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Use of stable isotope-labeled fatty acids to measure desaturase activities with negative chemical ionization GC-MS

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ABSTRACT

Fatty acid desaturases are key enzymes in lipid metabolism. They introduce double bonds between defined carbons of the fatty acyl chain and catalyze rate-limiting steps in the biosynthesis of polyunsaturated fatty acids. For decades, *in vitro* desaturase activities have been determined by using radiolabeled fatty acids as substrates, incubated with tissue or cell fractions containing membrane-bound desaturases. However, handling radioactivity is being increasingly complicated due to safety and regulatory concern. Radiolabeled fatty acids are also expensive and many of them are not commercially available. There is therefore a crucial need to develop new methods. Although methods using unlabeled fatty acids as substrates have recently been validated, they are well suited for large tissue samples and did not achieve the same sensitivity as the radioactive ones. Here, we show that negative chemical ionization GC-MS on stable isotope-labeled fatty acids, derivatized to pentafluorobenzyl esters, now offers this opportunity, because of its high sensitivity in the selected ion monitoring mode. By using this simple and affordable improved method, we measured the kinetic parameters of mouse liver $\Delta 6$ -desaturase for its two main substrates (C18:2 n-6 and C18:3 n-3; 10–13 μM). Moreover, this method enabled to compare $\Delta 5$ -desaturase apparent K_m values (19–22 μM) for its two main substrates (C20:3 n-6 and C20:4 n-3). Finally, we re-evaluated the controversial effect of freezing on desaturase activities by using both frozen rat tissues and cryopreserved human hepatocytes. This safe, reliable and sensitive method may be applied to other enzymatic activities involving fatty acids (elongation, hydroxylation) in miniaturized samples.

1. Introduction

Fatty acid (FA) desaturases are key enzymes in lipid metabolism (Nakamura and Nara, 2004). They introduce double bonds between defined carbons of the fatty acyl chain ($\Delta 9$ -, $\Delta 6$ - and $\Delta 5$ -desaturases in animals) and catalyze rate-limiting steps in the biosynthesis of polyunsaturated fatty acids (PUFAs) (Brenna and Kothapalli, 2022). The amount of synthesized unsaturated FAs, for instance the availability of n-6 and n-3 long chain PUFAs, thereafter regulates many physiological and pathophysiological pathways involved in health and diseases (Dyall et al., 2022). It is therefore critical to be able to determine at any time

the level of these enzyme activities and the biological parameters that regulate their activity.

However, in most studies analyzing the regulation of FA desaturase activities, usually linked to impaired lipid homeostasis or metabolic dysfunction, these activities are not really directly measured (Matsumoto et al., 2021; Moriyama et al., 2022; Vamadeva et al., 2022). Instead, estimated “desaturation index”, also called “desaturase enzyme indices”, are calculated by the FA product to FA substrate ratios and used as indirect proxies for each desaturase (Chajès et al., 2011). The reasons for the indirect use of ratios, often poorly related to real metabolic rate of conversion, are related to the relative complexity of the desaturase

Abbreviations: FAME, Fatty Acid Methyl Ester; GC-FID, Gas Chromatography-Flame Ionization Detector; NCI, Negative Chemical Ionization; PFB esters, Penta-FluoroBenzyl esters; PMS, Post-Mitochondrial Supernatant; SIM, Selected Ion Monitoring; ARA, Arachidonic Acid; TEA, TriEthylAmine; LOQ, Limit Of Quantification; SNR, Signal-to-Noise Ratio.

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assay at several levels (Park et al., 2016).

First, the reference method used for decades is based on radioactivity to achieve sufficient sensitivity (Brenner and Peluffo, 1966). Radiolabeled FA substrates are incubated with prepared post-mitochondrial supernatant (PMS) or microsome fractions, containing membrane-bound desaturases, in the presence of required cofactors (Brenner and Peluffo, 1966; D'andrea et al., 2002; Jan et al., 2004; Rioux et al., 2015). At the end of the incubation, radiolabeled remaining substrate and formed product of each desaturase are separated from each other and subjected to liquid scintillation counting. The product to substrate radioactive ratio is thereafter expressed as pmol substrate converted to product per minute and per mg of protein. In our laboratory, we ourselves have developed in the past a specific high-performance liquid chromatography (HPLC) method (Rioux et al., 1999), more resolutive than the older thin-layer chromatography on silver nitrate-impregnated silica plates (Legrand and Bensadoun, 1991), to separate radiolabeled FAs (substrate and product of desaturation) after their conversion to naphthacyl esters (Blanchard et al., 2013; Rioux et al., 2005). Although this method is reliable and showed high reproducibility to measure the activities of $\Delta 9$ -, $\Delta 6$ - and $\Delta 5$ -desaturases, the use of radiolabeled reagents remains complicated due to safety, regulation and cost. Moreover, these experiments are restricted to the decreasing number of radiolabeled FAs that are commercially available.

Second, in human studies, access to sufficient quantities of liver samples, which is the predominant tissue for desaturase activities (Valenzuela et al., 2024) is obviously a major constraint. Third, although this is still controversial (Rodriguez et al., 1998; Su and Brenna, 1998) because it is known that frozen tissues retain measurable desaturation activities (Valenzuela et al., 2024), membrane-bound FA desaturases are often described as not stable when frozen and it is recommended to measure activities extemporaneously by using fresh tissues or cells (Kato et al., 2006). Fourth, although alternative methods using unlabeled fatty acids as substrates have recently been validated (Metherel et al., 2024; Valenzuela et al., 2024), they are well suited for large tissue samples and did not achieve the same sensitivity as the radioactive ones. There is therefore a strong interest in finding other alternative methods, notably based on nonradioactive stable isotope-labeled FAs, to expand the fundamental and medical applications of metabolic research on FA desaturases.

In 1992, Pawlosky et al. (Pawlosky et al., 1992) actually described the beginnings of such an alternative method. Using deuterated linoleic and α -linolenic acids incubated with rat liver PMS, the FAs were derivatized to pentafluorobenzyl (PFB) esters, followed by a gas chromatography-mass spectrometry (GC-MS) analysis carried out in the negative chemical ionization (NCI) mode. This approach produced little fragmentation during ionization, revealing a large amount of an intact carboxylate [M-PFB] anion, instead of a large number of fragment ions and the loss of the molecular ion typically observed in electronic impact (EI) with PUFAs. Because of its high sensitivity in the selected ion monitoring (SIM) mode, this semi-quantitative method allowed the detection of very low abundant desaturated and elongated products of deuterated linoleic and α -linolenic acids. To date, this convenient tracer methodology has been used *in vivo* to decrypt essential fatty acid metabolism in animals (Lin and Salem, 2005) and humans (Salem et al., 1999). However, according to the scientific literature and to the best of our knowledge, the methodology has not yet been adapted to quantify *in vitro* desaturase activities without the use of radioactivity.

Therefore, we present here the adaptation and validation of this methodology to a routine and easy-to-use *in vitro* measurement of FA desaturase activities from different sources (mouse and rat livers, cryopreserved primary human hepatocytes in culture). We also measured the kinetic parameters of mouse liver $\Delta 6$ -desaturase for its two main substrates (C18:2 n-6 and C18:3 n-3). For the first time, this method additionally enabled to compare $\Delta 5$ -desaturase apparent K_m values for its two main substrates (C20:3 n-6 and C20:4 n-3).

2. Materials and methods

2.1. Chemicals

[17,17,18,18,18- 2D_5]-linoleic acid (2D_5 -C18:2 n-6), [17,17,18,18,18- 2D_5]- α -linolenic acid (2D_5 -C18:3 n-3), [8,9,11,12,14,15- 2D_6]-dihomo- γ -linolenic acid (2D_6 -C20:3 n-6), [8,9,11,12,14,15,17,18- 2D_8]-eicosatetraenoic acid (2D_8 -C20:4 n-3), [5,6,8,9,11,12,14,15- 2D_8]-arachidonic acid (2D_8 -C20:4 n-6), and [19,19,20,20,20- 2D_5]-eicosapentaenoic acid (2D_5 -C20:5 n-3) were obtained from Larodan (Solna, Sweden), each with chemical purity >99%. Triethylamine (TEA), α -bromo-2,3,4,5,6-pentafluorotoluene, NADH, ATP, CoA and unlabeled heptadecanoic acid (C17:0), γ -linolenic acid (C18:3 n-6) and stearidonic acid (C18:4 n-3) were purchased from Merck (Sigma-Aldrich, France). The solvents and other chemicals were obtained from Thermofisher Scientific (Elancourt, France).

2.2. Animals

All protocols complied with the European Union Guideline for animal care and use (2010/63/CEE). The experimental procedures on mice (#28583-2020-092917544848) and rats (#39726-2022-112509248162) were approved by the French Animal Care Committee (Rennes) and French Ministry of Higher Education. Male C57BL/6NHsd mice were purchased from Envigo (Gannat, France). Wistar rats were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Mice and rats were delivered to the animal facility (ARCHE, BioSit, Rennes, France) and housed 4 (mice) or 2 (rats) by cage with 12–12 h light-dark cycles and free access to food and water. To measure liver $\Delta 6$ - and $\Delta 5$ -desaturase activities, mice or rats were fasted overnight and euthanized through intracardiac puncture following deep sedation by isoflurane, in order to obtain clean tissues, with low blood contamination. Livers were promptly collected, additionally washed with phosphate-buffered saline solution (PBS) and immediately used for desaturase activities. Alternatively, livers were snap-frozen in liquid nitrogen and stored at $-80^\circ C$, to assess the effect of freezing on desaturase activity.

2.3. Cryopreserved primary human hepatocytes

Human hepatocytes were obtained from Biopredic International (St Gregoire, France). They were isolated by collagenase perfusion of histologically normal liver fragments from adult donors and cryopreserved by the distributor. Cells were thawed in a specific "MIL30 OneStep PHep Thawing Medium – Biopredic International" medium according to the protocol supplied by Biopredic International. Primary cultures were obtained by hepatocyte seeding at a density of 1.5×10^5 cells/cm² onto Primaria™ petri dishes in Williams' E medium supplemented with 10 % FBS (1 V Gibco™ Fetal Bovine Serum n°2389940RP / 3 V HyClone™ Fetal Bovine Serum n°AF29578030), 100 units/mL penicillin, 100 μ g/mL streptomycin, 1 mg/mL insulin, 2 mM glutamine, and 1 mg/mL hydrocortisone. The medium was renewed daily after cell seeding, during 4 days.

2.4. Desaturase activity assay with 2D -labeled FA substrates

Liver samples (approximately 1 g) were homogenized using a Potter-Elvehjem homogenizer in 3 mL of 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose. Human hepatocytes were sonicated in the same sucrose buffer after collection. The samples were then centrifuged at 10 000 g (30 min, 4°C). The resulting post-mitochondrial supernatant (PMS) containing microsomal desaturases was collected and diluted 4 times with a 50 mM phosphate buffer for the liver samples (Guillou et al., 2002) or used directly with no dilution for the hepatocytes (Jan et al., 2004). Aliquots were stored at $-20^\circ C$ for subsequent protein measurements.

Desaturase activities were performed using a 1 mL-assay mixture

containing 100 μL of post-mitochondrial supernatant (about 500 μg proteins), 6 mM MgCl_2 , 7.2 mM ATP, 0.54 mM CoA and 0.8 mM NADH in a 150 mM phosphate buffer (45 mM KH_2PO_4 , 105 mM Na_2HPO_4 , $2\text{H}_2\text{O}$, pH 7.16) (Blanchard et al., 2013; Rioux et al., 2015). The reaction was started with the addition of various amounts (10, 20, 40, 60 and 100 nmol) of ^2D -labeled fatty acid substrates dissolved in ethanol (Table 1). The reactions were carried out in a shaking water bath at 37°C for various times (from 1 min to 10 min). The incubation was stopped by adding 1 mL KOH 2 M in ethanol, followed by adding 2 μg heptadecanoic acid (C17:0, unlabeled), thereafter used as an internal standard for the quantification of fatty acids. Control assays were performed by adding KOH in ethanol, before adding the substrates, inhibiting thereby all enzymatic reactions. Each assay mixture was then heated at 70°C during 30 min in order to saponify the lipids. After acidification with 1 mL HCl 3 M, the released fatty acids were extracted twice with 2 mL diethyl ether. After solvent evaporation under a stream on N_2 flow at 55°C , samples were derivatized at room temperature for 15 min with 100 μL of 7 % pentafluorobenzyl bromide (PFB-Br) in acetonitrile and 10 μL triethylamine (Bronsard et al., 2023; Kuiper et al., 2018). The solvents were again removed under a stream of N_2 at 55°C and the PFB-esters of fatty acids were resolubilized in 150 μL of hexane.

2.5. GC-NCI-MS analysis of stable isotope fatty acids as PFB-esters

The analysis was carried out on a 7890B GC system coupled to a 5977B MS detector (Agilent, Les Ulis, France). The PFB-esters (1 μL) were usually injected in a 1/10 split ratio into a bonded silica capillary column (BPX 70, 60 m \times 0.25 mm; SGE, Melbourne, Australia) containing a polar stationary phase of 70 % cyanopropyl polysilphenylene-siloxane (0.25 μm film thickness). Helium was used as carrier gas (flow 1.8 mL/min). The column temperature program started at 190°C , then gradually ramped at $4^\circ\text{C}/\text{min}$ to 250°C and finally held at 250°C for 10 min. The transfer line, source and quadrupole temperatures were 280°C , 150°C and 150°C , respectively. The mass spectrometer was operated under NCI mode using methane as the ionization gas (flow 2 mL/min), with an average vacuum pressure of 1.5×10^{-4} torr.

Fatty acids were analyzed in the selected ion monitoring (SIM) mode using their specific [M-PFB] anions described in Table 1, as well as their [M-PFB+1] anions to take the natural abundance of ^{13}C into account. A dwell time of 10 msec was applied for each m/z . Fatty acids were identified both based on their retention times (R_t) compared to authentic PFB-ester standards and using their [M-PFB] specific carboxylate anion formed in the source. Peak area integration was performed with MassHunter Workstation Software Qualitative Analysis Version B.07.00 for Windows (Agilent Technologies) (Guillocheau et al., 2019).

2.6. Sensitivity and linearity of fatty acid standard curves

Standard curves were prepared from serial dilutions of stock solutions of stable isotope-labeled or unlabeled FAs, after their derivatization to PFB-esters (from 60 pmol injected to around 0.114 fmol) and analysis in the NCI mode, enabling a wide range to explore the linearity and sensitivity of the detector response. Calibration curves were analyzed by linear regressions and a Spearman correlation test was carried out to determine the statistical relevance of their differences

(GraphPad Prism software Version 8.0.2). The LOD (limit of detection) and LOQ (limit of quantification) were established by using the SNR (signal to noise ratio) obtained from MassHunter Workstation Software by calculating the peak height relative to the signal height of the baseline defined 1 min before the peak of interest. The LOD was defined by a SNR of 3 while the LOQ was defined by a SNR of 10 (Quehenberger et al., 2011).

2.7. Desaturase activity calculations

Chromatograms obtained in the SIM mode were used to integrate peak area. The $\Delta 6$ - and $\Delta 5$ -desaturase activities were determined by integrating the area of the deuterated desaturated fatty acid product ($^2\text{D}_5$ -C18:3 n-6 and $^2\text{D}_5$ -C18:4 n-3 for $\Delta 6$ -desaturase; $^2\text{D}_6$ -C20:4 n-6 and $^2\text{D}_8$ -C20:5 n-3 for $\Delta 5$ -desaturase) obtained at the end of the incubation ([M-PFB] anions described in Table 1). Indeed, the method is based on the total absence of stable isotope-labeled fatty acid product at the beginning of the incubation. Control assays were performed to verify that there was no co-elution at specific retention times. In order to take into account the natural abundance of ^{13}C in the FA products, [M-PFB+1] ions obtained in SIM mode were also used in the calculation. The areas obtained (arbitrary units) for each stable isotope were then converted into pmol relative to the area of heptadecanoic acid (C17:0) added as internal standard, with correction for the potential varying response factors.

2.8. Statistics

Statistical analyses were performed by using GraphPad Prism software (version 8.3.0). All data values represent the mean \pm SEM for the number of replicates (n) indicated in each figure legend. Spearman non-parametric correlation test was used to compare linear regression on standard curve data. One-way ANOVA or Mann-Whitney non-parametric test using substrate as parameter were performed to compare desaturase activities. Two-way ANOVA (substrate and frozen or not tissue) was performed to compare the effect of freezing on the desaturase activities.

3. Results

3.1. Sensitivity, linearity, limits of detection and quantification of the routine GC-MS-NCI method

In order to validate the routine GC-MS-NCI method parameters, calibration assays were realized by analyzing a mixture of deuterated and unlabeled fatty acids of interest, after their derivatization to PFB-esters (Table 2). The injected amounts (from 60 pmol injected to 0.1 fmol) were plotted against the peak area of the derivatives, in the SIM mode. These conditions were close to experimental reality of the desaturase assays (with multiple [M-PFB] anions analyzed at the same time in the SIM mode) and deliberately not specifically optimized to artificially lower detection limits and performance. Slope and coefficient of determination (r^2) were calculated from linear regression on standard curve data (Table 2).

In our hands and with the improved parameters described in the

Table 1

Deuterated fatty acids used as substrates for measuring $\Delta 6$ - and $\Delta 5$ -desaturase activities. The [M-PFB] anions (m/z) of the fatty acid pentafluorobenzyl (PFB) esters in negative chemical ionization (NCI) mode are described for each substrate and each expected desaturated product.

Desaturase activity	Fatty acid substrate	[M-PFB] anion of the substrate in SIM mode	Fatty acid product	[M-PFB] anion of the product in SIM mode
$\Delta 6$ -desaturase	[17,17,18,18,18- $^2\text{D}_5$]-C18:2 n-6	284	[17,17,18,18,18- $^2\text{D}_5$]-C18:3 n-6	282
$\Delta 6$ -desaturase	[17,17,18,18,18- $^2\text{D}_5$]-C18:3 n-3	282	[17,17,18,18,18- $^2\text{D}_5$]-C18:4 n-3	280
$\Delta 5$ -desaturase	[8,9,11,12,14,15- $^2\text{D}_6$]-C20:3 n-6	311	[8,9,11,12,14,15- $^2\text{D}_6$]-C20:4 n-6	309
$\Delta 5$ -desaturase	[8,9,11,12,14,15,17,18- $^2\text{D}_8$]-C20:4 n-3	311	[8,9,11,12,14,15,17,18- $^2\text{D}_8$]-C20:5 n-3	309

Table 2

Validation of the routine GC-MS-NCI method parameters. Standard curve data (range 60 pmol injected to around 0.114 fmol from serial dilutions) were obtained for deuterated ($^2\text{D}_5$ -C18:2 n-6, $^2\text{D}_5$ -C18:3 n-3, $^2\text{D}_6$ -C20:3 n-6, $^2\text{D}_8$ -C20:4 n-3, $^2\text{D}_8$ -C20:4 n-6, $^2\text{D}_5$ -C20:5 n-3) and unlabeled (C18:3 n-6, C18:4 n-3) fatty acids after their derivatization to PFB-esters. Slope and coefficient of determination (r^2) were calculated from linear regression. Limit of Detection (LOD) and Limit of Quantification (LOQ) were defined from the signal-to-noise ratio (SNR) being 3 and 10, respectively. Fatty acids were analyzed in the Selected Ion Monitoring (SIM) mode using the specific [M-PFB] and [M-PFB+1] carboxylate anions indicated in the table.

Fatty acid	Common name	m/z Ratio		Retention time (min)	Linear regression	r^2	SNR > 3		SNR > 10	
		[M-PFB]	[M-PFB+1]				LOD (fmol)	LOD (pg)	LOQ (fmol)	LOQ (pg)
$^2\text{D}_5$ C18:2 n-6	Linoleic acid	284	285	12.6	6E-06x	0.998	0.916	0.260	14.648	4.160
$^2\text{D}_5$ C18:3 n-3	α -Linolenic acid	282	283	13.6	1E-05x	0.993	7.324	2.065	58.594	16.523
C18:3 n-6	γ -Linolenic acid	277	278	14.4	6E-06x	0.920	0.229	0.063	3.662	1.014
C18:4 n-3	Stearidonic acid	275	276	15.4	2E-05x	0.902	7.324	2.014	29.297	8.057
$^2\text{D}_6$ C20:3 n-6	Dihomo- γ -linolenic acid	311	312	15.3	1E-05x	0.995	7.324	2.278	58.594	18.223
$^2\text{D}_8$ C20:4 n-3	Eicosatetraenoic acid	311	312	16.3	1E-05x	0.977	3.662	1.139	58.594	18.223
$^2\text{D}_8$ C20:4 n-6	Arachidonic acid	311	312	17.3	7E-06x	0.994	7.324	2.278	29.297	9.111
$^2\text{D}_5$ C20:5 n-3	Eicosapentaenoic acid	306	307	18.6	1E-05x	0.992	7.324	2.241	58.594	17.930

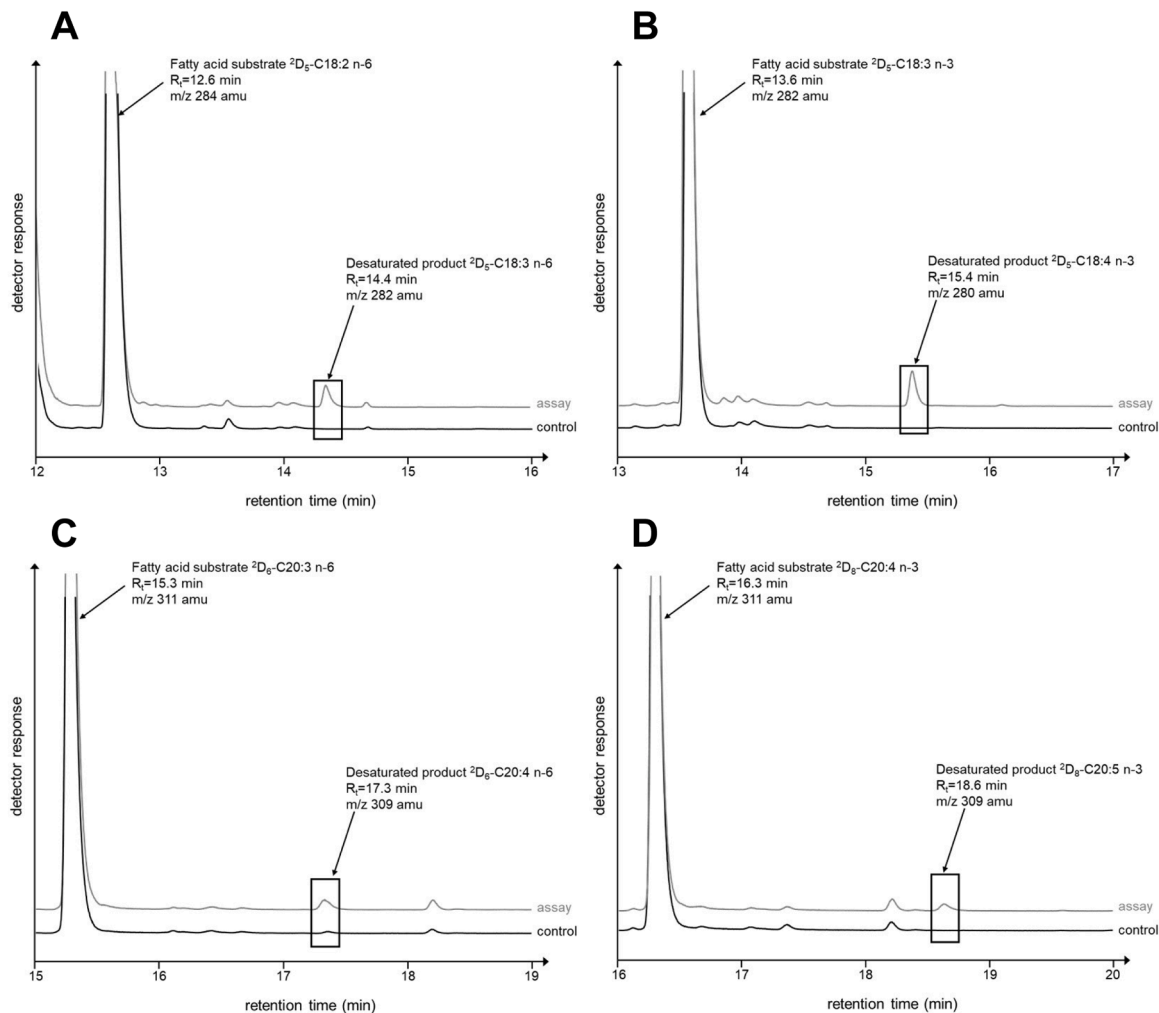


Fig. 1. Representative GC-MS chromatograms of fatty acid-PFB esters obtained after incubating mouse liver post-mitochondrial supernatant with deuterium-labeled fatty acids used as substrates of $\Delta 6$ -desaturase (A: $^2\text{D}_5$ -C18:2 n-6 and B: $^2\text{D}_5$ -C18:3 n-3) and $\Delta 5$ -desaturase (C: $^2\text{D}_6$ -C20:3 n-6 and D: $^2\text{D}_8$ -C20:4 n-3) activities. The MS detector was operated under Negative Chemical Ionization (NCI) mode. Fatty acids were analyzed in the Selected Ion Monitoring (SIM) mode using the specific [M-PFB] carboxylate anions indicated on each chromatogram (panels A-D) for the deuterated fatty acid substrate and deuterated desaturated fatty acid product. In order to undoubtedly detect and quantify the desaturated fatty acid product, each desaturase assay was run in parallel with a control where the desaturase activity was inhibited before adding the substrate.

Materials and Methods section, Table 2 shows that, for each analyzed fatty acid, the peak area was closely related to the amount of the injected fatty acid PFB-ester. Similar linear regression equations were obtained for each fatty acid, whatever the chain length, number of double bonds and presence of deuterated atoms. The linear concentration range was wide, with coefficients of determination >0.9 . The measured Limit of Detection (LOD) of the method, assuming a signal to noise ratio (SNR) of 3, was less than 10 fmol (less than 3 pg), and the measured Limit of Quantification (LOQ), with a SNR of 10, was less than 60 fmol (less than 20 pg).

3.2. Typical GC-MS-NCI profiles obtained after desaturase activity assays

Fig. 1 shows typical GC-MS profiles obtained using NCI in the SIM mode, after incubation of PMS with $\Delta 6$ -desaturase substrates (2D_5 -C18:2 n-6 and 2D_5 -C18:3 n-3, Fig. 1A-B) and $\Delta 5$ -desaturase substrates (2D_6 -C20:3 n-6 and 2D_8 -C20:4 n-3, Fig. 1C-D). Specific peak area of the desaturated products were easily integrated first because they were largely different from their potential unlabeled natural correspondent FA (5 or more deuterium atoms) and second because the desaturated products were totally absent in control assays where desaturase activities were inhibited before the addition of the substrate FA. In addition, due to the presence of several deuterium atoms, the retention times of the heavy fatty acids were shifted slightly earlier than unlabeled ones. Fig. 1B illustrates for instance the formation of 2D_5 -C18:4 n-3 produced after incubation of the PMS with 2D_5 -C18:3 n-3. Fig. 1B shows that there was absolutely no background signal at m/z 280 amu (2D_5 -C18:4 n-3) in control experiments where desaturase activity was inhibited before adding the substrate. Therefore, the signal detected at m/z 280 amu in incubated assay was only due to the *in vitro* synthesis of the reaction product. In these experimental conditions (only NADH and not NADPH or malonyl-CoA were added to the reaction as co-factors), only desaturase activity was shown and no elongated product was detected.

3.3. Application of the method to measure mice liver $\Delta 6$ - and $\Delta 5$ -desaturase kinetic parameters

By using this method, we were able to determine the apparent kinetic parameters of mouse liver $\Delta 6$ - and $\Delta 5$ -desaturases for their main known specific substrates (C18:2 n-6 and C18:3 n-3 for $\Delta 6$ -desaturase, C20:3 n-6 and C20:4 n-3 for $\Delta 5$ -desaturase). First, we verified that desaturated deuterated products were easily detected and quantified after only 1 min of enzymatic reaction and that desaturase activities were linear with incubation time up to 10 min (data not shown). Then, Fig. 2A shows that maximal velocities were obtained with substrate concentrations higher than 50–60 μM , whatever the desaturase and substrate considered. Measured $\Delta 6$ -desaturase activities were around 300 pmol/min/mg protein, whereas $\Delta 5$ -desaturase activities were less than 100 pmol/min/mg protein. The activity curves were statistically analyzed by one way ANOVA and showed a significant difference between $\Delta 5$ -desaturase and $\Delta 6$ -desaturase activities but no difference between the two substrates of the same enzyme (Fig. 2A).

Finally, Lineweaver-Burk plots (Fig. 2B) were deduced from the kinetic desaturation curves shown in Fig. 2A. Approximate K_m and maximal velocity (V_{max}) values were calculated from graphical data and indicated in the figure. The results showed values of K_m and V_{max} relatively close between each substrate of each enzyme but not between the enzymes. Indeed, a K_m of 10.27 μM and a V_{max} of 350 pmol/min/mg protein were obtained for C18:2 n-6, relatively close to the values calculated for C18:3 n-3 (K_m of 12.72 μM and V_{max} of 256 pmol/min/mg protein). Likewise, for the $\Delta 5$ -desaturase substrates, the K_m of 21.78 μM and V_{max} of 84 pmol/min/mg proteins for C20:3 n-6 were close to the values obtained for C20:4 n-3 (K_m of 18.85 μM and V_{max} of 91 pmol/min/mg proteins).

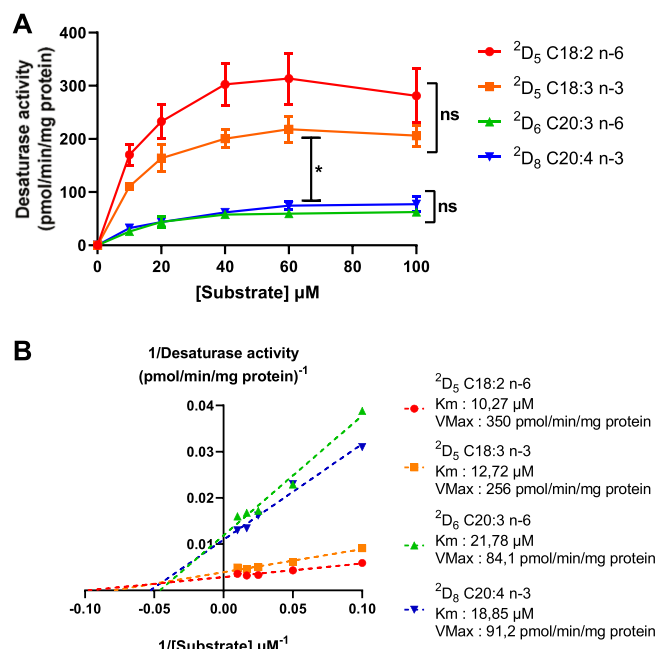


Fig. 2. Measurement of $\Delta 6$ - and $\Delta 5$ -desaturase activities in the post-mitochondrial supernatant of mice liver, by using deuterated substrates and quantification of desaturated fatty acid products with GC-MS in negative chemical ionization (NCI) mode. (A) Effect of increasing concentrations (from 10 to 100 μM) of 2D_5 -C18:2 n-6 and 2D_5 -C18:3 n-3 on $\Delta 6$ -desaturase activity and of 2D_6 -C20:3 n-6 and 2D_8 -C20:4 n-3 on $\Delta 5$ -desaturase activity. Curves were statistically analyzed by one-way ANOVA to demonstrate a statistical difference ($n=5$) between $\Delta 6$ - and $\Delta 5$ -desaturase substrate activities (* p -value < 0.05). (B) Lineweaver-Burk plots deduced from the kinetic desaturations shown in Fig. 2A. Approximate K_m and maximal velocities (V_{max}) values were calculated and indicated in the figure.

3.4. Other applications of the method

In order to demonstrate the utility and extensive possibilities of this easy-to-use method, several models were also designed for investigation.

We first wanted to answer the unresolved biological question concerning the controversial sensitivity of mammalian desaturases to freezing. Indeed, membrane-bound FA desaturases are often described as not stable when frozen and it is recommended measuring their activity on fresh samples. Fig. 3A shows comparative results on desaturase activities obtained extemporaneously from fresh rat liver samples and from pieces of the same livers snap-frozen in liquid nitrogen when collected, then stored for 3 months at $-80^\circ C$. $\Delta 6$ -desaturase activity (87.08 \pm 17.28 pmol/min/mg) on fresh livers was not statistically different from frozen ones (122.7 \pm 13.48 pmol/min/mg). Similarly, measured $\Delta 5$ -desaturase activity on fresh livers (45.38 \pm 7.93 pmol/min/mg) was not statistically different from frozen condition (37.11 \pm 4.32 pmol/min/mg).

We also investigated to what extent the initial biological sample quantity can be miniaturized. For that purpose, we used primary human hepatocytes and showed that about 100 μg protein in the incubated PMS was enough to still be able to measure sensitive desaturase activities (Fig. 3B). In this assay, we used 3 different batches of cells, showing that $\Delta 6$ -desaturase activities (54.30 \pm 25.77 pmol/min/mg protein) were largely dependent on the initial human donor and/or conditions of perfusion and preservation.

4. Discussion

Although discovered several decades ago, the NCI methodology

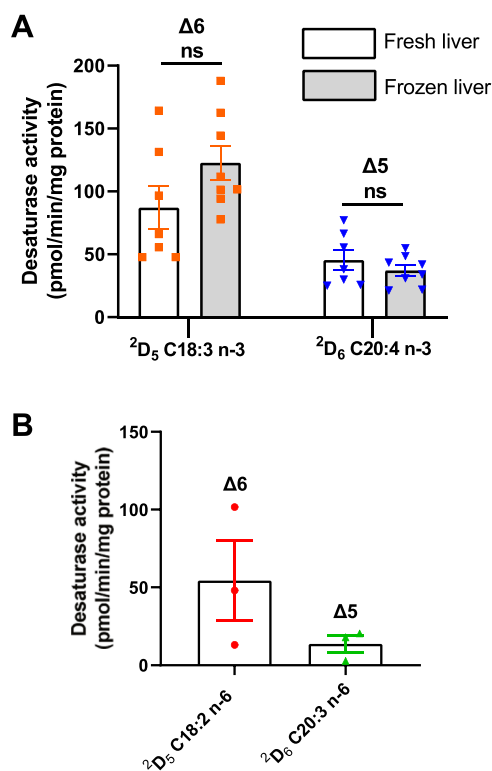


Fig. 3. Applications of the method to the measurement of $\Delta 6$ - and $\Delta 5$ -desaturase activities in different biological samples, by using deuterated substrates and quantification of desaturated fatty acid products with GC-MS in negative chemical ionization (NCI) mode. (A) Effect of freezing on desaturase activities by using both fresh rat liver samples and the same livers after 3 months of deep-freezing at -80°C . Results were analyzed by two-way ANOVA statistical test ($n=6$ or 7). (B) $\Delta 6$ - and $\Delta 5$ -desaturase activities measured in cryopreserved human hepatocytes ($n=3$). Results were analyzed by Mann-Whitney non-parametric test showing no significant difference due to high variability depending on the initial human donor.

applied to GC-MS analysis of FAs has remained relatively confidential, compared to the classical GC-MS-EI on FAMES. Yet, this soft ionization methodology combined with the derivatization of extracted FAs to PFB esters significantly increases detection limits and sensitivity. It has therefore already been applied to analyze the FA composition of samples with very low amounts of biological material (Kuiper et al., 2018; Quehenberger et al., 2011). Additionally combined with the use of stable-isotope tracers, it was also successfully used to study *in vivo* essential fatty acid metabolic pathways (Lin and Salem, 2005) as well as to quantify *in vitro* for instance Fatty Acid Synthase (FAS) activity (Rudolph et al., 2012).

In the present methodology paper, we wanted to evaluate if approaching quantitative *in vitro* analysis of desaturase activities is now possible “in routine” with stable isotope-labeled fatty acids and GC-MS-NCI, avoiding the undesirable issues of radioactive methods. It should be noted here that other non-radioactive methods have already been described to measure desaturase activities. Indeed, a simplified assay procedure for the measurement of recombinant desaturase activity using non-radiolabeled acyl-CoA as substrate and final detection as butylamide derivatives by common GC-FID was previously reported by Watanabe et al. (Watanabe et al., 2016). This assay was however well suited for overexpressed desaturase in yeast cells but not adapted for crude cellular and tissue extracts. Another method using GC-C-IRMS (gas chromatography-combustion-isotope ratio mass spectrometry) was described long ago to measure simultaneously desaturase activities using stable isotope tracers (Su and Brenna, 1998). Although incredibly sensitive, this method was limited by the price of the apparatus for

routine application. Finally, it has recently been shown possible to measure liver (and other tissue) desaturase and elongase activities by using unlabeled PUFAs as substrates, followed by classical derivatization into FAMES and basic GC-FID analysis (Metherel et al., 2024; Valenzuela et al., 2024). However, this useful method is well suited for large tissue samples, since it requires high amounts of highly purified microsomal proteins containing membrane-bound desaturases (1 mg) and long incubation time (30 min). These conditions are necessary to achieve sufficient synthesized amounts of PUFA products from the substrates in order to clearly detect and accurately quantified them by GC-FID. In addition, the presence of endogenous fatty acids, structurally similar to the ones that are produced by the desaturases when using GC-FID (for instance C20:4 n-6 or C20:5 n-6 that are products of $\Delta 5$ -desaturation) makes calculating the peak area difference from non-zero background potentially not easy and less precise. For all these reasons, this unlabeled method appears very interesting and useful when the biological sample is large but its sensitivity seems less than the previous ones using radiolabeled substrates.

Conversely, in our hands and with our apparatus, the NCI methodology was sensitive enough to routinely detect and quantify less than 60 fmol FA (about 20 pg) (Table 2), which is comparable with data described by others (Quehenberger et al., 2011). We notably found that the sensitivity was highly correlated with the cleanliness of the MS source in addition to its specific settings. Assuming that described liver desaturase activities are usually in the range 100–500 pmol/min/mg of microsomal or PMS proteins (typically 0.5 mg proteins), this result showed that the sensitivity was largely high enough to quantify desaturated FA product after only 1 min of *in vitro* incubation with the FA substrate. Indeed, Fig. 1B shows that we were able to detect and quantify adequately the specific production of 2D_5 -C18:4 n-3. Similarly, other products of $\Delta 6$ - and $\Delta 5$ -desaturases were easily detected (Fig. 1).

By using the described method and after calculation, we obtained $\Delta 6$ -desaturase activities around 200–300 pmol/min/mg of PMS protein and $\Delta 5$ -desaturase activities around 50–100 pmol/min/mg of PMS protein. These values are in the same range of magnitude than values previously described by us and others with ^{14}C -labeled substrates and substrate/product separation by TLC or HPLC (Rioux et al., 2005). In addition, we were able to determine the apparent kinetic parameters of mice liver $\Delta 6$ - and $\Delta 5$ -desaturases for their two main described substrates (Fig. 2). The $\Delta 6$ -desaturase apparent K_m values we obtained in mice liver PMS were similar for C18:2 n-6 and C18:3 n-3 (10–13 μM). These values are also in the same order of magnitude than the very first approximate Michaelis constants described for $\Delta 6$ -desaturase by Brenner and Peluffo in 1966 (9), by using rat liver microsomes and radiolabeled substrates (K_m between 3 and 10 μM for C18:2 n-6 and about 3 μM for C18:3 n-3). Moreover, this method enabled for the first time the comparison of $\Delta 5$ -desaturase apparent K_m values for its two main substrates (C20:3 n-6 and C20:4 n-3). In the present study, approximate K_m values for C20:3 n-6 and C20:4 n-3 were found similar (19–22 μM). It is usually described that desaturase enzymes have higher affinity for n-3 PUFA substrates than for n-6 PUFA substrates, leading to higher rates of conversion. In the present work, we however found no significant difference between apparent K_m values of $\Delta 6$ -desaturase for C18:2 n-6 and C18:3 n-3 nor of $\Delta 5$ -desaturase for C20:3 n-6 and C20:4 n-3, respectively. These apparent and approximate K_m values should however be used with caution, since we used a small number of mice ($n=5$) that may not be enough to obtain significant differences. In addition, we used the C57BL/6NHsd mice strain and this specific C57BL/6 substrain has never been previously studied for desaturase activities. Finally, they were obtained from a post-mitochondrial supernatant and not from a fully purified enzyme.

Taking advantage of the developed presented method, we showed that when collected tissues are promptly and correctly snap-frozen in liquid nitrogen, then stored at -80°C for at least 3 months, there was no significant effect of freezing on measured desaturase activities (Fig. 3A). In addition, PMS protein quantity as low as 100 μg was enough to

achieve the desaturase assay in primary human hepatocytes (Fig. 3B).

The method also has of course some limitations. The deuterated fatty acid substrates must be adequately chosen in the commercially available collection, by carefully looking at the number and position of the deuterated atoms, in order to have no or little impact on enzyme affinity. We also assumed that derivatization efficiency to PFB esters was complete and similar for each FA. Finally, accurate quantification greatly depends on the internal standard whose quantities must be adapted to the sample.

To conclude, this method is likely to be of broad utility in the lipid research community, to replace older methods using radioactivity, with a similar sensitivity. It may additionally be adapted to measure several other enzymatic reactions than desaturation, in which fatty acids are substrates, namely elongases (Rioux et al., 2007) and hydroxylation activities (Amet et al., 1995), previously quantified using radiolabeled substrates in miniaturized samples. The use of deuterated tracers additionally ensures the metabolic conversion link between the precursor (substrate) and product. Finally, the use of tracers also enables the discovery of unknown new minor conversion pathways by using structural data obtained from mass spectrometry and new potential substrates of these enzymes.

CRedit authorship contribution statement

Philippe Legrand: Writing – review & editing. **Catheline Daniel:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Manuel Vlach:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vincent Ciesielski:** Writing – review & editing, Investigation. **Lydie Hue:** Writing – review & editing, Investigation. **Vincent Rioux:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Youenn Launay:** Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Iwan Jan:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Thomas Guerbette:** Writing – review & editing, Investigation. **Karima Begriche:** Writing – review & editing. **Mélodie Succar:** Writing – review & editing, Investigation. **Léa Fret:** Writing – review & editing, Investigation.

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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.

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Data availability

Data will be made available on request.

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