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Cost-efficient and user-friendly $^{17}O/^{18}O$ labeling procedures of fatty acids using mechanochemistry†

Jessica Špačková, [ID] [a] Charlyn Fabra,[a] Guillaume Cazals,[b] Marie Hubert-Roux, [ID] [c]
Isabelle Schmitz-Afonso, [ID] [c] Ieva Goldberga, [ID] [a] Dorothée Berthomieu, [ID] [a]
Aurélien Lebrun,[b] Thomas-Xavier Métro [ID] *[b] and Danielle Laurencin [ID] *[a]

Two mechanochemical procedures for $^{17}O/^{18}O$-isotope labeling of fatty acids are reported: a carboxylic acid activation/hydrolysis approach and a saponification approach. The latter route allowed first-time enrichment of important polyunsaturated fatty acids (PUFAs) including docosahexaenoic acid (DHA). Overall, a total of 9 pure labeled products were isolated in high yields (≥80%) and with high enrichment levels (≥37% average labeling of C=O and C–OH carboxylic oxygen atoms), under mild conditions, and in short time (<half day).

Fatty acids (FAs) represent a substantial class of biomolecules with multiple applications. Because of their biological importance, they are widely studied in the fields of lipidomics and metabolomics, as well as in human nutrition research, especially the essential ω-3 and ω-6 PUFAs[1] like arachidonic acid (AA) or DHA.[2] Furthermore, because of their amphiphilic character they are also used in materials science, for example as surfactants in nanoparticles synthesis, where they can direct the final shape and properties of nanoparticles.[3,4]

The labeling of free FAs and their ester derivatives in stable isotopes (^{2}H, ^{13}C and $^{17}O/^{18}O$) is of high interest as it allows to better understand the structure and reactivity of the complex (bio)molecules and materials systems. For example, by deuteration, the influence of the chain length of free FAs on the phase behaviour of stratum corneum membranes could be explored by ^{2}H NMR.[5] Using ^{13}C labeling, *in vivo* ^{13}C NMR spectroscopy could be used to study the metabolism of polyunsaturated fatty acid methyl esters in the neonatal rat brains.[6] When labeled in ^{18}O, FA derivatives could be used as internal standards in mass spectrometry (MS) for metabolites quantitation or to elucidate reaction mechanisms.[7–9] Lastly, regarding ^{17}O labeling, it is

only very recently that we demonstrated that the ^{17}O enrichment of free FAs could be of great interest for (nano)materials science applications, by providing unique insight into the structure of metal soaps and in the surface reactivity of functionalized nano-particles.[10]

Despite the great potential that $^{17}O/^{18}O$-labeled FAs could offer to the research community, their use remains scarce compared to ^{2}H and ^{13}C labeled species. One of the reasons to this comes from the limited access to $^{17}O/^{18}O$-labeled FAs, which are not commercially available and require to be synthesized. The very few oxygen enrichment protocols found in the literature almost exclusively focus on the ^{18}O-labeling of specific FA derivatives used as standards in GC-MS analyses. In these protocols, the carboxylic oxygen atoms are labeled *via* hydrolysis of the corresponding esters (under basic or enzymatically catalysed conditions), or by acid catalysed oxygen exchange in labeled water.[7,8,11] These syntheses engage large excesses (>500 equiv.) of H_2O^* in order to produce only μg quantities of labeled products. Although such low amounts of product are adequate for applications in GC-MS analyses, they are absolutely insufficient for the synthesis of (nano)materials, and a simple scale-up of the protocols would be very costly considering the price of enriched water (up to ~2000 € for 1 mL of 90%-^{17}O H_2O^*, and ~120 € for 1 mL of >98%-^{18}O H_2O^*).

In order to address the need for efficient and scalable $^{17}O/^{18}O$ labeling protocols which would be generally applicable to a broad range of FA substrates, we have developed alternative synthetic approaches using mechanochemistry,[12] where the $^{17}O/^{18}O$ enrichment is performed at ambient temperature and pressure using only few μL of H_2O^*.[13] Recently, we adapted this methodology for the first time for the synthesis of ^{17}O and ^{18}O-labeled oleic and stearic acids, and also scaled-up producing up to 1 g of ^{17}O-labeled acids for less than 280 €.[10]

In this paper, we first demonstrate how by adapting our previous strategy, the scope of $^{17}O/^{18}O$-labeled free FAs can be extended to other saturated FAs with the chain-length varying between C12 to C18 and also to PUFAs containing up to four double bonds, and how detailed analyses of reaction media can help determine working conditions for future syntheses. Then,

[a] ICGM, Univ Montpellier, CNRS, ENSCM, Montpellier, France.
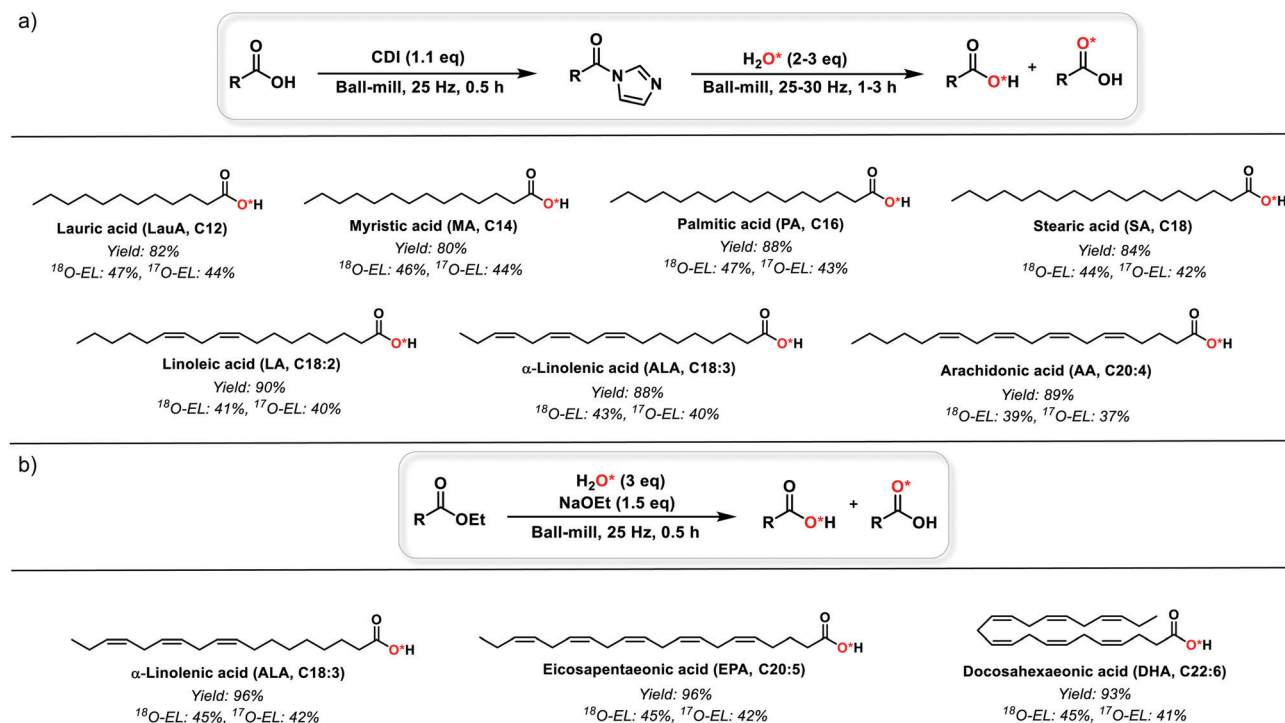 E-mail: danielle.laurencin@umontpellier.fr
[b] IBMM, Univ Montpellier, CNRS, ENSCM, Montpellier, France
  E-mail: thomas-xavier.metro@umontpellier.fr
[c] Normandie Univ., COBRA UMR 6014 and FR 3038 Univ. Rouen, INSA Rouen,
  CNRS IRCOF, Mont-Saint-Aignan, France
† Electronic supplementary information (ESI) available. See DOI: 10.1039/
d1cc02165f

Table 1 Fatty acids labeled in $^{17}O/^{18}O$ by mechanochemistry using (a) CDI-activation/hydrolysis, (b) saponification

a)

Lauric acid (LauA, C12)
Yield: 82%
^{18}O-EL: 47%, ^{17}O-EL: 44%

Myristic acid (MA, C14)
Yield: 80%
^{18}O-EL: 46%, ^{17}O-EL: 44%

Palmitic acid (PA, C16)
Yield: 88%
^{18}O-EL: 47%, ^{17}O-EL: 43%

Stearic acid (SA, C18)
Yield: 84%
^{18}O-EL: 44%, ^{17}O-EL: 42%

Linoleic acid (LA, C18:2)
Yield: 90%
^{18}O-EL: 41%, ^{17}O-EL: 40%

α-Linolenic acid (ALA, C18:3)
Yield: 88%
^{18}O-EL: 43%, ^{17}O-EL: 40%

Arachidonic acid (AA, C20:4)
Yield: 89%
^{18}O-EL: 39%, ^{17}O-EL: 37%

b)

α-Linolenic acid (ALA, C18:3)
Yield: 96%
^{18}O-EL: 45%, ^{17}O-EL: 42%

Eicosapentaenoic acid (EPA, C20:5)
Yield: 96%
^{18}O-EL: 45%, ^{17}O-EL: 42%

Docosahexaenoic acid (DHA, C22:6)
Yield: 93%
^{18}O-EL: 45%, ^{17}O-EL: 41%

For error bars see detailed Table in ESI (Table D-1). Average isolated yield (n = 3–4), ^{18}O-EL = average enrichment level of carboxylic oxygen atoms using 97%-^{18}O water (n = 2–3), ^{17}O-EL = enrichment level using 90%-^{17}O water (n = 1). Both oxygen atoms (C=O and C–OH) have the same probability of being enriched in the isolated product (only one is shown in red here for each labeled FA molecule). * For SA: K_2CO_3 (1 equiv.) was added at the hydrolysis step. Full work-up procedures leading to the pure labeled products are described in ESI.

we demonstrate how an original and efficient labeling scheme based on saponification of the ester derivatives can be used for the $^{17}O/^{18}O$-labeling of the longest and highly sensitive PUFAs with five and six double bonds (EPA and DHA), thereby expanding the scope of approaches for enriching FAs.

First, we looked into expanding the range of free FAs which can be labeled in $^{17}O/^{18}O$ focusing on other FAs which are not only of synthetic interest, but also through which we could learn more about the labeling mechanisms, using activation/hydrolysis (Table 1a). Here, the enrichment consists of two mechanochemical steps: activation with small excess of 1,1′-carbonyldiimidazole (CDI, 1.1 equiv.), followed by hydrolysis of the activated acyl–imidazole intermediate using labeled water (2–3 equiv.).[10] For each substrate, the oxygen isotope enrichment protocol was first optimized using $H_2^{18}O$, due to the lower purchasing price of ^{18}O-enriched water, before producing ^{17}O-labeled equivalents. The course of the reaction was monitored by FT-IR looking at the shift of carbonyl vibration band. The homogeneity of the reaction mixture was controlled by analysing samples from different parts of the reaction vessel at each synthetic step. After complete hydrolysis, products were recovered from the jar using non-labeled water, the medium was acidified and the labeled FAs were finally recovered by extraction with ethyl-acetate (see ESI† for detailed protocols). Enriched products were isolated in good synthetic yields (≥80%) and with high enrichment levels (≥39% average per carboxylic oxygen, for the ^{18}O-labeled products). The purity of

isolated phases was confirmed by solution ^1H and ^{13}C NMR spectroscopy and LCMS analyses. The ^{17}O-labeled solid FAs were also analysed by ^{17}O NMR, showing that on average, the ^{17}O-label is distributed equally among the two oxygen sites in the isolated product (see ESI,† Fig. B1-13, B2-13 and B3-13).

For the labeling of saturated FAs, concerning the activation step, full conversion was achieved for all molecules within 30 min of milling at 25 Hz. The hydrolysis step, in contrast, was found to be strongly substrate dependent, and needed to be optimized for each acid separately. Indeed, it was observed that the milling time required to achieve complete hydrolysis highly correlates with the increasing chain-length of activated acids. Specifically, milling for 1 h at 30 Hz was sufficient to fully hydrolyse lauroyl-imidazole (C12), whereas the complete hydrolysis of palmitoyl-imidazole (C16) was achieved only after 3 h at 30 Hz. In the case of activated stearic acid (C18), full conversion was not observed even after 6 h of milling at 30 Hz, and could only be achieved by adding K_2CO_3 (1 equiv.) (Table 2).

To further explain such differences, the melting points of the media isolated after activation were measured. These were found to follow the same trend as for the starting acids, with the melting points increasing with the length of the organic chain (Table 2). Additionally, it was found that the higher these melting points, the longer the time needed for hydrolysis. The evolution of the physical aspect of the reaction mixture is also worth mentioning: only when the reaction mixture had an oily aspect after hydrolysis was the hydrolysis found to be complete

Table 2 Melting points of non-labeled saturated FAs and their activated intermediate mixtures, and hydrolysis times used

Fatty acid	M.p. fatty acid [°C]	M.p. active. interm. mix. [°C]	Hydrolysis time[a] [h]
LauA (C12)	43.4–46.3	58.4–62.3	1
MA (C14)	54.2–56.0	66.3–70.9	2.5
PA (C16)	62.4–64.4	73.5–78.1	3
SA (C18)	67.3–71.0	77.0–82.0	3[b]

[a] Time necessary for complete hydrolysis at 30 Hz. [b] With the addition of K_2CO_3 (1 equiv.); see Table D-3 (ESI) for further details.

(see ESI,† Fig. D1). It can be hypothesized that the long milling times at 30 Hz required for the full conversion of the longer chain fatty acyl-imidazoles enabled to reach a temperature high enough to change the physical state of the reaction mixture, resulting in higher mobility of the molecules, which may accelerate the hydrolysis. These observations could be related to the progressive temperature increase during the ball-milling which has been described previously.[14–16]

For the labeling of PUFAs containing up to four double bonds, the same two steps protocol was applied. Compared to saturated FAs, no difference in reactivity related to the chain length was observed during hydrolysis, and overall reaction times were reduced. In particular, all PUFAs were completely activated in 20 min of ball-milling at 25 Hz and fully hydrolysed in 90 min of milling at 25 Hz. The reduction of reaction times is most probably caused by the different physical state of non-labeled starting compounds, with unsaturated FAs being viscous oils at ambient temperature. No impurities were detected in the isolated enriched products. However, slightly lower enrichment levels were determined (39–43%, compared to 44–47% for saturated fatty acids, for

^{18}O-labeled products), which can be explained by the choice of the milling equipment used for the labeling of PUFAs (see ESI,† Fig. D2). Hence, overall, we have proven here that the CDI-activation procedure can be easily optimized and generally applied to a variety of saturated and unsaturated FAs, allowing pure $^{17}O/^{18}O$-enriched compounds to be produced in high yields.

In order to reach other important PUFAs, such as eicosapentaeonic acid (EPA) and docosahexaenoic acid (DHA), and to further extend the substrate scope, a new enrichment procedure was developed: a one-pot mechanochemical saponification (Table 1b).[17,18] This appeared as a potentially attractive strategy considering that many fatty acids are commercially available in the form of esters. In this enrichment protocol, ethyl-ester derivatives of fatty acids were introduced into the reaction jar together with labeled water (3 equiv.) and sodium ethoxide (1.5 equiv.). After only 30 min of milling, full conversion was observed by FT-IR. In this one-pot synthesis, labeled hydroxide anions were most probably first formed *in situ via* acid–base reaction between labeled water and sodium ethoxide, and subsequently reacted with the ester function in the substrate, producing the labeled molecule (as carboxylate) and ethanol as the only side-product. Using the same work-up as for CDI-activation/hydrolysis, all labeled molecules were obtained in high yield (≥93%) with high enrichment levels (≥45% average per carboxylic oxygen, for ^{18}O-labeled products). The purity of PUFAs prepared *via* saponification was confirmed by ^1H and ^{13}C solution NMR and by LCMS analyses, and no degradation was observed (see ESI†). Hence, this work demonstrates for the first time that saponification reactions are highly attractive enrichment protocols for oxygen labeling of carboxylic functions of FAs, starting from the ester derivatives.
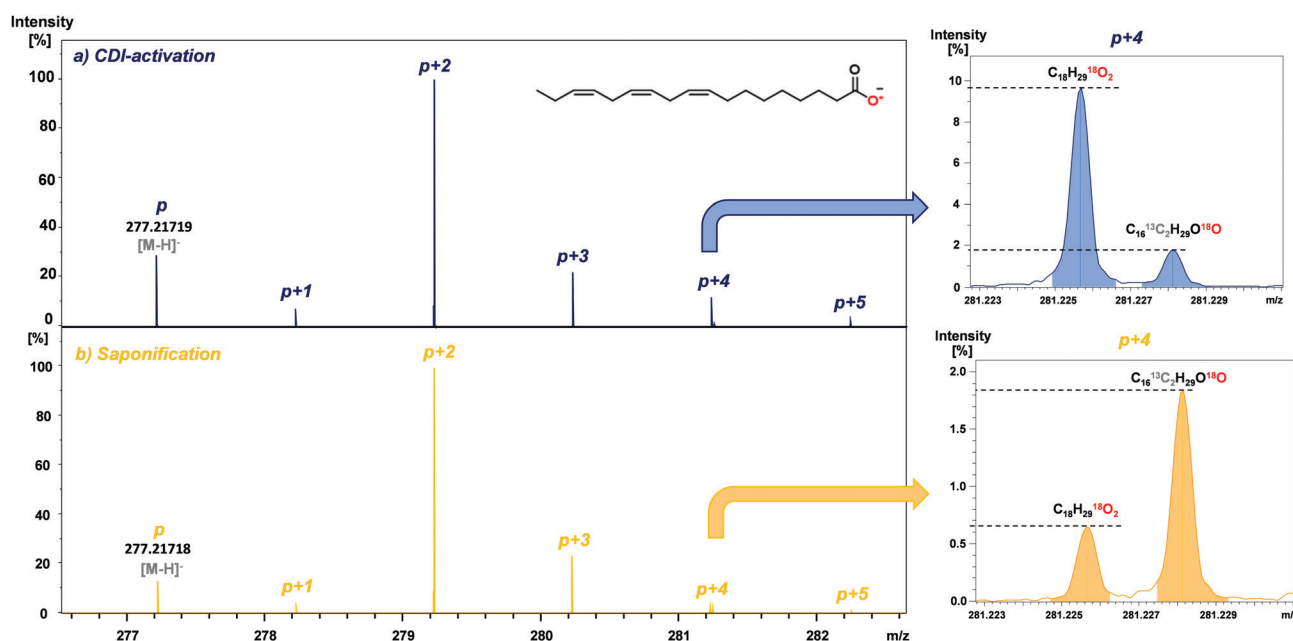
Fig. 1 Ultra-high resolution mass spectra in ESI negative mode of α-linolenic acid (ALA) enriched in ^{18}O (a) *via* CDI-activation/hydrolysis or (b) saponification procedure; relative content of doubly-labeled product is compared in $p + 4$ zoom on the right side; p = monoisotopic mass of ALA.

In comparison to previously published ^{18}O-enrichment protocols in which both oxygen atoms in carboxylic function get labeled (enrichment >80% per carboxylic oxygen), the reaction mechanisms of both our mechanochemical enrichment protocols allow only one oxygen isotope per carboxylic function to be introduced. This is apparent from the MS spectra of ^{18}O-labeled products, where the main molecular peak is shifted by two m/z units ($p + 2$), and from ^{13}C solution NMR analyses, where the main resonance of carboxylic function appears shielded by ~0.025 ppm in comparison with the non-labeled molecule due to the ^{18}O isotope effect (see ESI†). However, when looking at the whole MS pattern of unsaturated FA enriched via CDI-activation/hydrolysis procedure, we have noticed that a molecular peak shifted by four m/z units ($p + 4$) exhibits higher intensity than expected from simulations, suggesting a non-negligible presence of doubly labeled molecules. This becomes even more obvious when MS spectra of ^{18}O-labeled α-linolenic acid (ALA) prepared by the two mechanochemical protocols are compared. Indeed, as shown in Fig. 1, the ultra-high resolution FTICR mass spectra enabled us to resolve the "$p + 4$" peak (isotopic fine structures)[19] and to differentiate the relative contribution of doubly labeled molecules in each method. More specifically, it was observed that in the case of saponification the "$p + 4$" peak mainly consists of molecules containing two ^{13}C and one ^{18}O isotope as expected, with only very little amount of doubly ^{18}O-labeled product, whereas in the case of CDI-activation/hydrolysis, ~10 times more of doubly ^{18}O-labeled molecules was formed compared to saponification, representing the dominant contribution. It can be hypothesized that doubly labeled products result from a CDI-reactivation of already enriched acid molecules due to the presence of remaining unreacted CDI reagent in the reaction mixture.

Overall, it was shown here that mechanochemistry is a versatile approach that stands out from currently available oxygen enrichment protocols for carboxylic acids, thanks to the simplicity, rapidity and cost-efficiency of proposed procedures. It was shown that the reaction conditions can be easily optimized to extend the scope of ^{17}O/^{18}O-labeled fatty acids using CDI-activation/hydrolysis. A variety of saturated as well as unsaturated free FAs was for the first time enriched in ^{17}O/^{18}O and isolated pure in high yields (≥80%), with no need for column chromatography, in less than a half day. Moreover, the newly developed mechanochemical saponification enabled to reach other important and highly sensitive PUFAs, including EPA and DHA. Using simple work-up, ^{17}O/^{18}O-enriched PUFAs were isolated pure, in high yields (≥93%) and with high enrichment levels (≥45% average per carboxylic oxygen, for ^{18}O-labeling). Moreover, enrichment was found to be stable over a 1 year storage period (see ESI,† Table D-2). Although, in average, only one oxygen per carboxylic function is labeled, the enrichment levels provided by this methodology are perfectly suitable for (nano)materials science applications[20–22] for gaining deeper insight into the structure and reactivity of different systems via IR (using ^{18}O-labeled species) or high-resolution ^{17}O NMR analyses. More generally, saponification under ball-milling represents a new, simple, fast and cost-efficient ^{17}O/^{18}O labeling strategy, which has great potential to be applied for

oxygen enrichment of other organic molecules containing carboxylic functions. Among other advantages, it enables expanding the substrate scope of carboxylic molecules which can be enriched in ^{17}O, and may also offer more practical work-up procedures compared to the CDI activation/hydrolysis route. These are points we are currently looking into.

Conflicts of interest

There are no conflicts to declare.

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