Differentiation of frog fats from vegetable and marine oils by Fourier Transform Infrared Spectroscopy and chemometric analysis

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Summary

The agro-based production and consumption of frogs coupled with world-wide trading have been increased in the recent years giving rise to the risk of frog fat adulteration in expensive vegetable and marine oils. For the first time, we profiled here frog fats using Fourier Transform Infrared (FTIR) Spectroscopy coupled with multivariate principal component analysis (PCA). The comparison of the FTIR spectral absorbance intensities demonstrated linkage of frog fats to other edible fats and oils. Three commercially available marine oils and three vegetables oils were studied with frog fats and clear pattern of clusters with distinctive identifiable features were obtained through PCA modeling. PCA analysis identified 2922.21 cm^{-1}, 2852.88 cm^{-1}, 1745.45 cm^{-1}, 1158.29 cm^{-1} and 721.51 cm^{-1} FTIR-frequencies as the most discriminating variables influencing the group separation into different clusters. This fundamental study has clear implications in the identification of frog fat from its marine and vegetable counterparts for the potential detection of frog fat adulteration in various fat and oils.

Keywords: multivariate principal component analysis, edible fats and oils, edible frogs, agro-based production

Introduction

The recent scandals of horse-derivatives in school meals in Europe, porcine DNA in Cadbury chocolates in Malaysia and rat meat selling as lamb in China has given researchers, regulators, manufacturers and distributors a brain-storming apprehension on what to detect, when to detect and how to detect species ingredients to ensure transparency in the trading of foods (Ali et al., 2014; Ali et al., 2013). Frog meats have a great appeal to different communities because of their high-content of proteins and minerals and low-content of fat. Frog legs are highly appreciated in many European countries such as France, Belgium and United States because of their superior palatability and similarity in color and taste with their chicken counterparts (Tokur et al., 2008). Besides foods, frog-derived materials are used in traditional and homeopathic medicines to cure a number of diseases including respiratory infections, coughs, appendicitis and wound healing in Africa, North America, Britain, China and Germany (Mohneke et al., 2011). Consequently, frogs are traded world-wide and the major frogs' legs importing countries include France, the United States, Belgium, and Luxembourg (United Nations, 2008). In Asia, the predominant markets for frogs are Singapore, Hong Kong, and Malaysia (Kusrini and Alford, 2006). In 1950s, India and Bangladesh were the major exporters of frog legs. However, due to inhumane killing and adverse effects on natural control of agricultural pests, legal trade of frogs was banned in India in 1987 (Pandian and Marian, 1986).

The increasing demand for frogs in different countries encouraged its culture and agro-based production across the world. Frog culture is profitable since it is possible to cultivate huge number of frogs in a limited space with a small quantity of water and it brings a higher return over the most of the cultured freshwater fishes. The main producers of cultured frogs (capture and aquaculture) are Indonesia, Taiwan Province of China and Turkey (Ozogul et al., 2008). Several species of frogs such as American Bull frog, *Lithobates catesbeianus* and the Indian Tiger frog, *Hoplobatrachus tigrinus* (Daszdak et al., 2006) and several others are bred for consumption or medicinal uses (Oduntan et al., 2012a).

Ojewala and Udom (2005) studied the proximate and mineral composition of frog waste meal as an unconventional animal protein source and their findings suggested that it could be used as either partial or complete substitute of the conventional feed sources. Oduntan et al. (2012a; 2012b) proposed that the consumption of edible frog (*Rana esculenta*) to substitute bush meat is feasible since it has a competent source of animal proteins and other vital nutrients for human in great abundance and it is widely distributed in most of the West African countries, especially in the swampy, rainforest, and savannah eco-zones (IUCN, 2012).

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Tokur et al. (2008) studied the nutritional composition of frog waste meal that includes the head, viscera, skin and upper part of the body after the third vertebra. Up to 45% of the frog body parts ultimately go to the waste without being properly utilized for useful purposes. These wastes are rich in potentially valuable oils, minerals, enzymes, pigments and flavours having many applications in food, pharmaceuticals, agriculture, aquaculture and industries. They can be recycled and converted into several products of high economic value such as valuable protein, lipid fractions, vitamin, minerals, vehicle for lipid-soluble vitamins or ingredients in cosmetics. There might be limitless other uses for this material, and new uses are being invented over the time (Ozogul et al., 2008; Tokur et al., 2008).

Frogs are also rich in proteins, polyunsaturated fatty acids (PUFAs) and minerals (Ozogul et al., 2008; Tokur et al., 2008; Mendez et al., 1998) and are comparable with those of freshwater fish species. On the other hand, omega-3 fatty acids containing vegetable oils, such as canola oil, contain α-linolenic acid, which requires conversion to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) after ingestion (Gregory et al., 2014). On the other hand, PUFA are abundant in marine oils (Kolakowska et al., 2006). PUFA, especially EPA and DHA are known to have many health benefits including the reducing the risk of coronary heart disease (CHD), dementia and have anti-depressant factor among others. Though the uses and investigation of the frog fats are very scarce, studies are amounting due to the their potential as promising sources of new drugs (Mario et al., 2013) and utilization of frog fats as supplementation to increase the energy level of the diet for high producing animals (Aina et al., 2014). The former processing method requires extraction of body fat in the abdominal (ventral) regions of these frogs through Soxhlett Extraction (Mario et al., 2013), while the latter usage of frog fats is obtained by scrapping the subcutaneous fats from the frog or by roasting the frog over fire, in which fat droplets are then collected in container until completion of roasting process (Aina et al., 2014).

The PUFA content of frog fats in body and legs of frogs are 28 to 37% (Ozogul et al., 2008). In another experiment, the content of PUFA in marine oils was ranged from 29 to 40% and 28 to 70% in plant oils (Araujo et al., 2010; Jafari et al., 2009). Consequently, similarities of PUFA properties of frog fats with some marine oils and vegetable oils may pose a high risk of oil adulteration since frog waste particularly the body fats are cheap and readily available. Furthermore, frog consumption is also forbidden in Islam (Kusrini and Alford, 2006; Niekisch, 1986). According to the majority of the Islamic scholars, breeding of frogs for export purposes is unlawful (*haram*) since it is an amphibian animal. However, the mazhab Maliki doctrine of Islamic fatwa allows the breeding of harmless amphibians (e-fatwa, 2014).

Analytical techniques for distinguishing frog fats from those of marine and vegetables counterparts which are more expensive are not well documented. The application of spectroscopic and chemometric tools for food authentication are increasingly rising (Fadzlillah et al., 2014; Rohman et al., 2014; Mansor et al., 2012). The principle of such techniques is that chemical compositions of complex samples are characterized by multi-channel analytical signals and the useful information regarding the food quality could be extracted by multivariate analysis methods (Deng et al., 2012). FTIR spectroscopy is frequently used for this purpose as an analytical tool. Mid-infrared possesses some advantages, including rapid measurement, moderate instrument cost and relative ease of sample presentation, especially for liquid and paste samples (Deng et al., 2012; Fadzlillah et al., 2014; Rohman et al., 2014; Mansor et al., 2012). Despite multiple publications on FTIR differentiation of fats and oils, there is hardly any report on differentiation of frog fat from marine and vegetable oils. For the first time, we successfully applied here FTIR and chemometric based-on multi-variate principal component analysis (PCA) to profile frog fats from their vegetable and marine counterparts.

Materials and methods

Sample Preparation

Frog (*Hoplobatrachus rugulosus*) fats (n=3) were obtained in triplicates on three different days to eliminate species and farming variation effects on final data from Pasar Borong Pudu Raya located in Kuala Lumpur of Malaysia where farmed frogs are legally and commercially sold for consumption. Frog fats were extracted by rendering of the fat bodies. Melted fats were filtered through double-folded muslin cloth to remove impurities. Small proportion of anhydrous sodium sulfate was added to remove residual moisture. Samples were filtered through Whatman No.2 filter paper and stored at 4 °C. All chemicals used in this experiment were HPLC or analytical grade (Marikkar et al., 2001). Commercially edible oils (three types of marine oils namely cod liver oil (n=3), fish oil A (n=3) and fish oil B (n=3); and three types of vegetable oils namely corn oil (n=3), canola oil (n=3) and olive oil (n=3)) were purchased from local supermarkets (Jusco, Tesco and Giant) in Selangor, Malaysia.

FTIR Spectra Acquisition

All spectra were acquired using FTIR spectrometer of Nicolet 6700 (Thermo Nicolet, Madison, WI) instrument equipped with deuterated triglycine sulphate detector and potassium bromide beam splitter. The collected spectra were analyzed using OMNIC software (Version 7.0 Thermo Nicolet) included in the FTIR spectrometer. Using a Pasteur pipette, an approximately 1.0 mL of oil samples was properly placed on attenuated total reflectance (ATR) crystal. FTIR spectra were obtained at a frequency region of 4,000-650 cm^{-1} with accumulated 32 scans and 4 cm^{-1} resolution. FTIR spectra were displayed as absorbance values for at least in three different measurements (Fadzlillah et al., 2014).

Statistical Analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) Tukey's Test using MINITAB (version 14) statistical package at 0.05 probability level. Principal Component Analysis (PCA) was carried out on the FTIR frequencies using Unscrambler 9.7 (Camo, USA) software.

Results and discussion

FTIR Spectra of Frog fat

The major spectral features of frog fats are demonstrated in Fig. 1 and its band assignment is given in Table 1 according to Deng et al. (2012) and Che Man et al. (2011). These results match those observed in earlier studies, whereby three major features of edible fats and oils were reflected by CH stretching absorptions in 3050-2800 cm^{-1} region, the carbonyl absorption of the triglyceride ester at ~1741 cm^{-1}, and the bands associated with the fingerprint region at 1500-1000 cm^{-1} (Che Man and Mirghani, 2001; Van de Voort et al., 1994). The degree of unsaturation is often monitored based on several characteristic bands at ~1650 cm^{-1} (C=C stretching vibration of *cis*-olefins), ~1417 cm^{-1} (rocking vibrations of CH bonds of *cis*-disubstituted), and ~3001 cm^{-1} (CH stretching vibration of the *cis*-double bond). A shoulder peak at 1654 cm^{-1} (Fig. 1) reflected the presence of unsaturated fatty acids in frog fats.

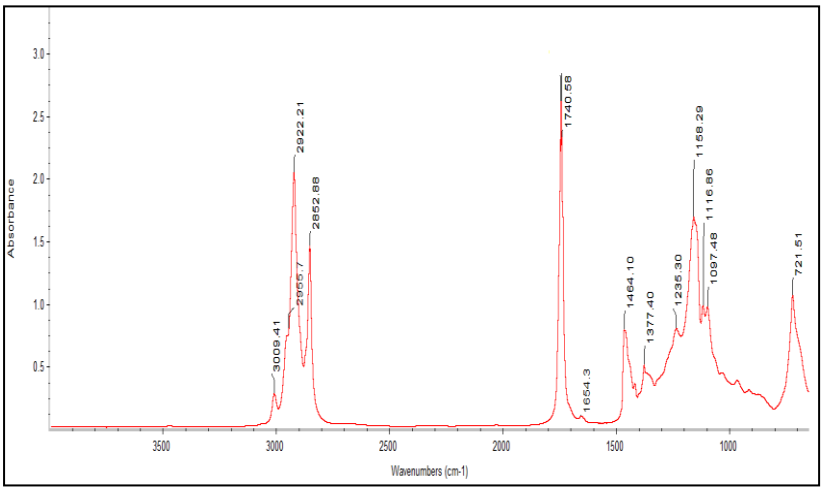

Fig. 1. FTIR spectra of frog fat (n=3) x: wavenumbers (cm^{-1}), y: absorbance

Table 1. Peak assignment of pure frog fat (Deng et al., 2012; Che Man et al., 2011)

Wavenumber (cm^{-1})	Stretching	Remarks
3009.4	cis C=CH	CH stretching vibration of the *cis*-double bond
2955.7	$-CH_3$	-CH3 stretching vibration
2922.1	$-CH_2$ asymmetric	Stretching of unsaturated –CH
2852.9	$-CH_2$ symmetric	Stretching of saturated –CH
1740.6	-C=O	Stretching of C=O
1654.3	cis C=C	Stretching vibration of *cis*-olefins
1464.1	$-CH_2$	Rocking vibrations of CH bonds
1377.4	$-CH_3$	Methyl symmetric bending
1235.3	-C-O	C–O–C Asymmetrical stretching vibration
1158.3	-C-O	-C-O from ester
1116.9	-C-O	-C-O from ester
1097.5	-C-O	-C-O from ester
966.0	-CH	Bending vibration of CH functional groups of isolated trans-olefin
721.5	cis –CH=CH	Out-of-plane rocking

Both asymmetric and symmetric stretching band for methylene appeared at ~2922.21 cm^{-1}, and 2852.90 cm^{-1} in frog fat, suggesting its similarities with other edible fats (Van der Voort et al., 1994). The C=O group of triglycerides showed the stretching vibration band at 1740.6 cm^{-1}. 1654 cm^{-1} band could be assigned to C=C stretching vibration of disubstituted cis C=C of acyl groups of oleic and linoleic acids. At ~1465 cm^{-1} band was due to the bending vibration of the methylene group. The peak at 1377 cm^{-1} could be due to the symmetrical bending vibration of methyl groups. Bands at 1158.3 cm^{-1}, 1116.9 cm^{-1} and 1097.5 cm^{-1} were assigned to the stretching vibrations of the C-O groups in esters. This vibration consists of two asymmetric coupled vibrations for C-C(=O)-O and O-C-C, while the former is more important (Guillen and Cabo, 1997).

The C-O stretching band at ~1158 cm^{-1} indicated the hydrolysis of fatty acids and glycerols (Siong et al., 2014). Frog fat showed two distinctive peaks at 1116.9 and 1097.5 cm^{-1}, which were inversely, related to the proportion of saturated acyl groups and oleic acyl groups (Che Man and Mirghani, 2001). In fresh oils, band at ~966 cm^{-1} indicates formation of trans-fatty acid due to isomerization of cis-unsaturated fatty acids upon bleaching, refining and deodorization (Siong et al., 2014). Since the frog fat in this experiment was freshly extracted, this could be the main reason for the occurrence of the peak. Finally, the band at ~721.5 cm^{-1} was due to the overlapping of the methylene rocking vibration and the out-of-plane bending vibration of cis-disubstituted olefins (Guillen and Cabo, 1997).

The comparative spectral features of frog fats with those of some marine and vegetable oils are shown in stack spectra in Fig. 2 and spectral intensity measurements are given in Table 2. To the naked eye, the FTIR of frog fat and vegetables and marine oils appeared very similar or identical. However, a comprehensive examination of the FTIR spectra of the fats and oils allowed the identification of certain peaks or ratios that help in the characterization, differentiation, and classification of frog fats from other fats and oils (Table 2). Nevertheless, it was still difficult and impractical to distinguish frog fat from the others using FTIR spectral features without any data treatment. Hence, the chemometric technique of PCA was used for such differentiation.

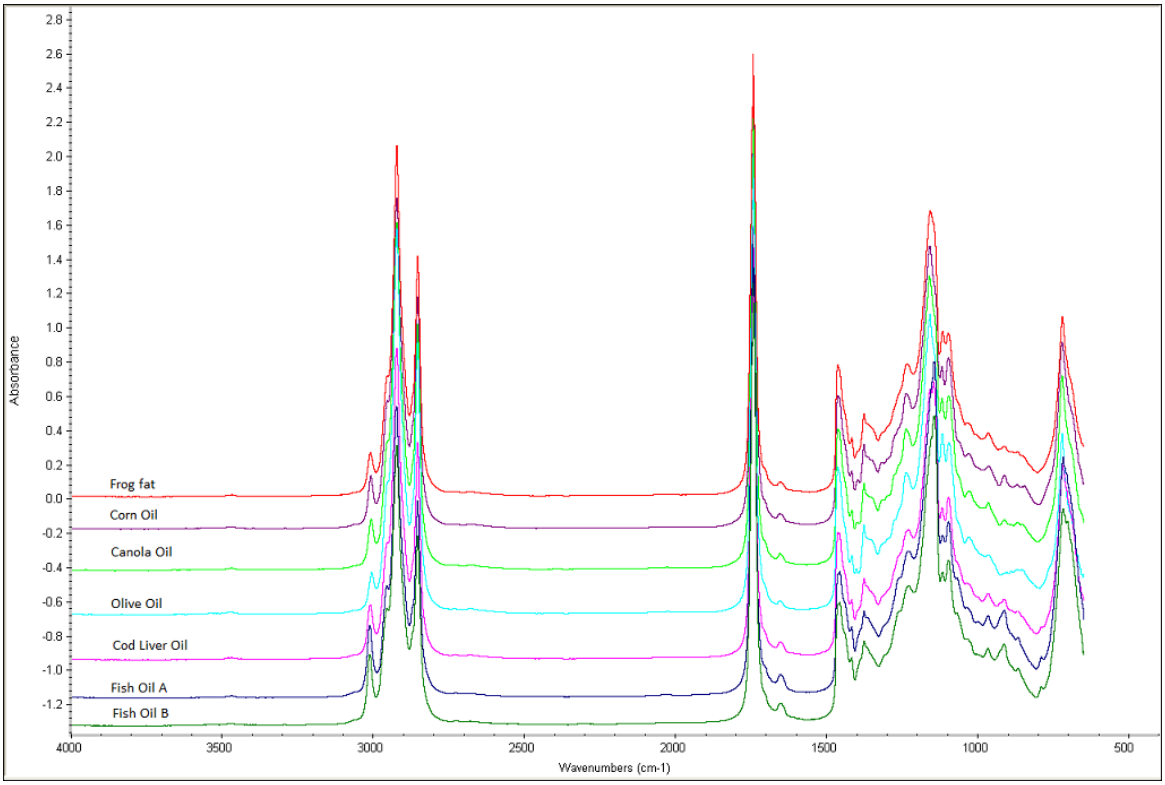

Fig. 2. FTIR spectra of frog fats and 3 vegetable oils (corn, canola and olive) and 3 marine 344 oils (cod liver oil, Fish Oil A, Fish Oil B)

Table 2. FTIR peak intensities (absorbances) at selected frequencies of frog fat and vegetable and marine oils for PCA analysis

No	Oils	Frequency						
		721.51	1097.48	1116.86	1158.29	1235.3	1377.4	1464.1
1	Frog fat	1.05±0.00^{d}	0.97±0.00^{a}	0.98±0.01^{a}	1.67±0.00^{b}	0.79±0.01^{ab}	0.50±0.01^{a}	0.78±0.02^{a}
2	Olive oil	0.98±0.01^{e}	0.92±0.01^{b}	0.97±0.00^{ab}	1.60±0.01^{d}	0.77±0.01^{bc}	0.48±0.00^{a}	0.79±0.01^{a}
3	Canola oil	1.06±0.01^{cd}	0.96±0.01^{a}	0.95±0.01^{b}	1.61±0.01^{cd}	0.78±0.01^{abc}	0.48±0.01^{a}	0.77±0.01^{ab}
4	Corn oil	1.09±0.01^{c}	1.00±0.00^{c}	0.94±0.01^{b}	1.64±0.02^{bc}	0.79±0.01^{ab}	0.50±0.00^{a}	0.78±0.00^{a}
5	Marine Oil A	1.14±0.02^{b}	0.94±0.01^{ab}	0.88±0.01^{c}	1.59±0.00^{d}	0.76±0.01^{c}	0.47±0.01^{a}	0.74±0.02^{b}
6	Marine Oil B	1.27±0.01^{a}	0.95±0.01^{a}	0.88±0.01^{c}	1.80±0.01^{a}	0.80±0.02^{a}	0.48±0.02^{a}	0.69±0.01^{c}
7	Marine Oil C	1.24±0.03^{a}	0.94±0.01^{ab}	0.87±0.01^{c}	1.77±0.02^{a}	0.79±0.01^{ab}	0.48±0.02^{a}	0.69±0.02^{c}

No	Oils	Frequency					
		1654.28	1745.45	2852.88	2922.21	2955.68	3009.41
1	Frog fat	0.09±0.01^{cd}	2.61±0.01^{a}	1.42±0.03^{ab}	2.03±0.03^{ab}	0.72±0.02^{ab}	0.27±0.00^{de}
2	Olive oil	0.08±0.00^{d}	2.45±0.06^{b}	1.45±0.02^{a}	2.04±0.05^{a}	0.71±0.01^{abc}	0.23±0.00^{e}
3	Canola oil	0.10±0.00^{c}	2.55±0.03^{ab}	1.36±0.02^{b}	1.92±0.04^{c}	0.72±0.02^{ab}	0.29±0.01^{cd}
4	Corn oil	0.10±0.00^{bc}	2.55±0.05^{ab}	1.35±0.02^{b}	1.92±0.01^{bc}	0.76±0.01^{a}	0.32±0.01^{bc}
5	Marine Oil A	0.11±0.00^{b}	2.44±0.06^{b}	1.27±0.02^{c}	1.84±0.04^{c}	0.70±0.02^{abc}	0.33±0.00^{b}
6	Marine Oil B	0.13±0.01^{a}	2.47±0.07^{b}	1.06±0.03^{d}	1.58±0.05^{d}	0.64±0.05^{c}	0.41±0.03^{a}
7	Marine Oil C	0.14±0.00^{a}	2.52±0.03^{b}	1.08±0.03^{d}	1.59±0.05^{d}	0.67±0.04^{bc}	0.41±0.02^{a}

Each value in the table represents the mean ± standard deviation of triplicate analyses and means within each rows with different superscript letters are significantly different at ($p < 0.05$)

Principle Component Analysis (PCA)

PCA modifies the most discriminating variables into new orthogonal axes, called principal components (PCs), by which the data presented in those axes are not associated with each other. It transfers the variation of the data into only a few principal components (Cordella et al., 2002). In this study, PCA was accomplished using FTIR spectral frequencies of 7 evaluated fats and oils at 13 different frequencies as shown in Table 2. The score plot of the frequencies is shown in Fig. 3(a). It represented the projection of samples defined by principle component 1 (PC1) and principle component 2 (PC2). The highest variation was explained by PC 1, while PC2 exhibited the second highest variation. It was found that 88% and 7% of the variation were described by PC1 and PC2, respectively, explaining 95% of the total variance.

Based on the group separation in Fig. 3(a), frog fat (denoted by f) was clustered in the upper right quadrant, while the marine oils (denoted m1-m3) were grouped in the left quadrant (m2 and m3) or lower middle quadrant. The cluster of vegetable oils (v1-v3) was found both in the upper (v2 and v3) and lower right quadrant (v1) in a distinct cluster separated from marine oils and frog fats. If a frequency region is further away from the origin of the variable point; the contribution towards the PCA model would be larger (Cordella et al., 2003), as reflected by the frequencies at 2922.21 cm^{-1}, 2852.88 cm^{-1}, 1745.45 cm^{-1}, 1158.29 cm^{-1} and 721.51 cm^{-1}. PC1 is influenced by frequencies 2922.2cm^{-1}, 2852.9cm^{-1} and 721.5cm^{-1} while PC 2 is influenced by 1745.5cm^{-1} and 1158.29cm^{-1}.

The loading plot for the determination of variables (frequency regions) contributing to the separation of the fats and oils is shown in Fig. 3(b). Five frequencies: 2922.21 cm^{-1}, 2852.88 cm^{-1}, 1745.45 cm^{-1}, 1158.29 cm^{-1} and 721.51 cm^{-1} were found as the most discriminating variables influencing the group separation into different clusters. It could conceivably be hypothesised that the frequencies of 721.51 cm^{-1} and 1158.29 cm^{-1} on the left side of the loading plot reflect on marine oils that are distributed on the left quadrant of the score plot. Frequency 2922.21 cm^{-1} and 2852.88 cm^{-1} right side of the loading plot indicate vegetable oils that is expressed on the right quadrant of the loading plot, while frog fats are indicated by frequency 1745.45 cm^{-1}, the furthest from PC 1. In PCA, objects that are projected close to each other in the score plots have similar characteristics. Besides, the location of samples (oils) in the score plot are highly correlated with the variables (frequencies) projected in the loading plot. Moreover, the variables that are projected close to each other in the loading plots are positively correlated (Cordella et al., 2003), as can be seen between these two frequencies: 721.51 cm^{-1} and 1158.29 cm^{-1} on the left side of both graphs; and 2922.21 cm^{-1} and 2852.88 cm^{-1} on the right side of both graphs. Hence, FTIR and PCA could be exploited in differentiation of marine, vegetable and frog fats.

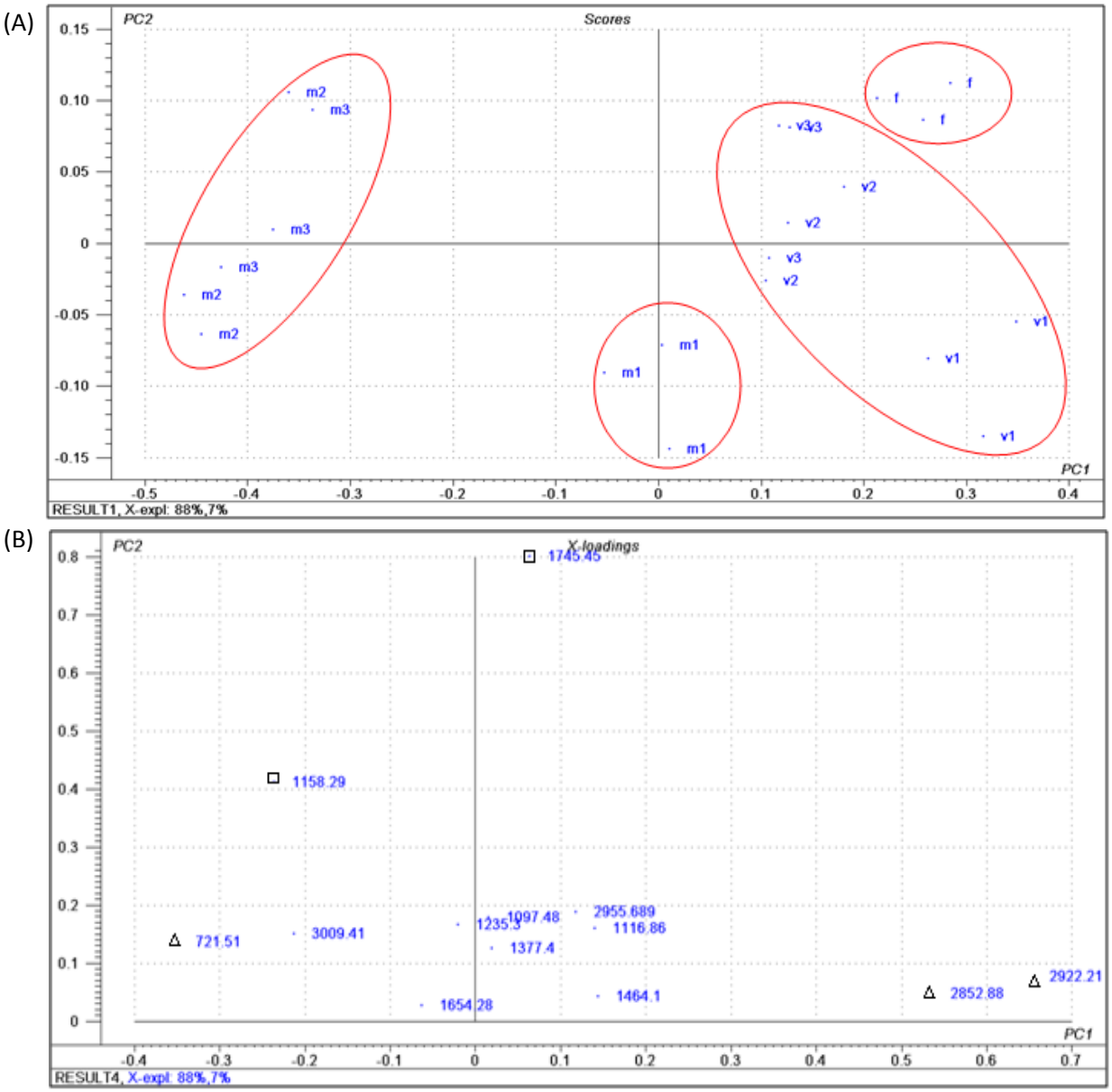

Fig. 3. Score plot (A) and loading plot (B) for PCA analysis of frog, vegetable and marine fats. In (A), shown are 3 frog fats, 3 vegetable and 3 marine oils, denoted by f: frog fat; m1: cod liver oil; m2: fish oil A; m3: fish oil B; v1: olive oil; v2: canola oil and v3: corn oil. In (B), it is shown that frequencies 2922.2cm^{-1}, 2852.9cm^{-1} and 721.5cm^{-1} are the most discriminating variables that separate frog fat from vegetable and marine oils in PC1. Frequencies 1745.5cm^{-1} and 1158.29cm^{-1} are the most discriminating variables that separate frog fat from vegetable and marine oils in the PC2. (△: PC1 variation; □: PC 2 variation)

Conclusions

FTIR spectroscopy and multivariate principal component analysis (PCA) was successfully applied to differentiate frog fats (n=3) from vegetable (n=3) and marine (n=3) oils. Three commercially available marine and three vegetables oils were studied with frog fats and clear pattern of clusters with identifiable features were obtained through PCA modeling. FTIR frequencies at 2922.21 cm^{-1}, 2852.88 cm^{-1}, 1745.45 cm^{-1}, 1158.29 cm^{-1} and 721.51 cm^{-1} were found to be the most discriminating variables influencing the group separation into different clusters of the studied fats and oils. This fundamental study has clear application in the identification of frog fat from its marine and vegetable counterparts for the potential detection of frog fat adulteration, as well as marine oils in various fat and oil brands.

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Conflict of Interest

All authors declare that this article does not have any content with conflicts of interest.

Compliance with Ethics Requirements

This study was conducted following all institutional and national guidelines for the handling of frog and fish fats used in this study.

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