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Original article

Differential scanning calorimetry evaluation of oxidation stability of docosahexaenoic acid in microalgae cells and their extracts

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Summary The aim of this work was to evaluate the oxidative stability of docosahexaenoic acid (DHA) from *Aurantiochytrium limacinum* SR21 microalgae cells and in their lipidic extract by differential scanning calorimetry (DSC). Besides, freezing was evaluated as a strategy for microalgal DHA long-term conservation by analysing changes in their thermal properties. As a first approach, mixtures of the most representative *A. limacinum* SR21-fatty acids were evaluated in model systems. DHA and palmitic acid were the major polyunsaturated and saturated fatty acids produced by the microalgae cells, respectively. Changes in DHA/palmitic acid ratio in model systems, in cells and their lipidic extracts, were detected by DSC through shifts in the oxidation onset temperature (OOT) values. However, OOT values of cells and lipidic extracts could be also influenced by cellular compartmentalisation, carotenoids and other components presence. Freezing was not a good strategy for DHA long-term conservation, as revealed by OOT values and thermal properties, which reflected the extensive changes that occurred during storage.

Keywords Carotenoids, DHA, differential scanning calorimetry, freezing, microalgae.

Introduction

Docosahexaenoic acid (22:6n-3) (DHA) is an essential long-chain polyunsaturated fatty acid (PUFA) that belongs to the omega-3 group. Human health benefits of PUFAs intake were recently reviewed by Gogus & Smith (2010). Several studies indicate promising anti-hypertensive, anticancer, antioxidant, antidepression, antiaging and antiarthritis effects (Siriwardhana *et al.*, 2012). The traditional main sources of omega-3 fatty acids have been fatty fish, but they have some disadvantages such as variable quality, contamination by environmental pollution (including dioxines, PCBs, synthetic estrogens) (Genius, 2008), unpleasant smell and taste and expensive purification (Carlson & Wilson, 1994; Gunstone, 2001). The opportunities to increase seafood harvest are limited; therefore, new alternative sources are required (Nichols *et al.*, 2010). Emerging sources include microbial oils (single cell oils), which offer interesting advantages: possibility to obtain oils with high and specific PUFAs content with higher oxidative stability, production from sustainable raw materials, lower purification costs, the absence of environmental man-made pollutants (Sijtsma & de Swaaf, 2004) and a more constant product quality (Mendes *et al.*, 2009). Among the oleaginous micro-organisms, thraustochytrids – common marine micro heterotrophs – can be cultured even at a large scale to produce high biomass, containing substantial amounts of lipids rich in PUFAs (Lewis *et al.*, 1999). The high DHA yields obtained with the thraustochytrid *Aurantiochytrium* (=*Schizochytrium*) (Yokoyama & Honda, 2007) resulted in a production of low cost oil (Raghukumar, 2008).

Biochemical composition and thus the nutritional value of lipids might be altered by freezing, drying and storage (Esquivel *et al.*, 1993; Babarro *et al.*, 2001; Ryckebosch *et al.*, 2011). The long-term conservation of intracellular microalgal lipids could be achieved by freezing at −20 °C (Babarro *et al.*, 2001) or spray- or freeze-drying (Ryckebosch *et al.*, 2011); in both cases, the amount of lipolysis decreased, probably by

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decreasing the amount of free water and due to a lower enzymatic kinetics at low temperature (Ryckebosch *et al.*, 2011). Lipid oxidation is one of the chemical changes in foods that leads to nutrient losses due to the alteration of essential PUFAs and to the reaction of oxidised lipids with proteins (Varela *et al.*, 1995), besides flavour deterioration, often resulting in food spoilage. These changes can occur regardless of the oil source, but are more pronounced and faster in rich polyunsaturated sources (Thurgood *et al.*, 2007). Therefore, oxidative stability is one of the most important indicators for maintaining the quality of edible oils (Tan *et al.*, 2002). A biological strategy adopted by microalgal cells to increase PUFAs stability against oxidation is the synthesis of carotenoids such as β-carotene, astaxanthin and canthaxanthin (Aki *et al.*, 2003; Burja *et al.*, 2006). The large-scale production of biomass, containing substantial amounts of lipids rich in PUFAs, requires practical and reliable methods to evaluate PUFAs oxidative stability. Many of the studies on the oxidative stability of omega-3 PUFAs are based on methods that rely on different fundamentals (such as the thiobarbituric acid reactive substances test (TBARs), weight gain, oxygen absorption and fatty acid analysis by gas chromatography), being some of them unspecific (Frankel *et al.*, 2002) and the results difficult to compare. Thermoanalytical methods, such as differential scanning calorimetry (DSC), are reliable for monitoring lipid oxidation because autoxidation of fats, fatty acids and lipids are attended by heat transport (Litwinienko *et al.*, 2000). DSC has been shown to provide a convenient alternative to determine the oxidative stability of various edible oils (Litwinienko & Kasprzycka-Guttman, 2000; Litwinienko *et al.*, 2000; Tan *et al.*, 2002; Thurgood *et al.*, 2007; Hammer, 2008; Chiavaro *et al.*, 2010b; Maggio *et al.*, 2012), revealing a high potential as a nonchemical and rapid method to determine oil quality parameters (Tan & Che Man, 1999a), also related to chemical composition (Tan & Che Man, 1999b; Chiavaro *et al.*, 2010a). Several authors correlated thermal properties obtained by DSC with known oxidation tests methods in various edible oils during isothermal (Kowalski *et al.*, 2004 – with Rancimat-; Tan *et al.*, 2002 – with OSI-) or nonisothermal oxidations (Torrecilla *et al.*, 2011; – with density and/or refractive index-). Also, changes in DSC thermal properties were found to be strictly correlated to those of standard oxidative stability indexes (peroxide and anisidine values, free fatty acids, etc.) (Chiavaro *et al.*, 2010b). However, until now, there are no reports on the use of DSC to study production and stability of DHA from microalgae.

The aim of this work was to evaluate the oxidative stability of DHA from *A. limacinum* SR21 microalgae cells in whole cells or in their lipidic extract by DSC. The obtained results were further compared with those obtained in models systems. Freezing was evaluated as a strategy for microalgal DHA long-term conservation.

Materials and methods

Materials

Micro-organism and culture

Aurantiochytrium limacinum SR21 used in this study was provided by the Institute of Fermentation of Osaka (Japan, strain number IFO 32693). The micro-organism was maintained in GPY (glucose-peptone-yeast extract) medium (Honda *et al.*, 1998) at −70 °C with the addition of glycerol 10% v/v. Two media formulated by Rosa *et al.* (2010) to enhance growth or lipid accumulation were used: a) growing medium (GM) with a C:N ratio of 6:1 (provided mainly by 2% w/v glucose and 1% w/v polypeptone); b) production medium (PM) with a C:N ratio of 55:1 (provided by 10% w/v glucose and 0.4% w/v NH_4CO-OCH_3) to obtain high PUFAs (mainly DHA) concentrations. Cultures were carried out in 100 mL of GM or PM in 500 mL Erlenmeyer flasks at 28 °C in an orbital shaker at 200 r.p.m. for 4 days, obtaining the so-called GM and PM cells, respectively. The inoculum (10% v/v) for both cell cultures was previously grown in GM for 4 days (Rosa *et al.*, 2010) in the same conditions of temperature and agitation described before. After harvesting (by centrifugation at 8000 ***g*** for 15 min), cells were washed twice with distilled water (fresh samples).

Lipid extract

Lipids extracts were obtained from GM and PM washed cells using a mixture chloroform:methanol (2:1) and 0.1 N KCl, as described by Kates (1998). The extracts were dried under nitrogen flux and immediately analysed by DSC.

Model systems

Docosahexaenoic acid:palmitic acid mixtures for oxidation onset temperature determination

Docosahexaenoic (C22:6, DHA) and palmitic acid (C16:0) standards were from Sigma–Aldrich, St. Louis, MO, USA. Different DHA:palmitic acid mixtures (75:25, 45:55 and 25:75 mass ratio) were prepared as model systems to evaluate their oxidative stability by DSC.

β-Carotene and oil mixtures for analyse the influence of carotenoids in oxidation onset temperature values

Ten parts per million of β-carotene (Warner Jenkinson Europe Ltd, Norfolk, UK) in n-hexane (Merck KGaA, Darmstadt, Germany; PROD.NER.109687) was added to pure DHA or commercial rose hip oil

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(Parafarm, Droguería Saporiti S.A.C.I.F.I.A, Buenos Aires, Argentina). The amount of β-carotene was selected based on its content in PM cells (see section Thermal stability of microbial DHA evaluated by DSC). Rose hip oil was selected as a complex oil system presenting between 75%–80% of polyunsaturated, 15%–20% of monounsaturated and around 5% of saturated fatty acids (Szentmihályia *et al.*, 2002).

Stability of frozen microalgal cells

Fresh samples were stored in a conventional freezer at −20 °C for 1, 8.5 and 30 months for short-, intermediate- and long-term conservation, respectively.

Oxidation onset temperature determination by differential scanning calorimetry

The oxidation onset temperature (OOT) was determined by differential scanning calorimetry (DSC) by means of a Mettler Toledo 822 equipment (Mettler Toledo AG, Switzerland) and STARe Thermal Analysis System version 3.1 software (Mettler Toledo AG). Temperature and melting enthalpy calibrations were performed using standard compounds of defined melting point (156.6 and 419.7 °C for indium and zinc, respectively) and heat of melting (28.45 J g^{-1} for indium). All measurements were taken at least in duplicate with 2–10 mg sample mass, using perforated aluminium pans of 40 μL inner volume (Mettler Toledo AG); an empty pan covered with a holed lid was used as a reference. The OOT was determined by heating the samples in oxidative conditions (with air flux) from 20 °C to 340 °C at 5 °C/min (standard method ASTM E2009) (ASTM, 2012). The onset was taken as the intersection of the baseline and the tangent to the oxidation peak. Prior to DSC determinations, both fresh and frozen-stored microalgal cells were dehydrated by freeze-drying in a Heto Holten A/S, cooling trap model CT 110 freeze-dryer (Heto Lab Equipment, Allerød, Denmark), to improve the intensity of the signal. It was reported that this method maintained microalgal lipid composition (Babarro *et al.*, 2001; Ryckebosch *et al.*, 2011). The chloroform extracts were dried with a nitrogen flux and were immediately analysed by DSC.

Determination of free fatty acids (FFA) content by gas chromatography

Cell samples were prepared as described in AOCS Official Method Ce 2-66/Ch 2-91 (AOCS, 1997) and subjected to gas chromatography/flame ionisation detector (GC/FID) (HP 6890; Hewlett-Packard Co., Palo Alto, CA, USA) equipped with a chrompak CP WAX de 50 m × 0.32 × 0.5 (Agilent Technologies Inc., Santa Clara CA, USA).

Determination of carotenoids content

The extraction of carotenoids was performed by adding 2 mL of hexane to 5 mL of cell suspension, followed by sonication (10 min at 40 kHz). The absorbance spectrum of the supernatant was determined in a UV-Vis spectrophotometer Jasco model V630 (Jasco Corporation Tokyo, Japan). The typical overall shape of the visible spectra obtained for the carotenoids extracted from cell suspension showed the three distinct peaks between 400 and 500 nm reported for carotenoids (Britton, 1995). The peak at 450 nm was selected for quantification. As β-carotene is one of the main carotenoids reported in *Aurantiochytrium* species (Yokoyama & Honda, 2007), carotenoids content was expressed as β-carotene, using its extinction coefficient in hexane ($A^{1\%1\ cm}$ = 2760 for β,Ψ-carotene (Britton, 1995)). Average results were presented in ppm (μg/g cell dry weight) from duplicate measurements.

Cell dry weight determination

Biomass production was estimated from cell dry weight (CDW) determination. Samples were washed with distilled water and dried at 90 °C for 24 h.

Statistic and fitting

Means of fatty acid content of GC determinations, unsaturated/saturated fatty acid ratio and carotenoids content were compared by a *t*-test at a 95% confidence level. Differences among means of OOT values were statistically evaluated by a multiple comparison procedure (Tukey's method) after one-way analysis of variance at a 95% confidence level ($P < 0.05$). All statistical analyses were performed using Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

A nonlinear regression was conducted between OOT data vs. composition (expressed as DHA:palmitic acid ratio), using a one-phase exponential decay equation.

All experiments described herein were repeated at least twice.

Results and discussion

Gas chromatography analysis of the fatty acids produced by *A. limacinum* SR21

DHA microalgal production is influenced by the growth phase, which depends on the C:N balance in the culture medium (Anderson & Wynn, 2001). To obtain cells with different DHA contents, cultures were grown in media with different C:N ratio (GM and PM). Table 1 shows the fatty acids content determined by GC for the lipidic extracts obtained both for PM and GM cells. As can be seen, DHA and palmitic acid

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Table 1 Fatty acid composition (as% of total fatty acids) determined by GC for the GM and PM lipid extracts. Standard deviation values are included.

Fatty acids	GM	PM	Significant differences[A]
C14:0 Myristic	2.1 ± 0.4	2.5 ± 0.4	ns
C15:0 Pentadecanoic	12.0 ± 0.2	2.1 ± 0.4	***
C16:0 Palmitic	42 ± 2	47 ± 3	ns
C17:0 Margaric	4.3 ± 0.4	1.0 ± 0.2	**
C18:0 Stearic	1.3 ± 0.3	2.3 ± 0.4	ns
20:5n3 Eicosapentaenoic (EPA)	0.62 ± 0.02	0.6 ± 0.1	ns
C22:5n3 Docosapentaenoic (DPA)	0.4 ± 0.2	0.4 ± 0.1	ns
22:6n3 Docosahexaenoic (DHA)	31 ± 2	42 ± 2	***
Other	2.5	1.6	
Saturated (S)	62 ± 4	55 ± 3	**
Monounsaturated (M)	1.2 ± 0.2	0.8 ± 0.2	ns
Polyunsaturated (P) - Total omega-3	32 ± 2	43 ± 2	***
Total fatty acids identified	98 ± 2	98 ± 2	ns

[A]A *t*-test was carried out for each row to check whether means were significantly different. $P < 0.001$ (***); $P < 0.01$ (**); $0.05 < P$ (not significant -ns-).

were the major polyunsaturated and saturated fatty acids, respectively, of *A. limacinum*, as previously observed (Fan *et al*., 2009; Taoka *et al*., 2009) and together represent between 73% and 89% of total fatty acids. Nitrogen limitations in cells grown in PM media led to a higher content of unsaturated fatty acids (mainly DHA) as expected (Rosa *et al*., 2010). The concentration of DHA standardised by total fatty acids increased 22% in the PM cells extract with respect to its concentration in GM extract. On the other hand, GM cells showed an increase of 15% of saturated fatty acids with respect to PM cells.

Oxidative stability of mixtures of the major microalgae fatty acids

Mixtures of the most representative fatty acids determined in PM cells (DHA and palmitic acid) were evaluated in a model system as a first approach to explore the feasibility of DSC to show how the relative concentrations of the fatty acids (different saturated/unsaturated ratios) affect the oxidation temperature of the systems. Although the real situation found in PM cells corresponded closely to the 45:55 DHA:palmitic acid ratio, the thermal oxidation of whole range DHA: palmitic acid ratio was analysed to confirm the adequate trend of the oxidation pattern. Thus, thermal oxidation was analysed for pure DHA and palmitic acid and for three DHA:palmitic acid mixtures: 25:75, 45:55 and 75:25 (Fig. 1a). The initial point of the exothermal shift of the oxidation peak was taken as the oxidation onset temperature (OOT) value. The OOT values of the pure fatty acids were well separated being 81 °C for DHA and 175 °C for the saturated fatty acid. A higher amount of palmitic acid concentration in the mixture conducted to an increase in the OOT value, following an exponential behaviour (Fig. 1b). These results indicated that changes in the relative amount of DHA/palmitic acid mixtures could be detected by DSC through shifts in the OOT values.

Thermal stability of microbial docosahexaenoic acid evaluated by differential scanning calorimetry

The stability to thermal oxidation of microalgal oils was analysed determining the OOT values. Figure 2 shows the thermograms obtained for GM and PM whole cells and for the lipidic extract of PM cells. The OOT value of the PM lipidic extract was more than 50 °C lower than the OOT for the PM cells. According to Table 1, PM lipidic extract contained a DHA: palmitic ratio of 47:53. However, the OOT value of this sample (118 °C) corresponds to a lower DHA content in the mixture (DHA:palmitic ratio of 30:70) (Fig. 1b). In the case of the whole cells, the presence of natural antioxidants, especially carotenoids (Aki *et al*., 2003; Burja *et al*., 2006), could play a role and modified OOT values, although cellular compartmentalisation as well as other cellular regulatory mechanisms could not be discarded. Table 2 shows the unsaturated/saturated fatty acids ratio, carotenoids content and OOT values determined for PM and GM cells and for their corresponding lipidic extract. The unsaturated/saturated ratio and the carotenoids content were significantly higher (50% and 70%, respectively) in the PM than in the GM cells (see Table 2). Then, it could be expected that the OOT of the PM cells would be much lower than those of the GM cells due to their higher proportion of unsaturated fatty acids. However, no significant differences were found between OOT values of PM and GM cells. This result could not be explained only by differences in DHA: palmitic ratio, but also by the presence of higher amount of carotenoids.

Effect of carotenoids in model systems

The effect of carotenoids was tested in model systems of DHA and of rose hip oil (which contains high levels

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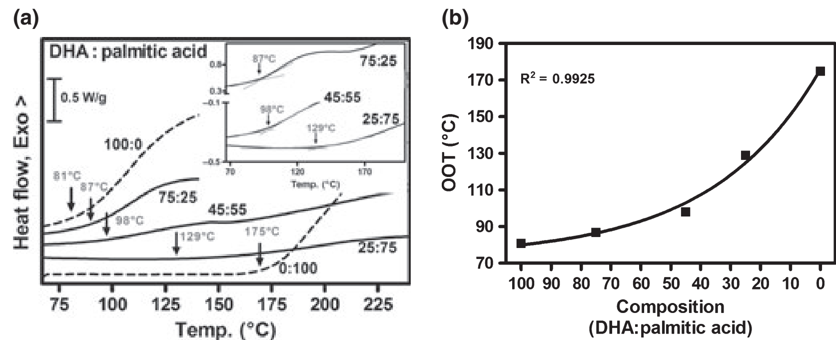

Figure 1 (a) DSC thermograms of DHA, palmitic acid and their mixtures (in mass ratio percentage). Arrows indicate oxidation onset temperature (OOT) values. The inserted graph shows a zoom of selected thermograms and their corresponding OOT evaluation. (b) Nonlinear regression showing the exponential behaviour of OOT values as a function of DHA:palmitic acid ratio.

Table 2 Unsaturated/saturated fatty acid ratio, carotenoids content and OOT values for PM and GM fresh cells and their corresponding lipidic extract. Standard deviation values are included.

	Unsaturated/ saturated[A]	Carotenoids content (ppm, µg/g cell dry weight)[A]	OOT (°C)[B]
PM cells	0.78 ± 0.04*	9.5 ± 0.8*	171 ± 1[b]
PM extract		-	118 ± 2[c]
GM cells	0.52 ± 0.04*	5 ± 1*	176 ± 8[ab]
GM extract		-	200 ± 7[a]

[A]A *t*-test was carried out for each column to check whether means were significantly different; 0.01 < P < 0.05 (*).
[B]Significant differences on means analysed by a multiple comparison procedure (Tukey's method) after one-way analysis of variance are indicated with different letters (a–c; P < 0.05).

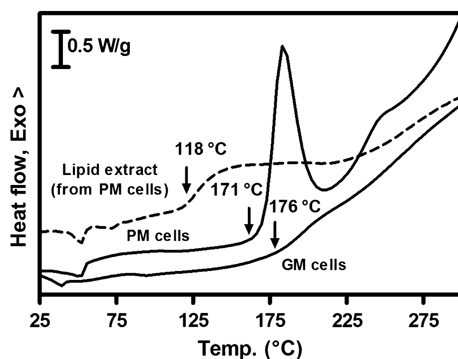

Figure 2 Oxidation onset temperature (OOT) determined by DSC on GM and PM cells and its lipidic extract. Arrows indicate OOT.

of PUFAs), supplemented with 10 ppm of β-carotene (see Table 2). The OOT value of DHA and rose hip oil supplemented samples shifted upward around 6 and 9 °C, with respect to the correspondent pure oils (data not shown). As stated before, further differences between OOT of GM and PM cells could then be attributed to the complex environment inside the cell, mainly cell compartmentalisation that protects

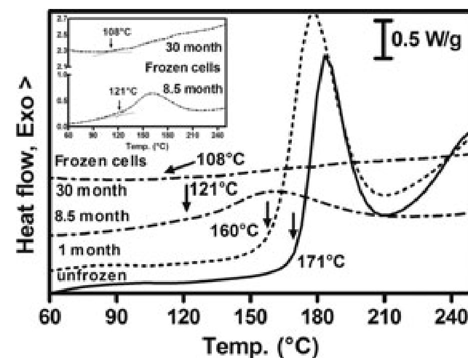

Figure 3 Oxidation onset temperature (OOT) determined by DSC for PM cells exposed to different freezing times at −20 °C. Arrows indicate OOT. The inserted graph shows a zoom of selected thermograms and their corresponding OOT evaluation.

sensitive molecules from the action of different enzymes acting on them (e.g. oxidases, hydrolases, etc.) and other different oxidative mechanism.

Oxidation onset temperature changes produced by frozen storage

Freezing was assessed as a strategy for long-term conservation of microalgal cells with high production of DHA. Figure 3 shows the thermograms obtained for PM fresh cells (unfrozen) and for PM cells frozen for 1, 8.5 and 30 months at −20 °C. These times allowed analysing short-, intermediate- and long-term conservation. As can be seen, the OOT values were lower for the frozen cells compared with the fresh ones and decreased as a function of frozen storage time. The shape of the thermogram obtained for fresh and 1 month frozen cells was similar. Babarro *et al.* (2001) observed that the lipid composition (including DHA) of another microalgal species remains unchanged in frozen cells after 3 months of storage at −76 °C. Moreover, Guevara *et al.* (2011) reported that freezing at -20 °C for 1 month was one of the most appropriate

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methods to maintain PUFAs (EPA and DHA) content of fresh microalgae to be used as feed for aquaculture. Thus, the slight decrease in the OOT values observed for the 1 month frozen cells could be probably related to the degradation of antioxidant compounds which protect lipids from oxidation (Ryckebosch *et al.*, 2011). After the longest storage (30 months at −20 °C), DHA content in PM cells decreased from 0.15 (fresh cells) to 0.06 g/g CDW. Therefore, the change in the shape of the thermograms of 8.5 and 30 month frozen cells in comparison to the fresh and 1 month frozen cells could be related to the decrease in DHA content and degradation of carotenoids and other protective compounds. Then, according to our results, freezing is not a good strategy for intermediate- and long-term conservation of microalgal DHA because the dramatic changes in the thermal properties after 8.5 months at −20 °C reflect the extensive changes which occurred during that time. The presence of some excipients such as biopolymers, an increment in the amount of antioxidants and microalgal drying, could be a more convenient strategies to achieve long-term conservation of microalgal DHA.

The decrease of the OOT with increasing storage time at −20 ° indicates that oxidant precursors had been formed under frozen conditions, which also cause antioxidants depletion (Passi *et al.*, 2005). The enhancement of oxidative reactions in frozen systems has been attributed to the increased oxygen solubility at low temperatures, to the reactants concentration in the unfrozen phase and to the catalytic effect of the ice surface (Nip, 2007).

Conclusions

- Changes in DHA/palmitic acid ratio in model systems were easily detected by DSC through shifts in the OOT values.
- Oxidation onset temperature values in microalgae cells were influenced not only by the unsaturated/saturated ratio, but also by other factors such as the presence of antioxidants like carotenoids, that prevent DHA from oxidation, compartmentalisation and metabolic status.
- Fatty acids stability in frozen cells could be evaluated by analysing the shift of OOT values. Freezing at -20°C was not a good strategy for intermediate- and long-term conservation of microalgal DHA because the dramatic changes in the thermal properties reflect the extensive changes which occurred during storage.
- Oxidation onset temperature isolated data obtained by the proposed methodology could be meaningless for analysing stability of complex systems. However, it should be taken into account that its applicability would be valid to study fatty acid stability in samples of the same composition during different processes.

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