EXTRACTION OF ORBITIDES FROM FLAXSEED

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By

Da Wang

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ABSTRACT

The goal of this project is to establish an efficient and economical industrial process for extraction of a Kaufmann and Tobschirbel orbitide (KT) mixture from flaxseed oil. KTs occur at a low level in flaxseed oil and must be concentrated at least 600 fold to produce a useful commercial concentrate. KT peptides are more polar than most lipids may be separated using solid- or liquid- phase extractants. Extraction protocols were investigated to determine a better approach for KT peptide extraction. Commercial solid-phase extraction methods would require the adaptation of bench-scale silica flash column chromatography. The first approach was to develop methods for separation of peptides using only silica, ethyl acetate and ethanol. Ethyl acetate is known to remove both oil and peptides from silica. Therefore, the ability of low temperature to decrease the peptide elution from silica was studied. The other method utilized liquid-liquid extraction. In order to measure the success of an extraction an analytical method was required to evaluate the separation of peptides from oil. An analytical procedure was developed that readily determined the relative concentration of peptide and lipid. Aqueous and anhydrous ethanol partitioning was used to extract the KT mixture from flaxseed oil. Ethanol solutions between 50 and 100% in water (v/v) were mixed with flaxseed oil. The oil and peptide content of the extracts were determined using ^{1}H-NMR. Liquid-liquid extraction using 70% aqueous ethanol at volume ratio (solvent to oil) of 0.25:1 produced a mole ratio of 2:1 (KTs to oil) making it the optimal solvent for KT extraction. In the second part of this project, the scale of liquid-liquid extraction was increased through several 10 to 30-fold steps to establish a potential industrial extraction process for recovery of the KT mixture. The feasibility of processing the solvent containing mixed peptides was investigated. Multiple evaporation and adsorption methods were also tested, including falling film evaporation, rotary evaporation, a combination of rotary evaporation and freeze drying, and a combination of rotary evaporation and spray drying. Various experimental methods to enrich and isolate KTs from water-rich fraction were performed. At the end of this project, 3328.89 g of KT mixture was produced that was suitable for commercial purposes. The increase of extraction scale was 140,000 fold.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CD	Circular dichroism
$CDCl_3$	Deuterated chloroform
KT	Kaufmann and Tobschirbel orbitide
CsA	Cyclosporin A
CSR	Chromolith® SpeedROD
DAG	Diacylglycerides
DMF	Dimethylformamide
ESI	Electrospray ionization
FAME	Fatty acid methyl ester
FDA	Food and drug administration
FFE	Falling film evaporator
FTIR	Fourier transform infrared spectroscopy
GMP	Good manufacturing practices
HPLC	High-performance liquid chromatography
HR-FABMS	High resolution-fast atom bombardment mass spectrometry
IC_{50}	The half maximal inhibitory concentration
IR	Infrared spectroscopy
MAG	Monoacylglycerides
MeOD	Deuterated methanol
Met	Methionine
MetO	Methionine *S*-oxide
$MetO_2$	Methionine *S*,*S*-dioxide
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information

NMR	Nuclear magnetic resonance
OSI	Oxidative stability index
PDE	Permitted daily exposure
PMA	Phosphomolybdic acid
Seg-A	Segetalin A
TAG	Triacylglycerides
TLC	Thin-layer chromatography
TOF	Time of flight

CHAPTER 1
INTRODUCTION

Flax (*Linum usitatissimum* L.) is one of the significant oilseed crops in Canada. On average, Canadian brown flaxseed has 41% lipid, 20% protein, 28% fibre, 7.7% moisture and 3.4% ash (Canadian Grain Commission, 2001). Flaxseed oil contains high levels of α-linolenic acid, a type of omega-3 fatty acid, which has been commercially available as a supplement with various health benefits.

Kaufmann and Tobschirbel orbitides (KTs) are a group of cyclic peptides identified and isolated from flaxseed, containing eight or nine amino acids. They belong to the family of Caryophyllaceae-type cyclopeptide (Tan and Zhou, 2006). Knowledge about KTs has been greatly expanded since the first isolation of [1–9-NαC]-KTA (**1**) in 1959. So far, sixteen KTs have been identified in flaxseed. Two gene accessions from flax gene database show embedded KT sequences (Sayers *et al.*, 2009). Many instruments were utilized for the identification and structural elucidation of KTs, including high-performance liquid chromatography (HPLC), HPLC-mass spectrometry (MS), ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, tandem MS, etc. (Naider *et al.*, 1971; Tancredi *et al.*, 1991; Morita *et al.*, 1999; Stefanowicz. 2001; Gui *et al.*, 2012; Olivia *et al.*, 2012; Jadhav, 2013). However, a systematic study of KT concentration in flaxseed and flaxseed oil was not achieved until recently (Gui *et al.*, 2012; Olivia *et al.*, 2013). Establishment of calibration curves using different KT standards enabled quantification of KTs in different flax cultivars, in different fractions of flaxseed and in processed flaxseed oils with high accuracy (Gui *et al.*, 2012). High-throughput and reliable HPLC analysis methods allowed screening of the world flax core collection for KT levels of both known and novel KT species (Olivia *et al.*, 2013).

Potential applications of KTs in industries can arise from their biological activities and chemical properties. Multiple biological activities of KTs were reported, including cytoprotective (Kessler *et al.*, 1986), antimalarial (Bell *et al.*, 2000), and immunosuppressive (Wieczorek *et al.*, 1991; Morita *et al.*, 1997; Morita *et al.*, 1999) activities. Recently, Sharav (2013) studied antioxidant activities of KT-containing fractions of flaxseed oil *in vitro*. Free radical scavenging

activity of [1–9-NαC]-KTA (**1**), [1–9-NαC]-KTB (**2**), [1–9-NαC],[1-MetO]-KTB (**3**), [1–8-NαC]-KTG (**14**), and [1–8-NαC],[1-MetO,3-MetO]-KTG (**15**) was studied using electron paramagnetic resonance (EPR) and was reported to be concentration and time-dependent. Jadhav and coworkers (2013) utilized chemically modified and activated [1–9-NαC],[1-MetO]-KTB (**3**) and [1–8-NαC],[1-MetO]-KTE (**9**) to bind with fluorescence tags, affinity chromatography media and bovine serum albumin (BSA). Due to their diverse biological activities, KTs may be potentially useful in food, cosmetic and pharmaceutical industries (Figure 1.1).

Recovery of KTs from flaxseed was achieved by solid-liquid extraction. Morita *et al.* (1997) extracted KTs from physically defatted flaxseed by using methanol. The methanolic extract was separated on a Diaion HP-20 column using a water-methanol gradient system. The fraction eluted by 100% methanol was collected and subject to silica gel chromatography using a chloroform-methanol gradient. Depending on the species of target KTs, fractions eluted by different chloroform-methanol composition was collected and further fractionated on columns (Morita. *et al.*, 1997; Morita. *et al.*, 1999; Matsumoto. *et al.*, 2001; Matsumoto *et al.*, 2002). KTs were recovered from flaxseed oil by extracting oil-laden silica with solvent of increasing polarity (Gui *et al.*, 2012; Reaney *et al.*, 2013a; Reaney *et al.*, 2013b). Briefly, non-polar components of flaxseed oil were removed from silica using varying percentage of ethyl acetate in hexane. KTs were eluted by ethyl acetate and 10% methanol in dichloromethane. Although efficient, this method employed hexane, methanol and dichloromethane, which are undesirable in food, pharmaceutical and cosmetic industries. Additionally, utilization of multiple solvents complicated solvent recovery. Therefore, optimization of KT recovery from flaxseed oil aims at establishing a simple, efficient and economical process using a low-toxic solvent(s).

Processing of KT-enriched liquid extract was essential in KT recovery. Rotary evaporation was the method of choice to produce solid KT mixture in literature after isolation and extraction. Other commonly applied techniques in bio-industries, such as spray drying, freeze drying, and falling film evaporation, have never been tested in drying KT mixture. Moreover, recovery of KTs from water-rich suspension has not been reported in literature.

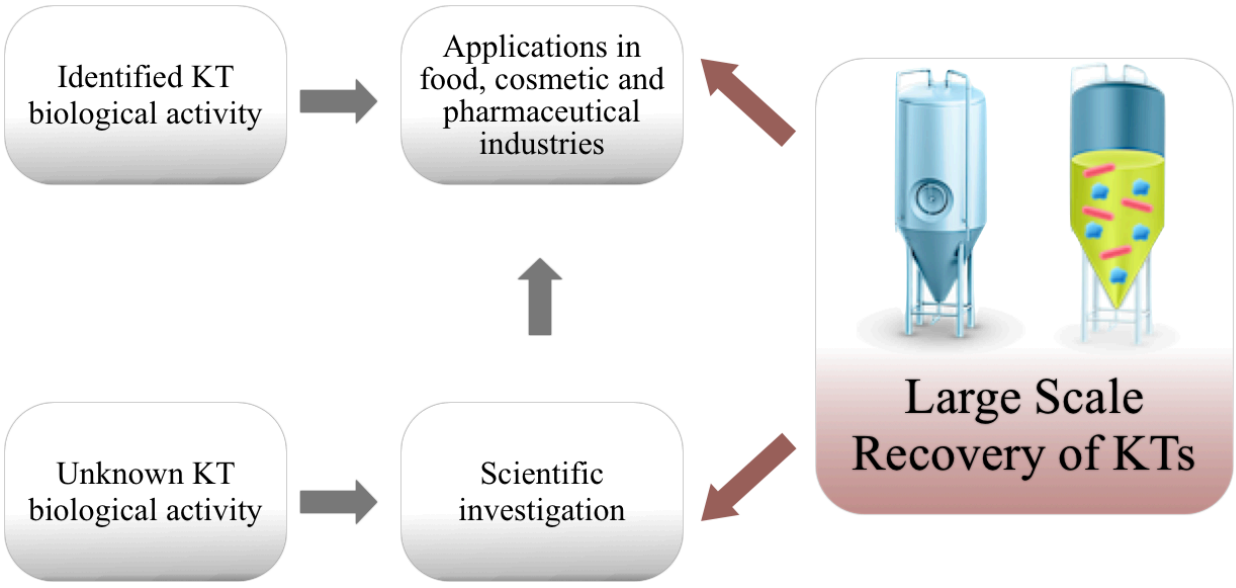

Figure 1.1 Developing markets for KTs

CHAPTER 2
OBJECTIVES

The goal of this project is to establish a simple, efficient and economical process for industrial extraction of a KT mixture from flaxseed oil (Figure 2.1). Specific objectives of this study are as follows:

- To determine the method of choice for bench-scale KT extraction. Sequential low and then room temperature (R.T.) elution of flaxseed oil/peptide-laden silica will be compared to liquid-liquid extraction of flaxseed oil using an array of aqueous ethanol solvent mixtures.
- To scale up the established bench-scale method to a scale sufficient for commercial production.
- To test the applicability of solvent removal technologies for production of a dried KT mixture in a powder form.
- To test the applicability of processing methods to isolate or enrich KTs in water-rich suspensions without water evaporation.

Hypotheses are as follows:

- NMR spectrometry may be used as a method for rapid and simultaneous quantitative analysis of KTs in flaxseed oil.
- Elution of peptide-laden silica at low temperatures using ethyl acetate may preferentially extract non-polar components of flaxseed oil including triacylglycerides (TAG) and diacylglycerides (DAG). Increasing the temperature and repeating the elution may enable the recovery of KTs from the silica.
- Liquid-liquid extraction of flaxseed oil using aqueous ethanol may effectively extract mixture of KTs and non-polar components of flaxseed oil. It is hypothesized that under optimal conditions, the amount of non-polar components of flaxseed oil extracted with aq. ethanol may be small relative to the amount of KT extracted.
- A bench-scale method can be scaled up by increasing the scale in steps to establish a suitable large-scale production protocol for KTs.
- A large-scale extraction protocol may be optimized in order to obtain satisfactory product in a short time with reduced production cost.

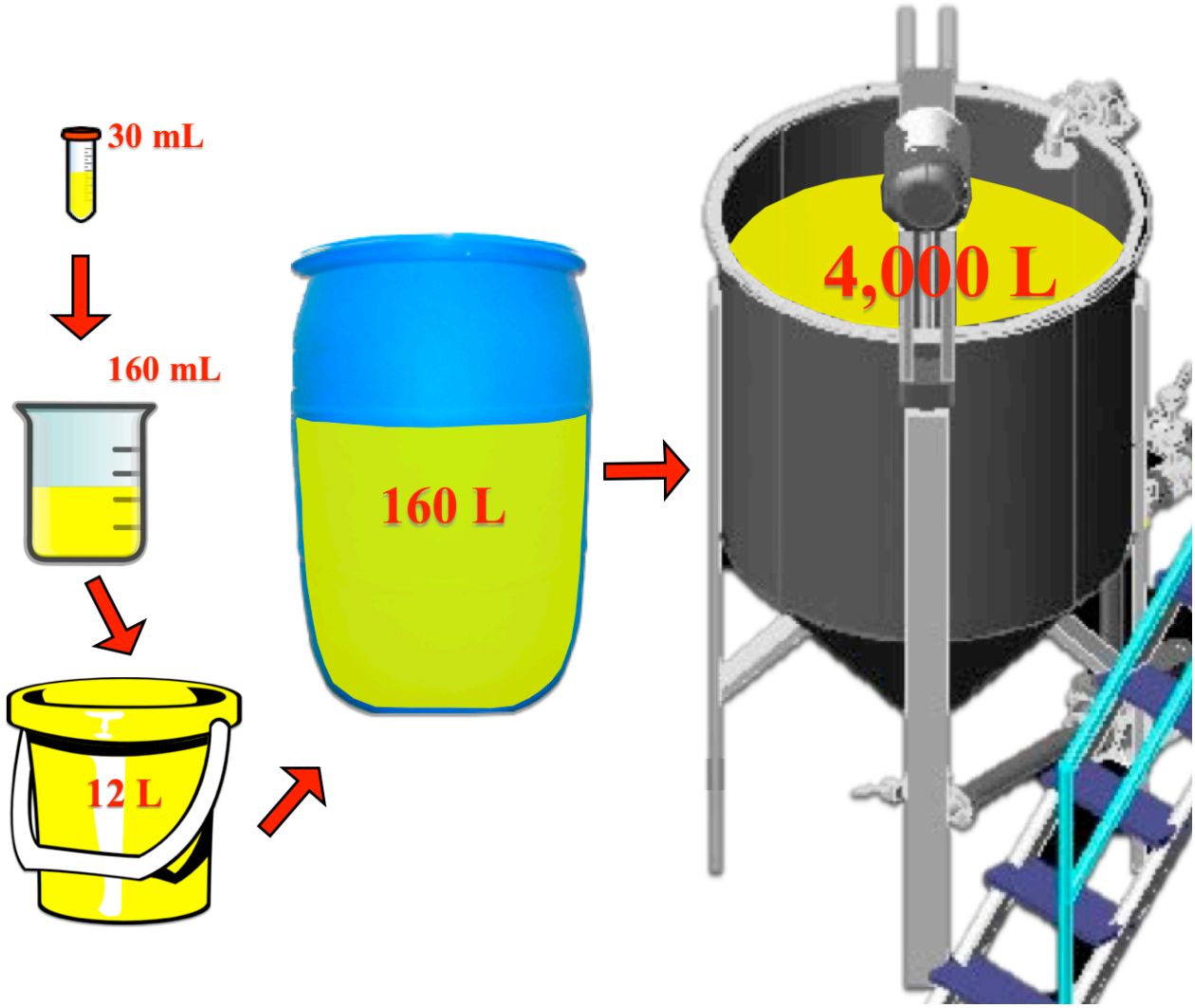

Figure 2.1 Scale-up stages in KT process development

CHAPTER 3
LITERATURE SURVEY

3.1 Flaxseed and Flaxseed Oil

Flax (*Linum usitatissimum* L.) is important to Canadian Agricultural industry. Canada is the number one producer of flax. Canadian farmers produced over 700,000 tonnes as an annual average from the years 2000–2010 (FAO, 2012). According to Statistics Canada, 368,000 tonnes of flaxseed were produced in 2011 (Statistics Canada, 2011). Flaxseed contains a large amount of oil, protein and dietary fibre. A study conducted by Canadian Grain Commission showed that, on average, Canadian brown flaxseed has 41% lipid, 20% protein, 28% fibre, 7.7% moisture, and 3.4% ash (Canadian Grain Commission, 2001). Other compounds were also identified, including phenolics, cyanogenic glycosides, phytic acid, trypsin inhibitors, phytoestrogens, and linatine (Bhatty, 1995). However, the composition of flaxseed can be affected by genetics, growing environment, seed processing and method of analysis (Daun *et al.*, 2003).

Flaxseed oil can be produced from flaxseed by pressing or a combination of pressing and solvent extraction. The major component of flaxseed oil is TAG comprised of linolenic acid (52%), linoleic acid (17%), oleic acid (20%), palmitic acid (6%), and stearic acid (4%) (Ley, 2003). Minor components of flaxseed oil include DAG, monoacylglycerides (MAG), phospholipids, tocopherols, sterols, sterol esters, free fatty acids, chlorophyll, carotenoids, and waxes (Gui, 2011).

3.2 Plant Cyclopeptides

Plant cyclopeptides, as defined by Tan and Zhou (2006), are “cyclic compounds formed mainly with peptide bonds of 2–37 protein or non-protein amino acids and discovered in higher plants, mainly L-amino acids”. [1–9-NαC]-KTA (**1**) in *L. usitatissimum* L. was the first plant cyclopeptide identified by scientists (Kaufmann and Tobschirbel, 1959). Tan and Zhou (2006) extensively reviewed the biology and chemistry of 455 cyclopeptides discovered in higher plants,

belonging to 26 families, 65 genera, and 120 species. They categorized plant cyclopeptides into two classes, five subclasses and eight types based on structural skeletons and distributions in plants. Details of the classification are shown in Table 3.1.

3.2.1 Chemistry and Biological Activities of Plant Cyclopeptides

Chemical structures of different types of plant cyclopeptides are shown in Figure 3.1. Cyclic di-, tri-, tetra-, penta-, hexa-, hepta-, octa-, nona-, deca-, undeca-, dodeca-, tetradeca-, octacosa-, nonacosa-, traconta-, hentriaconta-, tetratraconta-, and heptatraconta- peptides are found in plant cyclopeptides (Tan and Zhou, 2006).

Literature survey shows that plant cyclopeptides have a wide range of biological activities. It has been found that, within the type of cyclopeptide alkaloid, discarine-A, discarine-B, frangufoline, scutianine-B, nummularine-K, condaline-A, amphibine-H, nummularine-B, nummularine-R, nummularine-S, rugosanine-A, rugosanine-B, abyssenine-C, mucronine-F, mucronine-G, and mucronine-H possess antibacterial activity (Tschesche *et al.*, 1974; Pandey and Devi, 1990; Morel *et al.*, 2002; Giacomelli *et al.*, 2004; Suksamrarn *et al.*, 2005); Ziziphine-N and ziziphine-Q demonstrated antiplasmodial and antimycobacterial activity (Suksamrarn *et al.*, 2005); sanjoinine-A showed effective sedative activity (Han *et al.*, 1989); paliurine-A, paliurine-B, paliurine-C, paliurine-D, paliurine-F, and sativanine-G exerted immunostimulant activity (Lin *et al.*, 2000). FR900359, one of the two depsicyclopeptides, showed cytotoxicity to cultured rat fibroblasts and myelocytic leukemia cells (Fujioka *et al.*, 1988). Anti-angiotensin-converting enzyme and anti-renin activities have been reported for lyciumin A and lyciumin B, Solanaceae-type cyclopeptides (Yahara *et al.*, 1989; Yahara *et al.*, 1993). Celogentins A–J and moroidin of Urticaceae-type cyclopeptide were found to be antimitotic (Kobayashi *et al.*, 2001; Morita *et al.*, 2000; Suzuki *et al.*, 2003). Morita *et al.* (1992) investigated three antitumor compounds isolated from *Aster tataricus*, named astins A, B, and C. They belong to the Compositae-type cyclopeptide.

Tan and Zhou (2006) summarized a wide range of biological activities of Caryophyllaceae-type cyclopeptide including cytotoxic, antiplatelet, antimalarial, immunomodulating, immunosuppressive, Ca^{2+} antagonistic, inhibiting cyclooxgenase and tyrosinase, enhancing rotamase, and estrogen-like activity. *In vivo* and *in vitro* studies showed that some of Rubiaceae-type cyclopeptide possess antitumor activities (Jolad *et al.*, 1977; Itokawa

Table 3.1 Classification of plant cyclopeptide (Modified from Tan and Zhou, 2006)

Class	Subclass	Type		Number[1]
Hetero-cyclopeptide	Heteromono-cyclopeptide	Cyclopeptide alkaloid	(Type I)	185
		Depsicyclopeptide	(Type II)	2
		Solannaeae-type cyclopeptide	(Type III)	4
	Heterodicy-cyclopeptide	Urticaceae-type cyclopeptide	(Type IV)	13
Homo-cyclopeptide	Homomono-cyclopeptide	Compositae-type cyclopeptide	(Type V)	9
		Caryophyllaceae-type cyclopeptide	(Type VI)	168
	Homodi-cyclopeptide	Rubiaceae-type cyclopeptide	(Type VII)	23
	Homopoly-cyclopeptide	Cyclotide	(Type VIII)	51

[1] Number of cyclopeptides up to the year 2005.

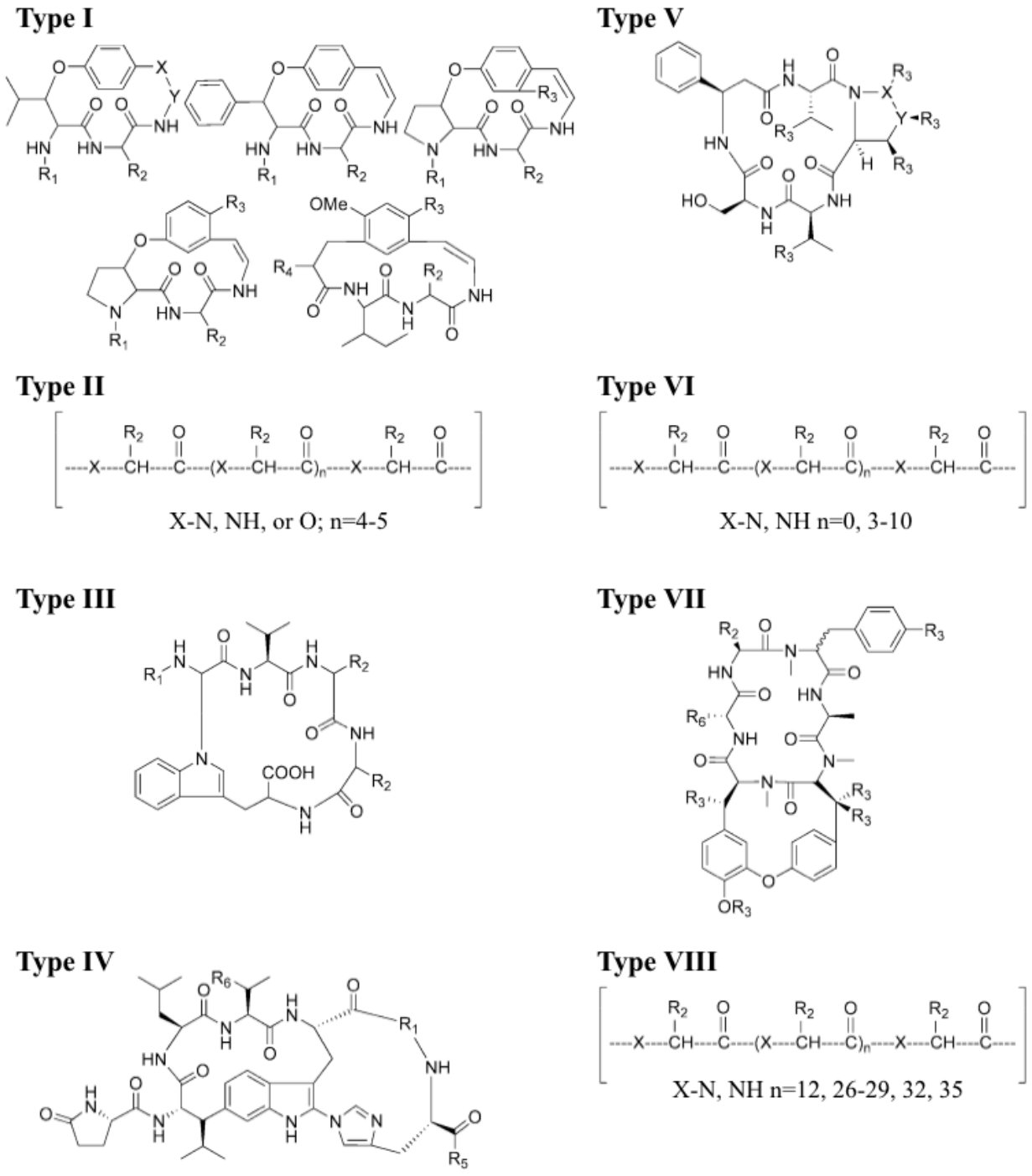

Figure 3.1 Structures of cyclopeptides (Modified from Tan and Zhou, 2006)

et al., 1986; Itokawa *et al.*, 1991; Morita. *et al.*, 1992). Among them, Itokawa *et al.* (1991) identified RA-VII as the effective anti-cancer drug with low toxicity. Cyclotides displayed a variety of bioactivities, of which anti-HIV is very unique in all types of plant cyclopeptides (Gustafson *et al.*, 1994; Gustafson *et al.*, 2000; Hallock *et al.*, 2000; Bokesch *et al.*, 2001;Tan and Zhou, 2006).

3.3 Kaufmann and Tobschirbel Orbitides

KTs are a group of cyclic, hydrophobic peptides with eight or nine amino acid residues. The molecular weights of KTs are approximately one thousand Daltons (Gui, 2011). According to the classification in section 3.2, KTs belong to Caryophyllaceae-type homocyclopeptides. Kaufmann and Tobschirbel isolated **1** in 1959 from slime filtered from crude flaxseed oil. Since then, 16 KTs have been identified from the seeds of *Linum usitatissimum* L. (Table 3.2 and Figure 3.2). The old naming system of KTs was established alphabetically according to the order of discovery. Shim *et al.* (2013) introduced a new naming system, which clearly reflects the oxidation status and location of methionine (Met) in KT molecule. The DNA database of flax (National Center for Biotechnology Information, NCBI) showed that the gene AFSQ01016651.1 contained embedded sequence of **1** (ILVPPFFLI), **2** (MLIPPFFVI), and **8** (MLVFPLFVI), while the gene AFSQ01025165.1 contained embedded sequence of **5** (MLLPFFWI), **11** (MLMPFFWV) and **14** (MLMPFFWI) (Covello, 2010).

3.3.1 Biological Activity of KTs

KTs have been reported to have a range of biological effects. However, the role of KTs in *Linum usitatissimum* is still unknown (Picur *et al.*, 2006). Among all the KTs, the bioactivity of **1** was most extensively studied. Kessler *et al.* (1986) discovered that **1** had cytoprotective ability, particularly with inhibition of cholate uptake into hepatocytes. The sequence -Phe-Phe-Pro- in **1**, which is similar to the functional block in antamanide and peptide hormone somatostatin, was responsible for the inhibition. Another group found that **1** can inhibit phalloidin transport into rat hepatocytes (Münter *et al.*, 1986). Wieczorek *et al.* (1991) investigated the immunosuppressant activities of **1**. The influence of **1** on the primary and secondary humoral response *in vivo* and *in vitro* was determined by plaque forming cell (PFC) test. The delayed-type hypersensitivity test, the skin-allograft rejection and the graft-vs.-host reaction was utilized to study the effect of **1**

Table 3.2 Amino acid sequences of KTs (Adapted from Shim *et al.*, 2014)

New name	Old name	Amino acid sequence (NαC-)	Chemical formula	Molecular weight
[1–9-NαC]-KTA (**1**)	CLA	Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile	$C_{57}H_{85}N_9O_9$	1040.34
[1–9-NαC]-KTB (**2**)	CLB	Met-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_9S$	1058.38
[1–9-NαC],[1-MetO]-KTB (**3**)	CLC	MetO-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_{10}S$	1074.38
[1–9-NαC],[1-MetO_2]-KTB (**4**)	CLK	MetO_2-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_{11}S$	1090.38
[1–8-NαC]-KTD (**5**)	CLD'	Met-Leu-Leu-Pro-Phe-Phe-Trp-Ile	$C_{57}H_{77}N_9O_8S$	1048.34
[1–8-NαC],[1-MetO]-KTD (**6**)	CLD	MetO-Leu-Leu-Pro-Phe-Phe-Trp-Ile	$C_{57}H_{77}N_9O_9S$	1064.34
[1–8-NαC],[1-MetO_2]-KTD (**7**)	CLD_{Msn}	MetO_2-Leu-Leu-Pro-Phe-Phe-Trp-Ile	$C_{57}H_{77}N_9O_{10}S$	1080.34
[1–8-NαC]-KTE (**8**)	CLE'	Met-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{51}H_{77}N_8O_8S$	961.26
[1–8-NαC],[1-MetO]-KTE (**9**)	CLE	MetO-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{51}H_{77}N_8O_9S$	977.26
[1–8-NαC],[1-MetO_2]-KTE (**10**)	CLJ	MetO_2-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{51}H_{77}N_8O_{10}S$	993.26
[1–8-NαC]-KTF (**11**)	CLL	Met-Leu-Met-Pro-Phe-Phe-Trp-Val	$C_{55}H_{73}N_9O_8S_2$	1052.35
[1–8-NαC],[1-MetO,3-MetO]-KTF (**12**)	CLF	MetO-Leu-MetO-Pro-Phe-Phe-Trp-Val	$C_{55}H_{73}N_9O_{10}S_2$	1084.35
[1–8-NαC],[3-MetO]-KTF (**13**)	CLI	Met-Leu-MetO-Pro-Phe-Phe-Trp-Val	$C_{55}H_{73}N_9O_9S_2$	1068.35
[1–8-NαC]-KTG (**14**)	CLM	Met-Leu-Met-Pro-Phe-Phe-Trp-Ile	$C_{56}H_{75}N_9O_8S_2$	1066.38
[1–8-NαC],[1-MetO,3-MetO]-KTG (**15**)	CLG	MetO-Leu-MetO-Pro-Phe-Phe-Trp-Ile	$C_{56}H_{75}N_9O_{10}S_2$	1098.38
[1–8-NαC],[1-MetO]-KTG (**16**)	CLH	MetO-Leu-Met-Pro-Phe-Phe-Trp-Ile	$C_{56}H_{75}N_9O_9S_2$	1082.38

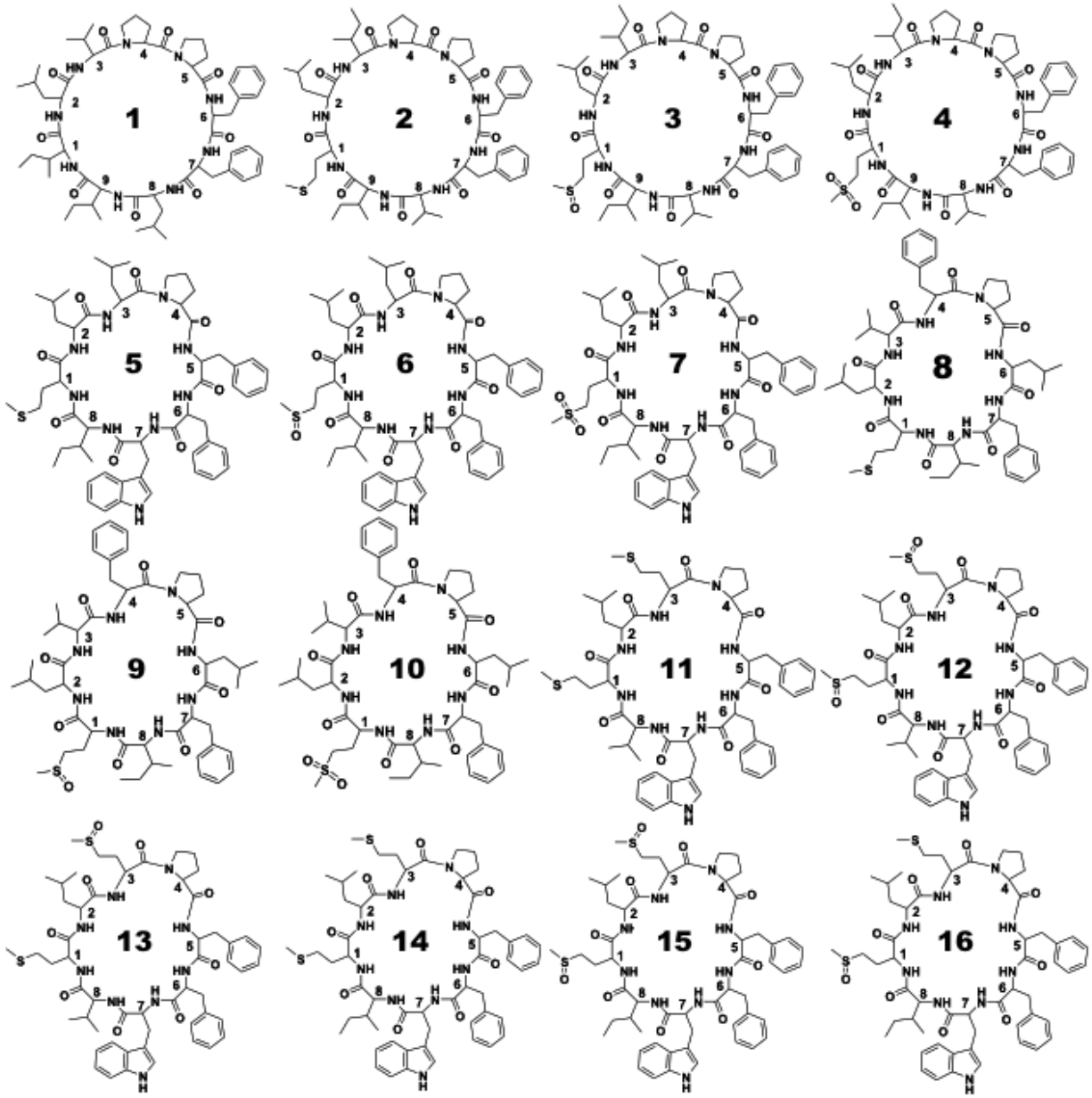

Figure 3.2 Chemical structures of KTs from *Linum usitatissimum* L. (Redrawn from Shim *et al.*, 2014)

the cellular immune response in mice. Human lymphocyte proliferation test *in vitro*, post-adjuvant polyarthritis test in rats and hemolytic anemia test in New Zealand Black mice were also conducted. The results indicated that **1** influenced both humoural and cellular immune response. Specifically, **1** increased the skin allograft rejection time, reduced the graft-vs.-host reaction index and inhibited human lymphocyte proliferation; and additionally showed pronounced protective effects on post-adjuvant polyarthritis test and hemolytic anemia test. Proliferation of human lymphocytes caused, *in vitro,* by phytohemagglutinin was also inhibited by **1**. The authors found that the immunosuppressive activity of **1** was comparable to that of cyclosporin A (CsA). At very low concentrations, **1** induced the same effects as CsA on T and B cell proliferation (Górski *et al.*, 2001). Gaymes *et al.* (1997) studied the mechanism of immunosuppressive activity of **1**. They concluded that **1** resembled the action of CsA through their inhibition of calcium-dependent T cell activation by binding to cyclophilin A, which is a peptidyl-prolyl *cis-trans* isomerase. However, the concentration of **1** required for complete inhibition was ten times higher than that of CsA. In another study, it was speculated that the amino sequence -Val-Pro-Pro-Phe-Phe- of **1** was responsible for the interaction between cyclophilin A and **1** (Gallo *et al.*, 1995). KT **1** also demonstrated antimalarial activity. Bell and coworkers (2000) utilized synthesized **1** and a series of its analogues to test the inhibition of human malarial parasite *Plasmodium falciparum* in culture. These authors did not determine a clear correlation between the structure of the peptides, immunosuppressive activity, and antimalarial activity. It was observed that the antimalarial activity of peptides was apparently connected to the strong hydrophobic nature of **1**; antimalarial activity was decreased or lost when a less hydrophobic residue was substituted into the peptide chain while the immunosuppressive properties were retained. A possible explanation for this observation was that the antimalarial effect of **1** and analogues resulted from their influence on cell membranes, rather than on some specific receptor such as cyclophilin. This hypothesis was later confirmed by experimental studies showing that binding of **1** to purified *Plasmodium falciparum* cyclophilin was only detected at very high concentrations. Decreased immunosuppressive activity was observed after introduction of D-aromatic residues into the **1** molecule, while this substitution had little effect on antimalarial activity. Rempel *et al.* (2010) investigated binding of **1** to human serum albumin (HSA), the most abundant protein in blood plasma. It was found that the formation of a complex was an endothermic and entropy driven reaction. The authors believed that the interaction

between **1** and HSA could be potentially utilized for developing a nano-delivery device in biomedical industries.

Wieczorek *et al.* (1991) evaluated the toxicity of **1** by three different administration methods, i.e. oral administration, intraperitoneal injection and intravenous injection. It was concluded that oral administration of **1** in olive oil, 2% gelatine solution at doses of 4 and 3 g/kg did not exert toxic effects on mice and rats, respectively. No toxic effects were observed on mice by intravenous injection of 230 mg/kg **1**. All the animals survived the oral toxicity test through 13 weeks at doses of 15, 45, and 135 mg/kg/day.

The immunosuppressive activity of other KTs was also reported. KT **2** had the inhibitory effect on mitogen (concanavalin A) induced response of human peripheral blood lymphocytes with IC_{50} of 44 ng/mL (Morita *et al.*, 1997). KTs **2** and **9** showed a moderate inhibitory effect of similar potency in mouse lymphocyte proliferation induced by concanavalin A with IC_{50} of 39 μg/mL and 43 μg/mL, respectively (Morita *et al.*, 1999). Reaney *et al.* (2013b) found that **1**, **3** and **9** could induce apoptosis in human lung epithelial cancer lines. KTs **4** and **10** were believed to be potent immunosuppressants with IC_{50} values of 28.1 and 25.2 μg/mL, respectively (Morita *et al.*, 2010).

Recently Sharav (2013) conducted extensive research on the antioxidant activity of KTs *in vitro*. Initially, five different fractions (fractions A to E) of flaxseed oil were obtained by eluting the oil-laden silica using solvents of increasing polarity. Fractions B to E were added to the silica-treated flaxseed oil for oxidative stability index (OSI) tests, which were then compared with OSI values of crude oil and silica-treated oil. It was found that silica-treated oil was less stable than crude oil. However, stability was improved after fraction D, containing **1**, **3**, β/γ- and δ-tocopherol was added to silica-treated oil. The study also showed that **1** could slow the oxidation of silica-treated oil with Ni^{2+} and Zn^{2+}, respectively, but not the oxidation of silica-treated oil with Co^{2+}, indicating the selective binding of **1** with metal ions. Electron paramagnetic resonance was utilized in *in vitro* antioxidant study. Using hydroxyl radical assay, it was discovered that **1**, **2** and **3** had concentration-dependent free radical scavenging activity. Using DPPH scavenging assay, it was proved that free radical scavenging activity of **1**, **2**, **3**, **14**, and **15** was concentration and time-dependent, which could also be affected by UV light irradiation.

3.3.2 Oxidation of KTs

As some species of KTs are Met/MetO containing peptides (Table 3.2), oxidation can result in the conversion of reduced KTs to their oxidized homologues. Brühl *et al.* (2007) identified **1**, **3**, **9**, **12**, and **15** in cold pressed flaxseed oil that were stored for up to 30 weeks while reduced KTs were absent. Oxidation of **8** to **9** was believed to contribute to the bitterness of flaxseed oil after long storage. Studies by Gui *et al.* (2012) and Olivia *et al.* (2012) confirmed that flaxseed stored for long periods contained only **1**, **3**, **6**, **9**, **12**, and **15**. Jadhav *et al.* (2012) detected **2**, **5**, **8**, **11**, and **14** in freshly ground flaxseed meal. After accelerated aging for 16 h, the amount of Met containing KTs (**2** and **8**) decreased significantly while their oxidized homologues (**3**, **4**, **9**, and **10**) increased over the same period of time. Olivia (2013) demonstrated that controlled oxidation of Met containing KTs to $MetO_2$ containing KTs can be initiated by addition of H_2O_2 to flaxseed methanol extracts and quenched by $Na_2S_2O_3$, which facilitated chromatographic separation.

3.3.3 Chemical Modification of KTs

Recently Jadhav (2013) reported modification of **3** and **9** through Met as point of attachment to form novel derivatives of KTs, while the basic core of KT structure was kept intact (Figure 3.3). KTs **3** and **9** were initially reduced to **2** and **8**, respectively, by reacting with $NaBH_4$, I_2 and THF. Using established chemical modification methods, activated groups (e.g. -CN, -OH, -COOEt, and $-NH_2$) were incorporated into the corresponding KT parent molecule. The structures of novel derivatives (**24**, **25**, **28**, and **29**) were elucidated by using a combination of HPLC-MS, tandem MS, 1D and 2D NMR analyses. Due to the reactive nature of amine and hydroxyl side chains, further chemical syntheses were possible. It was shown that KT analogs containing amine (**25** and **29**) could covalently link with coumarin succinimidyl ester dye to form fluorescent derivatives of KTs, which have higher quantum fields in methanol and ethanol than DMF and acetonitrile. A KT affinity matrix was formed by coupling amine KT with agarose gel matrix. The **3** conjugate was demonstrated to specifically bind with apolipoprotein A1 in chicken serum samples. The hydroxyl derivatives of KTs were utilized to couple with BSA. The following *in vivo* tests illustrated that polyclonal antibodies were produced in rabbits after they were immunized with the synthesized conjugates.

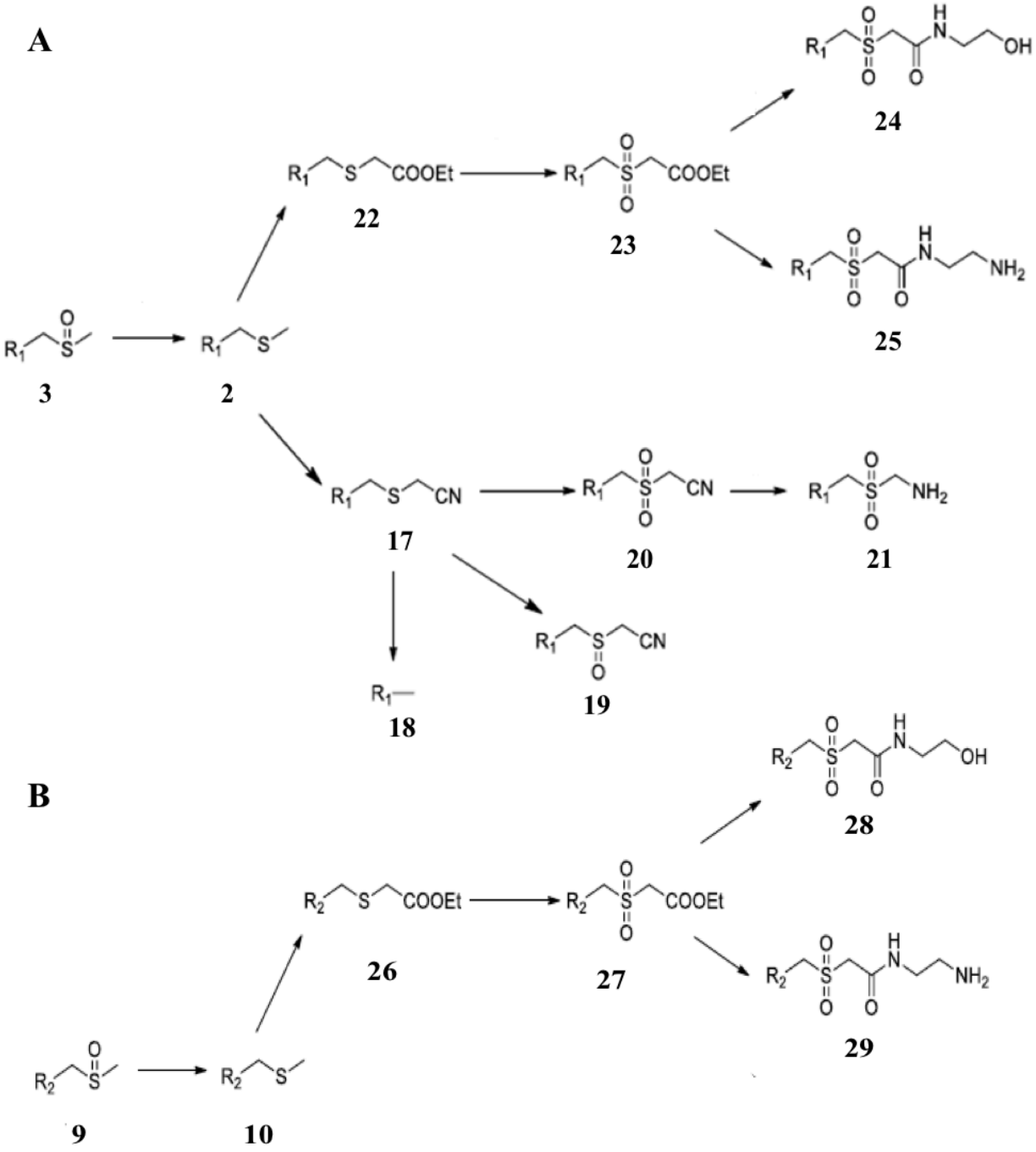

Figure 3.3 Synthesis routes of novel KT derivatives from (A) KT **3** and (B) KT **9** (Modified from Jadhav, 2013)

3.3.4 Extraction and Isolation of KTs from Flaxseed and Flaxseed Oil

Since the discovery of the first KT in 1959, several isolation methods have been developed. Morita *et al.* (1997, 1999) and Matsumoto *et al.* (2001; 2002) published a method using solid-liquid extraction and column chromatography. Briefly, seeds of *Linum usitatissimum* were squeezed to remove oil, followed by extraction three times with hot methanol. Four kilogram of a methanolic extract was obtained and loaded on a Diaion HP-20 column. A water-methanol gradient system (1:0–0:1) was used as the eluent. The fraction eluted by 100% methanol was collected and subjected to silica gel column chromatography employing a chloroform-methanol gradient (1:0–0:1). The following procedures were different depending on the types of KTs of interest: a) to isolate KTs, 1, 2, 3, 6, and 9, the fraction eluted with 10% methanol was loaded on reverse-phase ODS HPLC column which was eluted by 70–80% methanol and 40–60% acetonitrile gradient system; b) to isolate KTs 12, 13, 15, and 16, the fraction eluted with 15% methanol was loaded on reverse-phase ODS HPLC column which was washed by 40–60% acetonitrile gradient system. The yield of KTs was as follows: 0.007% **1**, 0.0004% **2**, 0.003% **3**, 0.0012% **6** and 0.0002% **9**, 0.0008% **12**, 0.00007% **13** 0.0024% **15**, and 0.0002% **16**.

A different method was developed to afford an extract containing a mixture of KTs (Stefanowicz, 2001). Briefly, five grams of ground flaxseed was extracted with 100 mL acetone at ambient temperature overnight. Vacuum concentration was conducted and the resulting mixture was dissolved in methanol and hydrolyzed with 10% NaOH. The solution was evaporated under reduced temperature, which was followed by ethyl acetate extraction and evaporation. KTs **1**, **2**, **6**, **9**, **12**, and **15** were shown to exist in the mixture.

A less laborious and more productive method for recovery of KTs from oils was recently developed (Reaney *et al.*, 2013b). This invention includes: a) efficient extraction of large amount of oils from plant materials from which substantial amounts of individual hydrophobic peptides can be extracted; b) separation of extracted oil into polar and non-polar fractions, thereby concentrating hydrophobic KTs; c) separation of KTs from oil by mixing silica with oils or non-polar fraction of oils and subsequently eluting with solvents of increasing polarity; and d) the application of chromatography, such as HPLC, in further separating and purifying eluted fraction obtained in c). Based on this patent, Gui *et al.* (2012) reported an improved solid-liquid extraction method with high KT recovery. Briefly, flaxseed was ground and passed through a 1.18-mm test sieve. Ground material (up to 5 g) was wrapped in filter paper and then placed in a

cellulose extraction thimble. Subsequently, extraction was conducted in a Goldfisch extractor using 50 mL acetone for 5 h. An oil sample was obtained after solvent evaporation of the extract. Silica gel flash column chromatography was employed for the separation of KTs in the oil extract. Silica gel (0.5 g) was loaded into the column and conditioned with hexane (2 mL) for 2 min. Oil (1 mL) and hexane (1 mL) was applied and then eluted with the following solvents: 100% hexane (10 mL), 20% ethyl acetate in hexane (v/v, 10 mL), 50% ethyl acetate in hexane (v/v, 10 mL), 100% ethyl acetate (10 mL) and 10% methanol in dichloromethane (v/v, 10 mL). The investigators found that no less than 95% of KTs in flaxseed oil were recovered in the very last two fractions. Sharav (2013) scaled up this method to recover KTs from 400 mL flaxseed oil. Flaxseed oil was introduced to a column with 80 g of silica, which was then washed with 100% hexane (250 mL), 20% ethyl acetate in hexane (v/v, 250 mL), 50% ethyl acetate in hexane (v/v, 250 mL), 100% ethyl acetate (250 mL), and 10% methanol in dichloromethane (v/v, 10 mL). Reaney *et al.* (2013a) mixed 40 kg Trysil silica with 1,860 kg flaxseed oil. The silica was collected and subject to multiple solvent washes in 0.5-2.0 kg lots. In one example, silica cake (500 g) was sequentially washed by hexane (1 L × 3), 50% ethyl acetate in hexane (v/v, 1 L × 3), and 10% methanol in dichloromethane (v/v, 1 L × 3). KTs were present in the fraction of 10% methanol in dichloromethane. The crude KT mixture could be further enriched using two methods: a) the crude mixture was dissolved in ethyl acetate, which was washed by saturated sodium bicarbonate solution to remove impurities; or b) the crude mixture was dissolved in methanol and cooled to –20^{o}C to precipitate less soluble compounds.

3.3.5 Instrumental Analysis of KTs

Many instruments have been applied for the analysis of KTs, including circular dichroism (CD) spectroscopy, infrared (IR) spectroscopy, Fourier transform infrared spectroscopy (FTIR), electrospray ionization-mass spectrometry (ESI-MS), electrospray ionization tandem mass spectrometry (ESI-MS/MS), electrospray ionization-time of flight-mass spectrometry (ESI-TOF-MS), high resolution-fast atom bombardment mass spectrometry (HR-FABMS), liquid chromatography-mass spectrometry (LC-MS), ^{13}C-NMR, ^{1}H-NMR and HPLC (Brewster and Bovey, 1971; Naider *et al.*, 1971; Morita *et al.*, 1999; Stefanowicz, 2001; Brühl *et al.*, 2007). Stefanowicz (2001) utilized ESI-MS and ESI-MS-MS in KT analysis. Samples were dissolved in methanol with 10 mM of ammonium acetate. MS analyses were conducted using a Finnigan

MAT TSQ-700 MS equipped with ESI source. The MS results of **1**, **2**, **6**, and **9** in that study corroborated the findings in a previous paper (Morita *et al.*, 1999). They also found two novel peptides, i.e. **12** and **15,** which were also reported by Matsumoto et al (2001). Their sequences were established based on collision induced dissociation spectra and structural similarities to **6**. NMR and MS techniques were also compared. It was concluded that MS analyses are much faster and less expensive than NMR analyses; however, the limitations of MS are that it cannot determine configuration of amino-acid residues and cannot distinguish between leucine and isoleucine residues.

Literature shows that CD and NMR are powerful tools for KT conformation determination. For example, Naider *et al.* (1971) successfully investigated conformation of **1** in different organic and organic-acid media, concluding that **1** exists in one set of conformations in trimethyl phosphate and trifluoroethanol and another set of conformations in hexafluoro-2-propanol. Brewster and Bovey (1971) obtained 220 MHz spectrum of **1** by decoupling, exchange of peptide NH protons with deuterium, and measurement of the temperature dependence of the NH chemical shift. They discovered that there was no intramolecular hydrogen bonds when **1** was dissolved in dimethylsuloxide. Tancredi *et al.* (1991) combined CD and NMR to study **1** conformers in acetonitrile and to explore the ion binding capacity of **1**.

Morita (1999) and Matsumoto *et al.* (2001) performed extensive analyses of **1**, **2**, **3**, **6**, **9**, **12**, **13**, **15**, and **16** using a combination of techniques, including HR-FABMS, ^{13}C-NMR, ^{1}H-NMR, IR spectroscopy and HPLC. Quasi-molecular ion peaks could be obtained from HR-FABMS, from which the molecular formula could be inferred. The mid IR absorption spectrum identified certain functional groups within the compound. After KTs were hydrolyzed under acidic conditions, the researchers conducted amino acid analysis by HPLC to identify the types of amino acids contained in KTs. ^{13}C-NMR and ^{1}H-NMR provided more information about the KT chemical structures. In this research, several NMR methods, such as rotating frame Overhauser effect spectroscopy, heteronuclear multiple quantum coherence and ^{1}H-^{1}H-correlation spectroscopy, were also applied in structure elucidation of KTs.

In ^{1}H-NMR spectra, peak intensities are proportional to the number of protons causing the signal (Kuhn, 1990). Therefore, quantitative analysis by ^{1}H-NMR is possible as long as the number of protons of representative peaks were known. Previous papers showed that protons from phenylalanine phenyl groups and tryptophan indole groups in KTs could generate peaks

within the chemical shift range of 7.00 to 7.40 ppm (Di Blasio *et al.*, 1989; Morita *et al.*, 1999; Matsumoto *et al.*, 2001; Gui *et al.*, 2012). However, the number of protons in the region 7.00 to 7.40 ppm varied for KTs with and without tryptophan (15 and 10 protons, respectively). It is worth noting that although protons of amines in KTs can also generate peaks in that same region (7.00 to 7.40 ppm,) these peaks would not appear in ^{1}H-NMR spectra if KTs are dissolved in MeOD due to hydrogen–deuterium exchange (Englander and Kallenbach, 1984).

In the study by Brühl *et al.* (2007), bitter isolates from flaxseed oil were subjected to structural analysis. KT **9**, which was identified as the key bitter compound in stored flaxseed oil, was identified utilizing MS/MS, FTIR, ESI-TOF-MS, NMR and amino acid analysis (Brühl *et al.*, 2007). The IR spectrum indicated the presence of a hydroxyl group, CH_3 and CH_2 groups; sulfoxide (vibration), C=O (vibration) and C-N, N-H vibration was also observed. Molecular mass was measured by ESI-MS/MS and ESI-TOF-MS, from which the molecular formula was inferred. The cyclic structure of the compound was proposed on the basis of hydrophobicity and the failure of the compound to produce a fragment representing the loss of an amino acid from the peptide chain C terminal upon MS/MS analysis. A bitter peptide of six amino acids was identified by ion-exchange chromatography, which was in agreement with the previously reported amino acid composition of **9** (Morita *et al*., 1999). Subsequent NMR experiments facilitated the final confirmation of structure. However, quantification was not achieved in this study.

A quantitative analysis method for **9** was developed by the same group (Brühl *et al.*, 2008). Briefly, one gram of flaxseed oil was diluted with 10 mL heptane. Then, the solution was loaded into an SPE cartridge (C18 SPE 1000-mg cartridges with 6 mL reservoir, J.T. Baker, The Netherlands) conditioned with 10 mL methanol. The non-polar fractions were eluted with three portions of 5 mL heptane, followed by recovery of polar fraction with 5 mL methanol. The liquid residue after solvent removal was dissolved in 0.5 mL methanol. Further separation of the sample (20 μL) was conducted using an HPLC system which consisted of a Basic Marathon autosampler (Spark, The Netherlands), an L7100 Merck-Hitachi gradient pump (Merck, Germany), a 250 × 4 mm, 5 mm, LiChrospher 100 RP-18 column (Merck, Germany) and a UVD 340-type UV/Vis detector (Gynkotek, Germany). The working wavelength of the detector was 210 nm. The authors reported a retention time of 12.43 min for **9**. An external calibration curve was established with R^2 of 0.998 for the concentration range 3–900 mg/mL. The concentration of KT **9** in oil from 21 flaxseed varieties over a storage time of 150 days was determined and reported.

A systematic study of KT quantification was accomplished recently (Gui *et al*., 2012). In this work, Segetalin A (Seg-A) was used as internal standard to verify the calibration curve. Standards of Seg-A and **1**, **2**, **3**, **6, 9**, and **12** were successfully separated in a single chromatographic run within 30 min. Linear standard curves, where KT concentration was plotted as a function of area ratios of KT/Seg-A, were obtained with R^2 value greater than 0.99. The accuracy of this HPLC method was satisfactory, as determined by using a set of quality control solutions. Using the analytical method, quantification of KTs was conducted in a series of experiments that a) measured the KT levels in different varieties of flaxseed, b) determined the KT distribution in flaxseed fractions, and c) measured the effects of processing on the distribution of KTs.

Further improvement in HPLC analysis of KTs was achieved by optimizing and comparing the performance of three monolithic C18 silica columns, i.e. Chromolith® SpeedROD (CSR), Chromolith® Performance, and Chromolith® High Resolution and two silica particulate columns, i.e. ZORBAX Eclipse XDB-C18 and POROS R1/20 (Olivia *et al.*, 2012). Standard solutions of **2**, **3** and **4**, together with desulfurized **3** as an internal standard, were utilized in analyses. Solvent gradient was optimized for each column. After comparing chromatographic data of several parameters including peak resolution, selectivity, retention times of KTs, repeatability, relative standard deviation, capacity factor, and peak width at half height, it was concluded that CSR was the column of choice for high-throughput screening of KTs in flaxseed. Further optimization of solvent gradient for CSR made it possible to separate **1**, **3**, **6**, **9**, **12**, and **15** within a 1.5 min chromatographic run without compromising resolution. Each chromatographic parameter of CSR was stable after 2000 injections of flaxseed extract, proving the longevity of the column. Using a previously established high-throughput HPLC analysis method, the flax core collection was screened for the concentration and composition of KTs (Olivia, 2013). Accessions with the highest total KT content, lowest total KT content, highest ratio of KTs coded by AFSQ01016651.1 to those coded by AFSQ01025165.1, and lowest ratio of KTs coded by AFSQ01016651.1 to those coded by AFSQ01025165.1 were identified. It was also found that there were no significant correlations between KT content and plant morphological and chemical traits, including plant height, stem branching, petal color, seed color, seed weight, seed oil content, and α-linolenic content.

Recently an HPLC-MS/MS method was established for KT characterization (Jadhav *et al.*,

2012; Olivia, 2013). The employed equipment included an Agilent HPLC 1100 series connected to a Bruker microTOF-Q II Mass Spectrometer (Hybrid Quadrupole TOF MS/MS, Bruker Daltonik GmbH, Bremen, Germany) with ESI source. The HPLC method was able to resolve KTs in flaxseed extracts within 16 min, while the fragmentation patterns of the peptides in MS/MS mode provided structural information for characterization of **4** and **10** (Jadhav, 2012), as well as identification of three novel KTs from accessions 'Hollandia' and 'Z 11637' of flax core collection (Olivia, 2013).

3.4 Low Temperature Chromatography

As chromatography techniques depend on the distribution of analytes between two phases, one common practice in silica chromatography is to change the polarity of eluent by modifying solvent composition, which may require the use of two or more solvents. Another option is to change the temperature of the chromatography process. It has been commonly recognized that temperature is a critical parameter controlling for the selectivity, reproducibility and efficiency of normal and reverse chromatography (Herbut and Kowalczyk, 1981; Dolan *et al.*, 1999). High temperature chromatography can easily be performed by heating the chromatography material and solvent, while low temperature chromatography is not commonly performed. Only a few papers on low temperature chromatography are available in literature, all of which dealt with thin-layer chromatography (TLC). Possible reasons are that TLC requires small plates, low flow rate and small amount of mobile phase, which makes it easier and less costly to achieve desired low temperatures (Zarzycki, 2008).

Malins and Mangould (1960) described an improved separation of certain fatty acids siliconized layers of silica gel under low temperature (4 to 6^{o}C). TLC separation of enantiomers were also affected by temperature (Xuan and Lederer, 1994). It was found that there was a large temperature effect when eluents containing α-cyclodextrin were used: the ΔR_f value decreased as much as five fold with an increase of temperature from 8 to 60^{o}C.

The group of Zarzycki published several papers describing temperature controlled TLC in the past decade. They conducted a systematic investigation into the influence of temperature on the retention and separation on cholesterol and bile acids using reverse-phase TLC (Zarzycki *et al.*, 1999). The parameters for retention and separation, R_f and $\sum \Delta hR_f$, respectively, were investigated both over a temperature range of 5 to 60 ^{o}C and a wide range of methanol-water

mobile phases. Similar methodology was employed to study the separation of testosterone and its derivatives on temperature-controlled micro TLC plates, controlling for temperature, mobile phase composition, chamber saturation and stationary phase (Zarzycki and Zarzycka, 2008). More recently, temperature controlled TLC was demonstrated to be useful in the analysis of volatile compounds from sage species (Sajewicz *et al.*, 2010a; Sajewicz *et al.*, 2010b; Sajewicz *et al.*, 2011). Initially, analytical TLC separations were conducted at -10^{o}C and 22^{o}C. After development, densitograms were obtained and compared. It was concluded that development at -10^{o}C provided better results and the densitograms at -10^{o}C were very useful in fingerprinting essential oils contained in the different sage species. At the same conditions, preliminary fractionation of essential oils was successfully performed on preparative TLC to facilitate GC-MS analysis that followed, although the separation performance of preparative TLC was not as satisfactory as analytical TLC (Sajewicz *et al.*, 2010b). In the following studies, this group utilized low temperature TLC in conjunction with MS and LC-MS with the help of a TLC-MS interface to obtain multiple fingerprints of essential oils from different sage species (Sajewicz *et al.*, 2010a; Sajewicz *et al.*, 2011).

As TLC can be used for flash column chromatography method development (Joseph, 2009), it can be reasonably expected that low temperature may also play a role in silica column elution.

3.5 Drying Techniques

Drying is always a method of choice to recover compounds from solution. Various drying methods are available in literature. Each method has advantages and disadvantages, which need to be thoroughly considered before application. Three types of the most commonly used drying techniques are reviewed as follows.

3.5.1 Freeze Drying

Freeze drying (lyophilisation) is the dehydration process in which water or another solvent in the substance is sublimated from solid-phase directly to gas-phase in a vacuum chamber (Figure 3.4). Literature shows that it is desirable to pre-concentrate the liquid material and increase the solids content to 30–50% to improve the economy of the process (Flink, 1975). Freeze drying comprises of three sequential steps: a) freezing in which the material is solidified under eutectic point, thereby freezing all free water; b) primary drying in which the sublimation

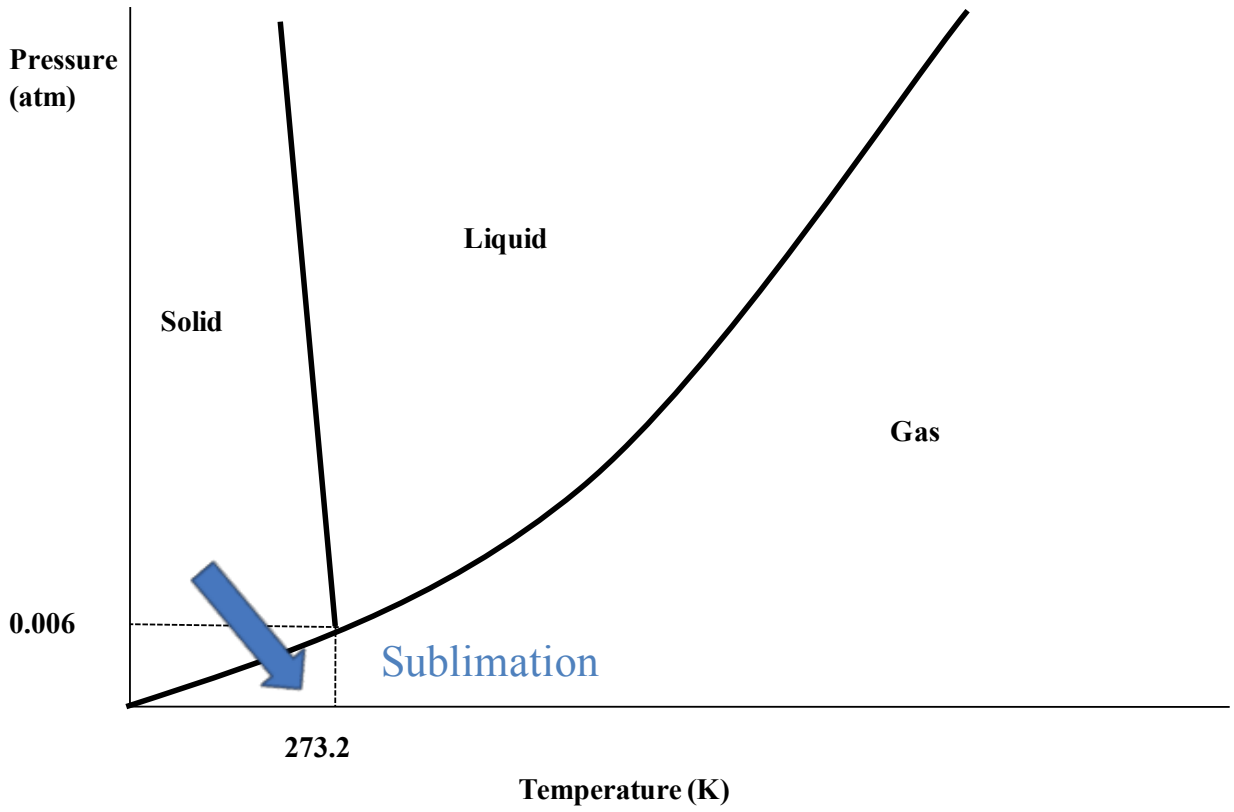

Figure 3.4 Phase diagram of water. Arrow shows phase change in sublimation (Redrawn from Barbosa-Cánovas *et al.*, 2005)

takes place under the temperature and pressure below the triple point of water; and c) secondary drying in which the moisture desorbed from the surface of porous material (Roberto and Athanasios, 2006; Yasuyuki and Tetsuya, 2010). It is believed that freeze drying can preserve chemical stability of products by minimizing the degradative reactions, such as oxidation, nonenzymatic browning, enzymatic reactions, protein denaturation, etc. (Roberto and Athanasios, 2006). Freeze drying is also expensive, energy extensive and time consuming due to the application of vacuum and slow drying rate (Franks, 1998; Roberto and Athanasios, 2006) which can be attributed to the resistance to heat and mass transfer in freeze drying process (Barbosa-Cánovas *et al.*, 2005). Therefore, it is suitable for the drying of high-value products (Ratti, 2001).

Since World War II, this technique has been widely applied in food and pharmaceutical industries (Roberto and Athanasios, 2006; Yasuyuki and Tetsuya, 2010). The commercial foodstuff that can be produced via freeze drying include, but are not limited to, powder from liquid extract, vegetables, instant coffee, meat product, and ready-to-eat meals (Flink, 1975). Sensitive pharmaceutical ingredients, such as antibiotics, antitoxins, antisera, vitamins, cancer chemotherapy drugs, diagnostic reagents, etc. are freeze dried in industrial production (Zdzis and Arun, 2006).

3.5.2 Spray Drying

Spray drying is a continuous processing technique that transforms liquid feed to dried particles by atomizing the feed and spraying the droplets to a hot drying medium. Solution, paste and suspension can be the subjects of spray drying (Huang *et al.*, 2006). A typical spray dryer contains an air heating system, feed transportation and atomization system, drying chamber and particle collection system (Figure 3.5) (Huang and Mujumdar, 2009). Note that if liquid feed is prepared in organic solvent, a closed-cycle spray dryer using inert gas should be utilized. The closed-cycle spray dryer reuses the inert gas for drying instead of exhausting directly out of the system. Condensation equipment is required to eliminate solvent vapour from the system (Figure 3.6). The advantages of spray drying include, but are not limited to: a) it being a continuous process using relatively simple equipment which allows high-tonnage production; b) its ability to spray dry thermolabile material; c) its product properties being readily controlled (e.g. moisture content, particle shape and particle size distribution); and d) its ability to set up as an aseptic

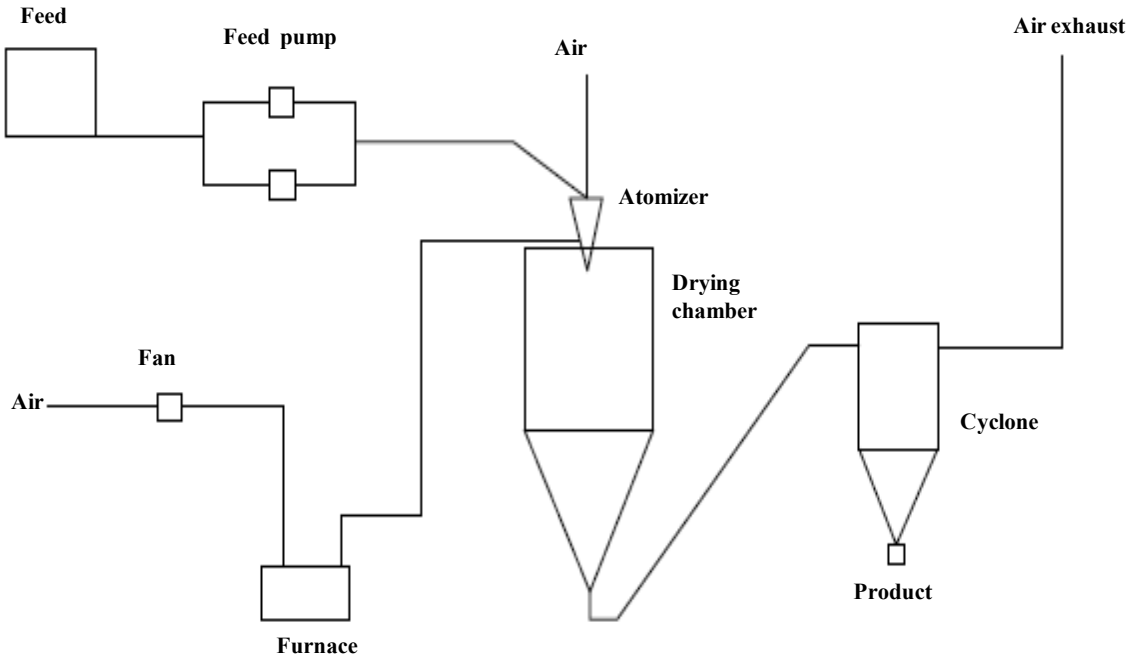

Figure 3.5 Layout of a typical spray dryer (open cycle) (Redrawn from Huang and Mujumdar, 2009)

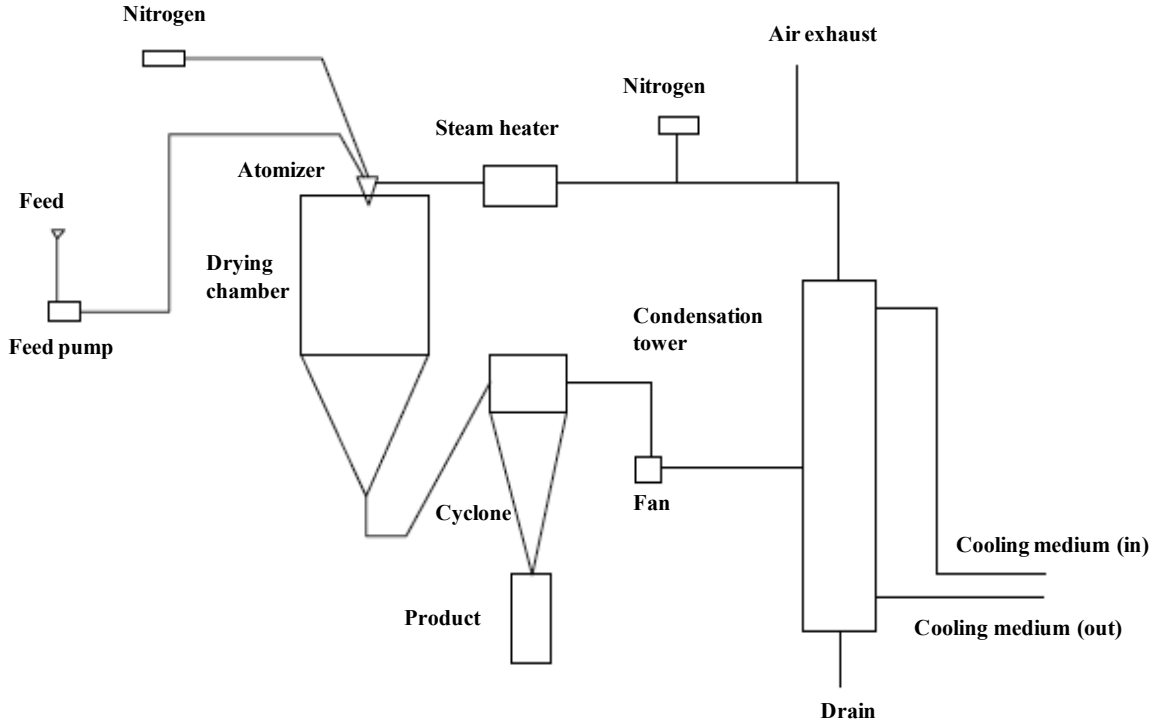

Figure 3.6 Layout of a closed-cycle spray dryer (Redrawn from Huang and Mujumdar, 2009)

process by using high-efficiency particulate air filters in the atomizer and sterilization of the nozzle and chamber walls (Huang and Mujumdar, 2009; Sollohub and Cal, 2010). Spray drying has been widely applied in large-scale production of a variety of products in food, pharmaceutical and biochemical industries (Table 3.3).

Particle size is directly related to the product recovery. Particles that are too fine may not be separated from the drying gas by the centrifugal force in the cyclone. For example, Maa *et al.* (1998) showed that a Büchi Mini Spray Dryer B-190 with standard cyclone could not recover particles smaller than 2 μm. Several parameters need be adjusted in spray drying, which can affect the quality of final products and efficiency of the process (Table 3.4). As shown in Table 3.4, higher solid concentration in feed and higher airflow rate can improve the separation of particles in cyclone. This is consistent with the findings of Prinn *et al*. (2002), in which the authors reported that feed concentration and gas pressure had the greatest effects on particle size.

3.5.3 Falling Film Evaporation

Falling film evaporator (FFE) is a type of efficient concentration device used in industrial processing (Figure 3.7). Feed is distributed at the top into vertical tubes. A thin film of liquid is formed inside the tubes so that the heat transfer rate is maximized and the residence time is minimized. Vapour and concentrated liquid is separated at the bottom of the device (Smith, 2011). The short residence time make it suitable for processing thermolabile fluid (Alhusseini *et al.*, 1998). FFE has been widely applied in food, pharmaceutical, desalination, refrigeration and air conditioning industries (Wiegand, 1971; Li *et al.*, 2011). Literature shows that in food products, such as milk, fruit juice, sugar and flavouring, material can be processed by this technique (Arndt and Scholl, 2011; Sharma *et al.*, 2012).

3.6 Liquid-Liquid Extraction

Liquid-liquid extraction is a process in which two immiscible liquid-phases come in contact so that compounds of interest are transferred from one phase to another (Don and Robert, 2008). The distribution of compounds of interest is caused by thermodynamic equilibrium between two phases, which follows Nernst Distribution Law (Wells, 2003; Don and Robert, 2008). Liquid-liquid extraction has been widely applied in food processing, separation of biomolecules, enrichment of pharmaceuticals, wastewater treatment, etc. (Don and Robert, 2008).

Table 3.3 List of products that can be produced *via* spray drying (Adapted from Huang *et al.*, 2006)

Industry	Products
Food industry	Milk, whey, egg, soya protein, glucose, total sugar, maltodextrin
Pharmaceutical industry	Penicillin, blood products, enzymes, vaccines,
Biochemical industry	Algae, fodder antibiotic, yeast extracts, enzymes

Table 3.4 Relationships of different parameters in spray drying process (Adapted from Cal and Sollohub, 2010)

Parameters	Air flow rate	Air humidity	Inlet temperature	Feed rate	Organic solvent	Solid concentration in feed
Outlet temperature	+[1]	+	+	–[2]	+	+
Particle size	NA[3]	NA	NA	+	–	+
Product moisture	–	+	–	+	–	–
Efficiency	+	–	+	+/–	+	+

[1]Denotes positive correlation

[2]Denotes negative correlation

[3]Not applicable

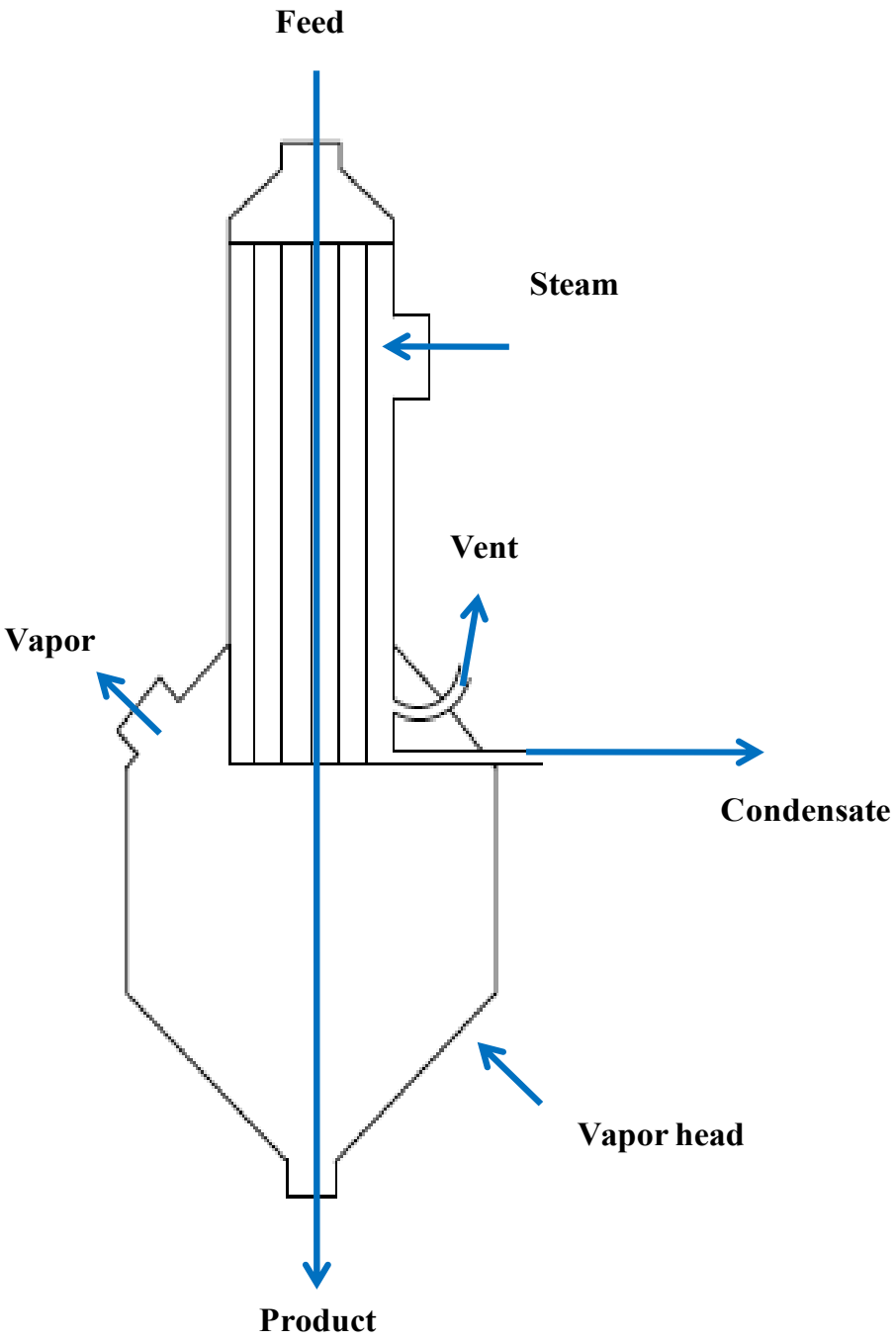

Figure 3.7 Layout of a FFE (Redrawn from http://nptel.iitm.ac.in/courses/103103027/pdf/mod3.pdf)

Solvent selection is significantly important when preparing for a liquid-liquid extraction process. Solubility of the compounds of interest in the extractant should be high enough so that the extractant to feed ratio can be controlled at a reasonable level. An extractant having a high selectivity for the compounds of interest is desirable so that compounds can be efficiently recovered, concentrated and purified to some extent. In industrial practice, the solvent should be readily recovered, recycled and stored. From an economical point of view, it is highly advantageous to use a single extractant to accomplish the extraction, which can be then recycled and reused for subsequent extractions and other purposes in the same process. Environmentally friendly solvents with limited or no toxicity is preferred. (Don and Robert, 2008).

Agitation force is a critical parameter when performing extraction. The mass transfer rate is determined by the force of agitation and interfacial area. Strong agitation force can disperse one liquid phase as small droplets into another phase, which may generate stable emulsion, especially when surfactants are present. In contrast, mild agitation generates large droplets of liquid in another phase, which can be readily separated into two phases. The phase separation rate is also related to the density difference and interfacial tension of the two phases, i.e. large density difference and high interfacial tension lead to swift phase separation (Tedder, 2008).

In industrial production, liquid-liquid extraction is conducted in extraction columns, stirred tanks and mixer-settlers (Don and Robert, 2008). Following extraction, other processing techniques, e.g. solvent evaporation, freeze drying, filtration, precipitation, distillation, are often applied to process the compounds of interest into acceptable purity and texture (Tedder, 2008).

3.7 Effects of Agitation and Freeze-Thaw on Suspension

The term used to describe the destabilization of a suspension by agitation is “shear-induced coagulation”. Vanni and Baldi (2002) reviewed the mechanism of shear-induced coagulation of colloidal particles. Two different forces are present among colloidal particles: attractive van der Waals force and repulsive electric double layer interaction. When agitated, the particles are forced within close proximity. If the attractive force (van der Waals force) outperforms the repulsive force (electric double layer interaction), and the net force can overcome thermal agitation and hydrodynamic drag, aggregation of particles takes place.

Coagulation may happen if a suspension is subjected to a freeze-thaw cycle. Nakamura and Okada (1976a) observed that fine particles in suspension tended to coagulate when freeze-thaw

treatment was carried out; particle concentration and freezing rate also had influence on this process. Further investigation of the underlying mechanism of the phenomenon showed that the growth of ice crystals, caused by slow freezing, had pushed the suspended particles close to each other. Coalescence occurred if the van der Waals force exceeded the energy barrier (Nakamura and Okada, 1976b).

3.8 Amberlite™ XAD™ Resin

Amberlite™ XAD™ resins are polymeric adsorbent products with highly porous structures manufactured by Rohm and Haas Company (PA, USA). On the basis of "like attracts like", the styrenic type resin exhibits hydrophobic behaviour in polar solvents and adsorbs organic compounds, while the acrylic type resin exhibits slightly hydrophilic behaviour in non-polar solvents and adsorbs polar compounds. As multiple products are available within each resin type, it is recommended that the selection of resin should be determined by nature of solvent, and the functionality, polarization, and size of solute. Regeneration of resin and desorption of solute can be achieved by increasing the temperature, solvent elution and changing pH. When studying the application of resin in concentrating a single solute, it is recommended to agitate certain amount of polymeric adsorbent in a solution for a certain amount of time so that adsorption curve and equilibrium capacity can be observed and calculated, respectively. It is also advised to use small columns packed with resin when investigating application in separating two or more compounds (Rohm and Haas Company, 2000).

Amberlite™ XAD™ 1600 resin is composed of white translucent beads made of macroreticular cross-linked aromatic polymers (Figure 3.8). It is designed for both batch operation and column operation in industrial scale. Rohm and Haas Company recommends the resin can be applied in a) the recovery and purification of antibiotics, water soluble steroids, amino acids and proteins; b) the absorption of non-polar compounds from polar solvents; and c) fruit juice upgrading. Recovery of hydrophobic compounds can be achieved by eluting the resin with water miscible organic solvents, including alcohols and acetone. It is claimed that this type resin has been proved for food applications under FDA Food Additive Regulation 21CFR173.65-Divinylbenzene Copolymer (Rohm and Haas Company, 2007).

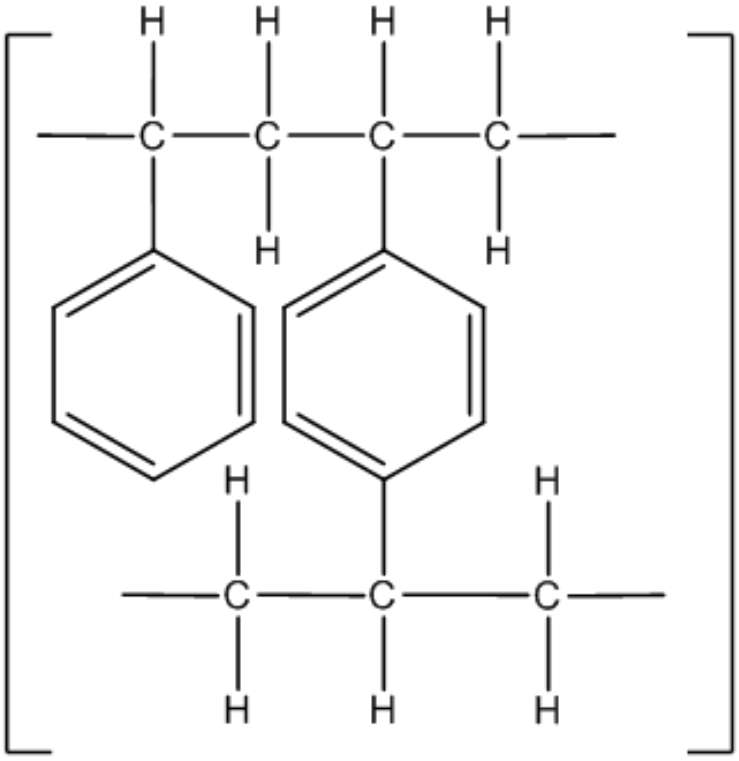

Figure 3.8 Chemical structure of Amberlite™ XAD™ 1600 resin (Redrawn from Rohm and Haas Company, 2007)

3.9 Industrial Production of Natural Products from Plants

Plants produce a diverse range of organic compounds in metabolism, classified as primary and secondary metabolites. Primary metabolites are indispensable for growth and survival of all plants, while the role of secondary metabolites, present in low quantity in plants, is not fully elucidated (Croteau *et al.*, 2000).

Extraction of natural products from plant sources was performed as early as Mesopotamian and Egyptian times. Presently, plants supply natural substances for food, pharmaceutical and cosmetic applications. These metabolites can be extracted from plant seed, root, leaves, bark, etc. (Bart, 2011). In general, plant primary metabolites are regarded as high-volume and low-value products, whereas plant secondary metabolites are more expensive due to scarcity (Balandrin *et al.*, 1985). Commercially available secondary metabolites include, but are not limited to, terpenoids, phenylpropanoids, flavonoids and alkaloids (Croteau *et al.*, 2000). Recently, there has been growing interest in commercialization of KTs, a type of secondary metabolites of flax, due to their biological activities (Section 3.3.1). Potentially, large quantities of these compounds may be needed for new product development in pharmaceutical, cosmetic and food industries. The commercial value of KT products is much higher than that of flaxseed and flaxseed oil (Reaney, personal communications).

Cultivation and extraction are two important aspects in the production of natural compounds. Concentrations of natural compounds are affected by cultivars, growth location, climate, and types of soil, etc. For example, oleanolic acid could be recovered from almond hulls, privet, rosemary, thyme, clove, lavender, olive, hawthorn, periwinkle, etc. High concentrations of oleanolic acid were only observed in a type of sage. Warm and wind-protected locations with light soil were ideal for the growth of the sage containing high level of oleanolic acid (Bart, 2011). With respect to KT content in flax, Olivia *et al.* (2012) recently screened the flax world core collection grown in two different locations, Saskatoon, SK and Morden, MB. Accessions with the highest content of KTs were identified. It was also reported that KT content was not significantly related to flax morphological and chemical traits. This knowledge is potentially helpful in cultivating flax accessions suitable for KT production.

Selection of solvents for industrial production is discussed in section 3.8. However, there are more stringent regulations for solvents utilized during natural product extraction. In food industry, the European Union allows the use of water, natural solvents, propane, butane, ethyl acetate,

ethanol, CO_2, N_2O, and acetone (Bart, 2011). In pharmaceutical production, organic solvents are graded into three classes. Class 1 includes solvents with unacceptable toxicity and, therefore, should not be used (FDA, 2012). Class 2 includes solvents that "should be limited in pharmaceutical products because of their inherent toxicity" (FDA, 2012) and must be regulated by permitted daily exposure (PDE) (Table 3.5). Class 3 solvents "may be regarded as less toxic and of lower risk to human health", although they need to "be limited by good manufacturing practices (GMP) or other quality-based requirements" (FDA, 2012) (Table 3.6). Currently, there is no published peer reviewed literature describing the industrial production of KTs. It is possible to scale up analytical methods such as those mentioned in Section 3.4. However, some of the aforementioned protocols involve multiple chromatographic separations and are laborious and, thus, may not be feasible for industrial process (Matsumoto *et al.*, 2001; Matsumoto *et al.*, 2002; Brühl *et al.*, 2008). Most methods were developed specifically for analytical purposes and require large amounts of solvent. A new isolation method developed by Reaney *et al.* (2013b) successfully separates KTs from flaxseed oil-laden silica, but utilizes undesirable solvents, including hexane, methanol, and dichloromethane. Although the removal of solvents can be achieved to satisfy the regulatory standards, the cost of quality control is appreciable. Besides, the use of multiple solvents not only increases production cost, but also makes it difficult to recycle the individual solvents.

3.10 Summary of Literature Survey

As discussed in this section, KTs are present in low concentration in flaxseed oil. Recovery of KTs from flaxseed oil can be laborious and time-consuming (Reaney *et al*., 2013b). Current method also utilizes solvents, which are undesirable for food, cosmetic and pharmaceutical applications. Recovery of KTs from liquid extract, although important in processing, has never been reported in literature other than rotary evaporation. Therefore, this project aims at establishing improved KT extraction protocol and selecting optimum KT recovery method.

Table 3.5 PDE of select Class 2 solvents (Modified from FDA, 2012)

Solvent	PDE (mg/d)
Acetonitrile	4.1
Dichloromethane	6.0
Hexane	2.9
Methanol	30.0
Chloroform	0.6

Table 3.6 Class 3 solvents (Modified from FDA, 2012)

Acetic acid	Heptane
Acetone	Isobutyl acetate
Anisole	Isopropyl acetate
1-Butanol	Methyl acetate
2-Butanol	3-Methyl-1-Butanol
Butyl acetate	Methylethyl ketone
Tert-butylmethyl ether	Methylisobutyl ketone
Cumene	Methyl-1-propanol
Dimethylsulfoxide	Pentane
Ethanol	1-Pentanol
Ethyl acetate	1-Propanol
Ethyl ether	2-Propanol
Ethyl formate	Propyl acetate
Formic acid	Tetryhydrofuran

CHAPTER 4
MATERIALS AND METHODS

4.1 Study of Effects of Low Temperature Development on Retention Factor of 1 in TLC

Dodecamolybdophosphoric acid (9.977 g) was weighed and dissolved in 100 mL ethanol to prepare phosphomolybdic acid (PMA) solution. A standard solution of **1** was prepared by mixing 47.0 mg of **1** powder with dichloromethane in a 10 mL volumetric flask. The starting line was drawn 1.0 cm from the bottom edge of the TLC plate (2.5 × 7.5 cm, EMD Chemicals Inc., Darmstadt, Germany) and the finishing line was drawn 0.5 cm from the top edge. The standard solution of **1** was applied as a spot on the starting line using a glass capillary. Ethyl acetate (10 mL) was added to two TLC developing jars that were lined with filter paper. One jar was placed at R.T. A spotted TLC plate was placed inside, after which the developing jar was capped. Another jar and plate were placed in a –20^{o}C freezer for 15 min. Then the plate was placed inside the capped jar for solvent development. To another jar that was lined with filter paper, 50% ethyl acetate in hexane (10 mL) was added. A spotted TLC plate was developed within the capped jar at R.T. All TLC plates were removed from their respective jar when solvent reached the finishing line and dried under airflow. The plates were then dipped into the phosphomolybdic acid solution for staining, followed by drying in the oven at 60^{o}C. The R_f values of **1** were calculated. Each TLC experiment was performed in duplicate.

4.2 Study of KT Profile of Fresh Flaxseed Oil

Bioriginal Food and Science Corp. (Saskatoon, SK, Canada) provided pressed flaxseed oil. Methanol (2 mL) was mixed with flaxseed oil (5 mL) by shaking in a 30 mL separatory funnel. After phase separation, the upper layer (primarily methanol) was removed and analyzed by HPLC (Agilent Technologies Canada Inc., ON, Canada) equipped with a quaternary pump (Agilent 1200) auto-sampler (Agilent 1100/1200), thermostat (Agilent 1100/1200) and diode array detector (Agilent 1200; wavelength range 190-950 nm). A monolithic C_{18}-bonded silica gel

column (Chromolith® SpeedROD, 50 × 4.6 mm I.D., MerckKGaA, Darmstadt, Germany) was utilized for analysis (Olivia *et al.*, 2012). The solvent gradient is provided in Table 4.1. The methanol extract was also analyzed by HPLC-MS, using Agilent 1200 series connected to MicroTOF-Q Mass Spectrometer (Bruker, Daltonik GmbH, Bremen, Germany). The analytical method of Jadhav *et al.* (2012) was applied. Briefly, electrospray ionization source was utilized to ionize eluting compounds. Separation of KTs was achieved using a Chromolith FastGradient RP-18e column (50 × 2 mm I.D., MerckKGaA, Darmstadt, Germany). The mobile phase consisted of H_2O with 0.1% formic acid and acetonitrile with 0.1% formic acid. The employed solvent gradient is listed in Table 4.2.

4.3 Study of Low Temperature Elution of Oil-Laden Silica

A bottle of ethyl acetate (Fisher Scientific, NJ, USA) was placed in the cold room (0–4^{o}C) overnight. One litre of freshly pressed flaxseed oil (Bioriginal Food & Science Corp., SK, Canada) was mixed with 40 g of silica by stirring for 2 h. After settling at R.T., the oil layer was decanted at R.T. and silica was loaded in a 2 L Buchner funnel, which was placed on top of a side-arm flask under vacuum and the oil was removed from the silica oil slurry. The Buchner funnel was placed in the cold room for 20 min. Four fractions of 400 mL cold ethyl acetate were used to sequentially wash the silica layer. The Buchner funnel was then placed in fume hood until it reached R.T. and was washed with two fractions of 400 mL ethanol to recover all remaining KTs. All collected fractions were evaporated under vacuum. All the flasks were weighed before and after evaporation so that the exact weight of each fraction could be determined. The 1st and 2nd fractions contained significant amounts of oil after evaporation and could not be analyzed using mass spectrometry or HPLC directly. Therefore, 50 and 10 mL methanol was then added to the oily residuals of 1st and 2nd fractions to extract KTs, respectively. The resulting two samples were then evaporated under vacuum and reconstituted in 2 mL methanol. Residuals from all the other four fractions were dissolved in 2 mL methanol after solvent removal. All samples were analyzed by HPLC according to the method described in section 4.2.

4.4 Study of aq. Ethanol as an Extraction Solvent of KTs

Flaxseed oil (5 mL) was added to 100% ethanol (5 mL) in a 30 mL separatory funnel. The funnel was shaken vigorously by hand to mix the two phases.

Table 4.1 HPLC solvent gradient for KT analysis (Olivia *et al.*, 2012)

Time (min)	Water (%)	Acetonitrile (%)
0.0	70	30
4.0	30	70
4.5	10	90
5.0	70	30
6.0	70	30

Table 4.2 HPLC-MS solvent gradient for KT analysis (Jadhav *et al.*, 2012)

Time (min)	Water (%)	Acetonitrile (%)
0.0	60	40
2.0	60	40
10.0	10	90
10.3	60	40
16.0	60	40

After shaking, the funnel was placed in a ring holder to allow phase separation. The upper layer of solution in the funnel (ethanol extract) was collected. Using a similar procedure, oil was contacted with 50, 60, 70, 80, and 90% of aqueous ethanol. HPLC analyses were performed according to the method in section 4.2.

4.5 Study of NMR as a Means to Estimate KTs to Oil Ratio

^{1}H-NMR (500 MHz) analyses were conducted for the 80% aq. ethanol extracts to determine the complexity of the extracts. Aqueous ethanol (80%, v/v; 2 mL) was mixed with flaxseed oil (5 mL) in a test tube using a vortex mixer. After the sample was allowed to settle, a portion of the upper liquid-phase (0.6 mL) was collected. The extract was dried under airflow in a glass vial. After drying dimethylformamide (0.5 mL; DMF; 5 mg/mL) standard solution in deuterated chloroform ($CDCl_3$) was added. The solution was then transferred to an NMR tube for analysis.

Solutions of **1** were prepared in deuterated methanol (MeOD) at concentrations of 20.50 mg/mL, 10.25 mg/mL, 5.12 mg/mL and 2.56 mg/mL. A DMF standard solution of 1.5 mg/mL in MeOD was also prepared. Individual solutions of **1** were then each mixed with 0.1 mL of the DMF solution in an NMR tube for analysis.

Fatty acid methyl ester (FAME) of flaxseed oil was prepared by base catalyzed transesterification as follows. Potassium hydroxide (0.9 g; KOH) was dissolved in 12 g methanol by agitation for 30 min, after which the solution was mixed with 100 g of flaxseed oil. The mixture was agitated overnight then transferred to a separatory funnel to allow phase separation. The upper phase of methyl esters was collected, mixed with additional KOH solution in methanol (0.5 g KOH in 8 g MeOH) and agitated overnight. The upper phase was collected and concentrated by rotary evaporation. Water (5 g) was added to the liquid upper phase, followed by vigorous shaking. Impurities in flaxseed oil FAME were removed by sequential contact with a sodium phosphate column and a silica column. Flaxseed oil FAME solutions were prepared in MeOD at concentrations of 34.2 mg/mL, 65.8 mg/mL, 99.8 mg/mL and 148.6 mg/mL with DMF as internal standard (1.5 mg/mL).

KT **1** and flaxseed oil FAME mixture samples were prepared by mixing 0.4 mL of **1** standard solution of varying concentrations described above, 0.1 mL of flaxseed oil FAME and 0.1 mL DMF solution in MeOD (20.0 mg/mL). ^{1}H-NMR (500 MHz) spectra were recorded for all samples using a Bruker 500 MHz ^{1}H-NMR spectrometer (Bruker, ON, Canada). Chemical

shift values were reported in parts per million (ppm) relative to the internal standard DMF. NMR analyses were conducted at R.T., where 128 scans were obtained for each spectrum.

4.6 Study of Extraction of Flaxseed Oil Using aq. Ethanol

Aqueous ethanol mixtures were prepared (50, 60, 70, 80, 90, and 100%) and added to flaxseed oil in 100 mL graduated cylinders. The cylinders were then covered with Parafilm™ and shaken by hand for 30 s. After phase separation, the volume of the extract layer (top phase) was recorded and divided by the initial volume of aq. ethanol to calculate solvent recovery. Volume ratios (solvent to oil) of 2:1, 1:1, 0.5:1, 0.25:1, and 0.125:1 were tested. The total KTs and oil within aq. ethanol extracts was determined as described below. The same set of aq. ethanol was prepared in triplicate within 50 mL centrifuge tubes. The volume of aq. ethanol and flaxseed oil added to the tubes is listed in Table 4.3. The tubes were shaken with a vortex mixer at 2200 rpm (IKA® Works Inc., NC, USA) for 15 s instead of shaking by hands. The tubes were placed on the rack for 4 h to allow phase separation. The extract layer of aq. ethanol was collected. Each extract sample (0.5 mL for volume ratios of 2:1, 1:1, and 0.5:1; 0.1 mL for volume ratios of 0.25:1) was transferred to a glass tube and dried under airflow. A solution of dimethylformamide (DMF) in MeOD (0.5 mL) was prepared and added to the tubes containing the dried extract. ^{1}H-NMR spectra of flaxseed oil before and after 70% aq. ethanol extraction were also obtained in $CDCl_3$.

4.7 Study of Agitation Force and Agitation Time in Liquid-Liquid Extraction

Flaxseed oil (160 mL) was measured and transferred into each beaker (250 mL), followed by addition of aq. ethanol (70% v/v; 40 mL). The beakers containing the samples covered with Parafilm™ (Pechiney Plastic Packaging, IL, USA) were placed on a magnetic stirrer (IKA, NC, USA) and then were agitated using stirring bars of the same size. The applied stirring speed settings were 140, 170, and 600 rpm. At the agitation speed of 140 rpm and 170 rpm, 0.2 mL of liquid extract was taken at 2, 6, 10, 14, 18, 22, and 26 h. Extracts were dried under airflow and reconstituted in 1 mL methanol. At the agitation speed of 600 rpm, flaxseed oil and aq. ethanol was stirred for 5 min, 30 min and 60 min. Part of the mixture was transferred into a 50 mL centrifuge tube (VWR, PA, USA) and was centrifuged at 4,500 rpm for 3 min (Allegra™ X-22R centrifuge, Beckman and Coulter, CA, USA).

Table 4.3 Volume of solvent and oil added to centrifuge tubes for extraction

Volume ratios (solvent to oil)	Volume of solvent (mL)	Volume of oil (mL)
2:1	20	10
1:1	10	10
0.5:1	10	20
0.25:1	5.0	20
0.125:1	2.5	20

A portion of the upper liquid extract (0.2 mL) was collected, dried under airflow and reconstituted in 1 mL of methanol. After extraction, part of the processed flaxseed oil was dried in a rotary evaporator (Rotavapor R-210, BUCHI Corporation, NY, USA) at 60^{o}C for 15 min and was cooled to R.T. Both the crude and processed flaxseed oil was then analyzed for KT content using the method of Gui *et al.* (2012). Briefly, a cotton ball was placed at the bottom of a 3 mL syringe, which was then covered by sand (1 cm in height SX0070-3, EMD, MA, USA). Silica gel 60 (0.5 g) was slurried in 1 mL hexane then poured into the syringe. Another layer of sand (0.5 cm in height) was placed on top of the silica. The syringe was inserted into Luer lock fittings (Becton Dickinson, MD, USA) of a Visiprep SPE vacuum manifold (Supelco Inc., PA, USA). Each silica column was conditioned with 2 mL of hexane before use. Flaxseed oil (1 mL) was mixed with hexane (1 mL) and loaded onto the column. The column was eluted sequentially with 10 mL aliquots of solvents of increasing polarity: 100% hexane, 20% ethyl acetate in hexane (v/v), 50% ethyl acetate in hexane (v/v), 100% ethyl acetate and 10% methanol in dichloromethane (v/v). The latter two fractions were combined, evaporated under reduced pressure and reconstituted in 1 mL methanol. All samples were analyzed according to the HPLC method described in section 4.2. Experiments were performed in triplicate.

4.8 Twelve Litre Scale Solvent Extraction and Peptide Recovery

Flaxseed oil (12 L) and 70% aq. ethanol (3 L) were transferred to a 20 L pail and agitated using a magnetic stirrer (Maxi-stirrer, Thermo Scientific, NC, USA) for 10 min. A sample of the mixture (50 mL) was removed while stirring then centrifuged at 4,500 rpm for 3 min. KT content of both the upper phase extract and lower phase oil were analyzed as described in section 4.7. Experiments were performed in duplicate.

This extraction process was repeated to supply additional upper phase liquid extract for drying. The flaxseed oil and aq. ethanol mixture was settled overnight to allow phase separation. The upper extract layer was collected and evaporated in a 1 L round bottom flask at 60^{o}C and 120 mbar. When condensation rate visibly decreased, 100 mL of 100% ethanol was added to the round bottom flask. This was repeated until no liquid was visible inside the flask. The total volume of 100% ethanol was recorded. Then, the pressure was reduced to 50 mbar and the evaporation temperature was increased to 70^{o}C for 30 min. The dried mixture was placed in a vacuum oven at 70^{o}C until the weight was constant.

Upper phase liquid extract was initially evaporated at 60°C and 120 mbar until the rate of solvent condensation in the condenser slowed appreciably. Then, the cloudy suspension and solids were transferred into an aluminum tray to form a thin layer. The tray was placed into a freeze dryer (Labconco, MO, USA) at –6°C and 0.02 mbar for 4 d, after which the material was weighed.

The upper phase liquid extract (ca. 2.5 L) from the flaxseed oil (12 L) was evaporated at 60°C and 120 mbar until there was ca. 700 mL extract remaining in the round bottom flask. The cloudy suspension was then subject to spray drying (Buchi Mini spray dryer B-290, Büchi Labortechnik AG, Switzerland). The alcoholic solution was subjected to spray drying under nitrogen gas to prevent oxidation and combustion. In an initial experiment, the aspirator was adjusted to 100%, the nitrogen gas flow was set at level 4, inlet temperature at 150–160°C, peristaltic feed pump at 20–30%, and the nozzle cleaner setting was adjusted to 4. The second experiment was conducted with adjusted settings: aspirator at 100%, nitrogen gas flow at level 5, inlet temperature at 220°C, pump at 40% and nozzle cleaner at 4. Powder was collected from the spray dryer sample receiver and weighed.

In an additional study, absorbent resin (Amberlite™ XAD™ 1600 resin, Rohm and Haas Canada, ON, Canada) was tested for its ability to remove lipids from the KT mixture. The effect of pre-treatment using resin on spray drying behaviour was studied. Initially, a small column was prepared using a 3 mL syringe. A cotton ball was placed at the bottom of the syringe, which was then covered by sand (1 cm in height). XAD 1600 resin (1 g) was slurried in 1 mL 70% aq. ethanol and was poured into the syringe. Another layer of sand (0.5 cm in height) was placed on top of the silica. The syringe was inserted into Luer lock fittings (Becton Dickinson, MD, USA) of the Visiprep SPE vacuum manifold (Supelco Inc., PA, USA). The column was conditioned with 2 mL 70% aq. ethanol prior to use. Fifteen fractions of 5 mL liquid extract were passed through the column and collected. Sample of each fraction (0.5 mL) were dried in airflow and reconstituted in MeOD with DMF as an internal standard. ^{1}H-NMR spectrum was obtained for each sample. Mole ratios of KTs to oil were calculated as described previously Section 3.1.4.6. In the next step, a column (16 × 0.5 cm I.D.) was prepared by loading 125 g of XAD 1600 resin. The column was conditioned with 70% aq. ethanol before use. Liquid extract (ca. 2.5 L) was loaded onto the column and the resulting solution was collected for evaporation. The spray drying conditions were as follows: aspirator at 100%, nitrogen gas flow at level 4, inlet temperature at

150–170°C, pump at 20–40%, and nozzle cleaner at 4. Dried powder was collected and weighed.

In another set of experiments, 2.5 L of liquid extract was evaporated to ca. 300 mL producing a cloudy suspension. The solution was transferred to 50 mL centrifuge tubes and centrifuged at 3900 relative centrifugal force (RCF) for 40 min. A sample of the supernatant was analyzed according to section 4.7.

4.9 One Hundred and Sixty Four Litre Scale Solvent Extraction and Peptide Recovery

Flaxseed oil (164 L) and 70% aq. ethanol (41 L) were mixed in a 208 L drum, which was then rolled on a drum roller (Fixed Speed Portable Drum Rollers 201/20-1, Morse Manufacturing Company, Inc., NY, USA) for 10 min. The mixture was settled for 7 d before liquid extract was collected. This process was repeated twice. KT content of both the liquid extract and processed oil were analyzed as described in section 4.7.

A total of 60 L upper phase liquid extract was subject to evaporation (Rotavapor R-200, BUCHI Corporation, NY, USA) in two batches (30 L per batch) as described below. Upper phase liquid extract concentrate (10 L) was initially transferred into a 20 L round bottom flask for evaporation. When ca. 4 L solvent was recovered from the condenser, another 4 L liquid extract was added to the flask under vacuum. When the last fraction of aq. ethanol was evaporated, 2 L of 100% aq. ethanol was added to speed up evaporation rate of water. The recovered solvent was collected and was tested for density and ethanol content. This process was repeated until ca. 4 L of cloudy suspension was produced for each batch. Evaporation conditions were as follows: water bath temperature at 60°C, rotation speed at 50 rpm, vacuum pressure at 120 mbar, and cooler (HX-20, Jeio Tech Co. Ltd, Republic of Korea) at 4°C. Evaporation produced a yellowish semisolid and a cloudy suspension. The semisolid was dissolved in 100% ethanol (4 L) and the suspension was collected for further analysis.

The cloudy suspension contained what appeared to be peptide particles. In order to coagulate the particles, a number of treatments were tested. The solution was subjected to: a) Freezing-thawing. Suspension (10 mL) was transferred into centrifuge tubes (15 mL). The tubes were placed into a refrigerated circulating bath (VWR, PA, USA) and frozen at –5, –15, and –25°C for 30 min. The tubes were thawed at R.T. It was observed that upon thawing solid material settled. Clear solution produced by thawing (0.2 mL) was dried in airflow and reconstituted in 1

mL methanol. In another set of experiments, 100 mL suspension was cooled to –25 ~ –30°C overnight. After thawing and settling in R.T., the mixture was filtered through glass filter paper (Whatman GF/A 70 mm, Whatman Inc., NJ, USA). Solid on the glass filter paper was dried at 70°C for 3 h. Part of the dried solid was dissolved in methanol. b) Filtration. The suspension was filtered through a number of filtration media. The suspension was filtered through a 13 mm syringe filter (0.45 μm PTFE membrane, VWR, PA, USA) or through glass filter paper (100 mL) in a Buchner funnel. Filtrates (0.2 mL) were dried under airflow and reconstituted in 1 mL methanol. c) Agitation. Suspension (100 mL) was agitated in a 250 mL beaker at 500 rpm for 5 h. The mixture was settled overnight, after which a sample of free solution (0.2 mL) was collected and dried under airflow then reconstituted in 1 mL methanol. d) Centrifugation. Suspension (500 mL) was transferred to a centrifuge bottle (500 mL, Beckman and Coulter, CA, USA) and centrifuged at 15,000 RCF for 80 min. A sample of the supernatant (0.2 mL) was dried under airflow and reconstituted in methanol (1 mL). Solid at the bottom of the bottle was collected, dried in an oven at 70°C and dissolved in methanol. All samples were analyzed by HPLC and the powder of KT mixture produced by spray drying was analyzed by HPLC-MS according to the method in section 4.2. Experiments were performed in triplicate.

4.10 Four Thousand Two Hundred Litre Scale Solvent Extraction and Peptide Recovery

Extraction and a portion of the processing were conducted at the Bio Processing Centre (SK, Canada). Flaxseed oil (4,201 L) was transferred into a stainless steel tank. In another tank, 70% aq. ethanol (1,050 L) was prepared from ethanol denatured with ethyl acetate (SDAG-13, Commercial Alcohols Inc., ON, Canada). Then, 70% aq. ethanol was pumped through the bottom of the tank containing flaxseed oil while the agitation was in progress. The time when the transfer of aq. ethanol was completed was recorded as the starting time of agitation. Agitation was conducted for 60 min and samples of the mixture of flaxseed oil and aq. ethanol were collected at 0 min, 30 min and 60 min. Samples were centrifuged to facilitate phase separation. The KT content in liquid extract, crude flaxseed oil, and processed oil was analyzed as described in section 4.7. The mixture was settled in the tank for 6 d before releasing the oil phase. Liquid extract was then evaporated in a FFE at 40–50°C and vacuum pressure at 25–29 mbar. The resulting cloudy suspension was collected and weighed. Denatured ethanol (SDAG-13) was

added to the evaporation system and it was circulated to dissolve any gels or solids that may have accumulated in the FFE. This was repeated twice with 74 and 49 L ethanol, respectively. The rinsing solution was collected and evaporated in two batches. For each batch, the ethanol solution was continuously added to the 20 L evaporation flask of a rotary evaporator using settings described previously (section 4.9). When solvent removal neared completion, 4 L of 100% ethanol was added to dissolve the material in the evaporator flask. The gel was then evaporated to dryness. Solid from both batches were combined, placed in a vacuum oven at 60^{o}C and 65 mbar for 2 d. The final product was obtained by pulverizing the dried solid in a coffee grinder to powder.

Recovery of KTs from dilute suspension was tested by subjecting the suspension to a freeze-thaw cycle. The suspension was transferred to a pail, which was frozen at –25 to –30^{o}C for 3 d and was thawed in R.T. Samples (0.2 mL) of the suspension before and after this treatment were dried under airflow and reconstituted in 1 mL methanol for HPLC analysis of KT content (section 4.2).

A hydrophobic adsorption resin (XAD^{TM} 1600) was also studied for its potential to recover KTs from suspension. Initially, a small column was prepared using 1 g of resin as described in section 4.8. Fifteen fractions of 10 mL suspension were loaded and eluting fractions collected. To scale up the process, 150 g of XAD^{TM} 1600 resin was added to pails containing 17.0–18.5 kg suspension. The resin and the suspension was agitated for 3 h. Samples were collected at the time of 0.5, 1, 2, and 3 h. The resin was collected by filtering the suspension through filter paper (Whatman No. 2, 150 mm, Whatman Inc., NJ, USA) in a Buchner funnel (2 L), in which the resin was washed by four fractions of ethanol (400 mL, 100%) for regeneration. The ethanol eluent was evaporated to dryness in a rotary evaporator. The partially dry solid was dried further in a vacuum oven for 2 d. The absorption-regeneration process mentioned above was repeated twice by adding regenerated resin to another pail of suspension. The experiment was duplicated. Six pails of suspension were processed. All samples (0.2 mL) were dried in airflow and reconstituted in methanol (1 mL) for HPLC analysis of KT content (section 4.2).

4.11 Analysis of KT Content in Products

All KT mixtures, except for the one from freeze-drying, were analyzed as follows. KT mixtures were dissolved in methanol to prepare solutions at concentrations of 0.2 to 0.3 mg/mL.

Samples of each solution (2 mL) were mixed with 30% hydrogen peroxide (0.2 mL) for 30 min. Desulfurized **3** solution (0.1 mL, 0.2 mg/mL in methanol) was added to 0.9 mL of the oxidized sample. All samples were analyzed by HPLC as described in section 4.2.

4.12 Statistical Analysis

Statistical analyses were conducted using IBM SPSS Statistics (version 13.0 for Windows®). The significance between mean values of total KT content was evaluated by Tukey's test at $P < 0.05$ level.

Chapter 5
RESULTS AND DISCUSSION

5.1 Study of Effects of Low Temperature Development on R_f Value of 1 in TLC

TLC is a simple, fast and economical chromatographic process. It was chosen in this study as a preliminary test to investigate the potential temperature effects on the retention of **1** on silica. PMA solution has been widely applied in TLC as a staining reagent which can stain compounds of low molecular weights with a wide range of functional groups (Zarzycki *et al.*, 2006). In addition, this reagent has been useful in staining KTs on silica TLC plates (Shen, personal communications). Typically, TLC plates that have been treated with this stain become light green, while the spots corresponding to compounds of interest turn dark green, as shown in Figure 5.1. Based on visual observation, it appeared that the retention of **1** on TLC plates changed with temperature. The retention factor (R_f) values were 0.146 and 0.221 for development under –20^{o}C and R.T., respectively. R_f values indicated stronger retention of **1** on silica plates at –20^{o}C compared to that at R.T. This finding was consistent with studies on TLC retention of other compounds under different temperatures, i.e. lower R_f value under lower temperature (Zarzycki *et al.*, 1999; Zarzycki, 2008). TLC plates developed in 50% ethyl acetate in hexane at R.T. achieved an R_f value of 0.148, which was very close to the value of low temperature development. Previous studies utilized 50% ethyl acetate in hexane to elute nonpolar components of flaxseed oil from silica, while retaining KTs (Gui *et al.*, 2012; Reaney *et al.*, 2013b). Therefore, it is possible that elution of flaxseed oil-laden silica using ethyl acetate under low temperature can achieve similar separation to using ethyl acetate and hexane mixture at R.T.

5.2 Study of KT Profile of Fresh Flaxseed Oil

Methanol has been proven to be suitable for extracting KTs from flaxseeds (Jadhav *et al.*, 2012; Olivia *et al.*, 2012). Due to the solubility of KTs in methanol and its immiscibility with flaxseed oil, methanol was utilized for liquid-liquid extraction to investigate KT profiles. The

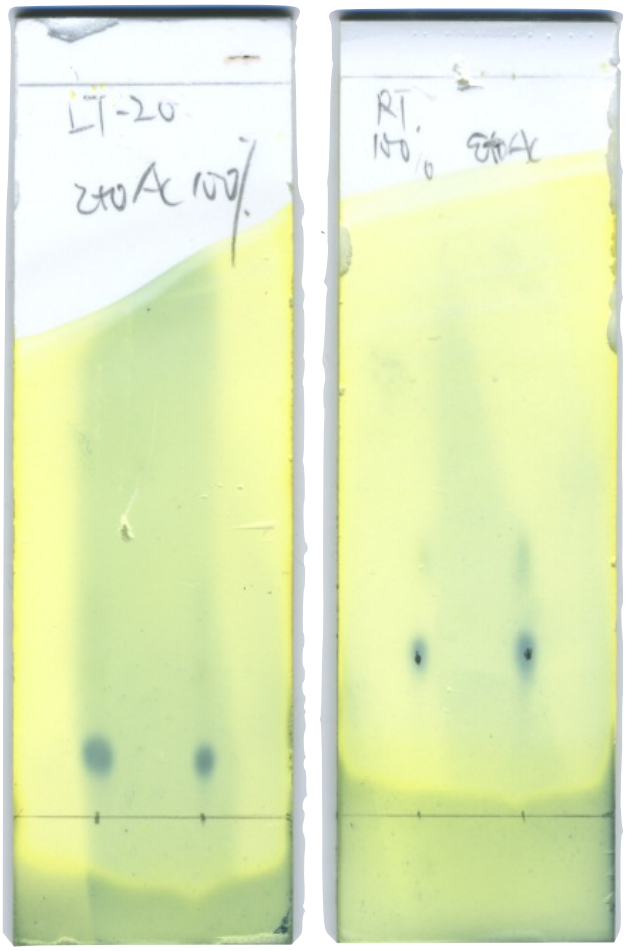

Figure 5.1 [1–9-NαC]-KTA (**1**) spots on silica TLC plates developed in ethyl acetate under –20°C (left) and R.T. (right)

HPLC chromatogram of the methanol extract (Figure 5.2) was compared with that of Jadhav *et al.* (2012). Identified peptides and corresponding retention times (parentheses) include **15** (2.73 min), **3** (2.83 min), **9** (3.27 min), **2** (3.94 min), **1** (4.28 min), **8** (4.28 min), **14** (4.42 min), and **5** (4.65 min). Mass of molecular ions of all the KTs above has been confirmed by HPLC-MS (Appendix A). However, the peak with the retention time of 3.66 min proved to be **16** instead of **6** reported by Jadhav *et al*. (2012). Additionally, HPLC-MS indicated the presence of trace amount of **12** and **13** with retention time of 1.6 and 5.1 min in the 16 min chromatogram, respectively. Neither **12** nor **13** showed any resolved peak in the 6 min chromatogram. The HPLC and HPLC-MS analysis results demonstrated that the methanol extract of fresh flaxseed oil contain KTs in both reduced forms and oxidized forms, which was in agreement with previous studies (Stefanowicz, 2001; Gui *et al.*, 2012; Jadhav *et al.*, 2012; Olivia *et al.*, 2012). It has been shown that freshness of flaxseeds and flaxseed oil contributes to the difference in KT species observed. More reduced KTs containing Met were present when fresh flaxseed was extracted. Stefanowicz (2001) detected **2**, **5**, **6**, **8**, **9**, **12**, and **15** in freshly extracted material. Jadhav *et al.* (2012) found that **2**, **5**, **8**, **11**, and **14** were present in one week old ground flaxseed and also showed that peaks corresponding to reduced KTs disappeared after an accelerated aging process while their oxidized homologues containing MetO and $MetO_2$ accumulated. Studies by Gui *et al.* (2012) and Olivia *et al.* (2012) confirmed that flaxseed stored for longer times contained only oxidized **3**, **6**, **9**, **12**, and **15**.

5.3 Study of Low Temperature Elution of Oil/Peptide-Laden Silica

Reaney *et al.* (2013b) showed that mixing 2,000 L flaxseed oil with 40 kg silica for 2 h allowed complete absorption of KTs by the silica. In this study, we applied a modified protocol where the ratio of silica to flaxseed oil (w/v) doubled and agitation time remained 2 h. The most recent and efficient KT recovery methods involved the use of ethyl acetate, hexane, methanol and dichloromethane (Reaney *et al.*, 2013b). From an industrial production point of view, application of multiple organic solvents complicates the process and increases the difficulty in recycling used solvents. Using multiple solvents therefore increases overall production cost. In addition, given the likely application of KTs in food, pharmaceutical and cosmetic industries, the use of hexane, methanol and dichloromethane are undesirable because these solvents possess some level of toxicity (FDA, 2012). In contrast, ethyl acetate and ethanol are less regulated (FDA, 2012).

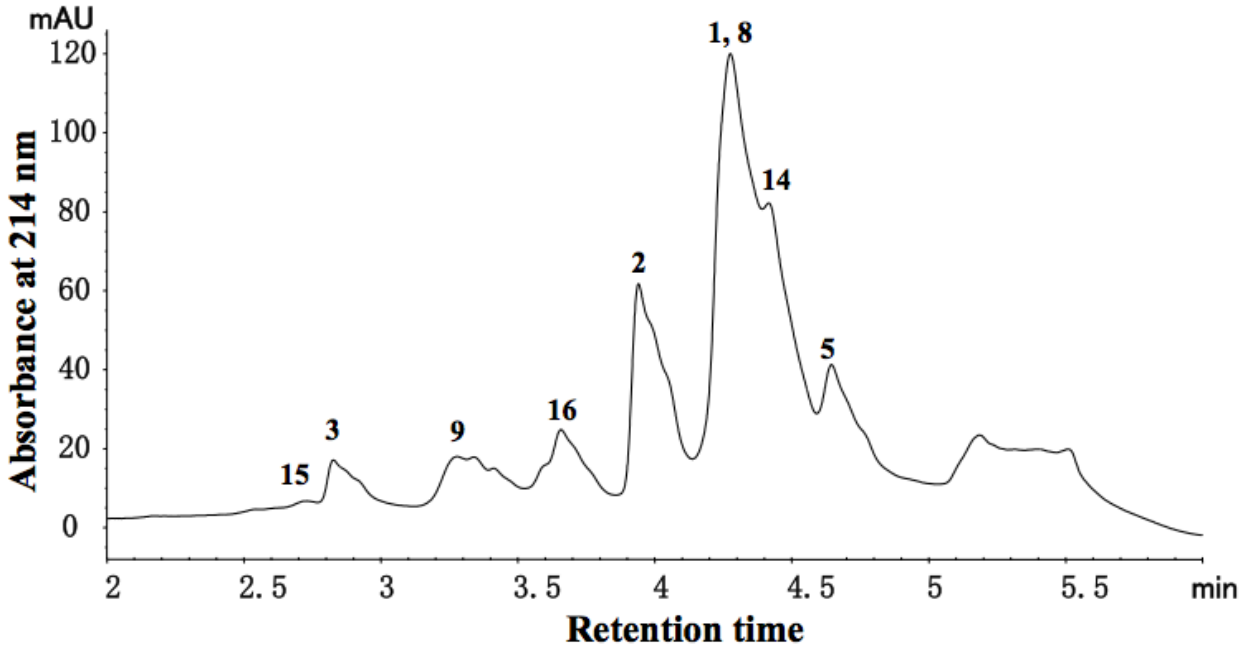

Figure 5.2 Chromatogram of methanol extract of fresh flaxseed oil. Identified KTs include [1–9-NαC]-KTA (**1**), [1–9-NαC]-KTB (**2**), [1–9-NαC],[1-MetO]-KTB (**3**), [1–8-NαC]-KTD (**5**), [1–8-NαC]-KTE (**8**), [1–8-NαC],[1-MetO]-KTE (**9**), [1–8-NαC]-KTG (**14**), [1–8-NαC],[1-MetO,3-MetO]-KTG (**15**), and [1–8-NαC],[1-MetO]-KTG (**16**)

Ethyl acetate was used to recover **1** in a rich polar fraction from flaxseed oil/peptide laden silica after prior elution with relatively less polar solvents to remove oil and other less polar compounds (Gui *et al.*, 2012; Reaney *et al.*, 2013b). Direct elution of flaxseed oil/peptide laden silica using ethyl acetate at R.T. can lead to co-elution of KTs and oil. TLC results from section 5.1 showed stronger retention of KTs on silica at –20^{o}C, which may indicate the possibility of improved separation of oil and KTs on a chilled silica column. Effective separation is possible if the retention of oil is not enhanced to the same extent as KTs. No previous literature is known comparing the retention of KTs on chromatography media at low temperatures.

The experiment was conducted at 0–4^{o}C as it was the only temperature range accessible to us for a small-scale column test. As shown in Table 5.1 the first and second fractions contained significant amounts of oil after solvent removal. In order to analyze and compare KT profiles of the oil rich fractions to other fractions, the oil was extracted using methanol. The first and second ethyl acetate fractions contained more of the lower polarity KTs (**1**, **2**, **5**, **8**, and **14**) (Figure 5.3) having retention times after 3.8 min. KT **16** was detected in trace amounts in both fractions. All KT species in flaxseed oil were present in the third and forth fractions. However, **3**, **9**, and **15** were detected in low intensities. Ethanol is more polar than ethyl acetate (Godfrey, 1972), meaning it has more elution strength in normal phase (e.g. silica) chromatography. It has been used to recover remaining KTs from silica, which are otherwise hardly eluted by solvents of lower polarity (Reaney *et al.*, 2013b). Data in this study confirmed previous findings. As shown in the chromatogram of the first ethanol fraction (Figure 5.3A), peaks corresponding to polar KTs that were only detected in low intensities in previous fractions emerged as major ones. Retention times of these KTs were less than 3.8 min. It is worth mentioning that **12**, which could not be detected by HPLC in the methanol extract of fresh flaxseed oil (Figure 5.2) and ethyl acetate fractions was observed in the chromatograms of both ethanol fractions. Previously, Jadhav *et al.* (2013) showed that reduced KTs previously adsorbed on silica could undergo oxidation and form their oxidized homologues. Therefore, appearance of **12** is possibly due to the oxidation of **13** during extraction.

The KT content of each fraction can serve as an estimate of loss vs. recovery of KTs during extraction. The second ethanol fraction only contained 6% of total KTs proving the efficiency of KT recovery by ethanol from silica (Table 5.2). Complete separation of oil and KTs was not achieved as the low polarity oil rich fractions contained 38% of total KTs. The ratio of oil to KTs

Table 5.1 Weight of fractions at 0–4°C and R.T. after solvent removal

Fractions	Weight after evaporation
1st Ethyl acetate (0–4°C)	68.25 g
2nd Ethyl acetate (0–4°C)	8.30 g
3rd Ethyl acetate (0–4°C)	0.67 g
4th Ethyl acetate (0–4°C)	0.36 g
1st Ethanol (R.T.)	0.59 g
2nd Ethanol (R.T.)	0.23 g

Table 5.2 Percentage of total recovered KTs in ethyl acetate fractions at 0–4°C and ethanol fractions at R.T.

Fraction	% of total recovered KTs	Sum
1st Ethyl acetate (0–4°C)	23[1]	38
2nd Ethyl acetate (0–4°C)	15	
3rd Ethyl acetate (0–4°C)	18	62
4th Ethyl acetate (0–4°C)	7	
1st Ethanol (R.T.)	31	
2nd Ethanol (R.T.)	6	

[1]Calculations were based on KT content of individual fraction and total KT content of all six fractions.

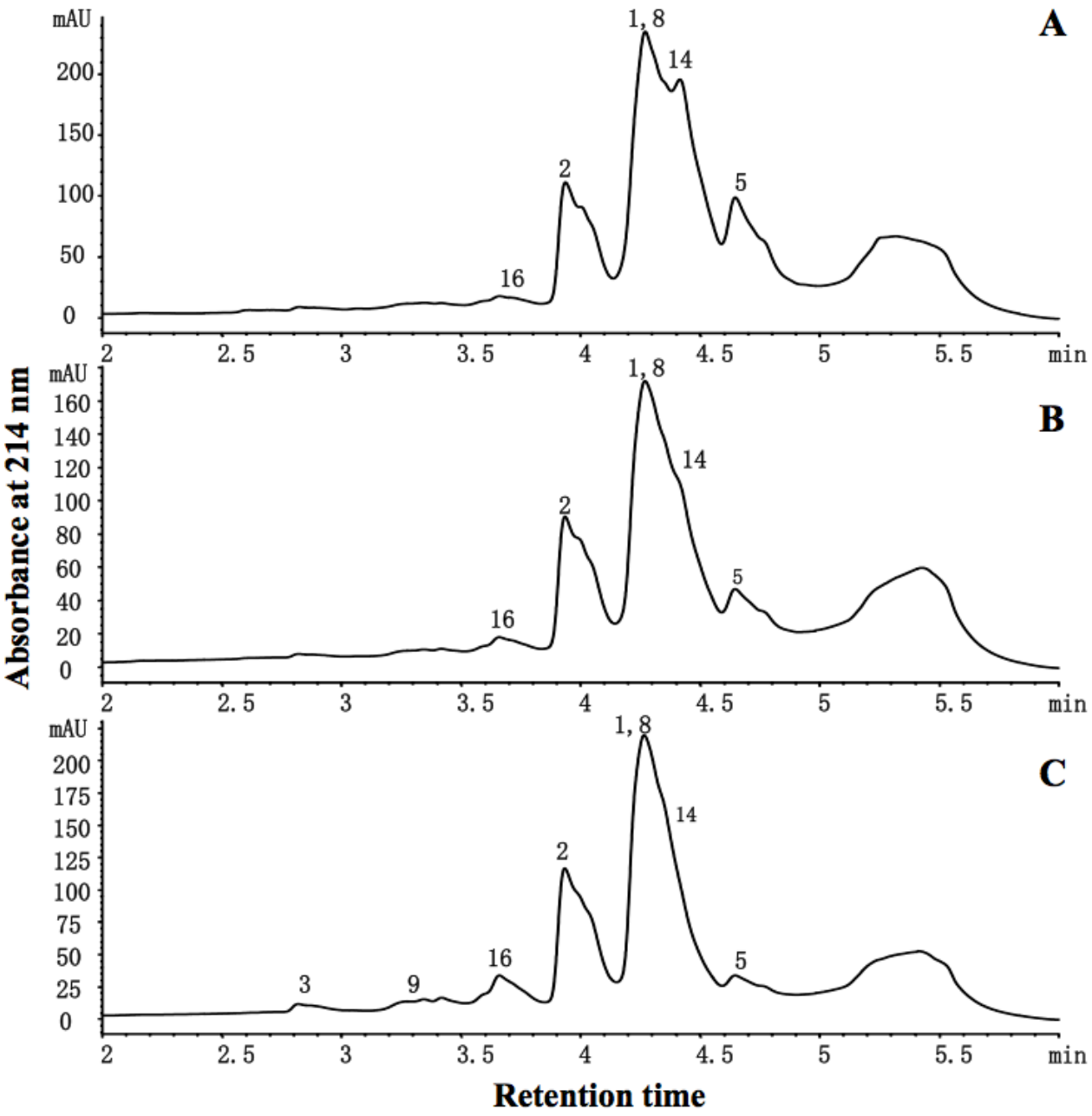

Figure 5.3 HPLC chromatograms of KT profile in different fractions. (A): 1st ethyl acetate fraction (obtained at 0–4°C), (B): 2nd ethyl acetate fraction (obtained at 0–4°C), (C): 3rd ethyl acetate fraction (obtained at 0–4°C), (D): 4th ethyl acetate fraction (obtained at 0–4°C), (E): 1st ethanol fraction (obtained at R.T.), (F): 2nd ethanol fraction (obtained at R.T.). Identified KTs include [1–9-NαC]-KTA (**1**), [1–9-NαC]-KTB (**2**), [1–9-NαC],[1-MetO]-KTB (**3**), [1–8-NαC]-KTD (**5**), [1–8-NαC]-KTE (**8**), [1–8-NαC],[1-MetO]-KTE (**9**), [1–8-NαC],[1-MetO,3-MetO]-KTF (**12**), [1–8-NαC]-KTG (**14**), [1–8-NαC],[1-MetO,3-MetO]-KTG (**15**), and [1–8-NαC],[1-MetO]-KTG (**16**) (**cont'd**)

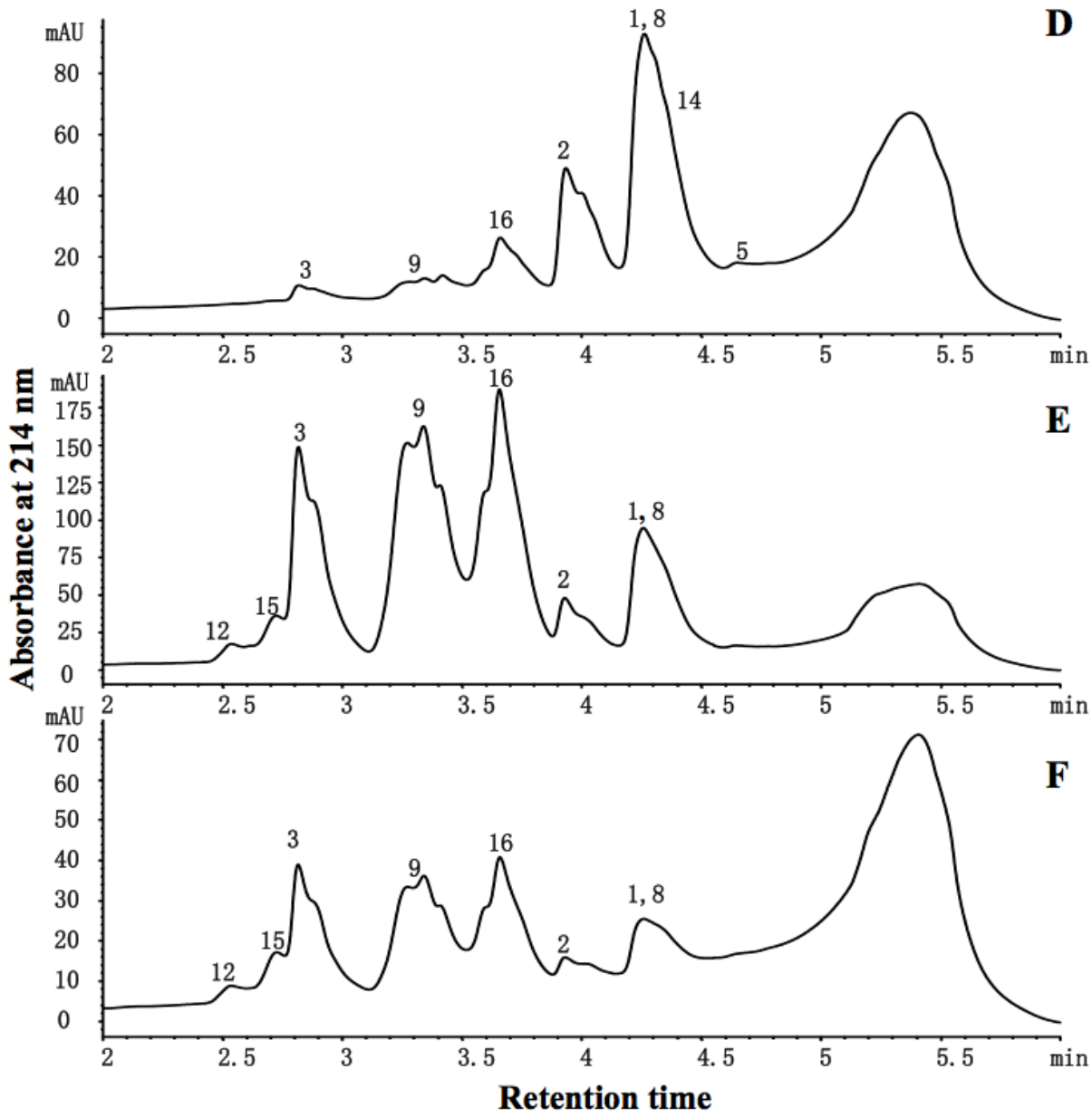

Figure 5.3 HPLC chromatograms of KT profile in different fractions. (A): 1st ethyl acetate fraction (obtained at 0–4°C), (B): 2nd ethyl acetate fraction (obtained at 0–4°C), (C): 3rd ethyl acetate fraction (obtained at 0–4°C), (D): 4th ethyl acetate fraction (obtained at 0–4°C), (E): 1st ethanol fraction (obtained at R.T.), (F): 2nd ethanol fraction (obtained at R.T.). Identified KTs include [1–9-NαC]-KTA (**1**), [1–9-NαC]-KTB (**2**), [1–9-NαC],[1-MetO]-KTB (**3**), [1–8-NαC]-KTD (**5**), [1–8-NαC]-KTE (**8**), [1–8-NαC],[1-MetO]-KTE (**9**), [1–8-NαC],[1-MetO,3-MetO]-KTF (**12**), [1–8-NαC]-KTG (**14**), [1–8-NαC],[1-MetO,3-MetO]-KTG (**15**), and [1–8-NαC],[1-MetO]-KTG (**16**)

in these fractions is too high for utilizing this fraction as a KT concentrate. It may be possible that loss of KTs can be minimized by mixing oil rich fractions with incoming fresh flaxseed oil for a second liquid-solid extraction. However, that hypothesis was not investigated in this current project. Although the bench-scale experiments demonstrated the potential for increasing the scale of this protocol, we did not have access to explosion-proof instruments and lab space that allowed accurate sub-ambient temperature control of large-scale column chromatography. Therefore, alternative extraction methods were the focus of further studies.

5.4 Study of aq. Ethanol as a Liquid-Liquid Extraction Solvent of KTs

Currently, extraction of KTs from flaxseed oil relies on organic solvent elution of oil laden silica (Reaney *et al.*, 2013b). Methanol extraction followed by column chromatography has been the method of choice to recover KTs from flaxseeds (Morita *et al.*, 1997; Matsumoto *et al.*, 2002). As discussed in section 5.3, methanol is not desirable for KT production due to its toxicity and strict governmental regulations. However, ethanol that differs from methanol by a methylene group is much less toxic (FDA, 2012) and has been consumed by people in various beverage products since ancient times. Its immiscibility with flaxseed oil makes it a possible solvent for liquid-liquid extraction. Water, a more polar solvent than ethanol, does not readily dissolve nonpolar compounds from flaxseed oil. Adding water to ethanol can adjust the polarity of the extraction solvent, which may influence KT recovery. Besides, aq. ethanol can be readily recovered, recycled and stored, which is highly advantageous from the economical viewpoint when conducting large-scale extractions (Don and Robert, 2008). HPLC analyses showed that an aq. ethanol extract of flaxseed oil (Appendix B) contained a similar KT profile to that extracted by methanol (Figure 5.2). Therefore, substitution of methanol with aq. ethanol in liquid-liquid extraction is feasible. However, the efficacy of aq. ethanol in KT extraction needs to be investigated systematically.

5.5 Study of NMR as a Means to Estimate KTs to Oil Ratio

The main goal of any KT recovery method is to maximize KT content while reducing co-extracted impurities. The major contaminants in liquid-liquid extraction of vegetable oils are TAG, DAG, and MAG. A high ratio of KTs to glycerides is desirable in method development and optimization. However, there was not any instrumental analysis method available in literature to

analyze ratio of KTs to glycerides.

^{13}C-NMR and ^{1}H-NMR are powerful tools to elucidate the structure of KTs (Morita *et al.*, 1999; Matsumoto *et al.*, 2001; Jadhav *et al.*, 2012), but these tools are rarely used for quantitation. In ^{1}H-NMR spectra, chemical shifts (in ppm) of the peaks demonstrate the electromagnetic environment of protons and peak intensities are directly proportional to the number of protons causing each peak (Kuhn, 1990). Theoretically, KT to oil ratio can be analyzed by ^{1}H-NMR if proper representative peaks can be chosen and integrated. Literature survey shows that peaks with chemical shifts of 7.00 to 7.40 ppm represent protons from phenylalanine phenyl groups and tryptophan indole groups in KTs (Di Blasio *et al.*, 1989; Morita *et al.*, 1999; Matsumoto *et al.*, 2001; Gui *et al.*, 2012), while chemical shifts between 2.20 and 2.38 ppm represent protons from alpha carbon next to the carbonyl group in fatty acids and fatty acid esters (Marcel *et al.*, 1995). Peaks corresponding to both chemical shift ranges can be readily identified in the ^{1}H-NMR spectrum of 80% aq. ethanol extract (Figure 5.4). However, residual chloroform in $CDCl_3$ has a peak at 7.26 ppm, which overlapped with the KT representative chemical shift range (Gottlieb *et al.*, 1997). Alternatively, MeOD was used for following ^{1}H-NMR analyses.

Analysis of the ratio of KT and fatty acyl moieties was tested using two representative standards and ^{1}H-NMR spectra. Flaxseed oil FAME was used instead of crude flaxseed oil because of the low solubility of flaxseed oil in MeOD. Conversion of TAG, DAG and MAG to FAME would not appreciably change the position of spectral peaks arising from protons attached to the alpha carbon next to the carbonyl group in fatty acid chain (Nie, 2012). ^{1}H-NMR spectra of **1** standard with DMF and flaxseed oil FAME with DMF in MeOD (Figure 5.5) clearly demonstrated that there was no overlap of peaks within the diagnostic chemical shift ranges of 7.00 to 7.40 ppm for KTs and 2.20 to 2.38 ppm for FAME. Therefore, interference of **1** signal and flaxseed oil signal would not occur. Although the peak contributed by olefinic protons (chemical shift of 5.40 ppm in Figure 5.5B) did not overlap with peaks of **1**, this peak was not selected for flaxseed oil estimation. The number of protons from carbon-carbon double bonds is a variable of genotype and environment and may also be altered with oxidative status of the oil. Satisfactory R^2 values ($\geq$ 0.949) were obtained in the investigation of ^{1}H-NMR response as a function of varying **1** content, flaxseed oil FAME content and **1** content with the presence of constant FAME content (Figure 5.6). Therefore, ^{1}H-NMR can simultaneously determine KTs and fatty acyl group ratios in flax oil extracts. This analysis method may be used in process development.

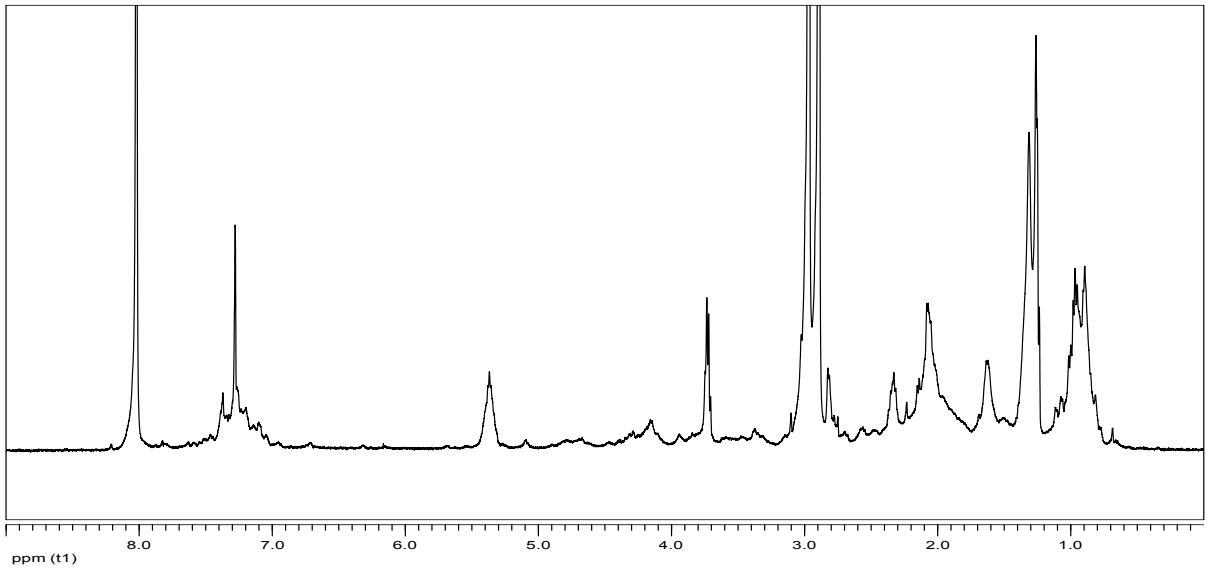

Figure 5.4 ^{1}H-NMR spectrum of 80% aq. ethanol extract in $CDCl_3$

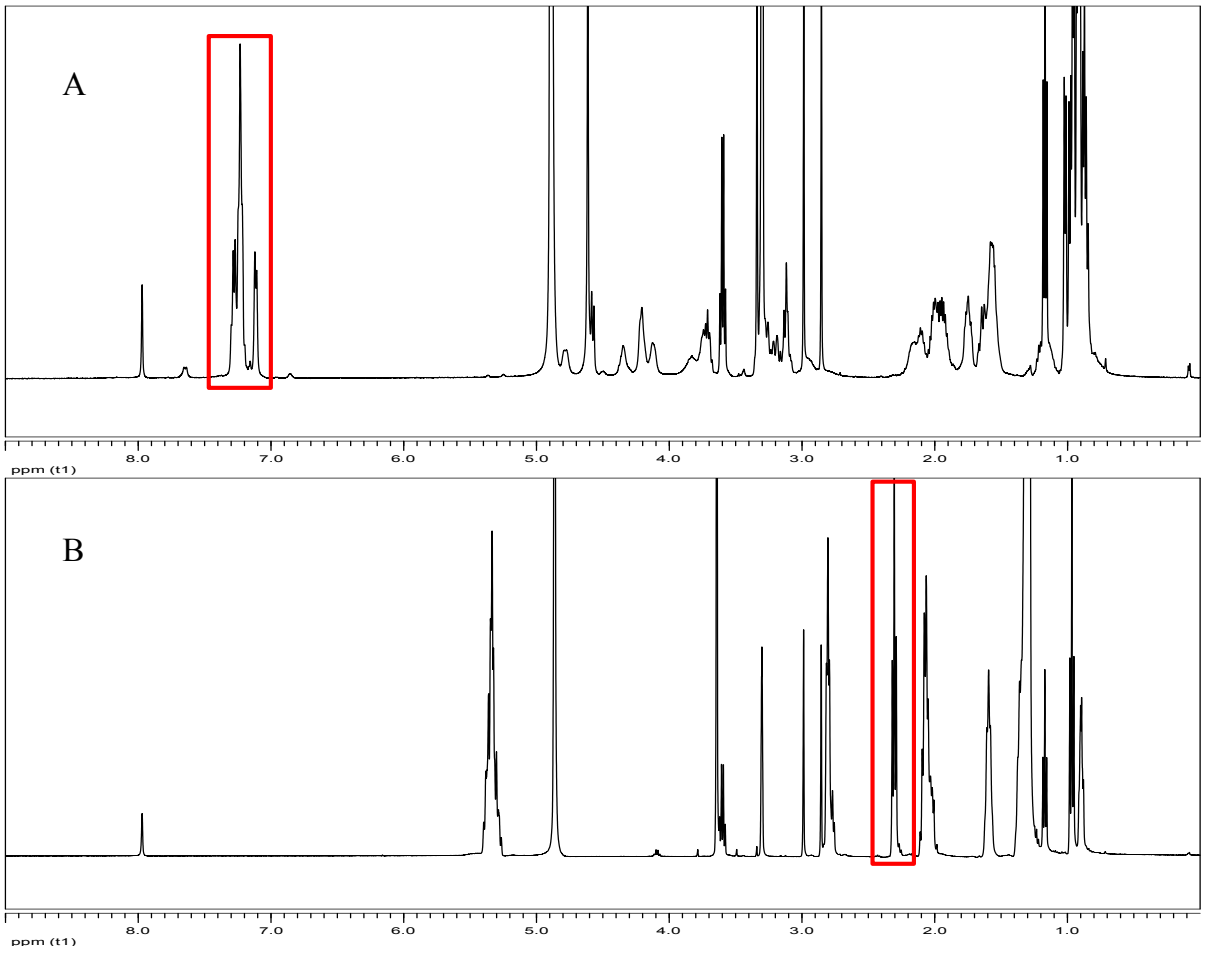

Figure 5.5 ^{1}H-NMR spectra of (A) [1–9-NαC]-KTA (**1**) standard with DMF in MeOD and (B) flaxseed oil FAME with DMF in MeOD

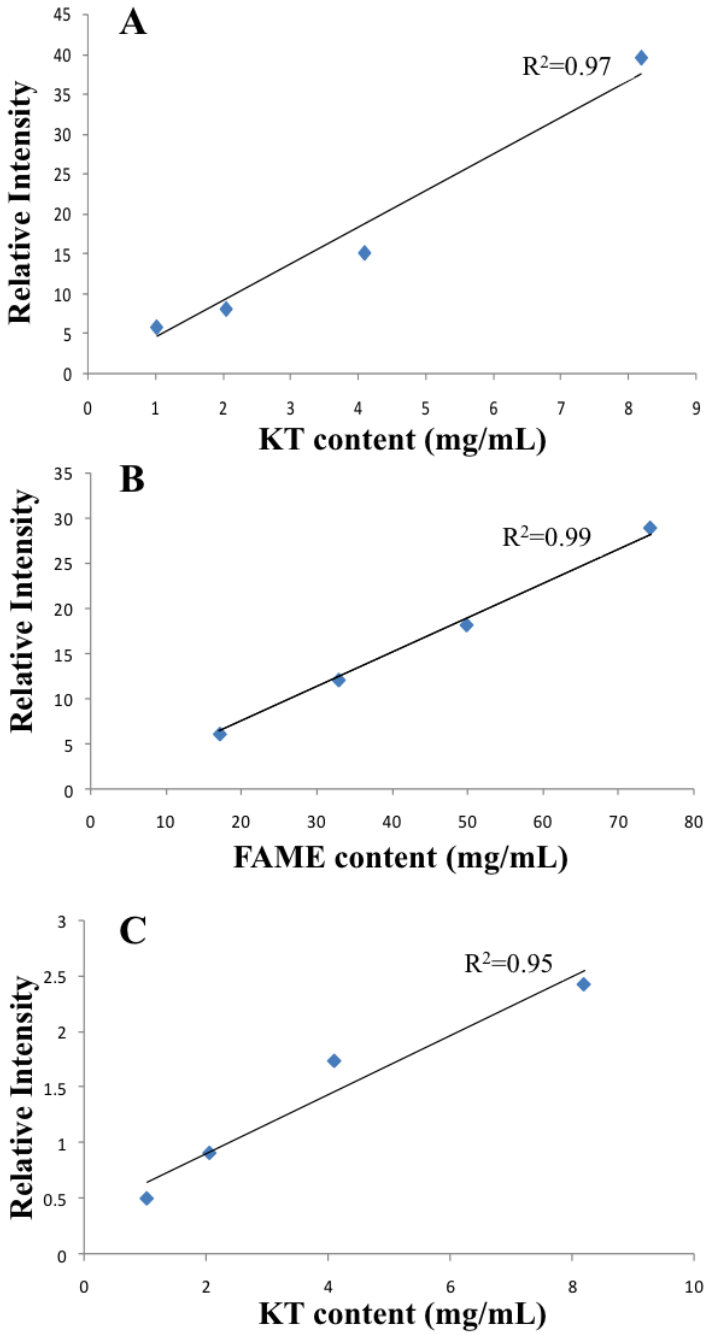

Figure 5.6 ^{1}H-NMR responses as a function of (A) varying [1–9-NαC]-KTA (**1**) concentration, (B) varying flaxseed oil FAME concentration, and (C) varying [1–9-NαC]-KTA (**1**) concentration in the presence of constant FAME

5.6 Study of Extraction of Flaxseed Oil using aq. Ethanol

Liquid-liquid extraction is a process in which the compounds of interest are separated from the matrix by partitioning between two immiscible liquids (Wells, 2003). Due to high efficiency and ease of operation, it has been commonly used in various industries, including in food processing, separation of biomolecules, enrichment of pharmaceuticals, wastewater treatment, etc. (Don and Robert, 2008). Several parameters in this process can affect the extraction efficiency and, therefore, need to be optimized to afford satisfactory recovery, e.g. the type of solvent, the amount of solvent relative to the matrix, temperature and pH value of the solvent (Gu, 2000; Wells, 2003). In this study, the effects of two factors were investigated, i.e. the percentage of aq. ethanol and the volume ratios of solvent to flaxseed oil. The optimal aq. ethanol should have high solubility and high selectivity for KTs so that compounds can be efficiently recovered, concentrated and purified to some extent (Don and Robert, 2008). As discussed in section 5.5, it is feasible to estimate the KT and oil content simultaneously in aq. ethanol extract using ^{1}H-NMR. Thus, the evaluation is based on the integrated signals representative of KTs and oil from ^{1}H-NMR spectra of alcohol extracts. Ideally, the highest KT content and highest KTs to oil ratio should be achieved under the optimal extraction condition.

During extraction, it was expected that certain amount of ethanol could enter the oil phase, while some oil components would be extracted into the solvent phase. This was confirmed by Figure 5.7 which showed an obvious increase in the peak representative of ethanol with the chemical shift of 3.72 ppm after extraction (Gottlieb *et al.*, 1997). As a result of the material transfer, the volume of the solvent phase, which can affect the calculation of KT and oil content, may also change. The volume of both phases without decanting and transfer after phase separation was determined by mixing both phases in 100 mL graduated cylinders and using these as extraction vessels. Results are listed in Appendix C. Some extraction conditions did not afford subsequent phase separation within the settling time of 4 h and are marked as “no separation”. Additionally, the solvent layer occurred above the flaxseed oil layer after phase separation when 60, 70, 80, 90, and 100% aq. ethanol was used. The exception was 50% aq. ethanol, which has higher density than flaxseed oil and, thus, became the lower phase in these extractions.

In section 5.5, we quantified KTs and oil acyl groups. However, as the signal intensity in ^{1}H-NMR spectra is based on the number of protons, certain assumptions need to be made in order

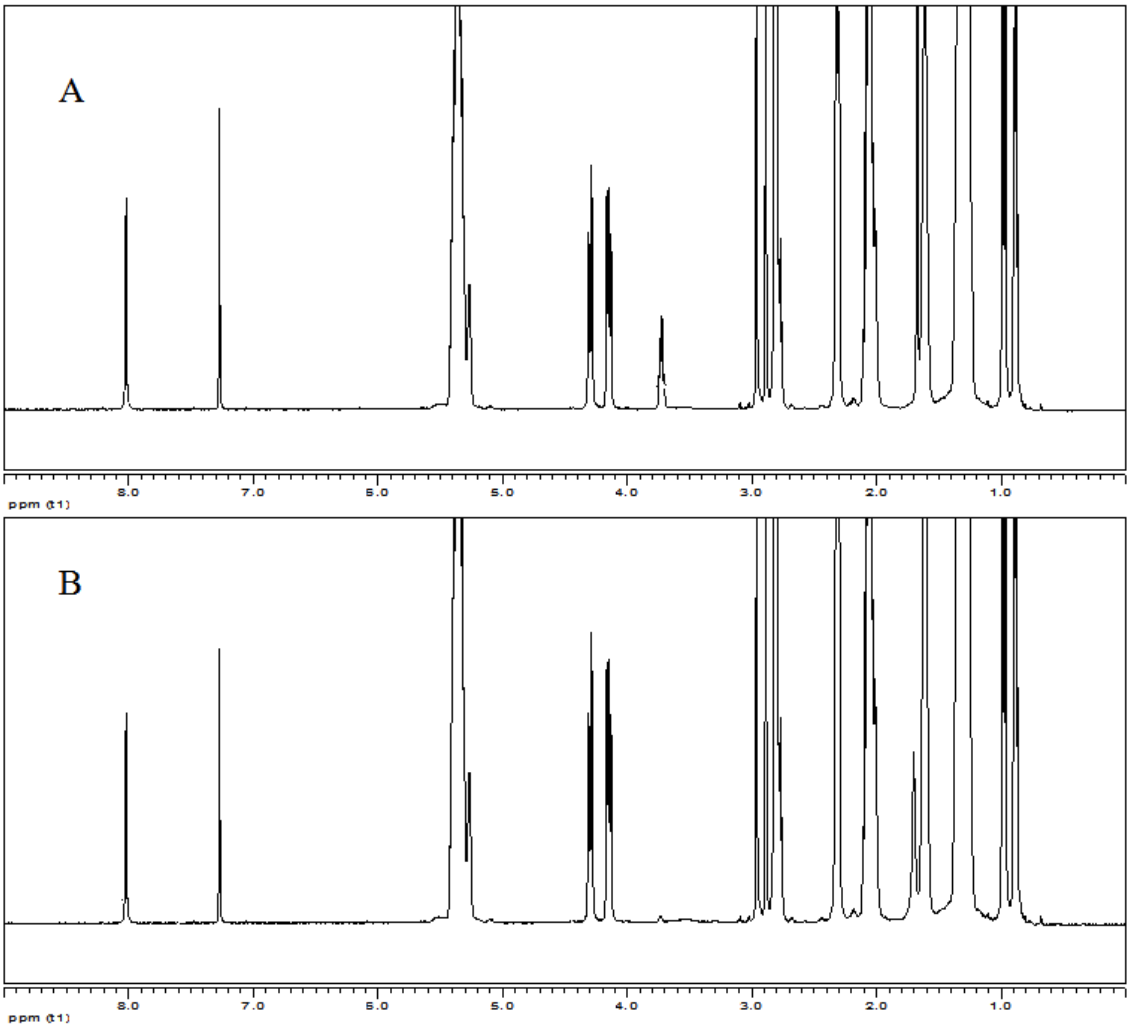

Figure 5.7 ^{1}H-NMR spectra of flaxseed oil in $CDCl_3$ (A) after and (B) before the extraction with 70% aq. ethanol

to proceed with calculations. For KTs with tryptophan, chemical shifts of 7.00 to 7.40 ppm represent 15 protons from phenylalanine phenyl groups and tryptophan indole groups, while for KTs without tryptophan, the same chemical shift range represents 10 protons from phenylalanine phenyl groups (Di Blasio *et al.*, 1989; Morita *et al.*, 1999; Matsumoto *et al.*, 2001) . Previous research showed that in commercial and lab-produced flaxseed oil, the amount of KTs without tryptophan was 2.4 times to 8.1 times as the amount of KTs with tryptophan (Gui, 2011). Therefore, to simplify the calculation, the number of protons within chemical shifts of 7.00 to 7.40 ppm is assumed to be 10. Also, as flaxseed oil is mostly triglycerides (Wanasundara *et al.*, 1999), it was assumed that this constituent was the major oil component. Thus, the number of protons used for oil calculation is 6 protons per triglyceride molecule. Calculations were conducted according to the following equations:

$$Total\ KT\ content = \frac{A}{N} \times \frac{V_S R}{V_A} \qquad [5.1]$$

where A is the integrated peak areas within the range of 7.00 and 7.40 ppm; N is the number of protons which is equal to 10; V_S is the volume of solvent added to the tubes for extraction; R is the ratio of solvent volume after and before the extraction; V_A is the volume of extract utilized for NMR analysis.

$$Total\ oil\ content = \frac{A}{N} \times \frac{V_S R}{V_A} \qquad [5.2]$$

where A is the integrated peak area within the range of 2.20 to 2.38 ppm; N is the number of protons from alpha carbons of triglycerides, which is equal to 6; V_S is the volume of solvent added to the tubes for extraction; R is the ratio of solvent volume after and before the extraction; V_A is the volume of extract utilized for NMR analysis.

The liquid-liquid extraction using 70 and 80% aq. ethanol with the volume ratios of 0.125:1 did not produce a phase separation for further analysis. In addition, peaks representative of flaxseed oil did not appear in ^{1}H-NMR spectra of 50% aq. ethanol extract with the volume ratios of 0.125:1, making it impossible to obtain a KT to oil ratio. Figure 5.8A showed a downward trend of oil content in 100, 90, 80, 60, and 50% aq. ethanol extracts when the volume ratios decreased from 2:1 to 0.5:1. It is possible that each of the solvents can bear a maximum amount of oil, which was reached when extraction occurred. As less solvent (relative to the oil) was used for extraction, less oil was dissolved in the extract layer. However, 70% aq. ethanol extract was an exception, in which the oil content observed showed minimal change over the same volume

ratio range. It showed the effectiveness of 70% aq. ethanol in expelling oil regardless of the volume ratio. There was slight increase in oil content for 70 and 80% aq. ethanol extracts when the volume ratios decreased from 0.5:1 to 0.25:1. However, the increase was not statistically significant. It is expected that water can help reduce oil in extracts, i.e. as water content in aq. ethanol increases, oil content decreases. This was true of 100, 90, 80, and 70% aq. ethanol extracts. However, 60 and 50% aq. ethanol extracts had a higher oil content compared to extracts with 80 and 70% aq. ethanol. This is potentially explained by the fact that small oil droplets can be formed in the aq. ethanol phase when strong agitation force is applied; small density difference and low interfacial tension between the two phases slow down phase separation (Tedder, 2008). As the density of 50% (0.926 g/mL) and 60% (0.905 g/mL) aq. ethanol is similar to that of flaxseed oil (0.931 g/mL as determined in lab at R.T.), these fine oil droplets may remain in the solvent-phase even after 4 h settling time, thereby increasing oil content in the collected samples.

Figure 5.8B demonstrated total KT yield under various extraction conditions. As the mechanism of aq. ethanol extraction of KTs from flaxseed oil is unknown, it is difficult to explain how the volume ratios and the percentages of ethanol can affect the total KT content in the extract layer. However, it is clearly shown that when the volume ratios decreased from 0.5:1 to 0.25:1 for 80 and 70% aq. ethanol, the total KT content recovered increased dramatically. KT content obtained using 80 and 70% alcohol at 0.25:1 ratio was significantly higher than that under any other conditions (Appendix D). The goal of this project is to recover the maximum amount of KTs from flaxseed oil. Therefore, total KT content is a dominant factor to be considered in extraction optimization. Figure 5.8C showed the changes of KT to oil ratio, which was a combined effect of KT and oil content variation. For all aq. ethanol solvents, the KT to oil ratio increased as the volume ratio decreased. This trend was consistent with downward trends of total oil content, although total KT content fluctuated over the volume ratio range. Given the complexity of components in the extract, analysis of this ratio can help determine the optimal extraction condition(s) with small amount of impurities (oil) relative to KT content. As illustrated in Appendix D, KT to oil ratio of six extraction conditions was statistically higher than those of other conditions.

Taking both tables of statistical analysis results into consideration, both 70 and 80% aq. ethanol with the volume ratio of 0.25:1 can result in high KT recovery and high KTs to oil ratio.

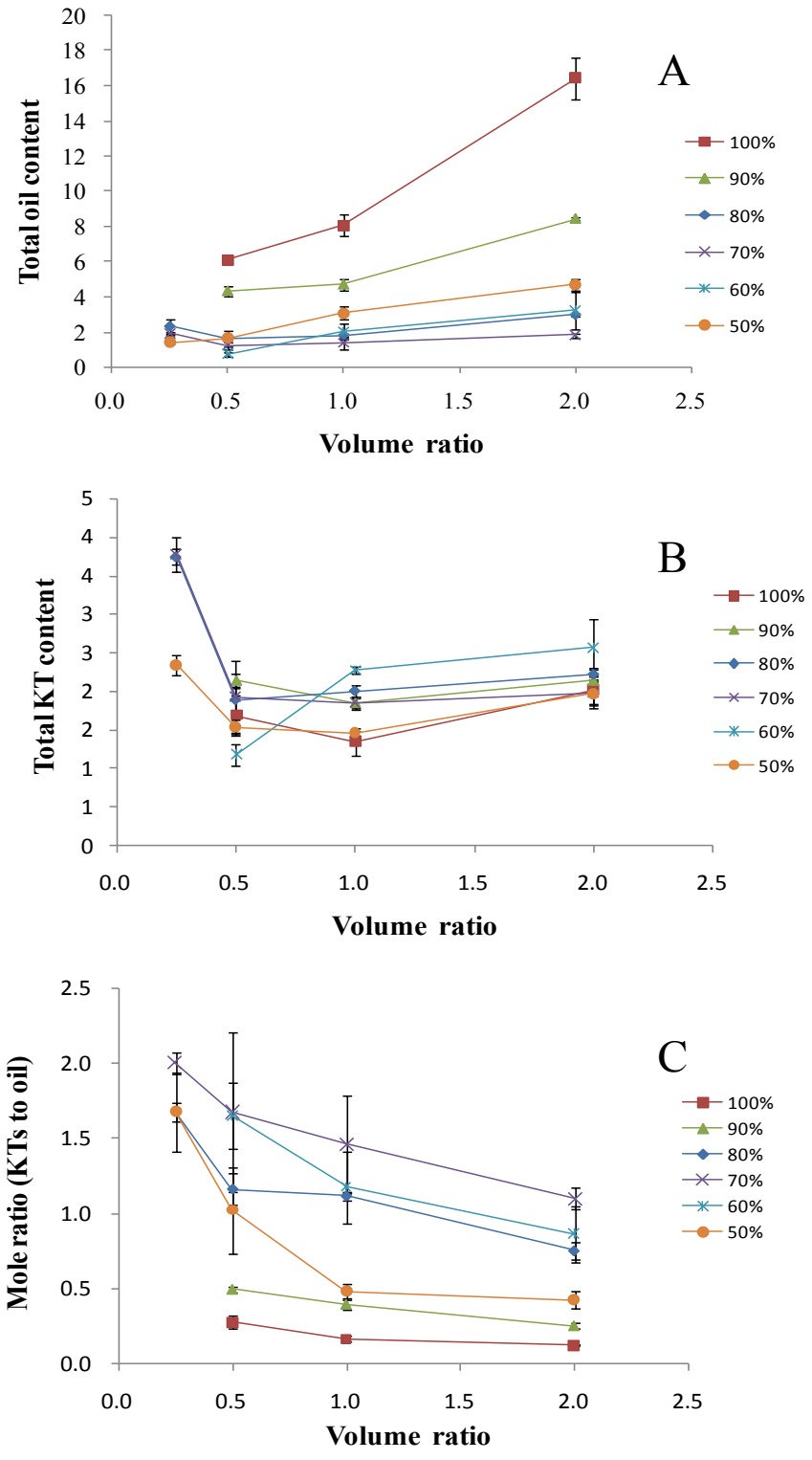

Figure 5.8 (A) total oil content, (B) total KT content, and (C) KTs to oil mole ratios of ethanol extracts (100, 90, 80, 70, 60, and 50% ethanol) as a function of volume ratios (n = 3)

However, the former condition was decided to be optimal based on the fact that it has the highest values of both parameters and that lower ethanol percentage can potentially save the cost of solvent in large-scale production.

5.7 Study of Agitation Force and Agitation Time in Liquid-Liquid Extraction

Liquid-liquid extraction relied on the distribution of compounds of interest between two phases, which is determined by the Nernst Distribution Law (Wells, 2003). Literature has shown that agitation can increase the surface area of interaction, thereby facilitating mass transfer (Wells, 2003). In this study, effects of agitation force and agitation time on KT recovery were investigated. However, agitation of samples that contain amphiphilic molecules may result in the formation of emulsions. It is known that the presence of an emulsion has negative effects on an extraction process as it can prevent formation of a clear boundary between two phases and, therefore, disrupt phase separation (Wells, 2003). Flaxseed oil contains numerous amphiphilic molecules, such as DAG, MAG, polyphenol, and sterols. Preliminary tests showed that aq. ethanol and flaxseed oil could form an emulsion if agitation was conducted in a vigorous manner, which corroborates the findings by Wells (2003). We observed that a long settling time (more than 6 h) was required to break the emulsion. Although centrifugation may be able to decrease or even eliminate emulsions efficiently, it was not considered to be part of the extraction process in this project for two reasons: a) no large centrifuge was available for larger-scale extractions; b) the capital cost of an explosion proof centrifuge is high. In order to determine optimal extraction conditions for KT recovery, the impact of extraction time and mild agitation conditions for extraction without formation of emulsions was studied.

Using mild agitation that did not mix the two phases together and did not create an emulsion layer, KT content in the liquid extract layer increased as extraction time lengthened (Figure 5.9A). Higher agitation speed lead to higher KT content in the extract. Both extraction time and agitation speed contributed to the extract KT content. However, when flaxseed oil and aq. ethanol was agitated in such a way that the two phases were thoroughly mixed, KT content in the extract layer reached equilibrium within 5 min as illustrated in Figure 5.9B. Agitation time did not significantly affect KT content from 5 to 60 min. The removal of KTs from crude flaxseed oil can be determined by the KTs remaining in the oil after extraction. Agitation at 140 and 170 rpm for 26 h only removed 36.2 and 55.8% of total KTs, while vigorous agitation at 600 rpm for 5 min

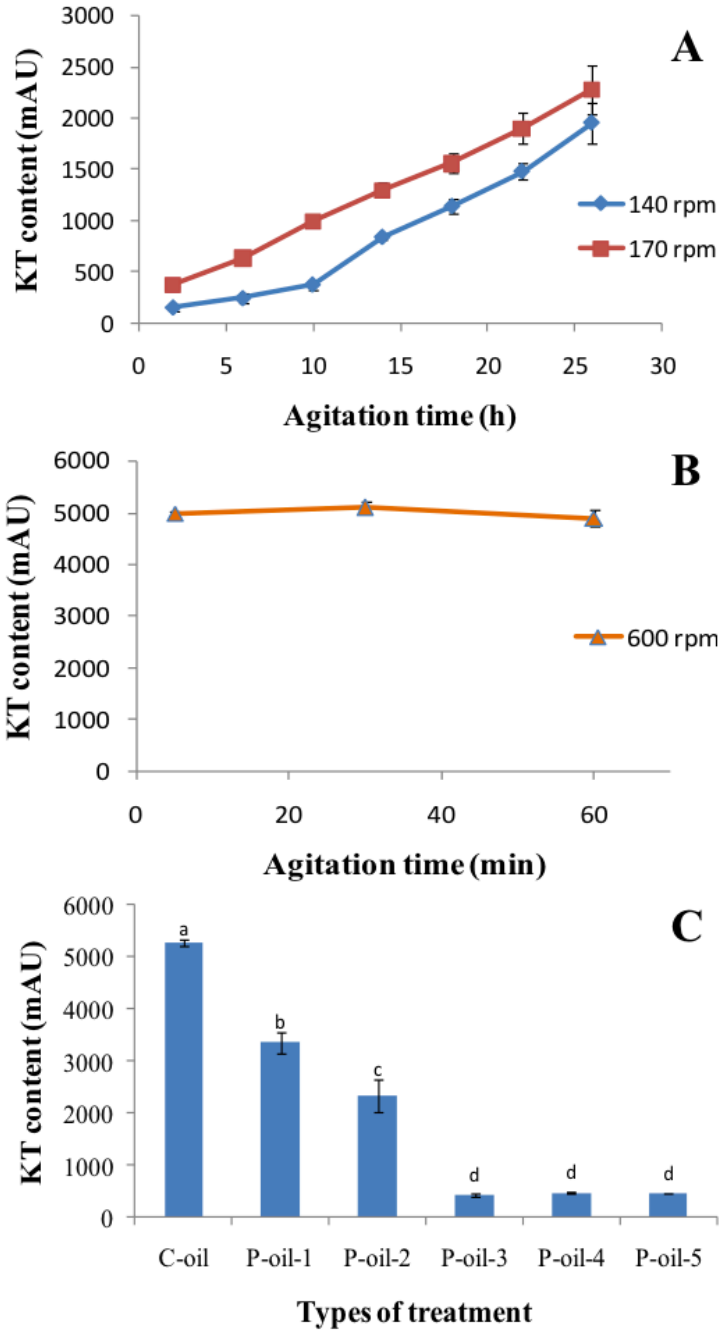

Figure 5.9 (A) KT content in extract as a function of extraction time. No emulsion was formed by agitation; (B) KT content in extract as a function of extraction time. Emulsion was formed by agitation; and (C) KT content in flaxseed oil and processed flaxseed oils. C-oil: flaxseed oil. P-oil-1: processed oil by agitation at 140 rpm for 26 h. P-oil-2: processed oil by agitation at 170 rpm for 26 h. P-oil-3: processed oil by agitation at 600 rpm for 5 min. P-oil-4: processed oil by agitation at 600 rpm for 30 min. P-oil-4: processed oil by agitation at 600 rpm for 60 min. Different letters on bars denote statistical difference by Tukey's test at $P < 0.05$ level.

was able to remove 91.8% of the total (Figure 5.9C). Content of KTs remaining after agitation at 600 rpm was significantly lower than crude oil and other treatments.

For reaction volumes of 200 mL, up to 6-hour settling time was required to afford phase separation due to emulsion formation following vigorous mixing of flaxseed oil with 70% aq. ethanol. However, vigorous agitation outperformed gentle agitation when considering both extraction time and KT recovery rate. Therefore, vigorous stirring was applied in following extractions.

5.8 Twelve Litre Scale Solvent Extraction and Peptide Recovery

Although the optimal extraction conditions have been determined in bench-scale experiments, the feasibility of the process needed to be tested through a series of extractions at increasing scale as unforeseeable factors may arise as new equipment is introduced. Additionally, process development after extraction is difficult to study at the bench-scale, as fractions can be small and not easily divided between treatments. Larger-scale studies can provide raw material for process research. When using 12 L volume of flaxseed oil and 3 L 70% aq. ethanol for extraction, it was observed that the volume of emulsion increased and longer settling times (ca. 12 h) were required to separate the phases. In spite of this, 92% of KTs were extracted out of flaxseed oil, which was comparable to that of the bench-scale extraction.

5.8.1 Rotary Evaporation

Rotary evaporation is a common and economical approach for drying in both the laboratory and small factory. When applied in this study, it was found that 60^{o}C and 120 mbar were the best conditions for continuous operation. Although higher evaporation temperature and lower vacuum pressure might accelerate solvent removal, severe boiling and foam production can occur, causing loss of product and contamination. Rotary evaporation was efficient until the ethanol content of the peptide fraction decreased. As evaporation proceeds, the ratio of water to ethanol increases. Continuous evaporation of the water rich fraction to dryness would require a long time and the properties of water supported the formation of foam. Adding 100% ethanol to assist such drying processes, called trituration, reduces the boiling point of the mixture (due to the azeotrope formed) and accelerates drying. It was found that adding 100% ethanol (400 mL) to the nearly dry liquid extract (2.5 L) before drying afforded an efficient route to producing a dry powder (18.92 g; Figure 5.10).

Figure 5.10 Dried KT mixture powder from rotary evaporation with adding 100% ethanol (400 mL)

Evaporation was accomplished in 4 h. HPLC analysis with an internal standard indicated that the content of KTs was 684.5 mg/g. Property changes of the liquid extract were observed as evaporation progressed. The light yellow liquid extract became a milky white suspension as evaporation proceeded. Further solvent removal resulted in the formation of numerous small yellowish clots, which eventually merged into a large yellowish clot. The clot was then dissolved with 100% ethanol. Ongoing evaporation and rotation helped evenly distribute the product on the walls of the flask. The product was recovered as a dried powder by scraping the flask walls.

5.8.2 Freeze Drying

Freeze drying is the method of choice for concentrating labile bioproducts, such as peptides, proteins and complex synthetic organic molecules often used in pharmaceutical applications (Franks, 1998). However, this technique is expensive, energy extensive and time consuming (Franks, 1998). Ethanol may not be used in a conventional freeze dryer as the condenser temperature (–50^{o}C) is much higher than the freezing point of ethanol (–117^{o}C); therefore, ethanol vapour will bypass the condenser and enter the vacuum pump, potentially causing damage. Therefore, prior to freeze drying, the liquid extract was evaporated in rotary evaporator until the rate of condensation in the rotary evaporator slowed substantially. The product of evaporation in the rotary evaporator was then assumed to contain mostly water and was subjected to freeze drying. After 4 d of freeze drying, the product was wet and pasty. It is possible that residual ethanol was still present after evaporation, which made it difficult to dry the product thoroughly. Based on the low drying rate and undesirable properties of products it was concluded that freeze drying was not suitable for further process steps.

5.8.3 Spray Drying

Spray drying is a continuous drying process in which the liquid feed is dried rapidly in a stream of hot air or other gas. Feed materials for spray drying may be in the form of a solution, a paste or a suspension (Huang and Mujumdar, 2009). This technology has been widely applied to food and pharmaceutical processing. Certain heat sensitive materials can be dried without losing their bioactivity as the exposure to heat can be very brief. Additionally the continuous operation facilitates processing larger volumes of wet material than possible with freeze drying (Huang and Mujumdar, 2009). Spray drying is potentially suitable for drying KT extracts. Liquid extract was

concentrated from 2.5 L to 700 mL (until the appearance of small solid clots in the suspension) to provide an alcohol reduced concentrated feed for spray drying.

Initial spray drying conditions were set according to the recommendation of the user's manual (described in section 4.8). However, after spray drying some of the feed using these conditions, the product was not observed in the collection vessel. Almost all the yellowish product was deposited on the glass wall of the drying chamber and cyclone. The recovered product from glass wall was not in the form of dry powder, but instead appeared as a wet paste (Figure 5.11A). Possible reasons for the failure of the initial conditions included: incomplete evaporation of moisture; the presence of oil components (e.g., TAG, DAG, and MAG) which could not form a powder; the applied gas flow rate was not sufficient to settle particles by centrifugal force. As product moisture is negatively correlated to gas flow rate and inlet temperature (Cal and Sollohub, 2010), operation conditions were modified substantially by increasing gas flow rate and inlet temperature (220^{o}C) for subsequent experiments. When spray drying was completed, minimal product was deposited on the glass wall of the drying chamber, while some was deposited on the glass of cyclone. The total product recovered, as a fine powder, from the cyclone and collection vessel totalled 9.6 g (Figure 5.11B). The KT content was 693.2 mg/g, which was comparable to that of product obtained by rotary evaporation. However, the yield was only 50.7% of that of rotary evaporation (18.92 g). Additionally, HPLC and LC-MS analysis showed that the KT profile of the extract did not change before and after spray drying at 220^{o}C (data not shown).

A huge loss of product still occurred even if the gas flow rate reached its maximum. Literature show that spray drying feeds that contain low concentrations of solids produces small-size particles that are difficult to separate using the cyclone (Vehring, 2008; Sollohub and Cal, 2010). Therefore, we believed particles from the KT mixture that were too small to be settled in the cyclone were carried by the gas to the exhaust filter and contributed to the loss observed. This hypothesis was confirmed when KT powder was observed accumulating on the outlet filter. This finding is consistent with that of Maa *et al.* (1998) in which protein (recombinant humanized anti-IgE antibody) particles smaller than 2 μm could not be collected in the standard cyclone of a Büchi Mini Spray Dryer B-190. Increasing the feed concentration can lead to larger particles. However, in this study, the amount of solid in solution was limited as solids precipitated during evaporation especially as ethanol content decreased, i.e., the concentration of feed was unlikely to

be sufficiently increased by pre-concentration to produce an alcohol free and high solid feed for a spray dryer. Product losses of 49.3% could not be addressed using existing equipment.

According to the literature, reverse-phase resin (Amberlite^{TM} XAD^{TM}) exhibits hydrophobic behaviour in polar solvents. Amberlite^{TM} XAD^{TM} 1600 is specially utilized for absorption of non-polar compounds from polar solvents (Rohm and Haas Company, 2000; Rohm and Haas Company, 2007). In aq. ethanol extract, it may preferentially absorb oil components which are more non-polar than KT. Additionally, the approval by FDA to apply this resin to food production eliminate the possible concerns for contamination (Rohm and Haas Company, 2007). Therefore, Amberlite^{TM} XAD^{TM} 1600 was tested as an alternative strategy to address problems associated with product texture and incomplete drying. It was hypothesized that the resin might remove non-peptide impurities in the liquid extract and facilitates the formation of a dry powder by a subsequent spray drying treatment. Tests on small columns showed that KT content and oil content of resin-treated samples shared the same trend: both parameters dropped dramatically in the 1st fraction as compared to untreated extract; then there was a sharp increase in the 2nd fraction, followed by steady increases for the rest of the fractions (Figure 5.12A and Figure 5.12B). Although KTs and oil were absorbed simultaneously by the resin, the extent of absorption was not the same. The hydrophobic resin preferentially absorbed oil over KTs (Figure 5.12C), resulting in the improvement of mole ratios of KTs to oil through the tested volume range. Subsequently, a ratio of resin to liquid extract (w/v) was set at 1:20 for a large column test. It was observed that oil content of the extract decreased by 48.8% and the mole ratio of KTs to oil increased by 58.9%. Spray drying of resin-treated liquid extract showed that without increasing drying temperature, white and fine powder was produced, indicating great improvement of product quality (Figure 5.11C). The color was also different from that of Figure 5.11B, probably due to the removal of pigment by the resin. Although KT content in this product was increased to 785.7 mg/g, only 0.70 g of product was recovered by this process. It was believed that absorption by resin and loss of material during spray drying jointly contributed to the low yield.

Despite the successful efforts to improve product properties by modifying spray drying conditions and resin pre-treatment, the product yield cannot be improved using current instruments. One disadvantage is that we did not have access to a closed-cycle spray dryer which is specially designed for feed in organic solvent. Although we managed to conduct spray drying of small-volume extracts safely by applying nitrogen and releasing exhaust to fume hood, volume

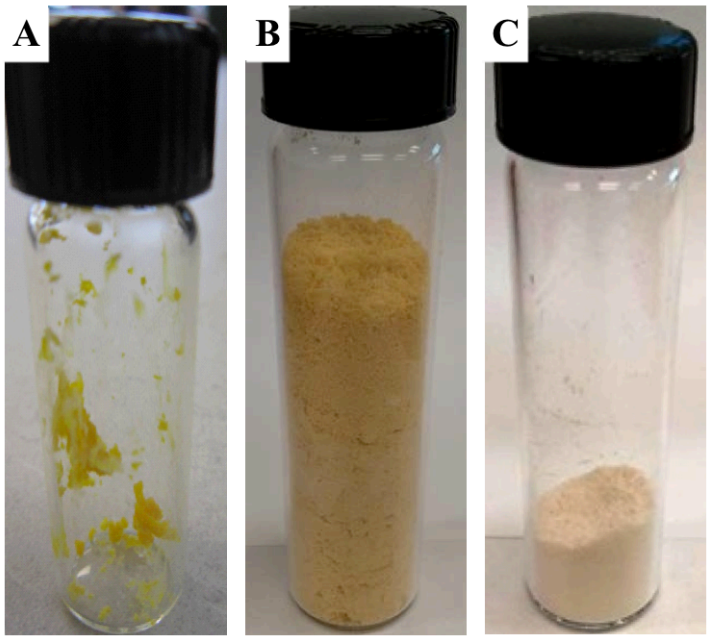

Figure 5.11 (A) KT mixture from freeze drying, (B) KT mixture from spray drying, and (C) KT mixture from spray drying after resin treatment

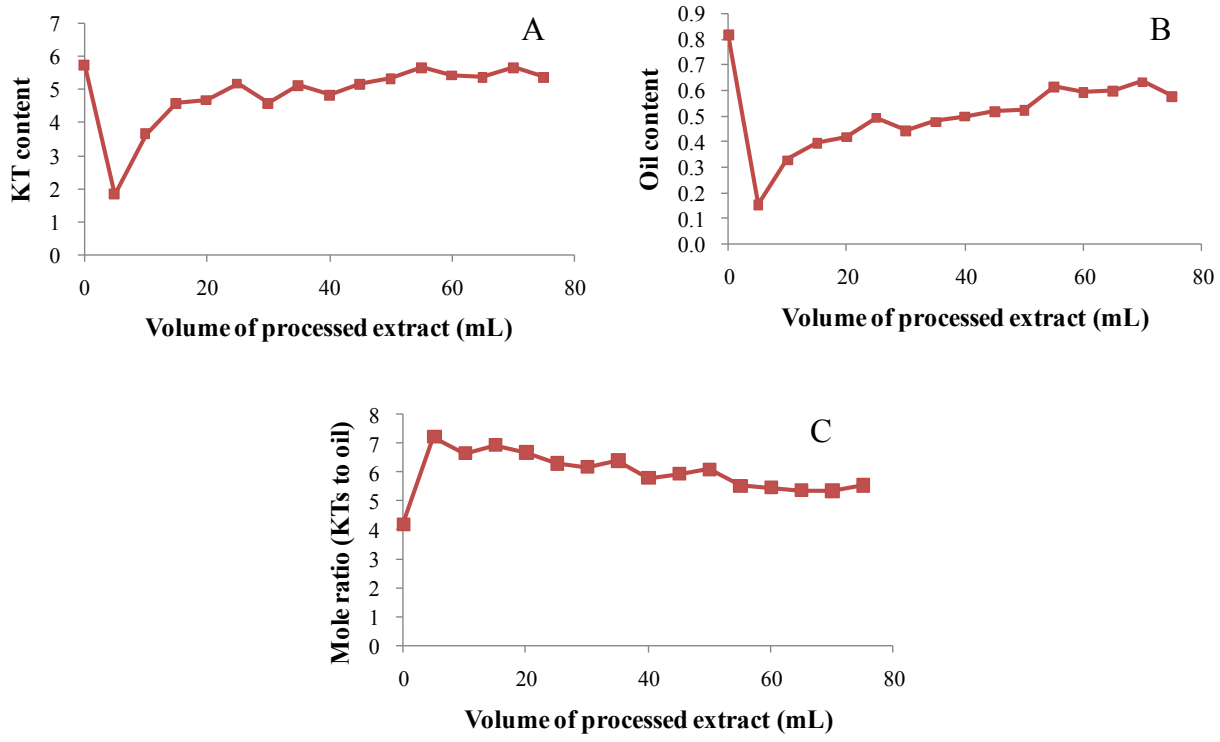

Figure 5.12 (A) KT content, (B) oil content, and (C) mole ratios of KTs to oil as a function of of processed extract by resin (n = 2)

the presence of ethanol in the feed might pose greater safety concern if large volumes of extract were loaded for drying. Considering product yield, complexity of operation and safety concerns, this protocol was not studied further.

5.8.4 Centrifugation

As described in section 5.8.1, properties of the liquid extract changed as evaporation progressed and ethanol content decreased. The formation of a suspension and solid is due to decreased solubility of KTs and other hydrophobic compounds in aq. ethanol of lower alcohol content. Centrifugation is often used to remove particles from liquid samples and, therefore, was tested as a means of processing the suspension. It was found that the KT profile of the suspension before and after centrifugation was significantly different (Figure 5.13). Originally, the suspension contained a variety of KTs in reduced and oxidized forms, including **1**, **2**, **3**, **5**, **8**, **9**, **12**, **15**, and **16** (Figure 5.13A). After centrifugation, only KTs of relatively higher polarity (**3**, **12**, and **15**) were present with relatively higher peak areas, all of which were in oxidized forms (Figure 5.13B). Peaks representative of **1**, **8**, and **9** were also shown in the chromatogram with decreased peak areas, while peaks representative of other KTs were almost eliminated by the treatment (Figure 5.13B). This finding indicated that KTs of relative higher polarity could still be dissolved in aq. ethanol of low percentage, while other KTs were precipitated out of solution and sedimented by centrifugation. This interesting discovery can be applied as a protocol of enriching **3**, **12** and **15** without sacrificing other KTs. Additionally, if purification of **3**, **12**, and **15** is required, this protocol can be potentially performed in conjunction with existing preparative HPLC method (Reaney *et al.*, 2013a) with reduced elution time. Furthermore, this protocol can be optimized with respect to evaporation volume and centrifugation conditions.

5.9 One Hundred and Sixty Four Litre Scale Solvent Extraction and Peptide Recovery

Extraction was scaled up to 210 L of flaxseed oil. Due to the increased volume, new flaxseed oil was used for this extraction. In section 5.7 it was shown that vigorous mixing of flaxseed oil and (70%) aq. ethanol for 5 min enabled the transfer of 91.8% of KTs present to the ethanol layer. The 210 L volume reactions were conducted in a sealed barrel that was placed on a barrel roller. As the vigour of the stirring was not known, the extraction time was increased to 10 min.

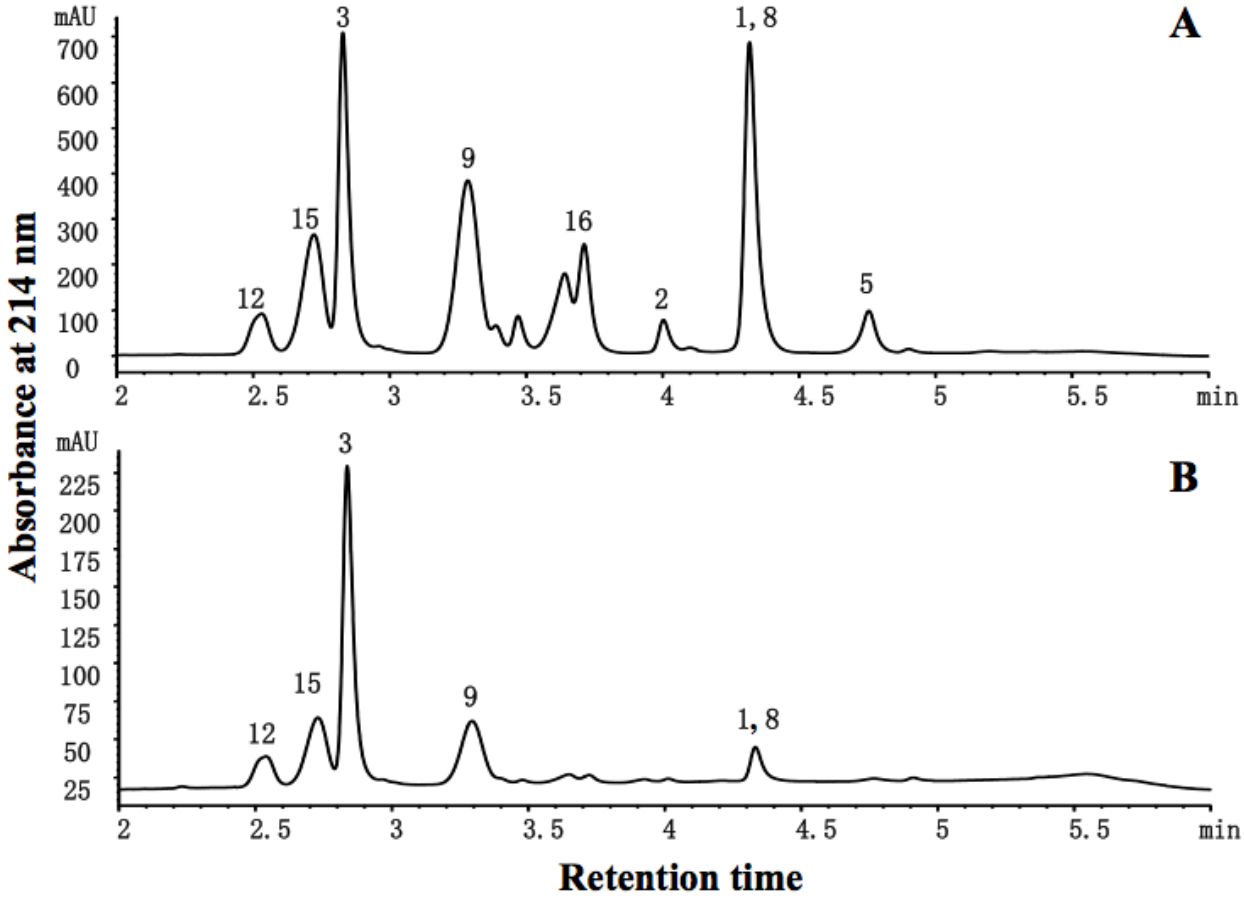

Figure 5.13 HPLC chromatograms of (A) suspension before centrifugation and (B) supernatant after centrifugation. KTs shown in chromatograms are [1–9-NαC]-KTA (**1**), [1–9-NαC]-KTB (**2**), [1–9-NαC],[1-MetO]-KTB (**3**), [1–8-NαC]-KTD (**5**), [1–8-NαC]-KTE (**8**), [1–8-NαC],[1-MetO]-KTE (**9**), [1–8-NαC],[1-MetO,3-MetO]-KTF (**12**), [1–8-NαC],[1-MetO,3-MetO]-KTG (**15**), and [1–8-NαC],[1-MetO]-KTG (**16**)

Following phase separation, analysis of the crude oil and processed oil demonstrated a recovery rate of just 61.2%, much lower than that achieved in the 250 mL beaker. The opacity of the drum hindered direct observation during the extraction process. Although sampling immediately after rolling the drum indicated that the aq. ethanol was thoroughly mixed with oil, potentially the agitation applied was not sufficient to completely contact the aq. ethanol with flaxseed oil in the drum. As the rolling speed of the drum roller is fixed, longer agitation time may result in better recovery rates.

In liquid-liquid extraction, extraction solvent is not only expensive to purchase, adding cost to the production, but also can pose environment concerns for disposal. Therefore, aq. ethanol recovery during evaporation was investigated. Evaporation conditions of 60^{o}C and 120 mbar were applied to maximize the evaporation efficiency while preventing boiling and flashing into the condenser. In both batches, as solvent removal progressed, the percentage of recovered aq. ethanol dropped significantly from ca. 85% in the beginning to 50–60% in the end (Table 5.3). Accordingly, low alcohol content can result in decreased vapour pressure. Using the constant evaporation conditions, the time required to evaporate the same volume doubled from the 1st fraction to 6th fraction. In the 7th fraction, although 100% ethanol was added to the mixture, less aq. ethanol was recovered over a longer time. Of all recovered aq. ethanol, 6 fractions have the ethanol content higher than 70% and can readily be used in subsequent extractions. The last fraction possesses ethanol of lower than 70%. Therefore, it is expected that a substantial volume of aq. ethanol having an ethanol content of no less than 70% can readily be reused for the next liquid-liquid extraction.

After evaporation of the ethanol, suspensions of ca. 4 L were obtained from each batch. As discussed in section 5.8.1, vacuum evaporation of liquid extract after the addition of 100% ethanol was found to efficiently produce a powdered KT mixture. However, when dealing with large volumes of water-rich suspensions in this section, this approach appeared to be slow. Thus, alternative strategies were investigated. Previously (section 5.8.4), the use of evaporation followed by centrifugation produces a fraction (2.5 L extract) enriched in **3**, **12**, and **15**. In this larger-scale process, suspensions were centrifuged in a larger volume centrifuge, using 500 mL centrifuge bottles. Under these conditions, the supernatant contained all KT species with reduced amounts of relatively low-polarity KTs (Figure 5.14). This phenomenon can be attributed to the higher ethanol content of the suspension and, therefore, higher solubility of low-polarity KTs in

Table 5.3 Ethanol content of rotary evaporator distillate during evaporation of a peptide extract

Fractions	Volume	Evap. time (min)	Density (g/mL)	EtOH% (v/v)
Batch One				
1	4 L	63	0.8389	85.5
2	4 L	80	0.8491	83.8
3	4 L	83	0.8570	81.3
4	4 L	85	0.8591	80.4
5	4 L	90	0.8638	78.7
6	4 L	125	0.8712	76.1
7	3 L	132	0.9071	60.9
Batch Two				
1	4 L	62	0.8396	86.3
2	4 L	67	0.8532	82.2
3	4 L	78	0.8656	77.8
4	4 L	86	0.8666	77.0
5	4 L	86	0.8773	73.3
6	4 L	113	0.8771	73.3
7	2 L	125	0.9281	50.6

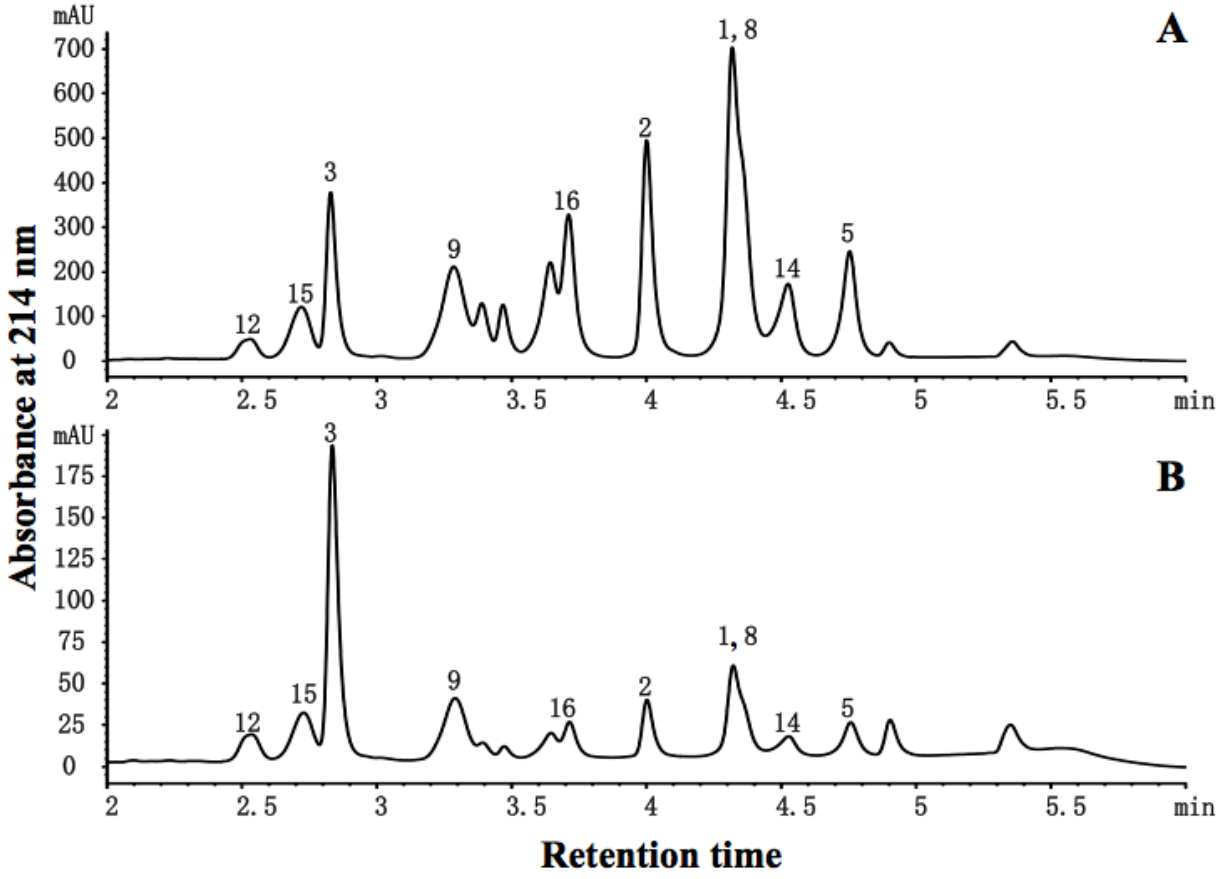

Figure 5.14 HPLC chromatograms of suspension (A) before centrifugation and (B) after centrifugation. KTs shown in chromatograms are [1–9-NαC]-KTA (**1**), [1–9-NαC]-KTB (**2**), [1–9-NαC],[1-MetO]-KTB (**3**), [1–8-NαC]-KTD (**5**), [1–8-NαC]-KTE (**8**), [1–8-NαC],[1-MetO]-KTE (**9**), [1–8-NαC],[1-MetO,3-MetO]-KTF (**12**), [1–8-NαC]-KTG (**14**), [1–8-NαC],[1-MetO,3-MetO]-KTG (**15**), and [1–8-NαC],[1-MetO]-KTG (**16**)

the larger-scale extracts compared with suspensions from small-scale tests. Analysis showed centrifugation was very efficient in precipitating particles from KT suspension. Of total KTs in the solution, 95.4% was present in the sediment (Table 5.4). The sediment contained all KT species that were present in the liquid extract (data not shown).

Filtration is a simple, commonly used and efficient method to eliminate particles from a suspension once an appropriate filtration medium is identified. Table 5.4 illustrated that syringe filters possessing pore size of 0.45 μm can remove 96.8% of KTs, while glass filter paper possessing pore size of 1.6 μm can only separate 15.6% of KTs from suspensions. The filtrate generated via the syringe filter was transparent by visual observation. HPLC analysis of this filtrate revealed a similar KT profile to that of the centrifuged suspension in which there was a dominant presence of **3**, **12**, and **15** (Figure 5.15). Unfortunately, despite the high efficiency and ease of operation of a syringe filter we did not conduct large-scale tests of filtration.

"Shear-induced coagulation" is the term used to describe the coagulation of suspensions when agitated. Once particles are brought into close proximity by fluid flow, the combined effects of two interparticle forces, attractive van der Waals force and repulsive electric double layer interaction, determine the movement of particles (vanni and Baldi, 2002). Coagulation occurs if the net force is attractive. In this study, after long periods of agitation and subsequent settling, the suspension remained cloudy although solid residues were observed at the bottom of suspension (54.1% of KTs were removed by this process). Further optimization of agitation methods may improve the recovery of KTs, though was not further investigated.

The effects of freezing then thawing on the stability of some emulsions and suspensions have been well studied. Nakamura and Okada (1976) studied the mechanism of particle coagulation in materials subjected to freeze–thaw process. They observed that growth of ice crystals facilitated the approaching of particles which then formed coagulates by van der Waals force. Table 5.4 showed that freezing at –15 and –25^{o}C followed by thawing was highly efficient in recovering KTs (97.2 and 97.6%, respectively), while freezing at –5^{o}C followed by thawing only recovered 25.4% of KTs. Likely, incomplete freezing at –5^{o}C contributed to the low peptide recovery. Figure 5.16 shows the separation of solid and liquid after the suspension was frozen and thawed. This protocol was easily performed and avoided removal of bulk liquid of high water content by extended periods of evaporation.

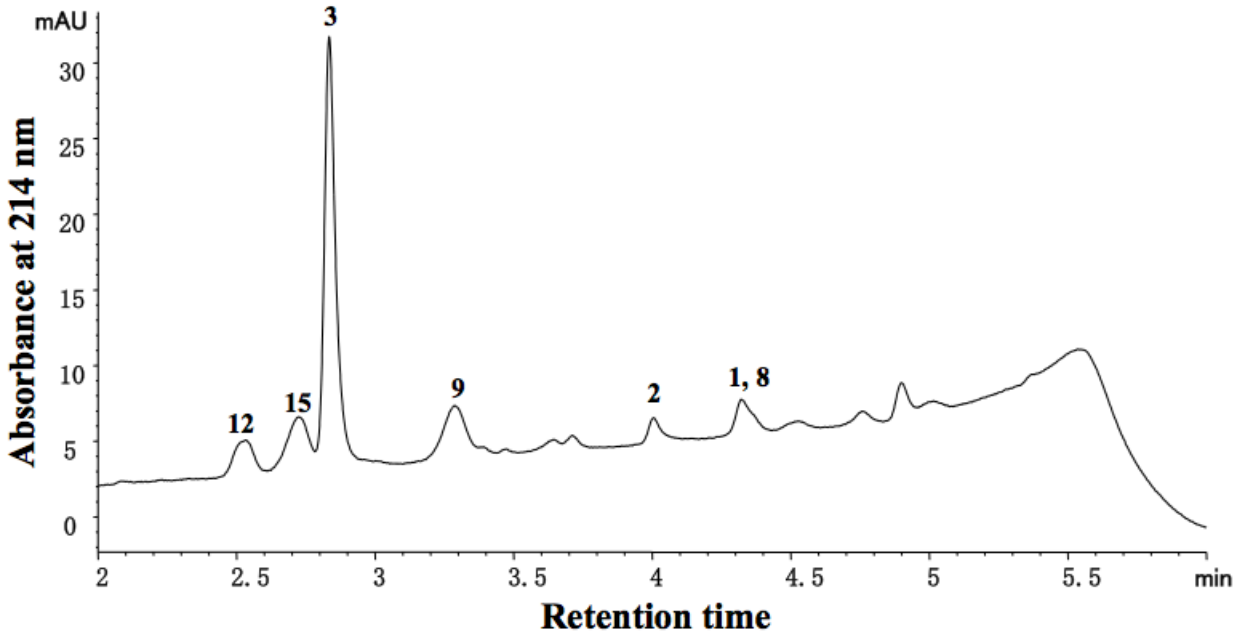

Figure 5.15 HPLC chromatograms of suspension after filtration through 0.45 μm syringe filter. KTs shown in chromatograms are [1–9-NαC]-KTA (**1**), [1–9-NαC]-KTB (**2**), [1–9-NαC],[1-MetO]-KTB (**3**), [1–8-NαC]-KTD (**5**), [1–8-NαC]-KTE (**8**), [1–8-NαC],[1-MetO]-KTE (**9**), [1–8-NαC],[1-MetO,3-MetO]-KTF (**12**), [1–8-NαC],[1-MetO,3-MetO]-KTG (**15**)

Table 5.4 Percentage of KTs removed from suspension by different processing methods (n = 2)

Processing methods	KTs removed from suspension (%)
Centrifugation	95.2
Filtration by filter paper	15.6
Filtration by syringe filter	96.8
Agitation	54.1
Freeze-thaw (–5^{o}C)	25.4
Freeze-thaw (–15^{o}C)	97.2
Freeze-thaw (–25^{o}C)	97.6

Figure 5.16 Suspension (right container) became clear (left container) after freezing at –25^{o}C and thawing at R.T.

5.10 Four Thousand Two Hundred Litre Scale Solvent Extraction and Peptide Recovery

As previously discussed, agitation time is an important factor in KT recovery from flaxseed oil. Therefore, the effects of agitation time were also investigated in this study. Figure 5.17A showed that KT content in extracts as a function of agitation time was not statistically different among 0, 30, and 60 min at $P < 0.05$ level. Equilibration of KTs between flaxseed oil and aq. ethanol was attained during the transfer of the solvent as larger-scale vessels required an appreciable length of time to load and mixing was initiated during transfer. The data are consistent with section 5.7 which showed that KT equilibration between the two phases was achieved within 5 min. The amount of residual KT in oil after extraction was not significantly different as the agitation time varied (Figure 5.17B). It was found that 79.5% of available KT could be extracted in the time required to fill the extraction vessel. The KT content of the flaxseed oil used in this study was 33.3% lower than the KT content of flaxseed oil used for extraction scale up research conducted in beakers and pails, and 46.6% lower than the KT content of flaxseed oil used for studies of extraction using drums. The difference in KT content may be explained by differences in the flaxseed pressing. The manufacturer utilized an Anderson International "Duo" for pressing flaxseed in contrast a series of Komet expeller presses were used for previous batches (Loutas, personal communications). The "Duo" press design filters expressed the majority of oil through the solids, while the Komet expeller press does not produce as thick a cake and, as such, the depth for filtration is smaller. In addition, the solids and oil have a longer residence time in the "Duo" press than the Komet press.

FFE is widely used in industry for evaporation and stripping of solvents. Previously, we were able to observe property changes while evaporating extracts in a glass rotary evaporation. The industrial FFE was constructed of stainless steel and, therefore, direct observation was not possible. Similar to section 5.9, a cloudy suspension (170.5 kg) was produced after evaporation was complete. However, KT concentration was considerably less than the crude liquid extract (data not shown). We infer that inside the tubing of FFE a thin layer of liquid feed was formed which maximized the heat transfer rate and efficiency (Smith, 2011). Therefore, FFE was very efficient in removing ethanol and, consequently, the ethanol content became so low that most of the KTs precipitated and were retained in the FFE. This was confirmed

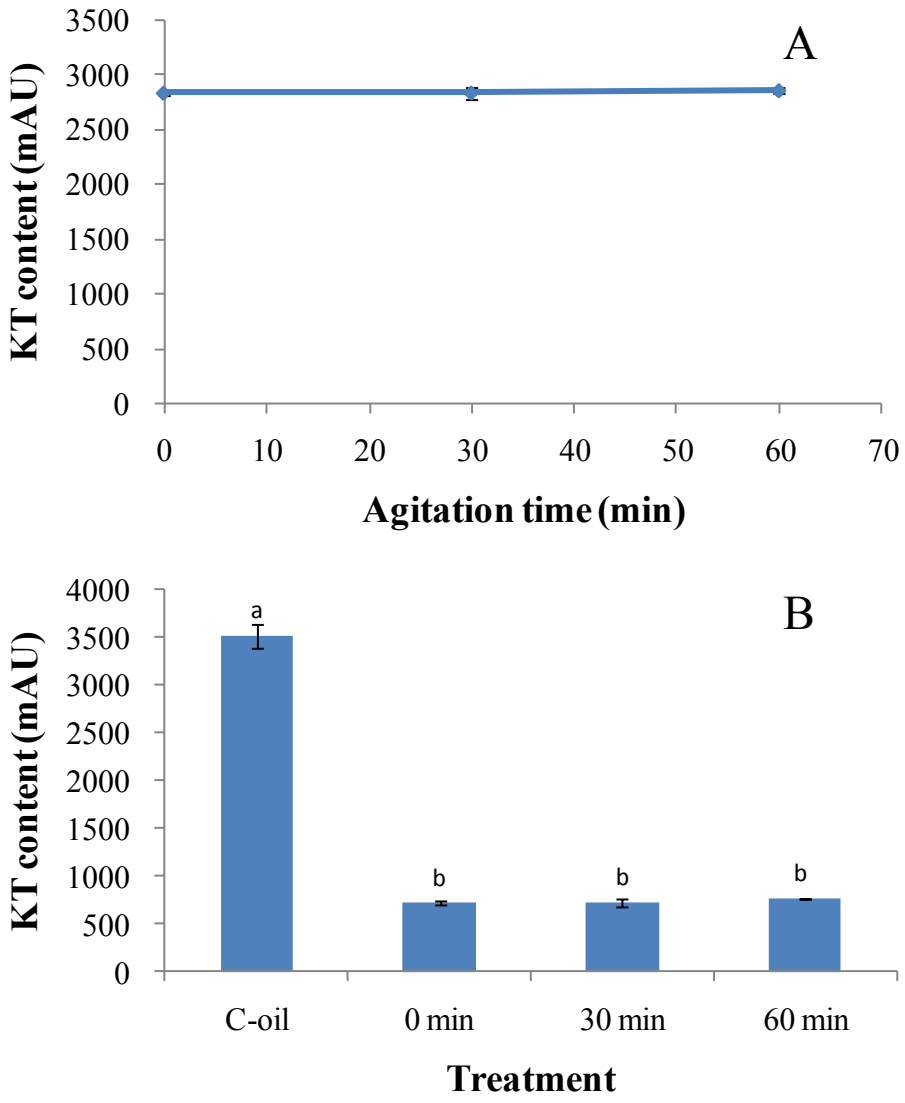

Figure 5.17 (A) KT content in extract as a function of agitation time (B) KT content in flaxseed oil and processed oils. C-oil: flaxseed oil; 0 min: flaxseed oil processed by agitation at 0 min; 30 min: flaxseed oil processed by agitation at 30 min; 60 min: flaxseed oil processed by agitation at 60 min. Different letters denote statistical difference by Tukey's test at $P<0.05$ level ($n = 3$).

when a concentrated KT solution was recovered by washing the FFE with two batches of 100% ethanol. In total, 97 kg of a yellow concentrated solution was collected.

The aforementioned ethanol solution was evaporated in two batches of equal volume in a large rotary evaporator. A thick layer of mixture formed when solvent was mostly removed (Figures 5.18A and B). The KT mixture was a hard solid. Drying in a vacuum oven at 60^{o}C and 65 mbar for 2 d decreased the weight of the product by 12.0%, affording 3328.89 g of a KT mixture. The solids in the evaporator were ground to a yellowish powder in a coffee grinder (Figure 5.18C). HPLC analysis with an internal standard showed the KT content of the powder was 796.3 mg/g. Product quality was comparable to that prepared according to Reaney *et al.* (2013b) (Olivia, personal communications).

KTs were readily recovered from suspension that was rich in water by freezing then thawing (section 5.9). When this approach was applied to suspensions prepared in the pilot plant only 40.4% of KTs were removed from the suspension by freezing and thawing the solution. In this study, KTs attached to the plastic wall of the jugs in the form of yellowish spots. In glass vessels, smaller extractions formed sediments. Therefore, alternative strategies or materials need to be developed for peptide recovery on larger-scale.

The hydrophobicity of XAD^{TM} 1600 resin gives it great potential in recovery of hydrophobic KTs from suspension of high water content. An initial absorption test using a small column showed that after loading 150 mL suspension on 1 g of resin, only a small portion of KTs (ca. 25%) remained in the suspension (Figure 5.19A). Further investigation is needed to study the adsorption curve and equilibrium capacity by agitating certain amount of polymeric adsorbent in a solution for a certain amount of time (Rohm and Haas Company, 2000). Therefore, 150 g of resin was agitated in vessels containing 17.0–18.5 kg suspension for up to 3 h. It was shown that resin was efficient in KT absorption. Agitation of the peptide solution in the presence of XAD for 1 h was able to remove more than 80% of KTs from suspension (Figure 5.19B). It was advised to use water miscible organic solvents (e.g. acetone and alcohol) to regenerate the resin and collect enriched fractions (Rohm and Haas Company, 2000). Previously, ethanol was utilized in KT extraction. From a viewpoint of economy, it is beneficial to use a single solvent (ethanol) for different purposes in a process (Don and Robert, 2008). Thus, resin regeneration by ethanol was tested and proved to be effective as demonstrated by the decrease in KT content in fractions of eluent (Figure 5.19C) and the high efficiency of absorption when regenerated resin was used for

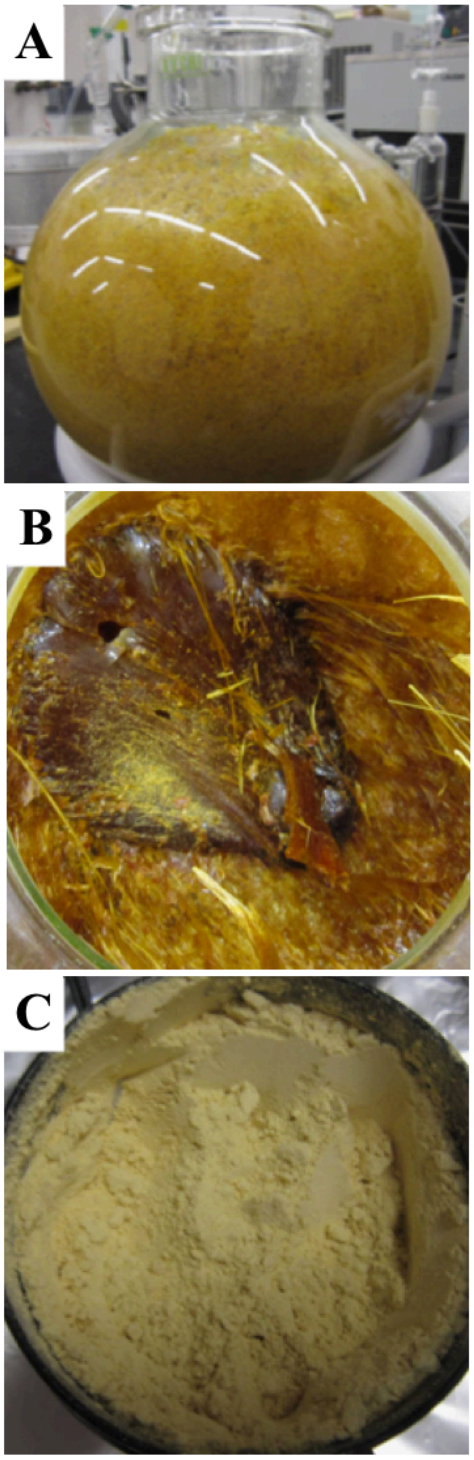

Figure 5.18 KT mixture after evaporation seen (A) outside the flask, (B) inside the flask, and (C) KT mixture after grinding

next batch of processing (Figure 5.19B). Processed suspension became less opaque and less yellowish (Figure 5.20A). In contrast to the usual yellowish color of KT containing solution, the 1st and 2nd fractions were dark red and light red, respectively (Figure 5.20B). Instead of forming dry and crunchy product, hard, elastic and dark brown solid was obtained by evaporation and vacuum drying (Figure 5.20C). The reason for the unusual texture and color of product was unknown. About 11 g of product could be recovered from each pail. HPLC analysis showed KT content in the product was 540.8 mg/mL.

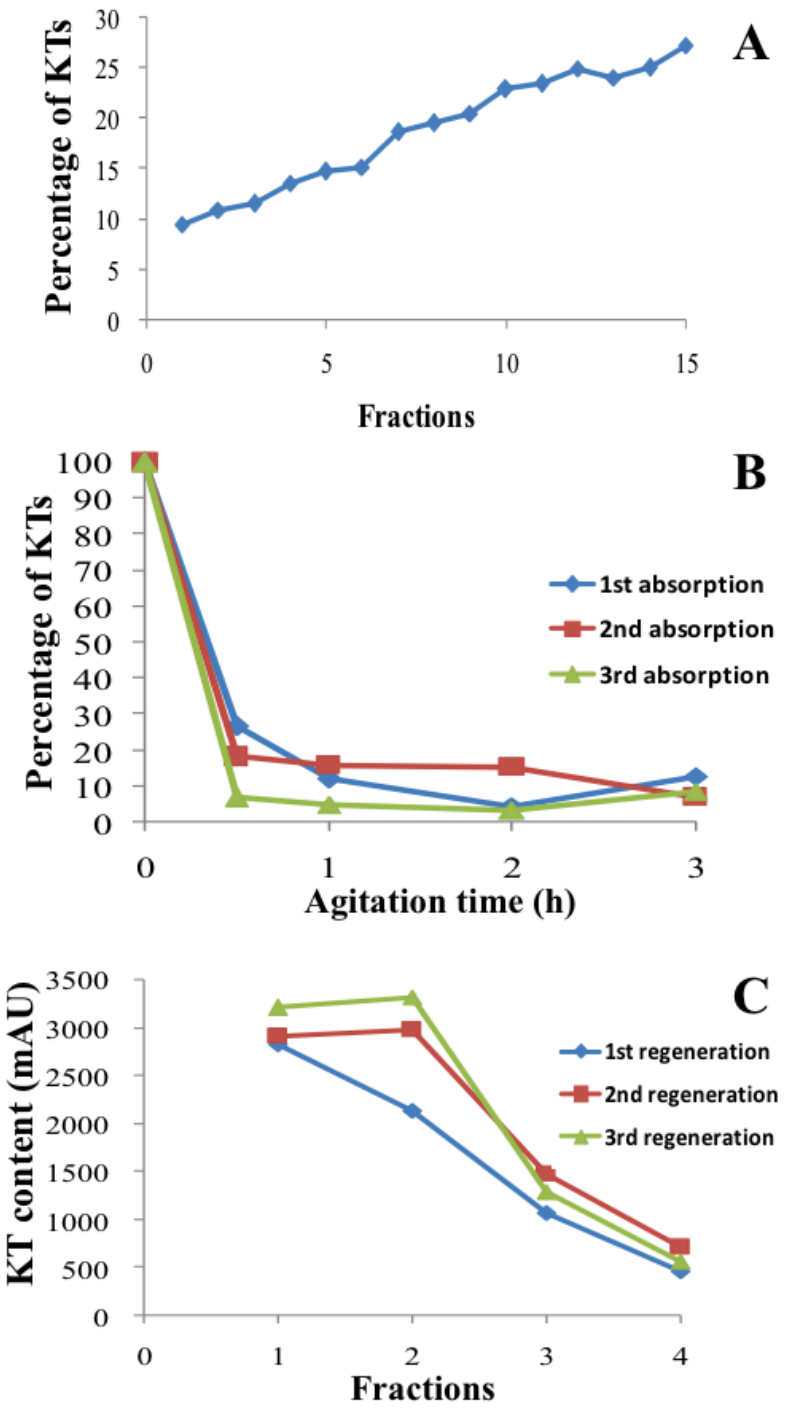

Figure 5.19 (A) Percentage of KTs in fractions of suspension after resin absorption in a small column, (B) percentage of KTs in suspension after agitating resin and suspension mixture in pails, and, (C) KT content recovered in ethanol regeneration (n = 1)

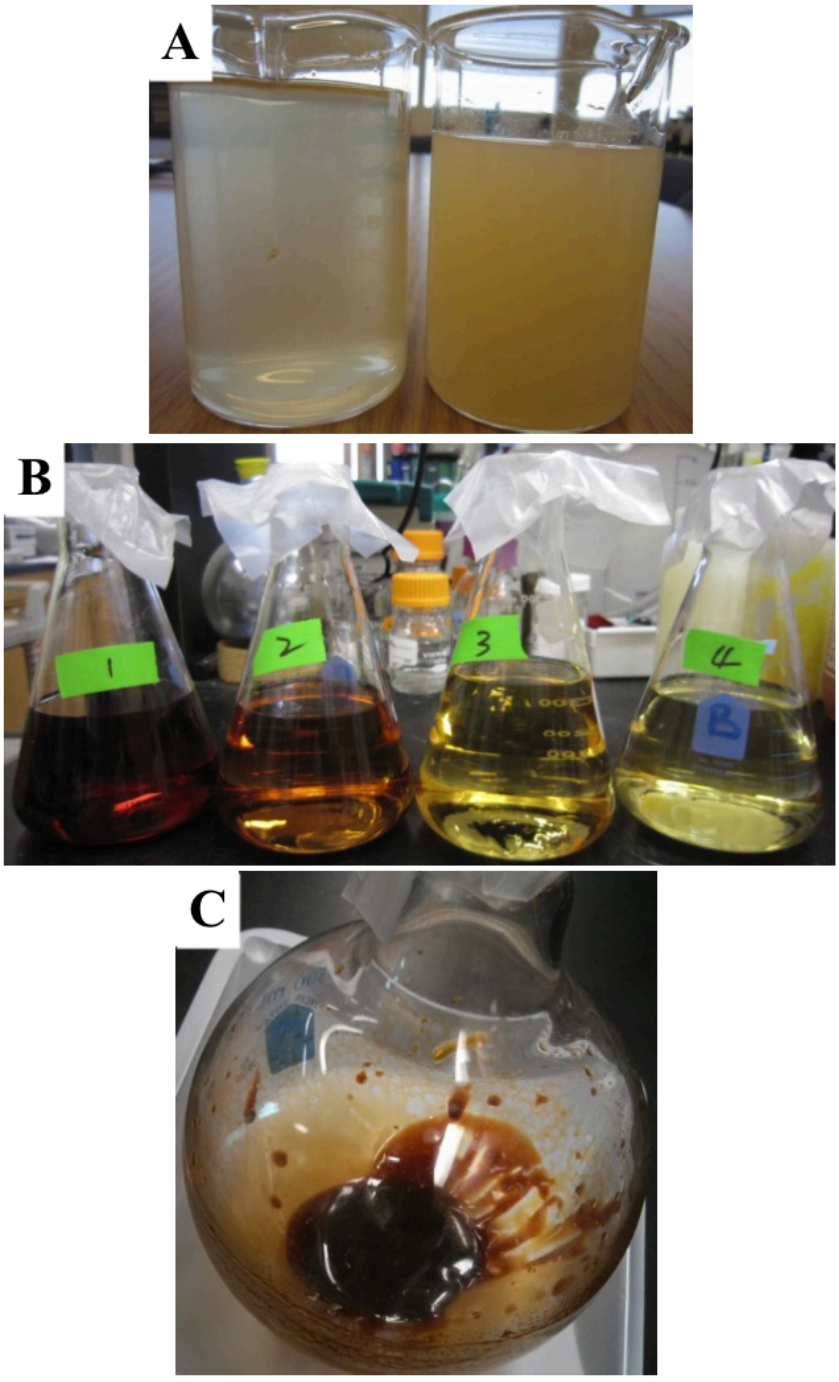

Figure 5.20(A) Color of suspension (right beaker) changed after resin treatment (left beaker), (B) color of ethanol solution after regenerating resin (1st to 4th fractions, from left to right), and (C) KT mixture yielded from resin regeneration

Chapter 6
SUMMARY AND CONCLUSIONS

KTs are a group of cyclic peptides within the family of Caryophyllaceae-type cyclopeptide, containing eight or nine amino acids (Tan and Zhou, 2006). Since the first discovery of **1** from slime filtered from crude flaxseed oil in 1959, sixteen species of KTs have been isolated and identified. Biological activities of KTs were investigated via various *in vitro* and *in vivo* studies. KTs can exhibit cytoprotective (Kessler *et al.*, 1986), antimalarial (Bell *et al.*, 2000), immunosuppressive (Wieczorek, *et al.*, 1991; Morita *et al.*, 1997; Morita *et al.*, 1999), and antioxidant (Sharav, 2013) activities. Chemically modified and activated **3** and **9** can bind with molecules or compounds of interest to form fluorescent adducts, affinity media and haptens for antibody production, which are important to biomedical research (Jadhav, 2013). Although there is growing interest in applying KTs in research and new product development in food, cosmetic and pharmaceutical industries, existing KT extraction and isolation methods in literature have various limitations, including laborious chromatography methods, use of multiple solvents, and use of solvents with toxicity levels that are undesirable to the potential market. Besides, processing and recovery of KT mixture from solvent extract, other than rotary evaporation, have never been reported.

Initially, this study aimed at improving solid-liquid extraction of KTs by reducing the temperature of chromatography process and eliminating the use of undesirable organic solvents. Bench-scale experiments showed that retention of KTs on silica was enhanced at low temperature. Specifically, elution of flaxseed oil-laden silica using ethyl acetate at 0–4^{o}C, followed by ethyl acetate and ethanol washes at R.T., could recover 61.21% of KTs from flaxseed oil. However, the lack of appropriate equipment hindered further testing. Alternatively, liquid-liquid extraction using aq. ethanol was investigated. Optimization of extraction regarding percentage of aq. ethanol and solvent to oil volume ratios was guided by ^{1}H-NMR analyses, which could determine the mole ratios of KTs to oil simultaneously. The use of 70% aq. ethanol with the solvent to oil volume ratio of 0.25:1 was considered to be the optimal condition on the basis of its highest KT

content and KTs to oil mole ratio among all the conditions. Subsequently, extractions were conducted using 160 mL, 12 L, 164 L, and 4,200 L flaxseed oil. Recovery rates of KTs from oil were 91.8, 92.0, 61.2, and 79.5%, respectively. Depending on the volume of liquid extract and practical limitations of instruments, different processing and recovery methods were tested for each scale of extraction. Based on product properties and ease of operation, the optimal methods were determined as follows: a) 12 L scale: rotary evaporation with addition of 100% ethanol; b) 164 L scale: a combination of rotary evaporation of liquid extract and freeze-thaw treatment of suspension; and c) 4,200 L scale: a combination of falling film evaporation and rotary evaporation of the ethanol-containing extract, in addition to reverse-phase resin treatment of the KT suspension.

Through several steps of scale-up experiments, this study demonstrated the large-scale extraction and recovery of KTs from flaxseed oil. The successful production of a KT mixture with satisfactory product properties demonstrated the promising application of this work in commercial extraction of KTs from flaxseed oil. However, future research on KT extraction, recovery and isolation from flaxseed and flaxseed oil is needed. Enriched KT mixtures and single species of KTs may be in demand by potential markets. Simplified and efficient KT enrichment and purification methods have not yet been established. Processing of ethanol-containing flaxseed oil after liquid-liquid extraction may be another direction for future research. Due to the antioxidant activities of KTs (Sharav, 2013), removal of these compounds is expected to alter the oxidation stability of flaxseed oil. It would be interesting to establish a process to eliminate ethanol, while preserving the original properties and health benefits of flaxseed oil. If the protocols suggested by this study were to be accepted by manufacturers, development of quality control protocols should be top priority as variation of flaxseed oil quality and processing equipment may lead to changes in product quality.

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APPENDIX A

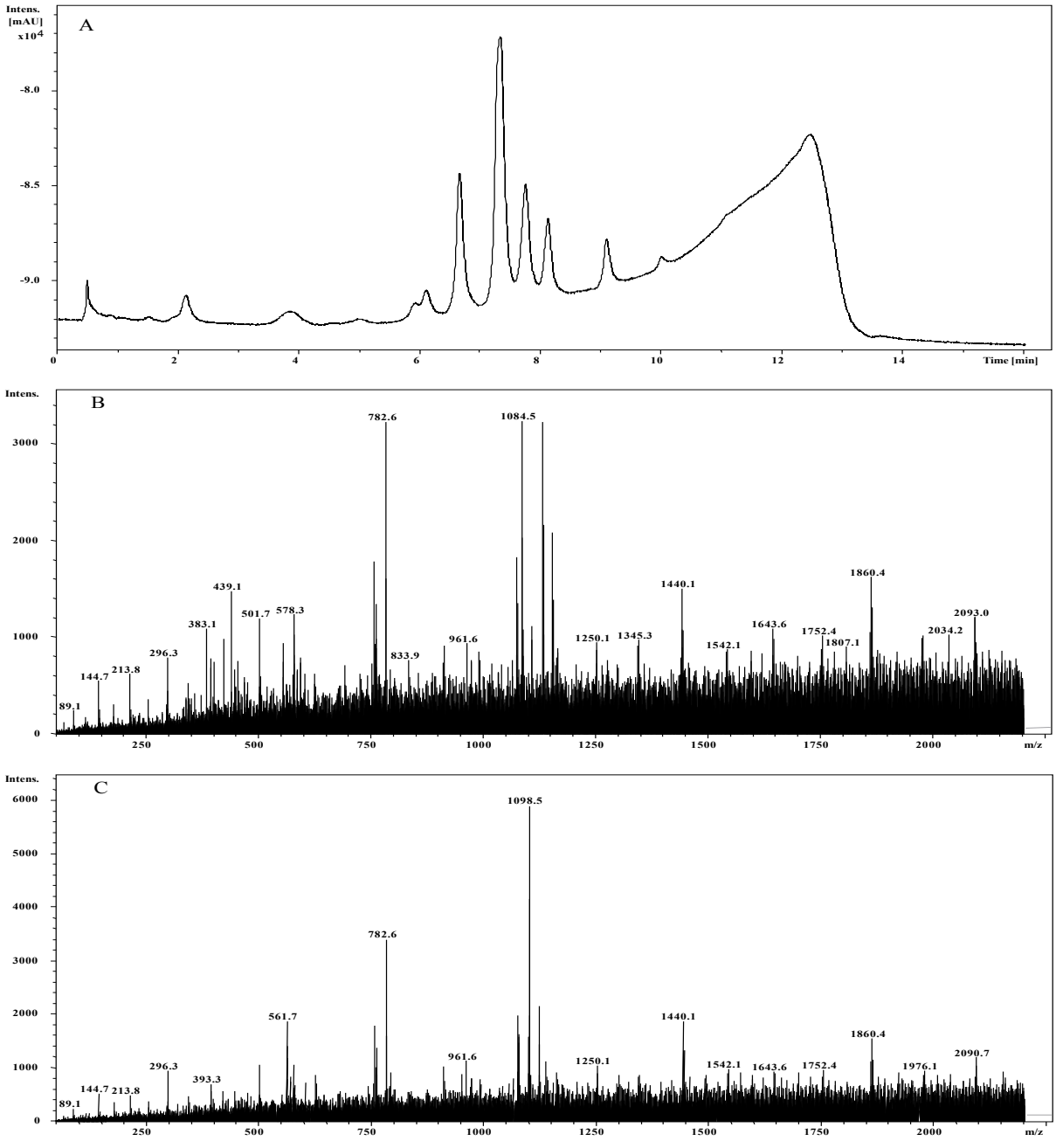
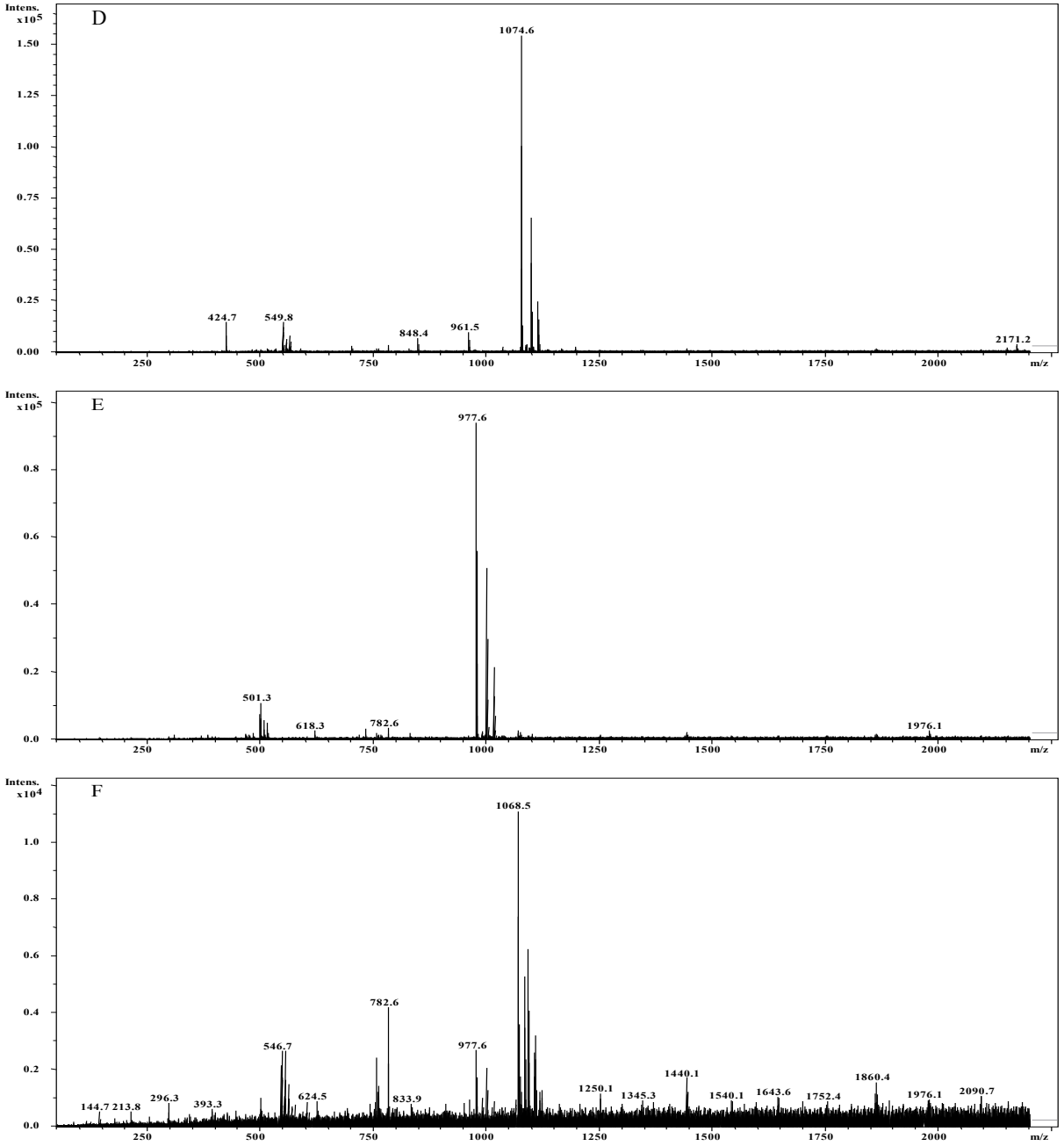


Figure A.1 (A) HPLC chromatogram of methanol extract of flaxseed oil and HPLC-MS spectra of methanol extract showing (B) **12**, (C) **15**, (D) **3**, (E) **9**, (F) **13**, (G) **16**, (H) **2**, (I) **1** and **8**, (J) **14**, and (K) **5** (**cont'd**)



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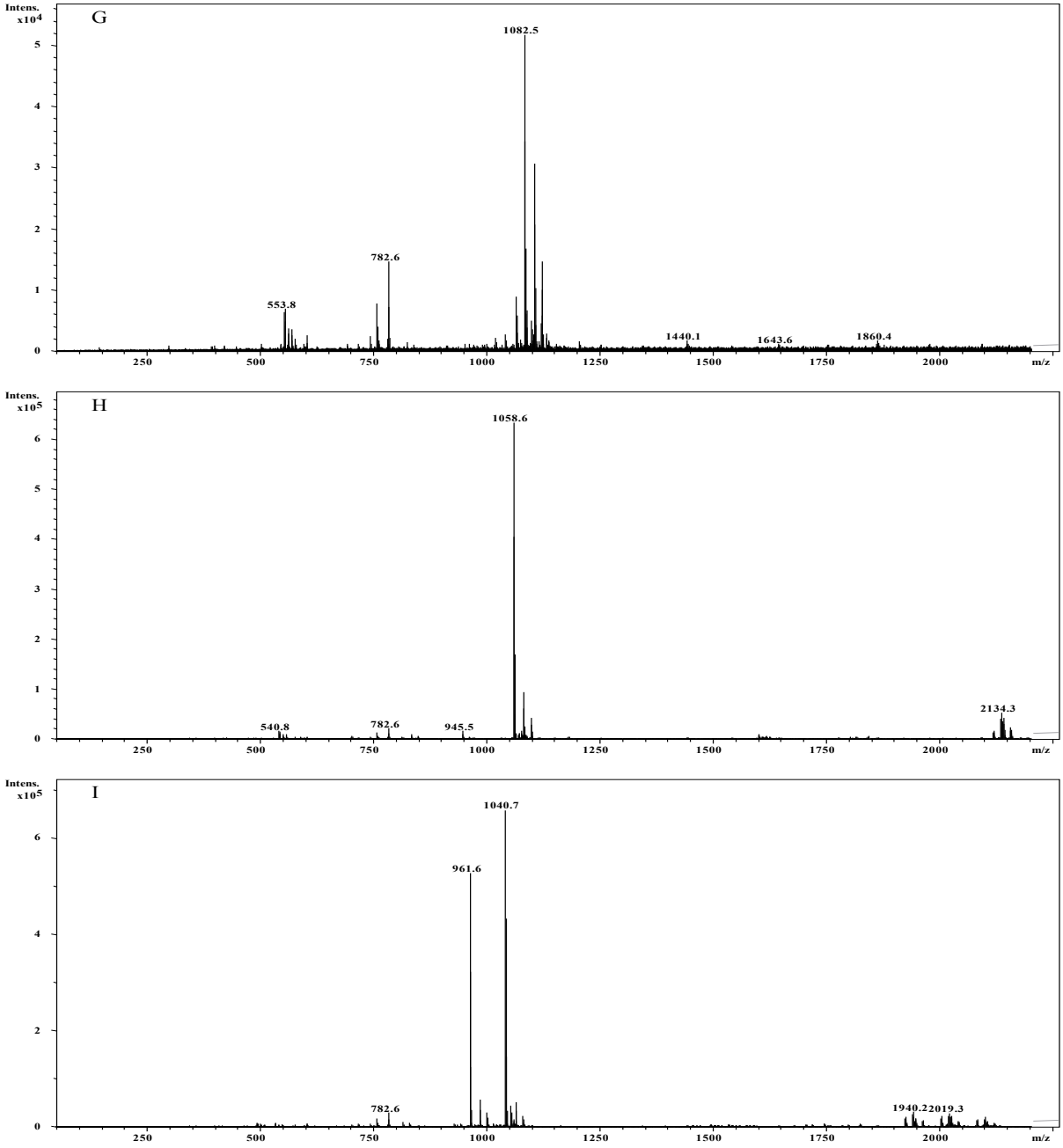

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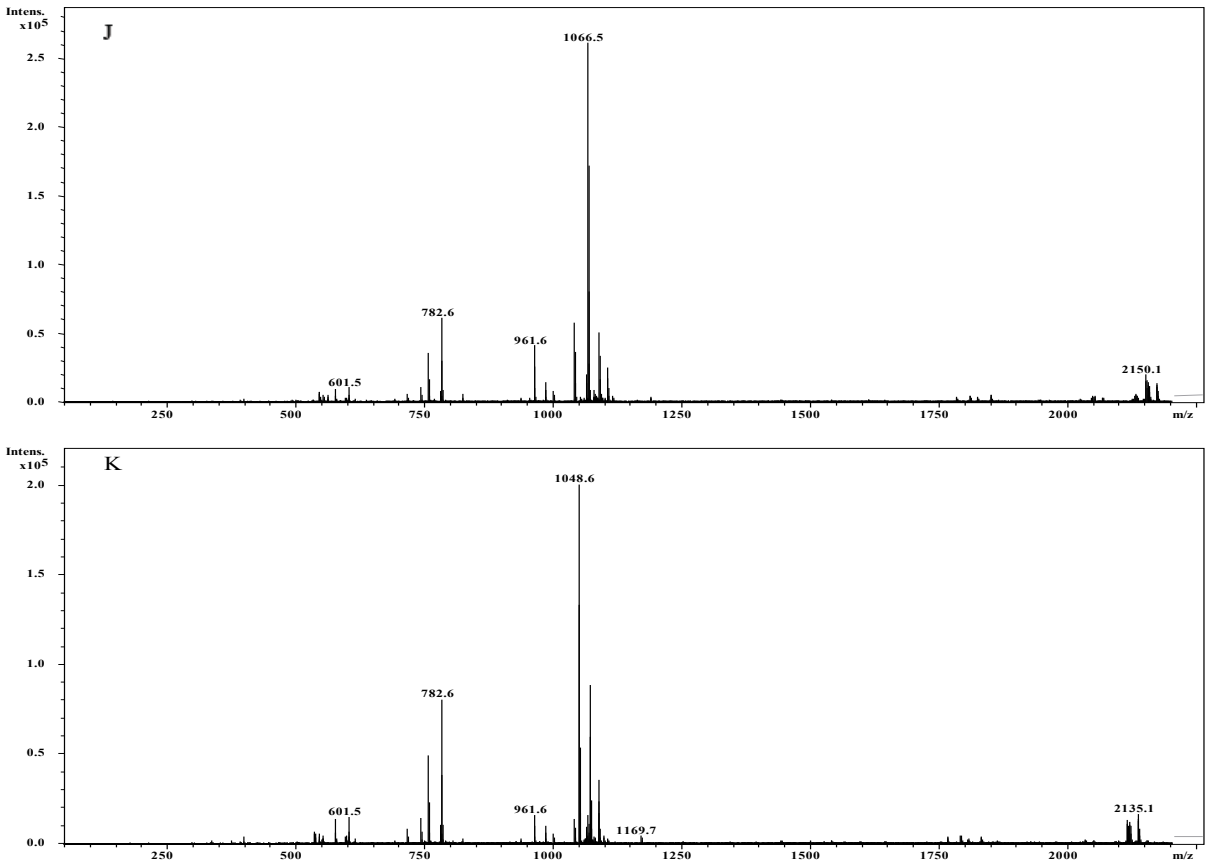

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APPENDIX B

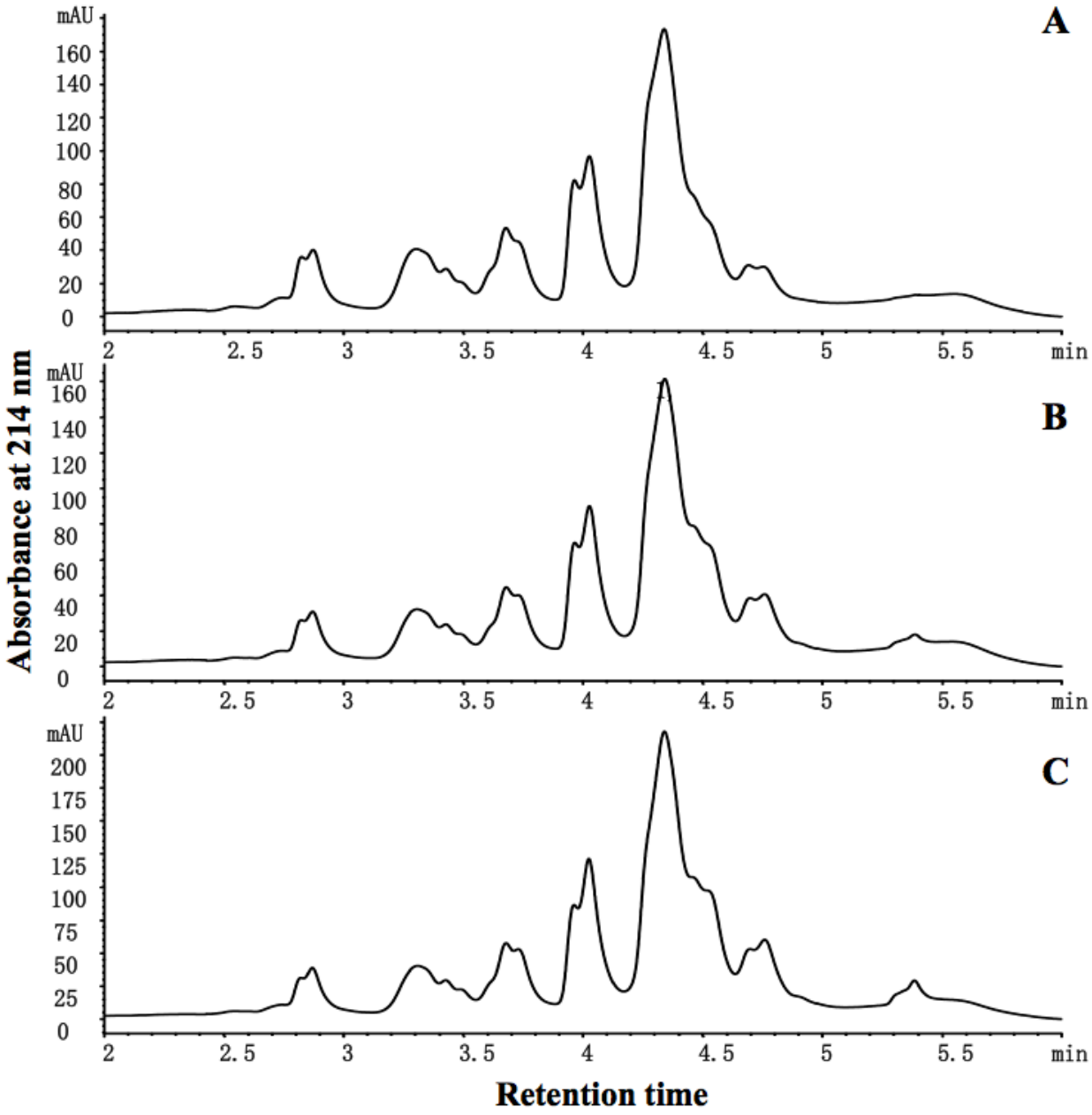

Figure B.1 HPLC chromatograms of (A) 50% aq. ethanol extract; (B) 60% aq. ethanol extract; (C) 70% aq. ethanol extract; (D) 80% aq. ethanol extract; (E) 90% aq. ethanol extract; and (F) 100% aq. ethanol extract (**cont'd**)

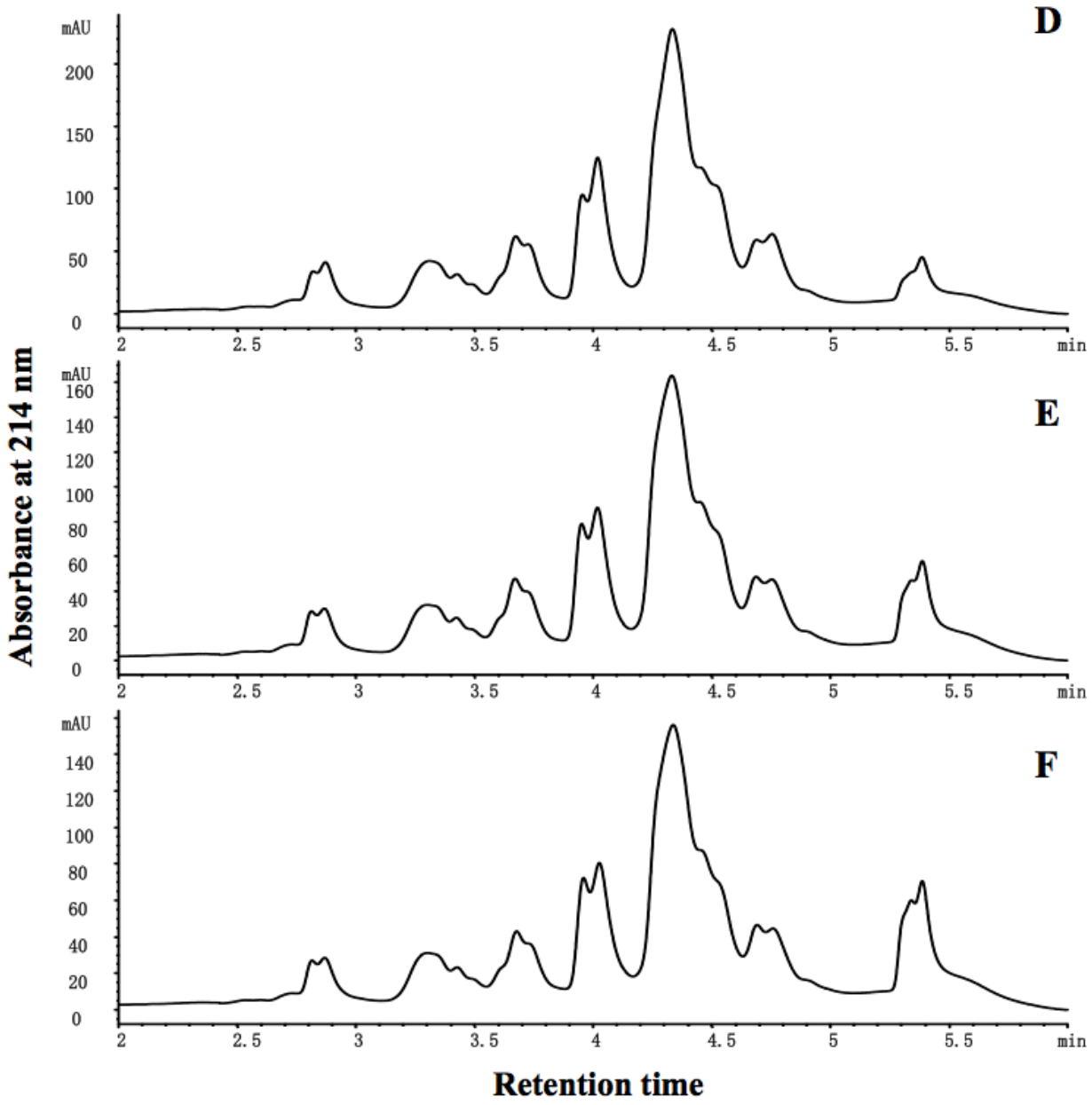

Figure B.1 HPLC chromatograms of (A) 50% aq. ethanol extract; (B) 60% aq. ethanol extract; (C) 70% aq. ethanol extract; (D) 80% aq. ethanol extract; (E) 90% aq. ethanol extract; and (F) 100% aq. ethanol extract

APPENDIX C

Table C.1 Recovery of aq. ethanol phase after extraction (n = 2)

Solvent: Oil	Percentage of Ethanol					
	100	90	80	70	60	50
2:1	0.98	1.00	1.03	1.05	1.20	0.98
1:1	0.84	0.94	1.00	1.03	1.25	0.89
0.5:1	0.60	0.83	0.93	0.97	0.85	0.97
0.25:1	NS[1]	NS[1]	0.87	1.00	0.80	0.88
0.125:1	NS[1]	NS[1]	0.90	0.30	NS[1]	0.70

[1]NS: no separation

APPENDIX D

Table D.1 Calculated KT content (n = 3)

Ethanol	Volume ratios	KT content	
		Mean[1]	S.D.
100%	2:1	2.03^{bcdef}	0.19
	1:1	1.35^{fg}	0.17
	0.5:1	1.69^{cdefg}	0.26
90%	2:1	2.15^{bcde}	0.14
	1:1	1.85^{cdefg}	0.09
	0.5:1	2.15^{bcde}	0.08
80%	2:1	2.22^{bcd}	0.09
	1:1	2.01^{bcdef}	0.07
	0.5:1	1.89^{bcdef}	0.16
	0.25:1	3.75^{a}	0.11
70%	2:1	1.97^{bcdef}	0.15
	1:1	1.85^{cdefg}	0.07
	0.5:1	1.93^{bcdef}	0.47
	0.25:1	3.78^{a}	0.22
60%	2:1	2.58^{b}	0.37
	1:1	2.28^{bc}	0.04
	0.5:1	1.18^{g}	0.15
50%	2:1	1.97^{bcdef}	0.19
	1:1	1.46^{efg}	0.03
	0.5:1	1.54^{defg}	0.09
	0.25:1	2.35^{bc}	0.13

[1]Means followed by the different superscript were statistically different by Tukey's multiple comparison test at 0.05 level.

Table D.2 Calculated KTs to oil ratios (n = 3)

Ethanol	Volume ratios	KTs to oil ratios	
		Mean[1]	S.D.
100%	2:1	0.12^{h}	0.00
	1:1	0.17^{gh}	0.02
	0.5:1	0.28^{gh}	0.05
90%	2:1	0.25^{gh}	0.02
	1:1	0.40^{fgh}	0.04
	0.5:1	0.50^{defgh}	0.02
80%	2:1	0.75^{defgh}	0.06
	1:1	1.12^{bcde}	0.03
	0.5:1	1.16^{bcd}	0.11
	0.25:1	1.68^{ab}	0.26
70%	2:1	1.10^{bcdef}	0.07
	1:1	1.46^{abc}	0.32
	0.5:1	1.68^{ab}	0.53
	0.25:1	2.00^{a}	0.07
60%	2:1	0.86^{cdefg}	0.19
	1:1	1.18^{bcd}	0.24
	0.5:1	1.65^{ab}	0.22
50%	2:1	0.42^{efgh}	0.06
	1:1	0.48^{defgh}	0.05
	0.5:1	1.02^{bcdef}	0.29
	0.25:1	1.68^{ab}	0.06

[1]Means followed by the different superscript were statistically different by Tukey's multiple comparison test at $P < 0.05$ level.