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► To cite this version:

E. Prado, Christophe Eklouh-Molinier, Florian Enez, David Causeur, C. Blay, et al.. Prediction of fatty acids composition in the rainbow trout *Oncorhynchus mykiss* by using Raman micro-spectroscopy. *Analytica Chimica Acta*, 2022, 1191, 10.1016/j.aca.2021.339212 . hal-03445774

HAL Id: hal-03445774

<https://hal.inrae.fr/hal-03445774v1>

Submitted on 6 Jan 2022

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Journal Pre-proof

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Received Date: 6 May 2021

Revised Date: 26 September 2021

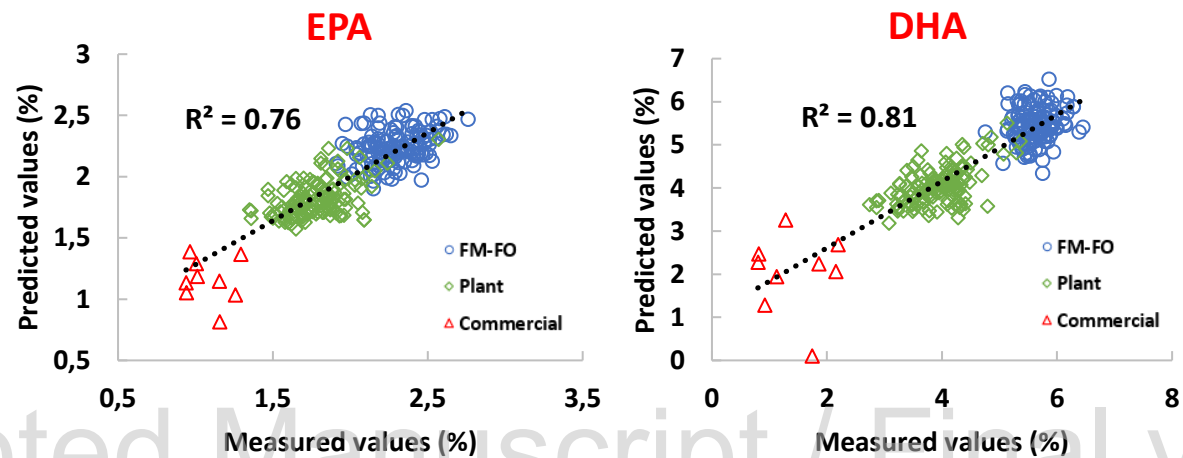
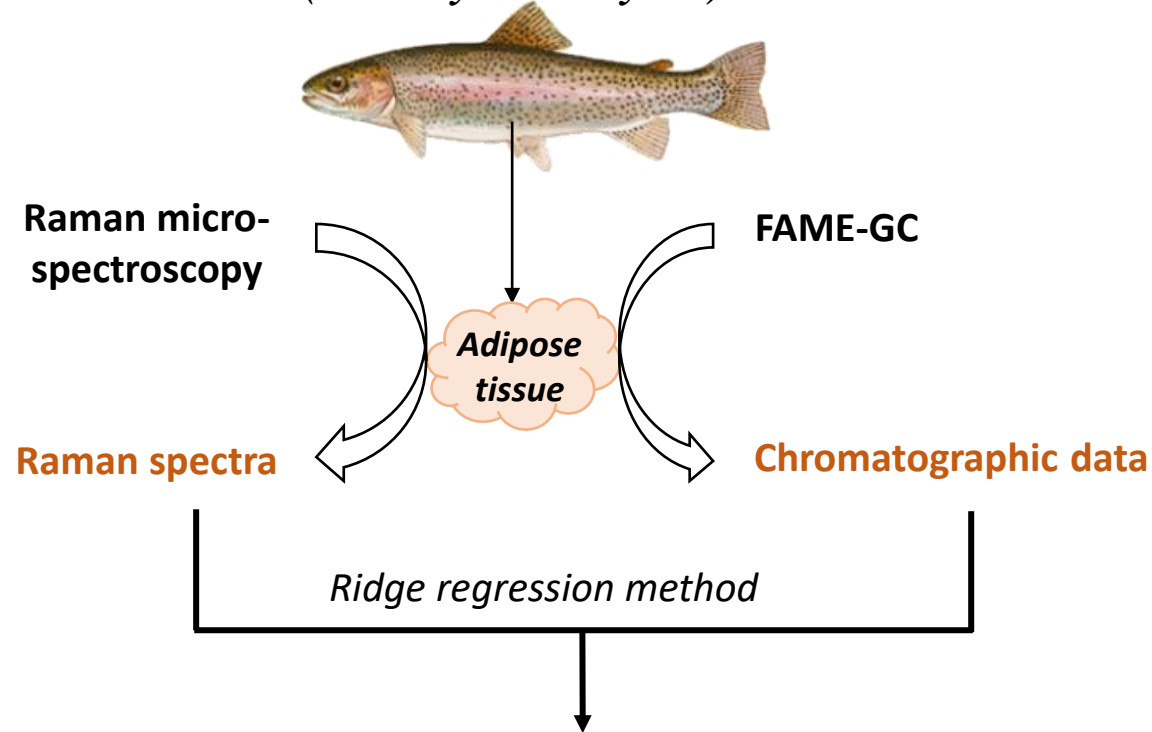
Accepted Date: 23 October 2021

Please cite this article as: E. Prado, C. Eklouh-Molinier, F. Enez, D. Causeur, C. Blay, M. Dupont-Nivet, L. Labbé, V. Petit, A. Moreac, G. Taupier, P. Haffray, J. Bugeon, G. Corraze, V. Nazabal, Prediction of fatty acids composition in the rainbow trout *Oncorhynchus mykiss* by using Raman micro-spectroscopy, *Analytica Chimica Acta*, <https://doi.org/10.1016/j.aca.2021.339212>.

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Rainbow trout fed with three different diets (*Oncorhynchus mykiss*)



Prediction of fatty acids composition in the rainbow trout

Oncorhynchus mykiss by using Raman micro-spectroscopy

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Keywords: Raman spectroscopy, fatty acids, rainbow trout, adipocytes, calibration model,
ridge regression method

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

44 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

103 **MATERIALS & METHODS**

104 **1. Raman calibration sampling**

105 The Raman calibration used 259 rainbow trout collected at the INRAE experimental facility of
106 PEIMA (Sizun, France). Trout from PEIMA belonged to the same batch and were fed with a
107 commercial feed until they reached an average weight of 600 g.

108 They were then splitted in two groups: one group (N=129) continue to be fed with a commercial
109 feed containing fish meal and fish oil (BioExtra F7, Le Gouessant), the second group (N=130)
110 was fed with a plant-based feed devoid of long chains PUFA Ω -3 (INRAE, Pisciculture de
111 Donzacq)) during 4 months. These two feeds differed in their FA composition and they were
112 used to induce changes in the fatty acid profiles of fish tissues. Fish were euthanized and
113 visceral fat samples were collected from the same “front” lobe of visceral adipose tissues,
114 placed in a coded aluminium foil (10 g) or in cryotubes (2 g), for GC or Raman measurements,
115 respectively, and were preserved in liquid nitrogen until further analyses. Nine additional
116 samples from rainbow trout, reared under commercial conditions (Aqualande group, Viviers de
117 la Hountine, Belin Béliet, France) and fed with a commercial feed (Viva Pro 7F, Aqualia), were
118 also analysed.

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

120 The fatty acid composition was determined for each calibration sample after lipid extraction by
121 cold biphasic method, methylation of FAs and analysis of the fatty acid methyl esters by GC
122 reference method (Eurofins Analytics, Nantes, France). The FAs proportion is defined by the
123 relative concentrations of the different FAs present in sample and generally compared to each
124 other by considering the major families of SFAs, MUFAs and PUFAs. The proportion of these
125 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:

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

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 R^2) 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

176 **RESULTS AND DISCUSSION**177 **1. Excitation wavelength selection and spectral assignment of vibrational bands**

178 A wide range of factors influences the intensity of Raman spectra of biological samples
179 including instrumental parameter as laser intensity, wavelength, and acquisition time. In this
180 study, three wavelengths were tested (532nm, 633nm and 785nm) on the sample in order to
181 select the most suitable for measuring lipids in adipose tissues of rainbow trout and to obtain
182 robust multivariate calibration models. Figure 1 shows the representative spectra of visceral
183 adipose tissue acquired with the three different wavelengths. In order to be comparable, the
184 spectra were just normalized by the acquisition time. The most characteristic features of Raman
185 spectra of lipids related to hydrocarbon chain were observed in the three spectra, notably C=C
186 bonds or CH₂ group vibration at ~ 1650 cm⁻¹ and ~1450 cm⁻¹, respectively [20, 21]. This strong
187 lipid fingerprint seems coherent with the Raman spectrum of a biological tissue containing a
188 large amount of lipids and associated molecules (FAs, triglycerides, etc) as adipose tissues. As
189 expected, this fluorescence contribution decreased when the 633 and 785 nm excitation
190 wavelengths were used. Nevertheless, the Raman intensity of high wavenumber vibrations was
191 dramatically reduced at 785 nm. Indeed, when considering the near-infrared 785 nm excitation,
192 CCD efficiency radically decreased for such high wavenumbers. Considering the above-
193 mentioned effects, the 785 nm wavelength was selected for the present study. Using this
194 wavelength will therefore allow to acquire informative spectral information from visceral
195 adipose tissues of rainbow trout.

196 The stability and homogeneity of the samples were tested by recording the Raman spectrum
197 at the same position at regular time intervals (of about 5') for 30' (physical replicas) or by
198 measuring the Raman spectrum at different positions in the sample (biological replicas). No
199 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 $\sim 1650 \text{ cm}^{-1}$ 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

400 **ACKNOWLEDGEMENTS**

401 This study was partly funded by The European Maritime and Fisheries Fund and
402 FranceAgrimer (Omega-Truite project, n° PFEA47 0017 FA 1000008). The authors thank the
403 INRAE's experimental facilities (PEIMA) for fish rearing and their technical assistance for
404 adipose tissue sampling.

405

406 **ETHICS STATEMENT**

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

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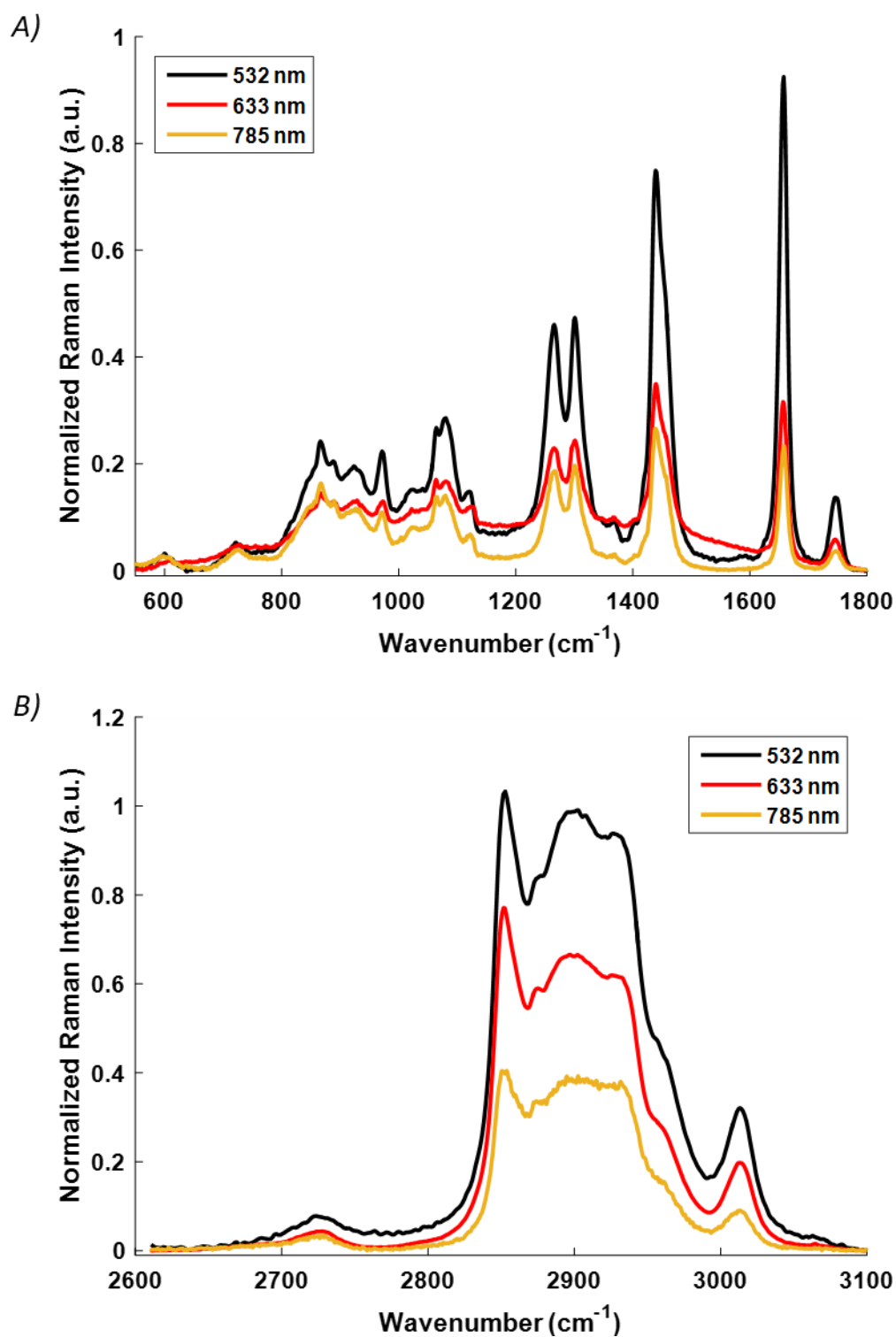
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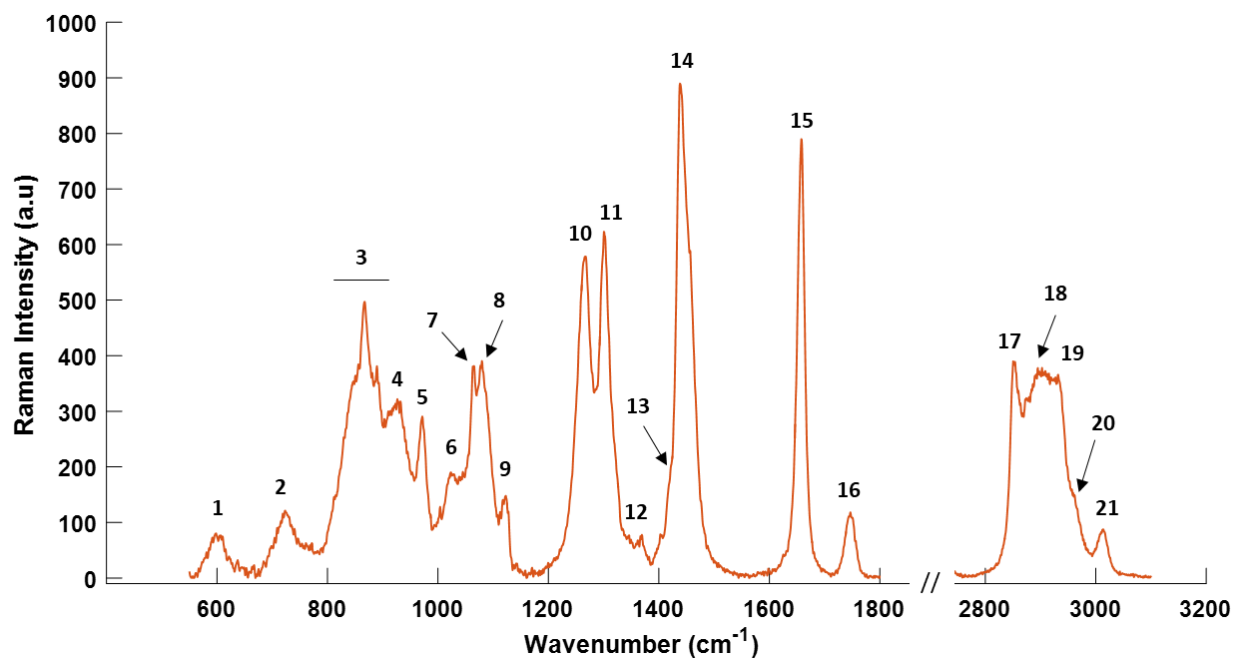


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555 **Fig. 1. Raman spectra of visceral adipose tissue from rainbow trout, acquired at**556 **different wavelengths: 532, 633 and 785 nm. (A) 550-1800 cm^{-1} , (B) 2600-3100 cm^{-1}**

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

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Table 1. Assignment of bands in the Raman spectra

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

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

576

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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580

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)

585

586 **Table 4. Summary statistics of the FA from visceral adipose tissues in rainbow trout**
 587 **(N=268), by FAME-GC analysis.**

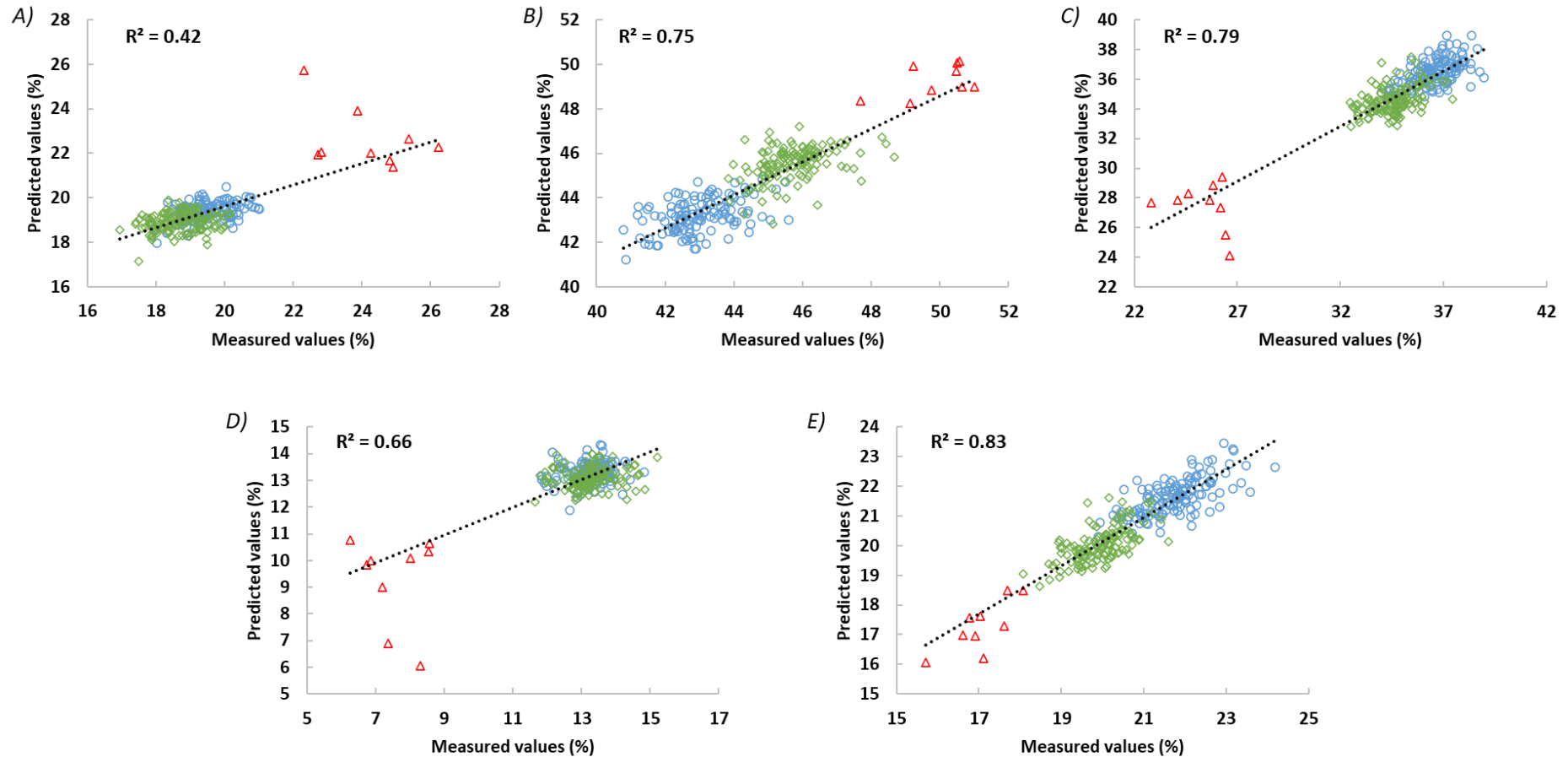
Fatty acid (FA)	$\mu^a (\pm \sigma^b)$	CV ^c	Range (min. - max.)	R ² ^d	RMSEP ^d
<i>Major FA group</i>					
SFA	19.27 ± 1.22	6.31	16.94 – 26.23	0.42	0.95
MUFA	44.55 ± 1.91	4.29	40.79 – 51.02	0.75	1.04
PUFA	35.23 ± 2.32	6.60	22.82 – 38.98	0.79	1.17
Total Ω-3	13.03 ± 1.19	9.19	6.26 – 15.22	0.66	0.79
Total Ω-6	20.72 ± 1.33	6.42	15.71 – 24.18	0.83	0.56
<i>Individual Ω-9 FA</i>					
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
<i>Individual Ω-6 FA</i>					
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
<i>Individual Ω-3 FA</i>					
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

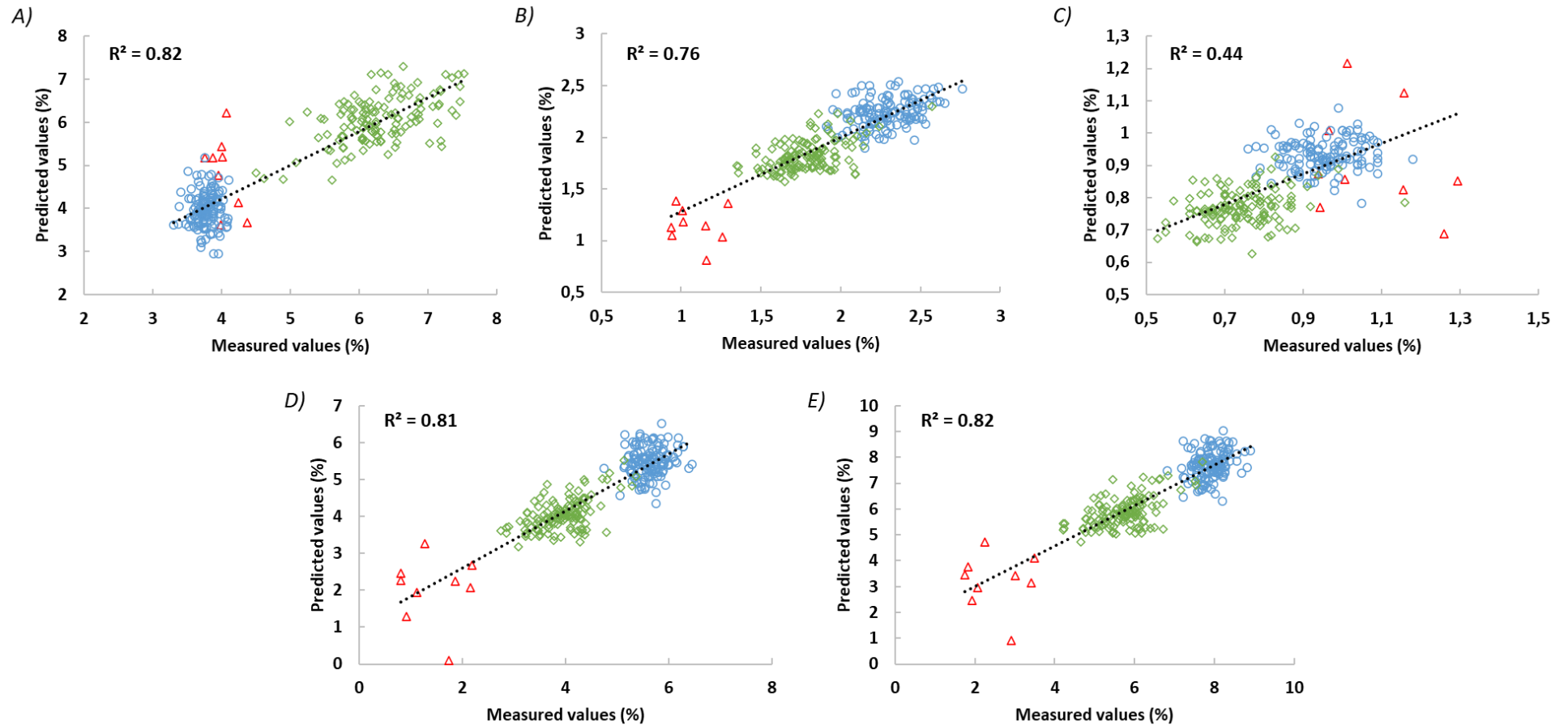
^c CV = coefficient of variation (SD /mean*100).

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



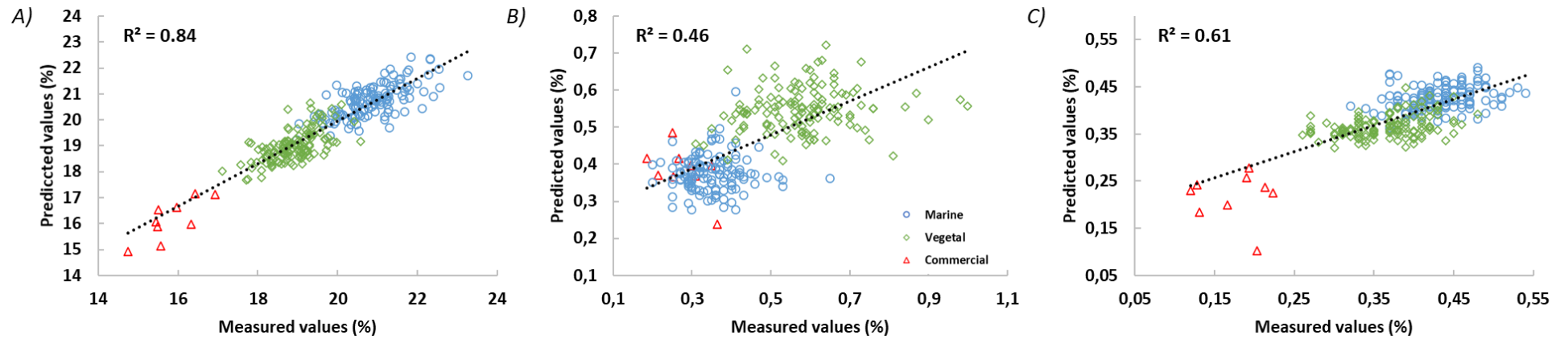
589

590 **Fig. 3. Predicted values using Raman spectra compared to measured values by using ridge regression, for each major FA group. (A)**591 **SFA; (B) MUFA; (C) PUFA; (D) total omega-3; (E) total omega-6. Rainbow trout individuals were fed with a FM-FO- (*blue circle*),**592 **plant- (*green diamond*), or commercial-based (*red triangle*) feed.**



593

594 **Fig. 4. Predicted values using Raman spectra compared to measured values by using ridge regression, for each individual omega-3 FA.**595 **(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***596 ***diamond*), or commercial-based (*red triangle*) feed.**



597

598 **Fig. 5. Predicted values using Raman spectra compared to measured values by using ridge regression, for each individual omega-6 FA.**599 **(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**
600 **(*red triangle*) feed.**

601

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: