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Strategy for specific isotope ratio determination by quantitative NMR on symmetrical molecules: application to glycerol

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Abstract—The strategy for the measurement of the site-specific isotope ratios by NMR within a symmetrical molecule is illustrated by the determination of the site-specific ^{13}C content in glycerol on the basis of (i) a selective derivatisation of glycerol, carried out by a lipase (*Candida antarctica*) to overcome the symmetry of the molecule, (ii) purification of the (*R*)-monobenzoate glycerol with a high enantiomeric excess and (iii) use of this derivative for quantitative ^{13}C NMR spectroscopy for the determination of ^{13}C isotopomer concentrations.

1. Introduction

Quantitative NMR spectroscopy has proven to be a unique tool for the determination of site-specific isotope ratios at natural abundance. Known as SNIF NMR™,[†] this method has been successfully applied to forensic[1] and metabolic investigations using ^{2}H NMR[2] and to the control of the origin of organic substances using ^{2}H NMR[3] and, more recently, ^{13}C NMR.[4] The ^{2}H or ^{13}C isotopomer concentrations can be retrieved from an NMR experiment, based on the double capacity of this technique to separate each isotopomer signal and to quantify them. Unfortunately, this approach fails when a symmetrical molecule is under investigation. Thus, for glycerol, the symmetry leads to a loss of isotopic information both for ^{2}H and ^{13}C isotopomers.[5] Despite the interest in glycerol as a molecular probe either for authenticity issues (genuineness of wines, botanical and geographical origins of vegetable oils and animal fats) or for metabolism studies (sugar fermentations), no strategy has so far been developed to express the full isotopic distribution within glycerol by NMR spectroscopy. Herein we report an enantioselective derivatisation of glycerol, which allows the unambiguous determination of the ^{13}C content of the three carbon atoms by ^{13}C NMR.

2. Results and discussion

Optically active glycerol derivatives can be obtained by transesterification under enzymatic catalysis. Consequently, efficient preparations of (*R*)-α-monobenzoylglycerol [(*R*)-MBG] have been reported[6,7] using *Candida antarctica* lipase, according to the reaction depicted in Figure 1. It was not our intention to improve the reaction conditions, but rather to use the most reproducible, robust and simple methodology for (*R*)-MBG synthesis. Extracted glycerol, from either wines or fats,[5] was benzoylated at room temperature (18–28 °C) using a carrier-fixed immobilised enzyme, NOVOZYM 435, until total consumption of the glycerol occurred.[16] Within our experimental conditions, 1,3-dibenzoylglycerol (DBG) may also be formed. This was not a disadvantage since the remaining '*pro R*' hydromethylene group on (*S*)-MBG reacted faster than the corresponding '*pro S*' remaining hydroxymethylene group of (*R*)-MBG (Fig. 1),[7] thus resulting in a decrease in MBG yield and in an enhancement of ee for (*R*)-MBG. Since the purpose of the derivatisation reaction

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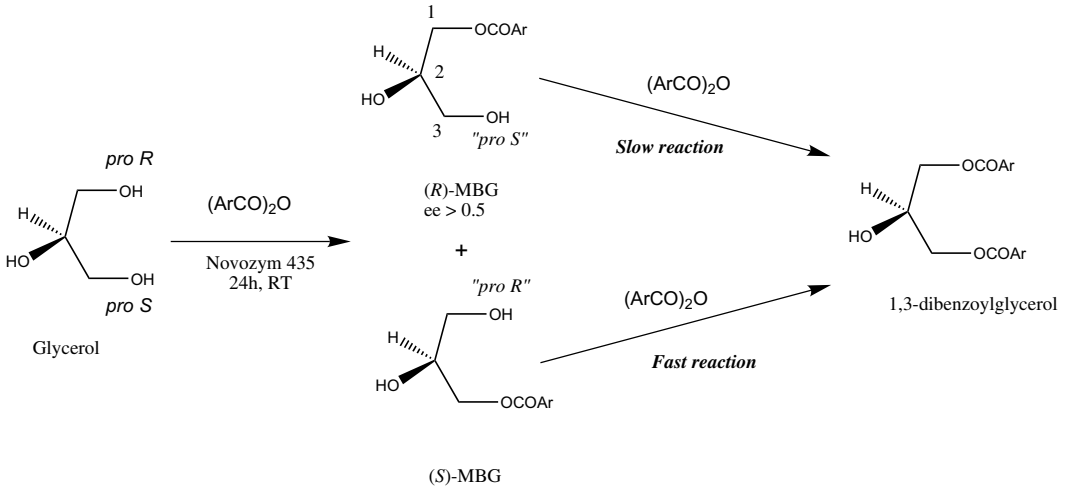

Figure 1. Schematic representation of the selective lipase-catalysed esterification of glycerol, where the carbon numbering is mentioned.

was to produce (*R*)-MBG with the highest enantiomeric excess, the yield was not so important as long as the amount of (*R*)-MBG was sufficient for ^{13}C NMR analysis. After separation and purification on a silica gel chromatography column, (*R*)-MBG showed an ee value higher than 60%. Then, a preferential recrystallisation[7,8] led to the target product having an average ee higher than 80%, with an average global yield higher than 30%. It should be pointed out that this rather low yield was not a drawback considering the influence of kinetic secondary effects on the values determined for the isotopic distributions. The latter can be neglected in the case of the esterification reaction as already shown for $^{2}H/^{1}H$ isotopic measurements.[9] When there is a very low influence of the yield of the esterification reaction on the $^{2}H/^{1}H$ isotopic distribution on each site of the esters, one can obviously discount such an effect on $^{13}C/^{12}C$ isotopic ratios.

The ^{13}C-SNIF NMR methodology was applied on the MBG samples under the best experimental conditions collected and improved so far.[4,10] The three carbons of the glycerol part were well separated and identified in the quantitative ^{13}C NMR spectrum.[17] As expected, the (*R*)-MBG and the (*S*)-MBG molecules presented the same ^{13}C NMR profile. Therefore, we used two numbering systems for the carbon atoms: (i) *a*, *b* and *c* were the peak attributions of the real MBG sample (^{13}C chemical shifts from TMS, $a = 66$ ppm, $b = 70$ ppm and $c = 63$ ppm), which contained mainly (*R*)-MBG and some (*S*)-MBG, while (ii) 1, 2 and 3 corresponded to the carbons in a pure (*R*)-MBG product, in agreement with the nomenclature used in the parent hexoses.[11] Consequently, the ^{13}C isotopic abundances *Ai*, which were calculated from the spectra, were not attributed to a specific site i: *Aa*, *Ab*, *Ac* were the experimental abundances while we were looking for *A*1, *A*2 and *A*3 (*Ai* are the actual isotopic abundances in pure (*R*)-MBG and, therefore, in glycerol). Corrections should be made as follows:

$$Aa = [R] \cdot A1 + [S] \cdot A3 \quad (1)$$

$$Ab = A2 \quad (2)$$

$$Ac = [S] \cdot A1 + [R] \cdot A3 \quad (3)$$

where [*R*] and [*S*] were the relative molar fractions of (*R*)-MBG and (*S*)-MBG, respectively. The proportions of (*R*)-MBG versus (*S*)-MBG were retrieved from chiral

Table 1. Site-specific ^{13}C content, expressed as the isotopic deviation $\delta‰$ and relative abundance of the (*R*)-MBG, obtained on glycerol samples from several origins

Sample no.	Origin glycerol	$\delta^{13}C$ (‰) Global on glycerol[a]	[*R*] (%)[b]	$\delta^{13}C$ (‰)[c] C1 MBG	$\delta^{13}C$ (‰)[c] C2 MBG	$\delta^{13}C$ (‰)[c] C3 MBG
1	Synthetic	−29.7	87.6	−33.1	−22.5	−33.6
2	Commercial	−24.3	89.4	−38.2	−12.4	−22.3
	Method repeatability[d]		87.4–91.2	1.7	0.5	1.3
3	Corn oil	−15.1	95.0	−37.9	3.0	−10.4
	^{13}C NMR repeatability[e]			0.6	0.7	0.8
4	Olive oil	−30.7	85.3	−49.4	−18.9	−23.8
5	Pork fat	−23.2	84.9	−34.3	−14.0	−21.3
6	Goose fat	−27.6	85.0	−49.8	−10.8	−22.2
7	French wine	−30.3	75.8	−48.4	−16.6	−25.8
8	German wine	−32.0	82.7	−51.5	−21.4	−23.0

[a] Isotopic deviation measured by IRMS.
[b] Molar fraction of (*R*)-MBG.
[c] Isotopic deviation calculated from *Ai*, see Refs. 5 and 12.
[d] Standard deviation of $\delta^{13}C$ calculated from three replicates of the whole method on glycerol 2.
[e] Standard deviation of δ ^{13}C calculated from five replicates of the ^{13}C NMR measurement on the same MBG obtained from glycerol 3.

HPLC experiments.[6,7] The resolution of the above Eqs. 1–3 led to

$$A1 = ([S] \cdot Ac - [R] \cdot Aa)/([S]^2 - [R]^2) \quad (4)$$

$$A3 = ([R] \cdot Ac - [S] \cdot Aa)/([R]^2 - [S]^2) \quad (5)$$

Since $A2$ corresponded to the central carbon, no correction was needed: the origin of this carbon was the same in *R*-MBG and *S*-MBG. The isotopic deviation $\delta‰$ of each carbon could then be calculated from the global ^{13}C content measured by IRMS (Isotope Ratio, Mass Spectrometry) on extracted glycerol, as has already been proposed for glycerol[5] and vanillin.[12] The glycerol samples studied included several commercial origins (pure chemicals, vegetable oils, animal fats) and authentic wines. In Table 1, the results from the three types of experiment are displayed: (i) the repeatability of the ^{13}C NMR measurement alone was assessed by five replicates over 3 months on the same MBG (sample 3). The standard deviation found was of the same magnitude as for the application of ^{13}C-SNIF NMR on other molecules.[13] (ii) The repeatability of the whole approach, from the glycerol to the ^{13}C NMR analysis on MBG via the synthesis and purification of MBG, was good enough for a routine application, as demonstrated by the three replicates of the methodology on the same glycerol (sample 2). (iii) The isotopic deviation observed for the three carbons of glycerol was very different for samples of a given origin and also between samples of different origins. This observation is in agreement with the previous work, which has shown that, either by NMR[5,14] or by IRMS,[11] in biogenic glycerol carbon 2 has a higher ^{13}C content than carbons 1 and 3. In synthetic glycerol (sample 1), the ^{13}C distribution was more homogeneous. The instrumental precision of the chiral HPLC allowing the determination of the relative percentage of (*R*)-MBG ([*R*]) was described by a relative standard deviation of 0.5%. Such small variations in [*R*] measurements had very little influence on the calculated isotopic abundance, as can be tested using Eqs. 4 and 5.

3. Conclusion

Work is currently in progress to delineate the significance of such an approach to study the isotope profile of glycerol wherever it is found. The present preliminary results indicate that large isotopic fractionations occur during the metabolism of glycerol, either in the fatty acid pathway or in the bio-transformation from glucose. The huge depletion of site 1 has been explained by both (i) an isotope balance where depletion is very large when a by-product, such as glycerol, is concerned and (ii) the kinetic isotope effect on the aldolase reaction,[15] supporting the origin of glycerol from the upper part of glucose.[11] By increasing the data on authentic glycerol samples, the origin of glycerol should be well characterised. The present methodology was easy to use and is robust. A similar strategy could be applied to other molecules, such as citric or tartaric acids, and to other isotopic determinations, such as site-specific $^2H/^1H$ ratios.

References

1. Hays, P. A.; Remaud, G. S.; Jamin, E.; Martin, Y.-L. *J. Forensic Sci.* **2000**, *45*, 552–562.
2. Robins, R. J.; Billault, I.; Duan, J.-R.; Guiet, S.; Pionnier, S.; Zhang, B.-L. *Phytochem. Rev.* **2003**, *2*, 87–102.
3. Remaud, G. S.; Martin, Y.-L. G.; Martin, G.; Martin, G. J. *J. Agric. Food Chem.* **1997**, *45*, 859–866.
4. Tenailleau, E. J.; Lancelin, P.; Robins, R. J.; Akoka, S. *J. Agric. Food Chem.* **2004**, *52*, 7782–7787.
5. Zhang, B.-L.; Buddrus, S.; Trierweiler, M.; Martin, G. J. *J. Agric. Food Chem.* **1998**, *46*, 1373–1380.
6. Kato, Y.; Fujiwara, I.; Asano, Y. *J. Mol. Catal. B: Enzym.* **2000**, *9*, 193–200.
7. Xu, J.-H.; Kato, Y.; Asano, Y. *Biotechnol. Bioeng.* **2001**, *73*, 493–499.
8. Collet, A.; Brienne, M.-J.; Jacques, J. *Chem. Rev.* **1980**, *80*, 215–230.
9. Rabiller, C.; Mazé, F.; Mabon, F.; Martin, G. J. *Analusis* **1991**, *19*, 18–22.
10. Akoka, S; Tenailleau, E. J. Presented in part at the 46th ENC, Providence, Rhode Island, USA, April 2005.
11. Weber, D.; Kexel, H.; Schimdt, H.-L. *J. Agric. Food Chem.* **1997**, *45*, 2042–2046.
12. Tenailleau, E. J.; Lancelin, P.; Robins, R. J.; Akoka, S. *Anal. Chem.* **2004**, *76*, 3818–3825.
13. Caytan, E.; Remaud, G. S.; Tenailleau, E. J.; Akoka, S. *Talanta*, in press.
14. Zhang, B.-L.; Trierweiler, M.; Jouitteau, C.; Martin, G. J. *Anal. Chem.* **1999**, *71*, 2301–2306.
15. Gleixner, G.; Schmidt, H.-L. *J. Biol. Chem.* **1997**, *272*, 5382–5387.
16. Chemicals: Typically, 1 g of glycerol was dissolved in 110 mL of 1,4-dioxane (VWR, France) with 3.7 g of benzoic anhydride (Alfa Aesar, France) and 0.9 g of carrier-fixed *Candida antarctica* Novozym 435 (Sigma, France). The mixture was stirred until there was complete consumption of glycerol, monitored by GC. After filtration (for enzyme recovery), the reaction mixture was evaporated under reduced pressure. The residue was dissolved in ethyl acetate (VWR, France)/*n*-hexane (VWR, France) (50/50) and loaded onto a silica gel (VWR, France) column. Dibenzoyl glycerol, benzoic acid and the remaining benzoic anhydride were eluted first by ethyl acetate/*n*-hexane (50/50). Then, MBG was obtained with pure ethyl acetate. To complete elution, methanol (VWR, France) may be added when monitored by GC. After evaporation to dryness, the refined MBG was dissolved in the minimum of boiling 2-propanol (VWR, France)/*n*-hexane (5/95). One crystal of (*R*)-MBG was added to initiate preferential crystallisation. The mixture was further chilled overnight at −15 to −20 °C. The white crystals of MBG were filtered and finally dried in an oven (40 °C max).
17. NMR experiments: Samples were prepared by dissolving 400 mg of purified MBG in 800 μL of acetone-D_6 (Eurisotop, France). Quantitative ^{13}C NMR spectra were recorded using a Bruker DRX 500 spectrometer fitted with a 5-mm-i.d. dual probe $^{13}C/^1H$ carefully tuned at the recording frequency of 125.76 MHz. The temperature of the probe was set at 303 K. The experimental parameters for ^{13}C NMR spectral acquisition were the following: pulse width 7.8 μs (90°), spectral width 30,000 Hz, sampling period 1 s, repetition delay 17 s, number of scans 400. Inverse-gated decoupling techniques were applied in order to avoid NOE. The decoupling sequence employed a cosine adiabatic pulse with appropriate phase cycles, as described in Ref. 10. Each sample was measured four times.