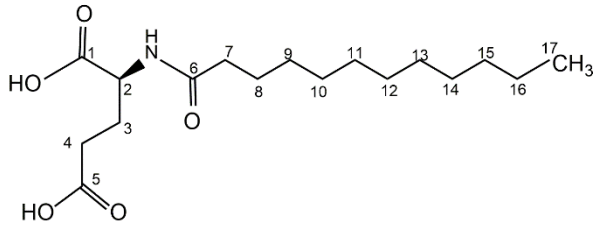
Figure 4.6 Structures of A) Nα-lauroyl lysine and B) Nε-lauroyl lysine.

Table 4.2 Chemical shifts observed for ^{1}H NMR and ^{13}C NMR of N-lauroyl amino acids products.

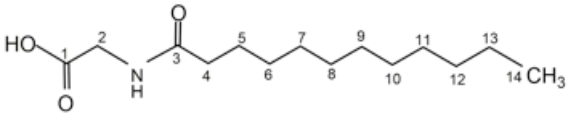
Compound	^{1}H NMR[1]	^{13}C NMR
N lauroyl glutamic acid Na^{+}	^{1}H NMR (D_2O), 500 MHz, δ ppm): 4.0 (m, NH), 3.66 (m, 1H, H-2, N-CH), 2.30 (t, 2H, H-7, CH_2), 1.94 -2.12 (m, 4H, H-3, H-4, 2xCH_2), 1.5 (m, 2H, H-8, CH_2), 1.20 – 1.30 (m, 16H, H-9 to H-16, 8xCH_2), 0.83 (t, 3H, CH_3)	^{13}C DEPTQ NMR (d-DMSO, 500 MHz, δ ppm): 181.43(C-6, C=O), 175.39 (C-1, COO^{-}), 54.69 (C-5, COO^{-}), 35.71 (C-4), 34.17 (C-3), 33.47 (C-7), 31.14 (C-8), 28.64 - 28.19 (C-9 to C-13), 27.39 (C-14), 25.25 (C-15), 22.00 (C-16), 13.38 (C-17)
N-lauroyl-L-glycine	^{1}H NMR (DMSO-d_6, 500 MHz, δ ppm): 12.45 (br, s, 1H, OH), 8.07 (t, 1H, NH), 3.71 (d, 2H, H-2, CH_2), 2.10 (t, 2H, H-4, CH_2), 1.47 (m, 2H, H-5, CH_2), 1.2 -1.3 (m, 16H, H-6 to H-13, 8xCH_2), 0.85 (t, 3H, H-14, CH_3,)	^{13}C NMR, UDEFT (DMSO-d_6, 500 MHz, δ ppm): 172.58 (C-1, COOH), 171.48 (C-3,C=O), 40.54 (C-2, CH_2-N), 35.10 (C-4, CH_2), 31.78 (C-5, CH_2), 29.12 (C-6, CH_2), 29.10 (C-7, CH_2), 29.04 (C-8, CH_2), 28.90 (C-9, CH_2), 28.80 (C-10, CH_2), 28.69 (C-11, CH_2),25.24 (C-12, CH_2), 22.17 (C-13, CH_2), 13.98 (C-14, CH_3)
N-lauroyl-L-leucine	^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm): 5.95 (d, 1H, NH), 4.62 (td, 1H, H-2, N-CH), 2.25 (t, 2H, H-7, CH_2), 1.55 - 1.78 (m, 5H, H-4, H-3, H-8, CH, CH_2, CH_2), 1.24 – 1.33 (m, 16H, H-15 to H-9, 8 x CH_2), 0.95 – 0.97 (m, 6H, H-5, H-5”, 2 x CH_3), 0.88 (t, 3H, H-17 CH_3)	^{13}C DEPTQ NMR ($CDCl_3$, 500 MHz, δ ppm): 176.44 (C-6, C=O of CO-NH), 174.55 (C-1, C=O of COOH), 50.96 (C-2, CH), 41.21 (C-7, CH_2), 36.38 (CH_2), 31.93(CH_2), 29.21-29.64 (6xCH_2), 25.71 (CH_2), 24.90 (C-4, CH), 22.70 (C-3, CH_2), 22.85 (C-5”, CH_3), 21.84 (C-5, CH_3), 14.14 (C-17, CH_3)
N-lauroyl-L-lysine	^{1}H NMR (D_2O), 500 MHz, δ ppm): 0.89 (t, 3H, CH_3), 1.2 -1.3 (m, 16H, 8xCH_2), 1.4 (m, 2H, CH_2), 1.6 (m, 2H, CH_2), 1.7 (m,2H, CH_2), 1.8 (m, 2H', CH_2), 1.9 (m, 2H'', CH_2), 2.3 (m, 2H, CH_2), 2.4 (m, 2H, CH_2), 3.3 (m, 2H, CH_2), 4.5 (m, 1H, CH), 6.2 (s, 2H, NH_2), 6.6 (m, 1H, NH), 10.0 (br, 1H, OH),	

N-lauroyl-L-proline	^{1}H NMR (d-DMSO, 500 MHz, δ ppm): 12.40 (s, 1H, OH), 4.19 (q, 1H, H-2, CH), 3.48 (td, 2H, H-5, CH_2), 2.23 (t, 2H, H-7, CH_2), 2.05 – 2.15 (m, 1H, H-3, CH), 1.8 – 1.9 (m, 3H, CH, CH_2), 1.41 -1.50 (m, 2H, H-8, CH_2) 1.17 – 1.20(m, 16H, 8 x CH_2), 0.85 (t, 3H, CH_3)	^{13}C DEPTQ NMR (d-DMSO, 500 MHz, δ ppm): 173.66 (C=O of CO-NH), 170.71 (C=O of COOH), 58.26 (CH, N-C-COOH), 46.51 (-N-$\underline{C}H_2$), 33.54 (-$\underline{C}H_2$-CO-N), 31.35 (-COOH-CH-$\underline{C}H_2$), 28.73-29.07 (7xCH_2), 24.39 (CH_2), 24.28 (CH_2), 22.15 (CH_2), 14.01 (CH_3)
N-lauroyl-L-valine	^{1}H NMR (MeOD, 500 MHz, δ ppm): 8.03 (d, 1H, NH), 4.32 (d, 1H, H-2, CH), 2.27(td, 2H, H-6, CH_2), 2.12- 2.21(m, 1H, H-3, CH), 1.58 – 1.64 (m, 2H, H-7, CH_2), 1.25 – 1.36 (m, 16H, H-15 to H-8, 8 x CH_2), 0.96 – 0.98 (m, 6H, H-4, H-4”, 2 x CH_3), 0.89 (t, 3H, H-16, CH_3)	^{13}C NMR (MeOD-d_4, 500 MHz, δ ppm): 176.65 (C-1, COOH), 175.10 (C-5, C=O), 59.07 (C-2, N-CH), 36.87 (C-6, CH_2), 33.22 (C-3, CH), 31.74 – 23.88 (C-7 to C-15, CH_2), 19.80 (C-4, CH_3), 18.53 (C-4”, CH_3), 14.62 (C-16, CH_3)

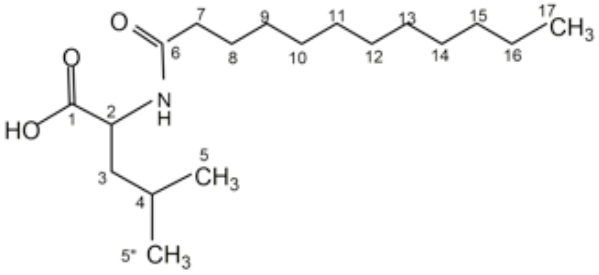
[1] d= doublet, m=multiplet, t=triplet, td=triplet x doublet



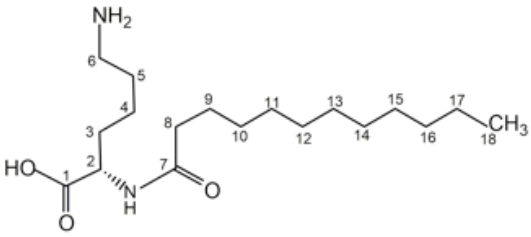
(A) N-lauroyl glutamic acid



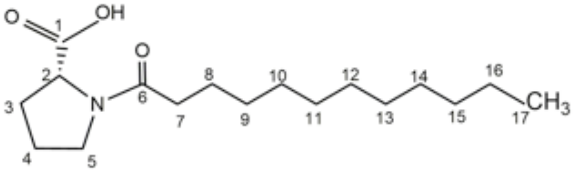
(B) N-lauroyl glycine



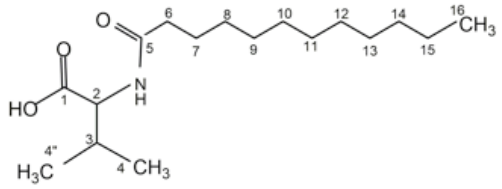
(C) N-lauroyl leucine



(D) N-lauroyl lysine



(E) N-lauroyl proline



(F) N-lauroyl valine

Figure 4.7 Structures of N-lauroyl amino acids prepared in the study.

4.5 Conclusions

Application of the Schotten Baumann reaction to attach a lauroyl (acyl) group to the nitrogen atom of L-glutamic acid, glycine, L-leucine, L- lysine, L-proline, and L-valine gave expected N-acylated

AA product with product yields in the range of 41% to 75%. The success of performing N-acylation reaction and product formation was confirmed by the structural features of the products revealed from FTIR and ^{1}H and ^{13}C NMR spectroscopies. The mixture of these AAs formulated according to the ratio of these AAs found in canola meal protein was also successfully derivatized to obtain a mixture of N-lauroyl derivatives of respective AAs.

4.7 Connection to the next study

This study confirmed the conditions and the process that can be employed in N-acylating glutamic acid, glycine, leucine, lysine, proline, and valine with a lauryl acyl group as individual AAs as well as in a mixture using the Schotten Baumann reaction. N-acyl AAs have functional properties, including surface tension reduction and antimicrobial activity. Particularly, the lauroyl group containing 12 carbons is a widely sought-after acyl group to functionalize molecules to be surfactants. Since the selected AAs for acylation is the abundant AAs in canola meal protein, knowledge on the properties of acylated AAs will be useful information to understand the behaviour of AAs in a canola protein hydrolysate upon acylation. Therefore, the next study was designed to evaluate the antimicrobial and surfactant properties of N-lauroyl derivatives of glutamic acid, glycine, leucine, lysine, proline and valine and their mixture prepared as described in Chapter 4.

5. SURFACE PROPERTIES AND ANTIMICROBIAL ACTIVITY OF N-LAUROYL DERIVATIVES OF SELECTED AMINO ACIDS

5.1 Abstract

Sodium N-lauroyl amino acids (sodium N-lauroyl AAs) were synthesized from six amino acids (AAs); glycine, L-glutamic acid, L-leucine, L-lysine, L-proline, and L-valine, which are abundant AA in canola meal proteins. The surface tension of solutions of N-lauroyl AAs were measured. Aqueous solutions (0.1 %, w/v) of these sodium N-lauroyl AAs were investigated individually and as a mixture for their ability to reduce the surface tension of pure water. Except for the proline derivative, the rest of the sodium N-lauroyl AAs had lower critical micelle concentration (CMC) than sodium dodecyl sulphate (SDS); whereas, the sodium N-lauroyl AA mixture, prepared according to the ratio at which they exist in canola protein, gave the highest surface tension lowering ability and the lowest CMC among the tested compounds. The foaming capacity of the sodium N-lauroyl AAs solutions was in the range between 117% to 276%, while SDS reported 331%. The foams prepared from solutions of sodium N-lauroyl-glutamate were the least stable, while foams from the solution of the N-lauroyl AA mixture had the highest stability, which was even superior to SDS. The effects of sodium N-lauroyl AAs on growth bacteria (*Escherichia coli, Pseudomonas fragi, Salmonella enteritidis*) and three Gram-positive bacterial strains (*Lactobacillus plantarum, Lactococcus lactis, Listeria monocytogenes*) were investigated. Both 25 mM sodium N-lauroyl glycinate and sodium N-lauroyl leucinate inhibited the growth of all six bacterial strains by >95%. The sodium N-lauroyl AA mixture was less soluble, and therefore, the tested concentration range was narrow. More than 90% inhibition was achieved for the growth of *E. coli, L. lactis and L. monocytogenes* at 250 ppm. These investigations show that sodium N-lauroyl AAs reduce surface tension ability, form stable foams, and have antibacterial activity against selected organisms.

5.2 Introduction

Surfactants are widely used in both food and non-food applications. The non-food applications include laundry detergents, pharmaceuticals, personal care, and oil recovery. Surfactant use is paramount, leading to large-scale production for daily use worldwide (Pinazo *et al*., 2011; Tripathy *et al*., 2018). Many of the surfactant production starts with petroleum industry-based or vegetable oil-based feedstocks. Along with the global movement to environmentally benign greener technologies, the surfactant industry has been dealing with the pressure from legislation and consumers on a transition towards eco-friendly products (Bordes & Holmberg, 2015; Infante *et al*., 2004). As a result, some established petroleum chemicals-based surfactant classes were replaced with new green alternatives. Finding green feedstocks, such as bio-based renewable materials, development of novel surfactant compounds with improved attributes, satisfying both functional and consumer expectations continuously challenge the field of surfactant research (Le Guenic *et al*., 2019; Morán *et al*., 2004; Pinazo *et al*., 2011). Within the last two decades, amino acid-based surfactants came into the market as bio-based surfactants with low environmental impact while providing excellent surface-active properties (Infante *et al*., 2004; Pinazo *et al*., 2011).

In the surfactant industry, detergents and personal care applications carry the largest market segment (Chican *et al*., 2019), and N-acyl AAs are known to provide biocompatible alternatives with mild and less skin irritant properties (Chen *et al*., 2020). Therefore, much interest has been found in investigations on their efficient synthesis (*i.e*., feedstock and processes), surface-active properties (*i.e*., surface tension lowering and aggregation activities), interfacial properties (*i.e*., foaming and emulsifying capacities), antimicrobial and cytotoxic properties of N-acyl AAs (Bajani *et al*., 2018; Chican *et al*., 2019; Gerova *et al*., 2008; Liu *et al*., 2018; Luo *et al*., 2020; Mhaskar *et al*., 1990; Perinelli *et al*., 2016). Synthesis of these amphiphiles was carried out with novel combinations of molecules for hydrophobic and hydrophilic groups to tailor their functionality. Fatty acids with different chain lengths as well as the mixture of fatty acids from plant-based oils (*e.g*., coconut and castor) were used as hydrophobic tail, and the effect of fatty acid chain length on functional differences of resulting compounds has been reported (Liu *et al*., 2018; Mhaskar *et al*., 1990; Rondel *et al*., 2009). Furthermore, the diverse nature of AA structure, along with its polarity and acidic or basic character, allows achieving a variety of hydrophilic head groups in the

surfactant molecules that result in functionally diverse products (Le Guenic *et al*., 2019). Molecular sizes, spatial structures, and availabilities of functional groups that form H bonds, as well as the presence of isomeric forms, were major factors that have been discussed in relation to their packing at the interface, aggregation, and diffusion rate, which influence surface activities and interfacial properties in the surfactant applications (Bajani *et al*., 2018; Gerova *et al*., 2008; Qiao & Qiao, 2012; Shinitzky & Haimovitz, 1993). The structure-function relationships of the structurally diverse AA-based surfactants are a proven observation that enable to understand their actual mechanism at the molecular level (Malik, 2015; Stubenrauch *et al*., 2017).

The surfactants with disinfecting abilities can expand their roles as a detergent. The development of antibiotic resistance of microorganisms has become a significant challenge in the health sector today (Bustelo *et al*., 2017). This urges the need to find alternatives that can compensate or work along with established antibiotics in fighting both spoilage and pathogenic microorganisms. Cationic surfactants are the most widely tested category, and their promising effect against both Gram-positive and Gram-negative bacterial strains and fungal species has been identified (Bustelo *et al*., 2017; McKellar *et al*., 1992; Pinazo *et al*., 2016a). However, comparatively a small number of studies report on antimicrobial activity of anionic surfactants compared to the reports of cationic surfactants (Sreenu *et al*., 2015b; Sreenu *et al*., 2014b).

AAs for surfactant development are obtained through enzymatic synthesis or protein hydrolysis (Pinazo *et al*., 2011). In this study, the interest was to identify a useful approach to enhance the functionalities of canola meal-derived AAs, which were obtained through acid hydrolysis of extracted meal protein. It was expected that investigating major AAs that comprise nearly 50% of canola protein, all in all, will enable us to understand their behaviour in the mixture in terms of properties useful as a surfactant. The study described in this chapter was designed as the first step in identifying the potential of AAs that are abundant in canola meal for their ability to generate sodium N-acyl derivatives, namely N-lauroyl derivatives, and their properties that are relevant to perform as a surfactant with an additional property of antibacterial activity. Therefore, the AAs were structurally modified to generate their sodium salt of N-lauroyl derivatives (described in Chapter 4), and the derivatives were evaluated for surface activity, foaming ability, foam stability and inhibitory activity for selected bacterial cultures.

5.3 Material and methods

5.3.1 Preparation of sodium salt of N-lauroyl derivatives of selected individual amino acids

Preparation of sodium salt of N-lauroyl AAs (L-glutamic acid, glycine, L-leucine, L-lysine, L-proline, and L-valine) was similar to the procedure described under Material and methods of Chapter 4.3.1 and 4.3.3.

5.3.2 Preparation of sodium salt of N-lauroyl amino acid mixture

Preparation of sodium N-lauroyl amino acid mixture was same as described in section 4.3.2 and 4.3.3 of Chapter 4.

5.3.3 Evaluation of surfactant properties

Surfactant properties of sodium N-lauroyl derivatives of L-glutamic acid, glycine, L-leucine, L-lysine, L-proline and L-valine and their mixture were evaluated by comparing with the properties of standard anionic surfactant, sodium dodecyl sulphate (SDS) at appropriate concentrations.

5.3.1.1 Surface tension

Surface tension at air/water interface was measured for 0.1% (w/v) solution of each test material using Wilhelmy ring method in a tensiometer (Lauda Scientific, Lauda-Königshofen, Germany) at room temperature. The ring was always cleaned and heated to a red/orange colour with a Bunsen burner before use. Three successive measurements were carried out to ascertain the reproducibility. The air/water surface tension was 72 mN/m at ambient temperature (25°C).

5.3.1.2 Conductivity

Electrical conductivity of the solutions of test materials (1 - 16 mM) prepared in triplicate was measured using a conductivity meter (Orion 5 Star, Thermo Scientific, Canada) at room temperature. Three successive measurements were carried out for each concentration level to ascertain the reproducibility. Before the measurement, the instrument was calibrated with 1,413 µS/cm and 12.9 mS/cm standard solutions (Orion, Thermo Fisher Scientific, Inc., Canada).

5.3.1.3 Critical micelle concentration

CMC was graphically determined using the slope of the graphs generated for conductivity (µS/cm) versus surfactant concentration (µS/cm) of each test materials.

5.3.1.4 Foaming capacity and foam stability

Foaming properties of 0.1% (w/v) solution of test material were measured as foaming capacity and foam stability. An aliquot of 15 mL of each solution was transferred to a 400-mL beaker and homogenized with a sawtooth probe attached to a Polytron-PT10/35 homogenizer (Kinematica AG, Luzernerstrasse, Switzerland) at speed 4 for 5 min. Then, the sample was transferred to a 50-mL graduated cylinder, and the foam volume was recorded immediately. The foam was allowed to stay undisturbed for 5 to 180 min, and foam volume was recorded after each 30 min. Calculation of foaming capacity (FC) and foam stability (FS) followed the equation 5.1 and equation 5.2, respectively.

$$\text{Foaming capacity, \%} = \left[\frac{\text{Initial foam volume in mL(time = 0)}}{\text{Sample volume in mL}}\right] \times 100\% \qquad \text{Equation 5.1}$$

$$\text{Foam stability, \%} = \left[\frac{\text{Foam volume in mL (time = t min)}}{\text{Initial foam volume in mL (time = 0)}}\right] \times 100\% \qquad \text{Equation 5.2}$$

5.3.4 Evaluation on anti-microbial properties

Antimicrobial activity was determined based on the minimum inhibitory concentration (MIC) and percentage growth inhibition of Gram-negative *Escherichia coli* TOP10F, *Pseudomonas fragi*, *Salmonella enteritidis* ATCC 4931, and Gram-positive *Lactobacillus plantarum* NRRL B-4496, *Lactococcus lactis* NRRL B-1821, *Listeria monocytogenes* bacteria. Except for *L. plantarum* and *L. lactis*, all other bacterial pre-cultures were prepared in lactose broth by incubating at 30°C for 24 h. Pre-cultures of *L. plantarum* and *L. lactis* were prepared by incubating at 37°C for 24 h in De Man, Rogosa and Sharpe broth (MRS broth) and Brain Heart Infusion (BHI) broth, respectively.

Test compounds were prepared in sterile water to obtain desired final concentration levels of 0.5 to 25 mM (144.19 - 7209.50 ppm) for SDS and sodium N-lauroyl product of glutamic acid, glycine, leucine, lysine, proline, valine, and 25 to 250 ppm for sodium N-lauroyl amino acid mixture of 6 AAs. Each test compound (100 µL) was added into a 96-well microplate followed by the addition of 100 µL of diluted bacterial culture containing 1.5×10^8 CFU/mL (equal to 0.5 McFarland standard). Ampicillin was used at 500 ppm (1.35 mM) as a standard for comparison purpose of bacterial growth inhibition. Negative control wells contained bacterial culture (100 µL) and 100 µL of sterile water instead of the test compounds. In addition, another control was carried out by applying only the test compounds. The experiment was carried out in triplicates. After seeding, contents were mixed by moving the plates slowly and incubated for 24 h at the desired temperature for the organism; *L. plantarum* and *L. lactis* were at 37°C, and all other cultures were at 30°C.

After the incubation period, 100 µL of the thoroughly mixed content from each well was taken and performed a ten-fold serial dilution up to 10^6. Aliquots (100 µL) of the diluted *L. plantarum* and *L. lactis* cultures were plated on MRS agar and BHI agar, respectively and incubated for 24 to 72 h at 37°C. All the other cultures were plated on nutrient agar and incubated at 30°C for 24 h. Colony count was recorded after the incubation and presented as CFU/mL. MIC was determined as the minimum concentration of the test compound, which inhibits the visible growth of bacteria. Percentage growth inhibition was determined as below (equation 5.3).

$$\text{Growth inhibition}, \% = \left[\frac{\frac{\text{CFU}}{\text{mL}} \text{ in negative control wells} - \frac{\text{CFU}}{\text{mL}} \text{in test wells}}{\frac{\text{CFU}}{\text{mL}} \text{in negative control wells}}\right] \times 100\%$$

Equation 5.3

5.3.5 Statistical analysis

All experiments were conducted as three replicated runs. Mean values and standard deviations are presented.

5.4 Results and discussion

5.4.1 Surfactant properties

Surface tension and CMC are the primary physiochemical properties employed in characterizing a surfactant in solution. SDS is the standard surfactant that is usually compared within assessing these properties. Many personal care products include SDS in the product formulation.

5.4.1.1 Surface tension

The surface tension of 0.1% (w/v) solutions of sodium N-lauroyl derivatives of L-glutamic acid, glycine, L-leucine, L-lysine, L-proline and L-valine and their mixtures at the air-water interface were compared with SDS at the same concentration (Table 5.1). Of individual AA derivatives, except N-lauroyl derivatives of glycine and glutamic acid, all other four were able to lower surface tension compared to that of SDS. It has been reported that a surfactant molecule with large side chains in the hydrophilic part is unable to pack closely at the interface, and consequently, its ability to lower surface tension decreases (Takehara, 1989). Findings of Mhaskar *et al*. (1990) support the same, that the presence of an additional carboxyl group (as in aspartic acid), an amine group (as in lysine) or a hydroxy group (as in serine) in the head component of surfactant molecule retards the surface tension lowering ability. This may be the reason that high surface tension was observed for sodium N-lauroyl glutaminate in the present study. However, this explanation may not apply to the high surface tension value obtained for glycine derivative, which has the smallest head group among the AAs. Interestingly, surface tension lowering ability reported by Mhaskar *et al*. (1990) for sodium N-lauroyl glycine was inferior to SDS, while leucine and lysine derivatives showed superior ability similar to the observation in the present study. The surface tension measured in the present study was at a concentration more than ten times lower than the CMC of respective compounds. Therefore, it is reasonable to expect higher surface tension values than the values they exhibited at CMC.

Several researchers have investigated the surface activities of sodium N-lauroyl glycinate, which has the simplest hydrophilic head group in the structure among the tested compounds. The present study reported a surface tension value of 47.35 mN/m for sodium N-lauroyl glycinate while lower values than that have been reported in previous studies; 41.17 mN/m (Qiao & Qiao, 2012), 38.5 mN/m (Kawase *et al*., 2010) and 32.29 mN/m (Chican *et al*., 2019) and the measurements were at

the CMC. It has been further reported that surface tension gave lower values in buffered systems resulting in 27 mN/m in 20 mM phosphate buffer at pH 7 (Bajani *et al*., 2018). Aqueous solutions (0.1% w/v) of sodium N-palmitoyl derivatives of isoleucine, phenylalanine, and proline (as the hydrophilic head) reported surface tension values of 31.4, 30.0 and 32.8 mN/m, respectively, and under the same conditions, SDS showed 29.8 mN/m (Sreenu *et al*., 2015b). The surface tension of sodium N-acyl proline prepared with a mixture of fatty acids from vegetable oils was inferior to SDS at the same concentration (Sreenu *et al*., 2015b). The surface tension of sodium N-lauroyl valine was at 37.04 mN/m in the present study, while Luo *et al*. (2020) reported 39.70 mN/m for the same compound at its CMC.

A surfactant that can reduce the surface tension of water from 72 mN/m to a value of 32-37 mN/m is considered to have good detergency, and such surfactant provides better wettability, a key requirement in dirt removal (Pradhan and Bhattacharyya, 2017; Sreenu *et al*., 2015b). The synthesized and tested compounds of the present study were able to reduce the surface tension of water even at 0.1% (w/v) solutions; therefore, they can be introduced as suitable surfactants to use as detergents.

5.4.1.2 Critical Micelle Concentration (CMC)

CMC, an important characteristic of surfactants, is the surfactant concentration in the bulk solution at which micelles start to form. CMC has been described in relation to controlling surface-active properties, such as solubilization, lytic action and interactions with biological membranes (Tripathy *et al*., 2018). The addition of surfactant molecules into an aqueous solution decreases its surface tension which is maintained as a constant even after approaching CMC (Patist *et al*., 2000). Different techniques are employed in determining CMC, *i.e*., conductivity, surface tension, spectroscopic, dye micellization. Therefore, the values obtained for CMC can be method-dependent (Bajani *et al*., 2018; Bustelo *et al*., 2017; Fu *et al*., 2015; Gerova *et al*., 2008; Patist *et al*., 2000; Shi *et al*., 2011). The present study measured the CMC using conductometry. Conductivity is related to the conductance of the ions in the solution, and an increase in conductivity occurs with increasing surfactant concentration. The plot of surfactant concentration against conductivity shows two straight lines with differences in slope (Examples are in Appendix A29]. The first line is due to the concentration range below CMC, where only monomers are

present in the solution. After the monolayer formation, with a further increase in concentration, self-assembly of the surfactants starts. At that point, the conductivity increase follows a different pattern, showing a change in the slope. The intersection of these two straight lines is taken as CMC (Fuguet *et al*., 2005; Li *et al*., 2013).

Table 5.1 Surface tension and CMC of six sodium N-lauroyl amino acids and their mixture.

Test compound[a]	Surface tension[b], mN/m	CMC
Sodium N-lauroyl glutaminate	64.49 ± 1.49	6.8 mM/ 2397 ppm
Sodium N-lauroyl glycinate	47.35 ± 0.19	7.5 mM/ 2095 ppm
Sodium N-lauroyl leucinate	32.33 ± 0.48	5.3 mM/ 1778 ppm
Sodium N-lauroyl lysinate	29.77 ± 0.17	5.1 mM/ 1787 ppm
Sodium N-lauroyl prolinate	33.86 ± 0.35	9.9 mM/ 3162 ppm
Sodium N-lauroyl valinate	37.04 ± 0.08	5.7 mM/ 1832 ppm
Sodium N-lauroyl amino acid mixture	29.46 ± 0.56	1100 ppm
SDS	45.44 ± 1.00	8.6 mM/ 2480 ppm

[a] 0.1% (w/v) solution (100 ppm) at 25ºC.
[b]Air/water surface tension was 72 mN/m at room temperature

Surfactants with lower CMC are more efficient in their surface activities (Sreenu *et al*., 2015b). CMC of AA-based surfactants is known to have lower values than conventional surfactants (Chandra & Tyagi, 2013; Tripathy *et al*., 2018). Compared to SDS, which reported a CMC of 8.6 mM, the AA-based surfactants of the present study had lower CMC with exceptions for proline derivatives (Table 5.1). Furthermore, Brito *et al*. (2011) reported that AA-derived amphiphiles based on serine, tyrosine and 4-hydroxyproline showed lower CMC than their corresponding conventional alkylammonium bromides, sodium alkyl sulphates and sodium alkyl carboxylates. In

the present study, all the synthesized surfactants have the same C12 hydrophobic tail component (from lauric acid); therefore, any differences in surface properties should be associated with the structural diversity in the hydrophilic head part from the AA residues and sulphate group. Self-assembly of surfactant molecules at the interface is governed by intermolecular interactions as well as the spatial structure of the molecule (Bajani *et al*., 2018). Shinitzky and Haimovitz (1993) introduced several factors that determine the formation of the micelles by N-acyl derivatives of AAs; packing of acyl chains in the trans or cis form, location of AA and amide bond in the micellar, distance between carboxylic residues due to charge repulsion, and the planes of amide bonds formed via intermolecular hydrogen bonds.

Inter-surfactant H bonding between amide groups has been evaluated in previous studies as the key role in the self-assembly process of N-acyl AA surfactants (Bajani *et al*., 2018; Bordes & Holmberg, 2011; Chican *et al*., 2019) but not conclusion agreed. Among the AA derivatives used in the present study, the proline derivative is the only surfactant that cannot form intermolecular H bonding at head groups because of its ring structure and reported the highest value of 9.9 mM, which is even higher than the CMC of SDS. This shows that the proline derivative favours self-assembly supporting the argument that the role of hydrogen bonding at the amide group is not a major factor affecting molecular packing. On the other hand, the higher CMC values of sodium N-lauroyl glycinate (7.5 mM) and sodium N-lauroyl glutamate (6.8 mM) can be attributed to their ability to form hydrogen bonds with the amide group resulting in tight packing at the interface. Furthermore, Chican *et al*. (2019) conducted a comparative analysis of CMC values of sodium lauroyl glycinate, forming intermolecular hydrogen bonds between the amide group in the head portion, with sodium lauroyl sarcosinate in which amide nitrogen is methylated, and therefore hydrogen bond forming is blocked. Although slightly higher CMC values of glycine derivative showed that aggregation is more favoured, no observation has been reported to support a significant difference between the CMC values of above-mentioned two surfactants to show that H bonding plays a significant role in aggregation (Chican *et al*., 2019). The study by Bordes and Holmberg (2011) reported close values for surface tension and CMC for both N-dodecanoyl glycinate and N-dodecanoyl sarcosinate, although their head groups were different. They reported a plateau value of surface tension above CMC was 40 and 41 mN/m for N-dodecanoyl glycinate and N-dodecanoyl sarcosinate, respectively, and their respective CMC values were 10 and 9.5 mM

from tensiometry and 14 and 13 mM from conductometry. These values demonstrate that blocking the hydrogen bonding ability of the amide group did not affect micellization and CMC. However, Bajani *et al*. (2018) conducted a similar study with the same two surfactants and had a different opinion. They reported the formation of bilayer aggregates of acid-soap dimer by sodium lauroyl glycinate in contrast to sodium lauroyl sarcosinate, which forms micelles in pH 7 buffer. Steric crowding due to the N-methyl group hindered the acid-soap dimer formation in sodium lauroyl sarcosinate resulting in weak molecular packing. The observed surface properties of these two surfactants supported the conclusion that sodium lauroyl glycinate molecules are tightly packed at the interface and more surface-active than sarcosinate derivatives (Bajani *et al*., 2018). Moreover, Liu *et al*. (2018) synthesized N-acyl amino acid surfactants using castor and cottonseed oil with glycine, alanine, and serine residues as head groups. According to geometry-based packing parameter theory, double-tailed surfactant tends to form vesicles and other bilayer structures while a surfactant with single-chain forms spherical micelles. Using structure characterization (FTIR and NMR), it has been shown that the self-assembly of these surfactants is aided by hydrogen bonding formed between amino moiety and carboxylic groups of the amide bond. Furthermore, the morphology of aggregates depends on the type of AA side chain and the functional groups on the hydrophobic tail (Liu *et al*., 2018).

Among the tested compounds of the present study, sodium N-lauroyl glutaminate, which has two carboxylic groups in the head group, showed the highest surface tension of 64.5 mN/m. The increased hydrophilicity of the head group can be presumed to provide better packing at the air-water interface. Bordes and Holmberg (2011) assessed the effect of two carboxyl groups on the polar head using sodium N-dodecanoyl glycinate and sodium N-dodecanoyl aminomalonate, which has an extra carboxyl group in the headgroup. The plateau value of surface tension above the CMC was 40 and 48 mN/m, respectively. For the same compounds, CMC values of 10 and 40 mM from tensiometry and 14 and 50 mM from conductometry, respectively, have been reported. The study by Bordes and Holmberg (2011) clearly showed the differences in the effect of the extra carboxylate group for key parameters that govern the self-assembly of the surfactant molecule.

According to the results of present study, sodium N-lauroyl glycinate gave CMC at 7.5 mM showing a better packing at the interface than leucine and valine, which have branched side chains

in the polar head. In previous studies, CMC values for sodium N-lauroyl glycinate were reported as 10 mM (Qiao & Qiao, 2012) and 0.29% (Chican *et al*., 2019). In sodium N-lauroyl glycinate, no side-chain groups are present in the head part, which may prevent any steric hindrance, increasing the possibility of tight packing at the interface. The number of methylene groups on the side chain of the polar head could be another influencing factor that brings differences in the surface properties of the tested molecules. Bordes and Holmberg (2011) compared sodium N-dodecanoyl derivatives of aspartic acid and glutamic acid to assess the effect of an extra methyl group in the head portion, which gave a plateau value of surface tension above CMC 48 and 51 mN/m, respectively. CMC from tensiometry was 50 mM for both compounds, and 73 and 74 mM from conductivity, respectively. Those values indicated no influence of having an extra methylene group in the head portion of the molecule for surface properties. In the present study, the surface tension of sodium N-lauroyl leucinate and sodium N-lauroyl valinate derivatives were 32.3 and 37.0 mN/m for surface tension and 5.3 and 5.7 mM for CMC, respectively, showing that the values were in a close range. The present observation also infers that having an extra methylene group does not play a role in their surface activities.

As mentioned in Chapter 4, proline derivative was identified to be in stereoisomers. Therefore, surface properties can be different from the pure enantiomer of the compound. Gerova *et al*. (2008) has studied sodium salt of various chiral N-palmitoyl AA surfactants derived from methionine, proline, leucine, threonine, phenylalanine, and dipeptide Phe-Gly for their aggregation in aqueous solution. In their study, no significant difference has been reported between the critical aggregation concentration of pure enantiomers and that of their racemic mixture. Also, no evidence was found for the formation of aggregation-induced chiral superstructures.

Inferior surface properties of the N-acylated derivative of glycine could be attributed to the smaller head group size, which brings the need for higher concentration to achieve better packing at the interface. This study measured the surface tension at a very low concentration of 0.1% (w/v), and the glycine derivative was not capable of lowering the surface tension of the solution at that concentration level. The same reason can cause having a higher CMC value for the solution of the glycine derivative. The lowest CMC value was reported with the surfactant produced using the mixture of AAs, demonstrating its promising surface active properties. The reported lowest values

for CMC and surface tension of the solutions of N-lauroyl mixture of AAs could be due to the synergistic contribution from N-lauroyl derivatives of individual AAs contained in the acylated mixture. There could be an increased number of inter-molecular bonding, such as H-bonding between the acylated AAs, supporting a better molecular arrangement at the interface, which lowers the surface tension of the solution. The acylated AAs in the mixture can have various spatial arrangements of their polar head groups or the AA moiety. These arrangements may result in occupying a larger surface area at the interface by a fewer number of molecules when compared to a surfactant of just one type of molecule. Therefore, monolayer formation can occur at a lower surfactant concentration helping CMC get lower values. Further, literature supported that CMC values of mixtures of surfactants are lower than their individuals. Chican *et al*. (2019) reported that CMC values obtained for the mixtures of sodium lauroyl glycinate and sodium lauroyl sarcosinate were lower as mixtures than other surfactant molecules, *i.e*., lauryl dimethyl amine oxide, cocamidopropylbetaine.

5.4.2 Foaming properties

Foaming capacity and stability of 0.1% (w/v) solutions of sodium N-lauroyl AAs were determined (Table 5.2). SDS had a higher foaming capacity (331.1%) and stability than all the tested compounds. Sodium N-lauroyl AA mixture showed a foaming capacity of 271.1%, and it showed higher stability than those of individual AA derivatives. Blagojević *et al*. (2016) reported that mixed surfactants had lower CMC and are more efficient candidates as cleaning and foaming agents.

Sodium N-lauroyl glutaminate showed the least foaming capacity (117.8%) and foam stability, disappearing all the foam bubbles in less than 5 min period. Low surface tension supports film foaming (Chen *et al*., 2019) and therefore, the high surface tension values of sodium N-lauroyl glutaminate solution may be responsible for its low foaming capacity. Furthermore, this observation can be associated with the diffusion rate of the molecule. The formation of inter-and intra-molecular H bonds by the surfactant molecules with more hydrophilic groups can reduce the number of molecules diffusing into the air-water interface (Pradhan and Bhattacharyya, 2017). However, it has also been mentioned that the requirement of H-bond formation between surfactant molecules for foam generation is still unclear since little information is known about the strength

of inter-surfactant H-bonding and the association behaviour of the molecules (Stubenrauch *et al*., 2017). The size of the surfactant molecule also governs the rate of diffusion. The molecules with high polarity in the head group form bulky structures after solvation (Beneventi *et al*., 2001). Surfactants larger in size make a loosely packed non-coherent interfacial film while creating an unstable foam (Azira *et al*., 2008). Therefore, less foam formation and low stability of sodium N-lauroyl glutamate can be attributed to its higher hydrophilicity in the polar head group and the large structure with two carboxylic groups. The effect of the spatial molecular structure on film formation was studied by Zhao *et al*. (2019). The reduced foamability of sodium N-acyl phenylalanine, compared to sodium N-acyl glycine, was related to the larger structure of phenylalanine moiety, which occupies a larger space at the interface than glycine and forms a thicker foam film in a reduced rate of foam formation. In addition, at the air-liquid interface, molecular spacing is increased, and interaction between the hydrophobic group is weakened, resulting in a loose interfacial film (Zhao *et al*., 2019).

Since all the tested surfactants of the present study have the same hydrophobic chain length, the differences in diffusion rate among different molecules are attributed to the bulkiness of the hydrophilic head group. The foam stability refers to the persistence or the life span of foam after the surfactant produces the foam (Zhao *et al*., 2019). Foam stability is governed by the balance between surface tension, surface activity and adsorption kinetics, while the rate of diffusion is not a major factor contributing to foam stability (Beneventi *et al*., 2001). The AA structure is also important; it has been noted that the effect of branched chains near the hydrophilic group decreases foam stability due to weak packing at the interface (Zhao *et al*., 2019).

In the present study, sodium N-lauroyl glycinate, which had surface tension close to the value of SDS, showed a foaming capacity of 235.6%. However, its stability was higher only to N-lauroyl glutamate. It has been observed that surfactants with low surface tension values result in high foam stability (Beneventi *et al*., 2001; Yea *et al*., 2018). Surfactant concentration is also an important parameter in foam formation, and increasing concentration can enhance the foam creation since it provides more surfactants to the interfacial film to stabilize the foam (Behera *et al*., 2014; Pradhan & Bhattacharyya, 2017). In the present study, foam generation was from 0.1% (w/v) surfactant solutions, a concentration below the CMC values of all the AA derivatives. Even at a lower

concentration, glycine, leucine, valine, and proline-derived surfactants showed foaming capacities higher than 200%. The observation of no foam formation for 0.05% aqueous solutions of sodium N-lauroyl glycinate was reported by Chican *et al*. (2019), and it can be due to low surfactant concentrations employed in their study. Zhao *et al*. (2019) reported on the formation of stable foam with sodium N-acyl glycinate; however, poor foaming stability was observed in the present study.

Generation of foam can be a desirable or undesirable attribute depending on the product application. For instance, foaming is not entertained for a detergent used for a washing machine or a dishwasher; however, it is desirable for shampoos and soaps for attracting consumers and giving a luxurious sensation (Behera *et al*., 2014). Furthermore, foaming has no association with the cleansing ability of a detergent. However, market products, such as personal care products and liquid dishwashing products, are introduced with surfactants that provide good foaming capacity and stability, primarily to receive customer attraction. Mostly customers have a perspective that products with more foam do a better cleaning (Blagojević *et al*., 2016; Pradhan and Bhattacharyya, 2017). Considering the dirt dispersion quality of a foaming surfactant solution, it is desirable to maintain surfactant concentration at a similar or higher concentration above CMC. Up to CMC, surfactants participate in making foams, and fewer surfactants are present in bulk solution. Therefore, more dirt is attracted to foam. After reaching CMC, excess surfactants are in the bulk solution, and they can keep the dirt in suspension. Dirt suspension is an important consideration since dirt remaining in the foam is hard to rinse off, and it is possible to re-deposit on the cleaned surface (Pradhan and Bhattacharyya, 2017). Surfactants with low foaming can be used for oil extraction, waste-water treatment, surface coating, dishwashing, and textile and paper industries (Sreenu *et al*., 2014a). On a practical note, salts of acyl glutamates prevent the formation of scum around bathtubs when incorporated into soap. Furthermore, their pH values are around 5-7, which is similar to human skin, and are reported to provide mildness to skin (Takehara, 1989).

A comparison between AA moieties for sodium N-oleyl AA surfactants has noted that foaming properties decrease with increasing hydrophobicity of the AA group, following the ascending order of phenylalanine, isoleucine, and proline as head groups (Sreenu *et al*., 2014a). Furthermore, the same research group reported that the proline derivative, an alicyclic head portion holder gave inferior foaming properties than SDS in sodium N acyl AA surfactants synthesized with a mixture

of fatty acids (Sreenu *et al.*, 2015a). However, the results for foaming properties in the present study did not follow the same pattern, and it could be assumed that contribution of other factors such as molecule size and spatial structure were in effect.

Table 5.2 Foaming capacity and stability of sodium N-lauroyl amino acids and their mixture in comparison with SDS[a].

Test compound	Foaming capacity, %	Foaming stability, %						
		5 min	30 min	60 min	90 min	120 min	150 min	180 min
Sodium N-lauroyl glutaminate	117.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sodium N-lauroyl glycinate	235.6	17.0	2.8	0	0	0	0	0
Sodium N-lauroyl leucinate	275.6	67.9	22.6	12.1	8.9	5.3	2.1	0.25
Sodium N-lauroyl lysinate	156.7	45.2	38.8	34.5	31.8	30.3	23.4	21.9
Sodium N-lauroyl prolinate	251.1	58.5	45.5	36.4	29.7	23.4	14.6	11.0
Sodium N-lauroyl valinate	260.0	65.5	33.0	22.7	15.5	8.5	5.3	3.9
Sodium N-lauroyl AA mixture	271.1	75.1	61.2	55.4	52.1	48.0	43.9	38.1
SDS	331.1	79.8	73.2	59.3	45.2	34.4	29.6	25.2

[a]All test solutions were at 0.1% (w/v) concentration

5.4.3 Antibacterial properties

Antibacterial properties of the produced sodium N-lauroyl AAs (surfactant molecules) were determined against pathogenic (*Salmonella Enteritidis*, *Listeria monocytogenes*) and non-pathogenic (*Escherichia coli*, *Pseudomonas fragi*, *Lactobacillus plantarum*, *Lactococcus lactis*) bacteria. Minimum inhibitory concentrations (MIC) of these compounds are shown in Table 5.3, and their percentage inhibition values are presented in Table 5.4.

Among the tested non-pathogenic bacterial strains, the sodium N-lauroyl derivatives of glycine, glutamic acid, leucine, proline, and valine had lower MIC than SDS against *E. coli*, a non-pathogenic, Gram-negative bacteria. As the responses for *P. fragi*, which is a non-pathogenic, Gram-negative strain, glutamic acid, lysine, and proline derivatives did not show inhibitory activities at the highest concentration tested (Table 5.3). Percentage growth inhibition was calculated based on the growth of bacteria, which were not treated with the test compounds. Inhibitory activity of glutamic acid, glycine, and lysine derivatives against the growth of *P. fragi* was higher than for *E. coli* at the lowest dose (0.5 mM; Table 5.4). While lysine derivative showed weak inhibitory effects against *E. coli* and *P. fragi*, the other derivatives showed more than 90% inhibitory effects within the range examined in this study. However, no significant inhibitory effect was reported with the N-lauroyl derivative of AA mixture against *P. fragi*.

When the compounds were tested on non-pathogenic Gram-positive bacteria, leucine, proline, and valine derivatives reported their MIC at 0.5 mM against *L. plantarum* and *L. lactis*. Sodium N-lauroyl AA mixture had MIC at a low value of 25 ppm for *L. lactis*, while it reported MIC above 250 ppm for all other bacteria tested. Growth of *L. lactis* was completely inhibited by all the surfactants except the sodium N-lauroyl AA mixture. Only the synthesized derivatives of lysine and AA mixture delivered lesser growth inhibition against *L. plantarum* than other tested compounds. Considering the non-pathogenic bacteria tested in the study, better growth inhibition was observed against Gram-positive organisms than Gram-negative bacterial strains.

It is apparent that pathogenic strains had more resistance to these tested compounds compared to non-pathogenic (Table 5.4). However, the glycine and leucine derivatives at 25 mM concentration exhibited complete inhibition against all the tested organisms. The growth inhibition by most of

the tested compounds exhibited a concentration-dependent manner of increasing percentages of growth inhibition, except for some degree of inconsistency showed by the lysine derivative and the AA mixture. It is assumed that the poor solubility, especially at high concentrations, and tendency to aggregate with time (in the culture media) led to the less effectiveness and inferior outcome of the derivatives of lysine and AA mixture. Therefore, testing of these two was carried out at lower doses than the other test compounds. When all the test compounds were compared, except the lysine derivative, virtually all others showed at least 80% growth inhibition against all the bacterial strains at 5 mM, except for sodium N-lauroyl glutaminate against *Salmonella* and *Listeria*.

The antibacterial activity of surfactants was widely discussed in the literature, focusing on cationic surfactants and their positive effects (Fait *et al*., 2015; Pinazo *et al*., 2016). Since bacterial cell wall is polyanionic, cationic surfactants interact easily and adsorb to the cell surface, weakening the cell wall integrity and penetrating through the cell membrane (Xia *et al*., 1995). The anionic surfactants of sodium N-palmitoyl AAs tested by Sreenu and group (2015b) against bacteria reported a lesser effect on Gram-negatives than Gram-positives. Gram-positive bacteria have a thicker peptidoglycan cell wall than the more complicated outer lipopolysaccharide layer of Gram-negative bacteria that protects bacteria cell (Sreenu *et al*., 2015b). Higher susceptibility of Gram-positive bacteria for acyl AA surfactants than Gram-negatives has also been reported by Xia *et al*. (1995). Mckeller *et al*. (1992) have reported the positive effect of fatty N-acyl AAs against Gram-positive pathogenic bacteria but not against Gram-negative pathogens. Test compounds of the present study showed a similar trend as reported by the latter study.

According to Xia *et al*. (1995), acyl AA surfactants show a broader antimicrobial effect by inhibiting the growth of both bacteria (Gram-positive and Gram-negative) and fungi compared to methyl-p-hydroxybenzoate, a commonly used antimicrobial compound. Furthermore, no correlation was observed between CMC and MIC of the test compounds, and MIC values were lower than their CMC. Xia *et al*. (1995) showed that antimicrobial activity is delivered in the monomeric form of the compound, not in micelle form. The test compounds of the present study delivered antimicrobial activities at low doses below CMC for certain bacterial cultures, while higher doses were needed to affect the growth of other strains.

Table 5.3 Minimum inhibitory concentration of different sodium N-lauroyl amino acids and their mixtures exhibited on the growth of *Escherichia coli, Pseudomonas fragi, Lactobacillus plantarum, Lactococcus lactis, Salmonella enteritidis* and *Listeria monocytogenes.* (non-shaded columns are for non-pathogenic strains and shaded columns are for pathogenic strains)

Test compound	Minimum inhibitory concentration (MIC)[a]					
	E. coli	***P. fragi***	***L. plantarum***	***L. lactis***	***S. enteritidis***	***L. monocytogenes***
Sodium N-lauroyl glutaminate	5 mM (1762.1 ppm)	>25 mM (8810.7 ppm)	5 mM (1762.1 ppm)	0.5 mM (176.2 ppm)	>25 mM (8810.7 ppm)	10 mM (176.2 ppm)
Sodium N-lauroyl glycinate	5 mM (1396.8 ppm)	0.5 mM (139.7 ppm)	>25 mM (6984.2 ppm)	0.5 mM (139.7 ppm)	5 mM (1396.8 ppm)	5 mM (1396.8 ppm)
Sodium N-lauroyl leucinate	10 mM (3354.7 ppm)	5 mM (1677.4 ppm)	0.5 mM (167.7 ppm)	0.5 mM (167.7 ppm)	>25 mM (8386.7 ppm)	10 mM (3354.7 ppm)
Sodium N-lauroyl lysinate	>10 mM (3504.9 ppm)	>10 mM (3504.9 ppm)	>10 mM (3504.9 ppm)	5 mM (1752.4 ppm)	>10 mM (3504.9 ppm)	>10 mM (3504.9 ppm)
Sodium N-lauroyl prolinate	5 mM (1597.2 ppm)	>25 mM (7985.7 ppm)	0.5 mM (159.7 ppm)	0.5 mM (159.7 ppm)	5 mM (1597.2 ppm)	>25 mM (7985.7 ppm)
Sodium N-lauroyl valinate	5 mM (1607.2 ppm)	0.5 mM (160.7 ppm)	0.5 mM (160.7 ppm)	0.5 mM (160.7 ppm)	10 mM (3214.5 ppm)	5 mM (1607.2 ppm)
Sodium N-lauroyl AA mixture	>250 ppm	>250 ppm	>250 ppm	25ppm	>250 ppm	>250 ppm
SDS	>25 mM (7209.3 ppm)	0.5 mM (144.2 ppm)	5 mM (1441.9 ppm)	0.5 mM (144.2 ppm)	>25 mM (7209.3 ppm)	10 mM (2883.7 ppm)

[a] MIC in mM and values in parenthesis indicate the exact concentration of compound in ppm

Table 5.4 Percentage inhibition of growth of *Escherichia coli, Pseudomonas fragi, Lactobacillus plantarum*, *Lactococcus lactis, Salmonella enteritidis* and *Listeria monocytogenes* at different concentrations of sodium N-lauroyl amino acids and their mixture. (non-shaded columns are for non-pathogenic strains and shaded columns are for pathogenic strains)

Test compound	Dose[a]	*E. coli*	*P. fragi*	*L. plantarum*	*L. lactis*	*S. enteritidis*	*L. monocytogenes*
Sodium N-lauroyl glutaminate	0.5 mM (176.2 ppm)	22.4	89.8	97.0	99.9	0	0
	5 mM (1762.1 ppm)	90.5	91.1	99.9	100.0	76.2	47.6
	10 mM (3524.3 ppm)	91.0	98.6	99.9	100.0	82.9	84.9
	25 mM (8810.7 ppm)	89.3	90.4	100.0	100.0	88.6	88.6
Sodium N-lauroyl glycinate	0.5 mM (139.7 ppm)	42.0	94.0	0	89.6	97.7	95.0
	5 mM (1396.8 ppm)	88.4	96.3	84.9	100.0	98.9	99.4
	10 mM (2793.7 ppm)	91.6	95.3	88.6	100.0	92.7	99.5
	25 mM (6984.2 ppm)	97.1	95.6	96.4	100.0	99.0	99.7
Sodium N-lauroyl leucinate	0.5 mM (167.7 ppm)	95.1	79.0	100.0	100.0	95.8	72.4
	5 mM (1677.4 ppm)	96.2	92.3	100.0	100.0	98.6	80.0
	10 mM (3354.7 ppm)	96.4	88.5	100.0	100.0	84.0	85.3
	25 mM (8386.7 ppm)	97.7	95.6	100.0	100.0	98.1	98.4
Sodium N-lauroyl lysinate	0.5 mM (175.2 ppm)	13.4	80.8	78.2	30.9	7.7	85.8
	5 mM (1752.4 ppm)	0	34.8	67.1	100.0	49.2	81.8
	7.5 mM (2628.7ppm)	0	64.4	67.8	100.0	0	85.8
	10 mM (3504.9 ppm)	0	0	34.4	100.0	0	81.4

Table 5.4 (Continued)

Test compound	Dose	*E. coli*	*P. fragi*	*L. plantarum*	*L. lactis*	*S. Enteritidis*	*L. monocytogenes*
Sodium N-lauroyl prolinate	0.5 mM (159.7 ppm)	56.3	39.1	99.5	100.0	0	8.7
	5 mM (1597.2 ppm)	90.6	89.0	100.0	100.0	93.7	95.4
	10 mM (3194.3 ppm)	90.8	94.0	100.0	100.0	91.9	94.8
	25 mM (7985.7 ppm)	91.1	94.8	100.0	100.0	91.8	94.9
Sodium N-lauroyl valinate	0.5 mM (160.7 ppm)	94.5	90.9	99.9	100.0	60.2	82.6
	5 mM (1607.2 ppm)	98.3	90.3	100.0	100.0	80.4	84.9
	10 mM (3214.5 ppm)	99.6	91.1	100.0	100.0	90.0	89.2
	25 mM (8036.2 ppm)	100.0	95.1	100.0	100.0	90.0	87.3
Sodium N-lauroyl AA mixture	25 ppm	90.4	28.0	67.9	68.5	52.2	73.9
	50 ppm	94.6	61.0	90.5	96.4	89.3	76.8
	125 ppm	95.8	36.5	78.7	86.6	77.5	91.0
	250 ppm	95.8	46.8	48.8	97.2	55.1	91.0
SDS	0.5 mM (144.2 ppm)	75.0	92.2	24.0	100.0	93.5	63.8
	5 mM (1441.9 ppm	78.0	94.4	100.0	100.0	92.8	74.5
	10 mM (2883.7 ppm)	63.7	96.4	100.0	100.0	82.0	89.9
	25 mM (7209.3 ppm)	87.2	97.2	100.0	100.0	95.8	90.2

[a] Tested concentrations of the compounds and the values in parenthesis are their concentration in ppm

It has been reported that the structure of AA surfactants has a profound effect on their antibacterial activity. The effect of the hydrocarbon chain length has been pointed out by Sreenu *et al*. (2015a) and Xia *et al*. (1995) as one of the most important considerations. In addition, the solubility of the test material plays a key role in giving sufficient dispersibility to facilitate adsorption to the bacterial cell wall (Xia *et al*., 1995).

5.5 Conclusion

All six sodium N-lauroyl AAs (glutamic acid, glycine, leucine, lysine, proline, and valine) and their mixture tested in this study lowered the surface tension of pure water at 0.1% (w/v), which was a lower concentration than their respective CMC. The foaming capacity of these compounds varied between 117% and 276%, while they showed different degrees of foam stability values. The mixture of six sodium N-lauroyl AA derivatives showed higher surface properties than their individual counterparts. It can be concluded that sodium salt of N-lauroyl derivatives of individual AAs can be introduced as potential surfactants for detergents and that sodium N-lauroyl AA mixture will be the best among them. All the compounds reported antimicrobial activity against Gram-positive and Gram-negative bacteria of the tested range of pathogenic (*Salmonella enteritidis, Listeria monocytogenes*) and non-pathogenic (*Escherichia coli, Pseudomonas fragi, Lactobacillus plantarum, Lactococcus lactis*) organisms. The effect on surfactant ability together with bacterial growth inhibitory activity makes these sodium N-lauroyl AAs (glutamic acid, glycine, leucine, lysine, proline, and valine) dual-action compounds. It is advantageous to use these molecules as disinfecting agents and add value in personal care or laundry detergent formulations.

5.6 Connection to the next study

This chapter demonstrated the surface-active and antibacterial properties of the sodium salt of N-lauroyl AAs that are abundant in canola meal protein. These activities lead to potential applications that N-lauroyl AAs can provide added functions. Interestingly, higher surface properties were observed for the sodium N-lauroyl AA mixture than the individual derivatives. The study presented in Chapter 5 provides evidence supporting the hypothesis that canola meal hydrolysate, which is a mixture of free AAs, will be able to generate compounds with promising functionalities required for a surfactant. The next chapter was designed to prepare the N-acyl derivative of canola

meal protein hydrolysate to evaluate the feasibility of the process and selected functional attributes of the resulting compound(s).

6. GENERATION OF N-ACYL PRODUCTS OF CANOLA MEAL PROTEIN HYDROLYSATES AND EVALUATION OF THEIR ANTIMICROBIAL PROPERTIES

6.1 Abstract

This study focused on preparing N-lauroyl products obtained from canola meal protein hydrolysis. Canola protein extracts were prepared in three ways using DT meal: i) pH 12 extracted and freeze-dried [45% protein, dry mass basis (dmb)], ii) pH 12 extracted, precipitated at pH 4 and freeze-dried (78% protein in dmb), and iii) pH 12 extracted, membrane separated (5 kDa MWCO) and freeze-dried (87% protein, dmb). Hydrolysis of these three canola protein preparations with 4 M H_2SO_4 resulted in Hydrolysate I, II and III, respectively. Chromatographic separation of Hydrolysate III yielded three separate fractions according to the charge and polarity consisting of AAs: Fraction 1 with negatively charged and polar AAs, Fraction 2 with AAs with hydrophobic side chains, and Fraction 3 with positively charged AAs. The unfractionated canola protein hydrolysate III and the three separated hydrolysate fractions (Fractions 1, 2 and 3) were reacted with lauric acid to obtain their respective N-lauroyl products following the Schotten Baumann reaction. Hydrolysate III and Fraction 1 provided sufficient yields of acylated products, and their respective sodium salts were prepared by reacting with 20% (w/v) ethanolic NaOH. The antimicrobial activity of these sodium salts of N-lauroyl products was tested against two pathogenic (*Salmonella enteritidis, Listeria monocytogenes*) and four non-pathogenic (*Escherichia coli, Pseudomonas fragi, Lactobacillus plantarum, Lactococcus lactis*) bacterial strains. The sodium N-lauroyl product of Hydrolysate III showed higher activity against the test strains than the sodium salt of N-lauroyl product of Fraction I. The highest effectiveness was observed against *L. lactis* (100%) and *S. enteritidis* (98%), reporting a MIC of 25 ppm for sodium N-lauroyl product of the unfractionated hydrolysate. The same product gave MIC of 125 ppm for

L. plantarum and *P. fragi* and 250 ppm for *E. coli* and *L. monocytogenes*. The sodium salt of N-lauroyl product of Fraction I required more than 250 ppm to provide any growth inhibition for all the organisms except *L. lactis*. All the tested concentrations of the acylated Fraction I prodcut were ineffective in inhibiting the growth of *P. fragi*. Experiments of this study showed a route for converting DT canola meal protein into an antibacterial agent that will have value in various applications.

6.2 Introduction

Protein hydrolysates obtained from different plant and animal tissues, particularly from by-products of biomaterial processing, are sources of AAs or peptides and have historical use in various food and non-food applications. During the last five to six decades, understanding the applicability of different hydrolyzing techniques suitable for various starting materials and ways to control hydrolytic reactions to obtain targeted product quality has been the topic of many scientific investigations (Alvarez *et al*., 2012; Lammens *et al*., 2012; Sari *et al*., 2014; Zhu *et al*., 2008). The content of peptides and free AAs of a protein hydrolysate depends on the hydrolysis methods since conditions and agents used in hydrolyzing decide the extent of cleaving the peptide bonds of the substrate protein (Calvo *et al*., 2014). The molecular composition of unfractionated protein hydrolysates consists of peptides of various lengths along with free AAs. Many of the studies reporting on protein hydrolysates have investigated functional attributes, such as solubility, emulsifying, foaming, oil holding, and biological activities that include antioxidative, antihypertensive, antiinflammatory, and antiaging (Alashi *et al*., 2014; Chabanon *et al*., 2007; Chang *et al*., 2007; He *et al*., 2013; Rivera *et al*., 2015). In addition, the use of protein hydrolysates as biostimulants for plant foliar applications, soil drenching or seed treatments to improve growth and yield, increasing resistance to abiotic stresses of crops have been reported, and commercial products with such functionalities are available in the market (Nardi *et al*., 2016; Paul *et al*., 2019). Protein hydrolysates are used for various nutritional purposes, including infant formula (EFSA, 2017; EFSA, 2021), geriatric nutrition (Nygard *et al*., 2018), sports nutrition, nutritional supplements (Huecker *et al*., 2019; Manninen, 2009), and in animal feed formulations (Gorissen *et al*., 2018; Hou *et al*., 2017). Free AAs are used as food flavouring agents (Leuchtenberger *et al*., 2005): glutamic acid (Halpern, 2000) and arginine are found to be particularly taste-active (Duan

et al., 2020). Moreover, in industrial uses, AAs are feed molecules to prepare bio-based surfactants (Pinazo *et al*., 2011).

Direct uses of free AAs obtained from hydrolysis of protein-rich substrates in foods, and feed applications have been reported. However, such direct uses in non-food non-feed applications are not reported without chemical modification of free AAs in the hydrolysates. Chi and group (2014) have reported on the modification of collagen hydrolysate with oleic acid chloride to prepare a surfactant, and they observed the effect of the degree of fatty acid grafting on the foaming, wetting and emulsifying capacity of the resulting compounds. Enzymatically hydrolyzed rapeseed protein was acylated with lauroyl chloride by Sanchez-Vioque *et al*. (2001) to obtain protein and surfactant stabilized foams. Small-sized peptides were ineffective in providing good foaming agents; therefore, improving hydrophobicity by conjugating long hydrocarbon chains has been successful in improving foaming ability (Sanchez-Vioque *et al*., 2001).

Chemically modified individual AAs have improved antimicrobial, foaming, emulsifying, and surfactant properties compared to unmodified individual AAs. The experiments described in Chapter 5 demonstrated that N-lauroyl derivatives of glutamic acid, glycine, leucine, lysine, proline, and valine possess surfactant and antimicrobial activities. Therefore, it can be assumed that a protein hydrolysate containing a mixture of free AAs, including the above-mentioned, can be converted to N-lauroyl derivatives to provide similar functions. A protein hydrolysate obtained by hydrolyzing mixed protein-containing substrates would contain free AAs, peptides of varying lengths and unhydrolyzed intact proteins. An increase in hydrophobicity of AAs and peptides of protein hydrolysate by attaching hydrocarbon chains via fatty acids have been demonstrated to improve their surface-active properties. The experiments of this chapter investigated antimicrobial activities of N-acylated (with lauric acid) AAs of protein hydrolysate obtained from DT canola meal to understand their potential use of canola-derived free AAs.

6.3 Material and methods

6.3.1 Preparation of protein extracts and hydrolysates

Three different hydrolysates (Hydrolysate I, II and II) were prepared from hydrolyzing the three different protein extracts obtained from DT canola meal (with ethanol pre-treatment). It was

expected that fewer non-protein components in the protein extract, a higher degree of peptide bond hydrolysis, and a cleaner hydrolysate could be obtained to proceed with the acylation reaction than unseparated proteins in the meal.

a. Hydrolysate I: Protein fraction obtained from alkali (pH 12) extraction without further separation

DT meal (ethanol-treated) was extracted at pH 12, meal-to-solvent (water) ratio of 1:10 (w/v) at room temperature for 1 h as described in Chapter 3 section 3.3.4. The extract was centrifuged at 12,429 ×g for 20 min at 4°C. The supernatant was decanted, and the same extraction process was repeated for the residual meal. The supernatants of two extractions were combined and freeze-dried. The resulting dry solid weight was recorded, and its nitrogen content was determined using the procedure described in section 3.3.2.2 in Chapter 3. Protein hydrolysate was prepared by hydrolyzing crude protein extract with 4 M H_2SO_4 (protein: acid ratio of 5 mg proteins:2 mL acid) at 110°C for 24 h. The hydrolysate was neutralized to pH 7 with 6 M NaOH, filtered under vacuum using 0.1-µm glass fibre filter paper, and stored at 4°C overnight to precipitate the salts. If no precipitation was observed after cold storage, a small amount of Na_2SO_4 was added to initiate precipitate formation. The hydrolysate was decanted, and precipitated salt was washed three times with water at 4°C to recover hydrolysate trapped in salt crystals. Recovered canola protein hydrolysate (Hydrolysate I) was then freeze-dried.

b. Hydrolysate II: Protein fraction obtained from alkali (pH 12) extraction and separated by precipitation at pH 4

DT meal (without ethanol pre-treatment) was extracted at pH 12 and recovered in a similar manner as described for Hydrolysate I preparation. The pH of the supernatant (meal extract) was adjusted to 4 with 1 M HCl, mixed well, and the solution was then centrifuged at 12,429 × g for 10 min. The solid fraction containing precipitated protein was recovered by decanting the supernatant and then freeze-dried. The recovery of dry solids was determined along with the crude protein content. Protein hydrolysate was prepared by hydrolyzing with 4 M H_2SO_4 acid and desalted as described for Hydrolysate I. Dry protein Hydrolysate II was obtained by freeze-drying.

c. Hydrolysate III: Protein fraction obtained from alkali (pH 12) extraction and separation by membrane filtration

DT meal (without ethanol pre-treatment) was extracted, providing similar conditions to Hydrolysate I. The protein extract was subjected to ultrafiltration by tangential flow membrane filtration unit consisting of Pellicon® 2 Mini Cassette with Ultracel® 5 kDa molecular weight cut off (MWCO) Membrane, C screen, Membrane area of 0.1 m^2 (Millipore Sigma, Oakville, ON, Canada). When the extract volume was reduced to one-third of the original and conductivity reached 100 µS, the retentate was recovered and freeze-dried. The recovered dry solid content was recorded, and its protein content was determined from N analysis. Protein hydrolysate was prepared by hydrolyzing with 4 M H_2SO_4 acid (protein-to-acid ratio 5 mg proteins:2 mL acid) at 110°C for 24 h. The hydrolysate was neutralized to pH 7 with $Ca(OH)_2$ slurry (40%, w/v) to precipitate excess sulphate ions as $CaSO_4$. The neutralized hydrolysate was filtered under vacuum using 0.1-µm glass fibre filter paper and stored at 4°C overnight to allow salts to precipitate. Liquid Hydrolysate III was decanted and used.

6.3.1.1 Determination of amino acid profile of the hydrolysates

Protein Hydrolysates I, II and III were further cleaned by passing through a conditioned Waters Oasis HLB cartridge (Waters Oasis, Waters Corp., Mississauga, ON, Canada) and derivatized according to Waters AccQtag amino acid analysis method, following the Waters AccQFlour Reagent Kit manual (Waters Corp., Mississauga, ON, Canada). Derivatized hydrolysates were separated on a Waters Aliance 2695 HPLC equipped with C18 AccQ-Tag Column (3.9 mm × 150 mm) and a fluorescence detector (Waters 2475, excitation at 250 nm and emission at 395 nm), using eluent A: Waters AccQTag eluent A buffer, Eluent B: HPLC grade acetonitrile, and Eluent C: water, at a flow rate of 1.0 mL/min.

6.3.1.2 Analysis of enantiomers in protein hydrolysates

Canola protein Hydrolysate III was assessed for enantiomer composition to understand the racemization status of AAs in the mixture. Marfey derivatives of the hydrolysate were prepared, and LC-MS study was performed to identify the AA enantiomers.

6.3.2 Separation of protein hydrolysate fractions based on amino acid charge

Protein hydrolysate was prepared according to the procedure for Hydrolysate III (Section 6.3.1.1 c) from ethanol-treated DT meal. It was separated using a cation exchange resin to obtain AA groups based on charge/polarity. The pH of the liquid hydrolysate was adjusted to 2.4 using 1 M citric acid. An aliquot of protein hydrolysate (5 mL per injection) was injected onto the cation exchange (CEX) column packed with Source 15S cation exchange resin [26 × 100 mm, column volume (CV) 53 mL] and separated using a medium pressure preparative liquid chromatography system (AKTA Explorer, Amersham Pharmacia, USA). The mobile phase employed in separation consisted of two buffers: 60 mM sodium citrate at pH 2.4 (Buffer A) and 60 mM sodium citrate, 1.0 M NaCl, pH 9.0 (Buffer B) and the flow rate were at 1.5mL/min. After equilibrating the column with 100% Buffer A for 2 column volumes (CV), the sample was injected, and the unbound sample was washed out with 6 CV of 100% Buffer A. Then, five linear gradual increments in the Buffer B concentration were applied to elute AAs by their charge. The gradient conditions were reaching 5% in 5 CV, 10% in 3 CV, 20% in 3 CV, 100% in 3 CV, followed by hold at 100% B for 3 CV. At the elution, the column was re-equilibrated with 100% Buffer A in 2 CV. Eluate of 20 mL was collected in 55 fractions, and AAs present in the fractions were quantitatively identified upon derivatization as described in section 6.3.1.1. Then, the fractions were pooled into three groups: 1) negatively charged and uncharged polar AAs, 2) AAs with hydrophobic side chains, and 3) positively charged AAs. These pooled fractions were freeze-dried. Then they were reconstituted in a 250-mL solution with deionized water to prepare solutions of known concentration and re-analyzed for AA type and quantity.

6.3.3 Preparation of N-lauroyl products of hydrolysates

6.3.3.1 Preparation of sodium N-lauroyl product of protein hydrolysates (Unfractionated)

N-acylation of AAs in hydrolysates with lauroyl chloride was achieved by performing the Schotten Baumann reaction in aqueous alkaline (pH 9) medium as described by Xia *et al*. (1996). The lyophilized hydrolysate was dissolved in water (20%, w/v) and was adjusted to pH 12 using 1 M NaOH. This solution was placed in a reaction flask, and acetone (50 mL) was added. While the mixture was stirring, lauroyl chloride was added dropwise. The amount of lauroyl chloride was determined based on the AA level of the solution. The reaction mixture was allowed to stir for 1 h at room temperature. The reaction was terminated by lowering its pH to 3 using 2 M HCl while

allowing the product to precipitate. The product precipitate was recovered by filtering (Whatman No 1 filter paper) and allowed to air dry under a fume hood.

6.3.3.2 Preparation of sodium N-lauroyl products of hydrolysate fractions

Hydrolysate fractions obtained as described in 6.3.2 were placed in reaction vessels with water (10 mL) to reduce the viscosity of the solution, and then pH was adjusted to 12 with 1 M NaOH. Then, acetone (50 mL) was added to the reaction mixture and mixed well. Lauroyl chloride (10% in excess than needed to react with amino groups in hydrolysate) was added dropwise into the reaction mixture while stirring at room temperature. After 1 h reaction time, 1 M HCl was added to bring pH between 2 and 3, and the solution continued to mix for a further 30 min. The crude product was filtered using Whatman 1 filter paper and air-dried under a fume hood. Each product was dissolved in hexane: ethyl acetate 1:1 (v:v) and separated by thin-layer chromatography (TLC) using hexane: ethyl acetate 1:1 (v:v) as the mobile phase to identify the presence of by-products and unreacted reactants. Then, the crude product of N-acylated Fraction 1 (Section 6.3.2) was separated on a silica gel column to separate the N-acyl derivative of negatively charged and uncharged polar AAs from unreacted lauroyl chlorides and formed lauric acid. The column eluate was fractionated, and they were monitored using TLC to identify the product fractions. The N-acylated crude products obtained from Fractions 2 (AAs with hydrophobic side chains) and Fraction 3 (positively charged AAs) were not proceeded for further purification steps because of the low yields resulting in the acylation processes of these fractions. The N-acylated product of Fraction 1 was then converted to its sodium salt by reacting with 20% ethanolic NaOH at room temperature for 1 h.

6.3.4 Evaluation of antimicrobial properties of N-lauroyl products of hydrolysates

Antimicrobial activity of prepared sodium N-lauroyl product of hydrolysate III and sodium N-lauroyl product of Fraction 1 was determined as percentage growth inhibition against Gram-negative [*Escherichia coli* TOP10F, *Pseudomonas fragi*, *Salmonella enteritidis* ATCC 4931] and Gram-positive bacteria [*Lactobacillus plantarum* NRRL B-4496, *Lactococcus lactis* NRRL B-1821, *Listeria monocytogenes*] and their minimum inhibitory concentrations (MIC). Pre-cultures of each strain were prepared as described in Chapter 5, section 5.3.4.

All test compounds were prepared in sterile water to obtain desired final concentration levels of 25, 50, 125, and 250 ppm. A 100-µL aliquot of each test solution was added into a 96-well microplate followed by the addition of 100 µL of diluted bacterial culture containing 1.5×10^8 CFU/mL (equal to 0.5 McFarland standard). Ampicillin was used at 500 ppm (1.35 mM) as a standard inhibitory compound for comparison purposes. Each concentration level of hydrolysate or AA derivative was tested in triplicate. Negative control wells contained bacterial culture (100 µL) and 100 µL of sterile water instead of the test compounds. A test compound control without organism was also carried out. After seeding, contents were mixed by shaking the plate slowly and incubated for 24 h at the desired temperature: *L. plantarum* and *L. lactis* were incubated at 37°C, and all other cultures were maintained at 30°C.

After the incubation period, the bacteria cultures were plated. A 100 µL of content from each well was taken and was serially diluted up to 10^6. The diluted *L. plantarum* and *L. lactis* samples (100 µL) were plated on MRS agar and BHI agar plates, respectively, and the plates were incubated for 24 to 72 h at 37°C. All the other organisms were plated on nutrient agar plates and were incubated at 30°C. Colonies of each plate were counted after the incubation period, and the live colonies in the diluted samples were determined in colony forming units per one millilitre of diluted samples (CFU/mL). Minimum inhibitory concentrations (MIC) of the test compound were determined from the least concentration from the dilution series, which were able to inhibit the visible growth of bacteria.

Percentage growth inhibition was determined as Equation 5.1.

$$\text{Growth inhibition}, \% = \left[\frac{\frac{\text{CFU}}{\text{mL}} \text{ in negaitive control wells} - \frac{\text{CFU}}{\text{mL}} \text{in test wells}}{\frac{\text{CFU}}{\text{mL}} \text{in negative control wells}}\right] \times 100\%$$

Equation 5.1

6.3.5 Statistical analysis

All analysis was carried out in triplicate and mean values of data are presented.

6.4 Results and Discussion

6.4.1 Preparation of protein hydrolysate suitable for acylation reaction

Preparation of N-acylated derivatives of canola meal-derived AAs necessitated obtaining a much cleaner AA mixture to avoid side reactions and unwanted products of other non-AA molecules. The first challenge was to obtain a protein-rich starting material with a lesser amount of non-protein components, *i.e*., increasing protein ratios. DT meal can be used for recovering AAs. In Chapter 3, it was observed that hydrolyzing of ethanol pre-treated DT meal protein (extracted at pH 12 contained 45% of the crude protein content of dbm) with 4 M H_2SO_4 provided extractions with ~65% of AA contents with a single extraction process. Also, it was clear from previous investigations (Chapter 3) that the ethanol pre-treatment did not improve the protein recovery of DT meal. Therefore protein product preparation for Hydrolysate II and III was carried out without ethanol pre-treatment, and it yielded protein products containing crude proteins at 78% (780 mg/g dbm) and 83% (830 mg/g dbm), respectively (Table 6.1). The ethanol pre-treatment of rapeseed meal, prior to protein extraction, was employed in the studies by Adem *et al*. (2014), Kalaydzhiev *et al*. (2019), Slawski *et al*. (2012) and Berot *et al*. (2005); however, these studies have not reported this pre-treatment as the method to obtain a clean protein product for further hydrolysis.

In the preliminary experiments, all three hydrolysates (Hydrolysate I, II and III) were continued to N-acylation reaction to select the most suitable hydrolysate preparation to move forward. Hydrolysate I had the lowest free AA level (Table 6.1) and did not result in a satisfactory product yield upon N-acylation reaction with lauroyl chloride. It was speculated that the abundance of non-protein substances in the starting protein extract (45% non-protein) ends up in the hydrolysate and negatively affects the reaction efficiency. Since the Hydrolysate I was prepared with a single alkali extraction to minimize processing steps, non-protein contaminants in this pH 12 extract can include canola meal fibre fraction, free sugars, oligosaccharides, phytates, phenolics, *etc*., that are soluble under the employed conditions (DasPukayastha and Mahanta, 2015). The solubilized non-protein components either get decomposed or chemically modified during acid hydrolysis and become part of the protein hydrolysate affecting its purity and N-acylated product formation. The dark brown colour of canola meal protein extract is considered a result of protein oxidation or polymerization with polyphenolic compounds at alkali pHs (DasPukayastha and Mahanta, 2015).

Since Hydrolysate II and III showed higher protein contents at 78% and 83% proteins, respectively, the methods employed for these hydrolysates, *i.e.*, the concentration step using precipitation at pH 4 and ultrafiltration with 5 kDa MWCO membrane, removed a considerable amount of non-protein compounds were removed. Content of AAs of the hydrolysates obtained with 4 M H_2SO_4 acid hydrolysis indicated a 13,730 pmol/µL for Hydrolysate II and 19,858 pmol/µL for Hydrolysate III (Table 6.1). Hydrolysate III prepared using membrane-separated (ultrafiltration with 5 kDa MWCO) protein extract was used for further separation and derivatization reaction because of the higher purity and yield resulted in than other two hydrolysates.

Preparation of protein hydrolysates from large-scale animal and plant processing industry by-products have been reported in literature; for example, porcine blood hemoglobin (Alarvez *et al*., 2012; Chang *et al*., 2007), poultry feathers keratin (Bouhamed and Kechaon, 2017), and canola/rapeseed meal protein (Alashi *et al*., 2014; Chabanon *et al*., 2007; He *et al*., 2013; Ma & Ooraikul, 1986; Salazar-Villanea *et al*., 2017). The major challenge in the present study was to produce DT meal protein hydrolysate containing high levels of AAs. Besides the amount of H_2SO_4 needed to achieve a high degree of hydrolysis, the hydrolysate gets diluted upon neutralization. In order to avoid this dilution, the neutralized hydrolysate was lyophilized and was reconstituted to obtain a higher concentration than the resulting liquid hydrolysate. However, the dry hydrolysate obtained from lyophilization was not completely soluble and made it not possible to reconstitute into a full hydrolysate.

Treatments under high alkali or heat are known to racemize the AAs in proteins (Klockeman *et al*., 1997; Friedman, 1999). The racemization of AAs or conversion between L and D enantiomer forms during the Hydrolysate III preparation was investigated, and it showed primarily L forms were present. Therefore, racemization of AAs has not happened during the extraction at pH 12 or the condition employed in protein hydrolysis. The canola protein hydrolysates prepared according to the above method did not produce a racemic mixture. It can be expected that the AAs in the hydrolysate III participated in the N-acylation reaction, and the products formed were of the L forms of AAs.

Table 6.1 Concentration of amino acids released (pmol/µL) from different protein preparations of DT meal upon hydrolyzing with 4 M H_2SO_4 (Mean values are presented).

Amino acid	Concentration of amino acids released (pmol/µL)[a]		
	Hydrolysate I	Hydrolysate II	Hydrolysate III
Ala	412.0	980.1	1377.7
Asp + Asn	473.5	610.5	717.9
Arg	301.5	1020.8	1610.4
Cysteic acid	0.00	0.0	0.00
Gly	615.5	1834.5	2222.1
Glu + Gln	1235.2	2175.1	3253.6
His	108.1	507.2	679.3
Ile	160.3	611.5	880.5
Leu	352.3	1140.2	1628.9
Lys	244.2	456.8	638.2
Met	0.0	0.0	52.2
Phe	86.4	523.8	939.8
Pro	428.2	1235.1	2440.7
Ser	319.5	405.2	479.7
Thr	229.0	1225.2	1610.4
Trp	0.0	0.0	0.0
Tyr	98.2	137.3	184.3
Val	278.1	867.2	1142.4
Total	5342.0	13730.5	19858.1

[a]Concentrations were expressed on the volume of the hydrolysates.
Protein content of hydrolysates were 45%, 78% and 87% on dmb for hydrolysate I, II and III, respectively.
For preparation methods, refer to Section 6.3.1.

6.4.2 Separation of amino acids of DT meal protein hydrolysate

DT canola meal protein Hydrolysate III obtained with 4 M H_2SO_4 was separated into fractions according to their charge and polarity using a cation exchange column. The purification step of low temperature (4°C) crystallization allowed excess salt removal as crystals while the liquid fraction retained free AAs. The chromatographic separation was expected to provide purified AAs based on their functional groups. In addition, non-protein and non-amino acid components of canola protein hydrolysates were removed during this process. This separation provided three fractions containing distinct AA profiles (Table 6.2). Fraction 1 contained negatively charged aspartic acid and glutamic acid and polar uncharged serine and threonine. Fraction 2 contained AAs with hydrophobic side chains. Among them, AAs with aliphatic side chains: leucine, isoleucine, and aromatic side chains: tyrosine and phenylalanine were observed. However, alanine, proline and valine were poorly separated into Fraction 2. The positively charged AAs arginine, histidine and lysine were well separated as Fraction 3 (Table 6.2).

Table 6.2 Amino acid composition and their levels in cation exchange chromatography separated fractions of DT meal protein hydrolysate III[a].

Amino acid	Amino acids (w/w, as a %of total amino acids in each fraction)[a]		
	Fraction 1	**Fraction 2**	**Fraction 3**
Ala	7.81	0.00	0.00
Asp	14.66	0.00	0.00
Arg	3 .10	8.32	58.50
Cysteic acid[b]	0.00	0.00	0.00
Gly	8.02	2.60	0.00
Glu	33.68	2.99	0.00
His	1.48	7.11	13.84
Ile	1.81	10.66	0.00
Leu	1.85	24.41	0.00
Lys	2.02	11.88	27.66
Met[b]	0.42	0.20	0.00
Phe	0.00	22.04	0.00
Pro	13.41	6.98	0.00
Ser	4.14	0.00	0.00
Thr	5.52	2.35	0.00
Trp[c]	0.00	0.00	0.00
Tyr	0.00	15.01	0.00
Val	6.65	1.61	0.00

[a]Hydrolysate III refer to Section 6.3.1 and Table 6.1.
[b]Fraction 1: Negatively charged and uncharged polar amino acids
Fraction 2: Amino acids with hydrophobic side chains
Fraction 3: Positively charged amino acids

6.4.3 N-Acylation of hydrolysate III (unfractionated)

N-acylation of Hydrolysate III provided a water-insoluble acylated product, and washing the product with hexane was expected to remove unreacted lauroyl chlorides or any of the free fatty (lauric) acid generated during the reaction. However, TLC analysis revealed that the N-lauroyl product was present in the hexane. Testing with the other organic solvents with different polarities: petroleum ether, chloroform, benzene, ethyl acetate, ethanol, acetone, acetonitrile, and dimethyl sulfoxide also demonstarted partial or complete solubility of the N-lauroyl product in them. The N-acylated canola protein hydrolysate was expected to be a mixture of N-acylated individual AAs with a possibility of presene of N-acylated peptides. These constituents of the hydrolysate can have different solubility profiles, and it may be the reason for not having a single solvent that can dissolve the N-acylated hydrolysate completely. Therefore, the soluble portion of the N-acylated hydrolysate in ethyl acetate:hexane 1:1 (v:v), which represented only a portion of the whole product upon acylation, was purified using silica gel. After eluting with ethyl acetate:hexane 1:1 (v:v), the fractions containing high concentrations of fatty acids and lauroyl chloride was removed, and the fractions rich in the N-acylated hydrolysate were collected and used for further testing.

Several studies have reported on the acylation of AAs in the crude hydrolysates of various protein sources. Sánchez-Vioque *et al*. (2001) reported Alcalase-catalysed hydrolysis from rapeseed meal to obtain peptides and their acylation with acyl (C6 to C16) chlorides to modify hydrophobic properties of molecules in the hydrolysate. The major drawback faced in this study was the instability of acyl chloride in aqueous media and its hydrolysis by water. Excess acyl chloride was used to overcome this issue. It was reported that the concentration of rapeseed hydrolysate in the reaction mixture was one of the most important parameters to obtain a reasonable yield of acylated product (Sánchez-Vioque *et al*., 2001). Higher the hydrolysate concentration (means reactive amino groups), lower the amount of excess acyl chloride required. A possible explanation for this observation was that the competition between water molecules and amino groups as reactive sites for acyl chloride becomes a considerable factor. The same researchers also noted that there could be an association between acyl chain length and the extent of modification because the reactivity and stability were different for different chain lengths in aqueous media (Sánchez-Vioque *et al*., 2001). Production of N-acyl AA surfactants using protein hydrolysates of industrial processing by-products of the cottonseed processing and the silk chrysalis processing was described by Xia

et al. (1996). Another study by Wu *et al*. (2014) reported on the production of N-acyl AAs from oil and protein hydrolysate obtained from silkworm pupa.

6.4.4 N-Acylation of hydrolysate fractions

The separation of canola meal hydrolysate into three factions (Fraction 1, 2 and 3) by ion-exchange chromatography showed that Fraction 1 contained more AAs than the other two fractions (Table 6.2). Acylation reaction was performed for all three fractions, and Fractions 2 and 3 gave extremely low product yields. In addition, these two fractions were smaller in mass quantity than Fraction 1 and needed several preparative chromatography runs to obtain a sufficient quantity of respective fractions for further reactions. Therefore, only Fraction 1 was used for N-acylation reaction and reaction products were separated on a silica gel column for further purification. Time was the major limitation to collect Fraction 1 for further work under the Covid-19 pandemic conditions (2020-2021) prevailed during this part of the experiment. Therefore only a limited amount of sodium salt of N-lauroyl product was generated from Fraction 1 of DT canola meal protein Hydrolysate III.

6.4.5 Antimicrobial activity

Antimicrobial activities of N-lauroyl products of Hydrolysate III and Fraction 1 were tested against six bacteria strains (the same organisms as Study 4 and 5) along with SDS as a reference compound. Both these samples were tested at a low concentration range from 25 to 250 ppm, considering the availability of limited product quantity. The sodium salt of the acylated derivative of the hydrolysate successfully inhibited the growth of both Gram-positive and negative pathogenic strains (Table 6.3). Among them, the most effective growth inhibition was against *L. lactis* (100%) and *S. enteritidis* (98%), reporting a MIC of 25 ppm (Table 6.3 & 6.4). More than 250 ppm of the Hydrolysate III derivative was required to reach 87% and 95% growth inhibition of *E. coli* and *L. monocytogenes*, respectively. Compared with SDS at 144 ppm, over 78% growth inhibition was observed for all organisms at 125 ppm of the Hydrolysate III derivative. The MIC of the Hydrolysate III derivative for *L. plantarum* and *P. fragi* was 125 ppm (Table 6.4) while *E. coli* and *L. monocytogenes* reported more than 250 ppm (Table 6.4)

Table 6.3 Percentage[a] growth inhibition of *Escherichia coli, Pseudomonas fragi, Lactobacillus plantarum, Lactococcus lactis, Salmonella enteritidis* and *Listeria monocytogenes* in the presence of sodium N-lauroyl products of canola protein hydrolysate III and Fraction 1 in comparison with SDS (non-shaded columns are for non-pathogenic strains and shaded columns are for pathogenic strains)

Test compound	Dose	*E. coli*	*P. fragi*	*L. plantarum*	*L. lactis*	*S. enteritidis*	*L. monocytogenes*
SDS	0.5 mM (144.2 ppm)	75.0	92.2	24.0	100.0	93.5	63.8
	5 mM (1441.9 ppm	78.0	94.4	100.0	100.0	92.8	74.5
	10 mM (2883.7 ppm)	63.7	96.4	100.0	100.0	82.0	89.9
	25 mM (7209.3 ppm)	87.2	97.2	100.0	100.0	95.8	90.2
Sodium N-lauroyl product of canola protein hydrolysate III	25 ppm	61.5	0	89.7	100.0	98.7	81.1
	50 ppm	69.4	38.1	99.3	100.0	98.7	84.9
	125 ppm	77.9	84.8	100.0	100.0	97.8	90.8
	250 ppm	87.7	96.7	100.0	100.0	97.6	94.7
Sodium N-lauroyl product of canola protein hydrolysate Fraction 1	25 ppm	34.8	0	52.3	74.3	80.2	34.6
	50 ppm	59.4	0	73.5	83.3	81.3	52.3
	125 ppm	70.0	0	74.6	100.0	85.1	53.7
	250 ppm	84.4	0	75.8	100.0	88.7	70.5

[a]Mean values are presented.

Table 6.4 Minimum inhibitory concentration (MIC) of sodium N-lauroyl products of canola protein hydrolysate III and Fraction 1 in comparison with SDS for the growth of *Escherichia coli, Pseudomonas fragi, Lactobacillus plantarum, Lactococcus lactis, Salmonella enteritidis* and *Listeria monocytogenes.* (non-shaded columns are for non-pathogenic strains and shaded columns are for pathogenic strains)

Test compound	Minimum inhibitory concentration (MIC)[a]					
	E. coli	***P. fragi***	***L. plantarum***	***L. lactis***	***S. enteritidis***	***L. monocytogenes***
SDS	>25 mM (7209.3 ppm)	0.5 mM (144.2 ppm)	5 mM (1441.9 ppm)	0.5 mM (144.2 ppm)	>25 mM (7209.3 ppm)	10 mM (2883.7 ppm)
Sodium N-lauroyl product of canola protein hydrolysate III	>250 ppm	125 ppm	125 ppm	25 ppm	25 ppm	>250 ppm
Sodium N-lauroyl product of canola protein hydrolysate Fraction 1	>250 ppm	>250 ppm	>250 ppm	>250 ppm	>250 ppm	>250 ppm

[a]Mean Values are presented.

The sodium salt of an acylated derivative of the hydrolysate Fraction 1 exhibited lesser inhibitory effects on the growth of all tested organisms than the acylated unfractionated Hydrolysate III containing all AAs of canola meal protein (Table 6.3). Except for *L. lactis*, all other organisms required more than 250 ppm concentration to show any growth inhibitory effect (Tables 6.3 and 6.4). The tested concentration levels were not adequate for the growth inhibition of *P. fragi*. At the highest concentration level tested (250 ppm), complete growth inhibition of *L. lactis* and 89%, 84%, 76%, and 71% growth inhibitory levels for *S. enteritidis*, *E. coli*, *L. plantarum* and *L. monocytogenes*, respectively, were observed. Compared to the mixed nature of AAs found in the unfractionated Hydrolysate III, Fraction 1 contained only a portion of selective AAs of the hydrolysate (Table 6.2). Therefore, it was reasonable to observe a lower effectiveness of negatively charged and uncharged polar AAs than the hydrolysate containing all AAs except tryptophan and sulfur-AAs (Table 6.1).

These results showed that adding another process step for separating amino acid fractions according to charge may not be more beneficial than using protein hydrolysate with all available AAs for generating N-acylated products that possess antibacterial activity. The diverse nature of reactive sites of the AAs could be advantageous to prepare the N-acyl derivatives of them, providing an intense antibacterial activity that could be useful in further applications. It was expected that the derivatives of individual AAs found in canola protein hydrolysate provide surfactant properties as observed with the AAs investigated in Chapter 5 that proved to act as surface tension reducing agents. Due to insufficient product quantity, surfactant property measurements were not continued as part of the present study. However, qualitative observation showed high foaming ability of the N-lauroyl product of hydrolysate III as well as the Fraction 1 products, indicating possible dual activities (antimicrobial and surfactant) of N-lauroyl derivatives of canola meal protein hydrolysates.

Amides of fatty acids were known to possess antimicrobial activity, and fatty N-acyl AAs, a type of amide, have been tested against microbial growth for many years (McKellar *et al*., 1992). In addition to antimicrobial activity, amino acid-based surfactant molecules have the additional importance of having low toxicity and biodegradability. The AAs can be obtained through fermentation or hydrolyzing various low-value materials from animal and plant processing

industry side streams. The source materials for AAs are renewable, and raw materials are widely available. The acyl component used in modifying AAs of the present study is derived from oil sources rich in lauric acid (C12 chain, dodecanoic acid). The oilseeds, such as coconut, are rich sources of C12 fatty acids and are widely available for fractionation and conversion into diverse compounds such as their chlorides (Busch *et al*., 2004; Dijkstra, 2016).

Synthetic cationic AA-based surfactants have been investigated as alternatives for conventional antimicrobial agents due to their promising effects (Pinazo *et al*., 2016). The present study showed the applicability of N-lauroyl products of canola protein hydrolysate as microbial growth inhibitors.

6.5 Conclusion

This study showed the possibility of using canola protein-based AA mixtures for converting into their N-acylated derivatives with a C12 chain attachment. Alkali protein extract of DT meal provided the chemically-cleaner protein preparation (feedstock of Hydrolysate I) contained ~50% non-protein material. Protein separation obtained with isoelectric precipitation (pH 4; feedstock of Hydrolysate II) had 78% of proteins; however, the precipitated proteins are poor in acid-soluble proteins of canola. Protein enrichment by removing low molecular weight components (retentate of 5 kDa MWCO; feedstock of Hydrolysate III) provided the highest free AA yield. The low solubility of the N-acylated product was the major hindrance to obtaining complete product recovery and high product purity. Amino acid fractions obtained from canola protein Hydrolysate III (separated with an additional process based on a charge of consisting AAs) had the same challenge of low solubility. The sodium salt of lauroyl derivative of unfractionated Hydrolysate III demonstrated a promising effect against the growth of *L. plantarum* and *L. monocytogenes* at 125 ppm concentration level, which was comparable with SDS at the same concentration. The MIC values of acylated products of unfractionated hydrolysate III for *P. fragi*, *L. plantarum*, *L. lactis* and *S. enteritidis* growth were 25 to 125 ppm, while MIC of the acylated product of Fraction 1 was higher than 250 ppm for all the tested strains. The lauroyl product prepared from Faction 1 (containing negatively charged and uncharged AAs) showed lesser effectiveness than the product of unfractionated Hydrolysate III. Although the effect was less, more than 70% growth inhibition was achieved with all tested bacterial strains, except *P. fragi* at a 250 ppm level concentration of

N-lauroyl derivatives from Fraction 1. The AAs obtained from acid-catalyzed hydrolysis of DT canola meal protein can be converted into N-lauroyl derivatives that possess antimicrobial activity. In order to increase efficecy of protein hydrolysis and AA derivative formation, obtaining protein enriched feedstock is a necessary step.

7. GENERAL DISCUSSION

7.1 Commercial canola meal as feedstock for protein extraction

Growing affluence and population are increasing the demand for food. In turn, the rising requirement for protein has led to increased exploitation of new protein sources and more efficient use of available sources; therefore, many protein-rich plant sources have become the mainstream starting materials for protein ingredient generation. The plant-based protein industry utilizes protein-rich legume crops such as soybean and pulses, as well as oil-rich crops. Canola/rapeseed (*Brassica napus*) is the world's second-largest oilseed crop in annual production. The protein industry sees it as a relatively new candidate crop than soybean. Canola is a botanically different plant from a legume, and also, in terms of human food use, it is different compared to the seeds of many legume crops. The conversion of industrial-grade rapeseed to food-grade vegetable oilseed, canola (double-low rapeseed), happened in the last 50 years. The canola industry has primarily focused on increasing oil yield per unit of cultivated area and unit weight of processed seed. Canola meal, the remaining seed residue after oil extraction, contains 36% - 40% protein in the dbm. The current use of canola meal is limited to animal feed, with the majority used in dairy cattle feed formulation. Canola meal contains all of the inherent chemical components of the seed except for oil and oil-soluble components. Commercial oil processing regimes employed for canola involve increased mechanical pressure, high temperature and exposure to hexane, resulting in several alterations to these components and leading to interactions that modify their native chemical, nutritional and functional characteristics. Positioning canola protein in the food protein ingredient market is challenging. Balancing the structural changes in the oil extraction process and the functional and nutritional values of the protein is a major challenge to use existing commercial canola meal as a starting material for protein extraction. The low extractability and yield and the undesirable colour and odour of the obtained proteins are the major factors hindering the use of industrially processed canola meal as a protein source or feedstock for protein extraction, irrespective of its abundance and nutritionally complete amino acid profile.

7.1.1 Suitability of commercial canola meals for protein extraction and recovering amino acids

The canola oil industry in Canada uses two main types of oil extraction processes, which generate meals differing in their compositional and quality characteristics. The majority of the canola oil extraction industry practices pre-pressed solvent extraction (PSE) for oil recovery producing desolventized-toasted (DT) meal (CCC, 2019). This oil extraction process generates extensively processed meals with a residual oil content of about 1%. Meanwhile, the increasing market for organic and non-GM canola oil encourages the use of expeller-pressing under low temperatures during the past decade. It is mainly employed in small-to-medium scale oil extraction facilities, and this process produces cold-pressed (CP) meal through expeller extraction. CP meal is distinctive from DT meals with a higher oil content (13% - 18%). The animal feed industry can be benefited from the extra energy of oil when CP meal is used in ration formulation. A few studies (Mosenthin *et al*., 2016; Mustafa *et al*., 2000) showed that the proteins of CP meal had retained more of their inherent/native properties than did the proteins of seed meal of PSE (DT meal). As a negative side for protein extraction, the high residual oil content of CP meal will reduce the solubility of protein and affect the purity of extracted protein due to co-extraction and contamination. When acid hydrolyzed, oil can also be hydrolyzed and contaminate protein hydrolysis products, lowering free AA yields. In order to reduce the level of oil in CP meal, a pre-treatment was employed in the present study. The solvent selected for pre-treatment was ethanol. Hexane is used as the industrial solvent for oil extraction. Ethanol (99%) was selected for two reasons: 1) ethanol is considered a food industry friendly solvent, and 2) ethanol solubilizes other compounds, such as phenolic compounds, glucosinolates and free sugars, that can interfere with hydrolysis and the resulting products. Although the first reason is applied to CP meal only, the latter is applied to both CP and DT meal. It was also expected that reducing residual oil, sugar, phenolic compounds and antinutrients in the meal through ethanol pre-treatment would positively affect protein extraction from a pre-treated meal. Past studies report that the extractability of phenolic compounds and glucosinolates from canola meal is highly dependent on the solvent type and organic solvent strength. For example, aqueous ethanol and methanol at ~70% strength have been used for reducing levels of total phenolics and free sugars and glucosinolates in canola meal (Kalaydzhiev *et al*., 2019; Adem *et al*., 2014). In the present study, extraction with 99% ethanol for 30 min at 50°C removed 88% of the oil from CP meal, producing a meal with a residual oil

content of 1.6% of the remaining oil content. Although the starting oil content of DT meal was quite low compared to CP meal, due to the process of oil extraction involved, some ethanol extraction conditions resulted in 78% oil removal producing a meal with <1% oil. It was beyond the scope of the present study to investigate the quality of the oil recovered from CP meal. However, considering the quantitative removal, 99% ethanol can be a suitable solvent for extracting oil for further use. Ethanol at this level can be recovered and recycled, although the recovered ethanol may carry some of the free water available in the meal. The moisture content of the CP meal used in the study was 6.5%, and a fraction of the free water may be extracted by ethanol. Reductions in total phenolics and free sugars levels were at 20% - 25% for CP meal and 22% - 23% for DT meal. This indicated a somewhat lesser amount of phenolic compounds and free sugars available in the pre-treated CP and DT meals as extractable or hydrolyzable during the acid hydrolysis step. Reduction of free sugars and total phenolics by 99% ethanol extraction was only up to 20% for both meals with 99% ethanol, and more polar solvents would be needed to increase the extent of their removal.

The effect of oil removal was reflected in the enhanced protein solubility of CP meal at alkaline pH. It had a negligible effect on DT meal because of the much lower oil content to start within the untreated meal (Figure 3.1). As mentioned before, CP meal protein was expected to have experienced a lesser degree of process-induced alterations than DT meal protein, and therefore it may have retained its native solubility characteristics to a greater extent. Even though ethanol pre-treatment reduced the levels of non-proteinaceous materials in DT meal that could be co-extracted with protein, the less soluble nature of the highly processed proteins in DT meal was not improved at alkaline pH, nor was the level of recovered protein that could be recovered.

Alkaline extraction followed by iso-electric precipitation is the general procedure for preparing protein isolates from oilseeds (Kinsella, 1979). Due to the diverse nature of constituent protein types and their inherent properties, canola proteins exhibit a wide range of iso-electric pHs and molecular weights, along with other characteristics. This is reflected in the differences in protein types soluble from *B. napus* meal at various pHs (Wanasundara *et al.*, 2012). An alkaline medium improves the extractability of oilseed meal proteins; however, alkaline conditions can lead to interactions between proteins and phenolic compounds and cell wall polysaccharides and

destruction and racemization of AAs that take place at high pHs (Klockeman *et al*., 1997). The cross-linkages constitute a significant concern where lysinoalanine is formed from lysine and dehydroalanine formation through degradation of cystine or serine (Klockeman *et al*., 1997). In the present study, extraction of more protein was the focus, but not a particular type of protein; therefore, pH 12 was employed in the bulk extraction of proteins from both CP and DT canola meals. The present study clearly showed the difference in protein recovery from these differently processed meals. Protein recovery at pH 12 reached nearly 83% for CP meal protein while only 30% for DT meal protein, even after two extraction steps. Adewole *et al*. (2016) and Mosenthin *et al*. (2016) have attributed the low solubility of DT meal proteins to the formed products formed via Maillard reaction and protein-protein cross-linking. There is the possibility of Maillard reaction occurring because the necessary reactants and conditions are readily available: free sugars in canola seed at ~9% of the seed dry weight (Slominski *et al*., 2012) and the epsilon amino group of protein-bound lysine, and the moist-heat treatment that usually reaches over 120°C for about 30 min at the desolventizing-toasting step. Even at the early stages of processing, cooking of flaked canola seed, where seed undergoes 85 - 95°C for 30 - 40 min in a seed cooker, is practiced in the oil extraction processes used for Brassica oilseeds to deactivate myrosinase enzyme. Mustafa *et al*. (2000) showed that the heat applied in the desolventizer-toaster influenced the proteins.

In this research, the extraction with ethanol resulted in non-protein substance (including oil) removal. The effect of this removal is evident in protein hydrolysis that achieved a higher AA liberation from ethanol pre-treated CP meal than from untreated meal. Following proteolysis can be achieved with different hydrolyzing agents: acid, alkali, or enzymes. Conventional acid hydrolysis of peptide bonds uses HCl, and it can lead to the destruction and conversion of some AAs into other chemical forms, being the major drawback associated with this process. The present study showed that H_2SO_4 is a suitable alternative to HCl for hydrolyzing peptide bonds and releasing AAs from canola protein; however, higher acid concentrations were needed. HCl was more expensive as it is food grade, and its use requires costly HCl-resistant equipment (Ma and Ooraikul, 1986). Amino acid destruction may be another disadvantage of using H_2SO_4. No tryptophan and fewer sulphur-AAs were detected upon hydrolysis with H_2SO_4 (Table 3.11 & 3.12). Alvarez *et al*. (2012) and Bouhamed and Kechaou (2017) showed that H_2SO_4 tended to form more peptides than free AAs in animal protein hydrolysis. In the present study, it was found that

concentrations of H_2SO_4 as low as 0.5 M tended to generate a greater proportion of peptides, which could represent up to 25% of the molecules in the hydrolysate (Figure 3.8, Table 3.13). Protein hydrolysates can contain both free AAs and peptides, and their proportions may depend on the type of acid and the concentration used. Enzymatic hydrolysis can be used to avoid AA destruction; however, the enzymatic reaction conditions required to achieve complete hydrolysis of seed storage proteins yielding free AAs are rarely reported in the literature. Free AAs through enzymatic proteolysis may require step-wise enzymatic hydrolysis, *i.e.*, once peptides are formed, the resulting peptides are further hydrolyzed with different enzymes (singular or combinations). Currently, AAs for food-grade utilizations are mainly produced through microbial fermentation. When using AAs as building blocks to prepare functional molecules, demand is always for less expensive sources of AAs. Therefore, the development of new, more economical routes for producing AAs will be encouraged.

Although DT meal showed limited potential for use in protein recovery and AA production by acid hydrolysis, due to the lower yield generated, it is still a possible starting material for preparing protein extracts and AA mixtures. One kilogram of DT meal can produce 361 g of proteins, and, in turn, hydrolysis of this protein with 4 M H_2SO_4 can yield 101 g of AAs. Although this yield is only half generated from CP meal, there was no notable difference in AA composition between CP and DT meal derived AAs. Due to the extensively processed nature of DT meal proteins, the meals are currently limited to animal feed use. Generation of AAs and converting them into new biomolecules could be an alternative approach to utilizing agri-industry by-products and co-products.

7.1.2 Amino acid derivatives as bifunctional (antimicrobial and surfactant) molecules

Twenty AAs found in nature have two functional groups - amino and carboxylic groups - in addition to the functional groups in the side chains. The major advantage of AAs molecules is the possibility of chemical modification via several pathways. Amino acids and peptides are assimilable upon digestion of food proteins. Upon assimilation, they can participate in various biochemical pathways. Free AAs as nutrition supplements is a billion-dollar industry today. Branched-chain AAs and the products they contain are widely used in sports nutrition, infant food formulation, meat, dairy, bakery, confectionery and other food/feed and health-related industries

(Kuesten & Hu, 2020; Shinde *et al*., 2018). Another major use of certain AAs in the food industry is taste and flavour enhancers (Pripis-Nicolau *et al*., 2000). L-glutamic acid (as sodium salt or glutamate) is known as the flavour enhancer, MSG, and L-aspartic acid and L-phenylalanine are used as starting materials to prepare the peptide sweetener L-aspartyl-phenylalanyl methyl ester (*i.e*., aspartame) (Bhalla *et al*., 2007; Leuchtenberger *et al*., 2005). Cysteine, methionine, and several AAs are key reactants in modulating the taste and aroma of plant-based, meat-like products by forming compounds with non-animal origin iron complexes such as leghemoglobin (Fraser et al., 2022). Fermented foods, such as soy sauce, cheese, fermented meat, wine, and sourdough, are particularly rich in bitter, umami or kokumi taste and flavour, generated by taste-active AAs and peptides or the AA-derived products (Zhao *et al*., 2016). Furthermore, AAs are important ingredients in the animal feed industry since they are needed for animal growth, development, reproduction, lactation, and health, and they play a key role in regulating food intake and nutrient metabolism in animals (Li *et al*., 2011; Wu *et al*., 2014). There should be the possibility of using AA mixtures derived from canola meal hydrolysis or AA fractions separated based on their polarity/charge (Chapter 6) in certain food applications and feed formulations.

Further to the direct application of free AA or AA mixtures, the current study showed the possibility of modifying them by attaching a C12 fatty acid chain using the Schotten Baumann reaction in an aqueous medium (Chapter 4). Lauric acid ($C_{12}H_{24}O_2$) has been used in commercial surfactants to provide a C12 hydrophobic chain to improve the hydrophobicity of molecules. The present study employed lauroyl chloride as the acylating agent to modify AAs with this hydrophobic chain, *i.e*., N-lauroyl AAs. N-acylated AAs, such as N-lauroyl-AA, are particularly important in the surfactant industry because of their suitability as mild surfactants with less skin irritability for incorporation into personal care products (Chen *et al*., 2020). Another reason for the attention on the synthesis of AA-based surfactants is the regulations of various jurisdictions that are coming into place on using environmentally benign surfactants. Market pull for greener surfactants constantly focuses on searching for alternatives to petroleum-based surfactants (Le Guenic *et al*., 2019; Morán *et al*., 2004; Pinazo *et al*., 2011). The majority of AA-based surfactants are readily biodegradable and non-toxic to aquatic organisms since their chemical bonds are easily broken by hydrolytic enzymes such as lipases and peptidases (Bordes & Holmberg, 2015; Infante *et al*., 2004). The properties of surfactants are based on their functional groups: co-presence of

hydrophobic and hydrophilic moieties in a single molecule. AA-based surfactants have functional groups (*i.e*., amino, carboxyl and side-chain groups of AA) that provide hydrophobicity, and the attached acyl group are a hydrophobic group depending on the length of the carbon chain. These groups yield the inter-and intra-molecular interactions in solution (Malik, 2015; Stubenrauch *et al*., 2017). Sodium salts of fatty N-acylated AAs have excellent solubility in water. Most AA-based cationic surfactants have been investigated and proven to have antimicrobial properties, but a comparatively lower number of studies are available on the antimicrobial activity of anionic surfactants (Bustelo *et al*., 2017; McKellar *et al*., 1992; Pinazo *et al*., 2016; Sreenu *et al*., 2015; 2014). The present study provides evidence for the possibility of using AA-based anionic surfactants that can inhibit the growth of both Gram-positive and Gram-negative bacteria. Moreover, AA-based surfactants have many uses as laundry detergents, emulsifiers, corrosion inhibitors, oil recovery aids and pharmaceuticals. Data presented in Chapters 4 and 5 confirmed that L-glutamic acid, glycine, L-leucine, L-lysine, L-proline, and L-valine as individual AAs or in a mixture can be converted to their respective N-lauroyl derivatives and these derivatives possess surface tension reducing ability, foam forming ability and antimicrobial activity (against selected organisms). In the current study, acylated derivatives of mixtures of AAs showed better functionality as surfactants and antimicrobials than most of the individual acylated-AA derivatives. Therefore, it was expected that a protein hydrolysate that contains above mentioned AAs would have superior properties due to the contributions from broad AA types.

In the present study, the canola meal protein hydrolysate after acylation had limited solubility, perhaps due to the solubility characteristics of acylated derivatives of different AAs in the mixture necessitating diverse solvent requirements. The actual hydrolysate contained more AA types than the mixture of selected six AAs prepared in Chapter 4 and assessed in Chapter 5. This diverse nature and the minor levels of other AAs may have resulted in only a portion of the N-acylated AAs in the final product being soluble in the solvent used.

The current study showed promising antimicrobial activities despite limited solubility and the fraction of soluble N-acylated AAs from the acylated hydrolysate of canola protein. The antibacterial activity of the prepared acylated products could be employed by the high-end, organic, greenhouse horticulture industry against plant pathogens. Moreover, the growth-

stimulating ability of protein hydrolysates has been reported and has been attributed to the activity of free AA and peptides as elicitors of certain biochemical pathways beneficial to plant health (Abdel-Mawgoud *et al.*, 2011; Murashev *et al.*, 2020; Radkowski *et al.*, 2020; Sarojnee *et al.*, 2009; Thomas *et al.*, 2009). As shown in the present study, the dual functionality of the synthesized N-acyl AA derivatives is advantageous because surfactant and antibacterial activities can act simultaneously in a single application. As an active compound in a cleaning product formulation, N-acyl AA derivatives can provide removal of harmful bacteria as well as lower the surface activity of lipid residues making them more water-soluble. This bi-functionality fits well with laundry, skin, or physical surface cleaning. The same bi-functionality can be advantageous against plant pathogens, particularly in formulations such as foliage sprays. The surface properties (*e.g.*, wettability, foaming) can provide uniform coverage over a higher surface area on the protective lipid/wax coating of leaves.

This study showed that canola meal could be utilized as a protein-rich, agri-processing by-product. The proteins can be recovered from the meals irrespective of the oil extraction method it has undergone, but pre-treatment with ethanol can enhance the applicability of meals for protein extraction. Furthermore, the generation of free AAs from extracted proteins can be achieved through acid hydrolysis, and resulting AAs are versatile building blocks for molecules/compounds with useful physicochemical and biological properties. Investigations carried out in this study demonstrated an alternative approach to utilizing CP and DT canola meals, which applies to other protein-rich, agri-processing by-products.

7.2 Future work

Based on the observations of the current project, the following suggestions are presented for continuation of the work.

- Scaling up the full process of converting canola meal into amino acids (AAs) is needed to prove the concept. Data from the present study showed that ethanol pre-treatment was beneficial for CP meal and offered no advantage for DT meal. To address complete utilization of CP meal, recovery of residual oil using ethanol (99%), followed by alkali extraction and hydrolyzing into AAs, should be considered. Implementing a process with measures to minimize cost for solvents, *e.g.*, reusing ethanol, the simultaneous

concentration of proteins, removing small molecules and liquid volume reduction of alkali meal extract, *e.g.*, ultrafiltration, and direct hydrolysis of concentrated protein, require further investigation. DT meal can be used without the ethanol washing step. Laboratory-scale acid hydrolysis in the current study demonstrated a reduction in acid concentration at the end of hydrolysis. Determination of the non-protein components in the protein extract, along with the assessment of the protein fraction recovered at pH 12 for non-protein components, such as phenolic compounds, sugars, phytates and fibre, prior to acid hydrolysis, will be useful in addressing this issue.

- In the scaled-up process of acid hydrolysis, a large quantity of meal residue (the acid-insoluble fibre fraction) is produced as a co-product. With DT meal, in particular, more protein and partially hydrolyzed protein will be retained with this fibre co-product because of its lower protein extractability compared to CP meal. Investigating this hydrolysis-modified protein and fibre fraction of canola meal will provide useful information.

- Investigations on the applicability of other protein de-construction methods prior to acid hydrolysis and to facilitate peptide bond breakage may be useful for reducing the use of highly concentrated acids in the process. Physical processes and methods, such as high-pressure homogenization and steam explosion, may be examples here.

- AAs recovered from canola meal protein can be investigated for different chemical modifications to improve hydrophobicity and functional characteristics. Reaction pathways to i) utilize the α-amino function of AAs (*e.g.*, alkylating with a long chain alkyl halide), ii) utilize the carboxylic group of AAs (*e.g.*, reacting with fatty alcohol or long-chain amine), iii) form dimeric or gemini-type structures, iv) form bola-amphiphiles, and v) form glycerolipid-like structures, can be studied to diversify functionalities. Since the length of the hydrocarbon chain attached to the AA has been reported to alter the activity of the resulting molecule, the same N-acylation reaction followed in the present study could be used to generate molecules with different alkyl chain lengths (short and long chains) to provide modified AAs with different physicochemical and biological properties.

- The applicability of the N-acylation reaction to hydrolysates containing mixed AAs should be studied further, improved and refined. Increasing product yields and product-solvent compatibility may be the main factors to study. Working with AA mixtures, sometimes from partially hydrolyzed proteins, caused problems in re-constituting after drying, even under milder drying conditions such as freeze-drying. Moreover, studies are needed to understand better the soluble portion of the acid hydrolysate, which may contain peptides, free sugars, partially hydrolyzed fibre, *etc*., which can participate in acylation reactions and affect product purity and yield.

- N-acylated derivatives of AA (individual and mixtures) should be further evaluated for parameters such as wettability, detergency, calcium tolerance, kraft temperature, and emulsification. In so doing, the suitability of these molecules as detergents can be further confirmed. Moreover, evaluation of their skin compatibility is needed for use in personal care applications by testing for cytotoxicity and skin irritability. The impact on the environment is another important criterion for evaluating novel compounds for use as surfactants. Therefore, evaluation of their biodegradability is required.

- N-acylated hydrolysates need to be evaluated for their surface activity. Due to the low product yield obtained and practical concerns related to project duration and the global pandemic, the current study could not be extended that far.

- The biological activity of the synthesized N-acylated derivatives of individual AA, AA mixtures, AA fractions and acid hydrolysates should be further evaluated. Studies on antimicrobial activity can be expanded using a broad spectrum of bacterial and fungal strains, which can be non-pathogenic, pathogenic to humans, animals or plants, or food-spoilage organisms, to understand the efficiency of and resistance to these compounds. Furthermore, it is necessary to investigate the risks associated with releasing novel compounds into the environment. Their toxicity profile, including mammalian toxicity, should be evaluated to help them move to the next step for use as drug candidates providing anti-bacterial or anti-fungal activity. Moreover, their suitability as plant growth simulants should be tested.

- Furthermore, it would be worthwhile to investigate the properties of canola meal hydrolysates and their fractions (separated according to the polarity and charge of AAs) in direct applications as surface-active agents and as anti-biologicals, *e.g.*, as antimicrobial agents. In such direct applications, it will be necessary to make the mixture of AAs pH neutral and salt-free to observe the effect of AAs.

- Refining and further development of processing steps should be carried out to develop an economical and feasible platform technology for enhancing the value of protein-rich materials with less value, *i.e.*, oilseed meals that cannot enter into food or feed markets due to inherent compositional limitations with respect to standards set by food and feed industry, *e.g.*, mustard and industrial oilseeds, such as castor bean, *etc*., or any other biomass that cannot enter into the food and feed use. This technology platform may be suitable for utilizing animal industry waste materials such as feathers and hides.

As mentioned above, the study can be extended to develop a scaled-up process which will be useful to understand the feasibility of moving the steps to large-scale production. Converting canola meal-derived AAs into useful functional molecules can be further supported by identifying possible other modification routes and expanding the testing for further biological activities. Moreover, identifying the functionality of N-acylated protein hydrolysate would provide potential routes of utilizing extensively processed DT canola meal with limited applicability.

8. OVERALL CONCLUSIONS

Canola/rapeseed (*Brassica napus*) is an oil, protein and fibre-rich seed containing other minor constituents such as simple and complex phenolic compounds, glucosinolates, phytates, sinapine, and free sugars. Commercially, the oil component brings the largest economic revenue to the canola seed industry. Investigations in the present study used the solid residue or meal generated from two different oil extraction processes used by the canola industry, namely cold-pressed (CP) meal and desolventized-toasted (DT) meal, as sources of amino acids (AAs) that can be further utilized. CP meal contains higher level of oil (133.9 ± 4.2 mg oil/g meal, dbm) than DT meal (43.3 ± 0.3 mg oil/g meal, dbm). Pre-treatment of canola meal with ethanol (99%) at a meal-to-solvent ratio of 1:4 (w:v), 50°C and for 30 min was able to reduce the oil content of CP by 88% resulting in a meal with 16.1 ± 0.2 mg oil/g meal. The same treatment conditions reduced the oil content of DT meal by 78%, resulting in a meal containing less than 1% oil. Concomitant removal of phenolic compounds and free sugars (reduction was limited to about 20%) was achieved for both meals during ethanol pre-treatment.

Pre-treatment with ethanol had a positive effect on protein purity when extracted at pH 12, because of the lesser amount of non-protein compounds in the meal that were co-extracted. This effect was clear with CP meal, where 14% increment in extracted protein amounts in the dry matter in a single extraction compared to the lower protein enrichment observed for DT meal - only a 3% increase in protein in the dry matter. Hydrolysis of untreated CP meals showed that the presence of more oil interfered with protein breakdown and release of AAs, as ethanol pre-treated CP meal yielded total of 884 mg AAs per g proteins as compared to untreated CP meal, which produced 766 mg AAs per g proteins. This means that ethanol pre-treatment facilitated protein extractability and hydrolysis.

Sulphuric acid (H_2SO_4) can be used as an alternative to hydrochloric acid (HCl) for canola meal

protein hydrolysis and releasing AAs; however, a higher concentration of H_2SO_4 was required than with HCl, despite the diprotic nature of H_2SO_4. Meal protein from DT meal required a higher concentration of H_2SO_4 to yield the same level of free AAs as protein from CP meal; protein from DT meal protein required 8 M H_2SO_4 to release 82% of the total AAs, while protein from CP meal yielded a similar amount with 4 M H_2SO_4. A lower acid concentration, such as with 1.5 M H_2SO_4, generated more peptides than free AAs than HCl hydrolysis at the same concentration. Mainly L-form AAs were found in the mixtures obtained from acid hydrolysis of canola protein. Two different oil extraction processes applied different external stresses on canola seeds. CP meal was not exposed to high heat, pressure, or non-polar solvent, unlike DT meal produced by PSE. Extensive modification of seed proteins and interactions with other seed components affected protein solubility and extractability and hydrolysis of the intact peptide bonds, which was observed in DT meal proteins.

The dominant AAs in canola seed protein, namely glutamic acid, lysine, leucine, proline, valine, and glycine, can be chemically modified using the Schotten Baumann reaction with lauroyl chloride to generate their N-acylated derivatives. The sodium salts of these derivatives have surface tension reducing ability (surface tensions of 29-64 mN/m and critical micelle concentrations of 5.1-9.9 mM) and possess foaming abilities ranging from 117% - 275%, which are important considerations for surfactant molecules. All of the derivatives possessed antimicrobial activity against both Gram-positive and Gram-negative bacteria of the tested pathogenic (*Salmonella Enteritidis, Listeria monocytogenes*) and non-pathogenic (*Escherichia coli, Pseudomonas fragi, Lactobacillus plantarum, Lactococcus lactis*) organisms compared to sodium dodecyl sulphate (SDS). When these modified AAs were in a mixture in the ratio found in canola protein acid hydrolysates, all of the properties were enhanced compared to those exhibited by the individual AAs.

The free AAs from the canola meal protein hydrolysates could be fractionated based on the charge and polarity of the AAs, which provided more purified reactants for derivatization than the crude hydrolysates. Free AA mixtures of both fractionated and unfractionated hydrolysates of DT meal protein had limited solubility and were somewhat restricted in generating N-lauroyl derivatives. Sodium salts of N-lauroyl derivatives of AAs in mixtures from the DT protein hydrolysate exerted

growth inhibitory effects on all of the aforementioned bacterial strains at lower doses than SDS, which was used as a reference surfactant molecule. Therefore, the lauroyl derivatives of canola AA mixtures can be both antimicrobial and surfactant molecules. The investigations of this study confirmed that industrially processed canola meal protein could be utilized to generate AAs that can be functionalized as surfactant and antimicrobial compounds. This may be an alternative way of adding value to DT canola meal protein which has limited use beyond the animal feed. CP canola meal protein is more readily amenable to such uses than DT canola meal protein which has been subjected to more severe processing stresses.

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APPENDICES

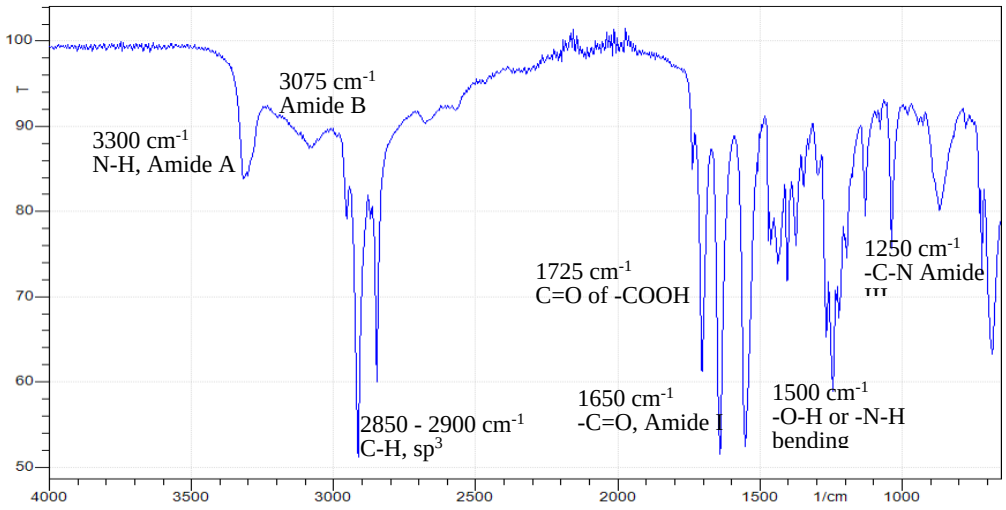


Figure A1: FTIR spectrum of N-lauroyl glycine

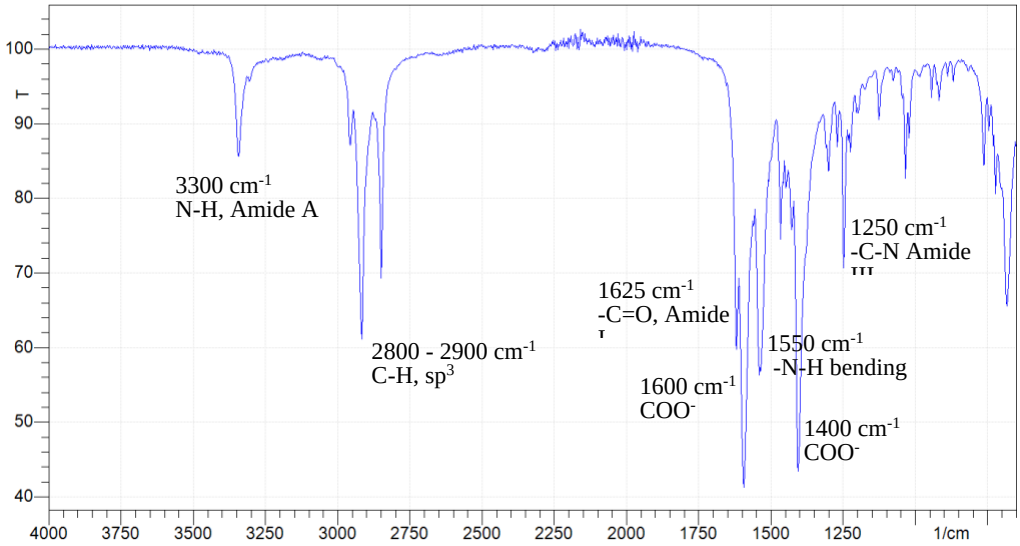


Figure A2: FTIR spectrum of sodium N-lauroyl glycinate

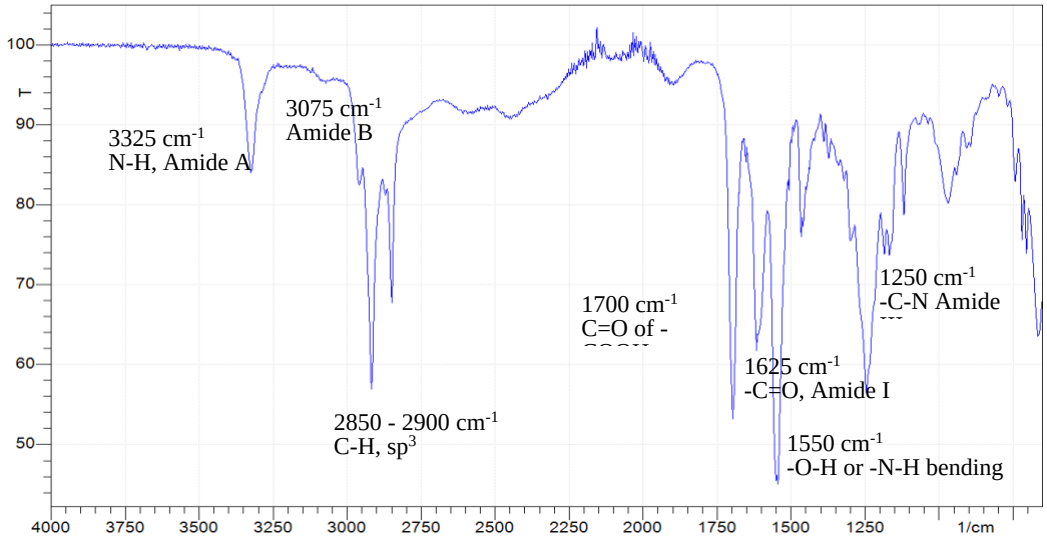


Figure A3. FTIR spectrum of N-lauroyl valine

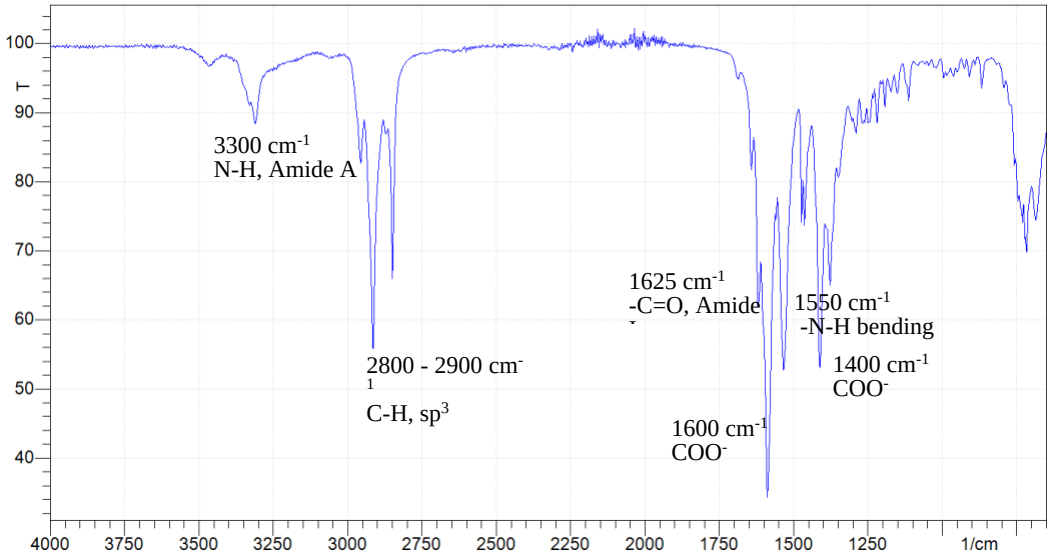


Figure A4. FTIR spectrum of sodium N-lauroyl valinate

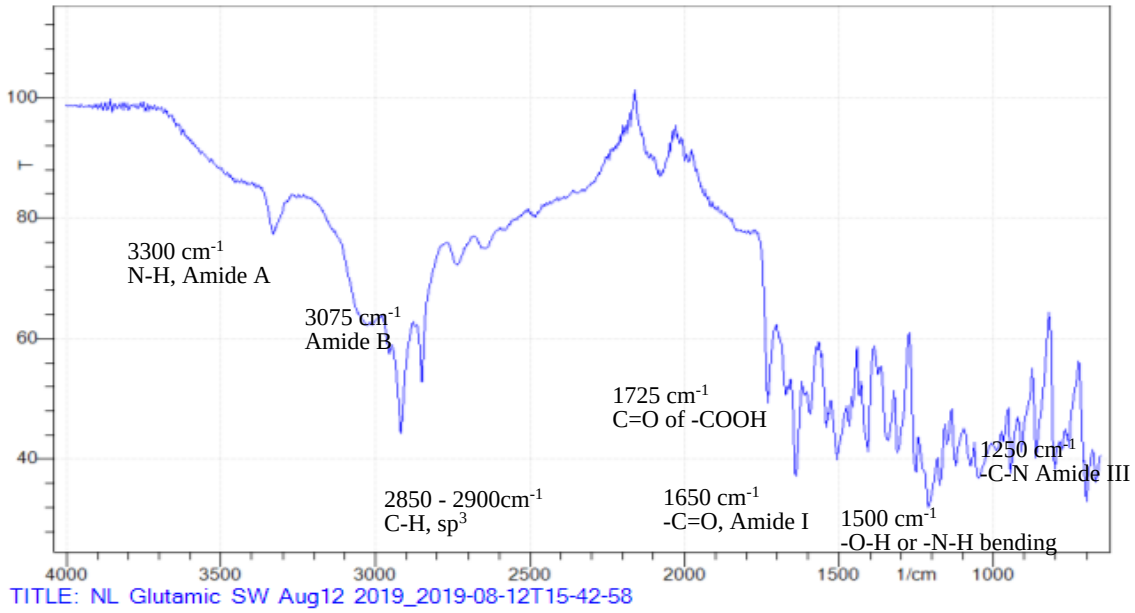


Figure A5. FTIR spectrum of N-lauroyl glutamic acid

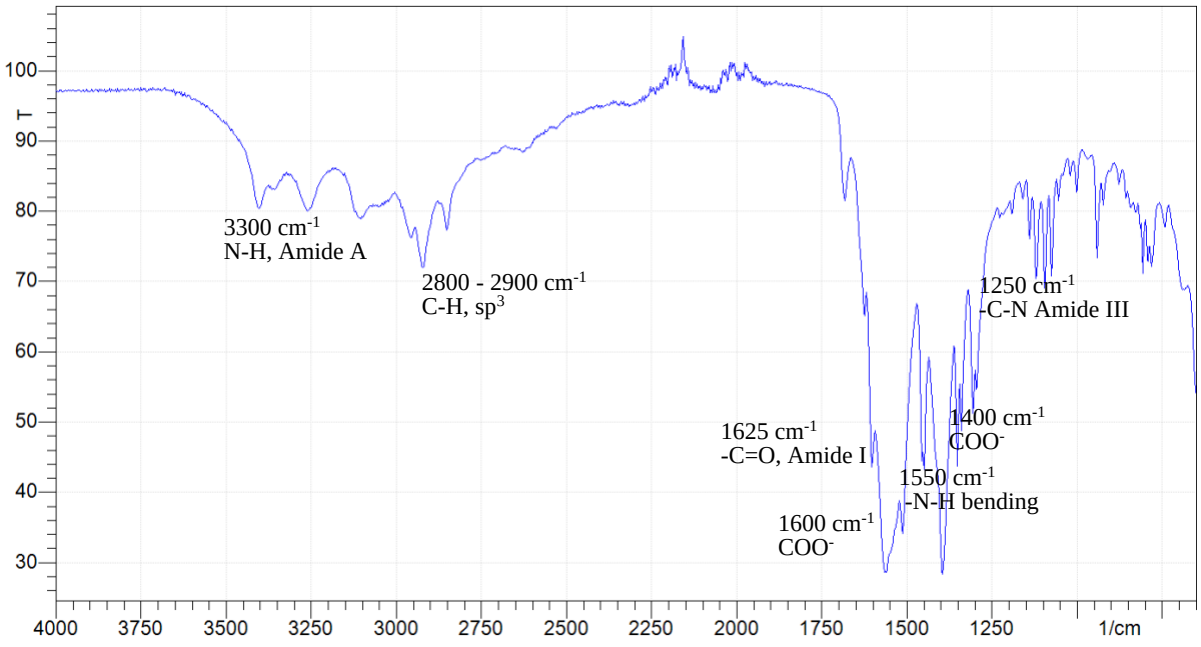


Figure A6. FTIR spectrum of Sodium N-lauroyl glutaminate

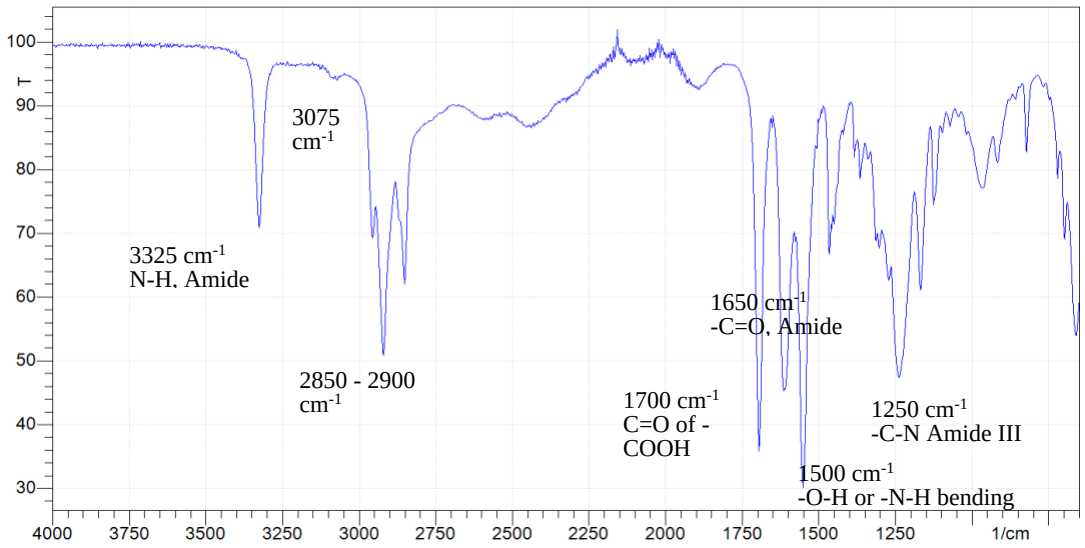


Figure A7. FTIR spectrum of N-lauroyl leucine

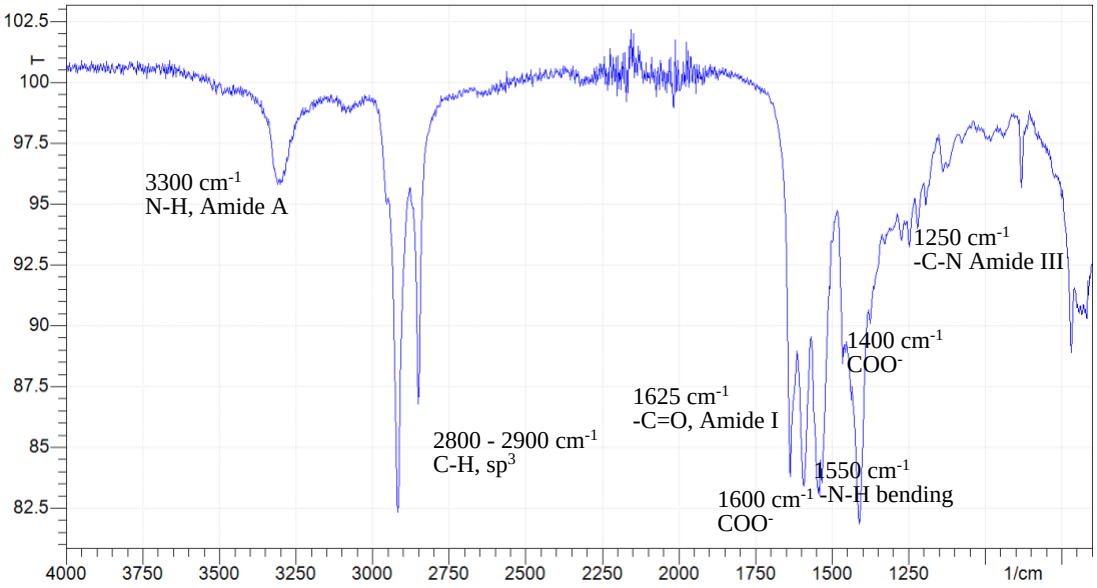


Figure A8. FTIR spectrum of Sodium N-lauroyl leucinate

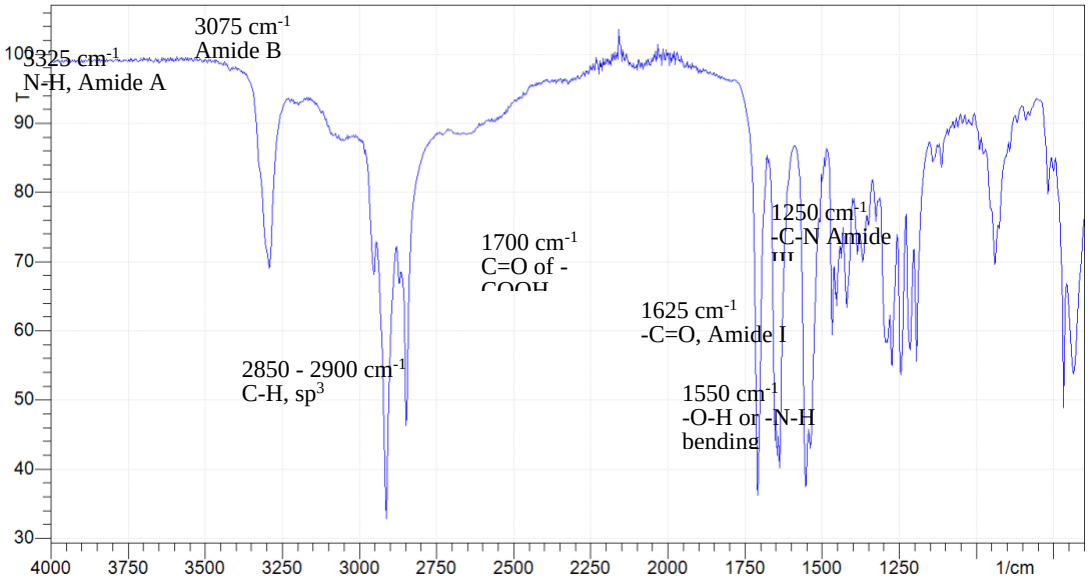
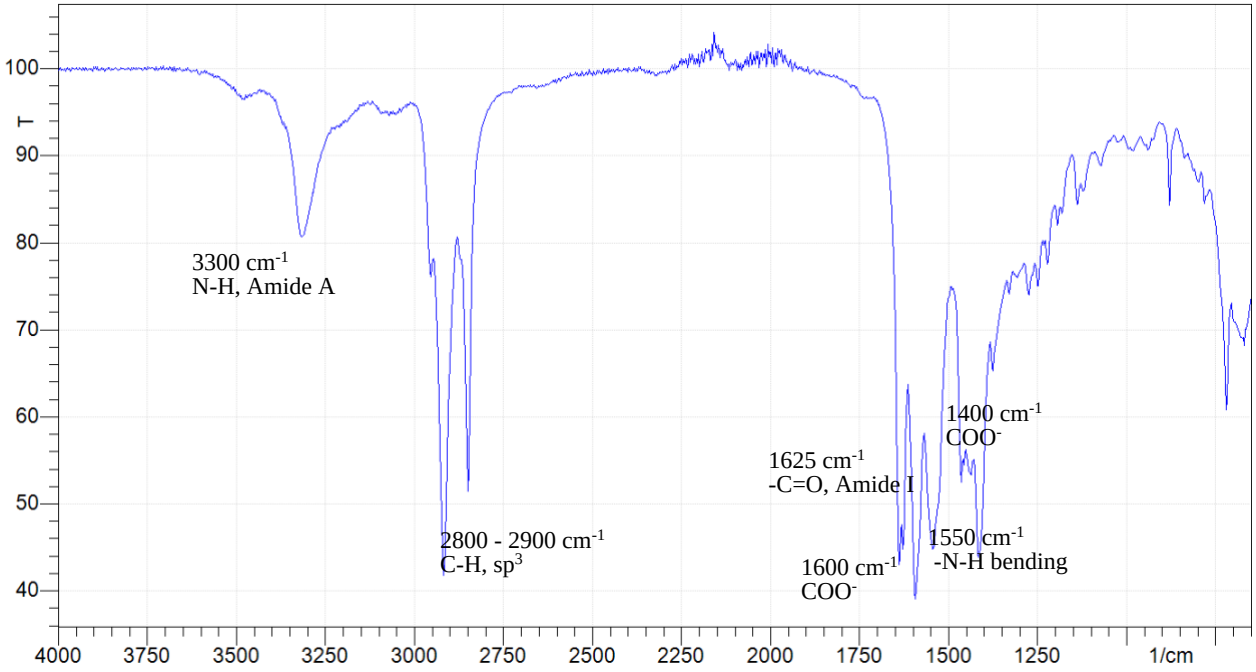


Figure A9. FTIR spectrum of N-lauroyl lysine



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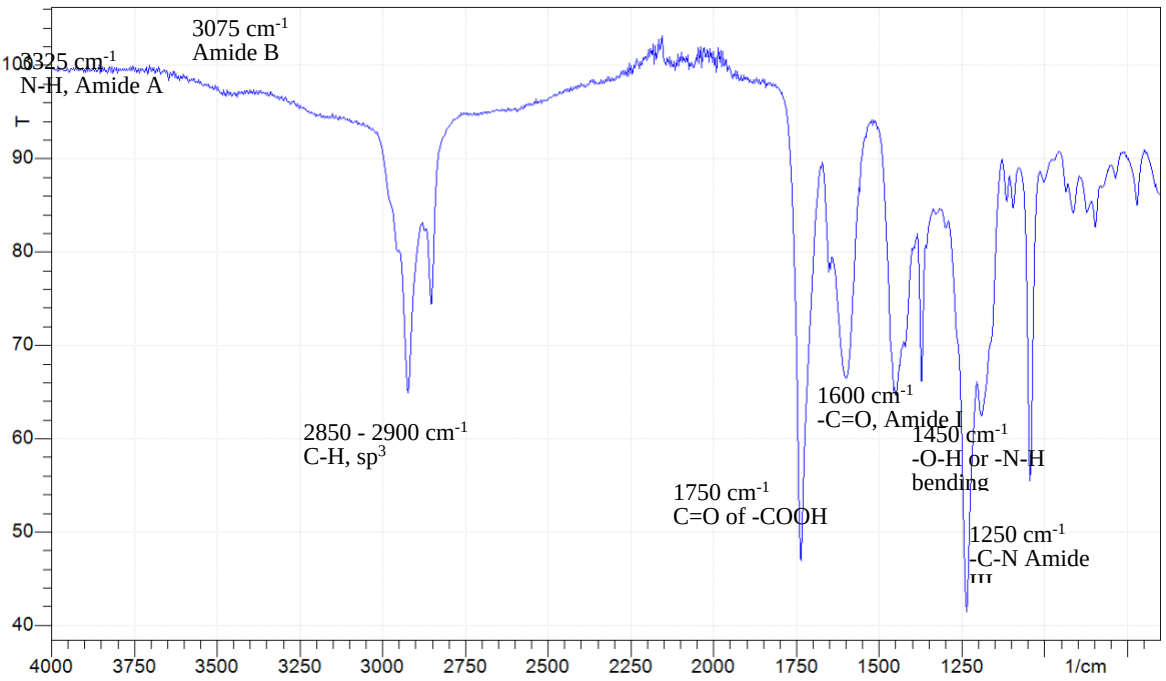


Figure A11. FTIR spectrum of N-lauroyl proline

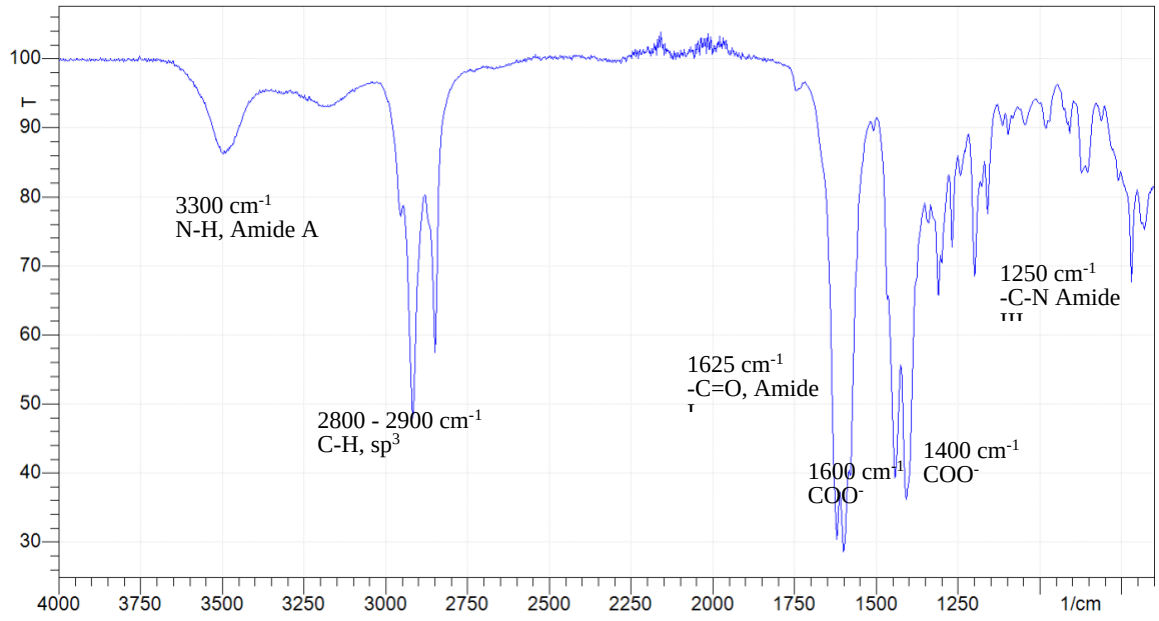


Figure A12.FTIR spectrum of N-lauroyl prolinate

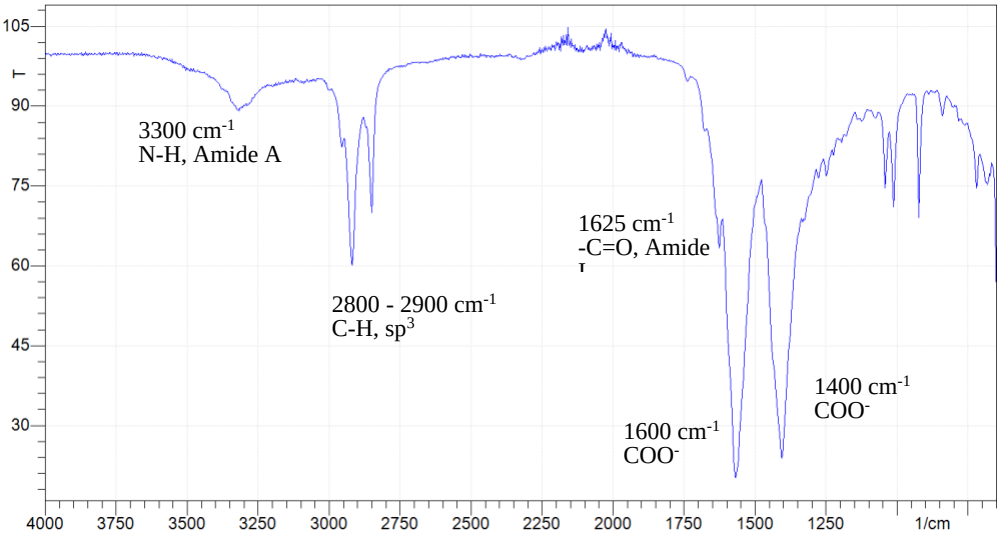


Figure A 13. Sodium salt of N-lauroyl mixture of amino acids

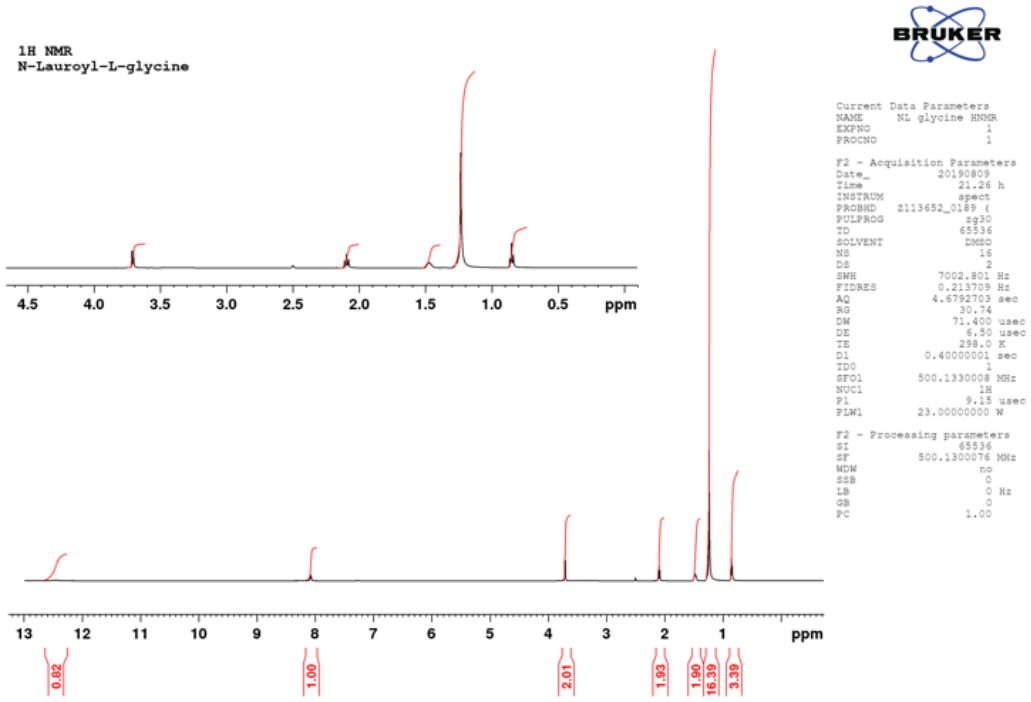


Figure A14. ^{1}H NMR spectrum of N-lauroyl glycine

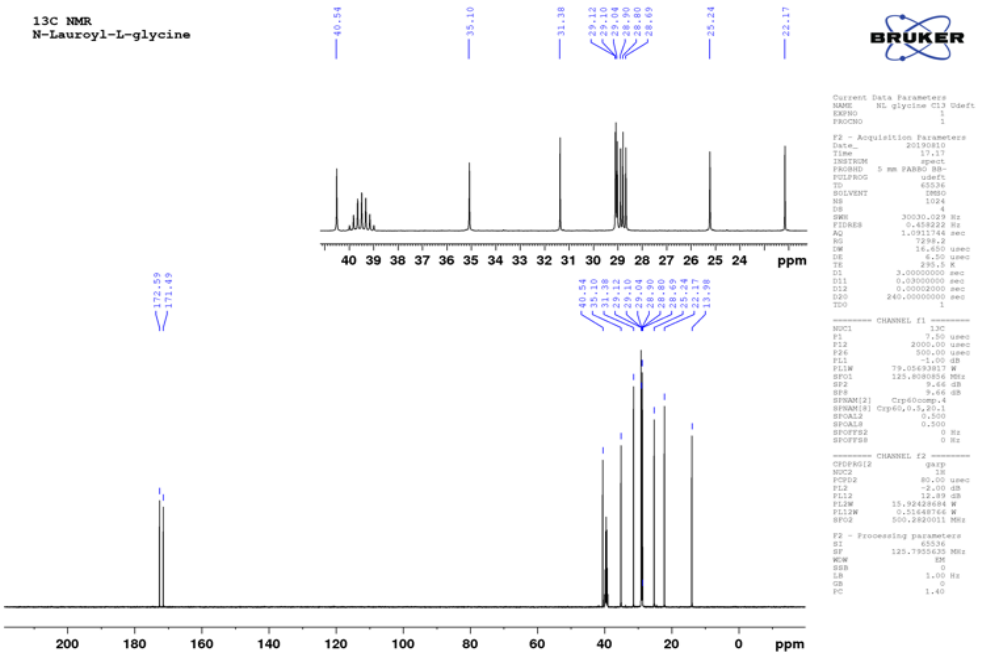


Figure A15. ^{13}C NMR spectrum of N-lauroyl glycine

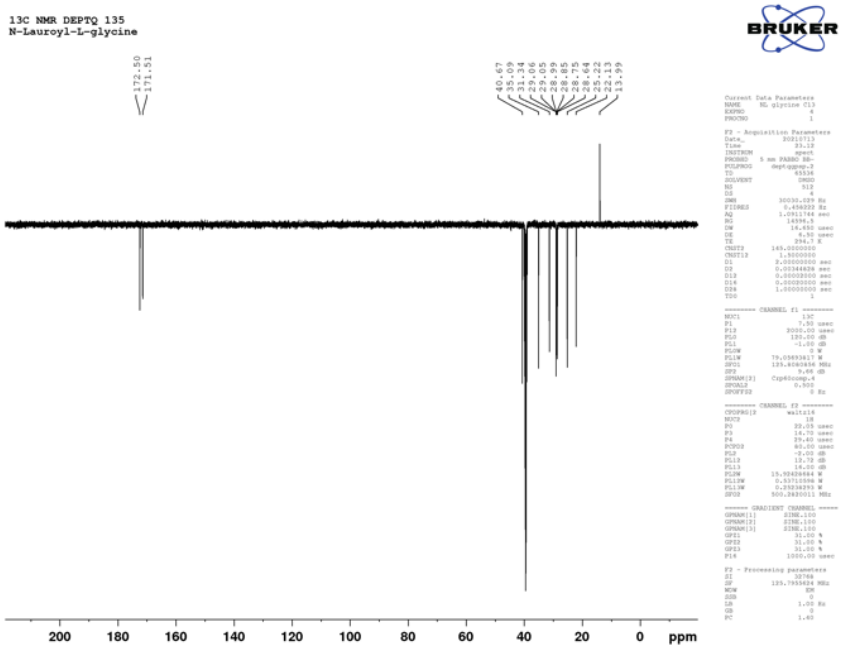


Figure A16. ^{13}C NMR DEPTQ 135 spectrum of N-lauroyl glycine

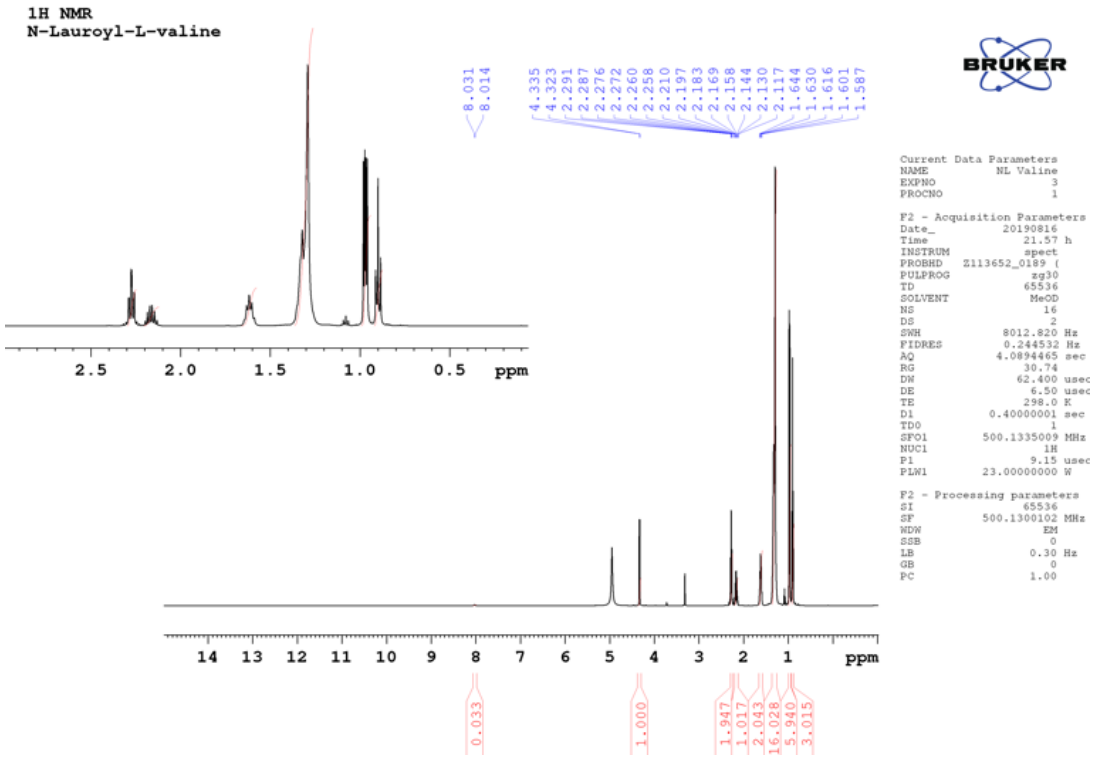


Figure A17. ^{1}H NMR spectrum of N-lauroyl valine

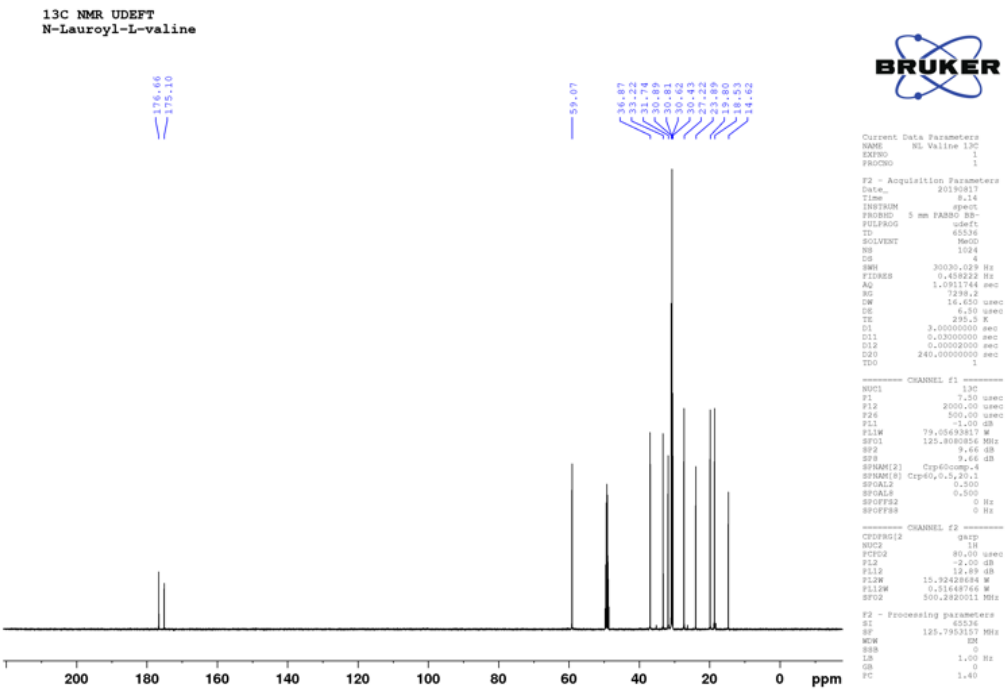


Figure A18. ^{13}C NMR UDEFT spectrum of N-lauroyl valine

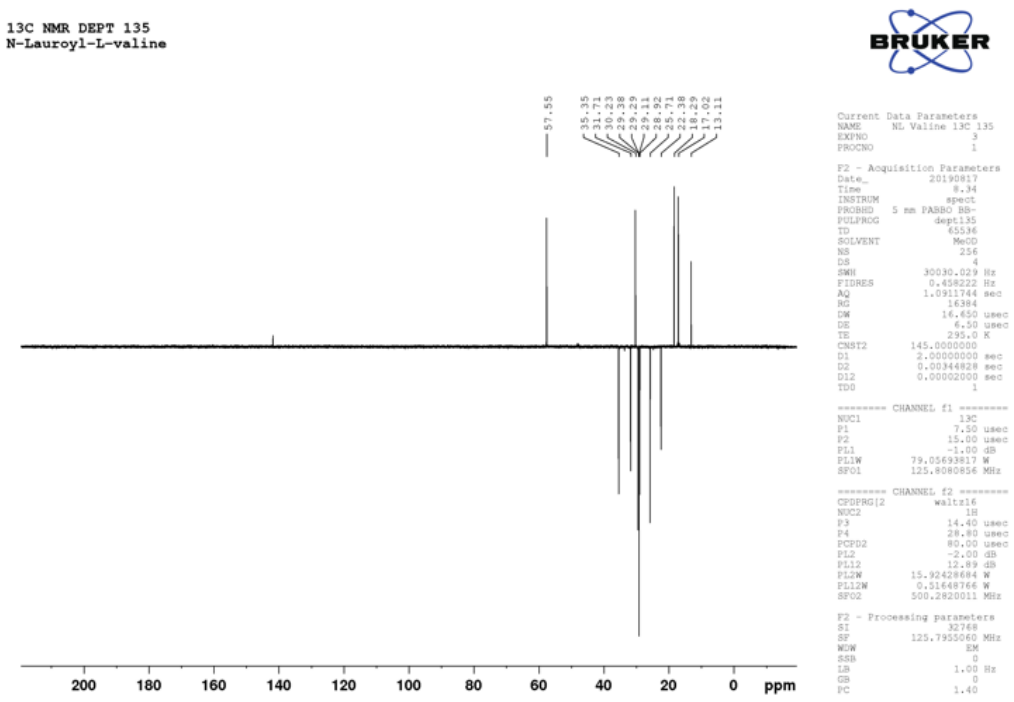


Figure A19. ^{13}C NMR DEPTQ 135 spectrum of N-lauroyl valine

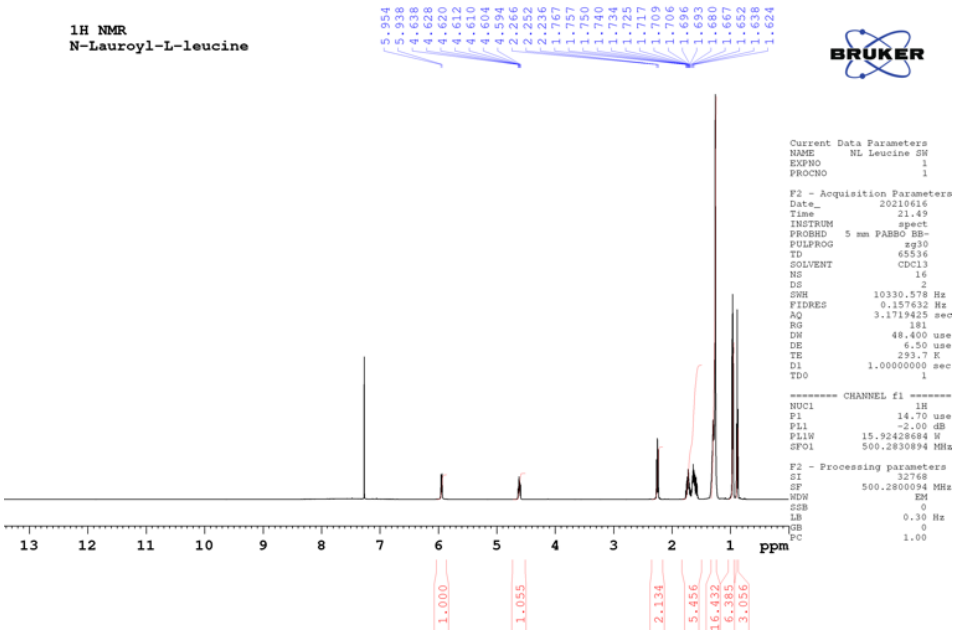


Figure A20. ^{1}H NMR spectrum of N-lauroyl leucine

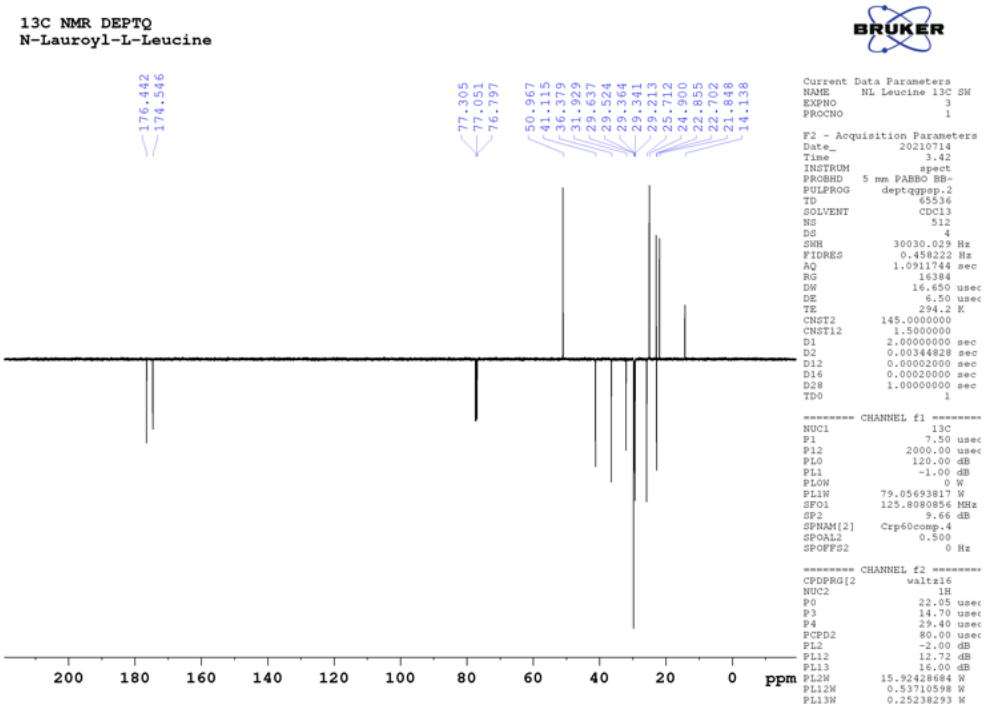


Figure 21. ^{13}C NMR spectrum of N-lauroyl leucine

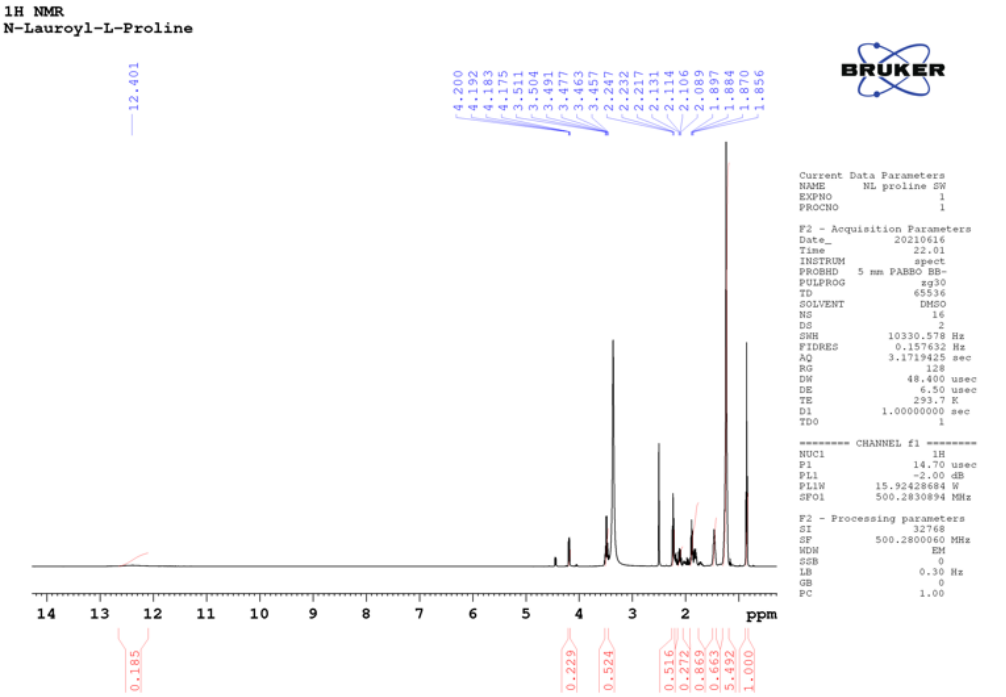


Figure A 22. ^{1}H NMR spectrum of N-lauroyl proline

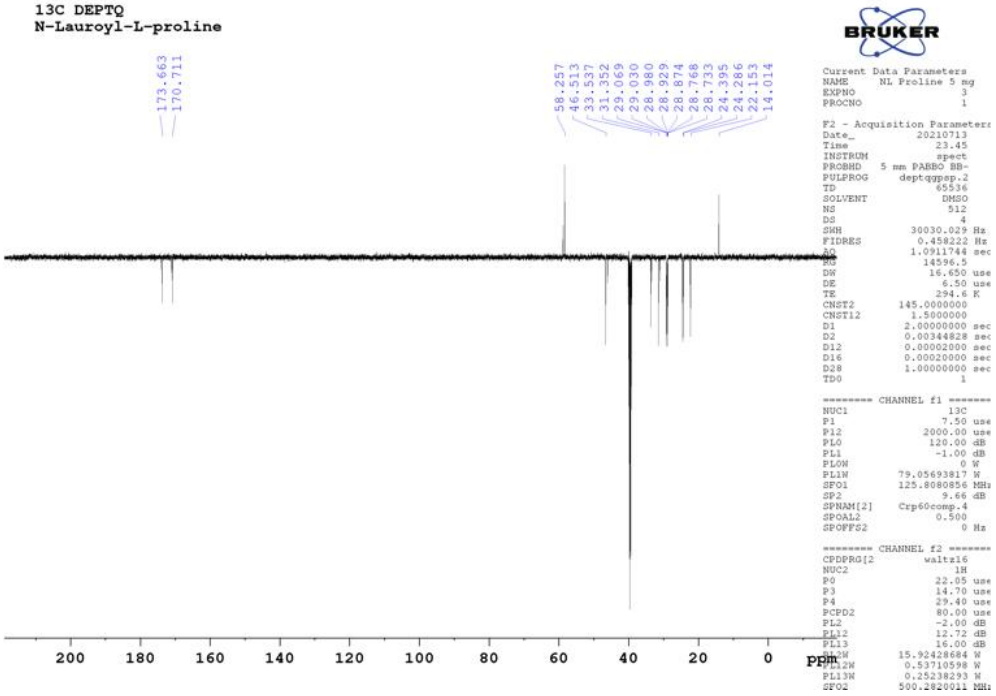


Figure A 23. ^{13}C DEPTQ NMR spectrum of N-lauroyl proline

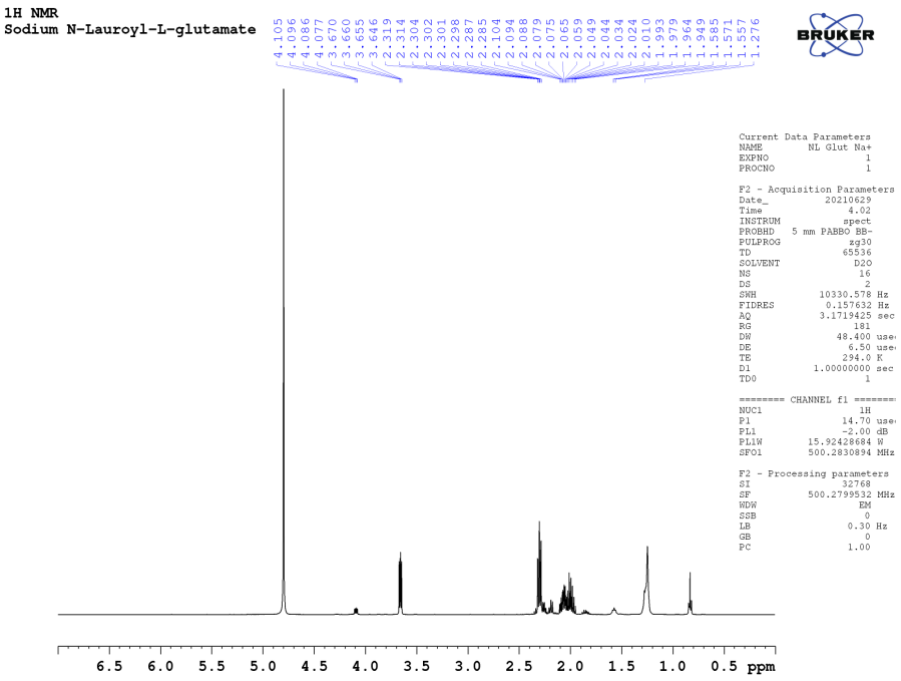


Figure A24. ^{1}H NMR spectrum of sodium N-lauroyl glutaminate

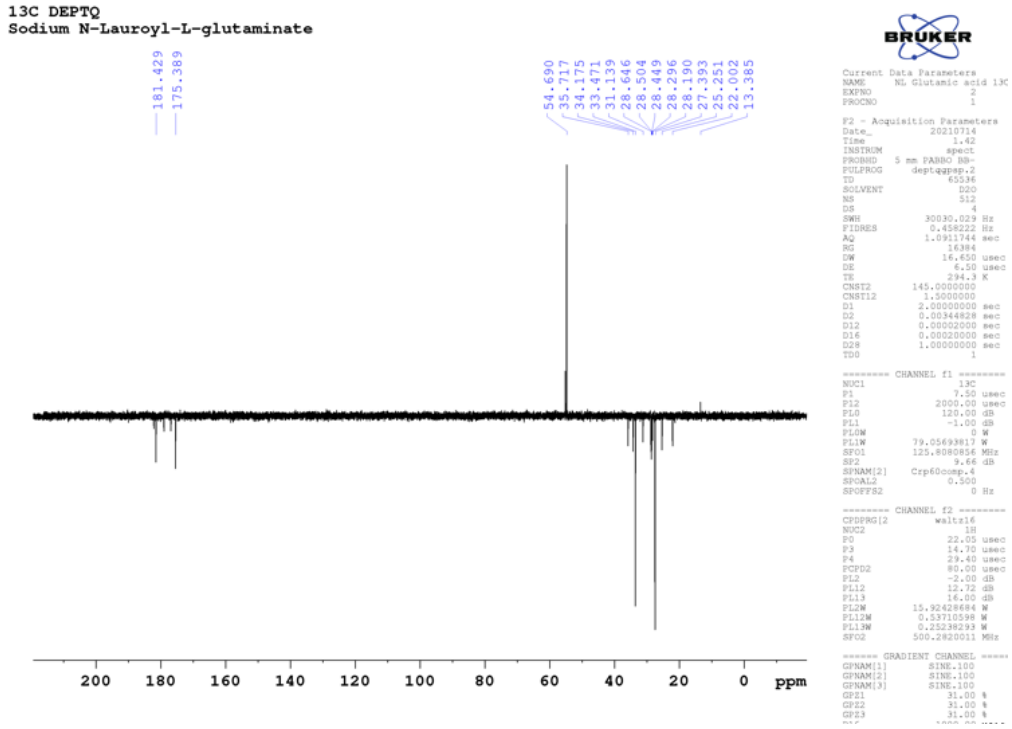


Figure A25. ^{13}C DEPTQ NMR spectrum of sodium N-lauroyl glutaminate

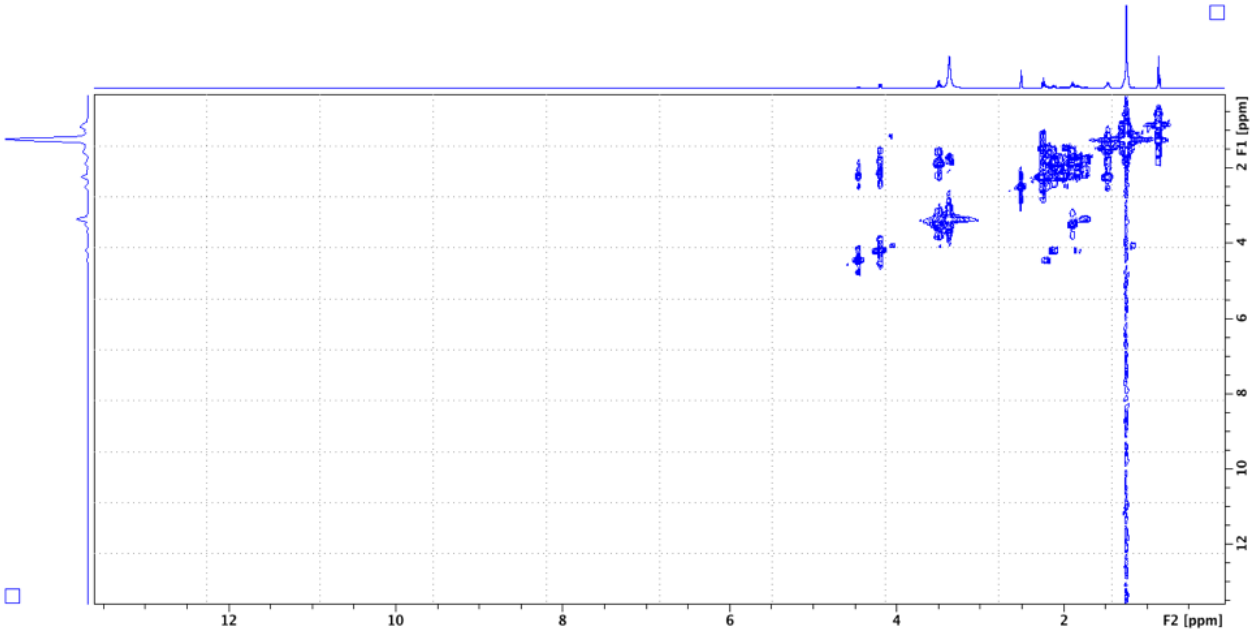
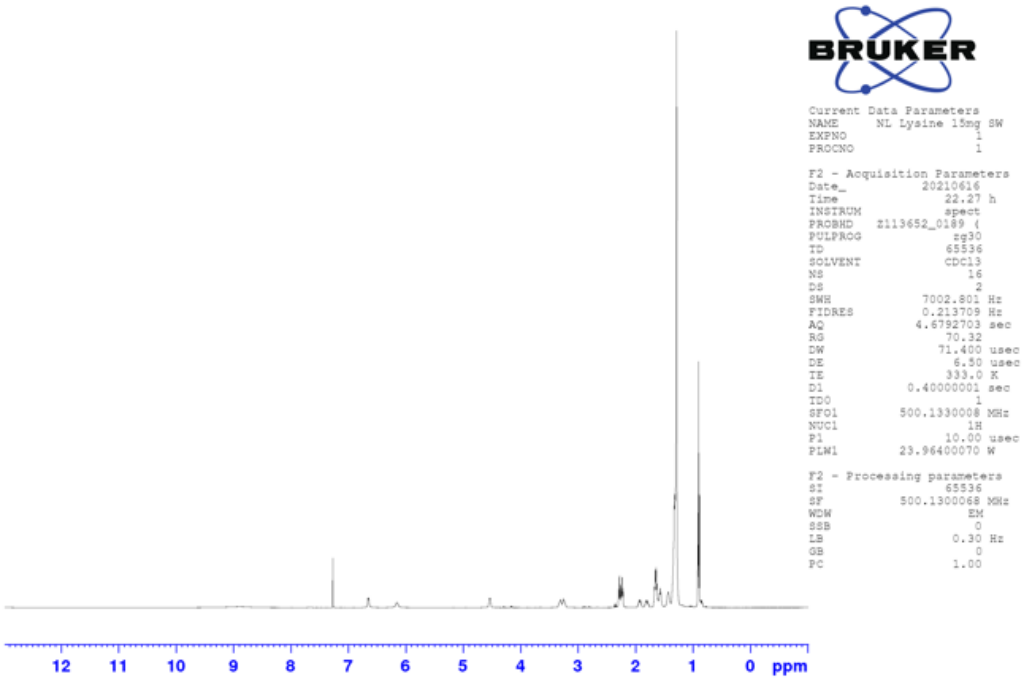


Figure A 26. COSY 2D NMR spectrum of N-lauroyl proline



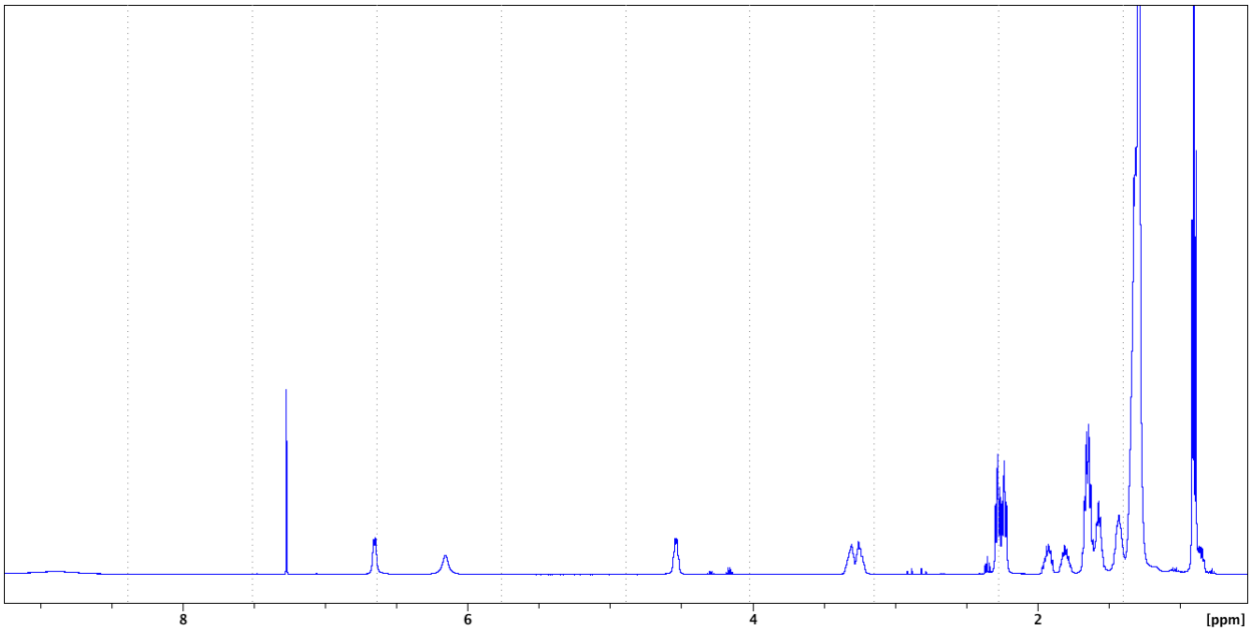


Figure A27. ^{1}H NMR spectrum of N-lauroyl lysine

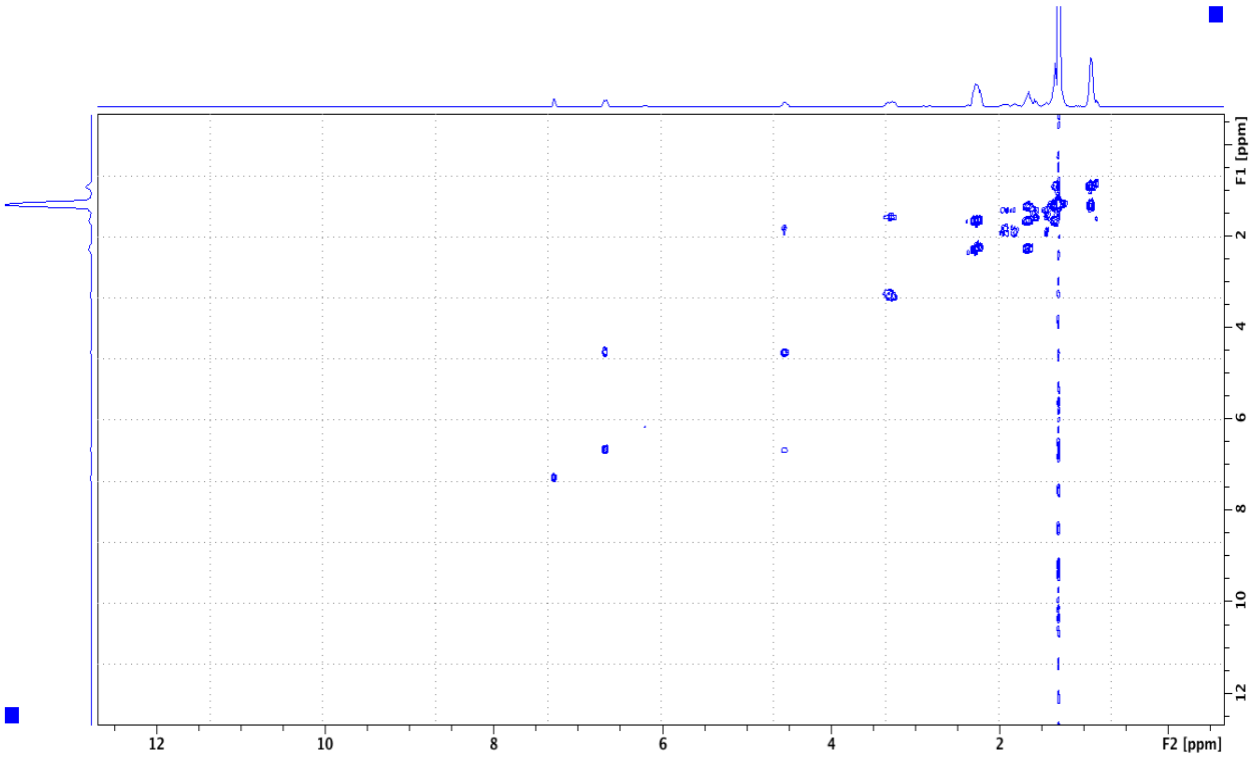


Figure A28. COSY 2D NMR spectrum of N-lauroyl lysine

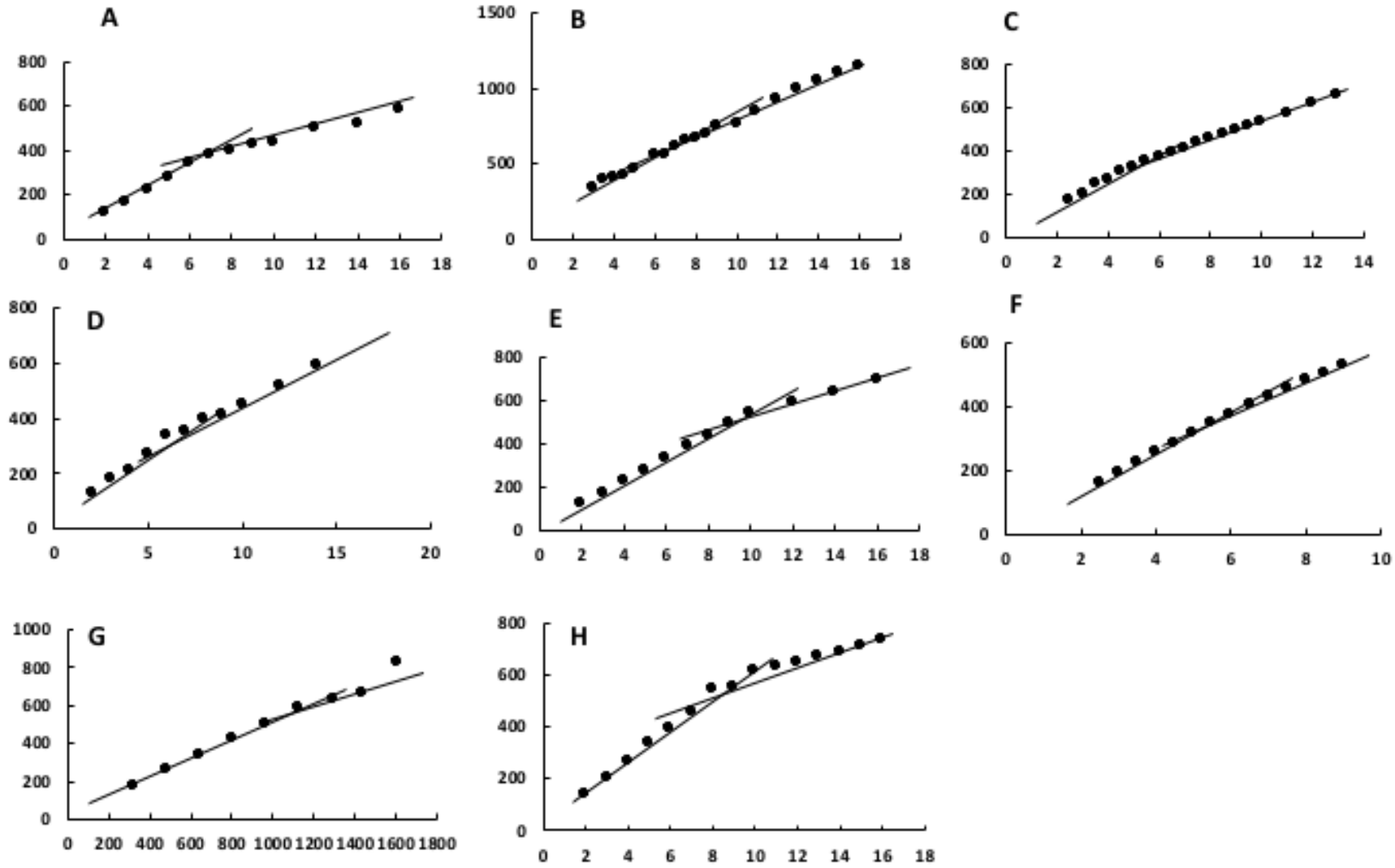


Figure A29. Conductivity curves for sodium salts of A) N-lauroyl glutaminate B) N-lauroyl glycinate C) N-lauroyl leucinate D) N-lauroyl lysinate E) N-lauroyl prolinate F) N-lauroyl valinate G) N-lauroyl mixture of AA H) SDS