CHAPTER TWO

LITERATURE REVIEW

2.1. Chromatographic techniques for analyses of edible oils and fats

2.1.1. Introduction

There are a number of specific analyses used to measure the chemical characteristics and quality of edible oils and fats. These tests include iodine value, acidity (free fatty acids), 1-monoglyceride content and peroxide value, which are based on classical wet chemical analyses[30,31]. Colorimetric and spectroscopic measurements have also been carried out[32-34], but these methods suffer from one inherent disadvantage whereby the analyte is not separated from the other compounds in the matrix. Consequently, the measurements are usually prone to interference.

The application of chromatographic separation techniques has provided a breakthrough in the analysis of edible oils and fats. One obvious advantage of using a chromatographic technique is that the compound of interest is usually separated from the other components in the sample matrix. This increases the detection sensitivity by reducing the background signal. In addition, the retention time of a compound also gives a higher degree of confidence for the purpose of identification.

The increase in the use of chromatographic techniques has also been rapid, from simple paper chromatography to more sophisticated instrumentation such as GC and high-performance liquid chromatography (HPLC). Today, thin-layer chromatography (TLC) is still extensively used for semi-quantitative screening applications because it is low cost and easy to use. With the development of different types of detectors, columns, stationary phases, and injection techniques, both GC and HPLC have become more versatile and indispensable in edible oil and fats analyses, some of which are briefly described in the following sections.

2.1.2. GC

2.1.2.1. Fatty acid methyl esters (FAMEs)

Methyl esters of fatty acids of edible oils and fats are almost exclusively analyzed by GC because of their ease of preparation and relatively high volatility. The preparation of various fatty acid derivatives has been reviewed[35]. The methyl esters from triglycerides can be prepared by acid-catalyzed transesterification in which an acidic reagent of HCl-methanol or H_2SO_4-methanol is usually used[36]. Sodium or potassium methoxide in anhydrous methanol is the most common reagent for base-catalyzed transesterification; although other reagents such as tetramethylguanidine (*m*-trifluoromethylphenyl)trimethylammonium hydroxide, trimethylsulphonium hydroxide and tetramethylammonium hydroxide have also been used[37].

A FAME is identified by comparing its observed retention time to that of a reference standard. However, where commercial standards are not available, particularly for unsaturated FAMEs and their isomers, the identities of the fatty acids can be deduced from their equivalent chain length (ECL) value. Because the retention characteristics of a FAME depends greatly on the number of double bonds and their distances from both ends of the fatty acid chain, ECL values of unknown fatty acids can be read directly from a straight line plot of the 'logarithms of retention times' of a homologous series of straight-chain saturated FAME standard against 'the number of carbon atoms' in the hydrocarbon chain of each compound[38]. The details on the recent applications of capillary GC for the analysis of FAMEs have been reviewed by Wolf[39].

2.1.2.2. Tri- and partial glycerides

Preliminary reviews of GC separation of tri-glycerides by Kuksis[40] and Litchfield[41] almost exclusively focused on the molecular weight differences using short packed cloumns. Grob[42,43] performed the first more detailed tri-glyceride analyses by means of capillary columns. A further development of the stationary phases opened new perpectives for high-performance capillary GC of complex tri-glyceride mixtures. The availability of medium polar gum phases for high temperature (>350°C) operation also enabled an excellent resolution of the tri-glycerides of the different types of fats[44,45]. The phenyl methyl silicone coated capillary columns used today can partly be heated up to 450°C allows the separation of saturated and unsaturated tri-glycerides and even positional isomers[46-49].

On the other hand, when analyzing mono- and di-glycerides using GC, a final temperature of 300°C is sufficient. The use of wall-coated open-tubular capillary columns has become generally accepted for such analyses. However, the problems associated with tailing and acyl migration must be expected during the analysis due to the adsorption and decreased resolution phenomena resulting from the hydroxyl groups[49]. Moreover, mono- and di-glycerides decompose and rearrange on the column, giving additional peaks corresponding to the di- and tri-glycerides[45]. Therefore, the conversion of the mono- and di-glycerides to the corresponding accetates trimethylsilyl ether or *tert*-butyldimethylsilyl derivatives is necessary for accurate analysis[50].

Similar to the tri-glyceride analyses on packed columns, the GC separation of the partial glycerides depend almost exclusively on the molecular weight, and there is no resolution on the basis of positional distribution of the fatty acids. In general, the di-glycerides elute with carbon numbers which are about 2 units lower than those of the corresponding tri-glycerides. However, with the use of a capillary column (SP-2330; 10 m x 0.25 mm i.d.), increased resolution of the saturated and unsaturated components of the *tert*-butyldimethylsilyl ether derivatives of the di-glycerides is also possible[51].

2.1.3. HPLC

2.1.3.1. Lipid classes

A normal-phase HPLC with a variety of different elution/detection systems is used for separating simple lipid classes. Under normal circumstances, there is very little separation by chain length or degree of unsaturation, which result in each class of lipid eluting as a single peak. For instance, an isocratic mobile phase made up of heptane, tetrahydrofuran and formic acid (90:10:0.5, vol/vol/vol) was employed by Greenspan and Schroeder[52] for resolving tri-, di-, mono-glycerides and a free fatty acid on a silica gel column within 1 hr. By increasing the polarity of the mobile phase slightly (heptane, tetrahydrofuran and formic acid, 80:20:0.5, vol/vol/vol), Ritchie and Jee[53] showed that the same analysis could be performed in shorter times.

In a separate approach, Christie[54] developed a rapid normal-phase HPLC system which employed an evaporative light scattering detection (ELSD). The method involved a ternary gradient elution system which successfully separated very diverse lipid classes from a complex lipid extract isolated from animal tissues. The three solvent mixtures in this gradient system were isooctane/tetrahydrofuran (99:1, vol/vol), isopropanol/chloroform (4:1, vol/vol), and isopropanol/water (1:1, vol/vol). In a subsequent paper, Christie[36,55] reported two improvements to this gradient system; isooctane was replaced by hexane to reduce the maximum operating pressure, and the water was buffered with 0.5 mM serine, adjusted to pH 7.5 with triethylamine, to sharpen the resolution of the minor acidic components.

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2.1.3.2. Free fatty acids

Even though GC methods are traditionally used for fatty acid analyses, they are accompanied by some disadvantages, particularly with respect to heat-labile or short-chain fatty acids, and, moreover, the separation of *cis-trans* isomers[56-58] is possible only with a capillary column.

In order to overcome some of these shortcomings, a number of HPLC methods have been introduced. These methods usually offer good resolution of the most important fatty acids, but the detection of underivatized fatty acids is neither sensitive nor selective because these compounds generally do not contain suitable chromophores. Absorption of underivatized fatty acids near 200 nm cannot be recommended because it is adversely affected by the properties of solvents and frequently impurities in organic solvents, which is specifically undesirable in gradient elution.

Derivatization of fatty acids can be performed pre- as well as post-column[59,60]. Post-column derivatization is more convenient for detection as the reaction does not have to be completed, provided it is reproducible. Besides requiring additional equipment such as an extra pump for adding reagent, the derivatization process is usually time consuming. On the other hand, the method that uses pre-column derivatization of fatty acids requires the reaction to be completed, and generally does not require any extra equipment.

Cooper and Anders[61] reported the reversed-phase HPLC analysis of unsaturated C_{18} and C_{20} fatty acids after conversion to naphthacyl esters. The mobile phase employed was methanol/water. [The free fatty acid (10 μmol), 2-

napthacyl bromide (20 μmol), and *N,N*-diisopropylethyalmine (40 μmol) were dissolved in 1 mL of dimethylformamide. The reaction mixture was heated at 60°C for 10 min, at which time the reaction was complete. An aliquot of the reaction mixture was injected into the HPLC].

Phenacyl esters of C_{12} to C_{24} fatty acids were prepared by Borch[62] for reversed-phase HPLC analysis with acetonitrile/water as the eluent. [Approximately 100 μg of fatty acid, 10 μL of phenacyl bromide solution (12 mg/mL in acetone), and 10 μL of triethylamine (10 mg/L in acetone) were combined and allowed to stand for overnight at room temperature. An aliquot of this solution was injected directly into the HPLC].

Miwa *et al.*[63,64] have demonstrated that both short- and long-chain fatty acids can be also converted into their 2-nitrophenylhydrazides and separated by reversed-phase HPLC with acetonitrile/water as the eluent. [To 0.2 mL of an ethanolic mixture of fatty acids, 0.2 mL of 2-nitrophenylhydrazine hydrochloride solution, 0.4 mL of 1-ethyl-3-(3-dimethylaminopro-pyl)carbodiimide hydrochloride solution in pyridine were added, and the resulting mixture was heated at 60°C for 20 min. After the addition of 0.1 mL KOH solution, the mixture was heated at 60°C for 15 min, then cooled. An aliquot of the mixture was injected directly into the HPLC].

Wolf and Korf[65] used bromomethylmethoxylcoumarin to react with the fatty acids to form esters labeled with bromomethyl group for HPLC-fluorescence detection. [1 mg/mL of 4-bromomethyl-7-methoxycoumarin in acetonitrile, 100 mg of potassium carbonate and 50 μL of water were added to 5 mL of a 20 mM solution of crown ether (18-crown-6) in acetonitrile. After sonication for ½ hr the

mixture was diluted with 5 mL acetonitrile. The supernatant was separated from the solid potassium carbonate on the bottom of the vial. The layer was then added with coumarin solution prior to analysis].

2.1.3.3. Partial glycerides

Maruyama and Yanese[66] have reported a study on the separation of various mono-glycerides directly on a reversed-phase HPLC with ultra-violet (UV) detection. Three types of reversed-phase columns were examined, namely C_2, C_8 and C_{18} with 7 μm particle size, to select a suitable phase for the separation of saturated mono-glycerides. The results showed that by using acetronitrile and water at the ratio of 80:20 (vol/vol) as a mobile phase, the reversed-phase C_{18} column gave good resolution between C_6 and C_{18} mono-glycerides whereas the reversed-phase C_8 column was only good for C_{10} and C_{18} mono-glycerides. However, the reversed-phase C_2 column was unable to resolve any mono-glyceride mixtures.

Takano and Kondoh[67] have demonstrated that by connecting six reversed-phase columns in series, the simultaneous determination of homologous distribution and the ratio of positional isomers of mono-glycerides in commercial products can also be achieved. However, the study demonstrated that only a few solvents could be used as a mobile phase because of the interference effects on the postcolumn reactor detector.

Kruger *et al.*[68] has described an HPLC-fluorescence detection method for the separation and determination of di-glycerides as their naphthylurethanes. [10 to 15 nmol of the standard mixtures of di-glycerides from the biological source were

solubilized in chloroform/methanol (2:1, vol/vol) and transferred into the reaction vials. After evaporating to dryness in a stream of nitrogen, the di-glycerides were dissolved in 100 μL of N,N-dimethylformamide. A 200-fold molar excess of α-naphthylisocyanate and a four-fold molar excess of 1,4-diazabicylo(2,2,2)octane were added to this solution. The stoppered vial was heated at 85°C for 2 hrs. After cooling to room temperature, the excess reagent was destroyed by addition of 10 μL of methanol. After 10 min, the reaction mixture was centrifuged and aliquots of 10 to 40 μL from the clear supernatant were analyzed].

Ryan and Honeyman[69] converted di-glycerides to the corresponding fluorescent Dns-phosphatidyethanolamines to be analyzed by HPLC. [0.1 ro 2.0 mg of di-glycerides was dissolved in 50 μL of anhydrous pyridine. Dns-ethanolamine phosphate (2 mequiv.) and 2,4,6-triisopropylbenzenesulfonyl chloride (5 mequiv.) were added and the reaction was allowed to proceed at 60-80°C for 24 hrs protected from light. The Dns-phosphatidyethanolamine was extracted with chloroform prior to analysis].

2.1.3.4. Tri-glycerides

The first paper on triglycerides analysis by using the reversed-phase HPLC was described by Pei *et al.*[70]. Since then, nowadays the only method used in the analysis of the tri-glyceride mixtures is reversed-phase liquid chromatography. Even though aqueous mobile phases are generally used with alkyl bonded phase columns, due to the lipophilicity of tri-glycerides, water could not be used in the mobile phase system for these particular applications; therefore the mobile phases

generally employed consisted of mixtures of acetone and acetonitrile and occasionally tetrahydrofuran, dichloromethane, or hexane.

By studying the effects of solvent composition upon tri-glyceride separations, Plattner[71] found that the mobile phase consisting of acetone/acetonitrile mixtures gave more interpretable HPLC chromatogram of tri-glyceride profile than the acetone/methanol mixtures. There were also studies occasionally employing tetrahydrofuran, dichloromethane or hexane[72-75] as the mobile phases. Additional studies[71,76-78] also found that reversed-phase C_{18} columns of small particle size gave the most efficient separations while having two columns connected in series improved the tri-glyceride profiles.

In addition, Plattner[79] and Takahashi *et al.*[80] also reported that on the reversed-phase HPLC analysis of tri-glycerides under an isocratic condition, the logarithm of the elution volume of a tri-glyceride is directly proportional to the total number of carbon atoms (CN) and inversely proportional to the total number of double bonds (DB) in the three fatty acyl chains. This elution behavior is controlled by the partition number (PN) of a tri-glyceride, which may be defined as PN = CN – *n*DB, where *n* is the factor for double-bond contribution and is normally close to 2. However, there is no distinction between tri-glycerides which are positional isomers.

Another problem associated with the analysis of tri-glycerides by using HPLC is that of detection which shows low sensitivity towards the tri-glycerides. UV-visible detectors are the most commonly used because most tri-glyceride moieties absorb in the 200 to 237-nm range. Therefore, only solvents that are transparent in this absorption window, such as hexane, acetonitrile, methanol, and

water, can be used. On the other hand, the refractive index (RI) detection mode is much less sensitive to tri-glycerides than the UV detection and is also incompatible with gradient elution system which gives serious baseline drift. However, recently an ELSD has been used extensively to overcome the problem associated with the use of gradient elution system for the analysis of tri-glycerides[81-87].

2.2. Trace analyses of contaminants in edible oils and fats

2.2.1. Inorganic contaminant

2.2.1.1. Metals

The analysis of Cr, Cu, Fe, Ni and Mg in edible oils and fats can be accomplished by atomic absorption spectrophotometry using a graphite furnace as described in the American Oil Chemists' Society (AOCS)[30] method Ca 18-79. The method requires the sample to be dissolved in methyl isobutyl ketone (MIBK) or MIBK and HNO_3 before being injected directly into a graphite tube furnace. On the other hand, the AOCS[30] method Ca 18b-91 and Ca 18c-91, and the Association of Official Analytical Chemists' International (AOAC)[31] methods 990.05 and 994.02 employed a direct vaporization of the test sample in a graphite furnace followed by sequential determination of metals at different absorption wavelengths.

The HPLC technique has also been used for the determination of trace metals in edible oils and fats[88]. The study showed that the interfering organic matrices of the vegetable oils and fats could be removed by saponification followed

by oxidative UV photolysis at 85±5°C. The determination of total Cl^-, PO_4^{3-} and SO_4^{2-} ions was accomplished by conductivity detection, using a carbonate-hydrogencarbonate eluent. Whereas, Pd(II) and Cd(II) can be determined by using an oxalate eluent, Fe(III), Cu(II), Ni(II), Zi(II) and Co(II) are determined by using a pyridine-2,6-dicarboxylic acid eluent followed by a variable-wavelength UV-visible detection system after a post-column reaction using 2-dimethylaminoethanol, NH_4OH and $NaHCO_3$ mixture.

2.2.2. Organic contaminants

2.2.2.1. PAHs

PAHs are very soluble in oils and fats. Since they are inert to caustic solution, many methods have been attempted to estimate the PAHs in the unsaponifiable fraction of the oils and fats. The PAHs have been extracted directly from the edible oils and fats by various liquid-liquid partition schemes, or in some cases preceded by a saponification step[89]. In order to obtain lower instrumental background noise, the extract has to be further cleaned up by column chromatography on silica gel, alumina or Florisil[3,90-92]. Although glass capillary GC analyses of PAHs involving cold on-column injection techniques are preferred, Kolarovic and Traitler[91] have shown that by complexing the oils and fats with caffeine-formic acid solution prior to the extraction step, a glass capillary GC with a split injection mode also gave satisfactory results.

Recently, the International Organization for Standardization (ISO)[93] has drafted 2 HPLC methods for the determination of benzo[α]pyrene and PAH contents in edible oils and fats; ISO/CD 15302:1998 and ISO/NP 15753:1999, respectively. The 2 methods employ different extraction procedures whereby in one case, benzo[α]pyrene was extracted from edible fats and oils using an alumina column chromatography, and in the other, PAHs were recovered from the unsaponifiable matters. In both techniques, the concentrated extracts obtained need to be chromatographed on a reversed-phase C_{18} column prior to fluorescence detection.

2.2.2.2. Organic solvents

Even though the flash point procedure reported by the AOCS[30] method Cc 9b-55 is widely used for the determination of organic solvents in oils and fats, the method is not only qualitative but also with slightly poor sensitivity (limit of detection, LOD of 300 ppm). On the other hand, the AOCS[30] methods Ba 13-87 and Ba 14-87Ca 3b-87, and the International Union of Pure and Applied Chemistry (IUPAC)[94] method 2.607 offer a more sensitive GC-headspace method for this purpose with the ability to detect organic solvents in the range of 10 to 1500 ppm levels. The principle of the headspace technique is that the volatile solvents are initially desorbed from the sample with heat (80-110°C) in a closed vessel. Then, the volatiles in the headspace are injected onto a packed or capillary GC column.

In addition to headspace techniques[95-97], several other sampling techniques have also been reported for the analyses of residual organic solvents namely solvent extractions[98-100] and direct GC methods[101,102].

The direct GC method is performed by introducing the oil sample directly in to the heated injection port of GC. Usually a pre-column system is used where the sample is pre-loaded on a pre-column, instead of onto a pre-heated GC inlet, to avoid deterioration before analysis. Consequently, by heating up the injection port to a comparatively high temperature, the organic solvent is rapidly evaporated and swept through the column. The advantage of such techniques is that less sample preparation is required, but continuous replacement of glass wool in the pre-column is necessary. Secondly, the direct GC method can also be performed by heating the oil samples in a pyrolyzer[103]. As organic solvents are evaporated in the chamber, they would be carried directly into the GC for analysis by the carrier gas and this minimizes the possibility of column contamination by the oil matrices.

In the solvent extraction technique, suitable extraction solvents are used to separate the oil sample to form 2 layers. In principle, a lower detection limit of the residual organic solvent such as hexane is possible by analyzing the concentrated aliquot of the solvent layer. However, the method suffers from the large co-eluent peak from the extraction solvent, which could mask the low-boiling-point volatile matters. Thus, choosing a correct solvent for such an extraction is extremely difficult.

2.2.2.3. Mineral oils

The analysis of mineral oil is usually performed after it has been extracted from the oil matrix, for instance, TLC as described in the IUPAC[94] method 2.611. Initially reference standards of oil samples containing 0.02, 0.05 and 0.10% of mineral oil were prepared. Then, a known quantity of the sample solution is streaked on a pre-conditioned silica plate. Once the plate is fully developed with heptane, the R_f value for mineral oils is confirmed by the reference oil value of *ca.* 0.7. The main drawbacks of the method are that the LOD of the mineral oil is largely dependent upon the condition of the TLC plate, and whether or not the mineral oil is completely dissolved in the oil sample. In addition, the method can only be applicable to paraffin oil, heavy and light fuel oil, petroleum jelly *etc.*, but not applicable to petrol or diesel fuel as they are too volatile.

The AOAC[31] method 945.102 has incorporated a saponification procedure for the extraction of mineral oils into the unsaponifiable fraction from the oil samples. The qualitative analysis can be carried out by adding water into the final extract. If mineral oil is present at more than 0.5%, a distinct turbidity is shown in the reaction solution. In order to determine the mineral oil content quantitatively, the extraction of unsaponifiable matter into petroleum ether layer is required. The extract is then further concentrated and centrifuged before the density of the mineral oil is obtained.

There have been studies[104-107] which described the use of column chromatography for the extraction of mineral oils from oil samples after saponification. However, the procedures are not suitable for the extraction of more

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volatile mineral oils such as diesel fuel as they could be easily lost during reflux. Therefore, to date, mineral oils are extracted directly from edible oils and fats through a column chromatography similar to those described by the AOCS[30] method Ca 6c-65 and the AOAC[31] method 966.17. In addition, different types of stationary phases have been used to obtain optimum recovery of mineral oils from edible oils and fats. Even though silica gel is commonly used as the stationary phase[28,29,108], alumina is gaining popularity because its performance is not seriously affected by the presence of moisture.

The main challenge attributed to the analysis of mineral oils in edible oils and fats is that many of the hydrocarbons found in petroleum are also found to be naturally present in animal and plant tissues[109], a situation that makes it difficult to distinguish between them. Nevertheless, mineral oils often contain *n*-alkanes with over 60 carbon atoms, though those with less than 40 carbon atoms are more typical[110,111]. Furthermore, GC chromatograms of lubricating oils or fractions that have been weathered are characterized by the presence of a 'hump' that cannot be resolved by a GC column into individual peaks. Consequently, contamination of edible oils and fats with mineral oil can normally be recognized by these method proposed by McGill *et al.*[28], Tan and Kuntom[29] and Parker *et al.*[112].

2.2.2.4. Thermal heating fluids

As mentioned earlier, the PCB-based thermal heating fluid has been banned[21]. However, there are several other brands of petroleum-based thermal heating fluids available in the market nowadays[21,24], which can be classified into 3

main classes namely eutectic mixtures of diphenyl oxide and biphenyl, partially hydrogenated terphenyls and synthetic/mineral oils. There are, at present, no specific methods for the determination of the partially hydrogenated terphenyls and synthetic oils in edible oils even though the IUPAC[94] (method 2.611) reported a standard method for detecting mineral oil to above the 300 ppm level. However, there are a number of studies describing various analytical procedures for determining the PCB-based and eutectic mixtures of diphenyl oxide and biphenyl in edible oils, which will be discussed briefly in the following sections.

2.2.2.4.1. PCB-based thermal heating fluid

The GC technique with electron-capture detection is the most sensitive method for the determination of PCBs. For the determination of PCBs in edible oil, the sample is first saponified before the PCBs are extracted into the hexane layer. The hexane layer is then concentrated by evaporation before passing through a silica gel column chromatography, using hexane as the eluent, for further clean-up to remove the interfering matrices[19,113-115].

2.2.2.4.2. Eutectic mixtures of diphenyl oxide and biphenyl

A crude method for detecting contamination of the fluid in a plant is based on a drop in the pressure of the closed system due to a leak or on its characteristic smell.

The GC technique provides a more reliable quantitative method to detect this eutectic mixture in edible oils. Takagi[116] reported a GC method that is capable of detecting the eutectic mixtures in the unsaponifiable materials of refined soybean oil down to 5 ppm level. However, after separating the hydrocarbon fraction from the unsaponifiable materials with TLC, the detection limit of the GC method was improved to 0.2 ppm of Dowtherm A™. Imai *et al.*[117], on the other hand, has incorporated a steam distillation technique to recover over 90% of the eutectic mixture from different edible oils followed by GC analysis. The detection limit reported was 0.2 ppm but the distillation process is rather long, taking about 2 hrs for a 10 g sample. Min and Wen[118] took it a step further by installing a U-shaped pre-column packed with potassium carbonate before the GC column to trap the oil sample, thus avoiding the sample preparation process. However, this technique could only measure the eutectic mixtures to as low as 5 ppm.

The AOCS[30] method Cd 25-96 has also recommended a solvent extraction method followed by GC analysis of the thermal heating fluid in both deodorizer distillate and finished oils. After multiple extractions of the samples with petroleum ether and acetonitrile, the petroleum ether fraction containing the fluid is further concentrated for GC analysis.