# APPENDIX

## APPENDIX A:

## Lists of equipments, chemicals, and reagents

| **Equipments** | **Source** |
|---|---|
| SpectroMetric®3200 | Shimadzu, Japan |
| Spectrometer 1600 | Shimadzu, Japan |
| PTFE membrane (13 mm diameter, 0.45 μm) | LIDA, Kenosha, WI, USA |
| Minisart® microsyringe | MS*R250, FUJI, JAPAN |
| HPLC column (Waters Spherisorb® S5 ODS2) (4.6x10 mm Guard Cartridge) | Water Corporation, USA |
| Guard column | Water Corporation, USA |
| Nylon membrane (0.45 μm) | LIDA, Kenosha, WI, USA |

| **Chemicals &Reagents** | **Source** |
|---|---|
| Acetonitrile (HPLC grade) | Merk, Lichrosolv®,Germany |
| Ammonium ferrous sulphate | BDH Chemical, Sydney |
| 2,2'-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid (ATBS) | Fluka, Swizerland |
| 2,2'-azobis(2-amidinopropane) dihydrochloride | Sigma, St.Louis, MO, USA |
| Bovine serum albumin (BSA) | Roche Diagnosis, Sydney |
| Buthionine sulfoximine (BSO) | Sigma, St.Louis, MO, USA |
| Butylated hydroxytoluene (BHT) | BDH Chemicals, Sydney |
| Catalase | Roche Diagnosis, Sydney |
| Diallyl disulfide (purity 90%) | Sigma, St.Louis. MO, USA |
| Dimethyl sulfoxide (DMSO) | Sigma, St.Louis. MO, USA |
| Di-potassium hydrogen phosphate ($K_2HPO_4$) | Merck, Germany |
| 3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium | Sigma, St.Louis, MO, USA |

| | |
|---|---|
| 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB) | Sigma, St. Louis, MO, USA |
| Folin-Ciocalteu's phenol reagent | Merck, Germany |
| Gallic acid | Fluka Chemika, Switzerland |
| Glutathione (GSH) | Sigma, St.Louis, MO, USA |
| Glutathione reductase (GRx) | Sigma, St.Louis, MO, USA |
| Guanidine hydrochloride | BDH Chemical, Sydney |
| Hexane (AR grade) | BDH Chemical, Sydney |
| Horseradish peroxidase (5,000 U) | Sigma, St.Louis, MO, USA |
| Hydrogen peroxide (30%, 9.79 M) | CARLO ERBA, Antibiotics SpA, |
| Immunytop (aged extract power capusule) | KHAO-LA-OR Laboratory Ltd., Thailand |
| Kyolic (aged garlic extract power) (NZ) | Nutra-Life Health & Fitness, NZ, Ltd, Auckland, New Zealand |
| L-glutamine (200 mM) | Trace Scientific, VIC |
| Liposome from soybean lipid | Provided by Dr.Gebicki's Lab |
| Lipopolyscharide (LPS) | Sigma, MO, USA |
| Methanol (HPLC grade) | Lab-Scan, Thailand |
| NADPH | Sigma, MO, USA |
| N-acetylcysteine (NAC) | Fluka, Buchsa, Switzerland |
| N-(1-Napthly)ehytlenediamine dihydrochloride | Metheson, Coleman&Bell, Norwood, Ohio |
| Perchloric acid (PCA) (70-72%) | Merck, Darmstadt, Germany |
| Phosphoric acid | Sigma, St.Louis, MO, USA |
| Potassium chloride (KCl) | BDH Chemicals, Sydney |
| Potassium iodide (KI) | Sigma, St.Louis, MO, USA |
| Potassium persulfate | Retert Pharmaceutical Co. Island |
| Quercetin | Sigma, St.Louis, MO, USA |
| Sodium nitrate | Sigma, St.Louis, MO, USA |
| Sulfanilamide | Fluka, Buchsa, Switzerland |
| Tetrahydrofuran (HPLC grade) | Lab-scan, Thailand |
| Thiazolyl blue (MTT) | Calbiochem, San Diego, CA, USA. |
| 2-Thiobarbituric acid | Sigma, St. Louis, MO, USA |

| | |
|---|---|
| Trichloroacetic acid (TCA) | Aldrick, Milwaukee, MO, USA |
| Trolox | Sigma, St. Louis, MO, USA. |
| Xylenol orange, sodium salt | Sigma, St.Louis, MO, USA |

**Cell line and Media**

| | |
|---|---|
| U937 (monocyte cell line) | Donated by Dr. Gebicki |
| J774.2 (mouse monocytic-macrophage cell) | Donated by Dr.Usanee |
| RPMI 1640 | Gibco, Island, USA |
| Fetal calf serum (FCS) | Trace Scientific, VIC |
| Penicillin/Streptomycin (5,000 IU/ml penicillin G) | Sigma, St. Louis, MO, USA |

**Culture vessels, serological pipettes and centrifuge tubes**

| | |
|---|---|
| 96-well microtitration plates | Greiner, Frickenhausen, Germany |
| 24-well cell culture plate | Costa®, USA |
| 6-well cell culture plate | Costa® USA |
| 60-mm diameter petri dishes | Falcon, NJ, USA |
| 25-$cm^2$ flasks with vented cap | Iwaki, Chiba, Japan |
| 75-$cm^2$ flasks with vented cap | Iwaki, Chiba, Japan |
| 10 ml sterile plastic pipettes | Sterilin, Staffordshire, UK |
| 50 ml sterile plastic pipettes | Sterilin, Staffordsshire, UK |
| 10 ml sterile centrifuge tubes | Iwaki, Chiba, Japan |
| 50 ml sterile centrifuge tubes | Iwaki, Chiba, Japan |

# APPENDIX B:

## Reagent preparation

### 1. Reagents for antioxidant activity assays

#### 1.1. ABTS decolorization assay solution

14 mM ABTS solution: 1 g of ABTS dissolved in 100 ml of deionized water (14 mM)

5 mM Potassium persulfate solution; 0.007g of potassium persulfate dissolved in 2 ml of deionized water; mixed ABTS and persulfate solution, kept in dark for 12-16 h at room temperature. The stock should be used within 3-5 days.

4.7 mM Standard gallic acid solution: 2 mg of gallic acid was dissolved in 100 ml of 40% ethanol.

5 mM Standard Trolox: 0.125 g dissolved in 100 ml of ethanol
1.5 mM Standard BHT: 0.033 g dissolved in 100 ml of deionized water.

#### 1.2. $H_2O_2$ scavenging assay solution

0.1 % ABTS: 0.1 g of ABTS was added in 100 ml of distilled water and keep in cool temperature at 4°C.

Peroxidase (10 U/ml): Diluted Horseradish peroxides (HRP) (5,000 U) with Phosphate buffer (pH 6.0)

$H_2O_2$ (0.003%): diluted $H_2O_2$ from stock $H_2O_2$ (30%) with deionized water

### 2. Reagents for protein and lipid hydroperoxide assays

#### 2.1. Tri-iodide assay solution

10% Potassium iodide (PI) solution: 20 mg of PI dissolved in 200 ml of acetic acid solution (50%) in anaerobic system.

#### 2.2. Ferrous oxidation-xylenol orange (FOX) assay solution

5 mM XO: 5 mM xylenol orange (XO) was made up in 110 mM $HClO_4$ and stored at room temperature.

5 mM Ferrous solution ($Fe^{2+}$): 5 mM ammonium ferrous sulphate was made up in 110 mM $HClO_4$ and stored at 4°C.

8 M guanidine HCl: this was made up in Milli Q water.

Methanol (20% BHT); 20 mg BHT was added in 100 ml of methanol (AR grade)

Chloroform (20% BHT): 20 mg BHT was added in 100 ml of Chloroform AR grade)

## 3. Reagents for glutathione and malonidaldehyde test in blood

### 3.1. AAPH solution

10 mM AAPH solution: working AAPH solution at 10 mM was prepared by dissolving 0.271 g of AAPH in 100 ml of phosphate buffer (pH 7.4). Fresh preparation of AAPH was used for experiments and was kept in -80°C until assay.

### 3.2. Erythrocyte glutathione assay solution

DTNB reagent: 40 mg of DTNB was dissolved in 100 ml of distilled water

Precipitating agent: 0.2 g of EDTA: 1.67 g of meta-phosphoric acid and 30 g of sodium chloride in 100 ml of distilled water

Phosphate buffer: 0.3 M $Na_2HPO_4$ was prepared in distilled water and adjusted pH to 7.4

Glutathione standard: dissolved stock GSH at 10 mg/100 ml in distilled water.

### 3.3. Plasma malondialdehyde assay

TBA reagent: 4% TBA was prepared by dissolving 0.4 gram of TBA in distilled water 100 ml and kept at 4°C.

TCA reagent: 100 % of TCA was prepared by 250 g of TCA dissolved in 250 ml of 6 M HCl.

Malondialdehyde standard: The standard malondialdehye at 100 mM was prepared by taking 20 µl from stock MDA and neutralized with 5-8 drop of concentrated HCl before being dissolved in distilled water to 100 µM.

## 4. Reagents for identification of bioactive compounds in HPLC

### 4.1. Phenolic analysis

Folin-Ciocalteu's phenol reagent

20% $Na_2CO_3$ was prepared by weighting $Na_2CO_3$ 20 g and dissolving in distilled water 100 ml

Standard gallic acid; 20 mg of gallic acid was dissolved in 25 ml 40% ethanol

### 4.2. Diallyl disulfide analysis

Mobile phase solution: mixed acetonitrile, water and terahydrofurane in 70/27/3 ratio (*v*:*v*:*v*) and degassed with vacuum equipment.

### 4.3. Allyl-2 propenethiosulfinate (allicin) analysis

Mobile phase solution was 50% methanol in distilled water and degassed with vacuum equipment.

## 5. Reagents for oxidative stress in U937 cells line

### 5.1. Cell culture media

RPMI 1640 (without L-glutamate) was supplemented with 2 mM L-glutamate, 10% (*v*:*v*) heat-inactivated FCS and 100 units/ml pencillin, 100 µg/ml streptomycin. The pH value of the medium was adjusted to 7.4 with 7.5% $NaHCO_3$. All solutions were handled under sterile conditions in a sterile laminar flow hood and stored at 4°C.

Phosphate-buffer saline (PBS): 137 mM NaCl, 2.7mM KCl, 1.5 mM $KH_2PO_4$, 10 mM $Na_2HPO_4$, pH 7.4 was sterilized by autoclaving and stored at room temperature.



### 5.2. MTT assay for cell survival assay

MTT stock solutions: MTT was dissolved in PBS at 5 mg/ml, filter sterilized and stored in the dark at 4°C. MTT stock solutions were discarded after one month.

**5.3. Intracellular glutathione assay**

Phosphate-EDTA buffer: 100 mM sodium phosphate, 5 mM EDTA, pH 7.5. It was stored at room temperature

DTNB stock solution: 4 mg/ml in phosphate-EDTA buffer. It was stored at 4°C and discarded after two weeks

NADPH stock solution: 4 mg/ml in phosphate-EDTA buffer. It was stored at 4°C and was discarded after two weeks.

3% PCA (*v*:*v*) solution: diluted from stock 70% PCA with distilled water.

GSH solution was prepared immediately before use in cold 3% PCA solution

**5.4. Griess reagent**

1% sulfanilamide solution: 1 g of sulfanilamide in 100 ml of 5% phosphoric acid

0.1% NED solution: 0.1 g N-1-napthlethylenediamine dihydrochloride in 100 ml of distilled water

Standard nitrate (0.1 M): 0.069 g of $NaNO_2$ in 10 ml of distilled water.

# APPENDIX C:

## The initial rates of reduction of standard antioxidant gallic acid and BHT

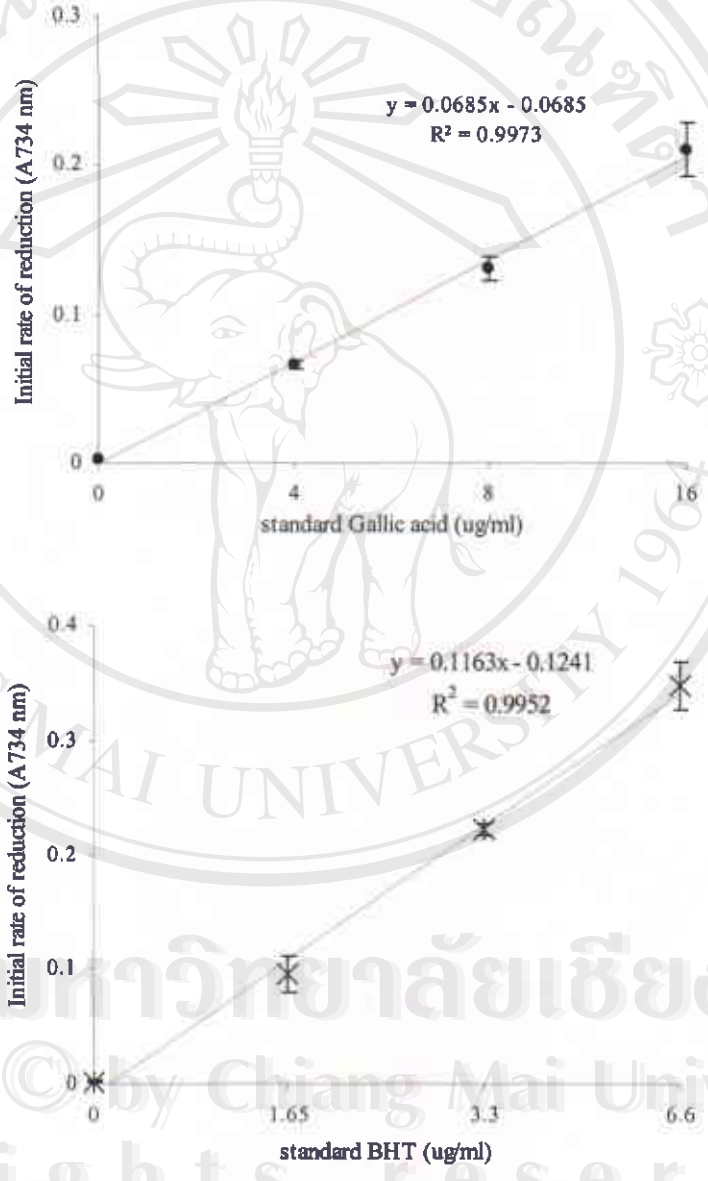


**Figure 72. Antioxidant activity from initial rates of reduction in ABTS cation radicals of standard gallic acid and BHT**. Initial rate of reduction (A734 nm) of standard antioxidants was determined with ABTS radical reduction within one minute. Each point represents the mean and standard deviation from triplicate trials.

# APPENDIX D:

## The percentage of cell survival from standard diallyl disulfide (DADS) in U937 cell line

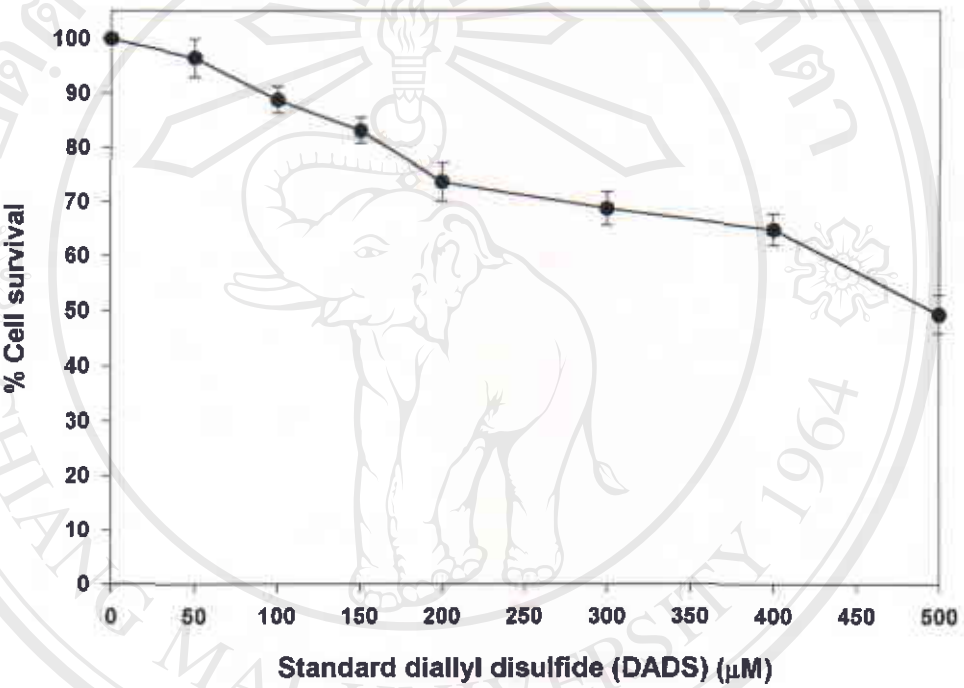


**Figure 73. Effect of diallyl disulfide (DADS) on U933 cell survival**. Percentage of cell survival was evaluated with MTT dye in U937 ($5x10^3$ cells/well) treated with standard DADS at 0-500 μg/ml and incubation for 24 hr at 37°C, 5% $CO_2$. Each point represents the mean and standard deviation from triplicate trials.

# APPENDIX E:

## The cell survival of $H_2O_2$–activated U937 cell line

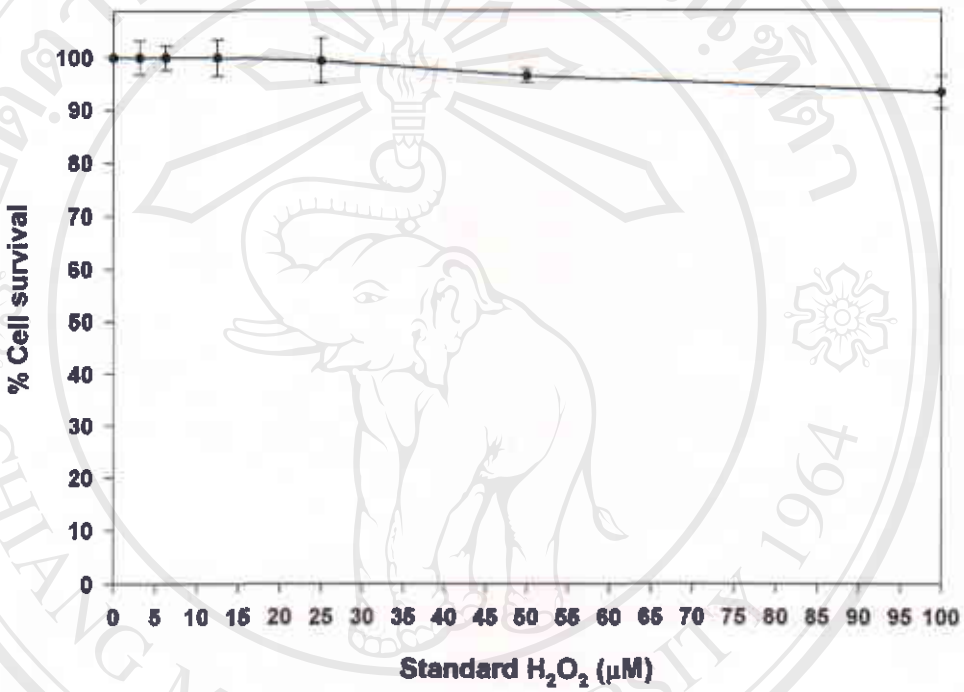


**Figure 74. Effect of $H_2O_2$ on U937 cell survival.** Percentage of cell survival was evaluated with MTT dye in U937 ($5x10^3$ cells/well). The cells were treated with $H_2O_2$ at 0-100 μM and incubation for 24 hr at 37°C, 5% $CO_2$. Each point represents the mean and standard deviation from triplicate trials.

# APPENDIX F:

## The standard curve of sodium nitrite.

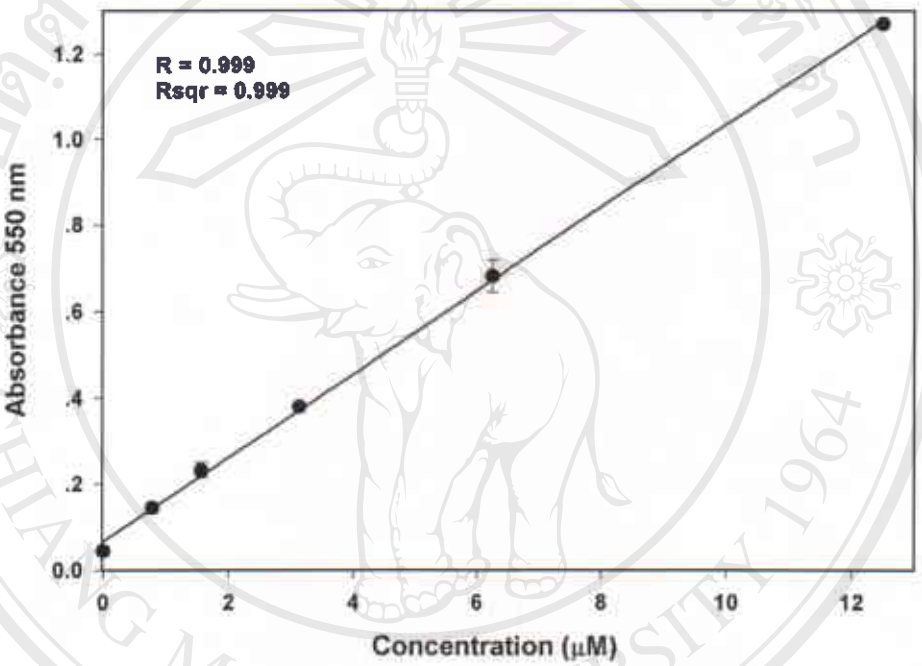


**Figure 75. Standard curve of sodium nitrite.** Nitrite levels was evaluated with Griess reagent in 96-well plate at 0-12 µM. The stock working sodium nitrate (100 µl) was mixed with 100 µl of NED and Sulfanilamide, the pink color was generated and read at 550 nm. Each point represents the mean and standard deviation from triplicate samples.

# APPENDIX G:

## The initial rate of reduction (ΔA/min) of standard glutathione

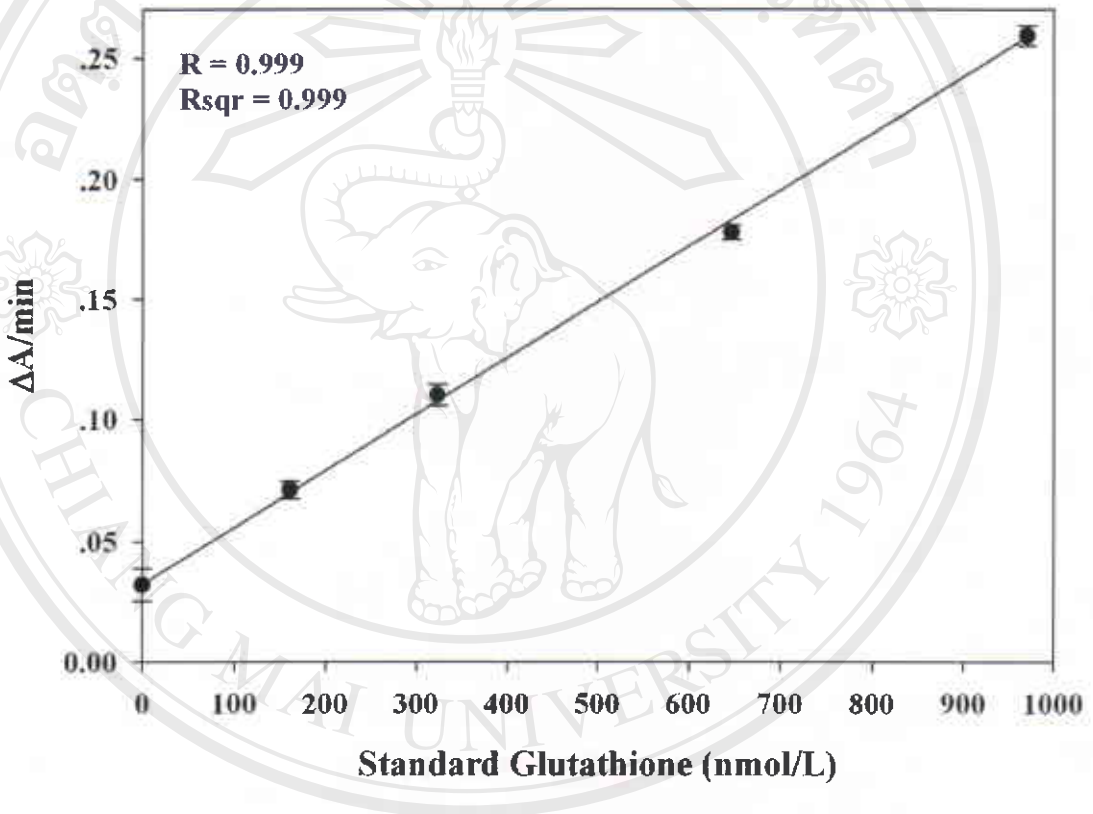


**Figure 76. The initial rate of reduction of standard glutathione**. The initial rate of reduction on ABTS cation radicals (734 nm) was evaluated with kinetic program. The glutathione at 0-1,000 nmol/L was prepared in 3% PCA and mixed with NADPH (4 mg/ml) and glutathione reductase. The yellow color production was recorded within 3 minutes and the initial rate of reduction (ΔA/min) was used. Each point represents the mean and standard deviation from triplicate samples.

# APPENDIX H:

## The standard curve of diallyl disulfide (DADS)

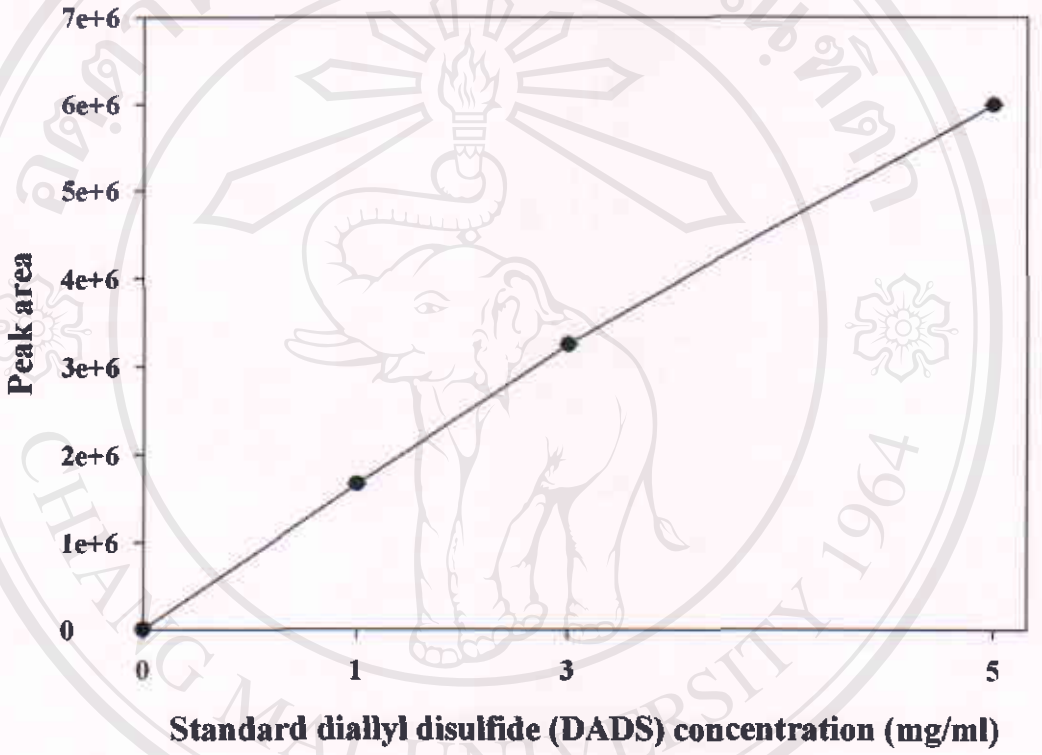


**Figure 77. Standard curve of diallyl disulfide (DADS).** The peak area of standard of DADS at 0-5.0 mg/ml are shown from HPLC chromatogram. Results from a UV detector at 240 nm at flow rate 1.0 ml/min (0.10 AUFS) eluted by acetonitrile:water: tetrahydrofuran mixture(70:23:3, *v:v:v*) as a mobile phase. Each point represents the mean and standard deviation from triplicate samples.

# APPENDIX I:

## The peaks of organosulfur compounds of standard diallyl disulfide (DADS) from gas chromatography-mass spectrum (GC-MS) analysis.

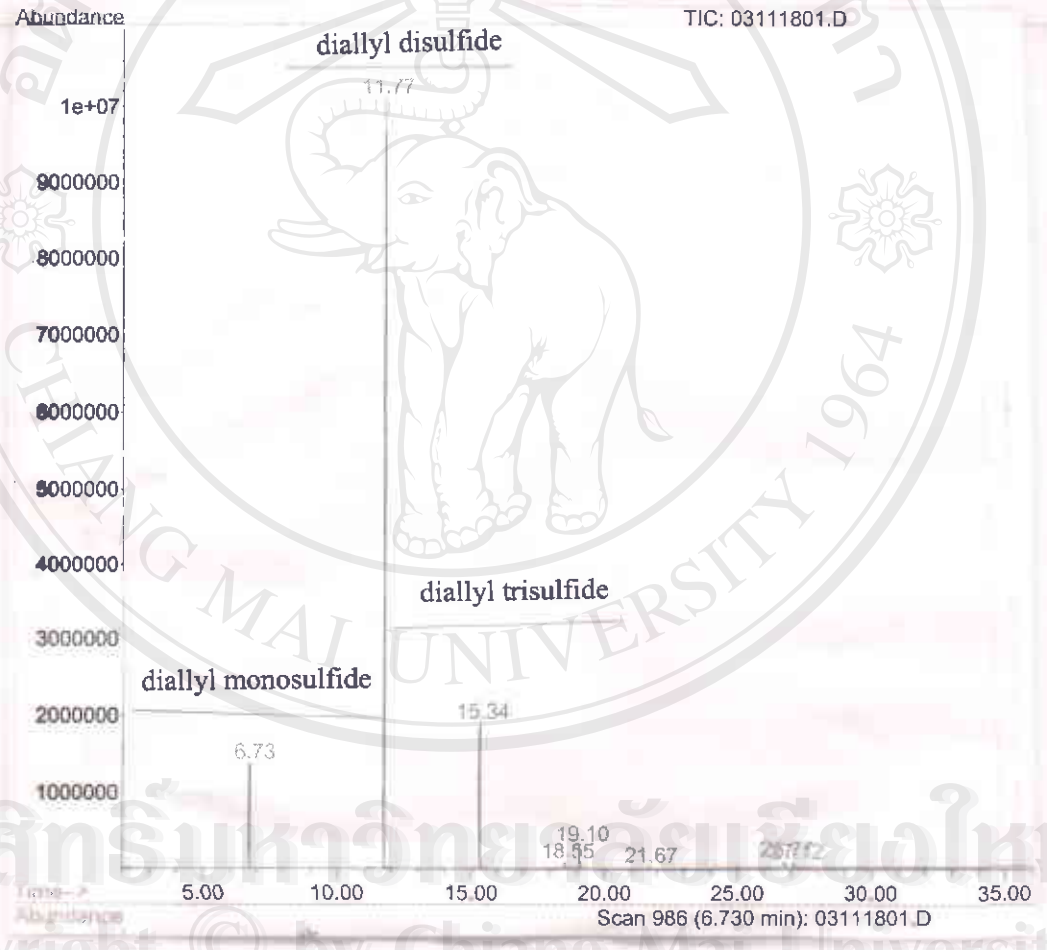




**Figure 78. GC-MS chromatogram of standard diallyl disulfide (DADS).** Three peaks of allyl sulfides; mono- (6.73 min), di- (11.77 min), and tri-sulfide (15.34 min) were identified with the Gases chromatography- mass spectrum (GC-MS). The 20 µl of standard DADS was taken in vial for GC-MS analysis.

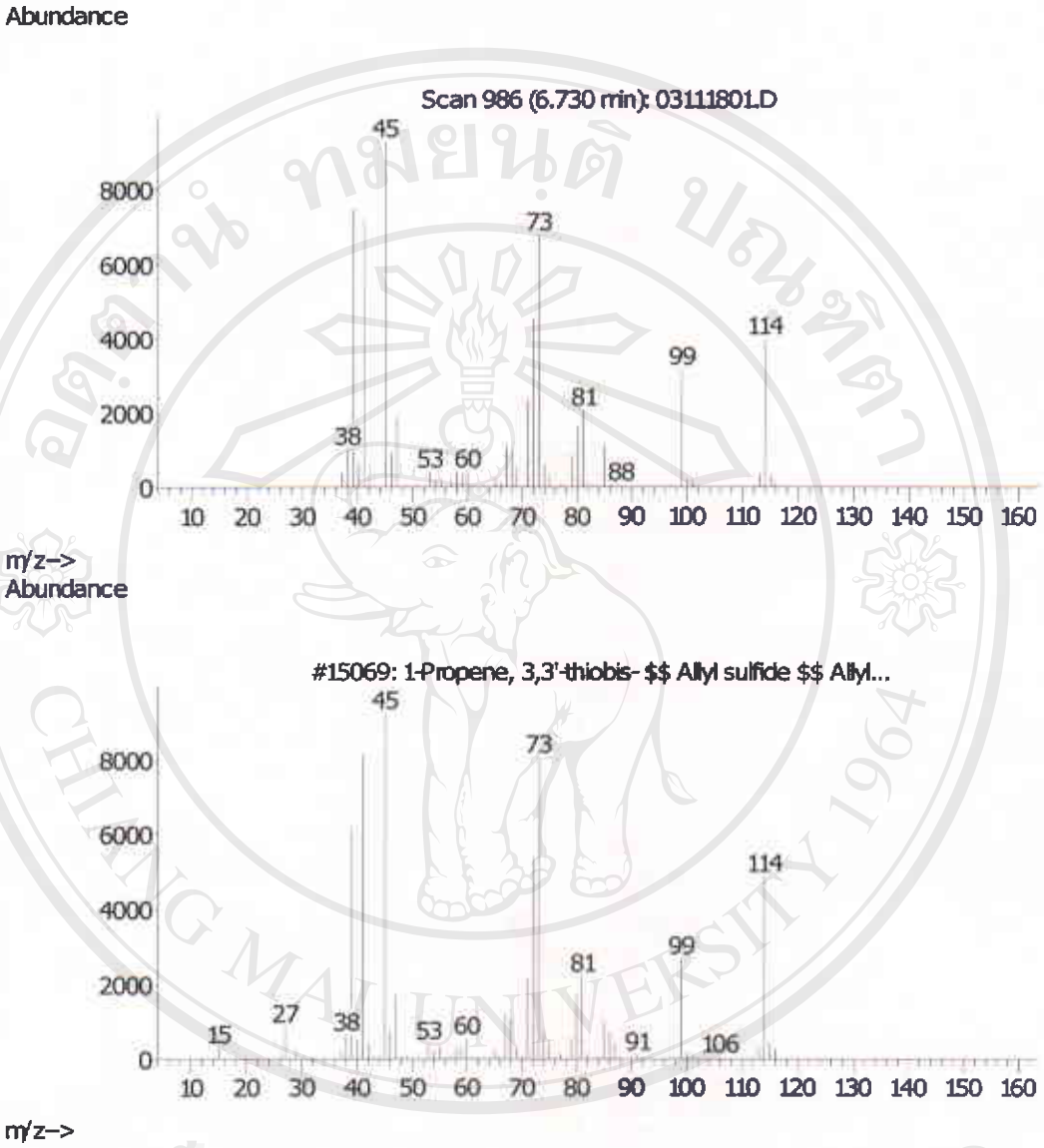


**Figure 79. GC-MS chromatogram and mass spectrum of diallyl monosulfide.** Mass spectra of diallyl monosulfide at 6.73 min from standard diallyl disulfide (DADS) (Sigma) that identified with the Gases chromatography- mass spectrum (GC-MS). The 20 µl of standard DADS was taken in vial for GC-MS analysis.

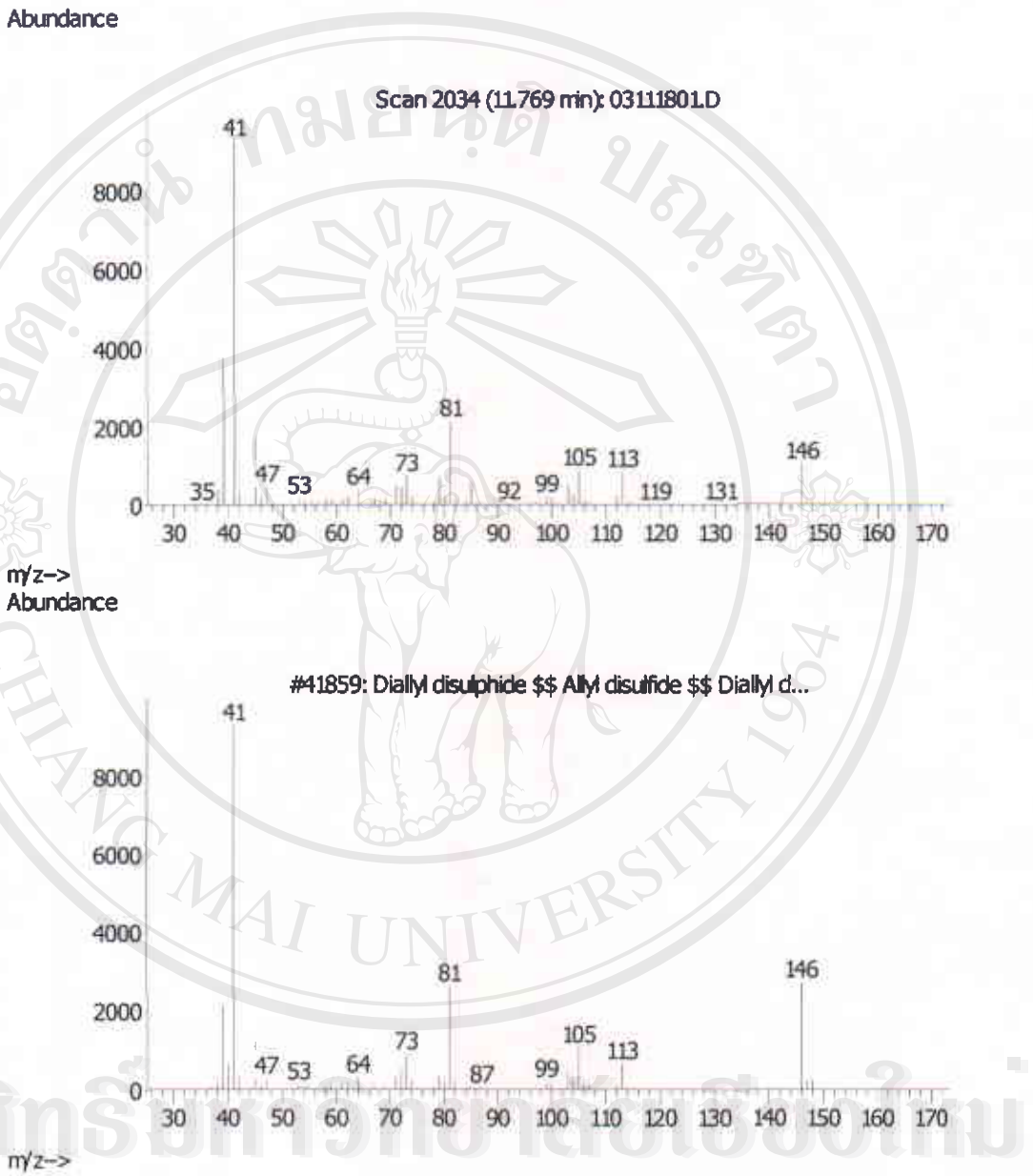


**Figure 80. GC-MS chromatogram and mass spectra of diallyl disulfide (DADS).** Mass spectra of diallyl disulfide at 11.76 min from standard diallyl disulfide (DADS) (Sigma) that identified with the Gases chromatography- mass spectrum (GC-MS). The 20 µl of standard DADS was taken in vial for GC-MS analysis.

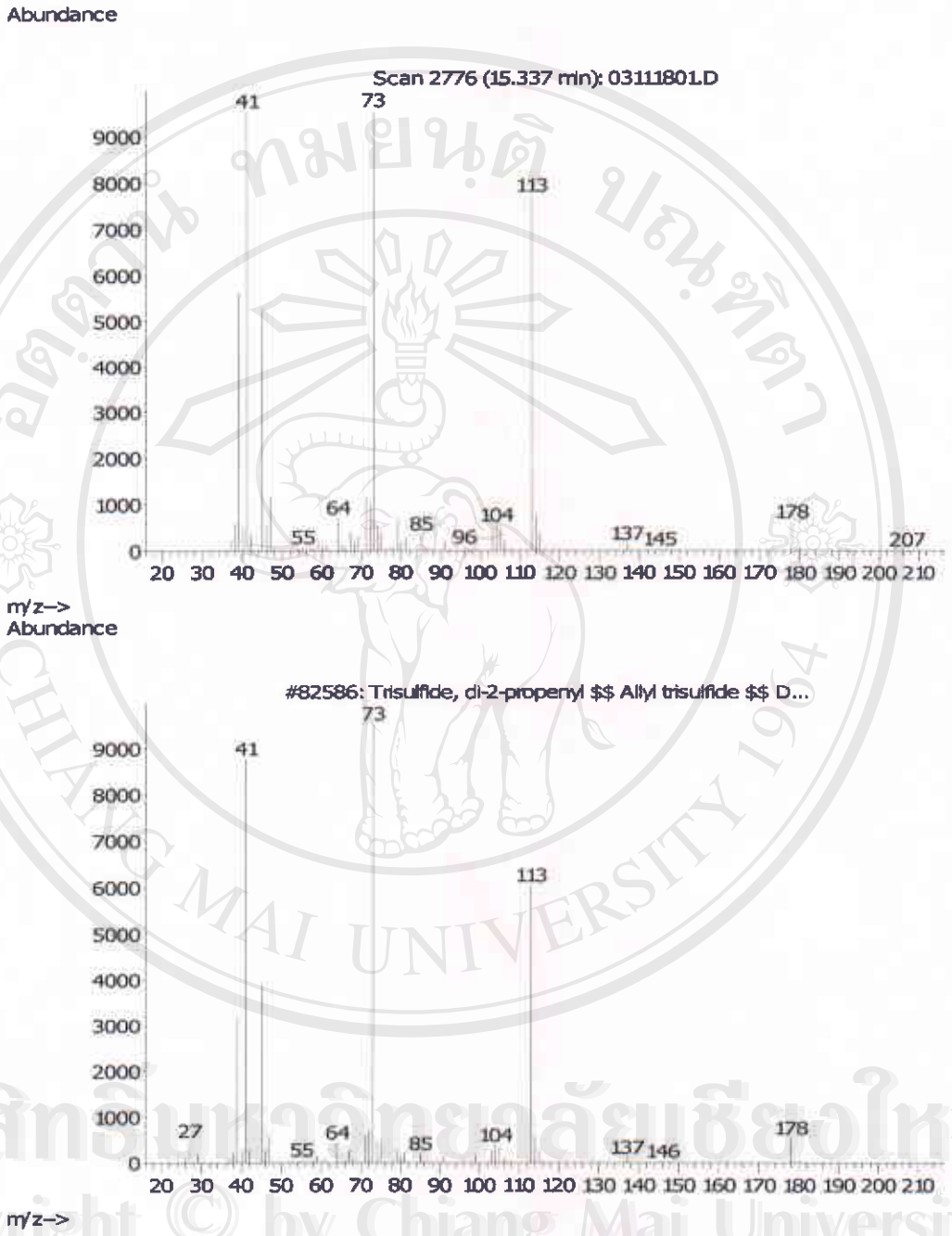


**Figure 81. GC-MS chromatogram and mass spectra of diallyl trisulfide (DATS).** Mass spectra of diallyl trisulfide at 15.33 min from standard diallyl disulfide (DADS) (Sigma) that identified with the Gases chromatography- mass spectrum (GC-MS). The 20 µl of standard DADS was taken in vial for GC-MS analysis.

| Pk# | RT | Area% | Library/ID | Ref# | CAS# | Qual |
|---|---|---|---|---|---|---|
| 1 | 6.73 | 11.03 | C:\DATABASE\WILEY275.L | | | |
| | | | 1-Propene, 3,3'-thiobis- (CAS) $$ | 11963 | 000592-88-1 | 98 |
| | | | 1-Propene, 3,3'-thiobis- (CAS) $$ | 11964 | 000592-88-1 | 98 |
| | | | 1-Propene, 3,3'-thiobis- (CAS) $$ | 11965 | 000592-88-1 | 97 |
| 2 | 11.77 | 73.61 | C:\DATABASE\WILEY275.L | | | |
| | | | Disulfide, di-2-propenyl (CAS) $$ | 32189 | 002179-57-9 | 78 |
| | | | Disulfide, di-2-propenyl (CAS) $$ | 32191 | 002179-57-9 | 30 |
| | | | Disulfide, di-2-propenyl (CAS) $$ | 32193 | 002179-57-9 | 10 |
| 3 | 15.34 | 10.86 | C:\DATABASE\WILEY275.L | | | |
| | | | Trisulfide, di-2-propenyl (CAS) $$ | 61720 | 002050-87-5 | 96 |
| | | | Trisulfide, di-2-propenyl (CAS) $$ | 61719 | 002050-87-5 | 64 |
| | | | 1-Propene, 3,3'-thiobis- (CAS) $$ | 11965 | 000592-88-1 | 43 |
| 4 | 18.55 | 0.45 | C:\DATABASE\WILEY275.L | | | |
| | | | Disulfide, di-2-propenyl (CAS) $$ | 32191 | 002179-57-9 | 38 |
| | | | Disulfide, di-2-propenyl (CAS) $$ | 32188 | 002179-57-9 | 22 |
| | | | Zinc, di-2-propenyl- (CAS) $$ ZINC | 32206 | 001802-55-7 | 14 |
| 5 | 19.10 | 2.35 | C:\DATABASE\WILEY275.L | | | |
| | | | 1,2-Benzenedicarboxylic acid, diet | 107342 | 000084-66-2 | 98 |
| | | | 1,2-Benzenedicarboxylic acid, diet | 107338 | 000084-66-2 | 98 |
| | | | 1,2-Benzenedicarboxylic acid, diet | 107340 | 000084-66-2 | 97 |
| 6 | 21.68 | 0.22 | C:\DATABASE\WILEY275.L | | | |
| | | | bis-TMS-C(13)-3-hydroxybutanoate $ | 132496 | 000000-00-0 | 42 |
| | | | Ethanedioic acid, bis(trimethylsil | 119112 | 018294-04-7 | 38 |
| | | | 1,3-Dioxolane (CAS) $$ 1,3-Dioxola | 1725 | 000646-06-0 | 27 |
| 7 | 26.77 | 0.93 | C:\DATABASE\WILEY275.L | | | |
| | | | 9-Octadecenamide, (Z)- (CAS) $$ OL | 162712 | 000301-02-0 | 86 |
| | | | 9-Octadecenamide, (Z)- (CAS) $$ OL | 162711 | 000301-02-0 | 62 |
| | | | 9-Octadecenamide, (Z)- (CAS) $$ OL | 162710 | 000301-02-0 | 60 |
| 8 | 27.12 | 0.55 | C:\DATABASE\WILEY275.L | | | |
| | | | Hexanedioic acid, dioctyl ester (C | 222005 | 000123-79-5 | 87 |
| | | | Hexanedioic acid, dioctyl ester (C | 222002 | 000123-79-5 | 55 |
| | | | Di(2-ethylhexyl)adipate | 22201 | 000103-23-1 | 49 |



**Figure 82. Comparative peaks and retention time of each diallyl sulfides.** The peaks and retention time of each diallyl sulfide were used to analysis the chemical structure and scientific name by using specific of mass spectrum in library database (Wiley275.L).

# CURRICULUM VITAE

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**Research projects**

Study of phenolic compound and total antioxidant capacity in fermented juices from Tomato, Carrot and Gum. Granted by Faculty of AMS (2006)

Study of response of hydroperoxide formation in blood from high intensity exercise between athletics and sedentary controls. Granted by Faculty of AMS (2005)

Study the effects of Thai shallot on total intracellular glutathione synthesis in oxidative stress and inflammation in human monocytic cell line (U937). Granted by SM.Pharmaceutical.Co (Thailand) (2002-2004)

Tendency of native shallot preparation for protective and inhibitory effects on oxidative stress in erythrocyte as commerce products. Granted by NSTDA (Northern Network), Thailand (2000-2001)

Comparative study of derivated N-acetycysteine drugs on oxidative stress in protein and lipid hydroperoxide formation in vitro. Granted by SM.Pharmaceutical.Co. (Thailand) (1999-2002)

**Publications**

A quantitative evaluation of the antioxidant properties of garlic and shallot preparation. Nutrition. 2006; 22: 266-274.

Exhaustive Exercise Test and Oxidative Stress Response in Athletic and Sedentary subjects. Chiang Mai University Journal . 2005; 4: 183-190.

Potential activity of Thai shallot (*Allium ascallonicum* L.) extract on the prevention of hemolysis and glutathione depletion in human erythrocyte from oxidative stress. . Chiang Mai University Journal. 2004; 3: 225-234.

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Anti-oxidative stress and anti-inflammatory effects of Thai shallot (*Allium ascanolicum* L.) extracts in human monocytic (U937) cells. . The Niigata Journal of Health and Welfare. 2004; 4: 11-19.

Free radical and Exercise Tolerance in Chronic Obstructive Pulmonary Disease: COPD . Bull Chiang Mai Assoc Med Sci 2000;33: 212-220.

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Chest Physical Therapy Techniques. Bull Chiang Mai Assoc Med Sci 2000; 33: 29-40.

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A case study; Therapeutic effects of physical therapy in a COPD patient on weaning from ventilator . Bull Chiang Mai Assoc Med Sci. 1999; 32: 31-42.

Abnormal pulmonary assessment in pediatric patients. Bull Chiang Mai Assoc Med Sci . 1999; 32: 25-30.

A new approach of respiratory physical therapy in a patient with empyema thoracis after decortication . Bull Chiang Mai Assoc Med Sci 1998; 31: 199-206.

Biochemistry and respiratory disorder in chest physical therapy. Bull Chiang Mai Assoc Med Sci . 1998; 31: 42-56.

Exercise and Glutathione under Oxidative Stress. Bull Chiang Mai Assoc Med Sci. 1998; 31: 225-230.

Respiratory Muscles Exercise in Chest Physical Therapy. . Bull Chiang Mai Assoc Med Sci. 1997; 30: 44-56.

Chest Physical Therapy for Abdominal Surgical Patients. . Bull Chiang Mai Assoc Med Sci 1996. 1996; 29: 19-29.

Exercise and Lung Disease. . Nursing Newsletter. 1996; 23: 26-33.

Lung atelectasis and Chest Physical Therapy. Bull Chiang Mai Assoc Med Sci. 1996; 30: 77-90.

**Books**

Chest radiography for Chest Physical Therapy (1997)

Chest Physical Therapy; Part II (2002)

**Academic Conference**

The effectiveness of Thai shallot (*Allium ascanolicum* L.) and phenolics compounds against protein and lipid peroxidation. International Colloquium 2004 Health Benefit and Application of Polyphenoids. Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand, 25 - 26 November 2004.

Effects of Thai shallot (*Allium ascalonicum* L.) extracts on oxidative stress under intracellular glutathione and nitric oxide. International Colloquium 2004 Health Benefit and Application of Polyphenoids. Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand, 24 - 26 November 2004.

Anti-oxidative stress and anti-inflammatory effects of Thai shallot (*Allium ascalonicum* L.) extracts in human monocytic, . The 28th Annual Scientific Meeting on Mahidol' s Day, Faculty of Medicine, Chiang Mai University, Thailand, 24 September 2004.

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