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High-Resolution ^{1}H Magic Angle Spinning NMR Spectroscopy of Intact Arctic Char (*Salvelinus Alpinus*) Muscle. Quantitative Analysis of *n*−3 Fatty Acids, EPA and DHA

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The lipid and small metabolite profiles from intact muscles of Arctic char were investigated using ^{1}H high-resolution magic angle spinning (^{1}H HR-MAS) NMR spectroscopy. Not only the total *n*−3 fatty acid content but also the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contents of the muscle were obtained from the ^{1}H HR-MAS NMR spectra without pretreatment of the tissue or lipophilic extraction. A number of small metabolites could also be observed, where creatine/phosphocreatine, anserine and taurine were the most abundant. Thus, the use of ^{1}H HR-MAS NMR led to simplified analysis techniques that can give direct information on the nutritional value of the fish.

KEYWORDS: Arctic char; *Salvelinus alpinus*; fatty acids; metabolites; muscle; tissue; ^{1}H HR-MAS NMR

INTRODUCTION

Both lipids and small metabolites are of relevance for the nutritional evaluation of fish. The main function of the small molecules is to stabilize the structure of proteins, protect cells against osmotic stresses and prevent oxidative damage (*1*, *2*). Such compounds are anserine (*β*-alanyl-1-methylhistidine), carnosine (*β*-alanylhistidine), taurine, choline and betaine. Regarding the lipid content, the levels of polyunsaturated *n*−3 fatty acids such as EPA (eicosapentaenoic acid, 20:5 *n*−3) and DHA (docosahexaenoic acid, 22:6 *n*−3) make marine lipids unique compared to other lipid sources. These polyunsaturated fatty acids (PUFA) are reported to reduce arterial disease (*3*), have a positive effect on brain and nervous system, and stimulate the immune system, and new health effects are still being discovered (*4*, *5*). Thus, the value is often linked to the content of EPA and DHA, and these compounds are present in relatively large amount in muscles of fatty fish like salmon and Arctic char. Due to the beneficial effect of fish on health, there is an increasing requirement for analytical methods capable of giving a picture of fish metabolites and to assess the nutritional quality of the product. These methods should be based on the analysis of a variety of metabolites and not only of a few specific classes of compounds.

Gas chromatography (GC) is among the most common techniques used for determining the fatty acid (FA) composition. A complementary method to GC is ^{1}H and ^{13}C NMR spectroscopy, which has proven to be useful to study metabolite profiles using either extracts or intact tissues (*6*, *7*). When using extracts, the compounds observed depend on the extraction procedure. Thus, with fish, the small metabolites are obtained from hydrophilic extracts, whereas the triglyceride and phospholipid fatty acid distribution is obtained from lipophilic extracts (*8*). The lipid profile contains information on different lipid classes, the global unsaturation level and *n*−3 polyunsaturated fatty acids. Intact tissue, on the other hand, can be analyzed by ^{1}H high-resolution magic angle spinning NMR (^{1}H HR-MAS NMR) without pretreatment, allowing high-resolution NMR spectra to be obtained for both small metabolites and lipids. ^{1}H HR-MAS NMR has been used in many biomedical studies on e.g. brain, liver, muscle tissue, gut and skin, mainly from rats and humans (*7*, *9*, *10*). Lipids have been observed by ^{1}H HR-MAS of whole vegetable seeds (*11*), of whole cells of *Thalassiosira pseudonana* (*12*) and of muscle of Atlantic salmon (*Salmo salar*) (*13*).

Arctic char is a relatively new aquaculture species, and to our knowledge, the small metabolites and the FA profile have not been investigated by NMR. The purpose of this work was to study intact muscle of Arctic char by ^{1}H HR-MAS NMR spectroscopy to obtain quantitative information on the PUFA composition and to detect the major small metabolites.

MATERIALS AND METHODS

Samples. Arctic char of the selected strain "Arctic superior", 0.5 year old, with an initial mean weight of 43.6 (SD ± 10.1) g were used in this study. The fish were raised from March to June at the Kälarne Research Station in Central Sweden and were fed under ambient water temperature until the fish had increased their weight 2-fold. On the day of final sampling, fish were anesthetized with ethyleneglycol monophenyl ether (5 mL/L) and killed by a blow on the head and the fillet was dissected from each fish. The left fillets were first stored on ice and then stored in −80 °C until further analyses. Triplicate samples from slices of the white muscle taken from the dorsal area of seven different specimens were subjected to ^{1}H HR-MAS NMR measurements.

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Lipid Analysis. The samples of white muscle tissue were extracted according to the method of Hara and Radin (*14*). Fatty acid methyl esters (FAME) were prepared according to a modified procedure originally described by Appelqvist (*15*). Lipids (2 mg) were dissolved in 0.5 mL of hexane and saponified with 2 mL of NaOH in dry methanol (0.01 M) and esterified with 3 mL of BF_3 (20% boron trifluoride−methanol complex). The FAME extracts were analyzed by gas−liquid chromatography (Varian CP3800, Varian AB, Stockholm, Sweden). The column used was a 50 m × 0.22 mm i.d., 0.25 μm film, BPX 70 capillary column (SGE, Austin, Texas). Helium was used as carrier gas (0.8 mL/min). The column temperature was programmed to start at 158 °C (held for 5 min) and then increased to 220 at 2 °C/min, where it remained for 8 min. The injector and detector temperatures were 230 and 250 °C, respectively. Individual methyl esters were identified by comparison with the fatty acid standard mixture GLC-461 (Nu-Check Prep, Elysian, MN). Peak areas were integrated using the Varian Galaxy software package, Version 1.0 (Varian AB, Stockholm, Sweden).

^{1}H HR-MAS NMR Spectroscopy of Muscle Tissues. The samples (*ca.* 15 mg) were inserted into zirconia rotors of 4 mm outer diameter, and a spherical sample volume of 20 μL (Bruker, Karlsruhe, Germany) and 20 μL of D_2O was added. The NMR analysis was performed on a Bruker Avance 600 spectrometer operating at a ^{1}H frequency of 600.13 MHz and using a 4 mm HR-MAS SB BL4 ^{1}H/^{13}C inverse detection probe equipped with *z*-gradient. Temperature of 25 °C and sample spinning rate of 4 kHz were used. All spectra were processed with the Bruker software, Topspin 2.0. For the ^{1}H spectra, the signal of the methyl group of lactate was used as reference (δ 1.34 ppm).

Four different types of ^{1}H NMR spectra were obtained for each tissue sample: (i) standard one-dimensional spectra with no water presaturation and 90° pulse angle. 64 transients were acquired with 64K data points. (ii) One-dimensional spectra with water presaturation using the NOESY-presat (noesygppr1d, Bruker library) pulse sequence in which the water peak is suppressed during the recycle delay of 5 s and mixing time (t_m) of 50 ms. (iii) Water-suppressed diffusion-edited ^{1}H NMR spectra allowing elimination of low molecular weight compounds. The spectra were acquired by using the bipolar-pair longitudinal-eddy-current-delay (BPP-LED) pulse sequence. Gradient strength was optimized (60−95%) to 95% of its maximum value (the maximum *z*-gradient was 52 G/cm) and a diffusion time of 200 ms, a gradient pulse length of 1.9 ms and a relaxation delay of 5 s were used. (iv) The water suppressed spin−echo Carr−Purcell−Meiboom−Gill (CPMG) pulse sequence (relaxation delay−90−$(t-180-t)_n$−acquire) was used to allow attenuation of broad signals from macromolecules. A total CPMG time of 70 ms (n = 100) was used after optimization between 35 and 140 ms. The water suppression was achieved by irradiation during the relaxation delay of 5 s. For the noesygppr1d, BPP-LED and CPMG experiments, 256 transients were collected into 32K data points with a spectral width of 8418 Hz. Total experimental times were typically less than 3 h, and no significant changes of the spectra were detected during this period of time.

Treatment of NMR Data for Quantification of *n*−3, EPA and DHA Amounts. The spectra were processed with a line broadening of 1 Hz and a zero filling factor of 1. Phasing was made automatically and baseline correction was automatically performed by subtraction of a polynomial of fifth degree. Integrals were measured between 0.869 and 0.935 ppm (signal 1, S1) (**Table 1**), between 0.941 and 1.002 ppm (S2), between 1.545 and 1.637 ppm (S4), between 1.646 and 1.684 ppm (S5), between 2.214 and 2.298 ppm (S7), and between 2.300 and 2.392 ppm (S8). These regions are slightly different from those used in the literature for lipophilic extracts (*16*, *17*) due to small differences in chemical shifts and linewidths observed with the intact tissues. For quantification purposes, the signal of the methyl group of *n*-3 fatty acids (S2) was used as reference (δ 0.971 ppm).

RESULTS AND DISCUSSION

A representative single pulse ^{1}H HR-MAS NMR spectrum of the muscle of Arctic char is shown in **Figure 1A**. The spectrum with water presaturation (**Figure 1B**) is dominated by signals from lipids, but small metabolites are also visible. The large molecules were separated from the low molecular weight components using spectral editing.

Table 1. Assignments of Signals of the ^{1}H NMR Spectra of Fatty Acids of Arctic Char Muscle

signal	functional group[a]	compound
1	$-CH_3$	all FA except *n*−3
2	$-CH_3$	*n*−3 FA
3	$-(CH_2)_n-$	all FA except DHA and EPA
4	$-C\underline{H}_2-CH_2-COOR$	all FA except DHA and EPA
5	$-C\underline{H}_2-CH_2-COOR$	EPA
6	$-C\underline{H}_2-CH{=}CH-$	unsaturated FA
7	$-CH_2-COOR$	all FA except DHA
8	$=CH-CH_2-CH_2-COOR$	DHA
9	$=CH-CH_2-CH=$	polyunsaturated FA
10	$-N(CH_3)_3$	phosphatidylcholine
11	$-CH_2-OCOR'$	glyceryl
12	$-CH_2-OCOR'$	glyceryl
13	$-CH-OCOR'$	glyceryl
14	$-CH{=}CH-$	unsaturated FA

[a] R = glyceryl unit, R′ = FA chain.

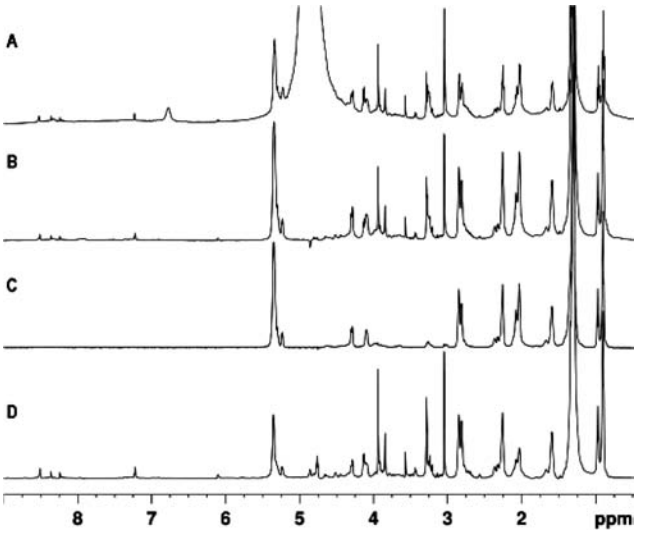

Figure 1. 600 MHz ^{1}H HR-MAS NMR spectra of 15 mg of muscle tissue at a rotation rate of 4 kHz: (**A**) no water presaturation, (**B**) Noesy-presaturation, (**C**) diffusion-edited with water presaturation, (**D**) CPMG with water presaturation.

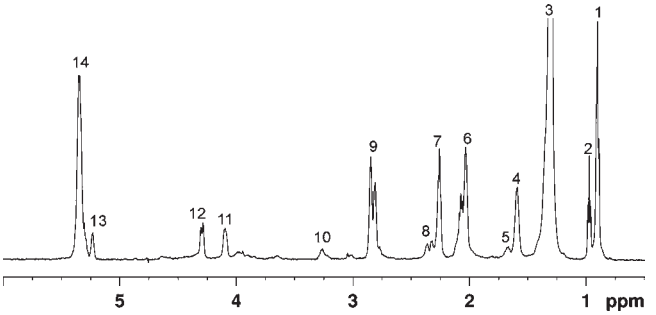

Figure 2. Diffusion edited ^{1}H HR-MAS NMR spectrum of 15 mg of muscle of Arctic char showing the fatty acid profile. The assignment of signals 1−14 is given in **Table 1**.

Fatty Acids. The diffusion-edited NMR spectrum (**Figure 1C**, **Figure 2**) allows filtering out the small metabolites, showing only signals from the triglycerides and phospholipids. The profile is similar to those reported in the literature for fish oils (*17*−*21*) or for lipophilic extracts (*8*, *22*, *23*), and signals from triglycerides, which have saturated and mono-, di-, and polyunsaturated

Table 2. *n*−3 Fatty Acids, DHA and EPA Content of Total Fatty Acids in White Muscle of Arctic Char Obtained by ^{1}H HR-MAS NMR Spectroscopy

	noesypr1d[a]			BPP-LED[a]			GC			
sample	*n*-3 mol %	DHA mol %	EPA[b] mol %	*n*-3 mol %	DHA mol %	EPA[b] mol %	*n*-3 mol %	DHA mol %	EPA mol %	AA mol %
1	31.1 ± 1.1	14.4 ± 0.7	11.8 ± 1.9	30.8 ± 2.6	15.6 ± 1.0	10.8 ± 0.9	31.3	16.7	9.0	0.7
2	28.7 ± 1.9	11.8 ± 0.5	8.5 ± 1.1	27.7 ± 0.5	13.8 ± 1.8	8.9 ± 0.9	29.9	14.2	8.6	0.7
3	25.6 ± 0.6	10.3 ± 0.5	8.0 ± 0.4	25.6 ± 0.3	10.5 ± 0.4	7.8 ± 0.5	28.6	12.0	9.7	0.8
4	25.0 ± 2.3	11.5 ± 1.1	8.6 ± 1.2	25.1 ± 1.4	10.6 ± 1.7	7.4 ± 1.4	31.4	15.9	9.1	0.8
5	28.2 ± 0.5	12.8 ± 1.0	11.4 ± 0.8	27.1 ± 0.9	15.2 ± 2.3	10.8 ± 1.5	31.3	15.4	9.1	0.8
6	27.5 ± 1.3	11.1 ± 2.6	9.2 ± 2.0	25.8 ± 2.1	15.0 ± 1.9	10.0 ± 2.4	29.5	14.2	8.7	0.8
7	28.8 ± 1.3	11.4 ± 0.6	11.0 ± 0.6	26.2 ± 1.9	16.8 ± 1.6	11.7 ± 1.1	29.1	12.8	9.5	0.8

[a] Values are means ± SD (n = 3). [b] Arachidonic acid (AA) contributes to the NMR signal used for the determination of EPA.

(EPA, DHA and other *n*−3 FA) acyl groups, are clearly seen. The assignment of the signals is given in **Table 1** and some of the, in total, 14 distinct peaks can be used to trace protons from *n*−3 fatty acids and from EPA and DHA (*17*, *19*−*22*). Signal 2 is, for example, due to the triplet of methylic protons of the *n*−3 polyunsaturated acyl groups. Signal 5 and signal 8 are typical of the fish lipids EPA and DHA and are absent in the ^{1}H NMR spectra of vegetable oils. Qualitative information on the degree of unsaturation can thus be obtained by simple observation of the NMR spectrum of the tissue.

More quantitative information on the amount of *n*−3 fatty acids can be obtained using the equation (*16*)

$$n-3\% = 100(S2)/(S1+S2)$$

with S1 being the methyl signal at 0.90 ppm representing all fatty acids where the first double bond after the omega end is more than 3 carbons away from it.

The proportion of DHA is evaluated using signal 8, which is due to the protons on the two methylene groups on the α and β carbons to the carbonyl group. The two protons on the α-group of all other fatty acids form signal 7. For determining the amount of DHA, the intensity of signal 8 must be divided by 2 as it represents four protons (*17*).

$$DHA\% = 100[(S8)/2]/[(S8)/2+S7]$$

The EPA content is determined using signal 5, which arises from the methylenic protons in the β-position to the carbonyl group. These two protons are also in the β-position to the double bond on carbon 5, which induces a downfield shift. This shift isolates signal 5 from signals of other β-methylene group protons, signal 4 (*20*, *21*).

$$EPA\% = 100(S5)/[S4+S5+(S8)/2]$$

β-Protons of arachidonic acid also contribute to signal 5 as this structural element is present there as well. However, only tuna oil has been shown to contain substantial amounts (1.8% of total FA composition) of arachidonic acid (*20*).

Integrations of the ^{1}H NMR signals were performed both on the original spectra and using deconvolution, and there was no significant difference in the calculations of the fatty acid composition. The error of repeated measurement for both the noesypr1d and the BPP-LED experiments was calculated from triplicate runs on the same sample and was found to be less than 1, 4 and 3% RSD for *n*−3 FA, DHA and EPA respectively. The content of *n*−3 FA, DHA and EPA calculated for triplicate samples of seven different specimens from the ^{1}H NMR spectra acquired and using diffusion-edited NMR experiment as well as noesypr1d experiment which is not affected by spectral editing are shown in **Table 2**. The calculated fatty acid composition did not change significantly depending on the types of ^{1}H NMR spectra. For comparison, the content of *n*−3 FA, DHA and EPA for the corresponding lipid extracts obtained by GC analysis is also reported in **Table 2**. Slightly higher *n*−3 fatty acid values are obtained with GC as already observed in previous studies (*13*, *16*, *17*, *20*). It has been proposed that some of the differences can be explained by the fact that, in the NMR, measurements are done on the heterogeneous lipid mixture containing phospholipids, sterols etc. while the additional procedures to which the lipid extract is submitted prior to GC may result in changes in the relative composition of the FAs (*13*).

Small Metabolites. The spectral contribution from macromolecules with short spin−spin relaxation times was attenuated using the spin echo CPMG pulse sequence (**Figure 1D**). The broad resonances from lipids were not completely suppressed by the CPMG experiment however, suggesting that the muscle tissue contains a fraction of highly mobile lipids.

Signals from lactate, anserine, choline, creatine/phosphocreatine, amino acids (alanine, glycine, taurine), β-glucose, acetate and inosine were identified by comparison of chemical shifts with the literature (*8*, *22*, *24*, *25*). Creatine/phosphocreatine, anserine and taurine were the most abundant metabolites (**Figure 3**, **Table 3**). The results are in good agreement with previous studies on the free amino acid composition in Arctic char white muscle, where alanine, glycine and taurine are found in highest concentration, together with the dipeptide anserine (*26*−*28*). Free amino acids are important for the osmoregulation of Arctic char (*26*) and contribute to the flavor of fish meat (*29*).

The metabolite profile is similar to that of intact white muscle of Atlantic salmon (*Salmo salar*), which was investigated by ^{1}H NMR spectroscopy (*8*). The same metabolites were found, with the exception of taurine, β-glucose and inosine. On the other hand, hypoxanthine, which is the degradation product of inosine, was observed. The presence of inosine and inosine monophosphate (IMP) versus hypoxanthine can be a marker of flesh quality, but the degradation rates differ between species (*30*). Thus some species end up with high hypoxanthine concentrations and others with high inosine concentrations. In Japanese char (*Salvelinus pluvius*) the concentration of inosine was found to be higher than that of IMP and hypoxanthine (*31*).

This study shows the potential of ^{1}H HR-MAS in studying the fatty acid profile and metabolite pattern in intact muscle of Arctic char. The *n*−3 FA, DHA and EPA contents obtained from ^{1}H HR-MAS NMR led to results similar to those obtained from GC analysis. The data are also comparable with those obtained in other studies on Arctic char (*32*). For some of the specimens however, several additional signals were observed in the NMR spectra (data not shown) probably as a result of sample degradation. Some of these signals interfered with the regions used for integration of the NMR signals of the fatty acids, thereby precluding calculation of their amount. Thus the quality of the spectra should be evaluated before calculating the fatty acid composition.

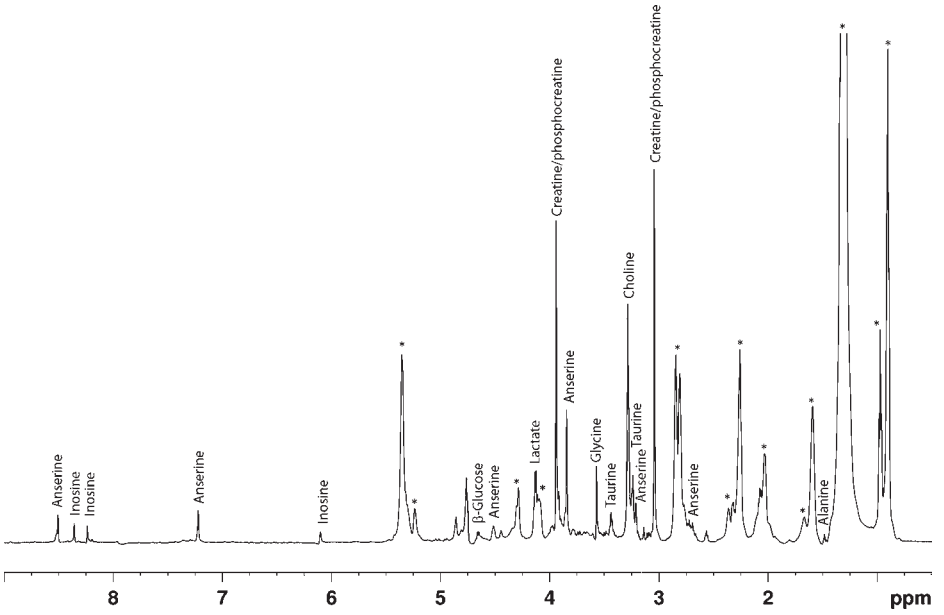

Figure 3. CPMG ^{1}H HR-MAS NMR spectrum showing the small metabolites. Asterisk (*) refers to signals from lipids.

Table 3. Assignment of Resonances from Small Metabolites in the ^{1}H NMR Spectra of White Muscle from Arctic Char

compound	group	^{1}H chemical shift (ppm)	^{1}H multiplicity
acetate	CH_3	1.94	s
alanine	βCH_3	1.49	d
anserine	CH_2	2.71	m
	CH_2	3.09	m
	CH_2	3.24	m
	NCH_3	3.84	s
	CH	4.51	dd
	CH (histidine)	7.23	s
	CH (histidine)	8.52	s
choline	NCH_3	3.29	s
creatine/phosphocreatine	NCH_3	3.04	s
	CH_2	3.94	s
β-glucose	C1H	4.66	d ($^3J_{C1H,C2H}$ = 7.9 Hz)
glycine	CH_2	3.57	s
lactate	αCH	1.34	d ($^3J_{CH,Me}$ = 7.3 Hz)
	βCH_3	4.13	q
inosine	C1′H (ribose)	6.11	d ($^3J_{C1'H,C2'H}$ = 5.8 Hz)
	C8H (ring)	8.24	s
	C2H (ring)	8.36	s
taurine	NCH_2	3.27	t
	SCH_2	3.44	t ($^3J_{CH_2,CH_2}$ = 6.7 Hz)

The relative or absolute quantification with ^{1}H HR-MAS NMR might be difficult requiring stable and repetitive experimental conditions (*33*), and more studies are necessary to investigate if the technique can be used to quantitate and compare the absolute amounts of metabolites and lipids in fish tissues. Since an internal reference is difficult to add and may interact with the tissue sample, an external electronic reference (ERETIC) has been developed to obtain absolute concentrations (*34*). Another issue is that a number of small metabolites are not detected in the 1D ^{1}H HR-MAS NMR spectra due to strong overlapping with signals from the fatty acids, especially in the region from 0.5 to 3 ppm where characteristic signals from amino acids such as valine, leucine or isoleucine appear. While the CPMG sequence allows suppressing signals from lipids from membranes, such as phospholipids and cholesterol that have very short T_2, it does not allow suppressing efficiently enough signals from triglycerides. Thus, the small hydrophilic metabolites might not be detected in the 1D HR-MAS NMR spectra of the white muscle with the same accuracy as it is done using extracts, due to overlap of signals with the fatty acids.

^{1}H NMR has been demonstrated to be a complementary method to GC for determination of the total $n-3$ FA composition as well as for the determination of EPA and DHA in fish oils (*18*). Aursand et al. have shown that the total $n-3$ FA amount could also be obtained on intact muscle tissue from Atlantic salmon by ^{1}H MAS NMR (*13*). In the present work, we show that the amount of DHA and EPA, in addition to the total $n-3$ FA amount, can be obtained from the ^{1}H HR-MAS NMR spectra of the intact muscles. Good agreement was observed between the data obtained from diffusion-edited experiments and the data obtained from single pulse NMR experiments. This will allow obtaining the fatty acid profile and the major metabolites without need for hydrophilic and lipophilic extractions, with fewer requirements on the amount of compound and minor risk of sample degradation.

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