Results in Chemistry 4 (2022) 100423

Contents lists available at ScienceDirect

Results in Chemistry

journal homepage: www.sciencedirect.com/journal/results-in-chemistry

^{31}P solid-state NMR on skeletal muscle of wild and farmed Atlantic salmon

Christian Totland [a], Signe Steinkopf [b], Lisa Tu Storhaug [c], John Georg Seland [c], Willy Nerdal [c,*]

[a] NGI – Norwegian Geotechnical Institute, Environmental Chemistry, Sognsveien 72, 0806 Oslo, Norway
[b] Western Norway University of Applied Sciences, Department of Safety, Chemistry and Biomedical Laboratory Sciences, Inndalsveien 28, 5063 Bergen, Norway
[c] University of Bergen, Department of Chemistry, Allégaten 41, 5007 Bergen, Norway

ARTICLE INFO

Keywords:
^{31}P
Solid-state NMR
Fatty acids
Phospholipids
Bilayer

ABSTRACT

Over the past 50 years, ^{31}P NMR has proven a powerful tool for obtaining information on cellular biochemistry. Here we use this technique for the first time to study intracellular phosphorous metabolites in skeletal muscle tissue of wild and farmed salmon, to investigate possible effects due to differences in diet and way of life. The wild salmon sample shows a significantly more diverse composition of metabolites compared to the farmed salmon sample. The differences are evident in the entire spectrum, including regions displaying resonances from phosphomonoesters and sugar phosphates, as well as other molecules important for phospholipid metabolism.

It is demonstrated that 31P MAS NMR can be used to study a broad range of phosphorus metabolites ex vivo, which can give useful information, both on its own or as a supplement to other extraction-based analyses. Further 31P MAS NMR investigations on farmed salmon raised under different controlled conditions may give important insights into the broad array of health issues seen in farmed salmon.

The mortality rate of farmed salmon is currently around 15 % in Norwegian fish farms, not including destruction of fish due to e.g. deformities. According to the annual Fish Health Report from the Norwegian Veterinary Institute, various infections are a significant cause of the baseline mortality. The reasons for the high baseline mortality are complex and not fully understood, and involves factors related to production and health, as well as environmental factors [1]. A better understanding of the biochemistry of the salmon is crucial to understand the relationship between mortality and factors related to salmon health. Here, we explore the use of the non-intrusive technique ^{31}P Magic Angle Spinning (MAS) NMR to obtain such information on tissues *ex vivo*.

In contrast to ^{1}H and ^{13}C NMR, ^{31}P NMR of tissues and cells show a limited number of resonances. Nonetheless, since the pioneering work in this field during the 1970′s [2,3], metabolites that contain phosphorous in sufficient levels have revealed crucial information on cellular biochemistry and physiology.

Inorganic phosphorous (P_i) is the most abundant phosphorous specie in the intracellular environment, constituting about 30 % of the total phosphorous content [4]. Other abundant metabolites include sugar phosphates, phosphocreatine (muscle tissues in particular) and nucleosides, as well as phosphomonoesters (PME) and phosphodiester (PDE), which are building blocks for phospholipid cellular membranes.

Deprivation of phosphorous in humans is known to manifest in a number of disorders, particularly in the musculoskeletal system [5]. Similarly, inadequate phosphorous content in the feed of fish has shown to have adverse effects on bone health and growth [6,7], as well as changing rates of expression of a number of enzymes [8] and causing deformities [7,9].

The metabolism of phosphorous depends on several other factors than the source of phosphorous itself. Fatty acids, in the form of diacylglycerol, make up the oleophilic acyl chains of phospholipids. Ingestion of high amounts of long-chain marine omega-3 fatty acids leads to high incorporation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into membrane phospholipids and a membrane packing that differs from a phospholipid membrane with low amounts of EPA and DHA. This in turn can affect membrane fluidity and function [10,11]. ^{13}C Magic Angle Spinning (MAS) NMR spectroscopy can quantify the most relevant fatty acids in salmon fat within 40 min, without use of chemical extractions.

Furthermore, it is well known that fatty acids in general, and marine omega-3s in particular, affect lipid metabolism in both humans and animals, as well as the phospholipid composition of cellular membranes [12]. Kellner et al. (2017) found that an omega-3 rich diet suppressed the mRNA abundance of genes involved in lipolysis in both adipose tissue and the liver of pigs [13]. Dagnelie et al. (1993) studied effects of varying dietary marine omega-3 on rats and humans using ^{31}P NMR, and

* Corresponding author.
E-mail address: Willy.Nerdal@uib.no (W. Nerdal).

https://doi.org/10.1016/j.rechem.2022.100423
Received 29 April 2022; Accepted 27 June 2022
Available online 3 July 2022

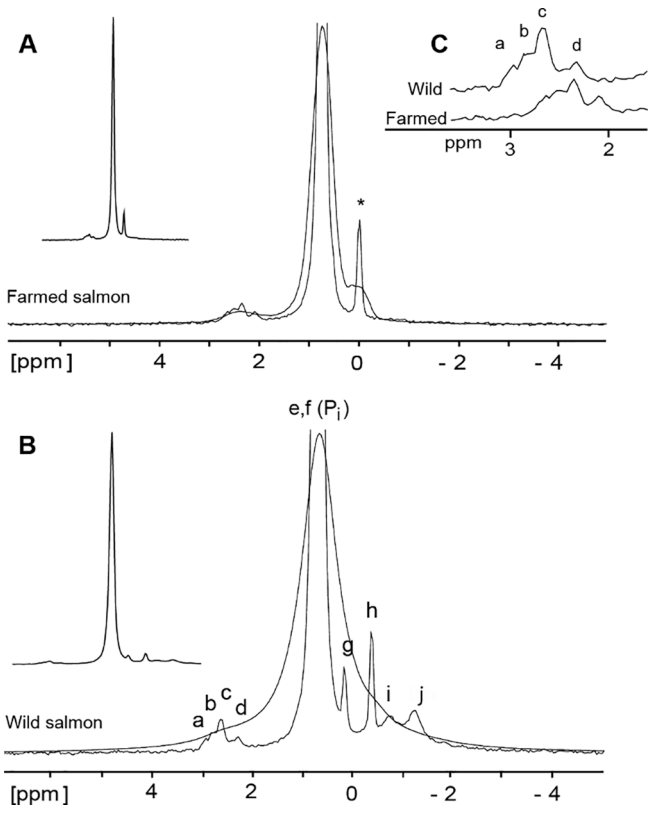

Fig. 1. ^{31}P spectra of: A; farmed salmon. B; wild salmon; C shows an enhancement of the 2–3 ppm region. Main spectra are static ^{31}P spectra and the corresponding MAS (spinning 3500 Hz) spectra. Inserted in both the farmed and the wild salmon spectra is the full height MAS spectrum. Labels a-j are at isotropic chemical shifts of phosphorous species, the intense peak labelled e, f corresponds to two resonances; one with spinning sidebands (not shown) and one without at the same chemical shift.

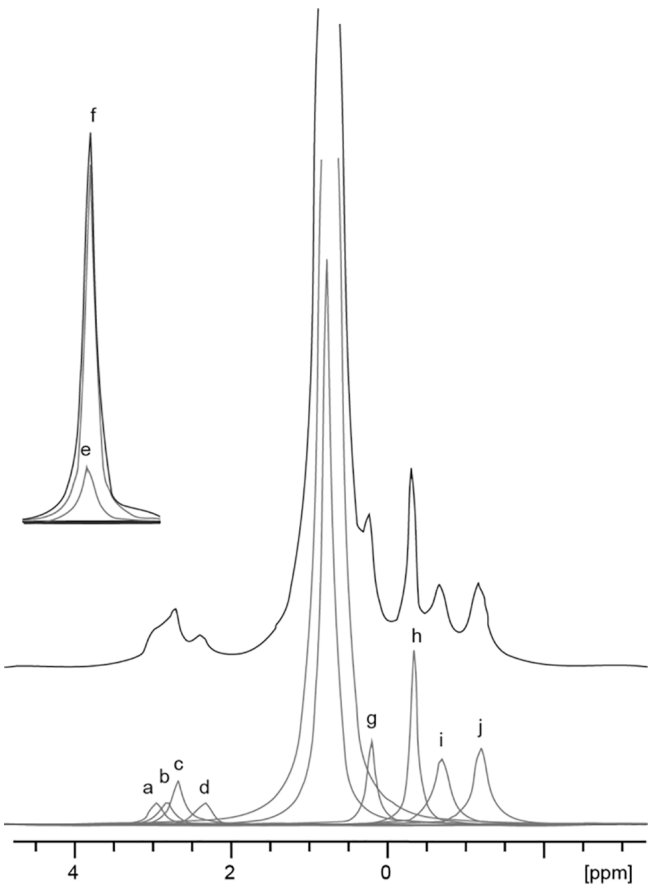

Fig. 2. A simulated MAS (spinning 3500 HZ) spectrum of the 10 species apparent in the wild salmon spectrum show good agreement with the experimental spectrum shown.

found markedly reduced phosphocholine (PC) levels in rat livers following high fish oil consumption, with a combined rise in glycerophosphocholine (GPC) [14]. This is consistent with a shift from the phospholipase C to the phospholipase A1/A2 pathway of phosphatidylcholine breakdown.

Levels of dietary marine omega-3 in farmed salmon feed have decreased markedly over the past two decades, which has changed the ratio of fatty acids in farmed salmon [15,16]. Marine omega-3 s have now to a large extent been replaced by vegetable fatty acids to increase sustainability of fish farming. In a previous solid-state 13C NMR study [16] it was found that the high omega-3 content in the farmed salmon tissue give cellular membranes that do not pack as well as in the wild salmon. This results in higher fluidity of the farmed salmon cellular membrane phospholipids compared to the omega-3 rich wild salmon, possible health effects will likely be investigated in the future.

Due to the widely different diet and way of life, it is reasonable to expect different metabolic profiles of intracellular phosphorous species for farmed and wild salmon. This may in turn directly or indirectly relate to salmon health. In this study we have used ^{31}P solid-state NMR for the first time to investigate differences in phosphorous metabolites in salmon. The chemical shift anisotropy (CSA) of phosphorous originates in reduced mobility of the molecules in that the effects of orientation dependent interaction with the magnetic field will not be averaged to one chemical shift value, the isotropic value. The statistical distribution of phospholipids will produce a characteristic broad line-shape for superimposed resonances, the CSA. The isotropic shifts of these phosphorous resonances are found from ^{31}P MAS experiments. When the sample spinning in Hz is less than the CSA (in Hz) the spectrum will contain spinning sidebands in addition to a resonance at the isotropic chemical shift.

Magic angle spinning (MAS) gives resolved spectra and allows metabolites to be studied directly in tissues *ex situ*. Farmed (Vågen Seafood, Norway) and wild (Alta River, Norway) Atlantic salmons are compared to identify possible differences. Samples are taken of skeletal muscle at the so-called Norwegian Quality Cut (NQT) [17]. The muscle sample was placed in 4 mm (80 μL) zirconia MAS rotors. Skeletal muscle inflammation is a common cause of mortality in farmed salmon [18], and it is thus interesting to **understand** the differences in intracellular biochemistry between farmed and wild salmon for such samples.

Fig. 1 shows resolved MAS (3500 Hz) and static ^{31}P spectra of skeletal muscle from a wild (A) and farmed (B) Atlantic salmon. The spinning spectra are cut at about the top level of the static spectrum. Labels a-j are at isotropic chemical shifts of phosphorous species, the intense peak labelled e, f corresponds to two resonances where one has spinning sidebands (not shown) and the other is without spinning sidebands at the same isotropic chemical shift.Fig. 1 C shows an enhancement of the 2–3 ppm region. It is obvious that there is a larger variety of phosphorous metabolites in the wild salmon sample, with ten observable resonances, compared to only six observable resonances in the farmed salmon sample.

^{31}P chemical shift values are referenced by external calibration with 85% H_3PO_4, where the resonance of H_3PO_4 is set to zero. The larger resonance at about 0.75 ppm in both spectra is inorganic phosphorous (P_i). It should be noted that the ^{31}P MAS NMR spectrum of muscle tissues will change with time after resection of the tissues. This is primarily due to hydrolysis of phosphocreatine, which also generates inorganic phosphate. Hence, a few hours following tissue resection, the

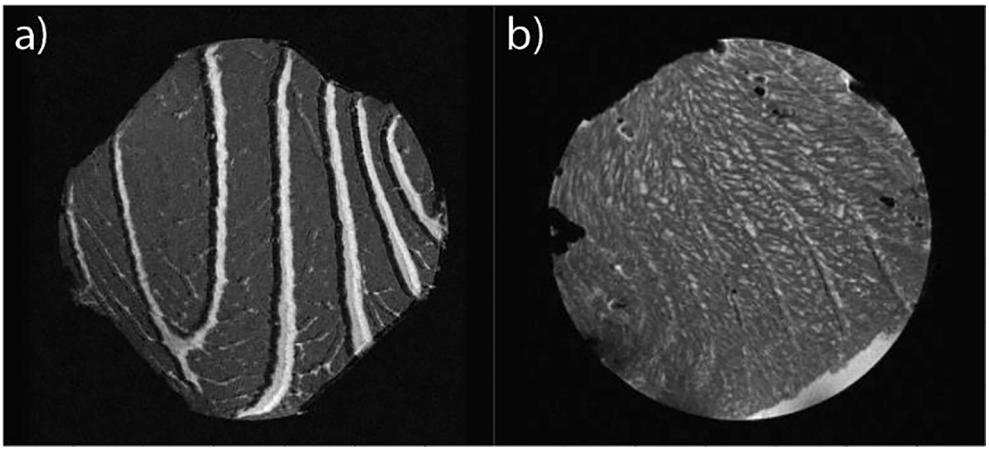

Fig. 3. MR images of salmon fillets (upper row) taken from farmed (a) and wild (b) species. In the fillets the average T_2 measured in the connective tissue has values of (54 ± 3) ms and (35 ± 3) ms for farmed and wild salmon, respectively. The corresponding T2 values for muscle tissue in the fillets were (40 ± 3) ms and (20 ± 3) ms, respectively.

phosphocreatine peak is not expected to be found in the spectrum, in favour of an increased signal from inorganic phosphorous [19]. A similar process will occur for adenosine triphosphate (ATP). Thus, in our spectra peaks from these molecules, which are involved in the energy production of the cells, will not give resonances. Instead, peaks will originate from molecules such as sugar phosphates, PME and PDE, including phosphatidylcholine (PC), GPC, *glycero*-phospho-ethanolamine (GPE) and phosphoenolpyruvate.

In Fig. 2, the MAS ^{31}P spectrum of the wild salmon sample is simulated to better visualize different peaks. A simulated spinning (3500 Hz) spectrum of the 10 species apparent in the wild salmon spectrum show good agreement with the experimental spectrum shown. The simulated spectrum was calculated in the "Topsolids" software (Bruker). It can be seen that the P_i resonance is made up of two overlapping resonances, which correspond to intracellular and extracellular P_i. This is evident by only one resonance giving spinning side bands, meaning only one resonance originates from intracellular motionally restricted P^i. The resonances downfield from P_i (*a-d*) originate from sugar phosphates and phosphomonoesters, including the important phospholipid precursors phosphoetanolamine and phosphocholine. Except for resonance *d*, which is common for both the wild and farmed sample, the spectra deviate. In particular, the wild sample has a pronounced resonance *c*, whereas the farmed sample lack both resonance *a*, *b* and has only a small peak at the position of resonance *c*. Based on literature [20], it is likely that resonances *a-c* are glucose-6-phosphate, fructose-6-phosphate and fructose 1-phosphate, although the precise assignment is unknown due to significant overlap of these resonances. Glucose-6-phosphate is the key to both glycolysis and the pentose phosphate pathway, which are two major metabolic pathways in the cells. Fructose-6-phosphate also play an important role in glycolysis as the isomerized product of glucose-6-phosphate. It should be noted that there is an additional sugar phosphate resonance in the farmed sample, which is not present in the wild sample. This resonance is located slightly up-field from resonance *d*, at 2.1 ppm (see Fig. 1 C). This resonance may be assigned to ribose-5-phosphate [20], which is the last step of the pentose phosphate pathway, and presence of this resonance may as such explain the reduction/absence of glucose-6-phosphate and fructose-6-phosphate in the farmed sample. However, this resonance is also located at the expected position of PC [14].

The spectral region with the most pronounced differences includes resonances *g-j*. Here, for example GPC, glycerophosphoetanolamine (GPE) and phosphoenolpyruvate will resonate. In this spectral region, there is no overlap of the wild and farmed spectra.

The chemical shift of resonance *g* and *h* relative to P_i indicates GPE and GPC, respectively [14]. The presence of these resonances in the wild

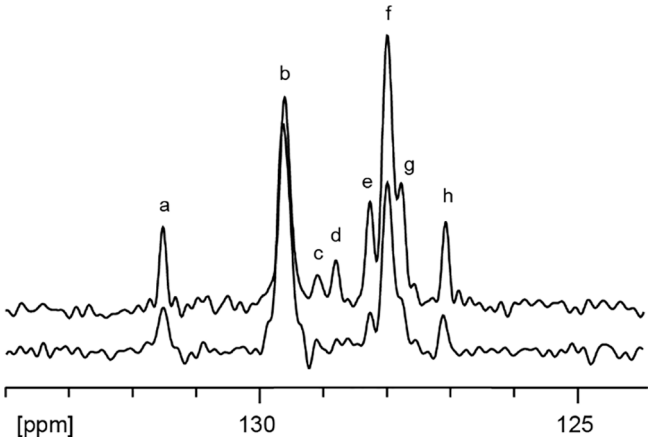

Fig. 4. ^{13}C NMR spectra of C = C region show: Top: Wild salmon resonances labelled a: EPA C18/DHA C20/ALA C16; b: monounsaturated fatty acid (f.a.); c: DHA C5; d: EPA C5-C6; e: highly unsaturated f.a.; f: polyunsaturated f.a.; g: not identified f.a.; h: EPA C17/DHA C19. Bottom: Corresponding resonances from farmed salmon.

sample, and corresponding absence in the farmed sample, may suggest different routes of phospholipid degradation. For example, [14] found that a diet rich in marine omega-3 promotes presence of GPC due to the phospholipase A1/A2 pathway being preferred for phosphatidylcholine breakdown.

Resonances *i* and *j* are broader, which means these originate from more motionally restricted molecules, such as phospholipids. In fact, line broadening is generally more pronounced in the spectra of the wild sample. T_2 values for muscle tissue, measured in a series of different region of interests in a MRI image (data not shown), were 40 ± 3 ms and 20 ± 3 ms for farmed and wild salmon, respectively (Fig. 3). MR images of salmon fillets (upper row) taken from farmed (a) and wild (b) species. In the fillets, the average T_2 measured in a series of different regions of interest (0.2 mm × 0.2 mm) in the connective tissue has values of (54 ± 3) ms and (35 ± 3) ms for farmed and wild salmon, respectively. The corresponding T_2 values for muscle tissue in the fillets were (40 ± 3) ms and (20 ± 3) ms, respectively. These values will primarily reflect H_2O mobility, where a lower value indicates lower mobility, and further demonstrate the different chemical environments of the two samples, causing differences in molecular mobility.

Fig. 4 shows the C=C region of ^{13}C MAS spectra recorded from the same salmon samples, showing resonances from unsaturated fatty acids.

The assignment of the resonances in the C=C region of the ^{13}C NMR spectra of wild and farmed salmon are as follows (see labelling in Fig. 4): a) EPA C18, DHA C20 and alpha-linolenic acid (ALA) C16; b) monounsaturated fatty acids (f.a.); c) DHA C5; d) EPA C5-C6; e) highly unsaturated f.a.; f) polyunsaturated f.a.; g): not identified f.a.; h) EPA C17 and DHA C19. Fatty acid methyl resonance at 14.1 ppm is used as chemical shift reference. Both spectra are obtained with high power broad band decoupling, a waiting time in between transients of 10 s and 256 transients. It is evident from the spectra that there are major differences in the amount and composition of fatty acids between the farmed and wild salmon sample, with a higher amount of PUFA in the wild sample, and more monounsaturated fatty acids in the farmed sample. Hence, the acyl chains would be expected to differ in phospholipids from the two samples, although further investigations are needed to fully explain the considerable deviations in the two ^{31}P spectra (Fig. 1).

In summary, our results indicate that there are significant differences in phosphorous metabolites between farmed and wild salmon. The results e.g. suggest that there may be deviations in phospholipid membrane composition, and thereby membrane fluidity and function. It is demonstrated that ^{31}P MAS NMR can be used to study a broad range of phosphorus metabolites *ex vivo*, which can give useful information, both on its own or as a supplement to other extraction-based analyses. Further ^{31}P MAS NMR investigations on farmed salmon raised under different controlled conditions may give important insights into the broad array of health issues seen in farmed salmon.

CRediT authorship contribution statement

Christian Totland: Writing – original draft. **Signe Steinkopf:** Data curation, Supervision. **Lisa Tu Storhaug:** Investigation. **John Georg Seland:** Methodology. **Willy Nerdal:** Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

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.

References

[1] V.H.S. Oliveira, K.R. Dean, L. Qviller, C. Kirkeby, B. Bang Jensen, Factors associated with baseline mortality in Norwegian Atlantic salmon farming, Sci. Rep. 11 (1) (2021).
[2] D.I. Hoult, S.J.W. Busby, D.G. Gadian, G.K. Radda, R.E. Richards, P.J. Seeley, Observation of tissue metabolites using 31P nuclear magnetic resonance, Nature 252 (5481) (1974) 285–287.
[3] R.G. Shulman, T.R. Brown, K. Ugurbil, S. Ogawa, S.M. Cohen, J.A. den Hollander, Cellular applications of 31P and 13C nuclear magnetic resonance, Science 205 (4402) (1979) 160–166.
[4] V.W. Dennis, Phosphate metabolism: contribution of different cellular compartments, Kidney Int. 49 (4) (1996) 938–942.
[5] T.O. Carpenter, Primary Disorders of Phosphate Metabolism, MDText.com Inc, South Dartmouth (MA), 2000.
[6] P.G. Fjelldal, T. Hansen, S. Albrektsen, Inadequate phosphorus nutrition in juvenile Atlantic salmon has a negative effect on long-term bone health, Aquaculture 334–337 (2012) 117–123.
[7] T.W.K. Fraser, P.E. Witten, S. Albrektsen, O. Breck, R. Fontanillas, L. Nankervis, T. H. Thomsen, W. Koppe, F. Sambraus, P.G. Fjelldal, Phosphorus nutrition in farmed Atlantic salmon (Salmo salar): life stage and temperature effects on bone pathologies, Aquaculture 511 (2019) 734246.
[8] Q. Liu, H. Ye, M. Xu, K. Mai, Z. Sun, X. Tan, C. Zou, S. Chen, N. Su, Y. Zhou, L. Chen, C. Ye, The effect and mechanism of dietary monocalcium phosphate restriction on hepatic lipid deposition and immune status in obscure puffer, Takifugu obscurus, Aquaculture 524 (2020) 735261.
[9] P.G. Fjelldal, T. Hansen, O. Breck, R. Sandvik, R. Waagbø, A. Berg, R. Ørnsrud, Supplementation of dietary minerals during the early seawater phase increase vertebral strength and reduce the prevalence of vertebral deformities in fast-growing under-yearling Atlantic salmon (Salmo salar L.) smolt, Aquac. Nutr. 15 (2009) 366–378.
[10] R.P. Mason, R.F. Jacob, S. Shrivastava, S.C.R. Sherratt, A. Chattopadhyay, Eicosapentaenoic acid reduces membrane fluidity, inhibits cholesterol domain formation, and normalizes bilayer width in atherosclerotic-like model membranes, Biochim. Biophys. Acta (BBA) – Biomembr. 1858 (12) (2016) 3131–3140.
[11] W. Stillwell, S.R. Wassall, Docosahexaenoic acid: membrane properties of a unique fatty acid, Chem. Phys. Lipids 126 (1) (2003) 1–27.
[12] R.C. Valentine, D.L. Valentine, Omega-3 fatty acids in cellular membranes: a unified concept, Prog. Lipid Res. 43 (5) (2004) 383–402.
[13] T.A. Kellner, N.K. Gabler, J.F. Patience, The composition of dietary fat alters the transcriptional profile of pathways associated with lipid metabolism in the liver and adipose tissue in the pig1, J. Anim. Sci. 95 (2017) 3609–3619.
[14] P.C. Dagnelie, J.D. Bell, I.J. Cox, D.K. Menon, J. Sargentoni, G.A. Coutts, S.C. R. Williams, Effects of fish oil on phospholipid metabolism in human and rat liver studied by 31P NMR spectroscopy in vivo and in vitro, NMR Biomed. 6 (2) (1993) 157–162.
[15] M. Sprague, J.R. Dick, D.R. Tocher, Impact of sustainable feeds on omega-3 long-chain fatty acid levels in farmed Atlantic salmon, 2006–2015, Sci. Rep. 6 (2016) 21892.
[16] C. Totland, J.G. Seland, S. Steinkopf, W. Nerdal, Analysis of wild and farmed salmon using 13C solid-state NMR and MRI directly on fillet tissue, Anal. Methods 9 (8) (2017) 1290–1296.
[17] C.A. Johnsen, Ø. Hagen, E.Å. Bendiksen, Long-term effects of high-energy, low-fishmeal feeds on growth and flesh characteristics of Atlantic salmon (Salmo salar L.), Aquaculture 312 (1-4) (2011) 109–116.
[18] G. Palacios, M. Lovoll, T. Tengs, M. Hornig, S. Hutchison, J. Hui, R.-T. Kongtorp, N. Savji, A.V. Bussetti, A. Solovyov, A.B. Kristoffersen, C. Celone, C. Street, V. Trifonov, D.L. Hirschberg, R. Rabadan, M. Egholm, E. Rimstad, W.I. Lipkin, B. Lindenbach, Heart and skeletal muscle inflammation of farmed salmon is associated with infection with a novel reovirus, PLoS ONE 5 (7) (2010) e11487.
[19] J. Chen, Y. Wu, P. DeCarolis, R. O'Connor, C. Somberg, S. Singer, Resolution of creatine and phosphocreatine H signals in isolated human skeletal muscle using HR-MAS H NMR, Magn. Resonance Med. 59 (2008) 1221–1224.
[20] J.D. Bentley, J.E. Jentoft, D. Foreman, D. Ambrose, 31P nuclear magnetic resonance (NMR) identification of sugar phosphates in isolated rat ovarian follicular granulosa cells and the effects of follicle-stimulating hormone, Mol. Cell. Endocrinol. 73 (2-3) (1990) 179–185.