Antioxidant activity of *Piper sarmentosum* Roxb. and its effect on the degradation of frying oils

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

Publications

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Kasemweerasan, P., Marshall, L. J. and Maycock, J. *Antioxidant Properties, Phenol Profile of Piper sarmentosum Roxb. Leaf Extracts and its Effect on Changes in Oils at Frying Temperatures*. The 12th Asian Congress of Nutrition, 14th -18th May 2015, Yokohama, Japan.

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(Ready for submission)

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2. Antioxidant Activity and Characterisation of *Piper sarmentosum* Roxb. Leaf Extract by UHPLC-PDA-ESI-MS

3. The Effect of *Piper sarmentosum* Roxb. Leaf Extract on Stability of Rice Bran Oil and Corn Oil during Storage and Frying

4. Pigment Behaviour of Piper sarmentosum Roxb. Leaf Extract in Rice Bran Oil and Corn Oil during Frying and Correlation to Total Polar Compounds

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Abstract

Synthetic antioxidants are commonly used for retarding rancidity in cooking oils. However, they are not effective at frying temperature and are potential carcinogens. To replace synthetic antioxidants with a natural source, which is safer and heat resistance, *Pandanus amaryllifolius* Roxb. (PD) and *Piper sarmemtosum* Roxb. (PS) were investigated. Total phenol content, antioxidant activity and synergistic effects of extracts of both leaves were determined. By comparing the results, PS leaf extract showed a higher total phenol content and antioxidant activity. So, PS was selected for further study on extraction conditions. PS extract extracted with 80 % ethanol (PSE extract) had the highest total phenol content. PS extract extracted with petroleum ether (PSL extract) possessed both highest total flavonoids and antioxidant activity. Compounds present in PSE and PSL extracts were identified using UHPLC-PDA-ESI-MS and quantified using HPLC. Seven compounds were identified as chlorogenic acid/neochlorogenic acid, caffeic acid, vitexin, *ρ*-courmaric acid, hydrocinnamic acid, quercetin and caffeine. The protective effects of PSE and PSL extracts on degradation of rice bran and corn oil were determined at accelerated storage and frying temperatures. In accelerated (60$\pm$3 °C), PSE extract inhibited lipid oxidation by lowering the peroxide, *ρ*-Anisidine, TBA and Totox values in both oils. The PSL extract did not retard lipid oxidation. However, butylated hydroxytoluene (BHT) showed a superior protective effect over PSE and PSL extracts throughout storage time. At frying (180 °C), PSE and PSL extracts had a significantly ($p<0.05$) positive protective effect on both rice bran and corn oil showing a lower photometric colour, acid value, peroxide value, TBARS value and total polar compounds than the control oils. The most effective extracts were

PSE 0.2 %, PSL 0.05 % and PSL 0.1 %. The *Piper sarmentosum* Roxb. leaf extract showed a better protective effect than BHT and therefore could be used as alternative natural antioxidants in frying oils.

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List of Abbreviations

°C .....degree Celsius

µL .....microlitre

µm .....micrometre

BHA .....butylated hydroxyanisole

BHT .....butylated hydroxytoluene

C .....corn oil

DFPS .....defatted PS (PS following the petroleum ether extraction)

DFPS20 % .....the defatted PS extracts extracted using 20 % ethanol

DFPS50 % .....the defatted PS extracts extracted using 50 % ethanol

DFPS80 % or DFPSE .....the defatted PS extracts extracted using 80 % ethanol

DFPSAbEtOH .....the defatted PS extracts extracted using absolute ethanol

DFPSW .....the defatted PS extracts extracted using water

DW …… dried weight

ESI .....Electrospray Ionisation

g.....gram

h ……..hour

kg.....kilogram

L .....litre

m/z .....mass-to-charge ratios

mg/L .....milligram per litre

mL.....millilitre

nm .....nanometre

PD.....*Pandanus amaryllifolius* Roxb. leaf

PDPS .....the mixture of extracts solution PS and PD

PG.....propyl gallate

PS.....*Piper sarmentosum* Roxb. leaf

PS20 % ……..the PS extracts extracted using 20 % ethanol

PS50 % .....the PS extracts extracted using 50 % ethanol

PS80 % or PSE or S .....the PS extracts extracted using 80 % ethanol

PSAbEtOH ……..the PS extracts extracted using absolute ethanol

PSL or L .....the PS extract extracted using petroleum ether

PSW .....the PS extract extracted using water

PV.....peroxide value

R.....rice bran oil

RBD .....refined, bleached and deodourised

rpm .....round per minute

RT ……retention time

SE.....standard error

SIM .....Selected Ion Monitoring

T .....BHT

TBHQ.....tertiary butylhydroquinone

w/v.....weight per volume

1 Introduction

1.1 Basic concepts of fats and oils

Fats and oils occur naturally in animal tissues, seeds and fruits. They have a major role in the human diet: provide satiety to foods (enhancing flavour, texture), provide energy and contain essential fatty acids which cannot be produced by the human body (O'Brien, 2004). In general, the term fats is used when they appear as a solid at room temperature. If they appear as liquid at room temperature, they are called oils (O'Brien, 2004). Fats and oils are comprised of triglycerides (which are glycerol esters of fatty acids as shown in Figure 1-1 and nonglyceride components such as phospholipids, tocopherols, sterols, trace metals and pigments (e.g. carotene, chlorophyll). The chemical and physical properties of fats and oils are influenced by the fatty acid and the attachment position of each fatty acid to the glyceride (O'Brien, 2004).

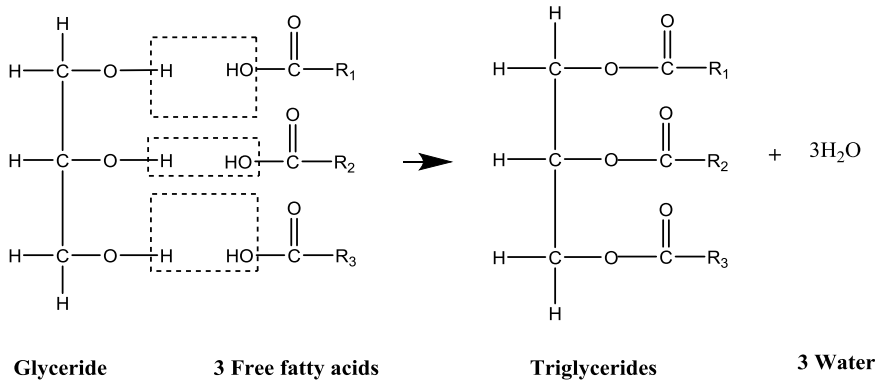


Figure 1-1: Esterification of triglycerides, adapted from Food Network Solution (2011)

If there was only one fatty acid attached, it is known as a monoglyceride whereas, it is a diglyceride if there are two fatty acids attached to the glycerol molecule (Figure 1-2). When there are three fatty acids attached, it is called a triglyceride.

It is a simple triglyceride when the three fatty acids in the molecule are the same and when two or three different fatty acids are present in the molecule, it is a mixed triglyceride, Figure 1-2 (a) & (b)(Lawson, 1995).

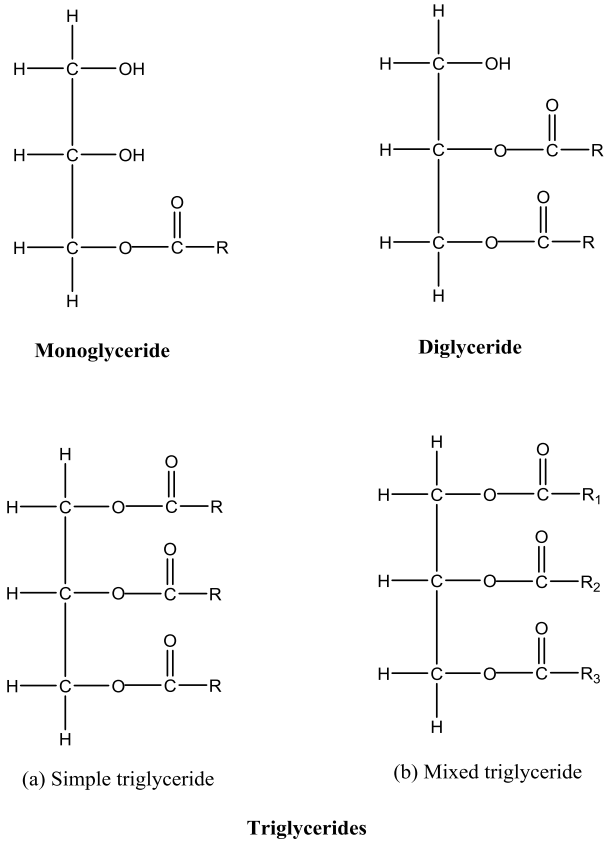


Figure 1-2: Structure of mono, di and triglycerides, adapted from Lawson (1995)

A fatty acid consists of a straight chain with an even number of carbon atoms, with hydrogen atoms along the length of the chain and at one end of the chain, a carboxyl group (−COOH). It is the carboxyl group that makes it an acid (carboxylic acid). Figure 1-3 shows the structure of saturated and unsaturated fatty acids. If the carbon-to-carbon bonds are all single, the acid is a saturated fatty acids (Sinha, 2014). The polyunsaturated fatty acids have two or more double bonds which are separated by a single methylene group, or 1,4-diene structure. For instance, linoleic acid with two *cis* double bonds in the 9,12

positions is designated as 18:2 n-6 (9,12) as shown in Figure 1-3 (c) and linolenic acid is 18:3 n-3 (9, 12, 15) as shown in Figure 1-3 (d), (Frankel, 1998b).

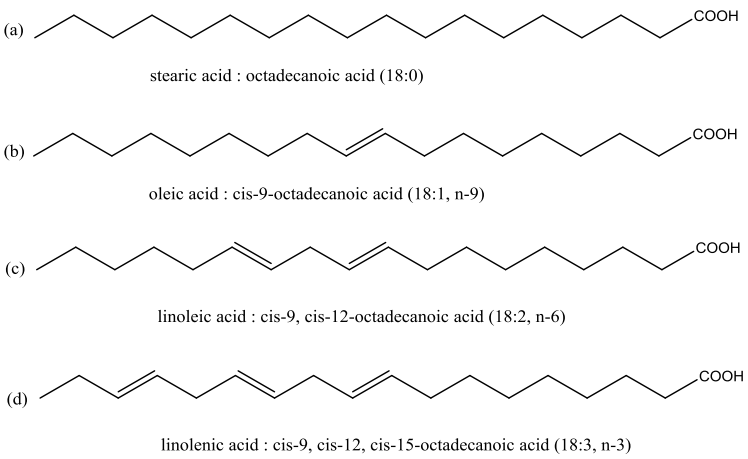


Figure 1-3: Structure of a saturated fatty acid (a), monounsaturated fatty acid (b) and polyunsaturated fatty acids (c, d), adapted from Frankel (1998b)

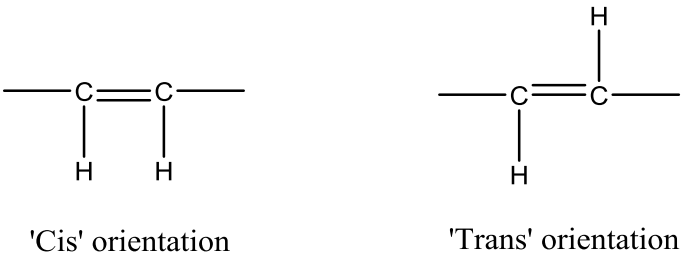


Figure 1-4: Orientation of the double bond in an unsaturated fatty acid, adapted from Child (2012)

There are 2 geometric configurations of an unsaturated fatty acid which depends on the position of the hydrogens of the double bonds (Child, 2012). In a 'cis-' orientation, the hydrogens are on the same side of the double bond, mostly found in natural fat and oils. In 'trans-' formation, the hydrogens are on opposite sides of the double bond (Figure 1-4). This phenomenon in fatty acid molecules gives rise to different physical properties and characteristics (Nettleton, 1994).

The chain length of fatty acid varies from short chains containing 4-6 carbons, medium chain length 8-10 carbons, long chain length 12 – 18 carbons or very long chain length containing 20 or more carbons (Rossell, 2001b; Nettleton, 1994). The saturated fatty acids commonly found are lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), arachidic (20:0), behenic (22:0) and lignoceric (24:0). Oleic acid (18:1) and erucic acid (22:1) are the most important monounsaturated fatty acids. Linoleic acid (18:2) and linolenic acid (18:3) are essential polyunsaturated fatty acids (O'Brien, 2004).

1.2 Frying oil

The quality of frying oils is very important due to it having an impact on the quality of fried food. It has influences over oil absorption and the types of by-products, and residues absorbed by food (Kochhar, 2001). The criteria for selecting frying oils and fats, particularly in the food industry, are high oxidative stability, high smoke point, low foaming, low melting point, bland flavour and high nutritional value. Many types of frying oils such as refined rapeseed oil, partially hydrogenated rapeseed oil, palm oil/rapeseed oil or soybean oil blends, and palm olein or super olein, are used in the food industry and in the household. Animal fats such as tallow or lard are also still used in specific products (Kochhar, 2001). Several new frying oils, both with good stability and a fatty acid composition similar to virgin olive oil, have been developed such as Nu-Sun; a mid-oleic sunflower oil (National Sunflower Association, 2015) or Good-Fry® edible oil, which is comprised of high-oleic sunflower oil blended with a small portion of refined sesame oil and rice bran oil (Silkeberg and Kochhar, 2000). The fatty acid composition of some types of the fats and oils used for frying are summarised in Table 1-1.

1.2.1 Chemistry of deep fat frying oils

Deep fat frying is one of the common cooking methods which gives specific desirable characteristics of fried foods such as the fried flavour, the golden brown colour and crisp texture. However, it can also give an undesirable off-flavour if perished oil is used. Heating the oil causes physical and chemical changes in the qualities of the oil (Warner, 2002). During frying, the oil is heated over a wide range of high temperatures from 130 °C up to 220 °C (Pokorny, 2001; Lawson, 1995). During this process hundreds of chemical reactions occur, in which 3 major reactions are categorised: hydrolysis, oxidation and thermal polymerisation (Paul *et al.*, 1997). From these reactions, volatile and non-volatile products are formed which greatly affect the functional, sensory and nutritional qualities of oils, thus affecting the food quality (Warner, 2002).

1.2.1.1 Hydrolysis reaction

When food is placed in oil at frying temperatures with the presence of air and moisture, the water and steam react (hydrolyse) with triglycerides resulting in the formation of decomposition products such as free fatty acids, monoglycerides, diglycerides (diacylglycerol) and glycerol (glycerine), a decrease in smoke point and a decrease in the stability and shelf life of the frying oils (Warner, 2002; Paul *et al.*, 1997).

Table 1-1: Typical fatty acid compositions of some frying oils, adapted from Rossell (2001a), Codex Alimentarius (1999) and Kochhar (2001)

Fatty acid (% wt)	Olive oil	Rice bran oil	Corn oil	Rapeseed oil	Palm oil	Soybean oil	Sunflower oil	Beef tallow	NuSun	Good-Fry® oil
Hexanoic (6:0)	-	-	-	-	-	-	-	-	-	-
Octanoic (8:0)	-	-	-	-	-	-	-	-	-	-
Decanoic (10:0)	-	-	-	-	-	-	-	-	-	-
Dodecanoic (12:0)	-	0-0.2	0-0.3	≤0.1	≤0.5	≤0.1	≤0.1	-	-	-
Tetradecanoic (14:0)	0.05	0-1.0	0-0.3	≤0.2	0.5-2.0	≤0.2	≤0.2	2.5	-	-
Hexadecanoic (16:0)	7.5-20.0	14-23	8.6-16.5	8.0-13.5	39.3-47.5	8.0-13.5	5.0-7.6	24.5	8.8	4.5
Hexadecenoic (16:1)	-	0-0.5	0-0.5	≤0.2	≤0.6	≤0.2	≤0.3	-	-	-
Heptadecanoic (17:0)	-	-	0-0.1	≤0.1	≤0.2	≤0.1	≤0.2	-	-	-
Heptadecenoic (17:1)	-	-	0-0.1	≤0.1	-	≤0.1	≤0.1	-	-	-
Octadecanoic (18:0)	0.5-5.0	0.9-4.0	0-3.3	2.0-5.4	3.5-6.0	2.0-5.4	2.7-6.5	18.5	2.3	3.7
Octadecenoic (18:1) (oleic acid)	55.0-83.0	38-48	20-42.2	17-30	36.0-44.0	17-30	14-39.4	40	64.5	78.7
Octadecadienoic (18:2) (linoleic acid)	3.5-21.0	21-42	34-65.6	48-59	9.0-12.0	48-59	48.3-74	5	22.1	10.8
Octadecatrienoic (18:3) (linolenic acid)	1.0	0.1-2.9	0-2.0	4.5-11	≤0.5	4.5-11.0	≤0.3	0.5	0.4	0.1
Eicosanoic (20:0)	0.6	0-0.9	0.3-1.0	0.1-0.6	≤0.1	0.1-0.6	0.1-0.5	0.5	-	-
Eicosenoic (20:1)	0.4	0-0.8	0.2-0.6	≤0.5	≤0.4	≤0.5	≤0.3	0.5	-	-
Eicosadienoic (20:2)	-	-	0-0.1	≤0.1	-	≤0.1	-	≤0.1	-	-
Docosanoic (22:0)	0.2	0-1.0	0-0.5	≤0.7	≤0.2	≤0.7	0.3-1.5	-	-	-
Docosenoic(22:1)	-	-	0-0.3	≤0.3	-	≤0.3	≤0.3	-	-	-
Docoasdienoic (22:2)	-	-	-	-	-	-	≤0.3	-	-	-
Tetracosanoic (24:0)	0.2	0-0.9	0-0.5	≤0.5	-	≤0.5	≤0.5	-	-	-
Tetracosenoic (24:1)	-	-	-	-	-	-	-	-	-	-

1.2.1.2 Oxidation reactions

The oxygen existing in fresh oil, at the oil surface and by addition of food can cause an oxidative reaction, which is a series of reactions, contributing to the formation of both volatile and non-volatile decomposition products, such as free radicals, hydroperoxides and conjugated dienoic acids (Warner, 2002; Paul *et al.*, 1997). The mechanism of oxidative reactions in frying oil occurs in 3 stages

Primary oxidation (initiation stage) $LH + R^{\bullet} \rightarrow L^{\bullet} + RH$

Secondary oxidation (propagation stage) $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$

$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH$

Tertiary oxidation (termination stage)

$LOO^{\bullet} + LOO^{\bullet} \rightarrow LOOL + O_2$ -----

$LOO^{\bullet} + L^{\bullet} \rightarrow LOOL$ ---------- } non-radical products

$L^{\bullet} + L^{\bullet} \rightarrow LL$ ----------

Secondary initiation $LOOH \rightarrow LO^{\bullet} + {}^{\bullet}OH$

$2\ LOOH \rightarrow LO^{\bullet} + LOO^{\bullet} + H_2O$

Metal-catalysed initiation $M^{(n)+} + LOOH \rightarrow LO^{\bullet} + {}^{-}OH + M^{(n+1)+}$

$M^{(n+1)+} + LOOH \rightarrow LOO^{\bullet} + H^{+} + M^{(n)+}$

LH is an unsaturated lipid and $R^{\bullet}$ is the initial oxidizing radical or pre-existent lipid hydroperoxides in oils (which may be formed by lipoxygenase action in the

plant prior to and during extraction of the oil). Secondary initiation by homolytic cleavage of hydroperoxides is a relatively low energy reaction, and it is normally the main initiation reaction in edible oils. This reaction is commonly catalysed by metal ions (Gordon, 2001). When the lipid is oxidized in the initiation stage, the alkyl radical ($L^{\bullet}$) is generated which is a highly reactive and can react with oxygen rapidly to form a lipid peroxyl radical ($LOO^{\bullet}$) in propagation stage (Frankel, 1998c). The reaction produces hydroperoxides and conjugated dienes which are unstable and rapidly decomposed further into secondary oxidation products, as affected by very high temperatures of frying (Warner, 2002). These peroxyl radicals ($LOO^{\bullet}$) can attack other unsaturated fatty acids to form more free radicals or they can extract a hydrogen from a fatty acid and yield a lipid hydroperoxide (LOOH) and alkyl radical ($L^{\bullet}$). The lipid hydroperoxides (LOOH) are very unstable, they can break down into a wide range of compounds including alcohols, aldehydes, carbonyls, free fatty acids, malonaldehyde, ketones, hydrocarbons and radicals including the peroxy (alkoxyl) radical ($LO^{\bullet}$). In the termination stage, the free radicals react with each other to form non-radical products. Numerous compounds formed at this stage produce the most decomposition products: oxidised monomers, oxidative dimers, trimers, polymers, epoxides, alcohols, hydrocarbons, also polar and non-polar compounds and also affect the physical properties by increasing viscosity and darkening of the oil (Frankel, 1998c; Gutierrez *et al.*, 1988; Lumley, 1988; Stevenson *et al.*, 1984). The distinctive off-odours of heated oil come from saturated and unsaturated aldehydes such as hexanal, heptanal, octanal, nonanal and 2-decenal being produced (Neff *et al.*, 2000). However, some of the volatile products from this stage, are desirable due to their contribution to the characteristic fried

flavour of fried food and in the oil. Such compounds are 2,4-decadienal, 2,4-nonadienal, 2,4-octadienal, 2-heptenal and 2-octenal (Warner *et al.*, 2001; Neff *et al.*, 2000).

1.2.1.3 Polymerisation

Polymerisation involves a great number of chemical reactions that result in the formation of various compounds with high molecular weight and polarity. The resultant effect is an increase in the viscosity of the oil. Polymers can form from free radicals or triglycerides by the Diels–Alder reaction. Cyclic fatty acids can form within one fatty acid; dimeric fatty acids can form between two fatty acids, either within or between triglycerides; and polymers which have high molecular weight are obtained through cross-linking of these molecules (Warner, 2002). If the cyclic compounds are formed with the oxygen, the polymers will contain oxygen in their structure. The presence of oxygen in the structure of a polymer, increases its polarity. The polymer formation scheme is illustrated in Figure 1-5.

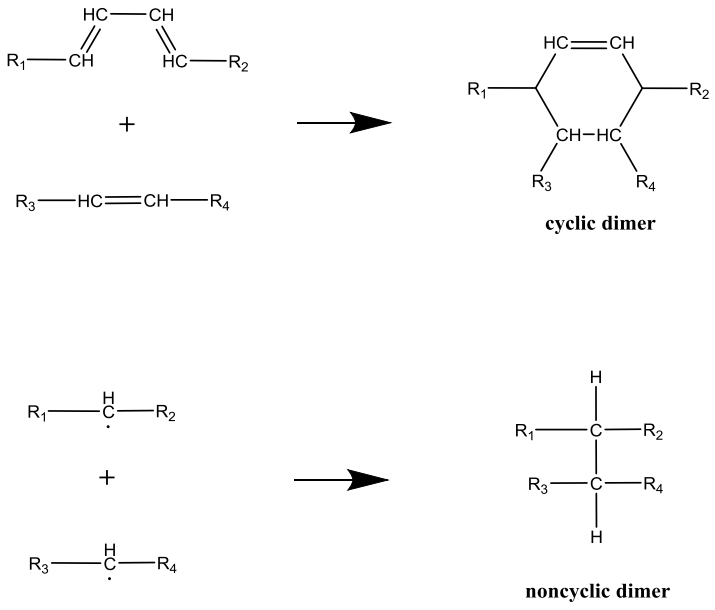


Figure 1-5: Polymer formation through Diels-Alder condensation mechanism, adapted from Frankel (1998c)

1.2.1.4 Decomposition products

In summary, high heated oils deteriorate and produce volatile and non-volatile decomposition products which change the physical and chemical properties of the oil. Figure 1-6 is a phenomenon of the production of the decomposition products during the frying process and Table 1-2 summarises the decomposition products found in frying oil. As discussed in chapter 1.3.2, some of the compounds can breakdown further and cause undesirable properties such as off flavour, colour, texture and can be potentially toxic.

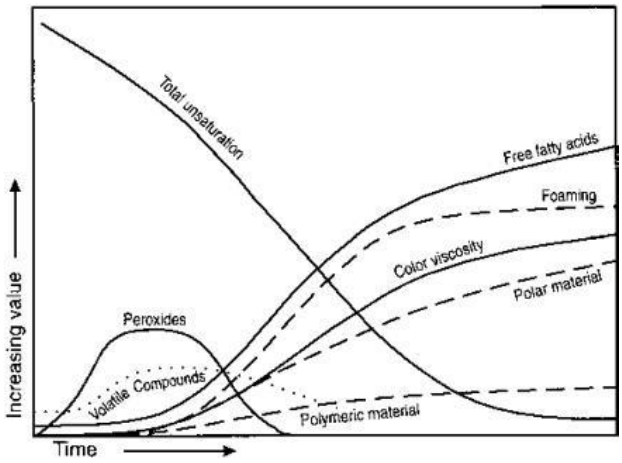


Figure 1-6: A phenomenon of production of the decomposition products during the frying process, adopted from Warner (2002)

Table 1-2: Volatile and non-volatile decomposition products produced during frying, adapted from Warner (2002)

Non-volatile	*monoacylglycerol, diglyceride, oxidised triglyceride, triglyceride dimer, triglyceride trimer, triglyceride polymer, free fatty acid*
Volatile	hydrocarbon, ketone, aldehyde, alcohol, ester, lactone

1.2.1.5 Health effects of deteriorated frying oil

There also have been many studies on the harmful compounds in heated frying oil and their impact to human health. For example, the study of Clark and Serbia (1991) reported that the degradation products of frying oil are harmful to human health, as they destroy vitamins, inhibit enzymes, potentially cause mutations and can cause gastrointestinal irritations. Excessive amounts of polar compounds found in cooking oil have also been related to hypertension (Soriguer *et al.*, 2003). In terms of legislation, in many European countries, polar compounds and triglyceride oligomers are used as the basis of discarding frying oil (Boskou, 2010). For example, Austria set an acceptable maximum for polar compounds at 27 %, France, Italy and Portugal accept 25 % as the maximum and in Germany it is set at 24 %. Gutierrez *et al.* (1988) and Rojo and Perkins (1987) found more cyclic monomers are formed in oils related with a higher content of linolenic acid. Cyclic monomers forming from the intramolecular cyclization of C_{18} polyunsaturated fatty acids are potentially harmful as they can join the body fat along with natural fatty acids (Rojo and Perkins, 1987). Dobarganes and Marquez-Ruiz (1998) found aldehydes have exhibited mutagenic properties. Moreover, Choe and Min (2007) found that acrolein formed from heating the oil reacted with asparagine which further formed acrylamide. Acrylamide has been found to cause genetic damage and cancer in laboratory animals (Boskou, 2010). Malonaldehyde is a secondary lipid oxidation product formed from hydroperoxide. It is reportedly mutagenic and carcinogenic. Malonaldehyde causes skin cancer in rats and can cross-link with lipids and proteins, inactivate ribonuclease and bind covalently to nucleic acids. In cultured mammalian cells, it induces chromosomal aberrations (Marnett, 1999; Madhavi and Salunkhe, 1995; Bird *et*

al., 1982). Crawford *et al.* (1965) reported acute toxicity or lethal dose (LD_{50}) of malonaldehyde in rats as 527 mg/kg. Draper *et al.* (1986) reported that feeding mice with drinking water containing malonadehyde 0.1-10.0 µg/g/day for 12 months produced dose-dependent hyperplastic and neoplastic changes in liver nuclei and increased mortality at the highest level.

1.2.2 Factors affecting oil deterioration and control measures

There are many factors that affect the deterioration of frying oil such as the unsaturated fatty acid content, oil temperature, oxygen absorption, catalysing metals or food matrix and this makes it complicate to understand the mechanism of deterioration (Warner, 2002).

1.2.2.1 Degree of unsaturation of free fatty acids

The degree of unsaturation of free fatty acids has a significant effect on the thermo-oxidative degeneration rather than chain length. The oxidation rate of oil increases as the content of unsaturated fatty acids of frying oil increases (Choe and Min, 2007; Warner *et al.*, 1994; Stevenson *et al.*, 1984). Shiota *et al.* (1999) and Mamat *et al.* (2005) stated that the blending of several oils can change the fatty acid composition and can decrease the oxidation of oils during frying (Choe & Min, 2007). Liu and White (1992) and Xu *et al.* (1999) found the content of linolenic acid is critical to the frying performance, the stability of oil and the flavour quality of fried food. Warner *et al.* (1997) observed that polar compound formation increased proportionally with the linoleic acid content in cottonseed oil during frying potato chips. Although, hydrogenation and genetic modification are used to improve stability of oil by decreasing the unsaturated fatty acids, it will generate *trans* fatty acids instead and may have a metallic flavour (Choe and

Min, 2007; Warner and Knowlton, 1997; Warner and Mounts, 1993). Consumption of *trans* fatty acids has been linked to increased low-density lipoprotein cholesterol and decreased high-density lipoprotein cholesterol in the human blood serum and coronary vascular disease (Willett *et al.*, 1993; Mensink and Katan, 1990).

1.2.2.2 Filtration

Daily filtration to remove the accumulated food particles e.g. charred food, charred batter or charred bread can help reduce the deterioration rate of the oil, excess colour formation and undesirable bitter flavours and odours. The filtration can be done by using metal screens, paper filters, plastic cloths, diatomaceous earth, filter aids etc. (Frankel, 1998c). The filtering of oil in the presence of adsorbents lowers free fatty acids and improves the quality of frying oil (Choe and Min, 2007). Maskan and Bagci (2003) filtered used sunflower oil with a mixture of 2 % pekmez earth, 3 % bentonite and 3 % magnesium silicate and found that the amounts of free fatty acids and conjugated dienoic acids of the oil decreased during frying at 170 ˚C. This was similar to the study by Bheemreddy *et al.* (2002), they used different adsorbents; a mixture of calcium silicate based hubesorb 600, magnesium silicate based magnesol and rhyolite and citric acid based fry powder. They found decreasing levels of free fatty acids and polar compounds formation and concluded the process can improve the frying quality of oil. Mancini *et al.* (1986) had reported that the treatment of shortening with bleaching clay, charcoal, celite or magnesium oxide can improve the quality of oil and the adding of ascorbyl palmitate to fresh oil can lower the amount of free fatty acid, but can change the dielectric constant and colour.

1.2.2.3 Replenishment of fresh oil

A high ratio of fresh oil to total oil can improve frying oil quality (Paul *et al.*, 1997). Frequently replenishing with fresh oil can decrease the formation of polar compounds, diacylglycerols and free fatty acids, thus increasing the frying life of the oils (Romero *et al.*, 1998). Stevenson *et al.* (1984) recommended replenishing 15 % to 25 % of the capacity of the fryer with fresh oil and the higher the amount replenished, the less antifoaming agent, such as silicones, needs to be used. Sanchez-Muniz *et al.* (1993) reported that replenishment can improve the quality of the frying oil after the 30th frying, while Cuesta *et al.* (1993) found frequent turnover of the oil can cause oxidative reactions rather than hydrolytic reactions during deep fat frying of potatoes.

1.2.2.4 Frying time and temperature

There are many studies reported showing that frying time and temperature has an effect on the quality of oil by accelerating thermal oxidation and polymerisation (Blumenthal, 1991). This will increase the production of free fatty acids, polar compounds and polymers (Tompkins and Perkins, 2000; Xu *et al.*, 1999; Romero *et al.*, 1998; Mazza and Qi, 1992). However, Cuesta *et al.* (1993) found the formation of polar compounds rapidly increases during the first 20 fryings, with no significant increase after the 30th frying ($P > 0.05$). Tyagi and Vasishtha (1996) reported the amount of conjugated dienes and *trans* fatty acids when frying potato chips at 170 °C was 3.09 % and 1.68 %. When the frying temperature was increased to 190 °C, the amounts of those compounds increased to 4.39 % and 2.60 %. Kim *et al.* (1999) found high frying temperature decreased polymers with peroxide linkage and increased the polymers with ether linkage or carbon to carbon linkage. Clark and Serbia (1991) found the intermittent heating and cooling of

the oils caused a higher deterioration than continuous heating, due to increasing solubility of the oxygen when the oil cools down from its frying temperature.

1.2.2.5 Food composition

The moisture in food affects the hydrolysis reaction during deep fat frying. The higher the moisture content of the food, the greater the hydrolysis of oils due to the moisture in foods creating a steam blanket over the fryer and reducing contact with air (Choe and Min, 2007; Paul *et al.*, 1997). Lecithin from food can cause foam formation at the early stage of frying (Stevenson *et al.*, 1984). Fat from fish can decrease the frying oil stability. Starch or battered food can degrade oil more quickly (Alvarez *et al.*, 2012). Artz *et al.* (2005) reported that transition metals such as iron, which is found in meat, were accumulated in the oil during frying and increased the oxidation rate and thermal degradation of the oil. Kim and Choe (2003) found a reduction in the formation of free fatty acids, conjugated dienoic acids and aldehydes in palm oil during frying at 160 ˚C by adding red ginseng extract (1 % and 3 %) to flour dough. Holownia *et al.* (2000) reported that an edible film coating on chicken strips (hydroxyl propyl methylcellulose film) can reduce free fatty acid formation in peanut oil during deep frying.

1.2.2.6 Fryer

Selection and maintenance of fryers are also important. A fryer with a large heating area enables faster and more uniform heating of the oil and can prevent the formation of hot spots (Paul *et al.*, 1997). A small surface to volume ratio of fryer minimizes air to oil contact at the surface which can reduce the oxidative

degradation, whilst copper, iron and alloys such as bronze and brass accelerates the oxidation of the oils (Choe and Min, 2007; Paul *et al.*, 1997).

1.2.2.7 Minor components of oils

Minor components in fats and oils such as γ-tocopherols, phospholipids (at less than 100 mg/kg), carotenoids (at low level), squalene and certain sterols (Δ^5-avenasterol) are beneficial to the stability of the oils (Kochhar, 2001). Sesamolin (antioxidant precursor), sesaminol and its isomers, sesamol and its dimer, and oryzanol (a group of ferulic acid esters of sterols present in rice bran oil) possess stabilizing effects during frying operations (Kochhar, 2001). Additions of some agents or compounds such as anti-foaming (dimethyl-polysiloxane) or antioxidants have also been reported to improve the stability of frying oil (Warner, 2002; Kochhar, 2001).

1.3 Antioxidants

Antioxidants are compounds even when present in trace amounts or at very low concentrations can delay or inhibit the oxidation processes which occur in the presence of oxygen or reactive oxygen species (Halliwell *et al.*, 1995; Wichi, 1988). Their role can improve the quality of foods and extend shelf life (Yanishlieva, 2001; Giese, 1996).

1.3.1 Mechanisms of antioxidants

Antioxidants can inhibit or retard oxidation in 2 ways: direct and indirect scavenging free radicals (Reische *et al.*, 2002; Michael, 2001).

- ***Direct scavenging free radicals***

In this case the compounds are called *primary antioxidants* or *chain breaking antioxidants* due to their action by converting free radicals to more stable products.

$$L^{\bullet} + AH \rightarrow LO^{\bullet}$$

$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet}$$

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$$

Primary antioxidants (AH) will donate one electron (hydrogen atom) to the free radicals (lipid radicals, $L^{\bullet}$, $LO^{\bullet}$, $LOO^{\bullet}$) and therefore, the free radicals are reduced. Polyphenols are very active in this action (Yanishlieva, 2001).

- ***Indirect scavenging free radicals***

In this case, the compounds do not directly involve scavenging of free radicals, but they will operate a variety of possible actions to slow the rate of oxidation. Therefore, the compounds are called *secondary antioxidants* or *preventive antioxidants*. Secondary or preventive antioxidants can act via several different mechanisms to retard the oxidation reaction by deactivating the active species and possible precursors of free radicals and suppressing the generation of free radicals and thus, reducing the rate of oxidation (Yanishlieva, 2001). They can chelate pro-oxidant metals and deactivate them, replenish hydrogen to primary antioxidants, decompose hydroperoxides to non-radical species, deactivate singlet oxygen, absorb ultraviolet radiation or act as oxygen scavengers (Reische *et al.*, 2002). The secondary or preventive antioxidants are often referred to as synergists because they contribute to the antioxidant activity of primary

antioxidants. Examples of these synergistic compounds are ascorbic acid, ascorbyl palmitate, lecithin and tartaric acid (Reische *et al.*, 2002).

1.3.2 Natural antioxidants

Antioxidants can occur as natural constituents of foods. Their source of origins can be plants, animal tissues, microorganisms, fungi or can be formed during processing (Pokorny, 2007; Simic, 1981). Examples of natural antioxidants, would be ascorbic acid, tocopherols, carotenoids, flavonoids, amino acids, proteins, protein hydrolysates, fermentation products, nitrosyl compounds from curing, maillard reaction products, phospholipids, sterols and enzymes. Numerous natural antioxidants are in plant sources and vegetable extracts. Phenolic compounds are the majority and the important groups are tocopherols, flavonoids and phenolic acids (Reische *et al.*, 2002; Yanishlieva, 2001).

There are advantages and disadvantages of naturally occurring or added antioxidants in oils and foods on the oil quality during frying. Several factors have influenced the efficacy of natural antioxidants, including fatty acid composition of the oil, the temperature of frying, the amount and type of natural antioxidant, the presence of synergists, chelators, sequesterants and pro-oxidant trace metals, light and product manufacturing conditions. For instance, the appropriate addition of tocopherols can improve oil stability and if adding in excessive amounts (more than 1000 mg/kg) can enhance oxidation (Rossell, 2001a; Frankel, 1998a).

Polyphenols and classification

Polyphenol or phenolic compounds are wide spread in all plants and add benefits to the human diet (Bravo, 1998). Fruits, vegetables and beverages are the main

sources of phenolic compounds (Landete, 2012) which have antioxidant activities to protect or reduce the risk of coronary heart disease, brain dysfunction and cancer (Honglian *et al.*, 2001; Gordon, 1996). Moreover, antioxidants from polyphenol have become an essential part of the food and cosmetic industries to be used as natural colourants and preservatives (Bravo, 1998). Polyphenol or phenolic compounds are secondary plant metabolites that play an important role in plant growth, reproduction, protection against pathogens and predators (Ignat *et al.*, 2011; Bravo, 1998). Flavonoids, phenolic acid, tannins (hydrolysable and condensed), stilbenes and lignans are the main groups of polyphenols (D'Archivio *et al.*, 2007). The polyphenol are synthesised from two pathways: the shikimic acid pathway and the acetate pathway (Bravo, 1998). In nature, the majority of polyphenol in plants are in a conjugated form known as a glycoside with different sugar unit and acylated sugars at different positions of the phenol skeletons (Tsao, 2010). They can be classified according to their source of origin, biological function or chemical structure (Tsao, 2010). The following main classes of polyphenol are divided by their chemical structures.

- ***Flavonoids***

Flavonoids are low molecular weight compounds and constitute a large group of naturally occurring plant phenolics. They are characterised by the carbon skeleton C_6-C_3-C_6. The basic structure of these compounds consists of two aromatic rings linked by three-carbon aliphatic chain which normally has been condensed to form a pyran or a furan ring (less commonly). There are 13 subclasses according to the difference in the degree of hydrogenation and hydroxylation of the three ring systems. The most important 6 subclasses are

flavonols, flavanols, flavones, flavanones, isoflavones and anthocyanins (or anthocyanidins). The basic structure and an example, are presented in Figure 1-7. Flavones and flavonols are found in almost every plant, particularly in the leaves and petals, with flavonols occurring more frequently than flavones. Flavonoids also occur as sulphated and methylated derivatives, conjugated with monosaccharides, disaccharides and form complexes with oligosaccharides, lipids, amines, carboxylic acids and organic acids (Maria, 2013). However, approximately 90 % of the flavonoids in plants occur as glycosides (Yanishlieva, 2001). Flavonoids have been reported in their ability to inhibit lipid oxidation by acting as antioxidants scavenging radicals (superoxide anions, lipid peroxyl radicals and hydroxyl radicals). Other mechanisms of action of selected flavonoids include singlet oxygen quenching, metal chelation and lipoxygenases inhibition (Ignat *et al.*, 2011; Tsao and Yang, 2003; Yanishlieva, 2001). The excellent antioxidant activity of flavonoids is related to the presence of hydroxyl groups in position 3' and 4' of B ring (Figure 1-7), which confer high stability to the formed radical by participating in the displacement of the electron, and a double bond between carbons C_2 and C_3 of the ring C together with the carbonyl group at the C_4 position, which makes the displacement of an electron possible from the ring B. Free hydroxyl groups in position 3 of ring C and in position 5 of ring A, together with the carbonyl group in position 4, are also important for the antioxidant activity of these compounds. The effectiveness or antioxidant activity of flavonoids decreases with the substitution of hydroxyl groups for sugars (glycosides) or in other word, flavonoids with glycosides are less effective antioxidants than the aglycones (no sugars) (Giada, 2013b). For maximum radical scavenging activity a flavonoid molecule needs to meet the following

criteria (1) 3',4'-dihydroxy structure in the B ring, (2) 2,3-double bond in conjunction with a 4-oxo group in the C ring and (3) presence of a 3-hydroxyl group in the C ring and a 5-hydroxyl group in the A ring. Flavonoids with free hydroxyl groups act as free radical scavengers and multiple hydroxyl groups, especially in the B ring, enhance their antioxidant activity. The hydroxyls in ring B are the primary active sites in interrupting the oxidation chain (Yanishlieva, 2001).

- ***Phenolic acids***

Phenolic acids are divided into 2 groups; benzoic acids and their derivatives, and cinnamic acids and their derivatives. The derivatives of cinnamic acid are more active antioxidants than the derivatives of benzoic acid (Yanishlieva, 2001). They are present in plants in free and bound forms, which bind to various plant components through ester, ether or acetal bonds. Their molecular structure consists of a benzene ring, a carboxylic group and one or more hydroxyl and/or methoxyl groups. The different forms of phenolic acids result in varying extraction conditions and susceptibilities to degradation (Maria, 2013; Ignat *et al.*, 2011; Ross *et al.*, 2009; Bravo, 1998). Structures of phenolic acid compounds are presented in Figure 1-8. The antioxidant activity of phenolic acids is generally governed by their chemical structures. The activity increases as the number of hydroxyl (OH) and methoxy groups increases, with the number of OH groups being more important. Thus, caffeic acid is more active than ferulic acid, which in turn is more active than coumaric acid (Pekkarinen *et al.*, 1999). Polyphenolic acids are more efficient than monophenolic acids and the introduction of a second hydroxyl group in the *ortho* or *para* position increases the antioxidative activity. The inhibition efficacy of monophenolic acid is

increased by one or two methoxy substitutions. The combination of two acid phenols increases the efficiency, such as rosmarinic acid (comprising of 2 molecules of caffeic acid) is more effective than caffeic acid. Esterification by sugar moiety decreases its activity, so chlorogenic acid is less effective than caffeic acid (Yanishlieva, 2001).

- ***Tannins***

Tannins are high molecular weight compounds, which can be divided into hydrolysable and non-hydrolysable or condensed tannins. Proanthocyanidins (condensed tannins) are polymeric flavonoids. Tannins are potential metal ion chelators, protein precipitation agents and biological antioxidants (Ignat *et al.*, 2011).

- ***Stilbenes and lignans***

Stilbenes are present in small amounts in food. An example of the compound is resveratrol which is mostly in the glycosylated form, and found in both *cis* and *trans* isomeric form (Ignat *et al.*, 2011). Lignans are mostly present in the free form. Their glycoside derivatives are only a minor form. Lignans and their derivatives are thought to have efficacy in cancer chemotherapy and various pharmacological effects (Saleem *et al.*, 2005).

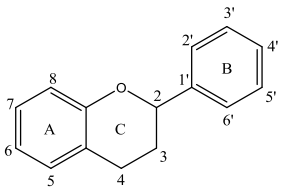
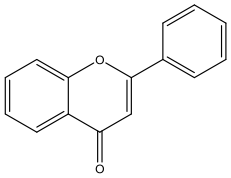
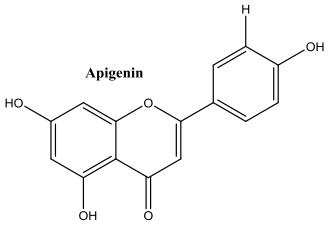
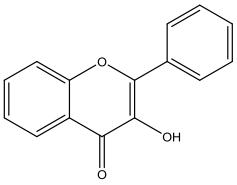
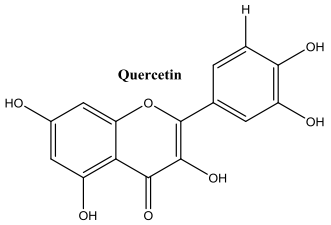
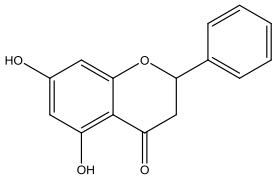
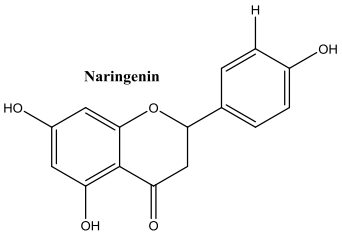
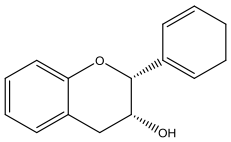
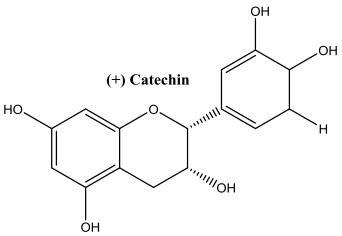
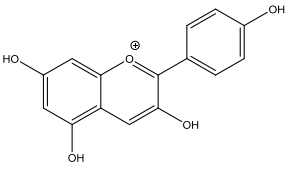
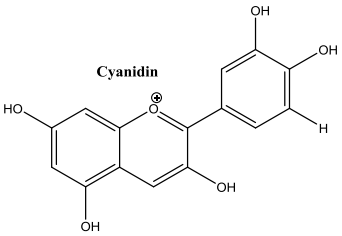
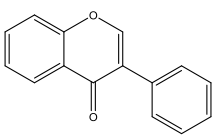
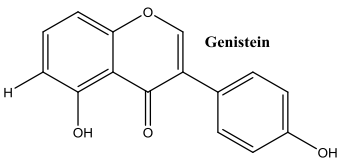
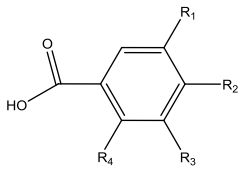
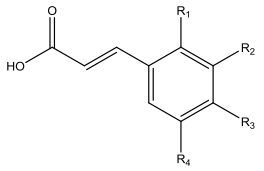
Flavonoid subclass	Basic structures	Sample flavonoid compounds
Structure and numbering system of flavonoids		-
Flavones		
Flavonols		
Flavanones		
Flavanols		
Anthocyanidins		
Isoflavones		

Figure 1-7: Basic structures of the main sub-class of flavonoids, adapted from Tsao (2010), Waterhouse (2005) and Bravo (1998)



(a) Basic structure of benzoic acid



(b) Basic structure of cinnamic acid

(a)	R_1	R_2	R_3	R_4	(b)	R_1	R_2	R_3	R_4
ρ-Hydroxybenzoic acid	H	OH	H	H	Hydroxycinnamic aicd	H	H	H	H
Vanillic acid	OCH_3	OH	H	H	*ρ*-Coumaric acid	H	H	OH	H
Gallic acid	OH	OH	OH	H	Caffeic acid	H	OH	OH	H
Syringic acid	OCH_3	OH	OCH_3	H	Ferulic acid	H	OCH_3	OH	H
					Sinapic acid	H	OCH_3	OH	OCH_3

Figure 1-8: Basic structure of phenolic acids, adapted from Maria (2013)

1.3.3 Synthetic antioxidants

Several synthetic antioxidants have been approved for use in food products, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), propyl gallate (PG), octyl gallate (OG), docedyl gallate (DG), Ionox-100, ethoxyquin, trihydroxybutyrophenone (THBP) and ascorbyl palmitate. Chemical structures are provided in Figure 1-9. The efficiency of synthetic antioxidants depends on their structures; the difference in structure leads to differences in physical properties and antioxidant activity (Reische *et al.*, 2002). Four synthetic phenolic compounds; BHA, BHT, TBHQ and PG, are the most active antioxidants and have been used in food products for over 50 years (Saad *et al.*, 2007).

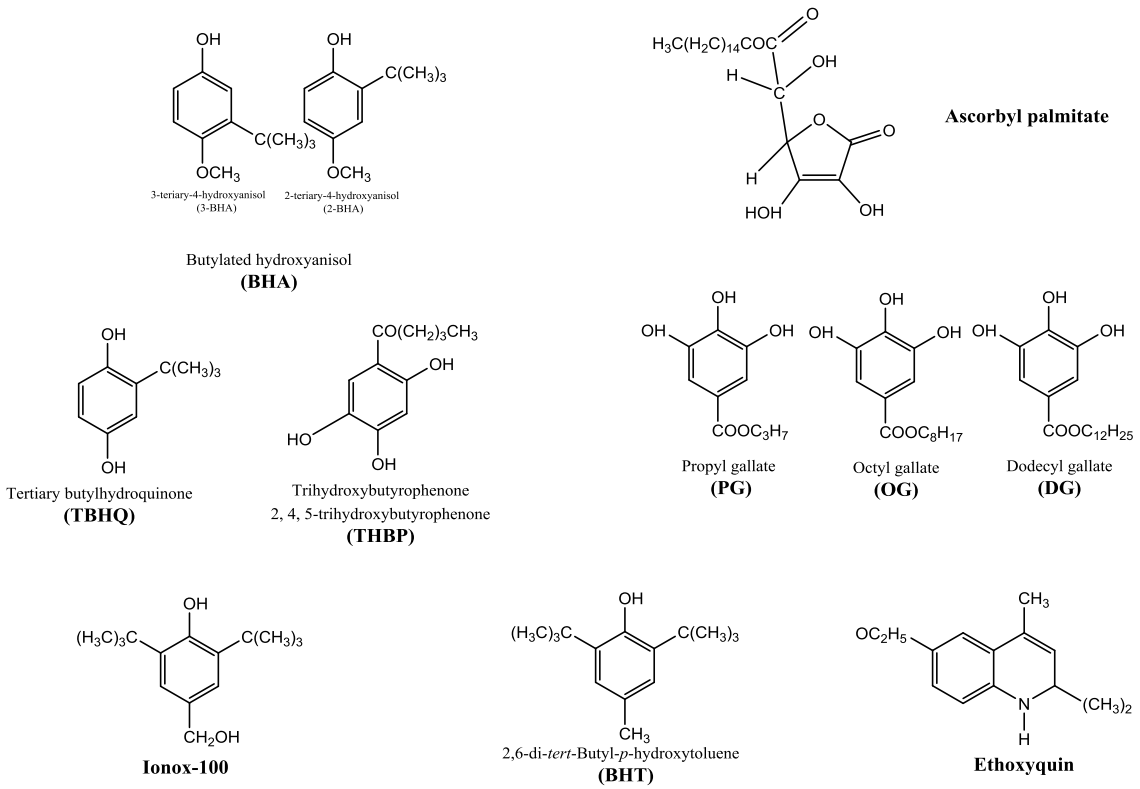


Figure 1-9: Structure of some synthetic antioxidants, adapted from Reische *et al.* (2002) and Yanishlieva (2001)

The limitation of usage had been set to a maximum of 0.02 % of the fat or oil content of the food. However, the type and allowance are different depending on the food product and also varies in regulatory guidelines among the countries (Shahidi, 2005a; Reische *et al.*, 2002). The regulatory approval of synthetic antioxidants in some countries is provided in Table 1-3

Table 1-3: Approval of synthetic antioxidants used in food and oil in some countries, adapted from Shahidi (2005a)

Country	Antioxidants			
	BHA	BHT	Gallates	TBHQ
Australia	/	/	/	/
Brazil	/	/	/	/
China	/	/	/	x
Denmark	/	/	/	x
France	/	/	/	x
Germany	/	/	/	x
Indonesia	/	x	x	x
Iran	/	/	/	/
Japan	/	/	/	x
Malaysia	/	/	/	/
Thailand	/	/	/	/
United Kingdom	/	/	/	x

X = not allowed to use / = allowed to use

For instance, BHA, BHT, PG, OG, DG and TBHQ are permitted by the European Union for use in frying oils and fats (except olive pomace oil). They are also permitted for using in oils and fats for manufacturing heat-treated foods individually or in combinations at maximum levels of 200 mg/kg oil or fat, while BHT is only permitted alone up to 100 mg/kg oil or fat (Marquez-Ruiz *et al.*, 2014; The European Union, 2011). BHA, BHT, PG and TBHQ have lower effectiveness at frying temperatures due to rapid decomposition and volatilisation (Marquez-Ruiz *et al.*, 2014). The stability of these synthetic antioxidants were reported differently. TBHQ has been noted as the most suitable for frying applications due to heat stability and it has good carry-through

(Allam and Mohamed, 2002; Yanishlieva, 2001; Gordon and Kourimska, 1995; Buck, 1991). In contrast to the study of Hamama and Nawar (1991), it was revealed the high temperature and the presence of steam from the food accelerated the evaporation and decomposition of the synthetic antioxidants. They reported TBHQ had the highest loss at 185 °C and the stability of 4 synthetic antioxidants against thermal oxidation in order was BHT>PG>BHA>TBHQ. This also contrasted with the finding of Augustin and Berry (1983) who reported BHT could not show a protecting effect on heated sunflower oil at 180 °C. The study of Tian and White (1994) and Frankel *et al.* (1985) showed the use of TBHQ alone did not extend the frying life of hydrogenated soybean oil and cottonseed oil. Moreover, the effect of BHA, BHT, TBHQ and PG in binary and ternary mixtures with ascorbyl palmitate and tocopherols studied by Allam and Mohamed (2002) showed negative and positive synergistic effects during high temperature treatment (180 °C). According to the study of Choe and Lee (1998), four synthetic antioxidants: butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and *tert*-butyl hydroquinone (TBHQ), slowed down oxidative deterioration at room temperature, however, they become less effective at frying temperatures due to loss through volatilisation. In other studies, Schroeder *et al.* (2006) found that carotenes reacted with oil radicals in red palm olein, but they cannot prevent thermal oxidation of the oil. Kim and Choe (2004) reported that lignin compounds in sesame oil: sesamol, sesamin and sesamolin are stable during heating and contribute to the high oxidative stability of roasted sesame oil during heating at 170 ˚C. This is similar with the findings by Kochhar (2000), where the addition of sesame oil and rice bran oil enhanced the oxidative stability and flavour stability of high oleic sunflower oil. This may be as a result

of avenasterol which is stable at high temperatures. Frankel *et al.* (1985) found that the combination of methyl silicone and TBHQ have a synergistic effect by decreasing the oxidation of soybean oil during deep frying 190 °C. Similar results were reported by Jaswir *et al.* (2000) and Che Man and Tan (1999), where a mixture of rosemary and sage extracts reduced oil deterioration during deep frying for 30 hours. Jaswir *et al.* (2000) indicated that rosemary, sage and citric acid showed the synergistic antioxidant effects on palm olein during frying potato chips. Kim and Choe (2004) also found that the hexane extract of burdock is a potential antioxidant of oil for deep frying and has a significant effect on decreasing the formation of conjugated dienes and aldehydes in lard at 160 ˚C.

1.3.4 Health impacts of synthetic antioxidants

Although, synthetic antioxidants have good efficiency, low cost and, high stability when used in foods, and have been tested for safety, some toxicity data shows that extended use may have an adverse effect to health. BHA, BHT, TBHQ, PG, OG and DG have been identified to cause dermatitis, urticarial, asthma, liver damage and carcinogenesis (Race, 2009; Reische *et al.*, 2002; Wichi, 1988; Grice, 1986). The study by Fisherman and Cohen (1973) reported seven people, who were given 250 mg of BHA and BHT by oral route, had the following symptoms within 2 hours: vasomotor rhinitis, headache, flushing, asthma, conjunctival suffusion, dull high retrosternal pain radiating to the back, diaphoresis (excessive sweating), or somnolence (sleepiness). In addition, their study found cross reactivity with aspirin where twenty-one people were intolerant to both compounds. BHA and BHT also cause liver damage and carcinogenesis in laboratory animals (Wichi, 1988; Grice, 1986). The study of Nera *et al.* (1984) reported that feeding TBHQ to rats and hamsters at 0.25 % for 9 days, had no

significant effect on inducing cellular proliferation. However, at a level of 1 % a significant increase in cell proliferation was observed. Also, there was a case reported of allergic contact dermatitis when in contact with vegetable oil which contained 0.1 % TBHQ (Aalto-Korte, 2000).

1.4 *Pandanus amaryllifolius* Roxb.

Pandanus amaryllifolius Roxb. (Figure 1-10) is commonly known as pandan leaf. This tropical plant belongs to the Pandanus genus, family Pandanaceae (Gangopadhyay *et al.*, 2004). The leaves are upright, green in colour and have a long-narrow blade-like shape. It has a very sweet fragrant aromatic odour, so widely used for enhancing flavour or as food colouring in sweet and savoury dishes in Southeast Asian cooking (Thai, Malaysian and Indonesian).

Figure 1-10: *Pandanus amaryllifolius* Roxb., adopted from Termsuk (2015)

The leaves are used as folk medicine and many studies had been reported to confirm its effect on health. It is used to refresh the body, reduce fever and relieve indigestion and flatulence (Cheeptham and Towers, 2002). Quisumbing (1951) described the essential oil from the leaf as a stimulant and antispasmodic, which also has effects against headaches, rheumatism and sore throats. Raj *et al.* (2014) reported the aqueous extract from root and leaves possess anticancer

activity. Various compounds are found in pandan leaf such as essential oils, carotenoids, tocopherols, tocotrienols (Lee *et al.*, 2004), quercetin (Miean and Mohamed, 2001) and alkaloids (Tan *et al.*, 2010; Takayama *et al.*, 2002; Busque *et al.*, 2002). Ghasemzadeh and Jaafar (2013) studied the profiling of phenolic compounds and antioxidant activity of pandan leaf (from 3 local areas) extracted using 80 % methanol (ratio 1:20) at 70 °C. They reported the amount of total flavonoids ranged from 1.12-1.87 mg/g dried weight (DW) and total phenol content ranged from 4.88-6.72 mg/g DW. The antioxidant activity was determined using free radical scavenging (DPPH) assay ranged from 50.10 % - 64.27 % and determined using Ferric reducing antioxidant power (FRAP) assay ranged from 314.8 – 517.2 µm Fe(II)/g DW. Phenolic compounds present in the extract were rutin, epicatechin, naringin, catechin, kaempferol, gallic acid, cinnamic acid and ferulic acid. Nor *et al.* (2008) studied antioxidant properties of pandan leaf and the potential use as a natural antioxidant. They extracted the leaf powder using ethanol (ratio 1:10) at 50 °C for 8 hours. The total phenol content was found to be 102$\pm$0.4 mg gallic acid equivalent/g extract. The antioxidant activity of pandan extract (50 mg/L and 100 mg/L) was determined using DPPH assay and found to be 45 % and 70 % respectively. The extract was also tested for antioxidant capacity using linoleic acid peroxidation system at 3 concentrations (1000 mg/L, 2000 mg/L and 3000 mg/L), the percentage of inhibition was 0.60 %, 0.65 % and 0.70 % respectively.

1.5 *Piper sarmentosum* Roxb.

Piper sarmentosum Roxb. as shown in Figure 1-11, belongs to the Piperaceae family. It has several names; locally known as Cha-plu in Thailand, Sirih duduk, Akar buguor or Mengkadak in Indonesia and Pokok Kadok or Kaduk in Malaysia

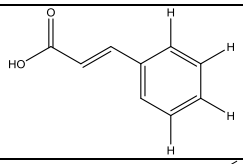
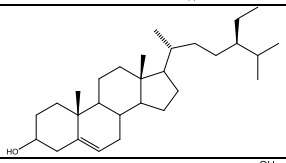
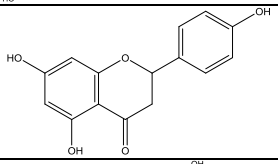
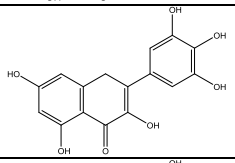
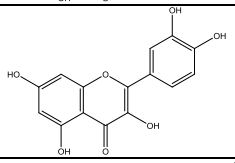
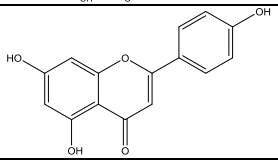
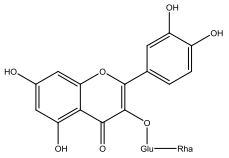
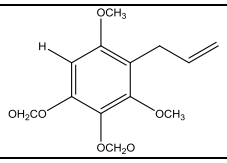
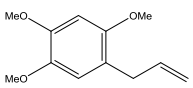
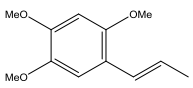
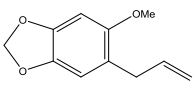
(Seyyedan *et al.*, 2013; Saralamp *et al.*, 1996; Rukachaisirikul *et al.*, 2004). The plant is about 60 cm in height, with a green trunk and jointed at the nodes. The leaves are thin, green, heart shape. It has a, spicy taste and pungent odour (Ridtitid *et al.*, 2007).

Figure 1-11: *Piper sarmentosum* Roxb., adopted from Frynn (2015)

In Thailand, the leaves are not only consumed as savoury snacks or in main dishes but also various parts of the plant are used as folk medicine. This plant has been used as an expectorant, a carminative, to sooth the throat, an anti-flatulence, to enhance the appetite, relieve asthma, treat muscle pain and coughs (Taweechaisupapong *et al.*, 2010; Sireeratawong *et al.*, 2010; Ridtitid *et al.*, 2007; Pongboonrod, 1976). In Malaysia and Indonesia, the plant is used for treating sickness such as toothache, fungoid, dermatitis on the feet, coughing, pleurisy, diabetes, hypertension and joint aches (Seyyedan *et al.*, 2013; Ridtitid *et al.*, 2007). A number of studies have been performed to identity the phytochemicals present within the various parts of *Piper sarmentosum* Roxb. The leaves, fruits and roots were found to contain flavonoids, alkaloids, amide, lignans, phenylpropanoids, tannins, phenolic, ascorbic acid, carotenes, tocopherol and xanthophylls (Hussain *et al.*, 2010; Sumazian *et al.*, 2010; Sim *et al.*, 2009;

Chanwitheesuk *et al.*, 2005). Other various compounds were found such as naringenin, hydroxycinnamic acid, sarmentosine, sarmentine, quercetin, rutin, sesamin etc. (Seyyedan *et al.*, 2013). The research done on chemical constituents of the leaf extracts are summarised in Table 1-4. Chanwitheesuk *et al.* (2005) studied antioxidant activity of *Piper sarmentosum* Roxb. (PS) using β-carotene bleaching method. The PS leaf was cleaned, cut, dried at 50 °C and pulverised, then extracted using methanol at ratio 1:20 w/v. They reported an antioxidant index of the extract to be 13.0±0.84, ascorbic acid content to be 16.6±0.06 mg/100 g and the total phenol content to be 123±0.12 mg/100 g. Ugusman *et al.* (2012) studied flavonoids and protective effects against oxidative stress of *Piper sarmentosum* Roxb. (PS) leaf. The leaf was cleaned, cut, sun dried and pulverised. Flavonoids were extracted from the leaf powder by soaking in water at ratio 1:9 w/v and incubated in a high speed mixer at 80 °C for 3 hours. They reported the amount of total phenol content present in the extract was 91.02±0.2 mg quercetin equivalent/g and total flavonoid content was 48.57±0.03 mg quercetin equivalent/g. The flavonoid compounds present in the extract were analysed using HPLC. Rutin and vitexin were reported as the main flavonoids in this water extract (75.70±0.05 mg/L and 51.93±0.55 mg/L respectively). Subramaniam *et al.* (2003) reported that the antioxidant activity of PS extract, extracted using methanol and determined by superoxide scavenging assay, was 87.6 %. The extract was also analysed for phenolic compounds using HPLC and found naringenin. Hussain *et al.* (2011) reported the antioxidant activity (DPPH assay) of the PS leaf extract extracted using ethanol (21.8 %) was more than using aqueous extract (5.3 %). Sumazian *et al.* (2010) determined the antioxidant activity of aqueous and boiled aqueous PS leaf extracts using FRAP and DPPH assays.

Table 1-4: Chemical constituents found in *Piper sarmentosum* Roxb. leaf extracts

Compound name	Structure	Extraction media	References
Hydrocinnamic acid		Petroleum ether	Niamsa and Chantrapromma (1983)
β-sitosterol			
Naringenin		Aqueous-methanol	Subramaniam *et al.* (2003)
Myricetin		Aqueous-methanol	Rukachaisirikul *et al.* (2004)
Quercetin			
Apigenin		Hexane and methanol	
Rutin		Aqueous-methanol	Miean and Mohamed (2001)
1-allyl-2,6-dimethoxy-3,4-methylenedioxybenzene		Methanol	Masuda *et al.* (1991)
1-allyl-2,4,5-trimethoxybenzene (γ-asarone)			
1-(1-E-propenyl)-2,4,5-trimethoxybenzene (α-asarone)			
1-allyl-2-methoxy-4,5-methylenedioxybenzene (asarone)			

The aqueous extract showed antioxidant activity 377.4 mg $FeSO_4$ equivalent/L using FRAP assay and 15.4 % of inhibition capacity using DPPH assay, while, the boiled aqueous extract showed 98.8 mg $FeSO_4$ equivalent/L and 40 % of inhibition capacity using FRAP and DPPH assays respectively. They reported the amount of total flavonoids was 3.05 mg/g in aqueous extract and 2.03 mg/g in boiled aqueous extract. The total phenol content in aqueous extract was 6.35 mg gallic acid equivalent/g and 7.66 mg gallic acid equivalent/g in boiled aqueous extract. Wan-Ibrahim *et al.* (2010) determined antioxidant activity of aqueous extract of PS leaf using DPPH and FRAP assay. The extract showed 24.3 % inhibition capacity with DPPH assay and 394$\pm$20.4 µmol $FeSO_4$ equivalent/L with FRAP assay. The amount of total phenol content was 430$\pm$3.1 mg gallic acid equivalent/g.

1.6 Rice bran oil and corn oil

Corn oil is derived from the germ or embryo of the corn kernel, while, rice bran oil is extracted from bran of rice paddy (Figure 1-12) (O'Brien, 2004; Gunstone, 2002).

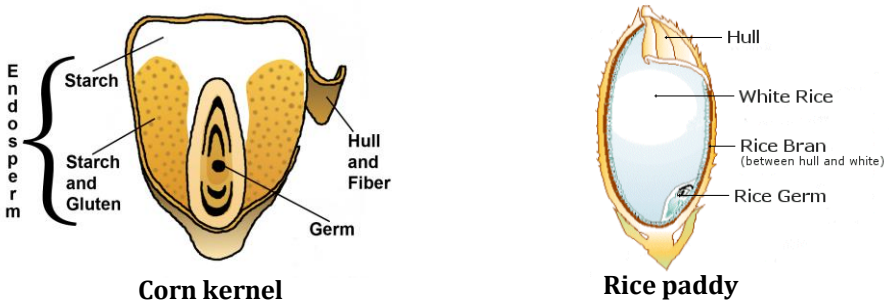


Figure 1-12: Diagram of the corn kernel and rice paddy, adopted from The Corn Refiners Association (2015) and Henan Kingman M&E Complete Plant Co. (2015)

1.6.1 Fatty acid composition of rice bran oil and corn oil

Both rice bran oil and corn oil contain long chain fatty acids, with high levels of unsaturated fatty acids (oleic and linoleic acids). Their typical fatty acid compositions are illustrated in Table 1-5.

Table 1-5: Fatty acid profiles of refined rice bran oil and refined corn oil, adapted from Gunstone (2002) and The Corn Refiners Association (2015)

Fatty acids	g / 100 g oil	
	Rice bran oil	Corn oil
Myristic acid (14:0)	0.2-0.7	-
Palmitic acid (16:0)	12-28	11-13
Palmitoleic acid (16:1)	0.1-0.5	-
Stearic acid (18:0)	2-4	2-3
Oleic acid (18:1)	35-50	25-31
Linoleic acid (18:2)	29-45	54-60
Linolenic acid (18:3)	0.5-1.8	1
Arachidic acid (20:0)	0.5-1.2	-
Paullinic acid (20:1)	0.3-1.0	-
Behenic acid (22:0)	0.1-1.0	-
Others	1	1

1.6.2 Natural antioxidants in rice bran oil and corn oil

As shown in Table 1-6, vitamin E and oryzanols are the main endogenous antioxidants presence in rice bran oil and the main antioxidant in corn oil is vitamin E.

Table 1-6: Natural antioxidants in rice bran oil and corn oil, adapted from Clifford (2001) and O'Brien (2004)

Natural antioxidant	mg / kg oil	
	Rice bran oil	Corn oil
Tocopherol	343	1,477 ± 183
α-Tocopherol	0-454	116-172
β-Tocopherol	0-10	0-22
γ-Tocopherol	16-358	1,119-1,401
δ-Tocopherol	0-42	59-65
Tocotrienol	265	355 ± 355
α-Tocotrienol	0-174	132.2
β-Tocotrienol	62-975	242.5
γ-Tocotrienol	0-104	-
Oryzanol	2,847	-

1.6.2.1 Tocopherols and Tocotrienols

Tocopherols and tocotrienols (tocols) are natural antioxidants found in plant-based oils (O'Brien, 2004). Their chemical structures are shown in Figure 1-13. Seed oils are rich sources of tocopherols while tocotrienols are prevalently found in palm oil, cereal oils such as barley and rice bran oil and legumes (O'Brien, 2004; David *et al.*, 2002). Tocopherols and tocotrienols, have 4 isomers; alpha (α), beta (β), gamma (γ) and delta (δ) (O'Brien, 2004). They inhibit lipid oxidation by acting as a free radical terminator in autoxidation reactions and their presence has a major effect on oil flavour quality (Shahidi, 2005b; O'Brien, 2004). They have a synergistic effect with ascorbic acid, citric acid and phospholipids. Their antioxidant activity depends on temperature and the type of isomer. α-Tocopherol has lower antioxidant activity in edible oils than others, whereas γ-tocopherol has been credited to have the highest antioxidant activity (Shahidi, 2005b; O'Brien, 2004; Yanishlieva, 2001; White, 2000). The amount of

tocols lost during processing are highest during the refining and deodorisation stage (O'Brien, 2004). An excessive addition of tocopherols can be a disadvantage due to its enhanced pro-oxidant effect, enhanced oxidation of unsaturated fatty acids and can cause haemorrhage (Reische *et al.*, 2002; White, 2000; Takahashi, 1995). Thus, the addition of tocopherols needs to be controlled (Shahidi, 2005b).

1.6.2.2 γ-Oryzanol

Rice bran oil is a rich source of γ-oryzanol which is not found in other plant oils. It is predominant in the germ/bran fraction of the rice kernel (Clifford, 2001). The term γ-oryzanol is usually referred to as a mixture of ferulic acid esters of phytosterols and triterpene alcohols. The following 5 compounds are sterol esters of ferulic acid:(1) cycloartenyl ferulate, (2) 24-methylene cycloartanyl ferulate, (3), campesteryl ferulate (4) β-sitosteryl ferulate and (5) cycloartanyl ferulate (Kochhar, 2001), of which 1-4 are the most abundant in rice bran oil (Figure 1-14) (Angelis *et al.*, 2011). Several studies found γ-oryzanol extracted from rice bran had a strong stabilizing effect during storage and frying (Mariod *et al.*, 2010; Chotimarkorn *et al.*, 2008).

1.6.2.3 Ferulic acid

Ferulic acid is another antioxidant presence in small amounts in corn oil but it contributes to the excellent oxidative stability of corn oil (O'Brien, 2004). The chemical structure of ferulic acid is provided in Figure 1-15.

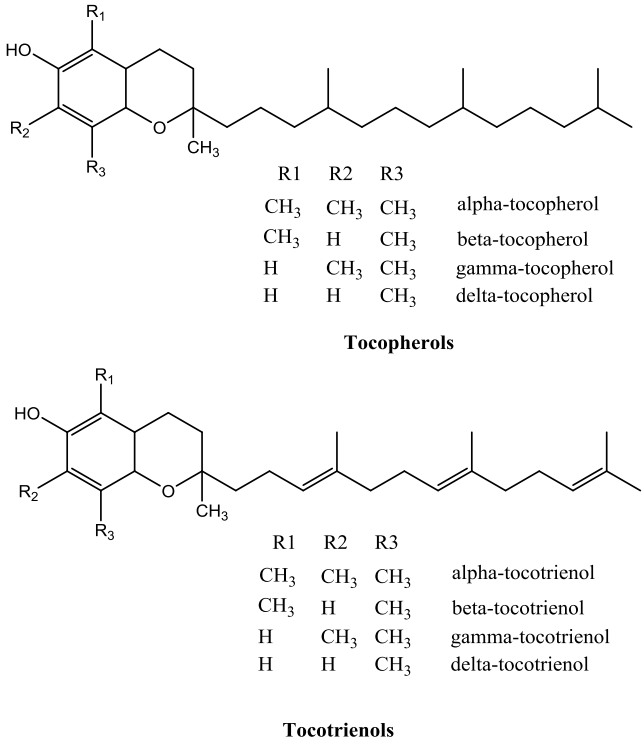


Figure 1-13: Structure of tocopherols and tocotrienols, adapted from Shahidi (2005b)

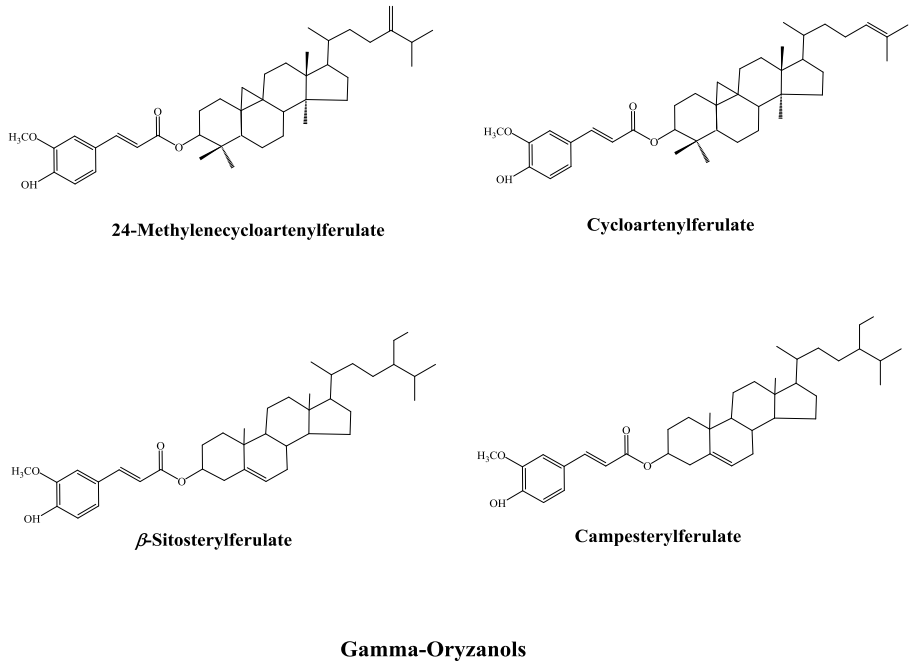
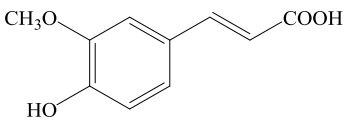


Figure 1-14: Structure of γ-oryzanols isolated from rice bran oil which are ferulic esters, adapted from Angelis *et al.* (2011)



Ferulic acid

Figure 1-15: Structure of ferulic acid, adapted from Balasundram *et al.* (2006)

1.7 Justification, aim and objectives of the study

The quality of frying oils certainly affects the quality of the fried food. During deep frying processes, the oil is heated repeatedly at high temperature. Under these conditions the oil is degraded, which changes the chemical and physical properties. Many factors affect the quality changes of oil during frying and numerous methods have been studied and proposals made to extend the life of frying oil. Synthetic antioxidant such as BHA, BHT, TBHQ and PG, are added to the oils to prevent autoxidation. However, the efficiency of those compounds are under room or mild temperature conditions. At frying temperatures, many studies have reported the synthetic antioxidants decompose and fail to protect the oil (Marquez-Ruiz *et al.*, 2014). The safety issue concerning the use of synthetic antioxidants, particularly, toxicity evidence involving carcinogenesis (Race, 2009) has given rise to numerous studies looking to replace them with new antioxidants from natural sources. Both *Pandanus amaryllifolius* Roxb. and *Piper sarmentosum* Roxb., which are used as food and folk medicine in South East Asia (particularly in Thailand and Malaysia), have shown antioxidant activity in a number of studies which have mostly emphasised on the pharmacological effects (Seyyedan *et al.*, 2013). The quantity and activity of such natural antioxidants

depends on both internal and external factors. External factors are those such as the extraction model; extraction conditions and different solvents at different concentrations; as well as differences in the assays used for determining their antioxidant activities. The internal factors for example, are variation of genotype, growing area, harvesting method, harvesting time (ripeness), climate condition etc. which can also cause differences in antioxidant activities (Apak *et al.*, 2013; Pokorny, 2010; Yanishlieva *et al.*, 2001). Numerous studies on the efficacy of the various natural extracts added in cooking oils, have been tested at storage or mild heating temperatures. For example, Hashemi *et al.* (2011) reported that sunflower oil with added essential oil extract of *Zataria multiflora* Boiss. at 0.025 %, 0.05 % and 0.075 % showed an antioxidant effect but lower than BHA and BHT during storage at 37 °C and 47 °C. Mariod *et al.* (2010) reported rice bran oil supplemented with rice bran extracts 0.1 % and 0.25 % showed a decreasing value of peroxide value, *ρ*-Anisidine value and TBARS value during storage at 70 °C compared to the negative control oil but higher than the oil with added BHA. The study of Merrill *et al.* (2008) reported the oxidative stability of conventional and high oleic vegetable oils with added rosemary extract 1000 mg/L, mixed tocopherols 200 mg/L and ascorbyl palmitate 1000 mg/L at 110 °C showed a significant oxidative stability compared to no antioxidant oils but it was not as effective as TBHQ. The effects of frying condition has rarely been studied. Therefore, it is essential to seek an antioxidant from a natural source that can be used to replace synthetic antioxidants in cooking oil, which have a good efficacy at frying temperature. The aim of this research was to analyse whether extracts from either *Pandanus amaryllifolius* Roxb. (PD) or *Piper sarmentosum* Roxb. (PS)

could be utilised as a natural antioxidant in frying oil. In order to achieve this aim, the objectives of the thesis were:

1) To determine the total phenol content, antioxidant activity and any synergistic effect of extracts of both PD and PS leaves.
2) To select the leaf extract that was higher in phenol content and antioxidant activity and further analyse the effect of extraction conditions.
3) To analyse the phenol profiles of selected leaf extracts in order to better understand their antioxidant activities.
4) To determine the effect of frying temperature on a commercial rapeseed oil, to gain a better understanding of the thermal deterioration pattern and to determine the heating conditions, analytical parameters and analytical methods to be used in further studies.
5) To evaluate the use of aluminium oxide to remove synthetic antioxidants present in palm olein oil and to then determine the oxidative stability of the stripped and unstripped oil.
6) To develop a HPLC method to determine which cooking oils are free of synthetic antioxidants and then select oils which were free of synthetic antioxidants for further experiments.
7) To determine the amount of extract to be used in frying oil by carrying out a preliminary investigation, measuring autoxidation.
8) To determine if there is a protective effect on thermal degradation of the chosen oils during mild heat and frying conditions.

2 Materials and methods

All the materials (chemicals, reagents and preparations) and analytical methods employed throughout the research are included in this chapter.

2.1 Chemicals and Reagents

All the chemicals and reagents were AR or ACS grade.

- Absolute ethanol - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- ABTS or 2, 2'-Azinobis-(3-ethylbenzothiozoline-6-sulfonic acid) - Calibiochem Co. Ltd (Darmstadt, Germany)
- Acetonitrile HPLC gradient - VWR International S.A.S (France)
- Acetonitrile LC/MS grade - VWR International S.A.S (France)
- Activated charcoal powder, NoRIT® SA 2 – ACROS Co. Ltd (New Jersey, USA)
- Aluminium chloride – Alfa Aesar Co. Ltd (Heysham, England)
- Ammonium molybdate – BDH Ltd (Poole, UK)
- Ammonium thiocyante – Alfa Aesar Co. Ltd (Heysham, England)
- *ρ*-Anisidine - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Barium chloride dehydrate - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Butanol or n-Butanol – Fluka Chemicals Ltd (Dorset, UK)
- Butylated hydroxytoluene (BHT) - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Caffeic acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Caffeine – Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Catechin- Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Catechol – Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Chloroform - Sigma-Aldrich Co. Ltd (St. Louis, USA)

- Chlorogenic acid (3CQA) - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- *ρ*-Courmaric acid- Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Cryptochlorogenic acid (4CQA) - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- DCPIP or 2, 6-Dichlorophenol indophenol sodium salt dehydrate >99 % – Fluka Chemicals Ltd (Switzerland)
- Diethyl ether - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- DPPH· or 1, 1-Diphenyl-2-picrylhydrazyl radical – Sigma Co. Ltd (Darmstadt, Germany)
- Epicatechin - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Epicatechingallate – Cambridge Bioscience Ltd (Cambridge, UK)
- Epigallocatechin – Cambridge Bioscience Ltd (Cambridge, UK)
- Epigallocatechingallate – Cambridge Bioscience Ltd (Cambridge, UK)
- Ethylenediaminetetraacetic acid (EDTA) – Fluka Chemicals Ltd (Dorset, UK)
- Ferric chloride – Sigma Co. Ltd (Steinheim, Germany)
- Ferrous (II) chloride or Iron (II) chloride – Sigma-Aldrich Co. Ltd (Steinheim, Germany)
- Ferrous (II) sulphate heptahydrate - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Ferrous chloride anhydrous - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Ferrous sulphate heptahydrate - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Ferulic acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Folin & Ciocalteu's phenol reagent - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Formic acid (99 %) - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Gallic acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Glacial acetic acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)

- Hesperidin - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Hydrochloric acid – Fisher Scientific Ltd (Leicestershire, UK)
- Hydrochloric acid 37 % - Fisher Scientific Co. Ltd (Leicestershire, UK)
- Hydrocinnamic acid- Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Hydrogen peroxide – BDH Ltd (Poole, UK)
- 4-Hydroxybenzoic acid - ACROS Co. Ltd (New Jersey, USA)
- Iron (III) chloride - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Iron (III) chloride hexahydrate - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- L-ascorbic acid - Fisher Scientific Ltd (Leicestershire, UK)
- Linoleic acid – Sigma-Aldrich Co. Ltd (Steinheim, Germany)
- Metaphosphoric acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Methanol HPLC grade - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Naringenin - LKT Laboratories Inc. (St. Paul, USA)
- Neochlorogenic acid (5CQA) - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- iso-Octane or 2,2,4- Trimethylpentane - Fisher Scientific Ltd (Leicestershire, UK)
- Oxalic acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Petroleum ether spirit 40°-60° - VWR International S.A.S (France)
- Phenol phthalein - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Phloridzin - Extrasynthese (Genay, France)
- Potassium dihydrogen orthophosphate - BDH Ltd (Poole, UK)
- Potassium ferricyanide – Aldrich Chemical Co. Ltd (Dorset, England)
- Potassium hydroxide – BDH Ltd (Poole, UK)
- Potassium persulphate - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- 2-Propanol or iso-Propyl alcohol - VWR International S.A.S (France)

- Rutin - ACROS Co. Ltd (New Jersey, USA)
- Sinapic acid – Aldrich Chemical Co. Ltd (Dorset, England)
- Sodium acetate trihydrate - Fisher Scientific Co. Ltd (New Jersey, USA)
- Sodium carbonate - Fisher Scientific Co. Ltd (New Jersey, USA)
- Sodium chloride - FSA supplies (Loughborough, UK)
- Sodium dehydrate orthophosphate - BDH Ltd (Poole, UK)
- Sodium hydroxide - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Sodium nitrite – Fisher Scientific Ltd (Leicestershire, UK)
- Sodium phosphate dibasic anhydrous – Sigma Co. Ltd (Darmstadt, Germany)
- Sodium phosphate heptahydrate (dibasic) – Sigma Co. Ltd (Steinheim, Germany)
- Sodium phosphate monohydrate (monobasic) – BDH Ltd (Poole, UK)
- Sulphuric acid - BDH Ltd (Poole, UK)
- Syringic acid – Alfa Aesar Co. Ltd (Lancaster, England)
- Tannic acid- Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Taxifolin - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- 2-Thiobarbituric acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- TMP or 1,1,3,3-Tetraethoxyopropate malonaldehyde bis (dimethyl acetal) - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Toluene - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- TPTZ or 2, 4, 6- Tri [2-pyridyl]-5-triazine - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Trans-cinnamic acid - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Trichloroacetic acid – Fisher Scientific (Loughborough, England)

- Trolox - Calibiochem Co. Ltd (Darmstadt, Germany)
- Tween 40 - Fisher Scientific (Loughborough, England)
- Vanillic acid – Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Vanillin - BDH Ltd (Poole, UK)
- Vitexin - Sigma-Aldrich Co. Ltd (St. Louis, USA)
- Water - Millipore grade – Food Analytical Laboratory, School of Food Science and Nutrition, University of Leeds (UK)
- Xylenol orange - Sigma-Aldrich Co. Ltd (St. Louis, USA)

2.2 Raw materials

2.2.1 Oils

- Alfa One® Rice bran oil - Hansells Foods UK Ltd (London, UK)
- King® Rice bran oil – Thai Edible Oil Co. Ltd (Bangkok, Thailand)
- Oleen® RBD Palm olein oil – Oleen Co., Ltd (Samutsakorn, Thailand)
- Morrisons® vegetable oil (rapeseed oil) – Morrisons supermarket (Leeds, UK)
- Sainsbury's® Corn oil – Sainsbury's supermarkets Ltd (London, UK)
- Sainsbury's® Rapeseed oil – Sainsbury's supermarkets Ltd (London, UK)
- Yorkshire® Rapeseed oil cold press – Wharfe Valley Farms (Collingham, UK)
- Yors® Rapeseed oil cold press – Wharfe Valley Farms (Collingham, UK)

2.2.2 Plants

- *Pandanus amaryllifolius* Roxb. leaves (Bai Taey leaf) – Nong Fern Thai supermarket (Leeds, UK)

- *Piper sarmentosum* Roxb. leaves (Cha Plu leaf) – Nong Fern Thai supermarket (Leeds, UK)

2.2.3 French fries

- McCain®, pre-fries French fries (90 x 5 x 5 mm) – Morrisons supermarket (Leeds, UK)
- Morrisons®, pre-fries French fries (95 x 8 x 8 mm)– Morrisons supermarket (Leeds, UK)

2.3 Instruments and apparatus

2.3.1 General apparatus

- Analytical balance (KERN KB) – KERN & SOHN GmbH Co. Ltd (Balingen, Germany)
- Analytical balance (Mettler Toledo Xs 104) – Mettler–Toledo Ltd (Beaumont Leys Leicester, UK)
- Burette 10 $\pm$ 0.02 mL borosilicate glass - School of Food Science and Nutrition, University of Leeds (UK)
- Chromatography column, reverse phase C_{18}; 5µm, 250 x 4.6 mm (Gemini 5µ C18 110A, S/NO 540974-22) – Phenomenex® (Cheshire, UK)
- Chromatography column, reversed phase C_{18}; 2.2 µm, 100 x 4.6 mm (Shim-pack XR-ODS , S/NO 70644748) - Shimadzu® (Shimadzu corporation, Japan)
- Erlenmeyer flask with glass stopper 250 mL - School of Food Science and Nutrition, University of Leeds (UK)
- Evaporator (Genevac EZ-2) – Genevac Ltd (Ipswich, UK)

- Extraction Manifold 20 positions, 16 x 100 mm tubes (Waters) – Waters Ltd (Hertfordshire, UK)
- Electrical deep fat fryer (4 L) - Kenwood Ltd (Havant, UK)
- Electrical deep fat fryer (3 L) - Russell Hobbs (Bristol, UK)
- Electrical deep fat fryer (0.5 L) – Coopers of Stortford (Hertfordshire, UK)
- Grinder machine (Kenwood Chef Classic KM336) – Kenwood Ltd (Havant, UK)
- Heated circulating bath (Grant TxF 200) - Grant Instruments (Cambridge, UK)
- pH Meter (Hanna Basic HI 2210) – Hanna Instruments Ltd (Bedfordshire, UK)
- Rhemometer (Kinexus ultra^{+}) – Malvern Instruments Ltd (Worcestershire, UK)
- Soxhlet (fat extraction apparatus) - School of Food Science and Nutrition, University of Leeds (UK)
- Spectrophotometer (Cecil CE 3021) – Cecil Instruments Ltd (Cambridge, UK)
- Ultrasonic water bath (Grant OLS 200) – Grant Instruments (Cambridge, UK)
- Vortex mixer (FB 15013) – Fisher Scientific Ltd (Loughborough, UK)

2.3.2 High Performance Liquid Chromatography (HPLC)

- High Performance Liquid Chromatography ($UFLC_{XR}$) consisting of binary pump, a photodiode array (PDA) with multiple wavelength SPD-20A and a LC-20AD Solvent Delivery Module coupled with an online unit degasser

DGU-20A3/A5, a thermostatised autosampler/injector unit SIL-20AC - Shimadzu Corporation (Kyoto, Japan)

The HPLC technique is used for separation and quantification of non-volatile compounds. This technique is comprised of three components: a mixture of compounds that need to be separated sample solution, a column (stationary phase) and a solvent (mobile phase). In general, the sample solution is injected into the system and moved through a column along with the mobile phase. The compounds in the sample will have different affinities and interactions with the material packed in the column, leading to separation of those components in the sample solution. The molecules with a stronger interaction with the stationary phase will move slowly or will be retained for longer in the column than components with weaker interactions. Therefore, the difference in interactions with the column will help separate sample components from each other (Kupiec, 2004). The separated components can be detected once eluted from the column by a detector. This HPLC technique can use a variety of stationary phases, among those the most widely used packing materials is silica-based. Reverse-phase HPLC is the most popular technique for separation and determination of polar or non-polar polyphenols with most common detection systems being diode array detector (DAD) and, mass or tandem mass spectrometry. The features of reverse-phase HPLC are a non-polar column packing material such as C_{18} coating on octadecyl-silica (ODS-silica) and a polar mobile phase, which is usually a mixture of water and a polar organic solvent such as methanol or acetonitrile. The HPLC technique is a highly sensitive, selective and reliable method, therefore this method is widely used for determination of polyphenol compounds (Agilent Technologies, 2011b).

2.3.3 Ultrafast-High Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS)

- Ultrafast-High Performance Liquid Chromatograph-Mass Spectrometry (UHPLC-MS, Nexera™) comprised of a system controller (CBM-20Alite), solvent delivery pumps (LC-30AD), a degasser (DGU-20A5), a Mixer MR 20 µL, a reservoir tray, an auto sampler (SIL-30AC), a column oven (CTO-30A), a UV-VIS detector (SPD-20a UFLC) and mass spectrometer (LCMS-2020) with an electron spray ionization (ESI) source and a single quadrupole - Shimadzu Corporation (Kyoto, Japan)

UHPLC is a special chromatographic method that is faster, gives better resolution of peaks and uses less solvent than conventional HPLC when it is used with a smaller column packed with smaller particles (usually less than 2 µm in diameter). The UHPLC-MS is a HPLC system coupled with a mass spectrophotometer detector. Although, a DAD detector offers good resolution, it may be difficult to identify or quantify any multicomponent which elutes at approximately the same time. In this case, mass spectrometry offers a better analysis technique due to its highly sensitive detection based on mass-to charge ratios of the molecule. Following the injection of the sample, the eluent is divided into two fractions. One fraction goes to a photodiode array detector (PDA, UV-Vis detector) at 200-600 nm to analyse the component compounds in the extracts. The other fraction is sent to a quadrupole mass spectrometer. The liquid is sprayed and ionized under atmospheric pressure by the atmospheric pressure ionization probe (ESI probe). Subsequently, the ions are separated in accordance with their mass-to-charge ratio (m/z) by a quadrupole mass filter and are detected. The detected ion signals are amplified and then processed by the LCMS

solution data processing software. MS data are acquired in the negative ionisation mode (Shimadzu Corporation, 2008).

2.4 Consumable materials

All these disposable materials were used throughout the studies.

- Cap (2 mL vial with PTFE/Neoprene septa) - Thermo Scientific Ltd (Waltham, USA)
- Cellulose extraction thimbles (30 x 100 mm)– Whatman® Fisher Scientific Co. Ltd (Leicestershire, UK)
- Centrifuge tube (15 mL and 50 mL) – School of Chemistry, University of Leeds (UK)
- Cuvette polystyrene (semi micro 1.5 mL) – Brand® (Wertheim, Germany)
- Eppendorf micro-centrifuge (2 mL) – Thermo®, Electron Corporation (Germany)
- Filter papers no.1– Whatman®, Fisher Scientific Co. Ltd (Leicestershire, UK)
- Magnetic stirrer – Stuart Scientific Co. Ltd (Surrey, UK)
- Pasteur pipette graduated (3 mL) - School of Chemistry, University of Leeds (UK)
- SPE silica cartridge (500 mg, 6 mL) – Thermo Scientific Ltd (Waltham, USA)
- Syringe 3 part polypropylene (1 mL and 5 mL Luer) -BD Plasticpak™, BD (Oxford, UK)
- Syringe filter membrane 0.45 µm (PTFE) - Fisher Scientific Co. Ltd (Leicestershire, UK)

- Syringe filter membrane, nylon 0.22 µm (PDVF)– Fisher Scientific Co. Ltd (Leicestershire, UK)
- Test tube (polypropylene, 16 mm x 100 mm) – Fisher Scientific Co. Ltd (Leicestershire, UK)
- Tip for pipette 10 µL, 200 µL, 1000 µL and 5000 µL – Starlab Ltd (Milton Keynes, UK)
- Vial amber glass (2 mL) – Thermo Scientific Ltd (Waltham, USA)

2.5 Reagents preparation

2.5.1 ABTS solution (7 mM)

ABTS (0.3841 g) was dissolved in water (100 mL) to make 7 mM ABTS solution.

2.5.2 ABTS radical cation ($ABTS^{\cdot+}$) solution

The $ABTS^{\cdot+}$ solution was a mixture of 7 mM ABTS solution and 2.45 mM potassium persulfate at 1:1 ratio (v/v). The mixture solution was mixed well and stored in the dark for 12-16 hours to complete the oxidation of the ABTS and given a bluish-green colour (Re *et al.*, 1999). The $ABTS^{\cdot+}$ solution was diluted, before used, until the diluted solution gave the absorbance 0.700 ± 0.02 at 734 nm.

2.5.3 Ammonium molybdate solution (5 % w/v)

An ammonium molybdate (5 g) was dissolved in distilled water (100 mL) to make 5 % ammonium molybdate solution.

2.5.4 Ammonium thiocyanate solution (30 %)

Ammonium thiocyante (30 g) was dissolved in water (100 mL) to make 30 % ammonium thiocyanate solution.

2.5.5 *ρ*-Anisidine in glacial acetic acid (0.25 %)

ρ-Anisidine (0.5 g) was dissolved in glacial acetic acid (200 mL) to make 0.25 % *ρ*-Anisidine reagent. The reagent was stored in a dark and cool place until used.

2.5.6 DPPH· solution (0.3 mM)

DPPH· (0.01183 g) was dissolved in absolute ethanol to make volume 100 mL. The solution was freshly prepared each time and kept in amber glass container to protect from light.

2.5.7 Elution solvent

Petroleum ether spirit 40-60 °C (900 mL) was mixed with diethyl ether (100 mL) to make elution solvent 9:1 v/v.

2.5.8 Ferric chloride solution (0.1 %)

Ferric chloride anhydrous (0.025 g) was dissolved in water (25 mL) to make 0.1 % ferric chloride solution.

2.5.9 Ferrous chloride (20 mM in 3.5 % hydrochloric acid)

Ferrous chloride (0.1268 g) was dissolved in water (30 mL). The concentrated hydrochloric acid (3.5 mL) was added. The solution was made to volume (50 mL) using water.

2.5.10 Hydrochloric acid (40 mM)

Concentrated hydrochloric acid (73 µL) was added to water (50 mL) to make 40 mM hydrochloric acid. The solution was kept at room temperatures 20-25 °C.

2.5.11 Iron chloride solution

Ferrous sulphate heptahydrate (0.25 g) was dissolved in water (25 mL). Barium chloride dehydrate (0.20 g) was dissolved in water (25 mL), then slowly added to ferrous sulphate heptahydrate solution with constant stirring. Hydrochloric acid (10 M, 1 mL) was added and mixed. The mixed solution was filtered with filter paper (Whatman no.1) and transferred into an amber glass container. The solution was stored in a dark at room temperatures 20-25 °C and was used within 1 month.

2.5.12 Linoleic acid emulsion

Linoleic acid (0.28 g) was mixed with Tween 40 (0.28 g) and 0.2 M PBS pH 7.0 (50 mL). The mixture was mixed well until homogeneous.

2.5.13 Metaphosphoric acid (6 %)

Metaphosphoric acid (6 g) was dissolved in water (100 mL) to make 6 % metaphosphoric acid. The solution was freshly prepared each time.

2.5.14 Mixed solvent acetic acid : chloroform (3:2)

Glacial acetic acid (1500 mL) was added in to chloroform (1000 mL) to make 3:2 mixture solvent.

2.5.15 Mixed solvent iso-propanol : toluene (1:1)

Iso-propanol (1000 mL) was added to toluene (1000 mL) and mixed well to make 1:1 mixture solvent.

2.5.16 Oxalic acid-EDTA solution

The solution was freshly prepared before used. Oxalic acid (0.05 M, 3.1518 g) and EDTA (0.2 mM, 0.0372 g) were dissolved in water to make volume 500 mL.

2.5.17 Phosphate buffer saline (0.2 M PBS, pH 6.6)

Sodium phosphate monobasic (5.52 g) was dissolved in water (200 mL) to make a solution. Sodium phosphate dibasic solution was also prepared using the same procedure (10.72 g in 200 mL). The PBS was a mixture of sodium phosphate monobasic solution (31.25 mL) and sodium phosphate dibasic solution (18.75 mL).

2.5.18 Phosphate buffer saline (0.2 M PBS, pH 7.0)

Sodium phosphate monobasic and sodium phosphate dibasic solution were prepared using the same procedure as 2.5.17. The PBS was a mixture of sodium phosphate monobasic solution (39 mL) and sodium phosphate dibasic solution (61 mL).

2.5.19 Potassium ferricyanide (1 %)

Potassium ferricyanide (1 g) was dissolved in water (100 mL) to make 1 % potassium derricyanide solution.

2.5.20 Potassium hydroxide (0.1 N)

Potassium hydroxide (6.80 g) was dissolved in water (950 mL) and brought to boil. The solution was cooled down and made to volume (1000 mL). To obtain the precise concentration, 0.1 N potassium hydroxide was titrated with potassium hydrogen phathalate solution. The potassium hydrogen phathalate solution was a mixture of potassium hydrogen phathalate (0.0105 g) and potassium iodide (0.15 g) in water (10 mL). The mixture was incubated in the

dark for 10 min before titrating with 0.1 N potassium hydroxide solution using starch solution as an indicator. The endpoint was reached when the blue colour disappeared. The precise concentration was calculated using the following equation.

$$N\ of\ potassium\ hydroxide\ = \frac{W_1\ \times 1000}{W_2\ \times 49.032}$$

W_1 = weight of potassium hydrogen phathalate, g

W_2 = weight of potassium hydrogen hydroxide, g

2.5.21 Potassium persulfate solution (2.45 mM)

Potassium persulfate (0.0662 g) was dissolved in water (100 mL) to make 2.45 mM potassium persulfate.

2.5.22 Starch indicator solution (1 %)

Soluble starch (1 g) was dissolved in water (100 mL) to make 1 % starch indicator solution.

2.5.23 Saturated potassium iodide solution

Potassium iodide (30 g) was dissolved in water (100 mL).

2.5.24 Saturated sodium carbonate solution

Sodium carbonate (100 g) was dissolved in water (400 mL). The solution was heated using hot plate and cooled down before dissolving more sodium carbonate to make it saturated. Then, the solution was filtered through filter paper (Whatman no.1).

2.5.25 Sodium acetate buffer (300 mM, pH 3.6)

Sodium acetate trihydrate (3.1 g) was dissolved in water (900 mL). Glacial acetic acid (16 mL) was added. The solution then was made up to 1000 mL. The pH was measured using a pH meter and adjusted to pH 3.6 with glacial acetic acid. The buffer solution was stored at 4 °C and used within 6 months.

2.5.26 Sodium thiosulphate (0.1 N)

Sodium thiosulphate pentahydrate (24.8200 g) was dissolved in water (1000 mL) to make 0.1 N sodium thiosulphate solution. To obtain the precise concentration, 0.1 N sodium thiosulphate was titrated with potassium hydrogen phathalate solution as described in chapter 2.5.20.

2.5.27 Standard phenols (1000 mg/L)

A standard phenol (1 g) was dissolved in 80 % ethanol (1000 mL) to make 1000 mg/L standard stock solution. The standard phenols were vanillin, vanillic acid, caffeine, caffeic acid, catechin, catechol, epicatechin, epigallocatechingallate, epicatechingallate, epigallocatechin, ferulic acid, gallic acid, 4-hydroxybenzoic acid, trans-cinnamic acid, hydrocinnamic acid, rutin, vitexin, *ρ*-courmaric acid, syringic acid, phloridzin, sinapic acid, chlorogenic acid (3CQA), cryptochlorogenic acid (4CQA), neochlorogenic acid (5CQA), taxifolin, hesperidin, tannic acid and naringenin (chapter 2.1).

2.5.28 Standard phenols (10 mg/L)

The standard stock solution (10 µL, chapter 2.5.27) was added to 80 % ethanol (9.99 mL) to make a working standard for identification 10 mg/L.

2.5.29 Standard synthetic antioxidants (50 mg/L)

The standard synthetic antioxidants BHA, BHT and TBHQ (50 mg, chapter 2.1) were dissolved in acetonitrile (1000 mL) to make concentration 50 mg/L. They were filtered through a filter membrane 0.45 µm PVDF (nylon) before injection into the HPLC system.

2.5.30 Sulphuric acid solution (5 % v/v)

Sulphuric acid anhydrous (5 mL) was dissolved in water (100 mL) to make 5 % sulphuric acid solution.

2.5.31 TPTZ solution (10 mM)

TPTZ (0.031 g) was dissolved in 40 mM HCl and made to volume (10 mL).

2.5.32 Trichloroacetic acid solution (10%)

Trichloroacetic acid (10 g) was dissolved in water (100 mL) to make 10 % trichloroacetic acid solution.

2.5.33 Working reagent for FRAP assay

The working reagent for the FRAP was a mixture of 300 mM sodium acetate buffer pH 3.6, 10 mM TPTZ in 40 mM hydrochloric acid and 20 mM of iron (III) chloride hexahydrate solution at ratio 10:1:1 respectively.

2.6 Methods used for the initial investigation of total phenol content and antioxidant activities of *Piper sarmentosum* Roxb. and *Pandanus amaryllifolius* Roxb. leaf extracts

2.6.1 Leaf preparation

Piper sarmentosum Roxb. (PS) leaves (chapter 2.2) were cleaned, trimmed and dried at 40 °C using an air oven for 24 hours, then pulverized using a grinder. The powder was stored at -20 °C until analysis. *Pandanus amaryllifolius* Roxb. (PD) leaves (chapter 2.2) were also prepared using the same procedure.

2.6.2 Antioxidant extraction procedure

An ultrasonic bath was used to assist extraction as described by Kim and Lee (2005a) with some modifications. PS leaf powder (chapter 2.6.1) was extracted using 80 % ethanol and absolute ethanol. The powder was mixed with the solvent at a ratio 1:20. The mixture was mixed using a vortex mixer for 1 min and extracted using an ultrasonic bath for 20 min (controlled temperatures not exceeding 40 °C). After that, the mixtures were centrifuged at 4,000 rpm for 10 min. The supernatants were filtered through a filter paper no.1. The residue was re-extracted twice by repeating the same procedure. The volume of the filtrates was reduced to 10 to 30 mL using a Genevac. The extract solution was made to volume (100 mL) using distilled water. This extract solution was then used for further analysis. PD leaf powder (chapter 2.6.1) was extracted using the same procedure.

A mixture of extracts was prepared to test the synergistic effect. The filtrates of PD and the filtrates of PS (which were reduced to 10-30 mL using a Genevac)

were mixed together at ratio 1:1. The mixed extracts solution (PDPS) was made to volume (100 mL) using distilled water and used for further analysis.

2.6.3 Determination of total phenol content

The total phenol content in the PS extract solution (chapter 2.6.2) was determined using the Folin-Ciocalteu method (Waterhouse, 2005). The PS extract solution (chapter 2.6.2, 10 µL) was added to distilled water (790 µL) and Folin & Ciocalteu's phenol reagent (50 µL). The solution was mixed well, incubated not exceeding 8 minutes and saturated sodium carbonate reagent (150 µL) was added. The absorbance was measured at 765 nm after incubating at 40 °C in the dark for 30 minutes. The PD extract solution (chapter 2.6.2) was determined using the same procedure as described above. A calibration curve was established using gallic acid as a standard solution (0, 50, 100, 250, 500 mg/L). The total phenol content was calculated using the following equation and expressed as milligram of gallic acid equivalent per gram of leaf powder.

$$Total\ phenolic\ content\ as\ mg\ gallic\ acid\ equivalent = \frac{A \times 100}{wt \times 1000}$$

A = concentration (mg/L) from standard curve
wt = mass of leaf powder in gram
100 = final volume (mL) of sample solution
1000 = constant value to change unit into mL

2.6.4 Determination of antioxidant activities

The difference in amounts, chemical structures, polarity of antioxidants and variation of food components and food systems result in different antioxidant

activities. Thus, the antioxidant activity of the extracts extracted using different solvent in this study was evaluated by various methods.

2.6.4.1 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay is determined the antioxidant capacity to reduce the ferric complex form to ferrous complex form at low pH, which was performed according to the method of Benzie and Strain (1999). This assay is a direct method for determining total antioxidant power of the extract which is fast, reliable and can be used for antioxidants in aqueous solutions. At low pH, the intense blue colour generated from the reduction of ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}) was measured as the absorption. The intensity of the blue colour is directly related to an increase in the absorbance and direct proportion to the concentration of the antioxidant compounds. The PS extract solution (50 µL, chapter 2.6.2) was added to working reagent (1.5 mL, chapter 2.5.33) and incubated at room temperatures 20-25 °C for 6 minutes before measuring the absorbance at 593 nm. Ferrous (II) sulphate heptahydrate was used as a standard solution (0, 20, 50, 100, 200 mg/L) to construct the calibration curve. The standard curve was used for calculating the ability of antioxidants in the extract to reduce ferrous to ferric ions. The FRAP was calculated using the following equation.

$$FRAP\ \ as\ mg\ ferrous\ (II) sulphate\ equivalent/g\ dried\ leaf\ = \frac{A\ \times\ 100}{wt\ \times\ 1000}$$

A = concentration (mg/L) from standard curve
wt = mass of leaf powder in gram
100 = final volume (mL) of sample solution
1000 = constant value to change unit into mL

2.6.4.2 DPPH radical scavenging assay

This assay is used for determining the ability of the antioxidant to scavenge the radical anion 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·), which based on the method proposed by Maizura *et al.* (2011). DPPH radical (DPPH·) is a stable free radical due to the delocalisation of the spare electron on the whole molecule. The DPPH· solution has an intense purple colour in absolute ethanol. Once the radical has accepted an electron or hydrogen atom (from antioxidant) to become a stable molecule, the mixed solution will show less purple colour. The loss of the colour is directly proportional to a decrease in the absorbance which is indirectly proportional to the amount/concentration of the antioxidant. Thus, the less/pale/decolourised colour, the higher the scavenging capacity and the higher the amount of antioxidant. This assay is strongly influenced by pH and the solvent properties, so it may suite lipophilic antioxidants. However, there is a solvent effect as it can compete with the antioxidant to scavenge hydrogen, so this can lead to false positive results. The DPPH· reagent (0.3 mM, 1 mL, chapter 2.5.6) was added to the extract solution (2.5 mL, chapter 2.6.2) and kept in the dark at room temperatures 20 to 25 °C for 30 minutes before measuring the absorbance at 517 nm. The blank was prepared using absolute ethanol (1 mL) instead of 0.3 mM DPPH. The control was prepared by using absolute ethanol (2.5 mL) instead of the extract solution. Percentage of DPPH· scavenging activity or percentages of inhibition activity of the extract were calculated using the following equation.

$$\%\ Inhibition\ or\ \%\ DPPH\ scavenging\ activity\ = \left(\frac{A_c\ -\ A_s}{A_c}\right) \times 100$$

Ac = absorbance of control

As = absorbance of the extract

2.6.4.3 $ABTS^{\cdot+}$ assay

The ABTS radical cation ($ABTS^{\cdot+}$) decolourisation assay is determined the antioxidant capacity to scavenge the radical which was performed based on the method published by Re *et al.* (1999). The bluish green colour of $ABTS^{\cdot+}$ solution was obtained from the reaction between ABTS and potassium persulfate. When the antioxidant compound is added to the $ABTS^{\cdot+}$ solution, the amount of radical is reduced, resulting in a variation of the colour which relates indirectly to the antioxidant concentration. Thus, the less colour, the higher scavenging capacity, and the higher the amount of antioxidant. This assay is suitable for both hydrophilic and lipophilic antioxidant compounds. An aliquot of the extract solution (20-40 µL, chapter 2.6.2) was added to $ABTS^{\cdot+}$ solution (2 mL, chapter 2.5.2), mixed and stood at room temperatures 20-25 °C for 1 min before measuring the absorbance at 734 nm. A range of standards 0-1000 mg/L (0, 50, 100, 500, 1000 mg/L) were prepared using Trolox in absolute ethanol solution to construct a standard curve. The ability of the extract to decolourise the $ABTS^{\cdot+}$ was calculated from the standard curve using the following equation and the data was expressed as milligrams of Trolox equivalent per gram of dry leaf powder.

$$Antioxidant\ activity\ = \frac{A\ \times\ 100}{wt\ \times 1000}$$

A = concentration from standard curve (mg/L)
wt = mass of leaf powder in gram
100 = final volume (mL) of sample solution
1000 = constant value to change unit into mL

2.6.4.4 Potassium ferricyanide reducing power assay

The potassium ferricyanide reducing power of the extract was determined using the method of Jayaprakasha *et al.* (2001). The antioxidant reacts with potassium ferricyanide to form potassium ferrocyanide and then later reacts with ferric trichloride result to form a ferric ferrocyanide complex. This complex compound gives a blue colour with a maximum absorbance at 700 nm. The increased absorbance of the mixtures indicates a higher reducing power of the extract, which directly relates to the higher amount or concentration of the antioxidant compound. The extract solution (100 µL, chapter 2.6.2) was mixed with 0.2 M phosphate buffer solution pH 6.6 (2.5 mL, chapter 2.5.17) and 1 % potassium ferricyanide (2.5 mL, chapter 2.5.19). The mixture solution was incubated for 20 min at 50 °C before adding 10 % trichloroacetic acid (2.5 mL, chapter 2.5.32), followed by centrifuging at 4000 rpm for 10 min. The supernatant (2.5 mL) was added to distilled water (2.5 mL) and 0.1 % ferric chloride solution (0.5 mL, chapter 2.5.8). The absorbance was measured at 700 nm.

2.7 Methods used for the study of the effect of solvent extraction method on total phenol content and antioxidant activity in *Piper sarmentosum* Roxb. leaf extracts

The PS leaf powder was prepared as described in chapter 2.6.1 and extracted using 3 different solvents (water, ethanol and petroleum ether). PS leaf powder following petroleum ether extraction was re-extracted using water and ethanol. The extract solution was used to determined total polyphenol, total flavonoid content, L-ascorbic acid and antioxidant activities. The experimental scheme is shown in Figure 2-1.

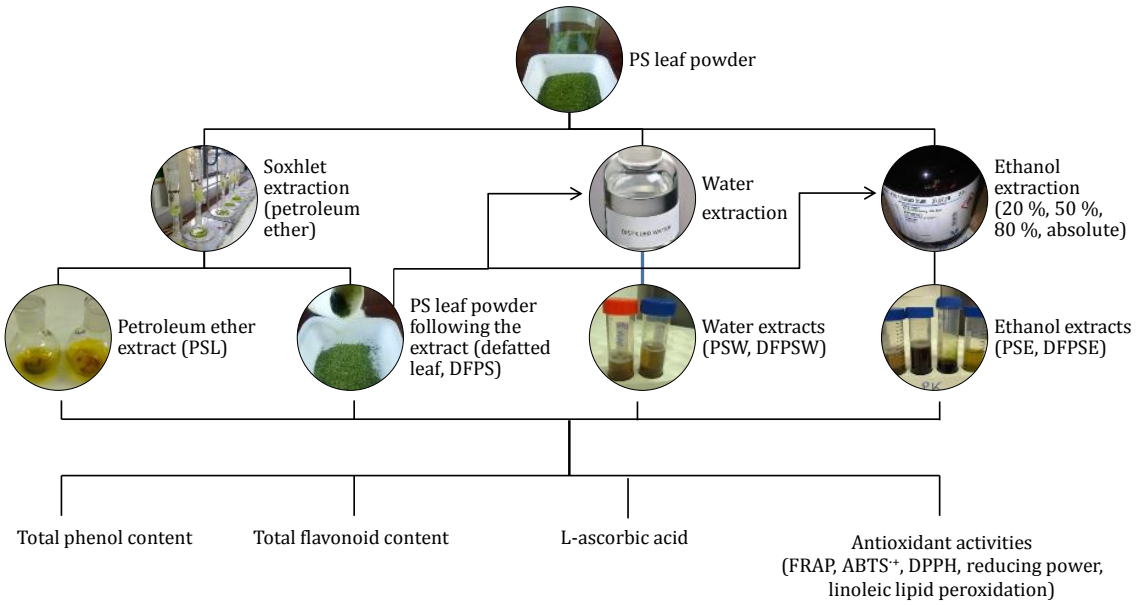


Figure 2-1: Experimental scheme of the study of *Piper sarmentosum* Roxb. leaf extract.

2.7.1 Preparation of the extracts

2.7.1.1 Petroleum ether extraction

The conventional soxhlet method (Nielsen, 2010) was used to extract antioxidants from the leaf powder using petroleum ether. The PS leaf powder (1-6 g, chapter 2.6.1) was placed into a thimble before placing in the soxhlet apparatus. An oven-dried round bottomed flask was weighed and assembled into the soxhlet device. Petroleum ether spirit 40-60 °C (250 mL) was used as extraction medium and extracted at 250 °C for 5 hours. The spirit was then removed, leaving only the petroleum extract residue (PSL) in the container. The container with the extract was dried in an oven 30±2 °C for 1 hour and cooled in a dessicator before weighing. The PSL extract was dissolved with absolute ethanol (10-30 mL) and made to volume 100 mL using distilled water for further

analysis. The leaf powder following the extraction (defatted leaf powder, DFPS) was used for further water and ethanol extraction.

2.7.1.2 Water extraction

PS leaf powder (chapter 2.6.1) and DFPS leaf powder (chapter 2.7.1) were extracted using distilled water by following the antioxidant procedure as described in chapter 2.6.2. These extract solutions (PSW and DFPSW) were used for further analysis.

2.7.1.3 Ethanol extraction

PS and DFPS leaf powder (chapters 2.6.1 and 2.7.1 respectively) were extracted using 20 %, 50 %, 80 % and absolute ethanol by following the antioxidant extraction procedure as described in chapter 2.6.2. These extract solutions (PS20%EtOH, PS50%EtOH, PS80%EtOH, PSAbEtOH, DFPS20%EtOH, DFPS50%EtOH, DFPS80%EtOH, DFPSAbEtOH) were used for further analysis.

2.7.2 Determination of total phenol content

The total phenol content of the extract solutions (chapters 2.7.1, 2.7.1.2 and 2.7.1.3) were determined using the Folin-Ciocalteu method (Waterhouse, 2005) as described in chapter 2.6.3.

2.7.3 Determination of total flavonoid content

The amount of flavonoids present in the extract solutions (chapters 2.7.1, 2.7.1.2 and 2.7.1.3) were determined using spectrophotometry as described by Floegel *et al.* (2011). The extract solution (500 µL) was added to distilled water (3.2 mL), followed by 5 % sodium nitrite (150 µL). The solution was mixed and left for 5 min before adding 10 % aluminium chloride (150 µL). The solution was mixed

well and stood for 6 min before adding 1 M sodium hydroxide (1 mL). The solution was mixed well and measured at 510 nm using a spectrophotometer. A calibration curve was established using catechin as a standard solution ranging from 0-500 mg/L (0, 50, 100, 500 mg/L). The total flavonoid content was calculated using the following equation and expressed as milligram of catechin equivalent per gram of leaf powder.

$$Total\ flavonoid\ content\ as\ mg\ catechin\ equivalent\ = \frac{A\ \times\ 100}{wt\ \times\ 1000}$$

A = concentration (mg/L) form standard curve
wt = mass of leaf powder in gram
100 = final volume (mL) of sample solution
1000 = constant value to change unit into mL

2.7.4 Determination of total L-ascorbic acid content

2.7.1.1 Spectrophotometric assay

L-Ascorbic acid content in PS leaf powder (chapter 2.6.1) and PS extract solutions (chapters 2.7.1 to 2.7.1.3) were determined by spectrophotometer according to Chanwitheesuk *et al.* (2005) with slight modification. The ammonium molybdate solution forms a complex with ascorbic acid where present. L -ascorbic acid content can be determined by measuring the molybdate blue complex at 760 nm using a calibration graph of standard ascorbic acid. PS leaf powder (0.5 g) or PS extract solutions (2 mL) were analysed by adding oxalic acid-EDTA solution (10 mL, chapter 2.5.16) and sonicating for 10 min at room temperatures 20-25 °C. The upper layer of the sample solution was filtered through a filter paper no.1 and the residue was re-extracted twice with oxalic acid-EDTA solution (5 mL). An aliquot of filtrated upper layer (2.5 mL) was transferred into a 25 mL volumetric flask.

The reagents, oxalic acid-EDTA solution (2.5 mL), metaphosphoric acid-acetic acid solution (0.5 mL, chapter 2.52.5.13), sulphuric acid solution (0.1 mL, chapter 2.5.30) and ammonium molybdate (2 mL, chapter 2.5.3) were added. The volume was then made up to 25 mL with distilled water, mixed and stood for 15 min before the absorbance was measured at 760 nm. A range of standards (0, 10, 50, 100, 200 mg/L) were prepared using L-ascorbic acid to construct a standard curve of absorbance against concentration. The L-ascorbic acid content was calculated using the following equation.

$$L - ascorbic\ acid\ content\ (mg/g) = \frac{A \times df}{1000 \times wt}$$

A = concentration from calibration curve, mg/L
df = dilution factor
wt = mass of leaf powder, g
1000 = constant value to change unit into mL

2.7.1.2 HPLC assay

The HPLC technique was used to determine L-ascorbic acid due to its high sensitivity, selectivity and reliability. The L-ascorbic acid present in PS leaf and the extract can be identified by comparing retention times with standard L-ascorbic acid. L-Ascorbic acid content in PS leaf powder (chapter 2.6.1), PS extract solutions (chapter 2.7.1 to 2.7.1.3) were determined using high performance liquid chromatography (HPLC) according to the method of Scherer *et al.* (2012) with slight modification. PS leaf powder (0.5$\pm$0.0001 g) and PS extract solutions (2 mL) were treated with 6 % metaphosphoric acid (5 mL) and sonicated for 5 min. The mixtures were centrifuged at 4000 rpm for 5 min and the supernatant was filtered through a filter paper no.1. The residue was re-extracted twice with the same procedure. The filtrate was made to volume 25 mL

using 6 % metaphosphoric acid before filtering through a 0.45 μm PVDF filter membrane. To determine the ascorbic acid present in the samples, 20 μL of the samples were injected into HPLC-PDA system, the $UFLC_{XR}$ (chapter 2.3). A reversed phase (C_{18}) column (100 x 4.6 mm., 2.2 μm) was used. The analytical conditions were isocratic elution with mobile phase 0.01 M potassium dihydrogen phosphate buffer solution pH 2.6, flow rate 0.5 mL/min, column temperatures 45 °C, PDA diode array UV detector at 250 nm and a cycle time 15 min. A range of standards (0, 10, 50, 100, 200 mg/L) were prepared using L-ascorbic acid to construct a standard curve of absorbance against concentration. The L-ascorbic acid content was calculated using the same equation as displayed in spectrophotometric method (chapter 2.7.1.1).

2.7.5 Determination of antioxidant activities

2.7.5.1 Ferric reducing antioxidant power (FRAP) assay

PSL extract solution (chapter 2.7.1.1), water extract solutions (2.7.1.2) and ethanol extract solution (chapter 2.7.1.3), were used to determine the antioxidant capacity using FRAP assay (chapter 2.6.4.1).

2.7.5.2 DPPH radical scavenging assay

PSL extract solution (chapter 2.7.1.1), water extract solution (chapter 2.7.1.2) and ethanol extract solution (chapter 2.7.1.3), were used to determine the inhibition capacity using DPPH assay (chapter 2.6.4.2).

2.7.5.3 $ABTS^{\cdot+}$ assay

PSL extract solution (2.7.1.1), water extract solution (chapter 2.7.1.2) and ethanol extract solution (2.7.1.3), were used to determine the antioxidant capacity using $ABTS^{\cdot+}$ assay (chapter 2.6.4.3).

2.7.5.4 Reducing power assay

PSL extract solution (chapter 2.7.1.1), water extract solution (chapter 2.7.1.2) and ethanol extract solution (chapter 2.7.1.3), were used to determine the antioxidant capacity using reducing power assay as described in chapter 0.

2.7.5.5 Linoleic lipid peroxidation inhibition assay or ferric thiocyanate method

This assay was performed according to Anesini *et al.* (2008) and Jayaprakasha *et al.* (2001). Any peroxides occurring from linoleic acid oxidation will react with ferrous chloride and ammonium thiocynate solution to form a complex that can be measured by spectrophotometry at 500 nm. This step was repeated every 24 hours until the control (phosphate buffer plus linoleic acid) reached its maximum absorbance value. High absorbance values indicate high levels of linoleic acid oxidation, and the lower inhibition capacity of the antioxidant. Therefore, the less intense the colour or the lower absorbance, the higher the inhibition capacity and the higher concentration of the antioxidant. The extract solution (500 mL, chapters 2.6.2 to 2.7.1.3) was mixed with 0.2 M phosphate buffer pH 7.0 (2.5 mL, chapter 2.5.18) and linoleic acid emulsion (2.5 mL, chapter 2.5.12). Following mixing, the mixtures solution was incubated at 37 °C for 120 hours. The incubated mixtures solution (100 µL) was sampled at 24 hours intervals. 75 % ethanol (5 mL), 30 % ammonium thiocyanate solution (0.1 mL, chapter 2.5.4) and 20 mM

ferrous chloride in 3.5 % HCl (0.1 mL, chapter 2.5.9) were added and mixed well and incubated at room temperatures 20-25 °C for 3 min before measuring the absorbance at 500 nm. The control was performed by the same procedure without adding the extract solution. The percentage inhibition of lipid peroxidation of linoleic acid was calculated by following equation.

$$Inhibition\ of\ lipid\ peroxidation\ (\%) = 100 - \left(\frac{A_s}{A_c} \times 100\right)$$

A_s = absorbance of the extract

A_c = absorbance of the control

2.8 Methods used for the study of the effect of decolourisation on total phenol content and antioxidant activity of the PSE extract

2.8.1 Decolourisation

The PS leaf powder (chapter 2.6.1) was extracted using 80 % ethanol by following the extraction procedure in chapter 2.7.1.3. The extract solution (20 mL) was mixed with activated charcoal (0.1 g or 0.5 % w/v, 0.2 g or 1 % w/v, 0.4 g, 2 % w/v). The mixture was stirred for 10 min at room temperatures 20-25 °C prior to centrifuge at 4000 rpm for 10 min. The supernatant was filtered through a filter paper no.1 and a 0.45 µm filter membrane PTFE respectively. The filtrated extract solution was used for further analysis. To determine the efficiency of the extraction method, gallic acid (50 $\pm$ 0.0001 mg) was added to PS dried leaf powder (chapter 2.6.1) prior to extraction using 80 % ethanol as described in chapter 2.6.4.2. The extract solution was decolourised using activated charcoal following the procedure as explained above. The spiked treated extract solution was used for

further analysis. The control was prepared the same procedure without treating with activated charcoal and no spiking.

2.8.2 Determination of total phenol content, antioxidant activity and efficiency of extraction model

The filtrated extract solution (chapter 2.8.1) was used to analyse total phenol content and antioxidant capacity using FRAP assay as described in chapter 2.6.3 and 2.6.4.1 respectively. The extract solution with spiked was analysed total phenol content and calculated percentage of recovery by the following equation.

$$\%\ Recovery\ = \frac{A\ - B}{C} \times 100$$

A = amount of total phenol as gallic acid in the extract solution with spiked

B = amount of total phenol as gallic acid in the extract solution without spiked

C = amount of gallic acid added

2.9 Methods used for the study of polyphenol profile of *Piper sarmentosum* Roxb. leaf extract

2.9.1 Preparation of PS and DFPS extracts

The PS and DFPS extract solutions were prepared using 80 % ethanol following the method in chapter 2.7.1.3. These solutions were filtered through a filter membrane 0.22 µm PVDF (nylon) prior to injection into an ultra-high performance liquid chromatograph mass spectrometer (UHPLC-ESI-MS), the Nexera™ (chapter 2.3).

2.9.2 Preparation of PSL extract

The PSL extract was prepared using petroleum ether for extraction (chapter 2.7.1.1). To extract polyphenol present in PSL extract, the methods of Saad *et al.* (2007) and Perrin and Meyer (2003) were used. Hexane (5 mL) and acetonitrile (3 mL) were added to the PSL extract and mixed for 1 min followed by centrifugation at 4000 rpm for 10 min. The acetonitrile layer was collected. The hexane layer was re-extracted twice with acetonitrile (3 mL). The acetonitrile layers were filtered through a filter membrane 0.22 µm PVDF (nylon) prior to injection into the Nexera™.

2.9.3 Identification of phenol compounds

The component compounds present in the extracts had very close retention times, so it was difficult to identify those compounds. Each polyphenol has a specific mass-to-charge ratio and therefore using this it is possible to determine the polyphenols. Thus, mass spectrometry was considered due to its high sensitivity and the detection based on mass-to-charge ratios of the molecule. An LC-MS, the Nexera™ (Shimadzu, chapter 2.3) operation system was used for identifying compounds present in the extract by comparing retention times and mass-to-charge ratios (m/z) with standard phenols. A reversed phase column (C_{18}), 5 µm particle size, 4.6 mm x 25 cm; Phenomenex®, was used. Solvents for the mobile phase were 0.1 % formic acid in water (mobile phase A) and 0.1 % formic acid in acetonitrile (mobile phase B). The injection volume was 10 µL with flow rate 0.5 mL/min of binary gradient mobile phase. Elution conditions were applied as following: the starting mobile phase condition was 10 % B, held isocratic for 2 min. Accordingly, solvent B was increased to 25 % (2-12 min) and then to 100 % B (linear gradient 12-32 min). The conditions were held at 100 %

B for 2 min (isocratic 32-35 min) prior to returning to 10 % B (linear gradient 35-38 min) with a final isocratic run to 10 % B from 38-45 min for reconditioning the column. The column oven was set at 25 °C. The operation was carried out at room temperatures 20-25 °C. The total analysis time was 45.01 min. Phenol compounds were measured at 275, 280, 320 and 360 nm. A single quadrupole mass spectrometer with atmospheric pressure ionization probe (ESI) was used. The liquid sample was sprayed and ionized under atmospheric pressure by the probe. The ions were separated in accordance with their mass-to-charge ratio (m/z) by a quadrupole mass filter and were detected by mass spectrophotometer. The detected ion signals were amplified and then processed by the LCMS solution data processing software (Shimadzu Corporation, 2008). The mass spectral data were acquired using SIM negative mode. Ionisation was performed using nitrogen as nebulizer with gas flow at 1.5 litre/min and drying gas (15 litre/min). The compounds were identified by comparing the retention time and m/z with 26 standards.

2.9.4 Quantification of phenol compounds

A series of standard curves (5, 10, 25, 50, 75, 100 mg/L) were constructed to quantify the amount of identified compounds (chapter 2.9.3). The extract solutions (10 µL, chapters 2.9.1 and 2.9.2), were injected into HPLC-PDA system, the $UFLC_{XR}$ (chapter 2.3). The amount of the identified compound was measured using the same condition as in chapter 2.9.3 at its specific wavelength which gave the highest absorbance. The amount of phenol compound presence in the PSE, DFPSE and PSL were calculated using the following equation.

$$mg\ of\ the\ compound\ \ /100\ g\ =\ \frac{A \times df}{wt}$$

A = concentration (mg/L) from standard curve
df = dilution factor = 10 for ethanol extract, = 1.2 for petroleum ether extract
wt = mass of leaf powder, g

2.10 Methods used for the study of the effect of repeated frying on the physical and chemical characteristics of the oils

2.10.1 Materials

Morrisons® vegetable oil (chapter 2.2.1), pre-fried French fries (McCain® and Morrisons®, chapter 2.2) and electrical deep fat fryer (4 L, chapter 2.3) were used for this preliminary frying.

2.10.2 Frying procedure

A batch of French fries (100 g) was fried in oil (3 L) at 190 ^{0}C, 3 min for McCain® French fries and 3.5 min for Morrisons® French fries. The frying process was continuously conducted for six days frying, ten batches a day. After frying 10 batches, the fryers were turned off and the oil was left to cool down. The oil (50 mL) was sampled, filtered through cotton wool for each day. The heated oil samples were kept in tight container at 20-25 ^{0}C for further analysis.

2.10.3 Analysis of physical changes in oils

2.10.3.1 Determination of colour changes

The changes in colours of the heated oils were measured the absorbance at 460, 550, 620 and 670 nm using the photometric method based on AOCS Cc 13c-50 (AOCS, 1998d). The heated oil (1 mL, chapter 2.10.2) was placed into a cuvette (chapter 2.4) and the absorbance measured at 4 different wavelengths (460, 550,

620 and 670 nm). The photometric colour of the heated oil was calculated by the following equation.

$$Photometric\ colour\ =\ 1.29\ A_{460} +\ 69.7\ A_{550} + 41.2\ A_{620} - 56.4\ A_{670}$$

A = absorbance at specified wavelength

Wavelengths between 400-700 nm are in the visible light wavelength and are associated with colour from violet to red (Loughrey, 2005). The four wavelengths at 460, 550, 620 and 670 nm reflect the light in the violet-blue, green-yellow, orange and red region respectively (McNicholas, 1935). In addition, these wavelengths are also responsible for pigment measurements for example, the wavelength between 400 and 500 nm are used for carotenoids pigments (Rodriguez, 2005). Flavonoids/carotenoids are normally measured at 510-550 nm which are suitable for yellow, red to brown colour (Floegel *et al.*, 2011; AOCS, 1998d). The wavelength between 490 to 580 nm are used for measuring anthocyanin pigment (red to purple to blue colour) which varies depending on pH of the pigment solution (Zhao and Yu, 2010; Giusti and Wrolstad, 2005) and 600 to 670 nm are used for chlorophyll pigments (Pokorny *et al.*, 1995; Pohle and Tierney, 1957; McNicholas, 1935). Within the plant kingdom, the most abundant pigments are the lipid soluble chlorophylls and carotenoids. The carotenoids pigments are often covered by green chlorophyll pigments. Anthocyanins are water soluble pigments in the flavonoid group which give blue, purple, red and orange colours of many plants and fruits. All these pigments are found in all higher plants due to their functions of photosynthesis and photoprotection. These pigments, their derivatives or their formation of pigment decomposition products contribute to food colour (Schwartz, 2005). Therefore, the colour of oil

is also influenced by pigments responding to these wavelength. The colour or pigments contained in the oil may be obtained from fried food or natural pigments such as carotenoids in crude palm oil (dark orange-red colour), chlorophyll and carotenoid in olive oil (Moyano *et al.*, 2010; Man *et al.*, 1996; Tan *et al.*, 1985). The above equation using four measurements was found to give a strong correlation of 0.993 with the Lovibond method. Therefore, this photometric method has been adopted to replace the Lovibond method (AOCS, 1950).

2.10.3.2 Determination of smoke point

The smoke point is the temperature that the oil begins to breakdown to glycerol and free fatty acids and is marked as the beginning of flavour and nutritional degradation (Bockisch, 1998). To investigate changes of the smoke point of each oil sample, the sample was analysed by following the AOCS Cc 9a-48 method (AOCS, 1998c). The oil (2 mL, chapter 2.10.2) was placed into a stainless plate (7 cm diameter) and heated. The smoke point temperature was recorded when the heating oil gave off a thin, continuous stream of smoke.

2.10.3.3 Determination of viscosity

The viscosity of the samples was determined using a rhemometer (chapter 2.3) to evaluate the impact of thermal degradation. The oil (6 ± 0.4 mL, chapter 2.10.2) was loaded into the cylindrical controlling cartridge before measuring the viscosity at room temperatures 20-25 °C using shear rate ($1\ s^{-1}$). The viscosity is expressed in terms of poise (η).

2.10.4 Analysis of chemical changes in oils

2.10.4.1 Determination of acid value and free fatty acid value

Exposing oil to prolonged high temperatures causes the triglyceride and diglyceride present to be converted to fatty acids and glycerol by a hydrolysis reaction resulting in the rancidity of the oils. This rancidity is measured by the acid value. The acid value was measured using the AOCS Cd 3a-63 (AOCS, 1998a). The acid value is represented by the number of mg of potassium hydroxide (KOH) to neutralize the free acids in 1 g of sample. The oil (0.0800 – 0.5000 g, chapter 2.10.2) was dissolved in mixed solvent (125 mL, chapter 2.5.15). Phenol phthalein indicator (2 mL) was added before titrating with standardised 0.1 N potassium hydroxide (chapter 2.5.20). The end point was determined by a fuchsia colour which persist for at least 15 sec. The acid value is expressed as mg KOH/g of oil and also can be expressed in terms of free fatty acid as a percentage of oleic acid by dividing the acid value by 1.99 as shown in following equations.

$$Acid\ value\ as\ mg\ KOH/g.of\ oil\ = \frac{mL\ KOH\ \times N\ \times 56.1}{w}$$

$$Free\ fatty\ acid\ as\ \%\ oleic\ = \frac{Acid\ value}{1.99}$$

N = normality of standardised KOH
w = weight of sample in g
56.1 = molecule weight of KOH
1.99 = conversion factor

2.10.4.2 Determination of peroxide value (Iodometric method)

Hydroperoxide, one of the primary products from the initial stage of lipid oxidation, can be determined using the iodometric method and colourimetric

methods. The iodometric method is based on the measurement of the iodine liberated from excess potassium iodide by the peroxides present in the oil. The results are expressed as milliequivalents of active oxygen per kilogram of oil (meq/kg). This method was performed as described by AOCS-Cd 8-53 (AOCS, 1998b). The oil (2.50 $\pm$ 0.05 g, chapter 2.10.2) was dissolved with a mixed solvent (15 mL, chapter 2.5.14). Saturated potassium iodide (250 µL, chapter 2.5.23) was added and continuous shaking for 1 min. Distilled water (15 mL) was added to the solution followed by titrating with standardised 0.1 N sodium thiosulphate (chapter 2.5.26) until a pale yellowish colour was achieved. The starch indicator solution (250 µL, chapter 2.5.22) was added (the solution turned to blue colour), the titration continued until the endpoint was reached (the blue colour disappeared). The peroxide value can be calculated using the following equation.

$$Peroxide\ value\ as\ meq\ oxygen/kg\ oil = \frac{(S - B)\ \times N\ \times 1000}{W}$$

S = mL of sodium thiosulfate used in titrating sample
B = mL of sodium thiosulfate used in titrating blank
N = normality of standardised sodium thiosulfate
W = weight of sample (oil), g
1000 = conversion unit to kg

2.10.4.3 Determination of the 2-thiobarbituric acid value (TBA)

Hydroperoxides, products from primary oxidation stage, break down into secondary products and produce an odour associated with rancidity (Pegg, 2005b). Monitoring the changes of secondary lipid oxidation of fats and oil, can be performed directly by measuring the TBA value based on IUPAC 2.531 (IUPAC, 1987b) or AOCS Cd 19-90 (AOCS, 1998e). Increasing TBA values indicate that lipid oxidation is proceeding (Pegg, 2005b). The TBA value is defined as the increase

of absorbance measured at 532 nm due to the reaction of the equivalent of sample (1 mg) per 2-thiobarbituric acid (1 mL) (Pokorny and Dieffenbacher, 1989). The oil (50-200 ± 0.1 mg, chapter 2.10.2) was dissolved in 1-butanol to make volume to 25 mL. The oil solution (5 mL) was taken to a screw cap test tube. 0.2 % TBA in 1-butanol (5 mL) was added and mixed well before heating in a water bath 95 ˚C for 2 hours. After cooling down (within 10 min), the absorbance of the test solution was measured at 532 nm. The TBA value was calculated using the following equation.

$$TBA\ value\ =\ (50\ \times A_{532})/m$$

50 = dilution factor

A_{532} = the absorbance of the test solution

m = mass of the test sample, mg

2.10.4.4 Determination of 2-thiobarbituric acid reactive substance (TBARS)

Malonaldehyde, the important product from the secondary stage of lipid oxidation was determined by following the method as described by Pegg (2005b). Two molecules of TBA react with one molecule of malonaldehyde forming TBA-MA complex which produces a red pigment with a maximum absorbance at 532 nm. The reaction is shown in Figure 2-2. The oil (50-200 ± 0.1 mg, chapter 2.10.2) was analysed using the TBA procedure (chapter 2.10.4.3). The absorbance was measured at 532 nm against the standard curve. A range of standards (0-1.0 mmol/L) were prepared using TMP (chapter 2.1) standard solution. The TBARS values expressed as malonaldehyde were calculated using the following equation.

$$TBARS\ value\ as\ mmol\ Malonaldehyde\ eq/g\ oil\ = \frac{A \times 5}{wt}$$

A = amount from standard curve, mmol

wt = oil mass in g

5 = mL of oil solution

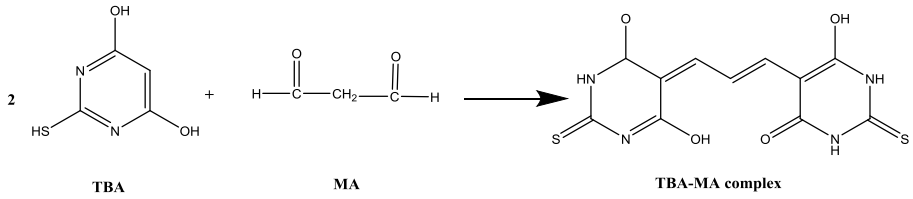


Figure 2-2: Reaction of 2-thiobarbituric acid (TBA) and malonaldehyde (MA) in TBA test, adapted from Pegg (2005b)

2.10.4.5 Determination of *ρ*-Anisidine value

The *ρ*-Anisidine value is another method to monitor the secondary stage of lipid oxidation. It measures the content of important aldehydes, principally, 2-alkenals and 2,4-alkadienals (Shahidi, 2005a; Rossell, 2001a). The reaction of *ρ*-Anisidine with aldehyde is shown in Figure 2-3. Malonaldehyde in an oil reacts with *ρ*-Anisidine under acidic condition and produces a yellowish product which absorb at 350 nm. According to IUPAC 2.504 (IUPAC, 1987c) and Steele (2004), *ρ*-Anisidine is defined as the absorbance of a solution resulting from the reaction of fat (1 g) in isooctane solution (100 mL) with *ρ*-Anisidine (0.25 % in glacial acetic acid). To analyse *ρ*-Anisidine, the oil sample (0.5 $\pm$ 0.0001 g, chapter 2.10.2) was dissolved in isooctane (25 mL). The oil solution (5 mL) with added 0.25 % *ρ*-Anisidine reagent (1 mL, chapter 2.5.5) was mixed well and stored in the dark for exactly 10 min before measuring the absorbance at 350 nm. The blank solution was prepared using isooctane (5 mL) instead of oil solution and treated

with the same procedure. The ρ-Anisidine value can be calculated from the following equation.

$$\rho - \text{Anisidine value} = \frac{25 \times (1.2A_s - A_b)}{m}$$

A_s = absorbance of the sample

A_b = absorbance of the blank

m = weight of the sample, g

25 = dilution factor

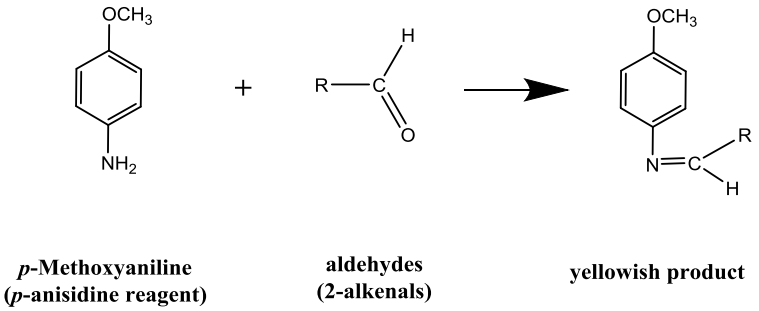


Figure 2-3: Reaction of ρ-Anisidine reagent with aldehyde to form a coloured product, adapted from Steele (2004)

2.10.4.6 Determination of total oxidation value (Totox value)

Totox value or oxidation value is a measurement of total oxidation including primary and secondary products, thus this value can be used for assessment of oxidation of oils (Fennema, 2008). The Totox value is derived from combining the ρ-Anisidine value with peroxide value. The value can be calculated using the following equation (Fennema, 2008; Shahidi and Wanasundara, 2008).

$$Totox\ value = 2PV + \rho AnV$$

PV = peroxide value

ρAnV = ρ-Anisidine value

2.10.4.7 Determination of total polar compounds

The total amount of new compounds formed during the early stages of degradation and polymerisation reactions of oil, can be determined as total polar compounds. The analysis was performed based on adsorption chromatography using a silica column to separate the oil into non-polar and polar compounds (IUPAC, 1987a) with slight modifications using silica cartridges for solid-phase extraction (Marmesat *et al.*, 2007). The silica cartridges were activated (preconditioned) using elution solvent (5 mL, chapter 2.5.7) before using. The oil (50-100 $\pm$ 0.1 mg, chapter 2.10.2) was dissolved with elution solvent (2 mL) and loaded into the preconditioned cartridge. The flow rate was adjusted to 0.5 mL/sec. The non-polar compound fraction was eluted with elution solvent (3 mL) and collected in an oven dried test tube (103$\pm$2 °C). The solvent was removed using the water bath at 60 °C, dried at 103$\pm$2 °C and cooled down before weighing. The total polar compounds was calculated using the following equation.

$$Total\ polar\ compounds\ (\%)\ = \frac{m - m_1}{m} \times 100$$

m = sample mass, g
m_1 = non-polar fraction, g

2.11 Methods used for the study of the oxidative stability of stripped and unstripped palm olein oil in the presence of *Piper sarmentosum* Roxb. leaf extract

2.11.1 Preparation of the PSE extract

The crude PSE extract was prepared by extracting PS powder (chapter 2.6.1) with 80 % ethanol using the same procedure in chapter 2.6.2. The solvent was removed from the filtrated extract solution using a Genevac. The residue or the crude PS extract (PSE) was used for further experiments.

2.11.2 Experimental procedure

The stripped oil was prepared by following the method of Atares *et al.* (2012). The stability of stripped oil in the presence of antioxidants was tested under accelerated temperatures following the method of Zhong and Shahidi (2012). The aluminium oxide was activated by drying at 200 °C for 5 hours before packing in a stainless steel column. The palm olein oil, Oleen® (chapter 2.2.1) was loaded into the column and passed through the aluminium oxide assisted by vacuum force to prepare stripped oil. The PSE extract from 2.11.1 (0 g, 0.02 g, 0.05 g, 0.1 g) was dissolved in the stripped oil (100 mL) to make concentrations 0 %, 0.02 %, 0.05 % and 0.1 % w/v respectively. These oils were incubated in the hot air oven at 60$\pm$2 °C for 120 hours and were sampled every 24 hours for further analysis. A set of control samples were prepared using unstripped oil instead and following the same procedure as the stripped oil.

2.11.3 Determination of *ρ*-Anisidine value

Oxidative stability of the stripped and unstripped Oleen® oil, in the presence of PS crude extract (PSE, chapter 2.11.1) were measured for the *ρ*-Anisidine value (chapter 2.10.4.5).

2.12 Methods used for the study of identification of synthetic antioxidants in cooking oils

2.12.1 Extraction of synthetic antioxidants from cooking oils

The variety of cooking oils: corn oil, rice bran oil, rapeseed oil and palm olein oil (Sainsbury's®, Yors®, King®, Yorkshire®, Oleen® and Alfa 1®, chapter 2.22.2.1) were extracted to analyse synthetic antioxidants. The synthetic antioxidants were extracted from these oils following the methods of Saad *et al.* (2007) and Perrin and Meyer (2003). The oil (0.5±0.0001 g) was dissolved in hexane (5 mL). Acetonitrile (3 mL) was added and mixed for 1 min followed by centrifugation at 4000 rpm for 10 min. The acetonitrile layer was collected. The hexane layer was re-extracted twice with acetonitrile (3 mL). The acetonitrile layers were pooled together and filtered through a 0.45 µm PVDF (nylon) filter membrane prior to injection into the HPLC system. Another set of oils, Oleen® and Alfa 1®, were passed through activated aluminium oxide chapter 2.112.11.2) followed by the same extraction. The synthetic antioxidant free oils (unstripped) were used as control samples and were prepared using the same procedure as the stripped oils.

2.12.2 Identification of synthetic antioxidants from cooking oils

The $UFLC_{XR}$ (HPLC-PDA system, chapter 2.3) operation system was used for identifying synthetic antioxidants presence in cooking oil by comparing retention times with standard synthetic antioxidants: BHA, BHT and TBHQ (chapter 2.5.29). The analytical conditions were trialled and the final optimised method (8^{th} trial conditions) was used. The samples (10 µL, chapter 2.12.1) were injected into the $UFLC_{XR}$. The analytical conditions were used a reversed phase column (C_{18}), 5 µm, 4.6 mm x 25 cm, Phenomenex®. Mobile phase A was 1 % acetic acid in water and mobile phase B was acetonitrile. The flow rate was 0.8 mL/min of binary gradient mobile phase A (10 %) to mobile phase B (90 %). The injection volume was 20 µL, column oven was set at 45 °C, PDA diode array UV detector at 280 nm and the cycle time was 20 min.

2.13 Methods used for the study of antioxidant activity of *Piper sarmentosum* Roxb. leaf extract in rice bran oil and corn oil at mild temperature

2.13.1 Preparation of PS extract and oils

The PSE and PSL extracts were used for this study. The PSE extract was prepared by extracting PS powder (chapter 2.6.1) with 80 % ethanol using the same procedure in chapter 2.6.2. The PSL extract was prepared using the same procedure in chapter 2.92.9.2. The PSL extract residue (after removing solvent) was weighed and ready to use. King® Rice bran oil and Sainsbury's® corn oil (synthetic antioxidants free, chapter 2.122.12.2) were used.

2.13.2 Schaal oven tests

The oxidative stability of the oils with added PSE and PSL extracts, were carried out by following the method of Zhong and Shahidi (2012). PSE and PSL extracts (chapter 2.13.1, 0.005 g, 0.01 g, 0.025 g, 0.05 g and 0.1 g) were mixed in rice bran oil (50 mL, chapter 2.13.1) and corn oil (50 mL, chapter 2.13.1) to make concentrations of 0.01 %, 0.02 %, 0.05 %, 0.1 % and 0.2 % w/v respectively. The control oils were prepared using BHT (0.1 g or 0.02 % w/v) as a positive control and the oils without adding antioxidants as a negative control. These oils were incubated in oven at 60±3 °C for 120 hours and were sampled every 24 hours. These samples were then analysed to determine the peroxide value, TBA value, *ρ*-Anisidine value, and Totox value (chapters 2.10.4.2, 2.10.4.3, 2.10.4.5 and 2.10.4.6 respectively).

2.14 Method used for the study of the performance of *Piper sarmentosum* Roxb. leaf extract on quality changes in rice bran oil and corn oil at frying temperatures

2.14.1 Preparation of PS extract and oils

The PSE extract used in this study was prepared by extracting PS powder (chapter 2.6.1) with 80 % ethanol using the same procedure in chapter 2.6.2. The PSL extract was prepared using the same procedure in chapter 2.92.9.2. The PSL extract residue (after removing solvent) was weighed and ready to use. King® Rice bran oil and Sainsbury's® corn oil (synthetic antioxidants free, chapter 2.122.12.2) were used.

2.14.2 Frying procedure

PSE and PSL extracts (0.5 g, 1 g and 2 g, chapter 2.14.1) were dissolved in rice bran oil (1000 mL, chapter 2.13.1) and corn oil (1000 mL, chapter 2.13.1) to make concentrations of 0.05 %, 0.1 % and 0.2 % w/v respectively. The control oils were prepared using BHT (0.1 g or 0.02 % w/v) as a positive control and the oils without antioxidants added were the negative control. These oils were consecutively heated in a thermostatic fryer (chapter 2.3) at 180 °C for 5 days, 5 hours a day and were sampled every day. Only the oil samples at 0, 5, 15 and 25 heated hours were used for further analysis.

2.14.3 Analysis of changes in oils at frying temperature

The heated oils were analysed to monitor the chemical and physical changes using the acid value, peroxide value, 2-thiobarbituric reactive (TBAR) substance as malonaldehyde, total polar compounds and photometric colour (chapters 2.10.4.1, 2.10, 2.10.4.4, 2.10.4.7 and 2.10.3.1 respectively).

2.15 Statistical analysis

All experiments were done in triplicate analysis. The data was reported as an average value with standard error (Mean$\pm$SE). IBM SPSS statistics software version 22 was used for statistical testing.

2.15.1 Statistics used for the study of initial investigation of the total phenol content and antioxidant activities of *Piper sarmentosum* Roxb. and *Pandanus amaryllifolius* Roxb. leaf extract

PD extract, PS extract, 80 % ethanol and absolute ethanol were considered as independent factors that may have an effect on total phenol content and antioxidant activity of the 4 assays (FRAP, $ABTS^{\cdot+}$, DPPH and reducing power assay). The data met assumption requirements (normality and equality of variance) by the Shapiro-Wilk test and homogeneity test respectively. Multivariate Analysis of Variance (MANOVA) was employed to analyse these multiple effects and the Tukey' s test was used for testing the difference between the groups at 95 % confidence (George, 2011a). The association between total phenol content and antioxidant capacity from each assay, was explored by Pearson Product-Moment correlation Coefficient (Wiredu, 2012; George, 2011b).

2.15.2 Statistic used for the study of effect of solvent extraction method on total phenol content and antioxidant properties in *Piper sarmentosum* Roxb. leaf extract

PS leaf powder, DFPS leaf, extraction solvents (water, 20 %, 50 %, 80 %, absolute ethanol and petroleum ether) were considered as independent factors which may have an effect on total phenol content and antioxidant activity from the 5 assays (FRAP, $ABTS^{\cdot+}$, DPPH , reducing power and linoleic lipid peroxidation assay). The data met assumption requirements (normality and equality of variance) by the Shapiro-Wilk test and homogeneity test respectively. Multivariate Analysis of Variance (MANOVA) was used to analyse the effect of

these multiple factors and the Tukey's test was used for testing the significant different between groups (George, 2011a).

2.15.3 Statistic used for the study of the effect of decolourisation on total phenol content and antioxidant activity of the PSE extract

The amount of activated charcoal (0 %, 0.05 %, 0.1 % and 0.2 % w/v) was considered as an independent factor that may have an effect on total phenol content and antioxidant capacity (FRAP assay) of the PSE extract. The data met assumption requirements (normality and equality of variance) by the Shapiro-Wilk test and homogeneity test respectively. An one-way ANOVA was used to analyse the effect of decolourisation process and the Tukey's test was used for testing the difference between the groups at 95 % confidence (George, 2011a).

2.15.4 Statistic used for quantification of the compounds present in *Piper sarmentosum* Roxb. leaf extract

A Tukey's test was used for testing the difference amounts of identified compound contained among the extracts (PSE, DFPSE and PSL) at 95 % confidence (George, 2011a).

2.15.5 Statistic used for the study of oxidative stability of stripped and unstripped palm olein oil in the presence of *Piper sarmentosum* Roxb. leaf extract

Three independent variables: stripped and unstripped oils, concentrations of the PS extract (0.01 %, 0.02 %, 0.05 %, 0.1 % and 0.2 % w/v) and sampling time (every 24 hours for 5 days), may have an effect on the changes of *ρ*-Anisidine value of the oil. The data met assumption requirements (normality and equality

of variance) by the Shapiro-Wilk test and homogeneity test respectively. Factorial Repeated Measures ANOVA was used to analyse the effect of these multiple factors and a Tukey's test was used to test the difference between the groups at 95 % confidence (George, 2011a)

2.15.6 Statistic used for the study of the performance of the *Piper sarmentosum* Roxb. leaf extract on quality changes in rice bran oil and corn oil at frying temperature

The PSE extract, PSL extract, concentrations of the extract (0.05 %, 0.1 % and 0.2 % w/v) and sampling time (every 5 hours for 5 days) were considered as independent factors that may have an effect on the changes in acid value and total polar compounds of the oils. The data met assumption requirements (normality and equality of variance) by the Shapiro-Wilk test and homogeneity test respectively. Factorial Repeated Measures ANOVA was used to analyse the effect of these multiple factors and the Tukey's test was used for testing the difference between the groups at 95 % confidence (George, 2011a)

3 Results and discussion

3.1 Initial investigation of total phenol content and antioxidant properties of *Piper sarmentosum* Roxb. and *Pandanus amaryllifolius* Roxb. leaf extract

Pandanus amaryllifolius Roxb. and *Piper sarmentosum* Roxb. have shown antioxidant activity in many studies. However, the variation of extraction protocols, methods of analysis and the plant sources, lead to variations in the results of the phenol content and antioxidant activities (Apak *et al.*, 2013; Pokorny, 2010; Yanishlieva *et al.*, 2001). Therefore, it is important to firstly explore the antioxidant properties of both plants under different extraction methods, prior to determining if they are suitable for use in frying oils. The aim of this experiment was to select either *Piper sarmentosum* Roxb. (PS) or *Pandanus amaryllifolius* Roxb. (PD) to use in further experiments as a potential natural antioxidant in frying oils.

3.1.1 The total phenol content and antioxidant activities of *Piper sarmentosum* Roxb. and *Pandanus amaryllifolius* Roxb. leaf extracts

The amount of phenols contained in the PD, PS and PDPS extracts solution were determined using the Folin-Ciocalteu method. The results were calculated using gallic acid as a standard curve. The standard curve for gallic acid ranging from 0 to 500 mg/L, is showed in Figure 3-1.

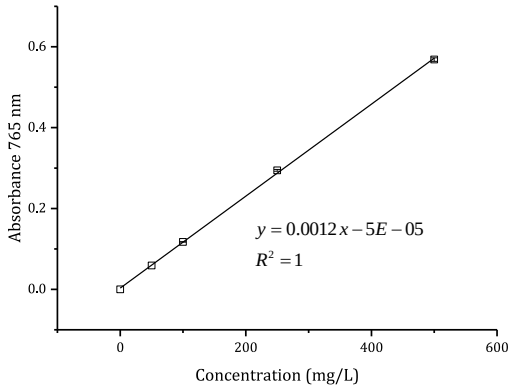


Figure 3-1: Gallic acid calibration curve (0-500 mg/L) for determining total phenol content using Folin-Ciocalteu assay, n=3, error bars represent the standard error (SE) of triplicate measurements.

Figure 3-2 shows the total phenol content in mg gallic acid equivalent (GAE) per gram of dried leaf extracting using 80 % ethanol and absolute ethanol. There are significant differences between different concentrations of the extraction solvent. The results show extracting using 80 % ethanol gives a significantly ($p<0.05$) higher phenol content than the extraction using absolute ethanol.

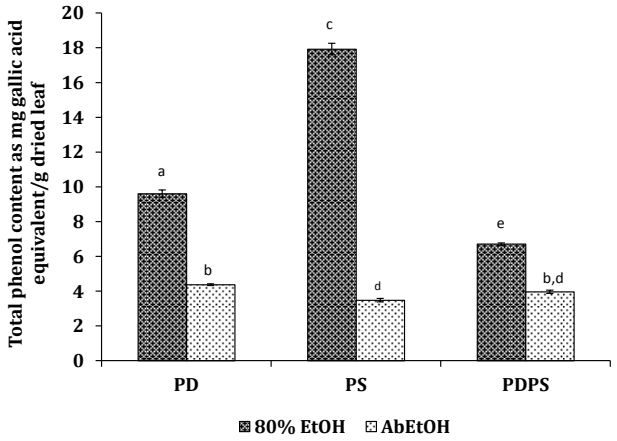


Figure 3-2: Total phenol content of *Piper sarmentosum* Roxb. (PS), *Pandanus amarylliforius* Roxb. (PD) and a 1:1 mixture of both leaves extracted (PDPS), extracted using 80 % ethanol (80%EtOH) and absolute ethanol (AbEtOH). The value is expressed as gallic acid equivalents (mg/g dried leaf), bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p < 0.05$)

The PD extract using 80 % ethanol (PD80%EtOH) has a total phenol content of 9.61±0.21 mg GAE/g which is higher than the extraction using absolute ethanol (PDAbEtOH) (4.37±0.05mg GAE/g). The amount of total phenol found in this study was more than the study by Ghasemzadeh and Jaafar (2013). They reported the total phenol content of the pandan extract ranged from 4.88-6.72 mg/g with the variation related to cultivar locations. The difference of total phenol content compared to this present study, is not only caused by different cultivar locations but also could be caused by the different extraction procedure. The extraction of this study was assisted using ultrasound (ultrasonic bath) at a temperature of 40 °C , while, Ghasemzadeh and Jaafar (2013) used reflux technique at 70 °C. Therefore, the extraction method used in this study could be optimised or some phenols might be lost during reflux at 70 °C. The PS extracted using 80 % ethanol (PS80%EtOH) has a total phenol content of 17.93±0.33 mg GAE/g which is higher than the extract using absolute ethanol (PSAbEtOH) (3.48±0.10 mg GAE/g). The total phenol content of the mixture (PDPS) is also higher in the 80 % ethanol extract (PDPS80%EtOH) (6.71±0.60 mg GAE/g) compared to the absolute ethanol extract (PDPSAbEtOH) (3.96±0.09 mg GAE/g). There is no synergistic effect of total phenol content of the PDPS mixture compared to PD or PS extract in the 80 % ethanol extract or absolute ethanol extract. Overall, the highest amount of phenols was detected in the PS80%EtOH extract. It has a significantly higher phenol content than the others ($p<0.05$). According to Waterhouse (2005), different types of plants have different phenol compounds (so are different in chemical structure) which gives different responses. The two plants leaves may have different phenol compounds leading to a difference in total phenol content and antioxidant capacity. This is in

agreement with a number of studies which have found variations in the phenol content and antioxidant activity in different plant sources (such as vegetables, fruits, seeds, spices or herbs), which were extracted using different solvents or different concentrations of solvent (Phomkaivon and Areekul, 2009; Lin and Tang, 2007; Tangkanakul *et al.*, 2006; Kahkonen *et al.*, 1999). The antioxidant capacity of the extracts determined by FRAP assay were measured against a ferrous (II) sulphate standard curve ranging from 0 to 200 mg/L. For the ABTS radical cation decolourisation ($ABTS^{\cdot+}$) assay, Trolox was used as the standard and the standard curve ranged from 0 to 600 mg/L (Figure 3-3).

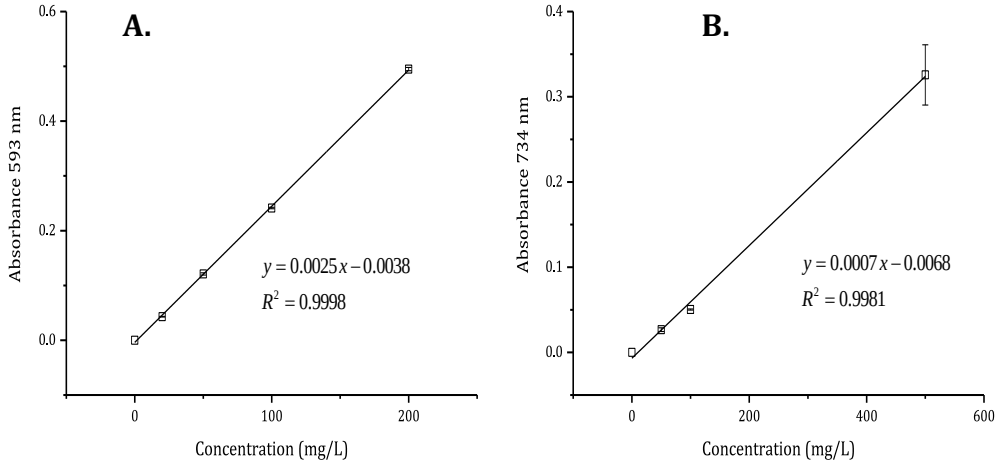


Figure 3-3: Ferrous (II) sulphate calibration curve 0-200 mg/L for determining antioxidant capacity (FRAP assay)(A) and Trolox calibration curve 0-500 mg/L for determining antioxidant capacity ($ABTS^{\cdot+}$ assay)(B), n=3, error bars represent the standard error (SE) of triplicate measurements.

Figure 3-4 illustrates the antioxidant capacity of the extracts determined by ferric reducing power (FRAP) assay as mg of ferrous sulphate equivalent per gram of dried leaf. The results show a significant difference between 80 % ethanol and absolute ethanol in their ferric reducing power ($p<0.05$) for both plants and the mixture.

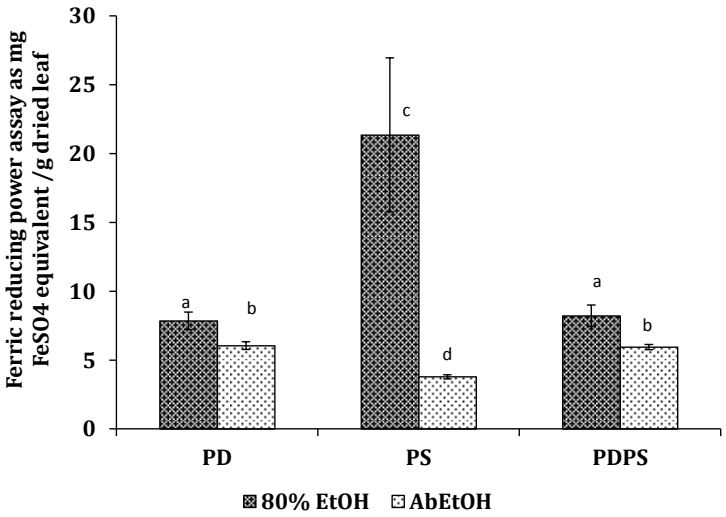


Figure 3-4: The antioxidant capacity (determined using ferric reducing power assay) of *Piper sarmentosum* Roxb. (PS), *Pandanus amarylliforius* Roxb. (PD) and a 1:1 mixture of both leaves extracted (PDPS), extracted using 80 % ethanol (80%EtOH) and absolute ethanol (AbEtOH). The value is expressed as ferrous (II) sulphate equivalents (mg/g dried leaf), bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p < 0.05$)

The highest ferric reducing power is found in PS80%EtOH extract (21.35±5.60 mg $FeSO_4$ eq/g). The PDPS80%EtOH extract has lower antioxidant capacity than PS80%EtOH and shows no significant difference, when compared to the PD80%EtOH ($p<0.05$). Therefore, there is no synergistic effect of ferric reducing power of the PDPS mixture.

The antioxidant capacity of the extracts determined using the $ABTS^{\cdot+}$ assay is shown in Figure 3-5. The $ABTS^{\cdot+}$ reducing power of the PD80 %EtOH, PS80 %EtOH and PDPS80 %EtOH extracts are 9.08±0.13, 20.94±0.69 and 14.77±0.07 mg Trolox equivalent/g respectively. These are significantly ($p<0.05$) higher than the PDAbEtOH, PS AbEtOH and PDPS AbEtOH extracts (7.60±0.15, 7.78±0.10, 8.62±0.16 mg Trolox equivalent/g respectively) which show no significant difference between themselves. The highest $ABTS^{\cdot+}$ reducing power is found in the PS80%EtOH extract. The PDPS80%EtOH extract has a significantly lower

reducing capacity than the PS80%EtOH extract but is significant higher than the PD80%EtOH extract.

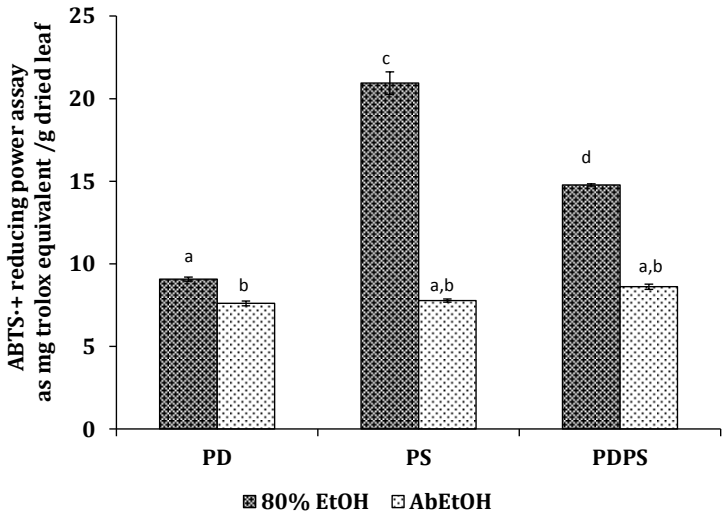


Figure 3-5: The antioxidant capacity (determined using ABTS$^{\cdot+}$ assay) of *Piper sarmentosum* Roxb. (PS), *Pandanus amarylliforius* Roxb. (PD) and a 1:1 mixture of both leaves extracted (PDPS), extracted using 80 % ethanol (80%EtOH) and absolute ethanol (AbEtOH). The value is expressed as Trolox equivalents (mg/g dried leaf), bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

Figure 3-6 shows the DPPH radical scavenging activity of the 80 % ethanol extracts were both significantly different for each plant and the mixture and were significantly higher than the absolute ethanol extracts ($p<0.05$). The highest percentage inhibition is found in the PS80%EtOH extract (76.63 %$\pm$0.15 %)

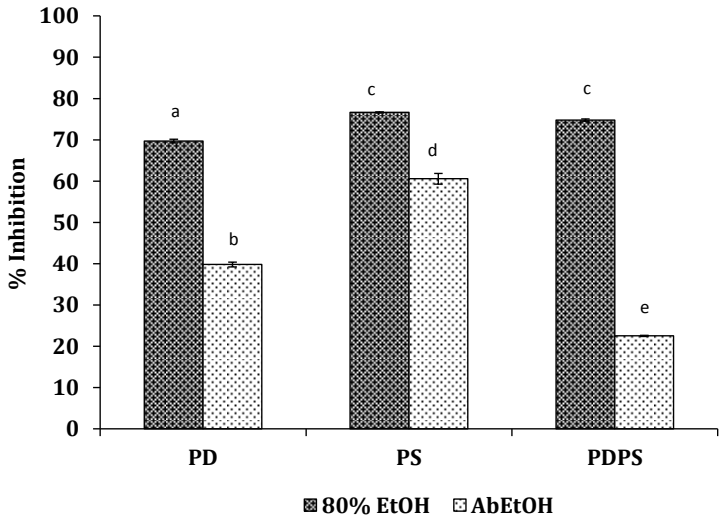


Figure 3-6: The antioxidant capacity (determined using DPPH assay) of *Piper sarmentosum* Roxb. (PS), *Pandanus amarylliforius* Roxb. (PD) and a 1:1 mixture of both leaves extracted (PDPS), extracted using 80 % ethanol (80%EtOH) and absolute ethanol (AbEtOH). The value is expressed as percentage of inhibition, bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

Reducing power is measured at an absorbance of 700 nm and the higher the absorbance, the higher the reducing power. The extracts using 80 % ethanol show a significantly higher reducing power (Figure 3-7) than the extracts using absolute ethanol ($p<0.05$). Again, the highest reducing power is obtained in the PS80 %EtOH extract and there is no synergistic effect of the PDPS mixture. The extraction yield and the antioxidant activity of the extracts from plants highly depend on the solvent polarity. From numerous literatures, it has been noted that methanol is a popular choice of solvent, mostly as a water mixture, due to it being efficient, having a high boiling point and low cost (Waterhouse, 2005).

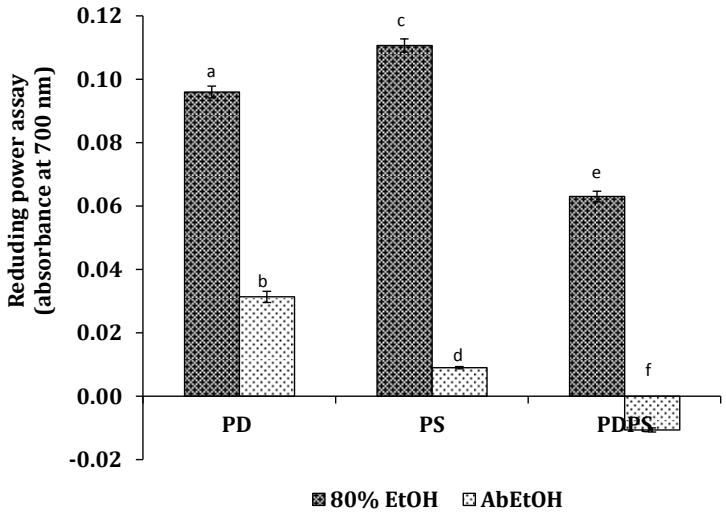


Figure 3-7: The reducing power of *Piper sarmentosum* Roxb. (PS), *Pandanus amarylliforius* Roxb. (PD) and a 1:1 mixture of both leaves extracted (PDPS), extracted using 80 % ethanol (80%EtOH) and absolute ethanol (AbEtOH). Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

Although, ethanol is less popular than methanol, it has been widely used because of its low toxicity and its polarity can be improved being used as a water mixture. The results of the present study show the extracts extracted using 80 % ethanol have a higher total phenol content and antioxidant activity than the absolute ethanol extracts. This is in agreement with the studies of Franco *et al.* (2008), Chizzola *et al.* (2008), Lafka *et al.* (2007) and Thaipong *et al.* (2006), where the effect of solvent polarity on antiradical power showed the highest total phenol and antioxidant activity from an ethanol/water mixture (aqueous ethanol). A 1:1 mixture of PD and PS was also extracted because the presence of natural antioxidants in plants and in combination with other antioxidants may have a synergistic effect. A synergistic effect is an effect which is greater than the individual or sum of the combination (Fuhrman *et al.*, 2000). Graversen *et al.* (2008) found antioxidant synergism between a mixture of black chokeberry juice and α-tocopherol. The study by Liu *et al.* (2008) also found antioxidant

properties of a mixture between lycopene, vitamin E, vitamin C and beta-carotene were superior to the sum of the individual antioxidant activities. However, the PDPS extracts in this study have not shown a synergistic effect. This could be due to Fuhrman *et al.* (2000) and Liu *et al.* (2008) using pure chemical antioxidants to study rather than using crude natural extracts as in this present work. The natural antioxidants presence in PDPS extracts could be bonding with various compounds such as sugars, amino acids etc. that can hinder the synergistic effect of the mixture (Pokorny, 2007). This is similar to the study by Maizura *et al.* (2011) which found no synergistic effect between the mixture of kesum, ginger and turmeric juice extracts. In addition, the ratio of the mixture (1:1) in the current study might not be an appropriate ratio to express synergism of the extracts (Liu *et al.*, 2008).

The difference in results of the 4 assays could be caused by the 4 assays work differently and therefore, depending on the compounds. The antioxidant compounds presence in the PS or PD extracts can vary in amounts of lipophilic or degree of hydrophilic compounds (Kim and Lee, 2005b). The difference in amount , type and degree of hydrophilicity or lipophilicity has an impact on the reacting or scavenging power of the free radicals in each assay (Apak *et al.*, 2013). Therefore, it comes to reason that the results from different assays were not comparable. However, the results from the 4 assays showed the same pattern, so they can give an idea of the protective potential of these plants and the use of more than one assay might be needed.

3.1.2 Correlation of total phenol content and antioxidant activity

Pearson's correlation coefficients (r) between total phenol content and antioxidant activity (4 assays) are shown in Table 3-1. The results show a

positively significant association so the higher the total phenol content, the greater the antioxidant capacity as determined by the assay.

Table 3-1: Pearson's correlation coefficients of total phenol content and antioxidant assays

Correlation coefficients	Total phenol content[A]	Total phenol content[B]
FRAP assay	0.964**	0.816**
DPPH assay	0.495	-0.544
ABTS$^{\cdot+}$ assay	0.721*	-0.044
Reducing power assay	0.870**	0.463

A = extraction using 80 % ethanol, B = extraction using absolute ethanol

*, ** significant at $p<0.05$ or 0.01 (2-tailed) respectively

The correlation coefficient between total phenol content and antioxidant activity determined by the FRAP assay, in 80 % ethanol extracts and absolute ethanol extracts, show the highest relationship ($p<0.01$) due to the very high *r* value (0.964 and 0.816 respectively). The high antiradical reducing power or the high percentage of scavenging of the 80 %EtOH extract could be explained by the positive correlation coefficients between the amount of total phenols and the antioxidant activity. They show a similar pattern and a strong association with a high significance ($0.495 \leq r \leq 0.964$, $p<0.01$), especially between the phenol content based on using the FRAP assay ($r=0.964$, $p<0.01$). This can support that extraction using 80 % ethanol can extract more phenol compounds and contribute to a higher antioxidant activity than using absolute ethanol. This is in agreement with several studies that reported phenol compounds in spices, herbs, fruits or vegetables significantly contributed to their antioxidant properties (Maizura *et al.*, 2011; Thaipong *et al.*, 2006; Wong *et al.*, 2006; Shan *et al.*, 2005;

Wu *et al.*, 2006). To summarise, the findings from the first investigation reveal that leaf extracts obtained from *Piper sarmentosum* Roxb. possess significantly higher amounts of total phenols and antioxidant activity ($p<0.05$) than *Pandanus amaryllifolius* Roxb. leaf extracts when extracted with 80 % ethanol. A very strong relationship was found between total phenol content and antioxidant activity determined using FRAP assay ($p<0.01$). Therefore, *Piper sarmentosum* Roxb. leaf will be selected for the future studies.

3.2 Effect of solvent extraction method on total phenol content and antioxidant properties in *Piper sarmentosum* Roxb. leaf extracts

The following study was designed based on the findings from chapter 3.1.1, where *Piper sarmentosum* Roxb. was selected for further study. Both water and ethanol at various concentrations were used to extract PS in order to determine the best solvent for extraction and alongside this, soxhlet extraction was carried out to observe the effects of petroleum ether extraction. The aims of the study were to determine the most effective solvent for extraction and to investigate whether defatting the PS leaf powder had an effect on the analytical results.

3.2.1 Effect of solvent extraction method on total phenol content, total flavonoid content and L-ascorbic acid content

Using a standard curve for gallic acid, the total phenol content in mg gallic acid equivalents (GAE) per gram of dried PS leaf extracts extracted with different solvents were calculated (Figure 3-8).

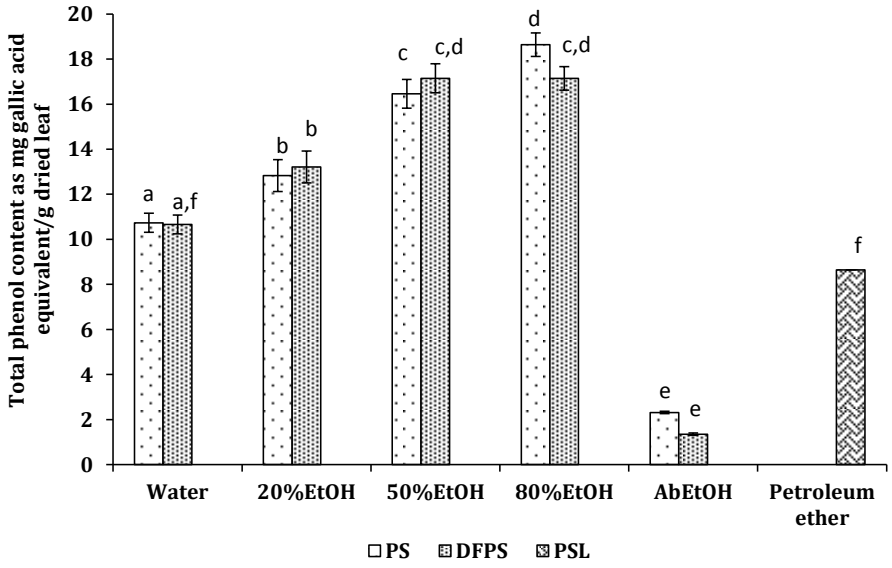


Figure 3-8: Total phenol content of *Piper sarmentosum* Roxb. leaf extracts. PS = the extracts from PS leaf powder extracted using water or ethanol (EtOH), AbEtOH = absolute ethanol, DFPS = the extracts from defatted PS leaf powder extracted using water or ethanol, PSL = the extracts from PS leaf powder extracted using petroleum ether. The value is expressed as gallic acid equivalents (mg/g dried leaf). Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p < 0.05$)

The total phenol content in PS and DFPS extracts, on the whole increased as the ethanol concentration increased up until 80 % concentration ($p<0.05$). The greatest amount of total phenols were obtained in PS80%EtOH, DFPS80%EtOH and DFPS50%EtOH extracts (18.64$\pm$0.13 mg GAE/g, 17.15$\pm$0.64 mg GAE/g and 17.15$\pm$0.52 mg GAE/g respectively). The smallest amount of total phenols were obtained in PSAbEtOH and DFPSAbEtOH extracts (2.32$\pm$0.07 mg GAE/g and 1.35$\pm$0.05 mg GAE/g respectively). The amount of total phenols in PSL extract extracted with petroleum ether at 250 °C for 5 hours was 8.64$\pm$0.00 mg GAE/g which is higher than PSAbEtOH and DFPSAbEtOH extracts, but, there was no significant difference when comparing with DFPSW extracts. There was no significant difference in the amount of total phenols between PS and DFPS

extracts. Figure 3-9 to Figure 3-10 are standard curves used for calculating the amount of flavonoid and L-ascorbic acid contained in the extracts.

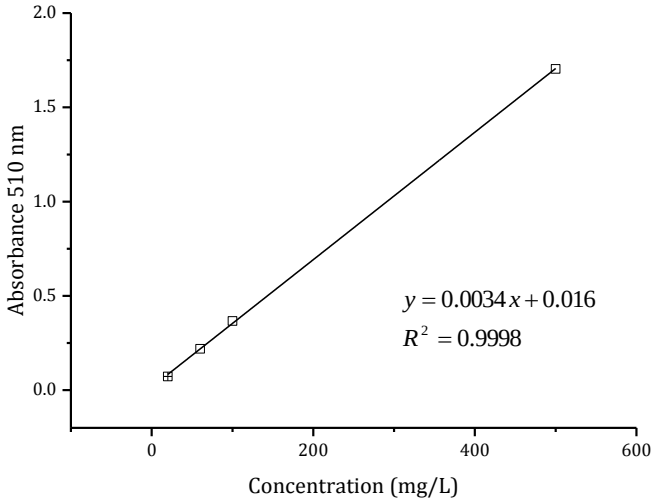


Figure 3-9: Catechin standard curve 0-500 mg/L for determining the amount of total flavonoid content, n=3, error bars represent the standard error (SE) of triplicate measurements

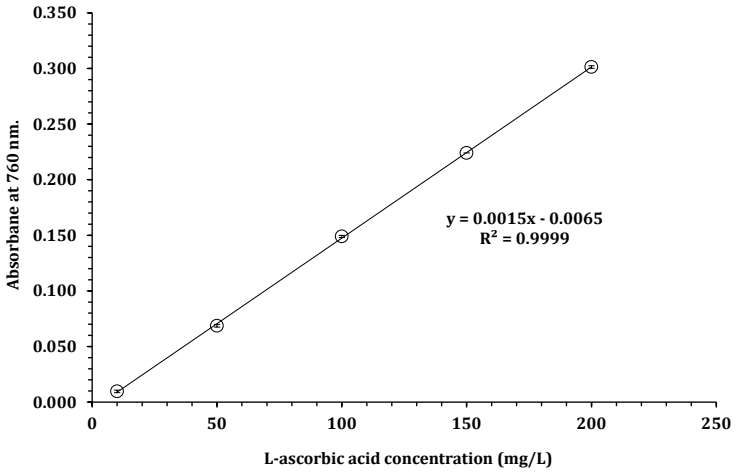


Figure 3-10: L-Ascorbic acid standard curve 0-200 mg/L for determining the amount of total L-ascorbic acid content, n=3, error bars represent the standard error (SE) of triplicate measurements

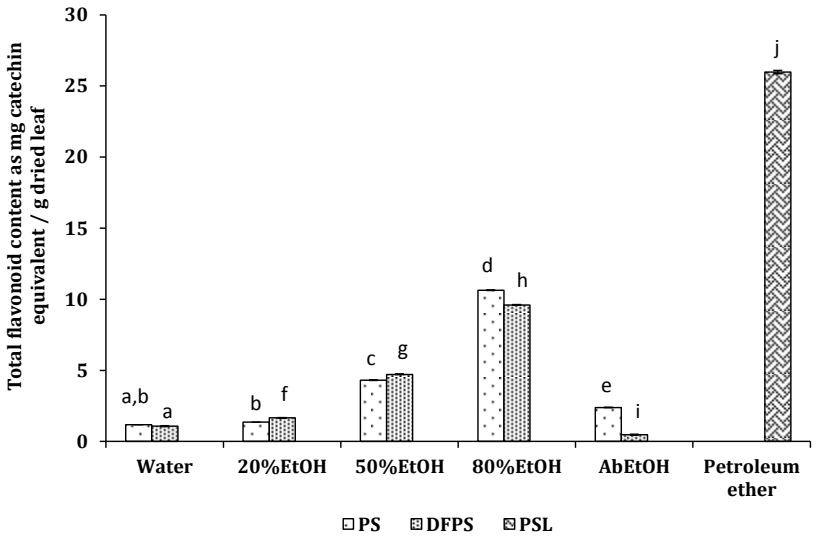


Figure 3-11: Total flavonoid content of *Piper sarmentosum* Roxb. leaf extracts, PS = the extracts from PS leaf powder extracted using water or ethanol (EtOH), AbEtOH = absolute ethanol, DFPS = the extracts from defatted PS leaf powder extracted using water or ethanol, PSL = the extracts from PS leaf powder extracted using petroleum ether. The value is expressed as mg chatechin equivalent/g dried leaf (mg CE/g). Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

Figure 3-11 shows the amount of total flavonoid contained in *Piper sarmentosum* Roxb. extracts extracted using different solvents and concentrations. The amount of total flavonoids are expressed as mg catechin equivalent/g of dried leaf (mg CE/g). The amount of total flavonoids in the extracts extracted with water or absolute ethanol increased significantly when extracted with aqueous ethanol (20-80 %EtOH) ($p<0.05$). Considering extraction using the same solvent, the amount of total flavonoids in the leaf extracts, PS and DFPS extracts, were found to be significantly different ($p<0.05$). However, the PS and DFPS extracts extracted using water were not significantly different (1.18 ± 0.00 and 1.08 ± 0.02 mg CE/g, respectively). In addition, the amount of total flavonoids in PS extracts extracted by water (1.18 ± 0.00 mg CE/g) and 20 % ethanol (1.13 ± 0.00 mg CE/g) were not found to be significantly different. The PSL extract had the highest

amount of flavonoid (25.98±0.12 mg CE/g) with a significant difference to all other extracts($p<0.05$). The lowest amount of total flavonoid was found in DFPS extract extracted using absolute ethanol (0.48±0.02 mg CE/g, $p<0.05$). Figure 3-12 shows the spectrophotometry result of the L-ascorbic acid contained in *Piper sarmentosum* Roxb. leaf extract. The amount of L-ascorbic acid in DFPSAbEtOH was lowest, followed by PS* leaf powder (before extraction with different solvents and concentrations) (15.92±9.08 mg/g, 18.21±0.65 mg/g respectively). The PS extracts using ethanol showed an increasing amount of L-ascorbic acid as concentration increased up to 80 % ethanol. The highest L-ascorbic acid content was found in PSL extract (413.19±119.18 mg/g). However, these results were found to contrast with the results analysed by HPLC assay as shown in Figure 3-13 to Figure 3-15 as no ascorbic acid detected in any extract .

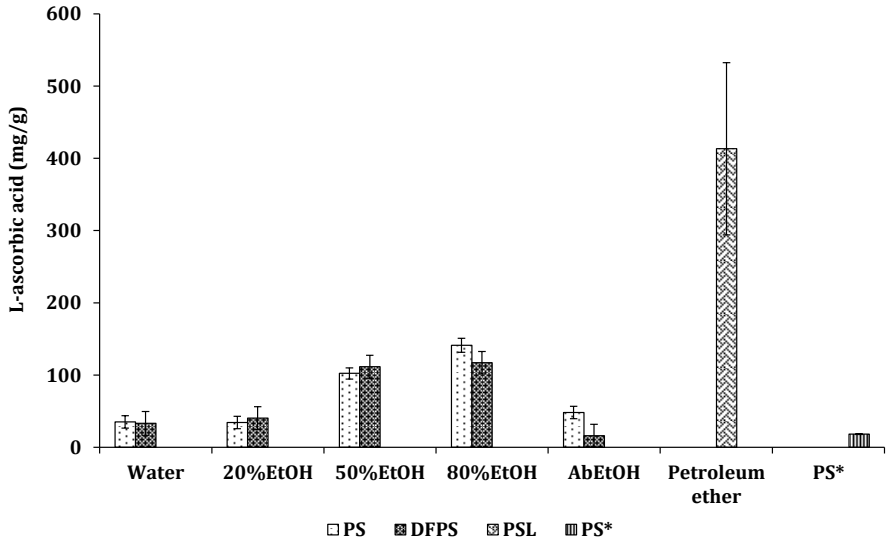


Figure 3-12: L-ascorbic acid of *Piper sarmentosum* Roxb. leaf extracts determined using spectrophotometry, PS* = PS leaf powder prior extracted using water, ethanol or petroleum ether, PS = the PS extracts extracted using water or ethanol (EtOH), AbEtOH = absolute ethanol, DFPS = the DFPS extract extracted using water or ethanol, PSL = the PS extract extracted using petroleum ether. The value is expressed as mg L-ascorbic acid/g dried leaf (mg/g). Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

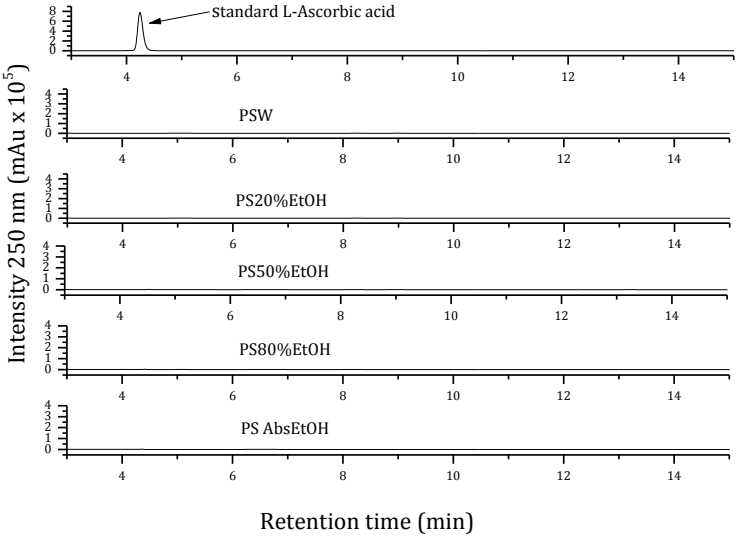


Figure 3-13: HPLC analysis of L-ascorbic acid content of *Piper sarmentosum* Roxb. leaf extracts, PS = the extracts from PS leaf powder extracted using water (w) or ethanol (20 %EtOH – 80 %EtOH) and absolute ethanol (AbsEtOH)

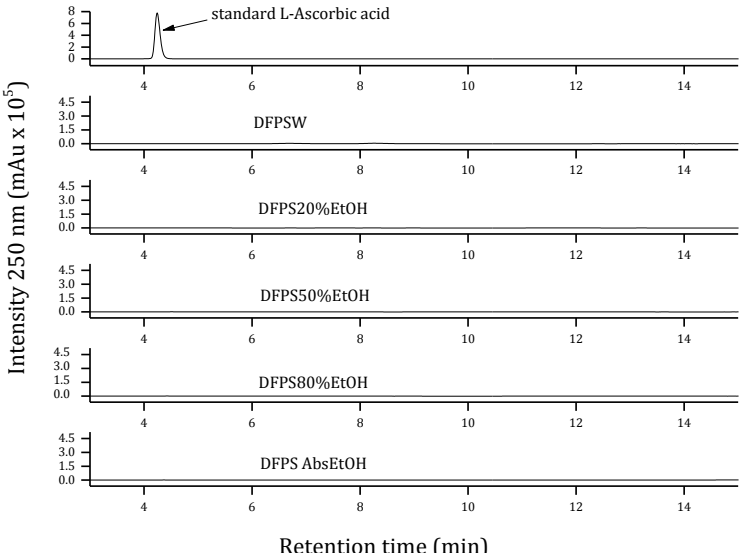


Figure 3-14: HPLC analysis of L-ascorbic acid content of *Piper sarmentosum* Roxb. leaf extracts, DFPS = the extracts from defatted PS leaf powder extracted using water (w) or ethanol (20 %EtOH – 80 %EtOH) and absolute ethanol (AbsEtOH)

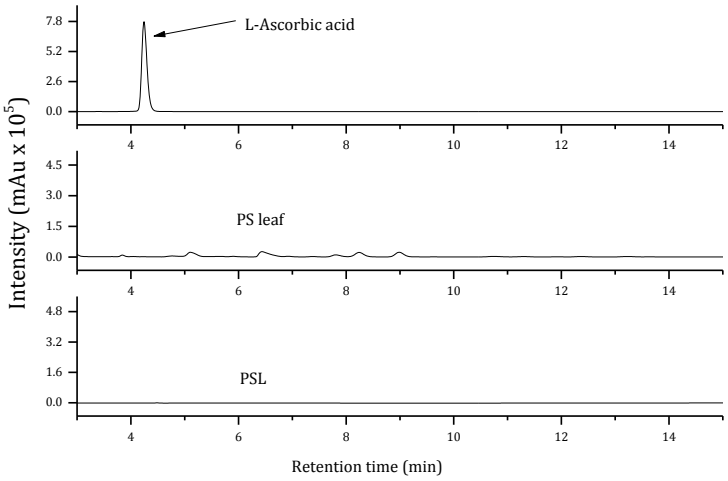


Figure 3-15: HPLC analysis of L-ascorbic acid content of *Piper sarmentosum* Roxb. leaf, PS leaf = PS fresh dried leaf powder, PSL = the extracts from PS leaf powder extracted using petroleum ether

The variation in the amount of total phenol content and flavonoid content in PS extracts results from using different extraction solvents. The highest amount of total phenols of the extracts was obtained by extraction using 80 % ethanol, while, the highest amount of total flavonoids in the extracts was achieved by extraction using petroleum ether, followed by 80 % ethanol. This indicates that extraction using different solvents will extract different types of phenol compounds due to their chemical structures. The compounds present in the extracts can be very lipophilic to very hydrophilic (Kim and Lee, 2005b). The study by Mahae and Chaiseri (2009) found more phenol compounds in the ethanol extracts (50 % ethanol) than water extracts. Also, Franco *et al.* (2008) found higher phenol content in extracts extracted using ethanol than water extracts. As shown in Figure 3-8 and Figure 3-11 the total phenol content and total flavonoid content increase with increasing concentrations of ethanol. This consents to a study by Sultana *et al.* (2009) that showed the extracts extracted

using 80 % methanol and 80 % ethanol had the highest amount of total phenols compared to absolute methanol and absolute ethanol. The study by Mahae and Chaiseri (2009) obtained less flavonoids when extracted using water compared to using 50 % ethanol. Kim and Lee (2005b) explained that phenol compounds are often most soluble in solvents less polar than water. In a study by Chanwitheesuk *et al.* (2005), the total phenol content in *Piper sarmentosum* Roxb. leaf extracts (PS) extracted using absolute methanol, was found to be 123±0.12 mg GAE/100 g dried leaf which was higher than the present study (2.32±0.07 mg GAE/100 g dried leaf). Also, a study by Ugusman *et al.* (2012) found higher total phenol and total flavonoid content in *Piper sarmentosum* Roxb. leaf extracts (91.02±0.02 mg GAE/g dried leaf and 48.57±0.03 mg quercetin equivalent/g dried leaf respectively) than the present study. The difference in amount of total phenol content and total flavonoids content could result from the variety of the plants and also the differences in extraction models. Chanwitheesuk *et al.* (2005) prepared the leaf extracts by soaking the dried leaf powder in methanol overnight. Ugusman *et al.* (2012) extracted the leaf using a high speed mixer at 80 °C for 3 hours. In case of total flavonoids, it is not appropriate to compare the amount of flavonoids as they were calculated from different standards. The amount of flavonoids in the present study are better extracted using petroleum ether. This could be due to the non-polarity of petroleum ether that can better extract less polar flavonoid compounds such as isoflavones, flavanones, while flavonoid glycosides which more polar are better extracted with alcohols or alcohol water mixtures (Marston and Hostettmann, 2006).

Using the spectrophotometric method, L-ascorbic acid was found in all PS extracts. The amount of L-ascorbic acid in *Piper sarmentosum* Roxb. leaf extract

reported by Chanwitheesuk *et al.* (2005) (16.3±0.06 mg/100 g dried leaf) was lower than this present study (18.21±0.65 mg/g in PS leaf powder). Although, the plants were cleaned, cut and dried similar to in the present study, the greater loss could be from the drying temperature used. They dried the leaf at 50 °C which was higher than the present study (40 °C). However, according to Moeslinger *et al.* (1995), the spectrophotometric method has some limitations on sensitivity and specificity because of interference due to the presence of sugars, amino acids or glucuronic acid which is usually found bonded with phenols (Landete, 2012). Therefore, it cannot be certain that these results are a true reflection of the L-ascorbic acid content in the extracts. Therefore a HPLC method was used due to high sensitivity and specificity. The HPLC chromatograms show L- ascorbic acid was not found in any of the extracts or the leaf powder itself (Figure 3-13 to Figure 3-15). This may be due to loss or decomposition during processing as a result of susceptibility to heat, light, pH and oxygen (Shahidi, 2005b). The leaves were cleaned, cut, trimmed and dried overnight. Moreover, grinding or pulverising to a fine powder can increase the deterioration rate of ascorbic acid. It would also be expected that the ascorbic acid in the defatted leaf (DFPS) or in PSL extracts would be destroyed, due to the high temperature (250 °C) of heating for 5-6 hours. Therefore, on the basis of the reasons of sensitivity and selectivity, the results obtained by the HPLC method were accepted as a true reflection of the L-ascorbic acid content.

The temperature used in the soxhlet extraction procedure, showed no effect on the amount of total phenols and total flavonoids. The results in Figure 3-8 and Figure 3-11, show the total phenol content and total flavonoid content obtained in defatted leaf powder (DF) was similar to that obtained in dried leaf (PS). This

suggests that the phenols in PS leaf are heat resistant as there was no loss on defatting, so PS leaf could be used in high temperature conditions. It also suggests that by using different extraction solvents, different amounts or types of compounds are extracted. The phenol compounds obtained by petroleum ether extraction might give a better solubility in fat or lipid matrix, so it could be easily dissolved in cooking oil.

3.2.2 Effect of solvent extraction method on antioxidant activity

Figure 3-16 shows the antioxidant capacity of the PS extracts as mg of ferrous sulphate equivalent per gram of dried leaf, which was determined by the FRAP assay. In terms of extraction solvent, the ferric reducing power in PS and DFPS extracts extracted with different solvents have been found to be significantly different ($p<0.05$). The results show an increase in ferric reducing capacity when extracted using an ethanol mixture (20-80 % ethanol, $p<0.05$). The highest ferric reducing power was obtained in both PS80%EtOH and DFPS80%EtOH extracts (34.14 ± 0.15 mg $FeSO_4$ equivalent/g, 32.41 ± 0.74 mg $FeSO_4$ equivalent/g, respectively). The lowest ferric reducing power was found in PS and DFPS extracts extracted using absolute ethanol (PSAbEtOH and DFPS AbEtOH, 6.28 ± 0.13 and 6.67 ± 0.47 mg $FeSO_4$ equivalent/g). PS extracts extracted using petroleum ether at 250 °C for 5 hours (PSL) found no significant difference in ferric reducing power (48.74 ± 2.93 mg $FeSO_4$ equivalent/g) compared with PS and DFPS extracts extracted using water or 20 % ethanol. PS and DFPS extracts, extracted using the same solvent, showed no significant difference in ferric reducing power for all extracts.

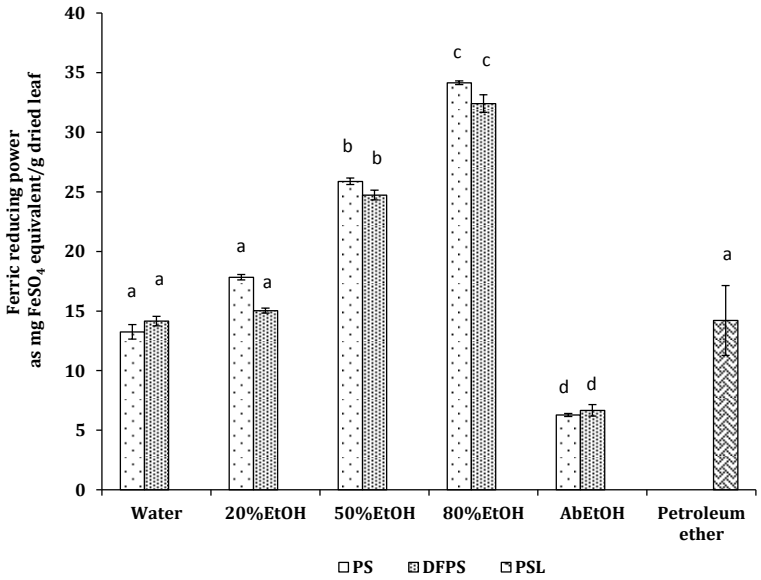


Figure 3-16: The antioxidant capacity (determined using Ferric reducing power assay) of *Piper sarmentosum* Roxb. leaf extracts. PS = the extracts from PS leaf powder extracted using water or ethanol (EtOH), AbEtOH = absolute ethanol, DFPS = the extracts from defatted PS leaf powder extracted using water or ethanol, PSL = the extracts from PS leaf powder extracted using petroleum ether. The value is expressed as ferrous (II) sulphate equivalents (mg/g dried leaf). Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

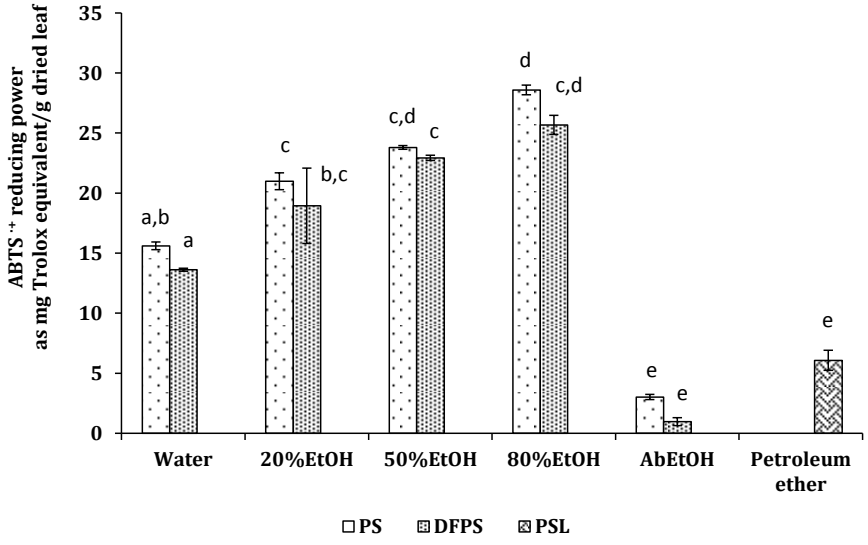


Figure 3-17: The antioxidant capacity (determined using $ABTS^{\cdot+}$ assay) of *Piper sarmentosum* Roxb. leaf extracts. PS = the extracts from PS leaf powder extracted using water or ethanol (EtOH), AbEtOH = absolute ethanol, DFPS = the extracts from defatted PS leaf powder extracted using water or ethanol, PSL = the extracts from PS leaf powder extracted using petroleum ether. The value is expressed as Trolox equivalents (mg/g dried leaf). Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

Figure 3-17 shows $ABTS^{\cdot+}$ reducing power of *Piper sarmentosum* Roxb. extracts, as mg Trolox equivalent/g dried leaf. The highest $ABTS^{\cdot+}$ reducing power was found in PS extracts extracted using 80 % ethanol (PS80%EtOH, 28.59±0.40 mg Trolox eq/g), showing no significant difference with DFPS80%EtOH extracts (25.68±0.79 mg Trolox eq/g). The lowest reducing capacities were PS and DFPS extracts extracted using absolute ethanol (PSAbEtOH and DFPSAbEtOH, 3.02±0.22 and 0.97±0.33 mg Trolox eq/g) and PSL extract (6.08±0.83 mg Trolox eq/g) which show no significant difference between them. PS and DFPS extracts, extracted using the same solvent, showed no significant difference in $ABTS^{\cdot+}$ reducing power for all extracts. Figure 3-18 shows the reducing power of *Piper sarmentosum* Roxb. extracts, measured at 700 nm by spectrophotometer. The higher the absorbance, the higher the reducing power.

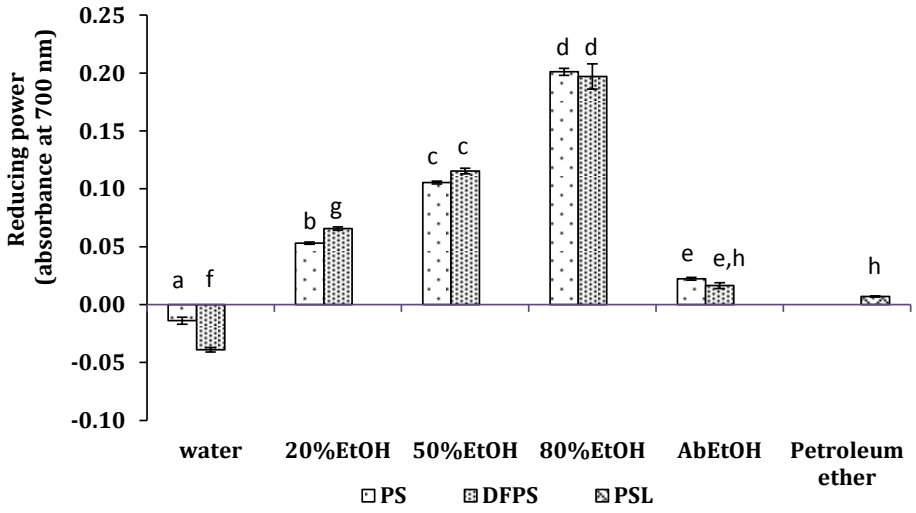


Figure 3-18: The reducing power of *Piper sarmentosum* Roxb. leaf extracts. PS = the extracts from PS leaf powder extracted using water or ethanol (EtOH), AbEtOH = absolute ethanol, DFPS = the extracts from defatted PS leaf powder extracted using water or ethanol, PSL = the extracts from PS leaf powder extracted using petroleum ether. Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

The reducing power in PS and DFPS extracts extracted using different solvents was found to be significantly different ($p<0.05$). The results showed an increase in reducing capacity when extracted using ethanol mixture ($p<0.05$). The highest absorbance was found in PS extracts extracted using 80 % ethanol (PS80%EtOH, 0.201 ± 0.002), which shows no significant difference with DFPS80 %EtOH extracts (0.197 ± 0.006). PS and DFPS extracts extracted using water, have not shown any reducing power with this assay. PSAbEtOH, DFPS AbEtOH and PSL extracts have very low absorbance (0.022 ± 0.001, 0.016 ± 0.001 and 0.007 ± 0.001 respectively) and no significant differences are found between them. Figure 3-19 shows the percentage inhibition of DPPH radical scavenging activity of *Piper sarmentosum* Roxb. extracts, measured at 517 nm. The higher the percentage, the higher the scavenging power. The scavenging power in PS and DFPS extracts extracted using water/ethanol mixtures have been found to be increasingly significant ($p<0.05$), as ethanol increases, up to 80 % ethanol. The highest scavenging power obtained in PS extracts was extracted using both absolute ethanol (PSAbEtOH, 93.15 %$\pm$0.009), and DFPS80%EtOH extracts (90.19 %$\pm$0.003) which found no significant difference between them. The extract with the lowest scavenging power was PS extracted using water (47.65 %$\pm$0.009). There was no significant difference in scavenging power between the PSL extract (59.05 %$\pm$0.002) and PS and DFPS extracts extracted using 20 % ethanol (59.34 %$\pm$0.011 and 60.41 %$\pm$0.003 respectively).

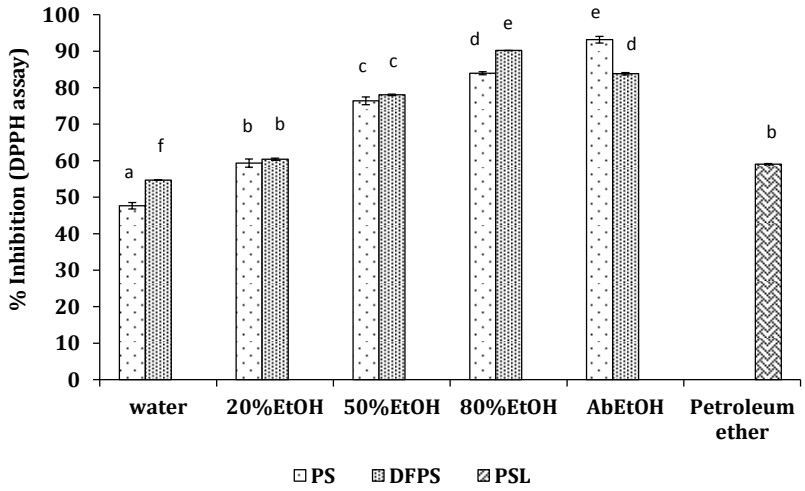


Figure 3-19: The antioxidant capacity (determined using DPPH assay) of *Piper sarmentosum* Roxb. leaf extracts. PS = the extracts from PS leaf powder extracted using water or ethanol (EtOH), AbEtOH = absolute ethanol, DFPS = the extracts from defatted PS leaf powder extracted using water or ethanol, PSL = the extracts from PS leaf powder extracted using petroleum ether. The value is expressed as percentage of inhibition. Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

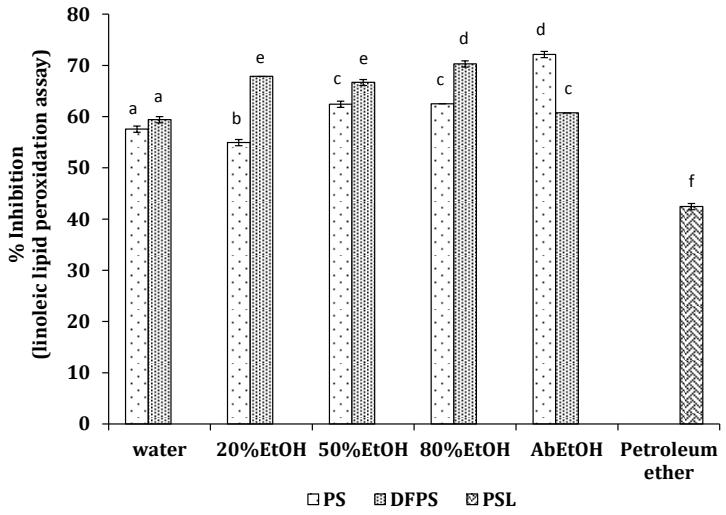


Figure 3-20: The antioxidant capacity (determined using linoleic lipid peroxidation assay) of *Piper sarmentosum* Roxb. leaf extracts. PS = the extracts from PS leaf powder extracted using water or ethanol (EtOH), AbEtOH = absolute ethanol, DFPS = the extracts from defatted PS leaf powder extracted using water or ethanol, PSL = the extracts from PS leaf powder extracted using petroleum ether. The value is expressed as percentage of inhibition. Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

Figure 3-20 shows the ability (percentage) of *Piper sarmentosum* Roxb. extracts to inhibit lipid peroxidation. The higher the percentage, the higher the inhibition. The inhibition of lipid peroxidation of PS and DFPS extracts extracted using different concentrations of ethanol showed an increase in trend with a range of 54-72 %. The highest inhibition capacity was obtained in PS extracts extracted using absolute ethanol (72.12 %$\pm$0.003), which had no significant difference with the DFPS80%EtOH extract (70.30 %$\pm$0.003). The lowest inhibition was found in PSL (42.42 %$\pm$0.003). There was a significant difference in inhibition of lipid peroxidation between PS and DFPS extracts, extracted using the same solvent, apart from between PSW and DFPSW extracts (57.58 %$\pm$0.003 and 59.39 %$\pm$0.003 respectively) ($p<0.05$).

The findings from these results show that the difference in extraction solvent and its concentration, have an effect on antioxidant activity of the extracts. For water and ethanol extracts, the antioxidant capacity demonstrates a similar pattern between all assays and gives similar trends to the total phenol content assay. The antioxidant capacity of the extracts determined by FRAP, $ABTS^{\cdot+}$ and reducing power assays found the highest antioxidant capacity in the extracts extracted by 80 % ethanol for both PS and DFPS leaf. Findings were similar in the study done by Ayusuk *et al.* (2009) where the extracts extracted by 70 % ethanol gave a higher antioxidant capacity with FRAP and $ABTS^{\cdot+}$ assays than the DPPH assay. The antioxidant capacity of the dried leaf (PS) and defatted dried leaf (DF) extracts increased when increasing the concentration of ethanol (Figure 3-17 and Figure 3-19). The results for antioxidant activity may be contributed by the amount of phenols present as there is a strong correlation between total phenol content and the 4 antioxidant assays (Table 3-1).

The antioxidant capacity of the extracts extracted by absolute ethanol and petroleum ether, obtained by DPPH and linoleic acid peroxidation assays are higher than FRAP, $ABTS^{\cdot+}$ and reducing power assays. This is similar to the findings by Phomkaivon and Areekul (2009). Similarly with the study by Maizura *et al.* (2011) reported that the plant extracts without using water had higher antioxidant capacity when determined with DPPH assay than FRAP assay. Franco *et al.* (2008) compared antioxidant capacity between ethanol extracts and water extracts by DPPH assay. The results showed that the ethanol extracts has higher inhibition ability than water extracts, which was also confirmed in the present study. These phenomena were explained by Kim *et al.* (2002). The $ABTS^{\cdot+}$ assay is based on an aqueous system which measures the intense of blue/green colour generated from $ABTS^{\cdot+}$. This assay is applicable to both hydrophilic and lipophilic antioxidants, whereas, the DPPH assay is based on an organic system, therefore, it has a higher response to hydrophobic (or lipophilic) antioxidants. Therefore, the majority of the compounds in the extract extracted by using absolute ethanol, may be lipophilic compounds. However, the present results contrast with the study by Floegel *et al.* (2011). They found the fruits, vegetable and beverage extracts (extracted by absolute methanol) measured using $ABTS^{\cdot+}$ assay had higher antioxidant capacity than DPPH assay due to the high pigmented and hydrophilic antioxidants were better reflected by $ABTS^{\cdot+}$ assay than DPPH assay. The results of the antioxidant capacity of PS extracts show effective antioxidant activity, particularly when tested by DPPH and linoleic lipid peroxidation assays. All the extracts tested by the linoleic acid peroxidation assay show good ability to inhibit lipid peroxide, although they were extracted using different solvents and concentrations. The system of linoleic acid

peroxidation assay is an emulsion system which is prepared by a mixture of linoleic acid in phosphate buffer (Tween 20 was used as emulsifier). Therefore, due to the emulsion system, the assay is applicable to both hydrophilic and lipophilic antioxidants. From the results of the study, it appears that the antioxidant capacity of defatted dried leaf (DF) extracts, show similar results as normal dried leaf (PS) extracts and in most case there is no significant difference. This suggests that temperature used in soxhlet extraction has no effect on the antioxidant capacity of the extracts. As different solvents and concentrations used for extraction result in different types and amounts of active compounds and therefore give a variety responses to different assays, different antioxidant assays should be employed when measuring antioxidant capacity.

In summary, according to the findings, the PS extracts extracted with 80 % ethanol (PSE) has the highest total phenol content and petroleum ether extracts (PSL) possess highest total flavonoids. They also exhibit high antioxidant activity as assessed by a various assays. Therefore, *Piper sarmentosum* Roxb. leaf will be extracted by 80 % ethanol and petroleum ether for future experiments. As there is no significant difference between defatted leaf (DFPS) extract and normal leaf (PS) extracts with each assay, it suggests that PS leaf and its extracts are heat resistant and so could be used in high temperature conditions. Also, as the PS, DFPS and PSL extracts demonstrate antioxidant capacity in linoleic lipid peroxidation system, it suggest that these extracts could also be used in oil or emulsion food matrices.

3.3 The effect of decolourisation on total phenol content and antioxidant activity of the PSE extracts

Chlorophyll present in oils, may have an effect on the autoxidation of lipids (Warner, 2002). Chlorophyll has been supposed to exert its pro-oxidative action on the deterioration of oils. It acts as a photosensitizer which accelerates the oxidation of oils when exposed to light (Endo *et al.*, 1985). Natural antioxidant extracts from plant leaves (crude extracts) contain chlorophyll pigments which take part in causing dark colour in fats or oils, and act as pro-oxidants in the light, particularly when present at higher concentrations (Pokorny, 2010; Hall *et al.*, 1994). Some approaches used to remove or reduce chlorophyll, pigment colour, odour or bitter substances from the crude extracts are using fractionation for purifying pigments of ethanol or methanol extracts, using activated carbon for bleaching the crude extracts prepared by polar or non-polar solvent or using ultraviolet irradiation with activated charcoal (Scheepers *et al.*, 2011; Pokorny, 2010; Chang *et al.*, 1977). However, all those applications have an impact on the yield of active substances of the crude extracts (Pokorny, 2010). The aim of this experiment is to observe the effect of a decolourisation process on the total phenol content and antioxidant capacity of the PS80%EtOH extracts (PSE) and to evaluate the efficiency of the extraction method. The results will determine if the crude extracts will be decolourised.

3.3.1 Effect of decolourisation on total phenol content and antioxidant activity of the PSE extract

The PSE extracts treated with activated charcoal at 0 %, 0.5 %, 1 % and 2 % w/v are shown in Figure 3-21. The colour of the bleached extracts become less green

in colour as the bleaching agent increases, turning to clear at 2 % w/v of activated charcoal. However, although the green colour in the bleached PSE extracts treated with 2 % w/v activated charcoal has disappeared, the noticeable black colour from the activated charcoal appears instead.

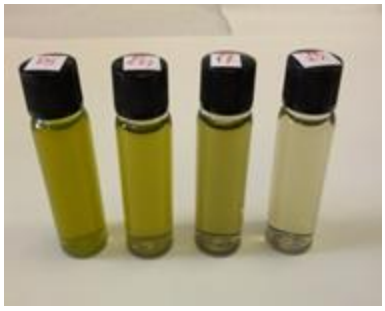


Figure 3-21: PSE extracts treated with activated charcoal from left to right 0 %, 0.5 %, 1 % and 2 % w/v, respectively

Using gallic acid to form the standard curve, the results of the amount of total phenol of PSE extracts treated with activated charcoal (0 %, 0.5 %, 1 % and 2 % w/v) show a significant decrease as the amount of activated charcoal increases ($p<0.05$), Figure 3-22. As the results show, remarkably, the amount of phenol has rapidly declined by 75 % from 21.10 mg GAE/g to 5.10 mg GAE/g with the 0.5 % w/v activated charcoal. The amount of total phenol is approximately 95 % reduction in the extracts treated with 2 % w/v activated charcoal. The results of the FRAP assay of PSE extracts treated with activated charcoal (Figure 3-23) also show a significant decrease as the amount of activated charcoal increased ($p<0.05$). The antioxidant activity of the extracts has rapidly decreased by 70 % from 25.86 mg $FeSO_4$ equivalent/g to 7.84 mg $FeSO_4$ equivalent/g with the 0.5 % w/v activated charcoal. The extracts treated with 2 % w/v activated charcoal demonstrate very low antioxidant capacity which is approximately a 95 % reduction.

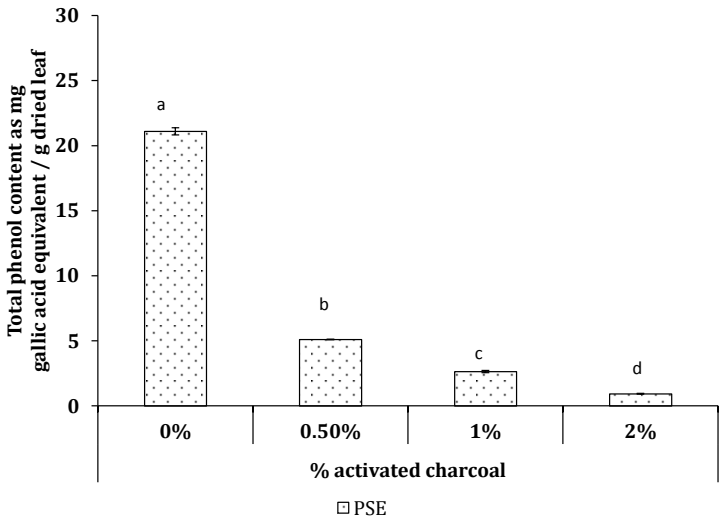


Figure 3-22: Total phenol content of *Piper sarmentosum* Roxb. leaf extracts. PSE = the extracts from PS leaf powder extracted using 80 % ethanol and treated using activated charcoal 0 %, 0.5 %, 1 % and 2 % w/v respectively. Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

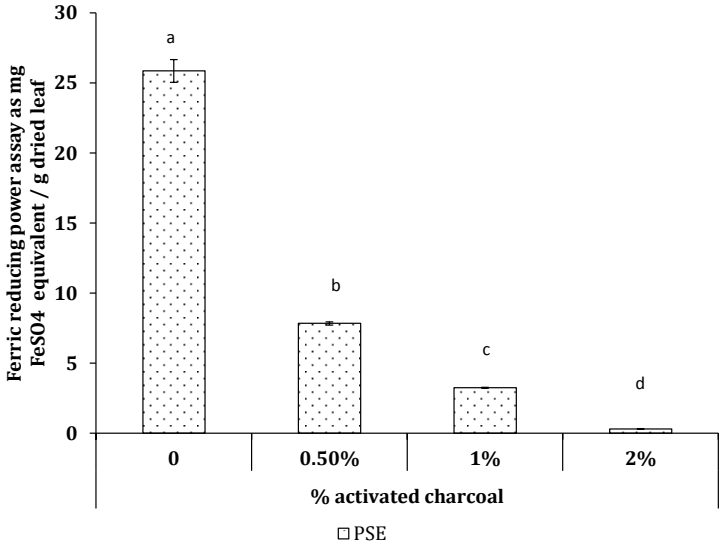


Figure 3-23: Antioxidant activity of *Piper sarmentosum* Roxb. leaf extracts (determined using ferric reducing power assay). PSE = the extracts from PS leaf powder extracted using 80 % ethanol and treated using activated charcoal 0 %, 0.5 %, 1 % and 2 % w/v respectively. Bars represent the mean±SE of triplicate analysis. Different letters indicate significant differences between samples by Tukey's test ($p<0.05$)

3.3.2 The efficiency of the extraction method

The efficiency of the extraction method is shown in Table 3-2 as percentage recovery. The highest recovery of gallic acid is obtained in PSE extracts without activated charcoal treatment (93.01 %), while the extracts treated with activated charcoal 0.5 %, 1 % and 2 % w/v reduce to 87.77 %, 74.87 % and 59.36 % respectively. According to the results, the decolourisation treatment has a negative effect on the amount of total phenols and antioxidant capacity of *Piper sarmentosum* Roxb. leaf extracts. A rapid reduction of total phenol content and ferric reducing power, is related to an increasing amount of activated charcoal.

Table 3-2: Recovery of total phenol content as mg gallic acid equivalent in PSE extracts, spiking with gallic acid standard 50 mg prior to the decolourisation and extraction process. The value represents the mean±SE of triplicate analysis

Extracts	Recovery (%)			
	activated charcoal (% w/v)			
	0 %	0.5 %	1 %	2 %
PSE	93.01	87.77	74.87	59.36

This agrees with the results of Chang *et al.* (1977). They bleached rosemary and sage crude extracts (extracted with various organic solvents) with activated charcoal. The bleached rosemary and sage extracts extracted with methanol showed a loss in antioxidant activity and the extracts of benzene and hexane had no antioxidant activity at all. North *et al.* (2012) used activated charcoal to reduce phenol compounds in culture media. They found that the activated charcoal significantly reduced the phenol content (53 % reduction) in culture media supplemented with activated charcoal. The reduction in the amount of phenols is caused by absorption by the bleaching agent. Activated charcoal has

differences in size, porous structure and different in functional groups (mainly oxygen). This characteristic contributes to its absorption property. Some phenols and their derivatives can be absorbed to carbon due to the functional group, hydroxyl group, on the phenol molecule (Dabrowski *et al.*, 2005). Although, the present study has examined a low amount of activated charcoal (0.5-2 % w/v), the extract appeared black in colour in the extracts treated with 2 % activated charcoal. Therefore, the increasing amount of activated charcoal might lead to other problems alongside the reduction of total phenol content and antioxidant activity. Moreover, the results of recovery show the efficiency of the extraction method. The normal sample (PSE) without bleaching has a 93 % recovery which is an acceptable result. The bleached extracts with activated charcoal demonstrate a reduction of recovery which is correlated to the level of activated charcoal added. The loss of gallic acid might be due to it being absorbed by the charcoal as gallic acid has 3 hydroxyl groups in a molecule.

To summarise, the results in this part demonstrate that the decolourisation process has a huge effect on the loss of phenol content and antioxidant activity. Therefore, the bleaching treatment is not appropriate for this study. However, when analysing the extraction efficiency it was clear that the original method using 80 % ethanol gave a good recovery which emphasises the effectiveness of this method of extraction.

3.4 Characterisation of polyphenol profile of *Piper sarmentosum* Roxb. Leaf extracts

According to the results from chapter 3.2, the *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE) possessed the highest total phenol

content and antioxidant activity. Defatted leaf extracts (DFPSE) extracted using 80 % ethanol had a slightly lower total phenol content but no significant difference was observed with antioxidant activity. Although, PS leaf extracted by petroleum ether (PSL) had a lower total phenol content and antioxidant activity than PSE and DFPSE, it had the highest total flavonoid content. As these solvents are likely to have extracted different active compounds, it would be useful to know the type of compounds present in the crude extracts that will be studied further. The aim of this study is to explore the antioxidants or polyphenols that are present in the PSE, DFPSE and PSL extracts.

3.4.1 Optimisation of the HPLC method

To find the best conditions for identifying the compounds present in the extracts, several trials were carried out. The conditions used for each trial are shown in Table 3-3 and the chromatograms are shown in Figure 3-24 to Figure 3-28. Reverse phase column (C_{18}), the $UFLC_{XR}$ (HPLC system) photodiode array (PDA) with multiple wavelengths and PSE extract were used for these trials (chapter 2.3). The 4^{th} trial shows the best peak resolutions of the PSE extract (Figure 3-27). Therefore, the 25 standard phenols and a standard caffeine were analysed using the 4^{th} trial conditions.

Table 3-3: Trial conditions used for optimising the HPLC method to identify polyphenols present in *Piper sarmentosum Roxb*. leaf extracts

Trial	Conditions	Chromatogram
1	Mobile phase A was 0.1 % formic acid in water, mobile phase B was 0.1 % formic acid in acetonitrile. The flow rate was 0.3 mL/min of binary gradients. Starting at 0.01 min with mobile phase B (10 %) hold for 5 min before increasing to 15 % at 9 min. Mobile phase B was increased to 95 % at 28 min and hold for 4 min before reducing to 10 % at 35 min until 45 min the system was completed a cycle time. The injection volume was 10 µL and column oven was set at 25 °C.	Figure 3-24
2	Only flowrate of the binary gradients was adjusted to 0.5 mL/min and column oven was set at 45 °C. Other conditions were the same as trial 1.	Peak resolutions are improved, Figure 3-25
3	The flow rate of binary gradient was 0.5 mL/min and adjusted with mobile phase B 10 % at 0.01 min hold for 5 min before increasing to 15 % at 9 min. Mobile phase B was increased to 95 % at 32 min and hold for 4 min before reducing to 10 % at 39 min until 50 min the system was completed a cycle time. The injection volume was 10 µL and column oven was set at 40 °C.	Peak resolutions are better than 2nd trial, Figure 3-26
4	The analysis was started with mobile phase B (10 %) at 0.01 min, reached to 25 % at 12 min. The increasing of mobile phase B to 100 % at 32 min was hold for 3 min before reduced to 10 % at 38 min. The cycle time was completed at 45 min. The column was set at 25 °C. The flow rate was 0.5 mL/min.	Peak resolutions are better than 3rd trial, Figure 3-27
5	The binary gradients were adjusted and started with mobile phase B (10 %) at 0.01 min reached to 25 % at 12 min. The increasing of mobile phase B to 100 % at 17 min was hold for 10 min before reduced to 10 % at 32 min. The cycle time was completed at 45 min. The flow rate was 0.5 mL/min.	Peaks resolution are worse, Figure 3-28

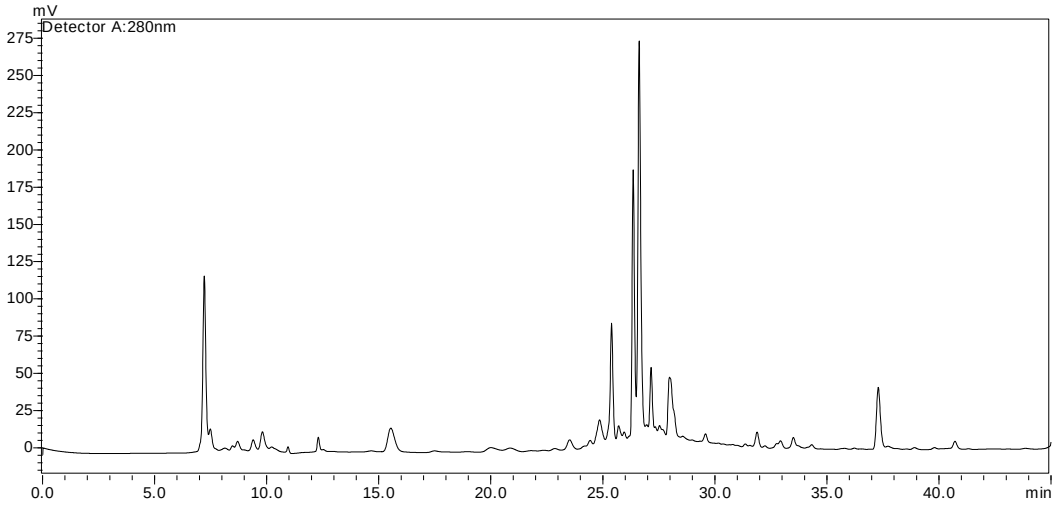


Figure 3-24: HPLC chromatogram of PSE extract (1st trial)

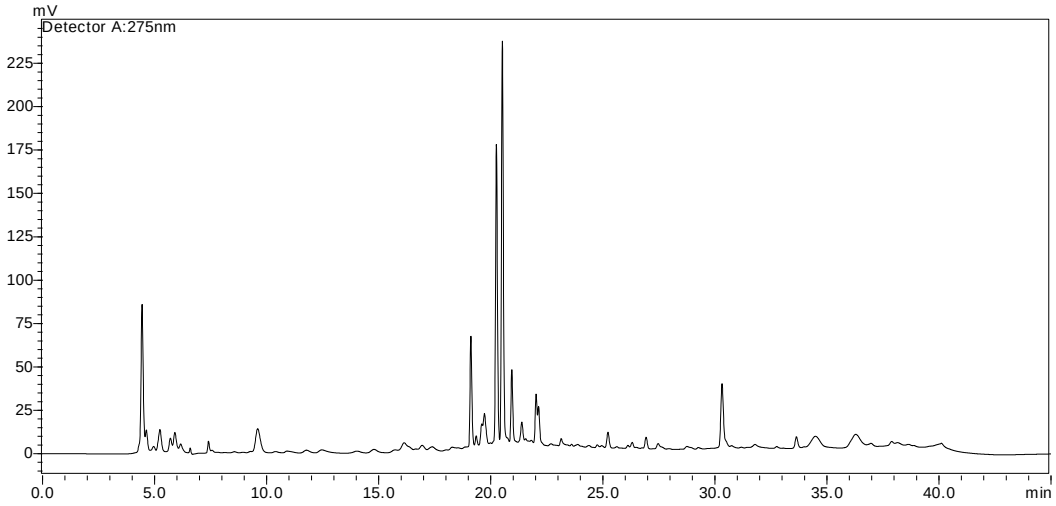


Figure 3-25: HPLC chromatogram of PSE extract (2nd trial)

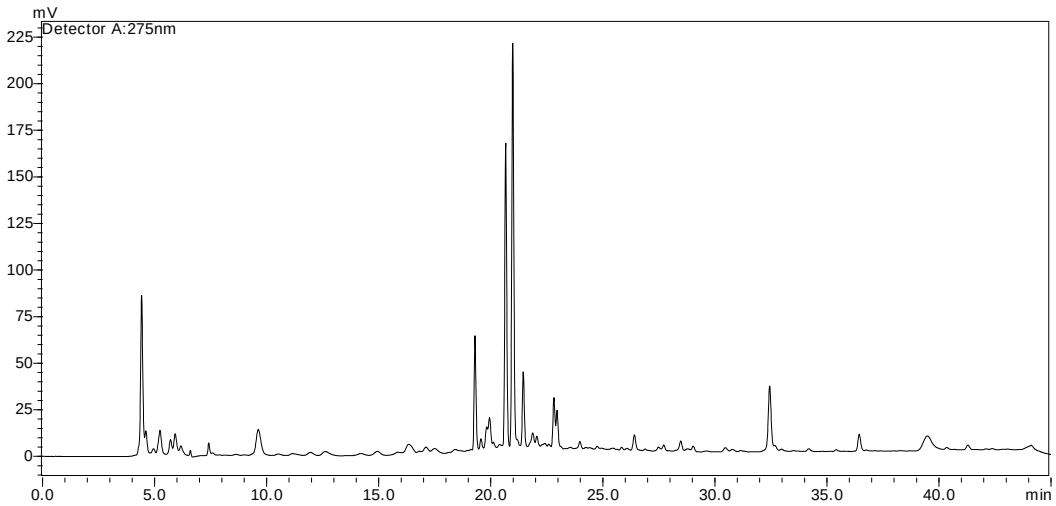


Figure 3-26: HPLC chromatogram of PSE extract (3rd trial)

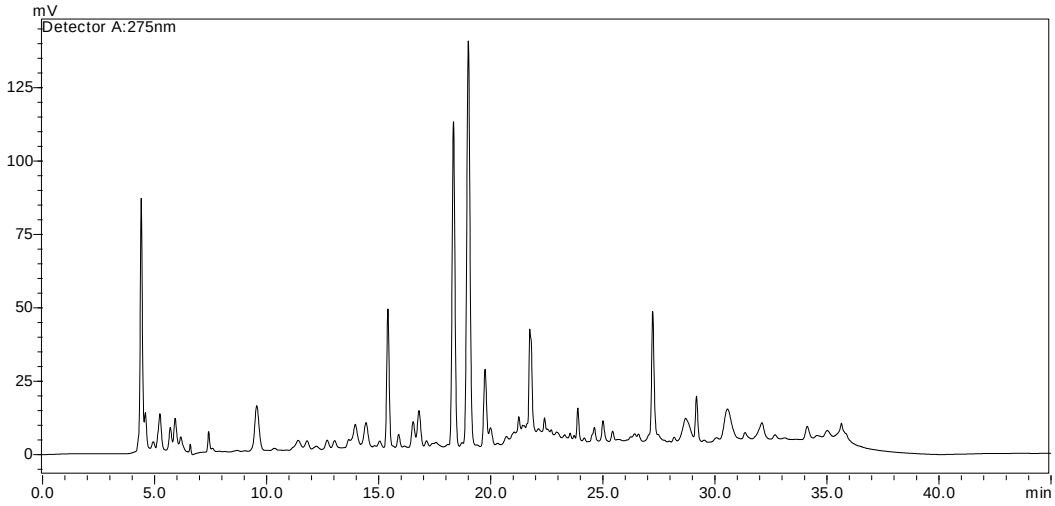


Figure 3-27: HPLC chromatogram of PSE extract (4th trial)

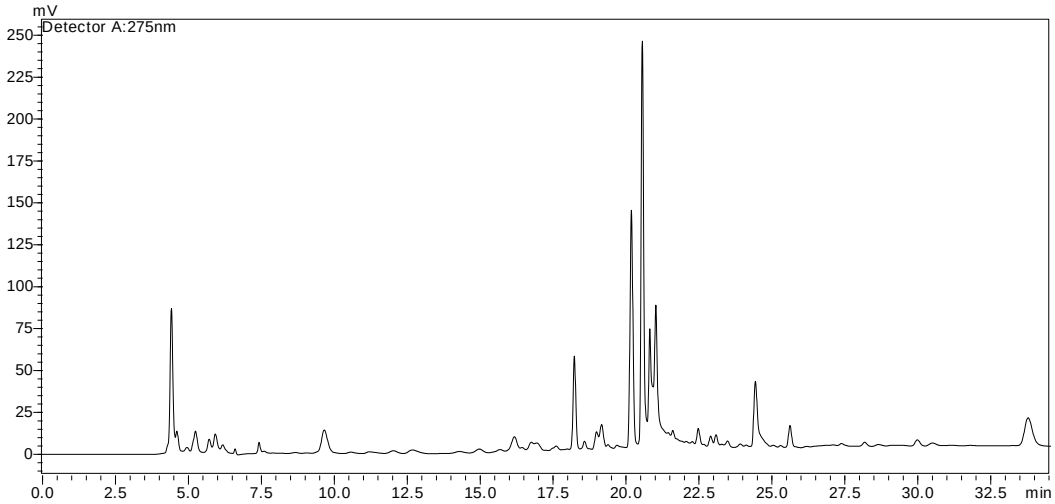


Figure 3-28: HPLC chromatogram of PSE extract (5th trial)

3.4.2 Identification of polyphenols in *Piper sarmentosum* Roxb. leaf extracts

Using the $UFLC_{XR}$ (HPLC-PDA), the retention time of the standards were found to be very close. So, it was necessary to use a mass spectrometer to assist in the identification of peaks. The UHPLC-ESI-MS, (Nexera™) coupled with a single quadrupole mass spectrometer equipped with an ESI probe, was used (chapter 2.3) and the analysis conditions were based on the 4th trial

(chapter 3.4.1). The characteristics of 25 phenol standards and a caffeine standard (retention time, maximum wavelength and m/z) analysed by UHPLC-ESI-MS are presented in Table 3-4. Following this, PSE, DFPSE and PSL extracts were analysed to see if any of these standard compounds were present. Each individual m/z ratio was observed and the retention times of the standards compared to the extracts to determine the presence of any of the standard polyphenols and caffeine. Profiling of the 3 extracts (PSE, DFPSE and PSL) obtained by UHPLC-ESI-MS, are shown in Figure 3-29.

Table 3-5 shows the identified compounds present in the extracts which have a retention time and m/z ratio match with the standards. The identified compounds present in PSE and DFPSE extracts are chlorogenic acid, caffeic acid, vitexin, *ρ*-courmaric acid, quercetin, hydrocinnamic acid and caffeine. Vitexin, hydrocinnamic acid and caffeine are also identified in PSL extract. The matching of retention time and m/z ratio between the standard and the peak observed in the extracts are shown in Figure 3-30 to Figure 3-36, mass chromatogram and mass spectra are shown in appendices A.1 to A.7.

Table 3-4: **Characterisation of standard compounds analysed using UHPLC-ESI-MS**

Retention time (min)	Compound	λ_{max}	$[M-H]^-$ m/z	Phenol group
4.074	trans-Cinnamic acid	275	147	Cinnamic acids
9.179	Gallic acid	275	169	Benzoic acids
14.414	Catechin	275	289	Flavanols
14.642	3-CQA (chlorogenic acid)	320	353	Cinnamic acids
14.805	5-CQA (neochlorogenic acid)	320	353	Cinnamic acids
15.403	4-CQA (cryptochlorogenic acid)	320	353	Cinnamic acids
15.940	Hydroxybenzoic acid	265	137	Benzoic acids
16.046	Epicatechin (EC)	275	289	Flavanols
16.783	Vanillic acid	275	167	Benzoic acids
16.797	Caffeic acid	320	179	Cinnamic acids
16.812	Epigallocatechin gallate (EGCG)	275	457	Flavanols
16.843	Syringic acid	275	197	Benzoic acids
19.364	Rutin	360	609	Flavones
19.614	Vitexin	320	431	Flavones
20.068	Vanillin	280	151	Benzoic acids
20.300	Epicatechin gallate (ECG)	275	441	Flavanols
20.539	*ρ*-Courmaric acid	320	163	Cinnamic acids
20.791	Sinapic acid	320	223	Cinnamic acids
20.817	Hesperidin	280	609	Flavanones
21.040	Ferulic acid	320	193	Cinnamic acids
21.325	Taxifolin	280	303	Flavanones
21.907	Phloridzin	280	471	Chalcones
24.467	Quercetin	360	301	Flavones
24.992	Hydrocinnamic acid	275	149	Cinnamic acids
25.692	Naringenin	280	271	Flavanones
27.748	Caffeine	275	193	Alkaloid

min=minute, λ_{max}=wavelength showed the maximum absorbance, $[M-H]^-$ = molecular ion in negative mode, m/z = mass to charge ratio

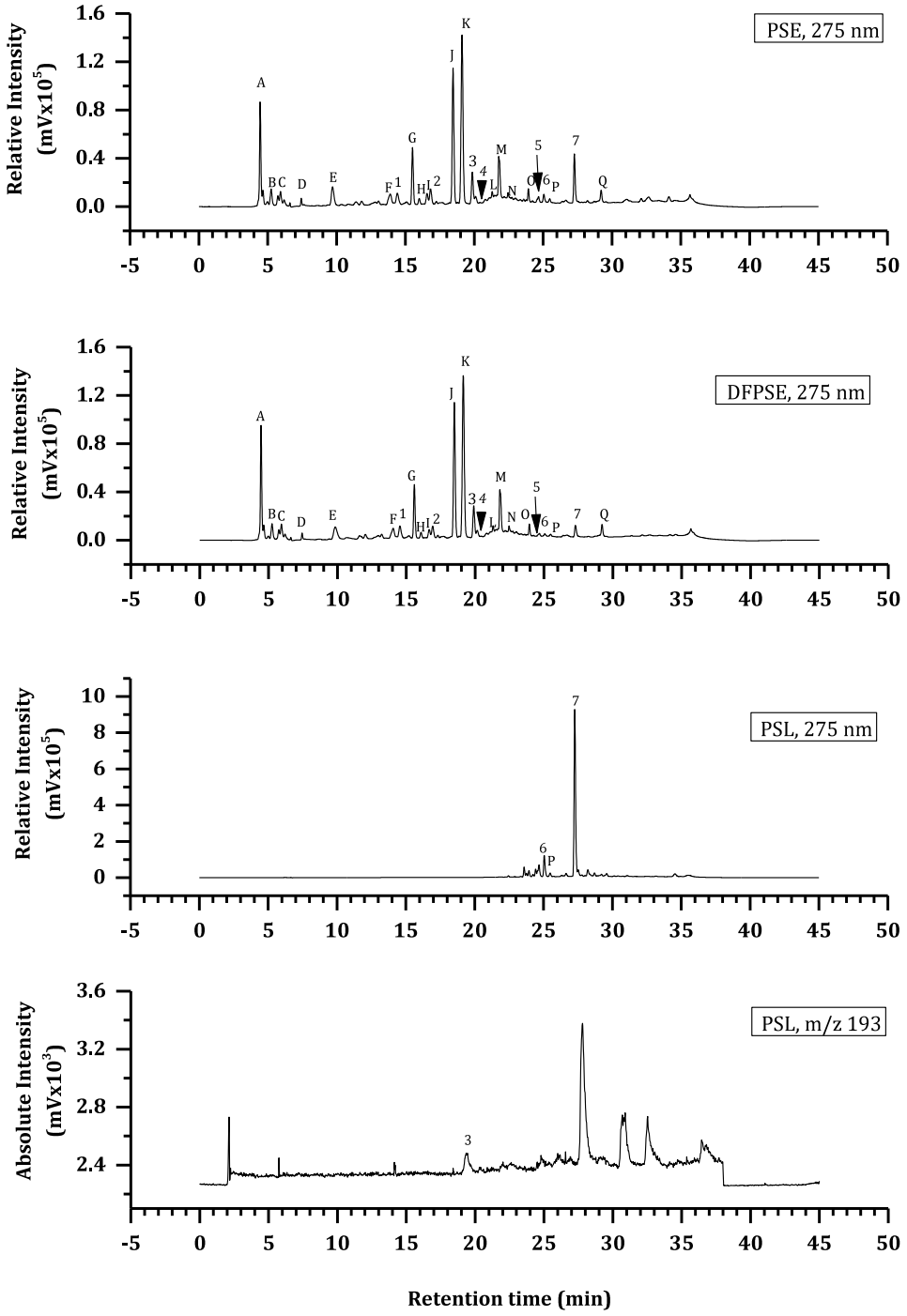


Figure 3-29: Profiling of *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE), defatted leaf extracts extracted using 80 % ethanol (DFPSE) and petroleum ether extracts (PSL) at 275 nm. m/z =193 is mass to charge ratio of vitexin. Analysed by UHPLC-ESI-MS using the same conditions. 1= chlorogenic acid, 2=caffeic acid, 3=vitexin, 4=*ρ*-courmaric acid, 5=quercetin, 6=hydrocinnamic acid, 7=caffeine. Letters A-Q =unidentified compounds

Table 3-5: **Identified compounds present in *Piper sarmentosum* Roxb. leaf extracts analysed using UHPLC-ESI-MS**

Peak	Retention time (min)	λ_{max}	$[M-H]^-$ m/z	Identified compound	Extract		
					PSE	DFPSE	PSL
1	14.68	320	353	Chlorogenic acid (3CQA)	•	•	
	14.81	320	353	Neochlorogenic acid (5CQA)	•	•	
2	16.82	275	179	Caffeic acid	•	•	
3	19.61	320	431	Vitexin	•	•	•
4	20.56	320	163	*ρ*-Coumaric acid	•	•	
5	24.44	360	360	Quercetin	•	•	
6	24.99	280	149	Hydrocinnamic acid	•	•	•
7	27.76	320	193	Caffeine	•	•	•

min=minute, λ_{max}=wavelength showed the maximum absorbance, $[M-H]^-$ = molecular ion in negative mode, m/z = mass to charge ratio, Extract = *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE), defatted leaf extracts extracted using 80 % ethanol (DFPSE) and petroleum ether extracts (PSL), ● = found

Figure 3-30 shows chromatograms of PSE, DFPSE and PSL extracts compared to the standards 3CQA, 4CQA and 5CQA. Chlorogenic acid (3CQA) was identified in PSE and DFPSE extracts according to the retention time (14.68 min) and m/z ratio (353) which matched the standard 3CQA. However, as the retention time of the standard 5CQA (neochlorogenic acid) (14.805 min) is close to the retention time of the peak and it has the same m/z ratio (353) , then the PSE and DFPSE extracts could also be identified as containing 5CQA (appendix A.1). However, the tiny peak observed at 15.2 min does not represent 4CQA present in the PSE and DFPSE extracts due to an absence of a peak at retention time (15.2-15.8 min) with the same m/z as the standard 4CQA (appendix A.2).

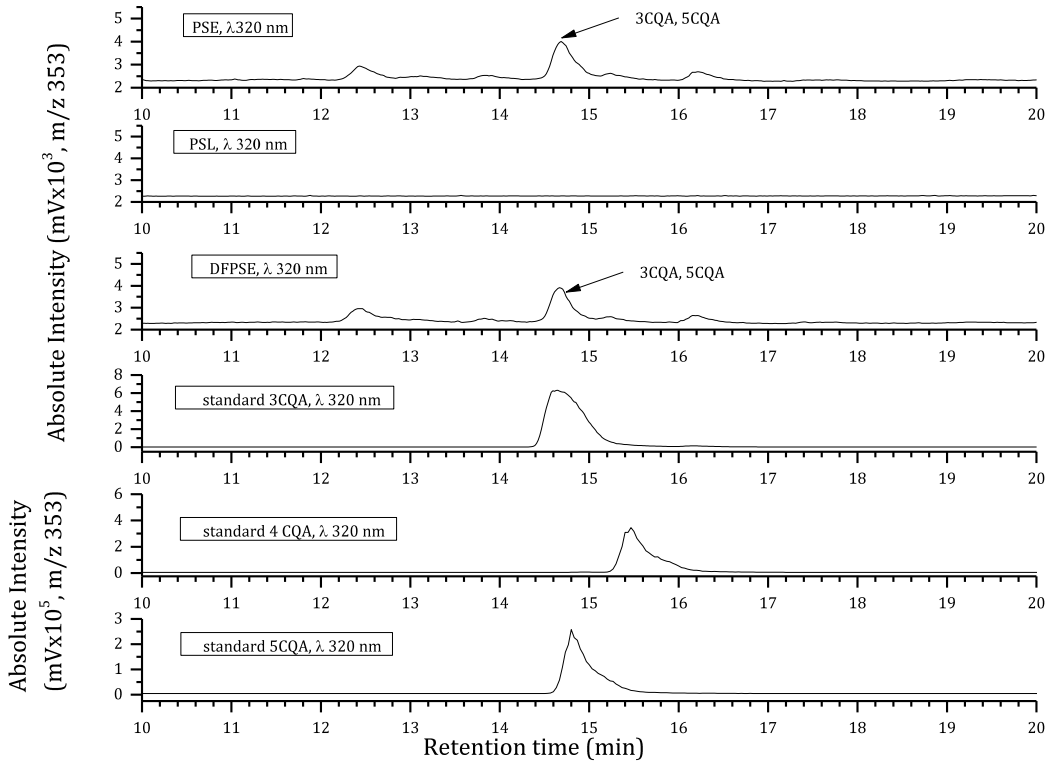


Figure 3-30: UHPLC-ESI-MS chromatograms of *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE), defatted leaf extracts extracted using 80 % ethanol (DFPSE) and petroleum ether extracts (PSL) comparing to standard chlorogenic acid (3CQA), cryptochlorogenic acid (4CQA), neoochlorogenic acid (5CQA), mass-to-charge ratio (m/z) =353, retention time 14.68, 15.40, 14.80 min respectively, wavelength 320 nm.

There are peaks shown at 16.82 min with a m/z ratio of 179 in PSE and DFPSE extracts, but not the PSL extract. Using Table 3-4, the PSE and DFPSE extracts therefore contain caffeic acid. The chromatograms are presented in Figure 3-31 and appendix A.3.

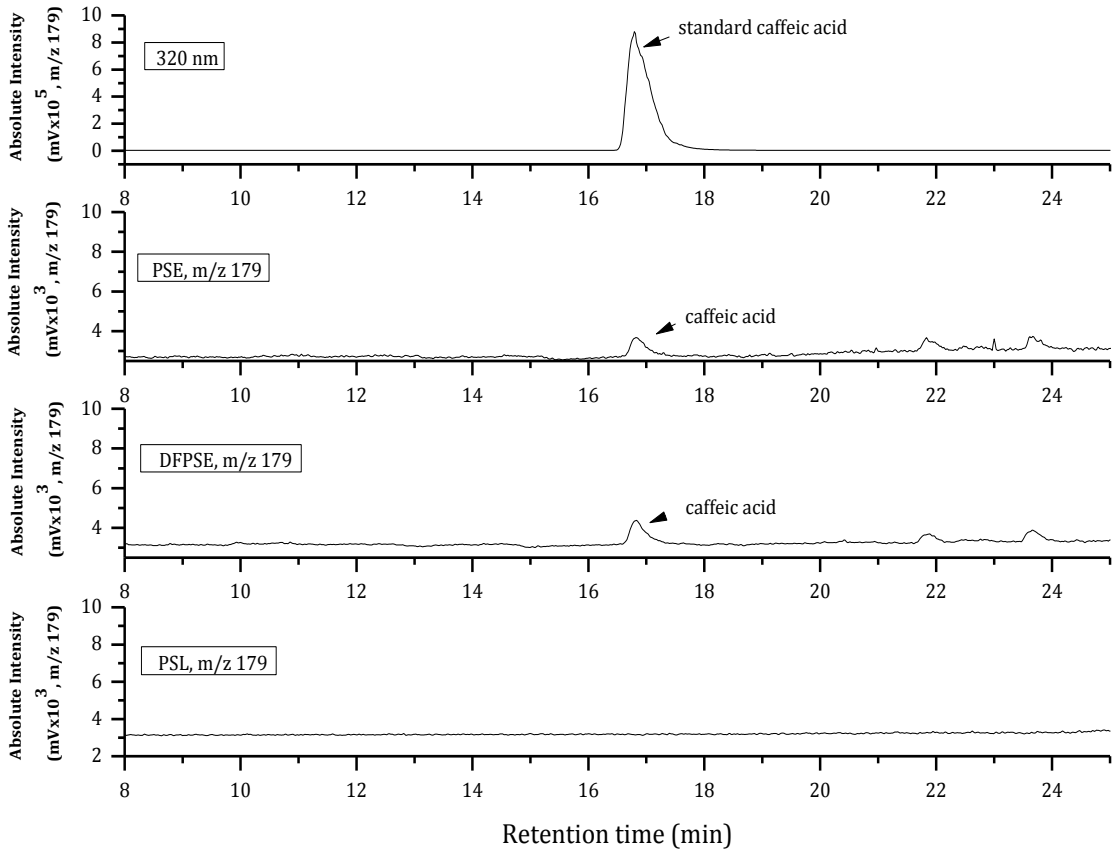


Figure 3-31: UHPLC-ESI-MS chromatograms of *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE), defatted leaf extracts extracted using 80 % ethanol (DFPSE) and petroleum ether extracts (PSL) comparing to standard caffeic acid, mass-to-charge ratio (m/z) =179, retention time 16.82 min, wavelength 320 nm

According to the retention time and m/z ratio, vitexin was also identified in the PSE, DFPSE and PSL extract. The chromatograms are presented in Figure 3-32 and mass spectra are shown in appendix A.4.

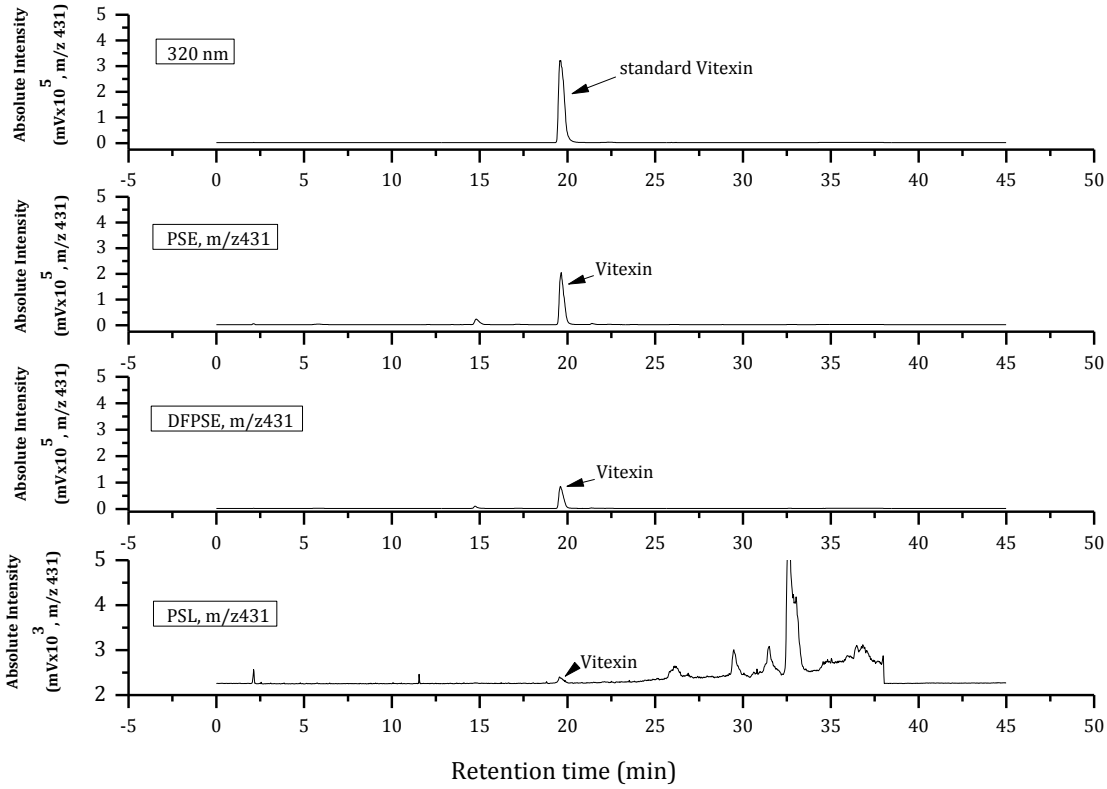


Figure 3-32: UHPLC-ESI-MS chromatograms of *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE), defatted leaf extracts extracted using 80 % ethanol (DFPSE) and petroleum ether extracts (PSL) comparing to standard vitexin, mass-to-charge ratio (m/z) =431, retention time 19.61 min, wavelength 320 nm

There were peaks in PSE and DFPSE extracts which had a retention time at 20.56 min and showed the same mass-to-charge ratio (m/z) of 163. This was identified as *ρ*-courmaric acid. PSL extract showed no peak at 20.56 min. The chromatograms are presented in Figure 3-33 and appendix A.5.

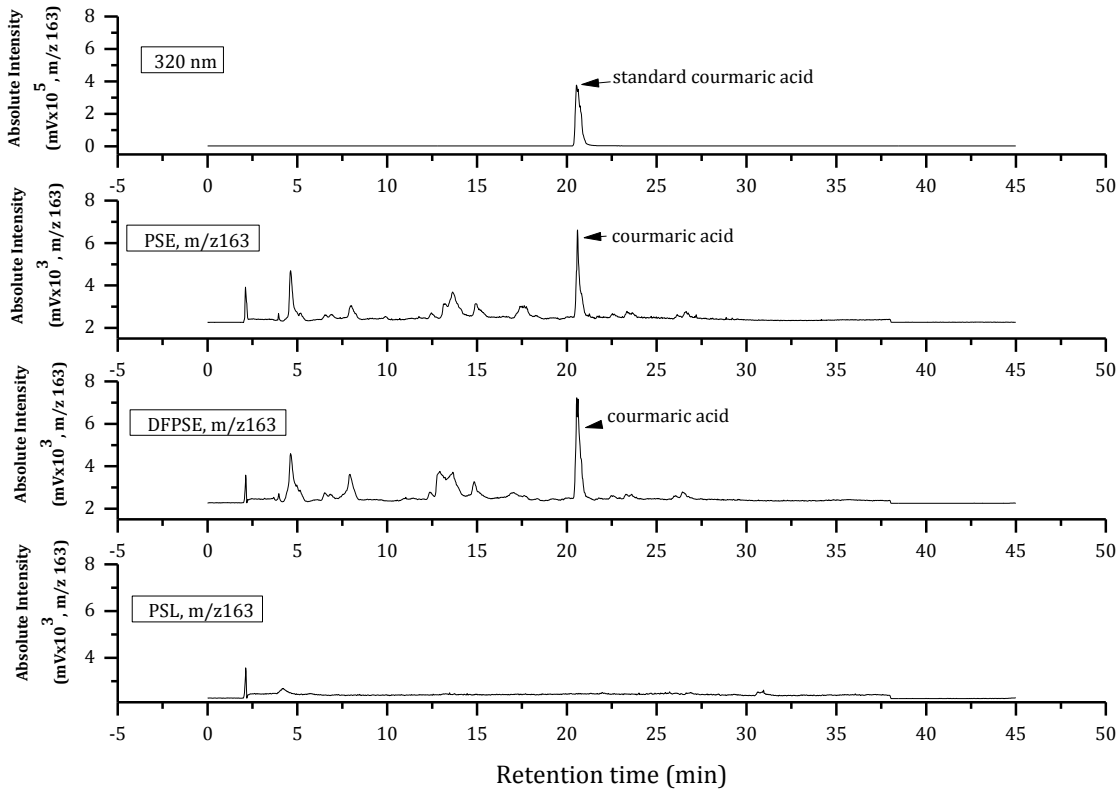


Figure 3-33: UHPLC-ESI-MS chromatograms of *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE), defatted leaf extracts extracted using 80 % ethanol (DFPSE) and petroleum ether extracts (PSL) comparing to standard *ρ*-courmaric acid, mass-to-charge ratio (m/z) = 163, retention time 20.56 min, wavelength 320 nm

Only PSE and DFPSE showed peaks which were identified as quercetin. The PSL extracts had no peak at 24.44 min, thus, no quercetin was present in PSL extracts. The chromatograms are presented in Figure 3-34 and appendix A.6.

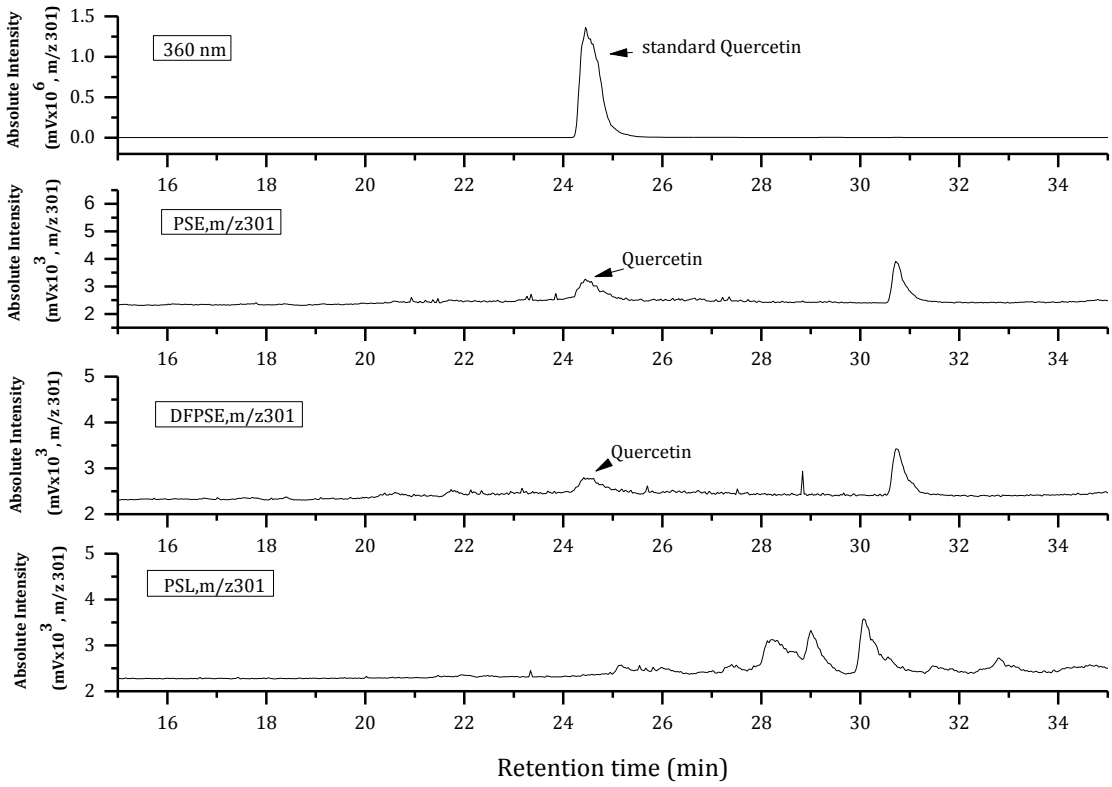


Figure 3-34: UHPLC-ESI-MS chromatograms of *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE), defatted leaf extracts extracted using 80 % ethanol (DFPSE) and petroleum ether extracts (PSL) comparing to standard quercetin, mass-to-charge ratio (m/z) =301, retention time 24.44 min, wavelength 360 nm

All extracts (PSE, DFPSE and PSL) have shown peaks at 24.99 min with the same m/z ratio of 149 which is hydrocinnamic acid. Therefore, all 3 extracts are found to contain hydrocinnamic acid. Their chromatograms are shown in Figure 3-35 and appendix A.7.

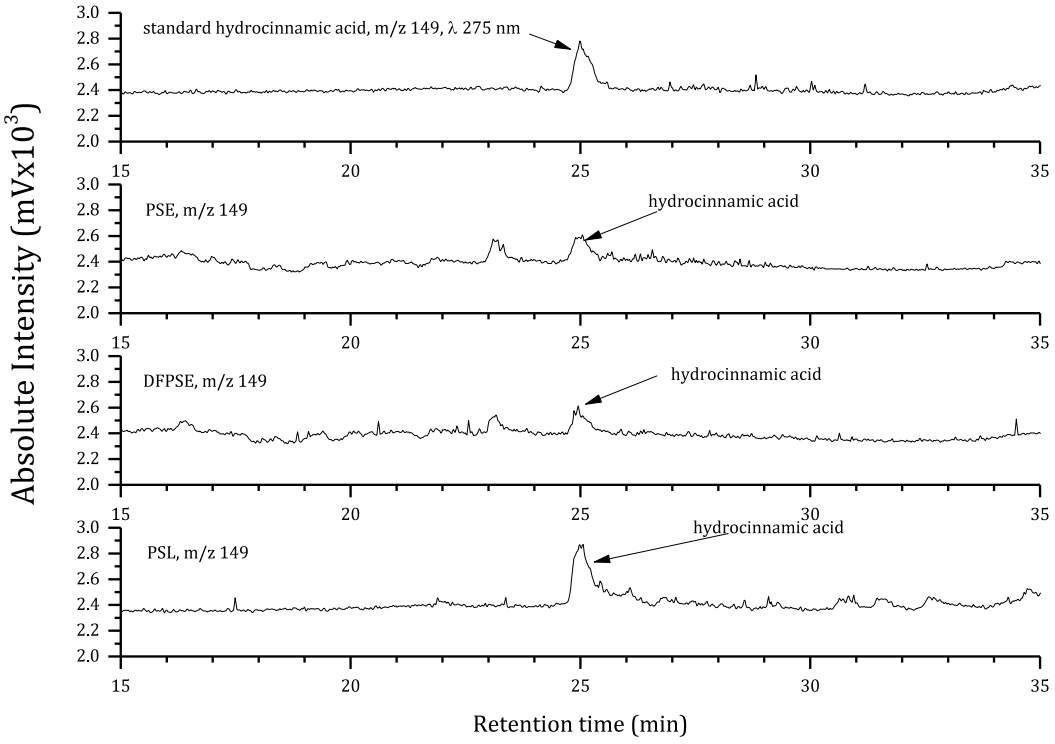


Figure 3-35: UHPLC-ESI-MS chromatograms of *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE), defatted leaf extracts extracted using 80 % ethanol (DFPSE) and petroleum ether extracts (PSL) comparing to standard hydrocinnamic acid, mass-to-charge ratio (m/z) =149, retention time 24.99 min, wavelength 275 nm

All extracts (PSE, DFPSE and PSL) have shown peaks at 27.75 min with the same m/z ratio of 193 which is caffeine. Therefore, all 3 extracts are found to contain caffeine. Their chromatograms are shown in Figure 3-36 and appendix A.8.

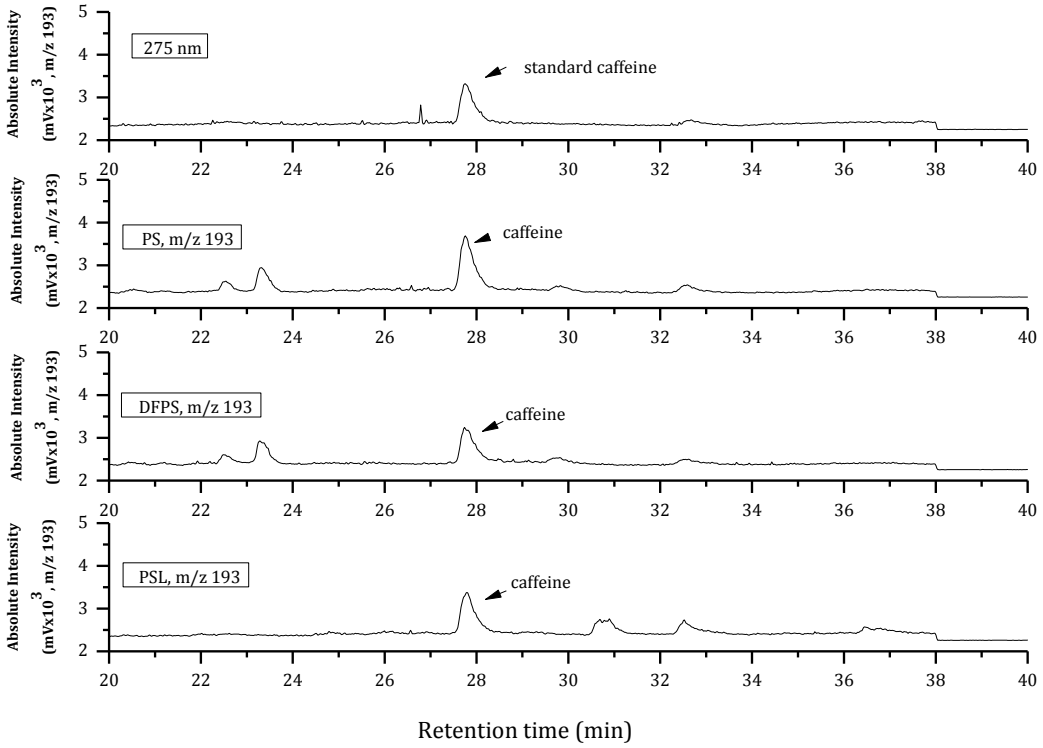


Figure 3-36: UHPLC-ESI-MS chromatograms of *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE), defatted leaf extracts extracted using 80 % ethanol (DFPSE) and petroleum ether extracts (PSL) comparing to standard caffeine, mass-to-charge ratio (m/z) =193, retention time 27.75 min, wavelength 275 nm

The profile of PS leaf extracts in Figure 3-29 also shows unidentified peaks A to Q. Some of them such as peak H, I, K, L, M and P, have retention times close to the epicatechin, vanillic acid, rutin, taxifolin, phloridzin and naringenin standards respectively (Table 3-4). The results in appendices A.9-A.14 clearly show that these standard compounds are not present in PS extracts due to the absence of peaks found at the same retention times with the same m/z ratio as the standards. With the limitation of a single quadrupole mass spectrometer which could not generate fragments, these unidentified compounds could not be defined directly. To try and elucidate the type of compounds these unknown peaks represent, tentative compounds could be proposed as based on the characterisation of maximum absorbance (λ_{max}) of the standards in Table 3-4.

The maximum absorbance of each unknown compound was determined (appendix A.15) and the proposed compounds are shown in Table 3-6. Peaks A, B, C and D are proposed to be cinnanic acid compounds due to their λ_{max} being in the range 275-280 and their elution times close to the trans-cinnamic acid standard. Peak E may be a benzoic acid compound due to its λ_{max} and elution time being similar to the gallic acid standard. Peak F could be a cinnamic acid compound due to its λ_{max} being over 300 nm and elution time was close to 3CQA. Peaks G and H are categorised as being cinnamic acid compounds due to their λ_{max} being over 300 nm and retention times between the 4CQA and caffeic acid standards. Peak I could be a benzoic compound due to its having an λ_{max} and elution time very close to vanillic acid standard. Peaks J and K could be major compounds present in the extracts. They are considered to be flavones compounds due to their λ_{max} (334 and 338 nm) and retention times close to rutin or vitexin standards. Peak L and M are proposed to be cinnamic acid compounds as their λ_{max} (319 and 317 nm) and retention time close to the *ρ*-courmaric acid standard. The flavanone compounds also could be either peak N, O or P due to their λ_{max} (290-300 nm) and elution time were between retention time of taxifolin and naringenin. Peak Q, is proposed to be a flavone compound due to its λ_{max} (352 nm) and elution time being similar to the pattern of quercetin or flavones group. According to the results, an alkaloid was identified as caffeine. Four cinnamic acids were identified as 3CQA or 5CQA, caffeic acid, *ρ*-courmaric acid and hydrocinnamic acid. Ten tentative cinnamic acid compounds and a tentative benzoic acid compound were proposed. Cinnamic acid and benzoic acid are subgroups of phenolic acids. Two flavones were identified as vitexin and quercetin. Three tentative flavones and 3 tentative flavanones compounds were

proposed. Flavones and flavanones are subgroup of flavonoids. Flavones J and flavones K are main compounds present in PSE and DFPSE extracts. Vitexin, flavones J and flavones K are in flavonoids groups. In total, an alkaloid, 15 phenolic acid compounds and 8 flavonoid compounds were identified, so this indicates that *Piper sarmentosum* Roxb. leaf extracts are a rich source of phenolic acids and flavonoids, so it is a good source of antioxidants.

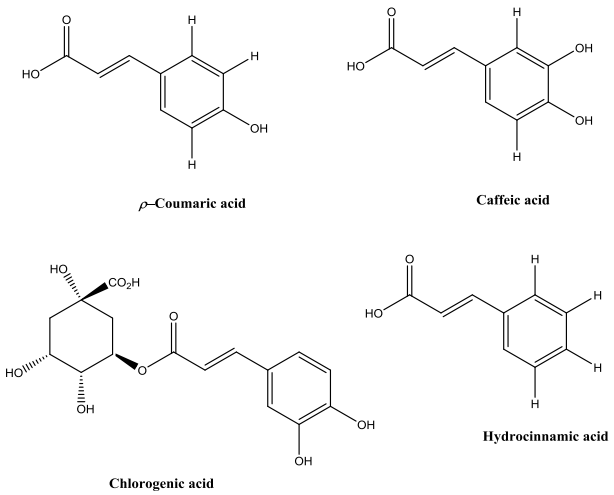


Figure 3-37: Chemical structure of the compounds found in *Piper sarmentosum* Roxb. leaf extracts which are in phenolic acid group, cinnamic acid subgroup. Adapted from Giada (2013a)

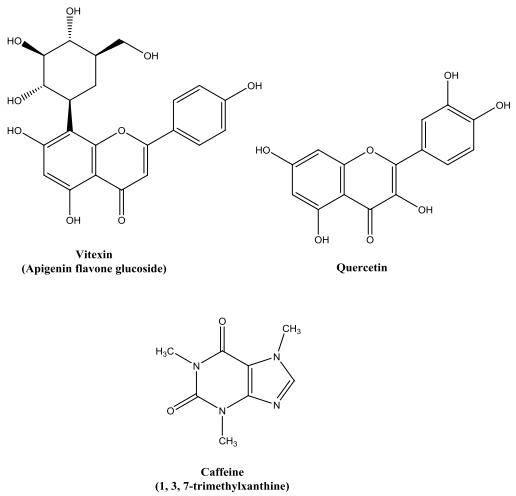


Figure 3-38: Chemical structure of the compounds found in *Piper sarmentosum* Roxb. leaf extracts. Vitexin and quercetin are in flavonoid group. Caffeine is an alkaloid compound. Adapted from Giada (2013a) and Azam *et al.* (2003)

Table 3-6: Propose tentative compounds present in *Piper sarmentosum* Roxb. leaf extracts analysed using UHPLC-ESI-MS

Peak	Retention time (min)	λ_{max}	Tentative compound	Extract		
				PSE	DFPSE	PSL
A	4.5	280	Cinnamic acid A	•	•	
B	5.2	263	Cinnamic acid B	•	•	
C	6.3	289	Cinnamic acid C	•	•	
D	7.5	352	Cinnamic acid D	•	•	
E	9.6	278	Benzoic acid E	•	•	
F	13.5	306	Cinnamic acid F	•	•	
G	15.5	323	Cinnamic acid G	•	•	
H	16.0	315	Cinnamic acid H	•	•	
I	16.5	277	Cinnamic acid I	•	•	
J	18.5	334	Flavones J	•	•	
K	19.1	338	Flavones K	•	•	
L	21.5	319	Cinnamic acid L	•	•	
M	21.9	317	Cinnamic acid M	•	•	
N	22.5	290	Flavanones N	•	•	
O	24.0	290	Flavanones O	•	•	
P	25.5	300	Flavanones P	•	•	•
Q	29.2	352	Flavones Q	•	•	

min=minute, λ_{max}=wavelength showed the maximum absorbance,
m/z = mass to charge ratio, Extract = *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE), defatted leaf extracts extracted using 80 % ethanol (DFPSE) and petroleum ether extracts (PSL), ● = found

3.4.3 Quantification of the compounds present in *Piper sarmentosum* Roxb. leaf extracts

Quantification of the identified phenol compounds and caffeine that were present in the *Piper sarmentosum* Roxb. leaf extracts (PSE, DFPSE, PSL) was performed by preparing standard curves as shown in Figure 3-39. The measurement was done in triplicate according to the maximum wavelength of each standard. As 3CQA could also be 5CQA, the quantification was carried out using 3CQA to represent its amount (using the standard curve of 3CQA). The results are presented in Table 3-7. According to the results, there was no significant difference in the chlorogenic acid present in the DFPSE extract compared with the PSE extract. Caffeic acid and *ρ*-coumaric acid are higher in the DFPSE extract than in the PSE extract with significance ($p<0.05$). Vitexin levels are found to be significantly higher among the extracts ($p<0.05$). The amount of vitexin in the PSE extract is higher than in DFPSE and found in only a small amount in the PSL extract. Quercetin presented in both PSE and DFPSE extracts are found to be significantly different ($p<0.05$). The amount of hydrocinnamic acid is found to be significantly different for all extracts ($p<0.05$). The highest amount is obtained in PSL extract. The amount of caffeine is highest in the PSL extract and found to be significantly different to PSE and DFPSE extracts ($p<0.05$), while the caffeine present in the DFPSE extract is less than in the PSE extract with no significant difference.

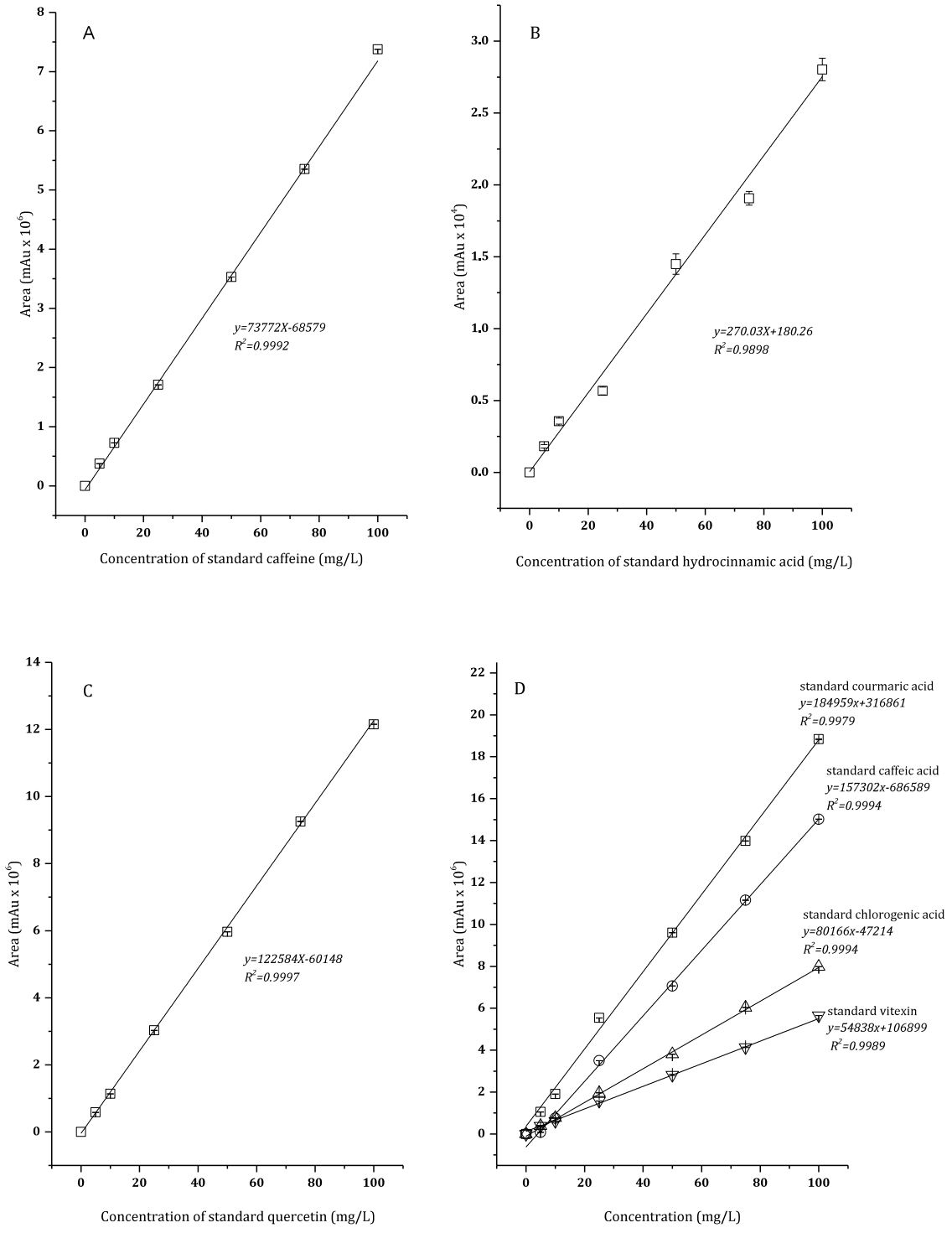


Figure 3-39: Calibration curves of standard caffeine 0-100 mg/L at 275 nm (A), standard hydrocinnamic aicd 0-100 mg/L at 275 nm (B), standard quercetin 0-100 mg/L at 360 nm (C), standard *ρ*-courmaric acid 0-100 mg/L at 320 nm (D), standard caffeic acid 0-100 mg/L at 320 nm (D), standard chlorogenic acid (3CQA) 0-100 mg/L at 320 nm (D) and standard vitexin 0-100 mg/L at 320 nm (D) for quantifying the identified compounds using UHPLC-ESI-MS. Results are expressed as mean±SE of triplicated analysis.

Table 3-7: The quantification of identified compounds contained in *Piper sarmentosum* Roxb. leaf extracts analysed by UHPLC-ESI-MS

Peak	Compound	RT (min)	λ max	$[M-H]^-$ m/z	mg / 100 g dried weight		
					PSE	DFPSE	PSL
1	3CQA, 5CQA	14.68	320	353	75.59±1.19^a	101.88±2.78^a	ND
2	Caffeic acid	16.82	275	179	75.42±0.02^a	81.15±0.17^b	ND
3	Vitexin	19.61	320	431	150.32±0.01^a	115.23±0.65^b	7.20±0.27^c
4	*ρ*-Coumaric acid	20.56	320	163	27.20±0.37^a	33.58±0.03^b	ND
5	Quercetin	24.44	360	301	5.58±0.01^a	5.13±0.01^b	ND
6	Hydrocinnamic acid	24.99	275	149	16.52±0.45^a	11.25±0.60^b	44.98±1.89^c
7	Caffeine	27.76	320	193	28.66±0.14^a	12.21±0.44^a	551.84±1.03^b

Results are reported as mean±SE mg/100 g of dried weight, RT= retention time (minute), λmax = wavelength shows the highest absorbance, $[M-H]^-$ = molecular ion in negative mode, PSE = *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol, DFPSE = defatted *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol, PSL = *Piper sarmentosum* Roxb. leaf extracts extracted using petroleum ether, ND = not detected. Values with similar letters within row are not significantly difference ($p<0.05$, n=3)

A number of studies have reported the presence of flavonoids, phenolic acids and alkaloids in different parts of *Piper sarmentosum* Roxb. (Ugusman *et al.*, 2012; Sim *et al.*, 2009; Subramaniam *et al.*, 2003). Caffeine (1, 3, 7-trimethylxanthine) is an alkaloid compound which can show both antioxidant activity and prooxidant activity (Yashin *et al.*, 2013; Farah and Donangelo, 2006; Azam *et al.*, 2003; Shi *et al.*, 1991). Standard phenolic compounds and caffeine which were available in the Food Chemistry Laboratory were used for this study. Of the standard polyphenols chosen for analysis, many of them have very close retention times. It is therefore essential to use an advance technique which is appropriate for identification. The technique of using mass spectrometry has been employed by a number of researchers to identify phenols in plants, food or beverage samples such as fruit, vegetables, seeds, wine, beverages etc.

(Puigventos *et al.*, 2015; Brito *et al.*, 2014; Ghasemzadeh and Jaafar, 2013; Zhang *et al.*, 2013; Jimenez *et al.*, 2011; Fattouch *et al.*, 2008; Alonso-Salces *et al.*, 2004; Ma *et al.*, 2004). This study used a single quadrupole mass spectrometer to detect the mass of the molecular ions of phenols present in PS leaf extracts. The chromatograms produced by the DFPSE extracts were almost like to those found in the PSE extracts. However, the peaks were different for the PSL extract (Figure 3-29). The different profiling of PSL extracts may result from using petroleum ether for extraction which is non polar. The compounds present in the PS leaf which are less polar or are non-polar will be extracted better using petroleum ether and will be eluted after higher polarity compounds (such as phenolic acids). However, with this method, it could detect a few compounds in the PSL extracts which reflect to ineffectiveness of binary gradients. This is also true for the 3CQA and its isomers (4CQA or 5CQA) which have the same molecular mass. So, it is not possible to distinguish them by using their m/z ratio and their retention time is almost the same. This may be improved by adjusting the binary gradient with a longer cycle time. Aladedunye and Matthaeus (2014) analysed phenolic compounds from rowanberry fruit extract using a reverse phase column and mobile phase the same as this present study. The binary gradient of mobile phase B (0.1 % formic acid in acetonitrile) was set to gradually increase and the cycle time was lengthened to 70 min. The 3CQA, 4CQA and 5CQA were then perfectly separated and eluted at different times. By improving the binary gradient such as gradually increasing the acetonitrile proportion and time, more compounds such as flavonoids may also be found in PSL extract due to these compounds being lipophilic. Several studies have identified component compounds of *Piper sarmentosum* Roxb. Ugusman *et al.* (2012) reported the

finding of rutin and vitexin (51.93 mg/100 g dried weight) in leaf extracted using water. The amount of vitexin in this present study are higher. Rukachaisirikul *et al.* (2004) reported the presence of quercetin and myricetin in leaf extracted using aqueous methanol and stigmasterol was found in fruit extracted using the same solvent. Subramaniam *et al.* (2003) reported naringenin present in methanol treated leaf extract. Myricetin, rutin and naringenin were not found in this study. Niamsa and Chantrapromma (1983) reported the finding of hydrocinnamic acid in leaf extracted using petroleum ether which was in agreement to this study. Likhitwitayawuid *et al.* (1987) reported the presence of β-sitosterol in leaf and fruit extracted using petroleum ether. Suzgec *et al.* (2005) also extracted *Helichrysum compactum* leaf using petroleum ether and reported an abundance of flavonoid compounds present in the extract. The difference of their findings to this study may attribute to many factors such as the variation of plant sources, extraction protocols (different concentrations or extraction procedures) and method of analysis. Likhitwitayawuid *et al.* (1987) extracted PS leaf powder using petroleum ether at 40-60 °C which was much lower than this study (250 °C), while Niamsa and Chantrapromma (1983) and Suzgec *et al.* (2005) did not state the temperature used. In their studies, they also treated their petroleum ether extracts further using different polarity solvent concentrations and passed the extracts through a chromatography column. The techniques used to identify compounds present in the extract were also different to this study. Rukachaisirikul *et al.* (2004) and Likhitwitayawuid *et al.* (1987) used a NMR technique. Ugusman *et al.* (2012) used HPLC and used similar mobile phases to this study but different binary gradients, flow rate and cycle time. Therefore, using different methods can result in different findings.

Figure 3-37 and Figure 3-38 show the 7 identified compounds (3CQA, caffeic acid, vitexin, *ρ*-courmaric acid, quercetin, hydrocinnamic acid and caffeine). It has been noted that phenolic acids and their esters are widely distributed in plant tissue at a cellular and subcellular level and they have a high antioxidant activity depending on the number of hydroxyl groups in the molecule, especially chlorogenic acid and caffeic acid. Chlorogenic acid has higher antioxidant activity than caffeic acid and *ρ*-courmaric acid. As seen in Figure 3-37, chlorogenic acid has 3 hydroxyl groups in the molecule while caffeic acid has 2 hydroxyl groups and *ρ*-courmaric acid has 1 hydroxyl groups in the molecule (Pandey and Rizvi, 2009). Flavonoids with free hydroxyl groups act as free radical scavengers and multiple hydroxyl groups, especially in the B-ring (Figure 1-7), enhance their antioxidant activity (Yanishlieva, 2001). The structure of vitexin has 7 hydroxyl groups and quercetin has 5 hydroxyl groups (Figure 3-38). The total phenol content, total flavonoid content and antioxidant capacity of the extracts examined in chapter 3.2 may result from some of these compounds including the tentative compounds A to Q. The flavonoid content and antioxidant activity of PSL extract may result from vitexin, hydrocinnamic acid, caffeine and tentative flavones Q. Based on the literature reviewed, it seems no studies had ever reported the presence of caffeine in this plant (*Piper sarmentosum* Roxb.) and also no one has reported finding vitexin and caffeine in petroleum ether extracts.

3.5 Preliminary studies of frying oil

The work in this section firstly looks to understand the behaviour of oil when exposure to frying temperature. The focus then moves to finding synthetic antioxidant free commercial oil. The information acquired will give a better understanding of the deterioration pattern of oil and give information on what is

occurring during heating, analytical parameters and analytical methods to be used in the final stage of the study.

3.5.1 Effect of repeated frying on the physical and chemical characteristics of the oils

The aim of this experiment is to understand the behaviour of oil when subject to frying temperature (190 ˚C). The information obtained will be used to create a frying method, as well as to determine the analytical parameters to be used for further experiments.

3.5.1.1 Effect of repeated frying on oil colour

A test was carried out to observe colour changes of the heated oils using a photometric method. The results of this test are shown in Figure 3-40. The colour of both oils used for frying McCain® and Morrison® chips sharply increased in darkness from the 1st day to the 6th day of frying compared to the un-used oil (day 0). In addition, the colour of the oil used to fry Morrison® chips was darker than the oil used to fry McCain® chips, possibly due to the longer frying time (3.5 min). The results of the present study are in agreement with many studies, such as the results reported by Plimon (2012). They found the colour of blended oil was darker as the number of fryings were performed. The study by Aladedunye and Przybylski (2009) reported canola oil which was heated at 185 ^{0}C and 215 ^{0}C, had increased in colour after frying each day. The study of Baixauli *et al.* (2002) showed an increase in darkness in sunflower oil colour after frying battered squid rings. Takeoka *et al.* (1997) had reported seven types of frying oils (soybean salad oil, corn oil, soybean liquid frying shortening, canola salad oil, cottonseed oil, canola liquid frying shortening, beef

tallow) which were heated at 190 °C for 8 days, and all had increased in photometric colour index as frying days increased. The darkening of the frying oil could be caused by substances from fried foods (sugar, starch, protein, phosphate, sulphur compound and trace metal) that collect in the oil during frying. These substances can brown themselves or react with the oil and cause the oil to darken (Lawson, 1995).

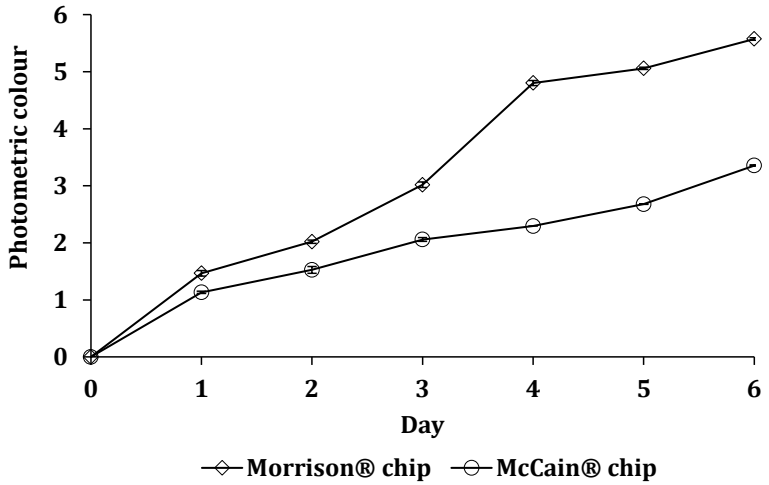


Figure 3-40: Changes of rapeseed oil colour at different frying days. The photometric colour value is expressed as mean±SE of triplicate analysis

3.5.1.2 Effect of repeated frying on oil smoke point

The smoke point of the oils was determined and the results are shown in Figure 3-41. The overall trend of the smoke point temperature of the oils was a decrease from day 1 to day 6 of frying when compared with the un-used oil (day 0). The smoke point of the oil used to fry Morrison® chips was lower than the oil used to fry McCain® chips, possibly due to the longer frying time (3.5 min). The smoke point of the oil was used for frying Morrison® chips had dropped in temperatures from 183.7 °C to 178 °C (day 1 to day 2) before rising up to 188 °C

in day 3, then the temperatures gradually decreased to 155 °C by day 6. The smoke point of the oil which was used for frying McCain® chips also showed fluctuation. The smoke point was in general decreased over time but was 145 °C on day 4, 160 °C on day 5 and then 155℃ on day 6. This result is in agreement with the study by Man and Hussin (1998). They found the smoking point of palm olein oil and coconut oil used for frying potato chips at 180 °C for 5 days, declined over the frying days. The declination of smoking point results from the oil breaking down to free fatty acids, which happens during heating the oil. The longer heating time, the more free fatty acids are produced (Bockisch, 1998). The amount of smoke released from the frying oil is directly proportional to the amount of free fatty acids and volatile compounds (low molecular weight decomposition products) in the degraded oil (Tarmizi and Ismail, 2008). The free fatty acids and other volatiles leaving the fat as vapours will appear as smoke when their concentration is high enough to permit aggregation to colloidal size particles (Man and Hussin, 1998). Whilst this assay is still used for monitoring the changes in frying oil as it can relate to free fatty acids, some researchers stated the disadvantage of using this method. According to Wu and Nawar (1986) and Warner (2002), the smoking point assay is a visual inspection which has less repeatability or reproducibility as it is difficult to notice the first temperature that produces continuous smoke from the oil, so they did not recommend this method for assessment of the quality of oil. Although, the present study has clearly results of decreasing smoking point as the days of frying increase, it also shows some difficulties as seen in day 4 to 5 for the frying oil used for frying Morrison® chips. Thus, the author agrees with the view of

Warner (2002) and Wu and Nawar (1986) as it is difficult to notice the first thin continuous smoke stream.

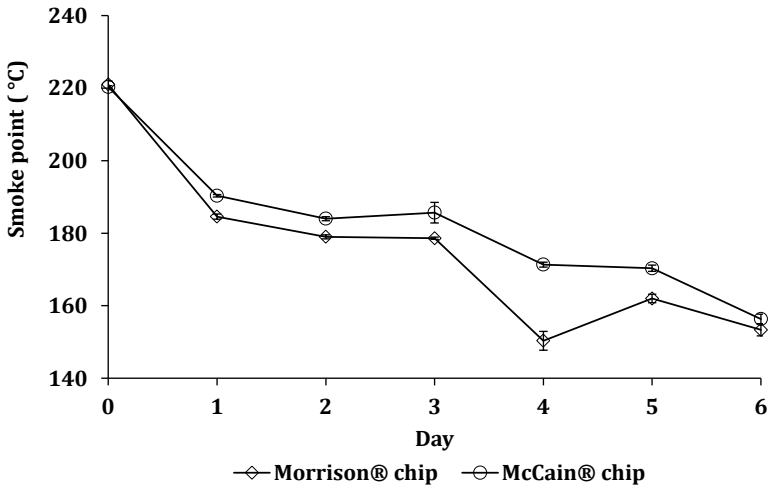


Figure 3-41: Changes of smoke point in rapeseed oil at different frying days. Temperature is expressed as mean±SE of triplicate analysis

3.5.1.3 Effect of repeated frying on oil viscosity

The oil samples were measured to evaluate the impact of thermal degradation on oil viscosity. The results, as shown in Figure 3-42, indicate that the viscosity of the oils increase with frying time. Both oils used for frying McCain® and Morrison® chips gradually increased in viscosity over time. The frying oil from the Morrison® chips was more viscous than the frying oil from the McCain® chips, possibly due to longer frying time (3.5 min). The increasing viscosity attributes to the formation of high molecular weight and polar compounds, such as polymers and cyclic fatty acids, which are products from a polymerisation reaction (Warner, 2002). These results are similar to the study of Santos *et al.* (2005). They heated cooking oils up to 190 ^{0}C for 8 hours and found that the viscosity of the cooking

oils increased in direct proportion to frying time and was caused by oxidation and polymerisation.

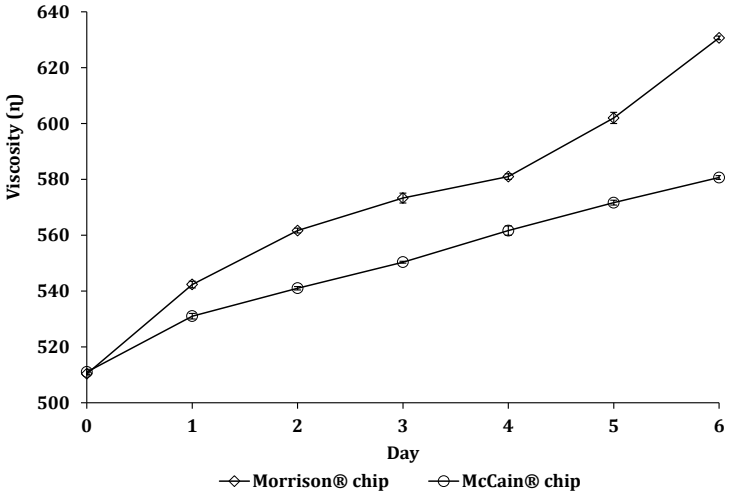


Figure 3-42: Changes of viscosity in rapeseed oil at different frying days. The value is expressed as mean±SE of triplicate analysis

3.5.1.4 Effect of repeated frying on acid value of oil

The hydrolysis reaction which occurs in frying oil causing triglycerides and diglyceride to be converted to free fatty acids and glycerol resulting in the rancidity of the oils. The changes of this reaction can be monitored by determining the acidity (or can be expressed as free fatty acids) of heated oils. As can be seen in Figure 3-43, the acid value of the oils used for frying McCain® and Morrison® chips increases over the frying time. The acidity of the oil used for frying Morrison® chips is higher than the oil used for frying McCain® chips from the 1st day till the last day of frying. The results of the present study similar to the studies of Tarmizi and Ismail (2008); Baixauli *et al.* (2002); Che Man and Tan (1999) and Man and Hussin (1998). They found the increasing acid value (or free fatty acids) corresponded to an increase in days of frying. The changes in

acidity of the heated oils are a result of a hydrolysis reaction which is generated by 3 factors: water, steam and oxygen (Choe and Min, 2007). Water will react with the ester linkage of triacylglycerol and produce di and monoaclyglycerol, glycerol and free fatty acids (Choe and Min, 2007). The acid value of the oil used for frying Morrison® chips is higher than the oil used for frying McCain® chips, possibly due to the water or moisture present in the chips. Higher amounts of water or moisture can hydrolyse the oil rapidly (Dana *et al.*, 2003) and faster than steam (Pokorny, 1989). The moisture present in Morrison® chips may be higher than McCain® chips due to the bigger size or volume of the Morrison® chips, so relating to a higher in acid value.

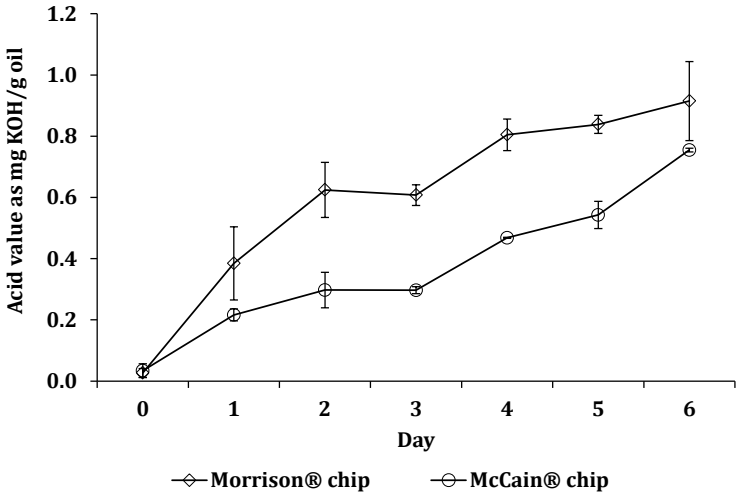


Figure 3-43: Changes of acid value in rapeseed oil at different frying days. The value is expressed as mg potassium hydroxide (KOH)/gram oil, mean±SE of triplicate analysis

3.5.1.5 Effect of repeated frying on peroxide value of oil

The samples were analysed for peroxide value (PV) to quantify primary oxidation. In general, the results show that the PV increased over frying time compared with the un-used oil (day 0). It was observed that the PV of the

samples fluctuated during frying time, as seen in Figure 3-44. There was a sharp increase on the first day of frying from 8.76 to 50 meq/kg for Morrison® chips and to 29.37 meq/kg for McCain® chips. After that the PV dropped for both oils but increased on day 5 for the oil which fried Morrison® chips before decreasing on day 6. The PV of Morrison® chips were higher than McCain® chips, this probably due to the longer frying time of Morrison® chips (3.5 min). The fluctuation of PV during frying time is similar to findings reported by Marinova *et al.* (2012), Farhoosh and Moosavi (2009), Baixauli *et al.* (2002) and Nawar (1984). They found that the PV of frying different vegetable oils had shown an increase during the early stages of frying followed by a decrease. The fluctuations of peroxides were caused by the frying temperatures as they are sensitive to high temperatures. Peroxides can be destroyed at the high frying temperature used because they can undergo further reactions such as oxidation, dimerisation and polymerisation (Choe and Min, 2007) but can reform during cooling (Fritsch, 1981). Therefore, peroxide value might not be a good indicator for analysing the oxidative reaction (Paul *et al.*, 1997; Fritsch, 1981).

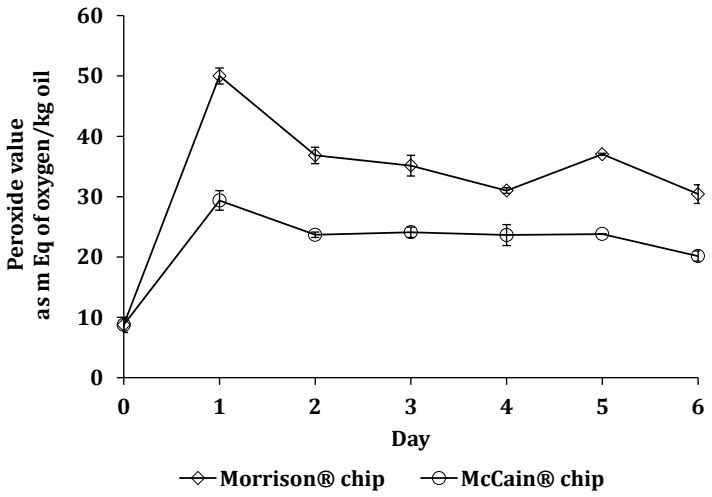


Figure 3-44: Peroxide value in rapeseed oil at different frying days. The value is expressed as milli equivalence (meq) oxygen/kilogram (kg) oil, mean±SE of triplicate analysis

3.5.1.6 Effect of repeated frying on *ρ*-Anisidine value and TBA value of oil

The secondary oxidative products of lipid oxidation can be monitored by *ρ*-Anisidine value or TBA value. The TBA assay can be also expressed as TBA value or TBA reactive substance (TBARS) as malonaldehyde equivalent. The standard curve used for malonaldehyde calculation range 0 to 1.20 µmol/mL is shown in Figure 3-47. The results determined by the 3 different methods show a similar increasing trend as seen in Figure 3-45 to Figure 3-46 and Figure 3-48. All methods show an increase in value over the number of frying days.

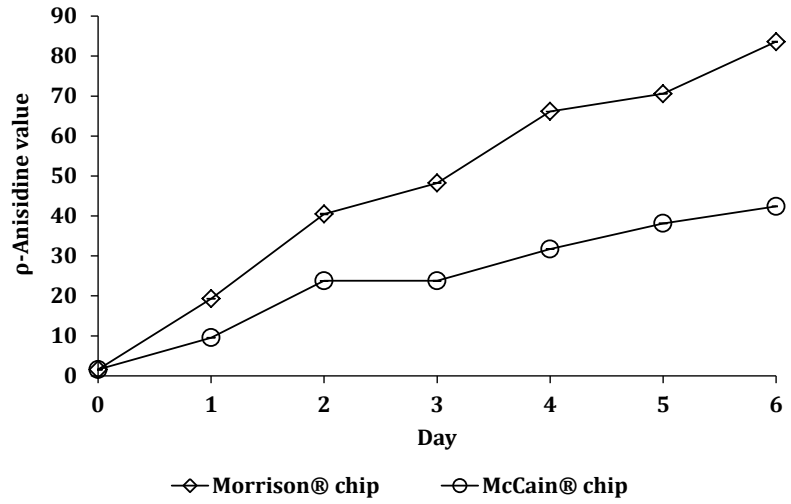


Figure 3-45: *ρ*-Anisidine value in rapeseed oil at different frying days. The value is expressed as mean±SE of triplicate analysis

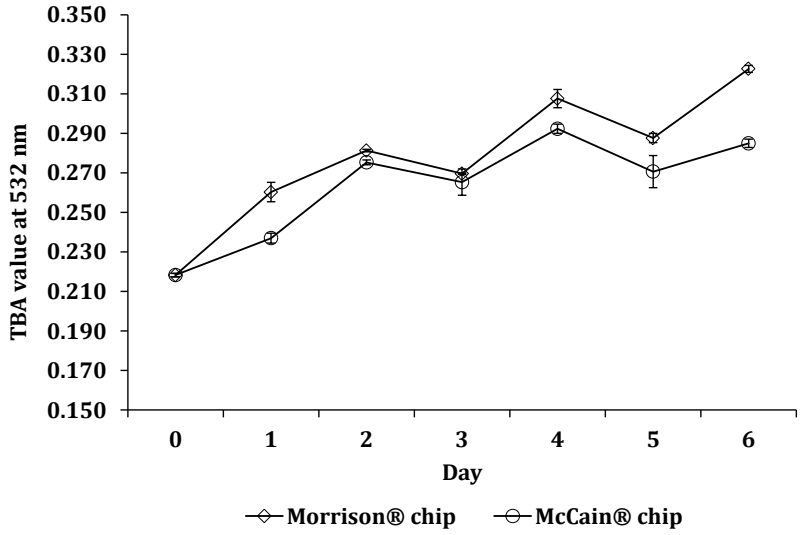


Figure 3-46: 2-Thiobarbituric acid value (TBA value) in rapeseed oil at different frying days. The value is expressed as mean±SE of triplicate analysis

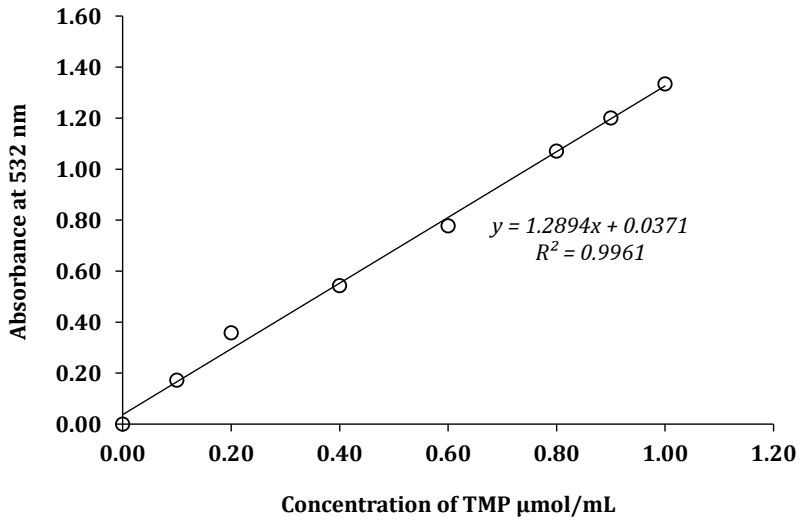


Figure 3-47: Standard curve of 1,1,3,3-tetrethoxypropane (TMP) 0-1.20 µmol/mL in 1-butanol for determining TBA reactive substances (TBARS) at a wavelength of 532 nm. Results are expressed as mean±SE of triplicated analysis

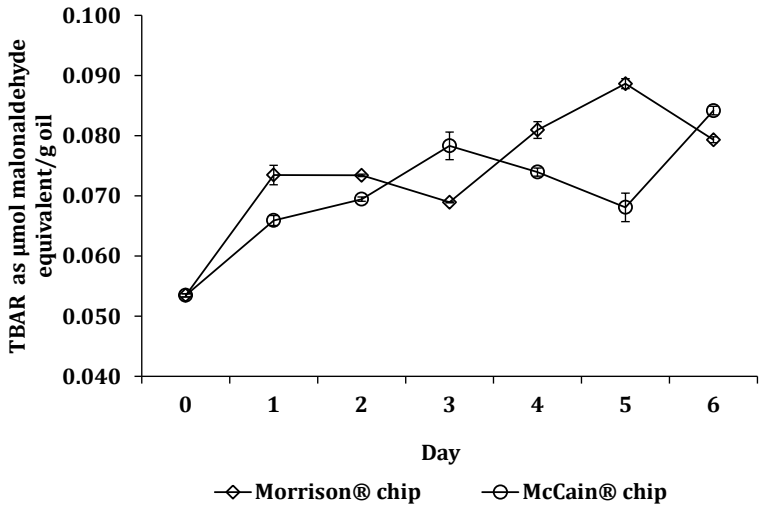


Figure 3-48: TBA reactive substance (TBAR) in rapeseed oil at different frying days. The value is expressed as mean of malonaldehyde equivalent±SE of triplicate analysis

The *ρ*-Anisidine value of the oil used for frying Morrison® chips is higher than the oil used for frying McCain® chips. Although, the TBA values have some fluctuations over frying time, they have similar behaviour as the *ρ*-Anisidine value in that the oil used for frying Morrison® chips has a higher TBA value than

the oil used for frying McCain® chips (due to Morrison® chips having a longer frying time). The fluctuation of TBA values were caused by frying temperature. The TBA values are representative to the secondary stage of lipid oxidation. The products which are unstable, can undergo further reactions such as oxidation and polymerisation (Choe and Min, 2007; Pegg, 2005a). The results of *ρ*-Anisidine value in heated oils of this study are similar to the studies reported by Ammari *et al.* (2012), Naz *et al.* (2004), Man and Hussin (1998) and Rade *et al.* (1997). Ammari *et al.* (2012) reported an increasing *ρ*-Anisidine value in sunflower oil which was heated at 140 °C as the heating time increased from 60 to 180 min. Naz *et al.* (2004) found the *ρ*-Anisidine value in olive oil, corn oil and soybean oil used for frying 158 French fries at 180 °C for 30, 60 and 90 min, increased as frying time increased. Man and Hussin (1998) reported the *ρ*-Anisidine value in palm olein oil frying potato chips at 180 °C for 5 days, increased as frying days increased. Rade *et al.* (1997) found an increasing of *ρ*-Anisidine value in palm olein oil used for frying French fries as frying hours increased. In addition, the increase of TBA value from this present study is also in agreement with the study by Man and Tan (1999). They reported the TBA value in palm olein used for frying potato chips at 180 °C for 7 consecutive days showed a continuous increase over the frying days. However, the study of frying French fries in canola oil at 185 °C and 215 °C by Aladedunye and Przybylski (2009) and the study of frying chips in coconut oil by Man and Hussin (1998) showed a different trend of *ρ*-Anisidine value from the present study. They found the *ρ*-Anisidine value increased in the first day of frying and declined in the following days. This may be attributed to the very low content of unsaturation of the oils they used (Arumughan *et al.*, 1984) or it might have already taken part in polymerisation as

aldehyde products are quite reactive (Berger, 1984). When, TBA values (Figure 3-46) are expressed as malonaldehyde equivalents (TBAR, Figure 3-48), the values also show some fluctuations over frying time which were affected by frying temperature, but both figures have a similar increasing trend. However, the TBA value is a semi-quantitative classical assay with some limitations and may give falsely high readings (Pegg, 2005b). The limitations are due to interferences by non-oxidation substances and are also affected by the method employed (extraction or distillation). For this reason, the limitations can be avoided by using a standard curve (Pegg, 2005b), so it is TBARS assay.

In summary, the results of these experiments show that the secondary degradation products are produced in the oils. This can be determined using the *ρ*-Anisidine assay and TBA assays (TBA and TBARS value). For further experiments in this study, the *ρ*-Anisidine assay will be used for determining Totox value.

3.5.1.7 Total oxidation value (Totox value) of repeated frying oil

Totox value is a combination of primary and secondary oxidation products. It is the summation of the peroxide value and *ρ*-Anisidine value, thus, this value will reflect the overall outcome of the oxidation reactions. In other words, if the peroxide value and/or *ρ*-Anisidine value is increased, the Totox value will be increased. The results for Totox value is shown in Figure 3-49. It shows an increasing trend versus length of frying period. The Totox value of the oil used to fry Morrison® chips is higher than the oil used for frying McCain® chips. The data shows the Totox value sharply increases in the 1st day of frying from 19 to 119 for the oil frying Morrison® chips, and from 19 to 68 for the oil frying McCain® chips. Then, the values of the 2 oils show a gradual increase over the frying time. This is the result of the sharp increase in the 1st day of frying of the

peroxide value as seen in Figure 3-49. The results of this study are in agreement with the study by Man and Hussin (1998). They reported the Totox value of palm olein oil used for frying potato chips increased over the frying time, while, the Totox value of coconut oil used for frying potato chips decreased after the second till the last day of frying.

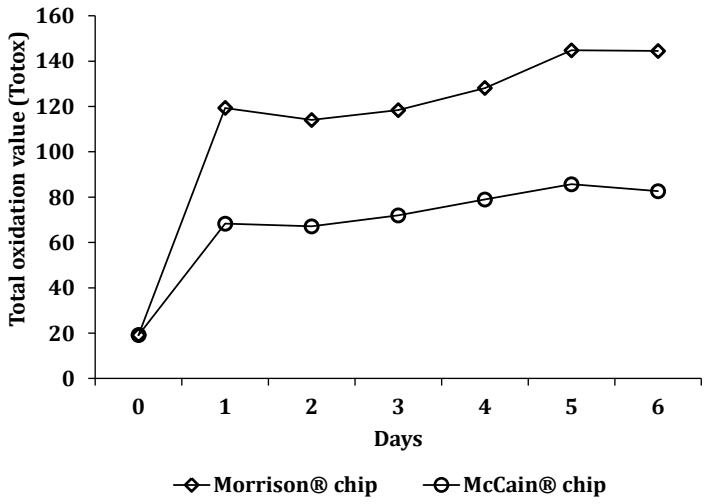


Figure 3-49: Totox value of the rapeseed oil at different frying days. The value is expressed as summation of 2(peroxide value) + *ρ*-Anisidine value

3.5.1.8 Effect of repeated frying on total polar compounds of oil

The amount of total polar compounds in the oil samples after frying McCain® and Morrison® chips are shown in Figure 3-50. The initial percentage polar compounds contained in the un-used oils are 1.16±0.11 % in the oil used for frying Morrison® chip and 1.21±0.25 % in the oil used for frying McCain® chip. The results also indicate that the rate of formation of polar compounds in the oil used for frying Morrison® chips (increased from 1.16 % to 65.21 %) was higher than the oil used for frying McCain® chips (increase from 1.21 % to 63.35 %). It shows that the two oils increased in percent polar compounds over the frying

days. This finding is similar to the researches by Aladedunye and Przybylski (2009), Tarmizi and Ismail (2008), Xu *et al.* (1999), Man and Hussin (1998), Rade *et al.* (1997) and Takeoka *et al.* (1997). Aladedunye and Przybylski (2009) found the total polar compounds increased almost linearly with the frying time at a rate affected by frying temperature. Tarmizi and Ismail (2008) found the polar compounds increased in plam olein oil and special palm olein as the frying time increased. Xu *et al.* (1999) reported total polar compounds in all 6 oil types increased significantly during frying and were strongly correlated with frying time ($p<0.001$, $r \geq 0.964$). Rade *et al.* (1997) found the polar compounds contained in the oils during frying French fries increased in relation to frying time. Takeoka *et al.* (1997) reported the amount of polar compounds contained in all 7 different cooking oils heated at 190 °C and 204 °C increased over the number of heating days, and the oils heated at 204 °C contained more polar compounds than the oils heated at lower temperature. Man and Hussin (1998) found increasing polar compounds in palm olein oil used for frying potato chips in respect to frying days, as well as in coconut oil. Polar compounds are a result of hydrolysis and oxidation reactions occurring during heating the oils. They are composed of breakdown products, non-volatile oxidised derivatives, polymeric and cyclic substances including non-triglyceride materials soluble in, emulsified in or suspended in oil (Rossell, 2001a; Xu *et al.*, 1999; Paul *et al.*, 1997; Stevenson *et al.*, 1984). Total polar compounds have been considered as a good and important indicator of frying oil quality. As cyclic monomers, decomposed products from oil degradation are highly harmful and so food authority agencies or food regulators in many countries have set the maximum amount of total polar

compounds present in the frying oil which varies from country to country, from 23-28 % (Rossell, 2001a; Paul *et al.*, 1997).

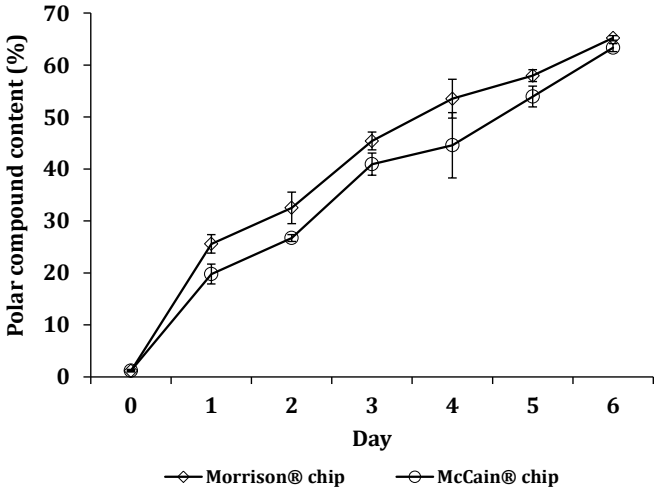


Figure 3-50: Percentage of total polar compounds in rapeseed oil at different frying days. The value is expressed as mean±SE of triplicate analysis

3.5.1.9 Correlations between total polar compounds and other oil quality indicators of rapeseed oil

The association between two indices were described by using Pearson's coefficient correlation. The Pearson's coefficient correlations (r) for all quality parameters of the oil used for frying Morrison® and McCain® chips are shown in Table 3-8 and Table 3-9. The total polar compounds in the oil used for frying both Morrison® and McCain® chips exhibit significantly strong correlation ($p<0.05$, $p<0.01$) with acid value, ρ-Anisidine value, TBA value, colour, smoke point and viscosity. They are all positively correlated apart from the smoke point which has negative correlation with all other indices. Therefore, the smoke point has decreased while the other indices have increased with increasing total polar compounds. In addition, there are significant strong correlations ($p<0.05$,

p<0.01) among indices of acid value, *ρ*-Anisidine value, TBA value, colour, smoke point and viscosity, but weak correlation with peroxide value. Therefore, these indicators could be chosen for monitoring quality changes in repeatedly heated oil in this experiment. While, the peroxide value could not be used alone itself due to it has weak correlation to other indices. The findings from this experiment are in agreement with the previous study by Takeoka *et al.* (1997) who found total polar compounds in cooking oils had high correlation with colour index. Xu *et al.* (1999) also found high positive correlation between total polar compounds with colour change and the acid value.

Table 3-8: Pearson's coefficient correlation (*r*) of the oil quality indicators used for frying Morrison® chips

Correlation (*r*)	TPC	AV	PV	ANI	TBA	COL	SMOKE
AV	0.979**						
PV	0.401	0.438					
ANI	0.978**	0.964**	0.249				
TBA	0.931**	0.960**	0.398	0.914**			
COL	0.978**	0.952**	0.265	0.986**	0.916**		
SMOKE	-0.953**	-0.968**	-0.489	-0.912**	-0.969**	-0.939**	
VISC	0.969**	0.949**	0.310	0.980**	0.919**	0.955**	-0.890**

**,* correlation is significant at the 0.01 level, 0.05 level (2-tailed) respectively. TPC = total polar compounds, AV=acid value, PV=peroxide value, ANI=*ρ*-Anisidine value, TBA=TBA value, COL=colour, SMOKE=smoke point, VISC=viscosity

In conclusion, the results revealed that the oils used for frying chips at 190 ˚C show deterioration which increases over the frying days. The oils have changed in physical and chemical properties. For physical changes, it shows an increase in colour (darker) and viscosity, while the smoke point decreases. For chemical

changes, the peroxide values show fluctuation, acid value increases over frying time as does the *ρ*-Anisidine value, TBA value and the total polar compounds.

Table 3-9: Pearson's coefficient correlation (*r*) of the oil quality indicators used for frying McCain® chips

Correlation (*r*)	TPC	AV	PV	ANI	TBA	COL	SMOKE
AV	0.959**						
PV	0.405	0.309					
ANI	0.985**	0.964**	0.351				
TBA	0.843*	0.813*	0.438	0.904**			
COL	0.996**	0.968**	0.439	0.983**	0.859*		
SMOKE	-0.954**	-0.959**	-0.547	-0.956**	-0.888**	-0.973**	
VISC	0.995**	0.973**	0.391	0.993**	0.859*	0.992**	-0.964**

**,* correlation is significant at the 0.01 level, 0.05 level (2-tailed) respectively. TPC = total polar compounds, AV=acid value, PV=peroxide value, ANI=*ρ*-Anisidine value, TBA=TBA value, COL=colour, SMOKE=smoke point, VISC=viscosity

In addition, the results from this study have revealed that deterioration of the frying oils is not only influenced by the number of frying days but also is attributed to the length of frying time (3.5 minutes for Morrison® chips and 3 minutes for McCain® chips). Although, it was the same food (chip) a different size related to a different frying time. It could also be that the food has moisture which relates to an increase in acid value. This shows that there is an influence of the food being fried on the deterioration rate of heated oil. Therefore, food was not taken into account in further experiments so that the deterioration of the oil alone can be monitored. The frying or heating temperature will also be adjusted to 180 °C according to the good frying practice which is recommended by DGF (2008). Based on the correlation results, it shows that the changes in colour, viscosity, acid value, *ρ*-Anisidine value, TBA value, Totox and total polar

compounds, can be used as indicators for deterioration of frying oil. It also shows that the testing methods used in the study are suitable too. However, there were concerns with the method of smoke point and peroxide value. Even though this study had a clear result for smoke point the method is based on visual inspection and so the results cannot be guaranteed to be accurate and/or precise. Thus, this method was not used in further experiments. For the peroxide value, as it is an intermediate compound in primary lipid oxidation, which can decompose further and also reform, it is not a good indicator for frying oil.

3.5.2 Oxidative stability of stripped and unstripped palm olein oil in the presence of *Piper sarmentosum* Roxb. leaf extracts

In order to gain a clear observation of the effect of using natural antioxidants in cooking oils, a synthetic antioxidant free oil is required. Oils stripped of natural antioxidants have been used by a number of studies (Atares *et al.*, 2012; Zhong and Shahidi, 2012; Khan and Shahidi, 2000) where the existing natural antioxidant originally present in the oil, such as tocopherol, were removed. Different adsorbents have been used such as silicic acid, celite 545, activated charcoal or aluminium oxide (Atares *et al.*, 2012; Khan and Shahidi, 2000). The study by Khan and Shahidi (2000) reported that the existing natural antioxidants cannot be guaranteed to be completely removed and the proportion removed for each compound may be different depending on the efficiency of the adsorbent material used. Nevertheless, the method used for removing natural antioxidants will be used to see if it can remove the synthetic ones in this present study. The aim of this study is to find oil free of synthetic antioxidants and use it for further experiments. The effect of PSE extract on oxidative stability of stripped and

unstripped palm olein oil using *ρ*-Anisidine as a representative assay are presented in Table 3-10. In general, the *ρ*-Anisidine value in the unstripped oil and stripped oil with and without adding PSE show a gradual increase, although, the values do fluctuate during the incubation time.

Table 3-10: Effect of *Piper sarmentosum* Roxb. leaf extracts (PSE) on *ρ*-Anisidine value of palm olein oil (Oleen®)which was passed and unpassed aluminium oxide

Oil	PSE (%)	*ρ*-Anisidine value				
		24 h	48 h	72 h	96 h	120 h
Un-stripped	0	$6.02\pm0.06^{a,b,A}$	$6.10\pm0.10^{a,A,B}$	$6.39\pm0.05^{a,B}$	$6.83\pm0.05^{a,C}$	$6.27\pm0.11^{a,b,B}$
	0.02	$5.86\pm0.27^{a,b,A}$	$6.60\pm0.05^{b,B}$	$6.53\pm0.09^{b,d,B,C}$	$6.83\pm0.05^{a,C}$	$6.89\pm0.14^{c,B,C}$
	0.05	$6.36\pm0.09^{a,A}$	$6.92\pm0.00^{c,B}$	$6.92\pm0.06^{d,B,C}$	$7.22\pm0.10^{b,C,D}$	$7.55\pm0.06^{e,D}$
	0.1	$6.33\pm0.37^{b,A}$	$7.05\pm0.05^{c,A,B}$	$6.80\pm0.09^{b,d,A,C}$	$7.14\pm0.09^{b,B,D}$	$6.86\pm0.09^{c,A,D}$
Stripped	0	$5.82\pm0.06^{a,A}$	$5.88\pm0.10^{d,A,B}$	$6.42\pm0.05^{a,C}$	$6.69\pm0.00^{a,D}$	$6.08\pm0.05^{a,B}$
	0.02	$6.29\pm0.00^{a,b,A}$	$6.56\pm0.00^{b,B}$	$6.72\pm0.06^{b,c,C}$	$7.17\pm0.05^{b,D}$	$7.18\pm0.06^{d,D}$
	0.05	$5.95\pm0.06^{b,A}$	$6.10\pm0.05^{a,B}$	$6.56\pm0.05^{a,c,C}$	$6.66\pm0.06^{a,C}$	$6.40\pm0.10^{b,C}$
	0.1	$6.30\pm0.05^{b,A}$	$6.23\pm0.05^{a,A}$	$6.73\pm0.06^{b,c,B}$	$6.80\pm0.05^{a,B}$	$7.19\pm0.05^{d,C}$

Un-stripped = oil was unpassed through the activated aluminium oxide, stripped = oil was passed through the activated aluminium oxide, h = hour. The value was expressed as mean ± SE of triplicate analysis. Different capital letters in the same row are significant difference at $p<0.05$. Different normal letters within each column are significantly different at $p<0.05$.

The *ρ*-Anisidine value of unstripped and stripped oils for each concentration, show a significant increase over incubation time after 72 hours onward compared to 24 h ($p<0.05$). Comparing, the effect of PSE extract, the unstripped oil and stripped oil without adding PSE (0 % PSE) shows no significant difference of *ρ*-Anisidine value at 24, 72, 96 and 120 hours. At 0.02 % PSE, the *ρ*-Anisidine value of unstripped and stripped oil shows no significant difference at 24, 48 and 72 hours, while, they show a significantly different *ρ*-Anisidine value at 96 and 120 hours ($p<0.05$). At 0.05 % PSE, the *ρ*-Anisidine values of unstripped and

stripped oil are gradually increasing. The stripped oil has a slightly lower *ρ*-Anisidine value than the unstripped oil with significant difference through the incubation periods ($p<0.05$). At 0.1 % PSE, the results are varied. On the whole the unstripped oil fluctuates over the storage time whilst the stripped oil shows a general increase over time and has a general slightly lower *ρ*-Anisidine value than the unstripped oil with a significant difference at 48, 72 and 96 hours of incubating time ($p<0.05$). The results of this experiment indicate that the PSE does not work well in stabilising the oil neither in the unstripped oil or stripped oil, compared to the control oil (0 % PSE). It implies the oils used in the experiment may contain other compounds which can interfere with the activity of the PSE extract or may have stronger protective effect over the PSE extract. These results differ from the study by Zhang *et al.* (2010). They found the *ρ*-Anisidine value in sunflower oil with added rosemary extract were lower than the unstripped oil under storage at 60 °C for 21 days. Hras *et al.* (2000) reported the *ρ*-Anisidine values in sunflower oil with added rosemary extract, ascorbyl palmitate and citric acid during storage at 60 °C for 12 days, were also lower than the unstripped oil. Mariod *et al.* (2010) reported the *ρ*-Anisidine values in rice bran oil with added defatted rice bran extract during storage at 70 °C for 168 hours, were lower than the control oil and were lower when the amount of the extract was increased. It has been noticed that they used synthetic antioxidant free oils for the studies which were supplied directly from oil manufacturers. In this study, the oils were purchased from a local supermarket and synthetic antioxidants were not declared on the label. Therefore, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ), may be present in oil and the stripping process may be unable to remove them.

Therefore, it is very important to have the synthetic antioxidant free oil for this research study to fully understand the effects of adding PSE extracts.

3.5.3 Determination of synthetic antioxidants in cooking oils

The common synthetic phenol compounds also known as synthetic antioxidants used in cooking oils are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ). They were added to prevent or delay the lipid oxidation (autoxidation) during processing and storage time (Saad *et al.*, 2007). Synthetic antioxidants are mostly unlisted in product labels (Dengate, 2015). The results in chapter 3.5.2, showed the *ρ*-Anisidine values of the unstripped oil and stripped oil with added PSE extracts are higher than the unstripped oil, as well as, there being no significant difference between the unstripped oil and stripped oil (0 % PSE) themselves. It means the unstripped oil (0 % PSE) had better oxidative stability than the oil treated with PSE extracts. The results led to the suspicion that the oils might contain active phenol compounds especially synthetic antioxidants which may have an impact on PSE activity. Therefore, the aim of this study was to prove the hypothesis that the oil used in chapter 3.5.2 contained synthetic antioxidants, to check that aluminium oxide cannot remove synthetic antioxidants present in commercial cooking oil and to find synthetic antioxidant free cooking oils for use in this research study. It is necessary to use a reliable detection method. A HPLC method was optimised and used to identify synthetic antioxidants by comparing with the relevant standards: butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ).

3.5.3.1 Optimisation of the HPLC method

To find the best conditions for identifying synthetic antioxidants, several trials were carried out. The conditions used for each trial and chromatograms are shown in Appendix B. Reverse phase column (C_{18}), photodiode array (PDA) at 280 nm and standard BHA were used throughout the trials (chapter 2.12.2). The final optimised HPLC method was achieved at the 8th trial conditions. By using this final method, chromatograms of standard TBHQ and BHT were also obtained. The chromatogram of the mixed standards of BHA, BHT and TBHQ 250 mg/L shows a good resolution with stable base line (Figure 3-51). Therefore, synthetic antioxidants (BHA, BHT and TBHQ) in cooking oil could be analysed.

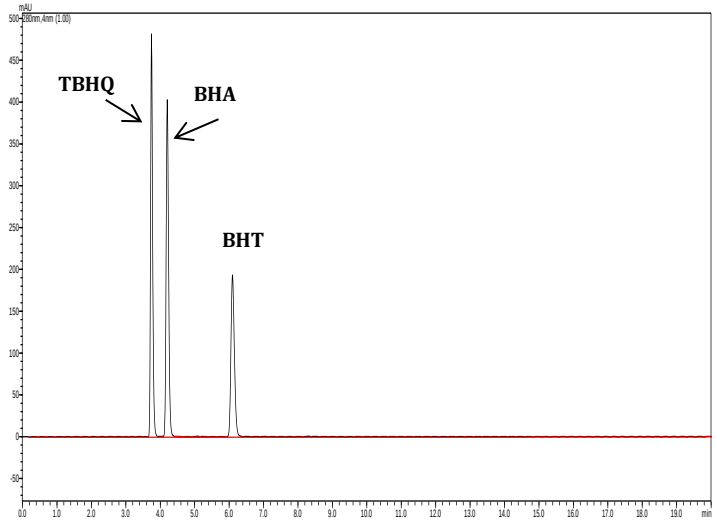


Figure 3-51: HPLC chromatogram of mixed standard TBHQ, BHA and BHT 250 mg/L, elution time at 3.60, 4.0 and 5.75 min, respectively. Mobile phase A was 1 % acetic acid in water, mobile phase B = acetonitrile. The flow rate was 0.8 mL/min of isocratic binary gradients (10 % A:90 % B). The cycle time was 20 min, injection volume was 20 µL and column oven was 45 °C.

3.5.3.2 Identification of synthetic antioxidants in cooking oils

Five brands of cooking oils: corn oil (Sainsbury's®), rapeseed oil (Yor®, Yorkshire® and Sainsbury's®) and rice bran oil (King®) were analysed to identify synthetic

antioxidants by comparing retention time with mixed standard BHA, BHT and TBHQ 250 mg/L. The retention times of the 3 standard synthetic antioxidants at 280 nm are 3.65 ± 0.08 min for TBHQ, 4.03 ± 0.08 min for BHA and 5.52 ± 0.05 min for BHT, as seen in Figure 3-52. The results are shown in Figure 3-53 to Figure 3-57.

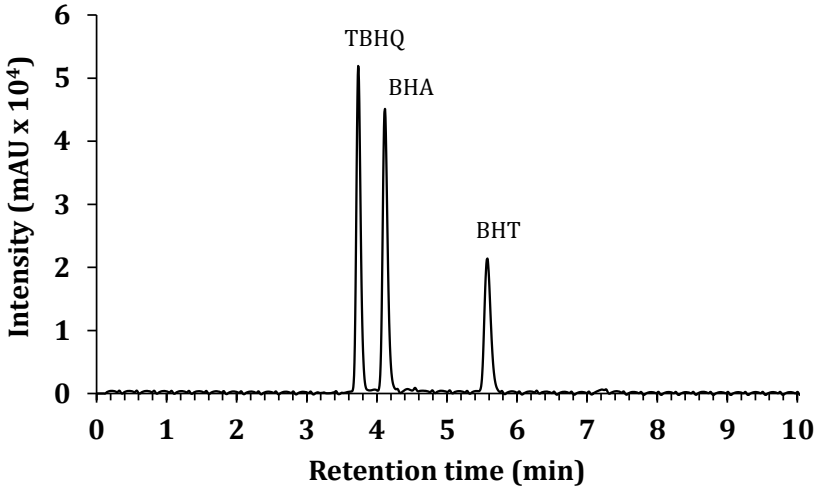


Figure 3-52: HPLC chromatogram of mixed standard synthetic antioxidants 250 mg/L: TBHQ (tertiary butyl hydroquinone, retention time 3.65±0.08 min), BHA (butylated hydroxyanisole, retention time 4.03±0.08 min) and BHT (butylated hydroxytoluene, retention time 5.52±0.05 min) at 280 nm

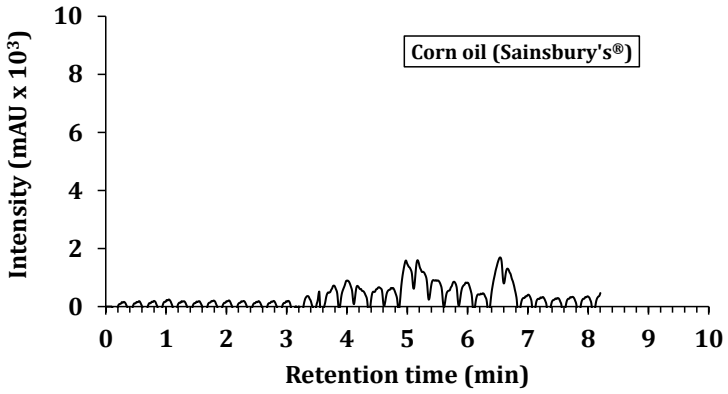


Figure 3-53: HPLC Chromatogram for identification of synthetic antioxidants in corn oil Sainsbury's® at 280 nm.

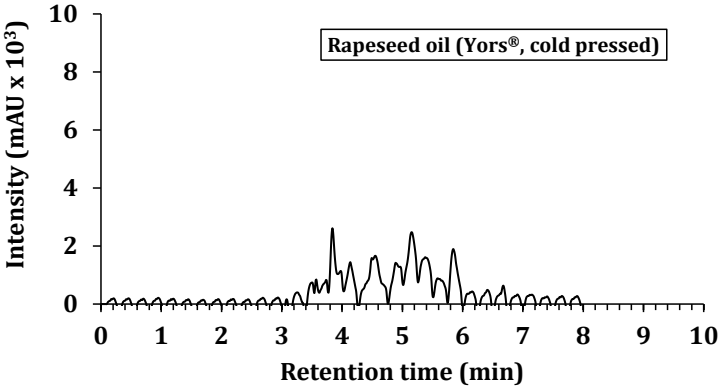


Figure 3-54: HPLC Chromatogram for identification of synthetic antioxidants in rapeseed oil Yor® at 280 nm

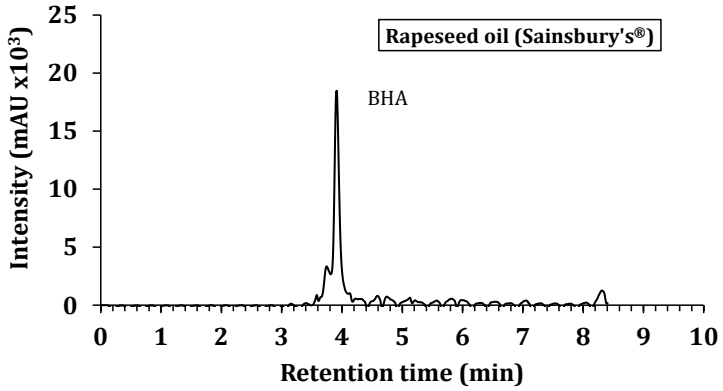


Figure 3-55: HPLC Chromatogram for identification of synthetic antioxidants in rapeseed oil, Sainsbury's® at 280 nm

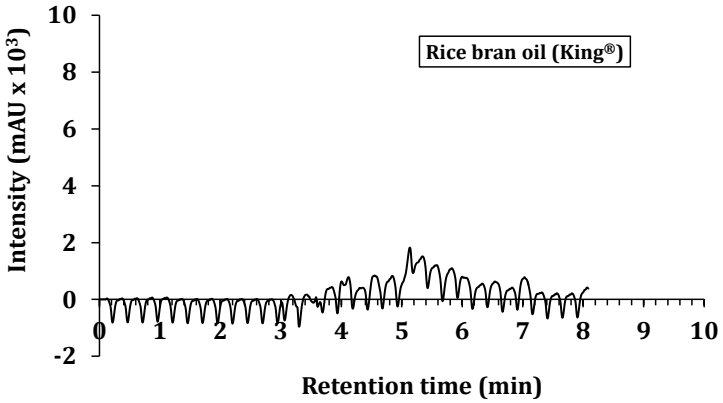


Figure 3-56: HPLC Chromatogram for identification of synthetic antioxidants in rice bran oil, King® at 280 nm

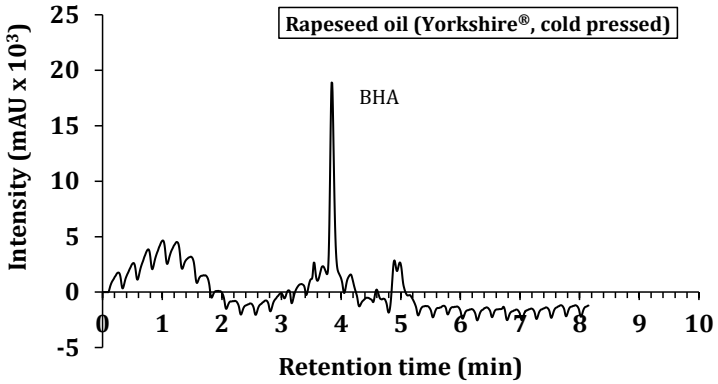


Figure 3-57: HPLC Chromatogram for identification of synthetic antioxidants in rapeseed oil, Yorkshire® at 280 nm

According to the results, there are only 3 oils free from synthetic antioxidants; corn oil (Sainsbury's®), rapeseed oil (Yor®) and rice bran oil (King®). The rest of them contained only BHA (rapeseed oil Sainsbury's® and Yorkshire®). Among the 3 oils which are free from synthetic antioxidants, the corn oil (Sainsbury's®), and rice bran oil (King®) will be selected for further experiments throughout the study because they are easier to source.

3.5.3.3 The effect of aluminium oxide on synthetic antioxidants in cooking oils

The effect of using aluminium oxide on synthetic antioxidants can be seen by studying Figure 3-58 to Figure 3-59. In both the normal Oleen® oil (not passed through aluminium oxide) and the stripped oil (passed through aluminium oxide) peaks which match with the retention times of the standard TBHQ, BHA and BHT are found as shown in Figure 3-58 and Figure 3-59.

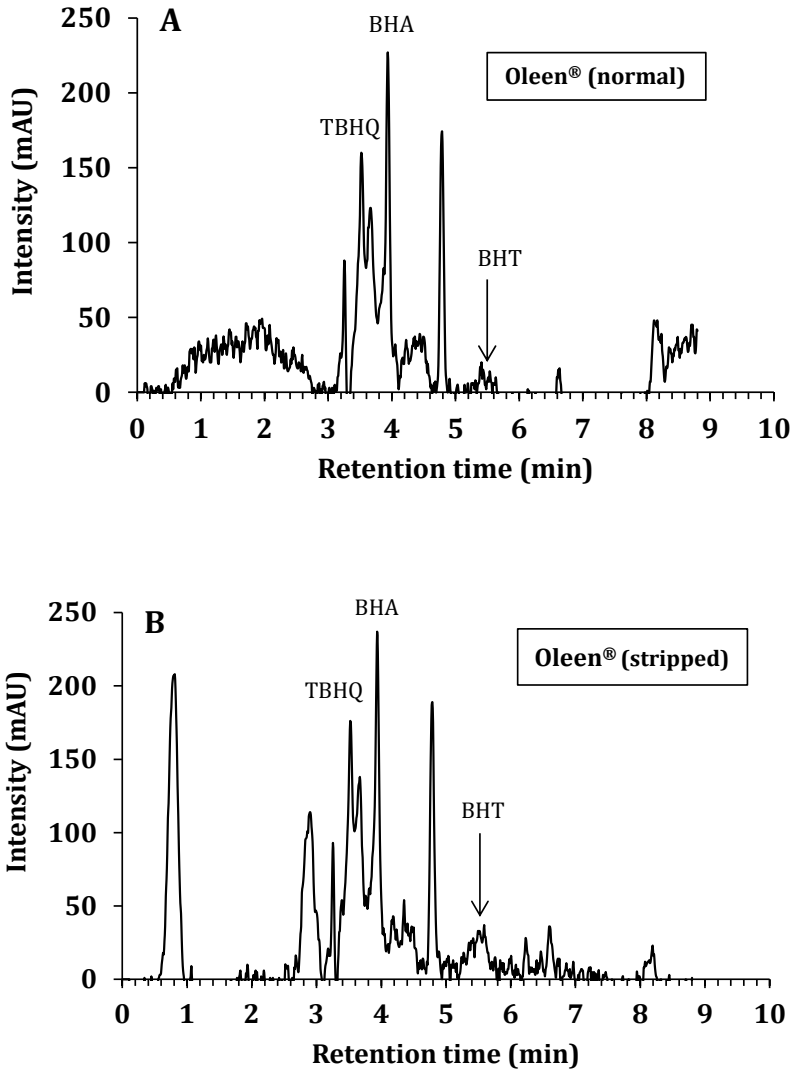


Figure 3-58: HPLC Chromatogram of normal palm olein oil (Oleen®)(A) which has not passed through aluminium oxide and stripped Oleen® oil (B) which has passed through aluminium oxide, at 280 nm

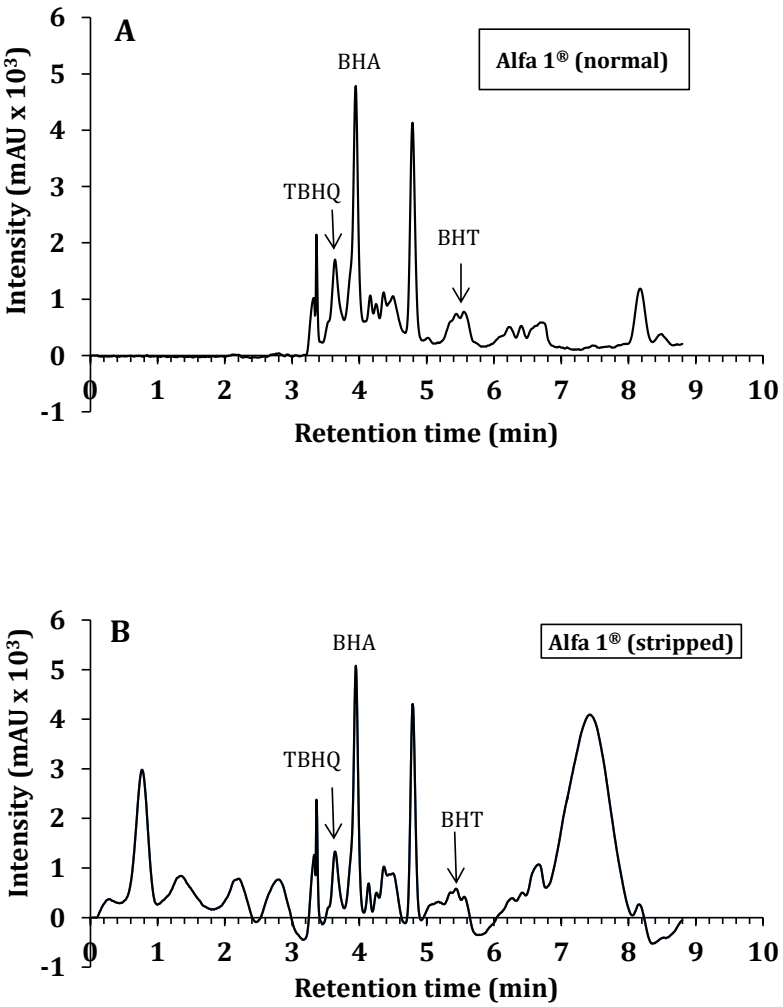


Figure 3-59: HPLC Chromatogram of normal rice bran oil (Alfa 1®)(A) which has not passed through aluminium oxide and stripped Alfa 1® oil (B) which has passed through aluminium oxide, at 280 nm

The normal Alfa 1® and the stripped oil also have peaks which match with the retention times of the standard TBHQ, BHA and BHT as shown in Figure 3-59. The investigation has shown that the Oleen® oil and Alfa 1® oil, both passed or not-passed through aluminium oxide contain all 3 synthetic antioxidants BHA, BHT and TBHQ. It indicates that the stripping process using aluminium oxide does not remove synthetic antioxidants. In addition, the evidence can elucidate

the doubt on the Oleen® oil used in the previous experiment (chapter 3.5.2) as it has been proven to contain BHA, BHT and TBHQ. Several studies have prepared purified edible oils for oxidative studies using aluminium oxide to remove natural antioxidants such as tocopherols in oils. The expectation of removing natural antioxidants is to determine the effects without them. However, the stripping process may not always be required, therefore, the natural antioxidants would be kept in the oil and the PS extract added for additional protection. The previous experiment (chapter 3.5.2) showed neither the unstripped oil nor the stripped oil with added PSE extracts has oxidative stability lower than the control oils (0 % PSE). In reality it may not be necessary to remove all natural antioxidants in cooking oils. Therefore, the synthetic antioxidant free oil used in further studies would not be stripped to determine the possibility of *Piper sarmentosum* Roxb. leaf extract replacing synthetic antioxidants in frying oil. According to the studies of Yasho Industries (2015), Omura (1995) and Sherwin (1972), they reported the antioxidants TBHQ, BHA and BHT demonstrated a synergistic effect when they were used in combination or mixtures. Thus, the use of all 3 synthetic antioxidants together in Oleen® oil is more effective in protect the autoxidation of the oil. This may lead to the results in the previous experiment (chapter 3.5.2) that showed neither the unstripped oil nor the stripped oil with added PSE extracts has an oxidative stability lower than the control oil (0 % PSE). The findings of this chapter support the notation of Dengate (2015) that synthetic antioxidants are the most hidden of all additive. The author had examined the labels of the oils used in this current study, and found no synthetic antioxidant be listed on the labels at all. This is due to exemptions of ingredients used in small quantities that need not be declared

according to the food labelling regulations 1996, UK (Food Standard Agency, 2015). According to the results, the reverse phase HPLC UV/Vis analytical method optimised for used in this study, showed a good separation of peaks for the 3 standards BHA, BHT and TBHQ. Therefore, the optimised conditions as well as the extraction procedure for sample preparation, illustrate that this method is suitable for the determination of synthetic antioxidants in edible oils. The attempt to remove synthetic antioxidants in the oils by passing them through aluminium oxide applying the stripping oil process failed. However, three brands of commercial cooking oils were found to be free from synthetic antioxidants. With both corn oil (Sainsbury's®) and rice bran oil (King®) being easy to source. These oils will be used for further experiments in this study.

3.6 Antioxidant activity of *Piper sarmentosum* Roxb. leaf extracts on quality changes in rice bran oil and corn oil under mild temperature

A trend in searching for natural antioxidants of polyphenol extracts from various parts of plants has been researched for decades. Numerous studies were reported on the antioxidant activities of the herb or spice extracts, such as rosemary, on oxidative stability of vegetable oils under storage conditions (Frankel *et al.*, 1996; Lee and Sher, 1984; Chang *et al.*, 1977). Regarding the results in chapter 3.2 and chapter 3.4, *Piper sarmentosum* Roxb. leaf extract can be a new source of natural antioxidant. No studies have reported the use of PS extract to inhibit lipid oxidation in edible oils. Therefore, the antioxidant activity of PS leaf extract on protecting lipid oxidation of vegetable oils (rice bran oil and corn oil) under storage condition would be the first examined by this study. The oxidative

stability of the edible oils can be evaluated by storing them at room temperatures 20-25 °C (time consuming) or accelerated storage at 60-63 °C in an oven (Schaal oven test) which is more rapid (Shahidi and Wanasundara, 1997). It has been observed that one day of storage under the Schaal oven condition is equivalent to one month's storage at room temperatures 20-25 °C (Pegg, 2005a; AbouGharbia *et al.*, 1996). The aim of this study was to know the protective effect of *Piper sarmentosum* Roxb. leaf extract on autoxidation of the oils.

3.6.1 Effect of *Piper sarmentosum* Roxb. leaf extracts on peroxide value in rice bran and corn oils under accelerated storage conditions Antioxidant activity of *Piper sarmentosum* Roxb. leaf

The influence of PSE and PSL extracts during accelerated storage on peroxide value in the rice bran and corn oils are presented in Figure 3-60 to Figure 3-63. As shown in Figure 3-60, the peroxide values of rice bran oil show an increasing trend with all samples over the storage time. The peroxide value of the oil with added BHT is the lowest. The oils with the PSE extracts at all levels illustrate a lower peroxide value than the synthetic antioxidant free oil from 72 hours onwards. The peroxide values of corn oil with and without PSE extracts and BHT show an increasing trend over the storage time, as shown in Figure 3-61. The synthetic antioxidant free corn oil has the highest peroxide value compared to all samples, while, the corn oil with added BHT is the lowest with exception of the oil with PSE 0.02 % at 72 hours. In general, the PSE extract at 0.02 % in rice bran oil and corn oil showed a lower peroxide value than at other concentrations.

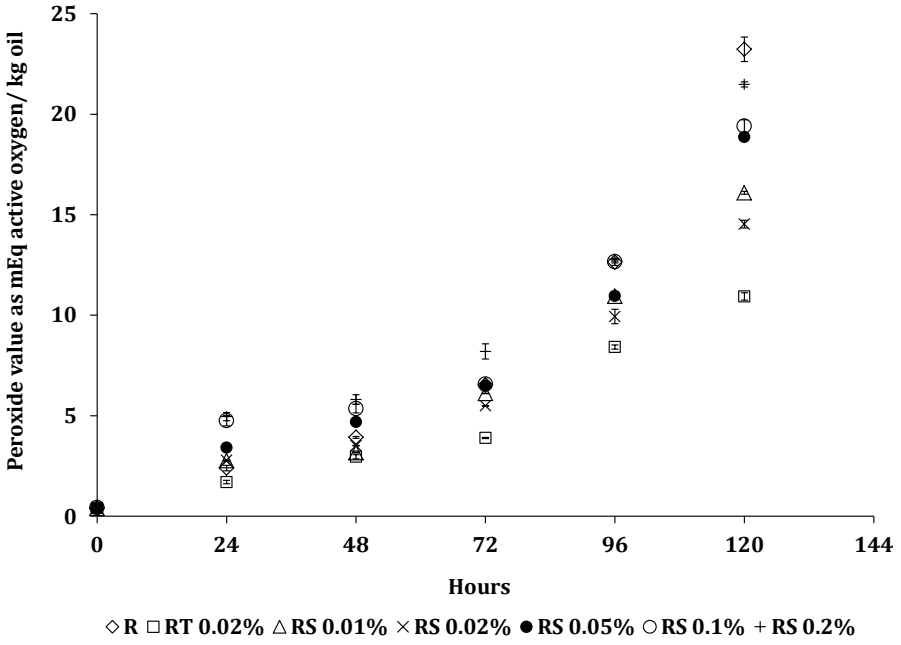


Figure 3-60: Effect of PSE extracts on peroxide value of rice bran oil during storage at 60±3 °C for 120 hours. The value is expressed as milli equivalence (meq) oxygen/kg oil, mean±SE of triplicate analysis. R = rice bran oil, S = PSE extract, T = BHT, % = percentage added

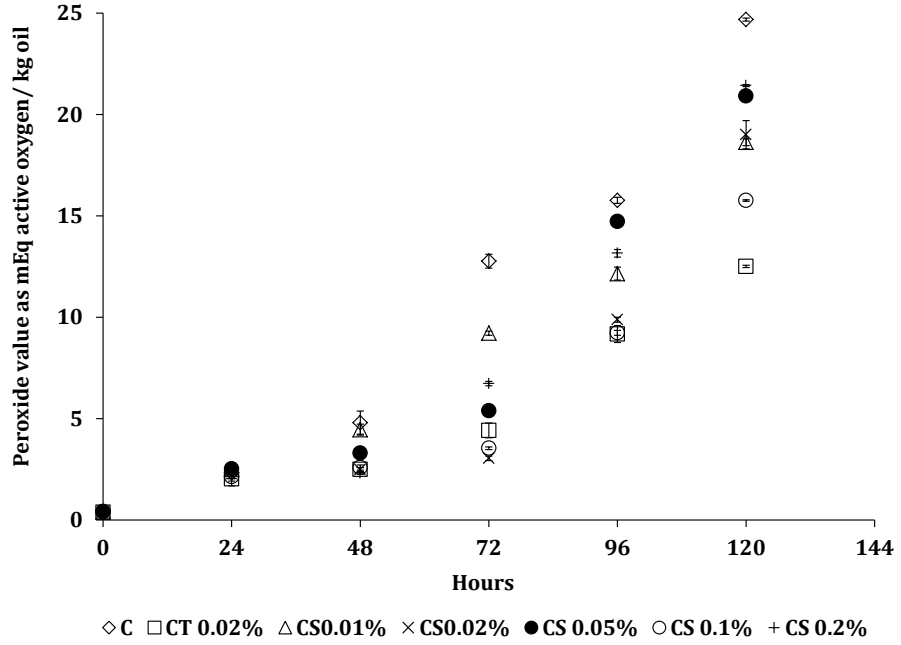


Figure 3-61: Effect of PSE extracts on peroxide value of corn oil during storage at 60±3 °C for 120 hours. The value is expressed as milli equivalence (meq) oxygen/kg oil, mean±SE of triplicate analysis. C = corn oil, S = PSE extract, T = BHT, % = percentage added

The peroxide value of the rice bran oil with and without PSL extracts also show an increasing trend with storage time, as seen in Figure 3-62. The lowest peroxide values are found in the rice bran oil with added BHT. The highest peroxide values are obtained in the oil with added petroleum ether extracts (PSL) at 0.2 %. After a storage time of 24 hours, the peroxide values in all the oils with added all amounts of the PSL extract are found to be higher than the control oil values.

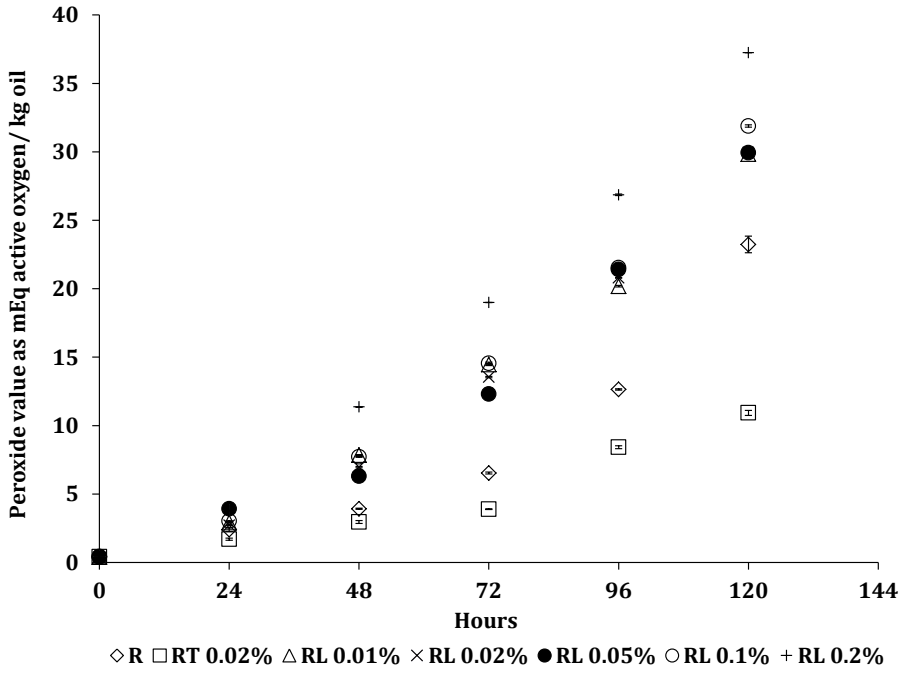


Figure 3-62: Effect of PSL extracts on peroxide value of rice bran oil during storage at 60±3 °C for 120 hours. The value is expressed as milli equivalence (meq) oxygen/kg oil, mean±SE of triplicate analysis. R = rice bran oil, L = PSL extract, T = BHT, % = percentage added

The peroxide value of the corn oil treated and untreated with PSL extracts are increasing over the storage time (Figure 3-63). The peroxide values in the positive and negative control oils are found to be lower than the oils with added the PSL extract from 24 hours thorough the time, with the lowest peroxide values

obtained by the oil with added BHT. Amongst the oils treated with PSL extract, the oils with 0.01 % PSL extracts seem to give a lower peroxide value than others.

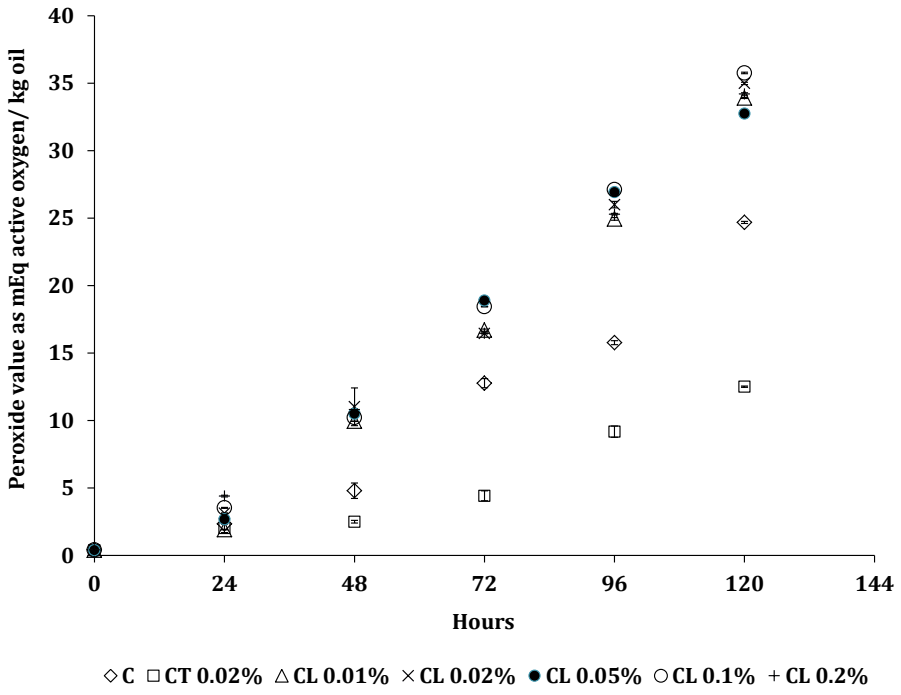


Figure 3-63: Effect of PSL extracts on peroxide value of corn oil during storage at 60±3 °C for 120 hours. The value is expressed as milli equivalence (meq) oxygen/kg oil, mean±SE of triplicate analysis. C = corn oil, L = PSL extract, T = BHT, % = percentage added

The results of the rice bran oils and corn oils treated with PSE extracts, are in agreement with the findings of Mariod *et al.* (2010). They reported the peroxide value of rice bran oil treated with the phenolic extracts from defatted rice bran increased as a function of time and were lower than the synthetic antioxidant free oil, but still higher than oil with added BHA. Similarly with the finding of Pimpa *et al.* (2009) on palm olein oil, the results showed that the peroxide values of palm olein oil with added α-tocopherol were lower than the synthetic antioxidant free oil but higher than the oil with added BHT. Also similarly, the

study by Aladedunye and Matthaeus (2014) reported the rapeseed oil with added polyphenolic fractions from rowanberry and crabapple fruit extract showed a lower increasing peroxide value than the synthetic antioxidant free oil but higher than the oil with added BHT throughout accelerated storage hours. They assumed the lower antioxidant activity of the extracts compared to BHT may be due to the influence of some compounds in the extracts acting as pro-oxidants.

The rice bran oils and corn oils with added PSE extracts demonstrate a lower peroxide value than the synthetic antioxidant free oils which is different to the results of the oils with added PSL extracts. The oils with added PSL extracts show a higher peroxide value than the synthetic antioxidant free oil and the oil with added BHT. This means the PSL extract does not exhibit a positive trend to protect oxidation in both oils which is opposite to the PSE extract. This may be due to the PSE extract having a variety and higher amount of polyphenols (flavonoids and phenolic acids) than the PSL extract, as seen in chapters 3.4.2 and 3.4.3, which resulted from using different extraction solvents. Also, the effectiveness of PSE extract may be due to its polarity as the effectiveness of phenolic antioxidants in bulk oil is dependent on their hydrophilic or lipophilic capacity (Yanishlieva, 2001). The PSE extract was extracted using 80 % ethanol, so it is more polar than the PSL extract which was extracted using petroleum ether (non polar). The PSL extract was found to have a high content of caffeine (Table 3-7) which may provide pro-oxidant activity when present in high amounts (Azam *et al.*, 2003; Shi *et al.*, 1991).

3.6.2 Effect of *Piper sarmentosum* Roxb. leaf extracts on *ρ*-Anisidine value and TBA value in rice bran and corn oils under accelerated storage conditions

3.6.2.1 The effect on *ρ*-Anisidine value

The *ρ*-Anisidine value of the rice bran oils treated with the PSE extracts and the controls are presented in Figure 3-64. The values of all the samples are increasing over the time, with the oil with added BHT illustrating the lowest *ρ*-Anisidine value. The rice bran oil treated with 0.01 % and 0.02 % PSE extracts demonstrate higher *ρ*-Anisidine values than rice bran oil treated with 0.05 %, 0.1 % and 0.2 %. Also, corn oil with added PSE extract 0.1 % and 0.2 % showed higher *ρ*-Anisidine values than corn oil with added PSE extract 0.01 %, 0.02 % and 0.05 %. It showed a lower *ρ*-Anisidine value in rice bran oil with added PSE extract 0.2 % and in corn oil with added PSE extract 0.01 %.

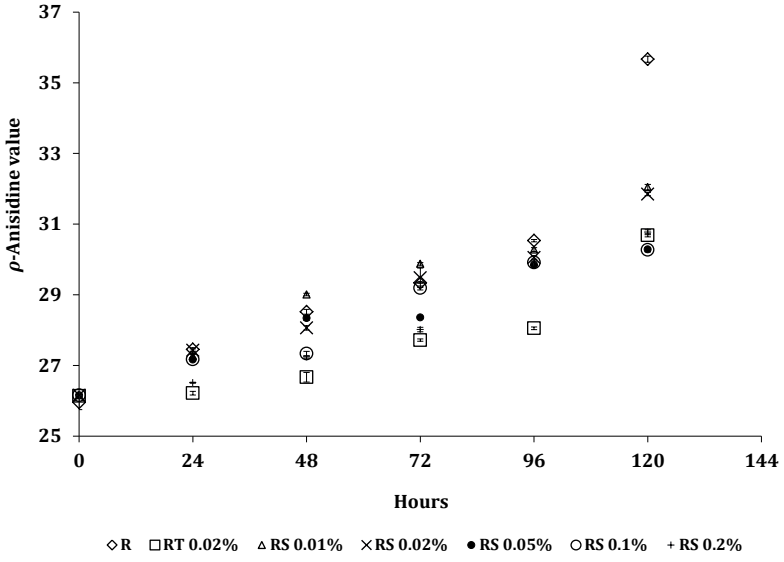


Figure 3-64: Effect of PSE extracts on *ρ*-Anisidine value of rice bran oil during storage at 60±3 °C for 120 hours. The value is expressed as mean±SE of triplicate analysis. R = rice bran oil, S = PSE extract, T = BHT, % = percentage added

The *ρ*-Anisidine value of the corn oils treated with PSE extracts including the controls show no clear difference between them from 0 to 48 hours of storage (Figure 3-65). Most of them show an increasing *ρ*-Anisidine value except the oil treated with 0.1 % PSE extract which shows fluctuation. The oil with added BHT has a fairly stable *ρ*-Anisidine value throughout the time. The negative control oil without PSE extracts shows the highest *ρ*-Anisidine value after 72 hours storage.

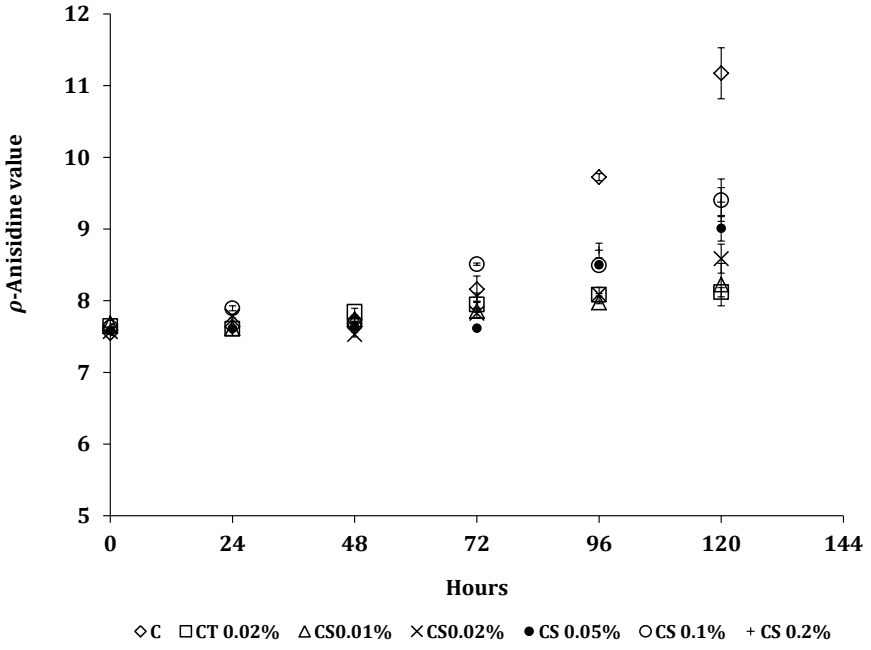


Figure 3-65: Effect of PSE extracts on *ρ*-Anisidine value of corn oil during storage at 60±3 °C for 120 hours. The value is expressed as mean±SE of triplicate analysis. C = corn oil, S = PSE extract, T = BHT, % = percentage added

The *ρ*-Anisidine values of the rice bran oil with added PSL extracts rise in the first 24 hours, then the values show fluctuation over the storage time, as seen in Figure 3-66. However, in general, all the rice bran oils treated with the PSL extracts and both the control oils show the *ρ*-Anisidine value increasing over the storage time. The oil with BHT has the lowest value, while the *ρ*-Anisidine value

in the oils with added the PSL extracts are higher than the synthetic antioxidant free oil through the 120 hours.

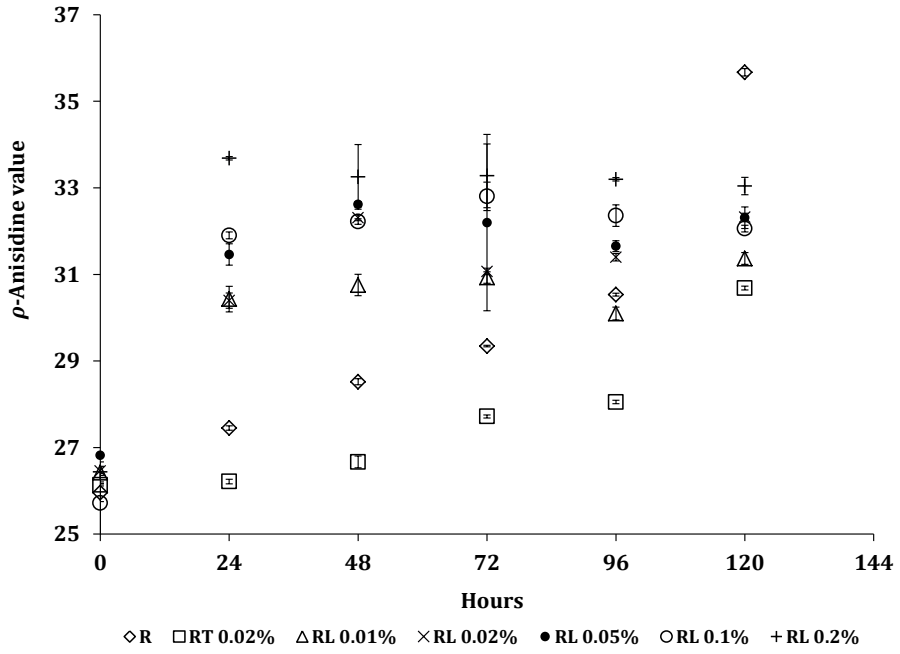


Figure 3-66: Effect of PSL extracts on *ρ*-Anisidine value of rice bran oil during storage at 60±3 °C for 120 hours. The value is expressed as mean±SE of triplicate analysis. R = rice bran oil, L = PSL extract, T = BHT, % = percentage added

The effect of PSL extracts on *ρ*-Anisidine value of corn oil are shown in Figure 3-67. The corn oils with added PSL extracts have an increasing *ρ*-Anisidine value which slightly fluctuates over the storage time. The oil with BHT is more stable in *ρ*-Anisidine value than the other samples. The corn oil with added 0.01 %, 0.02 % and 0.05 % PSL show a lower *ρ*-Anisidine value than the negative and positive control oils. The *ρ*-Anisidine value of the rice bran oils and corn oils treated with PSE and PSL extracts increased throughout the storage time. The results of the rice bran oil with added PSE extract showed a lower increase in *ρ*-Anisidine values than the negative control oil (without the extract added) but higher than the positive control oil (with BHT added) and the higher

amount of the extracts, the lower *ρ*-Anisidine value. These results are in agreement with the findings of Mariod *et al.* (2010). They reported that the *ρ*-Anisidine value of rice bran oil with added rice bran extract was higher than the oil with added BHA, but lower than the synthetic antioxidant free oil and the higher amount of the extract, the lower *ρ*-Anisidine value.

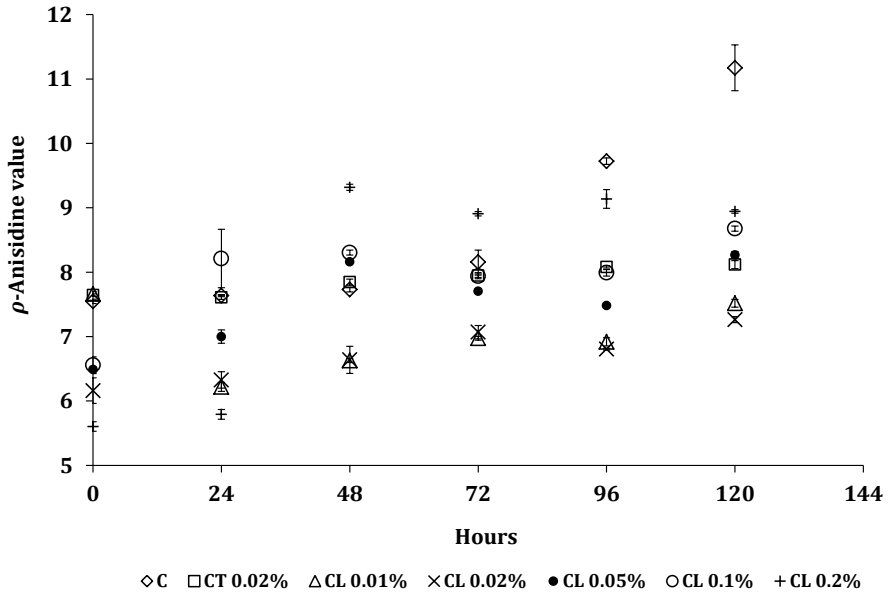


Figure 3-67: Effect of PSL extracts on *ρ*-Anisidine value of corn oil during storage at 60±3 °C for 120 hours. The value is expressed as mean±SE of triplicate analysis. C = corn oil, L = PSL extract, T = BHT, % = percentage added

However, in this study, the higher *ρ*-Anisidine value in the oils with added PSL extract compared to the synthetic antioxidant free oils, particularly in rice bran oil, indicated that the PSL extract showed no effective effect to protect lipid oxidation. It also showed the greater the amount of the PSL extract, the higher the *ρ*-Anisidine value.

3.6.2.2 The effect on TBA value

The 2-thiobarbituric acid (TBA) value of the rice bran oil and corn oil with added PSE extracts as well as the synthetic antioxidant free oils are shown in Figure 3-68 and Figure 3-69. They show an increasing TBA value over the storage time. The TBA value of the control oil with added BHT rice bran oil and corn oil are quite stable. The rice bran oils and corn oils treated with the PSE extracts show a lower TBA value than the synthetic antioxidant free oils but are higher than the control oils with added BHT. The lower TBA value in rice bran oil and corn oil were produced when 0.1 % PSE extract and 0.05 % PSE extract were added respectively.

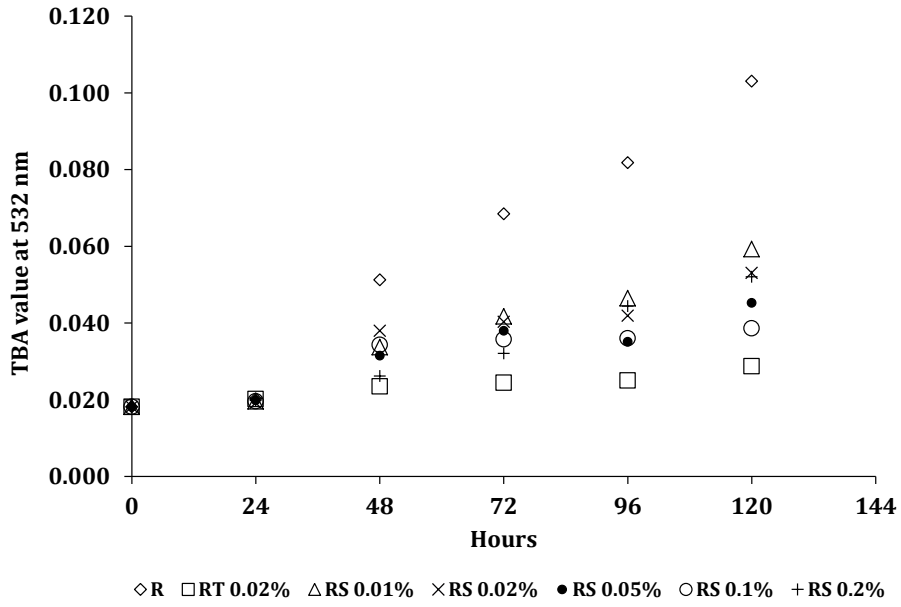


Figure 3-68: Effect of PSE extracts on 2-thiobarbituric acid value (TBA) of rice bran oil during storage at 60±3 °C for 120 hours. The value is expressed as mean±SE of triplicate analysis. R = rice bran oil, S = PSE extract, T = BHT, % = percentage added

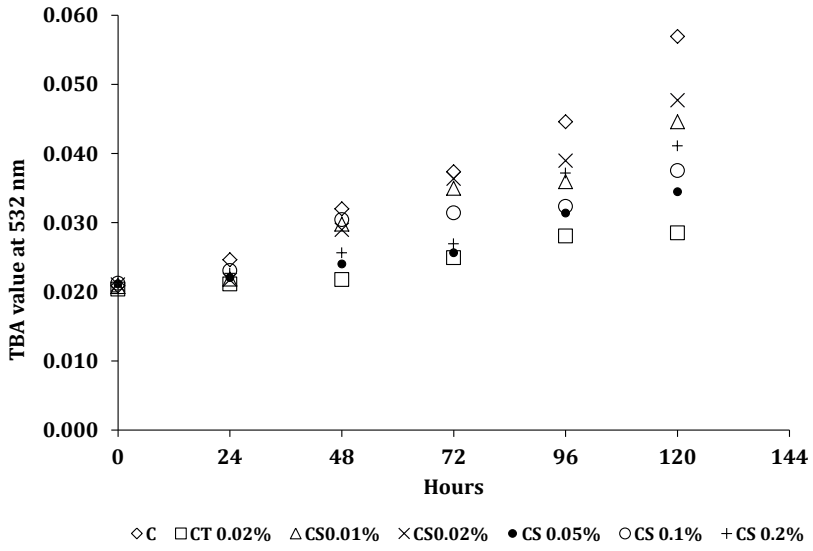


Figure 3-69: Effect of PSE extracts on 2-thiobarbituric acid value (TBA) of corn oil during storage at 60±3 °C for 120 hours. The value is expressed as mean±SE of triplicate analysis. C = corn oil, S = PSE extract, T = BHT, % = percentage added

The rice bran oils and corn oils with added PSL extracts show a slightly increasing TBA value over storage, as seen in Figure 3-70 to Figure 3-71. They have a higher TBA value than the control oils with added BHT but less than the synthetic antioxidant free oils after 48 storage hours. There were no clear differences in TBA values between 48 to 120 hours of both oils with added PSL extracts at all concentrations. The results of the TBA value in the present study show an increase over the storage time. The results clearly show that the oil with added PSE or PSL extracts have a lower increasing TBA value than the synthetic antioxidant free oil but higher than the oil with added BHT. These are in agreement with the study by Pimpa *et al.* (2009). They found rice bran oil with added α-tocopherol had an increasing TBA value which was lower than the synthetic antioxidant free oil but higher than the oil with added BHT. Zhang *et al.* (2010) reported the TBA value of sunflower oil with added rosemary extracts

increased over storage time which was lower than the synthetic antioxidant free oil and also lower than the oil with added BHT.

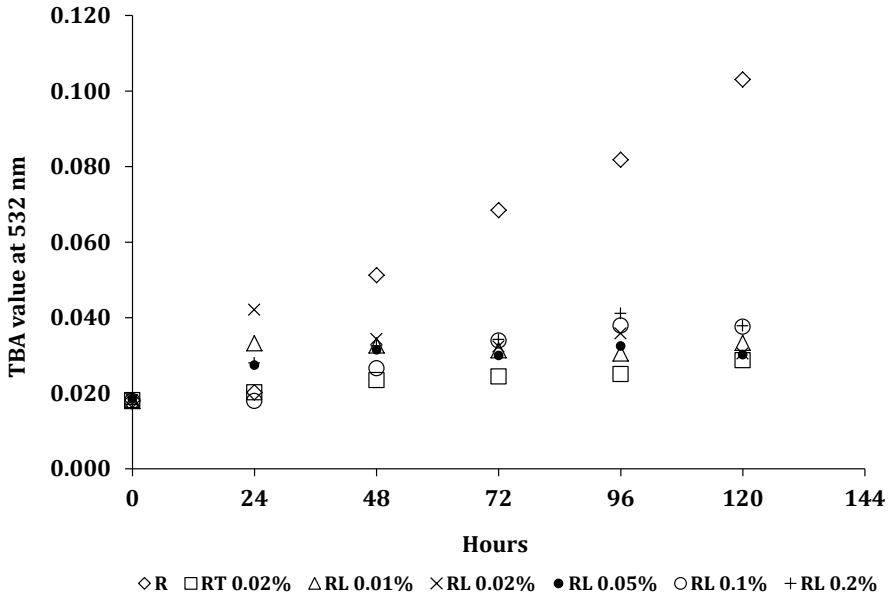


Figure 3-70: Effect of PSL extracts on 2-thiobarbituric acid value (TBA) of rice bran oil during storage at 60±3 °C for 120 hours. The value is expressed as mean±SE of triplicate analysis. R = rice bran oil, L = PSL extract, T = BHT, % = percentage added

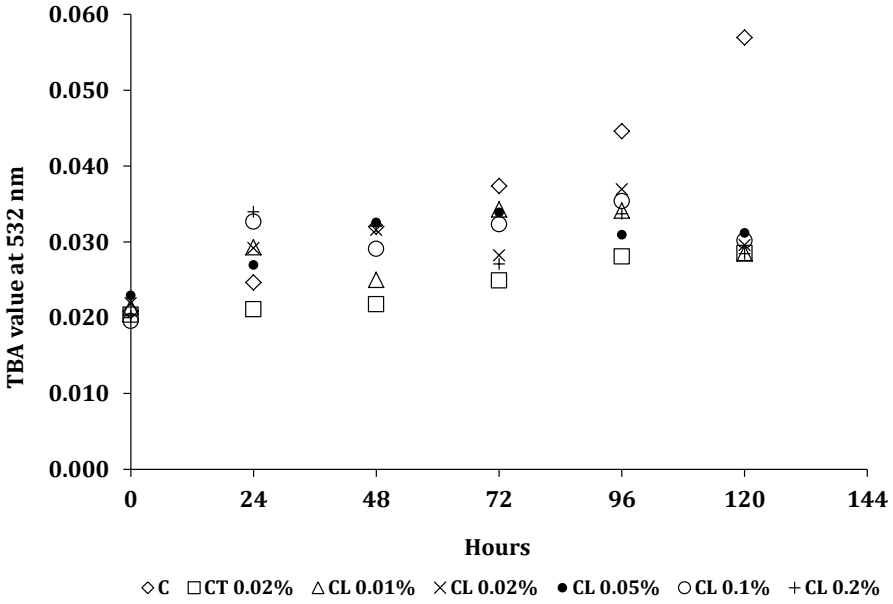


Figure 3-71: Effect of PSL extracts on 2-thiobarbituric acid value of corn oil during storage at 60±3 °C for 120 hours. The value is expressed as mean±SE of triplicate analysis. C = corn oil, L = PSL extract, T = BHT, % = percentage added

The results in this present study showed a relationship where increasing PSE extract gives a lower TBA value in both oils, which is in agreement with the study of Mariod *et al.* (2010). They reported the TBA value of rice bran oil with added defatted rice bran extract increased over storage time. The higher concentration of rice bran extract added the lower TBA value. However, the TBA value of oil with added rice bran extract were higher than the oil added with BHA, which was the same pattern as this present study.

3.6.3 Effect of *Piper sarmentosum* Roxb. leaf extracts on total oxidation value (Totox value) in rice bran and corn oils under accelerated storage conditions

As shown in Figure 3-72, the Totox value of rice bran oil with all samples shows an increasing trend throughout the storage time. Between 24 to 96 hours of storage, the Totox value of the oils with added PSE extracts at all concentrations are similar to the synthetic antioxidant free oil. At the storage time of 120 hours, the oils with added PSE extracts show a lower Totox value than the synthetic antioxidant free oils. This indicates that the PSE extracts have shown a positive effect on protecting the oil from oxidation. However, throughout the storage time, the oil with added BHT has exhibited a superior protective effect than the other samples. The slow increasing Totox values in the first 72 hours of storage and then the sharp increase at the end of storage time, may be due to the multiple effects from the inherent natural antioxidants (oryzanol or tocopherol) and PSE extract to protect against lipid oxidation. The longer storage time, the lower the concentration of antioxidants present to protect the oils. Thus, the Totox value showed a sharp increase at the end of storage time.

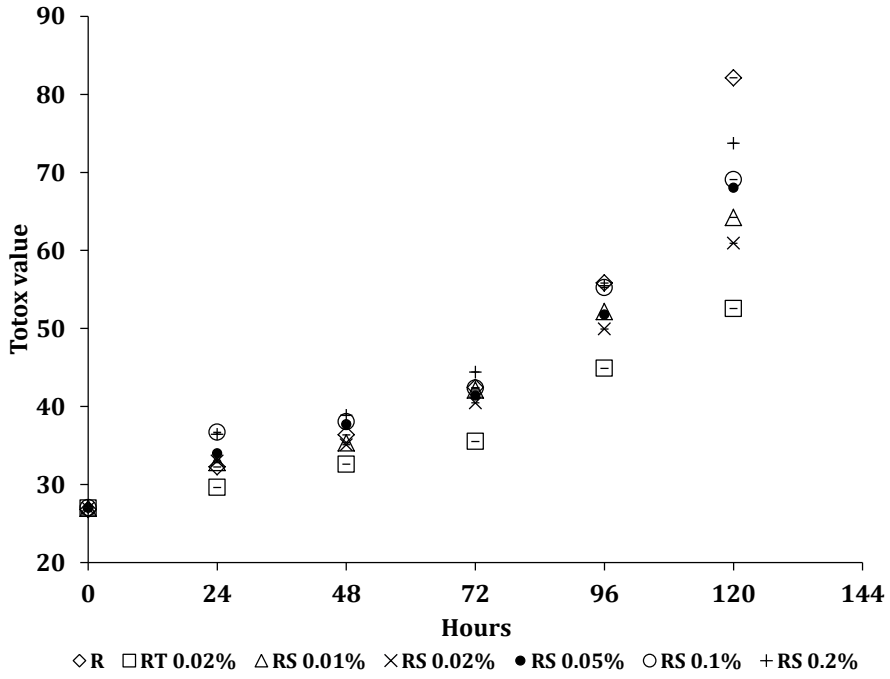


Figure 3-72: Effect of PSE extracts on Totox value of rice bran oil during storage at 60±3 °C for 120 hours. The value is expressed as 2(peroxide value)+ρ-Anisidine value. R = rice bran oil, S = PSE extract, T = BHT, % = percentage added

As shown in Figure 3-73, the Totox value of corn oils with added PSE extracts shows an increasing trend, with a lower value than the synthetic antioxidant free oil between 48 to 120 hours of storage time. Additionally, the PSE extracts at 0.02 % and 0.1 % exhibit no clear difference in protective activity over the oil with added BHT for the first 96 storage hours. They may have some positive effects to protect the corn oil from lipid oxidation and might be able to perform better than in rice bran oils. However, the oils with added BHT illustrates a low Totox value throughout the storage time, so its performance could still be considered better than that of the PSE extracts.

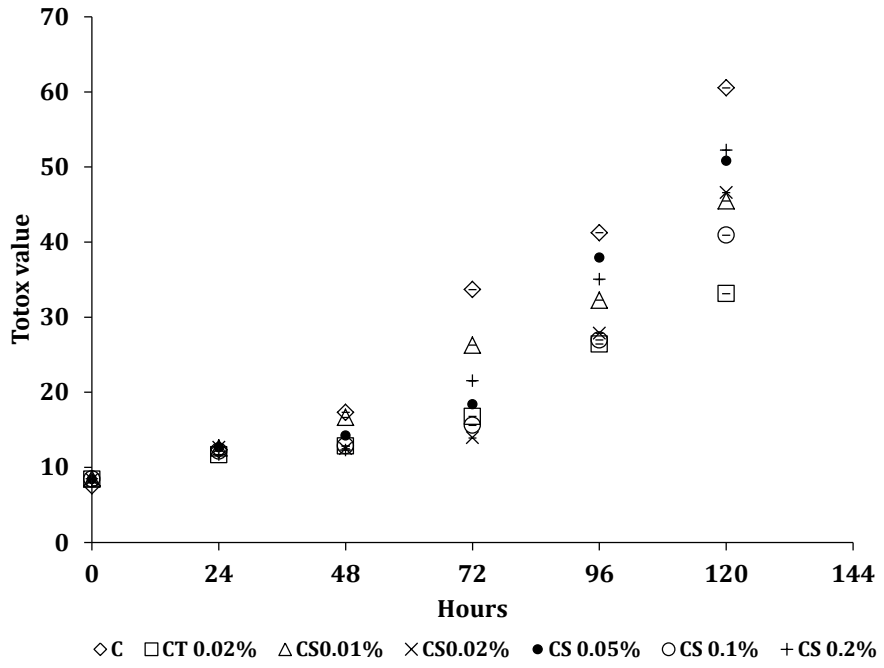


Figure 3-73: Effect of PSE extracts on Totox value of corn oil during storage at 60±3 °C for 120 hours. The value is expressed as 2(peroxide value)+ρ-Anisidine value. C = corn oil, S = PSE extract, T = BHT, % = percentage added

Figure 3-74 and Figure 3-75 show the effect of PSL extracts on the stability of rice bran oil and corn oil during storage time, respectively. The Totox values of rice bran oil and corn oil treated with PSL extracts are higher than the synthetic antioxidant free oils and the oils with added BHT. With synthetic antioxidant, BHT exhibiting a superior protective effect throughout storage time. This indicates that the PSL extracts do not show any positive effect on lipid oxidation to both oils throughout the storage time.

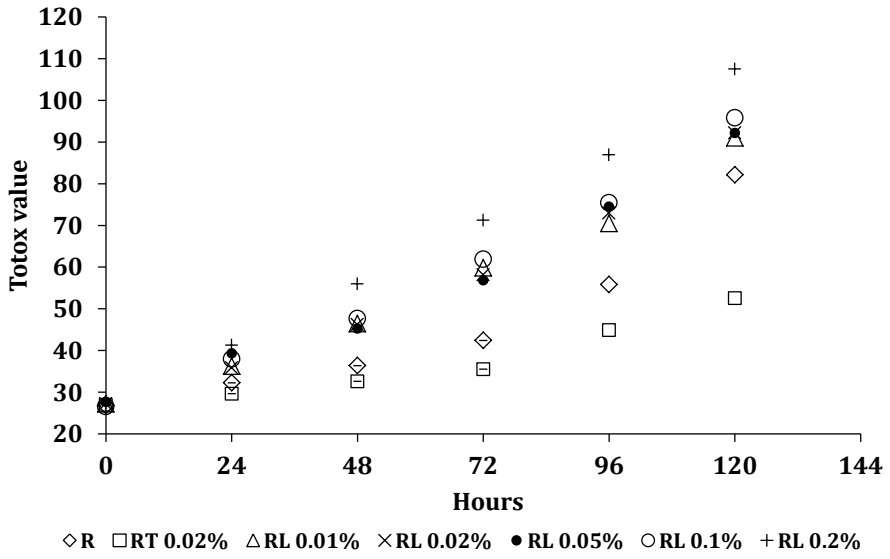


Figure 3-74: Effect of PSL extracts on Totox value of rice bran oil during storage at 60±3 °C for 120 hours. The value is expressed as 2(peroxide value)+ρ-Anisidine value. R = rice bran oil, L = PSL extract, T = BHT, % = percentage added

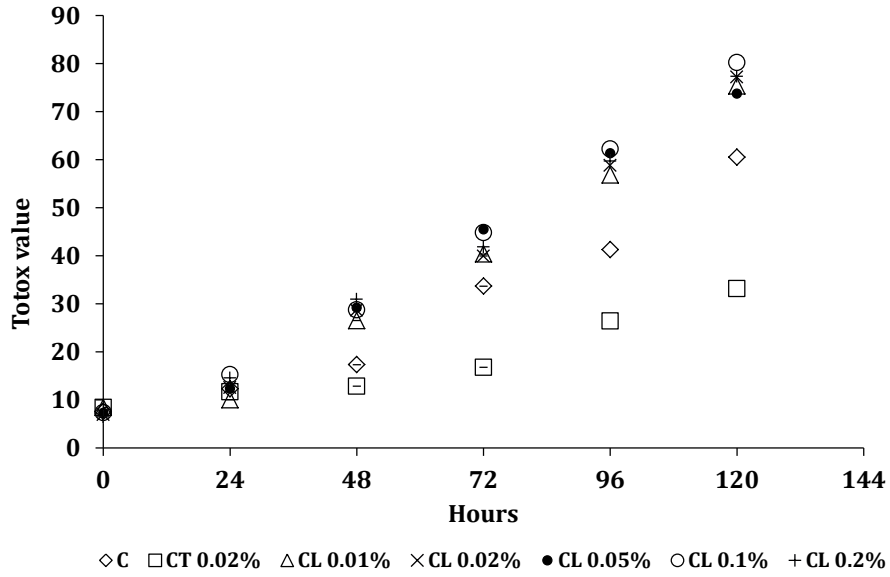


Figure 3-75: Effect of PSL extracts on Totox value of corn oil during storage at 60±3 °C for 120 hours. The value is expressed as 2(peroxide value)+ρ-Anisidine value, C = corn oil, L = PSL extract, T = BHT, % = percentage added

In addition, in rice bran oil, it also shows a negative effect relating to the amount of PSL extract added as a higher Totox value is found with higher amount of added PSL extract. The order of Totox value of the rice bran oil with added PSL extracts is 0.2 % > 0.1 % > 0.05 % > 0.01 %. However, it has been noticed that the Totox value in rice bran oils is quite high at the start. This was a resulted from the initial high *ρ*-Anisidine value in rice bran oils, as show in Figure 3-64 and Figure 3-66.

The effectiveness of the oils treated with PSE extracts are similar to the findings of Hashemi *et al.* (2011) on sunflower oil. They reported the Totox value of the oils with added *Zataria* multiflora extracts, increased over the storage time which was lower than the synthetic antioxidant free oil but higher than the oil with BHT. The Totox value found in rice bran oils with and without PSE extracts, are higher than corn oils. It is likely the extracts are working better in corn oil as can be seen by the close values of the 0.02 % and 0.1 % PSE extracts to the oil with added BHT. It is suspected that the phytochemical compounds present in the PSE extract (chapter 3.4) may have a greater effect on endogenous antioxidants in the corn oil than in rice bran oil. Corn oil is a rich source of tocopherols and tocotrienols (Table 1-6) particularly γ-tocopherol which has the highest antioxidant activity among other isomers (Shahidi, 2005b; O'Brien, 2004; Yanishlieva, 2001; White, 2000). Zhu *et al.* (2000) reported natural flavonoids (kempferol, morin, myricetin and quercetin) showed a protective activity against the depletion of α-tocopherol. Reblova and Okrouhla (2010) reported α-tocopherol was preserved during the heating of sunflower oil at 180 °C by phenolic acids; gallic acid, caffeic acid and gentisic. Jennings and Akoh (2009) reported no significant difference in the γ-oryzanol content in rice bran oil before

and after enzymatic modification which indicated that γ-oryzanol did not exert any antioxidant effects. Therefore, it is likely the PSE extracts work better in corn oil. In this study, the increasing concentration of PSE extract showed a positive trend by lowering the peroxide value, *ρ*-Anisidine value, TBA value and Totox value in both oils. In contrast, the increasing concentration of PSL extract showed the opposite effect by increasing these values which were higher than the synthetic antioxidant free oils. This pro-oxidant activity may be caused by some compounds which are present in the extracts such as caffeine. Caffeine has been reported to possess antioxidant activity but has shown pro-oxidant properties when present in high amounts (Yashin *et al.*, 2013; Azam *et al.*, 2003; Shi *et al.*, 1991). Due to the different polyphenol compounds present in the extracts, polyphenol compounds present in the PSE extract may have stronger antioxidant activity than in the PSL extract. It is important to note here that autoxidation can occur immediately in the presence of heat, light, metal, chlorophyll or several initiations under mild conditions (Gunstone, 2004; Frankel, 1998b). Pigments contained in PSE and PSL extract such as chlorophyll may therefore be involved in the ineffectiveness of the extracts due to photo-oxidation, which is an alternative route leading to the formation of hydroperoxides (Gordon, 2001). A final point to note is that the results in some test samples of this study showed the fluctuation of the peroxide value, *ρ*-Anisidine value and TBA values. This is due to the fact that lipid oxidation is a dynamic process, it tends to increase reach the maximum value and then decline. The products produced in these stages are unstable, so they can break down, reform or form new compounds (Pegg, 2005a).

3.7 Performance of the *Piper sarmentosum* Roxb. leaf extracts on quality changes in rice bran oil and corn oil at frying temperature

Synthetic antioxidants such as BHA, BHT, TBHQ and PG, are added to the oils to inhibit rancidity. These compounds give a good efficiency under room or mild temperature conditions but not at frying temperatures as they decompose, so fail to protect the oil (Allam and Mohamed, 2002; Hamama and Nawar, 1991). Also, from a safety issue, synthetic antioxidants promote carcinogenesis (Race, 2009). A large number of studies have been under taken to replace them with new antioxidants from natural sources but these mostly have been tested at storage temperatures, rather than during frying conditions. As *Piper sarmentosum* Roxb. leaf extracts extracted using 80 % ethanol (PSE) and extracted using petroleum ether (PSL) have the highest antioxidant activity and heat resistance (chapter 3), PS leaf extract could be a new source of natural antioxidant. No studies have applied the use of PS extract to inhibit thermal deterioration of frying oils. Therefore, the antioxidant activity of PS leaf extract on protecting thermal degradation of repeatedly heated oil at frying temperatures would be the first investigated by this study. According to the findings of preliminary studies of frying oil in chapter 3.5, the study by Brown (2013) and the study by Zhang and Taher (2012), frying temperature, frying time (number of frying and length of frying) and food being fried, have an effect on the degradation of the oils. The higher the temperature, the increase in number of fryings, the longer frying time and the more food being fried, the more degraded the oils become. Zhang and Taher (2012) also reported replenishing the oil could retard the oxidation but the acid values looked similar so there might not be an effect on hydrolysis.

Therefore, the frying model for this experiment will be based on these studies. The rice bran and corn oils will be treated with the PSE and PSL extracts and continuously heated at 180 °C without food particles and without replenishing. Also, the concentration of PSE and PSL extracts were chosen based on the finding in chapter 3.2. As some samples showed a positive trend when increasing the concentration of PSE, and PSL extracts at 0.05 % showed less fluctuation of TBA value than other concentrations in both oils, the concentrations of PSE and PSL extracts will be studied at 0.05 %, 0.1 % and 0.2 %. To compare the effectiveness between the PS extract and synthetic antioxidants, BHT was chosen as positive control due to most of countries allow to be used in the oils rather than TBHQ (Shahidi, 2005b). The aim of this study was to evaluate the ability of PSE and PSL extracts to stabilise the changes in rice bran oil and corn oil during frying and determine their possible use as natural antioxidant in these frying oils.

3.7.1 Effects of *Piper sarmentosum* Roxb. leaf extracts on acid value in rice bran and corn oils at frying temperature

Figure 3-76 to Figure 3-77, present the acid value results of rice bran oil and corn oil which were heated at 180 °C in total for 25 hours over 5 consecutive days. Both the rice bran oil and corn oil samples illustrate the acid values increased over the heating time. It indicates that high temperatures had an effect on the total acid value of the oils. The negative control oils (rice bran oil and corn oil without added extracts) had a higher acid value than other samples. The rice bran oil and corn oil with added PSE and PSL extracts are significantly ($p<0.05$) lower in acid value, compared to the both negative and positive control oils, after 5 hours of heating. The rice bran oil with added PSE extract at 0.2 % and the oil with added PSL extract at 0.1 % show a significantly lower and more stable acid

value than both the synthetic antioxidant free oil and the oil with added BHT after start of heating ($p<0.05$). The corn oils with added PSE and PSL extracts showed significantly lower acid values than the synthetic antioxidant free oil over the heating period and behaved as the oil with added BHT as no significant difference was found ($p<0.05$).

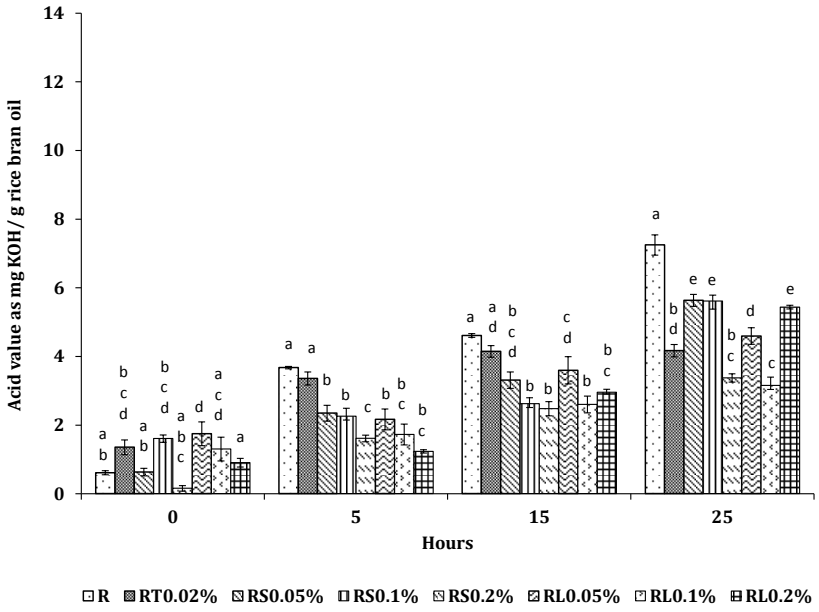


Figure 3-76: Effect of PSE and PSL extracts on acid values of rice bran oil heated at 180 °C for 25 hours. The values are expressed as mg potassium hydroxide (KOH)/g rice bran oil, mean±SE of triplicate analysis. Different letters for each heating hours are significantly different at $p<0.05$. R = rice bran oil, S = PSE extract, L = PSL extract, T = BHT, % = percentage added

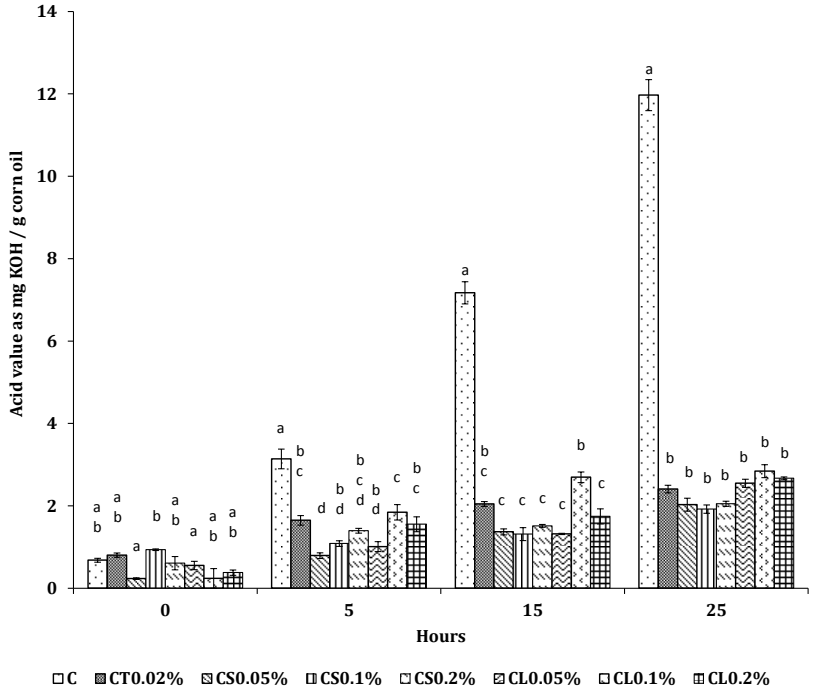


Figure 3-77: Effect of PSE and PSL extracts on acid values of corn oil heated at 180 °C for 25 hours. The values are expressed as mg potassium hydroxide (KOH)/g corn oil, mean±SE of triplicate analysis. Different letters for each heating hours are significantly different at $p<0.05$. C = corn oil, S = PSE extract, L = PSL extract, T = BHT, % = percentage added

From these results, it is evident that the PSE and PSL extracts have a protective effect in retarding the hydrolysis reaction, with some concentrations being equal or better than BHT. These are in agreement with the study by Misnawi *et al.* (2014). They reported the addition of 0.04 % polyphenol from cocoa extract in semi-purified crude palm oil resulting in significantly lower concentration of free fatty acid throughout frying times and lower than the oil without adding the extract.

3.7.2 Effects of *Piper sarmentosum* Roxb. leaf extracts on peroxide value in rice bran and corn oils at frying temperature

The ability of the PSE and PSL extracts to protect rice bran oil and corn oil during frying were assessed through the peroxide value. The increase of peroxide value

of the rice bran and corn oils are illustrated in Figure 3-78 to Figure 3-79 respectively. The protective effect of the PSE and PSL extracts in rice bran oil can be seen clearly after 5 hours of heating. The PSE and PSL extracts at all concentrations show an effective effect over the synthetic BHT (except the 0.1 % PSE extracts after 18 hours of heating) in rice bran oil. The most effective concentrations of PSE and PSL extracts in rice bran oils is 0.05 % which resulted in the lowest peroxide values throughout the heating procedure.

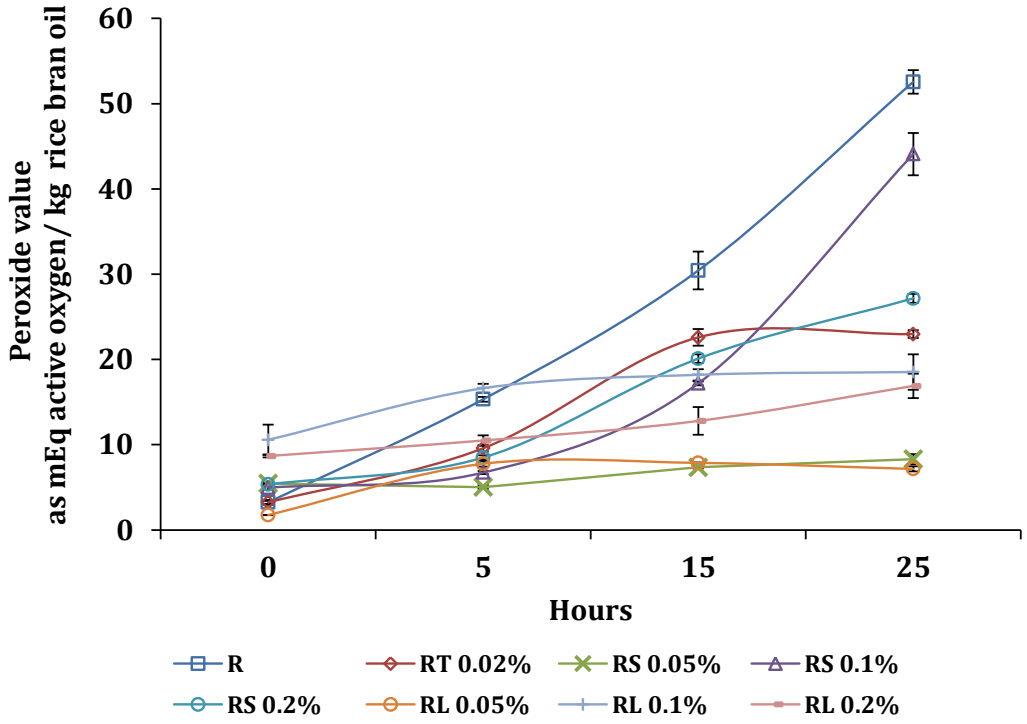


Figure 3-78: Effect of PSE and PSL extracts on peroxide values of rice bran oil heated at 180 °C for 25 hours. The values are expressed as milli equivalent (mEq) active oxygen/kg rice bran oil, mean±SE of triplicate analysis. R = rice bran oil, S = PSE extract, L = PSL extract, T = BHT, % = percentage added

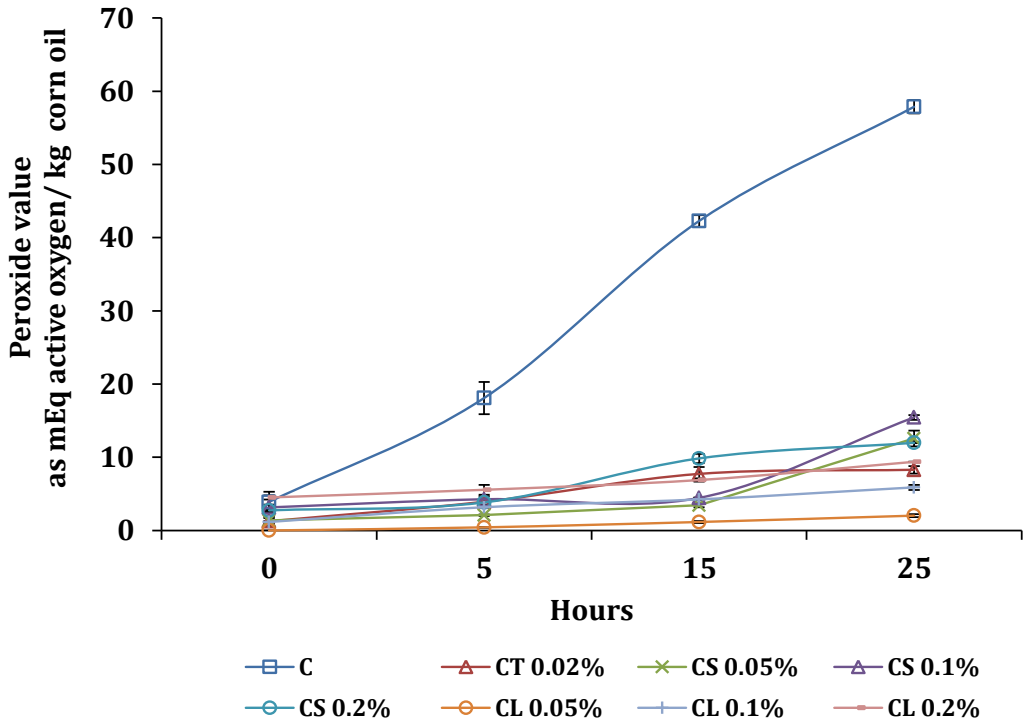


Figure 3-79: Effect of PSE and PSL extracts on peroxide values of corn oil heated at 180 °C for 25 hours. The values are expressed as milli equivalent (mEq) active oxygen/kg corn oil, mean±SE of triplicate analysis. C = corn oil, S = PSE extract, L = PSL extract, T = BHT, % = percentage added

The highest oxidation rate was found in the negative control corn oil, which was higher than the negative control rice bran oil. This is due to corn oil having a higher linoleic acid level than rice bran oil (Table 1-1). The oxidation rate of oil increases as the content of unsaturated fatty acids of frying oil increases (Choe and Min, 2007; Warner *et al.*, 1994; Stevenson *et al.*, 1984).

The protective effect of this study is in agreement with the study by Fukuda *et al.* (1986). They supplemented corn oil with 0.2 % sesamol and heated at 180 °C for 3 hours. The oil with added 0.2 % sesamol was significantly more stable than the normal corn oil. Similarly with the study by Misnawi *et al.* (2014), they reported the addition of cocoa extract ranging from 0–0.04 % significantly reduced peroxide values compared to the normal palm oil (0 % cocoa extract) and also

found with increasing concentrations of the extract, the peroxide values were lower, so they were more resistant to thermal oxidation of the oils. The effectiveness of PSE and PSL extract in this frying study is different to the accelerated storage results (chapter 3.6). In the accelerated storage study, BHT showed the most effective protection effect, PSE extract at 0.02 % showed a positive trend in both rice bran and corn oils, but not PSL extract. The positive protective effect from both extracts may be influenced by the temperature. The variation in temperature may change the mechanism of action of some antioxidants and result in their effectiveness (Yanishlieva, 2001). Marinova and Yanishlieva (1992) reported at 100 °C α-tocopherol exhibits greater effectiveness than at room temperature by reducing the rate of oxidation when the temperature increased. As seen in Figure 3-79 with the corn oils supplemented with PSE and PSL, all concentrations show a lower peroxide value than the synthetic antioxidant free oil. The PSL extract at 0.05 % offers the best inhibition effects. All concentrations of PSE extracts (0.05 %, 0.1 % and 0.2 %) lose their inhibition performance after frying for 20 hours compared to the oil with added BHT. It is likely PSL extracts work better at frying temperatures than the accelerated storage study. This is suspected as the effect of phytochemical compounds present in the extract such as quercetin or caffeine may show a greater effectiveness when the temperature is increased. Elhamirad and Zamanipoor (2012) reported that at 180 °C, quercetin had the most effective antioxidant activity compared to catechin, gallic acid and caffeic acid. The study by Bera *et al.* (2006) showed a small increase in peroxide value of flaxseed oil with added ajowan extract when the temperature was increased from 25-200 °C. While, the normal flaxseed oil without the extract had a sharp increase in

peroxide value when the temperature was increased. This meant the natural extract could have a greater protective effect on the lipid oxidation when the temperature increased. However, caffeine showed a negative effect when the concentration is increased.

3.7.3 Effects of *Piper sarmentosum* Roxb. leaf extracts on 2-thiobarbituric acid reactive substance (TBARS) value in rice bran and corn oils at frying temperature

According to the results in chapter 3.5.1.6, it showed the limitation of using TBA assay. Thus, in this experiment the formation of secondary lipid oxidation products was monitored using TBARS assay expressed as malonaldehyde instead. Using a standard curve of 1, 1, 3, 3-tetraethoxypropane (TMP) range 0-1.20 μmol/mL (Figure 3-47), the results of TBARS in rice bran and corn oils with added PSE and PSL extracts are presented in Figure 3-80 to Figure 3-81 respectively. The rice bran and corn oils supplemented with PSE extracts, PSL extracts and BHT are lower in malonaldehyde than the synthetic antioxidant free oils. Both synthetic antioxidant free oils have increasing malonaldehyde forming rates over the heating hours, whilst, the supplemented oils are lower with fluctuation throughout the heating time. Rice bran oil and corn oil with added PSE and PSL extracts at some heating hours have lower malonaldehyde formation than the oils with added BHT. This means PSE and PSL extracts show a positive protective effect over BHT.

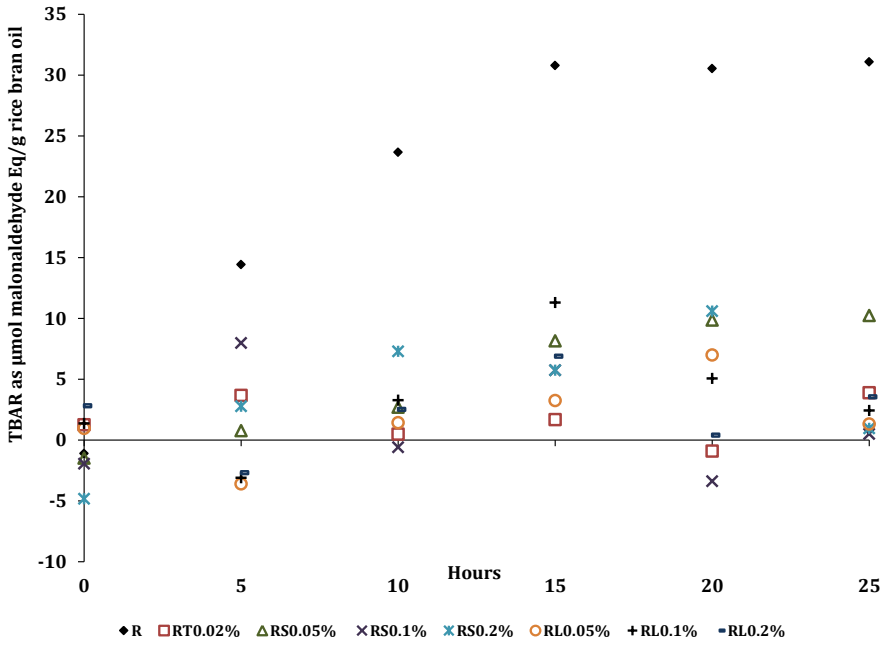


Figure 3-80: Effect of PSE and PSL extracts on 2-thiobarbituric acid reactive substances (TBARS) in rice bran oil heated at 180 °C for 25 hours. The values are expressed as µmol malonaldehyde equivalent/g rice bran oil, mean±SE of triplicate analysis. R = rice bran oil, S = PSE extract, L = PSL extract, T = BHT, % = percentage added

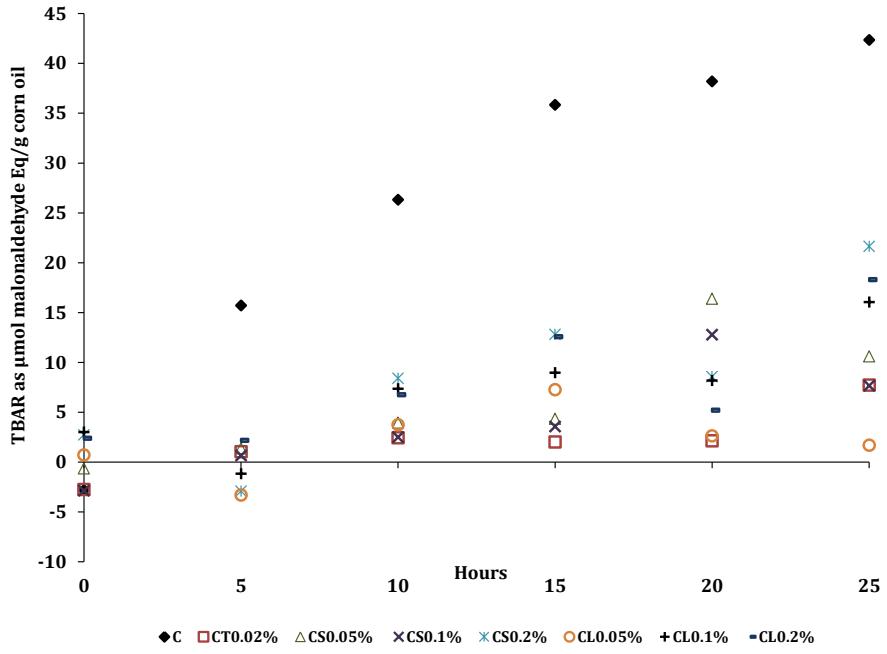


Figure 3-81: Effect of PSE and PSL extracts on 2-thiobarbituric acid reactive substances (TBARS) in corn oil heated at 180 °C for 25 hours. The values expressed as µmol malonaldehyde equivalent/g corn oil, mean±SE of triplicate analysis. C = corn oil, S = PSE extract, L = PSL extract, T = BHT, % = percentage added

The fluctuation of the supplemented oils may be the result of the protective effects of those extracts and BHT to inhibit the hydroperoxide compounds formed in the primary stage of oxidation. Polyphenols present in the PSE and PSL extracts may act as chain breaking antioxidants by scavenging free radicals; alkyl radicals or peroxyl radicals. These radicals will react further to produce hydroperoxide and conjugated dienes (Frankel, 1998c). Polyphenols donate hydrogen to these radicals to convert them into stable products (Yanishlieva, 2001), reducing the amount of hydroperoxides or conjugated dienes produced. Hydroperoxides are precursors for malonaldehyde formation. Thus, the less hydroperoxides, the less formation of malonaldehyde (Raharjo and Sofos, 1993). The amount of malonaldehyde forming throughout heating in both rice bran and corn oils is lower than the level to cause acute toxicity in rats (527 mg/kg or 37.98 mol/g) as reported by Crawford *et al.* (1965). It is also likely PSL extracts work better at frying temperatures as it did not exhibit protective effects in the accelerated storage study. As discussed in chapter 3.6.3, this is also suspected to be the effect of polyphenols present in the extract such as caffeine which may show a greater effectiveness when the temperature is increased. This hypothesis may be possible due to the finding of the study by Bera *et al.* (2006). They reported that the flaxseed oil with added ajown extract showed an increasing protective effectiveness as very low TBAR values arose when the temperature was increased from 100 °C, 130 °C, 160 °C, 190 °C and 220 °C at 1, 2 and 3 hours of heating. The normal flaxseed oil (without ajowan extract) showed a sharp increase in TBAR values throughout the heating time and temperatures. With a longer heating time (3 hours) and temperature increased to over 160 °C, the ajown extract showed a greater effectiveness over the oil with added BHT.

3.7.4 Effects of *Piper sarmentosum* Roxb. leaf extracts on total polar compounds in rice bran and corn oils at frying temperature

The major decomposition products of polymerisation of frying oil are non-volatile polar compounds and triacylglycerol dimers and polymers (Choe and Min, 2007). The determination of polar compounds contained in the frying oils is the most reliable parameter for monitoring the deterioration of the heated oils (Aladedunye, 2014; Shahidi, 2005a). As shown in Figure 3-82 to Figure 3-83, the rate of formation of polar compounds in rice bran oil and corn oil are found to significantly ($p<0.05$) increase over the heating time. This indicates that the high temperature has an effected on the formation of polar compounds. The rice bran oil with added 0.2 % PSE extracts and all concentrations of PSL extracts show significantly lower polar compounds than synthetic antioxidant free oils and the oils with added BHT at 5, 15 and 25 heating hours. The rice bran oil treated with 0.2 % PSE extracts and 0.1 % PSL extracts also have significantly ($p<0.05$) lower polar compounds than the oils with added BHT after frying for 5 hours. It is deduced that the 0.2 % PSE extracts and 0.1 % PSL extracts illustrate the highest protective effects on the rice bran oil.

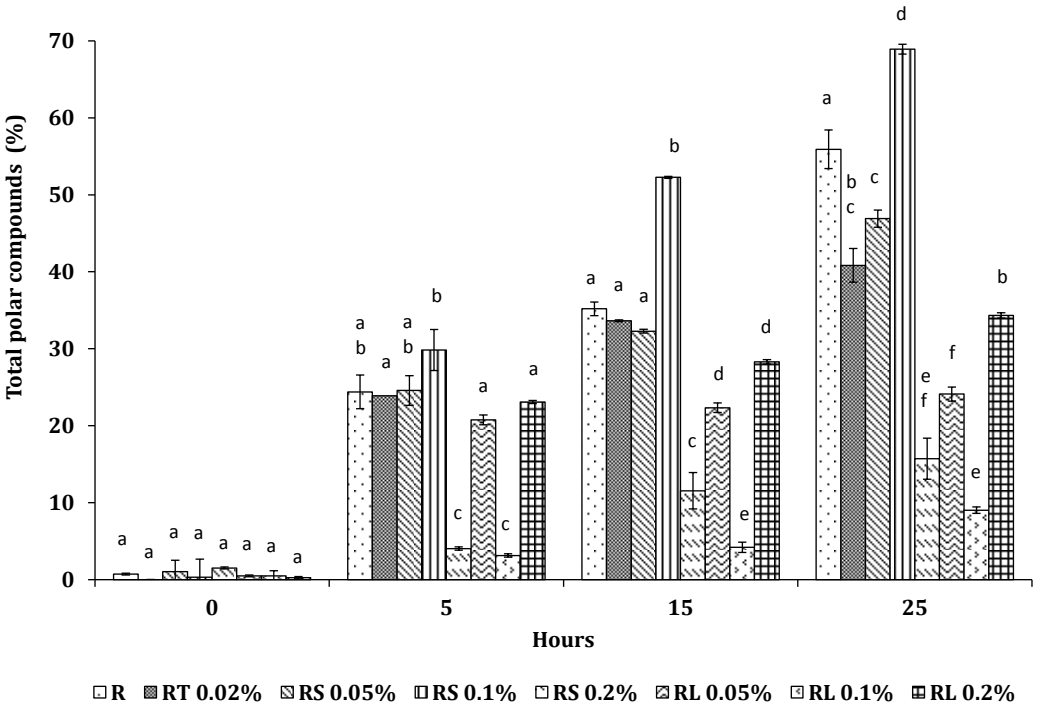


Figure 3-82: Effect of PSE and PSL extracts on total polar compounds in rice bran oil heated at 180 °C for 25 hours. The values are expressed as mean±SE of triplicate analysis. Different letters for each heating hours are significantly different at $p<0.05$. R = rice bran oil, S = PSE extract, L = PSL extract, T = BHT, % = percentage added

For the corn oil, as seen in Figure 3-83, the amount of polar compounds are increased over heating time. The lowest polar contents are found in the corn oil with added 0.2 % PSE extract and 0.05 % PSL extracts which are significantly different ($p<0.05$) from the other samples. So 0.2 % PSE extract and 0.05 % PSL extract demonstrate the highest protective effect on corn oil throughout the heating time. The results from this study reveal that PSE and PSL extracts have a positive protective effect inhibiting polar compounds formation. The effective amount of PSE extract in rice bran oil and corn oil was 0.2 %. While, the effective amount of PSL extract in both oils was different, (0.1 % in rice bran oil and 0.05 % in corn oil). With these effective amount of the extracts, it may enough to scavenge free radicals between 5-25 hours.

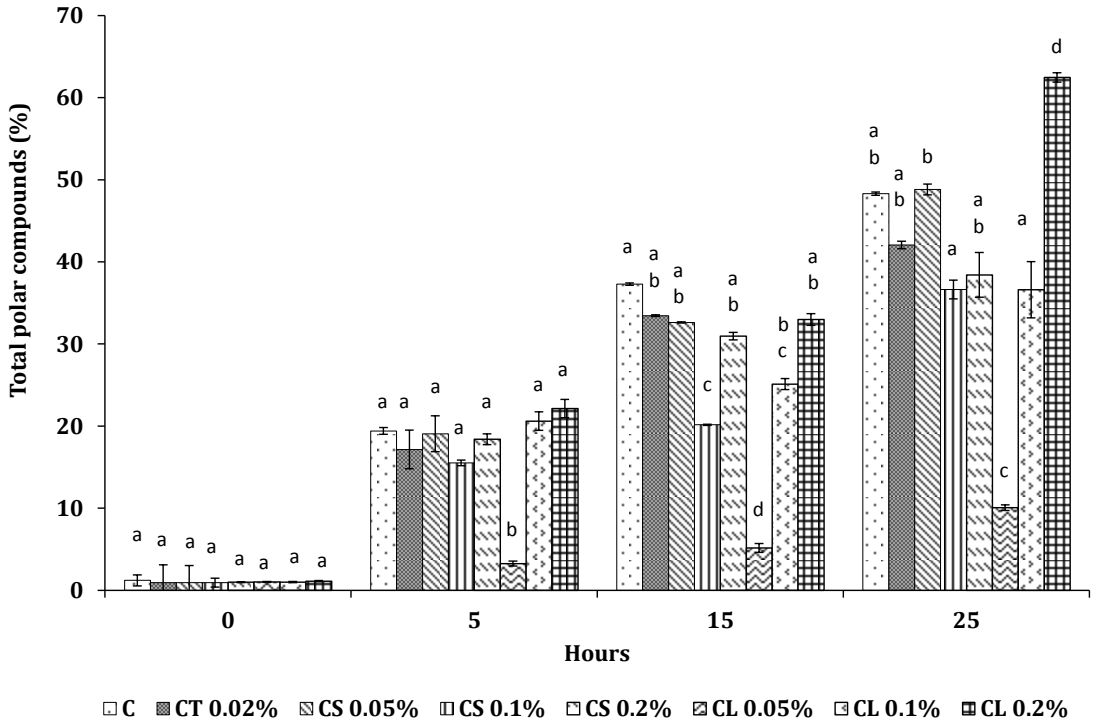


Figure 3-83: Effect of PSE and PSL extracts on total polar compounds in corn oil heated at 180 °C for 25 hours. The values are expressed as mean±SE of triplicate analysis. Different letters for each heating hours are significantly different at $p<0.05$. C = corn oil, S = PSE extract, L = PSL extract, T = BHT, % = percentage added

However, concentrations of PSE and PSL extracts used in this study may not reflect the best results as the wide range of concentrations can have either a positive or negative effect on polar compounds formation. The positive protective effect of PSE and PSL extracts in this study is in agreement with the study by Nor *et al.* (2008). They fortified palm olein oil with 0.2 % *Pandanus amaryllifolius* extract and fried at 180 °C for 40 hours. The oil with added extract showed an increase in polar compounds lower than the oil with added BHT which was significantly different after 24 hours of frying and also lower than the synthetic antioxidant free oil throughout frying times. Also similarly to the study by Aladedunye and Matthaeus (2014), they added phenolic fractions from rowanberry fruit extract and crabapple fruit extract to rapeseed oil. The oils

were heated at 180 °C for 16 hours. The results showed an increase in polar compounds over the heating hours. A smaller increase in polar compounds was found in the oils with both extracts added which was not found to be significantly different. These fortified oils showed a smaller increase in polar compounds than the oil with added BHT and normal rapeseed oil. It is likely that BHT will lose performance to inhibit thermal lipid oxidation in the oil as an increase in polar compounds were not found to be significantly different to the synthetic antioxidant free oils throughout the heating times. The failure of BHT during frying may be due to the losses through evaporation, decomposition and scavenging reactions (Augustin and Berry, 1983). The results of this study indicated that the polyphenols present in the PSE or PSL extracts are more thermally stable than the synthetic antioxidant, BHT. These results also illustrate that both extracts, particularly PSL extracts exhibit the protective effectiveness at frying temperatures or when the temperature was increased as it could not be seen from the previous accelerated storage study. The temperature variation therefore may change the mechanism of action of some compounds present in the extracts which results in their greater effectiveness at high temperature (Yanishlieva, 2001). This indicated by a number of studies such as the study by Fukasawa *et al.* (2009). They reported mixed tocopherols were more effective than the rooibos tea extract in soybean oil at 120 and 140 °C, but the extract was significantly effective compared to the mixed tocopherols at 160 and 180 °C. Similarly, Bensmira *et al.* (2007) reported lavender and thyme incorporated in sunflower oil showed no effect at 25 °C but the extracts showed a dramatic increase in effectiveness on oil stability at 150, 180 and 200 °C. Some polyphenols such as quercetin, catechin, gallic acid and caffeic acid, have been

compared in relation to their antioxidant activity when increasing the temperature to 180 °C (Elhamirad and Zamanipoor (2012). They reported at 120 °C, gallic and caffeic acids were more effective than catechin and quercetin in sheep tallow olein. At 180 °C, quercetin was more effective than catechin, gallic acid and caffeic acids respectively. So, the presence of quercetin in the PSE and PSL extracts in this present study may relate to the effectiveness of the extracts in retarding thermal degradation at frying temperature. The PSE and PSL extracts showed a better protective effect on thermal degradation in corn oil than rice bran oil. This may be due to the different endogenous components between corn oil and rice bran oil. Table 1-6 shows corn oil has an abundance of γ-tocopherol which is able to slow down the formation of hydroperoxides (Lampi *et al.*, 1999; Ochi *et al.*, 1989) and also has ferulic acid which contributes to the excellent oxidative stability of corn oil (O'Brien, 2004). Deepam *et al.* (2011) reported that stripped rice bran oil with added Tocols was more stable than the stripped rice bran oil with added oryzanol and sterol. This was in agreement with the findings by Jennings and Akoh (2009) and Nystrom *et al.* (2007) who reported that the γ-oryzanol component, stiosteryl ferulate, did not exert as antioxidants.

3.7.5 Effects of *Piper sarmentosum* Roxb. leaf extracts on colour changes in rice bran and corn oils at frying temperature

Figure 3-84 to Figure 3-87, illustrate the photometric colour changes in rice bran oil and corn oil supplemented with PSE and PSL extracts compared to synthetic antioxidant free oils and oils with added BHT. The colour of the rice bran oil and corn oil increased in darkness over the heating time. The colour of the rice bran oil with added BHT was darker than corn oil with added BHT. Nevertheless, the

colour of the both oils with added BHT were the darkest compared to the other oils heated.

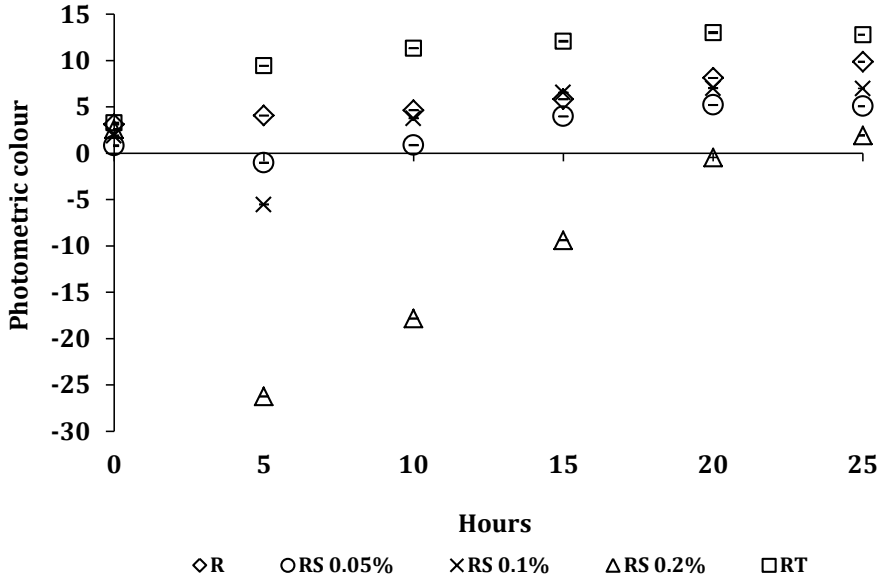


Figure 3-84: Effect of PSE extract on photometric colour changes in rice bran oil heated at 180 °C for 25 hours. The values are expressed as mean±SE of triplicate analysis. R = rice bran oil, S = PSE extract, T = BHT, % = percentage added

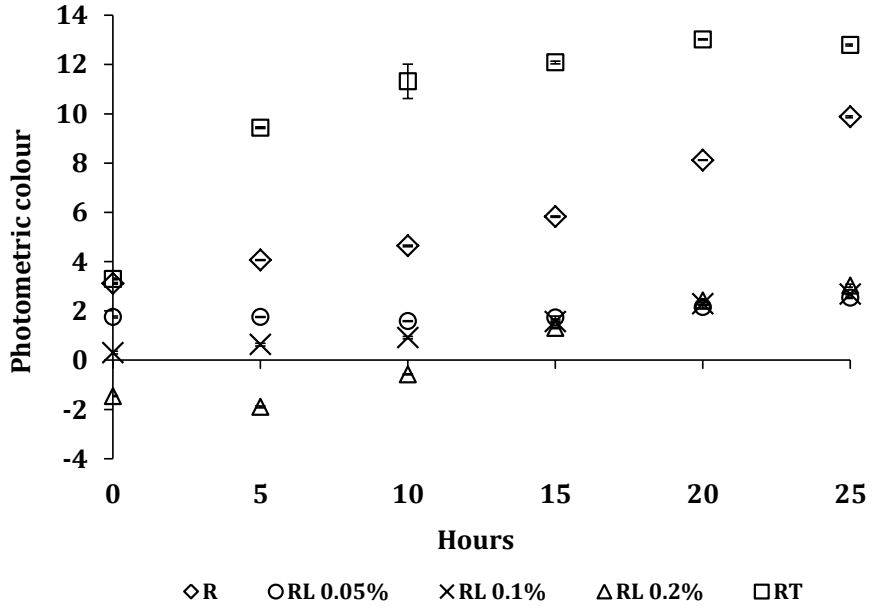


Figure 3-85: Effect of PSL extracts on photometric colour changes in rice bran oil heated at 180 °C for 25 hours. The values are expressed as mean±SE of triplicate analysis. R = rice bran oil, L = PSL extract, T = BHT, % = percentage added

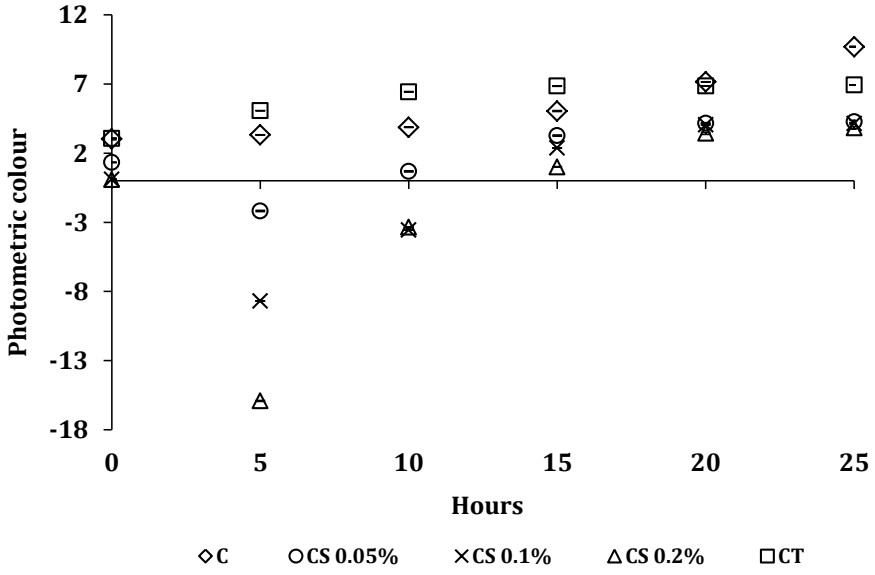


Figure 3-86: Effect of PSE extracts on photometric colour changes in corn oil heated at 180 °C for 25 hours. The values are expressed as mean±SE of triplicate analysis. C = corn oil, S = PSE extract, T = BHT, % = percentage added

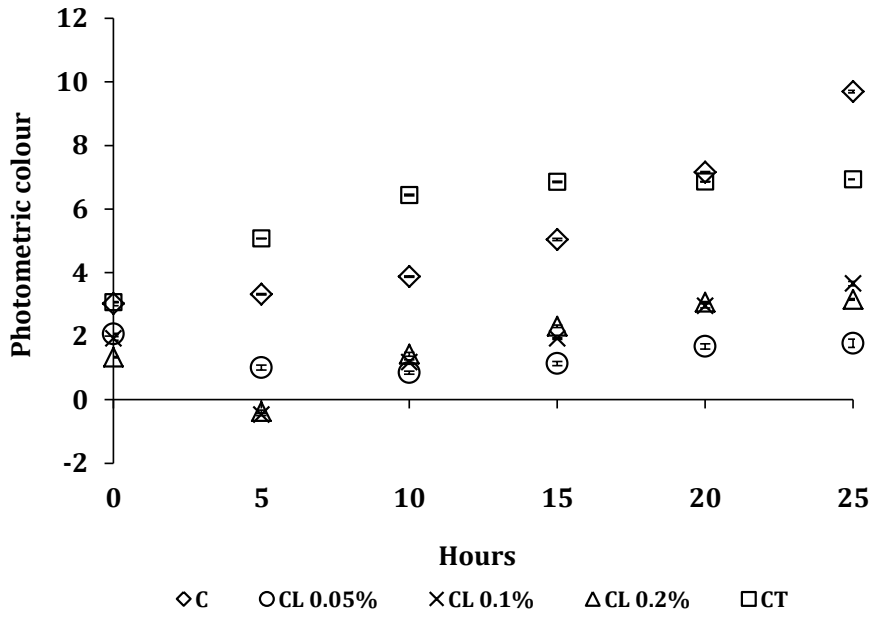


Figure 3-87: Effect of PSE extracts on photometric colour changes in corn oil heated at 180 °C for 25 hours. The values are expressed as mean±SE of triplicate analysis. C = corn oil, L = PSL extract, T = BHT, % = percentage added

The results of this present study have shown that the colour of the oils with added PSE and PSL extracts are lighter than the synthetic antioxidant free oils and the oil with added BHT. According to Das and Pereira (1990), they reported that the development of brown colour in frying oil is associated with oxidation and polymerisation, as well as, Augustin *et al.* (1987), who reported that the formation of polymers will promote the darkening of oils. Therefore, according to the results in chapter 3.7.2 to 3.7.4, the lower increase in darkness of the oils treated with the extracts indicate a protective effect of the extracts on thermal oxidation and polymerisation. This assumption can be supported by correlation results (chapter 3.7.6). It was found that the change in colour has strong correlation with total polar compounds with statistical significance ($p<0.05$). In this study, the PSE extract was dark green, the PSL extract was brownish-yellow.

So, the green pigments in PSE extract could be chlorophylls and brownish-yellow pigment in PSL extract could be carotenoids. It has been observed that the absorbance obtained from the oils with added PSE extracts measured at this wavelength (670 nm) was found highest at 0 hours (un-heated) and then decreased to the lowest as heating time increased. The colour of the oils became less green and more like the synthetic antioxidant free oil colour then became darker as heating time increased. These incidents are affected by the temperature. The high temperature will degrade chlorophylls and induce pheophytin formation. Thus, during heating the green colour is lost, turns to olive brown instead and also due to polymerisation of the oil. (Schwartz *et al.*, 2008; Boekel and Martinus, 2000). In this respect, the photometric colour indices from the equation are greatly affected by chlorophyll pigments (response at 670 nm) and hence why the colour index drops below zero. The more chlorophylls contained in the oil due to the concentration of PSE extracts, the lower the colour index will show from the start of heating until the oil turns brown. So, the low colour index of the oils with added PSE extracts is as follows: 0.2 %<0.1 %<0.05 % which relates to the amount of chlorophyll that would be present. Therefore, the findings from this study completely agree with Pohle and Tierney (1957) that high chlorophyll content oils have an unrealistic colour by this method due to the negative factor with excessive weight (constant number) given for measurement at this wavelength (670 nm) in the equation. The photometric colour index of the oils with added PSL extracts also show a similar pattern to the oils with added PSE extracts. The absorbance obtained from the oils with added PSL extracts measured at 550 nm were found to be lower than the absorbance measured at 670 nm. This implies that there might have also been some chlorophyll present

(Schwartz, 2005). Again the effects of the negative factor as mentioned above are seen when the concentration of PSL extracts added to the oils are higher, as noticed from the oils with added 0.2 % PSL extract.

These results show an effect of pigments on colour of the oil which were different from the results in the preliminary study of frying chips in chapter 3.5.1.1. Therefore, these findings agree with many researchers such as Bansal *et al.* (2010), Man *et al.* (1996) and Tan *et al.* (1985) that the colour of the degraded oil depends upon colour of the oil and types of fried food being fried. However, it was still clear to see that the oils all got darker with an increase in heating time.

3.7.6 Correlation between acid value, peroxide value, TBARS value, colour and total polar compounds in rice bran and corn oils at frying temperature

The relationship between monitored parameters in rice bran oil and corn oil with and without added PSE and PSL extracts and with added BHT, were examined by Pearson's correlation coefficient and the results are presented in Table 3-11 to Table 3-14. If the correlation coefficients $\geq$ 0.3, it means there is a relationship between the factors (Wiredu, 2012). The higher value, the stronger the correlation (Wiredu, 2012). As shown in Table 3-11, the formation of polar compounds in normal rice bran oil has a very strong correlation with the acid value ($p<0.01$) and peroxide value ($p<0.05$). In normal corn oil, the formation of polar compounds has a very strong correlation with TBARS value, peroxide value ($p<0.01$), and acid value ($p<0.05$). Both oils has a strong relationship between polar compound formation and colour changes but no significantly difference is found.

Table 3-11: Pearson's correlation coefficient (*r*) of normal rice bran oil and corn oil (without added extracts)

Correlation coefficients (*r*)	Rice bran oil				Corn oil			
	AV	PV	TBARS	Col	AV	PV	TBARS	Col
PV	.971*				.990*			
TBARS	.914	.895			.946	.980*		
Col	.933	.985	.806		.956*	.907	.810	
TPC	.999**	.980*	.928	.940	.972*	.991**	.995**	.862

*, ** correlation is significant at the 0.05 and 0.01 level (2-tailed) respectively, AV = acid value, PV = peroxide value, TBARS = 2-thiobarbituric acid reactive substance value, Col = colour, TPC = Total polar compounds

Table 3-12, the formation of polar compounds in rice bran oil with added PSE extracts shows a very strong relation with acid value ($p<0.01$) and peroxide value ($p<0.05$). Also in corn oil with added PSE extract, the formation of polar compounds has a strong correlation with acid value, peroxide value and TBARS value ($p<0.01$). Both oils show a weak correlation between formation of polar compounds and colour changes. Table 3-13, rice bran oil with added PSL extract show a very strong correlation between the formation of polar compounds and acid value ($p<0.05$), while corn oil with added PSL extract shows a very strong relationship between polar compounds formation with acid value, peroxide value and TBARS value ($p<0.01$). Rice bran oil with added PSL extract shows a very weak relationship between polar compounds formation with colour changes. No correlations are found between the formation of polar compounds with peroxide value, TBARS value in rice bran oil with added PSL extract and between colour changes in corn oil with added PSL extract.

Table 3-12: Pearson's correlation coefficient (*r*) of rice bran oil and corn oil with added PSE extracts

Correlation coefficients (*r*)	Rice bran oil				Corn oil			
	AV	PV	TBARS	Col	AV	PV	TBARS	Col
PV	.608*				.770**			
TBARS	.481	.071			.771**	.838**		
Col	.353	.218	.414		.599*	.392	.390	
TPC	.825**	.652*	.388	.484	.870**	.794**	.936**	.456

*, ** correlation is significant at the 0.05 and 0.01 level (2-tailed) respectively, AV = acid value, PV = peroxide value, TBARS = 2-thiobarbituric acid reactive substance value, Col = colour, TPC = Total polar compounds

Table 3-13: Pearson's correlation coefficient (*r*) of rice bran oil and corn oil with added PSL extracts

Correlation coefficients (*r*)	Rice bran oil				Corn oil			
	AV	PV	TBARS	Col	AV	PV	TBARS	Col
PV	.251				.445			
TBARS	.315	.357			.475	.819**		
Col	.798**	.183	.266		.148	.107	.500	
TPC	.702*	.064	.007	.325	.739**	.875**	.754**	.069

*, ** correlation is significant at the 0.05 and 0.01 level (2-tailed) respectively, AV = acid value, PV = peroxide value, TBARS = 2-thiobarbituric acid reactive substance value, Col = colour, TPC = Total polar compounds

Table 3-14, in rice bran oil with added BHT, the formation of polar compounds shows a very strong correlation with colour changes ($p<0.01$) and acid value ($p<0.05$). Corn oil with added BHT shows a very strong correlation between the formation of polar compounds with peroxide value ($p<0.01$) and acid value ($p<0.05$). Corn oil with added BHT also shows a strong relationship between the

formation of polar compounds with colour changes but no significant difference is found.

Table 3-14: Pearson's correlation coefficient (*r*) of rice bran oil and corn oil with added BHT

Correlation coefficients (*r*)	Rice bran oil				Corn oil			
	AV	PV	TBARS	Col	AV	PV	TBARS	Col
PV	.922				.946			
TBARS	.659	.410			.838	.946		
Col	.997**	.937	.666		.510	.747	.888	
TPC	.984*	.938	.695	.995**	.981*	.990**	.908	.652

*, ** correlation is significant at the 0.05 and 0.01 level (2-tailed) respectively, AV = acid value, PV = peroxide value, TBARS = 2-thiobarbituric acid reactive substance value, Col = colour, TPC = Total polar compounds

The correlation testing results of this study clearly indicate that the formation of polar compounds in both oils at frying condition 180 °C for 25 hours with or without antioxidant additions are related to peroxide values and TBAR values which are primary and secondary products from thermal lipid oxidation. However, some of the correlation coefficients (*r*) show a strong correlation with no significance or show no relationship at all (Table 3-13). These can support the fact that peroxide compounds or malonaldehydes which occurred in primary and secondary oxidation are not stable. They can decompose or form other compounds. Thus, these values (peroxide and TBARS values) may not suitable to use for investigating lipid thermal oxidation or monitoring quality of frying oil. It is interesting that the hydrolysis reaction has a very strong correlation with oxidative reaction (lipid oxidation) which occur during heating oil at frying temperatures, as the results (Table 3-11 to Table 3-14) show very strong

correlations between the formation of polar compounds and acid value with significance ($p<0.05$ and $p<0.01$) for both oils with all concentration of the extracts and with added BHT. The correlations between the formation of polar compounds with colour changes in both oils with and without added antioxidants show variations. Some are a strong correlation but no significant differences were found, some are weak relationships and some show no relationship between them. The results in chapter 3.7.5, revealed that the colour changes of the repeating heated oil are greatly influenced by pigments from the extracts.

This research determined changes in oils during frying through several indicators. It was manifestly observed that the indicators used for monitoring changes of primary and secondary product from lipid oxidation (peroxide value, *ρ*-Anisidine value, TBA value or TBARS value), should not be used for evaluating quality of the oil as the products measured in these tests are unstable so they can reform or decompose further (Paul *et al.*, 1997; Fritsch, 1981). Although, changes of colour had a strong correlation with total polar compound and the oils got darker as heating time increased, the changes in colour of the degraded oil is influenced by pigments contained in the oil and types of fried food being fried (Bansal *et al.*, 2010; Man *et al.*, 1996; Tan *et al.*, 1985). Therefore, the colour indicator can only be used if the acceptable value was specifically set for each oil and each food fried in it. The best indicators overall for monitoring quality changes in frying oil are total polar compounds and acid value (or free fatty acid). They showed a significant strongly correlation to each other despite them generating from different reactions. Free fatty acids develop from a hydrolysis reaction. Total polar compounds are end products of lipid oxidation. The higher frying temperature or the longer frying time, the more free fatty acids are formed

which also promotes the oxidation due to hydrolysis leading to an increase in the solubility of oxygen (Kochhar, 2001). Bhattacharya *et al.* (2008) and Kochhar (2001) also discovered the relationship between free fatty acids and total polar compounds in frying oil. According to Rossell (2001c), the International symposium on Deep Fat Frying in Germany in March 2000 recommended the combination of two tests is the best way of analysing suspect frying fats and oils. Therefore, based on this study, the best pair of indicators for monitoring thermal degradation of frying oils is acid value (free fatty acid) and total polar compounds.

4 Conclusion and Recommendation

4.1 Conclusion

The findings from the first investigation revealed that the *Piper sarmentosum* Roxb. (PS) leaf had a higher antioxidant activity and total phenol content than *Pandanus amaryllifolius* Roxb. (PD) leaf. It was found that 80 % ethanol had a better extraction efficiency than absolute ethanol and there was no synergistic effect of the mixture of both leaf extracts.

The results of the effect of the extraction method on total phenol content and antioxidant properties in PS leaf extracts clearly showed that the petroleum ether extracts (PSL) and the dried leaf (DFPS) following soxhlet extraction at 250 °C for 5 hours still contained phenols, flavonoids and had antioxidant activity with no significant difference when compared to the normal leaf extracts (PS). However, it was found that the 80 % ethanol extract still gave the highest total phenol content, total flavonoids and antioxidant activity. The decolourisation process had a huge effect on the loss of phenol content and antioxidant activity. The efficiency of the extraction was high with a 93 % yield. The PS, DFPS and PSL extracts demonstrated antioxidant capacity in linoleic lipid peroxidation system too, so these extracts showed the possibility for use in oil or emulsion food matrices. Based on these finding, it could be concluded that the *Piper sarmentosum* Roxb. leaf extracts possess a high antioxidant activity and are heat resistant because there was no loss in phenols, flavonoids or antioxidant activity when the leaf was defatted at such high temperatures for a long time.

The exploring polyphenol compounds that are present in the PSE, DFPSE and PSL extracts using HPLC-PDA-ESI-MS. Seven compounds were identified in

PSE and DFPSE extracts. They were 3CQA/5CQA, caffeic acid, vitexin, *ρ*-courmaric acid, hydrocinnamic acid, quercetin and caffeine. Vitexin, hydrocinnamic acid and caffeine were found in PSL extract. The quantified results revealed that the phenols which were found in PSE and DFPSE extracts showed no significant difference. Vitexin was found in the highest amount in PSE extract and caffeine was found in the highest amount in PSL extract. Nevertheless, unidentified compounds present in the extracts were proposed as tentative compounds which were 10 cinnamic acids, a benzoic acid, 3 flavones and 3 flavanones. Two flavones were main compounds in PSE and DFPSE extracts which are in the flavonoid group. Therefore, *Piper sarmentosum* Roxb. leaf extracts are rich source of phenolic acids, flavonoids and caffeine and therefore, it is a good source of antioxidants.

The study of the effect of repeated frying on the physical and chemical characteristics of the oils revealed that the oils used for frying chips at 190 ˚C show deterioration which increases over the frying days. It showed an increase in colour (darker) and viscosity, while the smoke point decreased, the peroxide values showed fluctuation, acid value increased over frying time as did the *ρ*-Anisidine value, TBA value and the total polar compounds. The results also revealed that deterioration rate of the frying oils were influenced by the length of frying time (a thicker chip required a longer frying time) and moisture from the food being fried. In addition, the findings by this study revealed that the following indicators: smoke point, peroxide value, *ρ*-Anisidine value, TBA value or TBARS value and colour changes should not be used to evaluate quality of repeated frying oil. The best pair of indicators to be used for evaluating degradation of frying oil are total polar compounds and total acid value (free

fatty acid). This information is very important for choosing the indicator to monitor the quality of repeated frying oils.

The results of oxidative stability of stripped and unstripped palm olein oil in the presence of PSE extract showed that the process of stripping the oil by using aluminium oxide may not have removed or completely eradicated the existing compounds present in the oils, especially, synthetic antioxidants. The attempt to find synthetic antioxidant free oil available in local shops was successful with confirmation using the HPLC analysis. Rice bran oil (King®), corn oil (Sainsbury's®) and rapeseed oil (Yor®) are synthetic antioxidant free oils. The study also showed that the palm olein oil (Oleen®) and rice bran oil (Alfa 1®), both stripped and unstripped using aluminium oxide contained 3 synthetic antioxidants BHA, BHT and TBHQ. It also proved that the stripping process using aluminium oxide does not remove synthetic antioxidants.

The study of antioxidant activity of PSE and PSL extracts on quality changes in rice bran oil and corn oil under mild temperature revealed that the effective concentration of the PSE extracts varies among the tests and among the oils throughout storage time. Thus, the effective amount of the extract could not be achieved from this study. The results also revealed that BHT exhibited a superior protective effect over PSE and PSL extracts. The PSL extracts did not show any positive effect to retard lipid oxidation in both oils over the storage time. PSE extracts showed a lipid oxidation inhibiting effect by lowering the peroxide value, *ρ*-Anisidine value, TBA value and Totox value in both oils. The reason PSL extracts showed different results to PSE extracts, may be due to the different amount and types of polyphenol compounds present in each extract.

At frying temperature, the results showed that the quality changes in the oils with or without added PS extracts are affected by high temperature and there is an increased deterioration as the heating time is increased. The results also indicate that the PSE extract and PSL extract have a significantly positive protective effect on both rice bran oil and corn oil during heating at frying temperatures. The most effective extracts were 0.2 % PSE, 0.05 % PSL and 0.1 % PSL because these concentrations show a significant decrease in acid value and polar compounds compared to oils with added BHT and of course lower than the synthetic antioxidant free oils. It means that 0.2 % PSE, 0.05 % PSL and 0.1 % PSL have a better performance than the synthetic antioxidant, BHT. The pigments contained in these natural crude extracts (PSE and PSL) did not seem to have had an impact from photo-oxidation due to they are degraded or destroyed at the frying temperatures.

To summarise, the results indicate that the PSE and PSL extracts could retard thermal degradation of repeatedly heated rice bran oil and corn oil at a frying temperature of 180 °C for 25 hours and the extracts had a protective effect better than BHT. Therefore, *Piper sarmentosum* Roxb. leaf extract shows high potential to be used as an alternative natural antioxidant in frying oils. Also, it is evident that the action of the antioxidants (either natural or synthetic) at frying temperatures, are not the same as at low or moderate temperatures. At high temperature, the loss of water or moisture from fried material can activate or enhance the antioxidant activity of the hydrophilic (polar) antioxidant, so this may be one reason why the polar antioxidants or PSE extracts showed more effective protective effect at frying temperature and the non-polar (lyophilic) antioxidants more effective in storage. Therefore, it is important to look at a

range of temperatures and oils as what might be successful in a storage test might not be successful at frying temperatures, and vice versa.

4.2 Recommendation for future work

1) The optimised analytical method using UHPLC-PDA-ESI-MS in this study showed a good resolution of peaks with the PSE extract only. This method could detect only a few compounds in the PSL extract. To improve this, further work should amend the analytical method so as to detect more compounds from the PSL extract. This could be done by changing the binary gradient of the acetonitrile content (or organic mobile phase). As the crude extracts are natural antioxidants which are complex and comprise of different compounds (Pokorny, 2010) and compounds in the PSL extract are likely to be nonpolar compounds, so by adjusting binary gradients, flavonoid and alkaloid compounds will be separated better. In addition there were a number of unidentified compounds which were found using the single quadrupole mass spectrometer. Without standard compounds and/or if the identified compounds have very close retention times with the same m/z ratio (isomer or derivatives), this method cannot identify or distinguish isomer compounds. To elucidate the proposed tentative compounds and their derivatives (or isomers) obtained by this study, more information is needed of molecular structure (Fulcrand *et al.*, 2008). To obtain structural information, the analyte ions are fragmented by a process known as collision-induced dissociation (CID) or collision-activated dissociation (CAD) (Agilent Technologies, 2011a). The CID is mostly associated with multi-stage MS (also called tandem MS or MS/MS or MS^n) which is a powerful way to obtain structural information. In triple-quadrupole (or quadrupole / quadrupole / time-of-flight instruments (Q-TOF)), the first quadrupole is used to select the

precursor ion. CID takes place in the second stage (quadrupole or octopole), then the third stage (quadrupole or TOF) will generate a spectrum of the resulting identify particular ions and derivatives (Agilent Technologies, 2011a). Some of the successive works using these multiple techniques can be found in the study by Puigventos *et al.* (2015). They used tandem spectrometry to analyse an authentication of fruit-based products and fruit-based pharmaceutical preparations. Alonso-Salces *et al.* (2004) used LC-MS with atmospheric pressure ionisation (APCI) to obtain molecular weight, number of hydroxyl groups, number of sugars and an idea about the substitution pattern of apple polyphenols. Oszmianski *et al.* (2011) used triple quadrupole mass spectrometer equipped with electrospray ionisation source to identified and quantified flavonoids and phenolic acids compounds in berry leaf extracts. The ion-trap mass analyser also has a very helpful in identifying unknown compounds (Fulcrand *et al.*, 2008). Fischer *et al.* (2011) used an ion-trap mass analyser for identification and quantification of phenolic compounds from pomegranate peel, mesocarp, aril and differently produced juices. Aladedunye and Matthaeus (2014) used Q-TOF mass spectrometer to identified phenolic compounds from rowanberry fruit extract and crabapple fruit extract. So, the further work can be done to find out the unidentified compounds or analyse other phytochemical compounds present in the PS leaf by using these techniques.

2) In accelerated storage conditions, the PSE and PSL extracts did not show a protective effect in both oils. This could be because of the pigments contained in the extracts. Chlorophyll can have an effect on the rancidity of oils (Pokorny, 2010; Hall *et al.*, 1994). When chlorophyll is in the presence of light autoxidation will occur via the photo-oxidation route leading to the formation of hydroperoxides

(Gordon, 2001). However, it was unable to decolourise the extracts as the results of decolourisation in chapter 3.3, where activated carbon was used to remove the pigments in the crude extract led to a huge loss of total phenol content and its antioxidant activity. However, in order to determine the effect of pigments contained in the extract on photo-oxidation, the experiment could be repeated by controlling the light throughout the storage times and the decolourised extracts should also be investigated. By comparing all the results, decolourised and non-decolourised, controlled and uncontrolled light, the influence of the pigments and the antioxidant activity of the extracts on lipid oxidation could be evaluated.

3) The PSE and PSL extracts were seen to retard thermal degradation of rice bran oil and corn oil. The PSL extract which contains non-polar compounds may have more advantage in terms of solubility in oil. Thus, the PSL extract can be used in oil and emulsion food systems. However, the concentration ranges of the extracts used in this study were limited. Future work should look at a wider concentration range of the extracts using the findings from this study as a guideline. Based on the results, PSE extract showed an increasing protective trend when the concentration increased, whereas PSL showed a pro-oxidant effect when the concentration increased. So, the range of concentrations used for PSE extracts should be increased and decreased for PSL extracts. Future work should trial both polar and non-polar antioxidants at low and high temperatures, and also should control the light throughout storage time. From this, the best effective concentration of PSE or PSL extract can be obtained.

Bibliography

Aalto-Korte, K. 2000. Allergic contact dermatitis from tertiary-butylhydroquinone (TBHQ) in a vegetable hydraulic oil. *Contact Dermatitis.* **43**(5), pp.303-303.

AbouGharbia, H.A.Shahidi, F.Shehata, A.A.Y. and Youssef, M.M. 1996. Oxidative stability of extracted sesame oil from raw and processed seeds. *Journal of Food Lipids.* **3**(1), pp.59-72.

Agilent Technologies. 2011a. *Basics of LC/MS.* [Online]. [Accessed 7 August 2014]. Available from: https://www.agilent.com/chem

Agilent Technologies. 2011b. *Fundamentals of Liquid Chromatography (HPLC).* [Online]. [Accessed 24 December 2015]. Available from: polymer.ustc.edu.cn/xwxx_20/xw/.../P020110906263097048536.pdf

Aladedunye, F. and Matthaeus, B. 2014. Phenolic extracts from Sorbus aucuparia (L.) and Malus baccata (L.) berries: Antioxidant activity and performance in rapeseed oil during frying and storage. *Food Chemistry.* **159**, pp.273-281.

Aladedunye, F.A. 2014. Natural antioxidants as stabilizers of frying oils. *European Journal of Lipid Science and Technology.* **116**(6), pp.688-706.

Aladedunye, F.A. and Przybylski, R. 2009. Degradation and Nutritional Quality Changes of Oil During Frying. *J. Am. Oil Chemistes'Soc.* **86**, pp.149-156.

Allam, S.S.M. and Mohamed, H.M.A. 2002. Thermal stability of some commercial natural and synthetic antioxidants and their mixtures. *Journal of Food Lipids.* **9**(4), pp.277-293.

Alonso-Salces, R.M.Ndjoko, K.Queiroz, E.F.Ioset, J.R.Hostettmann, K.Berrueta, L.A.Gallo, B. and Vicente, F. 2004. On-line characterisation of apple polyphenols by liquid chromatography coupled with mass spectrometry and ultraviolet absorbance detection. *Journal of Chromatography A.* **1046**(1-2), pp.89-100.

Alvarez, M.D.C.F.Molina-Hernandez, E.F.Concepcion Hernandez-Raya, J. and Elena Sosa-Morales, M. 2012. The Effect of Food Type (Fish Nuggets or French Fries) on Oil Blend Degradation during Repeated Frying. *Journal of Food Science.* **77**(11), pp.C1136-C1143.

Ammari, F.Jouan-Rimbaud-Bouveresse, D.Boughanmi, N. and Rutledge, D.N. 2012. Study of the heat stability of sunflower oil enriched in natural antioxidants by different analytical techniques and front-face fluorescence spectroscopy combined with Independent Components Analysis. *Talanta.* **99**, pp.323-329.

Anesini, C.Ferraro, G.E. and Filip, R. 2008. Total polyphenol content and antioxidant capacity of commercially available tea (Camellia sinensis) in Argentina. *Journal of Agricultural and Food Chemistry.* **56**(19), pp.9225-9229.

Angelis, A.Urbain, A.Halabalaki, M.Aligiannis, N. and Skaltsounis, A.-L. 2011. One-step isolation of gamma-oryzanol from rice bran oil by non-aqueous hydrostatic countercurrent chromatography. *Journal of Separation Science.* **34**(18), pp.2528-2537.

AOCS. 1950. Spectrophotometric method for vegetable oil colour adopted. *Chemical & Engineering News*. 22 May 1950, p.1743.

AOCS. 1975. *Official and tentative methods of the American Oil Chemists' Society.* 3rd ed. Champaign, Ill.: American Oil Chemists' Society (AOCS).

AOCS. 1998a. AOCS Official method Cd 3a-63. Acid value. In: Firestone, D. ed. *Official Methods and Recommended Practices of the American Oil Chemists' Society.* 5th ed. Champaign, Ill: American Oil Chemist's Society.

AOCS. 1998b. AOCS Official method Cd 8-53. Peroxide value. In: Firestone, D. ed. *Official Methods and Recommended Practices of the American Oil Chemists' Society.* 5th ed. Champaign, Ill: American Oil Chemist's Society.

AOCS. 1998c. AOCS Official method Cc 9a-48. Smoke, Flash and Fire Points. In: Firestone, D. ed. *Official Methods and Recommended Practices of the American Oil Chemists' Society.* 5th ed. Champaign, Ill: American Oil Chemist's Society.

AOCS. 1998d. AOCS Official method Cc 13c-50. Colour. Photometric method. In: Firestone, D. ed. *Official Methods and Recommended Practices of the American Oil Chemists' Society.* 5th ed. Champaign, Ill: American Oil Chemist's Society.

AOCS. 1998e. AOCS Official method Cd 19-90. 2-Thiobarbituric acid value. Direct method. In: Firestone, D. ed. *Official Methods and Recommended Practices of the American Oil Chemists' Society.* 5th ed. Champaign, Ill: American Oil Chemist's Society.

Apak, R.Gorinstein, S.Boehm, V.Schaich, K.M.Ozyurek, M. and Guclu, K. 2013. Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). *Pure and Applied Chemistry.* **85**(5), pp.957-998.

Artz, W.Osidacz, P. and Coscione, A. 2005. Acceleration of the thermooxidation of oil by heme iron. *J. Am. Oil Chemistes'Soc.* **82**, pp.579-584.

Arumughan, C.Bhat, K.K. and Sen, D.P. 1984. Evaluation of some chemical methods for the measurement of the progress of oxidative of oxidative deterioration in edible oils. *Journal of Food Science and Technology-Mysore.* **21**(6), pp.395-399.

Atares, L.Marshall, L.J.Akhtar, M. and Murray, B.S. 2012. Structure and oxidative stability of oil in water emulsions as affected by rutin and homogenization procedure. *Food Chemistry.* **134**(3), pp.1418-1424.

Augustin, M.A. and Berry, S.K. 1983. Efficacy of the Antioxidants BHA and BHT in palm olein during heating and frying. *J. Am Oil Chem Soc.* **60**, pp.1520-1523.

Augustin, M.A.Lee, K.H. and Yau, K.T. 1987. Comparison of the frying performance of market samples of palm olein corn oil and soy oil in Malaysia. *Pertanika.* **10**(3), pp.295-304.

Ayusuk, S.Siripongvutikorn, S.Thummaratwasik, P. and Usawaksesmanee, W. 2009. Effect of heat treatment on antioxidant properties of Tom-Kha paste and herbs/spices used in Tom-Kha paste. *Kasetsart J.* **43**, pp.305-312.

Azam, S.Hadi, N.Khan, N.U. and Hadi, S.M. 2003. Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. *Med Sci Monit.* **9**(9), pp.325-330.

Baixauli, R.Salvador, A.Fiszman, S.M. and Calvo, C. 2002. Effect of oil degradation during frying on the color of fried, battered squid rings. *Journal of the American Oil Chemists Society.* **79**(11), pp.1127-1131.

Balasundram, N.Sundram, K. and Samman, S. 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry.* **99**(1), pp.191-203.

Bansal, G.Zhou, W.Barlow, P.J.Lo, H.-L. and Neo, F.-L. 2010. Performance of palm olein in repeated deep frying and controlled heating processes. *Food Chemistry.* **121**(2), pp.338-347.

Bensmira, M.Jiang, B.Nsabimana, C. and Jian, T. 2007. Effect of Lavender and Thyme incorporation in sunflower seed oil on its resistance to frying temperatures. *Food Research International.* **40**(3), pp.341-346.

Benzie, I.F.F. and Strain, J.J. 1999. Ferric reducing antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Oxidants and Antioxidants, Pt A.* **299**, pp.15-27.

Bera, D.Lahiri, D. and Nag, A. 2006. Studies on a natural antioxidant for stabilization of edible oil and comparison with synthetic antioxidants. *Journal of Food Engineering.* **74**(4), pp.542-545.

Berger, K.G. 1984. The practise of frying. *PORIM Technology No.9.* Palm Oil Research Institute of Malaysia.

Bhattacharya, A.B.Sajilata, M.G. and Singhal, R.S. 2008. Lipid profile of foods fried in thermally polymerized palm oil. *Food Chemistry.* **109**(4), pp.808-812.

Bheemreddy, R.Chinnan, M.Pannu, K. and Reynolds, A. 2002. Active treatment of frying oil for enhanced fry-life. *J. Food Sci.* **67**, pp.1478-1484.

Bird, R.P.Draper, H.H. and Basrur, P.K. 1982. Effect of malonaldehyde and acetaldehyde on cultured mammalian cells: production of micronuclei and chrosomal aberrations. *Mutation Research.* **101**(3), pp.237-246.

Blumenthal, M. 1991. A new look at the chemistry and physics of deep-fat frying. *J. Food Technol.* **45**(2), pp.68-71, 94.

Bockisch, M. 1998. *Fats and Oils Handbook.* Champaign, IL: AOCS Press.

Boekel, V. and Martinus, A.J.S. 2000. Kinetic modelling in food science: A case study on chlorophyll degradation in olives. *Journal of the Science of Food and Agriculture.* **80**(1), pp.3-9.

Boskou, D. 2010. Frying Fats. In: Kolakowska, A. ed. *Chemical, Biological and Function Aspects of Food Lipids.* CRC Press, pp.429-454.

Bravo, L. 1998. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews.* **56**(11), pp.317-333.

Brito, A.Ramirez, J.E.Areche, C.Sepulveda, B. and Simirgiotis, M.J. 2014. HPLC-UV-MS Profiles of Phenolic Compounds and Antioxidant Activity of Fruits from Three Citrus Species Consumed in Northern Chile. *Molecules.* **19**(11), pp.17400-17421.

Brown, J.R. 2013. *An assessment of the effect of high heat temperature on specific plant frying oils.*

Buck, D.F. 1991. Antioxidants. In: Smith, J. ed. *Food Additives User's Handbook.* London, UK: Blackie and Son.

Busque, F.de March, P.Figueredo, M.Font, J. and Sanfeliu, E. 2002. Total synthesis of four Pandanus alkaloids: pandamarilactonine-A and -B and their chemical precursors norpandamarilactonine-A and -B. *Tetrahedron Letters.* **43**(32), pp.5583-5585.

Chang, S.S.Ostricmatijasevic, B.Hsieh, O.A.L. and Huang, C.L. 1977. Natural antioxdants from rosemary and sage. *Journal of Food Science.* **42**(4), pp.1102-1106.

Chanwitheesuk, A.Teerawutgulrag, A. and Rakariyatham, N. 2005. Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. *Food Chemistry.* **92**(3), pp.491-497.

Che Man, Y. and Tan, C. 1999. Effects of natural and synthetic antioxidants on changes in refined, bleached and deodorized palm olein during deep fat frying of potato chips. *J. Am. Oil Chemistes'Soc.* **76**, pp.331-339.

Cheeptham, N. and Towers, G.H.N. 2002. Light-mediated activities of some Thai medicinal plant teas. *Fototerapia.* **73**, pp.651-662.

Child, J. 2012. *The Fat Primer.* [Online]. [Accessed 29 January 2015]. Available from: http://www.raw-milk-facts.com/fat_primer_T3.html

Chizzola, R.Michitsch, H. and Franz, C. 2008. Antioxidative properties of Thymus vulgaris leaves: Comparison of different extracts and essential oil chemotypes. *Journal of Agricultural and Food Chemistry.* **56**(16), pp.6897-6904.

Choe, E. and Lee, J. 1998. Thermooxidative stability of soybean oil, beef tallow, and palm oil during frying of steamed noodles. *Korean J. Food Sci Technol.* **30**, pp.288-292.

Choe, E. and Min, B. 2007. Chemistry of Deep-Fat Frying Oils; Concise Reviews in Food Science. *Journal of Food Science.* **72**(5), pp.77-86.

Chotimarkorn, C.Benjakul, S. and Silalai, N. 2008. Antioxidative effects of rice bran extracts on refined tuna oil during storage. *Food Research International.* **41**(6), pp.616-622.

Clark, W. and Serbia, G. 1991. Safety aspects of frying fats and oils. *Food Technol.* **45**, pp.84-89.

Clifford, H. 2001. Sources of natural antioxidants: oilseeds, nuts, cereals, legumes, animal products and microbial sources. In: Pokorny, J., et al. eds. *Antioxidants in food.* Cambridge, England: Woodhead Publishing Limited, pp.180-188.

Codex Alimentarius. 1999. *Codex Standard for Named Vegetable Oils (CODEX-STAN 210 - 1999).* [Online]. [Accessed 12 February 2015]. Available from: www.codexalimentarius.org/input/download/standards/.../CXS_210e.pdf

Crawford, D.L.Sinnhube.RoStout, F.M.Oldfield, J.E. and Kaufmes, J. 1965. Acute toxicity of malonaldehyde. *Toxicology and Applied Pharmacology.* **7**(6), p826.

Cuesta, C.Sanchez-Muniz, F.Garrido-Polonio, M.Lopez-Varela, M. and Arroyo, R. 1993. Thermooxidative and hydrolytic changes in sunflower oil used in frying with a fast turnover of fresh oil. *J. Am. Oil Chemistes'Soc.* **70**, pp.1069-1073.

D'Archivio, M.Filesi, C.Di Benedetto, R.Gargiulo, R.Giovannini, C. and Masella, R. 2007. Polyphenols, dietary sources and bioavailability. *Annali dell'Istituto superiore di sanita.* **43**(4), pp.348-61.

Dabrowski, A.Podkoscielny, P.Hubicki, Z. and Barczak, M. 2005. Adsorption of phenolic compounds by activated carbon - a critical review. *Chemosphere.* **58**(8), pp.1049-1070.

Dana, D.Blumenthal, M.M. and Saguy, I.S. 2003. The protective role of water injection on oil quality in deep fat frying conditions. *European Food Research and Technology.* **217**(2), pp.104-109.

Das, N.P. and Pereira, T.A. 1990. Effect of flavonoids on thermal autoxidation of palm oil structure activity relationships. *Journal of the American Oil Chemists' Society.* **67**(4), pp.255-258.

David, W.R.Dorris, A.L. and Ronald, R.E. 2002. Antioxidants. *Food Lipids.* CRC Press.

Deepam, L.S.A.Sundaresan, A. and Arumughan, C. 2011. Stability of Rice Bran Oil in Terms of Oryzanol, Tocopherols, Tocotrienols and Sterols. *Journal of the American Oil Chemists Society.* **88**(7), pp.1001-1009.

Dengate, S. 2015. *BHA and other antioxidants.* [Online]. [Accessed 16 April 2015]. Available from: http://fedup.com.au/factsheets/additive-and-natural-chemical-factsheets/320-bha-other-antioxidants#labelling

DGF. 2008. Optimum deep-frying. Germany: Geutsche Gesellschaft Fur Fettwissenschaft e.V. (German Society for Fat Science e.V.), pp.1-22.

Dobarganes, M.C. and Marquez-Ruiz, G. 1998. Regulation of used frying fats and validity of quick tests for discarding the fats. *Grasas Y Aceites.* **49**(3-4), pp.331-335.

Draper, H.H.McGirr, L.G. and Hadley, M. 1986. The metabolism of malondialdehyde. *Lipids.* **21**(4), pp.305-307.

Elhamirad, A.H. and Zamanipoor, M.H. 2012. Thermal stability of some flavonoids and phenolic acids in sheep tallow olein. *European Journal of Lipid Science and Technology.* **114**(5), pp.602-606.

Endo, Y.Usuki, R. and Kaneda, T. 1985. Antioxidant effects of chlorophyll and pheophytin on the autoxidation of oils in the dark, a comparison of the inhibitory effects. *Journal of the American Oil Chemists Society.* **62**(9), pp.1375-1378.

Farah, A. and Donangelo, C.M. 2006. Phenolic compounds in coffee. *Brazilian Journal of Plant Physiology.* **18**(1), pp.23-36.

Farhoosh, R. and Moosavi, S.M.R. 2009. Evaluating the Performance of Peroxide and Conjugated Diene Values in Monitoring Quality of Used Frying Oils. *J. Agric Sci Technol.* **11**, pp.173-179.

Fattouch, S.Caboni, P.Coroneo, V.Tuberoso, C.Angioni, A.Dessi, S.Marzouki, N. and Cabras, P. 2008. Comparative analysis of polyphenolic profiles and antioxidant and antimicrobial activities of Tunisian pome fruit pulp and peel aqueous acetone extracts. *Journal of Agricultural and Food Chemistry.* **56**(3), pp.1084-1090.

Fennema, O.R. 2008. *Food chemistry.* 4th / edited by Srinivasan Damodaran, Kirk Parkin, and Owen R. Fennema. ed. Boca Raton ; London: CRC Press.

Fischer, U.A.Carle, R. and Kammerer, D.R. 2011. Identification and quantification of phenolic compounds from pomegranate (Punica granatum L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD-ESI/MSn. *Food Chemistry.* **127**(2), pp.807-821.

Fisherman, E.W. and Cohen, G. 1973. Chemical intolerance to butylated-hydroxyanisole (BHA) and butylated-hydrozytoluene (BHT) and vascular-response as an indicator and monitor of drug intolerance. *Annals of Allergy.* **31**(3), pp.126-133.

Floegel, A.Kim, D.-O.Chung, S.-J.Koo, S.I. and Chun, O.K. 2011. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *Journal of Food Composition and Analysis.* **24**(7), pp.1043-1048.

Food Network Solution. 2011. *Structure of Triglycerides.* [Online]. [Accessed 5 January 2012]. Available from: http://www.foodnetworksolution.com/home

Food Standard Agency. 2015. *Guidance notes on quantitative ingredient declaration (QUID). The food labelling regulations 1996.* [Online]. [Accessed 13/07/2015]. Available from: http://www.food.gov.uk/sites/default/files/multimedia/pdfs/quid.pdf

Franco, D.Sineiro, J.Rublilar, M.Sanchez, M. and jerez, M. 2008. Polyphenols from plant materials: Extraction and antioxidant power. *Electronic journal of Environmental, Agricultural and Food Chemistry.* **7**(8), pp.3210-3216.

Frankel, E.N. 1998a. Antioxidants. In: Frankel, E.N. ed. *Lipid Oxidation.* Dundee, Scotland: Oily Press, p.142.

Frankel, E.N. 1998b. Free radical oxidation. In: Frankel, E.N. ed. *Lipid Oxidation* Glasgow, Scotland.: The Oily Press LTD., pp.13-22.

Frankel, E.N. 1998c. Frying fats. In: Frankel, E.N. ed. *Lipid Oxidation* Glasgow, Scotland.: The Oily Press LTD., pp.227-248.

Frankel, E.N. 1998d. Classification of Lipids. In: Frankel, E.N. ed. *Lipid Oxidation* Glasgow, Scotland.: The Oily Press LTD., pp.1-6.

Frankel, E.N.Huang, S.W.Aeschbach, R. and Prior, E. 1996. Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion. *Journal of Agricultural and Food Chemistry.* **44**(1), pp.131-135.

Frankel, N.E.Warner, K. and Moulton, K. 1985. Effects of hydrogenation and additives on cooking oil performance of soybean. *J. Am. Oil Chemistes'Soc.* **62**, pp.1354-1358.

Fritsch, C.W. 1981. Measurement of Frying Fat Deterioration. *J. Am. Oil Chemistes'Soc.* **58**, pp.272-274.

Frynn. 2015. *Herb & Vegetable.* [Online]. [Accessed 5 January 2015]. Available from: http://frynn.com

Fuhrman, B.Volkova, N.Rosenblat, M. and Aviram, M. 2000. Lycopene Synergistically Inhibits LDL Oxidation in Combination with Vitamin E, Glabridin, Rosmarinic Acid, Carnosic Acid, or Garlic. *Antioxidants & Redox Signaling.* **2**(3), pp.491-506.

Fukasawa, R.Kanda, A. and Hara, S. 2009. Anti-oxidative Effects of Rooibos Tea Extract on Autoxidation and Thermal Oxidation of Lipids. *Journal of Oleo Science.* **58**(6), pp.275-283.

Fukuda, Y.Nagata, M.Osawa, T. and Namiki, M. 1986. Chemical aspects of the antioxidative activity of roasted sesame seed oil, and the effect of using the oil for frying. *Agricultural and Biological Chemistry.* **50**(4), pp.857-862.

Fulcrand, H.Mane, C.Preys, S.Mazerolles, G.Bouchut, C.Mazauric, J.-P.Souquet, J.-M.Meudec, E.Li, Y.Cole, R.B. and Cheynier, V. 2008. Direct mass spectrometry approaches to characterize polyphenol composition of complex samples. *Phytochemistry.* **69**(18), pp.3131-3138.

Gangopadhyay, G.Bandyopadhyay, T.Modak, B.K.Wongpornchai, S. and Mukherjee, K.K. 2004. Micropropagation of Indian pandan (Pandanus amaryllifolius Roxb.), a rich source of 2-acetyl-1-pyrroline. *Current Science.* **87**(11), pp.1589-1592.

George, D. 2011a. General Linear Models: MANOVA and MANCOVA. *SPSS for Windows step by step : a simple guide and reference, 18.0 update.* 11th ed. Boston, Mass. ; London: Allyn & Bacon, pp.294-306.

George, D. 2011b. Bivariate CORRELATION: Bivariate correlations, partial correlations and the correlation matrix. *SPSS for Windows step by step : a simple guide and reference, 18.0 update.* 11th ed. Boston, Mass. ; London: Allyn & Bacon, pp.123-132.

Ghasemzadeh, A. and Jaafar, H.Z.E. 2013. Profiling of phenolic compounds and their antioxidant and anticancer activities in pandan (Pandanus amaryllifolius Roxb.) extracts from different locations of Malaysia. *Bmc Complementary and Alternative Medicine.* **13**.

Giada, M.d.L.R. 2013a. *Food Phenolic Compounds: Main Classes, Sources and Their Antioxidant Power.*

Giada, M.d.L.R. 2013b. *Food Phenolic Compounds: Main Classes, Sources and Their Antioxidant Power.* [Online]. [Accessed 4 January 2014]. Available from: http://www.intechopen.com/books

Giese, J. 1996. Antioxidants: Tools for preventing lipid oxidation. *Food Technology.* **50**(11), pp.73-&.

Giusti, M.M. and Wrolstad, R.E. 2005. Characterisation and measurement of anthocyanins by UV-Visible Spectroscopy. In: Ronald E. Wrolstad, et al. eds. *Handbook of food analytical chemistry.* New Jersey: A John Wiley & Sons, Inc., Publication, pp.7-69.

Gordon, M.H. 1996. Dietary antioxidants in disease prevention. *Natural Product Reports.* **13**(4), pp.265-273.

Gordon, M.H. 2001. The development of oxidative rancidity in foods. In: Pokorny, J., et al. eds. *Antioxidants in food: practical application.* Cambridge, England: Woodhead Publishing Limited, pp.7-21.

Gordon, M.H. and Kourimska, L. 1995. The effects of antioxidants on changes in oils during heating and deep frying. *Journal of the Science of Food and Agriculture.* **68**(3), pp.347-353.

Graversen, H.B.Becker, E.M.Skibsted, L.H. and Andersen, M.L. 2008. Antioxidant synergism between fruit juice and alpha-tocopherol. A comparison between high phenolic black chokeberry (Aronia melanocarpa) and high ascorbic blackcurrant (Ribes nigrum). *European Food Research and Technology.* **226**(4), pp.737-743.

Grice, H.C. 1986. Safety evaluation of BHT in the liver, lung and gastrointestinal tract. *Food and Chemical Toxicology.* **24**, pp.1127-1130.

Gunstone, F.D. 2002. *Vegetable oils in food technology: composition, properties and uses.* New York: Blackwell Publishing Ltd.

Gunstone, F.D. 2004. Chemical properties related to unsaturated centres. *The chemistry of oils and fats.* Cornwall, UK: Blackwell Publishing Ltd., pp.146-188.

Gutierrez, R.Gonzalez, Q. and Dobaranes, M.C. 1988. Analytical procedures for the evalution of used frying fats. In: Varela, G., et al. eds. *Frying of Food, Principles, Changes, New Approaches.* England: Ellis Horwood, p.141.

Hall, C.Cuppett, S.Wheeler, D. and Fu, X.P. 1994. Effects of bleached and unbleached rosemary oleoresin and rosmariquinone on light sensitized oxidation of soybean oil. *Journal of the American Oil Chemists Society.* **71**(5), pp.533-535.

Halliwell, B.Aeschbach, R.Loliger, J. and Aruoma, O.I. 1995. The characterization of antioxidants. *Food and Chemical Toxicology.* **33**(7), pp.601-617.

Hamama, A.A. and Nawar, W.W. 1991. Thermal decomposition of some phenolic antioxidnats. *Journal of Agricultural and Food Chemistry.* **39**(6), pp.1063-1069.

Hashemi, M.B.Niakousari, M.Saharkhiz, M.J. and Eskandari, M.H. 2011. Influence of Zataria multiflora Boiss. essential oil on oxidative stability of sunflower oil. *European Journal of Lipid Science and Technology.* **113**(12), pp.1520-1526.

Henan Kingman M&E Complete Plant Co., L. 2015. *Rice bran oil project.* [Online]. [Accessed 5 January 2015]. Available from: http://www.oilmillmachinery.net/rice-bran-oil-project.html

Holownia, K.Chinnin, M.Erickson, M. and Mallikarjunan, P. 2000. Quality evaluation of edible film-coated chicken strips and frying oils. *J. Food Sci.* **65**, pp.1087-1090.

Honglian, S.Noriko, N. and Etsuo, N. 2001. Natural antioxidants. In: Pokorny, J., et al. eds. *Antioxidants in food: practical application.* Cambridge, England: Woodhead Publishing Limited, pp.147-158.

Hras, A.R.Hadolin, M.Knez, Z. and Bauman, D. 2000. Comparison of antioxidative and synergistic effects of rosemary extract with alpha-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. *Food Chemistry.* **71**(2), pp.229-233.

Hussain, K.Ismail, Z.Sadikun, A. and Ibrahim, P. 2010. Standardization and In Vivo Antioxidant Activity of Ethanol Extracts of Fruit and Leaf of Piper sarmentosum. *Planta Medica.* **76**(5), pp.418-425.

Hussain, K.Ismail, Z.Sadikun, A. and Ibrahim, P. 2011. Bioactive Markers Based Pharmacokinetic Evaluation of Extracts of a Traditional Medicinal Plant, Piper sarmentosum. *Evidence-Based Complementary and Alternative Medicine.* pp.1-7.

Ignat, I.Volf, I. and Popa, V.I. 2011. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry.* **126**(4), pp.1821-1835.

IUPAC. 1987a. Determination of polar compounds in frying fats (2.507). In: Paquot, C. and Hautfenne, A. eds. *Standard methods of the Oils and Fats. .* 7th rev. ed. Oxford : Blackwell Scientific: International Union of Pure and Applied Chemistry (IUPAC).

IUPAC. 1987b. Determination of the 2-thiobarbituric acid value: direct method (2.531). In: Paquot, C. and Hautfenne, A. eds. *Standard methods of the Oils and Fats. .* 7th rev. ed. Oxford : Blackwell Scientific: International Union of Pure and Applied Chemistry (IUPAC).

IUPAC. 1987c. Determination of the *p*-anisidine value (2.504). In: Paquot, C. and Hautfenne, A. eds. *Standard methods of the Oils and Fats. .* 7th rev. ed. Oxford : Blackwell Scientific: International Union of Pure and Applied Chemistry (IUPAC).

IUPAC. 1987d. *Standard methods of the Oils and Fats. .* 7th rev. ed. Oxford : Blackwell Scientific: International Union of Pure and Applied Chemistry (IUPAC).

Jaswir, I.Che Man, Y. and Kitt, D. 2000. Synergistic effects of rosemary, sage and citric acid on fatty acid retention of palm olein during deep fat frying. *J. Am. Oil Chemistes'Soc.* **77**, pp.527-533.

Jayaprakasha, G.K.Singh, R.P. and Sakariah, K.K. 2001. Antioxidant activity of grape seed (Vitis vinifera) extracts on peroxidation models in vitro. *Food Chemistry.* **73**(3), pp.285-290.

Jennings, B.H. and Akoh, C.C. 2009. Effectiveness of natural versus synthetic antioxidants in a rice bran oil-based structured lipid. *Food Chemistry.* **114**(4), pp.1456-1461.

Jimenez, P.Masson, L.Barriga, A.Chavez, J. and Robert, P. 2011. Oxidative stability of oils containing olive leaf extracts obtained by pressure, supercritical and solvent-extraction. *European Journal of Lipid Science and Technology.* **113**(4), pp.497-505.

Kahkonen, M.P.Hopia, A.I.Vuorela, K.J.Rauha, J.P. and Pihlaja, K. 1999. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* **47**, pp.3954-3962.

Khan, M.A. and Shahidi, F. 2000. Oxidative stability of stripped and nonstripped borage and evening primrose oils and their emulsions in water. *Journal of the American Oil Chemists Society.* **77**(9), pp.963-968.

Kim, D.K. and Lee, C.Y. 2005a. Ultrasound-assisted aqueous methanol extraction of polyphenolics. In: Ronald E. Wrolstad, et al. eds. *Handbook of food analytical chemistry.* A John Wiley & Sons, Inc., Publication, pp.471-472.

Kim, D.K. and Lee, C.Y. 2005b. Extraction and isolation of polyphenolics. In: Ronald E. Wrolstad, et al. eds. *Handbook of food analytical chemistry.* A John Wiley & Sons, Inc., Publication, pp.471-481.

Kim, D.O.Lee, K.W.Lee, H.J. and Lee, C.Y. 2002. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *Journal of Agricultural and Food Chemistry.* **50**(13), pp.3713-3717.

Kim, I. and Choe, E. 2003. Effects of red ginseng extract added to dough on the lipid oxidation of frying oil and fried dough during frying and storage. *Food Sci Biotechnol.* **12**, pp.67-71.

Kim, I. and Choe, E. 2004. Oxidative stability and antioxidant content changes in roasted and bleached sesame oil during heating. *J. Food Sci Biotechnol.* **13**, pp.762-767.

Kim, I.Kim, C. and Kim, D. 1999. Physicochemical properties of methyl linoleate oxidized at various temperatures. *Korean J. Food Sci Technol.* **31**, pp.600-605.

Kochhar, S. 2000. Stable and healthful frying oil for the 21st century. *Inform.* **11**, pp.642-645.

Kochhar, S.P. 2001. The composition of frying oils. In: Rossell, J.B. ed. *Frying: Improving Quality.* Cambridge England: Woodhead Publishing Limited, pp.86-114.

Kupiec, T.P. 2004. Quality-control analytical methods: high-performance liquid chromatography. *International journal of pharmaceutical compounding.* **8**(3), pp.223-7.

Lafka, T.-I.Sinanoglou, V. and Lazos, E.S. 2007. On the extraction and antioxidant activity of phenolic compounds from winery wastes. *Food Chemistry.* **104**(3), pp.1206-1214.

Lampi, A.M.Kataja, L.Kamal-Eldin, A. and Vieno, P. 1999. Antioxidant activities of alpha- and gamma-tocopherols in the oxidation of rapeseed oil triacylglycerols. *Journal of the American Oil Chemists Society.* **76**(6), pp.749-755.

Landete, J.M. 2012. Updated Knowledge about Polyphenols: Functions, Bioavailability, Metabolism, and Health. *Critical Reviews in Food Science and Nutrition.* **52**(10), pp.936-948.

Lawson, H. 1995. *Food oils and fats: technology, utilization, and nutrition.* London: Chapman&Hall.

Lee, B.L.Su, J. and Ong, C.N. 2004. Monomeric C-18 chromatographic method for the liquid chromatographic determination of lipophilic antioxidants in plants. *Journal of Chromatography A.* **1048**(2), pp.263-267.

Lee, M.H. and Sher, R.L. 1984. Extraction of green tea antioxidants and their antioxidant activity in various edible oils and fats. *J. Chin. Agric. Chem. Soc.* **22**, pp.226-231.

Likhitwitayawuid, K.Ruangrungsi, N.Lange, G.L. and Decicco, C.P. 1987. Studies on Thai medicinal-plants. Structural elucidation and systhesis of new components isolated from *Piper sarmentosum* (Piperaceae). *Tetrahedron.* **43**(16), pp.3689-3694.

Lin, J.-Y. and Tang, C.-Y. 2007. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. *Food Chemistry.* **101**(1), pp.140-147.

Liu, D.Shi, J.Ibarra, A.C.Kakuda, Y. and Xue, S.J. 2008. The scavenging capacity and synergistic effects of lycopene, vitamin E, vitamin C, and beta-carotene mixtures on the DPPH free radical. *Lwt-Food Science and Technology.* **41**(7), pp.1344-1349.

Liu, H.-R. and White, P. 1992. High temperature stability of soybean oils with altered fatty acid compositions. *J. Am. Oil Chemistes'Soc.* **69**, pp.533-537.

Loughrey, K. 2005. Overview of colour analysis. In: Ronald E. Wrolstad, et al. eds. *Handbook of food analytical chemistry.* New Jersey: A John Wiley & Sons, Inc., Publication, pp.201-215.

Lumley, I.D. 1988. Polar Compounds in Heated Oils. In: Varela, G., et al. eds. *Frying of Food, Principles, Changes, New Approaches.* Chichester, England: Ellis Horwood, p.166.

Ma, J.Yang, H.Basile, M.J. and Kennelly, E.J. 2004. Analysis of polyphenolic antioxidants from the fruits of three pouteria species by selected ion monitoring liquid chromatography - Mass spectrometry. *Journal of Agricultural and Food Chemistry.* **52**(19), pp.5873-5878.

Madhavi, D.L. and Salunkhe, D.K. 1995. Antioxidants, lipid peroxidation and health aspects of dietary lipid oxidation products. In: Maga, J.A. and Tu, A.T. eds. *Food additive toxicology.* New York: M. Dekker Inc.

Mahae, N. and Chaiseri, S. 2009. Antioxidant activities and antioxidative components in extracts of *Alpinia galanga* (L.)Sw. *Kasetsart J.* **43**, pp.358-369.

Maizura, M.Aminah, A. and Wan Aida, W.M. 2011. Total phenolic content and antioxidant activity of kesum (Polygonum minus), ginger (Zingiber officinale) and turmeric (Curcuma longa) extract. *International Food Research Journal.* **18**, pp.529-534.

Man, Y.B.C. and Hussin, W.R.W. 1998. Comparison of the frying performance of refined, bleached and deodorized palm olein and coconut oil. *Journal of Food Lipids.* **5**(3), pp.197-210.

Man, Y.B.C. and Tan, C.P. 1999. Effects of natural and synthetic antioxidants on changes in refined, bleached, and deodorized palm olein during deep-fat frying of potato chips. *Journal of the American Oil Chemists Society.* **76**(3), pp.331-339.

Man, Y.B.C.SuhardiyonoAsbi, A.B.Azudin, M.N. and Wei, L.S. 1996. Aqueous enzymatic extraction of coconut oil. *Journal of the American Oil Chemists Society.* **73**(6), pp.683-686.

Mancini, F.J.Smith, L.M.Creveling, R.K. and Alshaikh, H.F. 1986. Effects of selected chemical treaments on quality of fats used for deep frying. *Journal of the American Oil Chemists Society.* **63**(11), pp.1452-1456.

Maria, R.G. 2013. *Food phenolic compounds: Main classes, sources and their antioxidant power.* [Online]. [Accessed 20 May 2015]. Available from: http://dx.doi.org/10.5772/51687

Marinova, E.M. and Yanishlieva, N.V. 1992. Effect of temperature on the antioxidative action of inhibitors in lipid autoxidation. *Journal of the Science of Food and Agriculture.* **60**(3), pp.313-318.

Marinova, E.M.Seizova, K.A.Totseva, I.R.Panayotova, S.S.Marekov, I.N. and Momchilova, S.M. 2012. Oxidative changes in some vegetable oils during heating at frying temperature. *Bulgarian Chemical Communications.* **44**(1), pp.57-63.

Mariod, A.A.Adamu, H.A.Ismail, M. and Ismail, N. 2010. Antioxidative effects of stabilized and unstabilized defatted rice bran methanolic extracts on the stability of rice bran oil under accelerated conditions. *Grasas Y Aceites.* **61**(4), pp.409-415.

Marmesat, S.Valasco, J.Marquez-Ruiz, G. and Dobarganes, M.C. 2007. A rapid method for determination of polar compounds in used frying fats and oils. *Grasas Y Aceites.* **58**(2), pp.179-184.

Marnett, L.J. 1999. Lipid peroxidation - DNA damage by malondialdehyde. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis.* **424**(1-2), pp.83-95.

Marquez-Ruiz, G.Victoria Ruiz-Mendez, M. and Velasco, J. 2014. Antioxidants in frying: Analysis and evaluation of efficacy. *European Journal of Lipid Science and Technology.* **116**(11), pp.1441-1450.

Marston, A. and Hostettmann, K. 2006. Separation and quantification of flavonoids. In: Andersen, O.M. and Markham, K.R. eds. *Flavonoids: chemistry, biochemistry and applications.* CRC Press, New York: Taylor & Francis Group, p.2.

Maskan, M. and Bagci, H.I. 2003. The recovery of used sunflower seed oil utilized in repeated deep-fat frying process. *European Food Research and Technology.* **218**(1), pp.26-31.

Masuda, T.Inazumi, A.Yamada, Y.Padolina, W.G.Kikuzaki, H. and Nakatani, N. 1991. Constituents of piperaceae, antimicrobial phenylpropanoids from *Piper sarmentotsum*. *Phytochemistry.* **30**(10), pp.3227-3228.

Mazza, G. and Qi, H. 1992. Effect of after-cooking darkening inhibitors on stability of frying oil and quality of French fries. *J. Am. Oil Chemistes'Soc.* **69**, pp.847-853.

McNicholas, H.J. 1935. Color and spectral transmittance of vegetable oils. *Journal of Research of the National Bureau of Standards.* **15**(2), pp.99-121.

Mensink, R.P. and Katan, M.B. 1990. Effect of dietary trans-fatty-acids on high density and low density lipoprotein cholesterol levels in healthy subjects. *New England Journal of Medicine.* **323**(7), pp.439-445.

Merrill, L.I.Pike, O.A.Ogden, L.V. and Dunn, M.L. 2008. Oxidative stability of conventional and high-oleic vegetable oils with added antioxidants. *Journal of the American Oil Chemists Society.* **85**(8), pp.771-776.

Michael, H.G. 2001. Antioxidant effects. In: Pokorny, J., et al. eds. *Antioxidants in food: practical application.* Cambridge, England: Woodhead Publishing Limited, p.18.

Miean, K.H. and Mohamed, S. 2001. Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. *Journal of Agricultural and Food Chemistry.* **49**(6), pp.3106-3112.

Misnawi, T.W.Susijahadi, E.N.D. and Noor, A.F. 2014. The utilisation of cocoa polyphenol extract to improve the shelf life of bulk frying oil used in open and deep frying. *International Food Research Journal.* **21**(1), pp.111-118

Moeslinger, T.Brunner, M.Volf, I. and Spieckermann, P.G. 1995. Spectrophotometric determination of ascorbic acid and dehydroascorbic acid. *Clinical Chemistry.* **41**(8), pp.1177-1181.

Moyano, M.J.Heredia, F.J. and Melendez-Martinez, A.J. 2010. The Color of Olive Oils: The Pigments and Their Likely Health Benefits and Visual and Instrumental Methods of Analysis. *Comprehensive Reviews in Food Science and Food Safety.* **9**(3), pp.278-291.

National Sunflower Association. 2015. *NuSun.* [Online]. [Accessed 12 February 2015]. Available from: http://www.sunflowernsa.com/oil/nusun/

Nawar, W.W. 1984. Chemical changes in lipids produced by thermal processing. *J. Chem Edu.* **61**, pp.299-302.

Naz, S.Sheikh, H.Siddiqi, R. and Sayeed, S.A. 2004. Oxidative stability of olive, corn and soybean oil under different conditions. *Food Chemistry.* **88**(2), pp.253-259.

Neff, W.E.Warner, K. and Byrdwell, W.C. 2000. Odor significance of undesirable degradation compounds in heated triolein and trilinolein. *Journal of the American Oil Chemists Society.* **77**(12), pp.1303-1313.

Nera, E.A.Lok, E.Iverson, F.Ormsby, E.Karpinski, K.F. and Clayson, D.B. 1984. Short-term pathological and proliferative effects of Butylated hydroxyanisole and other phenolic antioxidants in the forestomach of Fischer-344 rats. *Toxicology.* **32**(3), pp.197-213.

Nettleton, J.A. 1994. *omega-3 Fatty acids and Health.* . New York: Chapman and Hall. A Thomson publishing company.

Niamsa, N. and Chantrapromma, K. 1983. Chemical constituents of Piper sarmentosum Roxb. *Songklanakarin J. Sci. Technol.* **5**, pp.151-152.

Nielsen, S.S. 2010. Determination of fat content. Soxhlet method. In: Nielsen, S.S. ed. *Food analysis laboratory manual.* 2nd ed. New York ; London: Springer Science, pp.31-32.

Nor, M.F.Mohamed, S.Idris, N.A. and Ismail, R. 2008. Antioxidative properties of Pandanus amaryllifolius leaf extracts in accelerated oxidation and deep frying studies. *Food Chemistry.* **110**, pp.319-327.

North, J.J.Ndakidemi, P.A. and Laubscher, C.P. 2012. Effects of antioxidants, plant growth regulators and wounding on phenolic compound excretion during micropropagation of Sterlitzia Reginae. *International Journal of the Physical Sciences* **7**(4), pp.638 - 646.

Nystrom, L.Achrenius, T.Lampi, A.-M.Moreau, R.A. and Piironen, V. 2007. A comparison of the antioxidant properties of steryl ferulates with tocopherol at high temperatures. *Food Chemistry.* **101**(3), pp.947-954.

O'Brien, R.D. 2004. Raw Materials. *Fats and Oils: Formulating and processing for applications.* 2nd ed. New York: CRC Press, p.9.

Ochi, T.Tsuchiya, K.Aoyama, M.Maruyama, T. and Niiya, I. 1989. Study on the mixing ratio of alpha-tocopherol and delta-tocopherol for enrichment of vitamin E in cookies. *Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi.* **36**(2), pp.103-107.

Omura, K. 1995. Antioxidant synergism between butylated hydroxyanisole and butylated hydroxytoluene. *Journal of the American Oil Chemists Society.* **72**(12), pp.1565-1570.

Oszmianski, J.Wojdylo, A.Gorzelany, J. and Kapusta, I. 2011. Identification and Characterization of Low Molecular Weight Polyphenols in Berry Leaf Extracts by HPLC-DAD and LC-ESI/MS. *Journal of Agricultural and Food Chemistry.* **59**(24), pp.12830-12835.

Pandey, K.B. and Rizvi, S.I. 2009. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity.* **2**(5), pp.270-278.

Paul, S.Mittal, S. and Chinnan, S. 1997. Regulating the use of degraded oil/fat in deep-fat/oil food frying. *Critical Reviews in Food Science and Nutrition.* **37** (7), pp.635-662.

Pegg, R.B. 2005a. Lipid oxidation/Stability. In: Ronald E. Wrolstad, et al. eds. *Handbook of food analytical chemistry.* A John Wiley & Sons, Inc., Publication, pp.513-627.

Pegg, R.B. 2005b. Determination of TBA reactive substances in oils or lipid extracts: direct method. In: Ronald E. Wrolstad, et al. eds. *Handbook of food analytical chemistry.* A John Wiley & Sons, Inc., Publication, pp.551-553.

Pekkarinen, S.S.Stockmann, H.Schwarz, K.Heinonen, I.M. and Hopia, A.I. 1999. Antioxidant activity and partitioning of phenolic acids in bulk and emulsified methyl linoleate. *Journal of Agricultural and Food Chemistry.* **47**(8), pp.3036-3043.

Perrin, C. and Meyer, L. 2003. Simultaneous determination of ascorbyl palmitate and nine phenolic antioxidants in vegetable oils and edible fats by HPLC. *Journal of the American Oil Chemists Society.* **80**(2), pp.115-118.

Phomkaivon, N. and Areekul, V. 2009. Screening for antioxidant activity in selected Thai wild plants. *Asian Journal of Food and Agro-Industry.* **2**(4), pp.433-440.

Pimpa, B.Kanjanasopa, D. and Boonlam, S. 2009. Effect of addition of antioxidants on the oxidative stability of refined bleached and deodorized palm olein. *Kasetsart J.* **43**, pp.370-377.

Plimon. 2012. *Stability study of naturfrie deep frying oil.* [Online]. [Accessed 5 May 2012]. Available from: www.plimon.com

Pohle, W.D. and Tierney, S.E. 1957. A spectorphotometric method for the evaluation of vegetable oil colours. *Journal of the American Oil Chemists Society.* **34**(10), pp.485-489.

Pokorny, J. 1989. Flavor chemistry of deep fat frying in oil. In: Min, D.B. and Smouse, T.H. eds. *Flavor Chemistry of Lipid Foods.* Champaign, IL: American Oil Chemists' Society, pp.113-115.

Pokorny, J. 2001. Changes during processes where oil is the heat transfer medium. In: Pokorny, J., et al. eds. *Antioxidants in food.* Cambridge, England: Woodhead Publishing Limited, pp.341-344.

Pokorny, J. 2007. Antioxidants in Food Preservation. *Handbook of Food Preservation, Second Edition.* CRC Press, pp.259-286.

Pokorny, J. 2010. Preparation of natural antioxidants. In: Pokorny, J., et al. eds. *Antioxidants in food: practical application.* Cambridge, England: Woodhead Publishing Limited, pp.311-327.

Pokorny, J. and Dieffenbacher, A. 1989. Determination of 2-thiobarbituric acid value-direct method-results of a collaborative study and the standardized method. *Pure and Applied Chemistry.* **61**(6), pp.1165-1170.

Pokorny, J.Kalinova, L. and Dysseler, P. 1995. Determination of chlorophyll pigments in crude vegetable oils- Results of a collaborative study and the standardized method (Technical report). *Pure and Applied Chemistry.* **67**(10), pp.1781-1787.

Pongboonrod, S. 1976. *The Medicinal Plants in Thailand.* Bangkok, Thailand: Kasembanakit Press.

Puigventos, L.Navarro, M.Alechaga, E.Nunez, O.Saurina, J.Hernandez-Cassou, S. and Puignou, L. 2015. Determination of polyphenolic profiles by liquid chromatography-electrospray-tandem mass spectrometry for the authentication of fruit extracts. *Analytical and Bioanalytical Chemistry.* **407**(2), pp.597-608.

Quisumbing, E. 1951. *Medicinal plants of the Philippines.* Manila: Bureau of Printing.

Race, S. 2009. *Antioxidants: The truth about BHA, BHT, TBHQ and other antioxidants used as food additives.* United Kingdom: Tigmor Books.

Rade, D.Mokrovcak, Z. and Strucelj, D. 1997. Deep fat frying of French fried potatoes in palm oil and vegetable oil. *Food Technology and Biotechnology.* **35**(2), pp.119-124.

Raharjo, S. and Sofos, J.N. 1993. Methodology for measuring malonaldehyde as a product of lipid peroxidation in muscle tissues-a Review. *Meat Science.* **35**(2), pp.145-169.

Raj, G.G.Varghese, H.S.Kotagiri, S.Vrushabendra Swamy, B.M.Swamy, A. and Pathan, R.K. 2014. Anticancer Studies of Aqueous Extract of Roots and Leaves of Pandanus Odoratissimus f. ferreus (Y. Kimura) Hatus: An In Vitro Approach. *Journal of traditional and complementary medicine.* **4**(4), pp.279-84.

Re, R.Pellegrini, N.Proteggente, A.Pannala, A.Yang, M. and Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine.* **26**(9-10), pp.1231-1237.

Reblova, Z. and Okrouhla, P. 2010. Ability of phenolic acids to protect alpha-tocopherol. *Czech J. Food Sci.* **28**(4), pp.290-297.

Reische, D.W.Lillard, D.A. and Eitenmiller, R.R. 2002. Antioxidants. In: Akoh, C.C. and Min, D.B. eds. *Food Lipids: Chemistry, Nutrition and Biotechnology.* 2nd, Revised and Expanded ed. New York, USA: Marcel Dekker.

Ridtitid, W.Ruangsang, P. and Reanmongkol, W. 2007. Studies of the anti-inflammatory and antipyretic activities of the methanolic extract of Piper sarmentosum Roxb. leaves in rats. *Songklanakarin J. Sci. Technol.* **29**(6), pp.1519-1526.

Rodriguez, G.A. 2005. Detection and measurement of carotenoids by UV/VIS Spectrophotometry. In: Ronald E. Wrolstad, et al. eds. *Handbook of food analytical chemistry.* New Jersey: A John Wiley & Sons, Inc., Publication, pp.84-90.

Rojo, J.A. and Perkins, E.G. 1987. Cyclic fatty acid monomer formation in frying fats; Determination and structural study. *Am. Oil Chemists'Soc.* **64**(3), p414.

Romero, A.Cuesta, C. and Sanchez-Muniz, F. 1998. Effect of oil replenishment during deep-fat frying of frozen foods in sunflower oil and high-oleic acid sunflower oil. *J. Am. Oil Chemistes'Soc.* **75**, pp.161-167.

Ross, K.A.Beta, T. and Arntfield, S.D. 2009. A comparative study on the phenolic acids identified and quantified in dry beans using HPLC as affected by different extraction and hydrolysis methods. *Food Chemistry.* **113**(1), pp.336-344.

Rossell, J.B. 2001a. The measurement of frying oil quality and authenticity. In: Rossell, J.B. ed. *Frying : Improving Quality.* Cambridge: Woodhead Publishing, pp.165-190.

Rossell, J.B. 2001b. Factors affecting the quality of frying oils and fats. In: Rossell, J.B. ed. *Frying: Improving Quality.* Cambridge England: Woodhead Publishing Limited, pp.115-164.

Rossell, J.B. 2001c. *Frying : Improving Quality.* Cambridge: Woodhead Publishing.

Rukachaisirikul, T.Siriwattanakit, P.Sukcharoenphol, K.Wongvein, C.Ruttanaweang, P.Wongwattanavuch, P. and Suksamrarn, A. 2004. Chemical constituents and bioactivity of Piper sarmentosum. *Journal of Ethnopharmacology.* **93**(2-3), pp.173-176.

Saad, B.Sing, Y.Y.Nawi, M.A.Hashim, N.Ali, A.S.M.Saleh, M.I.Sulaiman, S.F.Talib, K.M. and Ahmad, K. 2007. Determination of synthetic phenolic antioxidants in food items using reversed-phase HPLC. *Food Chemistry.* **105**(1), pp.389-394.

Saleem, M.Kim, H.J.Ali, M.S. and Lee, Y.S. 2005. An update on bioactive plant lignans. *Natural Product Reports.* **22**(6), pp.696-716.

Sanchez-Muniz, F.Cuesta, C.Lopez-Varela, M.Garrido-Polonio, M. and Arroyo, R. 1993. Evaluation of the thermal oxidation rate of sunflower oil using various frying methods. In: Applewhite TH, ed. *Proceedings of World Conference on Oilseed and Technology and Utilization, Champaigh, Ill.* American Oil Chemists Society. p.448-452, pp.448-452.

Santos, J.C.O.Santos, I.M.G. and Souza, A.G. 2005. Effect of heating and cooling on rheological parameters of edible vegetable oils. *J. Food Engineering.* **67**, pp.401-405.

Saralamp, P.Chauakul, W.Temsiririrkkul, R. and Clayton, T. 1996. *Medicinal Plants in Thailand.* Bangkok, Thailand: Amarin Printing and Publishing.

Scheepers, J.C.Malan, S.F.Du Preez, J.L. and Van Dyk, S. 2011. The high performance liquid chromatography (HPLC) analysis of ultraviolet (UV) irradiated chlorophyll a and secondary plant compounds. *African Journal of Biotechnology.* **10**(74), pp.16976-16985.

Scherer, R.Poloni Rybka, A.C.Ballus, C.A.Meinhart, A.D.Teixeira Filho, J. and Godoy, H.T. 2012. Validation of a HPLC method for simultaneous determination of main organic acids in fruits and juices. *Food Chemistry.* **135**(1), pp.150-154.

Schroeder, M.Becker, E. and Skibsted, L. 2006. Molecular mechanism of antioxidant synergism of tocotrienols and carotenoids in palm oil. *J. Agric Food Chem.* **54**, pp.3445-3453.

Schwartz, S.J. 2005. Pigments and colorants. In: Ronald E. Wrolstad, et al. eds. *Handbook of food analytical chemistry.* New Jersey: A John Wiley & Sons, Inc., Publication, pp.3-4.

Schwartz, S.J.Elbe, J.H.v. and Giusti, M.M. 2008. Colorants. In: Damodaran, S., et al. eds. *Fennema's Food Chemistry (4th Edition).* Taylor & Francis.

Seyyedan, A.Yahya, F.Kamarolzaman, M.F.F. and Suhaili, Z. 2013. Review on the ethnomedicinal, phytochemical and pharmacological properties of Pipr sarmentosum: scientific justification of its traditional use. *Tang Humanitas Traditional Medicine.* **3**(3), pp.1-32.

Shahidi, F. 2005a. Lipid Oxidation: Measurement methods. In: Shahidi, F. ed. *Bailey's industrial oil & fat products [electronic resource].* [Online]. 6th ed. Hoboken, N.J.: John Wiley & Sons, Inc., pp.357-385.

Shahidi, F. 2005b. Antioxidants: Regulatory Status. In: Shahidi, F. ed. *Bailey's industrial oil & fat products [electronic resource].* [Online]. 6th ed. Hoboken, N.J.: John Wiley & Sons, Inc.

Shahidi, F. and Wanasundara, U.N. 1997. Measurement of lipid oxidation and evaluation of antioxidant activity. In: Shahidi, F. ed. *Natural antioxidants: Chemistry, health effects, and applications.* Champaign, Illinois: AOCS Press, pp.379-396.

Shahidi, F. and Wanasundara, U.N. 2008. Methods for measuring oxidative rancidity in fats and oils. In: Casimir C. Akoh and Min, D.B. eds. *Food Lipids:Chemistry, Nutrition, and Biotechnology.* 3nd ed. CRC Press, p.465.

Shan, B.Cai, Y.Z.Sun, M. and Corke, H. 2005. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *Journal of Agricultural and Food Chemistry.* **53**(20), pp.7749-7759.

Sherwin, E.R. 1972. Antioxidants for Food Fats and Oils. *Journal of the American Oil Chemists Society.* **49**(8), pp.468-472.

Shi, X.Dalal, N.S. and Jain, A.C. 1991. Antioxidant behaviour of caffeine: Efficient scavenging of hydroxyl radicals. *Food and Chemical Toxicology.* **29**(1), pp.1-6.

Shimadzu Corporation. 2008. *High performance liquid chromatograph mass spectrometer. LCMS-2020. Instruction manual.* Kyoto. Japan: Shimadzu Corporation.

Shiota, M.Konishi, H. and Tatsumi, K. 1999. Oxidative stability of fish oil blended with butter. *J. Dairy Sci.* **82**, pp.1877-1881.

Silkeberg, A. and Kochhar, S.P. 2000. *Refining of edible oil retaining maximum antioxidative potency*. US Patent 6,033,706. 7 March.

Sim, K.-M.Mak, C.-N. and Ho, L.-P. 2009. A new amide alkaloid from the leaves of Piper sarmentosum. *Journal of Asian Natural Products Research.* **11**(8), pp.757-760.

Simic, M.G. 1981. Free-radical mechanisms in autoxidation processes. *Journal of Chemical Education.* **58**(2), pp.125-131.

Sinha, S. 2014. *Fatty acid.* [Online]. [Accessed 29 January 2015]. Available from: http://www.britannica.com/EBchecked/topic/202621/fatty-acid

Sireeratawong, S.Vannasiri, S.Sritiwong, S.Itharat, A. and Jaijoy, K. 2010. Anti-inflammatory, anti-nociceptive and antipyretic effects of the ethanol extract from root of *Piper sarmentosum* Roxb. *Journal of the Medical Association of Thailand.* **93** (7), pp.1-6.

Soriguer, F.Rojo, G.Dobarganes, C. and et al. 2003. Hypertension is related to the degradation of dietary frying oils. *Am. J. Clin. Nutr.* **78**, pp.1092-1097.

Steele, R. 2004. *Understanding and measuring the shelf-life of food.* Cambridge, England: Woodhead Publishing Limited and CRC Press LLC.

Stevenson, S.G.Vaisey-Genser, M. and Eskin, N.A.M. 1984. Quality control in the use of deep frying oils. *Journal American Oil Chemists Society.* **61**(6), pp.1102-1108.

Subramaniam, V.Adenan, M.I.Ahmad, A.R. and Sahdan, R. 2003. Natural Antioxidants: Piper sarmentosum (Kadok) and Morinda elliptica (Mengkudu). *Malaysian journal of nutrition.* **9**(1), pp.41-51.

Sultana, B.Anwar, F. and Ashraf, M. 2009. Effect of Extraction Solvent/Technique on the Antioxidant Activity of Selected Medicinal Plant Extracts. *Molecules.* **14**(6), pp.2167-2180.

Sumazian, Y.Syahida, A.Hakiman, M. and Maziah, M. 2010. Antioxidant activities, flavonoids, ascorbic acid and phenolic contents of Malaysian vegetables. *Journal of Medicinal Plants Research.* **4**(10), pp.881-890.

Suzgec, S.Mericli, A.H.Houghton, P.J. and Cubukcu, B. 2005. Flavonoids of Helichrysum compactum and their antioxidant and antibacterial activity. *Fitoterapia.* **76**(2), pp.269-272.

Takahashi, O. 1995. Hemorrhagic toxicity of a large dose of alpha-tocopherol, beta-tocopherol, gamma-tocopherol and delta-tocopherol, ubiquinone, beta-carotene, retinol acetate and L-ascorbic acid in the rat. *Food and Chemical Toxicology.* **33**(2), pp.121-128.

Takayama, H.Ichikawa, T.Kitajima, M.Nonato, M.G. and Aimi, N. 2002. Isolation and structure elucidation of two new alkaloids, pandamarilactonine-C and -D, from Pandanus amaryllifolius and revision of relative stereochemistry of pandamarilactonine-A and -B by total synthesis. *Chemical & Pharmaceutical Bulletin.* **50**(9), pp.1303-1304.

Takeoka, G.R.Full, G.H. and Dao, L.T. 1997. Effect of heating on the characteristics and chemical composition of selected frying oils and fats. *Journal of Agricultural and Food Chemistry.* **45**(8), pp.3244-3249.

Tan, M.A.Kitajima, M.Kogure, N.Nonato, M.G. and Takayama, H. 2010. Isolation and total syntheses of two new alkaloids, dubiusamines-A, and -B, from Pandanus dubius. *Tetrahedron.* **66**(18), pp.3353-3359.

Tan, Y.A.Ong, S.H.Berger, K.G.Oon, H.H. and Poh, B.L. 1985. A study of the cause of rapid colour development of heated refined palm oil. *Journal of the American Oil Chemists Society.* **62**(6), pp.999-1006.

Tangkanakul, P.Trakoontivakorn, G.Auttaviboonkul, P.Niyomvit, B. and Wongkrajang, K. 2006. Antioxidant activity of Northern and Northeastern Thai foods containing indigenous vegetables. *Kasetsart J.* **40**, pp.47-58.

Tarmizi, A.H.A. and Ismail, R. 2008. Comparison of the frying stability of standard palm olein and special quality palm olein. *Journal of the American Oil Chemists Society.* **85**(3), pp.245-251.

Taweechaisupapong, S.Singhara, S.Lertsatitthanakorn, P. and Khunkitti, W. 2010. Antimicrobial effects of *Boesenbergia pandurata* and *Piper sarmentosum*

leaf extracts on planktonic cells and biofilm of oral pathogens. *Pakistan Journal of Pharmaceutical Sciences.* **23**(2), pp.224-231.

Termsuk. 2015. *Safety diet.* [Online]. [Accessed 5 May 2014]. Available from: http://www.termsuk.com

Thaipong, K.Boonprakob, U.Crosby, K.Cisneros-Zevallos, L. and Byrne, D.H. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis.* **19**(6-7), pp.669-675.

The Corn Refiners Association. 2015. *The corn refining process.* [Online]. [Accessed 23 January 2015]. Available from: http://corn.org/process/

The European Union. 2011. Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. *Offial Journal of the European Union* **295**(54), p1.

Tian, L.L. and White, P.J. 1994. Antipolymerization activity of oat extract in soybean and cottenseed oils under frying conditions. *Journal of the American Oil Chemists Society.* **71**(10), pp.1087-1094.

Tompkins, C. and Perkins, E.G. 2000. Frying performance of low-linolenic acid soybean oil. *J. Am. Oil Chemistes'Soc.* **77**, pp.223-229.

Tsao, R. 2010. Chemistry and Biochemistry of Dietary Polyphenols. *Nutrients.* **2**(12), pp.1231-1246.

Tsao, R. and Yang, R. 2003. Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high-performance liquid chromatography. *Journal of Chromatography A.* **1018**(1), pp.29-40.

Tyagi, V. and Vasishtha, A. 1996. Changes in the characteristics and composition of oils during deep-fat frying. *J. Am. Oil Chemistes'Soc.* **73**, pp.499-560.

Ugusman, A.Zakaria, Z.Hui, C.K.Nordin, N.A.M.M. and Mahdy, Z.A. 2012. Flavonoids of *Piper sarmentosum* and its cyto-protective effects against oxidative stress. *Excli Journal.* **11**, pp.705-714.

Van Esch, G.J. 1986. Toxicology of *tert*-Butylhydroquinone (TBHQ). *Food and Chemical Toxicology.* **24**(10-11), pp.1063-1066.

Wan-Ibrahim, W.I.Sidik, K. and Kuppusamy, U.R. 2010. A high antioxidant level in edible plants is associated with genotoxic properties. *Food Chemistry.* **122**(4), pp.1139-1144.

Warner, K. 2002. Chemistry of Frying Oils. In: Casimir, A.C. and David, M.B. eds. *Food Lipids: Chemistry, Nutrition and Biotechnology.* 2nd ed. CRC Press, pp.205-221

Warner, K. and Mounts, T. 1993. Frying stability of soybean and canola oils with modified fatty acid compositions. *J. Am. Oil Chemistes'Soc.* **70**, pp.983-988.

Warner, K. and Knowlton, S. 1997. Frying quality and oxidative stability of high-oleic corn oils. *J. Am. Oil Chemistes'Soc.* **74**, pp.1317-1322.

Warner, K.Orr, P. and Glynn, M. 1997. Effect of fatty acid composition of oils on flavour and stability of fried foods. *J. Am. Oil Chemistes'Soc.* (74), pp.347-356.

Warner, K.Orr, P.Parrott, L. and Glynn, M. 1994. Effects of frying oil composition on potato chip stability. *J. Am. Oil Chemistes'Soc.* **71**(1117-1121).

Warner, K.Neff, W.E.Byrdwell, W.C. and Gardner, H.W. 2001. Effect of oleic and linoleic acids on the production of deep-fried odor in heated triolein and trilinolein. *Journal of Agricultural and Food Chemistry.* **49**(2), pp.899-905.

Waterhouse, A.L. 2005. Determination of total phenolics by Folin-ciocalteau colorimetry. In: Ronald E. Wrolstad ed. *Handbook of food analytical chemistry.* A John Wiley & Sons, Inc., Publication, pp.463-465.

White, P.J. 2000. Flavor quality of fats and oils. In: O'Brien, R.D., et al. eds. *Introduction of Fats and Oils Technology.* Champaigh, IL: AOCS Press, pp.354-357.

Wichi, H.P. 1988. Enchanced tumor development by BHA from the perspective of effect on forestomach and oesophageal squamous epithelium. *Food and Chemical Toxicology.* **26**, pp.717-723.

Willett, W.C.Stampfer, M.J.Manson, J.E.Colditz, G.A.Speizer, F.E.Rosner, B.A.Sampson, L.A. and Hennekens, C.H. 1993. Intake of trans-fatty-acids and risk of coronary heart-disease among women. *Lancet.* **341**(8845), pp.581-585.

Winter, R.A. 1999. *Food Additives: A consumer's dictionary.* 5th ed. Three Rivers Press.

Wiredu, E. 2012. *Common Statistics with SPSS: Chi-square, Ttest, ANOVA, Correlation & Regression.* Data Solutions Services.

Wong, C.C.Li, H.B.Cheng, K.W. and Chen, F. 2006. A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. *Food Chemistry.* **97**(4), pp.705-711.

Wu, C.Q.Chen, F.Wang, X.Kim, H.J.He, G.Q.Haley-Zitlin, V. and Huang, G. 2006. Antioxidant constituents in feverfew (Tanacetum parthenium) extract and their chromatographic quantification. *Food Chemistry.* **96**(2), pp.220-227.

Wu, P.F. and Nawar, W.W. 1986. A technique for monitoring the quality of used frying oils. *Journal of the American Oil Chemists Society.* **63**(10), pp.1363-1367.

Xu, X.Q.Tran, V.Palmer, M.WHITE, K. and Salisbury, P. 1999. Chemical and physical analyses and sensory evaluation of six deep-frying oils. *J. Am. Oil Chemistes'Soc.* **76**, pp.1091-1099.

Yanishlieva, N.V. 2001. Inhibiting oxidation. In: Pokorny, J., et al. eds. *Antioxidants in food: practical application.* Cambridge, England: Woodhead Publishing Limited, pp.22-57.

Yanishlieva, N.V.Sofia and Heinonen, I.M. 2001. *Sources of natural antioxidants: vegetables, fruits, herbs, spices and teas.* Cambridge, England: Woodhead Publishing Limited.

Yashin, A.Yashin, Y.Wang, J.Y. and Nemzer, B. 2013. Antioxidant and antiradical activity of coffee. *Antioxidants.* **2**, pp.230-245.

Yasho Industries. 2015. *Food Anitoxidants.* [Online]. [Accessed 16 April 2015]. Available from: http://yashoindustries.com/food-antioxidant-range.html

Zhang, A.Wan, L.Wu, C.Fang, Y.Han, G.Li, H.Zhang, Z. and Wang, H. 2013. Simultaneous Determination of 14 Phenolic Compounds in Grape Canes by HPLC-DAD-UV Using Wavelength Switching Detection. *Molecules.* **18**(11), pp.14241-14257.

Zhang, J. and Taher, A.B. 2012. *Effect of high temperature on the compostion of frying oils.* University of Leeds.

Zhang, Y.Yang, L.Zu, Y.Chen, X.Wang, F. and Liu, F. 2010. Oxidative stability of sunflower oil supplemented with carnosic acid compared with synthetic antioxidants during accelerated storage. *Food Chemistry.* **118**(3), pp.656-662.

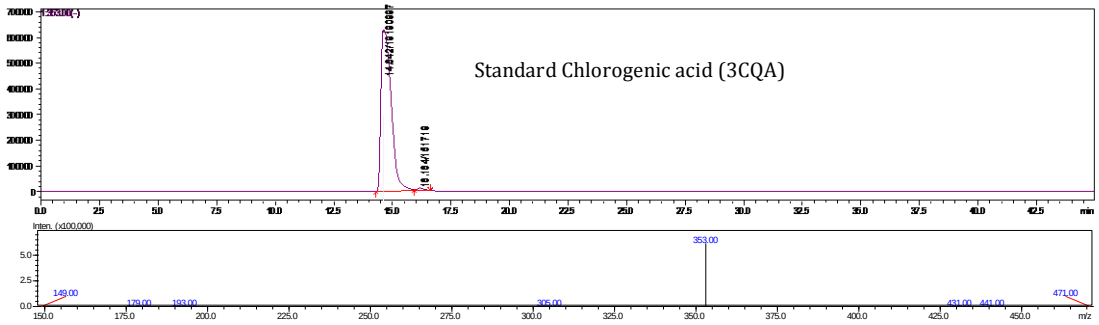
Zhao, T. and Yu, M.-q. 2010. Spectrum Characteristics and Stability of Anthocyanin Pigment from Hulless Barley (Hordeum vulgare ssp vulgare). In: Du, Z.Y. and Sun, X.B. eds. *Environment Materials and Environment Management Pts 1-3.* pp.2323-2328.

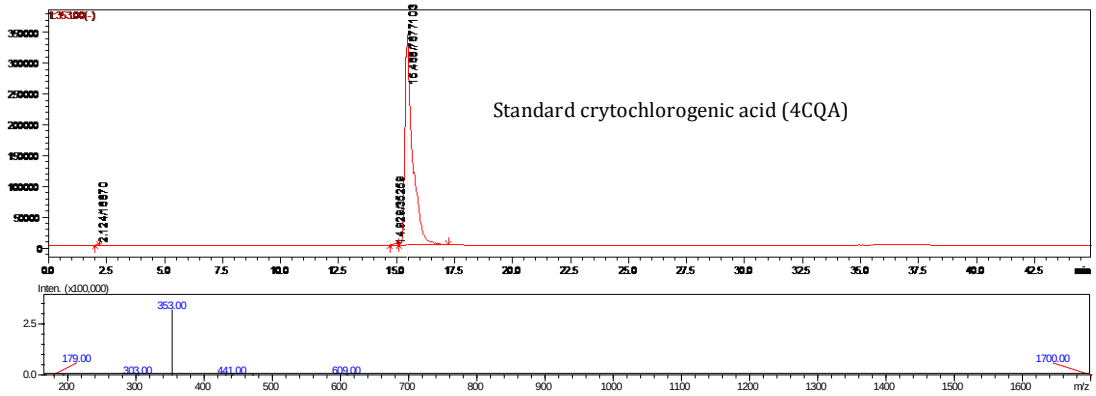
Zhong, Y. and Shahidi, F. 2012. Antioxidant Behavior in Bulk Oil: Limitations of Polar Paradox Theory. *Journal of Agricultural and Food Chemistry.* **60**(1), pp.4-6.

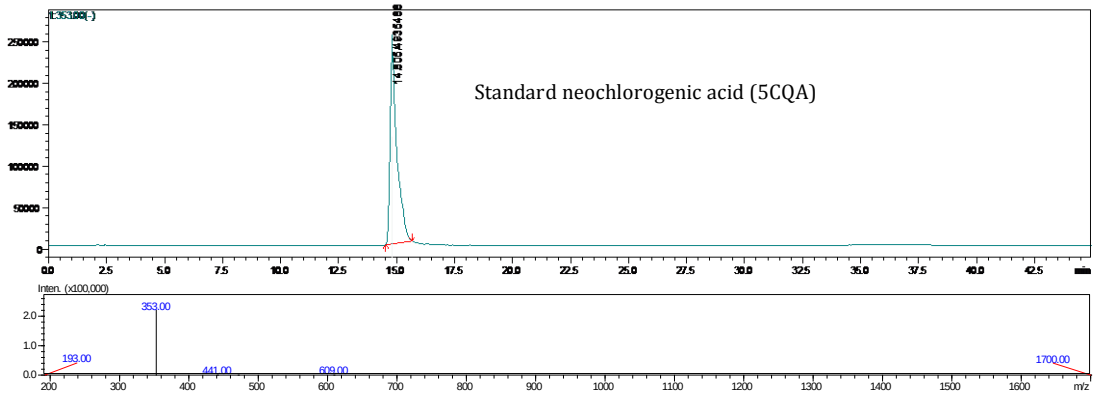
Zhu, Q.Y.Huang, Y. and Chen, Z.Y. 2000. Interaction between flavonoids and alpha-tocopherol in human low density lipoprotein. *Journal of Nutritional Biochemistry.* **11**(1), pp.14-21.

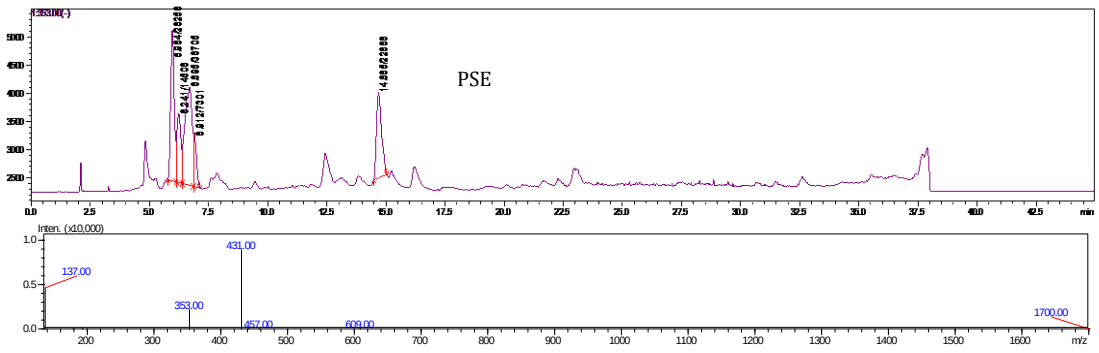
Appendix A

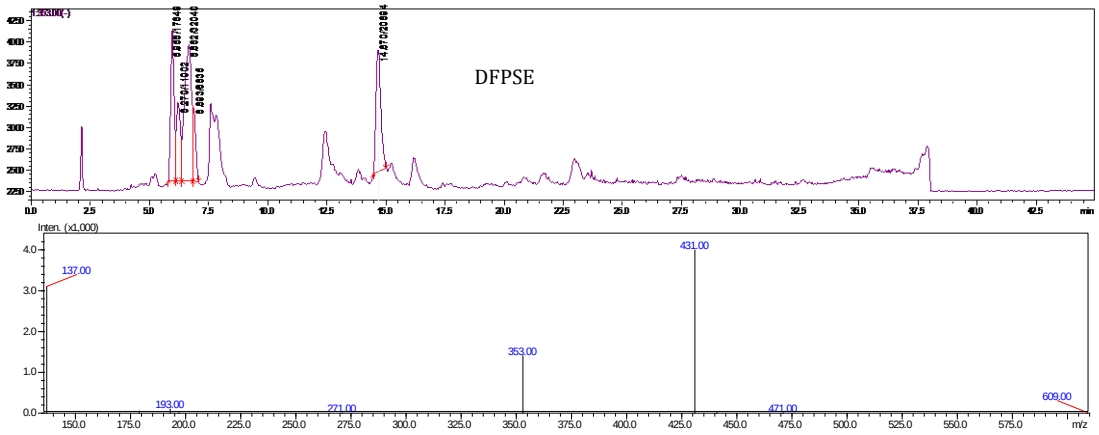
A.1 Mass chromatogram and mass spectrum of standard Chlorogenic acid compared to *Piper sarmentosum* Roxb. leaf extracts





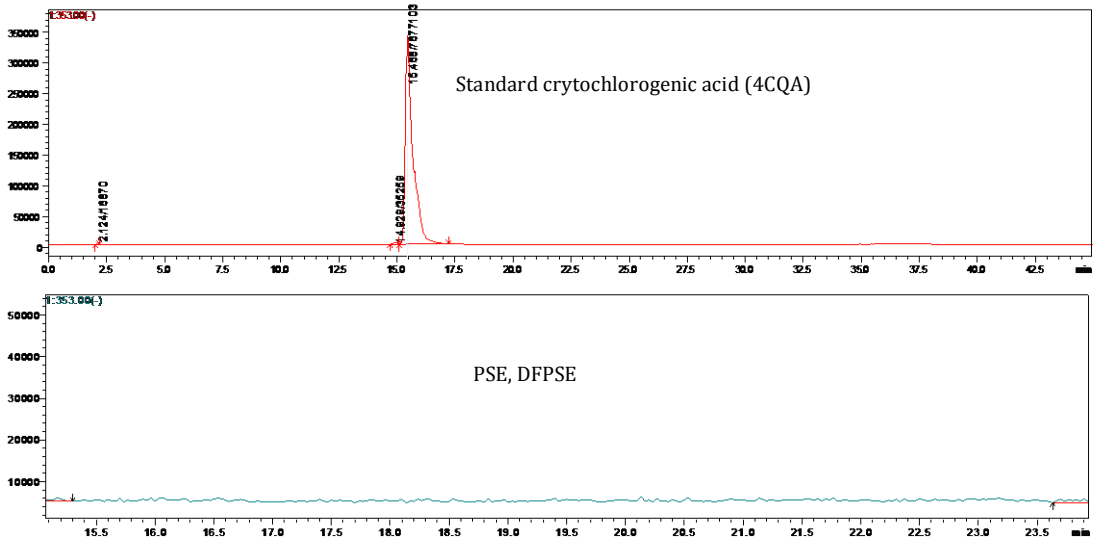






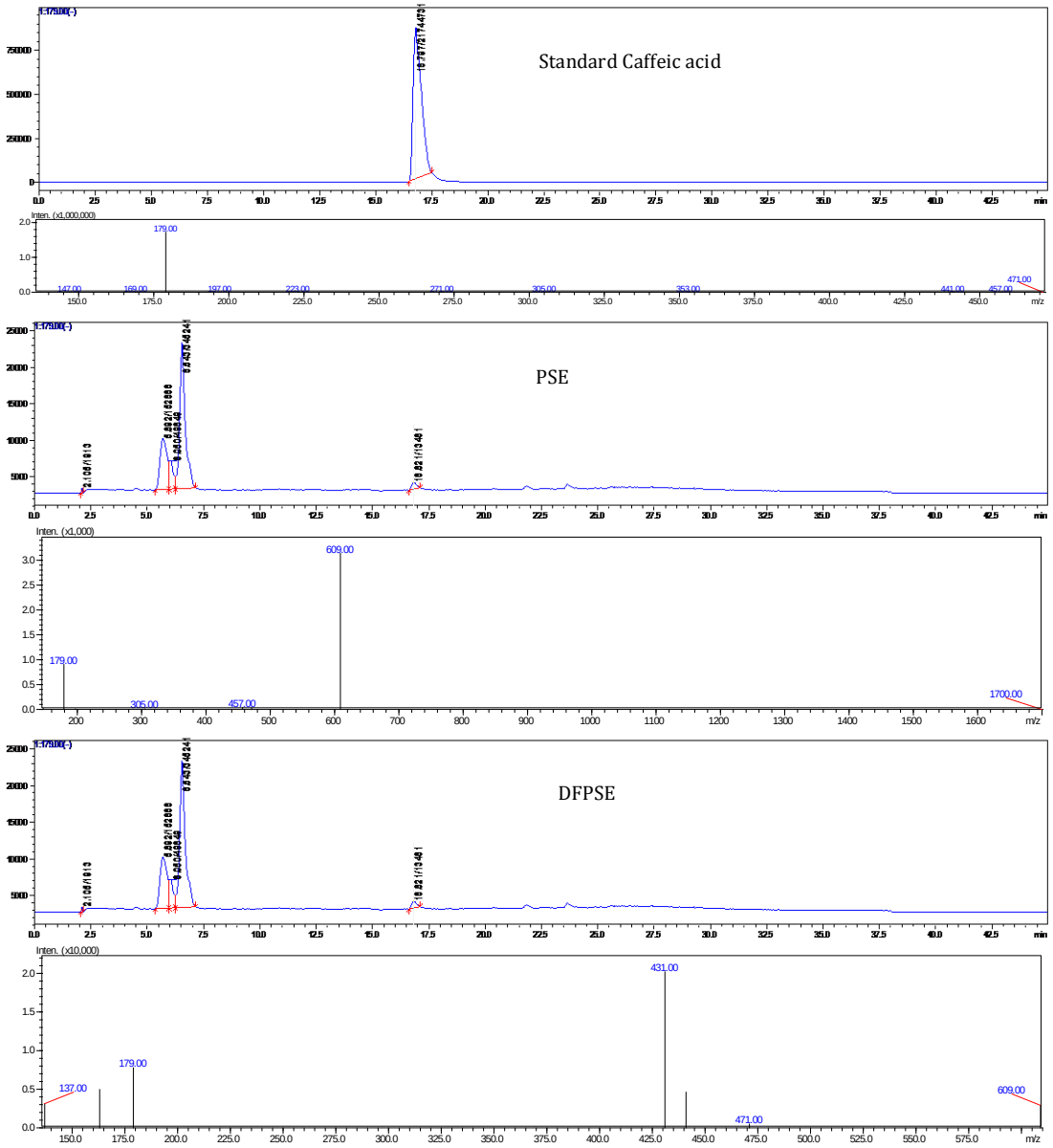
Peak	Retention Time	Area	S/N	Height	m/z	Peak Start	Peak End
3CQA	14.642	19190997	11284.82	627481	353	14.300	16.628
4CQA	15.468	7677103	4836.12	340376	353	15.100	17.233
5CQA	14.805	4935488	2336.68	251775	353	14.533	15.667
PSE	14.685	22868	18.07	1509	353	14.483	15.033
DFPSE	14.670	20694	19.32	1428	353	14.465	14.997

A.2 Mass chromatogram of PSE and DFPSE extracts at 15.468 min, m/z=353



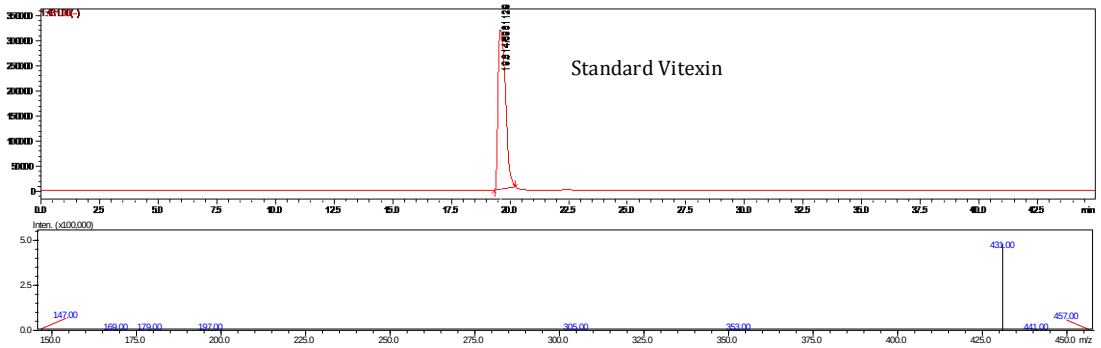
There is no peak found at 15.468 min, m/z ratio 353, so no 4CQA present in PSE and DFPSE extracts

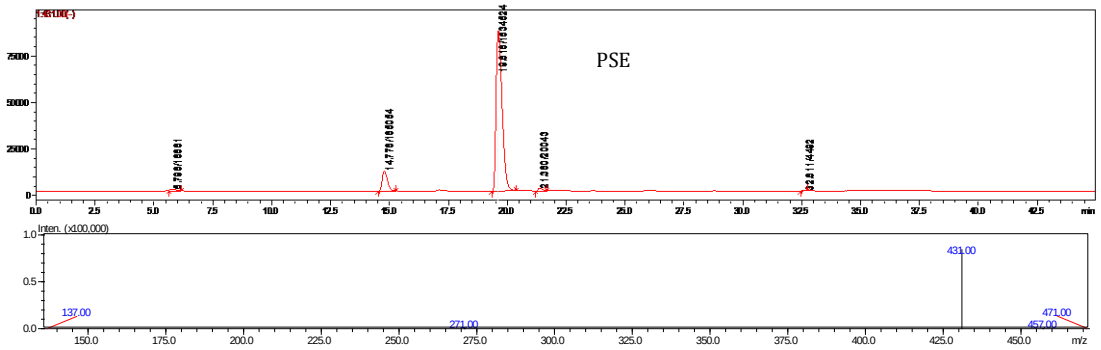
A.3 Mass chromatogram and mass spectrum of standard Caffeic acid compared to *Piper sarmentosum* Roxb. leaf extract

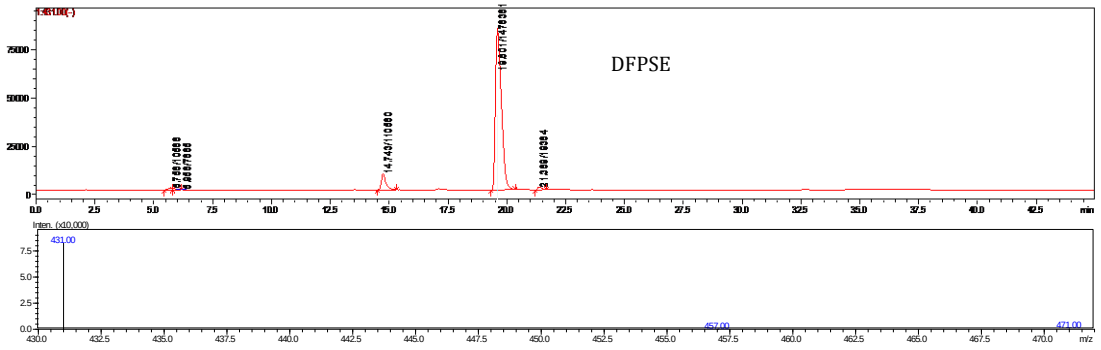


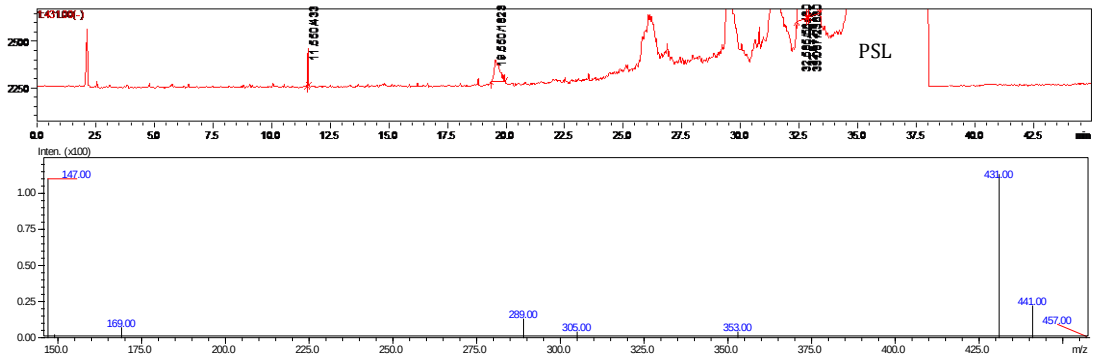
Peak	Retention Time	Area	S/N	Height	m/z	Peak Start	Peak End
Caffeic acid	16.797	21744731	3122.82	857855	179	16.500	17.508
PSE	16.821	13461	9.58	941	179	16.628	17.105
DFPSE	16.827	16068	10.65	1051	179	16.628	17.160

A.4 Mass chromatogram and mass spectrum of standard Vitexin compared to *Piper sarmentosum* Roxb. leaf extracts



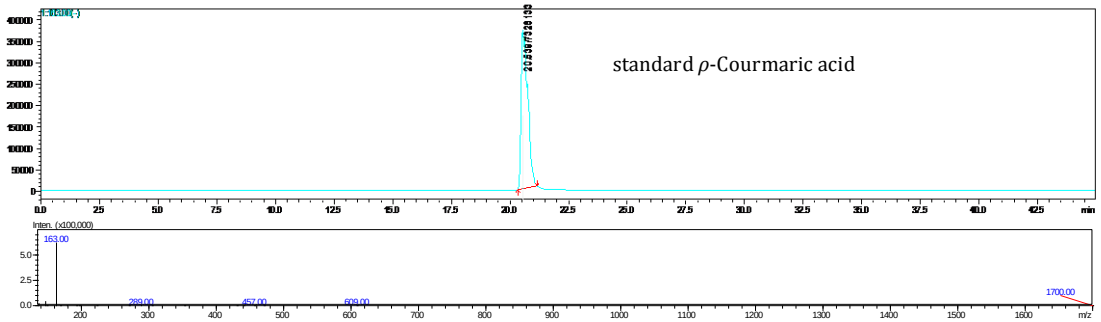


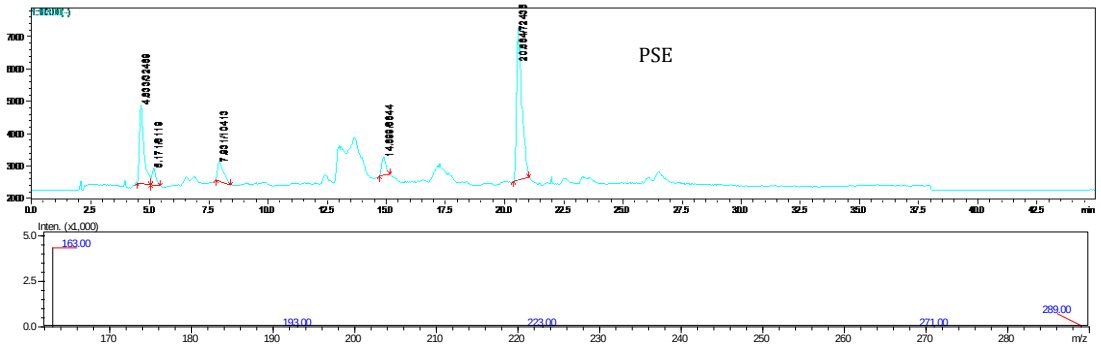


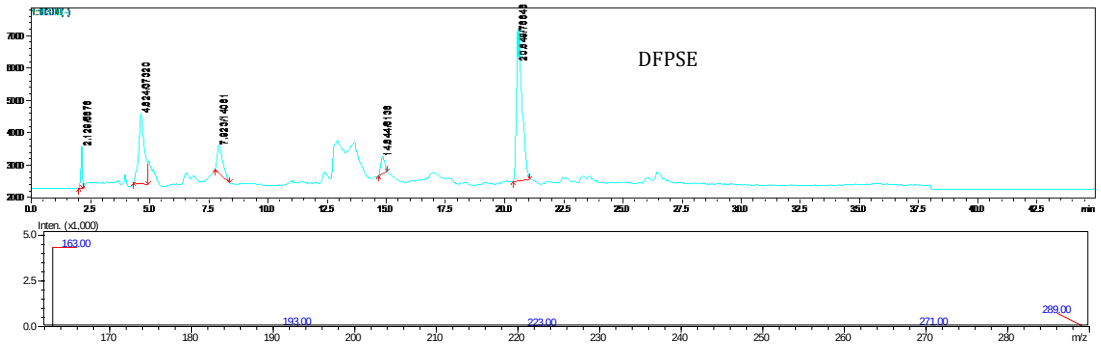


Peak	Retention Time	Area	S/N	Height	m/z	Peak Start	Peak End
Vitexin	19.614	6981129	3347.48	317383	431	19.342	20.240
PSE	19.616	1534524	1435.54	86295	431	19.342	20.387
DFPSE	19.601	1476361	1397.49	83529	431	19.323	20.368
PSL	19.550	1823	2.42	118	431	19.360	19.947

A.5 Mass chromatogram and mass spectrum of standard *ρ*-Courmaric acid compared to *Piper sarmentosum* Roxb. leaf extracts

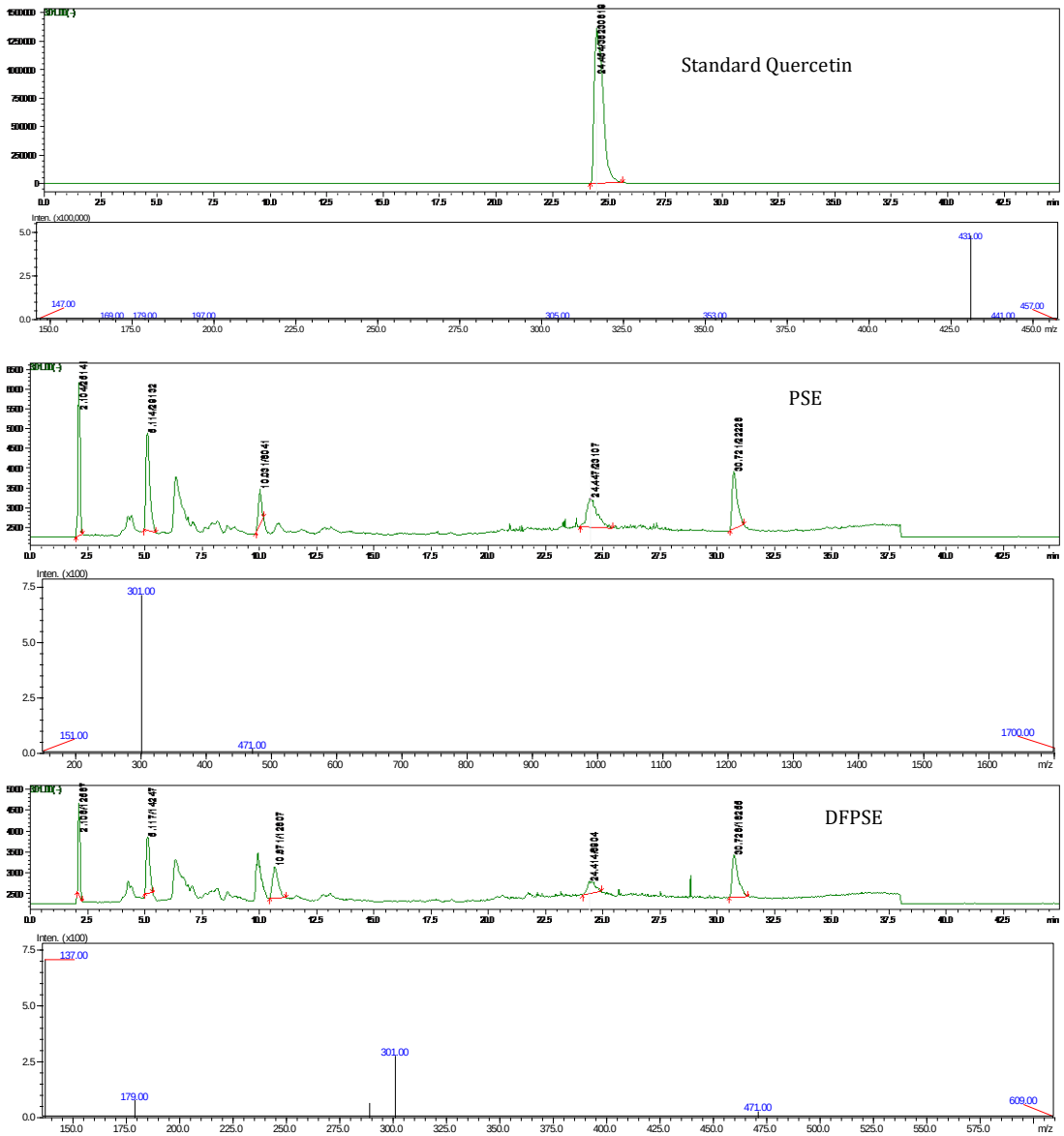






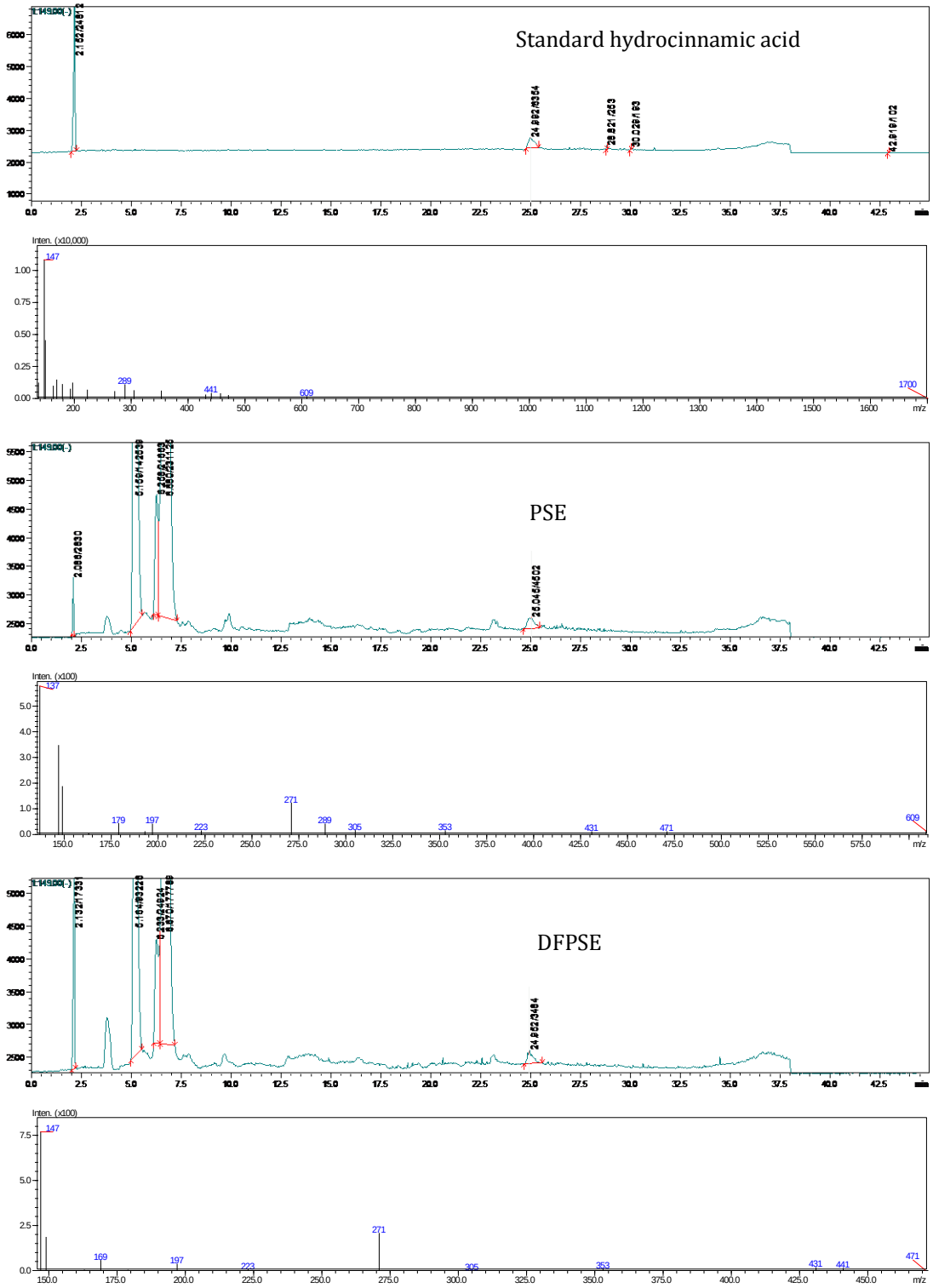
Peak	Retention Time	Area	S/N	Height	m/z	Peak Start	Peak End
ρ-Courmaric acid	20.539	7326133	4638.60	371800	163	20.332	21.157
PSE	20.564	72435	61.54	4683	163	20.368	21.028
DFPSE	20.549	76646	64.30	4712	163	20.350	21.065

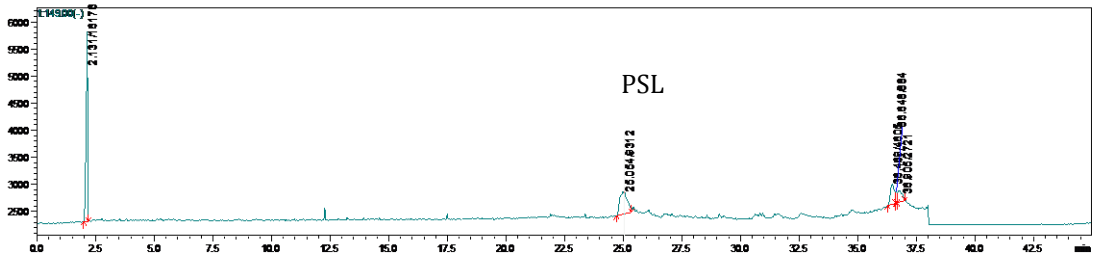
A.6 Mass chromatogram and mass spectrum of standard Quercetin compared to *Piper sarmentosum* Roxb. leaf extracts



Peak	Retention Time	Area	S/N	Height	m/z	Peak Start	Peak End
Quercetin	24.454	36230819	6531.62	1357502	301	24.167	25.600
PSE	24.447	23107	10.37	754	301	24.017	25.417
DFPSE	24.414	6904	3.83	291	301	24.117	24.950

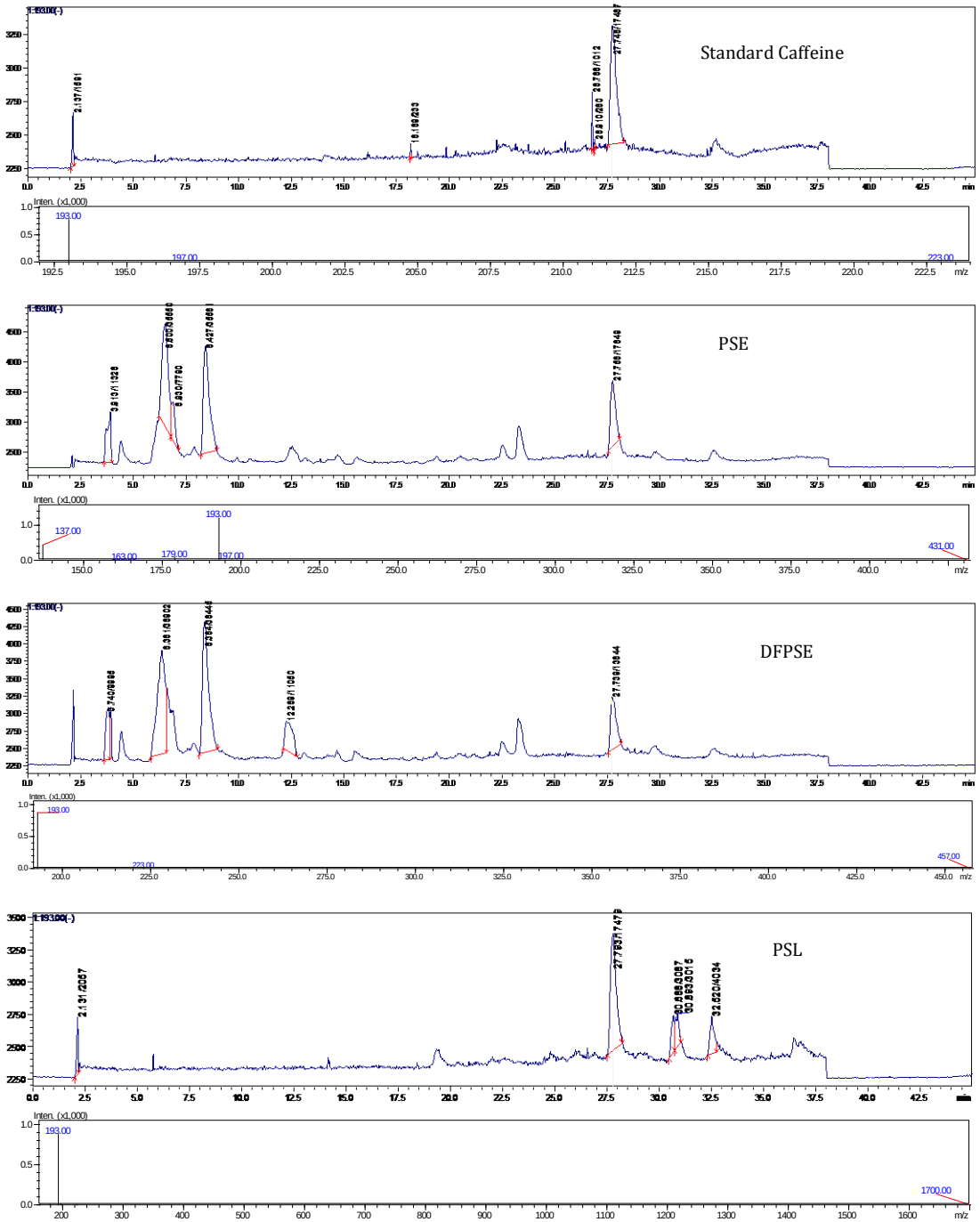
A.7 Mass chromatogram and mass spectra of standard hydroxycinnamic aicd compared to *Piper sarmentosum* Roxb. leaf extracts





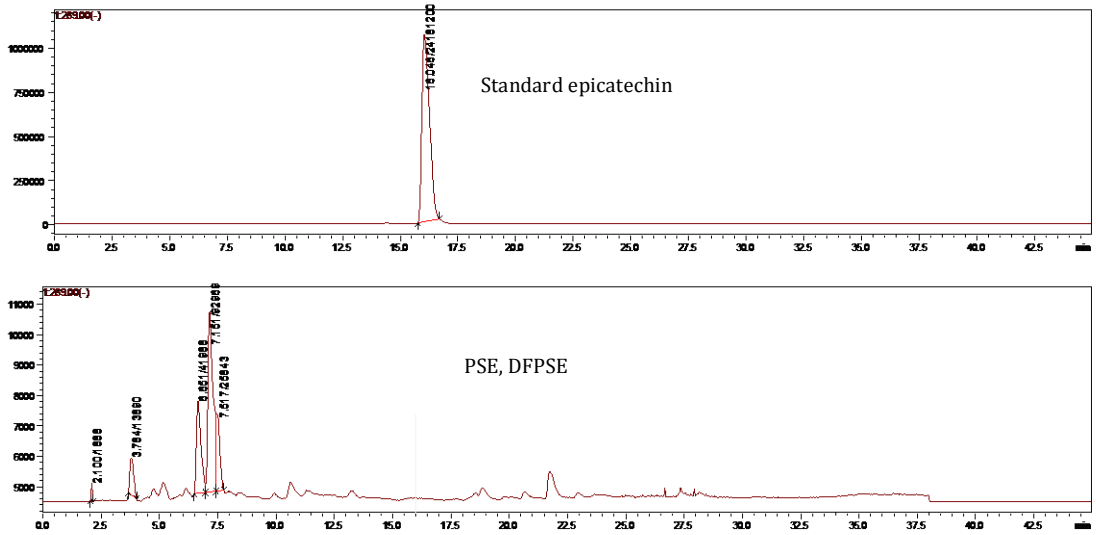
Peak	Retention Time	Area	S/N	Height	m/z	Peak Start	Peak End
Hydroxycinnamic aicd	24.992	6354	12.02	334	149	24.768	25.392
PSE	25.045	4502	4.31	197	149	24.64	25.447
DFPSE	24.952	3484	3.93	205	149	24.677	25.593
PSL	25.054	9312	11.62	415	149	24.713	25.337

A.8 Mass chromatogram and mass spectrum of standard Caffeine compared to *Piper sarmentosum* Roxb. leaf extracts



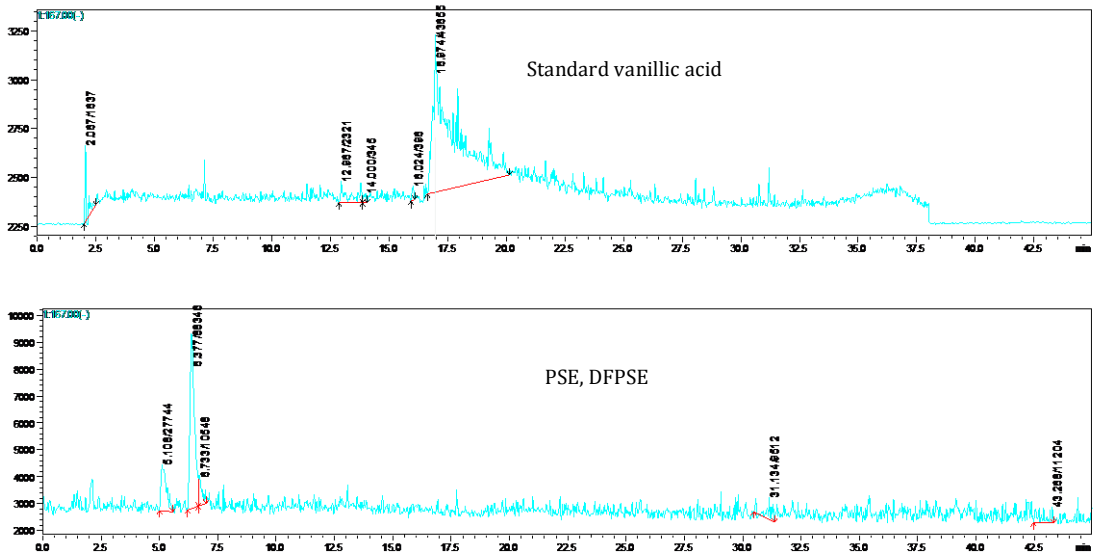
Peak	Retention Time	Area	S/N	Height	m/z	Peak Start	Peak End
Caffeine	27.748	17487	28.21	883	193	27.537	28.288
PSE	27.756	17849	19.70	1091	193	27.555	28.087
DFPSE	27.739	13644	10.86	761	193	27.555	28.160
PSL	27.793	17479	26.97	908	193	27.518	28.233

A.9 Mass chromatogram of PSE and DFPSE extract at 16.0 min, m/z 289



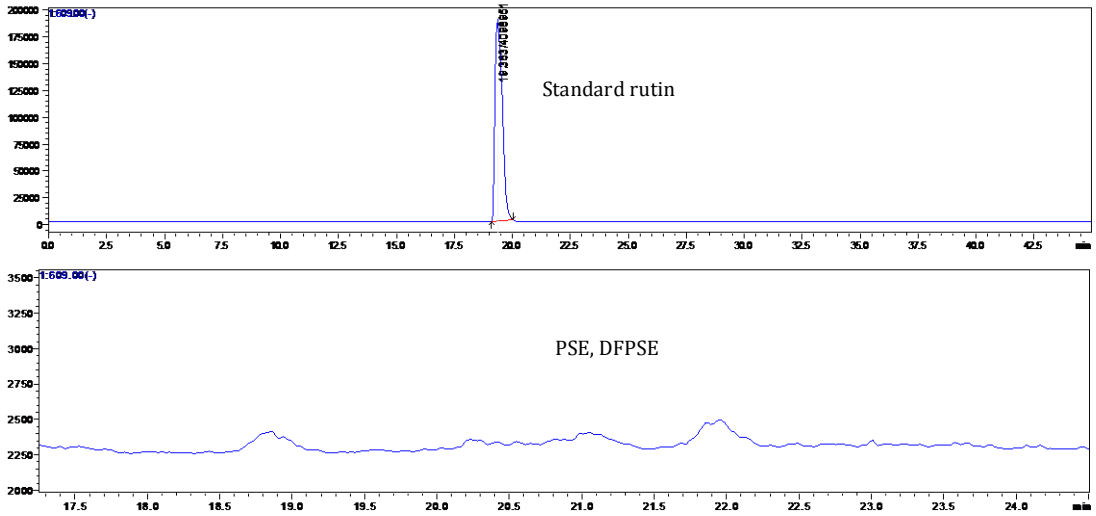
There is no peak found at 16.0 min, m/z = 289, so there is no epicatechin present in PSE and DFPSE extracts

A.10 Mass chromatogram of PSE and DFPSE extract at 16.5 min, m/z 167



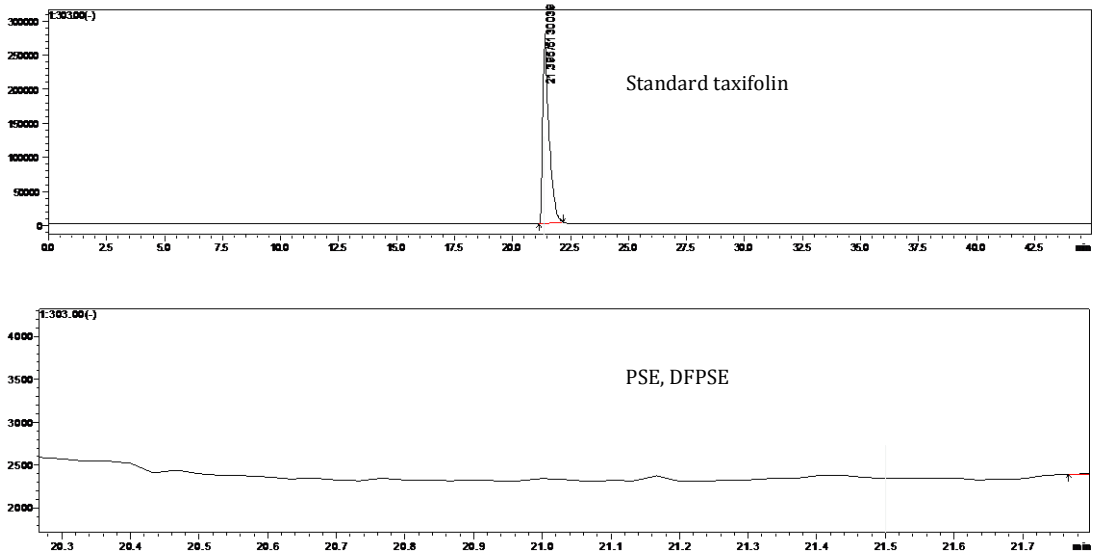
There is no peak found at 16.5 min, m/z = 167, so there is no vanillic acid present in PSE and DFPSE extracts

A.11 Mass chromatogram of PSE and DFPSE extract at 19.1 min, m/z 609



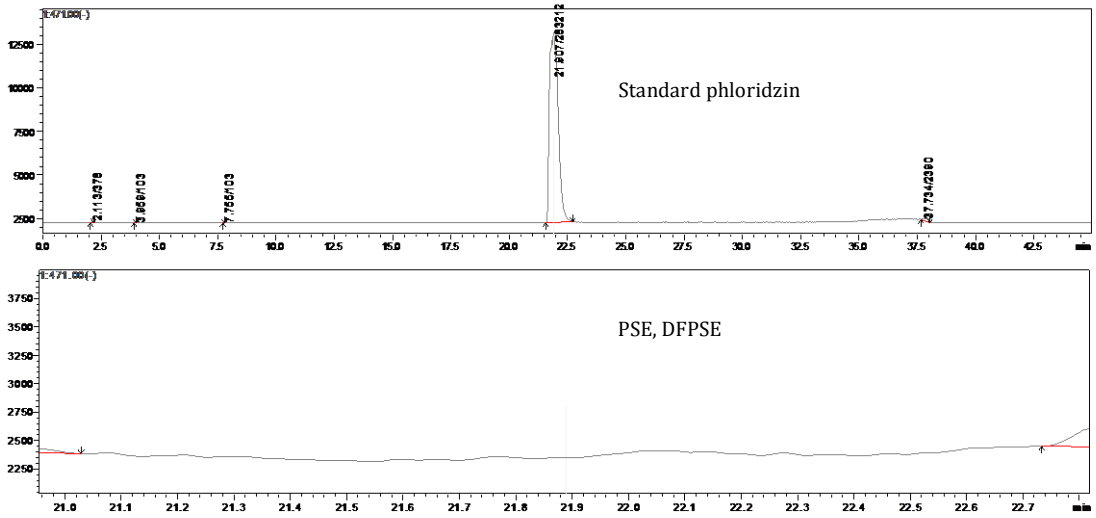
There is no peak found at 19.1 min, m/z = 609, so there is no rutin present in PSE and DFPSE extracts

A.12 Mass chromatogram of PSE and DFPSE extract at 21.5 min, m/z 303



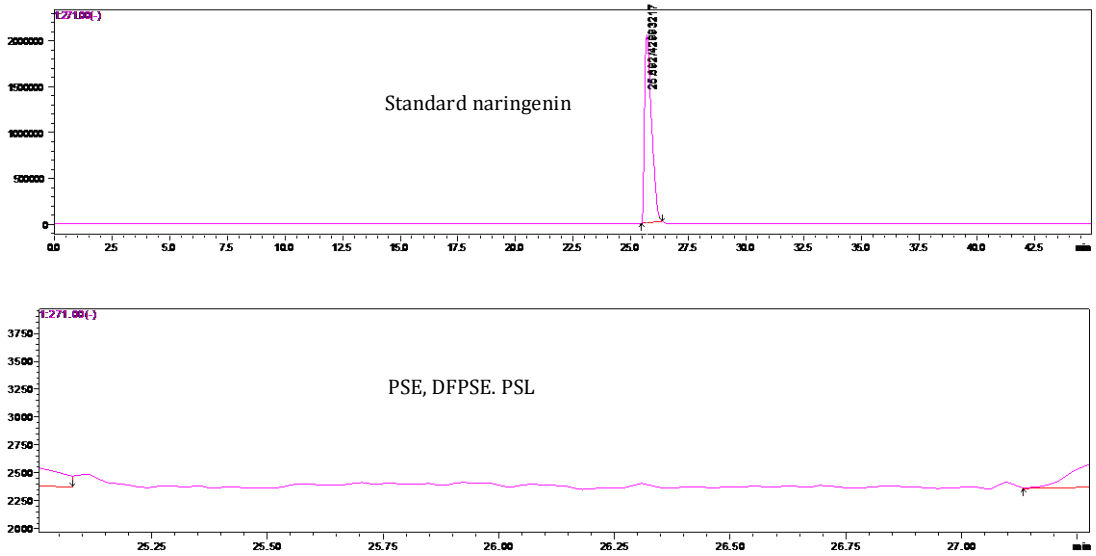
There is no peak found at 21.5 min, m/z = 303, so there is no taxifolin present in PSE and DFPSE extracts

A.13 Mass chromatogram of PSE and DFPSE extract at 21.9 min, m/z 471



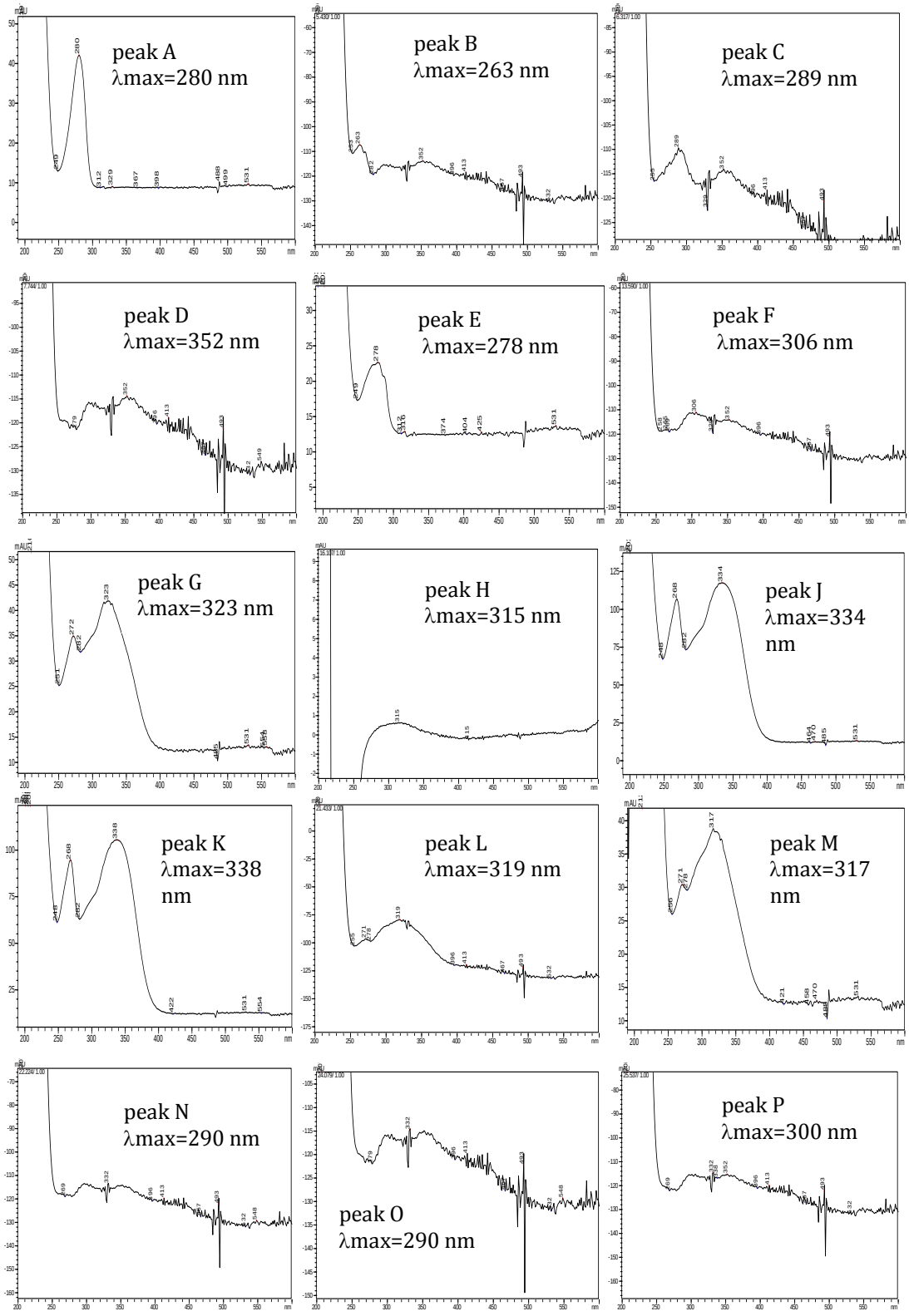
There is no peak found at 21.9 min, m/z = 471, so there is no phloridzin present in PSE and DFPSE extracts

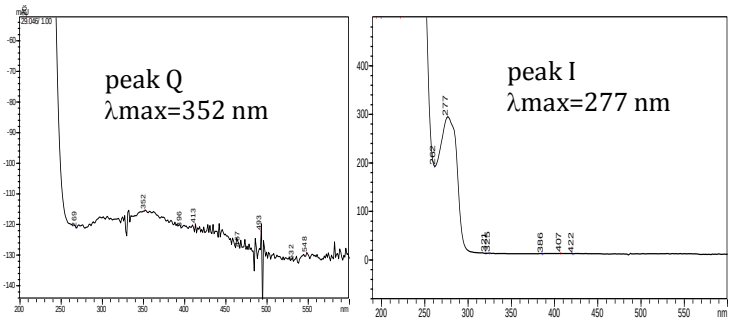
A.14 Mass chromatogram of PSE and DFPSE extract at 25.5 min, m/z 271



There is no peak found at 25.5 min, m/z = 271, so there is no naringenin present in PSE, DFPSE and PSL extracts

A.15 Maximum absorbance of unidentified peaks of *Piper sarmentosum* Roxb. leaf extracts



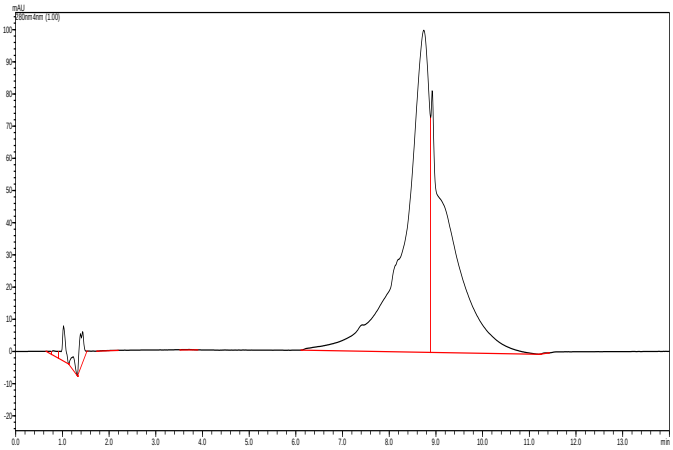

peak Q
λmax=352 nm
peak I
λmax=277 nm
277
386
407
422
nm

Appendix B

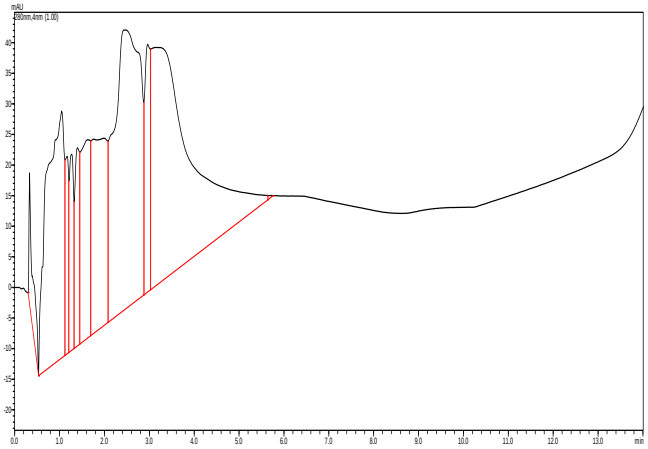
Summary of operating trial conditions of HPLC method to identify synthetic antioxidants in cooking oils

Trial	Conditions	Results
1	Mobile phase A was 0.02 % formic acid in water, mobile phase B was 70:30 (v/v) of acetonitrile:methanol. The flow rate was 0.5 mL/min of binary gradients. Starting at 0.01 min with mobile phase A (65 %) to mobile phase B (35 %), then mobile phase B was increased to 45 % within 2.52 min and hold for 2.40 min before increasing to 100 % within 2.5 min. At 10 min, mobile phase B was decreased to 35 % and hold for 3.5 min. The cycle time was 14 min, injection volume was 10 µL and column oven was set at 25 °C.	The standard BHA was eluted together with mobile phase
2	Mobile phase A and B were the same as trial 1. The binary gradients were started with mobile phase B 35 % at 0.01 min reached to 50 % at 7.00 min and hold for 3 min. Mobile phase B was then decreased to 35 % at 13.50 min and finished the cycle time at 14 min. The flow rate was set to 1.0 mL/min, injection volume was 20 µL and column oven was set at 45 °C.	Base line drifted
3	Mobile phase A and B were the same as trial 1. The binary gradients started with mobile phase B 30 % at 0.01 min reached to 35 % at 2.50 min, 45 % at 5.20 min and hold 45 % until reached to at 9 min. Mobile phase B was then increased to 100 % at 14 min and decreased to 70 % at 20 min, 30 % at 25 min. The cycle time was 30 min. The flow rate was set to 0.4 mL/min, injection volume was 20 µL and column oven was set at 45 °C.	Found 2 peaks,
4	Using mobile phase the same as trial 1. Binary gradient, injection volume and column oven the same as trial 3. The flow rate was changed to 1.0 mL/min.	The standard BHA was eluted at 16 min, base line drifted

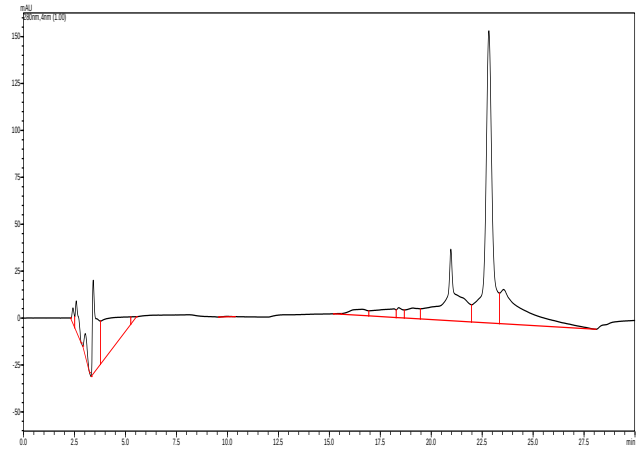
Trial	Conditions	Results
5	Mobile phase A and B were the same as trial 1. The binary gradients were started with mobile phase B 30 % at 0.01 min reached to 35 % at 2.50 min, 45 % at 5.20 min and hold 45 % until reached to at 9 min. Mobile phase B was then increased to 70 % at 14 min and hold for 6 min before decreased to 30 % at 25 min. The cycle time was 30 min. The flow rate was set to 0.4 mL/min, injection volume was 20 µL and column oven was set at 45 °C.	Found 3 peaks
6	Using mobile phases, binary gradients, injection volume and column oven the same as trial 5. The flow rate was changed to 1.0 mL/min.	The standard BHA was eluted at 16 min, base line drifted
7	Trial with a new set of mobile phase. Mobile phase A was 1 % acetic acid in water, mobile phase B was acetonitrile. The flow rate was 0.8 mL/min of isocratic binary gradients (10 % A:90 % B). The cycle time was 10 min. Injection volume was 20 µL and column oven was set at 45 °C.	The standard BHA was eluted at 3.85 min, base line more stable
8	Using mobile phases, flow rate, isocratic binary gradients, injection volume and column the same as trial 7. The cycle time was extended to 20 min.	The standard BHA was eluted at 4.0 min, base line was stable



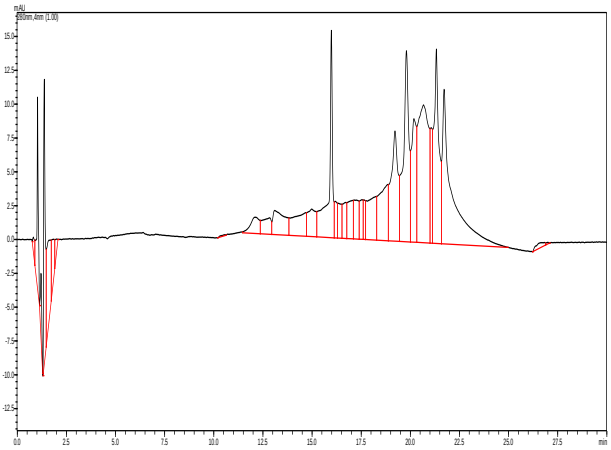
The 1st trial



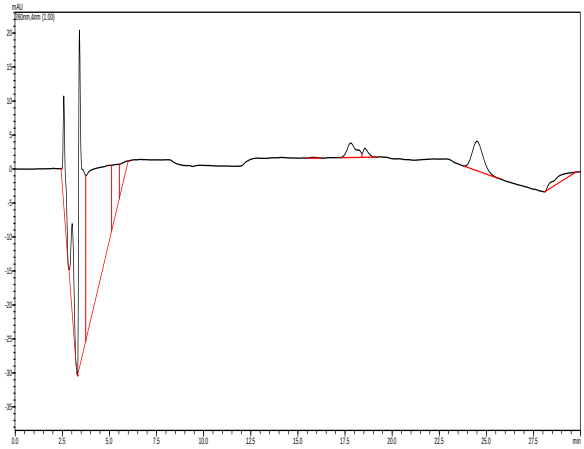
The 2nd trial



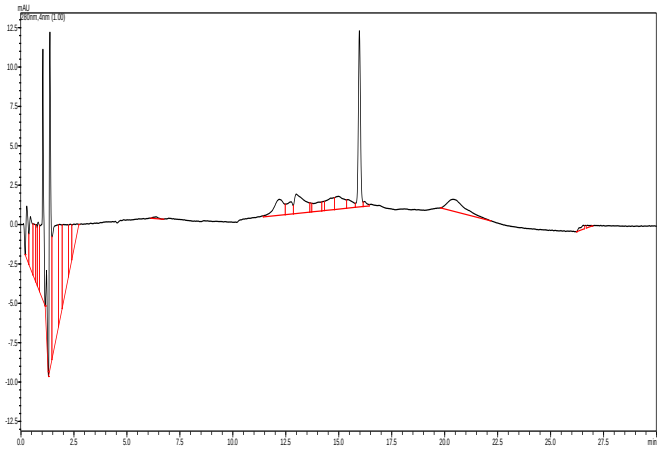
The 3rd trial



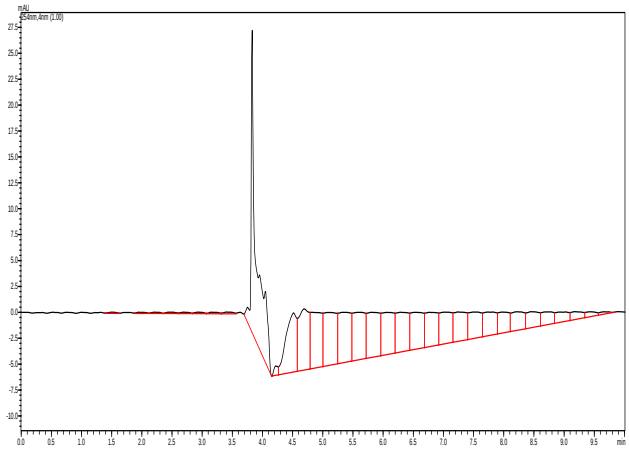
The 4th trial : chromatogram of standard BHA, retention time 16 min,



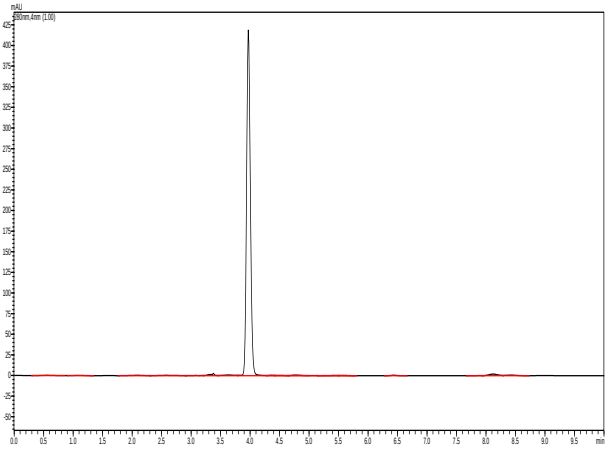
The 5th trial : chromatogram of standard BHA



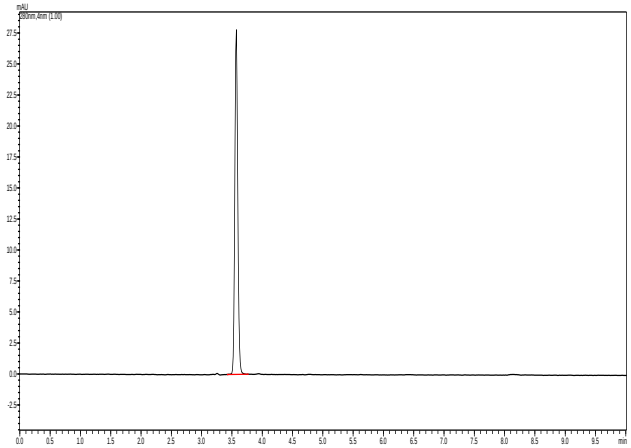
The 6th trial : chromatogram of standard BHA, retention time 16 min,



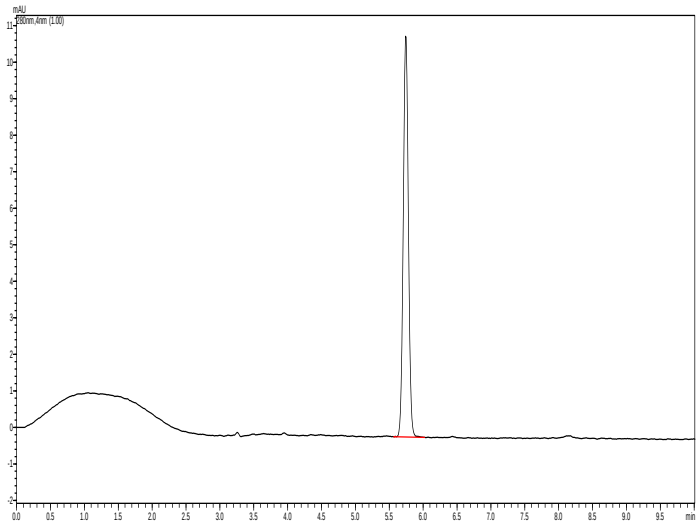
The 7th trial : chromatogram of standard BHA 100 mg/L, retention time 3.85 min,



The 8th trial : chromatogram of standard BHA 100 mg/L, retention time 4.0 min, 280 nm, the final method to identify synthetic antioxidants by HPLC method



Chromatogram of standard TBHQ 100 mg/L, retention time 3.60 min, 280 nm, using the final method (the 8th trial conditions) to identify synthetic antioxidants by HPLC method.



Chromatogram of standard BHT 100 mg/L, retention time 5.75 min, 280 nm, using the final method (the 8th trial conditions) to identify synthetic antioxidants by HPLC method.