Chemometrics Analysis Combined with GC-MS and NMR Spectroscopy Analysis of Fatty Acids as a Means of Discriminating Butterfat Adulteration

Nurrulhidayah Ahmad Fadzillah[1], Abdul Rohman[2], Amin Ismail[3], Yanty Noorziana Abdul Manaf[3], Arieff Salleh Rosman[4], Alfi Khatib[5], Norazian Mohd. Hassan[5] and Rashidi Othman[1,*]

[1]*International Institute for Halal Research and Training (INHART), Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environmental Design, International Islamic University Malaysia, 53100 Kuala Lumpur, Malaysia*

[2]*Research Center of Halal Products, Faculty of Pharmacy, Gadjah Mada University, 55281 Yogyakarta, Indonesia*

[3]*Laboratory of Halal Science Research, Halal Products Research Institute, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

[4]*Centre of Research for Fiqh Science & Technology, University Technology Malaysia, 81310 Johor Bahru, Johor Darul Takzim, Malaysia*

[5]*Department of Pharmaceutical Chemistry, Kulliyah of Pharmacy, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia*

Abstract: The adulteration of butter has become a major problem in food industries. Butter has the similar characteristic to lard which makes lard a desirable adulterant in butter due to economic advantages. Therefore, the method of detection to analyse the adulteration practice must be developed. This study used NMR spectroscopy in combination with chemometrics for the authentication of butter from lard. The presence of lard as an adulterant in butter has been analysed using Gas Chromatography–Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR) spectroscopy with the aid of chemometric of Principal Component Analysis (PCA) and Discriminant Analysis (DA). PC1 described 82% of the variation while PC2 accounted for 15% of the variation resulted in a model that described 80% of the total variance in the data. With 82% of the peak variation along the first PC, it was clear that all seventeen samples of butter in the market and pure lard sample were formed according to their own group and showed two well-defined and well-separated group. DA model classified 100% of all samples accurately according to its group (butter and butter adulterated with animal fats), meaning that no samples were misclassified into the wrong group. Lard was successfully determined at 2.63 ppm. In this study, NMR and PCA analysis has successfully discriminated between the market sample and lard and the results established that there is no lard being adulterated in all commercial butter samples. This could be a potential identification approach to determine if the product has been deceived in market.

Keywords: Fatty acid, triglycerides, nutrition, adulteration, butter, lard, chemometrics.

INTRODUCTION

Butter is a water-in-oil emulsion and essentially derived from milk fat. The principle constituents of normal butter are fat (82%), water (15.6-17.6%), salt (1.2%) as well as protein, calcium and phosphorus. Due to high price, butter is frequently a target of adulteration, primarily through the addition of less expensive animal fats or vegetable margarine [1]. Classification of pure and adulterated butter would protect consumer against deception and mislabelling. Spreadable butter containing blends of foreign fats can be commercialized, provided the denomination and the label stated the origin of the lipid material clearly. Many studies have been devoted to the fat composition of butter. In most cases, fatty acids are analysed after hydrolysis using GCMS. About 98% of the fat has been determined consists of triglycerides, and the most common fatty acids are palmitic, oleic, stearic, myristic and butyric [2]. The composition of butter is influenced by genetic factors [3] and feeding conditions [4]. Because of its overwhelming variety, the characterization of the natural sample is very difficult. Based on their fatty acid composition, the possible number of triglycerides in milk fat is calculated to be greater than 1,300 [5]. A review of the most commonly used methods for the detection of triglycerides composition of butter has been compiled [6]. Generally, it is considered as impossible to identify all triglycerides with only one chromatographic method [7]. Attempts

Address correspondence to this article at the International Institute for Halal Research and Training (INHART), Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environmental Design, International Islamic University Malaysia, 53100 Kuala Lumpur, Malaysia; Tel: +6012 6644772; Fax: +603 61964864; E-mail: rashidi@iium.edu.my

ISSN: 2223-3806 / E-ISSN: 1927-5951/19

have been made to detect vegetable or animal fats (beef tallow, lard) in butter by using a number of method, such as gas chromatographic analysis of fatty acids [8-10], triacylglycerol [4,11] or sterols [12-15], as well as thermo-analytical method such as differential scanning [16,17]. The legislation and analytical techniques are currently available for controlling the authenticity of dairy products in Europe [18]. However, while strict standards and criteria for product definition exist, practical means for judging product authenticity are not always available. The main approach for solving these kinds of problems is to look for a specific marker or indicator in the product, which could be a component (complex molecule, nucleic acid) or determine the ratio between some chemical constituents and assume that these ratios are a constant component of the particular dairy product. From this perspective, it seems to make sense that the addition of any substance to milk products will modify the value of these ratios or highlight an anomaly in their chemical composition. In this area, many pattern classification procedures can be applied to the dataset to compare similarities or differences in the sample data with the original data.

In food industry, especially in fats and oils analysis, GCMS has been used widely to assess a number of fats and oil samples [19]. Multivariate data analysis techniques such as principal component analysis (PCA) could be used to process fatty acid data if problems arise in the classification of data [20]. PCA is capable to identify patterns in data and express the data by emphasizing their similarities and differences [21]. However, from the literature search, there is no available report related to the application of PCA using fatty acid and NMR spectra as matrix variables of lard and other animal fats. Recently, an issue associated with the presence of lard in Halal butter was highlighted in the mainstream media and caused a lot of concern among consumers in Malaysia. This is where the importance of developing a rapid, non-destructive and sensitive method for Halal authentication lies. Therefore, this study aimed to assess the potential of GCMS and NMR to discriminate lard among other types of fats particularly (beef, chicken and mutton) and to discriminate adulterated butter with pure butter. Seventeen samples of commercial butter available in the market were also being considered for analysis.

MATERIALS AND METHODS

Sample Preparation

Lard was obtained by rendering the adipose tissue of pig [22] which were obtained from several local markets in Selangor, Malaysia and a butter sample was prepared according to the AOAC official method 920.118 [23]. Similar method applied to other animal fats. Prior to analysis, the samples were removed from frozen storage, and left static at room temperature for 1 h and then were thawed at 50^{o}C in water bath until they became completely molten. Sampling was done in triplicates and consisted of three pig samples. Adipose tissues were heated using a microwave according to the method proposed by De Pedro *et al*. [24]. The extracted lard was filtered through double-folded muslin cloth, and anhydrous sodium sulfate was added to the extract to remove residual moisture. The extract was then filtered through Whatman No. 2 filter paper and stored at 4°C before further analysis.

Fatty Acid Analysis

FA compositions of butter, lard and the mixture of butter-lard were determined using a headspace auto sampler (Model G1888, Agilent Technologies, Palo Alto, CA, USA). The transfer line from the headspace sampler was directly connected to the injector of the gas chromatograph (GC). The oven was set at 110°C. The extraction conditions in the headspace auto sampler were programmed as follows: 20.0 min for vial equilibration, 0.20 min for vial pressurization, 0.20 min for filling the injection loop, 0.05 min for loop equilibration and 1.0 min for sample injection. Helium with a purity of 99.999% was used for vial pressurization and as the carrier gas. Volatile compounds were analysed using a GC-MS (Model 7890, Agilent Technologies, Palo Alto, CA, USA) equipped with a non-polar column (J and W Scientific DB-5; 30 m, ID 0.25 mm, film thickness 0.25µm). The column temperature was kept at 40°C for 10 min, increased at 6°C/min to 240°C and maintained isothermally for 20 min. The mass selective detector (Model MSD59556, Agilent Technologies, Palo Alto, CA, USA) was used in electron ionization mode. A mass range between 30 and 550 m/z was scanned. The mass spectra obtained were compared to those of the National Institute of Standards and Technology (NIST) Mass Spectral Search Program for compound identification. 37 FAME standards (Sigma St. Louis, MO) were used as authentic samples to calculate the percentage of fatty acids based on peak area. The quantification of FAME was performed using a normalization internal technique.

NMR Measurement

A 10 mg sample of each sample was dissolved in 700 µl of deuterated methanol containing 0.5% TMS

and mixed using a vortex mixer. The sample was then sonicated for 1 h in a sonicator bath and left to solubilize for a further 12 h at room temperature. This was followed by centrifuging for 5 min to remove insoluble components, then the clear supernatant was transferred into NMR tubes. ^{1}H NMR spectra were recorded at 26˚C on a 500 MHz Varian INOVA NMR spectrometer operating at a proton NMR frequency of 499.887 MHz. Each ^{1}H NMR spectrum was acquired over a spectrum width of 20 ppm, consisted of 64 scans that require 3:15 min acquisition time. The resulting spectra were manually phased and base-line corrected using Chenomx software (v. 5.1, Alberta, Canada). The spectra were referenced to TMS at δ 0.00ppm.

Bucketing of ^{1}H NMR Spectra

^{1}H NMR spectra were automatically reduced to ASCII files using Chenomx software (v. 5.1, Alberta, Canada). Spectral intensities were binned by equal width (δ 0.04) corresponding to the region of δ 0.50–10.00. The region of δ 4.70–4.96 (water) and δ 3.28–3.33 (residual methanol) were excised from the analysis

Statistical and Chemometric Analysis

For fatty acid analysis, the statistical treatment using one-way analysis of variance (ANOVA), followed by Duncan multiple comparison using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) was used for differentiation of fatty acid composition levels in butter adulterated with lard. The significance value (*P*) of less than 0.05 was considered statistically different. All sample analyses (fatty acid composition and chromatogram) were performed as three replicates and averaged using Microsoft Excel 2007. In order to differentiate and classify lard, animal fats and butter, the chemometric of principal component analysis using the composition of fatty acid as matrix variable was performed with the aid of the Unscrambler software (version 9.7) from Camo, USA.

RESULTS AND DISCUSSION

Fatty Acid Composition

Fatty acid compositions of butter and other animal fats (lard, beef, chicken and mutton fat) are compiled in Table **1**. Fatty acid profiles were further subjected to PCA analysis in order to cluster the classification of butter and other animal fats. Figure **1A** show the PCA score plot of butter and animal fats representing the projection of samples defined by the first component (PC 1) and the second component (PC 2). This basic difference could be due to the diverse pattern of distribution of individual fatty acids among these animal fats. Using the projection of PC1 and PC2, it was clear that butter was well-separated from other animal fats. Using PC1 and PC2, butter was located in similar distance with other animal fats, therefore, all animal fats could be the potential adulterants in butter. Figure **1B** exhibits the loading plots for PC1 and PC2. The separation of butter was more influenced by fatty acids. According to the loading plot in Figure **1B**, six fatty acids (lauric, myristic, palmitic, stearic, oleic and linoleic acids) were the most discriminating variables that influence the group separation into three different clusters.

Table 1: Fatty Acid Compositions of Butter, Lard, Chicken Fat, Beef Fat and Mutton Fat

Fatty Acid	Lard (%)	Chicken Fat (%)	Beef Fat (%)	Mutton Fat (%)	Butter (%)
C12:0	0.18±0.0^{a,c}	0.64±0.01^{b}	0.66±0.04^{c}	0.28±0.01^{d}	1.63± 0.02^{a}
C14:0	2.64±0.04^{a}	1.62±0.01^{b}	6.43±0.08^{c}	5.08±0.08^{d}	1.63± 0.02^{a}
C15:0	0.16±0.0^{a}	ND	0.66±0.01^{c}	0.71±0.07^{b,d}	ND
C16:0	24.66±0.14^{a}	25.39±0.03^{b}	27.79±0.04^{c}	25.70±0.03^{a,d}	31.29±0.05^{c}
C16:1	1.10±0.06^{a}	5.32±0.03^{b}	1.55±0.1^{c}	0.71±0.04^{d}	ND
C17:0	0.80±0.04^{a}	ND	1.43±0.03^{c}	2.14±0.08^{d}	ND
C18:0	14.47±0.02^{a}	4.84±0.01^{b}	24.87±0.03^{c}	29.65±0.07^{d}	11.62± 0.04^{a,b}
C18:1	40.35±0.15^{a}	43.94±0.04^{b}	30.36±0.07^{c}	34.34±0.4^{b,d}	1.64± 0.00^{a}
C18:2	15.49±0.09^{a}	1.10±0.01^{b}	6.26±0.0^{c}	1.29±0.02^{d}	0.02±0.00^{b,c}

ND- Not detected.
Means within each column with different superscripts are significantly different at P <0.05.
Each value in the table represents the mean of duplicate analysis.

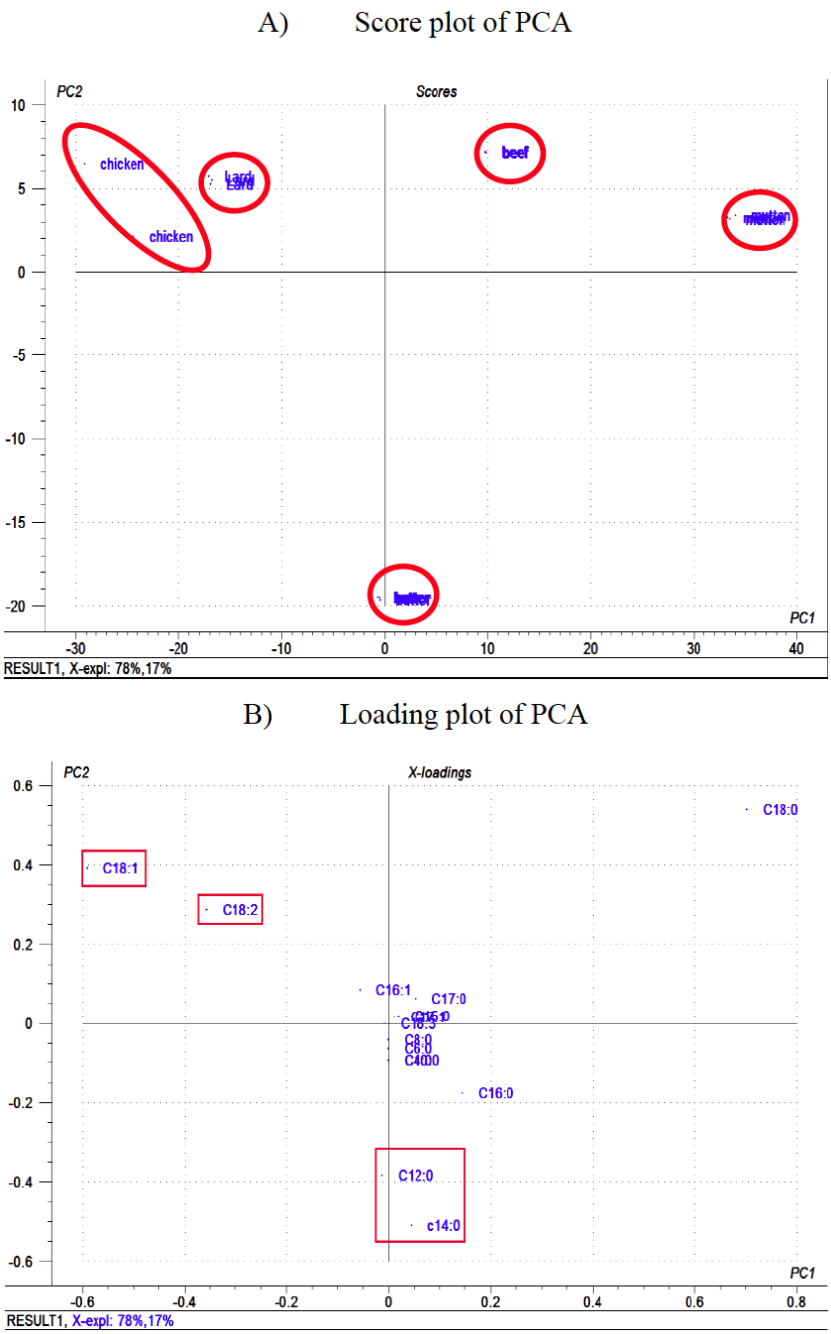

Figure 1: Score plot and loading plot of PCA of butter derived from animal fats based on fatty acid composition.

Discriminant Analysis

Discriminant analysis (DA) is one of the classification techniques which can be used to determine the class of butter similar to animal fats by computing the distance from each class centre in Mahalanobis distance units [23]. DA was carried out in two steps. Firstly, pure butter and butter mixed with animal fats (lard, beef fat and mutton fat) were classified into two groups known as pure butter and adulterated butter. Chemical shifts are compiled together with functional group of fatty acid used for DA, are compiled in Table **2**. The selection of chemical shift was performed in such a way that they gave no or the least misclassification between two groups (pure butter and butter adulterated with animal fats). A Coomans plot (Figure **2**) was applied for the classification of pure butter and butter adulterated with 1 – 50 % (v/v) of animal fats. The x-axis showed the Mahalanobis distance to butter, while the y-axis showed the distance to the adulterated butter with animal fats. The Mahalanobis distance is useful in assigning whether a set of unknown value samples is similar to a collection set of known measured samples. Coomans plot in Figure **2** clearly exhibited the separated group of pure

butter and animal fats-adulterated butter. In this study, DA model classified 100% of all samples accurately according to its group, meaning that no samples were misclassified into the wrong group, which could happen sometimes because of the close similarities in chemical composition between two groups.

Table 2: Tentative Identification of Functional Groups of Butter Product and Lard from ^{1}H-NMR Spectra

Chemical Shift (ppm)	Functional Group
0.93	-C**H**$_3$ (saturated, acyl chains)
1.03	-C**H**$_3$ (acyl chains)
1.32	-(C**H**$_2$)$_n$- (acyl chains)
1.70	-OCO-CH$_2$-C**H**$_2$- (acyl chains)
2.14	-C**H**$_2$-CH=CH- (acyl chains)
2.36	-OCO-C**H**$_2$-(acyl chains)

Principal Component Analysis of Market Sample of Butter with Lard

PCA was carried out based on the ^{1}H-NMR spectra data presented in Table **3**. PCA was carried out based on the ^{1}H-NMR spectra data from the seventeen sample of butter available in Malaysia. PCA is a technique that reduces the dimensions of the data. Reduction of the number of variables can lead to improved performance. Figure **3A** illustrates the score plot of PC1 versus PC2 from the ^{1}H-NMR spectra analysis. PC1 described 82% of the variation while PC2 accounted for 15% of the variation resulted in a model that described 80% of the total variance in the data. With 82% of the peak variation along the first PC, it was clear that all seventeen samples of butter in the market and pure lard sample were formed according to their own group and showed two well-defined and well-separated group (Figure **3A**). Figure **3B** shows the loading plot of PC1 versus PC2. The loading plot was used to determine which variables influence the separation of the samples. The absolute value of the loading in a component describes the importance of the contribution of a particular component. Thus, a variable further away from the origin means it has a greater contribution towards the model. As shown in Figure **3B**, it was clear that seventeen butter samples in the market were well separated from lard. The main compound that caused the separation in lard was =HC-C**H**$_2$-CH= (acyl chains). A high positive correlation between 1.32 and other chemical shift along PC1 showed that the market sample of butter indicated a higher proportion of -(C**H**$_2$)$_n$- (acyl chains), while a high negative score of lard indicated that =HC-C**H**$_2$-CH= (acyl chains) had a major influence on the differentiation of the product butter. According to the loading plot in Figure **3B**, out of the other chemical shifts, the peak at 2.63 was the most discriminating variable that influenced the group separation into two different clusters. This difference could be a potential marker to identify if a product contained lard especially for Halal authentication and verification. Based on previous study, there was lack of report in the identification of chemical marker in food products. Therefore, this work is considered new and could be useful in food industries and for Halal verification.

CONCLUSION

The presence of lard as an adulterant in butter has been analysed using GC-MS, and ^{1}H-NMR spectroscopy with the aid of chemometric of PCA and

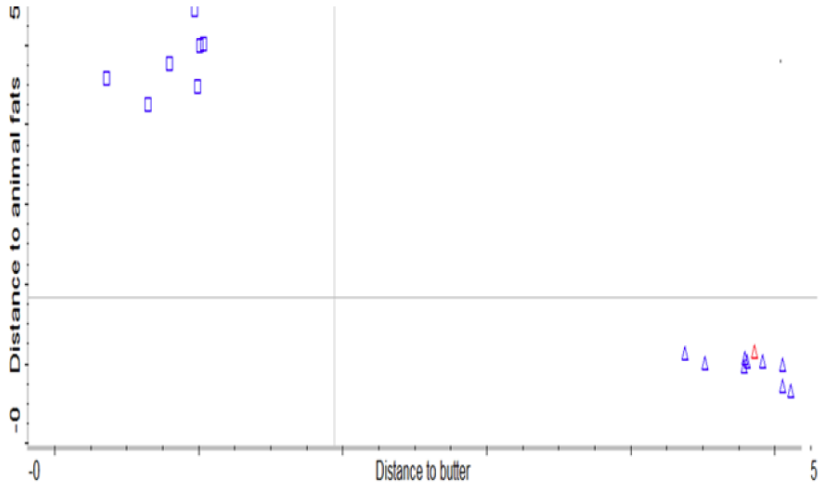

Figure 2: Coomans plot of butter and those adulterated with three animal fats; (□) pure butter; (Δ) butter sample adulterated with three animal fats.

Table 3: List of the Seventeen-Sample Product Brand of Butter Available in Malaysia

Sample	Product Brand	Country
1	Buttercup	Malaysia
2	Isigny St Mere bar	France
3	Isigny St Mere stick	France
4	Beurre De Surgeres	France
5	Dew Fresh	Northern Ireland
6	SCS	Australia
7	Golden Churn	Australia
8	Emborg	Belgium
9	Farmcows	Malaysia
10	Lescure Bar	France
11	Lescure Stick	France
12	Lurpark	Denmark
13	Ballantyne	Australia
14	Kerry Gold	Ireland
15	Ambassador	French
16	President	French
17	Tatura	Australia

DA. Lard was successfully determined at 2.63 ppm. The changes of fatty acid composition of butter adulterated with animal fats can complement the adulteration analysis of butter using ^{1}H-NMR spectroscopy. In addition, DA allows one to make a classification of pure butter and butter adulterated with animal fats. DA can classify butter and butter adulterated with animal fats without any misclassification. NMR and PCA analysis has successfully discriminated between the market sample and lard. This could be a potential identification approach to determine if the product has been deceived in market. Seventeen commercial butter samples available in Malaysia have been analysed using this method and the results show there is no lard being adulterated in these samples.

A) Score plot of PCA

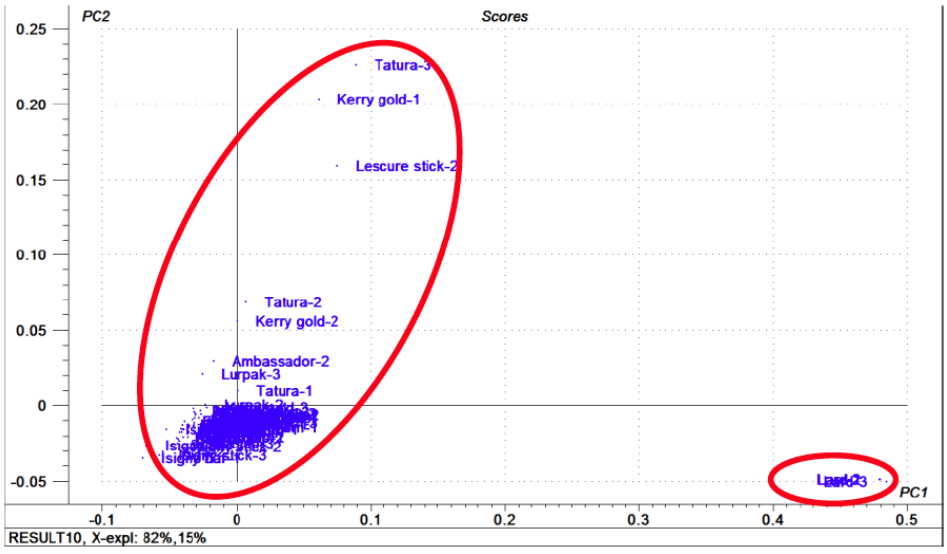

Figure 3. continued.

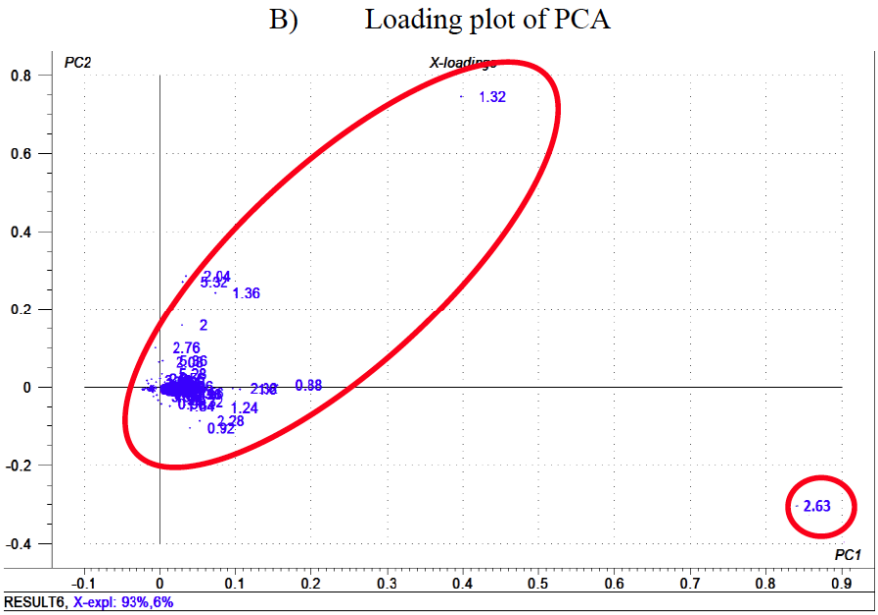

Figure 3: Score plot and loading plot of PCA market sample of butter and lard based on ^{1}H-NMR spectra.

ACKNOWLEDGEMENT

This work was supported by Ministry of Higher Education (MOHE) and International Islamic University Malaysia (IIUM) under Research Grant RIGS16-123-0287.

REFERENCES

[1] Picariello G, Sacchi R, Fierro O, Melck D, Romano R, Paduano A. High resolution 13C NMR detection of short-and medium-chain synthetic triacylglycerols used in butterfat adulteration. Eur J Lipid Sci Technol 2013; 115: 858-864. https://doi.org/10.1002/ejlt.201300018

[2] Jensen RG. The composition of bovine milk lipids: January 1995 to December 2000. J Dairy Sci 2002; 85(2): 295-350. https://doi.org/10.3168/jds.S0022-0302(02)74079-4

[3] German JB, Gibson RA, Krauss RM, Nestle P, Lamarche B, van Staveren WA, Steijns JM, de Groot LCPGM, Lock AL, Destaillats F. A reappraisal of the impact of dairy foods and milk fat on cardiovascular disease risk. Eur J Nutr 2009; 48(4): 191-203. https://doi.org/10.1007/s00394-009-0002-5

[4] Guyot A. Recent progress in reactive surfactants in emulsion polymerisation. Proceedings of the 2002 Macromolecular Symposia 2002; pp. 105-132. https://doi.org/10.1002/1521-3900(200203)179:1<105::AID-MASY105>3.0.CO;2-7

[5] Barron LJ, Hierro MTG, Santa-María G. HPLC and GLC analysis of the triglyceride composition of bovine, ovine and caprine milk fat. J Dairy Sci 1990; 57(4): 517-526. https://doi.org/10.1017/S0022029900029563

[6] Lipp M. Review of methods for the analysis of triglycerides in milk fat: application for studies of milk quality and adulteration. Food Chem 1995; 54: 213-221. https://doi.org/10.1016/0308-8146(95)00611-L

[7] Kuksis A, McCarthy MJ. Gas-liquid chromatographic fractionation of natural triglyceride mixtures by carbon number. Can J Biochem Physiol 1962; 40(5) :679-686. https://doi.org/10.1139/o62-077

[8] Echizen A, Diki M. Determination of butterfat in mixed fats. Relation between butyric acid and butterfat content in mixed fats. Rep Cent Customs Lab 1975; 15:111.

[9] Farag R, Abo-Raya S, Ahmed F, Hewedi F, Khalifa H. Fractional crystallization and gas chromatographic analysis of fatty acids as a means of detecting butterfat adulteration. J Am Oil Chem Soc 1983; 60: 1665-1669. https://doi.org/10.1007/BF02662429

[10] Iwaida M, Ito Y, Tonogai Y, Toyoda M, Watanabe T. Detection of adulterated milk fats by gas-liquid chromatographic determination of butyrate and caproate, Food Hyg Safe Sci 1979; 20: 328. https://doi.org/10.3358/shokueishi.20.328

[11] Timms RE. Detection and quantification of non-milk fat in mixtures of milk and non-milk fats. J Dairy Res 1980; 47(3): 295-303. https://doi.org/10.1017/S002202990002118X

[12] Gaydou EM, Bianchini JP, Ratovohery JV. Triterpene alcohols, methylsterols, sterols, and fatty acids in five Malagasy legume seed oils. J Agric Food Chem 1983; 31(4): 833-836. https://doi.org/10.1021/jf00118a039

[13] Harker M, Holmberg N, Clayton JC, Gibbard CL, Wallace AD, Rawlins S, Hellyer SA, Lanot A, Safford R. Enhancement of seed phytosterol levels by expression of an N-terminal truncated Hevea brasiliensis (rubber tree) 3-hydroxy-3-methylglutaryl-CoA reductase. Plant Biotechnol J 2003; 1(2): 113-121. https://doi.org/10.1046/j.1467-7652.2003.00011.x

[14] Holmberg K. Natural surfactants. Curr Opin Colloid Interface Sci 2001; 6:148-159. https://doi.org/10.1016/S1359-0294(01)00074-7

[15] Hubbard WD, Sheppard AJ, Newkirk DR, Prosser AR, Osgood T. Comparison of various methods for the extraction of total lipids, fatty acids, cholesterol, and other sterols from food products. J Am Oil Chem Soc 1977; 54(2): 81-83. https://doi.org/10.1007/BF02912395

[16] Freire E. Differential scanning calorimetry. Protein Stability and Folding: Theory and Practice. Methods Mol Biol 1995; 40: 191-218

[17] Kittur F, Prashanth KH, Sankar KU, Tharanathan R. Characterization of chitin, chitosan and their carboxymethyl

derivatives by differential scanning calorimetry. Carbohydr Polym 2002; 49: 185-193. https://doi.org/10.1016/S0144-8617(01)00320-4

[18] Lees M. Food authenticity and traceability. 1st ed. USA: Woodhead Publishing 2003.

[19] Rohman A, Sismindari Erwanto Y, Che Man YB. Analysis of pork adulteration in beef meatball using Fourier transform infrared (FTIR) spectroscopy. Meat Sci 2011; 88(1): 91-95. https://doi.org/10.1016/j.meatsci.2010.12.007

[20] Christy AA, Egeberg PK. Quantitative determination of saturated and unsaturated fatty acids in edible oils by infrared spectroscopy and chemometrics. Chemom Intell Lab Syst 2006; 82:130-136. https://doi.org/10.1016/j.chemolab.2005.06.019

[21] Shin EC, Craft BD, Peg RB, Phillips RD, Eitenmiller RR. Chemometric approach to fatty acid profiles in Runner-type peanut cultivars by principal component analysis (PCA). Food Chem 2010; 119(3): 1262-1270. https://doi.org/10.1016/j.foodchem.2009.07.058

[22] Rohman A, Che Man YB. Analysis of cod-liver oil adulteration using Fourier transform infrared (FTIR) spectroscopy. J Am Oil Chem Soc 2009; 86: 1149-1153. https://doi.org/10.1007/s11746-009-1453-9

[23] AOAC (Association of Official Analytical Chemists). Official methods of analysis of AOAC International (Official Method 920.118). 17th ed. Gaithersbug: MD 2000.

[24] De Pedro E, Casillas M, Miranda C. Microwave Oven Application in the Extraction of Fat from the Subcutaneous Tissue of Iberian Pig Ham. Meat Sci 1997; 45: 45-51. https://doi.org/10.1016/S0309-1740(96)00097-6

Received on 16-02-2019 Accepted on 11-03-2019 Published on 14-03-2019

DOI: https://doi.org/10.29169/1927-5951.2019.09.02.5