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INVESTIGATION ON FATTY ACID COMPOSITION OF JAPANESE BLACK WAGYU BEEF BY ATR-FTIR SPECTROSCOPY AND CHEMOMETRIC ANALYSIS

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ABSTRACT Japanese black Wagyu beef is known worldwide for its excellent marbling, tenderness and juiciness, which is caused by the fatty acid composition of beef fat. The objective of this study is to develop a non-destructive and rapid method for the determination of the percentages of oleic acid (C18:1), palmitic acid (C16:0), monounsaturated fatty acids (MUFA), and saturated fatty acids (SFA) in beef fat by attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy. In this study, ATR-FTIR spectroscopy and gas chromatography (GC) were used for the solvent-extracted fat and non-processed fat tissue. The results of GC analysis showed that Wagyu beef could have higher percentages of oleic acid and MUFA and lower content of SFA. The principal components analysis (PCA) result of fatty acid composition allowed the subcutaneous fat to be discriminated from intra- and inter-muscular fats but inter- and intra-muscular fats were indistinguishable. Moreover, the optimum PLS validation models of C18:1, C16:0, MUFA and SFA contents in solvent-extracted fat were obtained with the combination of 1500-1000, 1800-1620 and 3050-2800 cm^{-1} and coefficients of determination (R^2) were 0.890, 0.905, 0.961 and 0.974, respectively. For non-processed fat, the best PLS models for C18:1, C16:0, MUFA and SFA were with R^2 0.470, 0.482, 0.611 and 0.647, by use of the combination of 1400-1000 and 3050-2800 cm^{-1}.

Keywords: Monounsaturated fatty acids (MUFA), saturated fatty acids (SFA), partial least squares (PLS)

INTRODUCTION Japanese black Wagyu beef is renowned and expensive for fatty well-marbled texture and palatability. In Japan, the beef quality is graded according to the four item— marbling, meat color, firmness and texture of meat, color, luster and quality of fat on the beef carcass cross-section of 6[th]-7[th] ribs, which are formulated by the Japan Meat Grading Association. Nonetheless, fatty acid composition of beef is another critical influence factor in beef quality. More delicious beef has higher percentage of monounsaturated fatty acids (MUFA) and lower contents of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) in carcass fat, which cause unpleasant waxy feeling and flavour (Westerling & Hedrick, 1979).

Recently, rapid characterization of fatty acid profile of beef fat is of great interest. Gas chromatography (GC) analysis has been prevalently adopted as a standard method for the determination of fatty acid composition of edible oils and fats. However, this measurement is time-consuming and also requires noxious chemicals and trained operators.

Near infrared (NIR) and mid-infrared (MIR) spectroscopy has been proved to be an effective alternative to qualitative and quantitative chemical measurement in food. In recent years, attenuated total reflectance-Fourier transform infrared (ATR-FTIR) was applied to determine the potential adulterant of olive oil (Lai et al., 1995), fatty acid composition of pork fat (Ripoche & Guillard, 2001), relative amount of *n-3* and *n-6* polyunsaturated fatty acid species in vegetable oils and oil seeds (Yoshida & Yoshida, 2003), the contents of protein, fat and lactose in milk (Iñón et al., 2004), the spoilage of chicken breast muscle (Alexandrakis et al., 2009) and the contents of monounsaturated fatty acids and saturated fatty acids in Japanese Black Wagyu beef (Hu et al., 2010).

The objective of the present study was to investigate the feasibility of the application of ATR-FTIR spectroscopy to determine fatty acid profile of Japanese black Wagyu beef fat for two fat states— solvent-extracted fat and non-processed fat.

MATERIALS AND METHODS

Samples Meat samples from 22 Japanese black Wagyu cattle in this study, which were fed by Ito Ham (Co. Ltd) until 30 or 31 moths of age, were tested within 3 weeks after slaughter. According to the criterion on beef quality by Japan Meat Grading Association (Japan Meat Grading Association, 1988), two out of the twenty-two samples were graded to be at the “below average” level, twelve samples at the “average” level, six at “good” level and two at the “excellent” level.

Sample preparation Fat specimens (about 2.0g) were taken from subcutaneous, inter- and intra-muscular fat depots of the carcass cross-section between 6^{th}-7^{th} ribs respectively. The non-processed fat specimens were tested for ATR-FTIR measurement and subsequently preserved at -20℃ until the further experiment. The total lipid of each specimen was extracted by chloroform-methanol (2:1, by vol) and washed by 0.88% (w/w) potassium chloride solution according to Folch method (Folch & Sloane-Stanley, 1957). An aliquot of extracted lipid was tested for FTIR spectrum immediately and another aliquot was used for fatty acid analysis by GC as soon as possible.

ATR-FTIR spectrometry Triplicate spectra for each specimen were recorded between 4000 and 600 cm^{-1} at a resolution of 2 cm^{-1} with 32 scans on Bomen MB 3000 (ABB Co. Ltd., Tokyo, Japan) spectrometer mounted with a MIRacle-single horizontal ATR accessory (ZnSe crystal, PIKE Technologies Co. Ltd., WI, USA) and a high-pressure clamp (PIKE Technologies Co. Ltd., WI, USA). Specimens of two different states were used for FTIR detections: direct measurement of non-processed fat tissues and the measurement of solvent-extracted fat. For direct measurement, fat tissues (about 5mm thickness) were pressed with a high-pressure clamp to ensure a good contact between the sample and the surface of ATR crystal. For the solvent-extracted fat, 15 μL of extracted fat was placed on the surface of ATR crystal with mounting ring and trough insert. The surface of ATR crystal, pressure rod, and trough insert were cleaned by wiping with 2%

Triton X-100 and distilled water, and then dried with clean fat-free cotton before each measurement.

Fatty acid composition Fatty acid composition of the three kinds of fat (subcutaneous, inter- and intra-muscular fat) was determined by GC analysis. The extracted fat was first saponified and then esterified by modified BF_3-MeOH method (AOCS Method Ce 1b-89) before extraction into hexane added with BHT (50 μL/mL). A standard fatty acid methyl-esters mixture (catalog No. 07631-1AMP, Sigma Aldrich Chemical Co., Tokyo, Japan) and samples were then analyzed on a GC-2014 (Shimadzu Co. Ltd., Kyoto, Japan) equipped with an Omegawax^{TM} 250 capillary column (30m*0.25mm*0.25μm film thickness, Sigma Aldrich Chemical Co., Tokyo, Japan) and a flame ionization detector (FID). The temperatures of column, injection and detector were 180℃, 260℃ and 250℃, respectively. A split ratio of 1:100 was used in the analysis. Chromato-PRO software (Lab Lab Company Co. Ltd., Tokyo, Japan) was used for the integration of GC signals. The fatty acids of specimens was identified by comparing the retention time of standard fatty acid methyl-esters, and the relative proportions were determined as percentages of the summed peak areas according to the standard method for the analysis of fatty acids composition (JOCS Method 2.4.2.1-1996).

Statistical analysis Principal components analysis (PCA) and partial least-squares (PLS) regression were applied by PLS_Toolbox (Eigenvector Research Inc., WA, USA) with the Matlab software (The MathWorks Japan Inc., Tokyo, Japan) as follows: PCA was applied to the fatty acid composition in order to investigate the difference among the compositions of subcutaneous, inter- and intra-muscular fat. Outliers were identified using residual statistic (Q statistic), establishing a confidence limit value of 95%, such that the samples whose Q residual was greater than the set value. PLS regression was applied as the calibration method. The outliers of the calibration models were determined by Hotelling's T^2 statistic with a confidence limit value of 95%. RMSEC (V) (Root Mean Square Error of calibration/validation), R^2 (coefficient of determination) were used as criteria to evaluate validity of calibration and its leave-one-out cross-validation models whose formulas are:

$$RMSEC = \sqrt{\frac{\sum_{i=1}^{N}(Y_i^{ref} - Y_i^{pre1})^2}{N}} \quad (1) \qquad RMSECV = \sqrt{\frac{\sum_{i=1}^{N}(Y_i^{ref} - Y_i^{pre2})^2}{N}} \quad (2) \qquad R^2 = \frac{\sum_{i=1}^{N}\left(Y_i^{pre} - \overline{Y_i}\right)}{\sum_{i=1}^{N}\left(Y_i^{ref} - \overline{Y_i}\right)} \quad (3)$$

Where, N is the number of specimens, Y^{ref} is the fatty acid percentage by GC detection, Y^{pre1} is the predicted value of calibration models, Y^{pre2} is the predicted value of cross-validation models and $\overline{Yi}$ is the average value (Ripoche et al., 2001). To remove noise and enhance spectral differences, baseline correction, smoothing, second derivative transformation and normalization were conducted.

RESULTS AND DISCUSSION

Fatty acid composition of Japanese Black Wagyu fat As compared to earlier study (Oka et al., 2002), similar fatty acid composition was recognized (Table 1). Major fatty acids composing the beef fat were oleic acid (18:1) and palmitic acid (16:0). Moreover, MUFA content increased in the order of intra-muscular fat, inter-muscular fat and subcutaneous fat. SFA decreased in the same order. This tendency was consistent with

the results in the earlier investigations which indicated that bovine tissues near the body surface would have higher content of MUFA than internal tissues (Ozutsumi et al., 1983; Sturdivant et al., 1992; Oka et al., 2002). MUFA and oleic acid (C18:1) were demonstrated to be positively correlated with palatability of beef and also with fat softness (Westerling et al., 1979; Melton et al. 1982; Baublits et al., 2009). C18:1 was suggested to lower low-density lipoprotein without affecting high-density lipoprotein and thus decreases the risk of cardiovascular disease (Grundy et al., 1990; Lagrost, 1992; Fujiwara et al., 2005).To the contrary, stearic acid (C18:0) negatively correlated with beef flavor (Mandell et al., 1998; Baublits et al., 2009), and SFA has been proven to increase the level of cholesterol. As shown in Table 1, the subcutaneous fat of Japanese Black Wagyu could provide higher content of C18:1 (48.89%), lower content of C18:0 (5.87%), and higher ratio of MUFA to SFA (1.74) comparing the reported values of subcutaneous adipose tissue of American and Australian beef, which were 47.3%, 7.6% and 1.59 respectively (Huerta-Leidenz et al., 1993; Smith et al., 1998; Chung et al., 2006). In Oka's investigation (2002), it was presented that content of C18:1 was 51.7% from293 Japanese black Wagyu cattle, much higher than that in this study. Thus, it was suggested that Japanese Black Wagyu cattle could provide more flavorful and healthy beef than common American and Australian cattle breeds.

Table 1. Fatty acid composition of subcutaneous, inter- and intra-muscular fats measured by GC analysis for Japanese black Wagyu beef

Item	Subcutaneous fat	Inter-muscular fat	Intra-muscular fat
Fatty acid %			
Myristic acid (14:0)	2.51±0.55	2.46±0.52	2.76±0.56
Palmitic acid (16:0)	23.64±2.41	23.54±2.54	27.29±1.74
Palmitoleic acid (16:1)	6.85±1.19	5.03±1.14	3.89±1.09
Stearic acid (18:0)	5.87±1.66	9.16±2.52	10.50±2.52
Oliec acid (18:1)	48.89±3.27	49.42±3.41	46.71±2.81
Linoleic acid (18:2)	2.04±0.30	2.11±0.33	2.12±0.31
Saturated fatty acids (SFA)	32.02±3.77	35.16±3.94	40.54±2.69
Monounsaturated fatty acids (MUFA)	55.37±3.51	54.44±3.70	50.60±2.76
MUFA:SFA	1.74	1.55	1.25

Figure 1 shows the results of the classification model of principal components analysis (PCA) for the three adipose tissues of Japanese black Wagyu based on their fatty acid composition. The first principal component (PC1) in the PCA analysis accounted for 67.23% of the variance in the data and the second one accounted for 21.79% (Figure 1 (a)). Overall the subcutaneous fat could be distinguished from inter- and intra-muscular fats except two outliers and confusable three data of inter-muscular fat, however, inter- and intra-muscular fat could be indistinguishable [Figure 1 (a)].

Result of the relation among variables (various kinds of fatty acid) responsible for the observed differences in the sample (three kinds of fat) scores is shown in Figure 1 (b). It was indicated that in the discriminant analysis of these three kinds of fat, the total contents of unsaturated or saturated fatty acids (PC 1) and the percentages of C18:1 and C16:0 (the major components of PC2) were the most significant factors [Figure 1 (b)]. The results of PCA indicated that subcutaneous fat can be discriminated from inter- and intra-muscular fat but the latter two kinds of fat may not be distinguished from each other.

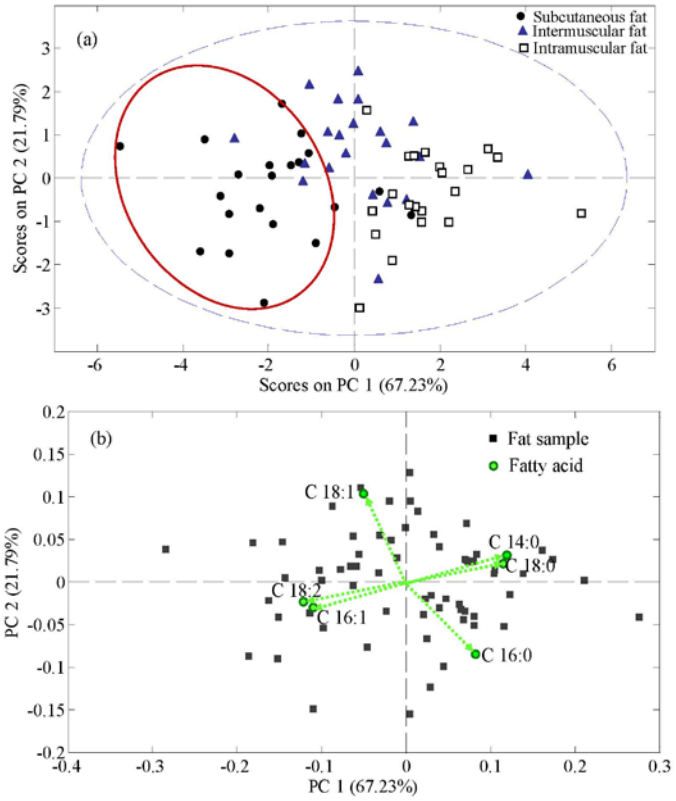

Figure 1. The results of PCA analysis: (a) Scores plot of first two PCs for the fatty acid composition of Japanese Wagyu beef fat; (b) Biplot for the fatty acid composition of Japanese Wagyu beef fat

Characterization of ATR-FTIR spectra of adipose tissue of Japanese Wagyu carcass

Strong absorptions of water (around 3300 and 1650 cm^{-1}) and protein (around 1550 cm^{-1}) were observed in IR spectrum of raw fat tissue (Figure 2), which were also observed in prior investigations (Koca *et al.* 2007, Yoshida *et al.* 2003). Furthermore, in the both spectra of raw fat tissue and extracted fat (Figure 2), there were major peaks representing triglyceride functional groups around 2925cm^{-1} (C-H asymmetric stretching), 2856 cm^{-1} (C-H symmetric stretching),1750 cm^{-1} (C=O stretching) as reported by Safar et al. (1994). The spectra also exhibited other fat-related peaks at 1465 cm^{-1} (C-H scissoring bending), and 1163 cm^{-1} (C-O stretching and C-H bending) (Christy et al., 2005; Chen et al., 1998; Guillén et al, 1997). In the second derivative spectra, the peak at around 3000 cm^{-1} corresponding to the stretching vibration of alkene was observed (Figure 3).

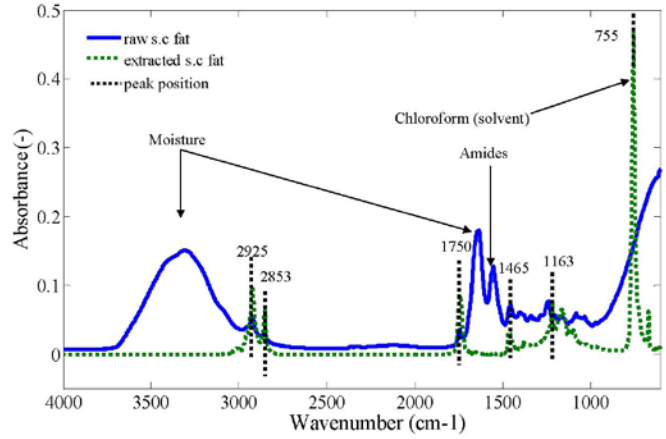

Figure 2. The IR spectra of extracted subcutaneous fat and subcutaneous fat tissue in the whole region (4000-600 cm^{-1})

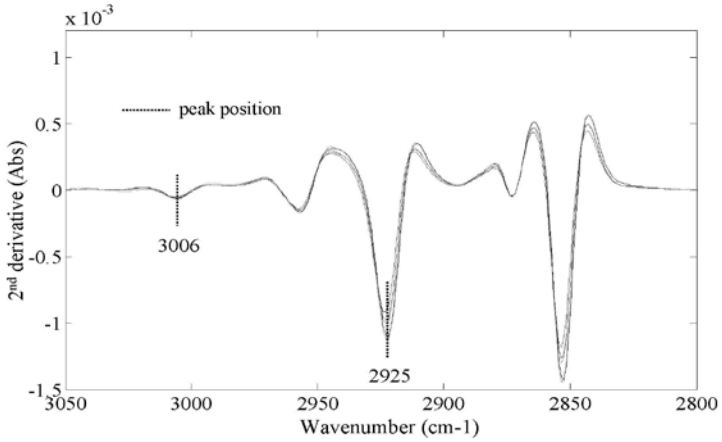

Figure 3 The second derivative spectra (11 points smoothed) of extracted inter-muscular fat in the alkene, methylene, and methyl bands areas between 2800 and 3050 cm^{-1}.

In this study, in order to investigate the relationship between ATR-FTIR spectra and fatty acid composition determined from GC analysis, Yoshida's method (Yoshida et al., 2003) was applied for beef fat. The averaged amounts of double bound ($\Delta-$avg), the averaged number of methylene (CH_2-avg) and the ratio of $\Delta-$avg to CH_2-avg, which is the percentage of the averaged number of double bond to methylene base by GC analysis, were calculated by the formulas of (5) and (6). Moreover, D-M factor from FTIR spectra is the product of the peak shift at around 3000 cm^{-1} and the ratio of the absorption intensities of alkene to methylene in the second derivative spectrum. Thus, this factor is correlated with the averaged unsaturation degree from ATR-FTIR spectra.

$$\Delta - avg = [1 \times (18:1 + 16:1)\% + 2 \times 18:2\%] \quad (5)$$

$$CH_2 - avg = [12 \times (18:2 + 16:1 + 14:0)\%] + [14 \times (18:1 + 16:0)\%] + 16 \times 18:0\% \quad (6)$$

$$Alkene\ \ shift = Alkene\ \ position - 3000 \quad (7)$$

$$D - M\ \ factor = Absorption\ \ ratio\ of\ (alkene\_2nd/methylene\_2nd) \times alkene\ shift \quad (8)$$

As illustrated in Figure 4, there was a linear positive correlation relationship(R^2=0.632) between the ratio of $\Delta-$avg to CH_2-avg and D−M factor. In the investigation of Yoshida et al. (2003), it was suggested that if the alkene shift from 3000 cm^{-1} was below 8, the major component of the oils was C18:1 and the oils contained a small amount of other polyunsaturated fatty acids [PUFA, (n-3 or n-6)]. In this study, the peak shift of beef fat was observed within 8 from 3000 cm^{-1}.This result may be caused by the major component in beef fat— C18:1. In Figure 4, the subcutaneous fat showed relatively higher D-M factor because of its higher unsaturated degree comparing inter/intra-muscular fat. This result implied that the unsaturated degree of fatty acids in beef fat can be qualitatively estimated from ATR-FTIR spectrum.

Evaluation of fatty acid composition by ATR-FTIR spectra with PLS In order to develop PLS models of oleic acid (C18:1), palmitic acid (C16:0), MUFA and SFA for solvent-extracted fat and raw fat, the spectral regions of 2800-3050 cm^{-1}, 1800-1500 cm^{-1},

1500-1000 cm^{-1} and the combinations of these regions were extracted to select the optimum region for PLS regression models.

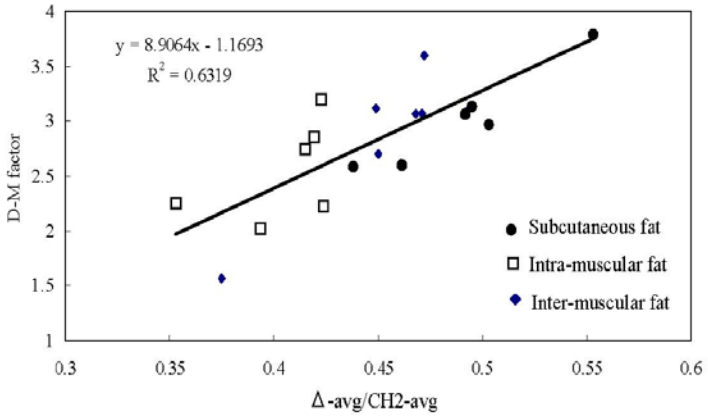

Figure 4 The relationship between the Δ-avg/CH_2 and D-M factor.

The optimized spectral region of PLS model of C18:1 for extracted fat is given in Table 2 (last row). It was suggested that the combination of 1500-1000, 1620-1800 and 3050-2800 cm^{-1} can provide the optimum PLS validation model for C 18:1 in extracted fat. In addition, by use of the combination of 1500-1000, 1620-1800 and 3050-2800 cm^{-1}, the optimum models of C16:0, MUFA and SFA for extracted fat were obtained, with coefficient determination (R^2) 0.905, 0.961 and 0.974 respectively. Whereas, for non-processed fat tissue, the best validation models for C18:1, C16:0, MUFA and SFA were established with coefficients of determination 0.470, 0.482, 0.611 and 0.647 respectively, when the combination of 1400-1000 and 3050-2800 cm^{-1} was adopted.

Table 2. The statistical results for PLS models of oleic acid (C18:1) for extracted fat determination developed by use of various spectral regions

Spectral region (cm^{-1})	PLS factors	R^2	RMSEC	RMSECV
4000-600	8	0.296	2.1502	2.8567
3050-2800	9	0.480	2.0384	2.4286
1800-1620	17	0.410	2.0867	2.6225
1500-1000	20	0.862	0.8767	1.2505
1500-1000, 1800-1620	19	0.871	0.8614	1.2095
1500-1000, 3050-2800	15	0.870	0.9594	1.2143
1800-1620, 3050-2800	20	0.688	1.2729	1.9104
1500-1000, 1620-1800, 3050-2800	20	0.890	0.8140	1.1125

As demonstrated in Figure 5 (a) and (b), the contents of C18:1 and C16:0 of extracted fat could be well estimated with high coefficient determination (R^2 larger than 0.890) and low error of the prediction (RMSECV lower than 1.1125). In contrast, the validation models for non-processed fat tissue [Fig. 5 (c) and (d)] were with worse validity: the coefficient determinations of C18:1 and C16:0 were 0.470 and 0.482, and RMSECV were 2.4234 and 2.0492, respectively.

Compared to the models for the solvent-extracted fat, the models for non-processed fat provided lower coefficients of determination (R^2) and higher predicted error [RMSEC

(V)], which was caused by inhomogeneous non-processed fat tissue, which contained some interference from water, protein and other organic matter.

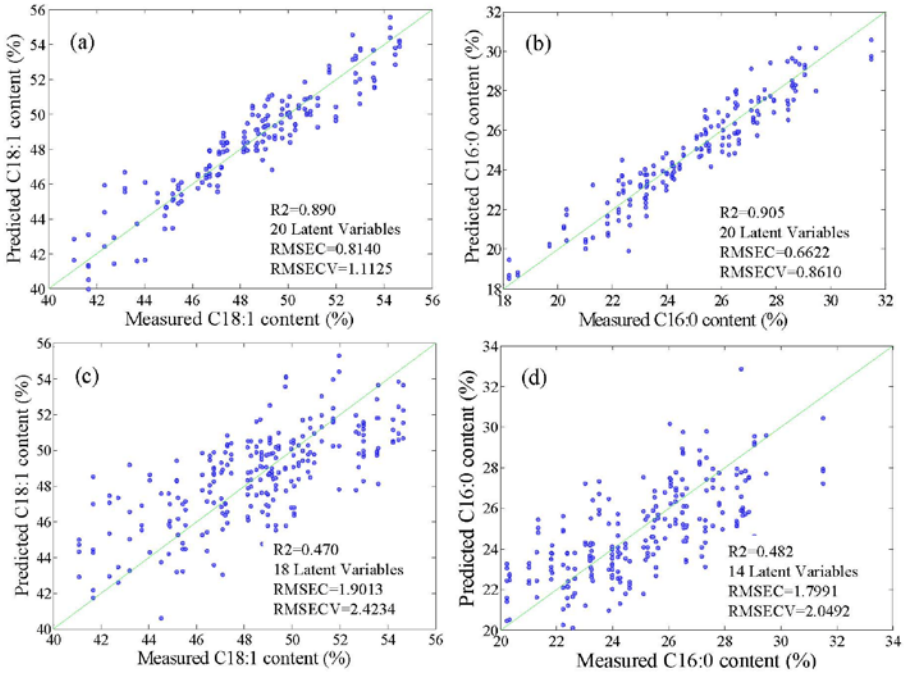

Figure 5. The results of PLS analysis with the optimum region: (a) and (b) are plots of predicted versus measured the contents of oleic acid (C18:1) and palmitic acid (C16:0) for extracted fat; (c) and (d) are plots of predicted versus measured the contents C18:1 and C16:0 for non-processed fat tissue.

CONCLUSION In this study, fatty acid composition of subcutaneous, inter-and intra-muscular fat of Japanese Black Wagyu beef were investigated by GC and IR spectra of non-processed fat tissue and solvent-extracted fat were compared and investigated. From the results of GC analysis, it was suggested that Wagyu beef could have higher percentages of oleic acid and MUFA and lower content of SFA than common American and Austrian beef, which implies Wagyu cattle could provide superior flavour beef. As a result of PCA, subcutaneous fat could be discriminated from intra- and inter-muscular fats but inter- and intra-muscular fats were indistinguishable. Moreover, a simple ATR-FTIR method could provide predictive models of the percentages of C 18:1, C16:0, MUFA and SFA of beef fat. For solvent-extracted fat, the optimum PLS models could be obtained by use of the combination of 1500-1000, 1800-1620 and 3050-2800 cm^{-1}. Whereas, with respect to non-processed fat tissue, when the combination of 1400-1000 and 3050-2800 cm^{-1} was used, the best validation models for C18:1, C16:0, MUFA and SFA can be established. In addition, the PLS validation models of C18:1, C16:0, MUFA and SFA contents for solvent-extracted fat showed higher correlations than those for fat tissue. Those coefficients (R^2) of determination were more than 0.890 for solvent-extracted fat and 0.470 for fat tissue. Further investigation is necessary to develop an in situ measurement method of fatty acid composition by ATR-FTIR to remove the interference by fat tissue heterogeneity.

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