Evidence in the formation of Conjugated Linoleic Acids from thermally induced 9t12t linoleic acid: A study by Gas Chromatography and infrared spectroscopy

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Abstract

Thermally induced isomerisation leading to the formation of Conjugated Linoleic Acids (CLA) has been observed for the first time during the thermal treatment of 9t12t fatty acid triacylglycerol, and methyl ester. Fifteen microlitre portions of the triacylglycerol sample containing 9t12t fatty acid (trilinoelaidin) were placed in micro glass ampoules and sealed under nitrogen, then subjected to thermal treatment at $250^{o}C$. The glass ampoules were removed at regular time intervals, cut open, and the contents were analysed by infrared spectroscopy using a single reflectance attenuated total internal reflectance crystal accessory. The samples were then subjected to derivatisation into their methyl esters. The methyl esters of the isomerised fatty acids were analysed by gas chromatography. The same procedure was repeated with methyl ester samples containing 9t12t fatty acid (methyl linoelaidate). Each sample was subjected to infrared measurements and gas chromatographic analysis after appropriate dilution in heptane.

The results show that the thermally induced isomerisation of 9t12t fatty acids from both triacylglycerol molecules and methyl esters give identical CLA profiles as those found for the thermally induced isomerisation of 9c12c fatty acids. The infrared spectrometry provides additional evidence confirming the formation of CLA acids during thermal treatment. A mechanism for the formation of the CLAs from 9t12t fatty acid molecules is also formulated for the first time. This mechanism complements the pathways of formation of CLAs from 9c12c fatty acids during thermal treatment.

Key words: Linoelaidin, Conjugated Linoleic Acids, Gas Chromatography, infrared spectroscopy, isomerisation.

Introduction

Conjugated Linoleic Acids (CLA) are natural components found in animal products such as meat and dairy products derived from ruminant animals. Conjugated Linoleic Acids have gained considerable attention in recent times because of their health benefits (Ip et al., 1991 and 1994; Nicolosi et al., 1993; Shanta et al., 1992). This interest has been followed by an explosion of research reports on the benefits of CLAs in the areas of cancer, heart disease, diabetes and many other areas (Parodi, 1996; Belury and Van den Heuvel, 1997; Schrezenmeir and Jagia, 2000; Pariza, 2000; Scimeca and Miller, 2000; Kritchevsky, 2000; Thom et al., 2001; Voorrips et al., 2002; Belury, 2002; Larsen et al., 2003; Tricon et al., 2004; Lin Yang et al., 2004; Zulet et al., 2005; Wang et al., 2006; Bhattacharya et al, 2006; Kelley et al, 2007; Fite et al., Amarù and Field, 2009; Coakley et al, 2009). The results in these reports are based on the studies on animals and the beneficial effects on humans are yet to be proven.

The conjugated isomers 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. Out of these 9c11t is the dominant conjugated linoleic acid. The second most abundant isomer is 7t9c if concentrates are fed to ruminants (Yurawecz et al., 1998), and 11t 13c if ruminants are pasture fed (Kraft et al., 2003). Among these, the isomer 9c11t is thought to be the most biologically active (Ip et al., 1991 amd 1994). The CLA isomers such as 9c11t and 10t12c can be found in hydrogenated vegetable oils (Cawood et al., 1983). The isomers 9c11t and 10t12c are formed in almost equal proportion in the alkali isomerised methyl linoleate (Berdeaux et al., 1996). The concentrations of the other isomers are negligible in the alkali isomerised methyl linoleate (Berdeaux et al., 1996).

The interest in conjugated linoleic acids in recent years has led to an increase in the investigation into the formation chemistry of the acids. The study of the chemistry of conjugated linoleic acids goes back more than a half a century to the investigation of the rearrangement of the 1,4 double bonds in polyolefinic compounds. The rearrangement was shown to occur during auto oxidation of poyolefins (Bolland and Koch, 1945; Farmer et al., 1943) and treatment in the presence of nickel catalyst (Bailey, 1945; Radlove et al., 1946) or in the presnce of alkali at high temperatures (Moore, 1939; Kass and Burr; 1939). Nichols and his co-workers (Nichols et al., 1951) showed that the isomerisation of linoleic acid to CLAs could be achieved by alkali isomerisation at high temperatures. Furthermore, with available literature and analytical methodology, UV spectrometry, they were able to show that the predominant products in the alkali isomerisation were 9c11t and 10t12c CLAs. In adition to this they were able to formulate a mechanism for the formation of conjugated double bonds. With sophisticated analytical techniques, several reseachers have confirmed the fact that the main products formed in the alkali isomerization of 9c12c fatty acid were indeed 9c11t and 10t12c. Nichols and his co-workers (Nichols et al., 1951) have also used theoretical considerations and predicted the formation of conjugated linoleic acids from the *cis/trans* isomers of methylene interrupted 9,12 linoleic acids.

Recent investigations have given us insight into the chemistry of conjugated linoleic acids. The chemistry and mechanisms related to the formation of CLAs are studied in depth. Berdeaux et al. (Berdeaux et al., 1998) have shown a simple method for the preparation of 9c11t and 10t12c in large quantities. In their work, they have clearly illustrated that the alkali isomerisation time and quantity of methyl linoleate used in the isomerisation are important factors that determine the composition of the CLA isomers formed. Destaillats and Angers have shown in their report (Deataillats and Angers, 2002) that the CLA acids 9c11t and 10t12c undergo [1,5] sigmatropic rearrangements to 8t10c and 11c13t respectively when they were subjected to high temperatures. Using these isomers of CLAs, they have identified the formation of CLA fatty acids in heated oils and methyl linoleate. In another report, Deataillats and Angers (Deataillats and Angers, 2003) have demonstrated that the whole series of CLA isomers could be produced starting from a mixture of 9c11t and 10t12c isomers. They (Deataillats and Angers, 2005) have again in one of their recent reports shown the formation of CLAs from the thermal induction of linoleic acid. They have also formulated a free radical and [1,3] sigmatropic

rearrangement mechanisms to explain the formation of CLAs in the heated methyl ester sample of 9c12c LA under innert conditions and oxidative conditions.

At the same time Kramer et al. (Kramer et al., 2004) have contributed in the analytical methodology of CLAs. They have compared conditions regarding the analysis of CLAs and given guidelines. A proper analytical methodology is an important tool in the analysis and study of the chemistry of CLAs. Furthermore, separation of CLA isomers, their identification and quantification are important in understanding the reaction pathways and formulating a mechanism for the isomerisation.

To this respect infrared spectroscopy can also play an important role. CLAs with *cis, trans* and *trans, cis* configurations give rise to two specific absorptions around 946 and 986 cm^{-1}. These absorptions arise due to the =CH out of plane deformation vibration (Mossoba et al., 1991). The CH out of plane deformation vibration of the *tt* (*trans, trans*) isomers absorb around 982 cm^{-1} and, *cis-cis* isomers do not absorb in this region. Furthermore, the =CH deformation vibrations of the methylene interupted *cis-trans, trans-cis* and *trans-trans* linoleic acid isomers absorb around 969 cm^{-1} (Mossoba et al., 1996; Belton et al., 1988; Dutton, 1974; Lancer and Emkem, 1988) which falls in between the two characteristic absorptions of the CLAs. This fact has been utilised in the simultaneous quantitative determination of isolated *trans,* and CLAs in oils and fats (Christy et al., 2003). The presence of absorption peaks at 946 and 982 cm^{-1} of the fatty acids formed during the isomerisation is a clear indication of the formation of CLA isomers.

The formation of CLAs during the alkali isomerisation of conjugated linoleic acids, subjecting the CLAs formed to higher temperatures to induce sigmatropic rearrangements and using selenium catalyst to obtain geometrical isomers of CLAs are now well known facts. However, the chemistry of formation of CLAs is not complete until all the possible pathways are explored and studied.

The aim of this paper is to demonstrate for the first time that the formation of CLAs can also be induced through the thermal treatment of 9t12t fatty acid. Furthermore, the mechanisms involving the formation of CLAs from the 9t12t fatty acid are also formulated. This finding will complement the possible pathways of CLAs formation and complete the mchanistic formulations already published in the literature.

Experimental
Samples and methods
The triacylglycerols and methyl esters of the 9c12c and 9t12t acids were purchased from Sigma Aldrich. The methyl esters containing the 9c11t and 10t12c (99%) and several other methyl esters of fatty acids were purchased from Larodan Chemicals, Sweden.

The heating experiments were carried out in micro glass ampoules. Several glass ampoules of length 4 cm were made from glass tubes of 1.5 mm internal diameter and a wall thickness of 1 mm. A propane oxygen flame was used to melting one of the ends of

each glass tube. 15 μl portions of the triacylglycerol containing the 9t12t fatty acid were injected in the tubes using a plastic syringe. Air in the remaining part of the glass tubes was flushed by a weak nitrogen flow and the glass tube end was melted and sealed. It is important to keep the glass tubes free of air. In the presence of air, the samples undergo oxidation and polymerisation. A second set of samples were prepared with the triacylglycerol containing 9c12c LA and a third set with methyl ester of 9t12t LA. The sealed glass tubes were then placed in a short 5 ml glass vial and placed in a chromatographic oven set at $250^{o}C$. The glass tubes were removed at regular time intervals.

Infrared spectroscopic measurements

Each glass tube was cut open and a portion of the sample was used to measure the representative infrared spectrum. A Perkin Elmer 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. The accessory requires only a thin layer of sample on the crystal to acquire the infrared spectrum. The sample was spread on the ATR crystal using the blunt side of a capillary glass tube. A background spectrum was scanned in the range of 4000-600 cm^{-1} before the application of a sample. A total of 30 scans at a resolution of 4 cm^{-1} were then measured on each sample. The samples from the experiment were measured immediately after their removal from the oven. The ATR crystal was washed with dichloromethane and acetone after each measurement. The same procedure was repeated for the thermally induced 9c12c LAs and samples with methyl ester of the 9t12t LAs.

All the infrared spectra were saved in absorbance format. The spectra were then doubly derivated and used in the quantitative analysis. Second derivatives of the spectra in the region 1000-900 cm^{-1} where the CLAs and all the *trans* LAs absorb were used for qualitative identification of the presence of CLAs in the heated mixtures.

Gas chromatographic analysis

The remaining triacylglycerol samples containing 9t12t fatty acid in the tubes after the infrared analysis were subjected to derivatisation. Each glass tube containing the sample was cut just above the liquid mark and crushed inside a 15 mL test tube. A solution of 0.5 M sodium hydroxide in methanol (2 ml) was then added to each test tube. The test tubes containing the mixtures were then placed in a water bath at 60 ^{o}C for 15 min. After cooling, each test tube was added 2 mL of BF_3/methanol and placed in the water bath again for 10 min. Each test tube was then added 2 mL of a saturated solution of NaCl and 1 mL heptane. The tubes were shaken to aid separation and dissolution of the FAMEs in the heptane layer. The glass tubes were allowed to stand for a few minutes, the upper heptane layers removed and then dried by the addition of anhydrous magnesium sulphate. The separated dried heptane layers were then carefully extracted and placed in small brown vials and kept in the dark for GC analysis. The same procedure was adopted with samples containing the triacylglycerol of 9c12c fatty acid. Methyl ester samples of the 9t12t fatty acid from the thermal treatment were diluted in heptane for GC analysis.

The GC analysis was carried out by using a Hewlett Packard 5890 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 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 $150^{o}C$ with equilibration time of 1 minute, then a temperature gradient of 5 ^{o}C/min up to $180^{o}C$. After a time of 50 min at $180^{o}C$ another teperature gradient of 5 ^{o}C/min was used to bring the final temperature to 220 ^{o}C. The final time at 220 ^{o}C was 30 minutes.

The peak identification was carried out by comparing the reported conjugated linoleic acid profiles formed during the thermal treatment of the methyl esters containing 9c12c acids in the literature (Eulitz et al., 1999) and using standards.

Results and discussion
Infrared spectroscopy
The infrared spectra measured on the samples taken after heat treatment are shown in Fig. 1 for methyl linoelaidate, trilinoelaidin and trilinolein. The infrared absorptions arising from the functional groups in LAs and CLAs are shown in table 1. The figures clearly show the variation of the peak at 969 cm^{-1} of the samples during the thermal treatment. The intensity of this peak decreases in the samples containing 9t12t fatty acids and increases in the sample containing 9c12c fatty acid molecules. Second derivatives of the spectral profiles in the region 1000-900 cm^{-1} are shown in Fig. 2a for pure and heat treated trilinolein samples. The difference spectrum is shown in Fig. 2b. The peaks at 987 and 946 cm^{-1} indicate the presence of CLAs in the heated sample. The peak at 969 cm^{-1} indicates the presence of *t,c* or *c,t* or *t,t* LAs or mixture of these isomers. The blue shift in the first peak clearly indicates that the CLAs in the heated sample is a mixture of *c,t/t,c* and *t,t* isomers of CLAs with a high percentage of *t,t* isomers.

The infrared spectroscopy can be used in following the real concentration of trilinoelaidin during the heat treatment. Oils and fats during thermal treatment can undergo several changes that lead to several different products. The mono and poly unsaturated fatty acid molecules can isomerise to *trans* fatty acids or undergo oxidation and degrade into other products or undergo intra cyclisation or polymerization (Christy et al., 2009). The doubly derivated infrared profiles of the heated samples were used for this quantification purpose. The pure trilinoelaidin was used as the reference and the peak at 969 cm^{-1} was used for quantitative determination of the remaining trilinoelaidin in the mixtures. The 2nd derivatives of the infrared profiles of the heated samples in the region 1015-915 cm^{-1} are shown in Fig 3. The length of the valley indicating the peak 969 cm^{-1} is measured from the zero line. This length is compared with the length of pure trilinoelaidin and the concentration of remaining trilinoelaidin in the sample is determined. After 10 days of thermal treatment, the concentration of trilinoelaidin reduces to 54%. A plot showing this decomposition is given in Fig. 4. The concentration profile of the 9t12t determined by infrared spectroscopy clearly indicates that the decrease in concentration is linear with heating time. This suggests that the degradation reaction might be of zeroeth order.

Gas chromatography

The gas chromatograms of the FAME of the heated trilinolein, trilinoelaidin and methyl linoelaidate are shown in Fig. 5,6 and 7 respectively. The main components are *t,c/c.t/t,t* isomers of LAs and CLAs. However, the proportions of these components are very different. The heated trilinolein sample isomerises to *c,t/t,c* isomers of LAs and these two isomers are in equal quantities. This proportion was observed in all the heated samples from Trilinolein. The formation of equal quantities of these isomers proves that the isomerisation of the 9,12 double bonds are equally probable and the double bonds are not perturbed by the carbonyl group lying in the acid unit or by any other neighbouring molecules or groups. Furthermore, the chromatograms of the heated trilinoelaidin have very small concentrations of *t.c/c.t/c,c* isomers of LAs indicating the stability of *t,t* isomer towards positional isomerisation.

The FAME profiles revealed by gas chromatography show only the fatty acids present in the thermally treated sample. These profiles exclude the degradation products, aldehydes and other compounds without an acidic group, because only the fatty acids in the triacylglycerol molecules and any other acid molecules formed during the degradation take part in the derivatisation process. As shown above under infrared spectroscopic analysis, trilinoelaidin undergoes degradation into other products. These products were not analysed in this work.

The parts of the chromatogram indicating the area where CLAs are eluted show that 9c12c and 9t12t LAs undergo isomerisation into CLAs. Parts of the chromatograms showing the profiles of the CLAs from heated samples of trilinolein, trilinoelaidin and methyl linoelaidate are shown in Fig.8. There is no doubt that the CLAs profiles are almost identical. The elution order of different CLAs is given in one of the chromatograms (Eulitz et al., 1999). The progress of formation of CLA during thermal treatment is shown in Fig. 9. The concentrations shown in the plot were based on the total fatty acid profiles analysed in the samples and represent relative concentration of total CLAs in the samples. The relative concentration of CLAs increases with the heating time in trilinolein, trilinoelaidin and methyl linoelaidate. The relative concentration of CLAs in the heated samples reaches a maximum of 5%. When the degradation of the trilinoelaidin is taken into account, the concentration of the total CLA in the heated sample amounts to around 2.2%. All the *t,t* CLAs isomers elute at the same time under these conditions and contribute a significant concentration to the CLA profiles. The relative concentration of *t,t* CLAs isomers amount to around 60% in all the heated samples.

The relative concentration of the 9t12t fatty acids in heated samples as determined by gas chromatography is also given in Fig. 4. The concentrations determined just by taking FAMEs into consideration overestimates the concentration of fatty acids in the sample. In this respect infrared spectroscopy provides concentrations in the heated mixtures where degraded components are also present. The use of peak at 969 cm^{-1} in quantifying the

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concentration of trilinoeladin in the mixture is reasonable because the total concentration of 9t12c and 9c12t and 9c12c is around 6-7% and therefore the error in the determination will be in that range.

Mechanism

The mechanisms proposed by Deataillats and Angers [22] can well be used here to explain the formation of CLAs from the 9t12t fatty acid moieties in the triacylglycerol molecules. Conjugated 9t11t and 10t12t fatty acids are formed as primary products through either 1) A free radical chain reaction mechanism or 2) An intramolecular [1,3] sigmatropic rearrangement mechanism. In the first mechanism a bis-allylic hydrogen at C-11 can migrate to C-9 or C-13 to form 9t11t and 10t12t fatty acids. The steps involved in the mechanisms are shown in Fig. 10. From these primary products all the isomers of CLAs are formed through a series of positional isomerisation and [1,5] sigmatropic rearrangements. These isomerisations are shown in Fig. 11.

The correctness of the proposed mechanisms can only be tested by analysing the products formed and comparing the results with the proposed mechanisms. Apart from 7c,9t, 7c9c, 12t,14c and 12c14c, all the other isomers can be identified in the chromatograms. Since, these CLAs are formed in the last stages of isomerisation, the concentrations can be too small to detect. Furthermore, the *trans,trans* isomers elute together and the presence of 7t9t and 12t14t fatty acids under the large peak is difficult to confirm.

Conclusion

In this paper, we have shown evidence in the formation of CLAs from trilinolein, trlinoelaidin and methyl linoelaidate during thermal treatment. The relative concentration evolution of CLAs shows that the concentration increases with the time of heating and reaches a value around 5% both in trilinolein and trilinoelaidin. The *trans,trans* isomers dominate in the CLA mixtures and constitute around 60% of total CLAs in the sample.

Furthermore, the profiles of the CLA concentrations show identical patterns in heated trilinolein, trilinoelaidin and methyl linoelaidate. This fact alone suggests that the mechanism for the formation of CLAs from these samples should follow identical paths.

The formulated mechanisms for the formation of CLAs during the thermal treatment pave the way for the identification of the CLA isomers in the heated samples of trilinoelaidin. Thanks are due to Destaillats and Angers for giving insight into the mechanisms through their research work. The mechanism accounts for all the *t,c/c,t* CLA isomers except two positional isomers of 7,9 CLAs and two positional isomers of 12,14 CLAs.

Apart from the formation of the positional isomers and CLAs, thermal induction also causes degradation of 9t12t fatty acid. The concentration of trilinoelaidin decreases almost to half in 10 days of heating.

We have used a special experimental procedure with micro glass ampoules for the study of thermal induction. Only a fraction of a millilitre sample is required for the preparation

of a series of samples. Similar procedure can be used in the study of reaction kinetics of high boiling organic compounds.

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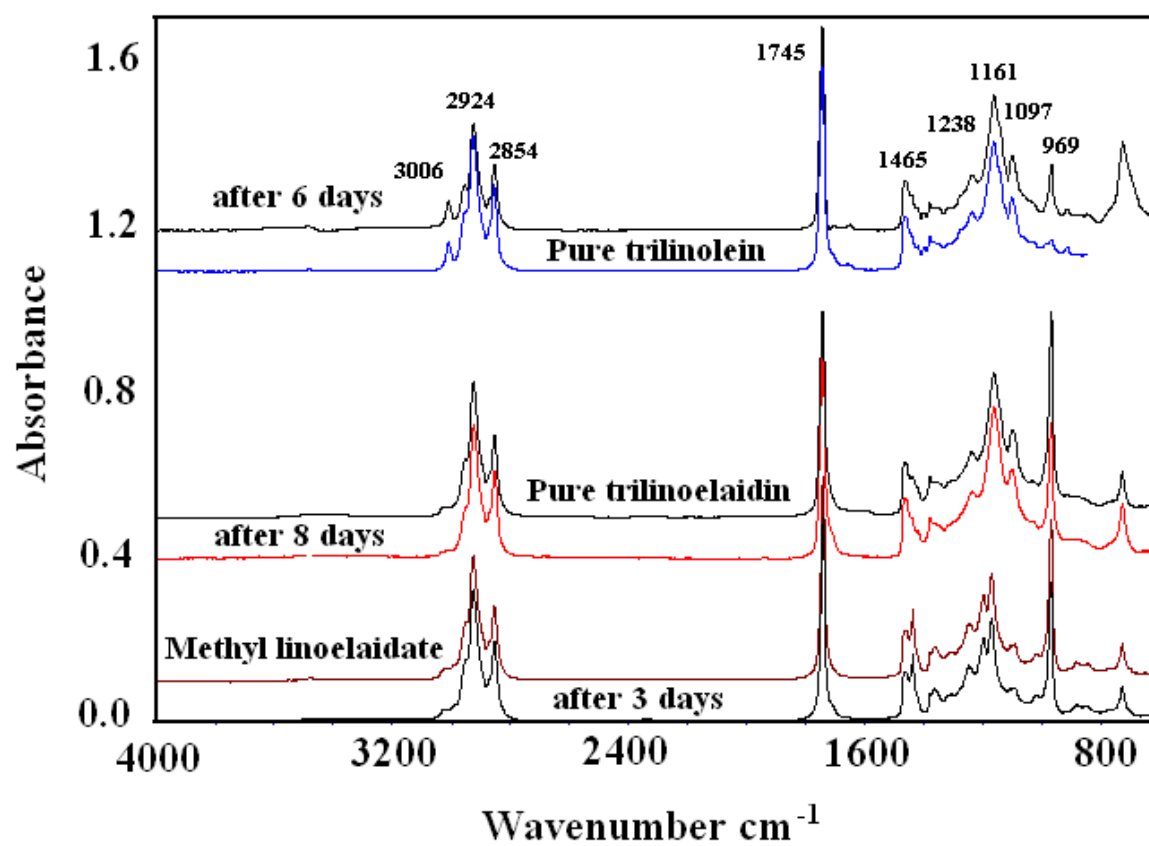
Fig. 1. Infrared spectra of some of the heat treated samples
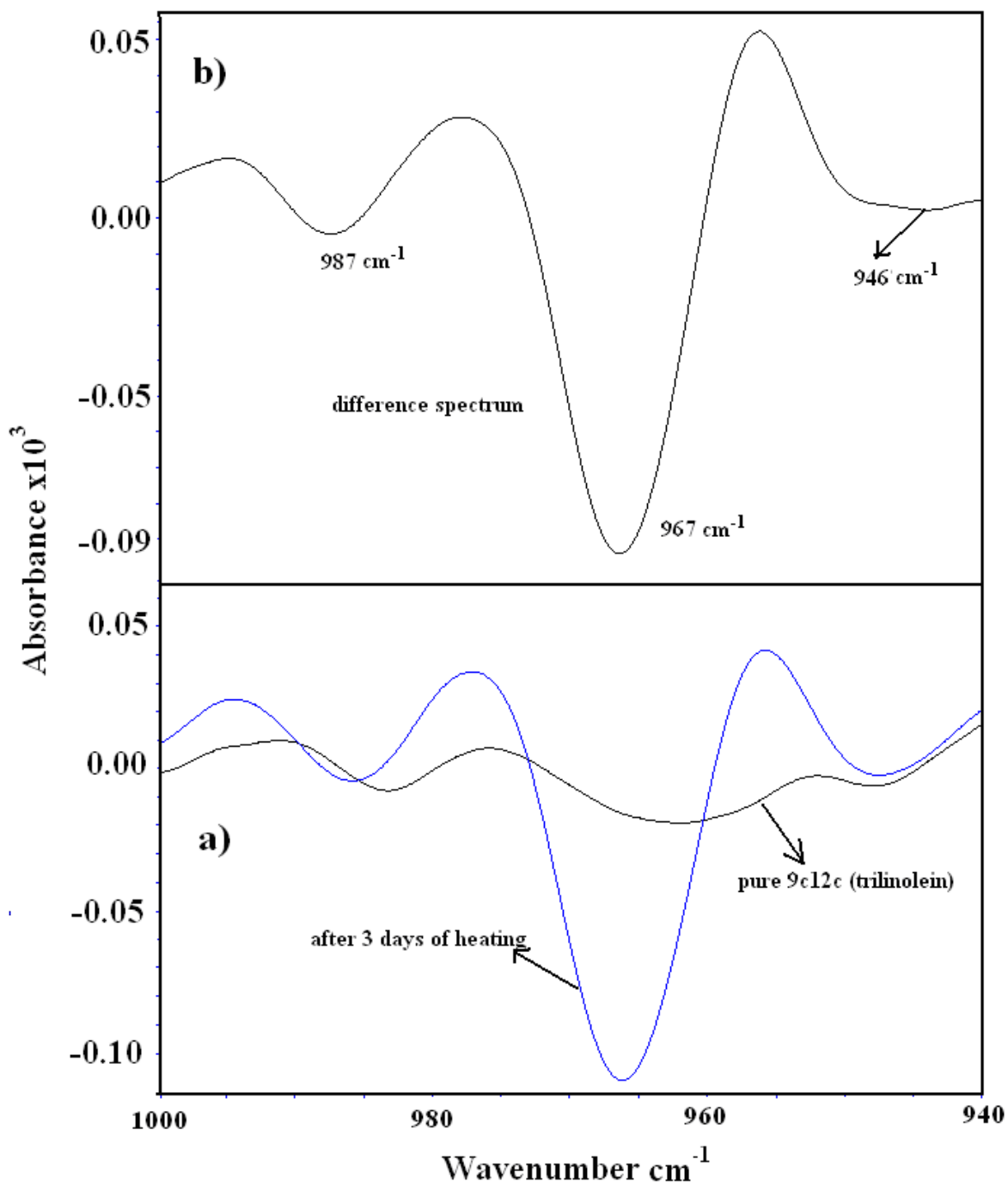
Fig. 2. a) The second derivated infrared profiles of pure trilinolein and a heat treated sample in the region 1015-915 cm^{-1}. b) The difference spectrum showing the absorptions arising from the formation of CLAs in the heated sample.
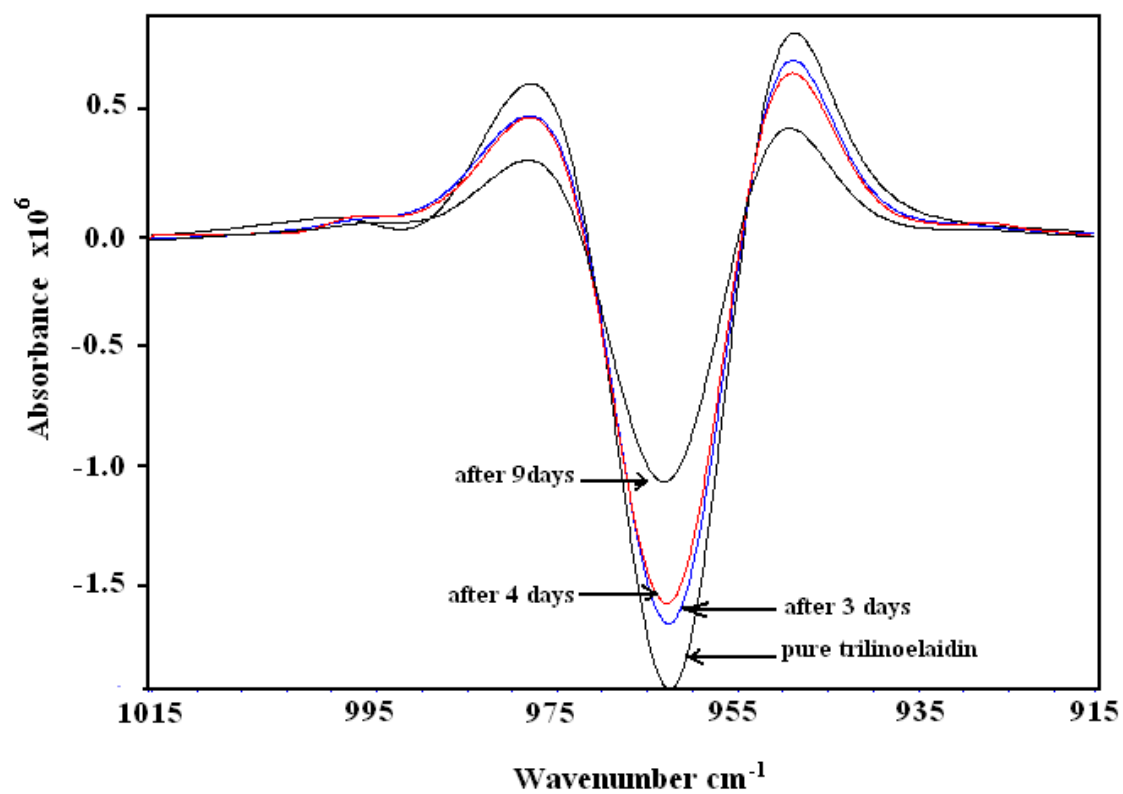
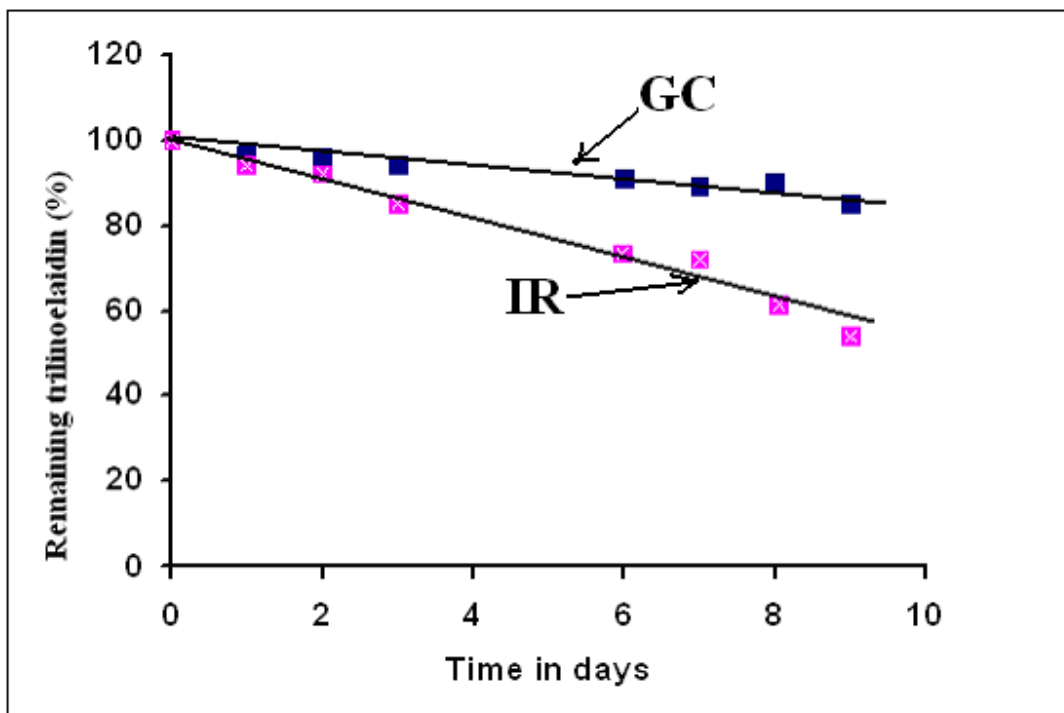Fig. 3. The second derivated infrared profiles of the heat treated samples in the region 1015-915 cm^{-1}.

Fig. 4. The concentration profiles of trilinoelaidin in the heated samples as determined by infrared spectroscopy and gas chromatography.
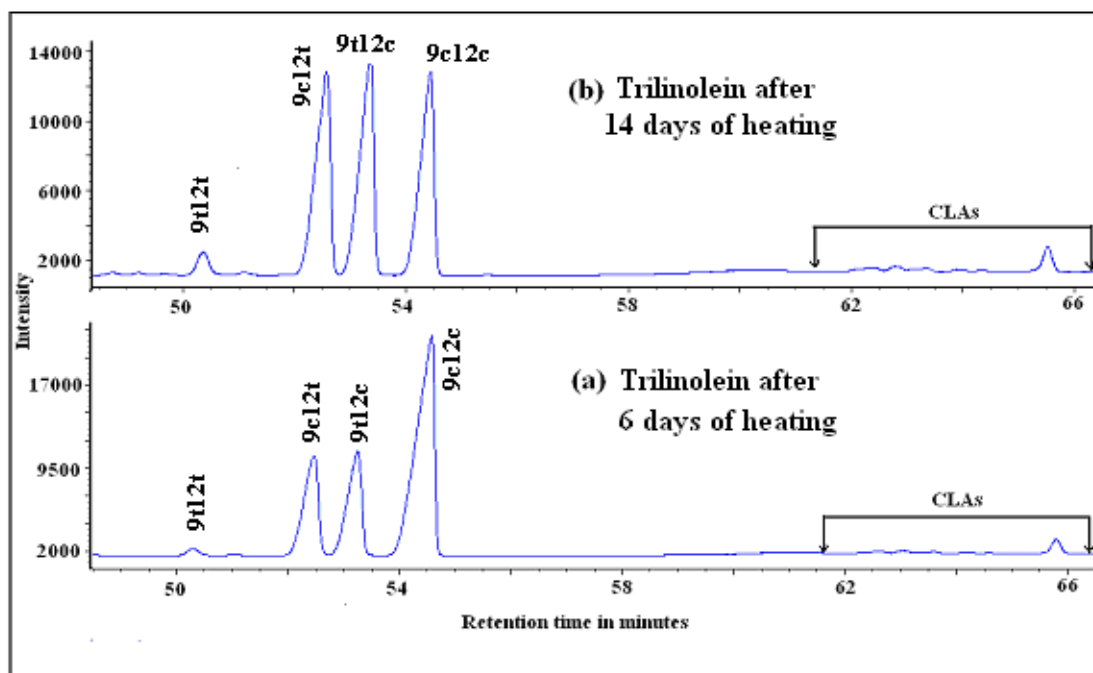
Fig. 5.Gas chromatogram of FAMEs prepared from heat treated trilinolein samples
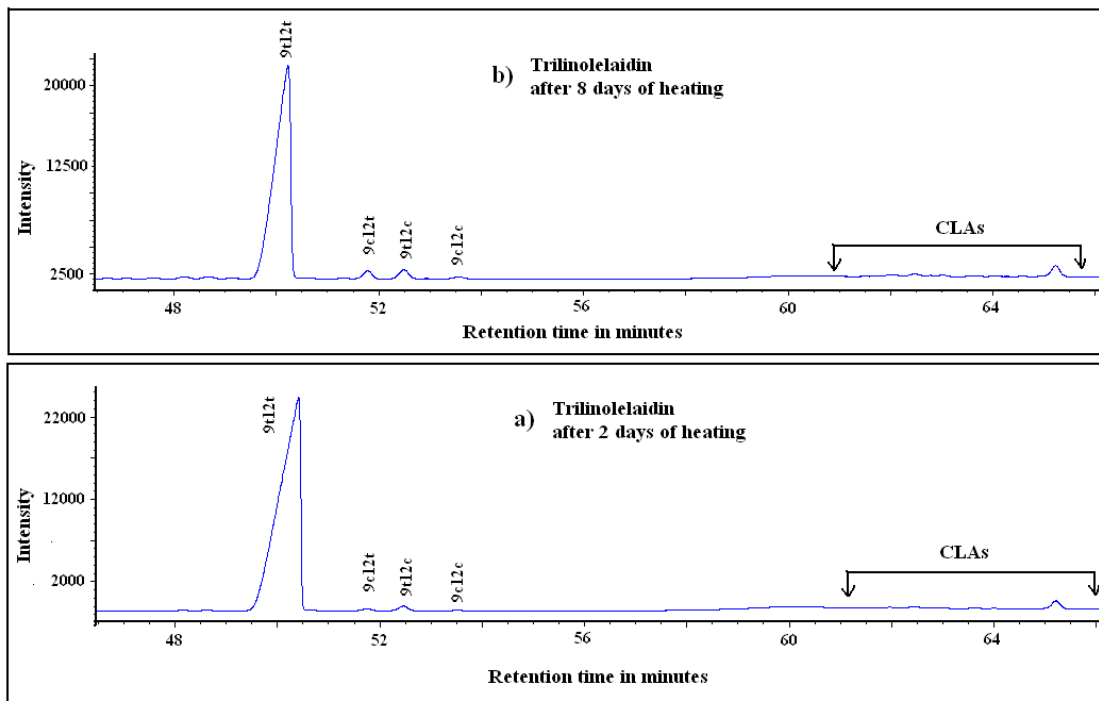
Fig. 6.Gas chromatogram of FAMEs prepared from heat treated trilinoelaidin samples
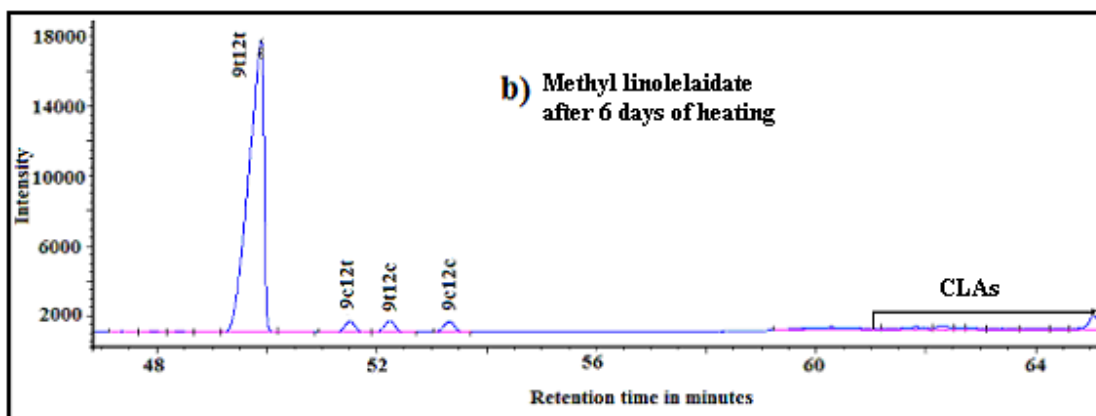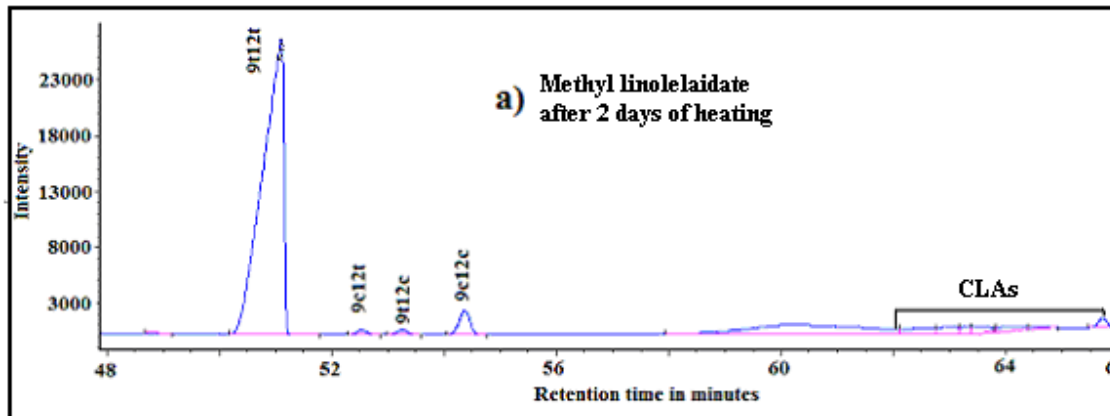
Fig. 7.Gas chromatogram of the samples prepared from heat treated methyl linolelaidate; 9t12t FAME.
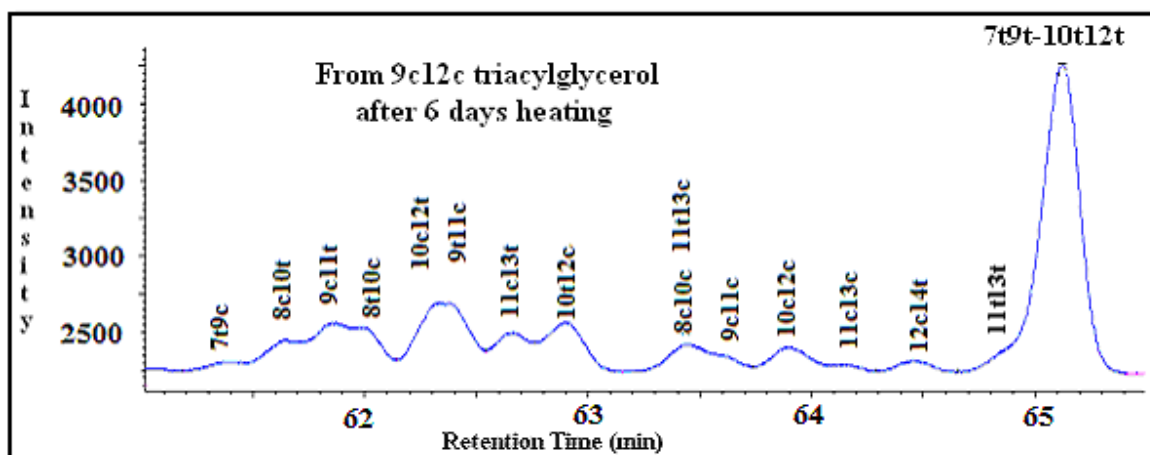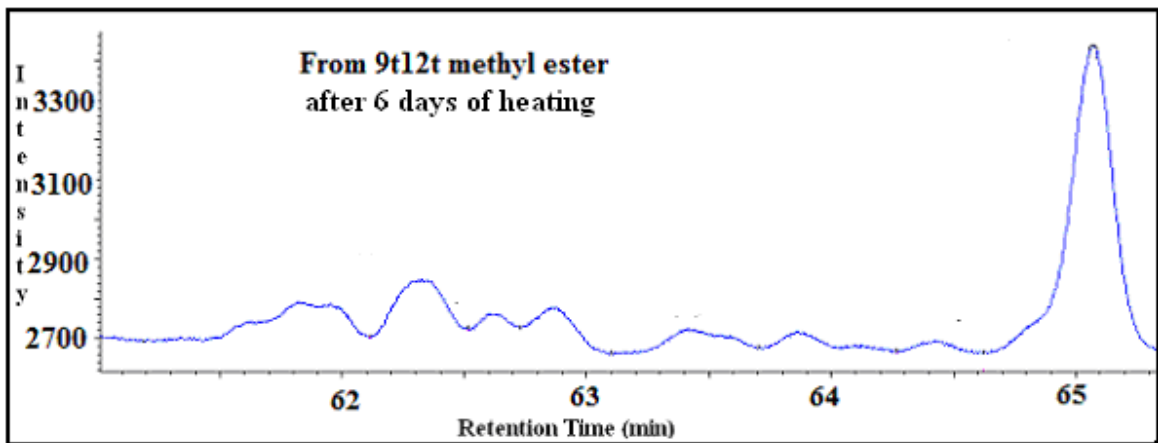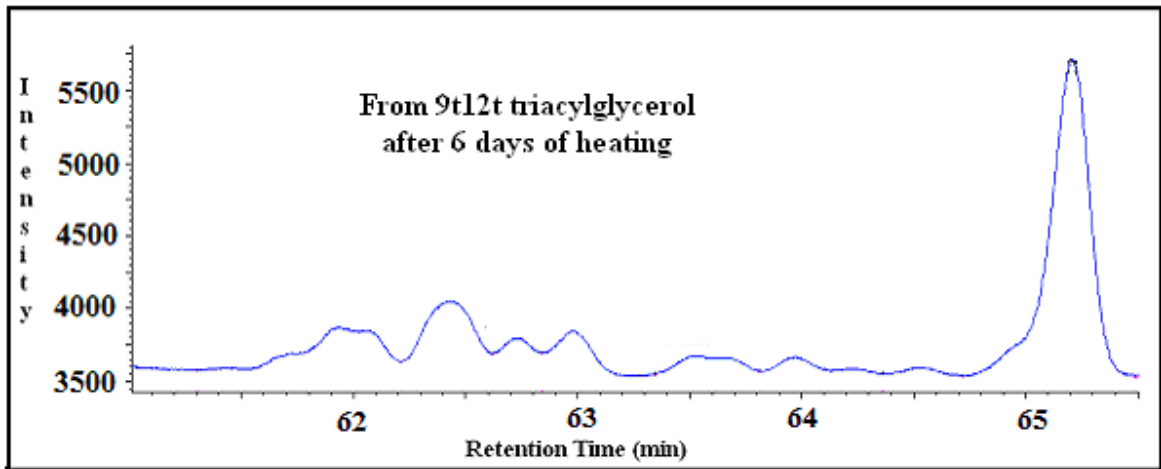
Fig. 8.Partial gas chromatogram of FAMEs of CLAs in the heat treated samples. The elution order of CLAs from Eulitz et al, 1999.
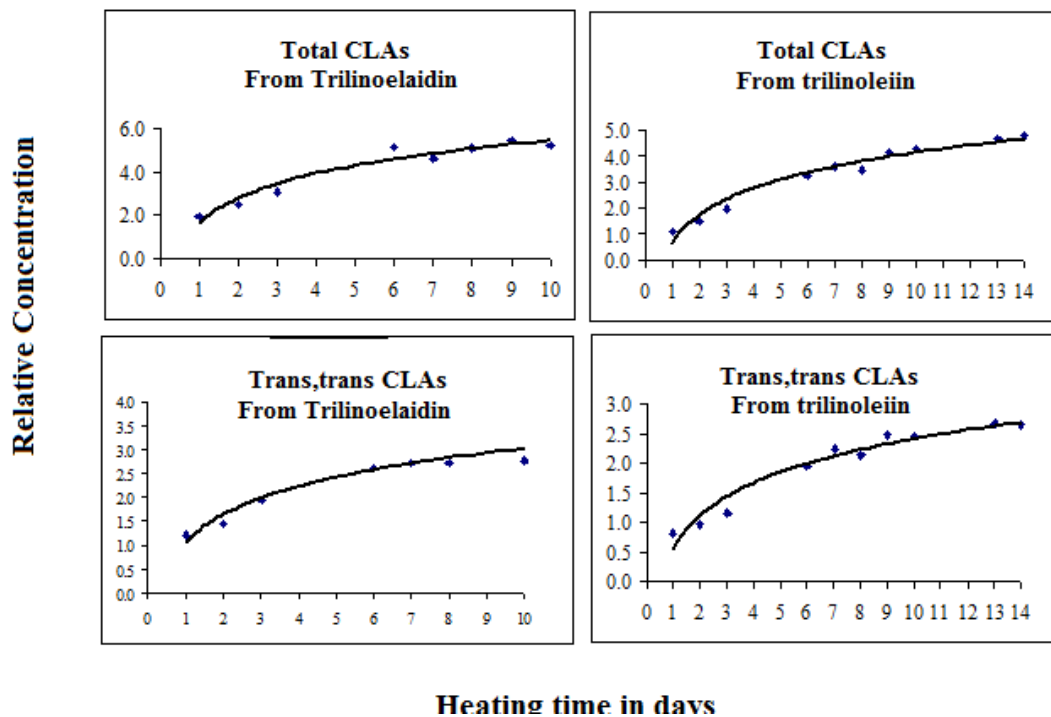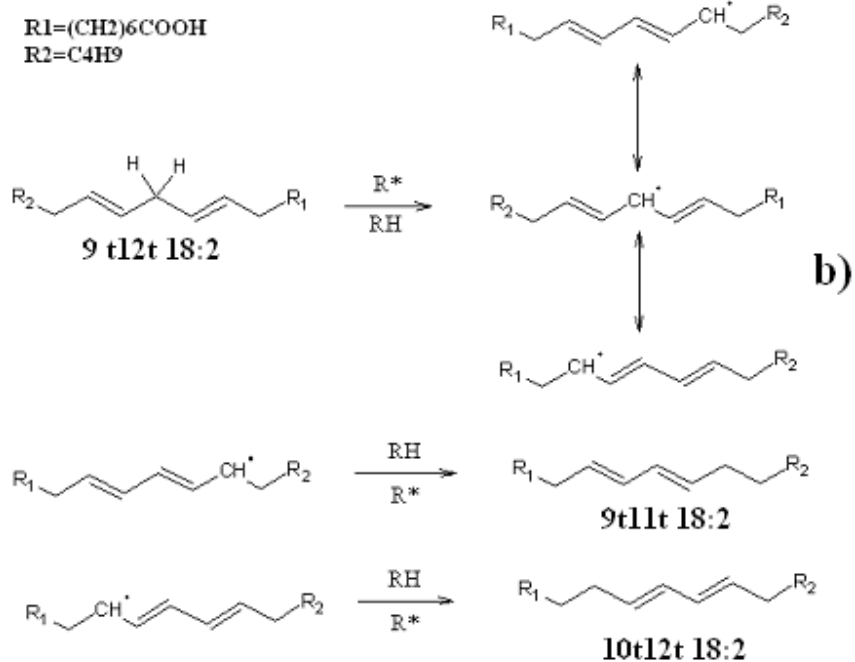
Fig. 9. Plots showing the concentration evolution of the total CLAs and *trans,trans* CLAs in the heated samples.
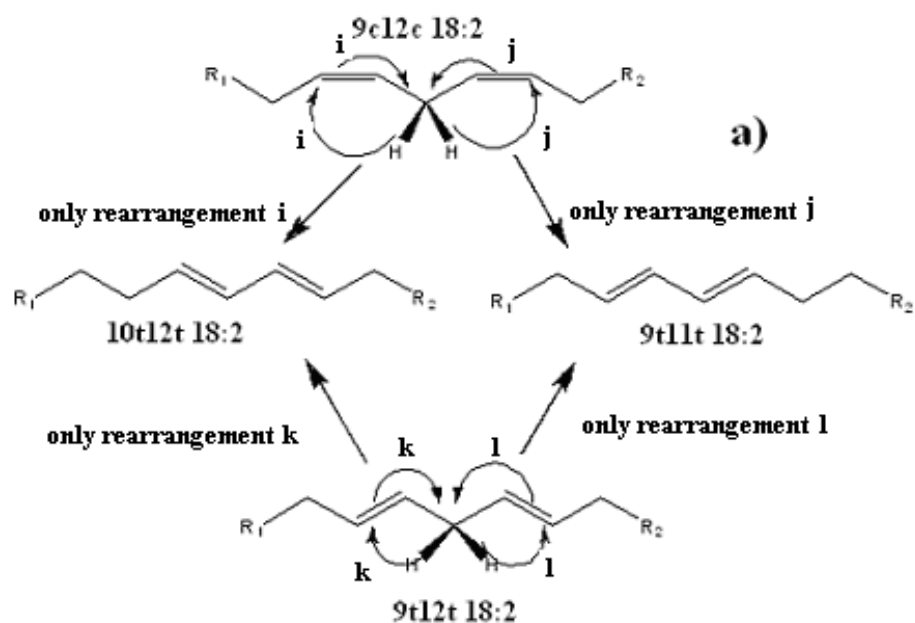
Fig. 10. Figures showing the radical and [1,3] sigmatropic rearrangement mechanisms leading to the formation of 10t12t and 9t11t CLAs from 9c12c and 9t12t LAs during thermally induced isomerisation. These rearrangements are theoretical and of no practical use for synthesising CLA isomers. The conversion is only about 5% from 9t12t to a mixture of CLA isomers.
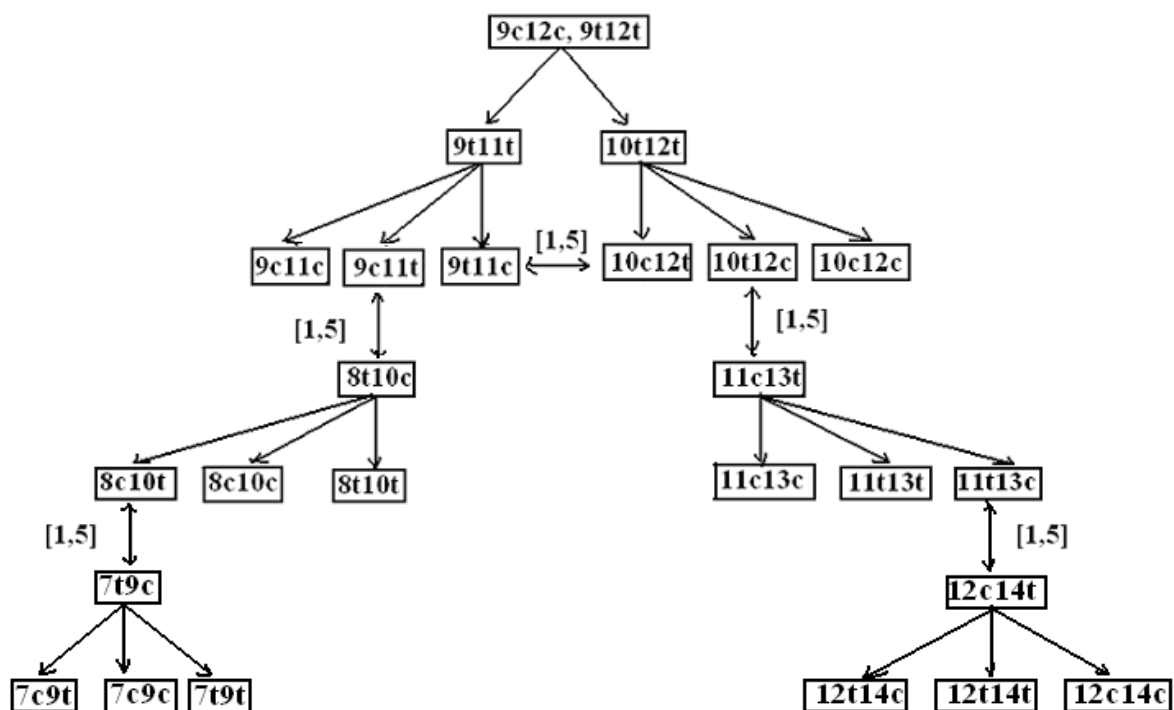
Fig. 11. An isomerisation tree showing the formation of CLA isomers in the heated samples.