CHAPTER IV

DIFFERENTIATION OF LARD FROM OTHER EDIBLE FATS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND CHEMOMETRICS

1. Introduction

The detection of fats and oils adulteration nowadays has became one of the major concerns in food industries from different aspects such as nutritional, economical, as well as religious concern. Because of the importance of oils and fats economically, the development of robust methods to confirm authenticity, to detect adulteration and to define the composition of blends is much-needed (Ashurt & Dennis, 1996; Carelli & Cert, 1993).

Edible fats and oils are complex mixtures containing a wide range of compounds. They are principally composed of free fatty acids (FFAs), diacylglycerols (DGs), triacylglycerols (TAGs), phospholipids, and other minor components (Janssen et al., 2003). On the other hand, the advantage of using TAG profile comparing to fatty acid (FA) profiles is that the stereospecific distribution of FA on the glycerol molecule is genetically controlled and thus, the information of intact TAGs is usually higher (Aparicio & Aparicio-Rui´z, 2000).

Since the early 1970s, the use of high-performance liquid chromatography (HPLC) has expanded rapidly (Lough & Wainer, 1996) During that time, each technique offered major improvements in convenience, speed, resolving power detection, quantification and applicability to new sample types (Synder, 2000; Yanagisawa et al., 1985).

Reverse-phase high-performance liquid chromatography (RP-HPLC) is considered as the most commonly method used for the separation of TAGs, because it function on the principle of both chain length and degree of unsaturation of fatty acids, therefore it offers a better separation of individual TAG molecules. The dietary TAGs constitute the major energy contribution from lipids and supply polyunsaturated fatty acids (PUFAs).

There are many sources of TAGs in the human diet including oils that originate from fruits such as olive oil and palm oil, or from seed such as soybean oil and corn-, rapeseed. In addition, fats from adipose tissues and intramuscular fat droplets from cattle, poultry, pigs, and lamb; and, marine sources such as whale oils, fish oils, and seal. These fats possess in general complicated fatty acid profiles involving a range of fatty acids, which may differ more than 30 fatty acids, with various chain lengths and unsaturation and fatty acid isomers.

In 1958, Mattson and Lutton described the nonrandom distribution of animal fats. They reported also that most of TAGs are synthesized by the glycerol-3-phosphate (G-3-P) pathway in both plants and animals. Specified acyltransferases acylate the *sn*-1 and *sn*-2 positions of *sn*-glycerol-3- phosphate with fatty acids activated with coenzyme A before the phosphatidic acid is hydrolyzed, and the *sn*-3 position acylated with transferases specific for this *sn* position. Acyltransferases are likely to have different affinities for different fatty acids since there is a high variation in TAG structure in fats and oils of different biological origin.

According to (Brockerhoff et al., 1966; Christie & Moore, 1970), in lard and in human milk fat palmitic acid is the predominant fatty acid in the *sn*-2 position (Christie, 1986). Differently, in beef tallow and other bovine adipose tissues, oleic acid comprises nearly 50% of the fatty acids in the *sn*-2 position, while palmitic, stearic, and oleic acids are the major fatty acids in the *sn*-1 and *sn*-3 positions (Smith et al., 1998).

Several analytical methods have been developed to analyze TAGs quantitatively. One of the most used techniques is high performance liquid chromatography (HPLC) (Rashood et al., 1996; Yoshida et al., 2009). In addition, many works intended to detect lard from other animal fats in food matrice have been already published including: (Norris, 1982); (Saeed et al., 1989); Lambelet & Ganguli (1983); (Marikkar et al., 2002b). (Che Man et al., 2005; Che Man et al., 2011); (Rohman et al., 2010).

Nevertheless, using literature searching, there is no obtainable article related to the application of principal component (PCA) and k-mean cluster analysis using TAG data

were added; vortexed for 30 sec and centrifuged at 5000 × g for 10 min, at room temperature. Using a Pasteur pipette the upper layer (methanol/water) was then removed, and the lower layer (methanol/chloroform) was transferred into a clear tube. Finally, the solvents were removed using a rotary evaporator under reduced pressure, at 60°C.

The extracted animal fat, lard, beef fat (BF), chicken fat (CF) and a series of 14 samples containing (0.5 - 5 % w/w) of lard in BF, and CF where analyzed. Samples containing lard were assigned as adulterated; while pure beef fat, pure chicken fat and pure lard were marked beef tallow (BT), chicken fat (CF) and lard (LD), and running theme using RP-HPLC system.

2.3 Analysis of Triacylglycerol (TAG) composition by HPLC

Triacylglycerol composition was obtained using shimadzu high performance liquid chromatography (Shimadzu HPLC, Japan) equipped with a borwin software and a refractive index detector. The TAG was separated using Purospher Star RP-C18 column (250 mm × 4.4 mm with a partical size of 5µm, Merck, Darmstadt, Germany) and was eluted from the column with a mixture of acetone/acetonnitrile 70:30 v/v) at the flow rate of 1ml/min. the TAG was detected with a refractive index detector Shimadzu RI-1530 RI detector, Japan). An amount of 0.1 g of the sample was dissolved in 1ml of HPLC grade acetone and 20 µl aliquots were injected into HPLC. The selected run time for total detection was fixed to equal 25 minutes.

2.4 Statistical Analysis

For differentiation and detection of lard from other animal fats, the peak area of TAGs of all samples was transformed to a multivariates data set. Then, the data was standardized using Microsoft Excel software 2007. Principal component analysis (PCA) and K-mean cluster were performed by using an Unscrambler software (X10) from Camo, USA. All analysis was done in triplicate.

3. Results And Discussion:

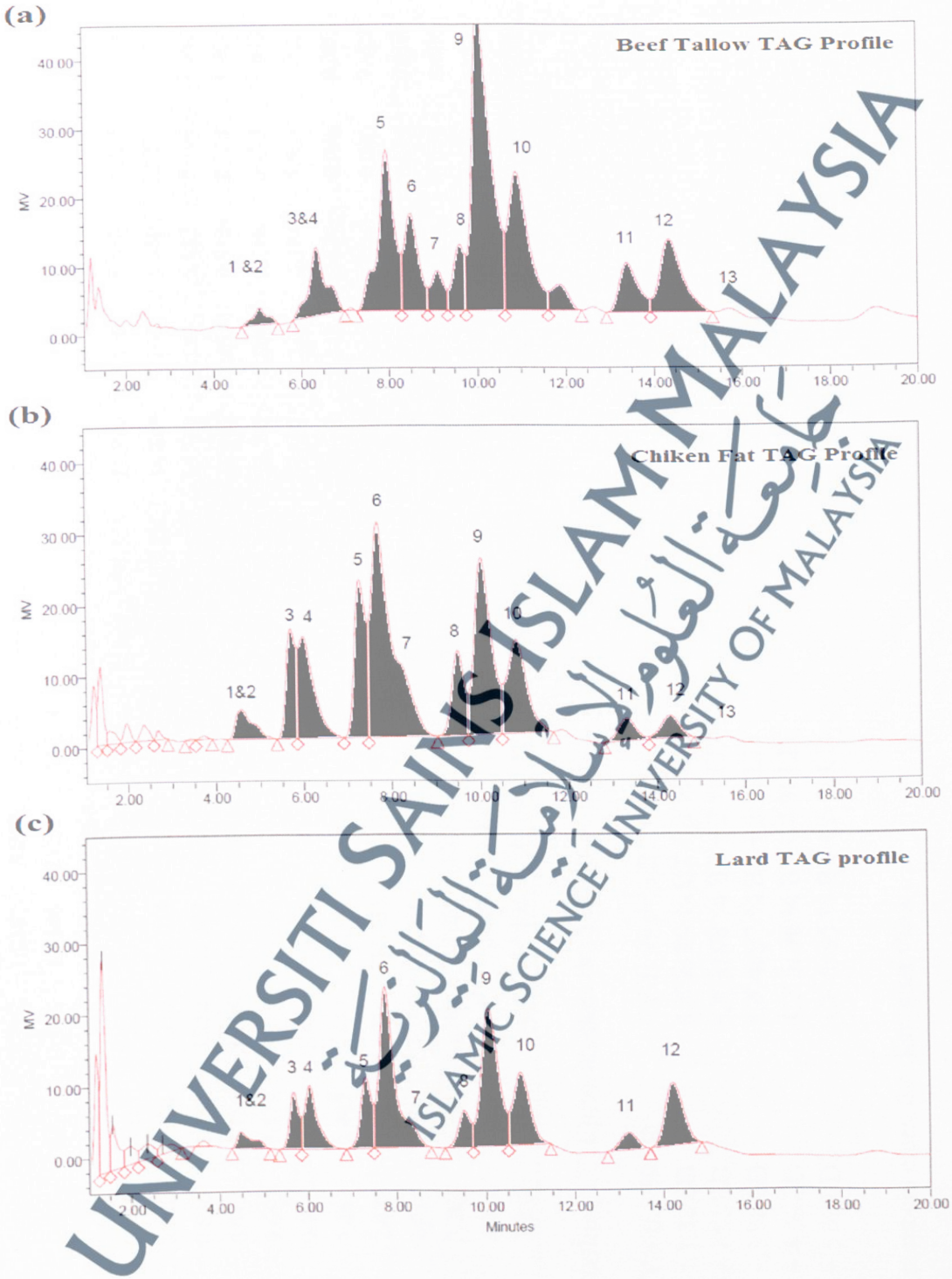

Fig. 3 The chromatogram of TAG as determined using HPLC with refractive index detector (a) BT, (b) LD, and (c) CF. peak 1 (MMM), peak 2(PLL), peak 3 (MPL), peak 4(OOL), peak 5 (PLO), peak 6 (PPL), peak 7 (OOO), peak 8 (OOP), peak 9 (PPO), peak 10 (PPP). peak 11 (OOS), peak 12 (POS), peak 13 (PPS) and peak 14(SOS) where; M, myristic; P, palmitic; O, oleic; L, linoleic; S, stearic .

Table 8: TAGs Results Of The Pure And The Adulterated Samples

TAGs	MMM	PLL	OOL	MPL	PLO	PPL	OOO	OOP	PPO	PPP	OOS	POS	PPS	SOS
BT	0.696	0.696	3.335	3.335	**14.287**	**8.342**	2.964	4.086	**30.810**	**14.967**	2.318	**5.302**	**8.840**	**0.970**
CF	1.669	1.669	3.376	3.376	**10.246**	**29.321**	**11.901**	6.777	**19.514**	**11.754**	2.623	**7.357**	0.000	0.000
LD	1.223	1.223	2.505	2.505	**8.242**	**25.566**	**7.810**	4.688	**23.730**	**13.140**	2.800	**10.768**	0.000	0.000
BT1	0.787	0.787	3.323	3.323	13.326	8.360	2.724	4.105	31.458	14.810	3.456	5.160	8.297	1.178
BT2	0.702	0.702	3.320	3.320	14.014	7.851	2.395	3.341	30.890	14.249	3.743	5.860	10.273	1.417
BT3	0.696	0.696	3.252	3.252	15.601	8.028	3.058	3.913	30.895	15.140	3.550	5.182	9.733	1.382
BT4	0.742	0.742	3.156	3.156	15.534	8.064	3.007	4.392	30.322	15.789	2.461	4.915	8.624	1.404
BT5	0.669	0.669	3.379	3.379	14.797	7.761	2.569	4.118	30.867	15.702	2.866	5.148	9.677	1.443
BT6	0.702	0.702	3.471	3.471	15.876	7.716	2.975	4.074	30.019	15.901	3.241	5.204	9.563	1.702
CF1	1.933	3.467	3.467	3.467	10.544	27.988	11.229	6.625	17.812	10.348	2.113	2.339	0.000	0.000
CF2	2.077	2.077	3.434	3.434	10.435	28.072	11.008	6.475	17.769	10.585	2.145	2.375	0.000	0.000
CF3	1.914	1.914	3.296	3.296	10.210	27.321	10.772	6.402	17.358	10.512	1.724	2.300	0.000	0.000
CF4	2.080	3.388	3.388	3.388	10.665	27.571	11.135	6.442	17.801	10.314	2.155	2.579	0.000	0.000
CF5	2.045	2.045	3.419	3.419	10.432	27.838	10.819	6.326	17.571	10.198	2.148	2.666	0.000	0.000
CF6	1.923	1.923	3.427	3.427	10.408	28.109	10.967	6.425	17.982	10.548	2.134	2.769	0.000	0.000

BT is pure beef tallow; CF is pure chicken fat; LD is pure lard;

BT 1: 99.5 % BT + 0.5 % LD
BT 2: 99 % BT + 1 % LD
BT 3: 98 % BT + 2 % LD
BT 4: 97 % BT + 3 % LD
BT 5: 96 % BT + 4 % LD
BT 6: 95 % BT + 5 % LD

CF 1: 99.5 % BT + 0.5 % LD
CF 2: 99 % BT + 1 % LD
CF 3: 98 % BT + 2 % LD
CF 4: 97 % BT + 3 % LD
CF 5: 96 % BT + 4 % LD
CF 6: 95 % BT + 5 % LD

Note: The bold numbers mean the dominant value

3.2 Principal Component Analysis (PCA)

In order to differentiate and to classify lard (LD) from beef tallow (BT), chicken fat (CF) and their different mixtures; the multivariates data matrix of 14 TAGs (i.e., the 14 TAGs in BT, LD, and CF as well as their mixtures) was subjected to principal component analysis (PCA). Shin et al. (2010) reported that PCA is a method of identifying patterns in data and expressing the data in such a way as to emphasize their similarities and differences. The authors also reported that PCA could designate interactions between groups of variables in a data set and show relationships that might exist between objects. In addition, to reduce the number of descriptors associated with the data set without losing the information of the original data. Scores and loadings matrices were generated using 4 principal components (PCs). An eigenvalue of about 98% was achieved using four PCs where PC1 accounted for 88% of the variation, while PC2 described 12% of the variation.

Figure 4 demonstrated the scores plot of PCA of 14 triacylglycerols (TAGs) representing the projection of samples defined by the first (PC1) and second (PC2) components. It explained the variation characteristics of the 14 TAGs' in the samples; there were some TAGS distributed very far from each other such as PPL, PPO and PPS. According to (Marina et al., 2010) distance makes the significant different between the samples. These TAGs are distributed reversely where PPO and PPS in the upper region in the figure (indicate BT), while PPL was at the negative side of the figure (indicate LD and CF). Therefore, this result can be justified by the stereospecificity distribution of fatty acids in TAGs molecules; where in beef tallow C16:0 is at *sn*-1 position and C18:1 is at *sn*-2 position. Contrary in lard, C16:0 is located exclusively at the *sn*-2 position, with an unsaturated fatty acid at *sn*-3, and almost same distribution of LD has been seen in chicken (Tilakavati et al., 2007). This distribution explained the difference between LD, CF and BT. Rohman et al., (2012) published quite similar result. In addition, Figure 4 showed that there are some TAGs with significant presence and they located oppositely of the figure regions, for instance: OOO (especially for LD), PLO, and POL. Furthermore, Figure 4 illustrated that other TAGs type show similar amount in all samples.

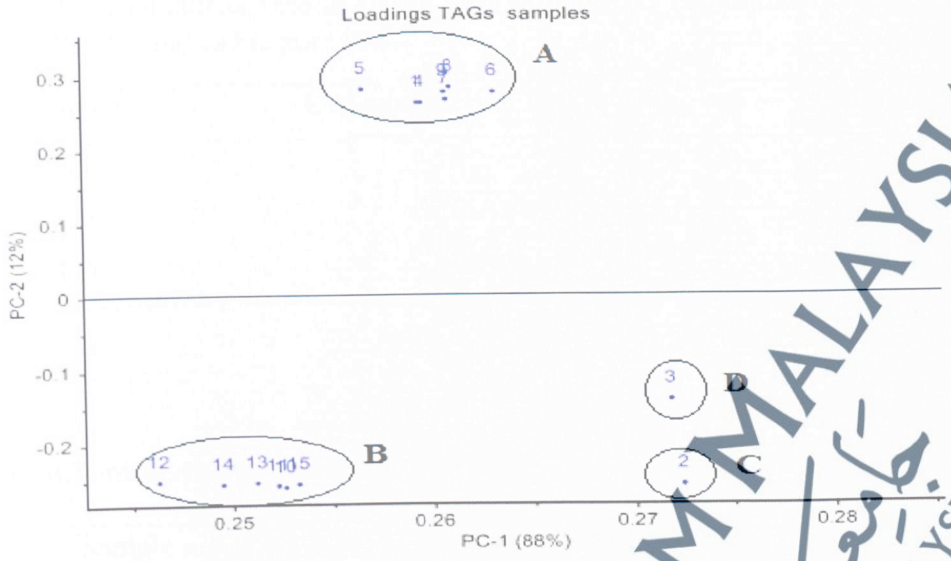

Fig.5 Principal component analysis (PCA) loadings plot of PC1 versus PC2 showing the pure and the adulterated samples, where: 1 refers to beef tallow (BT); 2 chicken fat (CF); 3 lard (LD); 4 BT+0.5 % LD; 5 BT+1 %LD; 6 BT+2 %LD; 7 BT+3 %LD; 8 BT+4 %LD; 9 BT+5 %LD; 10 CF+0.5 %LD; 11 CF+1 %LD; 12 CF+2 %LD; 13 CF+3%LD; 14 CF+4%LD; and 15 CF+ 5% LD.

3.3 Cluster Analysis

The original data set (Table 8), X (15, 14) consisted of 14 variables measured in 15 samples (3 samples are pure animal fats; BT, LD and CF and other 12 are BT+LD mixture, CF+LD mixture respectively), where each entry matrix was an average value of three replications. The data was standardized, normalized, and mean-centered prior to the analysis, and then they were subjected to cluster analysis (CA). The K-mean cluster analysis results are given in (Tables9, 10, and 11) that grouped all samples into three different classes class 1 for BF and BT+LD (Table 9 and Table 10); class 2 for CF and CF+LD (Table 9 and Table 11), while class 3 for LD. The mixtures were classified according to their main component of TAGs. This K-mean cluster results could not distinguish lard in small concentration (0.5-5 %) from other concentrations (more than 0.5%), but it may help to differentiate lard from other animal fats if it (lard) concentration in the mixtures is higher.

Table 9: K-mean cluster results for the pure samples BT is pure beef tallow; CF is pure chicken fat and LD is pure lard.

Sample	Concentration of lard %	Class
BT	0	1
CF	0	2
LD	100	3

Table 10:K-mean cluster results for BT+ LD

Sample	Concentration of lard %	Class
BT1	0.5	1
BT2	1	1
BT3	2	1
BT4	3	1
BT5	4	1
BT6	5	1

BT 1: 99.5 % BT + 0.5 % LD
BT 2: 99 % BT + 1 % LD
BT 3: 98 % BT + 2 % LD
BT 4: 97 % BT + 3 % LD
BT 5: 96 % BT + 4 % LD
BT 6: 95 % BT + 5 % LD

Table 11: K-means Cluster Results For CF+ LD

Sample	Concentration of lard %	Class
CF1	0.5	2
CF2	1	2
CF3	2	2
CF4	3	2
CF5	4	2
CF6	5	2

CF 1: 99.5 % BT + 0.5 % LD
CF 2: 99 % BT + 1 % LD
CF 3: 98 % BT + 2 % LD
CF 4: 97 % BT + 3 % LD
CF 5: 96 % BT + 4 % LD
CF 6: 95 % BT + 5 % LD

4. Conclusion

From the results have shown above, HPLC combined with the chemometric technique such as PCA and K-mean cluster can be employed to differentiate lard from beef tallow and chicken fat based on their TAGs particularly in PPL, PPO and PPS concentration. In addition, PCA has showed the ability of detection of lard within the mixtures of beef tallow- lard and chicken fat-lard as low as 0.5 % of lard. Meanwhile, K-mean cluster was only able to differentiate between pure beef tallow, pure chicken fat and pure lard.