CHAPTER III

DIFFERENTIATION OF LARD FROM OTHER HALAL ANIMAL FATS BY GAS CHROMATOGRAPHY-FLAME IONISATION DETECTOR (GC-FID) AND CHEMOMETRICS

1. Introduction

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). Since a long time, the trade of fats and oils has been facing a serious adulteration problem, which is usually motivated in a bid for maximizing profit by replacing expensive ingredients with cheaper ones (Cserháti et al., 2005); being versatile in providing the functional properties of the product. Lard in its original or its industrially modified fats might be mixed with other animals or vegetable oils to produce shortenings, margarines and other food oils and its detection as an adulterant in food systems is a major concern for many countries due to religious prohibitions and health reasons (Marikkar et al., 2005). The presence of lard in food is a serious issue for some religions like Islam and Judaism where consuming foods containing porcine and its derivatives is forbidden even at trace level (Regenstein et al. 2003). However, detection of lard when it exists as a minor component in other oils and fats is difficult. Hence, there is a great need for robust and trustworthy techniques for lard differentiation and detection for the practice of Halal authentication analysis by the relevant authorities. The use of gas chromatography coupled with chemometrics may provide a solution to this low concentration detection problem.

Haswell (1992) has defined chemometrics as the combination of mathematical, statistical, and other logic-based methods for managing and interpreting efficiently the chemically-derived data. Principal components analysis (PCA) is an unsupervised pattern recognition technique used in multivariate analysis (Che Man et al., 2011). It projects the original data to reduced dimensions in matrices called scores and loadings. Shin et al., (2010) has 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. PCA has been successfully used to define relationships that exist in fatty acid classification studies of food lipids, due to the capability to manage and interpret large data sets (Kadegowda et al., 2008; Matos et al., 2007). K-mean cluster analysis (CA) is calculated based on Euclidean distance from the object of each cluster to its centre by which pre-determined the number of cluster is K (Chen & Zhang, 2011). The object belongs to its cluster when the distance is near to the centre compared with others centres. CA has an advantage over PCA where CA information is more objective and provides numerical results. In addition, CA is able to reduce dimensionality, while retaining the required information (Che Man et al., 2011).

Thus, the objective of this study was to investigate the use of gas chromatography-flame ionization detector coupled with chemometrics techniques, as a means for distinguishing lard adulteration in very low concentrations from other animal fats such as beef tallow and chicken fats.

2. 0 Materials And Methods

The materials and methods used in Differentiation of Lard from Other Edible Fats by Gas Chromatography-Flame Ionisation Detector (GC-FID) and Chemometrics were discussed below.

2.1Materials

Adipose tissues of pig (lard), chicken and beef were obtained from local supermarkets. The analytical solvents used for fats extraction and GC analyses were: methanol 99.9 % (analytical, GC grade); acetone 99.9% (analytical, GC grade); *n*-hexane 99.0% (analytical, GC grade), chloroform 99.8% (analytical grade)and sodium methoxide 1% solution. All mentioned solvents were from Sigma- Aldrich, UAS.

2.2 Standards

The Supelco 37 Component FAME Mix from (Supelco, Sigma–Aldrich, USA) was used to identify retention time of the fatty acid composition of the samples.

2.3 Samples Preparation

The fats were extracted according to the Bligh-Dyer method (1959). Accurately, 300 to 400 mg of sample was weighed; then, 4 ml methanol, 2 ml chloroform and 0.4 ml water were added; homogenized and vortexed for 30 sec. After that, 2 ml of chloroform and 2 ml of water were added; vortexed for 30 sec and centrifuged at 3300 × g for 15 min, at 5° to 25°C. 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 - 10 % w/w) of lard in BF, and CF were analyzed. Samples containing lard were assigned as adulterated as the following: for BT (BT1 (99.5 % BT+ 0.5% LD, BT2 (99 % BT + 1 % LD), BT3 (98 % BT +2 % LD), BT4 (97 % BT +3 % LD), BT5 (96 % BT +4 % LD), and BT6(95 % BT +5 % LD); while for CF (CF1 (99.5 CF % + 0.5% LD, CF2 (99 % CF+ 1 % LD), CF3 (98 % CF +2 % LD), CF4 (97 % CF +3 % LD), CF5 (96 % CF +4 % LD), and CF6(95 % CF +5 % LD). Pure beef fat, pure chicken fat and pure lard were marked beef tallow (BT), chicken fat (CF) and lard (LD), and running theme using GC-FID.

2.4 Analysis Of Fatty Acid Methyl Esters (FAME) Using GC-FID

The fatty acid compositions were determined by conversion of the oil to fatty acid methyl esters (FAME) according to the method of Cocks and Van Rede (1966) with a slight modification. FAME was prepared by adding 950 µl of *n*-hexane into 50 mg of fat, followed by 50 µl of sodium methoxide. The mixtures were vortex for 5 sec and allowed to settle for 30–60 min. The top layer (1 µl) was injected into a gas chromatograph (Agilent 7890) equipped with a flame-ionization detector and a polar capillary column (HP88-Agilent Technologies, USA), 100 m, 0.25mm, and 0.25 µm film, internal diameter and film thickness, respectively, to obtain individual peaks of FAME. The temperature of the column was 90 ºC, programmed to increase to 220 ºC at

Table 3: FAME results of the pure and the adulterated samples.

FA/%	C4:0	C6:0	C10:0	C12:0	C14:0	C14:1	C15:0	C15:1	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1cis	C18:2trans
BT	**8.290**	ND	0.129	0.176	**7.349**	0.308	0.834	0.364	**56.038**	0.316	0.185	0.104	**20.335**	**3.248**	0.085
CF	**0.462**	0.069	0.243	0.509	**2.605**	0.497	0.177	ND	**71.461**	**1.192**	0.355	0.192	**15.643**	0.474	0.175
LD	**1.853**	ND	0.124	0.128	**1.864**	0.000	0.094	0.000	**35.374**	0.606	0.586	0.292	**20.491**	0.208	0.104
BT1	6.618	ND	0.141	0.177	6.481	0.247	0.700	0.293	55.833	0.442	0.153	0.089	22.609	2.715	0.067
BT2	5.700	ND	0.144	0.182	6.473	0.274	0.755	0.290	56.401	0.461	0.152	0.086	22.831	2.692	0.067
BT3	6.737	ND	0.139	0.177	6.370	0.242	0.687	0.091	55.780	0.456	0.156	0.085	22.828	2.681	0.067
BT4	6.732	ND	0.097	0.120	6.186	0.790	0.661	0.275	54.915	0.456	0.589	0.463	22.608	2.367	0.127
BT5	6.630	ND	0.144	0.176	6.333	0.237	0.678	0.183	55.670	0.467	0.153	0.084	22.485	2.608	0.067
BT6	7.102	ND	0.141	0.182	6.347	0.239	0.680	0.284	55.106	0.445	0.598	0.150	22.213	2.432	0.128
CF1	1.010	0.041	0.235	0.426	2.538	0.379	0.203	ND	66.777	1.132	0.495	0.260	19.644	0.460	0.157
CF2	1.061	0.038	0.228	0.423	2.538	0.362	0.198	ND	66.745	1.127	0.504	0.271	20.102	0.443	0.157
CF3	1.017	0.019	0.156	0.300	2.146	0.311	0.171	ND	58.939	0.997	0.459	0.258	18.274	0.386	0.141
CF4	1.181	0.053	0.226	0.411	2.557	0.345	0.194	ND	66.245	1.116	0.535	0.276	21.026	0.452	0.155
CF5	0.418	0.034	0.136	0.248	1.899	0.174	0.064	ND	49.629	4.772	0.318	0.182	15.181	15.627	0.739
CF6	1.073	0.040	0.228	0.400	2.580	0.338	0.193	ND	65.564	1.114	0.545	0.292	21.035	0.439	0.157

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: ND means not detected. The bold numbers mean the dominant value

(C20:0) showed almost similar amounts in pure samples with the range of 0.24 and 2.18 %. Table 3 showed a very interesting observation that there were some long chain fatty acids appeared only in lard such as Docosahexaenoic acid (C22:1n9), and Nervonic acid (C24:1), however their amounts was very small 0.39and 0.15 %. These two fatty acids could be used as markers for lard detection. In addition, Table 3 showed that the values of the C12–C18 fatty acids were quite closer in the three animal fats, but whereas small proportions of the C20–C24 PUFA were present in pork, these were not detected in beef, leading to the lack of incorporation of the long-chain PUFA triacylglycerols into ruminant (Enser et al., 1991).

Table 4: The Percentage Of Different Fatty acids In The Pure Samples

	BT	CF	LD
S SFA (%)	93.25 a	92.98 b	60.61 c
S MUFA (%)	5.60 a	4.54 ab	3.12 b
S PUFA (%)	1.20 c	2.45 b	36.14 a

Note: Means followed by the same latter on the row are not significantly ($p > 0.05$) different.

Duncan's multiple range test (DRMT) was performed to compare the total fatty acids contents of the samples (Table 4) (Appendix 1). BT showed the highest total saturated fatty acid content (TSFA) which was significantly different from that of CF (92.98 %) and LD (60.61%). Furthermore, Table 4 also illustrated that BT showed also be the highest total monounsaturated fatty acids (TMUFA) content which was significantly different form LD (3.12%), but it was not significantly different from CF (4.54%). This was because of the fatty acid, the ruminants, which is at high levels in concentrate feedstuffs (grains and oilseeds), is degraded into saturated and monounsaturated fatty acids in the rumen by microbial biohydrogenation and only a small proportion, around 10% of dietary 18:1n-6, is available for incorporation into tissue lipids (Wood et al., 2008).

However, from Table 4LD showed to be the highest total polyunsaturated fatty acids (TPUFA) content with (36.14%) which was very high significantly different from CF

(2.45%) and BT (1.20%). This according to (Wood et al., 2008) was due to the absence of the biohydrogenation phenomena inside pig's stomachs as monogasteric animal.

3.2 Principal Component Analysis (PCA)

In this study, the multivariates data matrix of 30 FAME (i.e., the 30 FAME in BF, LD, and CF as well as their mixtures) was subjected to PCA in order 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 seven principal components (PCs). An eigenvalue of about 98% was achieved using seven PCs where PC1 accounted for 1% of the variation, while PC2 described 97% of the variation; therefore, the remaining five (= 2% total) did not explain significant variability in the data.

Figure1 demonstrated the scores plot of PCA of 30 fatty acids methyl esters (FAME) representing the projection of samples defined by the first (PC1) and second (PC2) components. It explained the variation characteristics of the 30 FAME's in the samples; there were some fatty acids which are significantly different and which consist of medium chain and polyunsaturated fatty acids. These fatty acids were distributed in the upper left region in the figure for example C16:1, oleic acid (C18:1cis), and linoleic acid (C18:2cis) specifically in lard. This may be due to the unchanging structure of the fatty acids inside pigs' stomach (monogasteric animal) during digestion; oppositely to the ruminants with several stomachs have microbes that change fatty acids structure into saturated fats. Therefore, they can pass into the blood stream in the small intestine, and from there into tissues (Teye et al., 2006a; Teye et al., 2006b). In addition, the Figure 1 showed that there are some saturated fatty acids with significant presence and they were located in the lower left region in the figure, for instance: butyric acid (C4:0), myristic acid (C14:0), and stearic acid (C18:0). Furthermore, Figure 1 illustrated that palmitic acid (C16:0) which was cited on the right region and far from others acids. This means that it was significantly higher from other fatty acids and this especially in BT; and that may be due to the microbial biohydrogenation of fatty acids inside the ruminant's stomach; where the fatty acids are changed from poly and mono-unsaturated to saturated fatty acids (Wood et al., 2008).

Figure 2 showed the loading plot for the different concentrations of the samples (BT+ LD, and CF+LD) as well as the pure samples BT, CF and LD. The PCA loading plot described the different distribution of variables (concentrations) in two main groups. The first group (1, 4, 5, 6, 7, 8, and 10) which referred to BT and BT+LD; and it was located under X-axis. The second group (3, 11, 12, 13, 14, 15, and 17) referred to CF and CF+LD; and it was located up of X-axis. However, for the sample 2, which referred to lard sample, was very isolated from the other two groups. This grouping of samples was maybe because of the differentiation in their physic-chemical characteristics. Therefore, Figure 2 helped to differentiate BT and CF from LD, and it aids to distinguish very low adulterated concentration (0.5%) of lard in BT and CF.

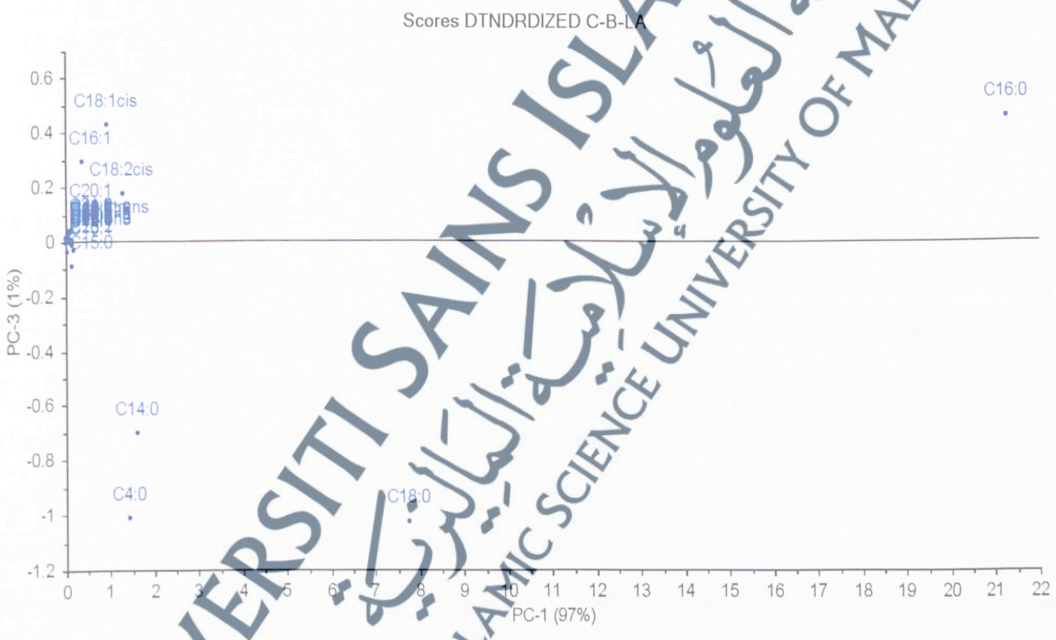

Fig. 1 The scores plot for the first two principal components (PC) for 30 FAME from the samples studied

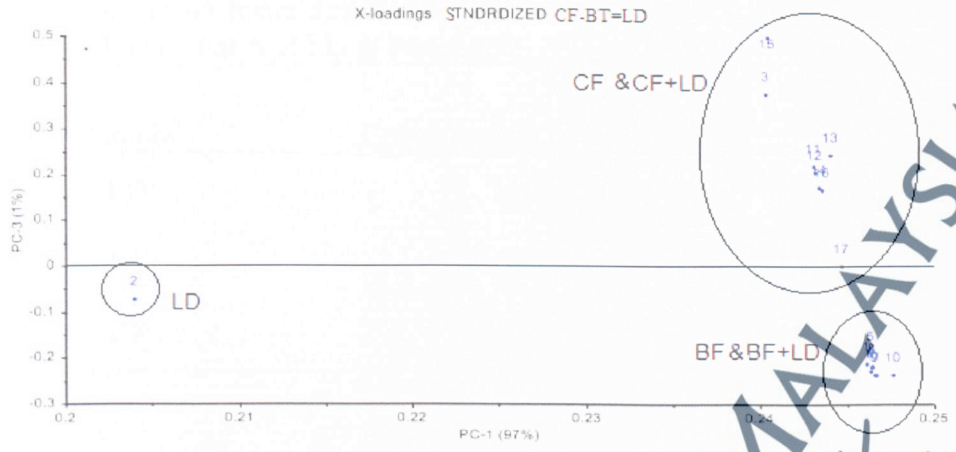

Fig.2 Principal component analysis (PCA) loadings plot of PC1 versus PC2 showing the pure and the adulterated samples, where: 1 refers to beef tallow (BT); 2 lard (LD); 3 chicken fat (CF); 4 BT+0.5 % LD; 5 BT+1 %LD; 6 BT+2 %LD; 7 BT+3 %LD; 8 BT+4 %LD; 9 BT+5 %LD; 10 CF+0.5 %LD; 11 CF+1 %LD; 12 CF+2 %LD;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

3.3 Cluster Analysis

The original data set (Table 3), X (15, 30) consisted of 30 variables measured in 15 samples (3 samples were pure animal fats: BT, LD and CF and other 12 were BT+LD, CF+LD 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 were given in (Tables5, 6, and 7) that grouped all samples into three different classes; class 1 for pure LD, BT, CF (Table 5), and class 2 for BT+LD (Table 6) ,while, class 3 for CF+LD (Table 7). The mixtures were classified according to their main component of fat; BT+LD in class 2 (BT), and CF+LD in class 3 (CF). Nevertheless, this K-mean cluster results could not distinguish lard in small concentration (0.5%) from other concentrations (more than 0.5%), but it may help to differentiate lard from other animal fats if it (lard) is dominant in the mixtures.

Table 5: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
LD	100	1
BT	0	2
CF	0	3

Table 6: K-mean Cluster Results For BT+ LD

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

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 7: K-mean Cluster Results For CF+ LD

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

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 above results, GC-FID was able to differentiate between lard, beef tallow, and chicken fat. For instance, BT and CF were higher in SFA and MUFA as compared with LD; while, the PUFA in LD was the highest. In addition, there were two fatty acids appeared only in LD (C22:1n9, and C24:1) and they could be used as markers for lard detection.

Furthermore, combining GC-FID with the chemometric technique such as PCA and K-mean cluster could be employed to differentiate lard from beef tallow and chicken fat. PCA 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. In future continuation from this study will look into more replications to elaborate more information about k-mean cluster analysis in intention of detection of lard in food matrix in very small possible amounts.