Rapid Near-Infrared Spectroscopic Method for the Determination of Free Fatty Acid in Fish and Its Application in Fish Quality Assessment

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A rapid, simple, and environmentally friendly near-infrared (near-IR) spectroscopic method has been developed to directly determine free fatty acids (FFA) in fish oil and for the assessment of mackerel quality. The use of first derivative mathematical treatment for near-IR spectra of fish oil samples provides better results than *N*-point smoothing and second derivative. Both Multiple Linear Regression (MLR) and Partial Least Squares (PLS) regression are used for calibration. However, the calibration equation for FFA obtained from PLS gives higher correlation coefficients of calibration and prediction (r_c and r_p), and lower standard errors of calibration and prediction (SEC and SEP). The relative errors of prediction for FFA in mackerel fat are less than 8% using the calibration equation obtained from PLS. In this research, FFA change in mackerel has the same trend as hypoxanthine (Hx) change as a fish freshness index. Therefore, near-IR spectroscopy could be a useful technique to determine FFA contents in fish oil and for the assessment of mackerel quality.

Keywords: *Near-infrared; free fatty acid; mackerel; fish quality assessment*

INTRODUCTION

Fish quality has traditionally been evaluated through sensory assessments. However, sensory assessments are subjective and a trained panel is needed to carry out this evaluation. The search for a simple objective method (physical or chemical methods) to assess fish quality has been receiving increasing attention. So far, several chemical compounds were found that related to the change of fish quality, such as ATP degradation products (e.g., hypoxanthine [Hx]), trimethylamine (TMA), total volatile base (TVB), free fatty acids (FFA), etc. (Martin et al., 1982; Kramer et al., 1987). The methods for determination of those compounds generally are colorimetric measurement and chromatographic measurement (Woyewoda et al., 1986). In 1988, Wong et al. reported a rapid method for TMA in fish using a test strip. In 1991, Ohashi et al. used a semiconductive trimethylamine gas sensor to detect trimethylamine to assess fish quality; Krzymien et al. (1990) used trimethylamine headspace analysis to determine fish freshness; Isono (1990) investigated a new colorimetric measurement of fish freshness using nucleoside oxidase; Greene et al. (1990) used patterns of nucleotide catabolism as freshness indicators in flatfish from the Gulf of Alaska. These methods however, require a lot of sample preparation and involve many chemical manipulations. Consequently, developing better methods to assess fish quality is an attractive field to researchers.

Free fatty acids as a fish and seafood quality index has been recognized for a long time (Ke et al., 1976; Woyewoda et al., 1980). FFA are the hydrolytic products from fish lipid. Fish lipid hydrolysis can occur either by heating or through enzyme action. Lipase, one of the important enzymes responsible for fish lipid hydrolysis, liberates fatty acids from tri-, di-, or monoglycerides. In frozen storage, lipids from fish skin and muscle tissues undergo lipolytic changes (enzymatic) resulting in an accumulation of free fatty acids in most marine species including cod, mackerel, tuna, etc. (Mazeaud et al., 1976). The effect of FFA formation or lipid hydrolysis on fish quality has been studied by Ohshima et al. (1984). They measured the binding of FFA to fish proteins and hypothesized that FFA caused protein denaturation, leading to fish muscle toughness. FFA may also cause a nutritional deterioration (Ke et al., 1975). Thus, FFA formation has often been used as a quality index for fish and other food products (Dyer, 1968; Rolden et al., 1985; Barassi et al., 1987).

Many techniques have been developed to assess the quality of fish and other food by measuring the FFA content. An organic solvent extraction of FFA followed by sodium hydroxide titration of the product (Ke et al., 1976, 1978; Richardson, 1985) is widely used because it is easy to perform and uses low-cost equipment. Although simple, the procedure is cumbersome, requiring substantial personnel time, substantial chemical reagents and glassware, and accurate preparation of a standard solution; the method is also dependent on a visual endpoint. Other analytical methods such as gas chromatography (GC) (Woo et al., 1980; May et al., 1993) or high-pressure liquid chromatography (HPLC) (Elliot et al., 1989) have been used to quantify individual FFA. These procedures are, however, expensive, time consuming, and complicated. Consequently, developing a new efficient, rapid, simple, nondestructive, and low-cost technique is needed to determine FFA quickly and accurately to assess mackerel quality.

Recently, near-IR spectroscopy has been recognized as a powerful analytical technique (McClure, 1994). Traditional near-IR methods allow for rapid determination of various constituents in food, agricultural, and other products (Williams et al., 1987; Osborn et al., 1986). However, advanced instruments capable of recording full near-IR spectra (basically between 1100 nm and 2500 nm) have recently become available for commercial use. Implementation of modern chemomet-

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ric methods for processing complex signals has extended the scope of NIR application to include all types of solid samples. Near-IR technology has two modes: reflectance and transmittance (Williams et al., 1987). In the reflectance approach, near-IR energy is directed against the surface of the sample. The energy scattered off the surface is measured by a suitable optical detector (usually made of lead sulfide). The sample must be ground into a consistent fine powder for meaningful measurements to be made. In the transmittance approach, near-IR energy intersects the surface of a sample. A portion of this energy is transmitted through the sample and exits from the rear of the sample. A silicon detector is used to measure this exiting scattered light. Measurements using transmittance mode can be made without any sample preparation, such as sample grinding. Near-IR spectroscopy offers four principal advantages: speed (less than 3 min), simplicity of sample preparation (sometimes no sample preparation), multiplicity of analyses from a single spectrum (determination of different components at the same time), and nondestruction of the sample (after analyses, the sample can be used for another purpose). Ben-Gera and Norris (1968) analyzed fat and moisture contents in emulsions of meat products by near-IR transmittance. Kim et al. (1990) reported the determination of starch and energy in feed grains by near-IR reflectance spectroscopy.

Mitsumoto et al. (1991) evaluated near-IR spectroscopy using reflectance, transmittance, and fiber optic modes as a means of determining physical and chemical characteristics of beef. Kamishikiryo et al. (1992) evaluated near-IR spectroscopic measurement of protein content in oil/water emulsions. Marquardt et al. (1993) determined the glucose in a protein matrix using the near-IR method. Hall et al. (1993) used near-IR spectroscopy to determine the total proteins, albumin, globulins in serum, and urea. The determination of the main components (protein, fat, and moisture) by near-IR technique on whole fish has been studied by Isaksson et al. (1995) and Lee et al. (1992). Lee et al. (1992) reported the use of a noninvasive short-wavelength near-IR spectroscopic method to estimate the crude lipid content in the muscle of intact rainbow trout. Isaksson et al. used near-IR diffuse spectroscopy for the nondestructive determination of fat, moisture, and protein in salmon fillets. However no reports were found in which near-IR spectroscopy has been used to determine FFA in fish oil and for mackerel quality assessment. The application of the near-IR spectroscopic method is very attractive as a simple, fast method for assessing fish quality. Unlike other food products, fatty fish contains more polyunsaturated fatty acids which are more susceptible to autoxidation than other unsaturated and saturated fatty acids. As a result, fatty fish loses its freshness faster than other food products.

The objective of our study was to develop a simple and fast near-IR spectroscopic method to directly measure the FFA content in fish oil and in mackerel for fish quality assessment.

MATERIALS AND METHODS

Materials. Fish oil (commercial menhaden oil) was obtained from Sigma Chemical Co. (St. Louis, MO). It contains long chain ω-3 polyunsaturated fatty acids which are abundant in fish oils. The average contents of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in this oil are 13% and 8%, respectively. Hypoxanthine (Hx) was obtained from Sigma Chemical Co. (St. Louis, MO). All other solvents were obtained from Fisher Scientific (Lexington, MA).

Mackerel (Boston mackerel) was obtained from a local seafood store (Captain's C, New Brunswick, NJ) during the winter. The average weight of the fish was 174 g and the average fat content was 6%. The flesh with skin was removed from the fish and prepared for experiments. It was ground using a Cuisinart Food Processor (Dlc-118B, Cuisinart, Inc., Norwich, CT). The ground fish flesh with skin was well mixed, and was kept at 4 and 24 °C for different time intervals.

Hypoxanthine Analysis. Hx measurement was conducted according to a procedure described by Burns et al. (1985). A 5-g homogenized fish sample was blended with 50 mL 0.6 mol/L perchloric acid ($HClO_4$) solution. The homogenate was filtered and diluted with distilled water to 100 mL. We took 1 mL of the diluted solution and mixed it with 1.0 mL potassium hydroxide-phosphate buffer (pH 7.6). The mixture was allowed to cool to 0 °C and stand for 15 min to permit potassium perchlorate ($KClO_4$) to crystallize out, and was then filtered. The reaction mixture was injected directly into the HPLC (Waters System, Marlborough, MA). Hx was determined on a reversed phase column (DELTA PAK C18, 300 Å, 3.9 mm × 15 cm, Waters, Marlborough, MA) with a UV absorbance detector at 254 nm. The instrument was calibrated by external standard.

Extraction of Total Fat from Mackerel. Total fat of mackerel was extracted from three 50 g ground fish samples using the procedure of Bligh and Dyer (1959). Following the concentration of the chloroform extracts of the fish samples, the total fat content of mackerel samples was determined gravimetrically.

Preparation of FFA Mixture for Calibration and Prediction of Near-IR Spectroscopy. A certain amount of fish oil (commercial menhaden oil) was saponified with sodium hydroxide (10 N) to produce FFA. The saponified oil was acidified to pH 5 with hydrochloric acid (3 N) to regenerate the FFA from the salts and was washed eight times with water in a separatory funnel to remove any salts and residual soaps. The remaining water was separated by centrifugation, and the FFA mixture was dried over anhydrous sodium sulfate. Then, the FFA mixture was added back into the original fish oil in varying amounts. The amount of FFA in fish oil was determined by the titration method, and prepared for calibration and prediction by near-IR spectroscopy.

Titration Method as a Reference Method for FFA Determination. The FFA determination is by a sodium hydroxide titration of free carboxylic acid groups present in fish oil. The fish oil was dissolved in the solvent mixture of chloroform, methanol, and isopropanol with a ratio of 2:1:2. Aqueous sodium hydroxide (0.05 N) was standardized by potassium acid phthalate (solid standard) and stored in a plastic bottle with desiccates. This standard sodium hydroxide solution was added to titrate the free carboxylic acid groups present in fish oil. The endpoint was indicated by a color change of meta-cresol purple indicator. FFA was reported based on the weight of oleic acid as percent by weight of oil (Ke et al., 1976, 1978).

Near-IR Spectroscopic Analysis for FFA in Fish Oil. All near-IR measurements were made with a Model 6500 Spectrophotometer (NIR Systems, Inc., Silver Spring, MD). This instrument contains a computer-based system with a single-scanning monochromator. The monochromator scans the range between 1100 and 2500 nm in a transmittance mode. Data were recorded at 2-nm intervals and 32 scans were averaged for every sample. An empty cuvette with 2 mm path length was used as a reference. A silicon detector was used for transmittance measurements. Spectral processing was performed on an IBM-386 interactive computer system. Regression computations were performed with an NSAS statistical software system (NIR Systems, Perstorp Analytical Co., Silver Spring, MD).

Regression Methods for Calibration of FFA in Fish Oil. Calibration of the near-IR instruments involved the establishment of a mathematical relationship between near-IR response and the FFA contents obtained from the referenced method. We used two multivariate procedures to perform the calibration: Multiple Linear Regression (MLR)

Table 1. Wavelengths Selected and Statistical Summary of Calibration and Prediction for FFA in Fish Oil by Near-IR Transmittance Analysis Using Multiple Linear Regression

		regression constants					regression wavelengths				SEC[g]		SEP[i]	
content	math	K_0	K_1	K_2	K_3	K_4	λ_1	λ_2	λ_3	λ_4	(%)	r_c[h]	(%)	r_p[j]
C[a]	N-P[d]	26.788	1538.520	−403.834	−1075.320	−53.102	2170	2132	2176	1886	0.0543	0.998	0.0593	0.998
	1st-D[e]	1.452	999.605	−1670.756	323.789	27.718	2024	2214	1684	2308	0.0546	0.998	0.0145	0.994
	2nd-D[f]	2.788	229.240	3922.260	4880.743	−1736.066	1398	2010	2024	1974	0.111	0.993	0.107	0.993
low[b]	N-P	7.827	−107.596	−455.328	408.950	148.237	1700	1208	1662	2140	0.0513	0.988	0.131	0.862
	1st-D	6.124	−1465.763	3381.953	−320.992	−297.133	2008	2032	2170	2210	0.0209	0.998	0.0317	0.988
	2nd-D	8.321	2154.264	80.928	−61.341	37.545	4014	1516	2378	2372	0.0248	0.997	0.0941	0.892
high[c]	N-P	−2.109	−14.163	16.092	−2018.363	2040.523	2352	2406	1902	1896	0.189	0.967	0.154	0.969
	1st-D	17.495	2624.772	−1086.707	−413.464	−2145.007	2022	2208	2056	1312	0.0448	0.998	0.0610	0.995
	2nd-D	16.278	2210.531	593.943	−3132.760	163.023	1944	2132	1810	2122	0.0625	0.996	0.0548	0.996

[a] FFA contents ranging from 0.098% to 3.46%. [b] FFA contents ranging from 0.098% to 1.07%. [c] FFA contents ranging from 1.13% to 3.46%. [d] *N*-point smoothing mathematical treatment. [e] First derivative mathematical treatment. [f] Second derivative mathematical treatment. [g] Standard error of calibration. [h] Correlation coefficient of calibration. [i] Standard error of prediction. [j] Correlation coefficient of prediction.

Table 2. Statistical Summary of Calibration and Prediction for FFA in Fish Oil by Near-IR Transmittance Analysis Using Partial Least Squares Regression

contents	math	range of wavelengths (nm)	factors	MSECV[g] (%)	r_c[h]	SEC[i] (%)	SEP[i] (%)	r_p[k]
C[a]	N-P[d]	2131−2176	5	0.019	0.998	0.0631	0.0575	0.998
	1st-D[e]	2024−2214	5	0.009	0.999	0.0447	0.0423	0.999
	2nd-D[f]	1398−2010	11	0.757	0.999	0.0289	0.0588	0.998
low[b]	N-P	1208−1700	4	0.039	0.977	0.0705	0.131	0.862
	1st-D	2008−2210	7	0.011	0.999	0.0121	0.0236	0.996
	2nd-D	2014−2378	2	0.165	0.737	0.2025	0.232	0.441
high[c]	N-P	1896−2406	5	0.040	0.995	0.0792	0.0783	0.992
	1st-D	2022−2208	6	0.007	0.999	0.0292	0.0283	0.999
	2nd-D	1810−2122	9	0.007	0.999	0.0156	0.0227	0.999

[a] FFA contents ranging from 0.098% to 3.46%. [b] FFA contents ranging from 0.098% to 1.07%. [c] FFA contents ranging from 1.13% to 3.46%. [d] *N*-point smoothing mathematical treatment. [e] First derivative mathematical treatment. [f] Second derivative mathematical treatment. [g] Mean square error of cross validation. [h] Correlation coefficient of calibration. [i] Standard error of calibration. [j] Standard error of prediction. [k] Correlation coefficient of prediction.

(Williams et al., 1987) and Partial Least Squares (PLS) regression (Martens et al., 1991).

The calibration equation should give a high correlation coefficient of calibration (r_c), and a low standard error of calibration (SEC). The standard error of calibration measures how well the instrument matches calibration samples. Once the calibration equation is established, the prediction samples are used to verify the equation. The correlation coefficient of prediction (r_p) and the standard error of prediction (SEP) demonstrate how well the calibration can be used to assess the samples.

RESULTS AND DISCUSSION

Preparation of the Calibration Set of FFA in Fish Oil. Chemical analysis usually consists of two steps: calibration step (first step) and prediction step (second step). The characteristics of an instrumental method are investigated in an attempt to match a behavior to a model [a model is a relationship $Y = f(X)$ between two groups of variables, often called dependent Y and independent X]. This is the calibration step. The data set used for this step is called a calibration set. The model parameters are called regression coefficients.

This paper used commercial menhaden oil as a model of fish oil to prepare the calibration set of FFA contents, using the saponification method for even distribution of FFA content. We prepared 60 samples in the calibration set. The range of FFA content in the fish oil samples was from 0.098% to 3.46%.

Mathematical Treatment for Raw Near-IR Spectral Data. Mathematical treatment is a modification of raw spectral data. It can correct the baseline, enhance spectral data, or assist in smoothing a spectrum. Applying a mathematical treatment for raw near-IR spectral data will prepare these data for use in a regression, and the subsequent development of a calibration equation. In the present research, we use *N*-point smoothing, first derivative, and second derivative mathematical treatments for the modification of raw near-IR spectra and compared the results of each mathematical treatment to the raw near-IR spectra to select the best one. From Tables 1 and 2, we can see that *N*-point smoothing, first derivative, and second derivative mathematical treatments gave the important effects for spectral regression of FFA in fish oil. The first derivative mathematical treatment provided better results than *N*-point smoothing and second derivative mathematical treatments of near-IR spectra of FFA in fish oil (a more detailed explanation is given later).

MLR for Calibration of FFA. To obtain quality MLR results, the choice of an appropriate wavelength is important. The wavelengths selected by the computer for *N*-point smoothing, first derivative, and second derivative mathematical processes to produce calibration equations with the lowest standard error of calibrations (SEC) and highest correlation coefficient of calibration (r_c) are given in Table 1. The standard error of prediction (SEP) and correlation coefficient of the prediction (r_p) by using these calibration equations to predict results are also shown in Table 1.

From Table 1, we can see that all of the r_c obtained from the first derivative mathematical process of the spectra give the highest value (0.998) for all the ranges (0.098−3.46%; 0.098−1.07%; and 1.13−3.46%) of FFA contents. There is a higher correlation using the first derivative mathematical process of the spectra for all ranges of FFA than with either *N*-point smoothing or the second derivative. In addition, almost all of the SEC obtained from first derivative mathematical treatment

of the spectra are lower than *N*-point smoothing and second derivative mathematical treatments.

We know that the standard error of calibration measures how well the instrument matches calibration samples. Once the calibration equation is established, prediction of samples is used to verify the equation. The correlation coefficient of prediction and standard error of prediction will show how well the calibration can be used to assess the samples. Therefore, if a calibration equation with a high correlation coefficient and a low standard error was used for prediction, it would provide a low correlation coefficient and high standard error of prediction. Obviously this equation is not suitable for unknown samples. Using the calibration equation obtained from MLR for prediction (Table 1), we can see that the first derivative mathematical procedure produces a high r_p and low SEP for all ranges of FFA contents. There is only one exception for the range of FFA content from 0.098 to 3.46%. Table 1 shows that the *N*-point smoothing mathematical procedure gives a high r_c and r_p, but its SEC is almost same as the first derivative mathematical procedure and the SEP is higher than the first derivative mathematical procedure.

The MLR was used for regression of FFA in fish oil to obtain a calibration equation with a high correlation coefficient (0.998) and a low standard error from the regression of the first derivative mathematical procedure of near-IR spectra.

PLS Regression for Calibration of FFA. PLS regression is different from MLR. The PLS regression is useful with samples that contain some experimental noise in the near-IR spectra, because the algorithm of PLS regression discards the noise.

PLS regression can be used for a full range of wavelength (1100−2500 nm) regressions. However, selecting the wavelengths to be used for quantification is helpful. Selection was made by eliminating those wavelengths that provide no information on the components in which you are interested, such as FFA. We have, however, only selected those wavelengths which provide information on the components. The PLS regression results for *N*-point smoothing, first derivative, and second derivative mathematical treatments therefore are from a selected range of wavelengths according to the results of the stepwise best wavelength selection.

Table 2 shows the statistical results of PLS using *N*-point smoothing, first derivative, and second derivative mathematical procedures for the selected wavelengths. From Table 2, we can see that the first derivative mathematical procedure also shows a higher r_c and a lower SEC than *N*-point smoothing that produced by the second derivative mathematical procedure for the range of FFA contents from 0.098% to 3.460%. A range of FFA content from 1.130% to 3.460% also produces high r_c and low SEC, as did the first derivative mathematical procedure.

When using the calibration equation obtained from PLS regression, the first derivative mathematical treatment was found to give a high r_p and a low SEP compared to *N*-point smoothing and second derivative mathematical processes.

Comparison of MLR and PLS for Calibration of FFA. From Table 1 and 2, we can see that the first derivative mathematical treatment of the near-IR spectra gives better r_c, r_p, SEC, and SEP for both MLR and PLS than *N*-point smoothing and second derivative mathematical treatments. However, the r_c and r_p from PLS regression are higher than those from MLR.

Table 3. Near-IR Prediction Results Using Multiple Linear Regression (MLR) and Partial Least Squares Regression (PLS) for First Derivative Mathematical Treatment of Near-IR Spectra of FFA Contents Ranging from 0.098% to 3.46% in Fish Oil

sample no.	chemical data (%)	near-IR (MLR) (%)	near-IR (PLS) (%)	RE_1 (%)[a]	RE_2 (%)[b]
1	0.098	0.104	0.030	5.90	113
2	0.170	0.020	0.034	78.9	110
3	0.309	0.311	0.294	0.65	4.98
4	0.350	−0.048	0.417	263	17.5
5	0.423	0.470	0.438	10.5	3.49
6	0.440	0.363	0.433	19.2	1.60
7	0.470	0.653	0.426	32.0	9.82
8	0.500	0.458	0.478	8.77	4.50
9	0.506	0.431	0.463	16.0	8.87
10	0.540	0.546	0.518	1.10	4.16
11	0.550	0.556	0.585	1.08	6.17
12	0.630	0.628	0.612	0.32	2.90
13	0.635	0.709	0.650	11.0	2.33
14	0.640	0.661	0.646	3.22	0.92
15	0.700	0.730	0.724	4.20	3.37
16	0.740	0.778	0.819	5.00	10.1
17	0.743	0.698	0.712	5.93	4.26
18	0.813	0.705	0.826	14.2	1.59
19	0.830	0.933	0.799	11.7	3.81
20	0.950	0.949	0.938	0.11	1.27
21	0.960	1.000	0.993	4.08	3.38
22	1.050	1.077	1.009	2.54	3.98
23	1.070	1.120	1.100	4.57	2.76
24	1.130	1.169	1.063	3.39	6.11
25	1.180	1.199	1.218	1.60	3.17
26	1.220	1.176	1.318	3.67	3.86
27	1.300	1.431	1.353	9.59	4.00
28	1.310	1.293	1.298	1.31	0.92
29	1.400	1.429	1.421	2.05	1.49
30	1.480	1.614	1.498	8.66	1.21
31	1.500	1.477	1.511	1.55	0.73
32	1.570	1.702	1.605	8.07	2.20
33	1.580	1.648	1.614	4.21	3.13
34	1.650	1.758	1.673	6.33	1.38
35	1.720	1.808	1.764	5.00	2.53
36	1.780	1.848	1.817	3.75	2.06
37	1.790	1.828	1.813	2.11	1.28
38	1.900	2.073	1.946	8.71	2.39
39	1.910	1.901	1.916	0.47	0.31
40	1.930	1.922	1.949	0.42	0.98
41	2.020	2.099	2.068	3.83	2.35
42	2.080	2.135	2.087	2.61	0.33
43	2.190	2.177	2.138	0.60	2.40
44	2.200	2.262	2.232	2.78	1.44
45	2.210	2.246	2.236	1.62	1.17
46	2.230	2.249	2.229	0.85	0.04
47	2.320	2.247	2.317	3.20	0.13
48	2.340	2.387	2.364	1.99	1.02
49	2.420	2.409	2.414	0.46	0.25
50	2.440	2.520	2.421	3.22	0.78
51	2.480	2.485	2.465	0.20	0.61
52	2.520	2.450	2.520	2.82	0.00
53	2.630	2.601	2.611	1.11	0.73
54	2.700	2.690	2.727	0.37	1.00
55	2.880	2.944	2.910	2.20	1.04
56	2.910	2.896	2.905	0.48	0.17
57	3.040	2.914	2.980	4.23	1.99
58	3.160	3.053	3.133	3.44	0.86
59	3.260	3.241	3.208	0.59	1.61
60	3.460	3.264	3.424	5.83	1.05

[a] Relative error by using MLR. [b] Relative error by using PLS.

Using Calibration Equation for Prediction of FFA in Fish Oil. Tables 3, 4, and 5 are the prediction results using the calibration equation obtained from PLS and MLR. These prediction results provided further information for selecting the regression method for our specific sample (fish oil). Table 3 shows the prediction results and its relative errors for FFA con-

Table 4. Near-IR Prediction Results Using Multiple Linear Regression (MLR) and Partial Least Squares Regression (PLS) for First Derivative Mathematical Treatment of Near-IR Spectra of FFA Contents Ranging from 0.098% to 1.07% in Fish Oil

sample no.	chemical data (%)	near-IR (MLR) (%)	near-IR (PLS) (%)	RE_1[a] (%)	RE_2[b] (%)
1	0.098	0.092	0.102	6.32	4.01
2	0.170	0.185	0.169	5.71	0.59
3	0.309	0.364	0.364	14.6	1.63
4	0.350	0.333	0.342	4.99	2.31
5	0.423	0.437	0.465	3.26	6.21
6	0.440	0.439	0.445	0.23	1.13
7	0.470	0.487	0.483	3.55	2.73
8	0.500	0.535	0.502	6.76	0.40
9	0.506	0.584	0.546	14.3	7.60
10	0.540	0.515	0.519	4.74	3.97
11	0.550	0.627	0.585	13.1	6.17
12	0.630	0.631	0.637	0.16	1.10
13	0.635	0.642	0.679	1.10	6.70
14	0.640	0.623	0.634	2.69	0.94
15	0.700	0.705	0.705	0.71	0.71
16	0.740	0.685	0.744	7.72	0.54
17	0.743	0.744	0.746	0.13	0.40
18	0.813	0.801	0.792	1.49	2.62
19	0.830	0.819	0.825	1.33	0.60
20	0.950	0.880	0.925	7.65	2.67
21	0.960	0.983	0.965	2.37	0.52
22	1.050	0.938	0.988	11.3	6.08
23	1.070	1.055	1.067	1.41	0.28

[a] Relative error by using MLR. [b] Relative error by using by using PLS.

tents ranging from 0.098% to 3.46% using the calibration equation obtained from PLS and MLR corresponding to FFA content ranging from 0.098% to 3.46%. From Table 3, we can see that the relative errors of chemical method (reference) and near-IR method using the calibration equation obtained from MLR are generally larger than the relative errors using the calibration equation obtained from PLS, especially for low FFA content. From Table 3, we also can seen that the relative errors of prediction for low FFA content (less than 1%) are much higher than those for high FFA content (more than 1%). The relative errors are higher than 10% for the 41% prediction samples of low FFA content using MLR and 18% prediction samples of low FFA content using PLS.

In order to improve the prediction results for low FFA content, we use the calibration equations obtained for the regression of the FFA content ranging from 0.098% to 1.07%. The prediction results are shown in Table 4. Table 5 also shows the prediction results using the calibration equation obtained from the FFA content ranging from 1.13% to 3.46%. The prediction results for the low FFA content and high FFA content were improved by dividing the calibration set into two groups and using MLR and PLS to obtain new calibration equations for each group. From Table 4, we can see that the relative errors are all less than 10% for PLS, the relative errors of 87% prediction samples for low FFA content are less than 10% for MLR. The relative errors shown in Table 5 are all less than 10% for high FFA content. The relative errors for assessment are acceptable. From Tables 4 and 5, we can see that the relative errors for PLS are less than those for MLR.

Therefore, we use the calibration equation obtained from PLS to predict the FFA content in mackerel.

FFA Content of Fish Samples at Different Storage Times. Table 6 shows the results of FFA content at different storage times. The FFA data were obtained from the average results of determining four fish samples. For the mackerel stored at 4 °C (refrigeration), the FFA content increased at a fairly moderate rate, reaching to 8.1% in mackerel fat (or about 0.48% in mackerel based on the fat content in mackerel) after 7 days refrigeration. The FFA level increased significantly between days 0 and 2, and 5 and 7. Unlike the mackerel stored at 4 °C, the FFA level increased much

Table 5. Near-IR Prediction Results Using Multiple Linear Regression (MLR) and Partial Least Squares Regression (PLS) for First Derivative Mathematical Treatment of Near-IR Spectra of FFA Contents Ranging from 1.13% to 3.46% in Fish Oil

sample no.	chemical data (%)	near-IR (MLR) (%)	near-IR (PLS) (%)	RE_1[a] (%)	RE_2[b] (%)
1	1.130	1.141	1.066	0.97	5.83
2	1.180	1.124	1.181	4.86	0.09
3	1.220	1.308	1.324	6.96	8.18
4	1.300	1.342	1.312	3.18	0.92
5	1.310	1.333	1.317	1.74	0.53
6	1.400	1.378	1.416	1.58	1.14
7	1.480	1.648	1.482	10.7	0.13
8	1.500	1.447	1.528	3.60	1.40
9	1.570	1.590	1.549	1.27	1.35
10	1.580	1.574	1.560	0.38	1.27
11	1.650	1.725	1.678	4.44	1.68
12	1.720	1.700	1.704	1.17	0.93
13	1.780	1.754	1.771	1.47	0.51
14	1.790	1.821	1.796	1.72	0.33
15	1.900	1.930	1.928	1.57	1.46
16	1.910	1.863	1.905	2.49	0.26
17	1.930	1.938	1.939	0.41	0.47
18	2.020	2.044	2.032	1.18	0.59
19	2.080	2.163	2.095	3.92	0.72
20	2.190	2.226	2.178	1.63	0.55
21	2.200	2.233	2.269	1.49	3.09
22	2.210	2.195	2.207	0.68	0.14
23	2.230	2.294	2.249	2.83	0.85
24	2.320	2.367	2.339	2.00	0.82
25	2.340	2.372	3.338	1.36	0.09
26	2.420	2.470	2.430	2.04	0.41
27	2.440	2.500	2.460	2.43	0.82
28	2.480	2.487	2.454	0.28	1.05
29	2.520	2.498	2.522	0.88	0.08
30	2.630	2.690	2.625	2.26	0.19
31	2.700	2.579	2.711	4.58	0.41
32	2.880	2.879	2.874	0.04	0.21
33	2.910	2.975	2.911	2.21	0.03
34	3.040	3.041	3.021	0.03	0.63
35	3.160	3.090	3.186	2.24	0.82
36	3.260	3.232	3.234	0.86	0.80
37	3.460	3.361	4.489	2.90	0.83

[a] Relative error by using MLR. [b] Relative error by using PLS.

Table 6. FFA Content in Mackerel Fat Determined by Chemical Method and Near-IR Method[a]

		FFA contents in mackerel fat				
storage temp (°C)	storage time (days)	chemical method (%)	near-IR $method_1$ (%)[b]	near-IR $method_2$ (%)[c]	RE_1 (%)[d]	RE_2 (%)[e]
	0	2.5	2.7	2.6	7.69	3.92
4	2	3.6	3.6	3.6	0.00	0.00
	3	4.1	3.9	4.0	5.00	2.50
	4	4.2	4.3	4.4	2.38	4.65
	5	4.7	4.7	4.7	0.00	0.00
	7	8.1	7.6	7.5	6.37	7.69
24	0	2.5	2.8	2.7	7.69	3.92
	1	4.5	4.5	4.5	0.00	0.00
	1.5	5.5	5.3	5.2	3.70	5.60

[a] The samples for near-IR determination were diluted by fish oil, the ratio is 1:1. [b] Using calibration equation obtained from PLS regression for FFA contents ranging from 0.098% to 3.46%. [c] Using calibration equation obtained from PLS regression for FFA contents ranging from 1.13% to 3.46%. [d] Relative error of chemical method with near-IR $method_1$. [e] Relative error of chemical method with near-IR $method_2$.

Table 7. Effect of Storage Times on the Hx Content and Sensory Value in Mackerel Stored at 4 and 24 °C

storage temp (°C)	storage time (days)	Hx content (μmol/g)	sensory test (overall smell at 24 °C)
4	0	1.14	1 (fresh)
	2	1.60	2
	3	1.62	3
	4	2.30	4 (beginning off-flavor)
	5	3.18	5 (unacceptable)
	7	5.14	6
24	0	1.14	1
	1	2.71	4
	1.5	4.99	6

more rapidly at 24 °C storage. These results showed that FFA increased with storage time and temperature and FFA contents in mackerel were in agreement with the report of Barassi et al. (1987).

Table 6 also shows the near-IR prediction results for FFA in mackerel fat using the calibration equation obtained from PLS regression. From Table 6, we can see that the relative errors are all less than 10%. These results show that the near-IR method can be used to determine the FFA content in mackerel fat to assess the quality of mackerel.

Hx Content of Mackerel Samples at Different Storage Times. The use of Hx as an index of fish quality was initially studied at the Torry Research Station (Jones, 1960; Jones et al., 1962). After that, researchers, such as Beuchat (1973), Spinelli et al. (1964), etc., reported that hypoxanthine levels in fish during iced storage had a good correlation to the degree of freshness. Generally, it is thought that Hx content in fish is an accurate marker of the degree of fish freshness. Therefore, we determined the Hx content of mackerel samples (same samples used for FFA determination) at different storage times as a comparison to FFA determination in the present research for assessing fish quality.

The Hx contents at different storage times are shown in Table 7. For the mackerel stored at 4 °C, the Hx increased at a fairly moderate rate, reaching 5.14 μmol/g after 7 days refrigerated storage. The Hx level increased significantly between days 0 and 2, and 3 and 4. Unlike the mackerel stored at 4 °C, the Hx level increased more rapidly at 24 °C storage. These results were in agreement with those of Jahns et al. (1976). According to an overall sensory smell test of mackerel, after 4 days of storage at 4 °C, the mackerel had developed a spoilage odor; after the second day of storage at 24 °C, the mackerel had developed a strong spoilage odor. These results are in keeping with those previously reported by Jhaveri et al. (1982). The average maximum Hx levels in seafood were reported as 5 μmol/g (Burt et al., 1976, 1977).

Based on above results, FFA change in mackerel has the same trend as Hx change under the same storage conditions. Therefore, we have demonstrated that FFA determined by near-IR method can be a useful marker to assess mackerel quality.

Conclusions. The developed near-IR method, which is simple, fast, nondestructive, and safe, is based on the use of the first derivative of the near-IR absorbance spectrum. The calibration equation obtained from PLS regression gave a high r_c and r_p, and low SEC and SEP. The calibration equation obtained from menhaden oil can be used for FFA prediction for mackerel. The developed method also minimizes the environmental concerns associated with the use of organic solvents and hazardous reagents for the determination of FFA.

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