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PAPER

Sustainable and efficient methodology for CLA synthesis and identification†

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Microwave-assisted organic synthesis and continuous-flow techniques have been successfully employed for the preparation of conjugated linoleic acids (CLA), compounds with high health beneficial effects. A good production rate of CLA was obtained. A sustainable methodology for the differentiation of both positional and geometrical CLA isomers (diene), based on the analysis by NMR spectroscopy of the resulting Diels–Alder cycloadducts with an appropriate dienophile, was developed.

Introduction

Due to their unique biological properties, conjugated linoleic acids (CLAs) have received a great deal of attention in the last few decades. These particular fatty acids show conjugated diene systems and are found in tissues of ruminant animals and, consequently, in meat and dairy products. They are formed as intermediate compounds in the biohydrogenation of linoleic acid (LA) carried out by microorganisms in the rumen. However, several positional and geometrical isomers (6,8- to 12,14-18:2) are formed. Among these, 9-*cis*,11-*trans*-octadecadienoic acid (9*c*,11*t*-CLA) is the main isomer and constitutes about 1% of the fatty acids of milk fat. Moreover, this isomer exhibits highly interesting specific physiological activities such as anti-obesity, anti-carcinogenic, anti-atherogenic, anti-diabetic, antioxidant, apoptotic, immunomodulatory and osteosynthetic effects.[1]

Several strategies can be followed for synthesizing CLAs. One of the employed methods is the alkali isomerization of linoleic acid, which leads to mixtures of geometric isomers of CLA.[2] Another approach is based on the dehydration of ricinoleic acid methyl ester (RAME) in the presence of $KHSO_4$ or other expensive dehydration reagents, such as DBU (1,8-diazabicyclo-(5.4.0)-undec-7-ene).[3] It is also possible to obtain 9*c*,11*t*-CLA from linoleic acid by means of microbial synthesis or chemo-enzymatic conversions.[4] However, the first method is the most widely used because of economic reasons.

As for the analytical tools, gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been extensively used to identify this kind of fatty acid.[5] Moreover, ^{13}C-NMR spectroscopy was reported as a tool to identify CLAs; however, only predicted data were given for some of the isomers.[6] On the other hand, the positions of double bonds in conjugated dienes can often be verified by mass spectrometry (MS) of dimethyloxazoline (DMOX) derivatives.[7] In addition, conjugated double bonds of CLAs can react specifically with certain dienophiles in Diels–Alder cycloaddition reactions. Thus, 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) readily forms stable adducts with such conjugated dienes and these adducts are sufficiently volatile to be analyzed by GC.[8] A related reagent to MTAD has been used for sensitive fluorescence detection of CLAs in human serum by HPLC.[9] However, all these methods only allowed the determination of positional isomers, not giving information on the double bond geometry.

Microwave irradiation has been successfully applied in organic chemistry.[10,11] Spectacular accelerations in comparison to conventional heating are attained, resulting in shorter reaction times. In this sense, Diels–Alder reactions, which are often limited by the reversibility of the reaction, have been accomplished with great success due to the reduction of reaction times.[12] It is worth noting that, under microwave heating, the selectivity (chemo-, regio- and stereoselectivity) can be modified in relation to that obtained with conventional heating.[13] Further improvements can be achieved by using neutral and recyclable ionic liquids (ILs), or under solvent-free reaction conditions, which give rise to environmentally friendlier protocols.[14]

We herein report the development of a new methodology to identify conjugated linoleic acids in a complex matrix. The study concerns the search and implementation of the most appropriate dienophile for identifying CLA isomers (diene) by NMR analysis of the corresponding formed Diels–Alder cycloadducts. The use of maleic anhydride in microwave-assisted solvent-free cycloaddition reactions of highly reactive CLAs permits the differentiation of both positional and geometrical CLA isomers by ^{13}C-NMR spectroscopy. Furthermore, two new sustainable approaches for the synthesis of CLAs are reported.

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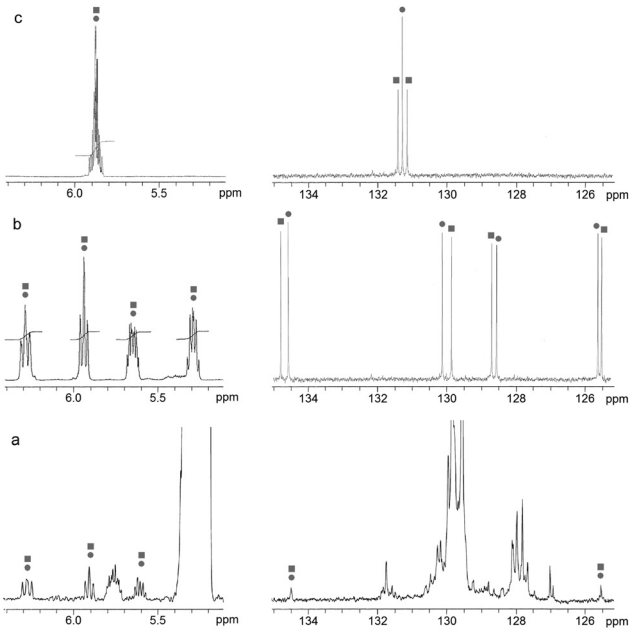
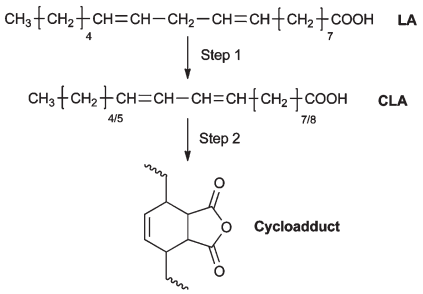


Fig. 1 Olefinic ^{1}H- and ^{13}C-NMR data of 9*c*,11*t* CLA (■) and 10*t*,12*c* CLA (●) isomers in a cheese organic extract sample (a), synthetic mixture isomers (b) and the corresponding formed Diels–Alder cycloadducts (c).

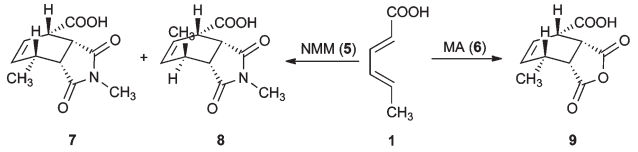


Scheme 1 General scheme of the methodology. Step 1: synthesis of CLA. Step 2: Diels–Alder cycloaddition reaction.

Results and discussion

Diels–Alder reactions of conjugated linoleic acids

The necessity of the development of a new methodology for differentiating the positional and geometrical isomers of CLA mixtures is illustrated by three different key facts. (i) The separation of the different CLA isomers is a rather difficult task by means of the commonly used purification techniques; (ii) their identification in a food complex matrix is also difficult because of their low natural abundance and the presence of other compounds (Fig. 1a); (iii) the different CLA isomers show up similar data in ^{1}H and ^{13}C-NMR spectra (Fig. 1b), even more, the chemical shifts in the ^{13}C-NMR spectrum depend on the presence of other isomers in the food matrix. Therefore, we focused our attention on the development of a methodology for such purpose implying the NMR analysis of the stereochemistry of the corresponding formed cycloadducts as a result of an appropriate Diels–Alder reaction (Scheme 1, step 2), presumably with simpler spectra, with differences in their ^{13}C-NMR chemical shifts, and provided that NMR spectroscopy allows the elucidation of the CLA geometric (*cis*/*trans*) isomerism in contrast to GC and HPLC analytical techniques.



Scheme 2 Diels–Alder reactions of sorbic acid (**1**).

Table 1 Diels–Alder reaction conditions of sorbic acid (**1**) with different dienophiles

Entry	Dienophile	*T* (°C)	*t* (min)	*P* (W)	Yield (%)
1	TCE (**3**)	80	15	30	—
2	PBQ (**4**)	150	15	120	—
3	NMM (**5**)	100	15	30	72 (**7**) + 18 (**8**)
4	MA (**6**)	150	15	100	93 (**9**)

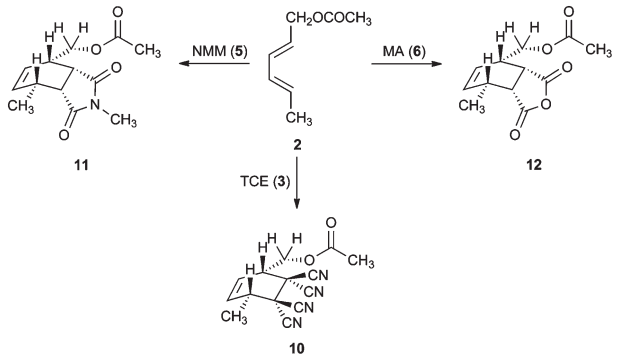
Thus, some compounds similar in structure to CLA were purchased to be used as dienes in order to understand the reactivity of CLAs in Diels–Alder cycloadditions for certain sustainable reaction conditions. Then, sorbic acid (**1**) and 2,4-*trans*,*trans*-hexadienyl acetate (**2**) were chosen. On the other hand, focused on finding the best dienophile, tetracyanoethylene (TCE) (**3**), *p*-benzoquinone (PBQ) (**4**), *N*-methylmaleimide (NMM) (**5**) and maleic anhydride (MA) (**6**) were selected, because they are known to be highly reactive in this type of reaction, stable and their products easily characterized.[15]

Reactions were optimized and carried out under solvent-free conditions in a monomode microwave reactor Discover CEM™. Different power and temperatures were used depending on the dienophile and in all reactions microwave irradiation was required for 15 min (Tables 1 and 2).

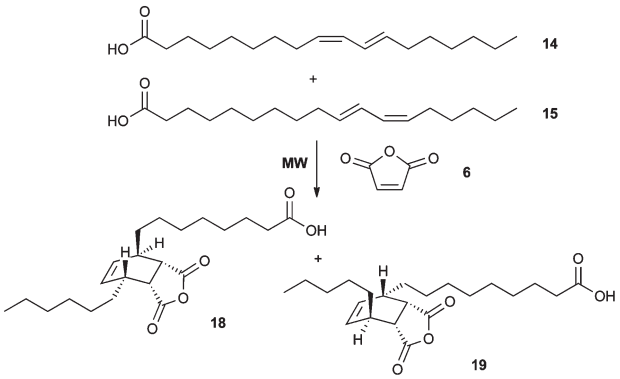
In the case of sorbic acid (**1**), excellent yields were obtained from *N*-methylmaleimide (**5**) and maleic anhydride (**6**) (Scheme 2). However, no products were obtained from tetracyanoethylene (**3**) because of an uncontrolled absorption of the microwave irradiation, resulting in a very quick increase of the reaction temperature and an undesirable reaction mixture. *p*-Benzoquinone (**4**) did not turn out to be a good dienophile since changes in the reaction parameters (time, temperature and power) did not result in products. Yields of the reaction are shown in Table 1. The pure products were identified by ^{1}H and ^{13}C-NMR spectroscopy as well as mass spectrometry.

The formation of stereoisomeric products should only be possible as a consequence of isomerization of the double bonds, which was not observed. However, this is not the case of reaction of sorbic acid (**1**) with NMM (**5**) (Table 1, entry 3) which produced diastereoisomers **7** and **8** in a 4 : 1 ratio; both result from the *endo* addition. The stereochemistry of these products was inferred by NOE difference experiments.

On the other hand, the previous set of cycloaddition reactions were carried out again but employing 2,4-*t*,*t*-hexadienyl acetate (**2**) as a diene (Scheme 3, Table 2). These reactions led to good results for all dienophiles except for *p*-benzoquinone (Table 2,



Scheme 3 Diels–Alder reactions of 2,4-*t*,*t*-hexadienyl acetate (**2**).



Scheme 4 Diels–Alder reactions of conjugated linoleic acid (**14–15**).

Table 2 Diels–Alder reaction conditions of 2,4-*t*,*t*-hexadienyl acetate (**2**) with different dienophiles

Entry	Dienophile	*T* (°C)	*t* (min)	*P* (W)	Yield (%)
1	TCE (**3**)	80	15	30	88 (**10**)
2	PBQ (**4**)	150	15	120	—
3	NMM (**5**)	100	15	30	93 (**11**)
4	MA (**6**)	150	15	100	95 (**12**)

entry 2), where changes in reaction parameters (time, temperature and power) did not result in the desired products. No isomerization of double bonds was observed. The pure products were identified by ^{1}H and ^{13}C-NMR spectroscopy as well as mass spectrometry. All reactions are 100% stereoselective and produce the *endo* stereoisomer according to the general requirements of the Diels–Alder reaction. The stereochemistry of these products was inferred by NOE difference experiments.

Based on such results, we can conclude that the best dienophile is the maleic anhydride (**6**) due to its high stereoselectivity and high reaction yields. Accordingly, the study of the reactivity of conjugated linoleic acids was carried out using maleic anhydride (**6**) as a dienophile.

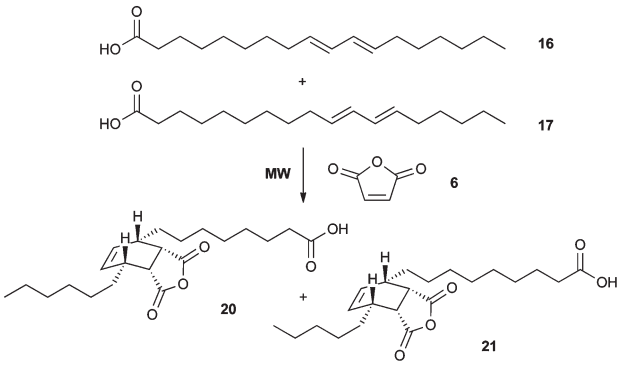
Two sustainable approaches, microwave-assisted organic synthesis as well as continuous flow techniques, were successfully employed (*vide infra*) for the synthesis of CLA, slightly modifying the reported procedure[2] of the alkali isomerization of linoleic acid (**13**) (Scheme 1, step 1). They resulted in a mixture of two positional and geometrical isomers of CLAs which showed very similar NMR data (Fig. 1b). In fact, no differences are found in the ^{1}H-NMR spectra, whilst slight differences are observed in the ^{13}C-NMR spectra (Fig. 1b). Due to the fact that these two methods can lead to the modification of the selectivity of the reaction,[13] the assumption of having obtained the same geometric isomerism as described[2] can not be made. Different methodologies such as column chromatography and HPLC were employed for the separation of the CLA isomers with unsuccessful results. Nevertheless, the purpose of this work was the development of a methodology for the identification of CLA isomers in a complex matrix, the accomplishment of the aforementioned separation being unnecessary.

After the selection of maleic anhydride (**6**) as a dienophile, Diels–Alder reactions were performed employing the initially obtained CLA (diene) mixtures (Scheme 1, step 2). The best results were achieved using microwave irradiation for 15 min at 150 °C in a 1 : 2 diene–dienophile molar ratio. It should be noted that all reactions occurred in the absence of solvent.

The pure products were identified by ^{1}H and ^{13}C-NMR spectroscopy (Fig. 1c) as well as mass spectrometry. A thorough NMR study was performed in order to elucidate the stereochemistry of the formed cycloadducts consisting of the following NMR experiments: g-COSY, g-HMQC, g-HMBC, TOCSY, g-HSQC-TOCSY and HMBC-TOCSY. Particularly, 1D-NOESY and 2D-NOESY experiments made it possible to unequivocally identify the different cycloaddition isomers. In addition, it allows us to conclude that the cycloaddition of maleic anhydride (**6**) with conjugated linoleic acids is also 100% stereoselective because the *endo* isomer is formed exclusively without any of the *exo* isomer.

Thus, cycloadducts **18** and **19** were obtained from the CLA isomers synthesized by the alkali isomerization procedure, with 100% conversion of reagents in the corresponding products and 86% of total reaction yield (Scheme 4). It is important to note that complete conversion is needed for the applicability of this methodology, in order to avoid the presence of NMR signals, corresponding to the starting material, in the region of study. At this point, it was possible to identify the different CLA geometrical isomers used as the starting material, after having elucidated the stereochemistry of the isolated cycloadducts. Accordingly, from linoleic acid isomerization 9*c*,11*t*-CLA (**14**) and 10*t*,12*c*-CLA (**15**) (1 : 1 ratio) were achieved, illustrating that microwave irradiation did not modify the selectivity because no differences were found with respect to the conventional reported method.[2] Note that cycloadduct **18** is derived from CLA **14** and cycloadduct **19** from CLA **15**. More importantly, the ^{13}C-NMR spectrum of the corresponding cycloadducts is simpler than that observed for the CLA isomers (Fig. 1c *vs.* 1b) due to the magnetic and chemical equivalency present in the reaction product. Moreover, the cycloadducts chemical shift values are independent of the presence of other isomers in contrast to that observed for the corresponding CLA isomers. These facts clearly illustrate the validity of the here reported method.

On the other hand, two other CLA isomers originated from isomerization of 9*c*,11*t*-CLA (**14**) and 10*t*,12*c*-CLA (**15**) catalyzed by iodine, expecting to observe the *trans*/*trans* geometry as reported.[16] The Diels–Alder cycloaddition of such dienes with maleic anhydride afforded the cycloadducts **20** and **21**,



Scheme 5 Diels–Alder reactions of conjugated linoleic acid (**16–17**).

with 100% conversion of the diene and 80% of total reaction yield (Scheme 5). After elucidation of the stereochemistry of the isolated cycloadducts, the geometric isomerism of the starting material was deduced to be *trans*/*trans*, particularly 9*t*,11*t*-CLA (**16**) and 10*t*,12*t*-CLA (**17**) (1 : 1 ratio). Cycloadduct **20** is derived from CLA **16** and cycloadduct **21** from CLA **17**.

In short, 9*c*,11*t*-CLA (**14**), 10*t*,12*c*-CLA (**15**), 9*t*,11*t*-CLA (**16**) and 10*t*,12*t*-CLA (**17**) are the CLA isomers obtained. Note that they represent the most interesting CLA isomers from a biological point of view, where 9*c*,11*t*-CLA (**14**) is significantly the most important.[1]

To verify the aforementioned conclusions, we performed the Diels–Alder reactions with some pure commercially available CLA isomers (9*c*,11*t*-CLA (**14**), 10*t*,12*c*-CLA (**15**) and 9*t*,11*t*-CLA (**16**)). For all cases, quantitative yields in the formation of the cycloadduct were obtained for maleic anhydride (**6**) as the dienophile in our reaction conditions. The pure products were identified by ^{1}H and ^{13}C NMR spectroscopy as well as mass spectrometry, confirming that the former relation found for each pair CLA–cycloadduct (*i.e.* cycloadduct **18** derived from 9*c*,11*t*-CLA (**14**)) was correct. The comparison of the NMR spectra of our resulting reaction mixture, in which some CLA isomers are present, with a purchased CLA isomer would be possible, but only in the case in which a limited number of isomers are present as illustrated in Fig. 1b (only two isomers and with slight differences in ^{13}C-NMR chemical shift values ($\Delta\delta_{13C\text{-}NMR}$ = 0.1–0.025 ppm)). However, this comparison can not be done in a complex matrix (Fig. 1a) since the presence of other compounds not only makes the identification a difficult task but also alters the NMR chemical shifts of the CLA isomers. These facts illustrate the utility of this reported methodology.

In the attempt to further confirm the stereochemistry of the different cycloadducts, theoretical calculations were performed. One of the most important uses of theoretical distance geometry is for deriving conformations that are consistent with the experimental distance information, especially distances obtained from NMR experiments as in this case.[17] Therefore, the corresponding structures at their ground state were optimized. At a first stage, Monte Carlo Multiple Minimum simulations were carried out to preliminarily evaluate the most stable conformers for each cycloadduct (see ESI† for the structure of all the conformers). The OPLS_2005 force field as implemented in MacroModel v.9 was used for all molecular modelling. As a second step, the selected conformers were optimized at the B3LYP(PCM)/6-31G* level, considering chloroform as the solvent. The most stable structures for each type of cycloadduct are depicted in Fig. 2. Both cycloadducts **18** and **19** adopted a conformation of half-chair (twisted form). In contrast, cycloadducts **20** and **21** exhibited a preference for a boat conformation. Nonetheless, in all cases the alkyl substituents on the central cyclohexene ring occupied equatorial positions in comparison with the less sterically demanding hydrogen atoms, which remained in the axial positions.

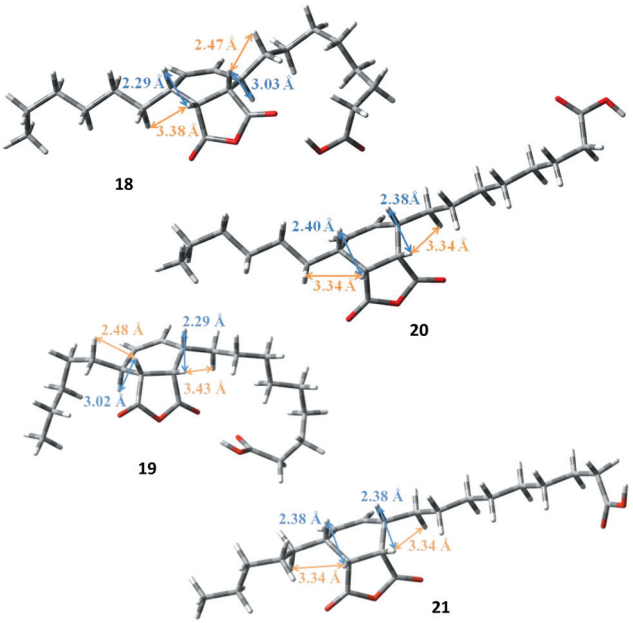


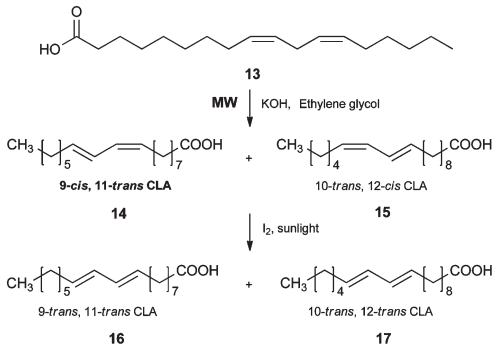
Fig. 2 Fully optimized B3LYP/6-31G* structures of the different Diels–Alder cycloadducts of CLA and maleic anhydride.

Table 3 Comparison of NOE experiment data (%) (after irradiating the protons of interest) with the distances (H–H) theoretically calculated (Å)

H–H	Cyc	NOE	d_{H-H}	Cyc	NOE	d_{H-H}
$H_{3'a}$–$H_{4'}$	**18**	1	3.03	**20**	5	2.38
$H_{7'a}$–$H_{7'}$		3	2.29		5	2.40
$H_{3'a}$–H_8		3	2.47		2	3.34
$H_{7'a}$–$H_{1''}$		1	3.38		2	3.34
$H_{3'a}$–$H_{4'}$	**19**	4	2.29	**21**	5	2.38
$H_{7'a}$–$H_{7'}$		2	3.02		5	2.38
$H_{3'a}$–H_9		2	3.43		2	3.34
$H_{7'a}$–$H_{1''}$		4	2.48		2	3.34

Moreover, it is worth noting that the structural differences among conformers corresponding to the same cycloadduct were related to the disposition of the alkyl chains, and not to the central bicyclic unit.

In order to verify the optimized structures, some relevant calculated proton distances and the corresponding NOE values after irradiating the protons of interest are collected in Table 3. As is shown for each compound, shorter distances are associated with higher NOE values and *vice versa*. In this sense, proton distances of more than 3.00 Å gave NOE values of 1–2%, meanwhile proton distances of *ca.* 2.40 Å led to NOE values of 3–5%. Therefore, this excellent agreement of theoretical distances and NMR data for each optimized structure of the cycloadducts



Scheme 6 Isomerization of linoleic acid (**13**) to CLA isomers.

confirms the obtained structures as the most stable ones. Additionally, it allows us to establish the value of the employed theoretical model for describing the geometrical parameters of the here investigated cycloadducts.

Synthesis of conjugated linoleic acids

Microwave-assisted organic synthesis and continuous flow techniques were employed for the synthesis of CLAs. Initially, basic isomerization of linoleic acid (**13**) was performed following a slightly modified reported method (Scheme 6).[2] Microwave irradiation was successfully applied. In comparison to conventional heating, reaction time was drastically reduced from 4 h to 15 min when employing microwave heating. Tedious and large steps in the preparation of the reaction were avoided because the addition of reagents was carried out in a one pot procedure. In this way, 9*c*,11*t* and 10*t*,12*c* conjugated linoleic acids (**14** and **15**) were obtained with a 1 : 1 ratio and high yields (90%). No changes in isomerization products ratio were observed by using conventional heating or microwave irradiation.

On the other hand, isomers 9*t*,11*t*-CLA (**16**) and 10*t*,12*t*-CLA (**17**) were obtained by isomerization from the mixture of products **14** and **15** (1 : 1 ratio) previously prepared. The reaction was carried out in the presence of iodine at room temperature during 5 h (Scheme 6).[16] An equimolecular mixture of these two isomers was provided with a 75% reaction yield. No isomerization towards *cis*,*cis* double bonds was observed.

As is shown in Scheme 6, pairs of isomers of CLA are obtained which could not be separated either by column chromatography or by HPLC to afford the necessary quantities to execute the Diels–Alder cycloadditions. Therefore, we have worked with pairs of isomers, as shown in the foregoing discussion for the CLA identification.

The same procedure was carried out in a flow system using a minifluidic reactor,[18] R2+R4 Vapourtec. Facile automation, secured reproducibility, improved safety and process reliability are several advantages when working on the continuous flow regime. Over the last 15 years, micro- and minifluidic reactors have entered the field of flow chemistry.[19] Minifluidic reactors present some advantages compared to microfluidic reactors such as improved flow capacities, lower pressure drop and no blocking of channels among others.[18] Optimization of reaction conditions is performed by control of residence time, and scalability of this kind of reaction is simply a matter of pumping, mixing

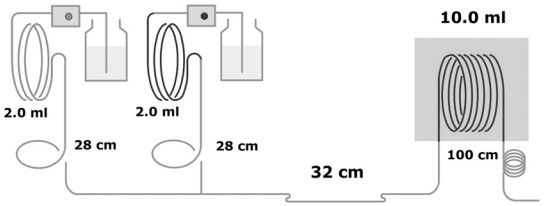


Fig. 3 Experimental setup of the flow system.

and quenching the reagents continuously through the microreactor. This approach permits rapid experimentation and scale-up, thus shortening the time from research to development and production. Furthermore, from an environmental point of view, production of hazardous waste is also reduced.[20]

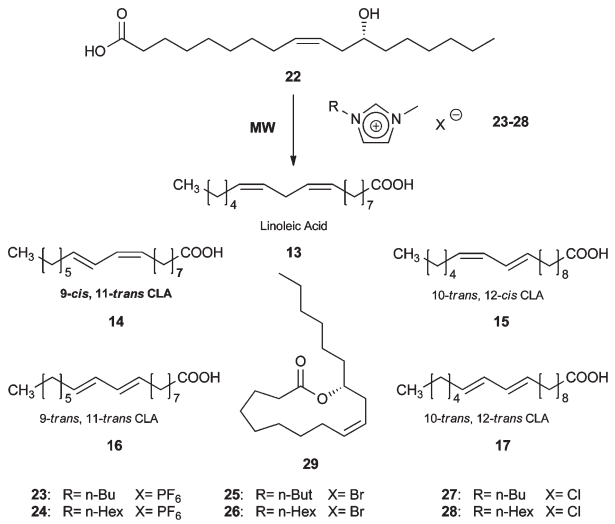
In this sense, in order to improve the synthesis of CLA by the alkali isomerization of linoleic acid (**13**) and to produce relatively high amounts of CLA isomers, a very valuable compound,[21] we envisaged the application of flow chemistry to this reaction. Thus, one of the lines was fed with linoleic acid in ethylene glycol and the other one with the catalyst (KOH in ethylene glycol). Both streams were mixed in a T-mixer and the reaction was carried out in a high temperature module. Then, the output was collected (Fig. 3).

Reaction conditions were optimized by modulating the temperature, time and equivalents of KOH. The best conditions were found at 180 °C and a residence time of 30 min, achieving a 100% conversion of LA to CLA. It should be noticed that the reaction was completed in 30 min instead of the 4 h reported with conventional heating.[2] The results were similar to the microwave approach, resulting in isomers 9*c*,11*t*-CLA (**14**) and 10*t*,12*c*-CLA (**15**) (1 : 1) (Scheme 6). The scale-up afforded 1 g of CLA isomers per hour. Note that the scale-up process is limited by the solubility of KOH in ethylene glycol. This good production rate makes it interesting to carry out epidemiological studies[1] usually limited by the low availability of this component.

Therefore, isomerization of linoleic acid (**13**) to CLA was successfully transferred to flow systems, further proving to be a valuable alternative to batch procedures and producing more product in a given time than an analogous batch reactor.[18]

Focused on the development of a more sustainable methodology for the synthesis of CLAs, which avoids the use of ethylene glycol, neutral and recyclable ionic liquids were effectively used as a green reagent and solvent in the dehydration of ricinoleic acid (RA) (**22**).[3] Currently, ionic liquids are widely used in synthesis due to their interesting properties such as low vapour pressure, high thermal stability and easy recyclability.[22]

First of all, ricinoleic acid (**22**) was heated with 1-butyl-3-methylimidazolium hexafluorophosphate (**23**) under microwave irradiation for 30 min at 140 °C and the corresponding CLA isomers were obtained in 32% conversion along with some side products (Scheme 7). We carried out various modifications on the reaction conditions, such as prolonged reaction times and increased reaction temperatures, to further increase the reaction performance, but to no avail. Consequently, a range of ionic liquids (Table 4) was investigated for their effects on the dehydration of RA (**22**). All the ionic liquids were



Scheme 7 Dehydration of ricinoleic acid (**22**) to CLA isomers.

Table 4 Effect of different ionic liquids on the dehydration of ricinoleic acid (**22**) under microwave irradiation

Entry	Ionic liquid	CLA (%) (**14–17**)	LA (%) (**13**)	RAL (%) (**29**)	RA (%) (**22**)
1	[bmim]PF_6 (**23**)	32	22	46	—
2	[hmim]PF_6 (**24**)	34	21	45	—
3	[bmim]Br (**25**)	32	12	9	47
4	[hmim]Br (**26**)	11	3	5	81
5	[bmim]Cl (**27**)	13	5	6	76
6	[hmim]Cl (**28**)	7	1	7	85
7[a]	[bmim]PF_6 (**23**)	57[b]	42[c]	<1	—

IL, ionic liquid; CLA, conjugated linoleic acid; LA, linoleic acid; RAL, ricinoleic acid lactone; RA, ricinoleic acid (unreacted). [a] Ricinoleic acid methyl ester. [b] CLA methyl esters. [c] LA methyl ester.

commercially available, except 1-hexyl-3-methylimidazolium hexafluorophosphate (**24**) and 1-hexyl-3-methylimidazolium bromide (**26**) which were synthesized as described in the Experimental section. Again, the procedure gave rise to mixtures of dienoic acids, such as LA (**13**) as well as positional and geometrical isomers of CLA (Scheme 7), which were identified and quantified by NMR. In addition, there was another secondary product, whose ^{1}H and ^{13}C-NMR spectra revealed that it was the lactone **29** from ricinoleic acid due to the intramolecular esterification between carboxyl and hydroxyl groups.

According to the results shown in Table 4, the nature of the counteranion in the ionic liquid seems to play a crucial role on the effect of the dehydration of ricinoleic acid (**22**). The best results were achieved with 1-butyl-3-methylimidazolium hexafluorophosphate (**23**) (Table 4, entry 1) and 1-hexyl-3-methylimidazolium hexafluorophosphate (**24**) (Table 4, entry 2), because they enhanced the concentrations of CLAs in comparison with other ionic liquids, and there was no unreacted RA. Nevertheless there was a significant increase in the formation of the lactone (**29**). We became interested in exploring ways to avoid the formation of compound **29**, so we have used ricinoleic acid methyl ester and 1-butyl-3-methylimidazolium hexafluorophosphate (**23**) in order to increase the conversion to CLAs. In this manner, the formation of the lactone (**29**) was almost suppressed and high conversions to CLA isomers were obtained (Table 4, entry 7). The small quantity of the lactone (**29**) detected in the mixture could be due to a possible transesterification reaction.

Furthermore, in order to check the recyclability of the ionic liquid, the products were extracted after completion of the reaction with ethyl ether, and the remaining ionic liquid was used again in a new reaction. There were no significant changes in the yields of reactions after three cycles.

In addition, water has been used as a solvent to conduct these reactions as an alternative to ionic liquids under the same experimental conditions. However, no reaction product was obtained.

In summary, three new sustainable approaches for the synthesis of CLAs are reported; microwave-assisted organic synthesis as well as continuous flow techniques have been successfully employed. Following our goal, it was noticed that it was much better to use the conjugated linoleic acids obtained by alkali isomerization of linoleic acid (**13**) than those obtained by dehydration of ricinoleic acid (**22**), because the latter provides a higher number of isomers. However, this is a mild, efficient and environmentally friendly alternative for the dehydration of ricinoleic acid.

Experimental

General

Microwave irradiations were performed in a CEM Discover microwave reactor. Temperature was monitored during the reaction by the standard IR pyrometer included in the microwave reactor. Analysis by layer chromatography was performed on aluminium oxide 60 F_{254} silica gel plates and visualised using short-wave ultra-violet light or with a $KMnO_4$ solution. All separations by column chromatography were carried out on silica gel Merck type 60 (0.040–0.063 mm).

Melting points are uncorrected. All NMR spectra were recorded on a Varian Inova 500 MHz spectrometer in $CDCl_3$ at 278 K and at 499.769 MHz and 125.678 MHz for ^{1}H and ^{13}C NMR, respectively. Chemical shifts were referenced to internal standard TMS. Assignment of spectra was carried out using NOESY-1D, NOESY-2D, g-COSY, g-HMQC, g-HMBC, TOCSY, g-HSQC-TOCSY and HMBC-TOCSY experiments. The NOESY-1D spectra were recorded with the following acquisition parameters: mixing time 800 ms and number of scans 256. Two-dimensional NMR spectra were acquired using 96 scans and 128 increments. The g-HSQC-TOCSY and HMBC-TOCSY experiments were recorded using 256 scans and different mixing times: 30, 80, 150 and 300 ms. The pulse programs were taken from the standard Varian pulse sequence library. All spectra were Fourier transformed with MestReNova 6.2.0 software.

High resolution mass spectrometry (HRMS) was carried out using the electronic impact technique at 70 eV on a VG AutoSpec spectrometer. In some cases a QStar pulsar i spectrometer with positive electrospray ionization was used.

Commercially available starting materials and solvents were used without previous purification.

Diels–Alder reactions of sorbic acid (1)

A mixture of sorbic acid (**1**) (5 mmol, 0.56 g) and *N*-methylmaleimide (**5**) (5 mmol, 0.60 g) in solvent free conditions was heated under microwave irradiation for 15 min at 100 °C. Purification of product **7** was achieved by column chromatography of silica gel using hexane–ethyl acetate (5 : 1) as the eluent; changing the solvent to ethyl acetate product **8** was obtained. Fractions containing products **7** and **8** were removed separately *in vacuo* and pure products were achieved.

(3a*R*,4*S*,7*R*,7a*R*)-2,7-Dimethyl-1,3-dioxo-2,3,3a,4,7,7a-hexahydro-1*H*-isoindole-4-carboxylic acid (7).[15] (0.80 g, 72%). Colourless solid. M.p. 176–177 °C. ^{1}H-NMR ($CDCl_3$, ppm) δ: 9.43 (s, COOH), 5.95 (1H, dd, J = 9.5, 6.6 Hz, 5-H), 5.90 (1H, dd, J = 9.5, 3.9 Hz, 6-H), 3.83 (1H, dd, J = 6.6, 2.6 Hz, 4-H), 3.66 (1H, dd, J = 9, 2.6 Hz, 3a-H), 3.19 (1H, dd, J = 9, 7.6 Hz, 7a-H), 2.97 (3H, s, NCH_3), 2.79–2.76 (1H, m, 7-H), 1.25 (3H, d, J = 7.3 Hz, CH_3). ^{13}C-NMR ($CDCl_3$, ppm) δ: 178.8 and 177.6 (C-1 and C-3), 176.4 (COOH), 136.7 (C-6), 123.8 (C-5), 43.1 (C-7a), 41.8 (C-3a), 40.3 (C-4), 28.9 (C-7), 24.8 (NCH_3), 16.6 (CH_3). HR-MS/EI $C_{11}H_{13}NO_4$: calcd 223.0845; found 223.0854.

(3a*R*,4*S*,7*S*,7a*R*)-2,7-Dimethyl-1,3-dioxo-2,3,3a,4,7,7a-hexahydro-1*H*-isoindole-4-carboxylic acid (8). (0.21 g, 18%). Colourless solid. M.p. 175–176 °C. ^{1}H-NMR ($CDCl_3$, ppm) δ: 9.45 (s, COOH), 6.32 (1H, dt, J = 9.5, 3.5 Hz, 5-H), 5.75 (1H, dt, J = 9.5, 3.4 Hz, 6-H), 3.76 (1H, dd, J = 8.8, 5.7 Hz, 3a-H), 3.19–3.16 (1H, m, 4-H), 3.15 (1H, t, J = 8 Hz, 7a-H), 2.91 (s, 3H, NCH_3), 2.47–2.45 (1H, m, 7-H), 1.44 (3H, d, J = 7.4 Hz, CH_3). ^{13}C-NMR ($CDCl_3$, ppm) δ: 177.2 and 176.6 (C-1 and C-3), 175.1 (COOH), 134.8 (C-6), 125.8 (C-5), 43.8 (C-7a), 43.7 (C-3a), 40.1 (C-4), 31.5 (C-7), 24.8 (NCH_3), 16.6 (CH_3). HR-MS/EI $C_{11}H_{13}NO_4$: calcd 223.0845; found 223.0854.

A mixture of sorbic acid (**1**) (5 mmol, 0.56 g) and maleic anhydride (**6**) (5 mmol, 0.50 g) in solvent free conditions was heated under microwave irradiation for 15 min at 150 °C. Purification of product **9** was achieved by column chromatography of silica gel using hexane–ethyl acetate (1 : 1) as the eluent. Fractions containing product **9** were collected separately and the solvent was removed *in vacuo*.

(3a*S*,4*S*,7*R*,7a*R*)-7-Methyl-1,3-dioxo-1,3,3a,4,7,7a-hexahydroisobenzofuran-4-carboxylic acid (9).[15] (0.98 g, 93%). Colourless solid. M.p. 174–175 °C. ^{1}H-NMR ($CDCl_3$, ppm) δ: 5.86 (1H, dt, J = 10, 2.9 Hz, 5-H), 5.56 (1H, dt, J = 10, 2.9 Hz, 6-H), 3.67 (1H, dc, J = 10.6, 2.9 Hz, 4-H), 3.50 (1H, dd, J = 10.6, 6 Hz, 3a-H), 3.07 (1H, t, J = 6 Hz, 7a-H), 2.56–2.51 (1H, m, 7-H), 1.15 (3H, d, J = 7.5 Hz, CH_3). ^{13}C-NMR ($CDCl_3$, ppm) δ: 172.6 and 172.3 (C-1 and C-3), 170.8 (COOH), 131.8 (C-6), 120.1 (C-5), 43.0 (C-7a), 41.1 (C-3a), 39.7 (C-4), 30.6 (C-7), 18.2 (CH_3). HR-MS/ESI $C_{10}H_{10}O_5$: calcd 210.0528; found 210.0546.

Diels–Alder reactions of 2,4-*trans*,*trans*-hexadienyl acetate (2)

A mixture of 2,4-*trans*,*trans*-hexadienyl acetate (**2**) (5 mmol, 0.72 g) and tetracyanoethylene (**3**) (5 mmol, 0.65 g) in solvent free conditions was heated under microwave irradiation for 15 min at 80 °C. Purification of product **10** was achieved by column chromatography of silica gel using hexane–ethyl acetate (5 : 1) as the eluent. Fractions containing product **10** were collected separately and the solvent was removed *in vacuo*.

[(1*S*,4*R*)-5,5,6,6-Tetracyano-4-methylcyclohex-2-en-1-yl]acetate (10). (1.17 g, 88%). Yellow oil. ^{1}H-NMR ($CDCl_3$, ppm) δ: 5.96 (1H, ddd, J = 10.8, 3.5, 2.8 Hz, 3-H), 5.74 (1H, dt, J = 10.8, 2.5 Hz, 2-H), 4.58 (1H, dd, J = 12.1, 5.3 Hz, CH_2(Ha)), 4.40 (1H, dd, J = 12.1, 8.4 Hz, CH_2(Hb)), 3.50–3.45 (1H, m, 1-H), 3.30–3.24 (1H, m, 4-H), 2.18 (3H, s, CH_3CO), 1,66 (3H, d, J = 7.5 Hz, CH_3). ^{13}C-NMR ($CDCl_3$, ppm) δ: 170.0 (CH_3CO), 128.9 (C-3), 121.0 (C-2), 111.2, 111.1, 109.8, 109.3 (4CN), 62.5 (CH_2), 43.0 (C-6), 41.1 (C-1), 39.2 (C-5), 37.5 (C-4), 20.5 (CH_3CO), 17.4 (CH_3). HR-MS/EI $C_{14}H_{12}N_4O_2$: calcd 268.0960; found 268.0966.

A mixture of 2,4-*trans*,*trans*-hexadienyl acetate (**2**) (5 mmol, 0.72 g) and *N*-methylmaleimide (**5**) (5 mmol, 0.60 g) in solvent free conditions was heated under microwave irradiation for 15 min at 100 °C. The further work-up was carried out as described above for product **10**.

[(3a*S*,4*S*,7*R*,7a*R*)-2,7-Dimethyl-1,3-dioxo-2,3,3a,4,7,7a-hexahydro-1*H*-isoindol-4-yl]acetate (11). (1.18 g, 92%). Colourless solid. M.p. 77–78 °C. ^{1}H-NMR ($CDCl_3$, ppm) δ: 5.75 (2H, m, 5-H and 6-H), 4.68 (1H, dd, J = 11.2, 7.2 Hz, CH_2(Hb)), 4.55 (1H, dd, J = 11.2, 8.4 Hz, CH_2(Ha)), 3.24 (1H, dd, J = 9.1, 6.5 Hz, 3a-H), 3.05 (1H, dd, J = 9.1, 7 Hz, 7a-H), 2.89 (3H, s, NCH_3), 2.61–2.59 (1H, m, 7-H), 2.45–2.42 (1H, m, 4-H), 2.09 (3H, s, CH_3CO), 1.45 (3H, d, J = 7.3 Hz, CH_3). ^{13}C-NMR ($CDCl_3$, ppm) δ: 177.1 and 177.0 (C-1 and C-3), 170.8 (CH_3CO), 135.5 (C-5), 129.0 (C-6), 64.4 (CH_2), 44.8 (C-7a), 42.4 (C-3a), 35.8 (C-4), 31.1 (C-7), 24.5 (NCH_3), 20.9 (CH_3CO), 16.6 (CH_3). HR-MS/EI $C_{13}H_{15}NO_4$: calcd 251.1158; found 251.1143.

A mixture of 2,4-*trans*,*trans*-hexadienyl acetate (**2**) (5 mmol, 0.72 g) and maleic anhydride (**6**) (5 mmol, 0.50 g) in solvent free conditions was heated under microwave irradiation for 15 min at 150 °C. The further work-up was carried out as described above for product **10**.

[(3a*S*,4*S*,7*R*,7a*R*)-7-Methyl-1,3-dioxo-1,3,3a,4,7,7a-hexahydroisobenzofuran-4-yl]acetate (12). (1.20 g, 97%). Colourless solid. M.p. 111–112 °C. ^{1}H-NMR ($CDCl_3$, ppm) δ: 5.88–5.86 (2H, m, 5-H and 6-H), 4.57 (1H, dd, J = 11.3, 7.8 Hz, CH_2(Hb)), 4.49 (1H, dd, J = 11.3, 7.7 Hz, CH_2(Ha)), 3.60 (1H, dd, J = 9.4, 6 Hz, 3a-H), 3.38 (1H, dd, J = 9.4, 7.3 Hz, 7a-H), 2.69–2.63 (1H, m, 4-H), 2.53–2.47 (1H, m, 7-H), 2.09 (3H, s, CH_3CO), 1.44 (3H, d, J = 7.4 Hz, CH_3). ^{13}C-NMR ($CDCl_3$, ppm) δ: 171.4 (CH_3CO), 170.9 and 170.8 (C-1 and C-3), 135.5 (C-5), 129.2 (C-6), 63.5 (CH_2), 45.5 (C-7a), 43.1 (C-3a), 35.0 (C-4), 30.4 (C-7), 20.7 (CH_3CO), 16.2 (CH_3). HR-MS/ESI $C_{12}H_{14}O_5$: calcd 238.0841; found 238.0836.

Diels–Alder reactions of conjugated linoleic acid (14–17)

General procedure. The corresponding conjugated linoleic acids (**14–17**) (0.75 mmol, 0.210 g) and maleic anhydride (**6**) (1.5 mmol, 0.155 g) in solvent free conditions were heated under microwave irradiation for 15 min at 150 °C. After the mixture had cooled to room temperature diethyl ether (50 mL) was added

and the organic layer was washed several times with water, in order to eliminate the excess of maleic anhydride. The organic layer was separated and dried with anhydrous sodium sulphate. After filtration the solvent was removed *in vacuo*. Purification of cycloadducts **18** to **21** was achieved by column chromatography of silica gel using hexane–ethyl acetate (5 : 1) as the eluent. Fractions containing pure products were collected separately and the solvent was removed *in vacuo*.

8-[(3a*S*,4*R*,7*R*,7a*R*)-7-Hexyl-1,3-dioxo-1,3,3a,4,7,7a-hexahydroisobenzofuran-4-yl]octanoic acid (18). From 9-*cis*,11-*trans*-octadecadienoic acid (**14**). (0.231 g, 43%). Yellow oil. ^{1}H-NMR ($CDCl_3$, ppm) δ: 5.93–5.85 (2H, m, 5′-H and 6′-H), 3.34 (1H, dd, J = 9.5, 7 Hz, 7′a-H), 3.13 (1H, dd, J = 9.5, 3.5 Hz, 3′a-H), 2.74–2.67 (1H, m, 4′-H), 2.62–2.56 (1H, m, 7′-H), 2.36 (2H, t, J = 7.5 Hz, 2-H), 1.70–1.61 (4H, m, 3-CH_2 and 1″-CH_2 (NOE assigned)), 1.60–1.52 (2H, m, 8-H), 1.50–1.27 (16H, m, 4–7 and 2″-5″-CH_2), 0.88 (3H, t, J = 7 Hz, 6″-H). ^{13}C-NMR ($CDCl_3$, ppm) δ: 179.6 (COOH), 174.4 and 171.8 (C-1′ and C-3′), 131.5 (C-5′), 131.2 (C-6′), 45.3 (C-3′a), 44.1 (C-7′a), 35.0 (C-4′), 34.6 (C-8), 33.9 (C-2), 33.6 (C-7′), 31.6 (C-4″), 29.2 (C-1″), 29.1 (C-3″), 29.0 (C-6), 29.0 (C-5), 28.8 (C-4), 27.8 (C-7), 27.2 (C-2″), 24.5 (C-3), 22.5 (C-5″), 14.0 (C-6″). HR-MS/EI $C_{22}H_{34}O_5$: calcd 378.2416; found 378.2446.

9-[(3a*S*,4*S*,7*S*,7a*R*)-1,3-Dioxo-7-pentyl-1,3,3a,4,7,7a-hexahydroisobenzofuran-4-yl]nonanoic acid (19). From 10-*trans*,12-*cis*-octadecadienoic acid (**15**). (0.229 g, 43%). Yellow oil. ^{1}H-NMR ($CDCl_3$, ppm) δ: 5.92–5.85 (2H, m, 5′-H and 6′-H), 3.35 (1H, dd, J = 9.5, 7 Hz, 3′a-H), 3.14 (1H, dd, J = 9.5, 3.5 Hz, 7′a-H), 2.75–2.68 (1H, m, 7′-H), 2.61–2.57 (1H, m, 4′-H), 2.35 (2H, t, J = 7.5 Hz, 2-H), 1.68–1.59 (4H, m, 3-CH_2 and 9-CH_2 (NOE assigned)), 1.57–1.52 (2H, m, 1″-H), 1.45–1.28 (16H, m, 4–8 and 2″-4″-CH_2), 0.89 (3H, t, J = 7 Hz, 5″-H). ^{13}C-NMR ($CDCl_3$, ppm) δ: 179.8 (COOH), 174.4 and 171.8 (C-1′ and C-3′), 131.4 (C-5′ and C-6′), 45.3 (C-7′a), 44.1 (C-3′a), 35.1 (C-7′), 34.6 (C-1″), 33.9 (C-2), 33.6 (C-4′), 31.6 (C-3″), 31.7 (C-9), 29.4 (C-7), 29.1 (C-6), 29.1 (C-5), 29.0 (C-4), 28.9 (C-8), 27.7 (C-2″), 24.6 (C-3), 22.5 (C-4″), 14.0 (C-5″). HR-MS/EI $C_{22}H_{34}O_5$: calcd 378.2416; found 378.2442.

8-[(3a*S*,4*S*,7*R*,7a*R*)-7-Hexyl-1,3-dioxo-1,3,3a,4,7,7a-hexahydroisobenzofuran-4-yl]octanoic acid (20). From 9-*trans*,11-*trans*-octadecadienoic acid (**16**). (0.222 g, 40%). Yellow oil. ^{1}H-NMR ($CDCl_3$, ppm) δ: 5.82 (2H, s, 5′-H and 6′-H), 3.37 (2H, dd, J = 4.5, 2 Hz, 3′a-H- and 7′a-H), 2.36 (2H, t, J = 7.5 Hz, 2-H), 2.27–2.20 (2H, m, 4′-H and 7′-H), 1.90–1.81 (2H, m, 1″-H), 1.80–1.72 (2H, m, 8-H), 1.64 (2H, q, J = 7 Hz, 3-H), 1.47–1.40 (4H, m, 7-CH_2 and 2″-CH_2), 1.39–1.32 (8H, m, 4–6 and 3″-CH_2), 1.31–1.29 (4H, m, 4″ and 5″-CH_2), 0.89 (3H, t, J = 7 Hz, 6″-H). ^{13}C-NMR ($CDCl_3$, ppm) δ: 179.8 (COOH), 171.6 and 171.5 (C-1′ and C-3′), 133.9 and 133.7 (C-5′ and C-6′), 44.9 (C-3′a), 44.8 (C-7′a), 36.3 (C-4′), 36.2 (C-7′), 33.9 (C-2), 31.7 (C-4″), 30.6 (C-8), 30.5 (C-1″), 29.2 (C-3″), 29.1 and 29.0 (C-5 and C-6), 28.9 (C-4), 27.9 (C-7), 27.8 (C-2″), 24.5 (C-3), 22.6 (C-5″), 14.0 (C-6″). HR-MS/EI $C_{22}H_{34}O_5$: calcd 378.2416; found 378.2444.

9-[(3a*S*,4*S*,7*R*,7a*R*)-1,3-Dioxo-7-pentyl-1,3, 3a,4,7,7a-hexahydroisobenzofuran-4-yl]nonanoic acid (21). From 10-*trans*,12-*trans*-octadecadienoic acid (**17**). (0.224 g, 40%). Yellow oil. ^{1}H-NMR ($CDCl_3$, ppm) δ: 5.82 (2H, s, 5′-H and 6′-H), 3.35 (2H, dd, J = 4.5, 2 Hz, 3′a-H and 7′a-H), 2.35 (2H, t, J = 7.5 Hz, 2-H), 2.28–2.20 (2H, m, 4′-H and 7′-H), 1.91–1.82 (2H, m, 1″-H), 1.81–1.74 (2H, m, 9-H), 1.68–1.60 (2H, m, 3-H), 1.44–1.40 (4H, m, 8-CH_2 and 2″-CH_2), 1.39–1.22 (12H, m, 4–7 and 3″-4″-CH_2), 0.91 (3H, t, J = 7 Hz, 5″-H). ^{13}C-NMR ($CDCl_3$, ppm) δ: 178.7 (COOH), 171.5 and 171.4 (C-1′ and C-3′), 133.9 and 133.8 (C-5′ and C-6′), 44.9 (C-3′a), 44.8 (C-7′a), 36.4 (C-4′), 36.3 (C-7′), 33.7 (C-2), 31.7 (C-3″), 30.9 (C-9), 30.6 (C-1″), 29.4 (C-7), 29.1 (C-6), 29.1 (C-5), 29.0 (C-4), 27.9 (C-7), 27.7 (C-2″), 24.6 (C-3), 22.6 (C-4″), 14.1 (C-5″). HR-MS/EI $C_{22}H_{34}O_5$: calcd 378.2416; found 378.2445.

Linoleic acid (13) isomerization

Linoleic acid (**13**) was isomerized to 9-*cis*,11-*trans* and 10-*trans*,12-*cis* conjugated linoleic acids (**14** and **15**) slightly modifying the previous work of Chin *et al.*[2] Linoleic acid (**13**) (0.87 mmol, 0.25 g) was mixed with KOH (12 mmol, 0.78 g) in 2.5 mL of ethylene glycol and refluxed at 160 °C for 15 min under microwave irradiation and an argon atmosphere. To the reaction mixture, 5 mL of methanol were added followed by acidification with 20 mL of HCl 3 N. The reaction mixture was extracted with hexane (3 × 5 mL), and then the organic layer was washed thrice with 30% methanol in water and three times with distilled water. Subsequently, it was dried over anhydrous sodium sulphate and hexane was removed in a rotary evaporator under vacuum. A colourless liquid was obtained corresponding with the mixture of 9*c*,11*t*-CLA (**14**) and 10*t*,12*c*-CLA (**15**) in equal proportions (0.23 g, 90%).

9-*cis*,11-*trans* Octadecadienoic acid (14). Colourless oil. ^{1}H-RMN ($CDCl_3$, ppm) δ: 6.32–6.26 (1H, m, 11-H), 5.94 (1H, t, J = 11 Hz, 10-H), 5.66 (1H, dt, J = 14.5, 7 Hz, 12-H), 5.29 (1H, dt, J = 11, 7.5 Hz, 9-H), 2.35 (2H, t, J = 7.5 Hz, 2-H), 2.15 (2H, ddd, J = 14.5, 7.5, 1.5 Hz, 8-H), 2.09 (2H, c, J = 7 Hz, 13-H), 1.63 (2H, q, J = 7.5 Hz, 3-H), 1.42–1.23 (16H, m, 4–7-CH_2 and 14–17-CH_2), 0.88 (3H, t, J = 7 Hz, 18-H). ^{13}C-RMN ($CDCl_3$, ppm) δ: 179.7 (C-1), 134.8 (C-12), 129.9 (C-9), 128.7 (C-10), 125.5 (C-11), 34.0 (C-2), 32.9 (C-13), 31.7 (C-16), 29.6 (C-7), 29.4 (C-14), 29.1 (C-15), 29.0 (C-5), 29.0 (C-6), 28.9 (C-4), 27.6 (C-8), 24.6 (C-3), 22.6 (C-17), 14.1 (C-18).

10-*trans*,12-*cis* Octadecadienoic acid (15). Colourless oil.^{1}H-RMN ($CDCl_3$, ppm) δ: 6.32–6.26 (1H, m, 11-H), 5.94 (1H, t, J = 11 Hz, 12-H), 5.65 (1H, dt, J = 14.5, 7 Hz, 10-H), 5.30 (1H, dt, J = 11, 7.5 Hz, 13-H), 2.35 (2H, t, J = 7.5 Hz, 2-H), 2.15 (2H, ddd, J = 9, 7.5, 1.5 Hz, 14-H), 2.09 (2H, c, J = 7 Hz, 9-H), 1.63 (2H, q, J = 7.5 Hz, 3-H), 1.41–1.25 (16H, m, 4–8-CH_2 and 15–17-CH_2), 0.89 (3H, t, J = 7 Hz, 18-H). ^{13}C-RMN ($CDCl_3$, ppm) δ: 179.5 (C-1), 134.6 (C-10), 130.1 (C-13), 128.6 (C-12), 125.6 (C-11), 33.9 (C-2), 32.8 (C-9), 31.5 (C-16), 29.4 (C-8), 29.3 (C-15), 29.2 (C-7), 29.1 (C-5), 29.1 (C-6), 29.0 (C-4), 27.7 (C-14), 24.6 (C-3), 22.5 (C-17), 14.0 (C-18).

Isomers 9-*trans*,11-*trans* and 10-*trans*,12-*trans* conjugated linoleic acids (**16** and **17**) were achieved using a common method of *cis*/*trans* isomerization catalyzed with iodine.[16] To a

solution of the previously obtained mixture of isomers 9-*cis*,11-*trans* and 10-*trans*,12-*cis* conjugated linoleic acids (**14** and **15**) (0.35 g, 1.25 mmol) in 80 mL of hexane, a small amount of iodine crystals was added. The mixture was stirred for 5 h at room temperature and sunlight. The reaction mixture was washed with Na_2SO_4 0.1 M (3 × 30 mL) to remove iodine, then the organic layer was washed thrice with distilled water and dried over anhydrous sodium sulphate and hexane was removed *in vacuo*. A white solid was obtained corresponding with the mixture of 9*t*,11*t*-CLA (**16**) and 10*t*,12*t*-CLA (**17**) in equal proportions (0.26 g, 75%).

9-*trans*,11-*trans* Octadecadienoic acid (16). White solid. ^{1}H-RMN ($CDCl_3$, ppm) δ: 6.07–5.95 (2H, m,10-H and 11-H), 5.64–5.50 (2H, m, 9-H and 12-H), 2.35 (2H, t, J = 7.5 Hz, 2-H), 2.05 (4H, c, J = 7.2 Hz, 8-CH_2 and 13-CH_2), 1.64 (2H, q, J = 7.5 Hz, 3-H), 1.44–1.20 (16H, m, 4–7-CH_2 and 14–17-CH_2), 0.89 (3H, t, J = 7 Hz, 18-H). ^{13}C-RMN ($CDCl_3$, ppm) δ: 179.5 (C-1), 132.5 (C-12), 132.2 (C-9), 130.4 (C-10), 130.3 (C-11), 33.9 (C-2), 32.6 (C-13), 32.5 (C-8), 31.7 (C-16), 29.4 (C-7), 29.3 (C-14), 29.1 (C-15), 29.0 (C-5), 29.0 (C-6), 28.9 (C-4), 24.6 (C-3), 22.6 (C-17), 14.1 (C-18).

10-*trans*,12-*trans* Octadecadienoic acid (17). White solid. ^{1}H-RMN ($CDCl_3$, ppm) δ: 6.06–5.96 (2H, m, 11-H and 12-H), 5.61–5.52 (2H, m, 10-H and 13-H), 2.35 (2H, t, J = 7.5 Hz, 2-H), 2.04 (4H, c, J = 7.2 Hz, 9-CH_2 and 14-CH_2), 1.64 (2H, q, J = 7.5 Hz, 3-H), 1.45–1.22 (16H, m, 4–8-CH_2 and 15–17-CH_2), 0.88 (3H, t, J = 7 Hz, 18-H). ^{13}C-RMN ($CDCl_3$, ppm) δ: 179.9 (C-1), 132.5 (C-13), 132.3 (C-10), 130.5 (C-11), 130.4 (C-12), 33.9 (C-2), 32.6 (C-14), 32.5 (C-9), 31.4 (C-16), 29.4 (C-8), 29.2 (C-15), 29.1 (C-7), 29.0 (C-5), 29.0 (C-6), 28.9 (C-4), 24.6 (C-3), 22.5 (C-17), 14.0 (C-18).

General procedure for the linoleic acid isomerization in flow. The manifold system (pumps, valves, PFA tubing and reactor) of a Vapourtec R2+R4 unit was dried with isopropyl alcohol (2 mL min^{-1}, 15 min) and ethylene glycol (0.5 mL min^{-1}, 15 min). For the previous optimization of the reaction conditions, a solution of the linoleic acid (**13**) (0.16 M) in ethylene glycol was loaded into a sample loop (2 mL) and a solution of KOH (0.44 M) in ethylene glycol was loaded into a second sample loop (2 mL). The two sample loops were switched in-line into streams of ethylene glycol, each flowing at 0.167 mL min^{-1} and mixed in the T-mixer. The mixture was then heated in the reactor (10 mL) at 180 °C and 30 min of residence time, and the output was collected. For the scale-up of the process, the concentration was increased 10 times and a longer residence time was needed for complete conversion (45 min). Only 23.5 ml of ethylene glycol was needed for the production of 1 g of CLAs. The further work-up was carried out as described above for the alkali isomerization.

Ricinoleic acid (22) dehydration

A mixture of ricinoleic acid (**22**) (0.33 mmol, 100 mg) and the corresponding ionic liquid (1 mmol), such as 1-butyl-3-methylimidazolium hexafluorophosphate (**23**), 1-hexyl-3-methylimidazolium hexafluorophosphate (**24**), 1-butyl-3-methylimidazolium bromide (**25**), 1-hexyl-3-methylimidazolium bromide (**26**), 1-butyl-3-methylimidazolium chloride (**27**) and 1-hexyl-3-methylimidazolium chloride (**28**), was heated with microwaves for 30 min at 140 °C under an argon atmosphere. After completion of the reaction, the mixture was cooled at room temperature and then extracted with ethyl ether or ethyl acetate (3 × 10 mL), and the ionic liquid was recovered in order to be used in the next cycle. The organic layer was washed with water, dried over anhydrous sodium sulphate and solvent was removed *in vacuo*. The same procedure was used for ricinoleic acid methyl ester. In all cases, yields were determined by ^{1}H-NMR.

(*R*,*Z*)-13-Hexyloxacyclotridec-10-en-2-one (29). ^{1}H-RMN ($CDCl_3$, ppm) δ: 5.48–5.41 (1H, m, 9-H), 5.37–5.28 (1H, m, 10-H), 4.88 (1H, q, J = 6.2 Hz, 12-H), 2.32 (2H, t, J = 7.5 Hz, 2-H), 2.27 (2H, t, J = 7.5 Hz, 11-H), 2.07 (2H, c, J = 6.8 Hz, 8-H), 1.59 (2H, q, J = 7.5 Hz, 3-H), 1.57–1.49 (2H, m, 13-H), 1.42–1.21 (18H, m, 4–7-CH_2 and 14–17-CH_2), 0.88 (3H, t, J = 6.1 Hz, 18-H). ^{13}C-RMN ($CDCl_3$, ppm) δ: 173.6 (C-1), 132.3 (C-9), 124.1 (C-10), 73.6 (C-12), 34.5 (C-11), 33.9 (C-2), 33.4 (C-13), 31.6 (C-16), 29.6 (C-7), 29.4 (C-15), 29.0 (C-5), 29.0 (C-6), 28.9 (C-4), 27.2 (C-8), 25.2 (C-14), 24.9 (C-3), 22.4 (C-17), 13.9 (C-18).

1-Hexyl-3-methylimidazolium hexafluorophosphate (**24**) was synthesized according to the method of Leadbeater *et al*.[23] On the other hand, 1-hexyl-3-methylimidazolium bromide (**26**) was obtained using the procedure of Varma and Namboodiri[24] slightly modified. A mixture of 1-methylimidazole (0.165 g, 2 mmol) and 1-bromohexane (0.383 g, 2.2 mmol) was heated under microwave irradiation at 80 °C for 15 min. After completion of the reaction, the mixture was washed with ethyl acetate and dried under vacuum.

1-Hexyl-3-methylimidazolium bromide (26) (0.416 g, 85%). Colourless viscous liquid. ^{1}H-RMN ($CDCl_3$, ppm) δ: 9.98 (1H, s, 2-H), 7.70 (1H, d, J = 1.8 Hz, 4-H), 7.54 (1H, d, J = 1.8 Hz, 5-H), 4.33 (2H, t, J = 7.5 Hz, 6-H), 4.11 (3H, s, 12-H), 1.91 (2H, q, J = 7 Hz, 7-H), 1.38–1.26 (6H, m, 8,9 and 10-CH_2), 0.87 (3H, t, J = 7 Hz, 11-H).^{13}C-RMN ($CDCl_3$, ppm) δ: 136.6 (C-2), 123.6 (C-4), 121.8 (C-5), 49.7 (C-6), 36.4 (C-12), 30.7 (C-9), 29.9 (C-7), 25.5 (C-8), 22.0 (C-10), 13.6 (C-11).

Computational studies

All calculations included in this paper were carried out using the GAUSSIAN 09 series programs,[25] with the standard 6-31G* basis set.[26] In order to include electron correlation at a reasonable computational cost, density functional theory (DFT)[27] was used. In this study, calculations were carried out by means of the three-parameter functional developed by Becke *et al*., which is usually denoted as B3LYP.[28] Zero-vibrational energies (ZPVEs) were computed at the B3LYP/6-31G* level and were not scaled. Solvent effects were estimated by means of polarization continuum models (PCM),[29] using chloroform as the solvent. MacroModel v.9[30] was used in its MCMM (Macromodel Monte Carlo Multiple Minimum) mode of conformational search in which a Monte Carlo method[31] is used to modify the input structures by random changes in the torsion angles and/or molecular positions. The OPLS_2005 force field[32] as implemented in MacroModel was used for energy calculation. MacroModel v.9 was run in

"perform automatic setup" mode, and the number of steps was set in 1000.

Conclusions

We herein report a sustainable and efficient methodology for the identification of different positional and geometrical CLA isomers in a mixture, by NMR analysis of Diels–Alder cycloadducts. Maleic anhydride was found to be the most appropriate dienophile to react with CLAs (diene) in solvent-free microwave-assisted Diels–Alder cycloadditions due to the high stereoselectivity and high reaction yields obtained. Note that it is not possible to differentiate among CLA isomers in a food matrix by NMR because of their low abundance and presence of other compounds which alter the CLA isomers chemical shift and overlap signals, however, the Diels–Alder cycloadducts show up differences in the ^{13}C NMR chemical shifts. Importantly, NMR allows the identification of both, positional and geometrical isomerism, in contrast to the reported analytical tools. Previous reports containing a complete NMR characterization, including all the methylene groups, of the CLA isomers could not be found, reporting here their first ^{1}H- and ^{13}C-NMR data. To develop this methodology, the synthesis of CLA is required. Thus, microwave-assisted organic synthesis and continuous-flow techniques are employed, enabling a reduction of the reported reaction times. The use of ionic liquids, a reduced amount of solvents and the simplicity of the isolation procedure make this method a green alternative for the preparation of this kind of compound. The continuous-flow process provides a good production rate for CLA isomers, valuable and important compounds from the biological point of view. Work is underway to broaden the applicability of this methodology for the identification of CLA isomers on a complex matrix (food, drugs).

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Notes and references

1 Y. L. Ha, J. Storkson and M. W. Pariza, *Cancer Res.*, 1990, **50**, 1097–1101; C. Ip, S. F. Chin, J. A. Scimeca and M. W. Pariza, *Cancer Res.*, 1991, **51**, 6118–6124; M. W. Pariza, *Am. J. Clin. Nutr.*, 2004, **79**(suppl), 1132S–1136S; R. Kumar, A. Bhatia and D. Arora, *J. Clin. Diagn. Res.*, 2009, **3**, 1953–1967; Y. Park, *J. Food Comp. Anal.*, 2009, **22S**, S4–S12.

2 S. F. Chin, W. Liu, J. M. Storkson, Y. L. Ha and M. W. Pariza, *J. Food Comp. Anal.*, 1992, **5**, 185–197.

3 L. Yang, Y. Huang, H. Q. Wang and Z. Y. Chen, *Chem. Phys. Lipids*, 2002, **119**, 23–31.

4 A. S. Demir and F. N. Talpur, *J. Agric. Food Chem.*, 2010, **58**, 1646–1652.

5 *Advances in Conjugated Linoleic Acid Research*, ed. J. L. Sebedio, W. W. Christie, R. Adlof, AOCS Press, Champaign, Illinois, 2003, vol. 2; A. Ruiz-Rodriguez, G. Reglero and E. Ibañez, *J. Pharm. Biomed. Anal.*, 2010, **51**, 305–326; O. Berdeaux, S. Fontagné, E. Sémon, J. Velasco, J. L. Sébédio and C. Dobarganes, *Chem. Phys. Lipids*, 2012, **165**, 338–347.

6 M. S. F. Lie Ken Jie, M. K. Pasha and M. S. Alam, *Lipids*, 1997, **32**, 1041–1044; A. L. Davis, G. P. McNeill and D. C. Caswell, *Chem. Phys. Lipids*, 1999, **97**, 155–165; M. S. F. Lie Ken Jie, *Eur. J. Lipid Sci. Technol.*, 2001, **103**, 594–632.

7 V. Spitzer, F. Marx and K. Pfeilsticker, *J. Am. Oil Chem. Soc.*, 1994, **71**, 873–876.

8 G. Dobson, *J. Am. Oil Chem. Soc.*, 1998, **75**, 137–142; M. J. T. Reaney, Y. D. Liu and W. G. Taylor, *J. Am. Oil Chem. Soc.*, 2001, **78**, 1083–1087.

9 G. Read, N. R. Richardson and D. G. Wickens, *Analyst*, 1994, **119**, 393–396.

10 V. Polshettiwar, M. N. Nadagouda and R. S. Varma, *Aust. J. Chem.*, 2009, **62**(1), 16–26; V. Polshettiwar and R. S. Varma, *Acc. Chem. Res.*, 2008, **41**(5), 629–639; A. K. Bose, M. J. Manhas, B. K. Banik and E. W. Robb, *Res. Chem. Intermed.*, 1994, **20**, 1; S. Caddick, *Tetrahedron*, 1995, **52**, 10403; S. A. Galema, *Chem. Soc. Rev.*, 1997, **25**, 233; P. Lidström, J. Tierney, B. Wathey and J. Westman, *Tetrahedron*, 2001, **57**, 9225; C. O. Kappe, *Angew. Chem., Int. Ed.*, 2004, **43**, 6250; B. L. Hayes, *Aldrichim. Acta*, 2004, **37**, 66; M. V. Gomez, R. Caballero, E. Vazquez, A. Moreno, A. de la Hoz and A. Diaz, *Green Chem.*, 2007, **9**, 331–336; R. B. N. Baig and R. S. Varma, *Chem. Soc. Rev.*, 2012, **41**, 1559–1584.

11 B. L. Hayes, *Microwave Synthesis: Chemistry at the Speed of Light*, CEM, Matthews, NC, 2002; *Microwave-Assisted Organic Synthesis*, ed. P. Lidström, J. P. Tierney, Blackwell Scientific, Oxford, 2005; C. O. Kappe and A. Stadler, in *Microwaves in Organicand Medicinal Chemistry*, ed. R. Mannhold, H. Kubinyi, G. Folkers, Wiley, Weinheim, 2005, vol. 25; *Microwaves in Organic Synthesis*, ed. A. Loupy, Wiley-VCH, Weinheim, 2nd edn, 2006.

12 F. Langa, P. de la Cruz, A. de la Hoz, A. Díaz-Ortiz and E. Díez-Barra, *Contemp. Org. Synth.*, 1997, **4**, 373–386; A. de la Hoz, A. Díaz-Ortiz, A. Moreno and F. Langa, *Eur. J. Org. Chem.*, 2000, 3659; A. Díaz-Ortiz, J. M. Fraile, A. de la Hoz, M. V. Gómez, J. A. Mayoral, A. Moreno, P. Prieto, L. Salvatella, A. Saiz and E. Vázquez, *Eur. J. Org. Chem.*, 2001, 2891–2899; A. de la Hoz, A. Díaz-Ortiz, A. Moreno and F. Langa, in *Microwaves in Cycloadditions in Microwaves in Organic Synthesis*, ed. A. Loupy, Wiley-VCH, Weinheim, 2002, ch. 9; A. Díaz-Ortiz, A. de la Hoz and A. Moreno, *Curr. Org. Chem.*, 2004, **8**, 903–918; K. Bougrin, M. Soufiaoui and G. Bashiardes, in *Microwaves in Cycloadditions in Microwaves in Organic Synthesis*, ed. A. Loupy, Wiley-VCH, Weinheim, 2nd edn, 2006, ch. 11.

13 A. de la Hoz, A. Díaz-Ortiz, J. M. Fraile, M. V. Gómez, J. A. Mayoral, A. Moreno, A. Sainz and E. Vázquez, *Synlett*, 2001, 753–757; A. Moreno, M. V. Gómez, E. Vázquez, A. de la Hoz, A. Díaz-Ortiz, P. Prieto, J. A. Mayoral and E. Pyres, *Synlett*, 2004, 1259–1263.

14 P. T. Anastas and J. C. Warner, *Green Chemistry: Theory and Practice*, Oxford University Press, New York, 1988; P. T. Anastas and T. C. Williamson, *Green Chemistry: Frontiers in Benign Chemical Synthesis and Process*, Oxford University Press, New York, 1988; D. Dallinger and C. O. Kappe, *Chem. Rev.*, 2007, **107**, 2563–2591; T. Razzarq and C. O. Kappe, *ChemSusChem*, 2008, **1**, 123–132; R. S. Varma, *Green Chem.*, 2008, **10**, 1129–1130; M. J. Hernáiz, A. R. Alcántara, J. I. García and J. V. Sinisterra, *Chem.–Eur. J.*, 2010, **16**, 9422–9437.

15 G. D. Khandelwal and B. L. Wedzicha, *Food Chem.*, 1997, **60**, 237–244; U. Biermann, W. Butte, T. Eren, D. Haase and J. O. Metzger, *Eur. J. Org. Chem.*, 2007, 3859–3862.

16 K. Eulitz, M. P. Yurawecs, N. Sehat, J. Fritsche, J. A. G. Roach, M. M. Mossoba, J. K. G. Kramer, R. O. Adolf and Y. Ku, *Lipids*, 1999, **34**, 873–877.

17 R. Leach, *Molecular Modelling: Principles and Applications*, Prentice Hall, 2nd edn, 2001.

18 J. Wegner, S. Ceylan and A. Kirschning, *Chem. Commun.*, 2011, **47**, 4583–4592.

19 M. V. Gomez, H. H. J. Verputten, A. Diaz-Ortiz, A. Moreno, A. de la Hoz and A. H. Velders, *Chem. Commun.*, 2010, **46**, 4514–4516.

20 *Microreactors: New Technology for Modern Chemistry*, ed. W. Ehrfield, V. Hessel and H. Löwe, Wiley-VCH, Weinheim, 2000; B. P. Mason,

K. E. Price, J. L. Steinbacher, A. R. Bogdan and D. T. McQuade, *Chem. Rev.*, 2007, **107**, 2300–2318; H. R. Sahoo, J. G. Kralj and K. F. Jensen, *Angew. Chem.*, 2007, **119**, 5806–5810; H. R. Sahoo, J. G. Kralj and K. F. Jensen, *Angew. Chem., Int. Ed.*, 2007, **46**, 5704–5708; C. Wiles and P. Watts, *Eur. J. Org. Chem.*, 2008, 1655–1671; *Microreactors in Organic Synthesis and Catalysis*, ed. T. Wirth, Wiley-VCH, Weinheim, 2008; T. Illg, P. Lög and V. Hessel, *Bioorg. Med. Chem.*, 2010, **18**, 3707–3719; R. L. Hartman, J. P. McMullen and K. F. Jensen, *Angew. Chem., Int. Ed.*, 2011, **50**, 7502–7519; J. M. de Muñoz, J. Alcazar, A. de la Hoz and A. Diaz-Ortiz, *Tetrahedron Lett.*, 2011, **52**, 6058–6060; J. M. de Muñoz, J. Alcazar, A. de la Hoz and A. Diaz-Ortiz, *Eur. J. Org. Chem.*, 2012, 260–263.

21 T. Tanaka, M. Hosokawa, Y. Yasui, R. Ishigamori and K. Miyashita, *Int. J. Mol. Sci.*, 2011, **12**, 7495–7509.

22 R. Kumar, A. Sharma, N. Sharma, V. Kumar and A. K. Sinha, *Eur. J. Org. Chem.*, 2008, 5577–5582.

23 N. E. Leadbeater, H. M. Torenius and H. Tye, *Tetrahedron*, 2003, **59**, 2253–2258.

24 R. S. Varma and V. V. Namboodiri, *Chem. Commun.*, 2001, 643–644.

25 M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, X. H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, Jr. J. A. Montgomery, J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, A. J. C. Burant, S. S. Iyengar, S. J. Tomasi, M. Cossi, N. Rega, N. J. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, O. A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski and D. J. Fox, *Gaussian 09, (Revision A.1)*, Gaussian, Inc., Wallingford, CT, 2009.

26 R. Ditchfield, W. J. Hehre and J. A. Pople, *J. Chem. Phys.*, 1971, **54**, 724–728; W. J. Hehre, R. Ditchfield and J. A. Pople, *J. Chem. Phys.*, 1972, **56**, 2257–2261; P. C. Hariharan and J. A. Pople, *Theor. Chim. Acta*, 1973, **28**, 213–222; P. C. Hariharan and J. A. Pople, *Mol. Phys.*, 1974, **27**, 209–215; M. S. Gordon, *Chem. Phys. Lett.*, 1980, **76**, 163–168.

27 R. G. Parr and W. Yang, *Density-Functional Theory of Atoms and Molecules*, Oxford University Press, New York, 1994.

28 W. Kohn, A. D. Becke and R. G. J. Parr, *Phys. Chem.*, 1996, **100**, 12974–12980; A. D. Becke, *J. Chem. Soc.*, 1993, **98**, 5648–5652; A. D. Becke, *Phys. Rev. A*, 1988, **38**, 3098–3100.

29 S. Miertus, E. Scrocco and J. Tomasi, *J. Chem. Phys.*, 1981, **55**, 117–129; B. Mennucci and J. Tomasi, *J. Chem. Phys.*, 1997, **106**, 5151–5158; R. Cammi, B. Mennucci and J. Tomasi, *J. Phys. Chem. A*, 2000, **104**, 5631–5637.

30 *MacroModel, version 9*, Schrödinger, LLC, New York, NY, 2007.

31 N. Metropolis, A. W. Rosenbluth, M. N. Rosenbluth, A. H. Teller and E. Teller, *J. Chem. Phys.*, 1953, **21**, 1087–1092.

32 W. L. Jorpensen, D. S. Maxwell and J. Tirado-Rives, *J. Am. Chem. Soc.*, 1996, **118**, 11225–11235.

