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METHODS

Isomerization of 9c11t/10t12c CLA in Triacylglycerols

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Abstract Isomers of conjugated linoleic acid from 7t9c through 12t14t can be induced by thermal treatment of triacylglycerol samples of 9c11t or 10t12c fatty acids in glass tubes. The formation of conjugated linoleic acids (CLAs) has been observed during thermal induction of the above-mentioned triacylglycerols at 250, 280 and 320°C. The concentrations of isomers formed in the mixture varied depending on the temperature and duration of the heating experiments. The objective of this study was to find a suitable thermal induction temperature and time that can produce most of the isomers of CLAs from the above-mentioned triacylglycerols. Such a mixture would give researchers a reference standard that can be used in the identification of CLAs in GC analyses of relevant samples. Fifteen-microlitre portions of the triacylglycerol samples containing 9c11t/10t12c fatty acid were placed in micro-glass ampoules, sealed under nitrogen and then subjected to thermal treatment. The glass ampoules were removed at regular time intervals, cut open, and part of the samples was analysed by infrared spectroscopy using attenuated internal reflectance technique. The remainder of the samples was subjected to derivatisation into their methyl esters. The methyl esters of the isomerised fatty acids were then analysed by gas chromatography after appropriate dilution in heptane. The results show that the thermally induced glyceride samples of 9c11t/10t12c fatty acids gave CLA profiles containing isomers ranging from 7t9c to 12t14t. However, the concentrations of the isomers are different depending on the duration of the thermal induction. It appears that [1,5] sigmatropic rearrangements and positional isomerisations take place in the heated mixtures. The rearrangements and positional isomerisations are accelerated by increasing temperature. The glyceride samples heated to 325°C form isomers within 30 min and provide a mixture of CLA isomers that can be used as reference sample containing the methyl esters of CLAs.

Keywords Thermal induction · Isomerisation · Conjugated linoleic acids · Gas chromatography

Abbreviations

CLA	Conjugated linoleic acids
ATR	Attenuated total internal reflectance
GC	Gas chromatography
FAME	Fatty acid methyl esters

Introduction

Research on conjugated linoleic acids (CLA) has become intense during the past few years because of their effects on carcinosis, atherosclerosis, the immune system, lipid metabolism and body composition. Furthermore, the focus on CLA is also due to their health benefits [1–9].

The conjugated isomers of linoleic acid in milk and fats are generally found in *trans*/*cis*, *cis*/*trans* and *trans*/*trans* forms. There are several conjugated linoleic acids present in milk and fat from the cud-chewing animals. Of these, 9c11t is dominant, with about 80–90% of all the conjugated linoleic acids. The isomer 7t 9c was found in most of the dairy products as the second dominant CLA fatty acid

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[10]. The isomer 9c11t is also thought to be the most biologically active [1, 2]. There are other isomers of CLAs in small concentrations [11]. These include *cis–cis*; *trans–trans*; *cis–trans* and *trans–cis* isomers of 7,9; 8,10; 9,11; 10,12 and 11,13 CLAs. There are 20 isomers from the above five CLA positional isomers. Several of these isomers have been identified in different food products. The 9c11t and 10c12t isomers are found in hydrogenated vegetable oils [11]. These are also found in equal proportion in the alkali isomerised methyl linoleate [13]. The concentrations of the other isomers are negligible [13].

The identification of different isomers of CLAs in samples of interest, in a chemical reaction aimed at synthesising CLA isomers or to prove or disprove the presence or absence of any CLA in a system of interest needs reference samples of CLAs. The two important CLAs namely 9c11t and 10t12c were prepared in large quantities by alkali isomerisation of 9c12c linoleic acid [14]. Eulitz et al. [14] synthesised eight CLA isomers, 8,10 through 11,13, by isomerising CLA mixtures by p-toluene sulfinic acid or iodine catalyst. Jain and Proctor [15] synthesised 9c11t, 9t11c, 10t12c and 10c12t CLA isomers in large quantities for the first time by UV photoirradiation of soya oil in the presence of iodine catalyst. The photoirradiation was carried out for 144 h to achieve this. Destaillats and Angers [16] were able to prepare 8t10c and 11c13t CLA isomers by heat teatment of 9c11t and 10t12c CLAs. Destaillats and Angers [11] demonstrated that the whole series of CLA isomers could be produced by starting from a mixture of methyl esters of 9c11t and 10t12c fatty acids. A two-step process was necessary in the preparation of these isomers: a heat-induced [1, 5] sigmatropic rearrangement in the first step and catalyst-induced positional isomerisation in the second. The formation of different isomers followed the mechanistic pathways outlined by Destaillats and Angers [11].

The chemistry and mechanistic pathways involved in the formation of CLAs as discussed in the research reports above spurred interest in looking at alternative ways of producing the isomers of CLAs. During preliminary investigations, it came to light that the [1, 5] sigmatropic transformations and positional isomerisations could be achieved by subjecting triacylglycerols of CLAs to a suitable high temperature. It was also clear that the duration of thermal treatment altered the concentrations of different isomers. The procedure adopted for the directed sequential synthesis of conjugated linoleic acids by Destaillats and Angers needed a total of 33 h (13 h for sigmatropic rearrangement reaction and 20 h for subsequent geometrical isomerisation) for the synthesis of CLA isomers ranging from $\Delta^{7,9}$ to $\Delta^{12,14}$. A straight-forward procedure that can reduce the isomerisation time and give a mixture that contains the same isomers should be preferred. The triacyl glycerols of 9c11t and 10t12c were selected because these can be easily prepared through alkali isomerisation, and then this mixture could be thermally induced to produce all the other isomers.

The aim of this paper is to demonstrate for the first time that the formation of CLA isomers ranging from 7,9 to 12,14 can be induced through the thermal induction of the triacylglycerol of 9c11t or 10t12c fatty acid. Furthermore, it is also the intention to look at the mechanistic aspects of the thermal induction for the formation of CLA isomers.

Experimental

Samples and Methods

The triacylglycerols of the 9c11t and 10t12c fatty acids were purchased from Larodan Chemicals, Sweden. The chemicals such as methanol, BF_3/methanol, NaOH, NaCl and $MgSO_4$ needed for derivatisation were purchased from Sigma-Aldrich. Methyl esters of 9c11t and 10t12c were prepared by esterification of the respective triacylglycerols.

The heating experiments were carried out in micro-glass ampoules as described in Ref. [17]. Three sets of glass ampoules containing the triacylglycerol samples were prepared. The first set of samples containing the triacylglycerols of the 9c11t or 10t12c fatty acids were placed in small glass vials and placed in a gas chromatographic oven (Agilent 5890) set at 250°C. Samples were then removed at 24-h intervals until the all the samples were exhausted. The experiment was repeated in the same manner with the second and third set of samples at 280 and 325°C except the samples treated at 325°C were removed at 30-min time intervals.

Infrared Spectroscopic Measurements

The samples from the thermal induction experiments were subjected to infrared measurements. A PerkinElmer Spectrum One FT-IR spectrometer equiped with a Harrick single reflectance ATR accessory and lead glycine sulphate detector was used in measuring the infrared spectra. Each sample was spread on the ATR crystal using a capillary glass tube. A total of 30 scans at a resolution of 4 cm^{-1} were then measured in the range of 4,000 to 600 cm^{-1} with previously scanned ATR crystal spectrum as the background. The ATR crystal was washed with acetone and dichloromethane after each measurement. A new capillary tube was used each time for the application of the sample to avoid cross contamination. All the infrared spectra were saved in absorbance format. Second derivative spectra of the absorption spectra were obtained using the derivative option in the scanning program of the instrument.

Gas Chromatographic Analysis

The glass ampoules containing the rest of the triacylglycerol samples were subjected to derivatisation as described in Ref. [17]. The methyl esters of the products of the thermal induction were extracted in heptane. The GC analysis was carried out by using a PerkinElmer auto XL system gas chromatograph. A 100-m capillary column with 0.25 mm internal diameter coated with 0.20-µm-thick 90%-bis-(cyanopropyl)-methyl polysiloxane stationary phase with a small amount of phenyl groups in the backbone of the polymer (HP 88) was used in the separation of the methyl esters of the fatty acid isomers. A temperature program with initial temperature 150°C with equilibration time of 1 min, then a temperature gradient of 0.5°C/min up to 170°C was used. The temperature was held at 170°C for 40 min. The peak identification was carried out by comparing the reported conjugated linoleic acid profiles in references [10, 11, 13, 14] and methyl esters prepared from the 9c11t and 10t12c triacylglycerols as described in the section "Samples and methods". The peaks in the chromatograms were then integrated, and their relative percentages were calculated using an excel spread sheet.

Results and Discussion

Infrared Spectroscopy

Infrared spectra of the CLA samples from the above experiments were recorded to confirm and complement the gas chromatographic analysis. CLAs with *cis–trans* and *trans–cis* configurations give rise to two specific absorptions at around around 946 and 986 cm^{-1} because of the =CH out-of-plane deformation vibration [18]. The CH out-of-plane deformation vibration of the *trans–trans* isomers absorb around 982–988 cm^{-1}, and *cis–cis* isomers do not absorb in this region. The =CH deformation vibrations of the methylene interupted *cis–trans, trans–cis* and *trans–trans* linoleic acid isomers absorb at approximately 967 at 4 cm^{-1} resolution at room temperature [19–22].

The second derivative profiles of the infrared spectra of the 9c11t/10t12c triaclylglycerol samples thermally induced at 250°C are shown in Fig. 1. The spectra show that the peak at 947 cm^{-1} decreases in intensity. The peak at 985 cm^{-1} also decreases, but because of the contribution of the absorption due to the *trans-trans* isomers of the CLA formed, the decrease is not parallel to the decrease in the intensity of the peak at 947 cm^{-1}. There is also a new peak appearing around 967 cm^{-1} indicating the formation of methylene interupted *cis–trans/trans–cis/trans–trans* isomers in the mixture.

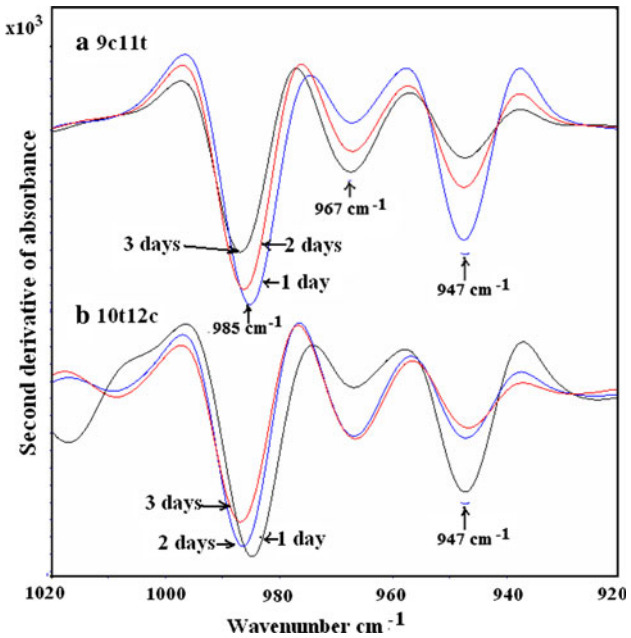

Fig. 1 Second derivative infrared profiles of thermally treated 9c11t and 10t12c triacylglycerol samples at 250°C

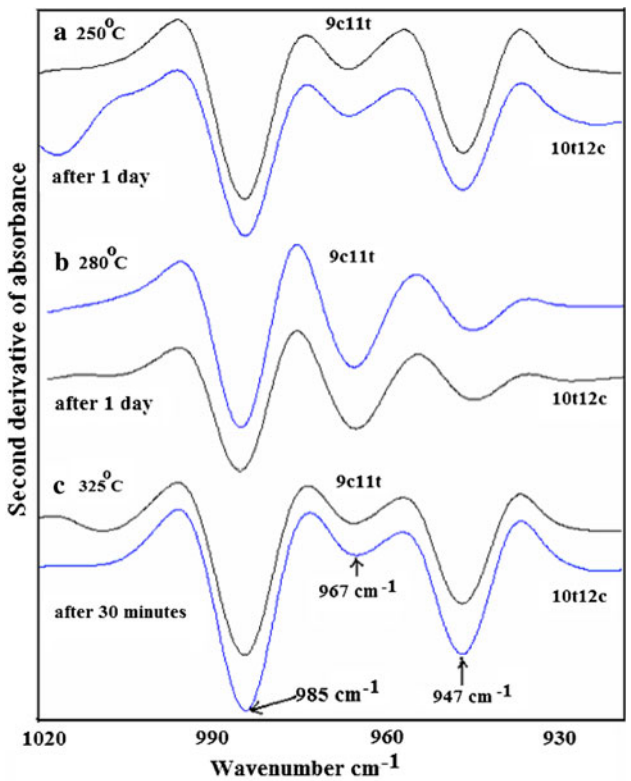

Fig. 2 Second derivative infrared profiles of thermally treated 9c11t and 10t12c triacylglycerol samples at 250, 280 and 325°C

The second derivative profiles of the infrared spectra of the 9c11t/10t12c triacylglycerol samples thermally induced at 250, 280 and 325°C are shown in Fig. 2. The spectra in

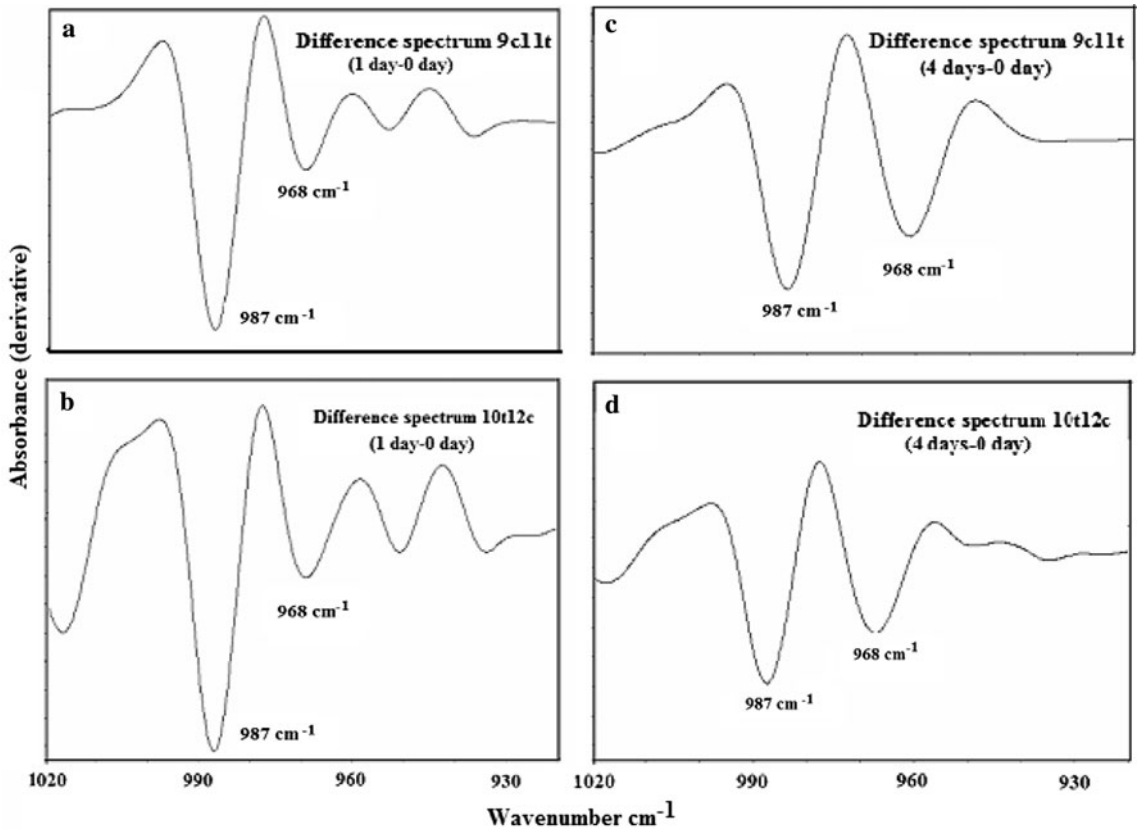

Fig. 3 Interactive substraction of the second derivative infrared spectrum of the starting material from the spectra of samples thermally induced at 250°C

the figure show that the intensity of the second derivative infrared peaks at 985 cm^{-1} of the samples thermally induced for 30 min at 325°C are higher than the peaks of the samples thermally induced for 24 h at 250 or 280°C. This is an indication of the formation of relatively higher concentrations of *trans–trans* CLAs in the thermally induced samples. This can be further confirmed by a proper interactive subtraction of the second derivative spectrum from the spectrum of starting material. The subtraction was carried out using the procedure found in the PerkinElmer instrument software. The results of the substraction are shown in Fig. 3.

Gas Chromatography

The gas chromatograms of the FAME of the thermally induced triacylglycerol samples containing 9c11t and 10t12c fatty acids are shown in Figs. 4 and 5, respectively, CLA profiles are identical to the FAME of most of the CLAs ranging from 7,9 through 12,14, except their relative concentrations. The effect of temperature is clear from the first two samples of the thermal induction. The samples heated at 280°C contain all the isomers of the CLAs, but their relative concentrations are small. The *trans–trans* isomers dominate in the sample. A comparison of the CLA profiles in the heated samples at 250, 280 and 325°C show that there are three pairs of CLA isomers standing out clearly from the rest of the profiles. They are 9c11t, 8t10c; 10c12t, 9t11c and 11c13t, 10t12c. The relationships between the area percentages of the components in the above pairs and thermal induction times are given in Fig. 6. For each starting material, the members in each pair have the similar relative concentrations in all the test samples. When the thermal induction experiments were carried out with 9c11t, the relative concentrations of 9t11c and 10c12t were higher than the concentrations of 10t12c and11c13t (Fig. 6). The thermal induction with triacylglycerol containing 10t12c shows again that the concentrations of 9t11c and 10c12t were higher than the concentrations of the pair 8t10c and 9c11t. It appears that the isomers 10c12t and 9t11c are at the intermediate position of the [1, 5] sigmatropic transformation sequence of the three pairs mentioned above.

Mechanism

A figure depicting the [1, 5] sigmatropic transformations and positional isomerisations in the CLAs from 7,9 through 12,14 is given in Fig. 7. The mechanisms related to the

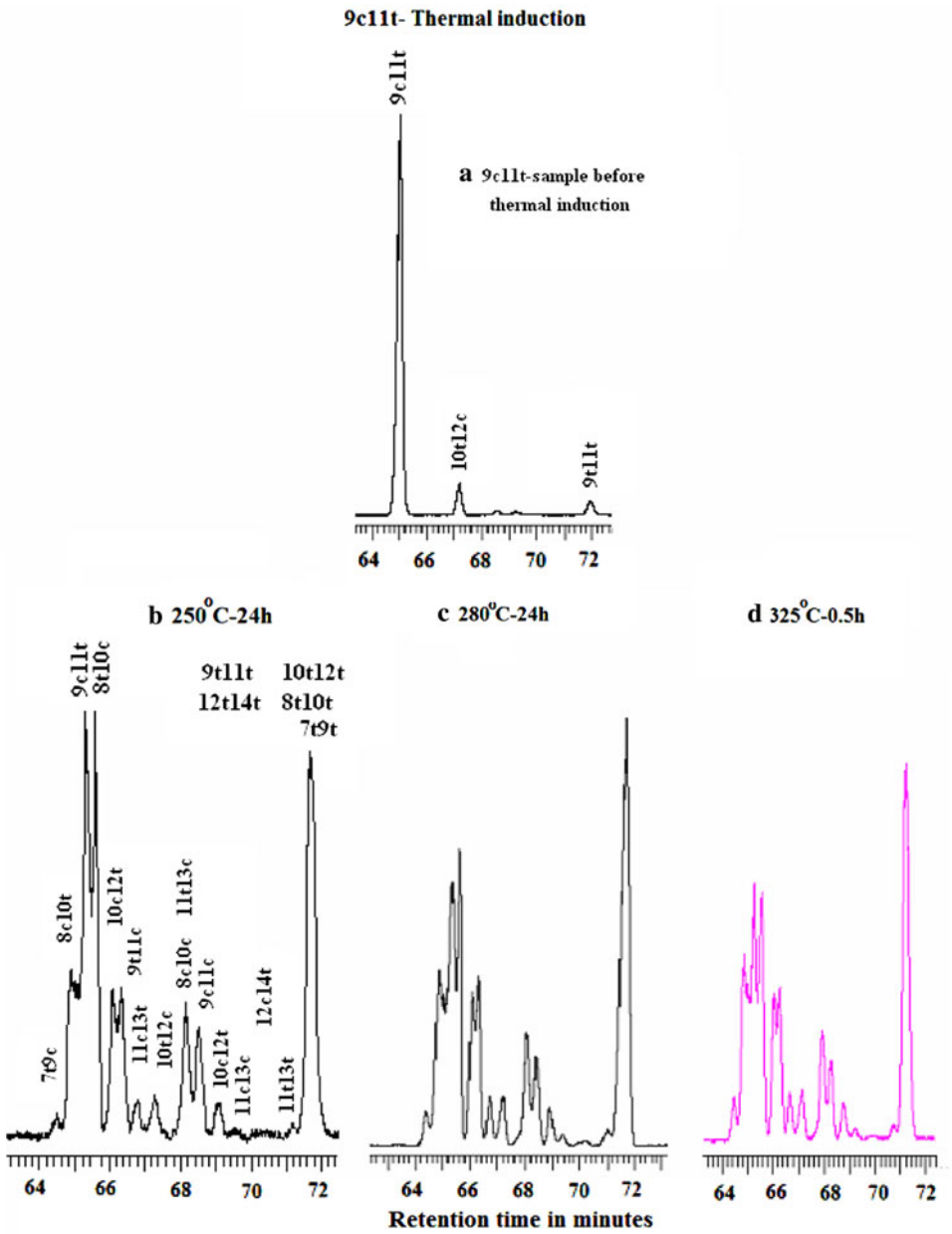

Fig. 4 Gas chromatograms of thermally induced 9c11t triacylglycerol samples

[1, 3] and [1, 5] sigmatropic transformations, and positional isomerizations can be found in Destaillats and Angers [11, 16] and Christy [23]. The figure is similar to the one formulated by Destaillats and Angers [11, 16] and Christy [23], but presented collectively in a manner to explain the appearance of the *cis–trans* and *trans–cis* isomers of CLAs that can be expected in a thermal induction experiment of another *cis–trans* CLA isomer. Thermal induction of 9c11t yields very little or no 11t13c and 12c14t isomers. The 9c11t isomer lies far away from this pair in the sequence. At the same time, the thermal induction of 10t12c, which lies closer to this pair in the sequence than 9c11t, shows the presence of 11t13c and 12c14t. Similarly, the weak appearance of the peaks representing 7t9c and 8c10t isomers during the thermal induction of 10t12c can be explained in the same manner. Furthermore, the 9c11t isomer lies closer to the 7t9c and 8c10t isomers in the sequence, and therefore thermal induction of 9c11t should show peaks representing these CLA isomers. It is interesting to note that the CLA fatty acids 9t11c and 10c12t are in the middle of the sequence of CLAs ranging from 7,9 to 12,14. It appears that a part of CLA isomers with relatively high concentrations compared to the rest of the CLA isomers can be prepared by selecting and thermally inducing a triaclyl glycerol containing a suitable *cis–trans* CLA fatty acid. Accordingly, thermal induction of either 9t11c or 10c12t should give CLA isomers with relatively higher concentrations of most of the isomers.

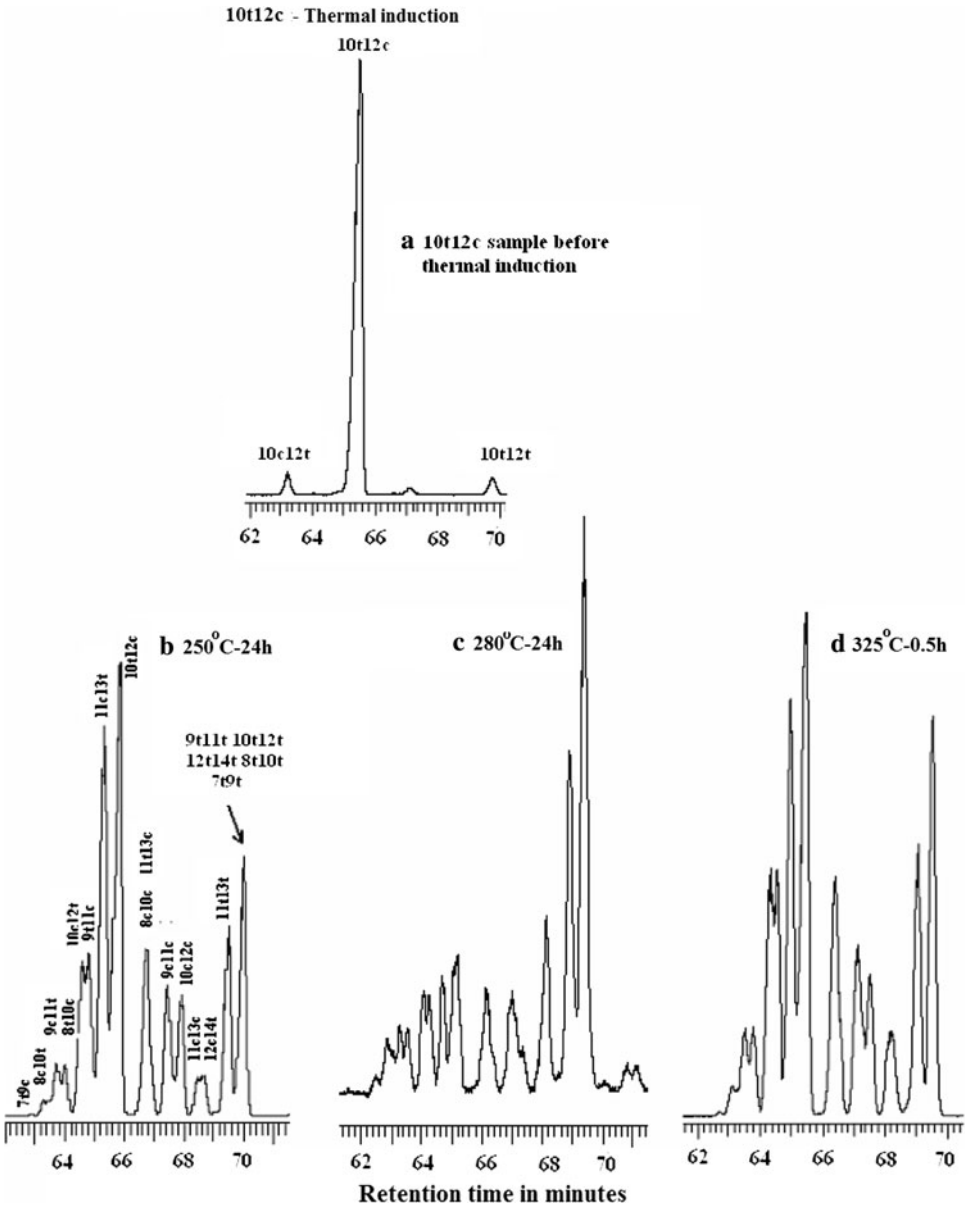

Fig. 5 Gas chromatograms of thermally induced 10t12c triacylglycerol samples

In addition to CLA isomers, the thermally induced mixtures also contain another peak accompanied by other very minor peaks in the chromatograms eluting 15 min earlier than the CLA isomers (Fig. 8). This peak is prominent in the samples thermally induced for 1 day at 250 and 280°C. At the same time, it is very small in the samples thermally induced for 30 min at 325°C. The identity of the relatively large peak in the group was confirmed as methylene interrupted 9t12t (18:2) fatty acid. In an earlier report [23], it was shown that the CLAs can also be formed by thermally inducing a triacylglycerol containing 9t12t fatty acid. Therefore, the formation of 9t12t (18:2) is possible during thermal induction of CLA fatty acids. The reaction takes place through an intramolecular [1, 3], sigmatropic rearrangement or a free radical chain reaction mechanism [16, 23]. The identification of 9t12t led to the inclusion of the [1, 3] sigmatropic transformation part in the mechanism presented in Fig. 7.

The peak in the infrared at 967 cm^{-1} also supports the identity. The absorption can be due to methylene interupted *cis–trans*, *trans–cis* and *trans–trans* isomers of fatty acids. The formation of 18:2 *trans–trans* fatty acid during the induction of either 9c11t or 10t12c fatty acids also stresses one point that the formation is specific of their standards. If the thermal induction of 9t12t can form 9c11t and 10t12c through an intermediate 9t11t, then the other CLA fatty acids can also be formed from *trans–trans* fatty acids such as 7t10t, 8t11t, 10t13t and 12t15t. However, it was not

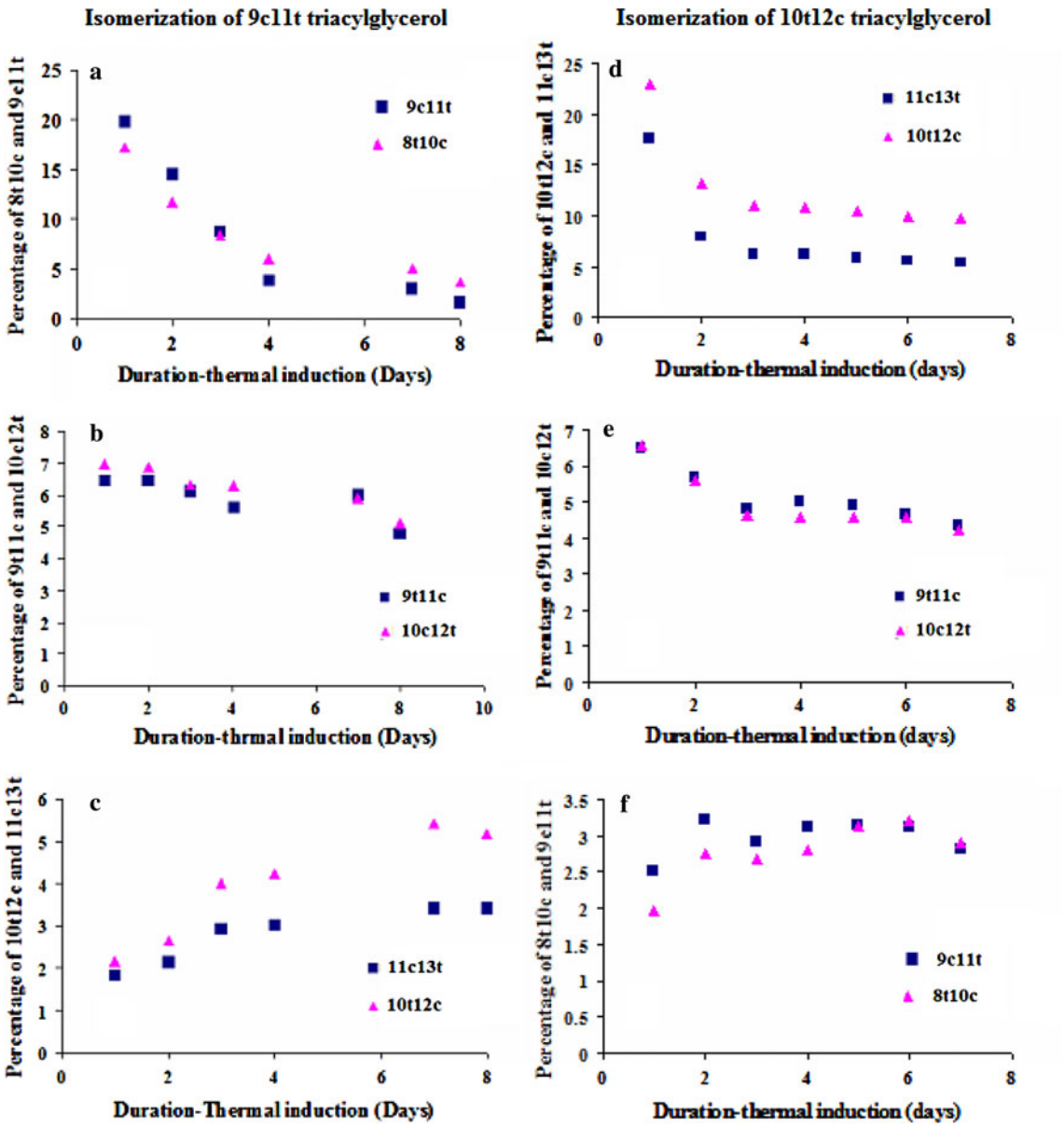

Fig. 6 Plots showing the relationships between relative percentages of the isomers formed in the mixtures during thermal induction of 9c11t and 1oc12t triacyl glycerol samples at 250°C

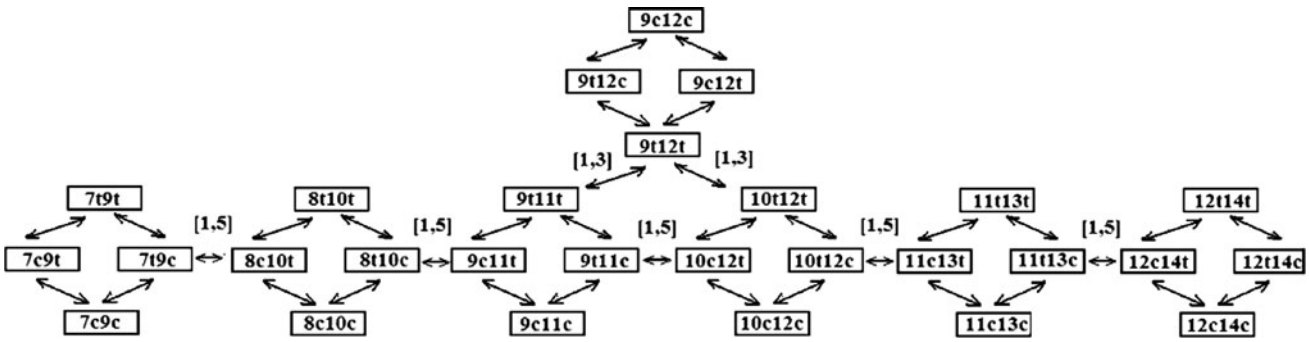

Fig. 7 Isomerisation sequence of conjugated linoleic acids

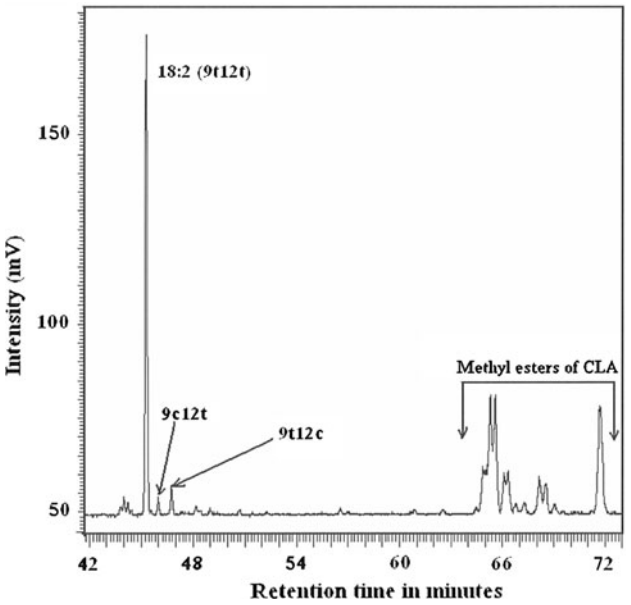

Fig. 8 Gas chromatogram of thermally induced 9c11t triacylglycerol sample

possible to confirm the presence of any of these *trans–trans* fatty acids because of the low concentrations and non-availability of the standards.

In this paper, it has been shown that the CLA isomers ranging from 7,9 through 12,14 can be prepared in one step by thermally inducing either 9c11t or 10t12c CLA. The formation of the isomers is relatively faster in the thermal induction of the samples at 325°C. The thermal induction of 10t12c CLA at 325°C for 30 min yields almost all the CLA isomers in detectable amounts that can be used as a reference sample. Furthermore, the preparation time of the isomers can be shortened from several hours to minutes, and several steps involving wet chemical reactions can be avoided. In addition, the isomerisation time used in the two-step synthesis forms high concentrations of *trans–trans* isomers of the CLAs, which elute together at the end as a very tall and broad peak (as shown in Ref. [11]) and dilute the other CLAs in the sample mixture. The isomerisation sequence presented in Fig. 7 and the experimental evidence provided in Fig. 6 clearly indicate the direction of isomerisation and the products one can expect in an experiment. Thermal induction of a CLA isomer is expected to give isomers that are close to the starting material. However, with the relative ease of obtaining either 9c11t or 10t12c CLA isomers, it is convenient to start with one of these isomers.

To illustrate the formation and the relative concentrations of the CLA isomers in the mixture, a thermal induction sequence diagram is presented utilising the pioneering work of Destaillats and Angers [12]. The figure gives an idea about which CLA isomer to select for the thermal induction to induce and form the CLA isomers one desires to prepare.

With the information presented in this paper, the mechanism involving the formation of CLA isomers has advanced one more step in the forward direction.

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