Prediction of fatty acids composition in the rainbow trout Oncorhynchus mykiss by using Raman micro-spectroscopy

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Rainbow trout fed with three different diets
(Oncorhynchus mykiss)
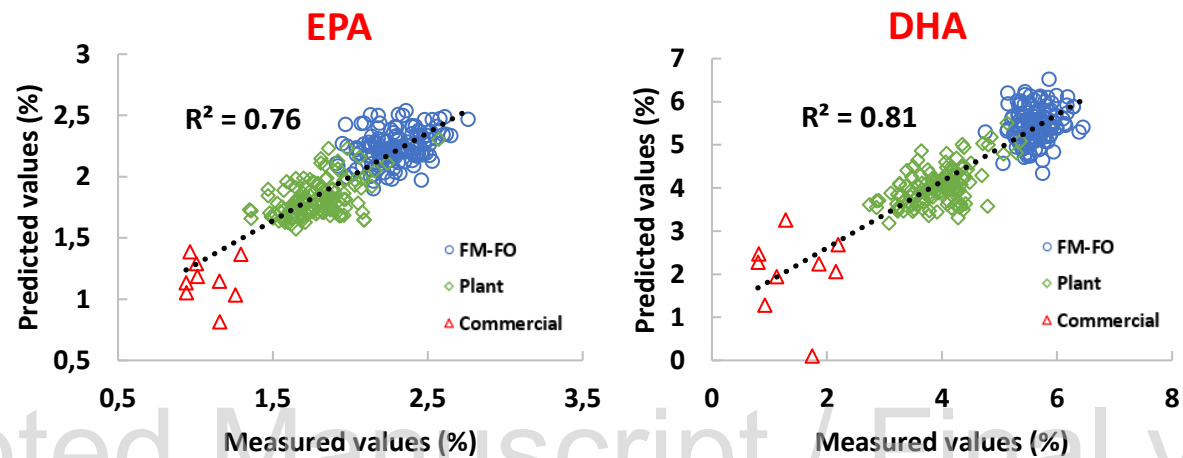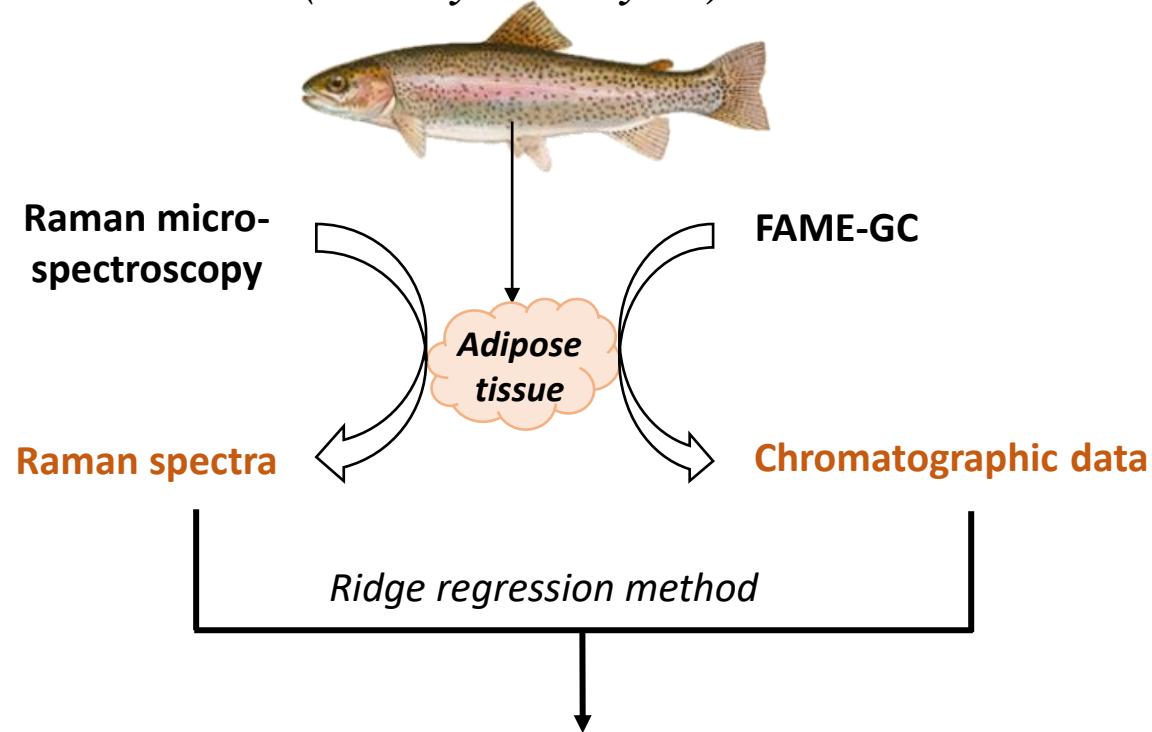
Raman micro-spectroscopy

FAME-GC

Adipose tissue

Raman spectra

Chromatographic data

Ridge regression method

EPA

$R^2 = 0.76$

Predicted values (%)

○ FM-FO
◇ Plant
△ Commercial

Measured values (%)

DHA

$R^2 = 0.81$

Predicted values (%)

○ FM-FO
◇ Plant
△ Commercial

Measured values (%)

Prediction of fatty acids composition in the rainbow trout

Oncorhynchus mykiss by using Raman micro-spectroscopy

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ABSTRACT

The importance of poly-unsaturated fatty acids (PUFAs) in food is crucial for the animal and human development and health. As a complementary strategy to nutrition approaches, genetic

27   selection has been suggested to improve fatty acids (FAs) composition in farmed fish. Gas

28   chromatography (GC) is used as a reference method for the quantification of FAs; nevertheless,

29   the high cost prevents large scale phenotyping as needed in breeding programs. Therefore, a

30   calibration by means of Raman scattering spectrometry has been established in order to predict

31   FA composition of visceral adipose tissue in rainbow trout *Onchorhynchus mykiss*. FA

32   composition was analyzed by both GC and Raman micro-spectrometry techniques on 268

33   individuals fed with three different feeds, which have different FA compositions. Among the

34   possible regression methods, the ridge regression method, was found to be efficient to establish

35   calibration models from the GC and spectral data. The best cross-validated R^2 values were

36   obtained for total PUFAs, omega-6 (Ω-6) and omega-3 (Ω-3) PUFA (0.79, 0.83 and 0.66,

37   respectively). For individual Ω-3 PUFAs, α-linolenic acid (ALA, C18:3), eicosapentaenoic acid

38   (EPA, C20:5) and docosahexenoic acid (DHA, C22:6) were found to have the best R^2 values

39   (0.82, 0.76 and 0.81, respectively). This study demonstrates that Raman spectroscopy could be

40   used to predict PUFAs with good correlation coefficients on adipocytes, for future on

41   adipocytes physiology or for large scale and high throughput phenotyping in rainbow trout.

42

43

INTRODUCTION

45   Nowadays, it is well known that fatty acids (FAs) play a major role as sources of metabolic

46   energy for growth and physiological functions in body. It explains the increased attention paid

47   in recent decades to the FA composition in human and animal nutrition. Among FAs, omega-3

48   (Ω-3) poly-unsaturated FAs (PUFAs), have a positive effect on the development and

49   maintenance of vital physiological processes such as brain function, immune and inflammatory

50   responses as well as a protective effect against cardiovascular diseases. Conversely, a higher

51   content of Ω -6 PUFA coupled with a deficit of Ω -3 promotes obesity or cardiovascular

52   diseases [1, 2]. The European Food Safety Authority has confirmed that nutritional intake of

53   Ω-3 PUFA (eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6))

54   have health benefits [2]. While saturated FAs (SFAs) and mono-unsaturated FAs (MUFAs) are

55   synthesized in the body, PUFAs of the Ω-6 and Ω-3 families, at least linoleic acid (LA – C18:2

56   – Ω-6) and α-linolenic (ALA – C18:3 – Ω-3) acid, must be provided by the diet. Indeed, LA

57   and ALA are Ω-6 and Ω-3 precursors, respectively, and the endogenous biosynthesis pathway

58   provides low yields of long chains PUFAs [3]. Thus, it is important that the nutritional intake

59   in humans of Ω-3 and Ω-6 with an adequate ratio be promoted by an appropriate diet.

60   Among the food products, fish represent the main source in terms of essential nutrients such as

61   PUFAs, particularly long chains PUFA Ω-3. In fish, their compositions and quantity are

62   influenced mostly by feed composition and feeding practices, environmental factors and genetic

63   determinisms [3, 4]. It is well known that the feed influence greatly the FA composition of fish

64   fillet, and therefore its nutritional value [3, 5]. Besides, it has also been proved that muscle FA

65   composition is partly controlled by genetic determinism [6]. To improve FA composition by

66   genetic selection it is necessary to estimate genetic parameters of FA composition in order to

67   optimize the design of breeding programs PUFAs bioconversion and/or retention abilities.

68   Among the most important farmed salmonid species reared worldwide, the production of

69   rainbow trout reaches 848 000 tons per year [7]. Its flesh is well known for its healthy

70   composition in PUFAs and for its lower lipid content than Atlantic salmon. If PUFAs

71   composition in farmed fishes is mostly determined by the feed composition, there is no

72   publication reported on estimation of genetic parameters of FAs composition in rainbow trout.

73   This estimation and/or investment in genetic selection will require the phenotyping, *i.e.* the

74   measurement of FA compositions on a large number of individuals and candidates. This is

75   necessary to rank candidates according to their estimated breeding value. This phenotyping will

76   require rapid affordable methods that can be applied to a large number of individuals.

77   Several conventional techniques with lipid extraction [8] and gas chromatography (GC) [9-

78   11] are usually used to perform FAs analyses. However, these techniques have several

79   disadvantages such as the use of solvents, the preparation time of samples before analysis and

80   their high cost, so, they are not applicable at a large scale. For, the development of breeding

81   programs to improve FAs composition, it is necessary to use more affordable technologies than

82   the FAME-GC as reference method.

83   Among different techniques, vibrational spectroscopic techniques like near-infrared (NIR) and

84   mid-infrared Fourier Transform Infrared (MIR FTIR) spectroscopy have been employed in

85   several studies to predict FA composition [12-15]. Another technique, the Raman spectroscopy,

86   is also a powerful analytical method applicable for differentiation and identification of

87   molecules of biological interest. Raman spectroscopy is also of significant interest because of

88   its low sensitivity to the water content of the samples, which affects much more severely other

89   vibrational spectroscopy techniques such as NIR and MIR FTIR spectroscopy. As an example,

90   concentrations of EPA, DHA and total Ω-3 FAs have been well predicted in fish oils, by using

91   infrared and Raman techniques [14]. Besides its ability to be used *in vivo*, Raman spectral bands

92   are thinner than those obtained by NIR spectroscopy, allowing more accurate band assignments

93   to molecular vibrational modes. To the best of our knowledge, no studies were realized yet on

94   the prediction of different FA classes and/or individual FAs in fishes using Raman

95   spectroscopy.

96   In this work, we present a calibration model using a statistical regression analysis by

97   combining Raman spectroscopy and GC data to allow the prediction of FAs in the visceral

98   adipose tissue of rainbow trout (*Oncorhynchus mykiss*). The objective of this study is to

99   determine the FAs composition of these adipose tissues of rainbow trout in order to evaluate

100   the efficiency of Raman spectroscopy as a routine FAs analysis method needed for breeding

101   programs.

102

MATERIALS & METHODS

1. Raman calibration sampling

The Raman calibration used 259 rainbow trout collected at the INRAE experimental facility of

PEIMA (Sizun, France). Trout from PEIMA belonged to the same batch and were fed with a

commercial feed until they reached an average weight of 600 g.

They were then splitted in two groups: one group (N=129) continue to be fed with a commercial

feed containing fish meal and fish oil (BioExtra F7, Le Gouessant), the second group (N=130)

was fed with a plant-based feed devoid of long chains PUFA Ω-3 (INRAE, Pisciculture de

Donzacq)) during 4 months. These two feeds differed in their FA composition and they were

used to induce changes in the fatty acid profiles of fish tissues. Fish were euthanized and

visceral fat samples were collected from the same "front" lobe of visceral adipose tissues,

placed in a coded aluminium foil (10 g) or in cryotubes (2 g), for GC or Raman measurements,

respectively, and were preserved in liquid nitrogen until further analyses. Nine additional

samples from rainbow trout, reared under commercial conditions (Aqualande group, Viviers de

la Hountine, Belin Béliet, France) and fed with a commercial feed (Viva Pro 7F, Aqualia), were

also analysed.

2. Gas chromatography (GC) analysis– reference analytical method

The fatty acid composition was determined for each calibration sample after lipid extraction by

cold biphasic method, methylation of FAs and analysis of the fatty acid methyl esters by GC

reference method (Eurofins Analytics, Nantes, France). The FAs proportion is defined by the

relative concentrations of the different FAs present in sample and generally compared to each

other by considering the major families of SFAs, MUFAs and PUFAs. The proportion of these

latter, as well as Ω-3 and Ω-6 FAs (total and individual), are calculated. The detection limit is

126   estimated at 0.05 % of the total fatty acids and the measurement uncertainty (MU) is calculated

127   from the following equation, provided by EuroFins:

128    $$MU = 0.25 + \sqrt{(0.1 * \%FA)}$$

129   where %FA correspond to the calculated proportion of FAs expressed in percent of total FAs.

130   Major FA group correspond to SFAs, MUFAs, PUFAs, total Omega-3 and total Omega-6.

131   SFAs were the sum of C14:0, C15:0, C16:0, C17:0, C18:0 and C20:0. MUFAs were the sum

132   of C16:1, C18:1, C20:1 and C22:1. PUFAs were the sum of C16:2, C16:3, C16:4, C18:2, C18:3,

133   C20:2, C20:3, C20:4, C18:3, C18:4, C20:3, C20:4, C20:5 and C22:6. Total Ω-6 FAs were the

134   sum of 18:2, 18:3, 20:2, 20:3 and 20:4. Total Ω-3 FAs were the sum of 18:3, 18:4, 20:5, 22:5

135   and 22:6.

136   **3. Raman instrumentation and spectra acquisition parameters**

137   A small piece of frozen sample (50-100 mg) was placed in a glass cup, cut rapidly with a scalpel

138   then rise at room temperature before placing under the microscope. The visceral adipose tissue

139   was selected as the first approach of our studies because adipocyte cells can be directly targeted

140   by the laser beam through a microscope objective in order to determine the FA composition.

141   The adipocyte cells were also studied to avoid bias, interference from unknown sources or lower

142   precision potentially associated with the cellular organization of the muscle in the fillet (mainly

143   connective tissues, muscle fibres or blood vessels).

144   Raman spectra were collected with a micro-spectrometer LabRAM HR800 (Horiba Scientific),

145   with a selected 600 g/mm grating suitable for the spectral resolution required for this study and

146   a charge-coupled device (CCD) detector cooled at -75°C. The Raman system was equipped

147   with three laser sources: 532 nm, 633 nm and 785 nm. In a first approach, although the

148   excitation laser at 785 nm should show less fluorescence for biological samples, all wavelengths

149   were tested and two spectral ranges were acquired through a 10x objective: 550 to 1800 cm^{-1}

150   and 2610 to 3100 cm^{-1} with two accumulations with acquisition times of 20 s and 30 s and,

151   respectively.

152   **4. Raman spectral treatment and statistical analysis by ridge regression.**

153   The first preliminary step consists to average replica spectra of reference samples. Then, a

154   baseline fitted by rubberband method to each spectrum was subtracted of all spectra to remove

155   the contribution of fluorescence and background noise from the spectra. The standard normal

156   variate transformation, which is basically a signal intensity normalisation, was applied on the

157   baseline-subtracted Raman spectra to eliminate variations in the general intensity.

158   Pre-processed Raman spectra covering two different frequency regions (550-1800 cm^{-1} and

159   2610-3100 cm^{-1}) were used to develop multivariate linear regression models based on partial

160   least squares (PLS) [16] and *ridge* regression methods [17]. Briefly, PLS constructs a set of

161   linear combinations of the inputs for regression, while the ridge regression also allows to apply

162   penalties on the least important features, *i.e.*, the ones that contribute little to the final result.

163   All treatments are realized with R using packages for the baseline [18], the PLS [16] and the

164   ridge regression method (*glmnet* package) [19]. Both methods are especially designed to

165   estimate linear prediction scores when the number of predicting variables, here the Raman

166   spectrum values at each wavenumber, exceeds the training sample size. The ridge method

167   introduces a shrinkage parameter whose optimization aims at finding the best compromise

168   between prediction bias and variance. The R package glmnet implements such an optimization

169   method.

170   A 10-fold cross-validation procedure was used to compare the prediction performance of each

171   model by the squared correlation between predicted and observed responses (cross-validated

172   R²) and the root mean square error of prediction (RMSEP).

173   Regarding their prediction performance, ridge regression showed the best results and was

174   finally selected to develop the calibration model.

175

RESULTS AND DISCUSSION

1. Excitation wavelength selection and spectral assignment of vibrational bands

A wide range of factors influences the intensity of Raman spectra of biological samples including instrumental parameter as laser intensity, wavelength, and acquisition time. In this study, three wavelengths were tested (532nm, 633nm and 785nm) on the sample in order to select the most suitable for measuring lipids in adipose tissues of rainbow trout and to obtain robust multivariate calibration models. Figure 1 shows the representative spectra of visceral adipose tissue acquired with the three different wavelengths. In order to be comparable, the spectra were just normalized by the acquisition time. The most characteristic features of Raman spectra of lipids related to hydrocarbon chain were observed in the three spectra, notably C=C bonds or CH_2 group vibration at $\sim 1650\,cm^{-1}$ and $\sim 1450\,cm^{-1}$, respectively [20, 21]. This strong lipid fingerprint seems coherent with the Raman spectrum of a biological tissue containing a large amount of lipids and associated molecules (FAs, triglycerides, etc) as adipose tissues. As expected, this fluorescence contribution decreased when the 633 and 785 nm excitation wavelengths were used. Nevertheless, the Raman intensity of high wavenumber vibrations was dramatically reduced at 785 nm. Indeed, when considering the near-infrared 785 nm excitation, CCD efficiency radically decreased for such high wavenumbers. Considering the above-mentioned effects, the 785 nm wavelength was selected for the present study. Using this wavelength will therefore allow to acquire informative spectral information from visceral adipose tissues of rainbow trout.

The stability and homogeneity of the samples were tested by recording the Raman spectrum at the same position at regular time intervals (of about 5') for 30' (physical replicas) or by measuring the Raman spectrum at different positions in the sample (biological replicas). No significant signal evolution was observed during the 30' acquisition of the physical replicas. In

200   the case of the biological replicas, the visceral fat front lobe was separated into five samples,

201   all measured independently. The similarity of the spectra obtained confirmed the high

202   homogeneity of the visceral tissue. As in the case of physical replicas, the variations in intensity

203   observed between 600 and 1200 cm^{-1} can be explained by a change in background noise that

204   may be due to a variation in the biological environment such as presence of traces of blood

205   and/or pieces of cell membranes.

206   The numbers in Figure 2 correspond to the Raman spectral band assignments provided in

207   Table 1 based on literature [21]. Among the major and characteristic spectral vibrations, the

208   bands 1 and 2, at 601 and 727 cm^{-1}, respectively, could be attributed to particular vibration

209   mode of phospholipids and trimethylamine, respectively, thus they are characteristic of lipidic

210   cellular membrane. The band 16 at 1748 cm^{-1} corresponded to (C=O) stretching vibration of

211   ester functional groups from lipids and FAs and the band 21 at 3013 cm^{-1} to (=C-H) stretching

212   of unsaturated fatty acids. Note that the assignment of band 15 at ~1650 cm^{-1} corresponded to

213   (C=C) stretching mode of unsaturation in *cis* conformation, *trans* conformation induced a band

214   shift at 1670 cm^{-1}. In the present study, no significant signal was observed at 1670 cm^{-1}, showing

215   a clear majority of the *cis* conformation of fatty acid unsaturation in visceral adipose tissue of

216   rainbow trout.

217   **2. Fatty acid composition of rainbow trout feeds**

218   FAME-GC measurements were realized to determine the FA composition of the different

219   feeds used in this study: the feed containing fish meal and fish oil (FM-FO feed) and the plant-

220   based feed fed by the trout from PEIMA, and the commercial feed fed by the trout from

221   Aqualande (Table 2). The predominant FAs in all fish feeds were C16:0, C18:1, LA (18:2 Ω-

222   6), ALA (C18:3 Ω-3), EPA (C20:5 Ω-3) and DHA (C22:6 Ω-3) fatty acids. Feeds contained

223   different proportions of SFAs: 27.31%, 21.71%, and 13.83%, for FM-FO, plant, and

224   commercial feeds, respectively. Differences between feeds were observed in the proportions of

225   MUFAs: 31.46%, 40.26%, and 56.24%, for FM-FO, plant, commercial feeds, respectively.

226   These differences were mainly due to 18:1.

227   The proportion of Ω-6 fatty acids in the FM-FO feed was 28.77% and this value was higher

228   than in the plant feed (19.25%), and commercial feed (19.33%). Differences were found

229   between all three feeds in the content of Ω-3 fatty acids. The proportion of Ω-3 fatty acids was

230   highest in the plant-based (18.73%) which contain only ALA, followed by 10.24%, in the FM-

231   FO feed, and 9.82% in the commercial feed. The plant-based feed was totally devoid of EPA

232   and DHA, whereas the proportions of EPA in the feeds were 2.21% (FM-FO feed) and 0.95%

233   (commercial feed), and those of DHA were 1.99% (FM-FO feed), and 1.15% (commercial

234   feed). These differences in FA composition reflected the nature of the ingredients incorporated

235   in the feeds, in particular the proportions of FM-FO, the main sources of EPA and DHA, versus

236   the proportions of vegetable ingredients, rich in 18:1, LA and ALA. It is now well known that

237   the FA composition of fish tissues reflected in a large manner that of the feeds [22-24]. So, we

238   expected in this study to have different FA profiles in perivisceral adipose tissue, the main lipid

239   storage site in rainbow trout, that allow to predict them by Raman spectroscopy after calibration.

240   3. **Prediction of fatty acid composition from visceral adipose tissue of rainbow trout**

241   Table 3 presented the data determined by FAME-GC analysis on the FA composition of

242   adipose tissue of the three groups of rainbow trout fed with the different feeds (FM-FO, plant-

243   based, commercial). Adipose tissue of fish fed with the commercial feed contains more SFAs

244   and MUFAs compared to the other two groups (FM-FO and plant-based feeds). The most

245   important SFAs and MUFAs in adipose tissue were the FAs 16:0 and 18:1 in accordance with

246   the proportions of these FAs in the feeds. These data are consistent with previously published

247   data on FA composition of adipose tissue in rainbow trout [24, 25].

248   LA is the main PUFA Ω-6 present in adipose tissue, reflecting the abundance of this FA in

249   the three feeds. However, the proportion of LA in adipose tissue of trout fed with the FM-FO

250   feed was 20.86%, which is lower than the proportion directly found in the feed (28.57%). This

251   seems to indicate that LA is more metabolized in trout fed with the FM-FO feeds, confirming

252   previous observations that LA is readily oxidized when present at high concentrations [3, 23].

253   For Ω-3 FAs, the higher proportion of ALA (6.29%) was found in fish fed with plant-based

254   feed, which is the richest in ALA (18.73%) whereas the fish fed with the FM-FO feeds had the

255   highest proportions of EPA and DHA compared to the other two groups. It is interesting to note

256   that DHA is present at 3.94% in visceral fat of trout fed with the plant-based feed, despite the

257   absence of this FA in the feeds. As these fish were previously fed with a commercial feed

258   containing FM and FO, this may be due to a selective retention of DHA and/or a biosynthesis

259   from the precursor ALA [3, 23, 26]. Overall, these data confirm that dietary FA composition is

260   minored in the fish's tissues; even the difference in the percentage of fatty acids between feeds

261   is greater than the difference observed in tissues of fish fed these feeds [27-29]. Besides dietary

262   effects, the incorporation of fatty acids into fish tissue is under various metabolic influences,

263   such as preferential incorporation, β-oxidation, lipogenic activity or fatty acid elongation and

264   desaturation processes [3, 23]. These metabolic influences can explain the individual variability

265   observed in FA composition and highlight the interest of a method for predicting FA

266   composition for breeding programs.

267   The coefficient of variation (CV) was also calculated for each FAs of interest, as

268   displayed in Table 3. It can be observed that the CVs calculated for the SFA in rainbow trout

269   are 0.82, 3.63, and 5.51%, for the FM-FO, plant- and commercial-based feeds, respectively.

270   Thus, the dispersion is very important for visceral adipose tissues from rainbow trout fed with

271   the commercial-based feed, compared to those from fed with FM-FO feed. This difference in

272   the distribution of values is clearly illustrated by the Figure 3.A, and could be explained by the

273   individual variability due to various metabolic influences, as mentioned above. The same

274   observation can be made for PUFAs: 0.55, 2.92, and 5.20%, for the FM-FO, plant- and

275   commercial-based feeds, respectively (Figure 3.C); and total Ω-3: 0.98, 5.05, and 11.27%, for

276   the FM-FO, plant- and commercial-based feeds, respectively (Figure 3.D). For individual Ω-3

277   FAs, the CVs were calculated for ALA: 4.51, 9.38, and 4.47%, for the FM-FO, plant- and

278   commercial-based feeds, respectively (Figure 4.A); for EPA: 3.49, 10.67, and 12.96%, for the

279   FM-FO, plant- and commercial-based feeds, respectively (Figure 4.B); and for DHA: 2.84,

280   12.43, and 39.16%, for the FM-FO, plant- and commercial-based feeds, respectively (Figure

281   4.D). Surprisingly, an important dispersion is observed for DHA from rainbow trout fed with

282   commercial-based feed. It could be hypothesized that all individuals could not metabolize in

283   an equal manner commercial feed in order to obtain long-chain PUFAs Ω-3, such as DHA,

284   explaining thus the lower composition compared to those obtained with FM-FO and plant-based

285   feeds. To add more weight to this hypothesis, the CVs were calculated for individual Ω-6 FAs.

286   It was obtained for LA: 1.87, 3.17, and 4.17%, for the FM-FO, plant- and commercial-based

287   feeds, respectively (Figure 5.A); for ARA: 16.28, 13.89, and 23.53%, for the FM-FO, plant-

288   and commercial-based feeds, respectively (Figure 5.C). It can be observed a more important

289   dispersion for C20:4 from rainbow trout fed with commercial-based feed. Thus, the

290   metabolization of the commercial feed by some individuals could be less effective for obtaining

291   long-chain PUFAs Ω-6, compared to other feeds.

292   The whole set of data used for calibration with mean values and standard deviations

293   regarding major FAs group and 18 individual FAs are given in Table 4. A large variability was

294   present in major FAs groups as demonstrated by the broad ranges and relative standard

295   deviation observed. This variability was expected since the fish were fed with feeds differing

296   in their FA composition. As shown in Table 4, the most abundant FAs identified are: 16:0, 18:1

297   and 18:2 Ω-6 (LA: linoleic acid) with an abundance upper 10%; and 18:3 Ω-3 (ALA: alpha-

298   linolenic acid), 20:1, 20:5 Ω-3 (EPA), and 22:6 Ω-3 (DHA) with an abundance between 1 to 10

299   %. FAs, which represented less than 1% of the total FA content, would not have signals

300   identifiable by Raman spectroscopy [30]. In our study, the FAs such as 15:0, 17:0, 20:0, 18:3

301   Ω-6, 20:2 Ω-6, 20:3 Ω-6, 20:4 Ω-6, 18:4 Ω-3 and 22:5 Ω-3 are concerned due to their low

302   abundance in visceral fat.

303   The first reason to choose visceral adipose tissue is that this tissue is the only available in

304   sufficient quantity in order to perform GC analyses on individual fish. This choice was also

305   planned to simplify interpretation of Raman spectra as visceral adipose tissue is mostly

306   composed of adipocytes to avoid contamination with, for example, the presence of muscle

307   proteins or blood in the flesh as in the muscle. The visceral adipose tissue presents the

308   advantages to be homogeneous and to not contain any or very few fluorescent molecules,

309   compared to the muscle tissue. Raman spectroscopy being a technique sensitive to the

310   fluorescence, masking usually the Raman signal. Raman measurements on a minced standard

311   portion of the muscle (known as Norwegian Quality Cut) as done by Difford et al., 2021 to

312   estimate total lipid content, implies in practice an additional step of mincing that was not

313   preferred for future applications as in breeding programs [31]. The Raman characterisation of

314   fat in the adipocytes from the visceral adipose tissue was also preferred because viscera seem

315   to be preferentially a lipid deposition site according to other studies [32, 33]. Hixxon et al.

316   showed that the lipid composition of the visceral adipose tissue most reflected that of the feed.

317   Indeed, most of the stored lipid came from the accumulation of lipids from the feed of rainbow

318   trout [34]. However recent advances have also reported that visceral or subcutaneous adipocytes

319   in Atlantic salmon may have different physiological functions (energy stocking and

320   immunology vs energy metabolism) and potentially different FA compositions. Thus, in tissues

321   with limited amounts of adipocytes (*e.g.* subcutaneous, dorsal fat or muscle myosepta), our

322   study may have a particular interest in the prediction of individual FAs [35].

323   Summary statistics for each major FA group and for each individual FA are displayed in

324   Table 4. Prediction performance using ridge regression is assessed by cross-validated R^2 and

325   RMSEP. Also, correlation plots for predicted values *vs* measured values on visceral fat are

326   shown in Figures 3, 4, and 5, in order to illustrate the prediction performance of each trait. SFA

327   group showed a weaker prediction performance (R^2=0.42). Yet, one of the most abundant SFAs,

328   16:0 (12.73%), presented a high R^2 value of 0.71. Although this FA represented about 66% of

329   the total SFAs, its good prediction performance is not enough to influence the overall prediction

330   of the total SFAs. Other less abundant SFAs, 14:0 and 18:0, represented about 10.6% and 17.3%

331   of the total SFAs, respectively. While 14:0 showed a good R^2 value of 0.70, the 18:0 displayed

332   a poor R^2 value of 0.44. It can be hypothesized that 18:0 could be mostly responsible to the

333   decrease of the prediction performance of the total SFAs. However, Behre et al., studying fatty

334   acids prediction in pork adipose tissues, obtained better R^2 results for the same SFAs: 14 :0

335   (1.1%), 16 :0 (22.4%) and 18 :0 (12.9%), for R^2 values of 0.67, 0.89 and 0.72, respectively [36].

336   Other studies suggested that vibrations of SFAs, depending on the polymorphic form of these

337   latter, could be split over multiple spectral regions [37, 38]. This could also explain the poor

338   prediction performance for SFAs. Good prediction performance was observed for MUFAs

339   (R^2=0.75) and PUFAs (R^2=0.79). Indeed, the more carbon double bonds will be present in the

340   FA chains of MUFAs and PUFAs, the more the Raman signal of these spectral vibrations, such

341   as at 1267 cm^{-1} (=C-H deformation) and 1658 cm^{-1} (C=C stretching) will increase. Thus, having

342   a high prediction performance for the MUFAs and PUFAs concentrations could indicate that it

343   would be an increase of the spectral vibrations' intensities within the Raman spectra [39].

344   Within the PUFAs, the total Ω-3 and total Ω-6 groups were distinguished and R^2 were obtained.

345   The total Ω-6 FA group shows a R^2 of 0.83, whereas the performance of total Ω-3 FA group

346   was weaker (R^2=0.66). R^2 were also determined for individual Ω-3 and Ω-6 FA. Poor to

347   intermediate prediction performances were obtained for 20:3, 20:2, and 18:3 : R^2 values were

348   0.02, 0.04, and 0.46, respectively. These Ω-6 FAs could explain their low R^2 by their mean

349   weight of the percentage being < 1%. Thus, considering limited predicting individual Ω-6 FAs

350   to predict total Ω-6 could explain the decrease of the overall prediction performance of this FA

351   group. The same hypothesis could be verified with the individual Ω-3 FAs. R^2 values of 18:4

352   and 22:5 were 0.04 and 0.44, respectively. These two Ω-3 FAs have a poor to intermediate

353   prediction performance certainly due to their mean weight of the percentage being < 1%, and

354   would not have signals identifiable by Raman spectroscopy [30]. Thus, taking into account

355   these two individual FAs could explain the decrease of the overall prediction performance of

356   the Ω-3 FA group. More precisely, the impact of the co-variance of individual FAs on the R^2

357   values of FA groups may be hypothesized. A recent study investigates the prediction of

358   individual FAs and total FA depending on variation of the iodine value in pork backfat, which

359   were fed with different dietary fat sources and levels. Good correlations were obtained ($R^2 =$

360   0.78–0.90) [36]. However, they report that the individual FAs predictions are indirect and

361   strongly depend on co-variance with the relative FAs composition. Based on this observation,

362   our results seem to show that low R^2 values of individual FAs could influence the prediction

363   performance of FA groups.

364   It is noted that moderate to very good R^2 values were found for the Ω-6 LA and arachidonic

365   acid, and the Ω-3 ALA, EPA, DHA: 0.84, 0.61, 0.82, 0.76, and 0.81, respectively. These FAs

366   displaying great prediction performance are usually well predicted in other studies [14, 18]. For

367   instance, high correlation coefficients (0.85-0.97) were previously obtained in commercial Ω-

368   3 PUFA oil supplements capsules enriched in FAs with much higher EPA (24.1 %-27.7%) and

369   DHA (18.6%-20.6%) concentrations [18]. Another study showed very good correlation values

370   of 0.97 and 0.9, for EPA and DHA respectively [14]. The much lower concentration in EPA

371   and DHA in visceral fat compared to concentrated fish oil may explain the lower prediction

372   performance obtained in this study (0.76, and 0.81). However, our correlation coefficients are

373   sufficiently high to enable high throughput estimation of FAs compositions in adipocyte cells.

374   As handheld Raman probes have already been used to estimate lipid composition of fish [40,

375 41] and fish oils [42], it will be interesting to associate this nondestructive technology with

376 minimally invasive biopsy in order to phenotype live or dead fishes for diverse aquaculture or

377 conservation applications (nutrition, reproduction, genetic improvement).

378

379 **CONCLUSION**

380 Our study was based on calibration of FA composition and prediction in visceral adipose

381 tissue as a first step of investigation and development for application of Raman spectroscopy in

382 fish and aquaculture breeding. The effective of individuals considered in the calibration will

383 gain to be extended for more broad and accurate predictions taking into account different FA

384 compositions, fish size and genotypes. The adipocyte cell was easily targeted by the laser ray

385 with our Raman apparatus equipped with a microscope. Transfer of the results to other Raman

386 device as portable device will need caution before extensive application. Our results also open

387 the way for fine surveys of adipocyte cell composition depending on tissues, biological factors

388 (age, sex, genotypes, polyploidy, species) or zootechnical practices (feeding strategy, feed

389 composition, rearing temperature, fish density) in decreasing potentially cost and time of

390 measurements. It also opens the way for *in vivo* measurement and repeated measurements based

391 on tissue coring on live animals in respecting welfare international recommendations and

392 regulations. Transfer of the results to other kind of tissues or aquaculture species may need

393 careful validation. Prediction performances are variable according to the FAs and/or groups or

394 sums or ratios investigated. If for quality control procedures, high performances of prediction

395 are required, the benefit/cost ratio needs estimation for the different predicted FAs. Thus, this

396 methodology shows that moderate to good correlation coefficients can be obtained to predict

397 PUFAs, and calibration models can be used to predict PUFAs contents for large scale and high

398 throughput phenotyping.

399

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ETHICS STATEMENT

This study was conducted in accordance with EU Directive 2010-63-EU on the protection of animals used for scientific purposes. The fish farmed at the INRAE experimental station of PEIMA (UE 0937) agreed under N° C29 -277 -02 were reared according to normal husbandry practices, and were not subjected to practices likely to cause pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice. As such, the experiment did not require approval by an Ethics Committee, in accordance with Article 2.5 of the EU Directive 2010-63-EU.

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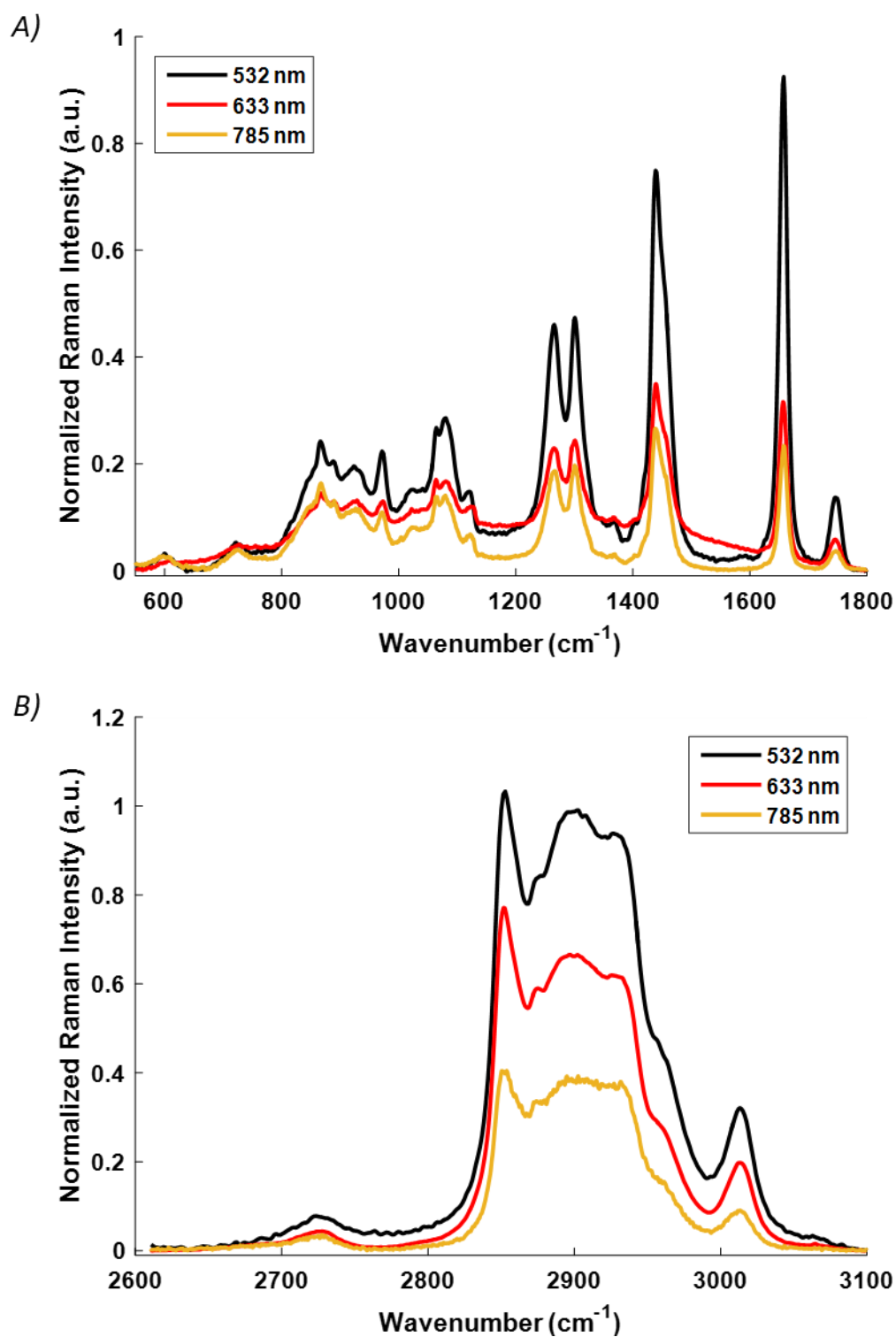539

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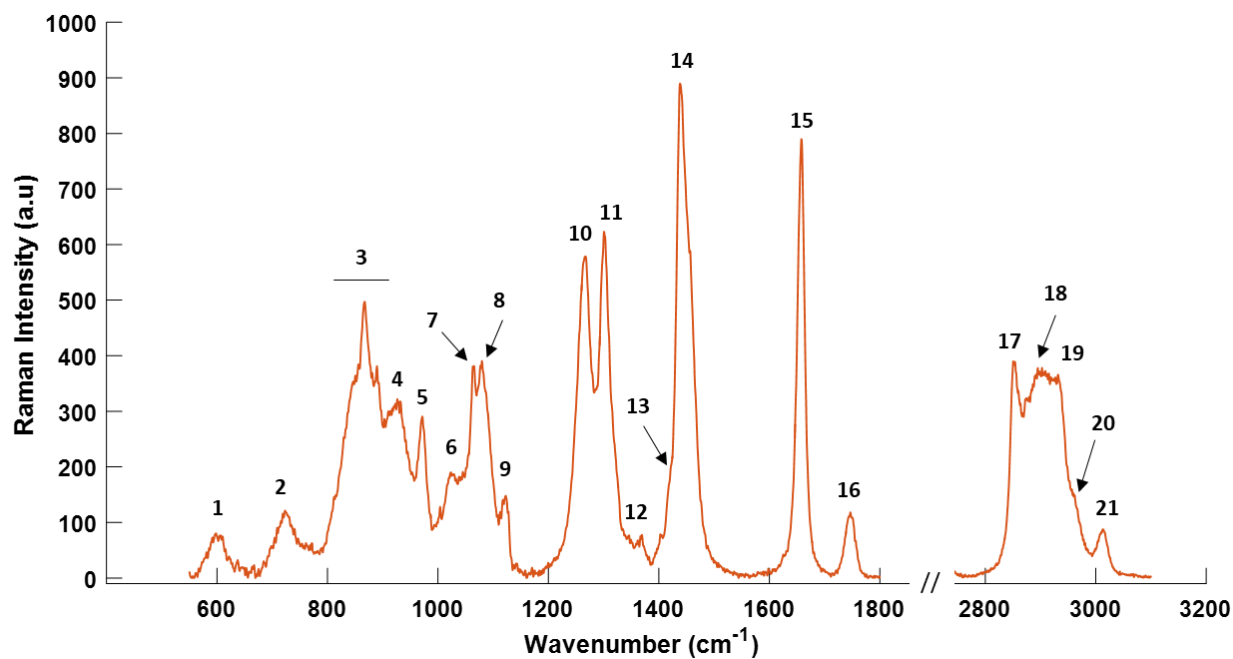545

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555 **Fig. 1. Raman spectra of visceral adipose tissue from rainbow trout, acquired at**

556 **different wavelengths: 532, 633 and 785 nm. (A) 550-1800 cm^{-1}, (B) 2600-3100 cm^{-1}**

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560 **Fig. 2. Raman spectrum of visceral adipose tissue acquired at 785 nm.**

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575 **Table 1. Assignment of bands in the Raman spectra**

Number	Raman shift (cm^{-1})	Assignment
1	601	δ(C=O-C)
2	727	ν_s(N$^+$(CH$_3$)$_3$)
3	845-889	ν(C-C)+ ν(C-O-C)
4	925	ν(CH)
5	972	ν(CH)
6	1025	ν(C-C)
7	1065	ν(C-C)
8	1080	ν(C-C)+ ν((PO^{4-})
9	1121	ν(C-C)
10	1267	δ(=C-H)
11	1303	δ_{as}(CH$_2$)
12	1367	δ_{as}(CH$_2$)
13	1420	δ_s(CH$_2$)
14	1438	δ(CH$_2$/CH$_3$)
15	1658	ν(C=C)
16	1748	ν(C=O)
17	2853	ν_s(=CH$_2$)
18	2895	ν_{as}(=CH$_2$)
19	2931	ν_s(=CH$_3$)
20	2960	ν_{as}(=CH$_3$)
21	3013	ν(=C-H)

ν. stretching; δ. bending; s. symmetric; as. asymmetric

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577   **Table 2. Fatty acid (FA) composition of the fish meal and fish oil- (FM-FO),**

578   **plant- and commercial-based feeds (% total FA) by FAME-GC analysis.**

	FM-FO feed	Plant-based feed	Commercial feed
12:0	0	0.90	0
14:0	4.96	0.73	1.49
15:0	0.40	0	0.18
16:0	18.22	17.65	9.75
17:0	0.27	0	0.16
18:0	3.30	2.27	1.97
20:0	0.15	0.16	0.29
SFA	27.31	21.71	13.83
16:1	4.18	0.25	1.81
18:1	24.35	39.65	53.02
20:1	1.67	0.36	1.08
22:1	1.10	0	0.22
MUFA	31.46	40.26	56.24
18:2 (LA)	28.57	19.25	19.21
20:4 (ARA)	0.20	0	0.12
PUFA Ω-6	28.77	19.25	19.33
18:3 (ALA)	4.90	18.73	7.41
18:4	1.14	0	0.31
20:5 (EPA)	2.21	0	0.95
22:6 (DHA)	1.99	0	1.15
PUFA Ω-3	10.24	18.73	9.82
PUFA	39.01	37.98	29.15

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581   **Table 3. Fatty acid (FA) composition of the FA from visceral adipose tissues in rainbow**

582   **trout, fed with FM-FO- (N=129), plant- (N=130) or commercial-based (N=9) feeds (%**

583   **total FA) by FAME-GC analysis. The coefficient of variation (CV) was calculated (CV =**

584   **(standard deviation/mean) * 100), and put into brackets.**

	FM-FO feed	**Plant-based feed**	**Commercial feed**
12:0	0.06 ± 0.01	0.11 ± 0.02	-
14:0	2.32 ± 0.05	1.67 ± 0.13	3.17 ± 0.33
15:0	0.23 ± 0.01	0.17 ± 0.02	-
16:0	12.60 ± 0.11	12.48 ± 0.48	18.08 ± 1.06
17:0	0.29 ± 0.01	0.23 ± 0.02	-
18:0	3.36 ± 0.01	3.40 ± 0.24	2.32 ± 0.22
20:0	0.23 ± 0.02	0.24 ± 0.02	-
SFA	19.49 ± 0.16 (0.82)	18.71 ± 0.68 (3.63)	24.15 ± 1.33 (5.51)
16:1	3.13 ± 0.13	2.57 ± 0.29	4.27 ± 0.39
18:1	31.66 ± 0.33	36.81 ± 1.04	44.32 ± 1.01
20:1	2.74 ± 0.06	2.08 ± 0.17	1.20 ± 0.31
22:1	2.94 ± 0.06	2.13 ± 0.40	-
MUFA	43.01 ± 0.44 (1.02)	45.72 ± 0.90 (1.97)	49.89 ± 1.05 (2.10)
18:2 (LA)	20.86 ± 0.39 (1.87)	18.90 ± 0.60 (3.17)	15.82 ± 0.66 (4.17)
20:4 (ARA)	0.43 ± 0.07 (16.28)	0.36 ± 0.05 (13.89)	0.17 ± 0.04 (23.53)
PUFA Ω-6	21.76 ± 0.24 (1.10)	19.93 ± 0.62 (3.11)	17.06 ± 0.69 (4.04)
18:3 (ALA)	3.77 ± 0.17 (4.51)	6.29 ± 0.59 (9.38)	4.03 ± 0.18 (4.47)
18:4	0.55 ± 0.08	0.55 ± 0.19	-
20:5 (EPA)	2.29 ± 0.08 (3.49)	1.78 ± 0.19 (10.67)	1.08 ± 0.14 (12.96)
22:6 (DHA)	5.63 ± 0.16 (2.84)	3.94 ± 0.49 (12.43)	1.43 ± 0.56 (39.16)
PUFA Ω-3	13.19 ± 0.13 (0.98)	13.25 ± 0.67 (5.05)	7.54 ± 0.85 (11.27)
PUFA	36.57 ± 0.20 (0.55)	34.58 ± 1.01 (2.92)	24.60 ± 1.28 (5.20)

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586   **Table 4. Summary statistics of the FA from visceral adipose tissues in rainbow trout**

587   **(N=268), by FAME-GC analysis.**

Fatty acid (FA)	μ[a] (± σ[b])	CV[c]	Range (min. - max.)	R²[d]	RMSEP[d]
Major FA group					
SFA	19.27 ± 1.22	6.31	16.94 – 26.23	0.42	0.95
MUFA	44.55 ± 1.91	4.29	40.79 – 51.02	0.75	1.04
PUFA	35.23 ± 2.32	6.60	22.82 – 38.98	0.79	1.17
Total Ω-3	13.03 ± 1.19	9.19	6.26 – 15.22	0.66	0.79
Total Ω-6	20.72 ± 1.33	6.42	15.71 – 24.18	0.83	0.56
Individual Ω-9 FA					
18:1	37.39 ± 2.82	7.55	32.64 – 45.27	0.85	1.18
20:1	2.50 ± 0.59	23.62	0.00 – 4.21	0.77	0.28
Individual Ω-6 FA					
18:2 (LA)	19.74 ± 1.39	7.04	14.73 – 23.26	0.84	0.57
18:3	0.46 ± 0.15	33.35	0.18 – 1.00	0.46	0.11
20:2	0.70 ± 0.41	59.31	0.00 – 1.27	0.04	0.41
20:3	0.47 ± 0.17	35.35	0.00 – 0.84	0.02	0.17
20:4	0.39 ± 0.07	17.69	0.12 – 0.54	0.61	0.05
Individual Ω-3 FA					
18:3 (ALA)	5.00 ± 1.32	26.45	3.30 – 7.52	0.82	0.55
18:4	0.52 ± 0.47	90.21	0.00 – 2.30	0,04	0.48
20:5 (EPA)	2.00 ± 0.35	17.60	0.94 – 2.76	0.76	0.18
22:5	0.86 ± 0.14	16.44	0.53 – 1.29	0.44	0.11
22:6 (DHA)	4.66 ± 1.10	23.69	0.80 – 6.45	0.81	0.51
EPA+DHA	6.67 ± 1.43	21.48	1.74 – 8.90	0.82	0.64

[a] μ = mean weight of the % total FA for all samples used within investigation

[b] σ = standard deviation of the % total FA

[c] CV = coefficient of variation (SD /mean*100).

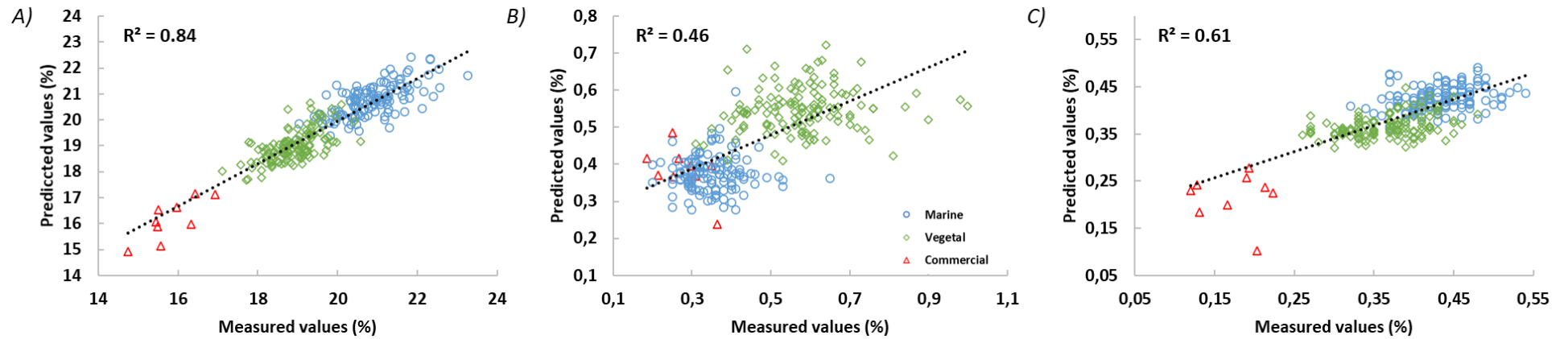[d] R² and RMSECV = statistical values from the ridge regression methods for the calibration

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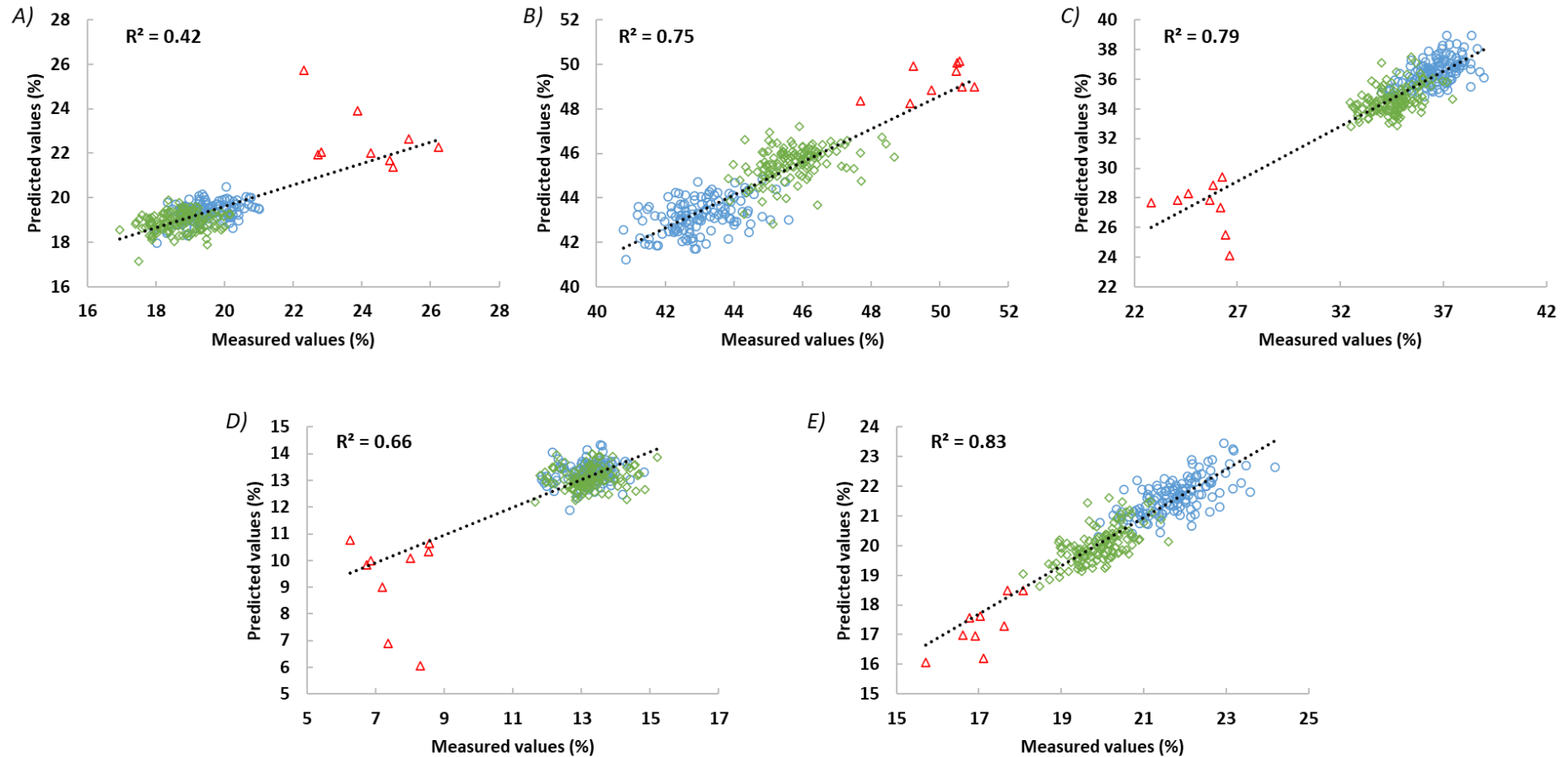
Fig. 3. Predicted values using Raman spectra compared to measured values by using ridge regression, for each major FA group. (A) SFA; (B) MUFA; (C) PUFA; (D) total omega-3; (E) total omega-6. Rainbow trout individuals were fed with a FM-FO- (*blue circle*), plant- (*green diamond*), or commercial-based (*red* triangle) feed.

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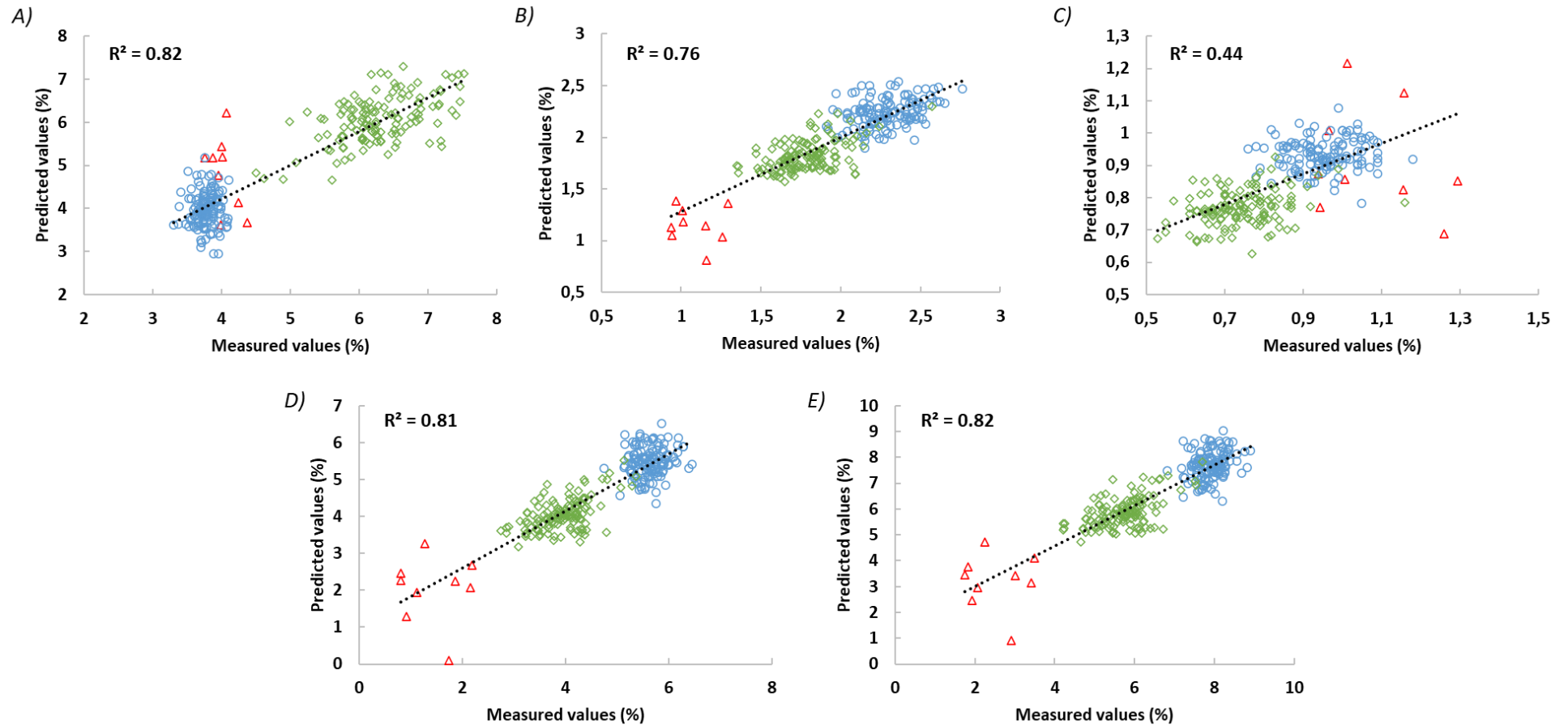
Fig. 4. Predicted values using Raman spectra compared to measured values by using ridge regression, for each individual omega-3 FA. (A) ALA ; (B) EPA ; (C) 22:5; (D) DHA ; (E) EPA + DHA. Rainbow trout individuals were fed with a FM-FO- (*blue circle*), plant- (*green diamond*), or commercial-based (*red* triangle) feed.

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Fig. 5. Predicted values using Raman spectra compared to measured values by using ridge regression, for each individual omega-6 FA.

(A) LA; (B) 18:3; (C) 20:4. Rainbow trout individuals were fed with a FM-FO- (*blue circle*), plant- (*green diamond*), or commercial-based

(*red* triangle) feed.

Highlights

- Fatty acids composition was predicted in rainbow trout *Onchorhynchus mykiss*.
- Three different feeds allowed to obtain different fatty acids composition.
- Raman spectroscopy was used to analyze adipose tissues from rainbow trout.
- Ω-3 eicosapentaenoic and docosahexenoic fatty acids have great R^2 values.
- Calibration models can be used for large scale and high throughput phenotyping.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: