

# A Whole Body, In Vivo, Fatty Acid Balance Method to Quantify PUFA Metabolism (Desaturation, Elongation and Beta-oxidation)

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**Abstract** Currently there are several contrasting methods utilized for estimating elongation and desaturation of fatty acids and their general metabolism. The majority of these methods involve an ex vivo approach, requiring expensive and sophisticated equipment, likely to result in considerable variation in enzyme activity between and within species. In the present paper we introduce a further development of the whole-body fatty acid balance method for the estimation of the elongation and desaturation of fatty acids. This method though receiving considerable attention because of its simplicity and reliability has yet to be presented in detail. Theoretically, the whole-body fatty acid balance method can potentially be applied to any organism and requires little more than a gas chromatography unit for fatty acid analysis and elementary calculations. As such in this paper we attempt to spell out in detail the theoretical basis and the methods of application drawing specific examples. Using the present method it is possible to measure the fate of individual fatty acids towards desaturation, elongation and oxidation and calculate the elongase,  $\Delta$ -6 desaturase and  $\Delta$ -5 desaturase activities.

**Keywords** Desaturase · Elongase · Fatty acids · Lipid metabolism · Oxidation

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## Introduction

Animals have the ability to convert 18:3n-3 ( $\alpha$ -linolenic acid) to 20:5n-3 (eicosapentaenoic acid) and ultimately 22:6n-3 (docosahexaenoic acid), and 18:2n-6 (linoleic acid) to 20:4n-6 (arachidonic acid) [1]. The first step in the fatty acid elongation and desaturation pathway is the production of 18:3n-6 from 18:2n-6 and of 18:4n-3 from 18:3, each catalyzed by the  $\Delta$ -6 desaturase enzyme. The two products can then be successively elongated by fatty acid elongase and further desaturated by the  $\Delta$ -5 desaturase enzyme to 20:4n-6 and 20:5n-3, respectively. The eventual production of 22:6n-3 requires the combination of a further elongation, a  $\Delta$ -6 desaturation and finally, a chain-shortening reaction [2].

A variety of methods exist for the assessment of fatty acid metabolism ex vivo and in vivo, recently reviewed in detail by Brown [3]. Of the ex vivo methods, the most widely used approach involves the isolation of whole cells (i.e. hepatocytes) or tissue microsomes and their subsequent incubation with labeled fatty acids (i.e. [1-<sup>14</sup>C]18:2n-6 or [1-<sup>14</sup>C]18:3n-3). These analytical methods require expensive and sophisticated equipment, generally unavailable to a majority of research institutions. Furthermore, these methods vary greatly between applications and may lack controlling factors (i.e. fatty acid-binding proteins) and other pathways (i.e.  $\beta$ -oxidation) which are important within the overall scheme of fatty acid metabolism. Alternately, in vivo methods employ a whole-body approach and offer an estimate of an organism's overall capacity to metabolize fatty acids within the context of an integrated system.

The whole-body fatty acid balance method, initially proposed by Cunnane and Anderson [4] on rats, and further developed and applied to finfish by Turchini et al. [5, 6] is

the latest and simplest in vivo method that addresses the capability of an organism to produce highly unsaturated fatty acids (HUFA). In the present note on methodology, information and computations required to carry out the whole-body fatty acid balance method are presented.

## Description of the Method

For the execution of the whole-body fatty acid balance method a feeding trial is a fundamental necessity. The basic concept on which the method is based on is the quantification of the initial and final fatty acid composition of the whole-body and the quantification of the net intake of dietary fatty acids, based on a feeding trial.

The feeding trial must be of a sufficient duration to guarantee an adequate weight gain in order to enable an accurate quantification of fatty acid elongation and desaturation. Moreover, as the present method is fundamentally a mass balance analysis, by increasing the difference between the factors, it is possible to increase the sensitivity of the measurement. It is crucial that the feed intake is measured accurately. The total collection of faeces or a corresponding digestibility estimation using an appropriate dietary marker is essential for the quantification of the net intake of fatty acids.

The type of diet used for the whole-body fatty acid balance method is unimportant given that its proximate and fatty acid compositions are known. However, for the quantification of elongase and desaturase activities a diet containing minimal amounts of these enzyme products (i.e. HUFA) is desired. Considering the method is based mainly on a mass balance analysis of individual fatty acids, a high intake of HUFA can mask the appearance of newly elongated and/or desaturated fatty acids [5].

The analyses required for the subsequent computations of the whole-body fatty acid balance method are: the initial and final body weight, initial and final quantitative fatty acid composition of the whole body, the total feed intake, the quantitative fatty acid composition of the diet and the fatty acid digestibility or the quantitative fatty acid composition of the total faeces produced during the experiment.

The computation of the whole-body fatty acid balance method is best dealt with in four steps.

The first step is to determine the partitioning of the dietary fatty acids amongst excretion, accumulation, appearance or disappearance, as described previously by Cunnane and Anderson [4].

The individual fatty acid amounts in diet, faeces, initial and final carcass need to be expressed in mg per animal. After calculating the individual fatty acid (FA) intake (= g of feed intake  $\times$  mg of FA per g of feed), excretion (= mg of FA intake  $\times$  FA digestibility; or = g of faeces  $\times$  mg of FA per g of faeces) and accumulation (= mg of FA in final carcass – mg of FA in initial carcass) it is possible to estimate the fatty acid appearance or disappearance (= FA accumulation – FA intake – FA excretion) (Fig. 1).

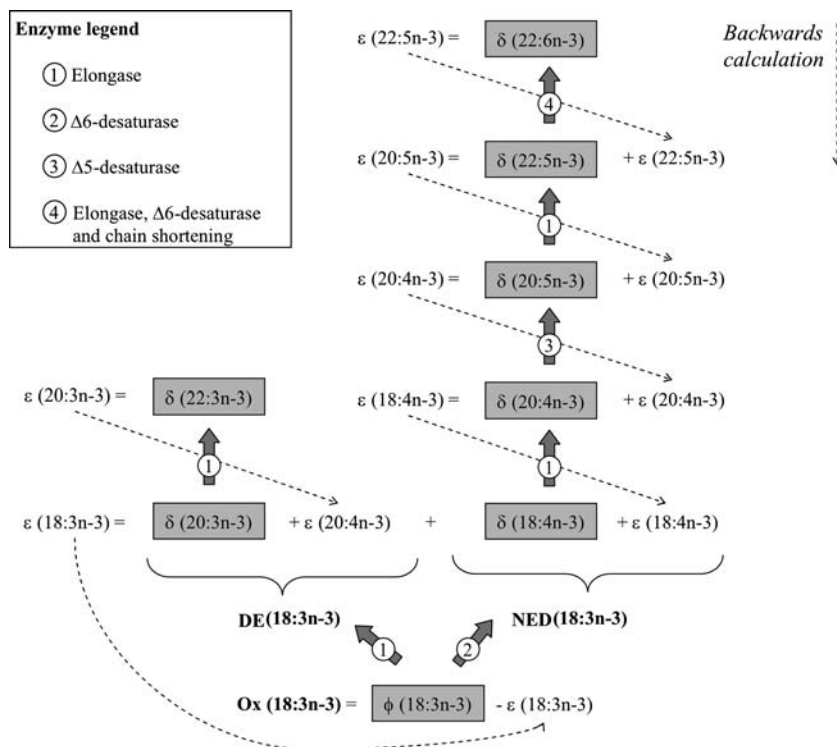
A disappearance of fatty acids may involve their elongation and desaturation to longer chain fatty acids or utilization of their carbon skeleton through  $\beta$ -oxidation for energy production. For purposes of this communication the metabolic pathway of  $\alpha$ -linolenic acid (18:3n-3) is presented. The metabolic pathways of other fatty acids are simpler and can therefore be deduced accordingly.

The common 18:3n-3 metabolic pathway can be subdivided into three categories: chain-shortening and oxidation (Ox), elongation towards dead end products (DE) and the normal elongation/desaturation pathway (NED). The fatty acids involved in the 18:3n-3 DE pathway are 20:3n-3 and 22:3n-3. While those involved in the 18:3n-3 NED pathway include 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3. The production 22:6n-3 involves a further elongation of 22:5n-3 to 24:5n-3, a  $\Delta$ -6 desaturation of 24:5n-3 to 24:6n-3 and a chain-shortening reaction from 24:6n-3 to 22:6n-3 [2]. However, 24:5n-3 and 24:6n-3 are seldom present in detectable amounts and therefore, the passage from 22:5n-3 to 22:6n-3 is in the present computation considered as a sole elongase,  $\Delta$ -6 desaturase and chain-shortening reaction.

The second step of the method involves the computation of the 18:3n-3 balance. The amount of fatty acids represented in the 18:3n-3 metabolic pathway needs to be converted from mg to mmol of appeared/disappeared FA per animal.

**Fig. 1** Schematic representation of the mass balance analysis of the individual fatty acid intake and accumulation. The first step in the whole-body fatty acid balance method

<b>Final fatty acid body content</b> - <b>Initial fatty acid body content</b> =		<b>Fatty acid intake</b> - <b>Fatty acid excretion</b> =	
<b>Fatty acid accumulation</b>	-	<b>Fatty acid net intake</b>	= <b>Fatty acid appearance/disappearance</b>



**Fig. 2** Schematic representation of the computation involved in the whole-body fatty acid balance method. Shaded figures [ $\delta$  values i.e.  $\delta(22:6n-3)$ ,  $\delta(22:5n-3)$ , etc.] represent the results obtained following the first step of the method (mmol of the specified FA appeared/disappeared), while the  $\phi$  value (for 18:3n-3 only) represents the total number of mmol of 18:3n-3 disappeared (given that no appearance is possible in vertebrates). Via a backwards calculation, starting from the end of the two pathways it is possible to calculate the  $\epsilon$  values,

which represent the mmol of specified FA converted to longer or more unsaturated homologues. Successively, it is possible to quantify the fate of  $\alpha$ -linolenic acid (18:3n-3) as (i) chain shortening and oxidation (Ox), (ii) elongation towards a dead end product (DE) or (iii) elongation and desaturation along the normal pathway (NED). Ultimately, it is possible to estimate the elongase,  $\Delta$ -5 and  $\Delta$ -6 desaturase activities

The number of mmol of longer chain fatty acids that appeared is subtracted from the number of mmol of the previous fatty acid in the fatty acid elongation/desaturation pathway. A diagram depicting the backwards calculation along the fatty acid metabolic pathways is provided (Fig. 2). The mathematical model used for the whole-body fatty acid balance computation for n-3 fatty acids is described by the following equations (Eqs. 1, 2, 3, 4, 5, 6) [where:  $\epsilon$  = mmol of specified FA converted (elongated or desaturated);  $\delta$  = mmol of the specified FA appeared/disappeared per animal; if  $\delta$  is a negative number (FA disappeared = oxidized), then  $\delta = 0$  for the following computation]:

NED pathway:

$$\epsilon(22:5n-3) = \delta(22:6n-3) \tag{1}$$

$$\epsilon(20:5n-3) = \delta(22:5n-3) + \epsilon(22:5n-3) \tag{2}$$

$$\epsilon(20:4n-3) = \delta(20:5n-3) + \epsilon(20:5n-3) \tag{3}$$

$$\epsilon(18:4n-3) = \delta(20:4n-3) + \epsilon(20:4n-3) \tag{4}$$

DE pathway:

$$\epsilon(20:3n-3) = \delta(22:3n-3) \tag{5}$$

Total 18:3n-3 balance:

$$\epsilon(18:3n-3) = \delta(20:3n-3) + \epsilon(20:3n-3) + \delta(18:4n-3) + \epsilon(18:4n-3) \tag{6}$$

The estimation of the fate (elongation, desaturation or oxidation) of each fatty acid can therefore be computed accordingly to its specific metabolic pathway; for example 18:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 22:4n-6 or 18:1n-9, 18:2n-9, 20:2n-9, 20:3n-9, and pathways involving the  $\Delta$ -9 desaturase enzyme such as 16:0, 16:1n-7, 18:1n-7 can be evaluated as well.

At this point (the third step of the method) it is possible to quantify the amount of 18:3n-3 used in  $\beta$ -oxidation or elongated and desaturated (Fig. 2). The total 18:3n-3 balance can therefore be delineated by the following equations (Eqs. 7, 8, 9, 10, 11, 12) [where: DE(18:3n-3) = total 18:3n-3 elongated to dead end products; NED(18:3n-3) = total 18:3n-3 converted through the normal elongation/desaturation pathway; Ox(18:3n-3) = total 18:3n-3 oxidized;  $\phi$  (18:3n-3) = total number of mmol of 18:3n-3 disappeared]:

$$\text{DE}(18:3n-3) = \delta(20:3n-3) + \varepsilon(20:3n-3) \quad (7)$$

$$\text{NED}(18:3n-3) = \delta(18:4n-3) + \varepsilon(18:4n-3) \quad (8)$$

$$\text{Ox}(18:3n-3) = \phi(18:3n-3) - \text{DE}(18:3n-3) - \text{NED}(18:3n-3) \quad (9)$$

The fate of 18:3n-3 can be further reported as a percentage relative to its total net intake (NI; calculated from the first step of the method; NI = FA intake – FA excretion and converted into mmol)

$$\begin{aligned} \text{\% of 18:3n-3 elongated to dead end products} \\ = \text{DE}(18:3n-3) \times \text{NI}(18:3n-3)^{-1} \times 100 \end{aligned} \quad (10)$$

$$\begin{aligned} \text{\% of 18:3n-3 elongated and desaturated} \\ = \text{NED}(18:3n-3) \times \text{NI}(18:3n-3)^{-1} \times 100 \end{aligned} \quad (11)$$

$$\begin{aligned} \text{\% of 18:3n-3 oxidized} \\ = \text{Ox}(18:3n-3) \times \text{NI}(18:3n-3)^{-1} \times 100 \end{aligned} \quad (12)$$

Ultimately, it is possible to estimate the total elongase,  $\Delta$ -5 and  $\Delta$ -6 desaturase activities with the fourth and final step of the method. The enzyme activity is expressed as mmol of products per gram of body weight (average body weight) per day with the following equations (Eqs. 13–16):

$$\begin{aligned} \text{Gram of BW day}^{-1} \\ = (\text{initial body weight} + \text{final body weight}) \\ \times 2^{-1} \times \text{no. of days}^{-1} \end{aligned}$$

$$\begin{aligned} \Delta\text{-6 desaturase} = [\delta(18:4n-3) + \varepsilon(18:4n-3) + \delta(22:6n-3)] \\ (\text{gram of BW day}^{-1})^{-1} \end{aligned} \quad (14)$$

$$\begin{aligned} \Delta\text{-5 desaturase} = [\delta(20:5n-3) + \varepsilon(20:5n-3)] \\ (\text{gram of BW day}^{-1})^{-1} \end{aligned} \quad (15)$$

$$\begin{aligned} \text{Elongase} = [\delta(20:3n-3) + \varepsilon(20:3n-3) + \varepsilon(18:4n-3) \\ + \varepsilon(20:5n-3) + \varepsilon(22:5n-3)](\text{gram of BW day}^{-1})^{-1} \end{aligned} \quad (16)$$

## Computation example

The following example is reported to further clarify the steps in the calculations for the execution of the presented method. The example is based on data reported previously [5] on the freshwater fish Murray cod (*Maccullochella peelii peelii*) fed a semi purified diet containing linseed oil as the principal lipid source for 112 days. The sample preparation is of paramount importance for the proper functioning of the method. To properly quantify the fatty acid content of the whole body, the entire specimen (or pooled specimens) needs to be chopped and subsequently finely minced to obtain a representative and homogenous sample. Admittedly, this is a limit of the method and it can be difficult for large animals or animals with tough tissues. With fish samples, we find the repeated utilization of a heavy duty meat mincer, with a reduction of the die size after each pass, well suited for homogenization. Lipid extraction should then be performed on a relatively large sample, on as many replicates as possible to improve the accuracy of the quantification and accordingly to standardized reliable protocols [7]. A second, equally important aspect is to implement a proper chromatographic quantitative procedure, as the simple, commonly adopted evaluation of fatty acids as a percentage value is not informative enough and can not be utilised. The use of a suitable internal standard and the correction by theoretical relative FID response factors of the resulting peak areas are essential for accurate FA quantification [8].

In Table 1, the results obtained from the calculation of the first step of the whole-body fatty acid balance method are reported. Subsequently, following the second step of the whole-body fatty acid balance method it is possible to calculate the  $\varepsilon$  values (Fig. 2) along the NED and DE pathways:

(Eq. 1):

$$\varepsilon(22:5n-3) = \delta(22:6n-3) = 12.8(\text{mmol per fish})$$

(Eq. 2):

$$\begin{aligned} \varepsilon(20:5n-3) = \delta(22:5n-3) + \varepsilon(22:5n-3) \\ = 123.2 + 12.8 = 136.0(\text{mmol per fish}) \end{aligned}$$

(Eq. 3):

$$\begin{aligned} \varepsilon(20:4n-3) = \delta(20:5n-3) + \varepsilon(20:5n-3) = -111.4 + 136.0 \\ = 24.6(\text{mmol per fish}) \end{aligned}$$

**Table 1** The first step of the whole-body fatty acid balance method; data is relative to the n-3 fatty acid balance during a 112-day period on Murray cod fed a linseed oil based diet [5]

	18:3n-3	18:4n-3	20:3n-3	22:3n-3	20:4n-3	20:5n-3	22:5n-3	22:6n-3
mg FA per fish								
Intake	4561.8	18.7	ND	ND	ND	46.2	10.4	72.3
Excretion	72.1	ND	ND	ND	ND	2.3	ND	4.7
Initial body content	17.5	21.7	1.7	ND	12.8	82.3	51.8	153.8
Final body content	2377.8	284.2	75.2	ND	137.4	92.5	102.9	225.7
Accumulation	2360.3	262.5	73.6	ND	124.6	10.2	51.1	71.9
Appearance/disappearance								
	18:3n-3	18:4n-3	20:3n-3	22:3n-3	20:4n-3	20:5n-3	22:5n-3	22:6n-3
mg FA per fish								
	-2129.4	243.8	73.6	ND	124.6	-33.7	40.7	4.2
	$\phi(18:3n-3)$	$\delta(18:4n-3)$	$\delta(20:3n-3)$	$\delta(20:3n-3)$	$\delta(20:4n-3)$	$\delta(20:5n-3)$	$\delta(22:5n-3)$	$\delta(22:6n-3)$
mmol FA per fish								
	7646.0	881.6	240.0	ND	409.3	-111.4	123.2	12.8

In the last row,  $\delta$  values represent the mmol of the specified FA appeared or disappeared (if value is positive or negative, respectively), while the  $\phi$  value (for 18:3n-3 only) represents the total number of mmol of 18:3n-3 disappeared

ND not detected

(Eq. 4):

$$\begin{aligned}\varepsilon(18:4n-3) &= \delta(20:4n-3) + \varepsilon(20:4n-3) = 409.3 + 24.6 \\ &= 433.9(\text{mmol per fish})\end{aligned}$$

(Eq. 5):

$$\varepsilon(20:3n-3) = \delta(22:3n-3) = 0(\text{mmol per fish})$$

Once the individual  $\varepsilon$  values which represent the mmol of specified FA converted to longer or more unsaturated homologues have been calculated, it is possible to calculate the total amount of 18:3n-3 elongated and desaturated through both of the pathways:

(Eq. 6):

$$\begin{aligned}\varepsilon(18:3n-3) &= \delta(20:3n-3) + \varepsilon(20:3n-3) + \delta(18:4n-3) \\ &+ \varepsilon(18:4n-3) = 240.0 + 0 + 881.6 + 433.9 \\ &= 1555.5(\text{mmol per fish})\end{aligned}$$

Through the third step of the method, it is possible to quantify the amount of 18:3n-3 used in  $\beta$ -oxidation or elongated and desaturated (Fig. 2).

(Eq. 7):

$$\begin{aligned}\text{DE}(18:3n-3) &= \delta(20:3n-3) + \varepsilon(20:3n-3) \\ &= 240 + 0 = 240(\text{mmol per fish})\end{aligned}$$

(Eq. 8):

$$\begin{aligned}\text{NED}(18:3n-3) &= \delta(18:4n-3) + \varepsilon(18:4n-3) \\ &= 881.6 + 433.9 = 1315.5(\text{mmol per fish})\end{aligned}$$

(Eq. 9):

$$\begin{aligned}\text{Ox}(18:3n-3) &= \phi(18:3n-3) - \text{DE}(18:3n-3) \\ &- \text{NED}(18:3n-3) = 7646.0 - 240 - 1315.5 \\ &= 6090.5(\text{mmol per fish})\end{aligned}$$

At this stage, with Eqs. 10, 11 and 12, the fate (DE, NED or Ox) of 18:3n-3 can be additionally reported as a percentage relative to its total net intake; in this example 1.5, 8.2 and 37.8%, respectively.

Ultimately, with the fourth and final step, through Eqs. 13, 14, 15, 16 it is possible to estimate the total elongase,  $\Delta$ -5 and  $\Delta$ -6 desaturase activities expressed as mmol of product per gram of body weight per day. In the example reported the initial fish weight was 21.3, final fish weight was 74.6 and the experimental period was 112 days, therefore (Eq. 13) the grams of BW day<sup>-1</sup> equates to 5,370.

(Eq. 14):

$$\begin{aligned}\Delta\text{-6 desaturase} &= [\delta(18:4n-3) + \varepsilon(18:4n-3) + \delta(22:6n-3)] \\ &(\text{gram of BW day}^{-1})^{-1} = (881.6 + 433.9 + 12.8) \\ &\times (5370)^{-1} = 0.24735(\text{mmol g}^{-1}\text{day}^{-1})\end{aligned}$$

(Eq. 15):

$$\begin{aligned}\Delta-5 \text{ desaturase} &= [\delta(20:5n-3) + \varepsilon(20:5n-3)] \\ (\text{gram of BW day}^{-1})^{-1} &= (-111.4 + 136.0) \times (5370)^{-1} \\ &= 0.00458(\text{mmol g}^{-1}\text{day}^{-1})\end{aligned}$$

(Eq. 16):

$$\begin{aligned}\text{Elongase} &= [\delta(20:3n-3) + \varepsilon(20:3n-3) + \varepsilon(18:4n-3) \\ &+ \varepsilon(20:5n-3) + \varepsilon(22:5n-3)](\text{gram of BW day}^{-1})^{-1} \\ &= (240.0 + 0 + 433.9 + 136.0 + 12.8) \times (5370)^{-1} \\ &= 0.15319(\text{mmol g}^{-1}\text{day}^{-1}).\end{aligned}$$

As previously reported [5], fish were housed in three replicate tanks (30 fish per tank) with four fish culled per tank and analyzed individually in duplicate. The analytical variability recorded was relatively low. For example, the average coefficient of variability ( $\text{st.dev} \times 100 \times \text{mean}^{-1}$ ) of DHA in the whole body within the replicates was 1.43%. However, the biological variability recorded between the four individuals was greater (8.43%), with the highest variability recorded in fish from the same tank ranging from 23 to 28 mg of DHA per g of lipid. Although this information is useful in understanding the analytical and biological variability within individual specimen, the actual replicate has to be considered the tank, hence  $N = 3$ , from a statistical viewpoint. If we were to focus our attention on the total  $\Delta-6$  desaturase activity (Eq. 14) evidenced using the whole body fatty acid balance method, we recorded for the three tanks the values 0.20068, 0.26297 and 0.27840  $\text{mmol g}^{-1} \text{day}^{-1}$ . Clearly this is to be considered as a broad indication for the estimation of appropriate sample size when fish are used. Understandably, the specific variability of an individual sample would need to be taken into consideration if the method was to be applied to a different animal.

## Discussion

A variety of methods have been developed for measuring elongase and desaturase activity. The methodology presented here employs an in vivo approach which provides a reliable estimation of an organism's overall capacity to metabolize fatty acids. To date, to the best of the authors' knowledge, this method has been applied only twice [5, 6], but not presented in detail. With respect to the quantification of elongase and desaturase activity, the results obtained in these studies are highly consistent and in general agreement within the highly variable range of results obtained using ex vivo counterparts. For example,

the  $\Delta-6$  desaturase activity reported on 18:3n-3 in the above mentioned separate studies and measured with the whole-body fatty acid balance method in juvenile fresh-water finfish Murray cod (*Maccullochella peelii peelii*) were  $0.147 \pm 0.006 \text{ mmol g}^{-1} \text{day}^{-1}$  and  $0.247 \pm 0.012 \text{ mmol g}^{-1} \text{day}^{-1}$  in fish fed semi purified diets containing only a blend of vegetable oils [6] and linseed oil [5] as the dietary lipid source. The differences in enzyme activity were ascribable to the difference in the percentage of 18:3n-3 in the diets, which were 25.7 and 55.4%, respectively.

Unfortunately, to date, it has not been possible to directly compare the results obtained by the utilization of the presented method with results from different ex vivo methods because they are generally reported as pmol per hour per gram of protein of the isolated cells, and not relative to the entire organism. Moreover, the two methods have not been implemented on a single species, and therefore a hypothetical comparison would be misleading.

Admittedly, there are certain limitations that can restrict accuracy and applicability of the proposed method. One variable that the whole-body fatty acid balance method does not take into consideration is the allowance of eicosanoid production. However, it is acceptable that the conversion of 20:4n-6 and 20:5n-3 is minimal, having little impact on the total balance of fatty acids [4]. A second variable not taken into consideration is the possible chain-shortening and oxidation of fatty acids previously elongated and desaturated [9]; for example if a given amount of 18:3n-3 is desaturated to 18:4n-3 and successively oxidized it will be considered as an oxidation process of 18:3n-3. However, although oxidation of longer and more unsaturated chain fatty acids is occurring to a lesser extent than their precursor [9], this is a possible occurrence, and hence a limit, also for other methods such as ex vivo approaches that employ whole cells and their incubation with labeled fatty acids. If for example [ $1-^{14}\text{C}$ ] labeled 18:3n-3 is utilised, the radioactive acid-soluble fatty acid oxidation products determined to quantify  $\beta$ -oxidation activity can derive from [ $1-^{14}\text{C}$ ] 18:3n-3 directly oxidized but also from [ $1-^{14}\text{C}$ ] 18:3n-3 previously desaturated to [ $1-^{14}\text{C}$ ] 18:4n-3 and successively oxidized.

In consideration that the method provides an accurate mass balance analysis it can quantify an animal's capability in depositing HUFA. We can therefore conclude that the whole-body fatty acid balance method is a simple, relatively inexpensive, routine laboratory technique and its utilization on different species will be instrumental in expanding our understanding of animal lipid metabolism.

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