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The Employment of FTIR Spectroscopy and Chemometrics for Classification and Quantification of Mutton Fat in Cod Liver Oil

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ABSTRACT

In pharmaceutical field, Cod Liver Oil (CLO) is one of the potential sources of long chain omega-3 fatty acids and fat soluble vitamins, especially vitamin A. In the fats and oils industry, CLO has high price. As a consequence, some market players try to blind CLO with other fats and oils to gain economics profit. Animal fats like Mutton Fat (MF) are potential to be mixed with CLO due to the similarity in terms of fatty acid composition. This study focused on the application of FTIR spectroscopy in conjunction with chemometrics for classification and quantification of MF as adulterant in CLO. The combined spectral regions of 3,010-2995 and 1,500-900 cm^{-1} were used for classification between CLO and CLO blended with MF at various concentrations, with the aid of Discriminant Analysis (DA). DA is able to classify CLO and CLO adulterated with MF without any mistakenly grouped. These frequency regions were also used for quantification of MF in CLO, offering the highest R^2 value (0.992) and the lowest root mean square error of calibration (RMSEC) value (1.31% v/v), compared with other studied spectral regions.

Key words: FTIR spectroscopy, cod liver oil, mutton fat, multivariate calibration, discriminant analysis

INTRODUCTION

Today, Cod Liver Oil (CLO) is one of the emerging fats and oils in the market owing to advantageous effects to human. It is well known that marine oils including CLO contain high levels of omega-3 fatty acids, especially cis-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA) and cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA). Some publications indicated that these fatty acids play preventive agents in severe diseases such as cardiovascular and breast cancer (Moghadasian, 2008; Jude *et al*., 2006). For this reason, CLO can be considered as functional food oils due to its capability to provide beneficial health effects (Kaur and Das, 2011). It is estimated that the production of CLO is nearly 10,000 ton per year and is originally marketed as potential sources of vitamin A and D (Gunstone, 2004). In pharmaceutical fields, CLO is persistently being sold as medicines or functional food oils, either in capsule or suspension formulations.

Confirmation of the authenticity of a food or food ingredient is an increasing challenge for food scientist. This is especially the case when an added-value claim, such as one relating to geographic origin or a particular processing history, is made on the food label. Regulatory agencies are concerned with the prevention of economic fraud while the food processor needs confirmation of such claims in order to protect a brand, the image of which could be severely damaged should an adulterated ingredient make its way into the branded food product (Aparicio and Aparicio-Ruiz, 2000).

For a long time, adulteration is a serious problem in the trade of fats and oils, because there is a price difference for different oil products. Adulterant is sometimes added deliberately and accidentally (Shukla *et al*., 2005). Adulteration involves the addition of cheaper oils. CLO may be adulterated with animal fats like mutton fat due to its similarity in terms of fatty acid profiles compared to plant oils. Besides, CLO is much more expensive than mutton fat; consequently, CLO is of adulteration target with cheaper fats and oils in order to gain economic benefit. Usually, complex, time-consuming and tedious chemical treatments of samples are required for the analysis of adulteration, typically using chromatographic and wet chemical methods (Aparicio and Aparicio-Ruiz, 2002). Today, with the huge issue in relation to green analytical technique, some scientist try to used environmentally friendly technique for chemical analysis. One of techniques is vibrational spectroscopic-based technique (Raman and infrared spectroscopy) (Namiesnik, 2001).

For authentication analysis of edible fats and oils, FTIR spectroscopy has been used, especially in combination with multivariate calibrations and classification techniques, for analysis of olive oil adulterated with palm oil (Rohman and Man, 2010), cod liver oil mixed with some vegetable oils (Rohman and Man, 2011a), sesame oil from palm oil (Rohman and Man, 2011b) and virgin coconut oil adulterated with palm oil (Rohman and Man, 2009). In this study, FTIR spectroscopy with the accessory of Horizontal Attenuated Total Reflectance (HATR) in conjunction with chemometrics techniques of discriminant analysis and multivariate calibration of partial least square was used for classification and quantification of CLO adulterated with mutton fat. In addition, the changes of Fatty Acid (FA) profiles of CLO after being adulterated with MF were also investigated.

MATERIALS AND METHODS

Sample preparation: CLO was bought from a local market in Jogjakarta, Indonesia (CLO was imported from Norway). Mutton fat was made by rendering process by heating adipose tissues of mutton in conventional oven (100±2°C) for 3 h. the MF obtained was further stored in refrigerator until being used for fatty acid and FTIR spectra analysis.

Gas chromatography analysis: For analysis of FA composition changes, CLO was blended with animal fats in the range of 5-60% (v/v) animal fats in CLO, namely 5, 10, 15, 20, 30, 40, 50 and 60%. In addition 100% CLO and animal fats were used as reference samples. These samples were kept in controlled room temperature (20°C) before and during FA analysis. Determination of FA compositions of all oil samples were done using gas chromatography with flame ionization detector (GC-FID) as Fatty Acid Methyl Ester (FAME), according to AOCS (1996) with slight modifications. Approximately 80 mg of oils were dissolved in 1.0 mL hexane and added with 0.2 mL 1 M $NaOCH_3$ in methanol. The mixture was vigorously shaken for 1 min with a vortex mixer, added with 5 drops of saturated NaCl and mixed again using vortex for 15 sec. Subsequently, 1 μL of the clear supernatant was taken and injected into a gas chromatograph (Shimadzu GC-2010, Shimadzu Corp., Tokyo, Japan). The column used is RTX-5 capillary column (0.25 mm internal diameter,

30 m length and 0.2 μm film thickness; Restex Corp., Bellefonte PA), oven was set at 50°C (hold for 1 min), then increased to 180°C (8°C min^{-1}), 180 to 240°C (8°C min^{-1}) and finally held at 240°C for 5 min, carrier gas of N_2, at 6.8 mL min^{-1}, FID was set 240°C and injector temperature used was 240°C; split ratio (1:20). Standard FAMEs of 37 compounds (C4 to C24) (Sigma Chemicals, St. Louis, MO, USA) were used to identify the retention times. Quantification analysis of FA was performed using normalization internal technique.

Discriminant analysis: CLO and MF were mixed to obtain a series of standard or trained sets of 25 pure and 20 adulterated samples containing 1-50% of MF in CLO. The samples containing MF were marked as adulterated, whilst a series of CLO in the neat form was assigned as pure CLO and classified with DA using FTIR spectra.

Quantitative analysis: For calibration model, a set of standards consisting of CLO and animal fats (LD, BF, CF and MF) was prepared by mixing of both at concentration ranges of 1-50% (v/v) animal fats in CLO. For validation/prediction, 20 independent samples which were different from calibration samples, were constructed. Pure CLO and animal fats as well as their blends were analyzed using FTIR. The spectral regions where the variations among them were observed were chosen for developing multivariate analysis.

FTIR spectra measurement: FTIR spectra was scanned using a FTIR spectrometer Nicolet 6700 (Thermo Nicolet Corp., Madison, WI) equipped with a detector of deuterated triglycine sulphate (DTGS) and connected to software of OMNIC operating system (Version 7.0 Thermo Nicolet). The procedure and condition of FTIR spectra measurement can be seen in Rohman and Man (2010).

Statistical analysis: For fatty analysis, the statistical treatment using one-way analysis of variance (ANOVA), followed with Duncan multiple comparison using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) was used for differentiation among fatty acid levels in CLO adulterated with MF. The significance value (p) of less than 0.05 was considered statistically different. During FTIR spectra analysis, Discriminant Analysis (DA) and PLS were accomplished using TQ Analyst™ software (Thermo electron Corporation). The leave-one-out cross-validation procedure was used to verify the calibration model. The values of root mean square error of calibration (RMSEC) and coefficient of determination (R^2) were used as the validity criteria for the calibration. The predictive ability of PLS calibration model was further used to calculate the validation or prediction samples. In discussing analytical techniques applicable for adulteration practice, it is generally assumed that adulterant will be present in the relatively high quantities (Smith and Bonwick, 2007); therefore, the evaluation of method sensitivity in terms of limit of detection (LOD) and quantification (LOQ) is not required (Swartz and Krull, 1997).

RESULTS AND DISCUSSION

Analysis of CLO adulteration using fatty acid composition data: Table 1 shows the changes of FA profiles of CLO subjected to adulteration with MF. As shown in Table 1, the main FAs in MF were palmitic (C16:0), stearic (C18:0) and oleic (C18:1) acids, accounting of 21.49, 28.06 and 33.94%, respectively. Compared with other animal fats, MF showed higher level of C17:0 (Man *et al.*, 2011). As a result, increasing the level of MF in CLO caused the increased level of

Table 1: Fatty acid composition of Cod Liver Oil (CLO) adulterated with different concentration of Mutton Fat (MF)

	Ratio (MF: CLO, % v/v)									
†FA compo-sition (% w/w)	(0:100%)	(5:95%)	(10:90%)	(15:85%)	(20:80%)	(30:70%)	(40:60%)	(50:50%)	(60:40%)	(100:0%)
C14:0	4.16±0.03^{a}	4.14±0.03^{a}	4.02±0.01^{ab}	3.93±0.05^{b}	3.92±0.06^{b}	3.71±0.01^{c}	3.57±0.08^{d}	3.29±0.11^{e}	3.15±0.14^{e}	2.65±0.01^{f}
C16:0	11.91±0.05^{a}	12.39±0.04^{a}	13.22±0.03^{b}	13.75±0.26^{b}	13.68±0.49^{b}	15.21±0.04^{c}	16.05±0.50^{c}	17.44±0.56^{d}	18.07±0.80^{d}	20.73±0.08^{e}
C16:1	7.02±0.04^{a}	6.80±0.09^{ab}	6.28±0.01^{bc}	5.87±0.20^{b}	5.78±0.32^{c}	4.89±0.02^{d}	4.33±0.32^{e}	3.37±0.44^{f}	2.90±0.53^{f}	0.96±0.01^{g}
C17:0	0.22±0.00^{a}	0.24±0.00^{a}	0.43±0.01^{b}	0.55±0.06^{b}	0.55±0.09^{b}	0.84±0.01^{c}	1.12±0.10^{d}	1.44±0.12^{e}	1.57±0.16^{e}	2.03±0.02^{f}
C18:0	2.30±0.01^{a}	3.13±0.06^{a}	5.66±0.08^{b}	7.23±0.78^{b}	7.19±1.37^{b}	11.35±0.08^{c}	13.79±1.55^{d}	18.14±1.81^{e}	20.06±2.31^{e}	28.06±0.14^{f}
C18:1 n9	21.14±0.05^{a}	21.32±0.23^{a}	22.81±0.04^{b}	23.54±0.42^{b}	23.44±0.66^{b}	25.80±0.01^{c}	26.88±0.67^{c}	29.05±0.86^{d}	30.11±1.11^{d}	33.94±0.19^{e}
C18:2 n6	2.08±0.04^{a}	1.89±0.01^{b}	1.84±0.07^{bc}	1.78±0.04^{bcd}	1.77±0.04^{cde}	1.72±0.01^{de}	1.67±0.05^{ef}	1.56±0.04^{f}	1.58±0.08^{f}	1.36±0.06^{g}
C18:3 n6	1.98±0.10^{a}	1.82±0.02f^{b}	1.74±0.01^{b}	1.69±0.02^{b}	1.69±0.04^{b}	1.27±0.01^{c}	1.07±0.13^{d}	0.73±0.14^{ef}	0.68±0.03^{f}	0.84±0.01^{e}
C20:1 n9	11.41±0.09^{a}	11.27±0.13^{b}	10.06±0.05^{bc}	9.36±0.37^{c}	9.38±0.57^{c}	7.61±0.04^{d}	6.41±0.81^{d}	4.58±0.77^{e}	3.51±1.29^{e}	0.10±0.01^{f}
C20:5 n3	16.75±0.06^{a}	16.39±0.18^{a}	14.75±0.15^{bb}	13.76±0.61^{b}	13.77±0.86^{b}	11.40±0.06^{c}	9.60±1.32^{d}	6.73±1.17^{e}	5.49±1.38^{e}	0.15±0.00^{f}
C22:5 n3	1.22±0.01^{a}	1.15±0.04^{ab}	1.06±0.01^{bc}	0.99±0.04^{c}	1.01±0.06^{c}	0.83±0.01^{d}	0.71±0.08^{e}	0.53±0.08^{f}	0.43±0.10^{f}	0.07±0.01^{g}
C22:6 n3	8.82±0.11^{a}	8.99±0.08^{a}	7.81±0.04^{b}	7.32±0.27^{b}	7.38±0.42^{b}	6.04±0.08^{c}	5.27±0.69^{c}	3.53±0.69^{d}	2.82±0.74^{d}	0.19±0.02^{e}

FA = Fatty acid; †Each value in the table represents the means from three replicates; SD is given afte±.Means within each row with different letters are significantly different at $p<0.05$

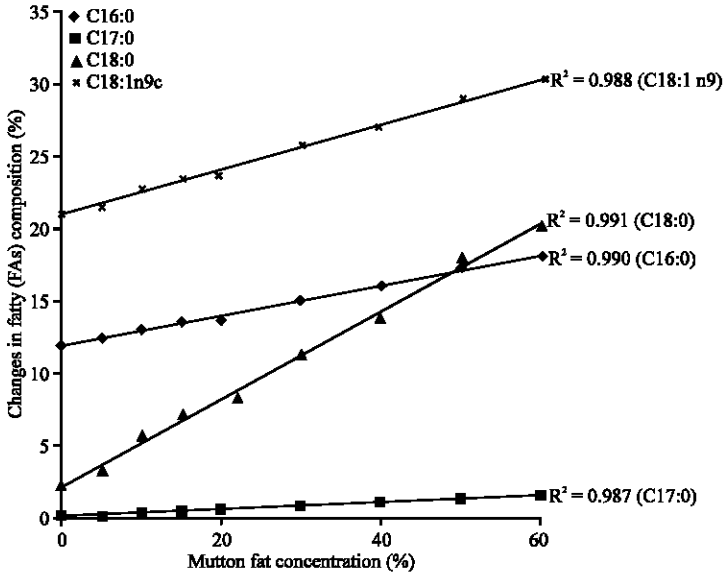

Fig. 1: The correlation between the increased levels of mutton fat (MF) (% v/v) in cod liver oil (CLO) and the fatty acid composition changes

C17:0. The presence of C17:0 in CLO can be an indicative that CLO may be adulterated with MF. The relationship between the increased levels of MF concentration with the changes of these four FAs (C16:0; C17:0; C18:0 and C18:1) during the adulteration of CLO with MF is shown in Fig. 1.

Meanwhile, some FAs mainly myristic (C14:0), palmitoleic (C16:1), EPA (C20:5 n-3) and DHA (C22:6 n-3) were significantly decreased from the level of 10% (v/v) MF in adulterated CLO. As indicated in Table 1, there was no significant difference among the concentrations of these FAs at MF levels of 5 and 10% ($p>0.05$). The R^2 values for the correlation between the increased levels of MF and the changes of FA compositions were as follows: 0.988 (for C14:0), 0.986 (C16:1 and EPA) and 0.978 (DHA). It is not surprising because these FAs are present in much higher level in CLO rather than in MF; consequently, the increasing levels of MF will decrease the concentrations of these FAs.

FTIR spectra analysis: FTIR spectra can be used as a potential tool which allows one to make a first differentiation among fats and oils due to its capability as fingerprint technique. Figure 2 shows FTIR spectra of CLO and mutton fat and having characteristic peaks of edible fats and oils spectra as described by Guillen and Cabo (1997) because the main components of fats and oils are triacylglycerols.

FTIR spectra of CLO and Mutton Fat (MF) appear very similar, however, they revealed slight differences in terms of band intensities and the exact frequencies at which the maximum absorbance were generated in each fats and oils, due to the different nature and composition of evaluated fats and oils (Guillen and Cabo, 1997), especially at wavenumber regions of 3006 (a), 2923 (b), 2852 cm^{-1}(c), 1654 (d), 1117 (e), 1098 cm^{-1} (f) and 965 cm^{-1} (g). These wavenumbers in which the peak intensities (absorbances) of MF and CLO were slightly different were further selected to be optimized for analysis of MF in CLO. Furthermore, the analysis of functional groups responsible for IR absorption in fat and oil samples can be found elsewhere (Lerma-Garcia *et al.*, 2010; Vlachos *et al.*, 2006; Guillen and Cabo, 1997).

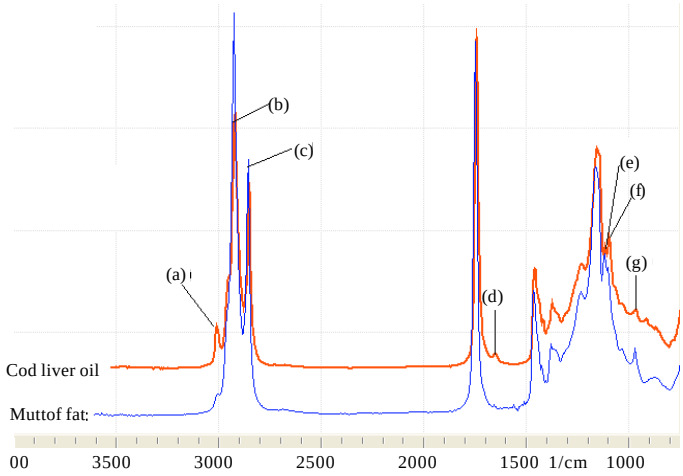

Fig. 2: FTIR spectra of cod liver oil (CLO) and other animal fats, scanned in mid infrared region (4,000-650 cm^{-1})

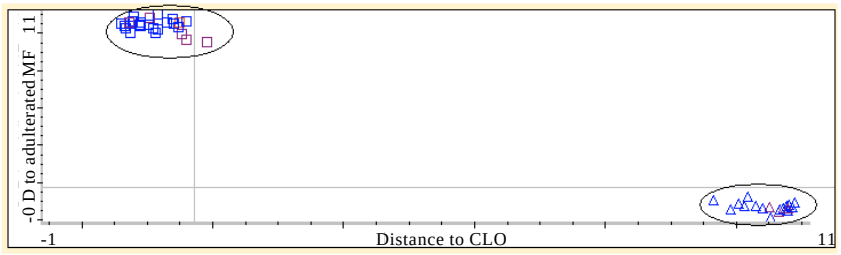

Fig. 3: The Cooman plot of cod liver oil (CLO) and CLO adulterated with animal fats: (□) CLO; (Δ) CLO samples adulterated with MF

Discriminant analysis: Discriminant Analysis (DA) is one of the chemometrics techniques widely used for classification of a group of samples (CLO and CLO adulterated with MF) by computing the distance from each class center in Mahalanobis distance units (Ballabio and Todeschini, 2009). DA is included in supervised pattern recognition. During discriminant analysis, pure CLO and CLO mixed with MF were classified into two groups, known as pure CLO and adulterated CLO. The frequency regions of 3,010-2995 and 1,500-900 cm^{-1} were selected during DA. The selection of frequency regions was performed in such a way that they give no or the least misclassifications between two groups (CLO and that adulterated with MF).

Figure 3 shows the Cooman plot for the classification of pure CLO and that adulterated with 1-50% (v/v) of MF. The x-axis showed the Mahalanobis distance to CLO, while the y-axis showed the distance to the adulterated CLO with MF. The Mahalanobis distance is useful in assigning whether a set of unknown value samples is similar to a collection set of known measured samples. The Cooman plot clearly exhibited the separated group of pure CLO and that adulterated with MF. 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 (Manaf *et al*., 2007).

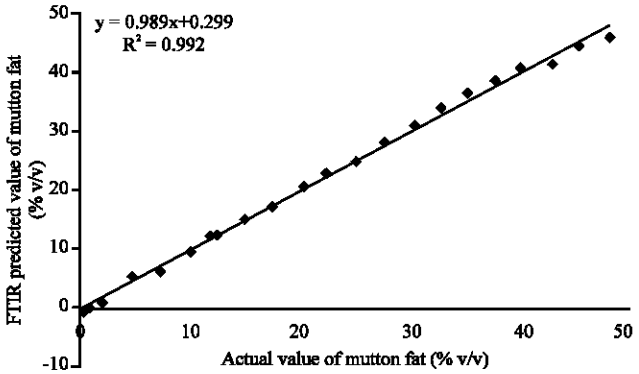

Fig. 4: The equation for the relationship between actual value of Mutton Fat (MF) and FTIR predicted value using PLS calibration model at frequency regions of 3,010-2995 and 1,500-900 cm^{-1}

Quantitative analysis of MF in CLO: Quantitative analysis of MF in CLO was performed with the aid of Partial Least Square (PLS) regression. In PLS calibration model, the absorbances of MF with concentration range of 0-50% (v/v) in CLO were recorded at mid infrared region of 4,000-650 cm^{-1}. PLS calibration was employed for making the relationship between actual value (x-axis) and FTIR predicted value of animal fats (y-axis) in CLO. The presence of MF in CLO was determined at the combined spectral regions of 3,010-2995 and 1,500-900 cm^{-1}. The selection of frequency region is performed such a way that give the high value of R^2 and the low value of RMSEC by taking into account the overfitting which may takes place during PLS calibration. These regions (the combined frequencies of 3,010-2995 and 1,500-900 cm^{-1}) have the capability to provide the highest R^2 value (0.992) and the lowest RMSEC value (1.31% v/v), compared with other studied spectral regions. The frequency regions at 3010-2995 give RMSEC value of 1.45% v/v, whereas frequency regions at 1,500-900 cm^{-1} give RMSEC value of 1.33% v/v. Using PLS calibration model, the relationship between actual value and FTIR predicted value of MF in CLO is shown in Fig. 4.

The performance of PLS calibration model was further assessed with cross validation using leave-one-out technique. Cross validation using leave one out technique was exploited to evaluate the performance of PLS model. The number of factors or Principal Components (PCs) used for developing PLS model was based on the Predicted Residual Error Sum Square (PRESS) value (Sedman *et al.*, 1997). The PRESS value is a direct measure on how well a calibration predicts the concentrations left out during a cross validation (Smith, 2002). Based on the PRESS value, the optimum number of factors used was 3. The root mean square error of cross validation (RMSECV) value obtained from cross validation of PLS calibration model was 1.76% (v/v). This indicated that FTIR spectroscopy was reliable enough for the analysis of MF in CLO. PLS calibration model was further used to calculate the prediction samples. The equation obtained for the relationship between actual and FTIR predicted values of MF in prediction samples was $y = 1.024x-0.678$, with R^2 and RMSEP values were 0.991 and 1.47% v/v, respectively.

CONCLUSION

We concluded that certain fatty acids could be used as an indicative for adulteration practice of CLO with MF. The presence of MF as adulterant in CLO has been successfully analyzed using FTIR spectroscopy with the aid of chemometrics of PLS at combined frequency regions of

3,010-2995 and 1,500-900 cm^{-1}. This frequency regions was also used for DA which classify CLO and that adulterated with MF with accuracy level of 100%.

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