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# Accelerated shelf-life test of alkamides in Echinacea purpurea root aqueous ethanol Soxhlet extracts

Avery Kent Sia Solco  
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**Accelerated shelf-life test of alkamides in *Echinacea purpurea*  
root aqueous ethanol Soxhlet extracts**

By

**Avery Kent Sia Solco**

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

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Program of Study Committee:  
Patricia A. Murphy, Major Professor  
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2007

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## Abstract

*Echinacea* species are purported to have anti-inflammatory, anti-viral and other medicinal values. *Echinacea* alkamides, which are unsaturated fatty acyl isobutylamides/methylbutylamides, are thought to be the characteristic compounds responsible for some of these medicinal values. *Echinacea* species are rich in anti-oxidant phenolic acids, thus may protect the alkamides from oxidation. Our hypothesis is antioxidant phenolic acids protect degradation of alkamides in *Echinacea purpurea*. Alkamide stability was evaluated in ethanol extracts of *E. purpurea*: phenolics-rich and phenolics-poor extracts. The stability of the alkamides in extracts was evaluated in dry form at 70, 80 and 90°C and in DMSO solution at 90, 100, and 110°C. Analytical HPLC with a photodiode array detector was used to measure alkamide concentrations. The relative abundance of the alkamides are as follows: Alkamide 2 > 9 > 8 > 3 > 4 > 1 > 7 > 5 > 10 > 11 (See Figure 2-2 for structures of alkamides). Alkamides in phenolic acid-rich extracts were more stable than in phenolics acid-poor extracts in dry form. However, the alkamide were more stable in phenolics-poor extracts than in phenolics-rich extracts when dissolved in DMSO. In addition, the alkamides, with or without phenolic acids, were more stable in DMSO than in dry form. The degradation of the alkamides followed apparent first order kinetics. The order of the degradation of the alkamides in dry was: 1 ≈ 2 > 9 ≈ 8 > 3 ≈ 4 ≈ 5 ≈ 7 ≈ 10 ≥ 11, which appeared to have followed singlet oxygen oxidation mechanism. The order of the degradation of the alkamides in DMSO was: 9 ≈ 8 > 2 > 1 > 5 ≈ 7 ≈ 4 ≈ 10 ≥ 11 ≈ 3, which appeared to have followed free radical oxidation

mechanism. Alkamides are very stable compounds. There is a difference in the energy of activation ( $E_a$ ) of degradation of the alkamides in dry form and in DMSO.  $E_a$  (kJ/K-mol) for alkamides 1, 3, 9 and 8 in phenolics-rich dry form were 83, 74, 66 and 70 respectively and in phenolics-rich DMSO solution were 163, 120, 111 and 120, respectively, suggesting a difference in degradation mechanism. However, the change in the Gibb's free energy of activation ( $\Delta G^\ddagger$ ) of the alkamides are the same in all treatments except for phenolics-rich DMSO solution, which suggests a similar degradation mechanism of the alkamides in all treatments, except for phenolics-rich DMSO solution. The main difference between the DMSO and the dry form is probably  $O_2$  availability.

## Chapter 1

### Introduction

*Echinacea* is one of the most popular herbal supplements in the United States and parts of Europe (Bauer, 1998). The plant genus *Echinacea* belongs to the plant family Asteraceae. The genus *Echinacea* contains 9 species, all of which are indigenous to North America (Bauer, 1998). Purple coneflower and eastern purple coneflowers are scientifically named *Echinacea purpurea*, while narrowleaf coneflowers are *Echinacea angustifolia*, Topeka purple coneflower are *Echinacea atrorubens*, smooth coneflower and smooth purple coneflowers are *Echinacea laevigata*, pale purple coneflowers are *Echinacea pallida*, yellow coneflower and bush's purple coneflower are *Echinacea paradoxa*, Sanguine purple coneflowers are *Echinacea sanguinea*, wavyleaf purple coneflowers are *Echinacea simulata* and Tennessee coneflowers are *Echinacea tennesseensis*. Most of the flowers of these species are purple, hence almost all of them have the word purple in their names (See Figure 1). Other common names of *Echinacea*, which may refer to one or multiple species of *Echinacea*, which also depends on the locale, may include any one or more of the following names: American coneflower, purple coneflower, black Sampson, black Susan, cock-up-hat, combflower, hedgehog, igelkopf, Indian head, Kansas snake root, kegelblume, narrow-leaved purple coneflower, red sunflower, rudbeckia, scurvy root, snakeroot, solhat and sun hat (MayoClinic.com 2007). Table 1 on the next page lists the species name with some of their common names. Figure 1 on the next page shows the pictures of *Echinacea* with their species names.

**Figure 1-1:** *Echinacea* species in the following order: *E. angustifolia*, *E. angustifolia* var. *angustifolia*, *E. atrorubens*, *E. angustifolia* (mutant ray type), *E. laevigata*, *E. pallida*, *E. pallid*, *E. paradoxa*, *E. purpurea*, *E. sanguinea*, *E. simulata*, and *E. tennesseensis*.



(McCoy and Widrlechner 2005)

**Table 1-1:** Scientific names and some common names of species belonging to the genus *Echinacea*.

Scientific Name	Common Names
<i>E. angustifolia</i>	Narrow-leaf coneflower
<i>E. atrorubens</i>	Topeka purple coneflower
<i>E. laevigata</i>	Smooth coneflower, smooth purple coneflower
<i>E. pallida</i>	Pale purple coneflower
<i>E. paradoxa</i>	Yellow coneflower, bush's purple coneflower
<i>E. purpurea</i>	Purple coneflower, eastern purple coneflower
<i>E. sanguinea</i>	Sanguine purple coneflower
<i>E. simulata</i>	Wavyleaf purple coneflower
<i>E. tennesseensis</i>	Tennessee coneflower

Native Indian Americans have traditionally used various *Echinacea* species to cure many ailments (Bauer, 1998). The knowledge of medicinal uses of *Echinacea* came from the Cheyenne, Dakota (Sioux, Oglala), Fox (Meskwaki), Kiowa, Crow, Omaha, Pawnee, Ponca, Teton, Winnebago, Choctow, Delaware and Comanche tribes, where they used them for wounds, burns, mumps, insect bites, toothache, neck pains, headaches, stomach cramps, coughs, chills, measles, gonorrhoea, snake bites and other poisoning (Bauer and Wagner 1991). It is believed that the Native American Indians used mainly *Echinacea angustifolia* because the areas where these tribes lived are in the range where *Echinacea angustifolia* is distributed, but the Native American Indians are also known to have used *Echinacea pallida* and *Echinacea purpurea* (Bauer and Wagner 1991).

Frederick Gronovius reported that European settlers were already using *Echinacea* in the 18<sup>th</sup> Century. In his report, *Flora Virginica*, plant number 417, he called *Echinacea purpurea* *Obeliscotheca barbulis pallide rubentibus*, and reported that *Echinacea* “possessed a sharp-tasting root and is very effective in the treatment of saddle sores on horses (Bauer and Wagner 1991).” However, the use of *Echinacea angustifolia* was not popularized to white settlers until a German man named H. C. F. Meyer who lived in Pawnee City, Nebraska, sold it as Meyer’s Blood Purifier and claimed to cure or reduce the effects of rheumatism, neuralgia, headache, toothache, erysipelas, dyspepsia, tumors, boils, wounds, vertigo, scrofula, bad eyes, rattlesnake bites and plant poisoning (Bauer and Wagner 1991), which is not too different from what the Native American Indian used it for.

In 1998, there were at least 800 drugs that contain *Echinacea* in Germany mainly, from the roots of *E. purpurea* and aerial parts of *E. pallida* and *E. purpurea*; while in the United States, *Echinacea* remedies are being sold in tinctures and in powdered form mainly from the roots (Bauer, 1998). According to a report done at the University of Montana in 1997, annual retail sales of *Echinacea* were about \$4 million dollars in the United States (Montana Native Plant Society). A more recent report in 2005 by MSNBC.com reports that annual *Echinacea* sales are \$300 million and in 1997 represented about 10% of the dietary supplement market (MayoClinic.com 1997).

*Echinacea* is primarily sold as a cold medicine. Oddly, the Native American Indians and H. C. F. Meyer, who sold *E. angustifolia* as “Meyer’s blood purifier,” apparently did not use *Echinacea* as cold medicine.

There is controversy on whether *Echinacea* is truly an effective medical treatment. There are several scientific articles reporting that there is no evidence on the healing effects of *Echinacea* when it comes to colds. A study done by Turner et al. (2005) concluded that *E. angustifolia* had no effect on the treatment of colds by using a Type 39 Rhinovirus to infect participants in their experiment. But, a meta-analysis done by Schoop et al. (2006), which includes the experiment by Turner et al. (2005), concluded that the overall results of experiments conducted by different scientists suggested that there was a 55% higher probability of getting a cold in the placebo treatment in comparison to when *Echinacea* was being taken. Another study by Goel et al. (2005), found that a propriety extract from *E. purpurea* enhances systemic immune response during a common cold by increasing the total white blood cells, monocytes, neutrophils,

and NK cells. Another report by Goel et al. (2004) concluded that *E. purpurea* lowered the symptoms of colds. Another contradiction arose in a study done by Schwarz et al. (2005) who reported that *Echinacea* did not have any effect on the common cold in their experiments.

However, there are many other scientific articles that reported on healing properties of *Echinacea* other than on colds. These healing properties found in *Echinacea* include anti-HIV by the inhibition of HIV Type I integration into the host genome *in vivo* and by the competitive inhibition of HIV-1 integrase *in vitro*, (Reinke et al. 2004); anti-viral through cytokine activity (Senchina et al. 2006); phagocytosis stimulatory activity (Bauer, 1998), anti-*Herpes simplex* virus (HSV-10) by inhibiting growth of HSV-10 when activated with light and UV-A light (Binns et al. 2002<sup>b</sup>); anti-inflammatory properties (Bauer, 1998; Facino et al 1993); adjuvant therapy for respiratory and urinary tract infections (Bauer, 1998); and immune stimulating properties as reported by Bauer in three separate experiments (Woelkart and Bauer 2007). Senchina et al. (2005) and Raduner et al. (2006), also found immune stimulating properties of *Echinacea* in separate studies by the modulation of pro-inflammatory cytokine TNF- $\alpha$  and anti-inflammatory cytokines IL-10 and IL-12; and by inhibition of LPS-induced inflammation respectively.

The difference in scientific findings may be due to the different species, varieties, accessions, growth conditions, age or plant parts of *Echinacea* used; methods of preparation; dosage; and target illness/vector in the study (Bauer 1998; Blumenthal and Farnsworth 2005; Leach 2005; Gray et al. 2003; Seemannova et al 2006, Binns et al 2002<sup>a</sup>; Binns et al. 2002<sup>c</sup>). Some scientific articles did not report the species or plant part

used, or worse, the wrong species used (Upton 2004). *Echinacea* is divided into 9 species, namely, *E. purpurea*, *E. angustifolia*, *E. pallida*, *E. tennesseensis*, *E. sanguinea*, *E. atrorubens*, *E. laevigata*, *E. simulate*, *E. paradoxa*, only the first three (3) are believed to have the greatest medicinal value (Bauer 1998; MayoClinic.com 2007).

Another possible difference in effectiveness of *Echinacea* preparations is due to formations of micelles in alkamides. According to Raduner et al. (2007), alkamides 11 and 9 form micelles in aqueous solutions at minimum concentrations of 200-300 nM and 7400-10000 nM respectively; and once these micelles are formed, they could no longer act as stimuli for cannabinoid type-2 receptor activation, which is believed to be related to immunomodulatory effects such as the inhibition of tumor necrosis factor alpha. As such, difference in concentration of alkamides as well as method of delivery, since micelles are not known to form in nonaqueous solutions (Raduner, 2007), could account for the difference in the effectiveness of *Echinacea* as herbal supplements.

Due to the growing popularity of herbal supplements like *Echinacea*, the United States government decided that it has to regulate and standardize the contents, discover the efficacy and determine the toxicity of these complementary alternative medicines (NCCAM 2007). Grants were given to several research centers that specialize in this area of research, including the Iowa State University Botanical Supplements Research Center (from now on referred to simply as Botanicals Center). This center is concentrating its research on two main herbal supplements, *Echinacea* and *Hypericum* (St. John's Wort), with *Prunella* being included in the grant renewal. This thesis will address some of the research done on *Echinacea* and not *Hypericum* and *Prunella*. The

author was given the responsibilities of extraction, fractionation and analysis of *Echinacea* and extraction of *Hypericum*.

## Chapter 2

### Literature review

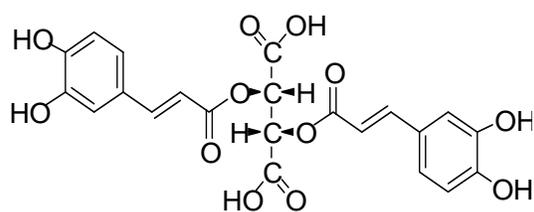
#### Chemistry of *Echinacea*:

The biochemicals in *Echinacea* species are composed mainly of glycoproteins, polysaccharides, phenolic caffeic acid derivatives, highly unsaturated fatty acyl amides commonly referred to as alkamides; and polyacetylenes, which are highly polyunsaturated ketones (Bauer, 1998). These compounds are believed to be the ones having medicinal properties as some of these compounds are characteristic of some members of the genus *Echinacea*. Figures 2-1 and 2-2 illustrate the chemical structures of the six caffeic acid derivatives, nineteen alkamides and seven polyacetylenes that have been detected by several researchers in the genus *Echinacea*. There are other caffeic acid derivatives that have been reported. Not all of these compounds are found in every species of *Echinacea* and not in every plant part either. Table 2-1 lists three of the most commonly used *Echinacea* species, namely *E. purpurea*, *E. pallida* and *E. angustifolia* with compounds that are known to be present in the roots of these species.

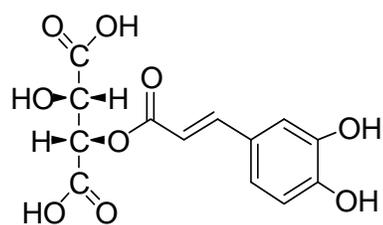
In addition, different *Echinacea* species may or may not contain any of the above mentioned compounds depending on the plant part, age and plant accession; however, species is the most important determining factor for composition. For example, Bauer (1998) reported that *E. purpurea* roots contain cichoric acid, dicaffeoyl tartaric acid and caftaric acid in significant quantities, while echinocside is not found in the roots; however, echinocside is found in tissue cultures of *E. purpurea*. They also reported that

*E. pallida* and *E. angustifolia* contain echinocside. *E. purpurea* contains alkamides, but no polyacetylene. On the other hand, some *E. pallida* contains polyacetylenes, but not alkamides. *E. purpurea* leaves may contain only some alkamides, while the roots contain a greater variety of alkamides (Bauer 1998). Overall, caffeic acid derivatives, alkamides and polyacetylenes are distributed unevenly in different parts of *Echinacea* plants (Bauer 1998).

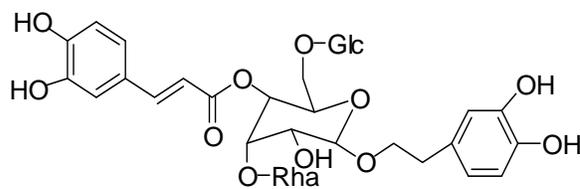
**Figure 2-1:** Structures of the caffeic acid derivatives found in the genus *Echinacea* (Pellati et al. 2005).



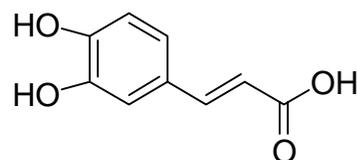
Cichoric acid  
(2,3-*O*-dicaffeoyl tartaric acid)



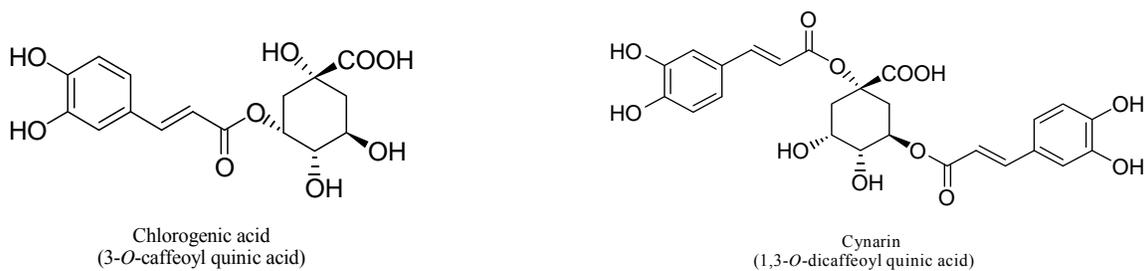
Caftaric acid  
(2-*O*-caffeoyl tartaric acid)



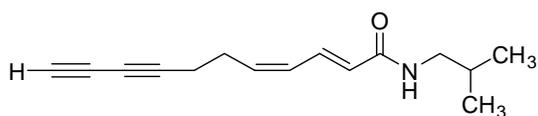
Echinacoside



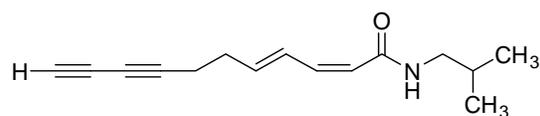
Caffeic acid



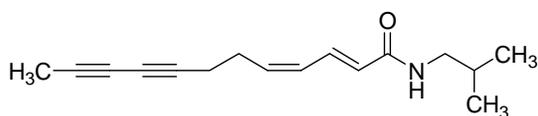
**Figure 2-2:** Structures of alkamides and polyacetylenes found in the genus *Echinacea*, following Bauer's naming system (Bauer, R. and Reminger, P. 1989).



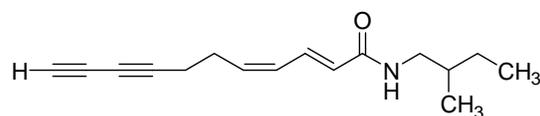
1 undeca-2*E*, 4*Z*-diene-8, 10-diynoic acid isobutylamide



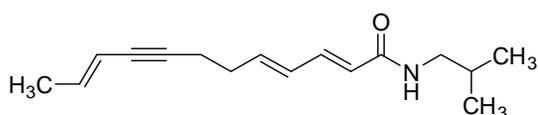
2 undeca-2*Z*, 4*E*-diene-8, 10-diynoic acid isobutylamide



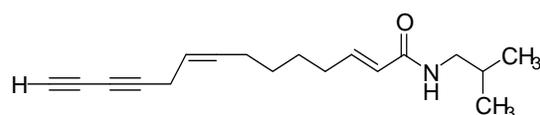
3 dodeca-2*E*, 4*Z*-diene-8, 10-diynoic acid isobutylamide



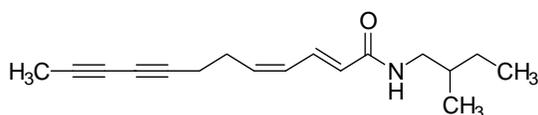
4 undeca-2*Z*, 4*E*-diene-8, 10-diynoic acid 2-methylbutylamide



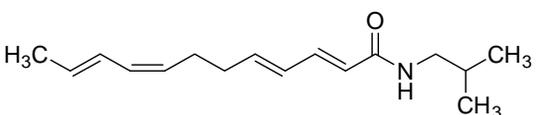
5 dodeca-2*E*, 4*E*, 10*E*-trien-8-ynoic acid isobutylamide



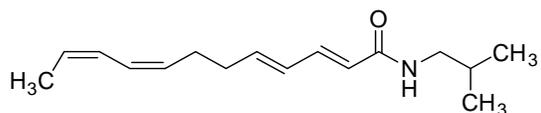
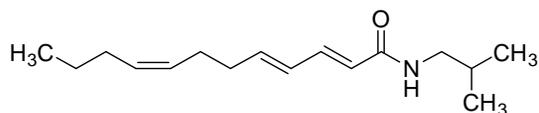
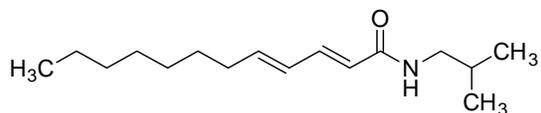
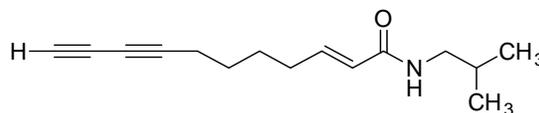
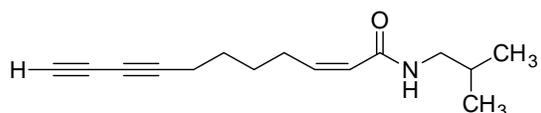
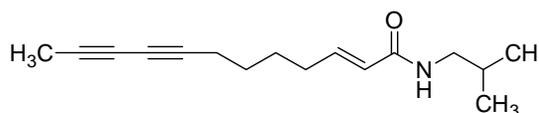
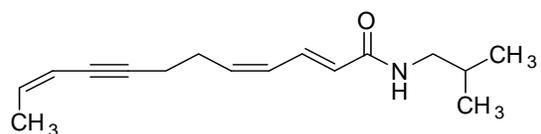
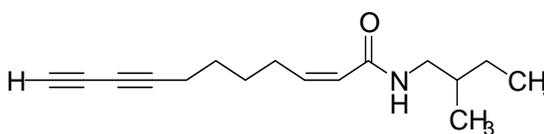
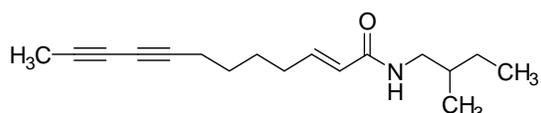
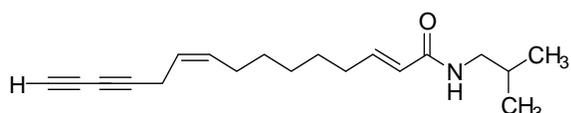
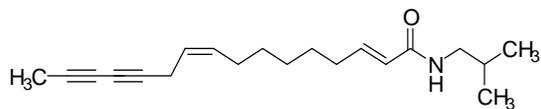
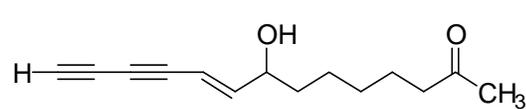
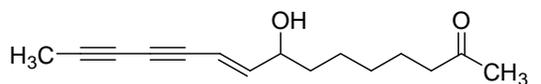
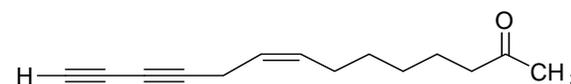
6 trideca-2*E*, 7*Z*-diene-10, 12-diynoic acid isobutylamide

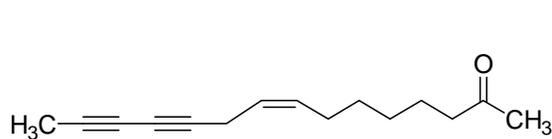


7 dodeca-2*E*, 4*E*-diene-8, 10-diynoic acid 2-methylbutylamide

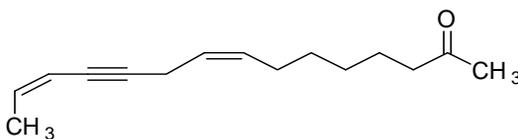


8 dodeca-2*E*, 4*E*, 8*Z*, 10*E*-tetraenoic acid isobutylamide

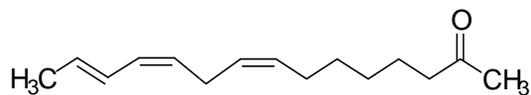
9 dodeca-2*E*, 4*E*, 8*Z*, 10*Z*-tetraenoic acid isobutylamide10 dodeca-2*E*, 4*E*, 8*Z*-trienoic acid isobutylamide11 dodeca-2*E*, 4*E*-dienoic acid isobutylamide12 undeca-2*E*-ene-8, 10-diynoic acid isobutylamide13 undeca-2*Z*-ene-8, 10-diynoic acid isobutylamide14 dodeca-2*E*-ene-8, 10-diynoic acid isobutylamide15 dodeca-2*E*, 4*Z*, 10*Z*-triene-8-ynoic acid isobutylamide16 undeca-2*Z*-ene-8, 10-diynoic acid 2-methylbutylamide17 dodeca-2*E*-ene-8, 10-diynoic acid 2-methylbutylamide18 pentadeca-2*E*, 9*Z*-diene-12, 14-diynoic acid isobutylamide19 hexadeca-2*E*, 9*Z*-diene-12, 14-diynoic acid isobutylamide20 8-hydroxytetradeca-9*E*-ene-11, 13-diyn-2-one21 8-hydroxypentadeca-9*E*-ene-11, 13-diyn-2-one22 tetradeca-8*Z*-ene-11, 13-diyn-2-one



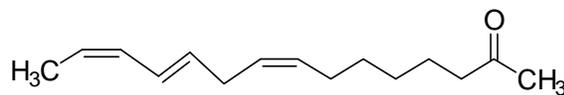
23 pentadeca-8Z-ene-11, 13-diyne-2-one



24 pentadeca-8Z, 13Z-diene-11-yn-2-one



25a pentadeca-8Z, 11Z, 13E-trien-2-one



25b pentadeca-8Z, 11E, 13Z-trien-2-one

**Table 2-1:** Constituents of root extracts of in different *Echinacea* species by different researchers in  $\mu\text{g/mL}$  of extract. An M means that at least one researcher has found the compound in the root portion of the *Echinacea* species and the content is minute.

Compound	<i>E. purpurea</i>	<i>E. angustifolia</i>	<i>E. pallida</i>
Chlorogenic Acid	0.0157	0.0137	0.0079
Caftaric Acid	0.1568		0.0535
Caffeic Acid	M		
Echinocside		0.0310	0.0885
Cynarin		0.0223	
Cichoric Acid	1.0147		0.0141
Alkamide 1	0.0456	M	
Alkamide 2	0.0159	0.0059	
Alkamide 3	0.0310	0.0059	
Alkamide 4	M	0.0059	
Alkamide 5	0.0156	0.0124	

Alkamide 6			
Alkamide 7	0.0092		
Alkamide 8	0.2912	0.2798	
Alkamide 9	0.0396	0.0343	
Alkamide 10	0.0102	0.0368	
Alkamide 11	0.0106	0.0486	
Alkamide 12		0.0371	
Alkamide 13		0.0449	
Alkamide 14		0.0141	
Alkamide 15		M	
Alkamide 16		M	
Alkamide 17		M	
Alkamide 18		M	
Alkamide 19		M	
Ketone 20			M
Ketone 21			M
Ketone 22			0.0540
Ketone 23			M
Ketone 24			0.1688
Ketone 25a			M
Ketone 25b			M

(Bauer et al. 1988<sup>a</sup> and <sup>b</sup>; Gotti et al. 2002; Binns et al. 2002<sup>a</sup> and <sup>c</sup>; Nigel et al. 2001; Pomponio, et al. 2001; Pietta et al. 1998 ; Senchina et al. 2006).

#### *Compounds with Medicinal Properties*

Some compounds found in *Echinacea* are confirmed or considered to have medicinal properties. For example, the caffeic acid derivative, L-cichoric acid has been reported in at least three different experiments to have in vitro anti-HIV activity by inhibiting HIV integrase from mediating integration of the HIV viral DNA into the host DNA (Reinke et al 2004, McDougall et al. 1998; Neamati et al.1997); while alkamides, polyacetylenes and glycoproteins are reported to have anti-inflammatory activity by inhibiting LPS-mediated activation of macrophage cells (Bauer, 1998; Chen et al 2005). The anti-inflammatory activity is important because prolonged inflammation is detrimental to normal development of the human body (Khovidhunkit et al. 2000). The hexane extract of eight species of *Echinacea*, which contains the alkamides and polyacetylenes, are reported to inhibit completely infectious *Herpes simplex* viral particles in vitro when exposed to visible and UV-A light (Binns et al. 2002<sup>b</sup>). Three different experiments by Bauer and his colleagues claimed that it is the alkamides that have immunological activities (Woelkart and Bauer, 2007).

According to the data presented in Table 2, in the root extracts, *E. pallida* do not contain alkamides, but does contain polyacetylenes. *E. angustifolia* contain the most numerous types of alkamides. *E. purpurea* contain almost all the same alkamides as *E. angustifolia*, except that *E. purpurea* do not contain alkamides with lambda max at 211 nm in the range of 190 to 600 nm as *E. angustifolia* does i.e. alkamides 12, 13 and 14.

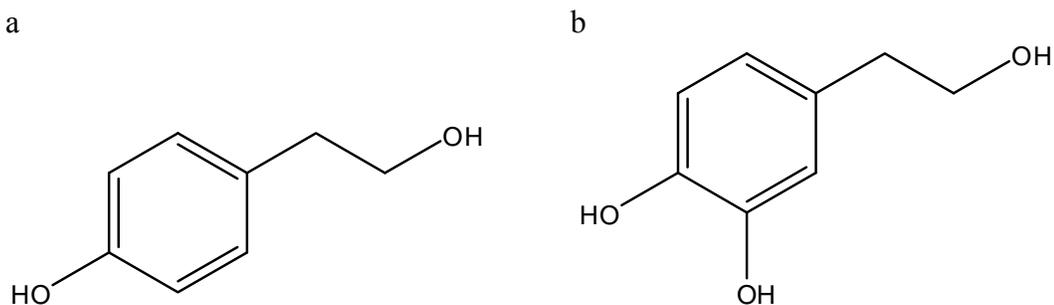
Bauer, et al. (1989) reported that diene moiety can result in maximum absorbance at 260 nm, while a monoene moiety can result in maximum absorbance at 211 and 119 nm in the UV spectra. The lambda max increases as the number of double bonds in conjugation increases, with a single conjugated double bond having lambda max of 217 nm; three double bonds in conjugation having lambda max at 258 nm and four double bonds in conjugation having a lambda max at 290 nm (McMurray 2004). Both *E. purpurea* and *E. angustifolia* alkamides contain diene moiety, however, only *E. angustifolia* alkamides contain monoene moiety and hence the reason why, alkamides with lambda max at 211 nm is not found in *E. purpurea*, but is found in *E. angustifolia*. Although some alkamides are found in *Echinacea angustifolia* and not found in *Echinacea purpurea*, there are more alkamides found in both *E. purpurea* and *E. angustifolia*; for example, both *E. purpurea* and *E. angustifolia* contain alkamides 8 and 9. According to Bauer, et al. (1989), *E. purpurea* roots contain 0.004-0.039% alkamides 8 and 9 while *E. angustifolia* contain more from about 0.009-0.151% (wt/v).

Other than plant species, another reason for the difference in composition in *Echinacea* is due to accession. Binns, et al. (2002<sup>a</sup>) found that *E. angustifolia* plants from different geographic regions do not all contain the same amount and types of both caffeic acid derivatives and alkamides, and concluded that the variety of the biochemicals within the same species is great as well as the possibility of inter-species hybridization, which would have made it difficult to determine the species by chemical analysis. For example, in 1 year old *Echinacea purpurea* roots, no echinocside was detected but in wild-harvested *Echinacea purpurea* roots, 0.10 mg of echinocside per gram dry weight was

found and in the roots of wild *Echinacea purpurea* transplants, 0.54 mg of echinocside per gram dry weight was found. In the roots of *Echinacea purpurea* transplants, caffeic acid was detected, but not in the wild-harvested and 1 year old roots of *Echinacea purpurea* (Binns et al. 2002<sup>c</sup>). Since there is a wide difference of these biochemical compounds within the same species, one can conclude that perhaps the best way to standardize herbal supplements is to analytically measure the active ingredient(s) for its specific use, rather than to claim purported medical benefits by species differentiation. In addition, according to Hudaïd et al. (2002), plant infections can cause a huge variability in the concentration of these compounds in *Echinacea*.

Caffeic acid derivatives have a phenol moiety in their structures. Phenols are known to act as anti-oxidants by localizing free radicals in their aromatic rings (Frankel 1998). However, phenolic compounds are not equivalent as anti-oxidants. For example, butylated hydroxyanisole (BHA) is not as effective an anti-oxidant compared with butylated hydroxytoluene (BHT), which in turn is not as effective an anti-oxidant in comparison with tertiary butylhydroquinone (TBHQ) (Frankel et al. 1994). A study done by Tsimidou et al. (1992) found that there was no correlation between the stability of olive oil and the phenolic compound, tyrosol (Figure 2-3). Previous work done by Papadopoulos and Boskou (1991) also found low anti-oxidant activity in tyrosol compared to BHT, a commonly used anti-oxidant in food preparations. Tsimidou et al. (1992), however, found a correlation between the ratio of hydroxytyrosol : tyrosol and the stability of olive oil.

Figure 2-3. <sup>a</sup>Tyrosol, the major phenolic compound in olive oil. <sup>b</sup>Hydroxytyrosol, another phenolic compound found in olive oil.



Since the phenolic compounds in *Echinacea* are believed to have anti-oxidant properties, it is important to know how strong an anti-oxidant they are. A study done by Pellati et al. (2004) found that caffeic acid derivatives in *Echinacea* had radical scavenging activity in vitro based on the reduction of an alcoholic 2,2-diphenyl-1-picrylhydrazyl (DPPH<sup>•</sup>) radical solution with cichoric having the greatest anti-oxidant activity followed by echinocside, cynarin, caffeic, chlorogenic then by caftaric, which is consistent with literature stating that the more aromatic rings with hydroxyl groups, the more radical scavenging activity it has. A biological type evaluation done by Facino et al. (1995) also reported the same order of antioxidant activity of the caffeic acid derivatives on the free radical-induced degradation of Type III collagen. In addition, a study by Dalby-Brown et al. (2005) also reported that cichoric acid, followed by echinocside then by caffeic acid, had the most antioxidant activity in *E. purpurea* extracts through the inhibition of Cu(II)-catalyzed oxidation of human low-density lipoprotein in vitro and had a synergistic effect with alkamides and polysaccharides. The study of Pellati et al. (2004) and Sloely et al. (2001) concluded that in *Echinacea* extracts, *E. purpurea* has the

most phenolics, followed by *E. pallida* then by *E. angustifolia*, which is also the order of the extract with the most radical scavenging activity.

A further study by Pellati et al. (2005) reported that the other species of *Echinacea* also show the same correlation with regards to phenolic contents and radical scavenging properties *in vitro* using DPPH· radical scavenging method. These caffeic acids derivatives, cichoric acid, echinocside, cynarin, caffeic, chlorogenic, and caftaric acid may act as anti-oxidant in the stability of the alkamides and polyacetylenes. Hu and Kitts (2000) also concluded that *Echinacea* root extracts have hydroxyl radical scavenging properties in a solution of hydrogen peroxide, EDTA and ascorbic acid in which deoxyribose was degraded by ·OH radical as well as anti-oxidant activity *in vitro* and are able to chelate transition metals.

Although caffeic acid derivatives are abundant in *Echinacea*, they are also commonly found in other plants. However, alkamides are characteristic of *Echinacea*, a member of the *Asteraceae* family. Some members of the plant families, *Rutaceae*, *Piperaceae* (peppers) and *Aristolochiaceae* also contain alkamides (Pengelly 2004), but not necessarily the same ones. Of the one hundred fifty (150) isolated alkamides, eighty (80) are from *Compositae* to which *Echinacea* belongs to (Bauer and Wagner, 1991).

These *Echinacea* alkamides have some unique properties. *Echinacea* alkamides are insecticides (Bauer and Wagner, 1991) and are also believed to be the main compounds responsible for some of the purported healing properties of *Echinacea*. For example, alkamides give a sharp sensation and cause saliva production in human tongues which is then followed by a numbing effect. This may be the reason why several plant

families that contain alkaloids are used to control toothaches and are known as “toothache plants” in their native lands (Pengelly 2004).

### **Stability of Biological Activity in Echinacea**

Since many herbal supplements including *Echinacea* have long expiration dates listed or none at all (Lee et al. 2004, Edwards and Draper 2003), it is important to know how long its purported medicinal benefits would last. A report done by Senchina et al. (2006) stated that after sixteen months of storage, *Echinacea* root extracts from 7 different species still contained significant quantities of alkaloids and caffeic acid derivatives and still had their immunostimulatory activities. Senchina et al. (2005) reported that it is likely that either or both alkaloids and caffeic acid derivatives may be responsible for the immunostimulatory effect.

However, another study performed by Senchina (McCann et al. 2007) in which they compared fresh extracts and the same extracts in ethanol tinctures stored for two years at -20°C, reported that six out of seven *Echinacea* species tested increased proliferation of IL-10, IL-12 and TNF- $\alpha$  after a 2-year storage, in contrast to the cytokine production, which was higher in the fresh extracts than in extracts stored for two years which meant that some activity was lost while some activity remained. Five of the seven 2 year old extracts also increased in IL-10 production compared to only two of the seven extracts tested when they were fresh. Their report also showed that *Echinacea* in alcoholic tinctures stored for two (2) yrs at -20°C still had significant amounts of alkaloids and polyacetylene ketones in them, sometimes, even greater than when they

were first extracted but was most likely due to the evaporation of the ethanol that was used as the solvent for storage. *E. purpurea*, *E. simulata* and *E. tennesseensis* extracts all showed increase in alkamide concentrations compared to when they were first extracted, while *E. angustifolia*, *E. simulata* and *E. sanguinea* extracts showed no changes in alkamide concentrations; however there was no correlation that could be drawn between the changes in alkamides and the changes in biological activity (McCann et al. 2007).

### **Stability of Alkamides and Caffeic Acid Derivatives in *Echinacea purpurea***

Since alkamides and caffeic acid derivatives may be responsible for some medicinal properties of *Echinacea* and *Echinacea* supplements may have a long or no expiration dates printed on the labels (Lee et al. 2004, Edwards and Draper 2003), it is important to find out if these highly unsaturated alkamides are stable, and if antioxidant caffeic acid derivatives help in the stability of these alkamides. Most of the research done so far is on *Echinacea purpurea*.

Dalby-Brown (2005) conducted a storage stability study that compared storage of *Echinacea purpurea* as dry root material and as alcoholic extracts stored in the dark at 8°C. The researcher found no degradation of cichoric acid and alkamides for at least 15 weeks in ethanol solution. But in dry powdered root form, Dalby-Brown (2005) reported that the alkamides degraded, but did not provide degradation rates or percent reduction or state which alkamides degraded, while cichoric acid degraded only if in contact with moisture. This is in line with what Perry et al. (2001) reported for their extraction procedure. Perry et al. (2001) reported that if water is added prior to ethanol during a

shaker-extraction procedure at room temperature, cichoric acid, cynarin, chlorogenic acid, echinocside and caftaric acid degraded by greater than 50% in water in a matter of minutes, but suggested that it might have been due to enzymatic reactions, in which the enzymes degraded the caffeic acid derivatives whereby o-quinones are formed when the ethanol content was less than 70%. These enzymes are reported to be polyphenol oxidases that require oxygen, but these enzymes can be inactivated by some processing as evident in consistently high cichoric acid content of some *Echinacea* preparations (Nusslein et al 2000). Nusslein et al. (2000) found that cold pressed *Echinacea* juices, which do not inactivate enzymes, are devoid of cichoric acid, which is consistent with the findings of Perry et al. (2001) and Dalby-Brown (2005). Another study by Li and Wardle (2001) found that an increase in the drying temperature (45°C) over 3-5 days of *E. purpurea* freshly harvested roots also increased the cichoric acid content, which is consistent with polyphenol oxidase inactivation at higher temperatures. Wills and Stuart (2000) also reported that lowered moisture content of roots or blanching be done in order stabilize cichoric acid contents before storage at 5, 20, and 30°C, in order to denature PPO. When cichoric acid is hydrolyzed by polyphenol oxidase, it can form caftaric acid and caffeic acid (Nusslein et al. 2000). See Figure 2-1 for structures of caffeic acid derivatives. This is probably the reason why caffeic acid detected in *E. purpurea* is not uncommon as Pietta et al. (1998) hypothesized that caffeic acid may be a degradation product but may also possibly be a difference in the variety of *Echinacea purpurea* plants.

Nusslein et al. (2000) reported that in *Echinacea purpurea* aqueous extracts in which PPO had been inactivated, an increase in caffeic acid was observed. They hypothesized that it may be a different enzyme - an esterase - that was not inactivated in the condition that inactivated PPO. This enzyme then catalyzed the hydrolysis of caftaric acid into caffeic acid and tartaric acid. Caftaric acid is caffeic acid esterified with tartaric acid. Wills and Stuart (2000) reported that alkalamide levels in *E. purpurea* increased in their experiment in which they intentionally damaged the harvested plant materials. The reason for this increase is unclear.

Since *Echinacea* can be processed in several ways, the caffeic acid derivatives and alkalamides may vary according to the process.

#### *During Drying of Echinacea Roots*

Drying of whole *Echinacea purpurea* roots did not cause loss of alkalamides (Perry et al. 2000). Drying of whole *Echinacea purpurea* roots at different temperatures (40 to 70°C) did not result in a decrease in alkalamide levels either, however, cichoric acid decreased when an increase in drying temperature was done (Stuart and Wills 2003). This is contradictory to the report of Li and Wardle (2001) in which they found that an increase in drying temperature of freshly harvested roots caused an increase in the cichoric acid content.

#### *During Storage of Echinacea Roots*

Wills and Stuart (2000) did not report any loss of alkalamides or cichoric acid when air-dried whole *E. purpurea* plant materials at 12% moisture were stored in ambient conditions for 60 days. They also observed that alkalamides in air-dried (<12% moisture)

mechanically crushed (not powdered) *E. purpurea* roots did not decrease when stored at 5°C in the dark for 60 days. This is consistent with the findings of Dalby-Brown (2005) in which no decrease in alkaloid levels were found when pulverized (powdered) freeze-dried *E. purpurea* root powder was stored at 8°C for 16 weeks. But when Wills and Stuart (2000) stored air-dried (8-12% moisture) mechanically crushed (not powdered) *E. purpurea* at 20°C and 30°C, a rapid degradation of alkaloids was observed, especially when held in visible light. This is not surprising since light is known to cause photo-oxidation of double bonds in the presence of sensitizers (Frankel 1998). The difference between the reports of Dalby-Brown (2005) and Wills and Stuart (2000) may be due to water content which caused the activation of enzymes that are released during crushing of the roots (8-12% moisture) which may cause degradation in the report of Wills and Stuart (2000); but in the report of Dalby-Brown (2005), there was no water (0% moisture) available for enzymatic reactions to occur after freeze-drying. Perry et al. (2000) found similar results in which *E. purpurea* plant materials (7% moisture) stored at 24°C had degradation of alkaloids by over 80% in 64 weeks and plant materials that were stored at -18°C had degradation of alkaloids by 40% in 64 weeks. In the case of Perry et al. (2000), none of their processing, in which they dried the plant materials at 30-32°C for 64 hours, could have caused inactivation of enzymes in *Echinacea*, which may have caused the degradation of some compounds by enzyme that were still active. A report by Rogers et al. (1998) stated that powdered *Echinacea angustifolia* roots stored at 25°C for 8 weeks caused alkaloid levels to decrease by 13%.

*During Storage of Echinacea Aqueous Ethanol Extracts*

Cech et al. (2005) detected caftaric acid, cichoric acid and alkamide 2 at 0.7, 0.71 and 2.0 mg/ml, respectively, in 18 month old alcohol tinctures of *E. purpurea* stored at room temperature. They also detected alkamides 1, 4, 7, 8, 9, 10, and 11 (as shown in Figure 2-2), but were unable to quantify them because standards were not available; however, the initial concentrations of these compounds are unknown. In any case, it can be implied that the alkamides have great stability since after 18 month of storage, they are still detectable. A study by Livesay et al. (1999) confirmed that *E. purpurea* extracts in aqueous ethanol did not result in significant reduction of alkamides at -20, 25 and 40°C for 7 months, but cichoric acid did decline at 25 and 40°C with the higher temperature resulting in greater degradation of cichoric acid. This suggests that alkamides are very stable in ethanol tinctures. In the dry form, the alkamides concentration decreased as temperature increased from -20, 25 and 40°C, but cichoric acid did not decrease during storage for 7 months (Livesey et al. 1999). This suggests that the alkamides were oxidizing in the powdered form and the cichoric acid was unable to act as an anti-oxidant in a dry system.

*Accelerated Shelf-Life Test on Alkamides in E. purpurea aqueous ethanol extract*

A 10-day accelerated shelf-life test (ASLT) was done by Liu and Murphy (2006) with *E. purpurea* extracts. Accelerated shelf-life test is a method of determining shelf-life at room temperature without having to wait for degradation at room temperature. This is done by storing the subject of interest at accelerated temperatures and using the Arrhenius plot to extrapolate shelf life at another temperature, i.e. room temperature.

The results from the experiments done by Liu and Murphy (2006) indicated that alkaloids were very stable in their model systems. Their hypotheses were: 1. the alkaloids degrade or oxidize faster when in dry film since there is more surface area for oxygen to interact with the alkaloids in comparison to alkaloids dissolved in dimethyl sulfoxide (DMSO); and 2. the phenolic caffeic acid derivatives in *Echinacea purpurea* 95% ethanol Soxhlet extracts act as anti-oxidants and protect the alkaloids from oxidation. Their study had a total of 4 conditions: 1. extract that was dissolved in dimethyl sulfoxide (DMSO) with all the caffeic acid derivatives intact after extraction (DMSO Rich); 2. extract that was dissolved in DMSO but with all the caffeic acid derivatives removed (DMSO Poor); 3. extract that was dried (Dry) with all the caffeic acid derivatives intact after extraction (Dry Rich); and 4. extract that was dried with all the caffeic acid derivatives removed (Dry Poor). Their results showed that all the alkaloids followed an apparent 1<sup>st</sup> order degradation reaction mechanism in all conditions, with half-lives at room temperature in Dry in the thousands of days, while in DMSO, it was even longer. The low energies of activation in dry poor and dry rich were the same which suggested that temperature did not significantly affect the degradation and also that the oxidation mechanism was the same for both conditions. The low energies of activation also implied rapid reaction taking place. Their results showed that the alkaloids are more stable in DMSO than in dry, which supported their first hypothesis. Their results also showed that alkaloids in Dry Poor degraded faster than in Dry Rich, which supported their second hypothesis that phenolic acids act as antioxidants and prevent alkaloid from degradation. However, when the extract was dissolved in

DMSO, the opposite is true, alkamides in DMSO Poor were more stable than alkamides in DMSO Rich. They were not able to give an explanation for this, but proposed a hypothesis which stated that pro-oxidants that may have been present in the *Echinacea purpurea* extracts may have different mobility in dry and DMSO solutions, and these pro-oxidants were not removed when the phenolic acids were removed.

Liu and Murphy (2006) did not find any significant 3 way interaction between phenolics, temperature and alkamide in their 3-way ANOVA test for the degradation constant (k) in the Dry. This meant that an overall ranking of the k values of the alkamides was the same for both Dry Rich and Dry Poor. Liu and Murphy (2006) had mislabeled alkamide 4 and referred to it as alkamide 6; which is now referred to as alkamide 4 throughout this thesis. The mislabeling was confirmed with the findings of our colleagues here at the Iowa State University Botanicals Supplement Research Center (Senchina et al. 2006, Kraus et al. 2006, Wu et al. 2004). Liu and Murphy (2006) reported that the degradation rate constants (k) in Dry for alkamides 1, 2, 4 and 9 were not statistically different from each other and were higher than the k for alkamide 8; with the exception that alkamides 8 and 9 were not statistically different from each other; followed by k's for alkamides 3, 5, 7, with the exception that alkamides 8, 3, and 5 were not statistically different from each other. In DMSO, Liu and Murphy (2006) did not find significant degradation of the alkamides in DMSO Poor. The degradation rates in DMSO Rich had the order: alkamides  $1 \approx 2 \approx 4 \approx 8 \approx 9 > 3 \approx 5 \approx 7$ , except for alkamides 1, 7 and 8 which were not significantly different from each other. What this means is that alkamides had more or less the same ranking in Dry and in DMSO.

However, Liu and Murphy (2006) wrote that their standard errors were too huge in some instances to get values with smaller standard errors and this is due to the short incubation period of only 10 days when some of the alkaloids did not degrade significantly. In addition, the small number of half-lives reached and the analytical precision being low in such cases due to detection of microscopic amounts, the standard error becomes too huge to obtain more significant results. Therefore a longer accelerated shelf life test should be done, preferably one that is long enough for the alkaloids to go through 3 half-lives for better standard errors.

In summary, enzymes and moisture play an important role in the stability of compounds found in *Echinacea*. These enzymes, which can be denatured upon sufficient heating or adding sufficient ethanol, mainly act on the phenolic caffeic acid derivatives which as a result, provide less anti-oxidant capacity to protect alkaloid from oxidation. A proper preparation of *Echinacea* for medicinal purpose to enhance the shelf-life of the caffeic acid derivatives and alkaloids should be examined.

### **Accelerated Shelf-Life Testing:**

Accelerated shelf-life test (ASLT) can be used to determine the shelf-life of foods, chemicals and pharmaceuticals. The more accurate way of determining the shelf-life of a product is for a researcher to store the product at its intended storage condition and monitor its degradation. However, since manufactured goods cannot be allowed to sit in storage while the scientist determines the shelf-life due to economic reasons, accelerated shelf-life tests were developed to compensate the long storage tests (Labuza

and Schmidl 1985; Saguy and Karel 1980; Taoukis and Labuza 1996, Labuza and Riboh, 1982).

ASLT is usually done by keeping the product at three (3) different temperatures to simulate an abuse. Although more than 3 experimental storage accelerated temperatures can be used, the better the shelf-life prediction will be, but the consequences associated with having more than 3 experimental temperatures mean more oven, more experimental and analysis time, and therefore greater monetary cost may be prohibitive (Labuza and Riboh, 1982). Other parameters in ASLT modeling that can be changed are: oxygen pressure, moisture level and any combinations of these parameters (Saguy and Karel 1980; Labuza 2000, 1984). Factors that may affect prediction of shelf-life at lower temperatures are analytical errors, phase changes, reactants concentrating at freezing temperatures, carbohydrate crystallization, multiple reactions which masks one over another reactions at different temperatures, protein/enzyme denaturation, microbial death, gas/oxygen solubility, pH changes, moisture loss, and water activity (Labuza 2000, 1984). Most foods constituents undergo apparent zero order (frozen foods overall quality, Maillard browning) or apparent first order reactions (vitamin loss, oxidative color loss, microbial growth and inactivation) (Saguy and Karel 1980), which is similar with what Liu and Murphy found (2006).

The parameters that can be obtained from performing an accelerated shelf life experiments are the degradation rate constant  $k$  at experimental temperature, the extrapolated  $k$ , shelf life, energy of activation, enthalpy of activation ( $\Delta H^\ddagger$ ), entropy of

activation ( $\Delta S^\ddagger$ ) and free energy of activation ( $\Delta G^\ddagger$ ) of the degradation (Labuza and Riboh 1982, Kirk 1981).

#### *Accelerated Shelf Life Test and Stability of Lipids*

Since the alkamides found in *Echinacea purpurea* are highly unsaturated fatty acyl isobutyl amides or 2-methylbutylamides, with multiple ethylene and/or acetylene bonds, these alkamides have very similar chemical structures as unsaturated fatty acids found in foods. Accelerated shelf-life test (ASLT) has been conducted on oils and other oily compounds in the past. Lipid oxidation has been reported to follow a zero-order reaction in the case of lipid oxidation in potato chips (Labuza and Bergquist 1983), lipid oxidation in very pure lipids is found to be half-order (Labuza 1971), lipids with phenolics anti-oxidant may be first order and may also be zero order. In pure lipids systems, Ragnarsson and Labuza (1977) and Ragnarsson et al. (1977) found that ASLT done based on higher than room temperatures to predict the degradation rate constant and therefore shelf-life fits the typical Arrhenius equation, which is the equation used to predict chemical reactions. This means that ASLT is a reliable way for predicting shelf-life (Labuza and Bergquist 1983). Energies of activation ( $E_a$ ) reported for lipid oxidation were in the range of 41-100 kJ/mol (Labuza 1971). Food systems without antioxidants usually have an  $E_a$  of around 46-54 kJ/mol; while food systems with added antioxidants, the energy of activation increases to 84-92 kJ/mol (Ragnarsson et al. 1977). What this means is that even at very low temperature, oxidation of lipids cannot be completely halted since such low energies are typically readily available. Labuza and Bergquist (1983) found that the lipid oxidation of potato chips with anti-oxidants had an  $E_a$  of  $60 \pm$

9 kJ/mol, which is more typical of a system without antioxidants. They hypothesized that the added anti-oxidants, BHA and BHT, in these potato chips prior to frying, may have volatilized off during frying.

### **Autoxidation**

There are two ways for oxidation of lipid to occur. These are the autoxidation and the photo-oxidation mechanism (Frankel 1998). The more likely oxidation that occurs in alkamide degradation is autoxidation since there is no UV source to catalyze the formation of singlet oxygen from triplet oxygen. For the autoxidation mechanism, a free radical must be formed. There are 3 steps in autoxidation: initiation, propagation and termination (Frankel 1998). The following explanations of these steps are taken from Frankel (1998), unless noted otherwise.

#### *Initiation*

According to Frankel (1998), during initiation, there are 2 ways in which free radicals are formed: (1) is in the presence of an initiator (I) and a free radical (R·) which abstract a hydrogen from unsaturated lipid ( $\text{LH} \xrightarrow{\text{I}, \text{R}\cdot} \text{IH} + \text{L}\cdot$ ) and (2) triplet state oxygen is converted to biradical singlet oxygen by a radical formation through 3 possible mechanisms. These three mechanisms are: 1. the thermal decomposition of naturally present hydroperoxides ( $\text{LOOH} \rightarrow \text{LO}\cdot + \cdot\text{OH}$ ), 2. the decomposition of hydroperoxides catalyzed by metals with variable valences (a.  $\text{LOOH} + \text{M}^{2+} \rightarrow \text{LO}\cdot + \text{OH}^- + \text{M}^{3+}$  or b.  $\text{LOOH} + \text{M}^{3+} \rightarrow \text{LOO}\cdot + \text{H}^+ + \text{M}^{2+}$ ) and 3. light reacting with a sensitizer such as ketone. The excited state ketone then reacts with triplet oxygen to form singlet oxygen,

which can act abstract hydrogen from unsaturated lipids, forming an alkyl radical on the lipid. This process is called initiation.

### *Propagation*

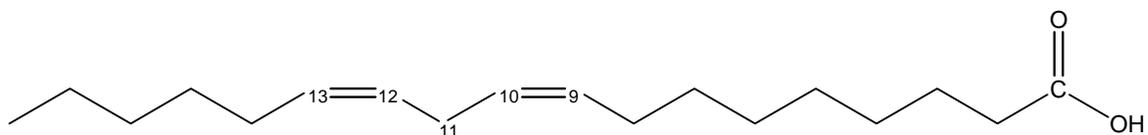
The lipid alkyl radical on the allylic carbon ( $L\cdot$ ) then reacts with molecular oxygen ( $O_2$ ) and forms peroxy radicals ( $LOO\cdot$ ) which in turn reacts with an unsaturated lipid to form hydroperoxide ( $L\cdot + O_2 \rightarrow LOO\cdot \rightarrow LOO\cdot + LH \rightarrow LOOH + L\cdot$ ). This second step is slow and so hydrogen is abstracted selectively from the most weakly bonded allylic hydrogen. The free radical on the lipid resonates between the 3 carbon atoms where the double bonds were and the alkyl carbon. Oxygen attacks the free radical on either end of the 3 carbon atoms and form peroxy radicals which in turn can react with other molecules propagating radical formation.

### *Termination*

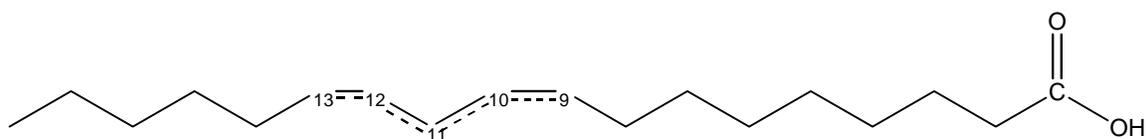
When the peroxy radicals react with one another, non-radical products are formed; at 1 atmosphere, non-radical products are formed through a tetroxide intermediate ( $LOO\cdot + LOO\cdot \rightarrow [LOOOOL] \rightarrow$  non-radical product). Another way for termination to occur is simply the reaction of any 2 radicals forming a stable molecule (Frankel 1998).

### *Dismutation*

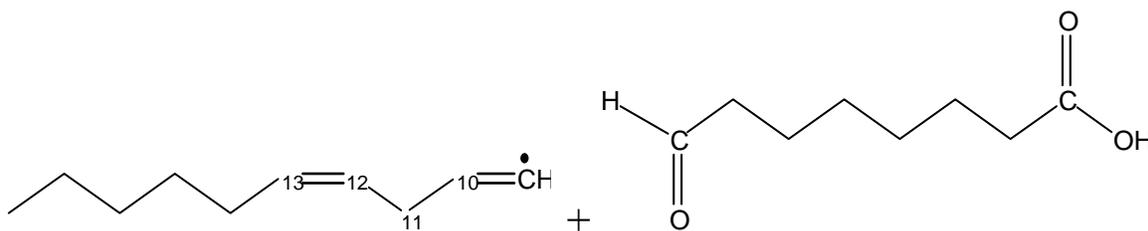
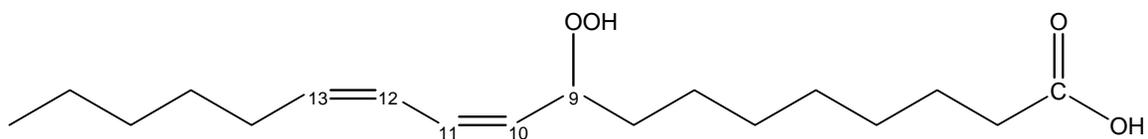
During propagation, dismutation can occur. This is when the free radical ( $RO\cdot$ ) reacts with another lipid thereby forming another radical and a non-radical such as ketones, aldehydes and alcohols. These non-radicals can result in the perceptible sensory rancidity of oils (Hamilton 1994, Frankel 1998).

*Auto-oxidation mechanism of linoleic acid (Frankel 1998)*

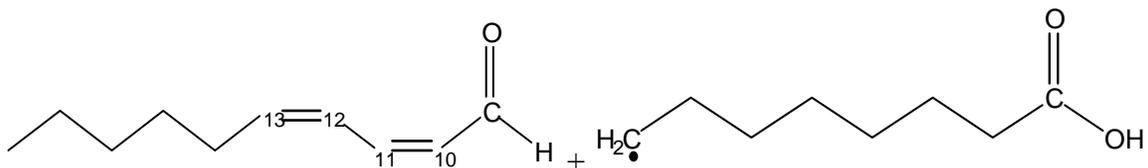
H· is abstracted from the allylic carbon in between the 2 double bonds and the free radical electron can move around forming a hybrid.



Of the five carbons that share an electron is attacked by oxygen. Carbon 9, 11 and 13 are the carbon that are usually attacked by oxygen, forming 3 possible products. The diagrams below shows the products when carbon 9 is attacked.



OR



## Photooxidation

Electromagnetic radiation can react with sensitizers such as chlorophyll, myoglobin, riboflavin, metals, and erythrosine to form singlet oxygen in photo-oxidation (Hamilton 1994). Singlet oxygen, which is 1,500 times faster in reaction than the normal state of triplet oxygen, can then react with unsaturated lipids and form hydroperoxide. The exact mechanism of which is unclear, but with no free radical mechanism in the initial stage (Hamilton 1994, Frankel 1998). Frankel (1998) explained that ultraviolet light "...catalyzes the decomposition of hydroperoxides (ROOH) and other compounds such as peroxides (ROOR), carbonyl compounds (RCOR) or other oxygen complexes of unsaturated lipids." This is followed by the free radical reactions in autoxidation (Frankel 1998). There are two types of photo-oxidation: Type I and Type II, which is based on the 2 types of sensitizers, both of which are not inhibited by anti-oxidants of free radical oxidation (Frankel 1998), but can be inhibited in whole or in part by other competitive inhibitors such as tetramethylethylene, tetraphenylcyclopentadiene, diphenylisobenzofuran and beta-carotene (Rawls and Santen 1970). Type I sensitizers in triplet state react with lipids by abstraction of hydrogen atom or electron transfer thereby forming radicals, which can react with oxygen, while type II sensitizers also in the triplet state reacts with oxygen via energy transfer forming singlet oxygen which reacts further

with unsaturated lipids; both of which can be inhibited by the reaction of carotenoids such as beta-carotene and alpha-tocopherol which quench the singlet oxygen and by forming stable addition products, however beta-carotene is highly sensitive to autoxidation and therefore must be protected by anti-oxidants like delta and gamma-tocopherols, otherwise beta-carotene will act as a pro-oxidant (Frankel 1998). The other difference between photo-oxidation and free radical autoxidation is the by-products formed; the distribution of the hydroperoxides formed are different between photo-oxidation and autoxidation (Frankel 1998). Free radical oxidation does not produce nonconjugated hydroperoxides, but singlet oxygen oxidation does (Rawls and Santen 1970).

It is known that the more unsaturated a lipid is, the more it is prone to oxidation. Linolenic acid reacts 100 times faster than oleic acid and linoleic acid reacts 64 times faster than oleic acid (Allen and Hamilton 1994). This means that the highly polyunsaturated alkamides found in *Echinacea purpurea* should be very prone to oxidation and more prone to oxidation compared to fatty acids, which is contrary to the report of Liu and Murphy (2006).

### **Lipid Oxidation**

Lipid oxidation can occur in two ways – autoxidation and singlet oxygen oxidation (Frankel 1998).

In the autoxidation of methyl oleate, Frankel et al. (1977a) reported that under heat, methyl oleate autoxidized primarily into 8 and 11- hydroperoxides than 9 and 10-

hydroperoxides, which is consistent with the most accepted mechanism for oleate autoxidation in which hydrogen abstraction occurs at the 8 and 11 positions. The unequal distribution of the four hydroperoxides were proposed to be due to allylic rearrangements of hydroperoxides (Frankeel et al. 1977a). Frankel et al. (1977a) further reported that minor products were also form in the autoxidation of methyl oleate and these are allylic enones, epoxy-octadecanoates (8,9-, 9,10-, and 10,11-), dihydroxyoctadecenoates (8,9-, 9,10-, and 10,11-), and dihydroxyoctadecanoates (8,9- 8,10-, 8,11-, 9,10-, 9,11-, 10,11-), in which they proposed that these minor products were the results of secondary reactions.

In the autoxidation of methyl linoleate, formation of equal amounts of 9- and 13-hydroperoxides were reported by Frankel et al. (1977b), which is consistent with hydrogen abstraction at the allylic carbon 11 forming a pentadiene with carbons 9 and 13 becoming susceptible to oxygen attack. Minor products of methyl linoleate autoxidation include keto dienes, epoxyhydroxy monoenes, di- and tri-hydroxy monoenes. Frankel et al. (1977b) further reported that in mixtures of oleate and linoleate autoxidation experiments, linoleate autoxidized faster as comparison to the formation of 13-hydroxyperoxides to the 9-hydroperoxides formed since 13-hydroperoxides are only from the degradation of linoleate and not oleate. At about a mixture of 90% oleate and 10% linoleate, which is when amounts of 9-hydroxyperoxides formed from the degradation of linoleate and oleate equally. This suggests a 1:10 autoxidation rate between oleate and linoleate.

Experiment by Frankel et al. (1977c) on the autoxidation of linolenate yielded 75-81% 9- and 16-hydroperoxides, with the remainder as 12- and 13-hydroperoxides.

Frankel et al. (1977c) reported that latter two products underwent secondary reactions that resulted in cyclic peroxides, cyclic peroxide-hydroperoxides and prostaglandin-like endoperoxides, which would cause the consumption and therefore lower concentrations of 12- and 13-hydroperoxides. Frankel et al. (1977c) discovered this after formation of 9,10,12- and 13,15,16-trihydroxyoctadecanoate when highly oxidized linolenate was hydrogenated. The formation of these secondary products from the autoxidation of linolenate, may result in a greater hydroperoxide formation by linoleate than by linolenate at lower peroxide values (Frankel 1977c). On the autoxidation of soybean oil, Frankel and Neff (1979) also reported the same, that higher hydroperoxides from linoleate than from linolenate were found and again was more likely due to the secondary reactions that hydroperoxides from linolenate underwent, which inevitably decreased their amounts.

This suggests that alkamide oxidation would also most likely result in the formation of several secondary products since there are more unsaturated bonds in *Echinacea* alkamides and that direct monitoring of alkamide rather than monitoring of degradation products, since secondary reactions could possibly diminish or increase the amounts of primary degradation products, would be more appropriate to determine shelf-life of alkamides.

Evolution of 12-hydroperoxide in the autoxidation of methyl soybean oil (Frankel and Neff 1979) and also in methyl cottonseed, safflower and cottonseed oils (Frankel et al. 1979) were unexpectedly high. The formation of high amounts of 12-hydroperoxide is most likely due to photoxidation of linoleate as the latter three oils do not contain

appreciable amounts of linolenate (Frankel et al. 1979) and the same product was unexpectedly high in comparison to the linolenate concentration in soybean oil esters (Frankel and Neff 1979). This suggests that at low levels of oxidation, singlet oxygen oxidation may be predominant over autoxidation (Frankel et al. 1979).

In singlet oxygen oxidation, methyl oleate produced equal amounts of 9 and 10-hydroperoxides (Frankel et al. 1979), in comparison to the primarily autoxidation products 8 and 11-hydroperoxides with lower levels of 9 and 10-hydroperoxides (Frankel et al. 1977a). In methyl linoleate, singlet oxygen oxidation products were 66% 9- and 13-hydroperoxides and 34% 10- and 12-hydroperoxides (Frankel et al. 1979), while its autoxidation products were 9- and 13-hydroperoxides (Frankel et al. 1977b). In methyl linolenate, singlet oxygen oxidation products were 75% 9-, 12-, 13- and 16-hydroperoxides and 25% 10- and 15-hydroperoxides (Frankel et al. 1979), while its autoxidation products were 75-81% 9- and 16-hydroperoxides, with the balance being 12- and 13-hydroperoxides Frankel et al. (1977c). Further experiment by Frankel et al. (1981) found that when autoxidized, products from singlet oxygen oxidation of methyl oleate resulted in positional isomerization into autoxidation products of methyl oleate to give all four hydroperoxides found in autoxidation of methyl oleate; while those of methyl linoleate and linolenate also resulted in positional isomerization and changed the compositional amounts of oxidation products.

In the experiments done by Frankel et al. (1980) on  $\Omega$ -3 fatty acid methyl esters, they found that hydroperoxide formation was more prevalent in C-16 and C-17 than C-14 and C-15 of 9,15-octadecadienoate, while in 12,15-octadecadienoate, in which

hydroperoxide formation is expected to occur at positions C-12 and C-16, C-16 was more prone to hydroperoxide formation, which means that double bonds at the  $\Omega$ -3 position was more prone to autoxidation than double bonds that are more internalized.

From the experiments done by Gunstone and Hilditch (1945), it was found that methyl oleate autoxidation reached a peroxide value of 500 at 20°C in 25 days, at 50°C in 15.8 days, at 80°C in 1.54 days, at 90°C in 0.15 days (9 hours) and at 120°C in 0.04 days (2.5 hours); with rates of increase in peroxide values of 1.2, 2.9, 28, 110, 330, and 450 units per hour respectively of temperatures mentioned at the linear portion of peroxide increase, discounting the induction period. Although the peroxide value is not directly proportion to the degradation of oleate with one of the reasons being that at moderate peroxide values the peroxides may have degraded (N. P. Analytical Laboratories 2007), there is no better way to approximate the degradation of oleate. I did not come across any literature that measured directly the disappearance of oleate. For simplicity, we will assume that the peroxide value is directly proportional to oleate degradation with a 1:1 reaction, this would give oxidation rate of 0.8%, 2%, 19%, 74% and 223% of oleate oxidized per day at 20, 50, 80, 100 and 120°C respectively based on Gunstone and Hilditch's (1945) experiment. As can be seen here, oleate is very prone to autoxidation especially at high temperatures. This is equivalent to about 99%, 98%, 81%, 26% and below 0% oleate remaining per day at respective temperatures.

Gunstone and Hilditch (1945) also reported that at higher temperatures of 100 and 120°C, methyl oleate autoxidation resulted in formation of not just 8 and 11-hydroperoxides as what they observed in autoxidation of methyl oleate at 20°C, but also

of 9 and 10- hydroperoxides, which they concluded that must have been a direct oxygen attack on the double bonds.

From Gunstone and Hilditch's (1945) experiment, linoleate autoxidation would have been 91%, 41% and less than 0% remaining linoleate per day at 20, 50 and 80°C respectively, assuming that one hydroperoxide would form per linoleate before the second double bond of linoleate would form hydroperoxide.

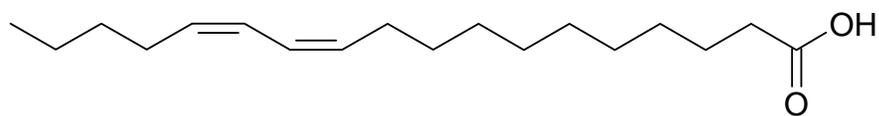
### **Stability Studies on Biodiesel.**

Methyl esters are mainly used as biodiesel. They are formed by reacting triglycerides with methanol. Just like food lipids, methyl esters are prone to oxidation. A study was done by Du Plessis et al. (1985) on methyl and ethyl esters of sunflower seed oil, which is rich in linoleic acid, reported that methyl and ethyl esters are just as prone to oxidation as lipids, but the induction period is only 1/3 that of sunflower oil. Du Plessis et al. (1985) also reported that ethyl esters are more unstable than methyl esters, but did not provide an explanation for it. The addition of antioxidant *tert*-butylhydroquinone (TBHQ) increased stability of biodiesel at 20 and 30°C but at 50°C TBHQ no longer protected biodiesel from oxidizing (Du Plessis et al. 1985). Mittelbach and Gangl (2001) also found that biodiesel made from rapeseed oil and used frying oil was unstable under thermal oxidative stability test and peroxide value test, but their induction period increased when anti-oxidants were present and oxidative stability decreased in presence of light. The stability of methyl esters can be compared with that of alkamides since both have similar structures.

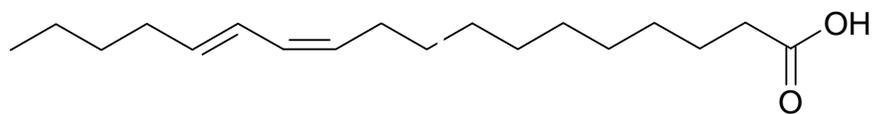
### Stability Studies on Conjugated Linoleic Acid

Zhang and Chen (1997) reported that conjugated linoleic acid (CLA) is more unstable than non-conjugated linoleic acid. Zhang and Chen (1997) also reported that CLA, which has 2 conjugated double bonds, are more unstable than linoleic acid, which has 3 double bonds; and arachidonic acid, which has 4 double bonds; but is just as unstable as docosahexaenoic acid (DHA) which has 6 double bonds that are not conjugated. This implies that conjugation imparts instability to oxidation. Yang et al. (2000) found that more than 80% of CLA oxidized within 110 hours in air at 50°C. Among 16 *cis-trans* isomers (see Figure 2-4) of CLA's, Yang et al. (2000) reported that the four *cis-cis*-CLA was most unstable followed by the four *cis-trans* CLA's then by the four *trans-trans*-CLA, which was relatively stable compared to linoleic acid, the reason that Yang et al. (2000) gave was that *cis*-double bonds are chemically less stable than *trans*-double bonds because *cis*-double bond has a higher free energy level and more vulnerable to oxygen attack. Yurawecz et al. (1995) reported that the oxidation products of conjugated octadecadienoic acid are furan fatty acids. These results suggest that the conjugated double bonds are less stable than non-conjugated double bonds and that *cis-trans* isomerism are important in the stability of lipids. A thermal stability/illumination stability study by Chen et al. (2001) with methyl CLA, methyl linoleate, methyl oleate and methyl stearate found them to be least stable in that order.

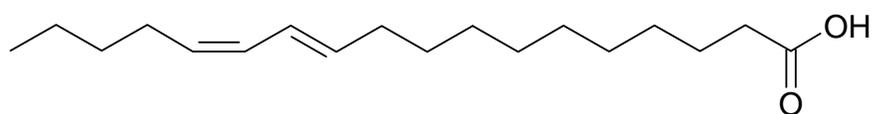
Figure 2-4. Chemical structure of 16 cis-trans isomers of CLA.



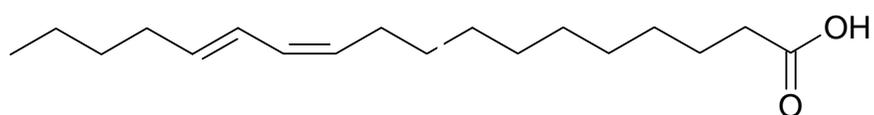
octadeca-11Z,13Z-dienoic acid



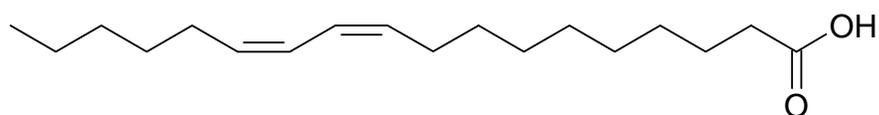
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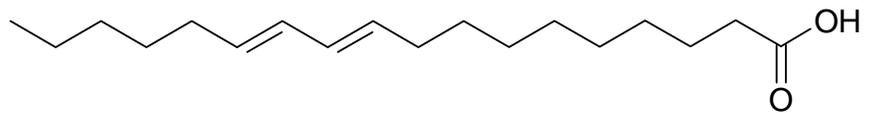
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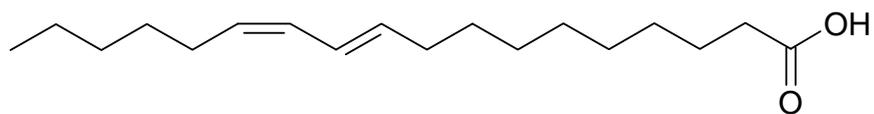
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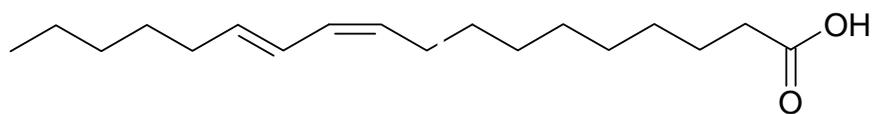
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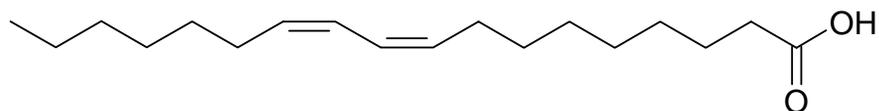
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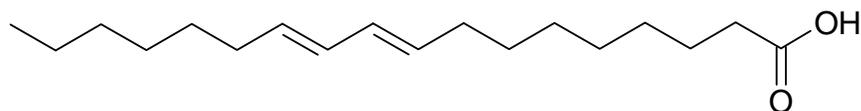
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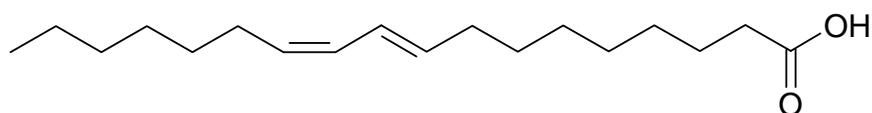
octadeca-10Z-12E-dienoic acid



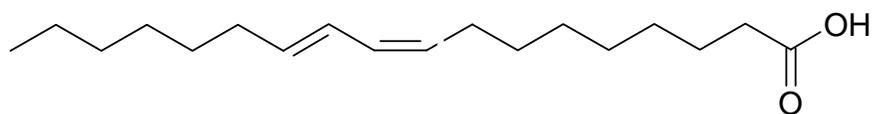
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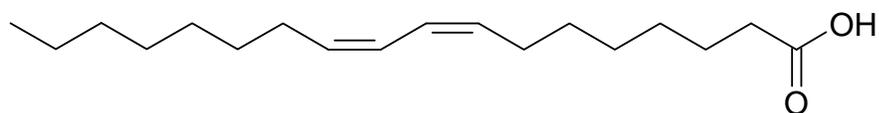
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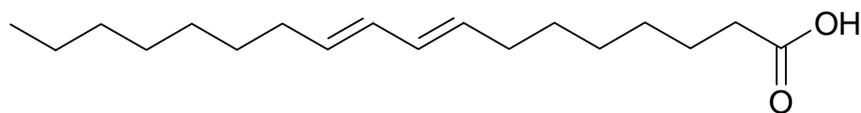
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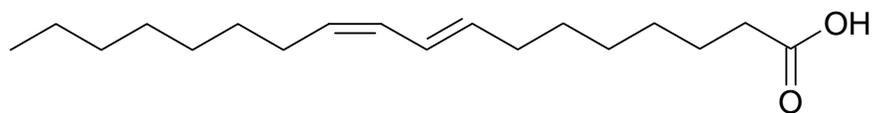
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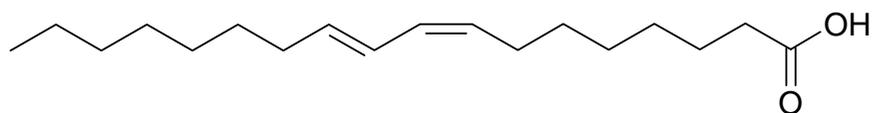
octadeca-8Z,10Z-dienoic acid



octadeca-8E,10E-dienoic acid



octadeca-8E-10Z-dienoic acid



octadeca-8Z-10E-dienoic acid

Since all the alkamides in *Echinacea purpurea* are highly conjugated, it would be a good idea to compare the stability of these to the CLA's mentioned above, and perhaps a correlation can be drawn. According to the experiment of Liu and Murphy (2006), alkamide 9, which has 2 *cis*-bonds and 2 *trans*-bond, was more prone, although not statistically significant which is perhaps due to the number of replications, to oxidation than alkamide 8, which has 1 *cis*-bonds and 3-*trans*-bond, which is supported by the report of Chen et al. (2001) in which they concluded that more *cis*-bonds results in greater oxidation.

### **Stability of Conjugated Acetylene Bonds**

*E. purpurea* alkamides contain several conjugated acetylene bonds, alkamides 1, 2, 3, 4 and 7, it would be interesting to know if conjugation in diacetylenes is the cause for the very high stability of alkamides found in *Echinacea* as found by Liu and Murphy (2006). Rogers et al. (2004) found that conjugated polyacetylenes do not have greater stability compared to non-conjugated polyacetylenes, which is contrary to conjugated dienes which are more stable than their non-conjugated counterparts; which is opposite from the results of Yang et al. (2000) and Liu and Murphy (2006). Rogers et al. (2004) also reported that terminal acetylene bonds are less stable than internal ones in their G3(MP2) calculations.

### **Stability of Terminal Double and Triple Bonds**

Cumpston and Jensen (1998), found that poly(phenylene acetylene), which has a terminal acetylene bond is more stable to photo-oxidation than poly(phenylene vinylene) (PPV) which has a terminal ethylene bond. Perhaps it is the terminal acetylene bonds that add stability to some alkamides in comparison to terminal ethylene bonds. In addition, Cumpston and Jensen (1998) reported that terminal ethylene bonds are less stable than internal ethylene bonds. Perhaps this is the reason why Liu and Murphy (2006) found that generally alkamides 8 and 9, both of which have terminal methyl groups are more stable than alkamides 1, 2 and 4, in dry storage with and without phenolic acids all of which have terminal acetylene bonds. Cumpston and Jensen (1998) further reported that an additional electron-withdrawing moiety added to terminal ethylene bonds causes greater stability, the reason for this is because singlet oxygen is very electrophilic which is attracted to the large electron density around the ethylene terminal. The addition of the an electron-withdrawing moiety to the terminal ethylene group disperses that electron density, causing more stability; another way of protecting the ethylene terminal bond from singlet oxygen is by protecting the terminal ethylene bond with a bulky group to sterically block singlet oxygen from attacking, which is what the terminal methyl group in alkamides 8 and 9 may have done. Perhaps it can be deduced from the report of Cumpston and Jensen (1998) that methylated acetylene bonds are more stable than methylated ethylene bonds, which support the results from the experiment of Liu and Murphy (2006) in which they reported alkamides 3 and 7, both of which have methyl

capped acetylene bonds, were more stable than alkamides 8 and 9, both of which have methyl capped ethylene bonds, in Dry Rich and Dry Poor.

### **Stability of Hydrocarbons with Nitrile Moiety**

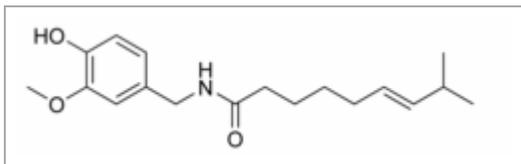
The other main difference between food lipids and *E. purpurea* alkamides are that the alkamides have amides attached to them. It would be interesting to know if such a moiety can increase stability. Cumpston and Jensen's (1998) research showed that addition of a nitrile group to vinylene moiety in PPV made it more stable to singlet oxygen (Cumpston and Jensen 1998), which could suggest that the nitrogen, however even when not in nitrile form, in alkamides may be increasing the stability of alkamides. Bauer, et al. (1988) report that *E. pallida* polyacetylenes, which did not contain amides, degraded significantly when stored in a few days (2-8) at room temperature in root powder form. This suggests that perhaps the amide portion of the *Echinacea purpurea* alkamides give them extraordinary stability since the only main difference structurally between the *Echinacea purpurea* alkamides and the *Echinacea pallida* polyacetylenes is the presence of amide in *Echinacea purpurea* alkamides.

### **Stability of Other Alkamides not found in Echinacea**

Capsaicin, 8-Methyl-N-vanillyl-trans-6-nonenamide (See Figure 2-5), is an alkamide responsible for the hotness in some peppers (The Chile Pepper Institute 2007), which is reported to have anti-prostate cancer properties by inducing apoptosis of cancer cells (Mori et al. 2006). Kopec et al. (2002) reported capsaicin when stored at 4°C

protected from light at higher concentrations of 4.0-128  $\mu\text{M}$  solutions were stable for 1 year, with little or no degradation; however, if the store at lower concentrations of 0.5 – 2.0  $\mu\text{M}$  at the same condition, a 40% reduction was observed. Capsaicin stored at room temperature exposed to light had a much greater degradation of about 90% after 1 year, but when stored at room temperature protected from light a reduction of 80% was observed; and when stored frozen at  $-20^{\circ}\text{C}$  protected from light, capsaicin degraded by 15% after 1 year. (Kopec et al. 2002). This suggests that alkamides are very stable as the experiment of Liu and Murphy (2006) also reported, light can play a role in the degradation of alkamides, most likely causing photooxidation and freezing does not necessarily preserve the chemical integrity of alkamides.

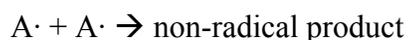
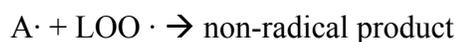
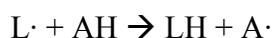
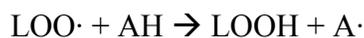
Figure 2-5. Chemical structure of capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide)



### Effects of Phenolic Antioxidants on Food Lipid Oxidation

Phenolic acids are usually natural present in foods. Artificial phenolic acid can also be added in foods. Different phenolic acids have different anti-oxidant capacities. Usually, phenolic compounds with electron withdrawing moiety in the *ortho* and *para* positions are more effective than those in *meta* position (Frankel 1998). According to Frankel (1998), they usually “...inhibit or retard lipid oxidation by interfering with either

chain propagation or initiation by readily donating hydrogen atoms to lipid peroxy radicals.” Reactions, include (Frankel 1998):



Polyphenols are antioxidants and naturally present in food oil, but are removed if the oil is refined. Virgin oils, such as virgin olive oil, retain these polyphenols since they are not refined. Gutierrez et al. (2001) reported that about 50% of the stability in virgin olive oil is contributed by polyphenols particularly hydroxytyrosol and oleuropein aglucone.

Overall, there is a wide range of articles relating to the medical efficacies or absence of efficacies in *Echinacea*. In the stability of the alkamides, most papers do agree that alkamides are relatively stable, however, there are a few papers that contradicts this.

The objective of the experiment of this thesis is to test the stability of the alkamides in *Echinacea purpurea* root aqueous ethanol Soxhlet extracts. This is accomplished through the modeling of an accelerated shelf-life test on the alkamides through temperature abuse. Similar to the experiment of Liu and Murphy (2006), but with 3 half-lives instead of 10 days pass the starting concentrations of the alkamides monitored using HPLC through the measurement of peak heights, *Echinacea purpurea* root extracts will be dried with and without the anti-oxidant caffeic acid derivatives and another set of extracts will be dissolved in DMSO also with and without the caffeic acid

derivatives and stored at accelerated temperatures. The result should give a better estimate of the half-lives at room temperature (25°C) of the alkamides.

## Chapter 3

### Materials and Methods

#### Materials:

##### *Plant Materials*

For the accelerated shelf-life study, only *Echinacea purpurea* roots were used. Plant materials were provided by Dr. Kathleen Delate and Fredy Romero, Core A, Iowa State University Botanical Supplements Research Center; and by Dr. Mark Widrlechner and Joe-Ann McCoy of the USDA North Central Region Plant Introduction Station in Ames, Iowa. All plant materials were stored in -20°C under nitrogen in zip-lock bags prior to use.

The *Echinacea purpurea* plant materials used were all dried root powders. They were previously washed with cold running water to remove the dirt and soil then completely dried at 40°C forced air conditions, followed by grinding through a 40-mesh screen Wiley grinder by Fredy Romero prior to delivery to the Murphy Lab (Romero 2006).

The *Echinacea purpurea* plant material used for the accelerated shelf-life test belonged to the accession “Johnny’s Seeds”, harvested on Oct 28, 2005 with ID numbers EPURNA102805KDE017, EPURNA102805KDE019, EPURNA102805KDE020, EPURNA102805KDE021, EPURNA102805KDE022, EPURNA102805KDE022,

EPURNA102805KDE023, EPURNA102805KDE024, EPURNA102805KDE026, EPURNA102805KDE027, EPURNA102805KDE028, EPURNA102805KDE029, EPURNA102805KDE029, EPURNA102805KDE030, EPURNA102805KDE033, EPURNA102805KDE034, EPURNA102805KDE034, EPURNA102805KDE035, EPURNA102805KDE037, EPURNA102805KDE039, EPURNA102805KDE039, EPURNA102805KDE040, EPURNA102805KDE041, EPURNA102805KDE042, EPURNA102805KDE043, EPURNA102805KDE044, EPURNA102805KDE045, EPURNA102805KDE087, EPURNA102805KDE092, and EPURNA102805KDE093.

#### *Chemical Reagents*

Reagents used were glacial acetic acid ( $\text{CH}_3\text{COOH}$ ) certified A.C.S. PLUS; acetonitrile –ACN- ( $\text{CH}_3\text{CN}$ ) HPLC Grade; chloroform ( $\text{CHCl}_3$ ) HPLC Grade; dimethyl sulfoxide - DMSO – ( $\text{CH}_3$ )<sub>2</sub>SO certified A.C.S.; hexanes HPLC grade; methanol ( $\text{CH}_3\text{OH}$ ) HPLC Grade; Nochromix (proprietary peroxygen oxidizer, Godax Labs. Inc., Takoma Park, MD) used as directed for cleaning of glassware; o-Phosphoric Acid (85%) ( $\text{H}_3\text{PO}_4$ ) NF/FCC; Sulfuric Acid ( $\text{H}_2\text{SO}_4$ ) certified A.C.S. PLUS; and trifluoroacetic Acid - TFA – ( $\text{C}_2\text{HF}_3\text{O}_2$ ) reagent grade were purchased from Fisher Scientific (Fair Lawn, NJ) through Iowa State University Chemistry Stores (Ames, IA). Ethanol ( $\text{CH}_3\text{CH}_2\text{OH}$ ) 100% (200 Proof) was purchased from Iowa State University Chemistry Stores (Ames, Iowa).

#### *Chemical Standards*

Six (6) caffeic acid derivatives found in *Echinacea* genus were purchased: synthetic chlorogenic acid and caffeic acid (Sigma-Aldrich, Inc. St. Louis, MO.), isolated

caftaric acid, echinococside, cynarin, and cichoric acid from *Echinacea* spp. (Chromadex Santa Ana, CA). Five (5) alkamides (undeca-2*Z*, 4*E*-diene-8, 10-diynoic acid isobutylamide (alkamide 2); dodeca-2*E*, 4*E*, 8*Z*, 10*E*-tetraenoic acid isobutylamide (alkamides 8); dodeca-2*E*, 4*E*, 8*Z*-trienoic acid isobutylamide (alkamides 10); dodeca-2*E*, 4*E*-dienoic acid isobutylamide (alkamides 11) and dodeca-2*E*-ene-8, 10-diynoic acid isobutylamide (alkamide 14)), and four (4) polyacetylene (8-hydroxytetradeca-9*E*-ene-11, 13-diyne-2-one (ketone 20); 8-hydroxypentadeca-9*E*-ene-11, 13-diyne-2-one (ketone 21); tetradeca-8*Z*-ene-11, 13-diyne-2-one (ketone 22); and pentadeca-8*Z*-ene-11, 13-diyne-2-one (ketone 23)) were provided by Dr. George Kraus and Jaehoon Bae (Project 2, Iowa State University Botanical Supplement Research Center). All standards were used as external standards to determine relative retention times and used for spiking of samples for confirmation of peak identification. The standards were also used for the creation of the UV spectral library for confirmation of peak ID through the similarity of the UV spectra.

All caffeic acid derivative standards and standard solutions dissolved in DMSO were stored before and after use in desiccators at -20°C. All alkamides and polyacetylene standards in methanol as provided were stored before and after use in -80°C under nitrogen in solution.

#### *Water*

Culligan system deionized distilled filtered water maintained by Department of Food Science and Human Nutrition at Iowa State University (Ames, IA) underwent

another purification through the Milli-Q water filtration system (0.45  $\mu\text{m}$ ) as maintained by the laboratory technician Cathy Hauck (Millipore Co., Bedford, MA).

### *Equipment*

Buchi rotovaps model R-144, R-110, R-111 and R-200 (Switzerland) with water bath set at 30°C for evaporation of 95% aqueous ethanol and 100% ethanol extracts. No heat was applied when chloroform, hexane or methanol solvents were evaporated.

A Labconco freeze-drier model number 7750020 (Kansas City, Mo) set at automatic, which is -50°C and 0.120nBar were used for freeze-drying of pre-frozen samples. Dryness was confirmed by the disappearance of both ice and of coldness on the flasks.

Standard ovens (Precision Scientific, Aloha, OR; Fisher Scientific, Fair Lawn, NJ; and NAPCO) were used for the stability study with a thermometer immersed in mineral oil through a rubber stopper placed inside. This is used for temperature taking at sampling days.

### *Analytical HPLC System:*

The system used by Liu and Murphy (2006), which has same model used by Kraus et al. (2006), were used by the author. All of the following parts are from Beckman Coulter (Fullerton, CA): System Gold 126 Pump module; System Gold 508 autosampler; System Gold 168 detector (photodiode array); and 32 Karat Software version 5.0.

The columns used were YMC-PACK ODS-AM 250mm length \*4.6mm I.D. s-5 $\mu\text{m}$ , 12nm, reversed-phase C<sub>18</sub> columns.

### *Other Supplies*

HPLC 2 mL glass vials with Teflon and red rubber septa covers from National Scientific, Fisher Scientific (Fair Lawn, NJ) were used as sample vials for HPLC analysis as well as for storage vials for the accelerated shelf-life testing on *Echinacea purpurea*. HPLC 13 mm and 25 mm 0.45 µm PTFE hydrophobic filters from VWR International (West Chester, PA) were used to filter samples prior to placing inside the vials for HPLC analysis. Whatman cellulose extraction thimbles of 43\*123 mm (England, Britain) were used to contain the plant materials inside the Soxhlet which was topped with Pyrex glass wool from Corning (Corning, NY) to prevent the powdered plant materials from floating out of the extraction thimbles. Syringe of 1 and 3 mL sizes from Becton Dickinson (Franklin Lakes, NJ) and from VWR International (West Chester, PA). Silica cartridge PrepSep™ Solid Phase Extraction Product Silica 10 g /60 mL from Fisher Scientific (Fair Lawn, NJ) was used for the preparation of phenolics-poor Echinacea extracts.

## **Methods:**

### *Preparation of vials for use in ASLT*

A special cleaning procedure to ensure removal of metals and organic molecules that may interfere in our studies was done on the HPLC vials for use in storage of samples in the ovens. This cleaning procedure entailed rinsing of the vials with distilled water, followed by submersion in Nochromix oxidizing solution formulated as the directed on the package, followed by another rinsing with distilled water then oven dried. The lids and septa were left as is as they were not used in the stability storage.

### *Extraction Procedure of Echinacea Plant Materials*

All plant materials were extracted using Soxhlet apparatus for at least 6 hours for exhaustive extraction, following the protocol created by Liu (2006). Solvents used for Echinacea extractions were 95% ethanol. A maximum of about 50 g of plant material was used for each extraction. All extracts were stored at -20°C. A total of about 1.3 kg of dried powdered *E. purpurea* roots was used. After the extraction of *Echinacea purpurea* the 95% aqueous ethanol solvent was removed using a Rotavap to obtain the dried extract weight.

#### *Preparation of phenolic rich samples*

For the DMSO Rich treatment, after extraction and drying, the dried extracts were redissolved in DMSO in an amount depending on what showed up in the HPLC chromatograms. They were redissolved in the minimum amount of DMSO to have a maximum peak of 2000 mAU, then placed in pre-marked HPLC vials for storage at experimental temperatures of 90, 100 and 100°C in the ovens.

For the Dry Rich treatment, after extraction and drying, the dried Echinacea purpurea extracts were redissolved in methanol in an amount depending on what showed up in the HPLC chromatograms. They were redissolved in the minimum amount of methanol to have a maximum peak of 2000 mAU then transferred in 2 ml HPLC vials at 1.5 mL per vial.

#### *Drying of Echinacea Extracts in 2 mL HPLC Vials*

Drying of Echinacea purpurea extracts in 2mL vials can be tedious and extremely time consuming. Since a centrifuge evaporator that can hold 100 test-tubes at a time was not available, an improvised apparatus had to be created. This apparatus is a desiccator in

which a hole is attached to a rotavap in order to dry several smaller 2 mL HPLC vials, at the same time. After this is done, the samples were then stored at experimental temperatures of 70, 80 and 90°C.

#### *Preparation of Phenolics-Poor samples*

A method modified from that used by Liu and Murphy (2006) for the preparation of phenolics-poor was employed. After extraction of *Echinacea purpurea* roots for at least 6 hours and the solvent removed using a Rotavap, the extract was redissolved in water and hexane (1:1). The flask containing the extract was then stored at -20°C overnight to minimize any possible degradation. Work was continued the next day. This has the added effect of freezing the water phase, thereby making the top portion- the hexane phase – which remained in liquid phase, easily separated from the frozen water phase. The hexane phase was then quickly poured off once the flask is removed from the freezer, while the water phase remains frozen.

The Prep-Sep silica cartridge was pre-treated with chloroform then the hexane phase that contained the alkaloids was poured inside the cartridge. The alkaloids were then eluted with chloroform. To facilitate elution, vacuum by water aspiration was used. The chloroform was then evaporated using a Rotavap, leaving dry residue in the flask.

For the DMSO Poor treatment, the dry residue was then redissolved in DMSO in an amount depending on what showed up in the HPLC chromatograms. They were redissolved in the minimum amount of methanol to have a maximum peak of 2000 mAU and transferred in to marked 2 ml HPLC vials at 1.5 mL per vial for storage at experimental temperatures of 90, 100 and 110°C in the ovens.

For the Dry Poor treatment, the dry residue was then redissolved in hexane in an amount depending on what showed up in the HPLC chromatograms. They were redissolved in the minimum amount of methanol to have a maximum peak of 2000 mAU and transferred in to 2 ml HPLC vials. They were dried using the same procedure and apparatus as the samples for the Dry Rich treatment. After which, the samples were then stored at experimental temperatures of 70, 80 and 90°C in the ovens.

#### *Preparation of phenolics rich purpurea extract*

The phenolics-rich *Echinacea purpurea* extract was prepared in the same manner as the usual regular extraction mentioned in the “Extraction Procedure” described above . After extraction, the solvent was evaporated using the Rotavap. For storage in DMSO, the extracts were redissolved in DMSO then transferred and put to mark on pre-marked 2 mL HPLC vials. Each vial had approximately 1.2 mg of extract.

### **HPLC Analysis**

#### *Sample preparation for HPLC analysis of Echinacea purpurea*

Samples taken from the ovens for HPLC analysis were filtered using hydrophobic 0.45 um PTFE 13mm filters prior to analysis in the HPLC system.

#### *Preparation of Mobile Phases for HPLC*

For the 3 HPLC methods: alkamide, caffeic and comprehensive; 3 kinds of mobile phases were used; with each method having 2 mobile phases. Each method used acetonitrile, the more hydrophobic mobile phase, for Pump B. As for Pump A, alkamide method used 100% Milli-Q water that was degassed using a water aspirator until no more bubbles were visible in the water which took approximately 2 hours; and both caffeic and

comprehensive methods used 0.1% (85%) o-phosphoric acid, which was eventually replaced with 0.1% acetic acid due to the crystals formed by phosphoric acid which shortens the life of the column by building up blockage. Both acids were made by volume in a volumetric flask for accuracy and degassed using a water aspirator.

#### *Analytical HPLC methods*

The method used to detect alkamides (from now on referred to as the alkamide method) were the same as employed by Liu and Murphy (2006), Kraus et al. (2006), Senchina et al. (2006) and Wu et al. (2004). The conditions are described below. The method used to detect caffeic acid derivatives (from now referred to as the caffeic method) was similar to Senchina et al. (2006); and Liu and Murphy (2006). The conditions for HPLC are described below. Senchina et al. (2006) used formic acid instead of phosphoric acid. Liu and Murphy (2006) had a flow rate of 1.5 ml/min instead of 1.0 ml/min.

A third method was developed to detect both caffeic acid derivatives and alkamides in a single run (from now referred to as the comprehensive method), which is similar to the caffeic method with an extended time to allow the alkamides to elute.

#### *Alkamide HPLC Analysis Method:*

The mobile phase for the alkamide method was degassed water for Pump A and acetonitrile for Pump B. The flow rate was at 1.0 mL/min. Injection volume per sample was 10  $\mu$ l. UV Scan was collected in the range of 190-600 nm. Echinacea alkamides were viewed at 260 nm.

## Time Program:

Time (min)	Module	Function	Value	Duration
0	Pump	%B	40	0
0	Pump	%B	80	45
45	Det 168	Stop Data		
46	Pump	%B	40	2
50	Det 168	Alarm		0

The time program is read as follows: at time 0 min, Pump B is pumping 40% of the volume (while Pump A pumps 60% of the volume). This lasts for 0 min. At time 0, Pump B starts pumping to reach 80% (while Pump A deduced to 20%) and it takes Pump B a duration of 40 minutes to go from 40% to 80%. At 45 min, the computer stops taking data. At time 46 min, Pump B starts going back down to 40% (while Pump A goes to 60%) and it takes 2 min to do this. The system continues pumping at 40% B then it reaches 50min when the alarm stops. This means from time 48-50, there is a steady 40% B Pumping.

*Caffeic acids HPLC Analysis Method:*

The mobile phase used for the caffeic method were 0.1% (85%) phosphoric acid, which was eventually replaced with 0.1% acetic acid due to crystallization which blocks the column for Pump A; and acetonitrile while for Pump B. The flow rate was at 1.0 mL/min. Injection volume was at 15  $\mu$ L. UV Scan was collected in the range of 190-600 nm. Caffeic acid derivatives were viewed at 330 nm.

## Time Program:

Time (min)	Module	Function	Value	Duration
0	Pump	%B	10	0
0	Pump	%B	22	13
13	Pump	%B	40	1
14	Pump	%B	40	7
21	Pump	%B	10	0.5
21.5	Pump	%B	10	12.5
34	Det 168	Stop Data		
34	Det 168	Alarm		

*Comprehensive Method:*

The mobile phase used for the comprehensive method were, for Pump A, 0.1% (85%) phosphoric acid, which was eventually replaced with 0.1% acetic acid due to crystallization of phosphoric acid which blocked the column; while for Pump B, acetonitrile. The flow rate was at 1.0 mL/min. The injection volume was at 10  $\mu$ l. UV scan was collected in the range of 190-600 nm. Alkamides were viewed at 260 nm, while caffeic acid derivatives were viewed at 330 nm.

## Time Program:

Time (min)	Module	Function	Value	Duration
0	Pump	%B	10	0
0	Pump	%B	40	30
30	Pump	%B	50	2
32	Pump	%B	85	40
72	Pump	%B	100	2
76	Pump	%B	10	2
80	Det 168	Stop Data		

At the end of the sequence of runs, methanol was run through the column in order to minimize loss of column performance by washing out very hydrophobic material. Perry et al. (2000) also found that flushing with methanol also extends the life of the column.

Identification of peaks was made possible with the collaboration of Senchina et al. (2006), Kraus et al. (2006), Wu et al. (2004) and Liu and Murphy (2006), who also worked on the same project at the Iowa State University Botanical Supplement Center. Peak identification was based on relative retention time, similarity of UV spectra, internal standards, spiking and chromatogram fingerprinting. Individual standards were run to check for relative retention times and UV spectra. *Echinacea purpurea* sample with identified peaks was also run to obtain the finger print of the chromatogram, the relative

retention times of the alkamides and caffeic acid derivatives, and to obtain the UV spectra of identified compounds.

### **Statistics**

Statistical analysis was done using the statistical software SAS 9.1 (Cary, NC). Experiments were carried out in triplicates, with each condition and temperature done three times. In order to determine the rate order of the degradation of the alkamides in *Echinacea purpurea* extracts, regression analyses were conducted on the zero, 1<sup>st</sup> and 2<sup>nd</sup> reaction orders using PROC REG. For zero order, peak height was regressed against time, for 1<sup>st</sup> order, the natural logarithm of peak height was regressed against time, and for the 2<sup>nd</sup> order, the reciprocal of peak height was regressed against time. After the best fit rate order was determined based on the R<sup>2</sup> values. 3-way analysis of variance (ANOVA) was conducted separately for each solvent to compare the average k values between phenolics rich and phenolics poor, as well as the average k values between alkamides within a set condition of temperature and phenol system using PROC MIXED. In order to satisfy the equal variance assumption, the natural logarithm of k values was used as the response variable. Adjusted means were obtained from the LSMEANS statement and the Tukey-Kramer method was employed for the multiple comparisons. By using the Arrhenius plot of the natural log of k against 1/temperature (in Kelvin), PROC REG was used to extrapolate and obtain the standard error of the predicted k values of each alkamide at each condition of solvent and phenol at 25°C. The slope and y-intercept of this regression was used to calculate the Ea and ln (A). Using the estimated values and standard errors, t-values were computed to test whether the k values and half-lives at

25°C, and  $E_a$  are significantly different between phenolics-rich and phenolics-poor in the same solvent for each alkamide. PROC REG was used to regress the natural log of  $(k/T)$  against  $1/T$ , where  $T$  is temperature in Kelvin, in order to obtain  $\Delta H^\ddagger$ .  $\Delta H^\ddagger$  was then used to calculate  $\Delta S^\ddagger$  and  $\Delta G^\ddagger$ . T-values were used to compare  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  of the alkamides between phenolics-rich and phenolics-poor within the same solvent.

Preliminary accelerated shelf-life temperature was carried out at 90, 100 and 110°C for the extracts in DMSO. The reason for this is because previous work by Murphy and Liu (2006), were carried out at 70, 80 and 90°C and there was not significant degradation of alkamides in some instances at these temperature in a reasonable amount of time. Preliminary data showed that at storage temperature of 90, 100 and 110°C for extracts in DMSO was sufficient for degradation to occur in a reasonable amount of time.

## Chapter 4

### Results and Discussion

Echinacea is one of the most popular herbal supplements in the United States and Europe. It is believed that alkamides may have anti-inflammatory activity (Bauer, 1998; Chen et al 2005), anti-Herpes Simplex Virus properties (Binns et al. 2002) and immunological activities (Woelkart and Bauer 2007). These biological activities may be due to the alkamides found in Echinacea (Woelkart and Bauer 2007, Binns et al. 2002), as such, it is important to determine if these alkamides are stable.

The purpose of this experiment was to determine the stability of the alkamides found in *Echinacea purpurea* root aqueous ethanol extracts. This experiment had two hypotheses, the first one was that the phenolics found in *Echinacea purpurea* root extracts act as antioxidants and protect the alkamides from oxidation; the second was that the alkamides are more stable during storage when dissolved in DMSO than when prepared as dry films because there is limited oxygen concentration in DMSO to oxidize the alkamides.

#### **On the Removal of Phenolics**

In order to study the effect of the phenolics caffeic acid derivatives on the stability of the alkamides in found in *Echinacea purpurea*, these caffeic acid derivatives must be removed in order to compare the stability of alkamides with and without these phenolics. After 95% aqueous ethanol extraction of *Echinacea purpurea* roots, the aqueous ethanol solvent was evaporated using a rotary evaporator, after which the dry residue was dissolved, as much as possible, in water then hexane was added. This was done in order

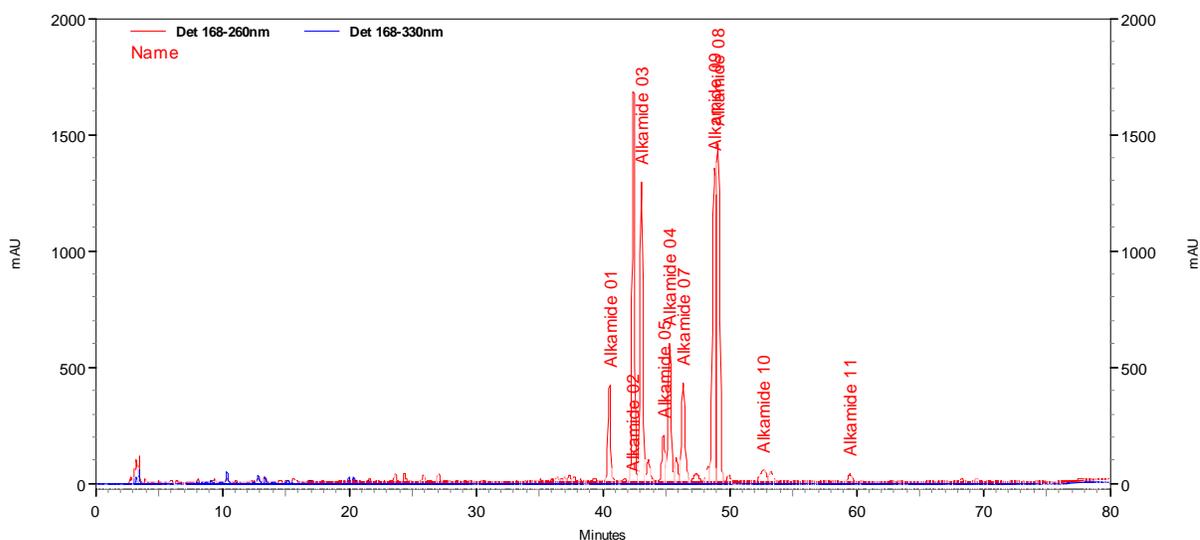
to separate the hydrophilic caffeic acid derivatives which were in the water phase and the lipophilic alkamides which were in the hexane phase. In order to facilitate phase separation and minimize degradation of the alkamides, the hexane/water phase system was placed in the freezer overnight in order that the water would freeze by the following day after which the hexane phase was poured off the flask as quickly as possible before the water phase which had turned into ice started to melt. It was found that there was no difference in the HPLC chromatograms between the hexane phase, in the water/hexane phase separation of phenolic acids and alkamides that underwent further removal of phenolic acids using a silica cartridge and one that did not undergo further removal of phenolic acids using a silica cartridge (See Figure 4-1). Both had the same amount of what is presumed to be cichoric acid, the least hydrophilic of all the six (6) caffeic acid derivatives (data not shown). It could not be ascertained that any of the peaks in the HPLC chromatograms were indeed caffeic acid derivatives since the peaks were below the detection limit of the phenolic acid standards, suggesting that the caffeic acid derivatives have indeed been removed. The caffeic acid derivatives would have eluted between 5-25 minutes in the comprehensive method. The fingerprint of the caffeic acid derivatives portion of the chromatogram has disappeared as a result of the removal of all or almost all of the caffeic acid derivatives, which means that the hexane phase contained the alkamides with almost no caffeic acids left. But in order to be consistent with the work of Liu and Murphy (2006), the hexane phase was further treated to remove phenolics by using the PrepSep<sup>TM</sup> silica cartridge. The peaks in red were taken at 260 nm, near the absorbance maximum of most of the alkamides that are conjugated in *Echinacea*

*purpurea* alkamides and the few peaks or possibly just some noise in blue traces were taken at 330 nm, near the absorbance maximum of the caffeic acid derivatives found in *Echinacea purpurea*. As can be seen in Figure 4-1, the chromatograms a and b are quite similar. During the PrepSep silica cartridge treatment, we discovered that if hexane was used to elute the alkamides rather than chloroform, the alkamides would not elute out of the silica cartridge (results not shown). Chloroform had to be used for this purpose.

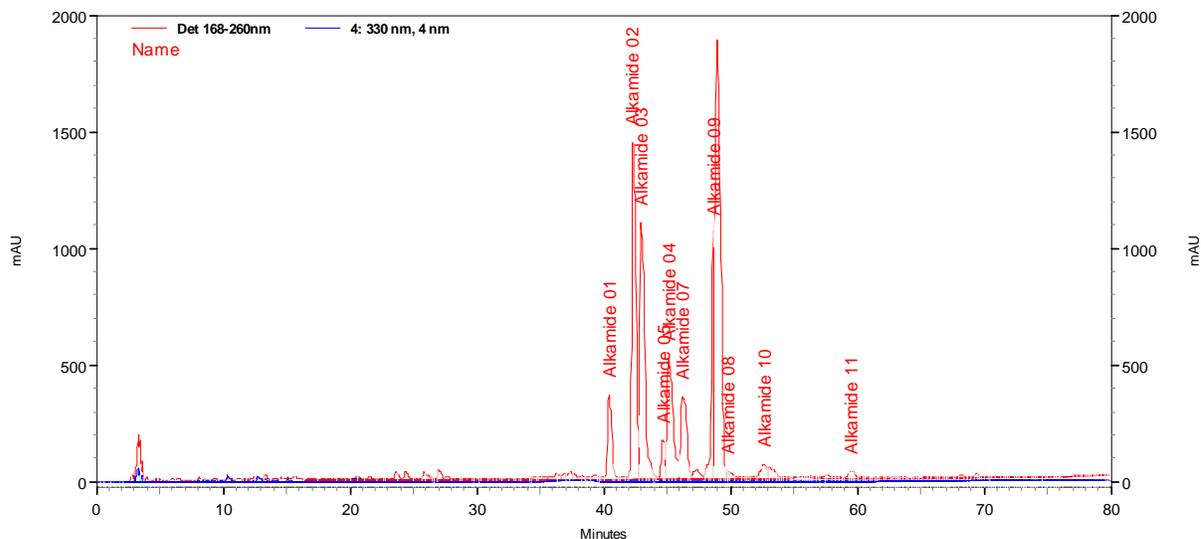
**Figure 4-1** a. Chromatograph using the comprehensive HPLC method in the analysis of the hexane phase of *Echinacea purpurea* root 95% aqueous ethanol extract.

b. Chromatograph using the comprehensive HPLC method in the analysis of the hexane phase of the *Echinacea purpurea* root 95% aqueous ethanol extract that was further cleaned up by elution through a PrepSep™ silica cartridge.

a



b



### Accelerated Shelf-Life Test on Alkamides of *Echinacea purpurea* root Extracts

It is important to determine the shelf-life of *Echinacea purpurea* alkamides because these alkamides are purported to have medicinal values as discussed in the literature review chapter of this thesis (Woelkart and Bauer 2007, Binns et al. 2002). However, it is not known how stable these alkamides are. The preliminary accelerated shelf-life test done by Liu and Murphy (2006) reported the  $k$  values at experimental temperatures of 70, 80 and 90°C; Liu (2006) also reported the energies of activation of the alkamides, as well as their half-lives at room temperature. Liu and Murphy (2006) reported that the alkamides were stable specially when dissolved in DMSO compared to dry; however, they reported huge standards of errors because some of the alkamides did not degrade far enough to have better standard errors, which also resulted in other valuable information that could not be obtained. Information such change in enthalpy of

activation for alkamide degradation, entropy of activation for alkamide degradation, Gibb's free energy of activation for alkamide degradation, and more accurate half-lives of the alkamides at room temperature were not obtained in their study because there was insufficient degradation of the alkamides in some parts of their experiment. For example, the degradation of the alkamides in DMSO Dry were not statistically different from 0 (Liu and Murphy 2006).

As such, another accelerated shelf-life test similar to the methods of Liu and Murphy (2006) which were based on the methods of Taokis and Labuza (1996), was done on the alkamides in *Echinacea purpurea* root aqueous ethanol extract so that the thermodynamics parameters could be obtained, but this time, the alkamides underwent 3 half-lives of oxidation. According to Perry et al. (2001), alkamides in Echinacea roots dropped to about 20% after 64 weeks of storage at 24°C. This suggests that the half-lives of the alkamides were about 200 days at room temperature.

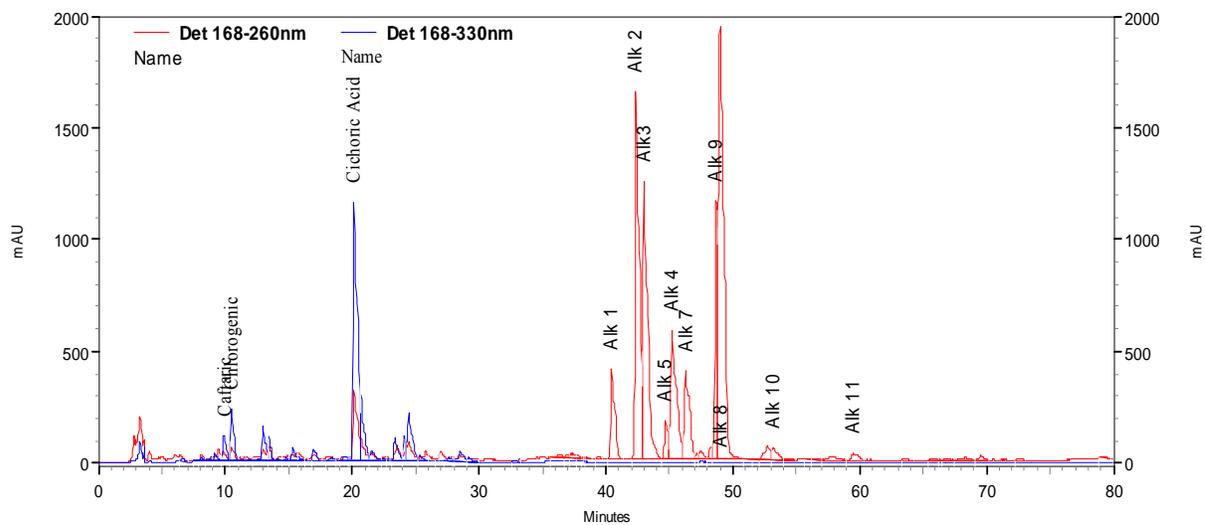
For this experiment, the accelerated shelf-life test on the alkamides of *Echinacea purpurea* root 95% aqueous ethanol Soxhlet extracts required approximately 1.3 kg of plant material, which were prepared separately and as time allowed. The extraction procedure obtained an average extraction yield of 10%. This was divided into 4 treatments, (1) extracts dissolved in DMSO with all of its caffeic acid derivatives (DMSO Rich); (2) extracts dissolved in DMSO with all or most (since the amounts are below the limits of detection) of its caffeic acid derivatives removed (DMSO Poor); (3) stored as dry residue with all of its caffeic acid derivatives (Dry Rich); and (4) stored as dry residue with all or most of its caffeic acids removed (DRY Poor). These conditions were

chosen because one of the hypotheses was that the caffeic acid derivatives in *Echinacea purpurea* root extracts act as antioxidants and minimize the oxidation of the alkamides. The other hypothesis was that in dry films, there is limited oxygen concentration in DMSO than in air therefore there existed a higher probability for oxygen to react with the alkamides, causing the alkamides' oxidation to be greater in Dry than in DMSO.

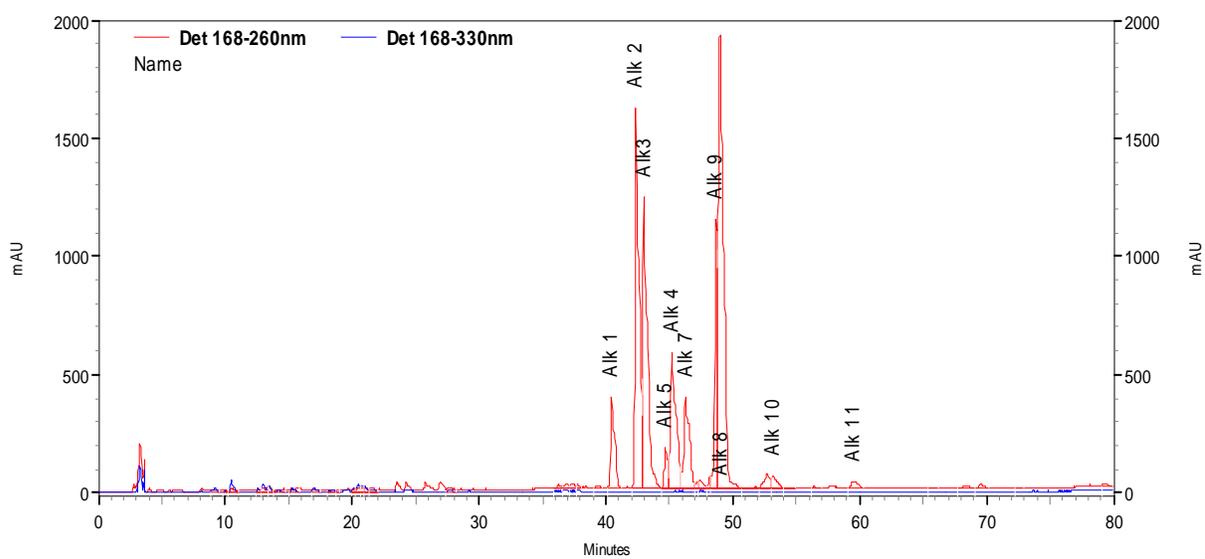
At time zero of the accelerated shelf life test, the representative chromatograms of each treatment at Day 0 are shown in Figure 4-2. Liu and Murphy (2006) reported about alkamide 4, which they have mislabeled as alkamide 6. This paper refers to the compound with its true identification, alkamide 4. Identification of the alkamides was done in collaboration with our colleagues (Senchina et al. 2006, Kraus et al. 2006, Wu et al. 2004) at Iowa State University Botanical Supplements Research Center. The chromatograms show the caffeic acid derivatives in red which were measured at 330 nm and show the alkamides in blue which were measured at 260 nm. The chromatograms show the caffeic acid derivatives absent in the phenolics poor treatment and show the caffeic acid derivatives to be present in the phenolics rich treatment. Figure 4-3 shows the UV absorbance spectra of the caffeic acid derivatives and alkamides in *Echinacea purpurea* root extract which were used to verify the alkamides in addition to the relative retention time (Senchina et al. 2006, Kraus et al. 2006, Wu et al. 2004) here at Iowa State University Botanicals Supplements Research Center. The UV spectra were obtained using commercial chemical standards as well as previously identified compounds by Senchina et al. (2006), Kraus et al. (2006) and Wu et al. (2004).

**Figure 4-2.** Representative chromatograms of DMSO Rich, DMSO Poor, Dry Rich and Dry Poor treatments, at time 0.

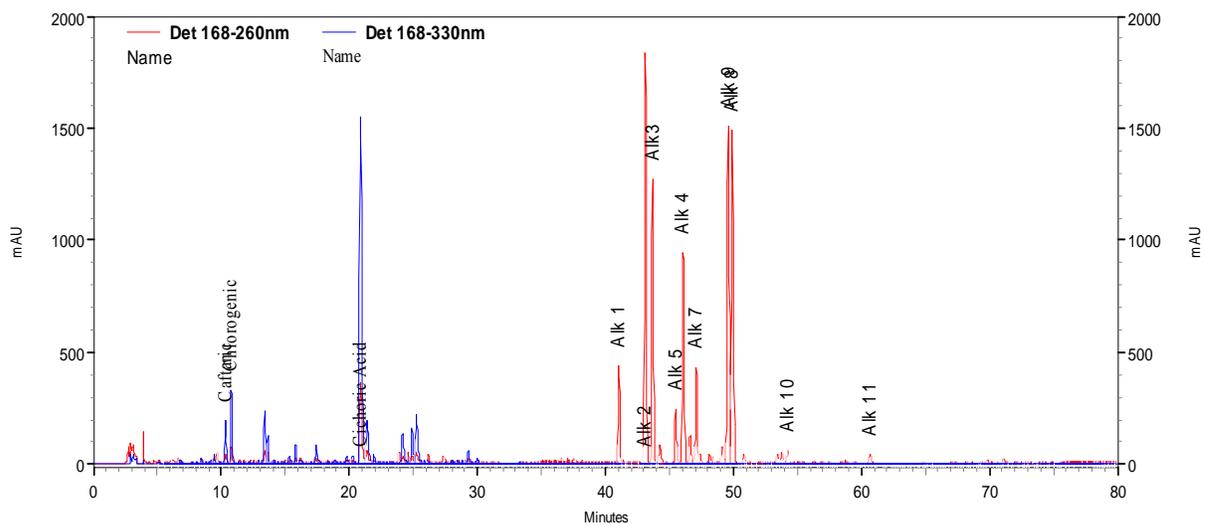
a). Chromatogram of DMSO Rich at Day 0.



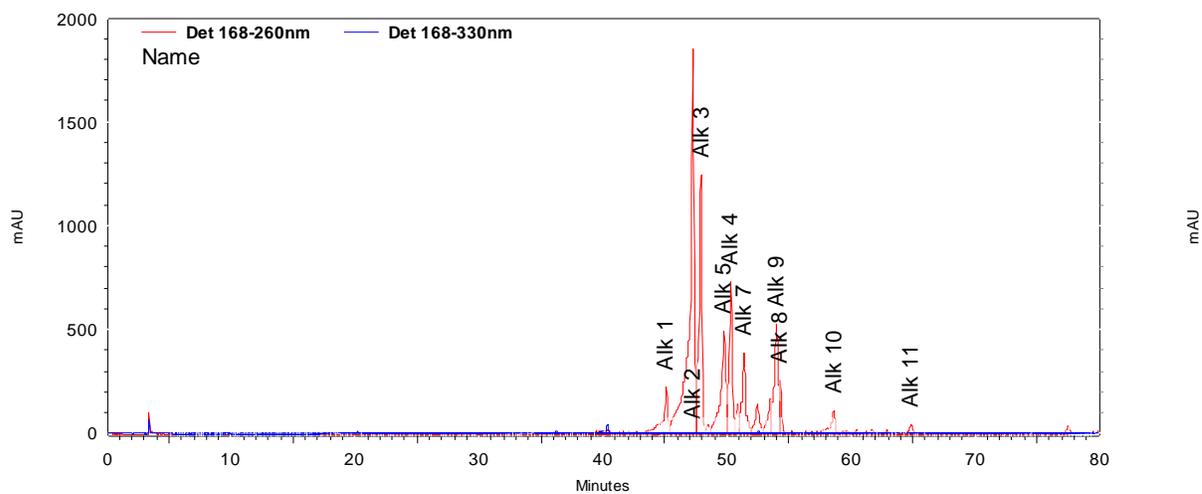
b). Chromatogram of DMSO Poor at Day 0.



## c.) Chromatogram of Dry Rich at Day 0

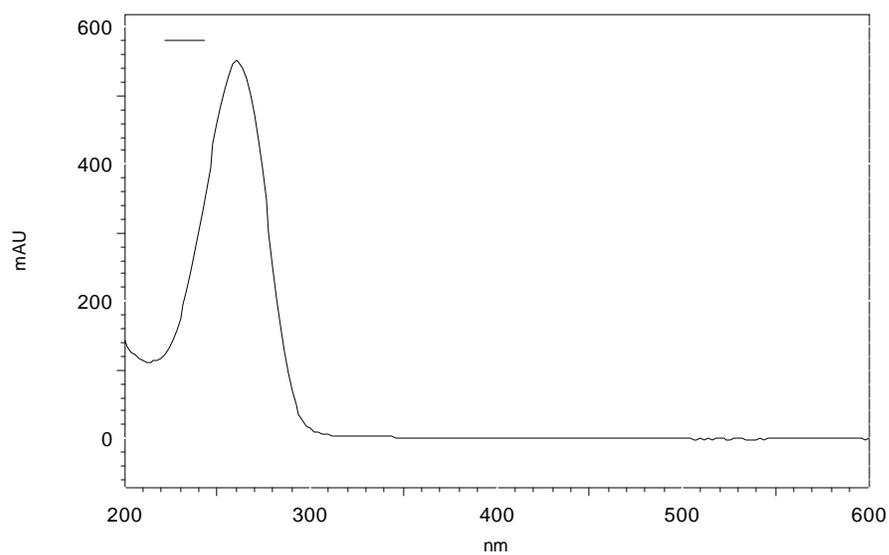


## d). Chromatogram of Dry Poor at Day 0.

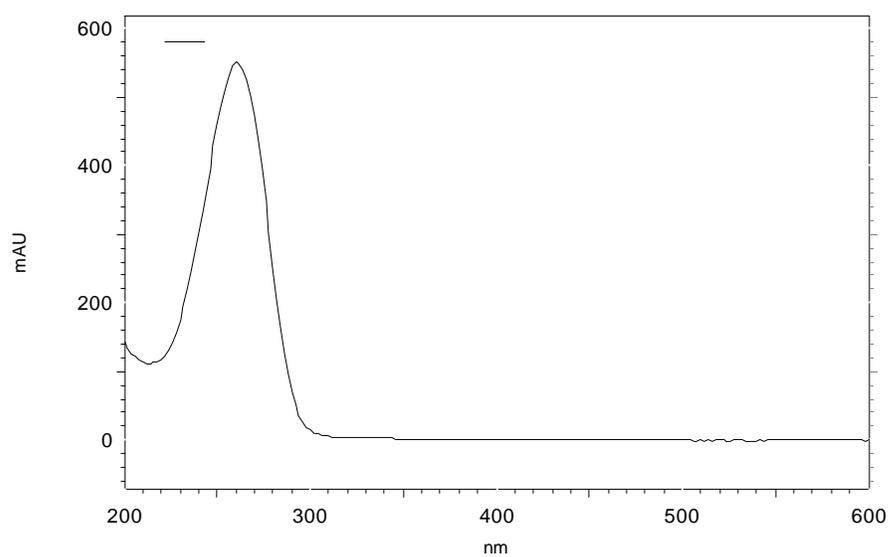


**Figure 4-3.** UV absorbance spectra of the alkamides in the aqueous ethanol extracts of *Echinacea purpurea* root from 200-600 nm

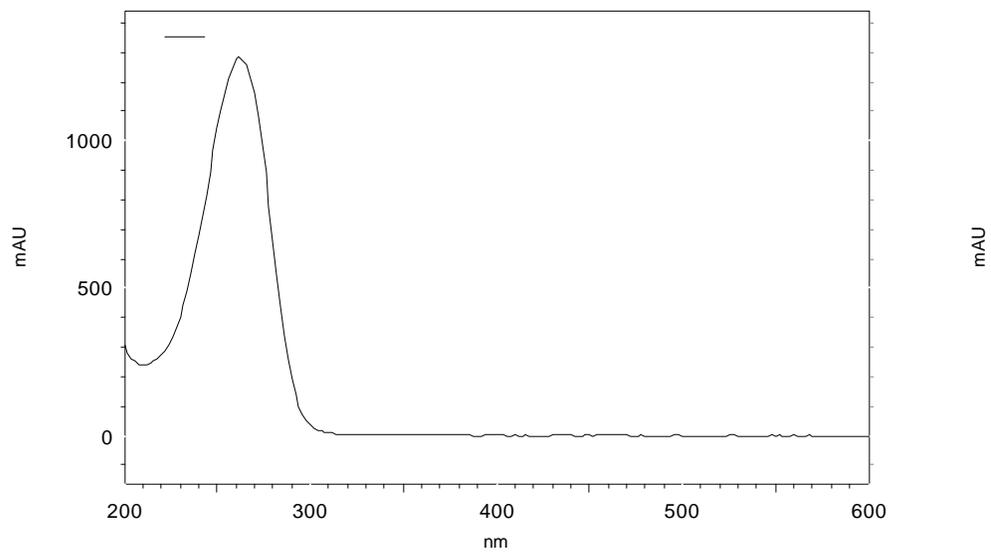
a). Alkamide 1



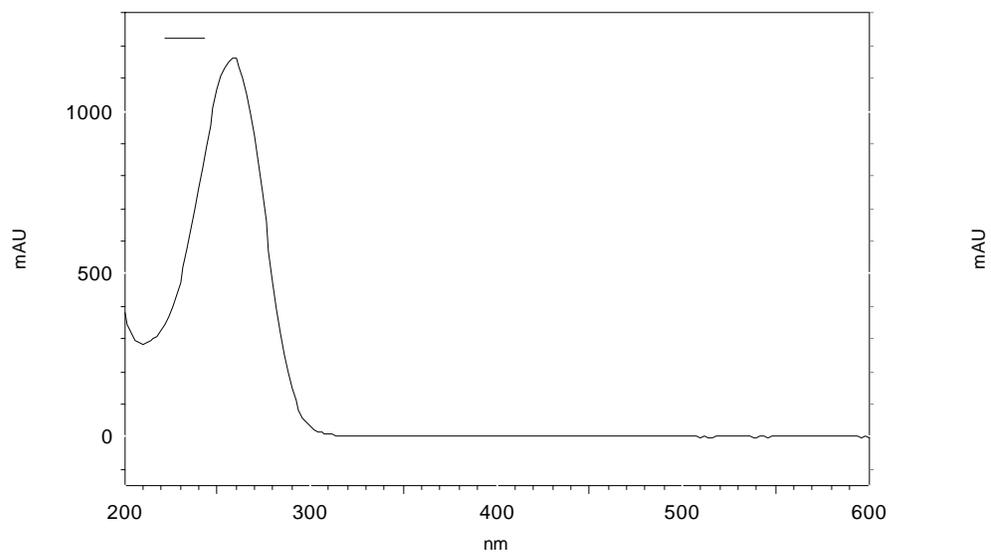
b.) Alkamide 2



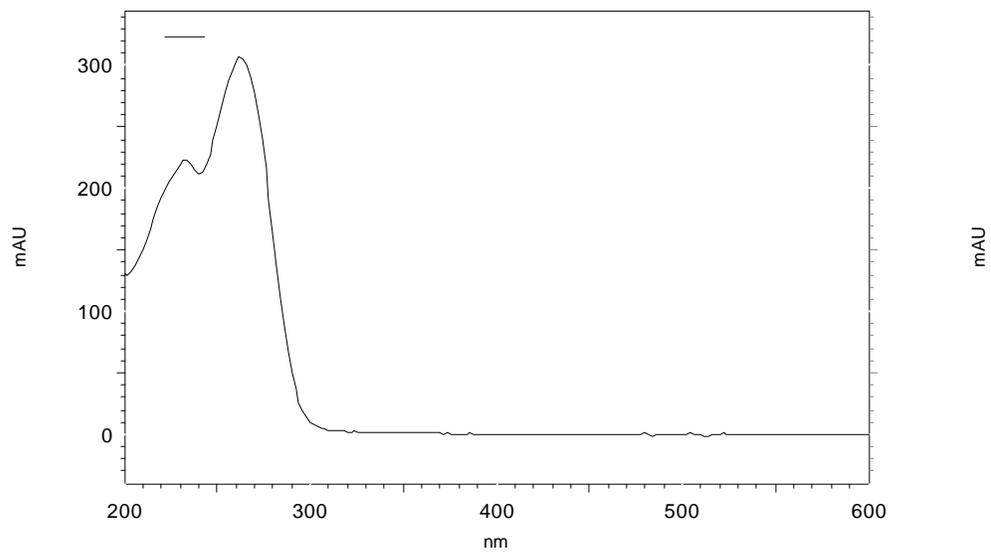
c). Alkamide 3



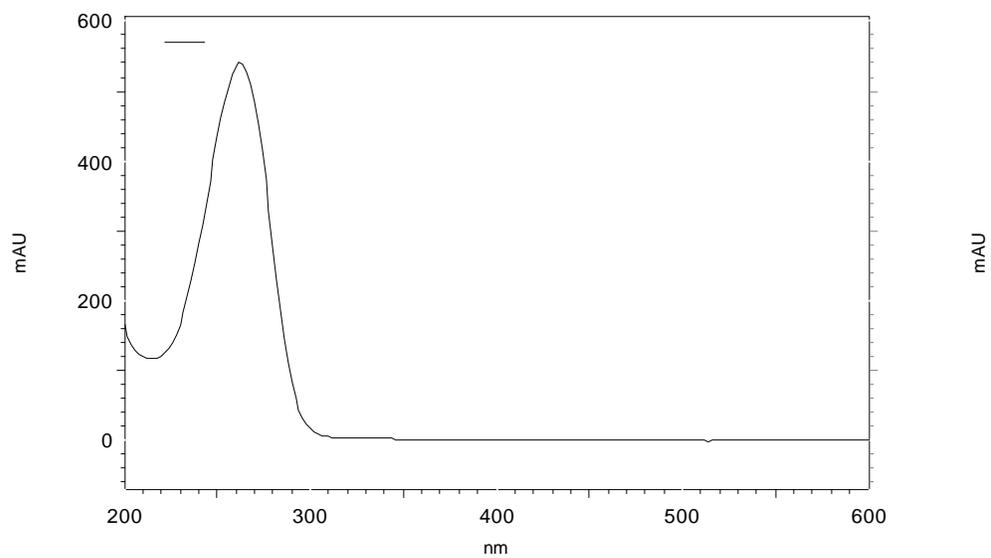
d). Alkamide 4



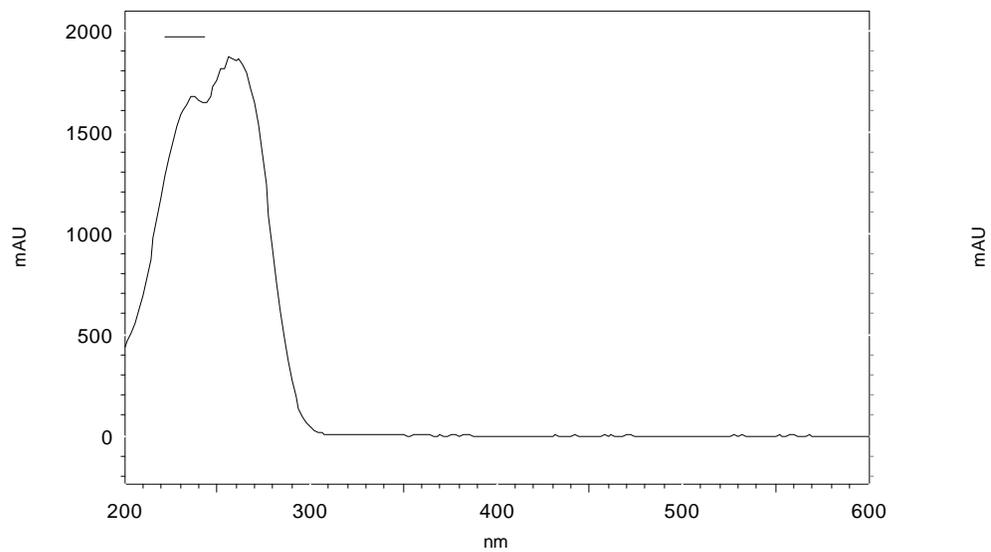
e). Alkamide 5



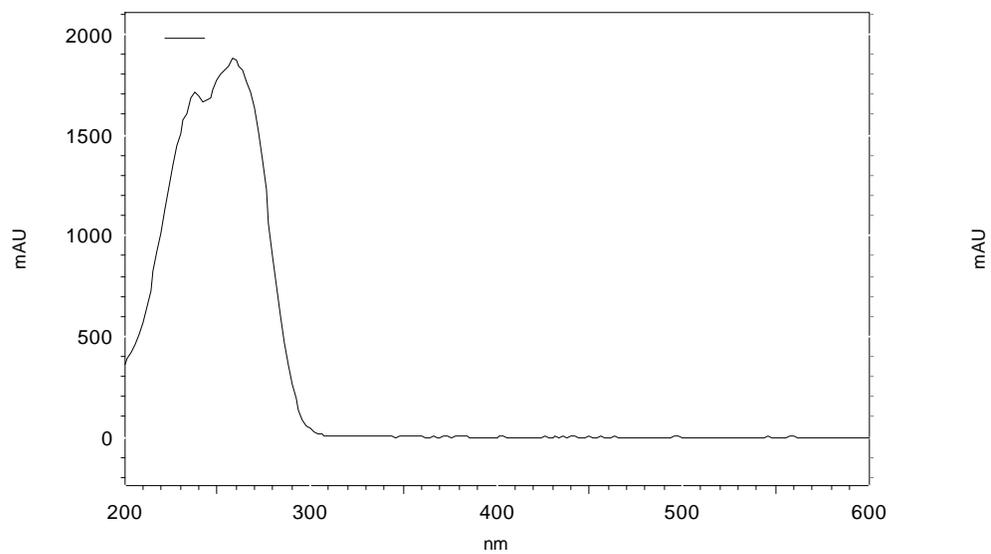
f). Alkamide 7



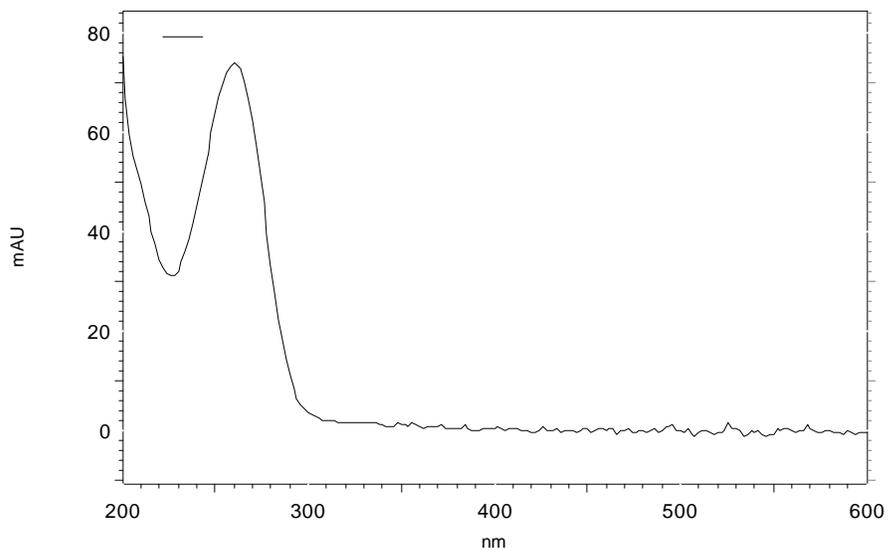
g). Alkamide 8



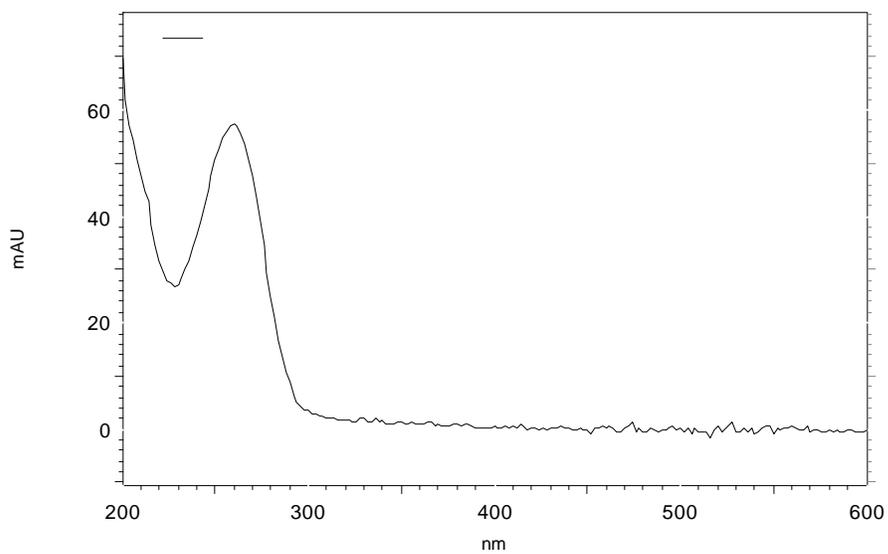
h). Alkamide 9



i). Alkamide 10



j). Alkamide 11



The DRY Rich and Dry Poor extracts were incubated at accelerated storage temperatures of 70, 80, and 90°C, while the extracts dissolved in DMSO were incubated at accelerated storage temperatures of 90, 100, and 110°C. The difference in the temperatures was necessary in order to complete the accelerated shelf-life test in a reasonable amount of time. The temperatures for the Dry were chosen as such because Liu and Murphy (2006) reported significant degradation at 70, 80, and 90°C, therefore a longer time of up to 3 months of storage at accelerated temperatures would be sufficient for the alkamide to have undergone 3 half-lives of degradation at these temperatures. Time of storage of 3 half-lives was the target because according to Benson (1960), the standard error of the extrapolation of the degradation values using the Arrhenius plot to  $k$  values at room temperature becomes smaller with a coefficient of variance of analysis of 10%.

Since Liu and Murphy (2006) reported that alkamides in DMSO stored at 70, 80 and 90°C barely degraded especially in DMSO Poor, a preliminary experiment of 10 days was done to determine storage temperatures for the DMSO treatments that would result in a degradation of 3 half lives in a reasonable period of time. The result of this preliminary experiment indicated that at 110°C, the alkamides would undergo degradation of 3 half-lives in approximately 2 weeks.

In the accelerated shelf-life experiment done by Liu and Murphy (2006), it was discovered that the alkamides are much more stable when stored in solution in DMSO, with and without the caffeic acid derivatives, than stored as dry residues. This is probably due to the larger surface area available for oxygen to react with the alkamides in the Dry

compared to the limiting oxygen concentration in DMSO. Therefore, the extracts in DMSO were incubated at higher temperatures in order to accelerate their oxidation. The ten alkamides, undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (alkamide 1), undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide (alkamide 2), dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (alkamide 3), undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide (alkamide 4), dodeca-2*E*,4*Z*,10*E*-trien-8-ynoic acid isobutylamide (alkamide 5), dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide (alkamide 7), dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide (alkamide 8), dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (alkamide 9), dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide (alkamide 10), and dodeca-2*E*,4*E*,dienoic acid isobutylamide (alkamide 11), commonly found in the extracts of *Echinacea purpurea* roots were monitored using HPLC with photodiode array detector. The changes in their peak heights in the HPLC chromatograms measured at 260 nm were analyzed using the HPLC alkamide method. The peak heights were considered directly proportional to the concentration of individual alkamides in the *Echinacea purpurea* extracts.

The caffeic acid derivatives were believed to act as antioxidants and minimize the oxidation of the alkamides. For samples submitted to the biological groups of the Iowa State University Botanicals Supplement Research Center, the caffeic acid derivatives are at times removed through semi-preparative HPLC fractionation. It was important to determine whether the alkamides in these caffeic acid-free fractions are stable since they are at times stored for over a year prior to biological testing, which is a coincidental reason for having phenolics-poor conditions in the accelerated shelf-life experiment. One

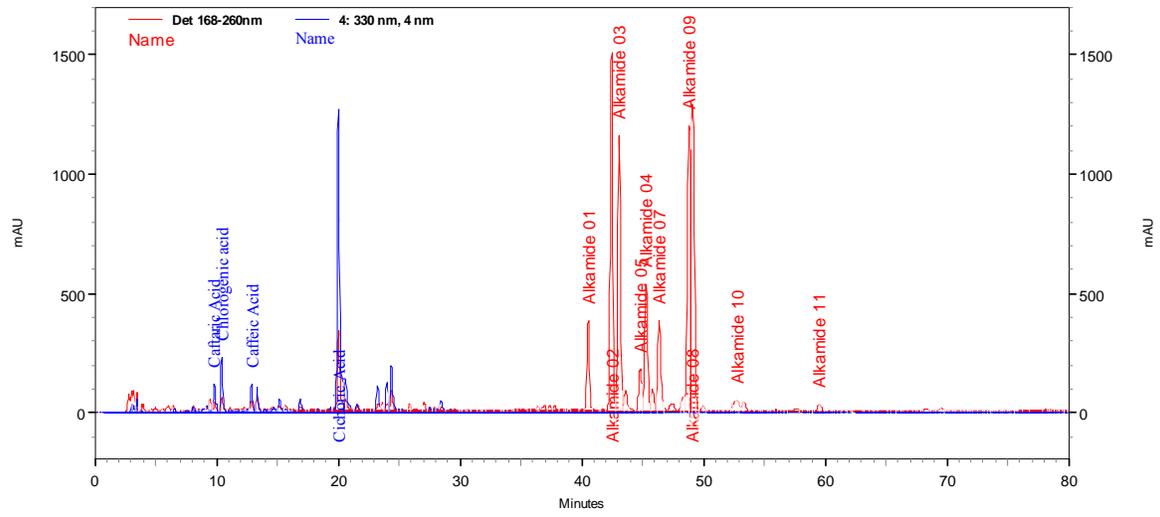
of the solvents used to dissolve *Echinacea* extracts and fractions for biological testing was DMSO. It was a coincidence too that DMSO was used in the accelerated shelf life experiment since *Echinacea purpurea* extracts and fractions were dissolved in DMSO for biological testing. The reason that DMSO was used in the biological test was because DMSO did not interfere with the specific biological assays done by several laboratories at the center, unlike ethanol, the predominant solvent used to dissolve *Echinacea* extracts in the botanical supplement industry. To determine the stability of the alkamides of *Echinacea purpurea* root 95% aqueous ethanol Soxhlet extracts with the caffeic acid derivatives intact, a faster separation of the caffeic acid derivatives from the alkamides was used instead of semi-preparative HPLC fractionation. This separation technique used a silica cartridge (PrepSep) to trap the more hydrophilic compounds such as the phenolic caffeic acid derivatives, while the more hydrophobic compounds such as alkamides were eluted out of the silica cartridge when chloroform was used as the mobile phase.

Figure 4-4 shows the HPLC chromatograms for both phenolics-rich and phenolics-poor extracts *Echinacea purpurea* roots. The peaks in red, taken at 260 nm, represented the alkamides as labeled and the peaks in blue or the absence thereof, taken at 330 nm, the lambda max for the caffeic acid derivatives, represented the caffeic acid derivatives as labeled.

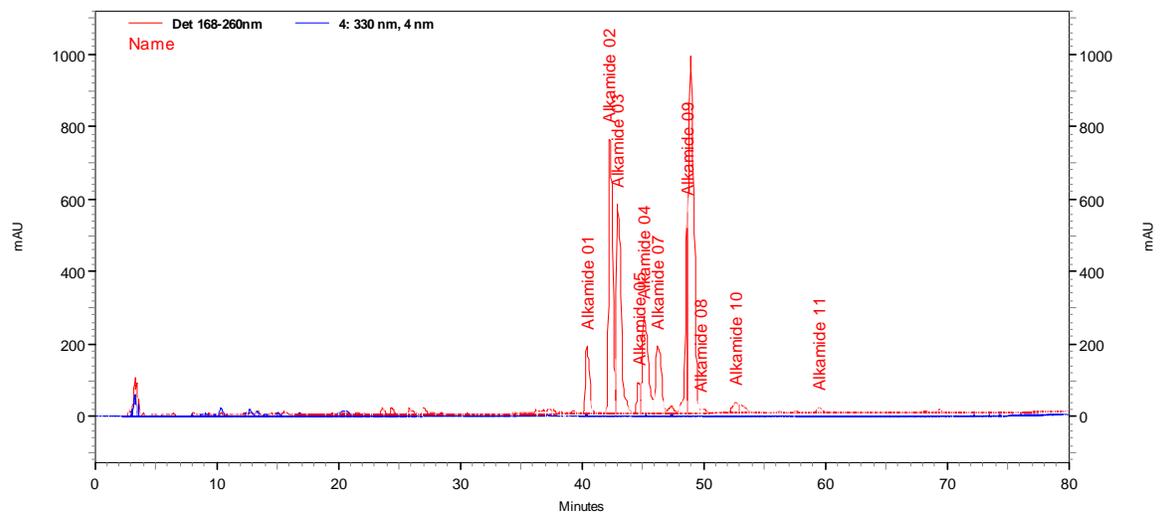
**Figure 4-4.** a. HPLC Chromatogram of Phenolics-Rich *Echinacea purpurea* root extracts prior to incubation at accelerated storage temperatures using the comprehensive

method. b. HPLC chromatogram of phenolics-poor *Echinacea purpurea* root extracts prior to incubation at accelerated storage temperatures.

a.



b.



After the *Echinacea purpurea* root extracts were prepared for the accelerated shelf-life experiment, the peak heights of the alkamides were monitored over time. Initially, samples were evaluated and analyzed on a daily basis for all experimental conditions. It was apparent that the extracts stored at higher temperatures were degrading fast enough to warrant daily sampling; this was done for Dry at 90°C and DMSO at 110°C. At the second highest temperatures, 80°C for Dry and 100°C for DMSO, samples were taken twice a week; and at the lowest temperature, 70°C for Dry and 90°C for DMSO, samples were taken once every 1-2 weeks.

#### *Degradation Rate Constant k*

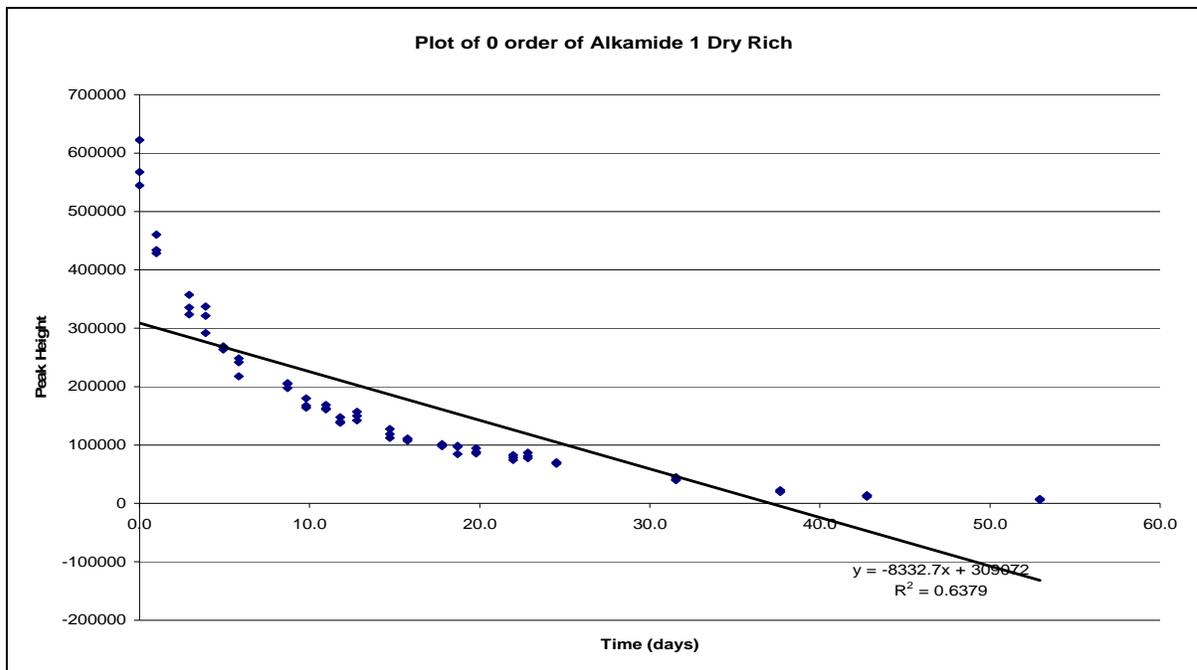
Liu and Murphy (2006) presented data that the alkamides followed an apparent 1<sup>st</sup> order degradation. However, in order to be certain that the alkamides do indeed follow an apparent 1<sup>st</sup> order degradation, the data collected from the degradation of the alkamides were plotted in the 0, 1<sup>st</sup> and 2<sup>nd</sup> order, which is the only way to determine the apparent reaction order for any compound (Liu 2006, Chang 2001<sup>a</sup>). For 0 order degradation rate, the peak heights were plotted versus time; for 1<sup>st</sup> order degradation rate, the natural log of the peak heights were plotted versus time; and for 2<sup>nd</sup> order degradation rate, the initial height minus the current height divided by the product of initial height and current height were plotted versus time. The plot for an apparent 1<sup>st</sup> order degradation rate gave the best fit line. See figure 4-5 to compare the 0, 1<sup>st</sup> and 2<sup>nd</sup> order degradation rate.

After the HPLC analysis of samples of *Echinacea purpurea* root extracts for the accelerated shelf-life test, the natural log of height (ln of height) was plotted against time in days, since this gave the best fit line. The negative slope of the regression is the

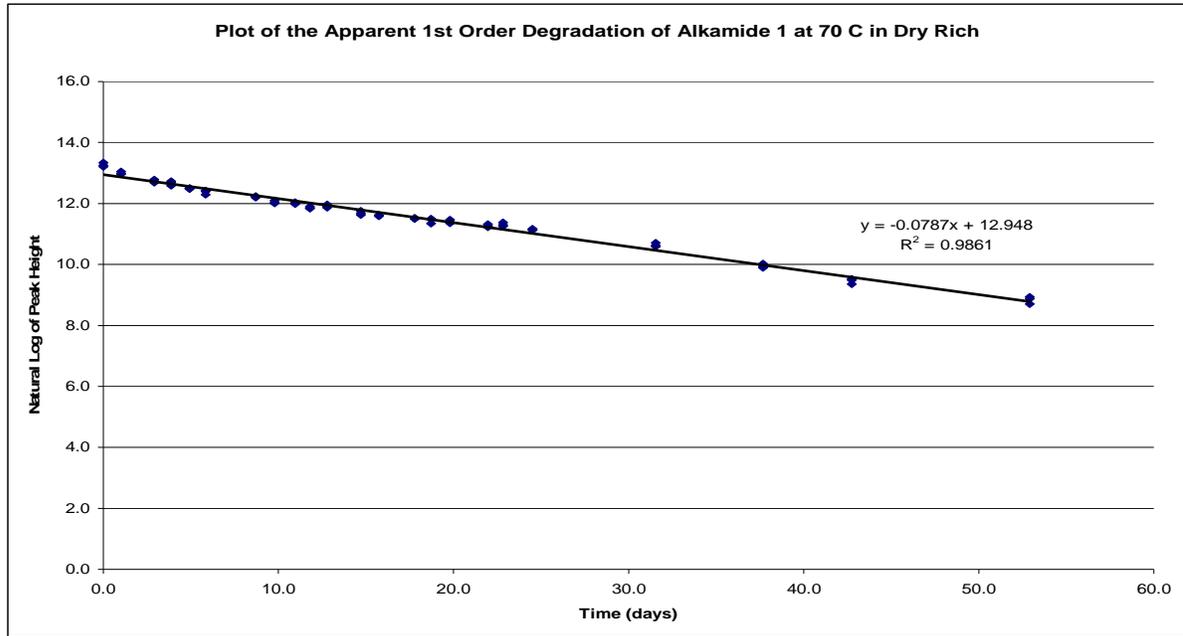
degradation rate constant  $k^{-\text{days}}$ . Since the residual plots of  $k$  were not evenly distributed, log transformations of  $k$  values had to be done to obtain the least square means of the degradation rate constant  $k$  for all the alkamides, otherwise the results may become statistically incorrect.

**Figure 4-5.** Plot of zero order degradation rate for alkamide 1 in Dry Rich

a



b



c

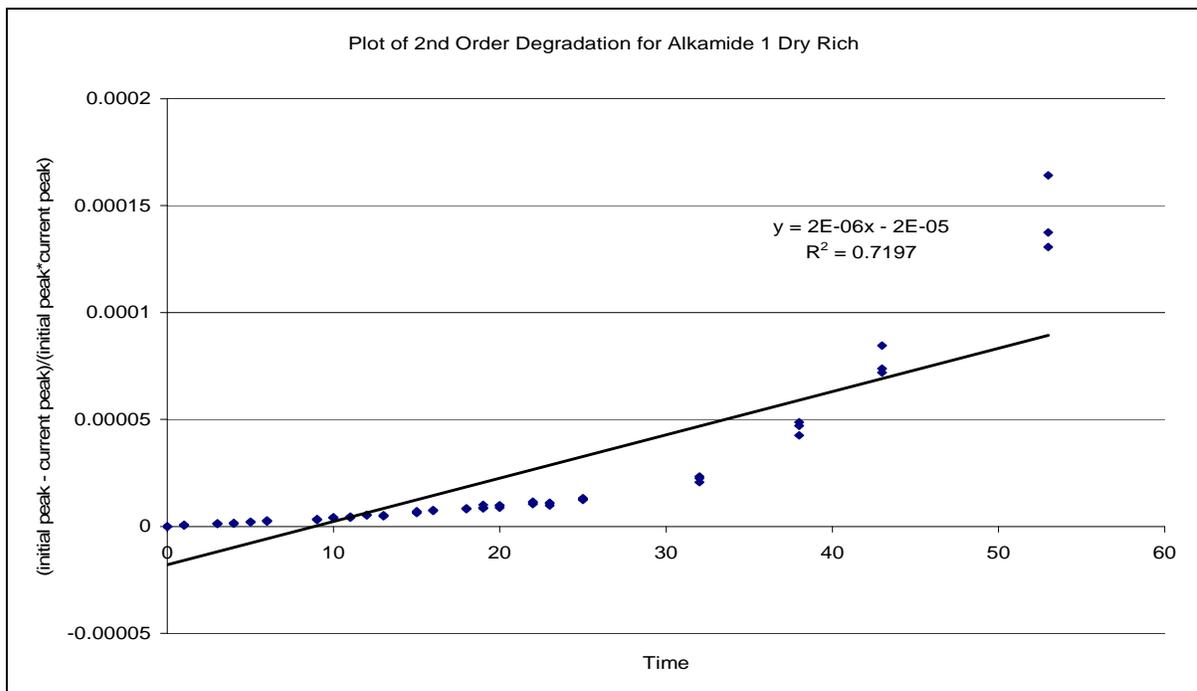


Figure 4-6 illustrates that the degradation rate constants increased as the temperature increased which is what was expected. A 3-way analysis of variance (ANOVA) was performed separately for the log transformed  $k$  for the Dry and DMSO. Statistical comparisons between the phenolics rich and phenolics poor for each alkamide within the same solvent system and temperature were done using multiple comparison  $t$ -test with Tukey adjustment. Statistical differences between Rich and Poor were observed for most of the  $k$  values at 80 and 90°C. However, at 70°C, no statistical differences between phenolics-rich and phenolics-poor for each alkamide were found. This is due to the large standard errors at 70°C in comparison to the small  $k$  values at 70°C which makes the difference between the  $k$  values statistically insignificant.

When comparing the degradation rate constants ( $k$ ) of the alkamides of slight higher than 0 to 0.7 per day at all conditions, versus methyl oleate and methyl linoleate from the experiments for Gunstone and Hilditch (1945), it is apparent that alkamides are less stable than methyl oleate, but more stable than methyl linoleate. From the experiments of Gunstone and Hilditch (1945) the half-life of methyl oleate was approximately 2.5 days, while methyl linoleate was completely degraded in less than one day at 80°C. In comparison, alkamides stored at 80°C in the dry form, with and without phenolic acids, had half-lives which ranged from about 1.4 to about 2.3 days. Alkamides in DMSO are even more stable as reported in this experiment. It is very likely that methyl oleate is more stable than the alkamides because methyl oleate contains only one double bonds, while the alkamides contain multiple unsaturated bonds, including acetylene bonds.

Figure 4-6 a. Degradation Rate Constants of Alkalamides

Apparent 1st Order Degradation Rate Constants of Dry Phenolics-Rich

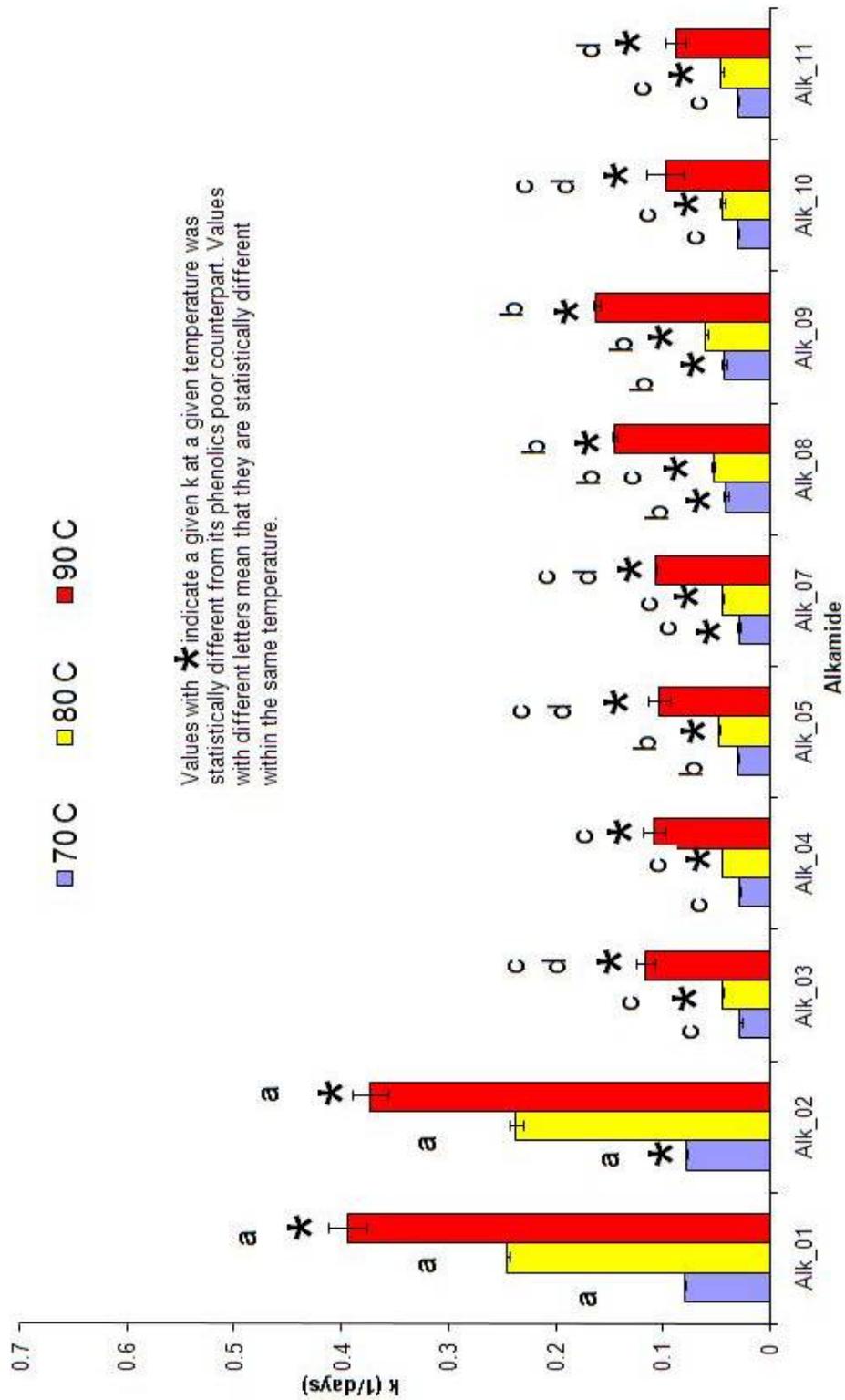
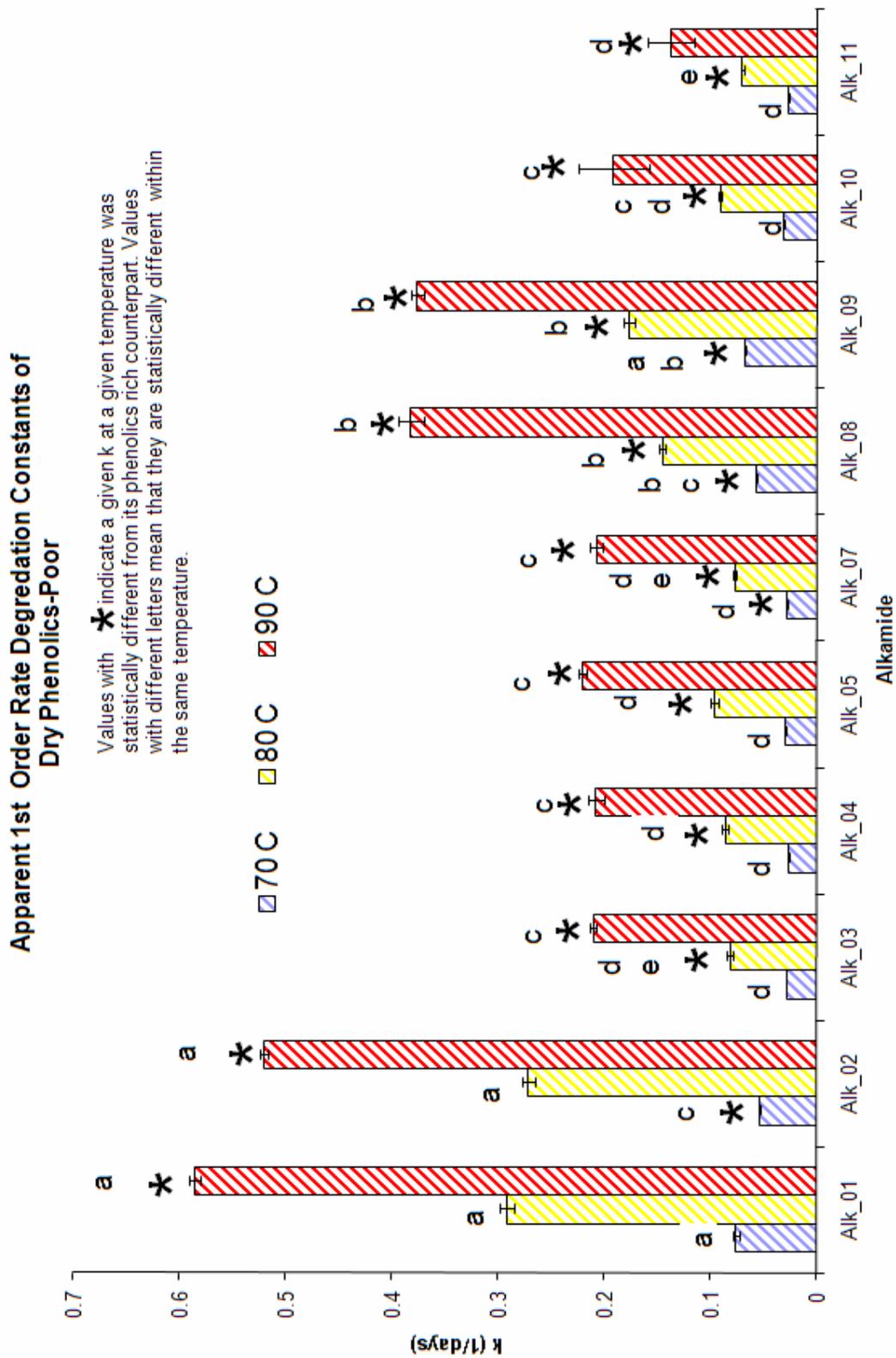
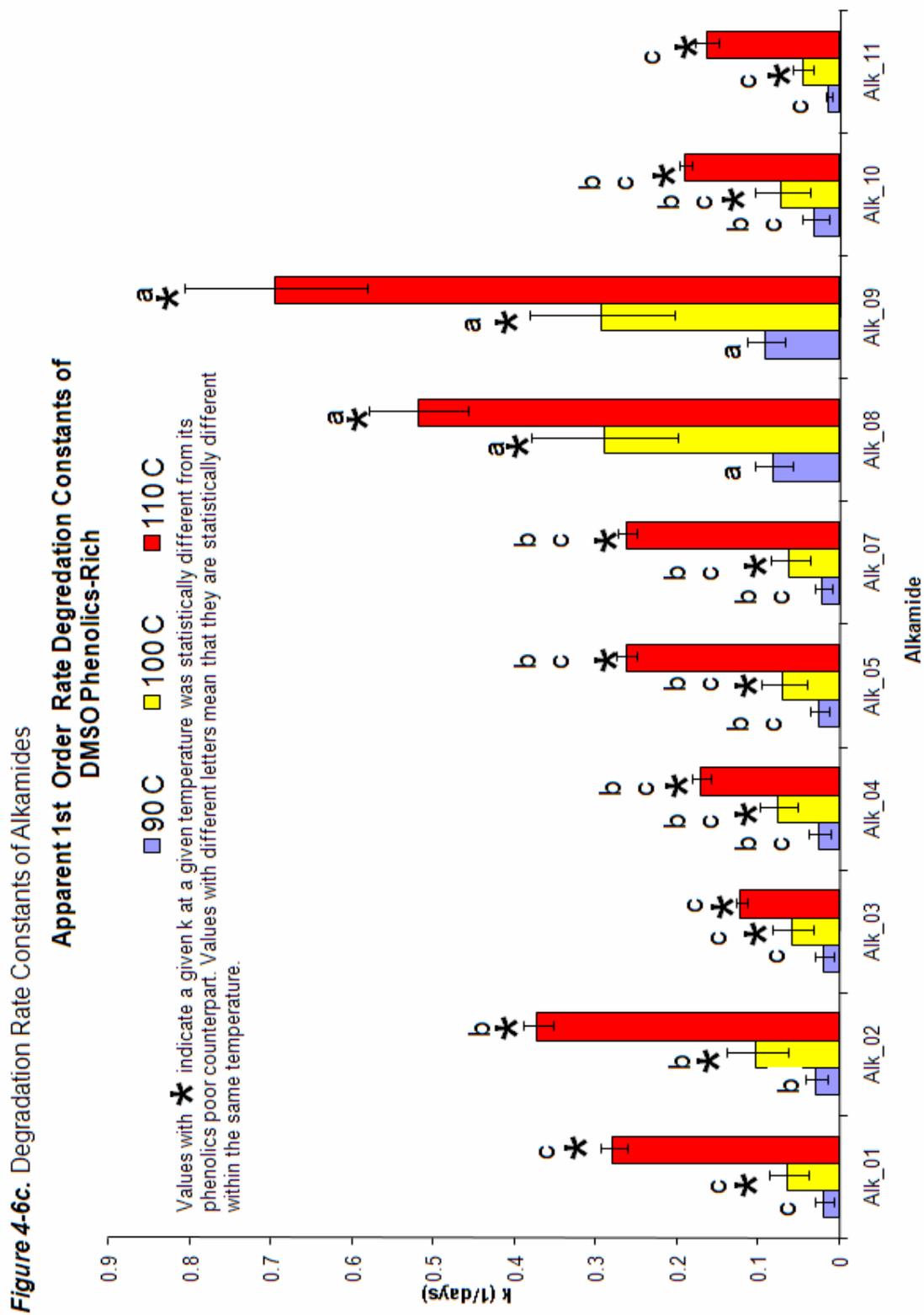


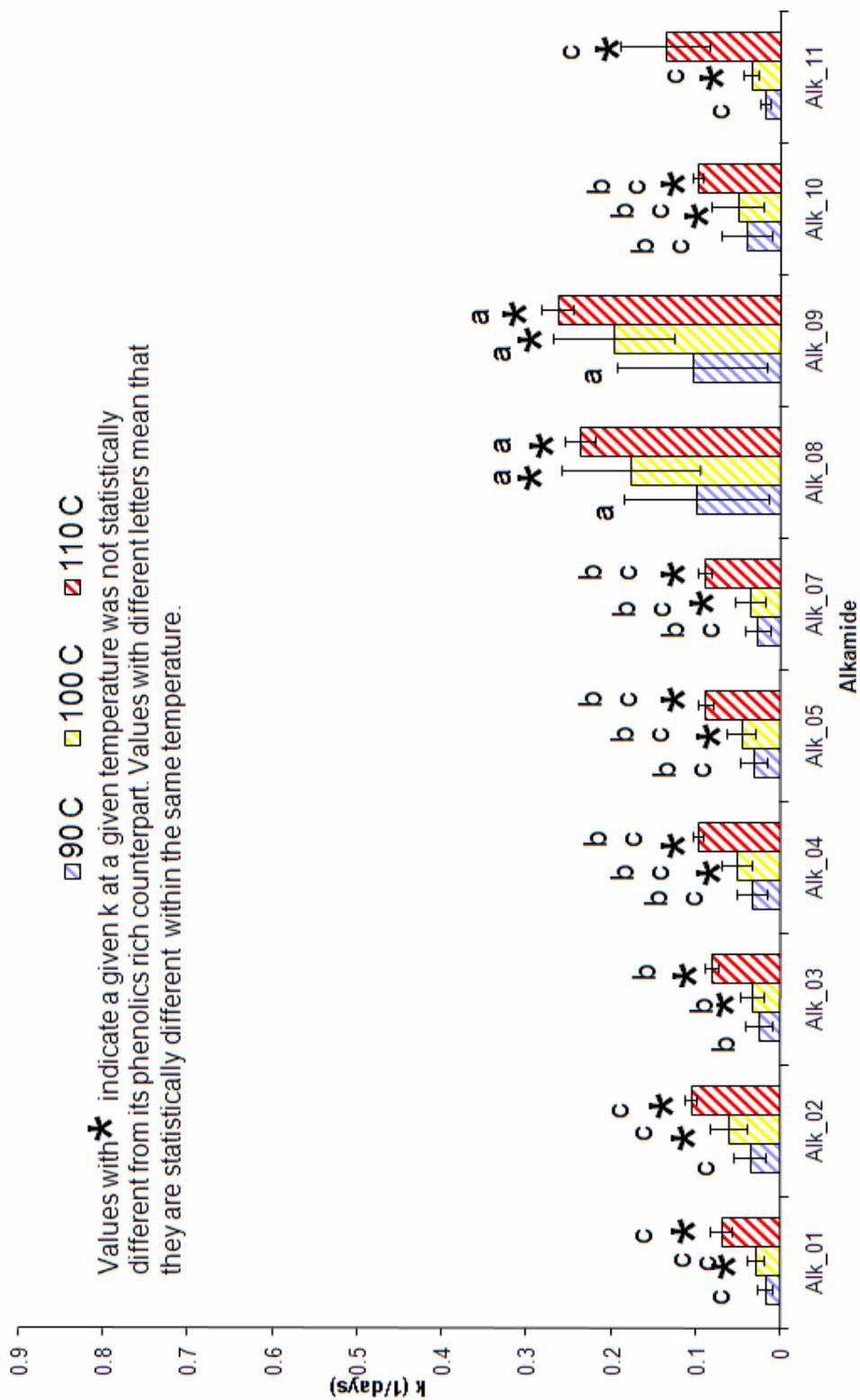
Figure 4-6b. Degradation Rate Constants of Alkamidides





**Figure 4-6d.** Degradation Rate Constants of Alkamide

**Apparent 1st Order Rate Degradation Constants of DMSO Phenolics-Poor**



In DMSO however, phenolic Rich had larger k values than phenolic Poor. The reason why the k values between the DMSO Rich and DMSO Poor at 90°C were not statistically different is the same reason given for Dry Rich and Dry Poor at 70°C. Liu and Murphy (2006) also reported k values between Rich and Poor at 70°C were not statistically different from each other. It is unknown why in DMSO Rich the k values are larger than in DMSO Poor. A possible explanation for this is due to the rapid degradation of the cichoric acid at 90, 100 and 110°C (results not shown). Within 2 days of storage at all of the experimental temperatures, the major phenolic acid, cichoric acid, the peak that represented chicoric acid was no longer detected. The degradation of cichoric acid probably gave rise to caffeic acid, since the peak for what was apparently caffeic acid increased, which eventually degraded as well. Since the major and most effective known anti-oxidant found in *Echinacea purpurea*, cichoric acid, (Pellati et al. 2004, Facino et al. 1995, Dalby-Brown, et al. 2005, Sloely et al. 2001) degraded in the DMSO treatments, it is doubtful that there is still significant anti-oxidant activity left which would have protected the alkamides from oxidation. In addition, at high temperatures of 100°C or more, phenolic acids are known to potentially act as pro-oxidants by abstracting a hydrogen from a hydroperoxide ( $\text{LOOH} \rightarrow \text{LOO} \cdot + \text{AH} \cdot$ ) (Frankel 1998). It has been known that some anti-oxidants can act as pro-oxidants in certain other conditions, for example, beta-carotene acted as a pro-oxidant in the absence of  $\gamma$  and  $\delta$ -tocopherol in the photo-oxidation of soybean oil; as well as a pro-oxidant when soy bean oil was stored in the dark (Frankel 1998). Another possibility is that there might have been pro-oxidants that were not removed in the phenolic acids-poor samples (Poor) which behaved

differently when dissolved in DMSO as compared to when the *Echinacea purpurea* extracts were dried.

Statistical analysis done for the k values of the Dry samples showed that there was a three-way interaction between alkamides, temperature and the presence or absence of the caffeic acid derivatives (this treatment is herein referred to as “phenol”). Therefore, a pair by pair comparison between each alkamide was performed at each temperature within a set phenol and solvent system treatment in order to obtain the ranking of the k values of the alkamides, using Bonferroni adjustment. A pattern was observed in the degradation of the alkamides in both Dry Rich and Dry Poor which was alkamides  $1 \approx 2 > 9 \approx 8 > 3 \approx 4 \approx 5 \approx 7 \approx 10 \geq 11$ . This is similar to the pattern reported by Liu and Murphy (2006), and the magnitudes of the k values were similar in this experiment and that of Liu and Murphy (2006) for all observed alkamides except for alkamide 4, in which they reported higher k values. Alkamides 10 and 11, which have the least number of unsaturated bonds, were the most stable. This was not surprising as the less unsaturated bonds there are, the less prone to oxidation the compound is.

Cumpston and Jensen (1998) reported that a terminal ethylene bond that has an electron withdrawing moiety attached or a bulky moiety attached on the terminal unsaturated bond leads to more stability in photo-oxidation. This is because the electron withdrawing moiety decreases the electron density on the unsaturated bonds, making the unsaturated bonds less attractive to electrophilic species such as singlet oxygen, thereby making the compound more stable to electrophilic attacks. According to Wang and Hammond (2006), singlet oxygen attacks the double bonds in lipids while free radicals

attack the allylic carbon. In this study, the results suggested that the alkamides were attacked by electrophilic species such as singlet oxygen more so than free radicals. If it has been the free radicals that were attacking, the alkamides with more acetylene bonds would have been more prone to oxidation due to the attraction of electrons by the acetylene bonds from allylic carbons (McMurry 2004) which would make the hydrogen atoms of the allylic carbons more susceptible to abstraction by free radicals. However, the results of this experiment reported here concluded that the more acetylene-rich alkamides such as alkamides 3, 4, 5, and 7 were more stable than the acetylene-free alkamides 8 and 9, which implied that an electrophilic attack, such as singlet oxygen oxidation, on the double bonds was more likely.

Even if the acetylene bonds were more stable to auto-oxidation than the ethylene bonds, then the order of degradation should have been alkamides  $8 \approx 9 > 5 \approx 10 >$  other alkamides, which was not the case as reported in this experiment. A reason why singlet oxygen might have been responsible for the oxidation more so than free radicals in the Dry is because the alkamides were spread out on a very thin film, allowing maximum exposure to oxygen; as well as possibly immobilizing to an extent, the free radicals that would have caused autoxidation through the abstraction of hydrogen from allylic carbons. This immobilization may be due to the adhesion of the free radicals to the walls of the storage HPLC vials, which would be the same force responsible for the alkamides adhering to the walls of the storage HPLC vials rather than flowing down to the bottom of the vials.

If indeed singlet oxygen is responsible for oxidation, there must be a source for singlet oxygen to form from triplet oxygen. It is known that singlet oxygen can be formed from triplet oxygen in gaseous state by microwave (Schaap et al. 1975) and radio-wave (Rawls and Santen 1970) discharges; however, this accelerated shelf life experiment did not use either radiation for the oxidation of the alkamides. So there might have been a different source of singlet oxygen. It may be possible that infra-red light (heat) might have caused the formation of singlet oxygen from triplet oxygen in gaseous state since microwave is just one type of electromagnetic radiation away from infra-red radiation in the electromagnetic radiation spectrum.

Other than direct breakdown of triplet oxygen to singlet oxygen through electromagnetic radiation, chemical reactions such as the reaction of sodium hypochlorite with hydrogen peroxide; thermal decomposition of ozonides of triphenyl phosphate and the reaction of potassium superoxide in water; and biological processes such as adrenodoxin reductase-adrenodoxin enzyme system, xanthine oxidase; rat liver microsomes, NADPH and O<sub>2</sub>; and human polymorphonuclear leukocytes upon phagocytosis, may also cause the formation of singlet oxygen from its ground state triplet oxygen (Schaap et al. 1975), although both chemical reaction and biological processes were unlikely to have caused the formation of singlet oxygen, if any were formed, in this experiment. It is, however, possible for certain frequencies of electromagnetic radiation to cause sensitizers to react with triplet oxygen to form singlet oxygen; while the sensitizers also change their state between triplet to singlet states (Rawls and Santen 1970; Rabek and Ranby, 1975; Porter et al. 1995; Frankel 1984). For examples, organic

matter in water can absorb sunlight and transfer this energy to dissociate triplet oxygen to form singlet oxygen (Haag and Hoigne 1986). Cumpston and Jensen (1995) reported that visible light can also cause singlet oxygen formation in the presence of specific sensitizers.

It may be possible that there are compounds in the *Echinacea purpurea* extracts that may absorb some form of electromagnetic radiation and catalyze the dissociation of triplet oxygen to singlet oxygen; or perhaps a series of cascading electromagnetic radiation transformation as it is absorbed and release by different molecules which eventually caused the dissociation of triplet oxygen to singlet oxygen. It is also known that some catalysts cause singlet oxygen oxidation while a different, but similar catalyst can cause free radical oxidation. For example, Reddy et al. (1995) reported that a particular cobalt containing compound catalyzed oxidation of cyclic alkenes to form epoxides while a different but similar cobalt containing compound catalyzed oxidation of cyclic alkenes to form allylic alcohols or enones at 25°C in acetonitrile under dioxygen balloon. Perhaps, there is a compound found in *Echinacea purpurea* alcohol extracts that prefers the catalysis of alkamide oxidation via singlet oxygen oxidation rather than autoxidation.

It has been reported in literature that chlorophyll can act as sensitizers that cause formation of singlet oxygen (Lee and Min 1988, Fakourelis et al. 1987, Telfer et al. 1994). Telfer et al. (1994) reported that illumination of P680, chlorophyll a molecules of the primary electron donor, lead to the formation of singlet oxygen. The carotenoids, however, act as anti-oxidants by consuming singlet oxygen and returning them to their

steady state triplet oxygen (Lee and Min 1988, Fakourelis et al. 1987, Telfer et al. 1994). The formation of singlet oxygen due to chlorophylls was unlikely, as the roots of higher plants such as Echinacea, do not contain chlorophyll. Chlorophylls are found in chloroplast, however, in part of the plants that do not undergo photosynthesis, chloroplasts are not formed (Oyama 1997, Magill 2007). The proplastids that chlorophylls are developed from, do not differentiate into chloroplasts in plant parts that do not undergo photosynthesis, but do differentiate into other plastids such as amyloplasts, which is used as starch storage; chromoplast, which gives carrots its color, elaioplast, which is used as fat storage; and other forms of plastids (Magill 2007).

However, a more likely cause for singlet oxygen oxidation were the termination of secondary hydroperoxides from the oxidation of alkamides via a tetraoxide intermediate which can decompose into a ketone, an alcohol and oxygen, just as when lipids undergo singlet oxygen oxidation (Frankel et al. 1979); and oxygen reaction with metals of variably valencies (Frankel 1998).

A further study by Cumpston et al. (1997) revealed that UV and visible light are not necessary to induce singlet oxygen formation with specific sensitizers, that perhaps heat, which is radiation in infra-red range of the electromagnetic radiation spectrum, is responsible for singlet oxygen formation in their experiment. However, it might also be not necessary for triplet oxygen to form singlet oxygen for singlet oxidation to occur. According to Labuza (1971), singlet oxygen reacted about  $10^3$  to  $10^4$  times faster than normal oxygen with methyl linoleate. Perhaps this is the reason why the alkamides oxidized slowly in both this experiment and that of Liu and Murphy (2006).

Bulky moieties, on the other hand, provide more stability by sterically blocking the attack of electrophilic species like oxygen (Cumpston and Jensen 1998); in this case, the methyl group is certainly bulkier than a hydrogen atom. According to Masuda et al. (1985), methylated acetylenes are more stable than non-methylated acetylenes. This is perhaps the reason why in the Dry, alkamides 1 and 2, which have terminal acetylene bonds oxidized faster than alkamides 3, 5, 7, 8, 9, 10 and 11 all of which do not have terminal acetylene bonds. This is consistent with the experiments done by Frankel et al. (1980), in which they reported that in fatty acyl methyl esters, the double bonds that is more external were more prone to oxidation than double bonds that were more internal. However, it does not explain why alkamide 4, which also has an acetylene terminal end, was more stable than alkamides 1 and 2. But, the report given by Liu and Murphy (2006) stated that alkamides 1, 2 and 4 (6) have the same rate of oxidation, which would support that terminal acetylene bonds are more susceptible to oxidation than methylated acetylene bonds. Cumpston and Jensen (1998) reported that ethylene bonds were more prone to electrophilic attacks than acetylene bonds. It is very likely that the ethylene bonds in alkamides are the ones degrading instead of acetylene bonds. Cumpston and Jensen (1998) reported that terminal acetylene bonds are more stable than terminal ethylene bonds. Since acetylene bonds are more stable than ethylene bonds and unsaturated bonds that have a bulky moiety are more stable, it is also likely true that terminal acetylene bonds with a methyl attached are more stable than terminal ethylene bonds with a methyl attached. Alkamides 8 and 9, both of which have methylated terminal ethylene bonds are

less stable than alkamides 3 and 7, both of which have methylated terminal acetylene bonds.

It is very unlikely that these alkamides underwent decomposition into individual elements rather than oxidation as it has been reported that disubstituted acetylenes are stable in air at 120°C for at least 20 hours (Masuda et al. 1985) and oleate hydroperoxides were formed when triolein was heated at 192°C (Frankel et al. 1981). In addition, many experiments on fatty acids have been carried out at 140°C and the initial products are oxidation products (Frankel 1998) and not molecular decomposition into elements. Moreover, Masuda et al. (1985) reported that none of the acetylene polymers that they experimented on, including methylated and non-methylated acetylenes, degraded under vacuum up to 200°C, which means that oxygen is required for degradation to occur; in addition, they found oxygen carbonyl and hydroxyl absorption bands in the degradation products of these acetylenes under IR spectroscopy, which implies that oxygen is required in the degradation of these acetylenes in alkamides.

In the DMSO treatment, the 3-way interaction between phenol, alkamide and temperature was not statistically different. Therefore, a general ranking order can be established for the  $k$  values of the alkamides. The order of degradation for the alkamides in DMSO was  $9 \approx 8 > 2 > 1 > 5 \approx 7 \approx 4 \approx 10 \geq 11 \approx 3$ . This is remarkably different from the DRY treatment, in which alkamides 9 and 8 degraded faster than alkamides 1 and 2. The reason for this is unknown, but it may be possible that the DMSO somehow made the bulkier methyl ends of alkamide 8 and 9 less effective in sterically blocking electronegative species from attacking the ethylene bonds close to the terminal in

alkamides 8 and 9. The rest of the alkamides in DMSO had almost the same rate of degradation compared to each other and are the most stable. Another possible reason is that in DMSO, alkamides undergoes autoxidation rather than photo-oxidation, which would make Alkamides 8 and 9 degrade faster than the rest of the alkamides, however, the pattern is still not perfect as some alkamides with more double bonds are more stable than some alkamides with fewer double bonds. Since there is limited oxygen in DMSO especially at such high temperatures of 90, 100 and 110°C, it is more likely that free radical oxidation of the alkamides were taking place in DMSO rather than singlet oxygen oxidation; however, the result does not support this since the alkamides with more acetylene bonds, alkamides 1, 2, 3, 4, 5, and 7, should have been more susceptible to free radical oxidation, which contradicts the report in this experiment. Then perhaps another electrophile may be responsible for oxidation in DMSO. This electrophile may have been given off by the solvent, DMSO, through the release of oxygen, or sulfur oxides.

According to the oxidation experiment of Yang et al. (2000) on conjugated linoleic acid free fatty acid, *trans* bonds are stable while *cis* bonds are not; and a mixture of *trans* and *cis* bonds are more stable than all *cis* bonds. This report by Yang et al. (2000) supported the results of all 4 treatments of this stability study and also the stability study of Liu and Murphy (2006) on the oxidation of the alkamides; where alkamide 9, which has 2 *cis*-bonds and 2 *trans*-bonds, was always more prone to oxidation than alkamide 8, which has 1 *cis*-bond and 3-*trans*-bonds. This is because *cis* bond has a higher free energy level and more vulnerable to oxygen attack (Yang et al. 2000). As for alkamides 1 and 2, both of which have the same chemical structure except for the

interchange of the *cis-trans* position of the 2 double bonds; alkamide 2 in Dry is more stable than alkamide 1 in Dry, which may be because the more unstable *cis* bond in alkamide 1 is farther away from the more protective electronegative amide than alkamide 2, making alkamide 1 more prone to oxidation than alkamide 2. As for DMSO, alkamide 1 and 2 had an opposite result. It is unknown why this happened.

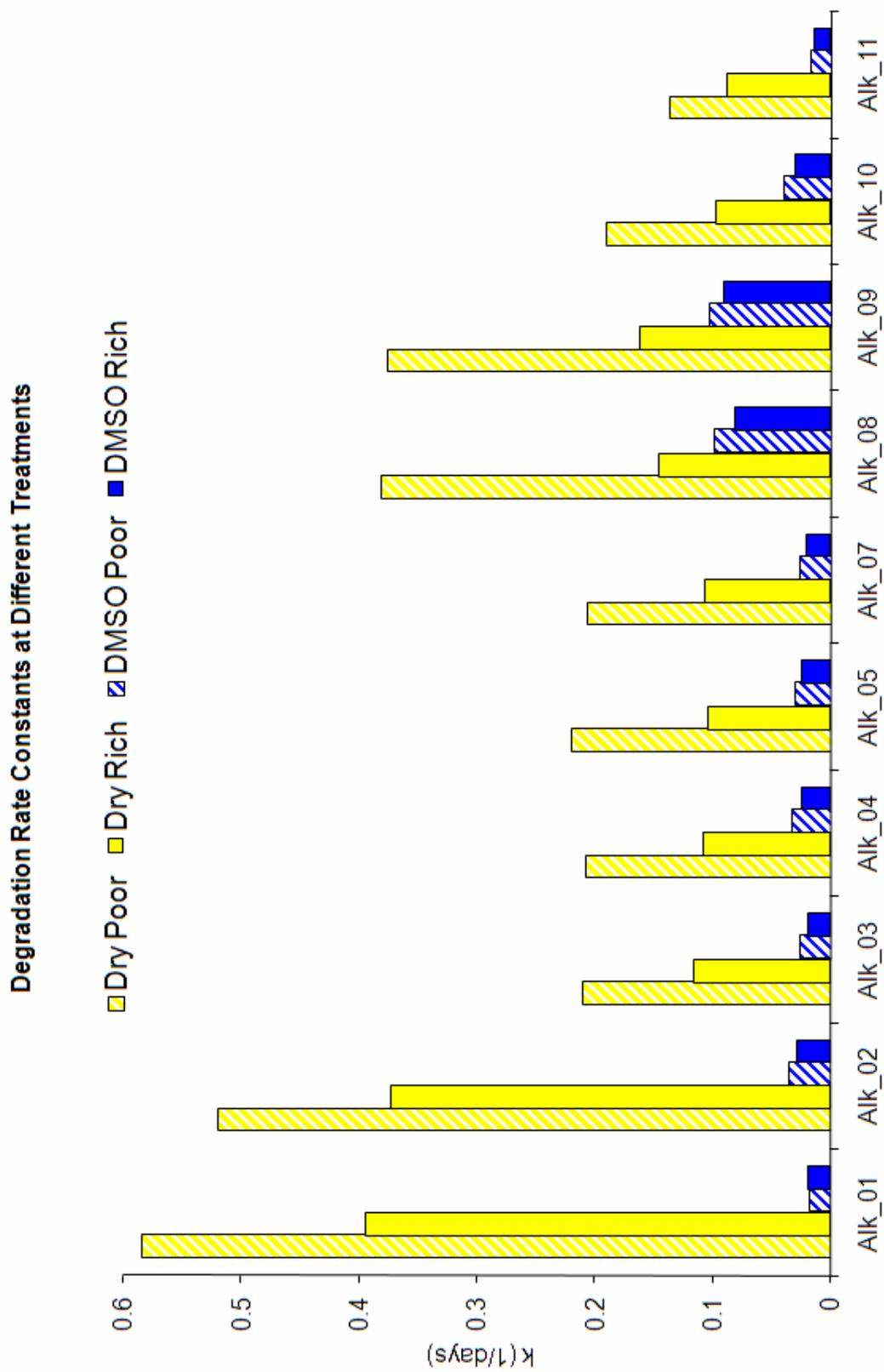
Since all the treatments had 1 storage temperature in common (90°C), comparison can be made between conditions for each alkamide at this temperature. Figure 4-7 shows the k values of each alkamide for each treatment at 90°C.

Figure 4-7 illustrates that all the alkamides at 90°C were least stable in the following order: Dry Poor > Dry Rich > DMSO Poor = DMSO Rich. As expected, the alkamides are least stable in Dry Poor since the protective anti-oxidants have been removed; followed by Dry Rich since there is more surface area for the oxygen to react with the alkamides. DMSO Rich and DMSO poor were expected to be most stable since there is limited oxygen for oxidation to occur.

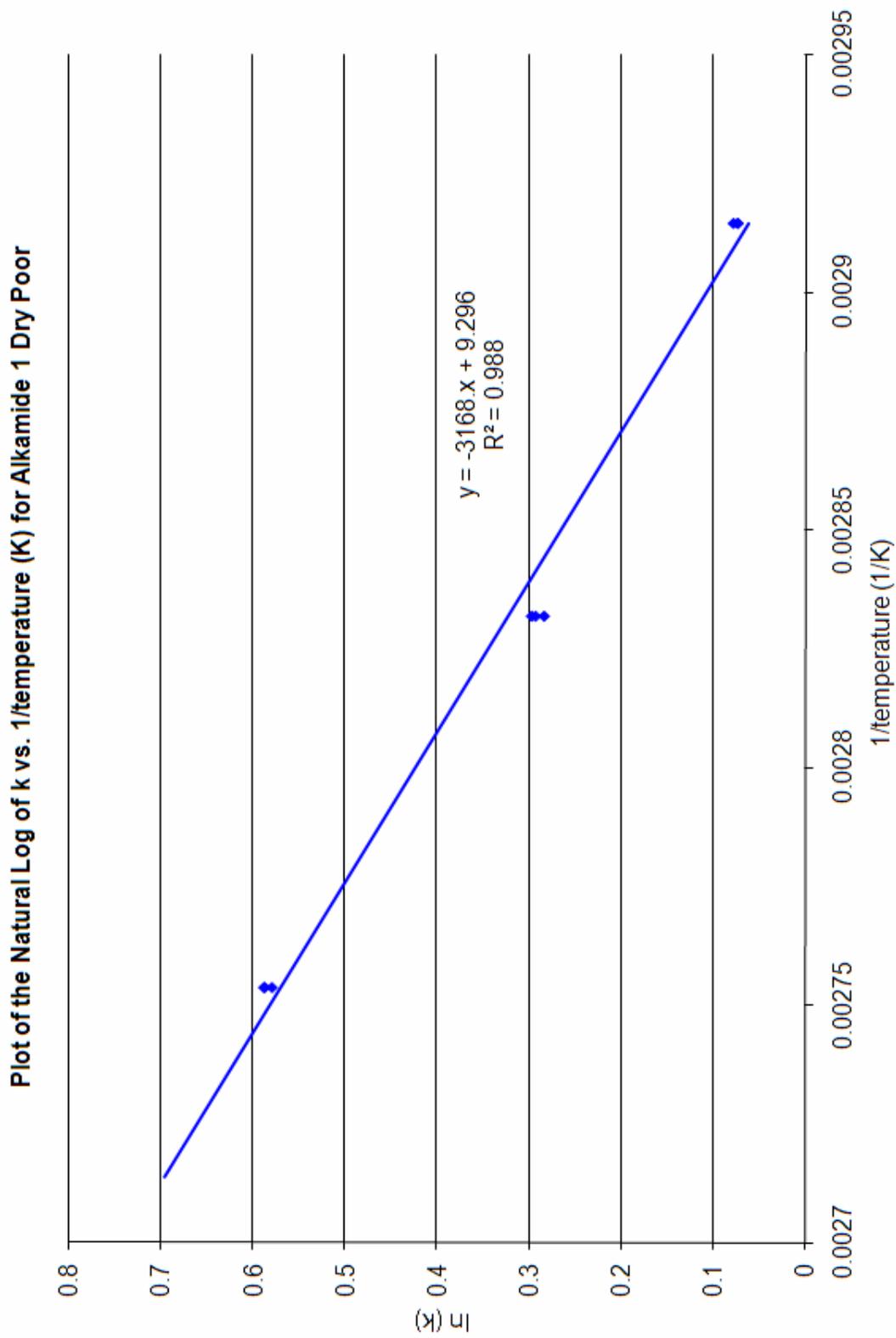
#### *Extrapolation of degradation rate constant k and half-life at 25°C*

An Arrhenius plot of the natural log of k versus the reciprocal of temperature in Kelvin for each alkamide for each treatment of phenol (Rich / Poor) and solvent (Dry / DMSO) was obtained to extrapolate the degradation rate constants k in order to calculate the half-lives at 25°C (see Figure 4-8). From the rate constants, the half-lives were calculated using the formula  $\ln 2/k$ . Figures 4-9 and 4-10 illustrate the k values at 25°C for Dry and DMSO respectively.

**Figure 4-7.** Degradation rate constants at 90°C for alkamides at each condition



**Figure 4-8.** Arrhenius plot of  $\ln k$  versus  $1/\text{temperature (}^\circ\text{K)}$  of Alkamide 1 Dry Poor



The results shown in Figures 4-9 and 4-10 for the degradation rate constant at 25°C have very large standard errors of up to 228% relative to  $k$ . This is due to the extrapolation to a temperature that is far from experimental temperatures. According to Benson (1960), analytical precision and extent of the degradation are both important in determining the standard error of the measured reaction rate. The analytical precision in this experiment was at best 10% due to the measurement of minute quantities and the errors inherent in measuring volumes as was needed for dissolving the Dry extracts prior to HPLC analysis and the errors associated with adding DMSO to bring back up the volume that was reduced due to evaporation in DMSO Rich and DMSO Poor. From Table 4-1, it can be concluded that there is at least 30% error with an analytical precision of 10% and extent of degradation of 1 half-life. This error represents only the error of analysis. It does not yet include random errors associated with any scientific experiment. From Figure 4-6 a-d, which shows the rate constants  $k$  at experimental temperatures, the errors associated with the measured  $k$  values can be seen on all bars. In addition, the farther away the temperature used for extrapolation, the greater the standard error as can be seen in Figure 4-11. This is the reason why more rate constants  $k$  at more temperatures plotted would help lower the standard error of the extrapolated  $k$  value at room temperature. Labuza and Riboh (1982) found huge statistical errors in the accelerated shelf life experiment for lysine loss in whey, thiamin loss in pasta and lipid oxidation in potato chips. They suggested that more data points and at more temperatures be taken, but both costs money (Labuza and Riboh 1982).

**Figure 4-9.** Extrapolated degradation rate constant  $k$  at 25°C in Dry

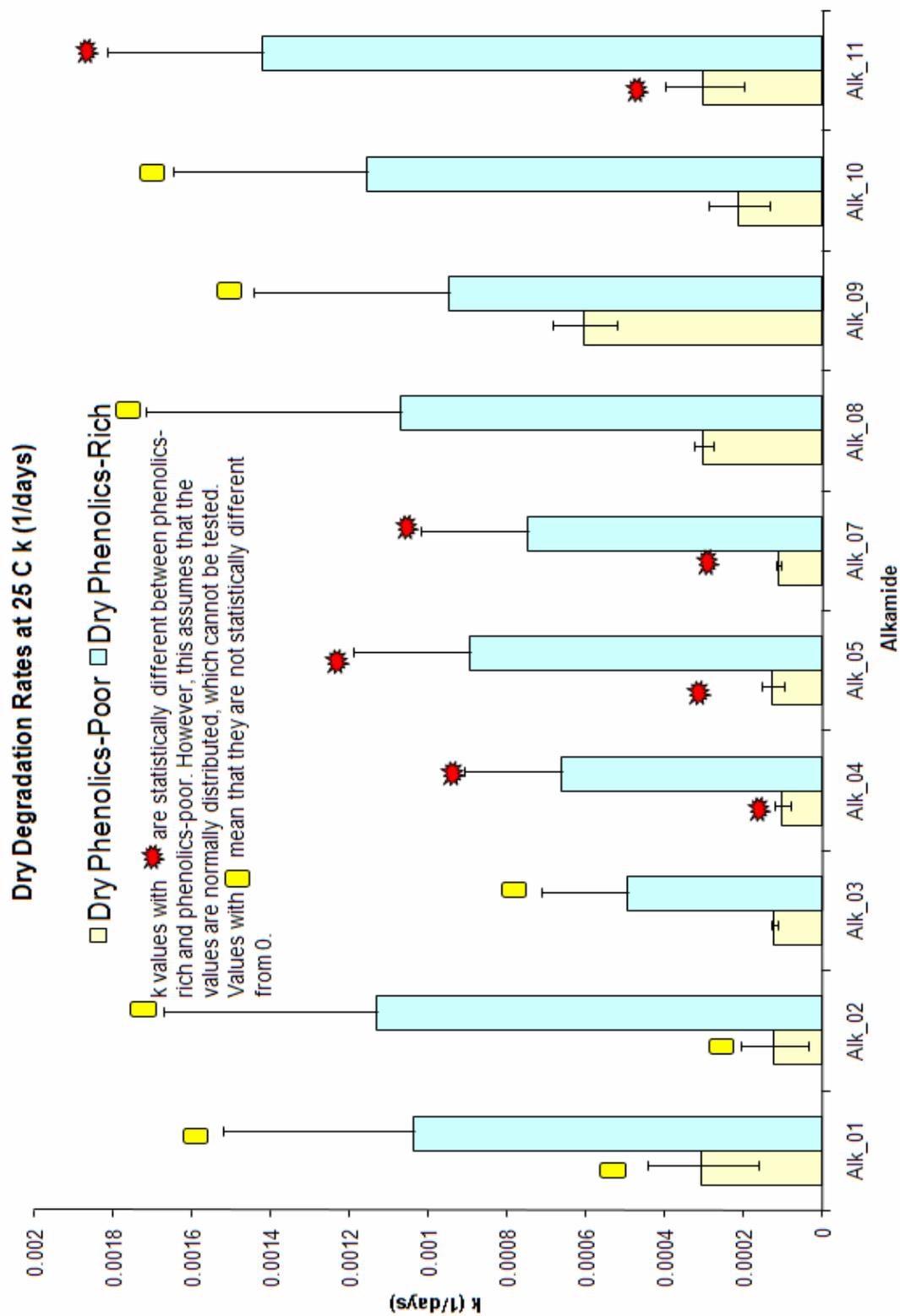


Figure 4-10. Extrapolated degradation rate constant  $k$  at 25°C in DMSO

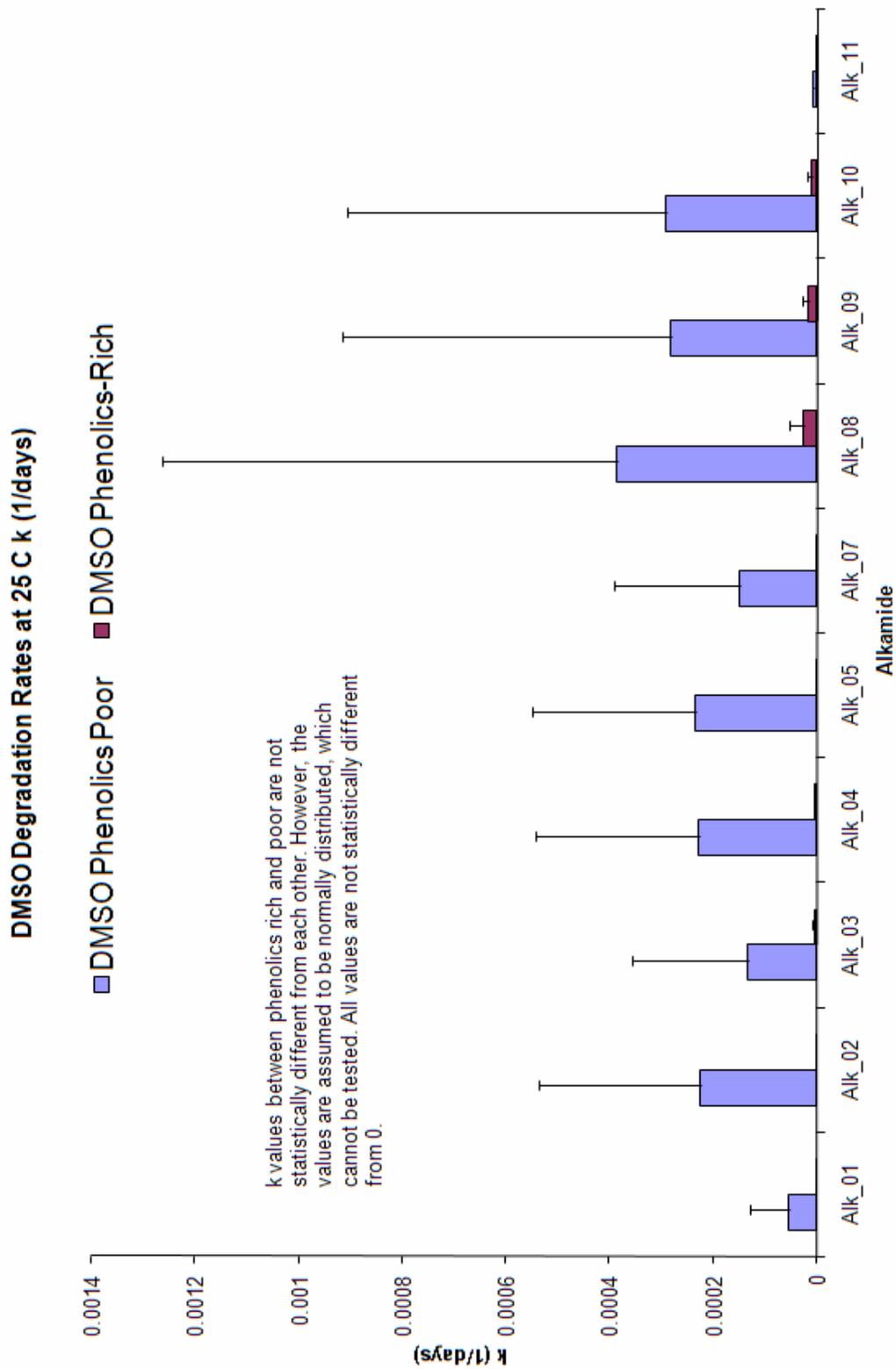


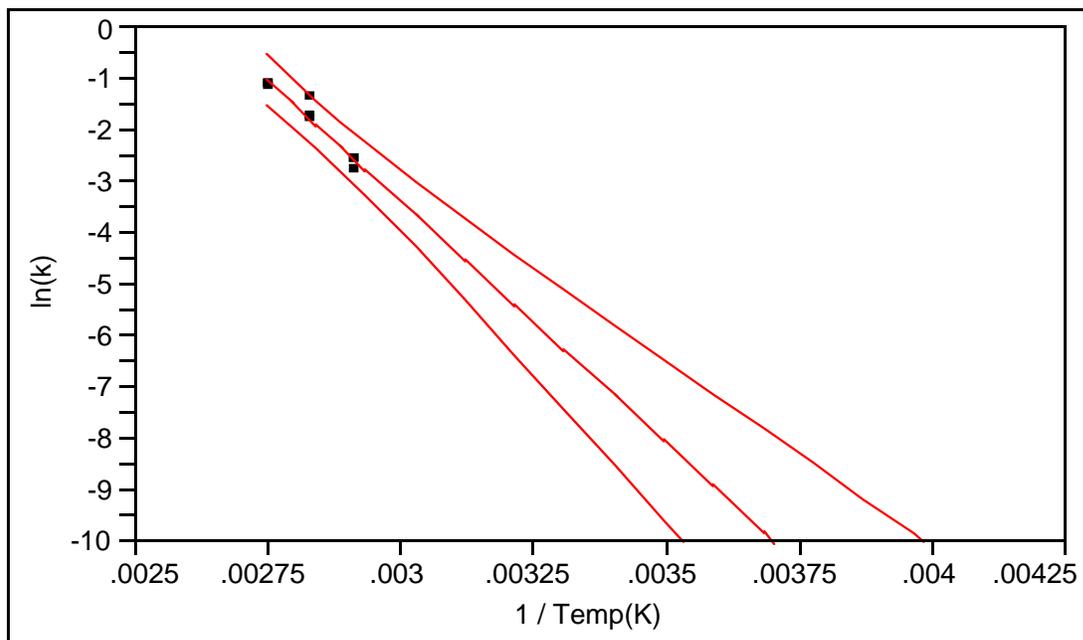
Table 4-1. Errors in calculated rate constants caused by analytical errors.

## Effect of Analytical Precision of the Method on the Estimation of the Rate Constant for Chemical Reactions

analytical precision%	Percent Error in reaction rate constant k at a given percent change in reaction species monitored						
	1%	5%	10%	20%	30%	40%	50%
0.1	14	2.8	1.4	0.7	0.5	0.4	0.3
0.5	70	14	7	3.5	2.5	2	1.5
1.0	>100	28	14	7	5	4	3
2.0	>100	56	28	14	10	8	6
5.0	>100	>100	70	35	25	20	15
10.0	>100	>100	>100	70	50	40	30

(Taoukis and Labuza 1996, Benson 1960 )

**Figure 4-11.** Extrapolation of the degradation rate constant k at 25°C for alkamide 1 in Dry Poor. The dashed lines represent the standard errors, while the solid line represents the calculated values of k.

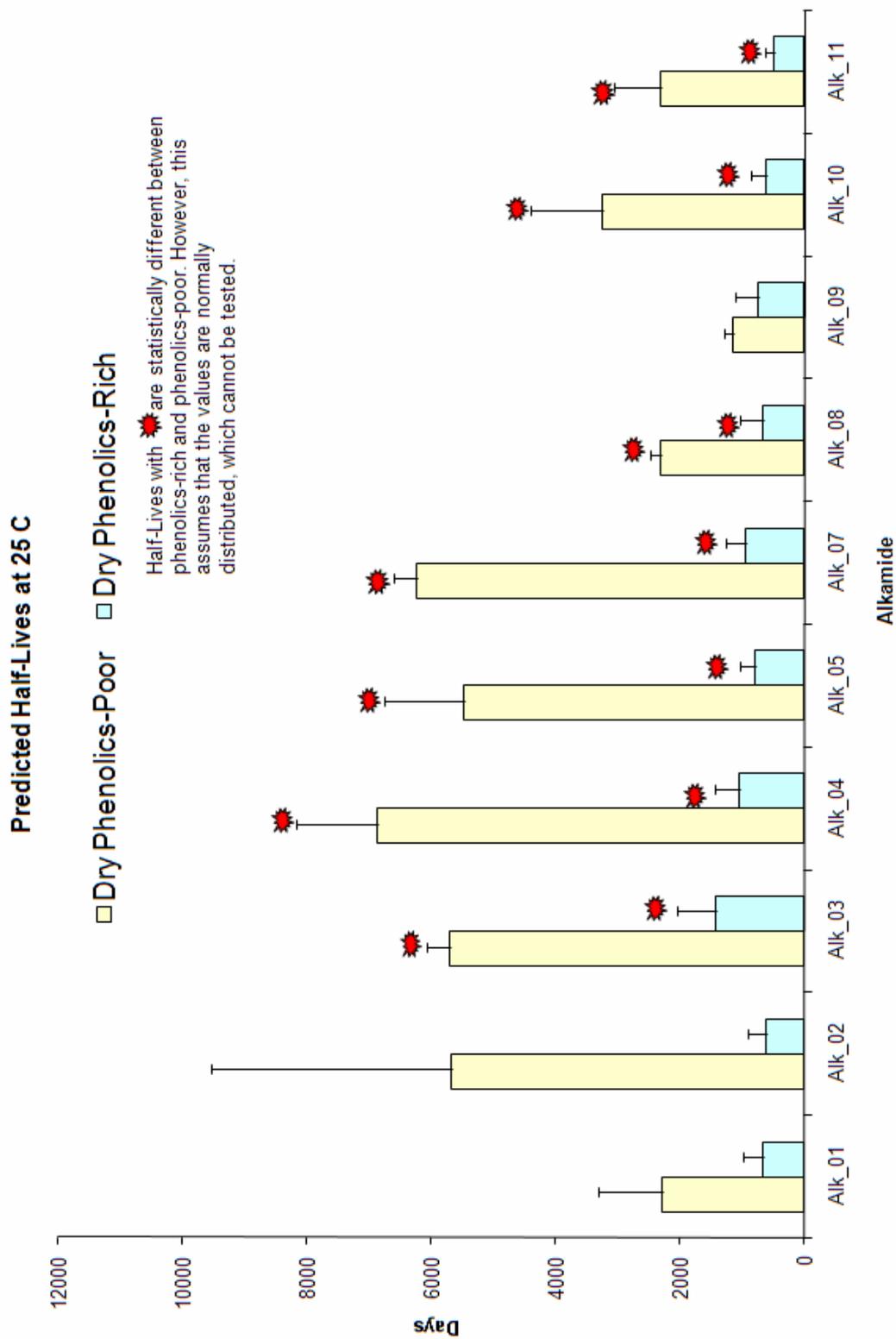


In Figure 4-11, even though the  $r^2$  value of 0.9238 for this slope is good as it is very close to 1.0, the mere extrapolation of the degradation rate constant  $k$  to a much lower or higher temperature causes the standard error to be large. The further away from the experimental temperature, the greater the standard error is. As a result, most of the  $k$  values in Figure 4-9 and 4-10 are not statistically different from 0, in addition, most  $k$  values are also not statistically different from Dry Rich versus Dry Poor and DMSO Rich and DMSO Poor.

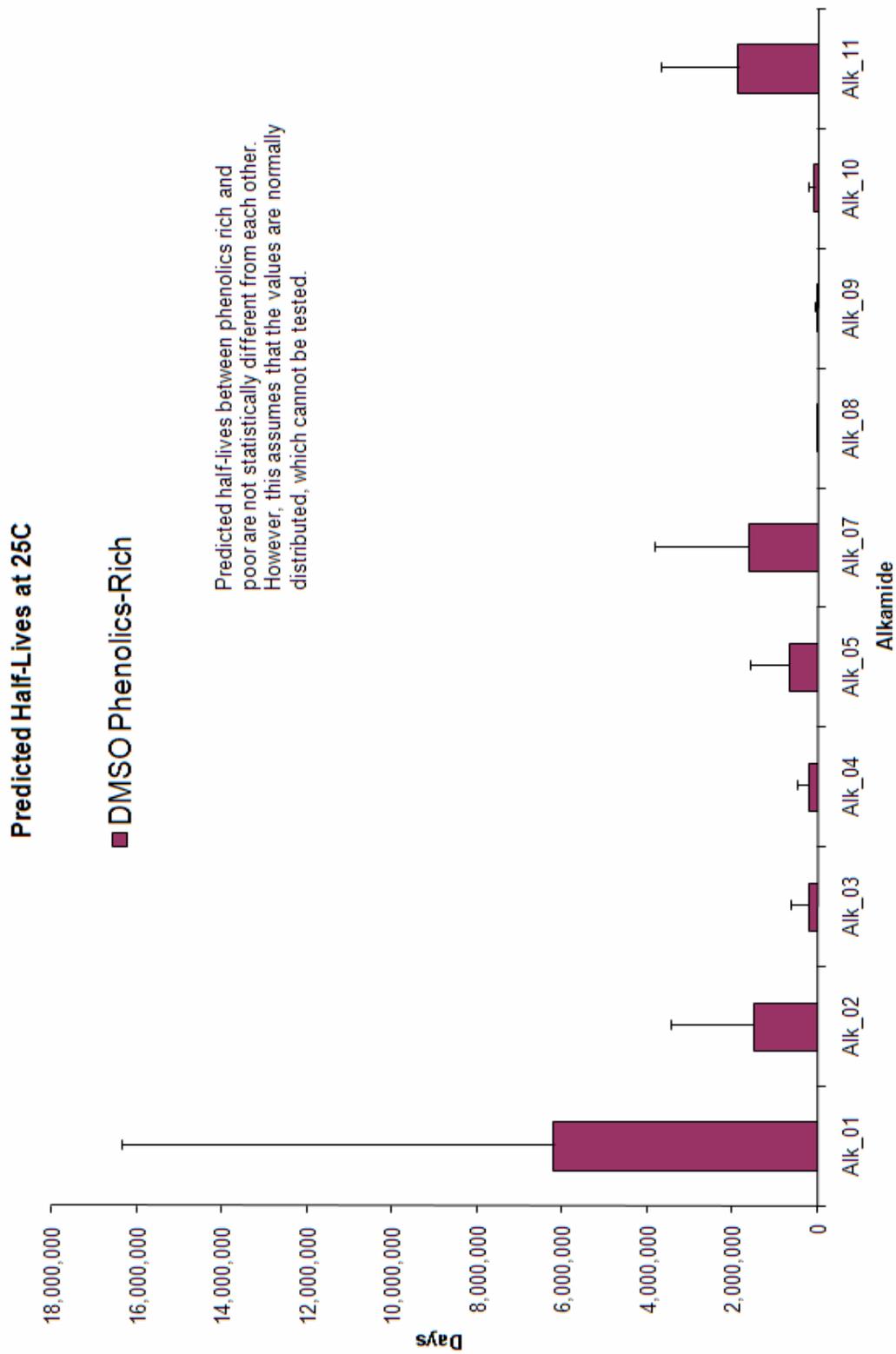
The degradation rate constants  $k$ 's can be converted to half-lives by using the formula  $\ln 2/k$  for 1<sup>st</sup> order reactions. Figures 4-12 and 4-13 show the half-lives at room temperature (25°C) for each alkamide in Dry and in DMSO, respectively.

From Figure 4-12, it can be seen that the half-lives of the alkamides for Dry Rich are much shorter than for Dry Poor, which is contrary to the half-lives at experimental temperatures in which the half-lives of the alkamides for Dry Rich are longer than for Dry Poor. Figure 4-13 illustrates that the half-lives of the alkamides for DMSO Poor are much shorter than for DMSO Rich, which is also contrary to the half-lives at experimental temperatures in which the half-lives of the alkamides for DMSO Poor are longer than for DMSO Rich. This is caused by the shifting of the slope in the Arrhenius plot, as will be discussed in the Energy of Activation section of this chapter. Liu (2006) also reported that the half-lives for Dry Rich are larger than the half-lives for Dry Poor. However, Liu (2006) reported that the half-lives for DMSO Rich are also larger than DMSO Poor, which contradicts the report of this experiment.

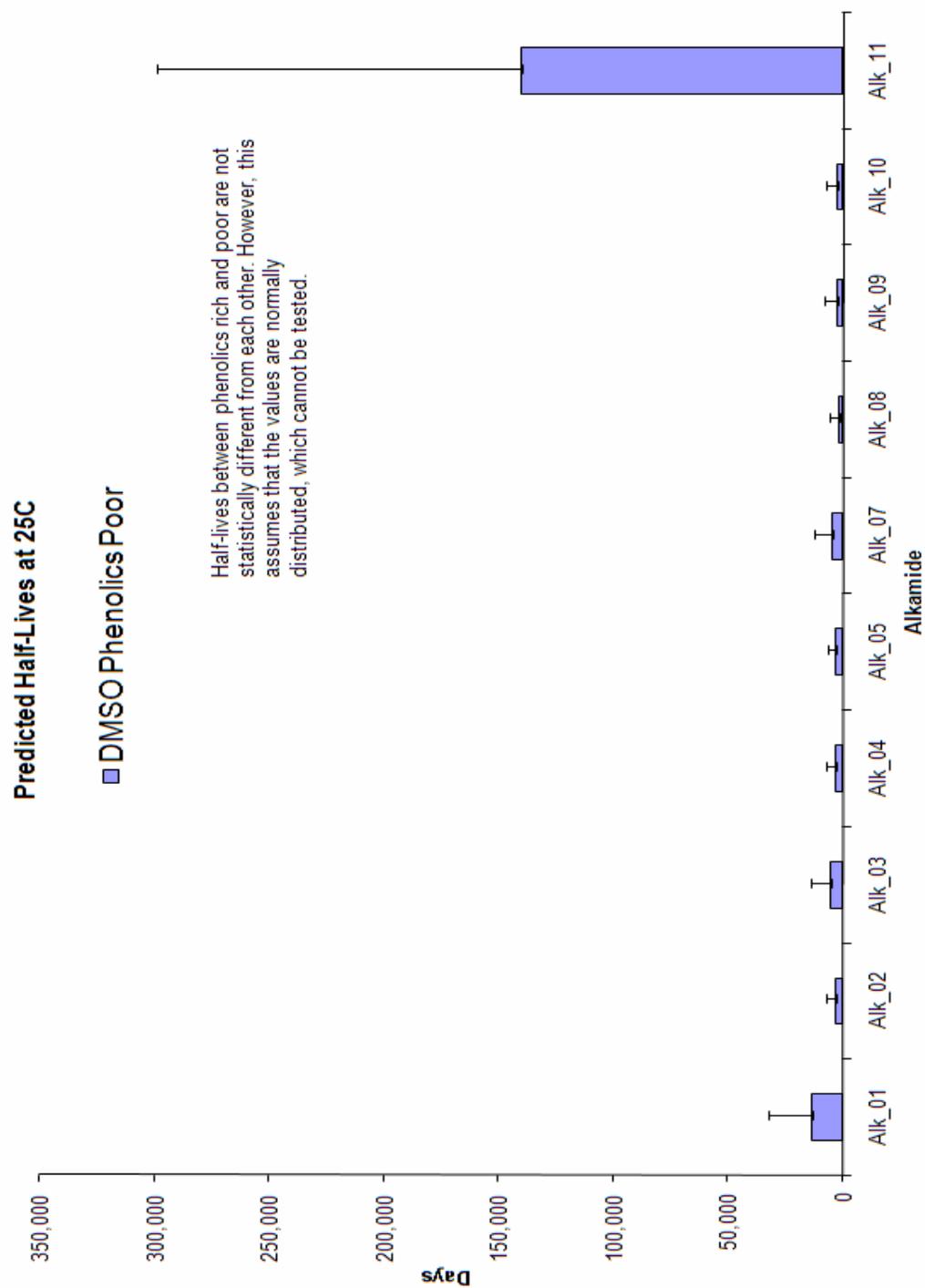
**Figure 4-12.** Predicted half-life of each alkamide in Dry to 25°C



**Figure 4-13a.** Predicted half-life of each alkamide in DMSO to 25°C.



**Figure 4-13b.** Predicted half-life of each alkamide in DMSO to 25°C.



At 25°C, the half lives of the alkamides in Dry Poor, which were in the range of 1000 – 7000 days, are much longer than the half-lives of the alkamides in Dry Rich, which are under 2000 days. Similarly, Liu (2006) reported that at 20°C, the half-lives of the alkamides in Dry Poor, which were in the range of 3000 to 60,000 days, are much larger than the half-lives of the alkamides in Dry Rich, which were in the range of 2,000 – 49,000 days. The half-lives of the alkamides in Dry Poor are not statistically different from each other, except for alkamide 8 and 9 which are statistically different from alkamide 3, 4, 5 and 7, in which alkamides are still more susceptible to oxidation than alkamides 3, 4, 5 and 7 as at the experimental temperatures. In Dry Rich, the half-lives of the alkamides are not statistically different from each other. In DMSO, the half-lives of the alkamides in DMSO Poor, which are in the range of 1800 – 140,000 days, are much shorter than the half-lives of the alkamides in DMSO Rich, which is in the range of 26,000 – 6,000,000 days. This is in contrast with Liu's (2006) report that the half-lives of the alkamides in DMSO Poor, which were in the range of 600 – 522,000 days, were longer than the half-lives of the alkamides in DMSO Rich, which were in the range of 280-2,700 days. Neither in DMSO Poor nor in DMSO Rich are the half-lives of the alkamides statistically different from each other.

Since the half-lives of the alkamides were longer in Dry Poor than in Dry Rich, it means that the alkamides are more stable in Dry Poor than Dry Rich, which has been the opposite at experimental temperatures and as hypothesized. Once again, the reason for this is due to the shifting of the slope of the Arrhenius equation, as will be discussed in the next section.

### *Energy of Activation*

The Arrhenius plot used for extrapolation of the degradation rate constants at room temperature can also be used to calculate the energy of activation ( $E_a$ ) of the degradation. Figure 4-8 illustrates the plot of  $\ln k$  versus the reciprocal of temperature in Kelvin for alkamide 1 in Dry Poor. The slope of the line is equal to  $-E_a/R$ , where  $R$  is the gas constant  $8.314472 \times 10^{-2}$  kJ/ mole\*K. Another important value that can be obtained from this is  $\ln(A)$ , which will be used to calculate  $\Delta S^\ddagger$ . The y-intercept of this Arrhenius plot is the  $\ln(A)$ . Figure 4-14 illustrates the  $E_a$  of each alkamide at every condition.

The  $E_a$  for the degradation of all the alkamides in *Echinacea purpurea* 95% aqueous ethanol Soxhlet extraction in Dry Poor is higher than in Dry Rich, which is the opposite of what is expected. Liu (2006) also reported higher  $E_a$  in Dry Poor than in Dry Rich as a general trend. According to Frankel (1998) and Ragnarsson et al. (1977), lipids in foods with anti-oxidants have higher energies of activation than lipids without anti-oxidants. Since the  $E_a$  is dependent on the slope of the Arrhenius plot, errors in  $E_a$  can be attributed to the large standard errors associated with the experimental  $k$  values as well as any possible shifting of the slope of the Arrhenius plot. There are multiple possible reasons as to how the slope of the Arrhenius plot could have shifted. One is that the slope of the Arrhenius plot may have shifted is due to changes in phase state at 1 or 2 experimental temperatures, which would have lead to erroneous prediction of half-life and  $E_a$ . Another possible explanation for this is that at 90°C, pro-oxidants, that may be present in both Dry-Rich and Dry-Poor, degraded, causing the degradation rate constants to be lower than expected at 90°C, thereby causing the slope to shift with Dry-Poor

having a steeper slope and Dry-Rich having a shallower slope. A third possible reason for this is more anti-oxidants or stronger anti-oxidants were created from the degradation of other compounds at 90°C in Dry Rich, causing the k value to be lower than expected and therefore forcing the slope to be shallower. From the data of this experiment as well as from the data from Liu (2006), there is a sudden increase in the k values at 90°C for Dry Poor, which would have caused a steeper slope than expected. A possible explanation for this sudden increase in k is the increase of pro-oxidants or the decrease of unknown anti-oxidants at 90°C in Dry Poor. A sixth possible explanation for the shifting of the slope in the Arrhenius plot is the degradation of pro-oxidants in Dry Rich at 90°C which would have caused the slope to be shallower.

It has been reported that some anti-oxidants without the presence of another anti-oxidant act as pro-oxidants as in the case of beta-carotene and  $\delta$ - and  $\gamma$ -tocopherol in soybean oil (Frankel 1998). It is possible that at 90°C in Dry Poor, an anti-oxidant that was necessary to prevent another anti-oxidant from becoming a pro-oxidant deteriorated which would have caused a faster oxidation than what would have been expected thereby increasing the degradation rate constant k and while at 90°C in Dry Rich, the phenolic caffeic acid derivatives may have been sufficient in off-setting the increase of the anti-oxidant that became pro-oxidants. While in DMSO, the energy of activation is higher in DMSO Rich than in DMSO Poor, which is also the opposite of what the result at experimental temperatures say about the degradation rate constant k – DMSO Rich has higher k values than DMSO Poor.

Frankel (1998) reported that the  $E_a$  of lipid oxidation in foods is between 62 and 105 kJ/mol, which is lower if pro-oxidants are present and higher if anti-oxidants are present. The  $E_a$ 's for alkamide oxidation reported in this study are similar to the  $E_a$  of lipid oxidation in foods. The difference in the  $E_a$  between Rich and Poor in a set solvent system as well as the  $E_a$  between solvent systems with a set phenol condition may represent a change in degradation mechanism, so thermodynamic parameters have to be calculated to investigate this further (Kirk 1981).

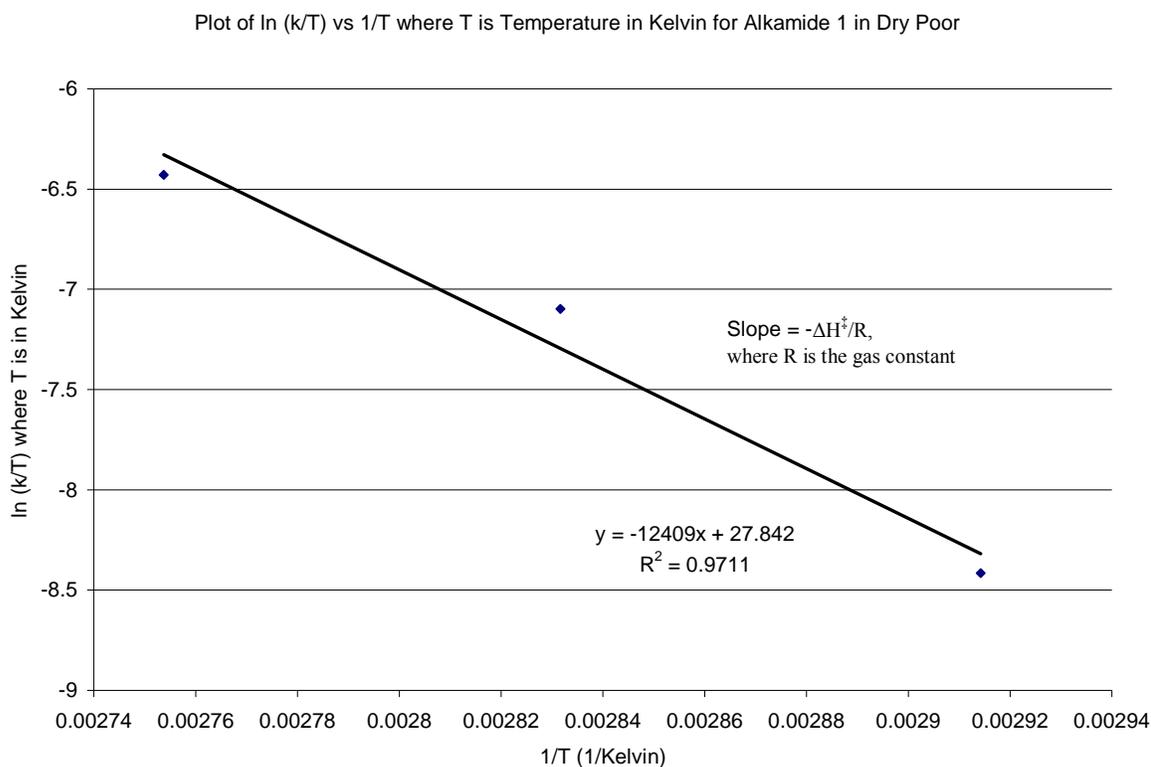
#### *Thermodynamics of degradation*

Other than the Arrhenius plot, another plot can be drawn using the degradation rate constants  $k$ 's obtained in the experiment. This plot is  $\ln(k/\text{temperature})$  versus the reciprocal of temperature, where the temperature is in °Kelvin. Figure 4-15 illustrate the plot of  $\ln(k/\text{temp})$  versus the reciprocal of temperature in °Kelvin for alkamide 1 in Dry Poor. From this plot (Figure 4-15), the  $\Delta H^\ddagger$ , which is the change in enthalpy of the reactants going to the intermediate molecule prior to going into products (Song 2007) can be obtained. The slope of the regression is equal to the negative of  $\Delta H^\ddagger/R$ , where  $R$  is the gas constant  $8.314472 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$

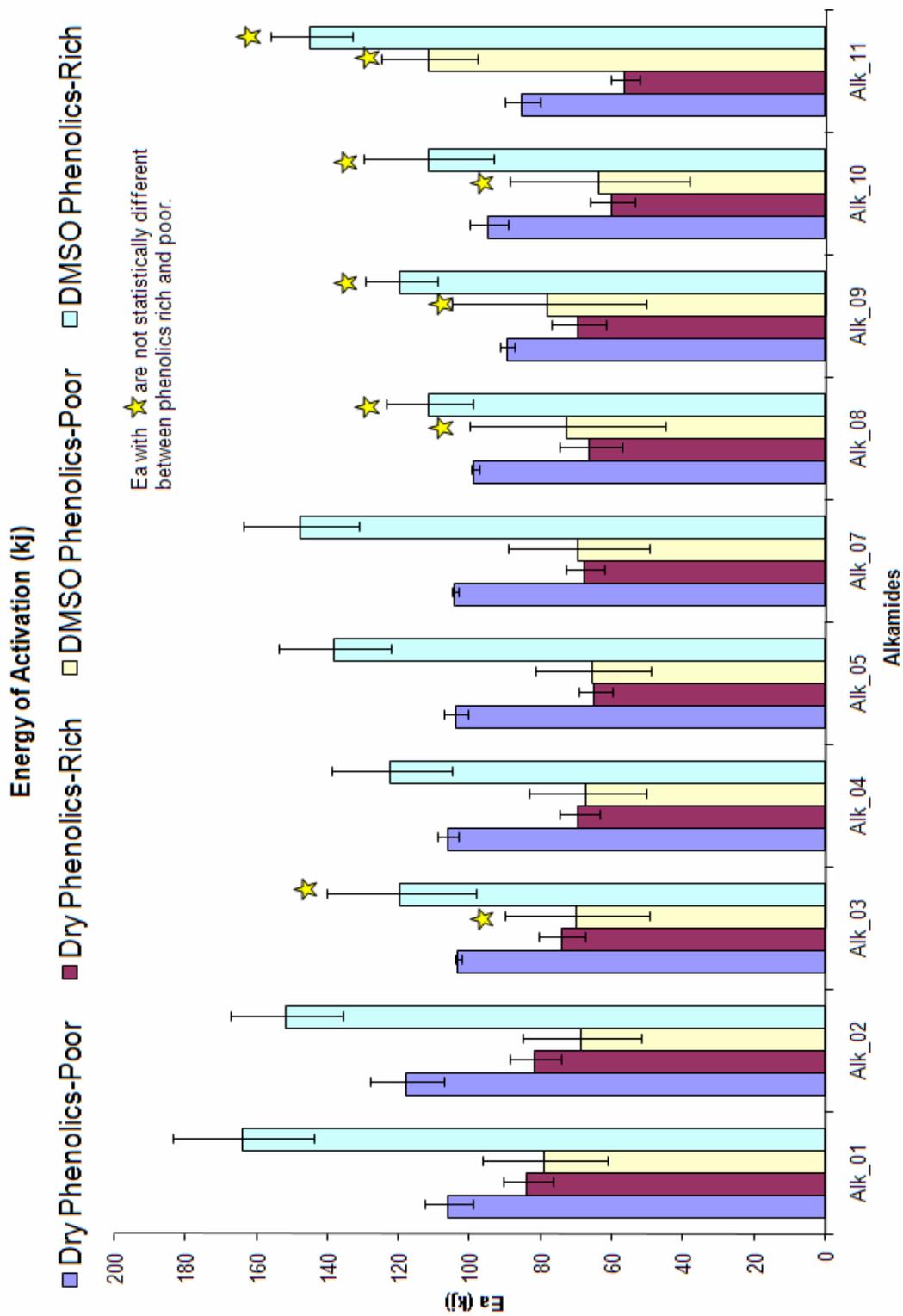
Figure 4-16 illustrates the  $\Delta H^\ddagger$  of each alkamides in Dry and in DMSO. In this study, all alkamides in all treatments have a positive  $\Delta H^\ddagger$ . If  $\Delta H^\ddagger$  is positive, it means that the reaction is endothermic, which means that the reaction can be controlled by lowering the temperature, i.e. cold storage (O'Keefe et al. 1991). However, because the  $E_a$  with a range of 60 – 150 kJ/mol is extremely low, which is similar to the  $E_a$  of lipid oxidation in foods in the range of 62 - 105 kJ/mol and higher if anti-oxidants are present

(Frankel 1998), the oxidation of alkamides cannot be halted all together. From figure 4-16,  $\Delta H^\ddagger$  had the greatest magnitude in DMSO Rich for all alkamides, followed generally by Dry Poor, DMSO Poor then by Dry Rich, all of which are different from each other. The reason for this is unknown but it suggests alkamide oxidation is following a different pathway in DMSO rich compare to the other 3 model systems.

**Figure 4-15.** Evaluation of enthalpy of activation ( $\Delta H^\ddagger$ ) for alkamide 1 in Dry Poor model system.



**Figure 4-14.** Energy of activation for degradation of each alkamide at each condition



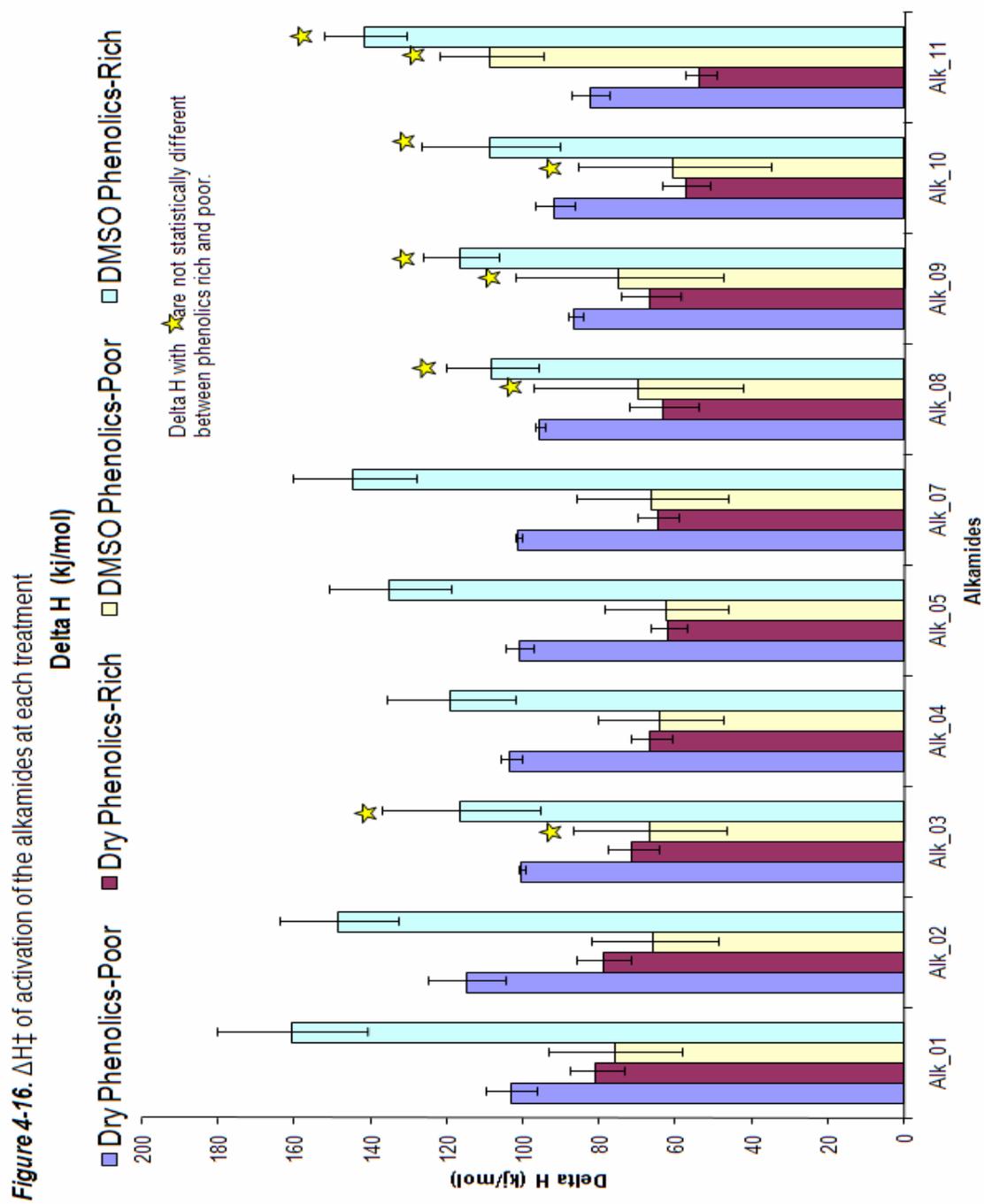
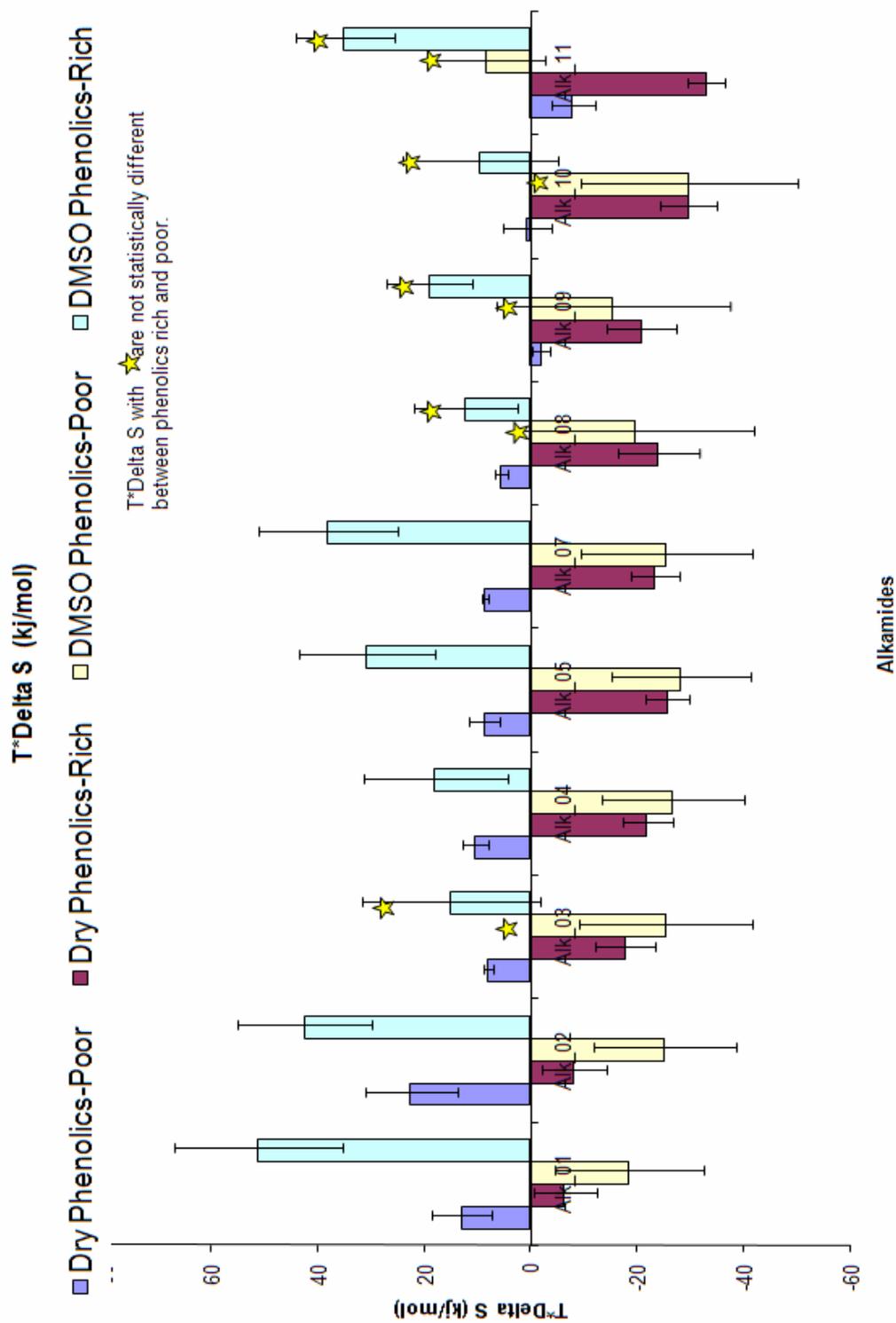


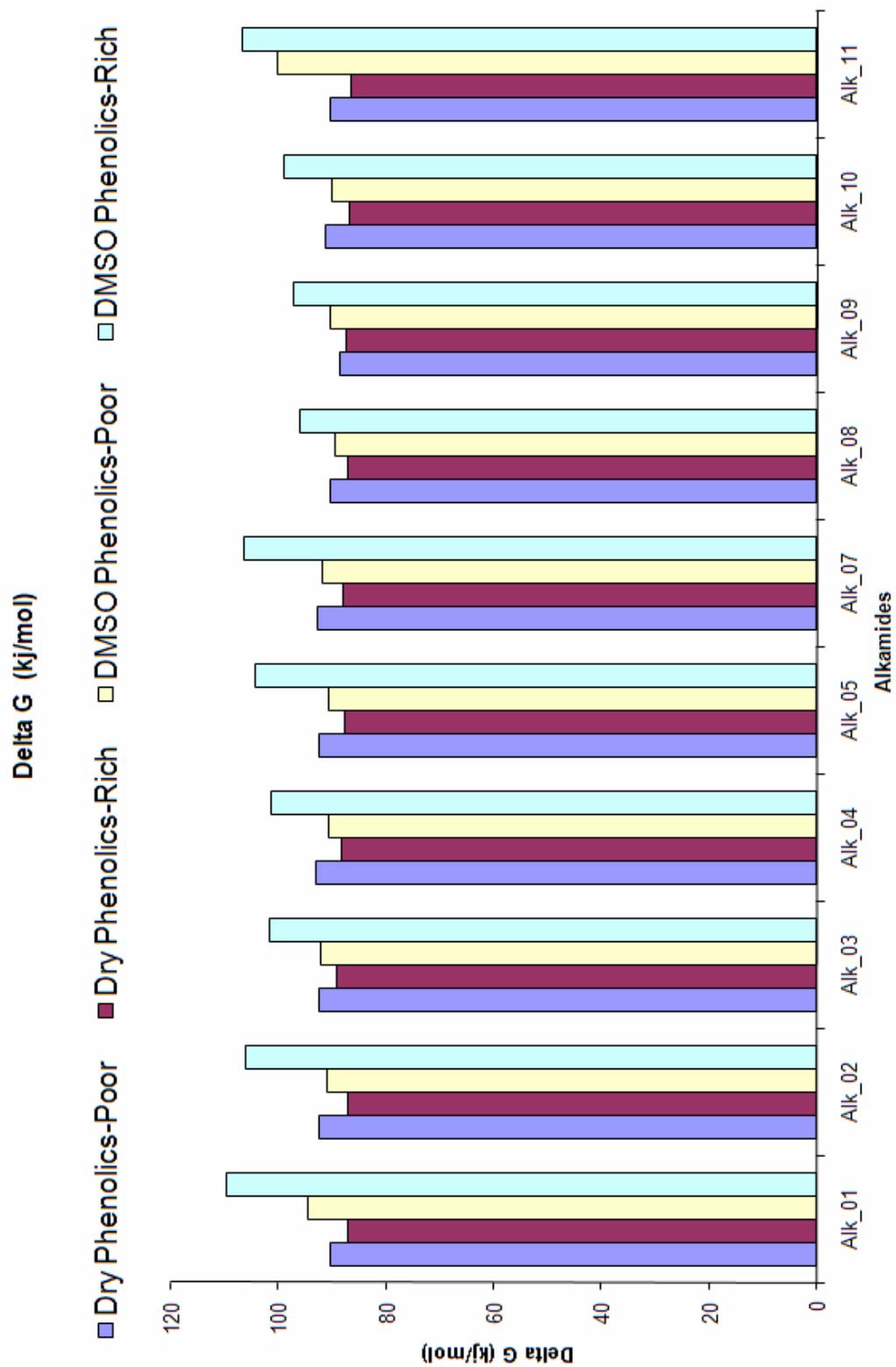
Figure 4-17.  $T^*\Delta S^\ddagger$  of activation of the alkamides at each treatment



$\Delta S^\ddagger$ , the change in entropy of activation of the reactants going to the intermediate molecule prior to going into products (Song 2007), can be calculated using the equation  $\Delta S^\ddagger = R [\ln(A) - \ln(K_b T/h)]$ ; where  $\Delta S^\ddagger$  is the change in entropy in kilojoules per Kelvin, R is the gas constant,  $K_b$  is Boltzmann's constant  $1.38 \times 10^{-26}$  in kJ/K and h is Planck's constant  $6.626 \times 10^{-37}$  in kJ\*s,  $\ln(A)$  is y-intercept in the Arrhenius plot. Figure 4-17 illustrates the  $T\Delta S^\ddagger$  of each alkamide in Dry and in DMSO.

In the report of Kirk (1998) on the thermodynamics of activation of retinyl acetate, there is a compensation effect between  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  of retinyl acetate stored at 2 different conditions, which resulted in a constant  $\Delta G^\ddagger$  thereby indicating that same degradation mechanism of retinyl acetate was taking place at the 2 different storage conditions. It can be deduced that since from Figure 4-16, all the alkamides have a positive  $\Delta H^\ddagger$  in Dry Poor and DMSO Rich, except for alkamide 9 and 11 in Dry Poor, while in Dry Rich and DMSO Poor all of the alkamides have a negative  $T\Delta S^\ddagger$ , except for alkamide 11 in DMSO Poor. The reason for the difference is the compensation effect. The entropy is balancing the differences in enthalpy of activation to give a relatively constant free energy of activation. It also has consequences in the calculations of  $\Delta G^\ddagger$ , the change in Gibb's free energy. The change in Gibb's free energy,  $\Delta G^\ddagger$ , is obtained using the formula  $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$ , where T is temperature in Kelvin and  $\Delta G^\ddagger$  is in kJ. Figure 4-18 illustrates the  $\Delta G^\ddagger$  of each alkamide in Dry and in DMSO.

**Figure 4-18.**  $\Delta G^\ddagger$  of activation of the alkamides at each treatment



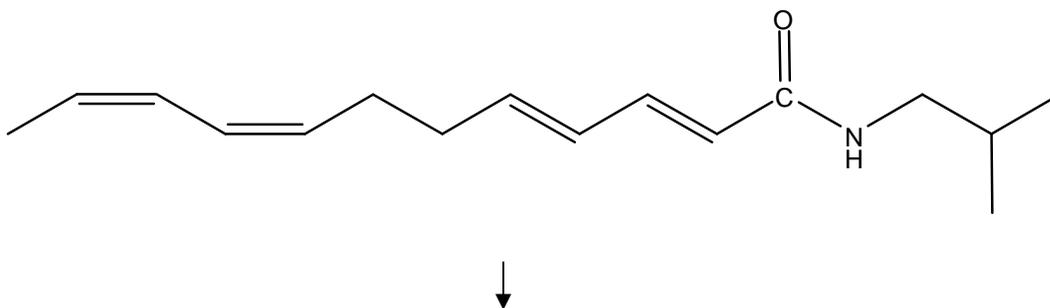
If  $\Delta G^\ddagger$  of activation is positive, it means that the reaction is not spontaneous (Chang 2002), and there is some other species that is initiating the reaction other than enthalpy and entropy, that is driving the reaction, which fits with the lipid oxidation initiation mechanism. Since all of the  $\Delta G^\ddagger$  for all alkamides at all conditions are positive, it means that the alkamides do not spontaneously undergo the reaction to reach the activated state. Another molecule or cause is needed for the alkamide to turn into intermediates. In this study, it seems that an electrophile, most likely singlet oxygen, is responsible for this reaction.

If the enthalpy of activation ( $\Delta H^\ddagger$ ) is bigger than the entropy of activation ( $\Delta S^\ddagger$ ), then it is the enthalpy that is driving the reaction (Chang 2001). What this means is the heat is the one responsible for converting the reactants to products. On the other hand, if entropy is bigger than enthalpy, it is the entropy that is driving the reaction. What this means is that the natural tendency of the reactants wanting to go to products is what is driving the reaction. In Figures 4-16 and 4-17, it is apparent that  $\Delta H^\ddagger$  has a larger magnitude than  $T\Delta S^\ddagger$ , which means that enthalpy has a larger effect in driving the reaction than does entropy.

As discussed in the Energy of Activation section of this chapter, the  $E_a$  between Rich and Poor in both Dry and DMSO are different, which signifies a change in degradation mechanism; however, this is not necessarily true and the thermodynamics of the degradation of the alkamides should be looked at. Since the  $\Delta G^\ddagger$ ,  $\Delta H^\ddagger$ , and  $\Delta S^\ddagger$  have been obtained, this can now be verified or discredited. As can be seen in Figures 4-16 and 4-17, in all conditions for every alkamide, as  $\Delta H^\ddagger$  increases in magnitude,  $T\Delta S^\ddagger$  decreased

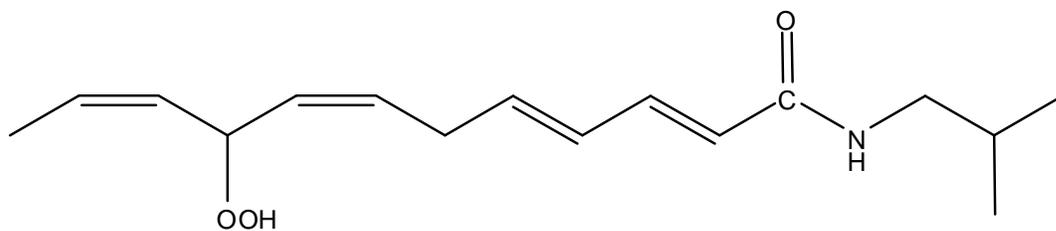
in magnitude, therefore there is an apparent compensation effect between the  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$ , which results in  $\Delta G^\ddagger$  to be the same for every alkamide in every condition except in DMSO Rich, thereby indicating that the degradation mechanism in all of the conditions are the same, except DMSO Rich are the same.

*Hypothesized mechanism of singlet oxygen oxidation of alkamide 9*

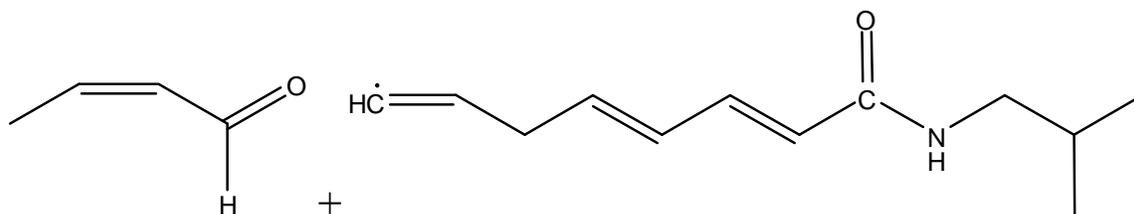


Alkamide 9 contains 2 pairs of conjugated double bonds. It is likely that the conjugated double bonds farther from the isobutylamide moiety are the ones oxidizing since it is assumed that the stability of alkamide 9 in comparison to fatty acids is due to the isobutylamide moiety. As with the photo-oxidation of linoleic acid, singlet oxygen attacks a carbon with a double bond and shifts the double bond (Frankel 1998). Since it is more energetically unfavorable for 2 bonds to shift compared to only 1 bond to shift, it is unlikely that the oxygen will attack on either ends of the conjugated double bonds, leaving the 2 internal carbons of the conjugated double bonds more susceptible to singlet oxygen attack. In addition, since double bonds are electronegative, the 2 internal carbons of the conjugated double bonds should be more electronegative than the 2 external carbons of the conjugated double bonds because they are mutually closer to each other's electronegative properties. The hydroperoxide formed is now in between the double

bonds.



Further reactions then could cause the break down of the alkamide chain into 2 by-products as shown below.



## Chapter 5

### Conclusion & Recommendations for Future Work

Plants belong to the *Echinacea* species are one of the most popular herbal supplements in the United States and in Europe. Alkamides are characteristic of the genus *Echinacea* and are believed to have medicinal values. Alkamides are purported to have anti-inflammatory, anti-viral, and immunostimulatory properties. The three most popular *Echinacea* species used as medicine are *Echinacea purpurea*, *Echinacea pallida* and *Echinacea angustifolia*. The most widely used and cultivated in the United States is *Echinacea purpurea*. Iowa State University Botanicals Supplement Research Center has been evaluating the efficacies of these 3 *Echinacea* species as medicinal plants.

The roots of *Echinacea* species have been extracted at Iowa State University using 95% aqueous ethanol Soxhlet extraction for at least 6 hours. The extracts, as a whole, which includes the alkamides and includes several of the caffeic acid derivatives, caffeic acid, chlorogenic acid, echinocside, caftaric acid, cichoric acid and cynarin are generally stable.

Accelerated shelf life experiments were done on the alkamides of *Echinacea purpurea* with and without phenolics as dry films and as dissolved in DMSO. The four treatments were Dry Rich, Dry Poor, DMSO Rich and DMSO Poor. The experiment was carried out at 70, 80 and 90°C for the dry film and 90, 100 and 110°C as dissolved in DMSO. It was determined that the alkamides followed an apparent 1<sup>st</sup> order degradation

rate. The alkamides were most stable when dissolved in DMSO without phenolics, followed by when they are dissolved in DMSO with phenolics, in dry film with phenolics then dry film without phenolics. Our 1<sup>st</sup> hypothesis that alkamides are more stable with the anti-oxidants caffeic acid derivatives was true in dry films, but opposite when dissolved in DMSO. Our 2<sup>nd</sup> hypothesis that alkamides are more stable when dissolved in DMSO compared to as stored as dry films, due to the greater surface area of oxygen to react with the alkamides in dry films, was true. The degradation rate constant  $k$  of the alkamides at 25°C were extrapolated using the Arrhenius equation and the alkamides were found to be stable, with half lives in the 1000's of day in dry films and in 100,000 to millions of days when dissolved in DMSO. The energy of activation for the degradation of the alkamides were similar to food lipids, but are much more stable. The  $\Delta G^\ddagger$ ,  $\Delta H^\ddagger$ , and  $\Delta S^\ddagger$  were calculated and it was found that enthalpy played a greater role than entropy in the degradation of the alkamides, however, both  $\Delta H^\ddagger$ , and  $\Delta S^\ddagger$  compensated each other to give a similar  $\Delta G^\ddagger$  for each alkamide at every treatment, indicating a similar degradation mechanism in all 4 treatments.

Recommendations for future work include determination of degradation products of both caffeic acid derivatives and alkamides, through the use of mass spectrometry, IR spectroscopy and nuclear magnetic resonance.

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