Discriminant analysis of terrestrial animal fat and oil adulteration in fish oil by infrared spectroscopy

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Abstract: In order to improve the effective utilization of animal fats and oils, and then ensure the feeding quality and safety in the field of animal husbandry engineering, the discriminant analysis of different species of terrestrial animal fats and oils in fish oil based on Fourier transform infrared spectroscopy was explored in this study. Twenty-seven different species of animal fat and oil materials including fish oil, lard, chicken oil, tallow and suet were studied. The experimental calibration and validation samples were prepared by adulterating different proportions of terrestrial fat and oil in fish oil. Results show that, it is easy to discriminate different species of raw material of fish oil, lard, chicken oil and ruminant fats based on the infrared spectral characteristics, while the distinction of tallow from suet samples is difficult. For the adulterated samples with percentage range of 1%-60% (w/w), ideal results were obtained to discriminate the terrestrial fat and oil ingredients (lard, chicken oil, tallow and suet) in fish oil, the correct discriminant rates for the independent validation set were all higher than 95%. It was proved by further study that the detection limits for the discriminant analysis of lard, chicken oil, tallow and suet in fish oil were 0.8%, 0.6%, 2% and 3%, respectively.

Keywords: infrared spectroscopy, fish oil, animal fat and oil, discriminant analysis, detection limit

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1 Introduction

As a kind of high-energy agro-product, fats and oils have been widely used in the feed of livestock and poultry. It has also been proved that the addition of fats and oils could supplement the feed energy, improve the feed palatability, etc., and then promote the animal growth effectively[1-7]. Currently, the commonly used animal fats and oils in feed are fish oil, lard, beef tallow and poultry oil. Compared with the terrestrial animal fats and oils (lard, beef tallow and poultry oil), fish oil is superior on the feeding efficiency with a corresponding higher price, which always leads to the inferior products adulteration in the feeding fat and oil market. In addition, in view of aftermath of BSE crisis, China has regulated the use of animal by-products in animal feeds by defining Feed and Feed Additive regulations in 2013. It recommends that animal derived ingredients except milk and dairy products should not be used as an ingredient in ruminant feed. Thus, there is a high security risk of using animal fats especially ruminant fats in feed. At present, with the increasing of the variety and quantity of feeding fats, effective quality control and reliable discriminant method is becoming more and more important for feeding purposes[8].

Infrared spectroscopy is a molecular vibrational

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spectroscopic technique, which is capable of structural identification and qualitative determination of the 'fingerprint' of organic compounds. As a nondestructive and fast technique, Fourier transform infrared (FT-IR) spectroscopy is one of the most popular methods used for fats and oils authentication analysis, especially for classification and quantification analysis of vegetable oils[9-12]. Compared with the complex information of frequency doubling and combination band of molecular vibration given by near infrared spectroscopy, FT-IR can supply the information of fundamental frequency vibration. Therefore, FT-IR is more advantageous in qualitative analysis and is expected to be promising for the species identification. Rohman et al.[13] firstly investigated the feasibility of FT-IR for distinguishing the adulteration of lard in cod liver oil for food safety. In the following years, further analysis was performed to discriminate cod-liver oil from beef and mutton fats on the basis of their FT-IR spectra[14-15]. These initiated a chemical-free approach for the discriminate analysis of animal fats and oils, which is a valuable reference for the present study although the lipid composition and structural characteristics of cod liver oil and feeding fish oil are totally different.

In order to ensure the effective and safe utilization of animal by-product resources, FT-IR combined with attenuated total reflectance (ATR) was firstly applied by our team to classify different processed animal proteins based on their fat characteristics[16-17]. This research further explored the discriminant analysis of different species of feeding terrestrial animal fats and oils in fish oil and corresponding detection limit based on FT-IR-ATR and partial least squares discriminant analysis (PLS-DA).

2 Materials and methods

2.1 Samples and preparation

An overall number of 27 different species of animal fat and oil materials, including five fish oil samples and 22 different species of terrestrial animal fats and oils classified six lard samples, five chicken oil samples, five tallow samples and five suet samples were adopted. All the raw materials were collected from reliable feeding fat and oil factories of a particular variety in China.

Pure fish oils were blended with lard, chicken oil, tallow and suet samples separately in a pair combination way at designed concentration ranges of 1%-60% (w/w). Specific steps of mixing process were as follows: firstly, pre-heated terrestrial animal fat samples into liquid, added an appropriate amount of sample into a test tube, weighed M_1. Then added a certain amount of fish oil, weighed M_2, calculated the actual mixing proportion, $Z=M_1/M_2\times100\%$. The mixtures were well-mixed by vortex for 2 min. Consequently, 64 blended samples of fish oil adulterated with lard, tallow, 56 blended samples of fish oil adulterated with chicken oil, suet, were prepared in this study.

2.2 Fatty acid characteristic analysis

In this study, 37 distinct fatty acids were analyzed via classical gas chromatography (GC-2014C, Shimadzu, Japan) equipped with a flame ionization detector (FID). Firstly, made ready the sample for GC analysis by preparing fatty acid methyl ester (FAME), and then injected it into the inlet using a split/split less injector at 225°C and adopted helium as the carrier gas. The specific parameters were: injection volume, 1 μL; oven program, heating 4°C/min from 100°C to 240°C (35 min). The fatty acid analyses were performed induplicate and were expressed as the normalized percentage of total fatty acids identified in the sample.

Fatty acid methyl ester standards were purchased from Sigma-Aldrich Chemicals (Deisenhofen, Germany).

2.3 FT-IR analysis and calibration and validation set

Fourier transform instrument made by Co. PerkinElmer (Spectrum 400 FT-IR/FT-NIR, Seattle, USA) was used for FT-IR analysis. Spectra were acquired using a deuterated triglycerine sulphate (DTGS) detector and an attenuated total reflectance mode (ATR) sample presentation instrument. For the scanning, ATR accessory was carefully cleaned with ethanol and pure chloroform to eliminate the presence of fat residues between two measurements and to ensure a clean crystal surface. All reagents and solvents used were of analytical grade. The FT-IR spectra were collected in frequency (4000-550) cm^{-1} by co-adding 32 scans with a resolution of 8 cm^{-1}. A new reference air background

spectrum was taken before each scan. Scanning was performed three times per sample and the average of the three spectra was recorded as log 1/R. The RSD (Relative Standard Deviation) of the FT-IR analysis is 4.1%. Besides the five raw pure fish oils, another 10 fish oil samples were obtained by pairwise mixing them.

Therefore, totally 45 pure fish oil spectra were got in the study, in which 30 were selected randomly for calibration and 15 for validation. For the fish oil samples adulterated with lard and tallow, 192 spectra were got, in which 144 for calibration and 48 for validation. For the fish oil samples adulterated with chicken oil and suet, 168 spectra were got, in which 120 for calibration and 48 for validation.

2.4 Statistical and chemometrics analysis

Matlab (R2014a, Mathworks, USA) was used for the statistical and chemometrics analysis in this research.

Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were used for discriminant analysis. Three indicators, sensitivity, specificity and classification error, were used to evaluate the discriminant results. The values of sensitivity and specificity were closer to 1, and the values of classification error were closer to 0, the better the discriminant analysis was[18]. For the discrimination of adulterated samples, the rate of correct discrimination (correct discrimination rate=number of correct discriminant samples/total sample number×100%) was calculated. If a pure fish oil sample was judged to be an adulterated sample, it was denoted as a false positive; if an adulterated sample was judged to be pure fish oil, it was denoted as a false negative. The total number of samples minus false positive and false negative number was defined as the number of correct discriminant samples.

3 Results and discussion

3.1 FT-IR spectral characteristics of raw materials

Figure 1 shows the FT-IR spectra of raw material of test fat and oil samples in the study.

Infrared spectroscopy can mainly represent the information of lipid structure and functional group. From the detected spectral peaks, the infrared spectra of lard, chicken oil, tallow and suet are quite similar. There are some obvious differences between the spectra of fish oil and terrestrial animal fats and oils, which is identified at the band of 3006 cm^{-1} (locates in the functional group region) and 722 cm^{-1} (locates in the fingerprint region). According to the assignments of the major FT-IR bands of fats reported by Guillen et al.[19] and Wójcicki et al.[20], both the higher band at 3006 cm^{-1} and 722 cm^{-1} characterized the vibrations of cis-unsaturated structures. This is consistent with the GC results of fatty acid constitution of different species of animal fat and oil samples presented in Table 1. It was indicated that the unsaturated fatty acid especially the polyunsaturated fatty acid content of fish oil samples was significantly higher than the other animal fat and oil materials. Note that among the materials of terrestrial animal fats and oils, the unsaturated fatty acid content of chicken oil was much higher than that of lard, tallow and suet. This was also represented by an evident higher peak at about 3006 cm^{-1} in the FT-IR spectra.

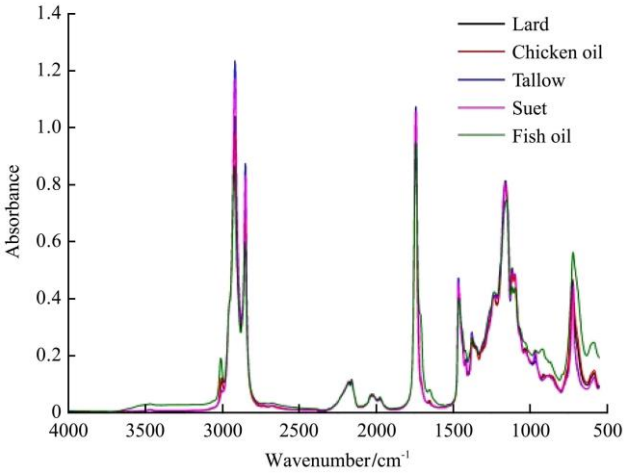

Figure 1 FT-IR spectra of different species of animal fat and oil materials

Table 1 Fatty acid composition and statistical analysis of animal fat and oil materials

Compounds	Normalized percentage/%				
	Lard	Chicken oil	Tallow	Suet	Fish oil
SFA	43.95±1.39^{b}	30.22±1.47^{c}	63.87±1.71^{d}	63.57±2.36^{d}	37.47±0.27^{a}
MUFA	41.53±0.86^{b}	45.95±1.88^{c}	34.26±1.54^{a}	34.08±2.22^{a}	32.39±0.46^{a}
PUFA	14.53±0.60^{b}	23.83±3.01^{c}	1.86±0.25^{d}	2.35±0.25^{d}	30.14±0.22^{a}
UFA	56.05±1.39^{b}	69.78±1.47^{c}	36.13±1.71^{d}	36.43±2.36^{d}	62.53±0.27^{a}

Note: SFA is saturated fatty acid; MUFA is monounsaturated fatty acid; PUFA is polyunsaturated fatty acid; UFA is unsaturated fatty acid; Mean values in the same row with different superscripts are significantly different (p<0.05).

3.2 Discriminant analysis result of different species of animal fat and oil materials

Figure 2 displays the PCA visualization of the tested animal fat and oil materials based on FT-IR spectra characteristics, in which the two strongest principal components explains 81.11% and 14.68% of the total variance, respectively. It is obvious that fish oil, non-ruminant fat and oils (lard and chicken oil), ruminant fat and oils (tallow and suet) are grouped into three separate clusters corresponding to their different species origin, while there is big overlap for tallow and suet and nearly no overlap for lard and chicken oil samples. What is also worth noticing is that this is completely consistent with the statistical analysis of fatty acid constitution. Beyond the data demonstrated in Table 1 above, there was no significant difference for saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and unsaturated fatty acid (UFA) contents between tallow and suet fats. By contrast, there was significant difference for SFA, MUFA, PUFA and UFA contents between the lard and chicken oils.

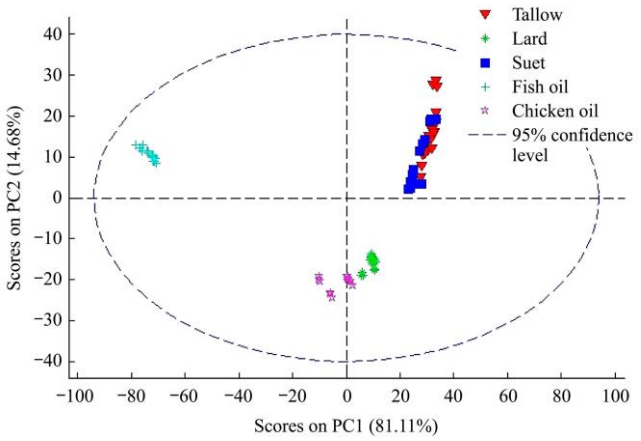

Figure 2 Principal component analysis visualization of FT-IR spectra of animal fat and oil materials

Table 2 shows the corresponding PLS-DA discrimination results containing the cross validation values of sensitivity, specificity and classification error.

Table 2 Results of PLS-DA discrimination of tested materials based on FT-IR spectra

Species	Lard	Chicken oil	Tallow	Suet	Fish oil
Sensitivity (CV)	1.00	1.00	0.89	0.87	1.00
Specificity (CV)	1.00	0.99	0.94	0.94	1.00
Classification error (CV)	0	0.01	0.09	0.10	0

Note: CV is cross validation.

Note that the sensitivity and specificity to discriminate the fish oil, lard and chicken oil samples are all higher than 0.95, which indicate a great potential to distinguish these tree species of animal fat and oil materials. This is consisting with the PCA results. Identification of tallow and suet samples was proved to be a little difficult with the sensitivity of between 0.85-0.90.

3.3 Qualitative discriminant analysis of terrestrial animal fats and oils in fish oil

3.3.1 Calibration and validation set

According to the designed concentration ranges of 1%-60% (w/w), the actual adulteration percentage range of calibration and validation set for qualitative discriminant analysis is presented in Table 3.

Table 3 Calibration and validation samples for qualitative discriminant analysis

Adulterated sample	Calibration set		Validation set	
	Sample number	Percentage range/%	Sample number	Percentage range/%
Fish oil+lard	174	1.00-60.79	63	1.00-60.39
Fish oil+Chicken oil	150	1.00-60.90	63	1.00-60.29
Fish oil+ tallow	174	0.99-60.68	63	1.00-59.81
Fish oil+ suet	150	1.00-60.75	63	1.00 -59.32

3.3.2 Discrimination of adulteration based on FT-IR spectral characteristics

PLS-DA qualitative discriminant models of terrestrial animal fats and oils in fish oil were established, separately, and then an independent validation set was predicted. The results of calibration and validation are displayed in Table 4.

Table 4 Results of PLS-DA discrimination based on FT-IR spectral characteristics for adulteration percentage range of 1%-60%

Adulterated sample	Calibration		Validation			
	R_{CV}^2	RMSECV	RMSEP	Number of false positive	Number of false \negative	Correct discrimination /%
Fish oil+lard	0.89	0.1275	0.1120	0	0	100
Fish oil+ Chicken oil	0.94	0.0976	0.0857	0	0	100
Fish oil+tallow	0.81	0.1691	0.1912	0	1	98.41
Fish oil+suet	0.82	0.1743	0.2171	0	3	95.24

Note: R_{CV}^2 is the correlation coefficient of cross calibration; RMSECV is the root mean square error of cross calibration; RMSEP is the root mean square error of prediction.

With the highest R_{CV}^2 value of 0.94 and the lowest

RMSECV value of 0.0976, the qualitative discriminant model of chicken oil in fish oil reveals the best calibration result. Results of independent validation showed that, correct discrimination rates of lard and chicken oil in fish oil are both 100%, with none false positive and false negative sample. Correct discrimination rates of tallow and suet in fish oil are both higher than 95%, with one and three false negative samples for tallow and suet, respectively. Further analysis indicated that the adulterated percentage false negative samples were all 1%, not only for tallow adulteration but also for suet adulteration in fish oil.

Getting rid of the adulterated samples of 1% tallow and suet fats in fish oil, PLS-DA qualitative discriminant models were re-established for the adulteration percentage range of 5%-60% (Table 5). Good validation results were acquired with none detected false positive and false negative sample, and the correct discrimination rates of tallow and suet oil in fish oil were both 100%.

Based on these results, it can be concluded that infrared spectroscopy can be successfully used to discriminate the terrestrial fat and oil ingredients including lard, chicken oil, tallow and suet in fish oil. The detection limits for the lard and chicken oil adulteration in fish oil should be between 0% and 1%, and the detection limits for the tallow and suet adulteration in fish oil should be between 1% and 5%.

Table 5 Results of PLS-DA discrimination based on FT-IR spectral characteristics for adulteration percentage range of 5%-60%

Adulterated sample	Calibration		Validation			
	R_{CV}^2	RMSECV	RMSEP	Number of false positive	Number of false negative	Correct Discrimination /%
Fish oil+tallow	0.94	0.0978	0.1167	0	0	100
Fish oil+suet	0.94	0.1058	0.1545	0	0	100

Note: R_{CV}^2 is the correlation coefficient of cross calibration; RMSECV is the root mean square error of cross calibration; RMSEP is the root mean square error of prediction.

3.4 Detection limit for the discriminant analysis of terrestrial animal fats and oils in fish oil

Based on the above research results, samples of fish oil adulterated with lard and chicken oils in the level of 0.4%, 0.6%, 0.8% (w/w) was prepared for further study of the detection limit. Simultaneously, samples of fish oil adulterated with tallow and suet in the level of 2%, 3%, 4% (w/w) were prepared. PLS-DA discriminant calibration and validation were performed in the same way presented above, whose results are shown in Tables 6 and 7.

Table 6 Results of detection limit for lard and chicken oil adulteration in fish oil

Adulterated sample	Calibration		Validation			
	R_{CV}^2	RMSECV	RMSEP	Number of false positive	Number of false negative	Correct discrimination/%
Fish oil+lard (0.4%-60%)	0.86	0.1246	0.1975	0	4	95.06
Fish oil+lard (0.6%-60%)	0.86	0.1321	0.1473	0	1	98.67
Fish oil+lard (0.8%-60%)	0.90	0.1142	0.1123	0	0	100
Fish oil+Chicken oil (0.4%-60%)	0.89	0.1159	0.1667	0	2	97.53
Fish oil+Chicken oil (0.6%-60%)	0.91	0.1109	0.1282	0	0	100
Fish oil+Chicken oil (0.8%-60%)	0.94	0.0951	0.1066	0	0	100

Note: R_{CV}^2 is the correlation coefficient of cross calibration; RMSECV is the root mean square error of cross calibration; RMSEP is the root mean square error of prediction.

Table 7 Results of detection limit for tallow and suet adulteration in fish oil

Adulterated sample	Calibration		Validation			
	R_{CV}^2	RMSECV	RMSEP	Number of false positive	Number of false negative	Correct discrimination/%
Fish oil+Tallow (2%-60%)	0.87	0.1251	0.1299	0	0	100
Fish oil+ Tallow (3%-60%)	0.88	0.1298	0.1357	0	0	100
Fish oil+ Tallow (4%-60%)	0.89	0.1278	0.1357	0	0	100
Fish oil+Suet (2%-60%)	0.84	0.1532	0.1908	0	3	96.00
Fish oil+Suet (3%-60%)	0.92	0.1083	0.1367	0	0	100
Fish oil+Suet (4%-60%)	0.92	0.1077	0.1444	0	0	100

Note: R_{CV}^2 is the correlation coefficient of cross calibration; RMSECV is the root mean square error of cross calibration; RMSEP is the root mean square error of prediction.

Results showed that the adulterated lard ingredients of 0.8%-60% in fish oil could be identified completely with the correct discrimination rate was 100%. However, for the discrimination of adulteration percentage range of 0.4%-60% and 0.6%-60%, false negative samples were detected. Therefore, it is safe to draw a conclusion that the detection limit for the discriminant analysis of lard in fish oil is about 0.8%. Similarly, for the discrimination of chicken oil in fish oil, the correct discrimination rates were 100% for both percentage range of 0.6%-60% and 0.8%-60%, while two false negative samples were identified by the PLS-DA model of percentage range of 0.4%-60%. Therefore, the detection limit for the discriminant analysis of chicken oil in fish oil was proved to be about 0.6%.

All the correct discrimination rates of the PLS-DA qualitative discriminant models of tallow in fish oil were 100% for adulteration level of 2%-60%, 3%-60% and 4%-60%. Combined with the results in Table 4, the detection limit for the discriminant analysis of tallow in fish oil was about 2%. Considering the discriminant analysis of suet in fish oil, only three false negative samples were found for the percentage range of 2%-60%, which proved that the detection limit should be about 3%.

From what has been demonstrated above, it was easier to discriminate lard and chicken oil ingredients in fish oil than tallow and suet adulteration. The detection limit for the discriminant analysis of lard and chicken oil in fish oil was much lower than that of tallow and suet. One basic point that needs to be taken into consideration was that the fatty acid characteristics of terrestrial animal fat and oil materials with different species origin were different. According to Table 1, there was significant difference for all the SFA, MUFA, PUFA and UFA contents between the lard, chicken oil and fish oil, while there was no significant difference for MUFA content between the tallow, suet and fish oil. This can also be supported by the results of PLS-DA discrimination of tested materials based on FT-IR spectra. Big difference of lipid composition and structural characteristics of raw materials contributed to the big difference of FT-IR spectral characteristics, and then contributed to the better discrimination of adulterated samples. What's more, it was also worthwhile to note that the fatty acid characteristics of lard and chicken oil were significantly different with that of the tallow and suet, which indicated a possibility to further explore the discriminant analysis of ruminant fats in non-ruminant fats and oils. All these work will improve the effective utilization of animal fat and oil resources and ensure the effective supervision of feeding quality and safety especially for the consideration of BSE crisis.

4 Conclusions

The present investigation studied the discriminant analysis of different species of terrestrial animal fats and oils in fish oil based on infrared spectroscopy and partial least squares discriminant analysis. The following conclusions can be drawn from this study:

a) It was easy to discriminate different species of fish oil, lard, chicken oil and ruminant fat materials based on the infrared spectral characteristics, while it was difficult to distinguish tallow from suet samples.

b) It was feasible for infrared spectroscopy to discriminate terrestrial fat and oil adulterations (lard, chicken oil, tallow and suet) in fish oil, and it was easier to discriminate lard and chicken oil ingredients in fish oil than tallow and suet adulterations.

c) The detection limits for the discriminant analysis of lard, chicken oil, tallow and suet adulterations in fish oil were proved to be about 0.8%, 0.6%, 2% and 3%, respectively.

More reliable samples is expected to be further added in the established models for validation, and research on further refinement of detection limits for the discriminant analysis is also worthwhile.

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