Research Article

Magnetic Resonance in Chemistry

Received: 14 September 2011 Revised: 16 February 2012 Accepted: 25 February 2012 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/mrc.3814

^{13}C NMR quantification of mono and diacylglycerols obtained through the solvent-free lipase-catalyzed esterification of saturated fatty acids

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In the present investigation, we studied the enzymatic synthesis of monoacylglycerols (MAG) and diacylglycerols (DAG) via the esterification of saturated fatty acids (stearic, palmitic and an industrial residue containing 87% palmitic acid) and glycerol in a solvent-free system. Three immobilized lipases (Lipozyme RM IM, Lipozyme TL IM and Novozym 435) and different reaction conditions were evaluated. Under the optimal reaction conditions, esterifications catalyzed by Lipozyme RM IM resulted in a mixture of MAG and DAG at high conversion rates for all of the substrates. In addition, except for the reaction of industrial residue at atmospheric pressure, all of these products met the World Health Organization and European Union directives for acylglycerol mixtures for use in food applications. The products were quantified by ^{13}C NMR, with the aid of an external reference signal which was generated from a sealed coaxial tube filled with acetonitrile-d3. After calibrating the area of this signal using the classical external reference method, the same coaxial tube was used repeatedly to quantify the reaction products. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: esterification; acylglycerols; lipases and quantitative ^{13}C NMR

Introduction

Partial acylglycerols, such as monoacylglycerols (MAG) and diacylglycerols (DAG), are well-known biodegradable, biocompatible, non-toxic and non-ionic surfactants that are widely used in food, pharmaceutical and industrial applications.[1,2] Compared with traditional chemical glycerolysis, lipases demand milder reaction conditions, which minimize energy costs, allow better reaction control and provide higher-quality products.[3] Several studies have shown that partial acylglycerols can be produced from unsaturated fatty acids by lipase-catalyzed reactions.[4–7] However, to the best of our knowledge, very few studies have explored enzymatic esterification from saturated fatty acids (SFA) in a solvent-free system.[8–12]

In recent years, a growing number of scientific reports on the successful application of ^{1}H NMR and ^{13}C NMR to the quantification of esterification and trans-esterification products have been published.[13–18] Compared with traditional chromatographic-based techniques (GC and HPLC),[19] NMR offers the following advantages: (i) sample preparation is easy, (ii) the technique is non-destructive and non-invasive, (iii) multiple target analytes in a mixture can be directly quantified without isolation, (iv) additional structural information is readily available in a single analysis and (v) a reference of the compound of interest is not required.

Quantification by NMR, sometimes referred to as qNMR is based on the premise that the signal produced by exciting a nucleus from a fully relaxed state is directly proportional to the number of molecules that contain the nucleus of interest. qNMR can be performed by comparing the area of a selected analyte peak with a standard compound at a known concentration.[19,20]

Among several basic requirements, a suitable standard for NMR quantification must produce a sharp signal, preferably a singlet, to avoid peak overlap. For both internal (the standard compound is co-dissolved with the analyte) and external (the standard is added to a coaxial insert) calibration methods, the peaks corresponding to the calibration compound and the analyte must not overlap.[21,22] Although peak overlap can be circumvented by altering the external calibration method (the spectrometer response can be calibrated with standards that are independently measured in separate precision tubes), this approach does not guarantee that the analyte and reference will be analyzed and processed under identical conditions.

Alternatively, the benefits of both methods can be combined, and a generic liquid compound in a sealed coaxial insert, such as regular or deuterated solvent, can be used as a suitable internal reference for quantification. After calibrating the signal from the internal reference against an external standard, the coaxial insert can be switched from one tube to another after each

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NMR analysis, allowing accurate and robust quantification. Based on this technique, a simple method for the ^{13}C NMR quantification of monoacylglycerols and diacylglycerols produced via solvent-free, lipase-catalyzed esterification of SFA was developed, and an external reference signal was generated from a sealed coaxial tube filled with acetonitrile-d3.

Methodology

Lipase-catalyzed reactions

Solvent-free esterification reactions were evaluated by varying four parameters, including (i) the type of lipase[23–27]: Novozym 435, Lipozyme RM IM or Lipozyme TL IM; (ii) the type of free fatty acids (FFA): stearic acid (C18:0), palmitic acid (C16:0) or an industrial residue (containing 87% palmitic acid); (iii) the reaction time: 3 or 6 h and (iv) vacuum application: no vacuum or 3 mm Hg. The FFA industrial residue and all of the lipases used in the present study were kindly donated by Agropalma A/S and Novozymes A/S, respectively. The remaining reagents and solvents described herein were analytical grade and were acquired from a local distributor (Vetec, Brazil).

The substrate mixture, which consisted of FFA (0.025 mol for stearic and palmitic acid and 0.022 mol for the industrial residue, relative to palmitic acid), glycerol (0.0375 mol) and lipase (1.0 g), was introduced into a jacket reactor under magnetic agitation, which was maintained at 65 °C (for stearic acid and the industrial residue) or 60 °C (for palmitic acid) via water circulation. All reactions were carried out in duplicate. At the end of the reaction period, the resultant mixture was dissolved in dichloromethane, filtered to remove the catalyst and washed with distilled water (3 × 15 ml) to remove residual glycerol. The organic phase was extracted with dichloromethane (3 × 15 ml), and the solvent was removed under reduced pressure. The products were dried over anhydrous sodium sulfate and were stored in a refrigerator in an amber flask. The reactions were performed in parallel under the same conditions, except for the presence of the enzyme.

^{13}C NMR quantification

To shorten the relaxation time, a $CDCl_3$ stock solution of $Cr(AcAc)_3$ (0.1 M) and 0.1% TMS was used to prepare standards and samples. The standard and sample solutions were transferred from volumetric flasks to the annular space between the stem coaxial insert (no. WGS-0BL) and the 10-mm precision NMR tube (no. 513-7PP-7, Wilmad-LabGlass, USA). The volume of liquid added to the coaxial insert and sample tube is not important because the tube extends well past the sensitive area of the receiver coil.[28,29] All deuterated solvents, standards and relaxation agents were obtained from Sigma Aldrich.

Internal reference

A solution of $Cr(AcAc)_3$ (0.1 M) in acetonitrile-d3 (99.8%) was placed in the coaxial insert, and the 10-mm sample tube was fitted to the insert prior to ^{13}C NMR analysis. The C≡N group of acetonitrile-d3, which appeared at 118.7 ppm, produced a non-overlapping, constant-amplitude reference signal on each spectrum. After NMR analysis, the coaxial insert was carefully rinsed with acetone, completely dried and switched to another sample tube.

Calibration

Calibration of the reference signal was performed using six samples of L-menthol (≥99%, 0.106×10^{-3}–0.731×10^{-3} mol/ml in a $CDCl_3$ stock solution). To assess the stability of the system, three calibration samples were prepared and analyzed prior to the two product quantification runs. Using the quantitative ^{13}C NMR spectra, the calibration model was established according to the linear relationship $Conc = b_0 + b_1*(Area/Area^{ref})$, where *Conc* is the calibration sample concentration (mol/ml), and *Area* and $Area^{ref}$ are the areas of the calibration and reference peaks, respectively. The CH group of menthol at 49.2 ppm was arbitrarily selected as the calibration peak. The intercept (b_0) and angular coefficient (b_1) were estimated by least squares regression using Origin ver.6.0 software (OriginLab, USA). Unlike the NMR precision tubes, the stem coaxial inserts are not manufactured with absolute rigid tolerances; thus, the developed calibration model can only be applied to the insert employed in the present study. Otherwise, a new calibration model must be constructed.

Quantification

Samples from each esterification product were prepared from the same $CDCl_3$ stock solution at a concentration of approximately 30% (*m/v*) and were analyzed by quantitative ^{13}C NMR in two different runs, which were separated by a 30-day period. The acylglycerols were identified using the C-2 groups along the glycerol backbone, which resonates in the characteristic spectral region from 68 to 76 ppm, according to Gunstone[15]: 1-MAG = 70.37; 2-MAG = 75.02; 1,2-DAG = 72.24; 1,3-DAG = 68.35 and TAG = 69.02 ppm. After integrating the internal reference peak and the ^{13}C NMR peaks of the glycerol backbone of MAG, DAG and TAG, the concentrations were predicted with the previously prepared calibration model. In the same manner, the residual FFA concentrations were also measured using the carboxylic peak[30] at 179 ppm. To verify the reliability of the method, the FFA concentrations obtained by ^{13}C NMR were compared with those measured by NaOH titration.

Acidity by titration

The FFA concentration was determined by titration, which was executed according to a modified Ca 5a-40 method.[31] Approximately 0.5 g of the sample was diluted in 50 ml of an ethanol/ethyl ether 1 : 1 (*v/v*) mixture and was titrated with a NaOH solution (0.05 M) using phenolphthalein as the end-point indicator. The acidity indicates the percentage of fatty acids remaining at the end of the reaction or the degree of esterification.

Data acquisition and processing

NMR experiments were performed on a 11.75-Tesla VNMRS (Varian, USA) spectrometer equipped with two 10-mm channel broadband probes for ^{15}N-$^{31}P\{^{1}H$-$^{19}F\}$. Quantitative ^{13}C NMR spectra were recorded at 35 °C without spinning the sample, and an inverse-gated decoupling sequence[32] with a recycle delay of 3.2 s (more than five times the longest T_1) and a WALTZ16 proton decoupling scheme were employed. Free-induction decays (FIDs) were collected using 42.779 data points over a spectral width of 220 ppm, and a 90° pulse was applied for 12 μs until a signal-to-noise level of 200 (relative to the larger signal between 68 to 76 ppm) was obtained. The samples were identically positioned, individually tuned and matched before each experiment. Prior to multiplication by a 5.0-Hz exponential function and Fourier transformation, the FID was zero-filled to 143.360 points. The phase and baseline were corrected manually.

The peak areas were measured by Voigt function deconvolution using MestReNova ver.6.0.2 (Mestrelab, Spain).

Results and Discussion

^{13}C NMR quantification

Internal reference calibration

After collecting quantitative ^{13}C NMR spectra of the six menthol calibration samples, which were split in two different runs separated by a 30-day period, a calibration model was developed by performing a linear regression on the peak areas ratios from menthol (CH at 49.2 ppm) and acetonitrile-d3 (C≡N at 118.7 ppm) *versus* the menthol concentration, as shown in Fig. 1.

Although the spectrometer configuration was switched several times between the two runs (the probes were changed and the system was recabled), the correlation coefficient ($R^2 = 0.9997$, $p = 0.0001$) for the regression was high, which indicates that the response for the two runs was linear, even after 30 days. The ruggedness of the configurational changes is an interesting feature, considering the context of most NMR facilities, in which multipurpose spectrometers cannot be scheduled for long uninterrupted periods. The regression statistics also showed that the intercept of the calibration model was not significantly different from 0 ($b_0 = -0.0028 \pm (0.0054)$), which is a good indication that the adopted baseline correction and line fitting procedure were suitable for avoiding instrumental artifacts.[33]

Acetonitrile-d3 was chosen as an internal reference instead of non-deuterated acetonitrile solely because it is readily available in the solvent inventory of most NMR laboratories. However, a regular solvent is preferable as an internal reference for ^{13}C NMR quantification because, in addition to reducing sensitivity, peak splitting due to scalar coupling between ^{2}H and ^{13}C can interfere with the peak of interest.

Regarding the external calibration standards, even those that produce too many NMR peaks are suitable for calibrating the internal reference signal. Aside from the purity of the standard, which must be analytical grade, the only prerequisite is that the peaks of the standard must not overlap with those of the internal reference. For example, menthol is not the most appropriate compound for acylglycerol quantification via internal and external (by coaxial inserts) calibration because its C-1 peak (CH at 71.3 ppm) lies in the middle of the glycerol backbone region.

In addition to the CH group at 49.2 ppm, other menthol ^{13}C signals were also evaluated (data not shown); however, the results were virtually identical. When the ^{13}C NMR spectra are acquired under quantitative conditions, the peak area must be equivalent regardless of which peak is selected.

Quantification performance evaluation

After collecting and processing the 36 ^{13}C NMR spectra of the esterification products, the peaks were integrated relative to FFA, and their concentrations (mol/ml) were calculated by applying the internal reference and respective peak area ratios ($Area/Area^{ref}$) to the previously developed calibration model. To verify the reliability of this method, the FFA concentrations (*m/v*) obtained by ^{13}C NMR were compared with the reference values measured by NaOH titration, as shown in Fig. 2.

In addition to the high correlation coefficient ($R^2 = 0.9945$, $p = 0.0001$) of the linear regression of the response curve, the intercept and slope were not significantly different from 0 ($b_0 = -0.1079 \pm (0.6202)$) and 1 ($b_1 = 0.9922 \pm (0.0130)$), respectively, suggesting that the NMR response was not biased (by additive or proportional systematic errors) compared with the titration. Regarding the accuracy of NMR, the root-mean-square error, a measurement of the average difference between the predicted and measured response, suggested that the FFA concentration can be measured with an average error of 1.8%, which can be extrapolated to estimate the quantification error of acylglycerols. The performance of NMR was compatible with the expected analytical errors involved in the preparation of samples that are difficult to handle, such as SFA derivatives, which require extra care to ensure sample homogeneity and avoid losses.

The overall performance of NMR measurements also suggested that the quantitative experimental parameters were well adjusted, especially the relaxation delays, which were selected to ensure ^{13}C was detected from a completely relaxed spin state (at least 99%).

Synthesis evaluation

From the ^{13}C NMR spectra of the esterification products, the peaks were unambiguously assigned and integrated relative to

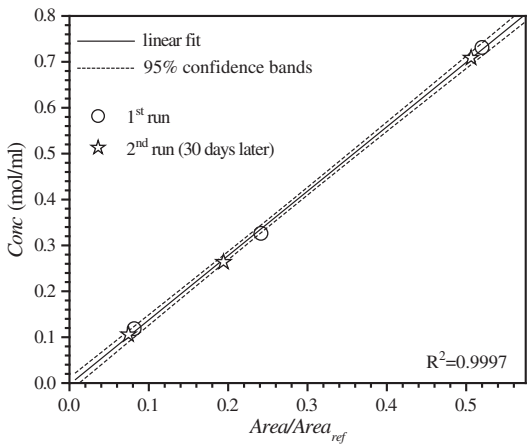

Figure 1. Regression between menthol (CH at 49.2 ppm) and acetonitrile-d5 (C≡N at 118.7 ppm). ^{13}C NMR peak area ratios and menthol solution concentrations were acquired in two different runs, which were separated by a 30-day period. The resultant internal reference calibration model was $Conc = -0.0028(\pm 0.0054) + 1.4035(\pm 0.0165)*(Area/Area^{ref})$.

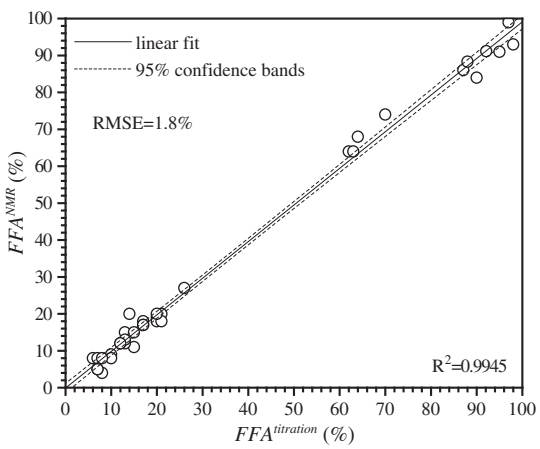

Figure 2. Regression between the FFA concentrations measured by NaOH titration ($FFA^{titration}$) and predicted by NMR (FFA^{NMR}). The resultant model response curve was $FFA^{NMR} = -0.1079(\pm 0.6202) + 0.9922(\pm 0.0130) * FFA^{titration}$.

the C-2 groups on the glycerol backbone of the acylglycerols. Figure 3 presents a typical spectrum of acylglycerols produced from lipase-catalyzed esterification and an expansion of the glycerol backbone region, where the MAG, DAG and TAG C-2 peaks were clearly observed.

The MAG, DAG and TAG concentrations (mol/ml) were calculated by computing the internal reference and respective C-2 peak area ratios ($Area/Area^{ref}$) and applying the results to the previously developed calibration model. Table 1 summarizes the acylglycerol quantification data according to the enzyme and reaction

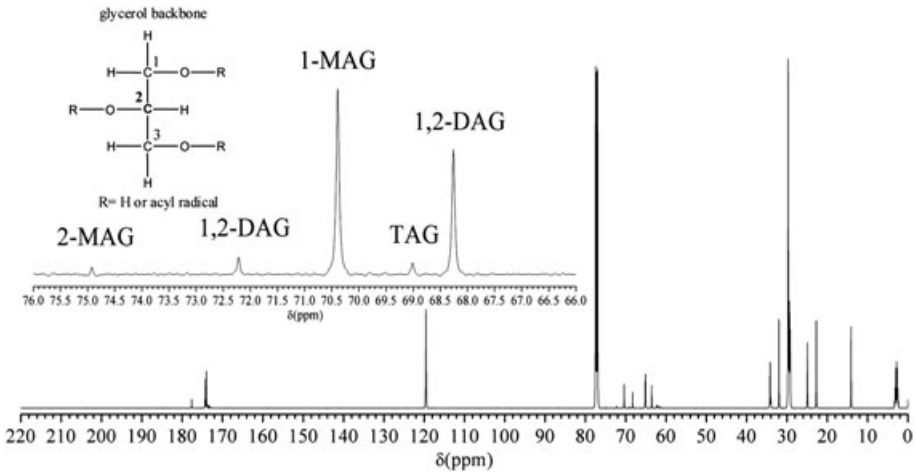

Figure 3. Typical ^{13}C NMR spectrum of a mixture of acylglycerols. The insert shows the C-2 region of acylglycerols, which was used for quantification.

Table 1. Lipase-catalyzed esterification reaction of pure stearic acid, palmitic acid and industrial fatty acid residue containing 87% palmitic acid, excluding Lipozyme TL IM

Substrate (lipase type)	Conditions		Acylglycerol composition (%)[a]					DAG/MAG	Conversion (%)
			MAG		DAG		TAG		
	Vacuum	Time (h)	1-	2-	1,2-	1,3-			
Stearic acid (C18:0)									
(Novozym 435)	No	3	20	1	14	35	20	2.3	91
	No	6	27	1	15	34	14	1.8	92
	Yes	3	25	0	1	54	12	2.2	92
	Yes	6	22	0	5	49	19	2.4	95
(Lipozyme RM IM)	No	3	35	1	5	35	3	1.1	80
	No	6	29	1	6	35	9	1.4	80
	Yes	3	29	9	14	33	3	1.2	88
	Yes	6	34	1	6	38	5	1.2	85
Palmitic acid (C16:0)									
(Novozym 435)	No	3	20	0	6	53	9	3.0	88
	No	6	17	0	3	54	14	3.2	83
	Yes	3	19	0	7	44	12	2.7	82
	Yes	6	16	0	9	41	16	3.2	81
(Lipozyme RM IM)	No	3	34	0	5	37	5	1.2	82
	No	6	32	1	7	34	10	1.2	85
	Yes	3	38	1	5	43	4	1.2	92
	Yes	6	36	2	7	43	6	1.3	95
Fatty acid residue									
(Novozym 435)	No	3	7	0	8	59	14	10	88
	No	6	11	0	16	51	14	6	92
	Yes	3	23	1	12	35	13	2	83
	Yes	6	26	1	11	32	17	1.6	87
(Lipozyme RM IM)	No	3	26	1	9	28	9	1.4	74
	No	6	25	1	10	30	14	1.5	80
	Yes	3	32	1	4	43	8	1.4	88
	Yes	6	32	0	12	38	10	1.5	92

[a]Determined by ^{13}C qNMR.

conditions for reactions with conversion rates greater than 20%. All reactions with Lipozyme TL IM were below this limit, and the corresponding results are not reported in Table 1.

Regarding the FFA conversion, Novozym 435 and Lipozyme RM IM showed superior performance. This result is in good agreement with that of Freitas *et al.*,[8] who found that the conversion degree of Lipozyme RM IM (86%) in the esterification of lauric acid (C12 : 0) was superior to Lipozyme TL IM (45%). To our knowledge, reports describing the application of Novozym 435 to the solvent-free synthesis of partial acylglycerols from SFA have not been previously published.

Aside from the natural predominance of 1-MAG over 2-MAG, 1,3-DAG was the main diester produced under all of the reaction conditions. This behavior was expected because the enzymes are 1,3-specific; however, the DAG-to-MAG ratio also suggested that Novozym 435 was more specific for diester formation. In general, Novozym 435 also presented a 1,3-DAG-to-1,2-DAG ratio that was equal to or greater than that of Lipozyme RM IM, indicating that the regioselectivity of the studied reaction system was higher.

Considering the application of acylglycerol mixtures in the food industry, Lipozyme RM IM provided the best performance under the reaction conditions evaluated in the present study. Except for the reaction of industrial residue at atmospheric pressure, all of the esterifications catalyzed by Lipozyme RM IM resulted in a final mixture that fulfilled the World Health Organization (WHO) and European Union (EU) directives.[34,35] According to the WHO and EU, acylglycerol mixtures used as food emulsifiers must contain at least 70% MAG + DAG and 30% MAG and less than 10% of glycerol and TAG.

Although some of the reaction conditions resulted in the formation of products with specifications that did not fulfill the prerequisites for use in foodstuffs, good conversion rates were obtained, and the products can be employed in non-food applications, such as cosmetic creams, pharmaceutical preparations, cement and concrete additives, polishes, shoe waxes and plastics.

Conclusions

A mixture of MAG and DAG was obtained with high conversion via lipase-catalyzed esterification of SFA under solvent-free reaction conditions. Due to the simplicity and efficiency of the proposed methodology, this method can be used as an alternative to alkaline-catalyzed chemical glycerolysis to obtain MAG and DAG on an industrial scale.

We demonstrated that the utilization of a constant natural reference signal from an analytical grade solvent in a sealed coaxial insert, combined with classical external calibration, was an extremely simple and reliable approach for routine NMR quantification. Methods such as electronic reference to access *in vivo* concentrations and quantification by artificial signal also use artificial internal reference signals for quantification; however, dedicated hardware and software are required.[36–38]

Regarding the quantification of acylglycerol products, ^{13}C NMR can compete with GC and HPLC, as highlighted in the introduction. However, in general, quantitative ^{13}C NMR is more time-consuming and expensive than chromatographic-based techniques.

Acknowledgements

We are grateful to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for a research grant and Agropalma A/S and Novozymes A/S for donating the industrial FFA residue and lipases, respectively. We would also like to acknowledge two anonymous reviewers for their technical and editorial comments, which improved the paper.

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