



HAL
open science

Chemical Synthesis and Isolation of trans-Palmitoleic Acid (trans -C16:1 n-7) Suitable for Nutritional Studies

Étienne Guillocheau, Gaëtan Drouin, Daniel D. Catheline, Clement Orione,
Philippe P. Legrand, Vincent V. Rioux

► To cite this version:

Étienne Guillocheau, Gaëtan Drouin, Daniel D. Catheline, Clement Orione, Philippe P. Legrand, et al..
Chemical Synthesis and Isolation of trans-Palmitoleic Acid (trans -C16:1 n-7) Suitable for Nutritional
Studies. European Journal of Lipid Science and Technology, inPress, 10.1002/ejlt.201900409 . hal-
02572244

HAL Id: hal-02572244

<https://institut-agro-rennes-angers.hal.science/hal-02572244>

Submitted on 20 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Short communication

Chemical synthesis and isolation of *trans*-palmitoleic acid (*trans*-C16:1 n-7) suitable for nutritional studies.

Etienne Guillocheau^{1,2}, Gaëtan Drouin¹, Daniel Catheline¹, Clément Orione³, Philippe Legrand¹, Vincent Rioux^{1*}

¹Laboratory of Biochemistry and Human Nutrition, Agrocampus-Ouest – Rennes, France

²French Dairy Interbranch Organization (CNIEL), Technical and Scientific Department – Paris, France

³Centre Régional de Mesures Physiques de l'Ouest (CRMPO), Université de Rennes 1 – Rennes, France

***Correspondence:**

vincent.rioux@agrocampus-ouest.fr

Laboratory of Biochemistry and Human Nutrition, Agrocampus-Ouest

65, rue de Saint-Brieuc – 35042 Rennes Cedex (France)

RUNNING TITLE

Chemical synthesis of *trans*-palmitoleic acid.

KEYWORDS

Chemical synthesis; Nutritional studies; Purification; *Trans*-palmitoleic acid.

Received: 11 11, 2019; Revised: 03 18, 2020; Accepted: 04 24, 2020

ABBREVIATIONS

CPA: *cis*-palmitoleic acid

CVA: *cis*-vaccenic acid

FAEE: Fatty acid ethyl ester

GC-MS: Gas chromatography-mass spectrometry

LC: Liquid chromatography

NMR: Nuclear magnetic resonance

TPA: *trans*-palmitoleic acid

TVA: *trans*-vaccenic acid

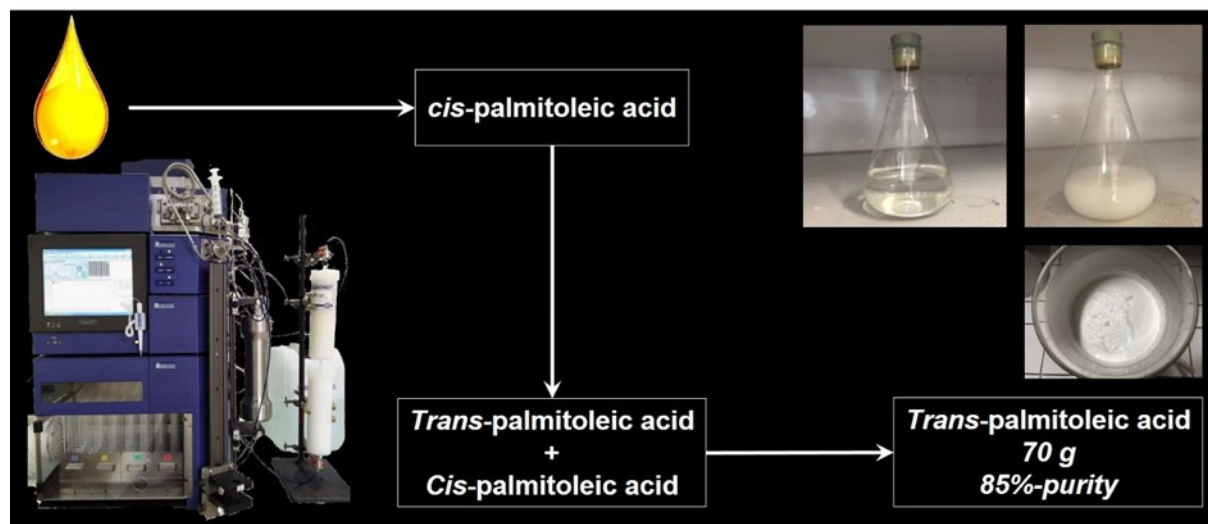
ABSTRACT

Trans-palmitoleic acid (*trans*-9 C16:1, or *trans*-C16:1 n-7, TPA) is typically found in ruminant-derived foods (milk and meat). Of note, previous epidemiological studies associated high levels of circulating TPA with a lower risk of type 2 diabetes and metabolic syndrome in humans. At the current time, TPA intakes in humans are ensured by ruminant-derived foods. However, due to the very low commercial availability of TPA, there are no supplementation studies carried out so far. Therefore, the ability for dietary TPA to prevent type 2 diabetes and metabolic syndrome has never been experimentally assessed. Here, we report a method (among others) to get dozens of grams of pure TPA as ethyl ester, to perform dedicated supplementation studies. For that purpose, we started from food sources containing high amounts of *cis*-palmitoleic acid (*cis*-9 C16:1, or *cis*-C16:1 n-7, CPA), dealing with fatty acids ethyl esters all along with the experiment. CPA was purified with flash LC, then submitted to a *cis/trans* isomerization step. Finally, TPA was separated from CPA by low-temperature crystallization in methanol. The final product was fully characterized by ¹H and ¹³C nuclear magnetic resonance spectrometry. We were able to produce approx. 70 g of 85%-purity TPA suitable for nutritional studies.

PRACTICAL APPLICATIONS

The synthesised *trans*-palmitoleic acid may serve for supplementation (or nutritional) studies aiming at unravelling its physiological impacts suggested by epidemiological work. Depending on the amount of synthesised *trans*-palmitoleic acid, one may carry out nutritional studies on rodents or even on humans.

GRAPHICAL ABSTRACT



A method (one among others) is described in this manuscript to get pure *trans*-palmitoleic acid ethyl ester in enough amounts to carry nutritional studies out. The dietary complement Provinal[®] was used as a source of *cis*-palmitoleic acid, in combination with flash LC. Isomerisation and large scale *cis/trans* fractionation were applied afterwards.

1. INTRODUCTION

Trans-palmitoleic acid (*trans*-9 C16:1, or *trans*-C16:1 n-7, TPA) belongs to the family of natural *trans* fatty acids, as reviewed by our research group [1]. Since TPA arises from ruminal biohydrogenation, its presence has been recently reported in ruminant-derived foods by our research group: for instance, TPA accounts for 0.04% of total fatty acids in dairy fat [2]. Interestingly, many epidemiological studies associated high circulating levels of TPA in humans with a lower risk of type 2 diabetes and metabolic syndrome [3–10]. Undoubtedly, these epidemiological findings had considerable impact, making TPA an intriguing dietary fatty acid of high nutritional interest. However, supplementation studies are still lacking to unravel accurate mechanisms of action of dietary TPA. In fact, TPA is believed to prevent insulin-resistance by targeting insulin secretion and improving pancreatic β -cell function [8–10], but such a hypothesis remains to be confirmed by supplementation studies.

Getting pure TPA in enough amounts to perform nutritional studies is quite challenging, and many strategies are available. First, using dairy products as a raw material may be useful but TPA only accounts for 0.04% of total fatty acids in dairy fat [2–11–12]. Besides, dairy fat contains other natural *trans* fatty acids such as *trans*-vaccenic acid (*trans*-11 C18:1, or *trans*-C18:1 n-7, TVA) and rumenic acid (*cis*-9,*trans*-11 C18:2, RMA) for which benefits have been reported [13]. Thus, neither conventional dairy fat nor butter enriched in natural *trans* fatty acids [14] is convenient. Second, TPA was found in partially hydrogenated fish oils. Not only are these oils neither found nor consumed anymore [15], but the presence of other *trans*-C16:1 isomers does not meet the purity criterion [11]. Third, partially hydrogenated vegetable oils are suspected to contain TPA [16]. However, (1) the content is lower than that of dairy fat [17], (2) other *trans*-C16:1 isomers that would theoretically be here as well would bias the results, and (3) such oils cannot be found anymore due to the FDA ban [18]. Fourth, a recent clinical trial relied on sea buckthorn oil augmented in TPA (about 10% of fatty acids): such increase in TPA content was achieved by partial hydrogenation of sea buckthorn oil (isomerization of *cis*-palmitoleic acid, *cis*-9 C16:1, or *cis*-C16:1 n-7, CPA), but again the purity criterion is not met as other *trans* fatty acids were supplemented along with TPA despite minimizing the other positional isomers [19]. Fifth, one could rely on well-known chemical companies, but to the best of our knowledge, the commercially available amounts (several hundreds of mg) are not suitable for nutritional studies. Therefore, the only remaining solution lies in the chemical synthesis of TPA, which has never been described so far but still appears to be the most convenient one for high-quality studies.

Like TVA, TPA is a *trans* monounsaturated fatty acid. Fortunately, some nutritional studies involving chemically-synthesized TVA were carried out either on humans [20–21] or rodents [22–26]. Therefore, any methodology used for getting high amounts of pure TVA might be translated to TPA. To the best of our knowledge, two ways were used to obtain TVA. The first one consists of alkali isomerization of vegetable oils [22–26]. According to the second way, *cis*-vaccenic acid (*cis*-11 C18:1, or C18:1 n-7 *cis*) is chemically synthesized first, then a *cis/trans* isomerization and a *cis/trans* fractionation are carried out [20–21–27–31].

Therefore, taking advantage of the methods established for the chemical synthesis of TVA, our study aimed at translating these methods in the case of chemically synthesized TPA.

2. MATERIAL & METHODS

2.1. Dietary sources of CPA

We took advantage in this study of food sources that contain reliable amounts of CPA. Two sources of CPA were assessed here: macadamia nut oil, and the dietary complement Provinal[®] [32].

Macadamia nut oil is known to be a good source of CPA which accounts for approx. 15% of total fatty acids [33]. Besides, such oil is easily available [34]. As for Provinal[®], it is a CPA-enriched dietary complement, which consists of purified Peruvian anchovy oil. CPA accounts for more than 50% of total fatty acids. Such a dietary complement was designed since CPA is thought to have benefits on human health [35–36]. Like macadamia nut oil, Provinal[®] is easily available [37].

Fatty acids contained in Provinal[®] are directly supplied as fatty acid ethyl esters (FAEE). FAEE being a suitable derivative for both nutritional studies and analytic purposes, we chose to deal with such derivatives during the whole experiment. Consequently, lipids from macadamia nut oil were first extracted with hexane/isopropanol (3/2, v/v) [38], and fatty acids were obtained as FAEE by ethanolic HCl.

At this stage two main criteria were defined to assess the best dietary source: (1) the amount criterion (*i.e.*, the content of CPA in the dietary source) and (2) the purity criterion (*i.e.*, the relative amount of *cis* C16:1 isomers that will be isomerized during the following isomerization step).

2.2 Purification of CPA by flash LC

Flash LC was performed with the PuriFlash[®] 4250 system (Interchim[®], Montluçon, France) (**Supplementary Figure 1**). As for the columns, an association between two Flash cartridges was settled in this accurate order: (1) a CHROMABOND[®] Flash RS 330 C₁₈ ec column (27.0 cm length, 60,0 mm internal diameter, 40-63 μm mean particle size; ref. 732817; Macherey-Nagel, Düren, Germany), and (2) a PuriFlash[®] PF-15C18HP50-F0330 column (22.6 cm length; 60.0 mm internal diameter; 15 μm mean particle size; Interchim[®]). Conditions of elution were the following: an isocratic mode consisting of methanol/water (95/5), at a constant flow rate of 130 mL/min. Data were acquired with Interchim[®] soft V5.1c.06 for Windows.

Considering these operating conditions, approx. 20 mL of FAEE (either from Provinal[®], or macadamia nut oil) could be injected each time in the Flash LC device. Fractions of interest were collected, and the excess of methanol was removed by vacuum evaporation. As described in the “Results” section, we were able at this stage to assess the best dietary source of CPA.

2.3. *cis/trans* isomerization

After choosing the best dietary source and isolating the CPA, the isomerisation step was performed as previously described [28] directly on CPA as FAEE. Removal of reagents was carried out by Flash LC relying on the same conditions as described above.

2.4. Large-scale cis/trans fractionation

Three methods were compared to carry out large-scale separation of CPA and TPA. First, we performed low-temperature differential crystallisation in methanol. Based on previous research, 20 g of FAEE were dissolved in 1 L of methanol then refrigerated at -80 °C for 2 hours. The solution was afterwards filtered on a Büchner funnel (porosity 4): crystals were harvested, kept at ambient temperature until melting, and the excess of methanol was removed by vacuum evaporation.

Second, we attempted urea complexation in the solvent, according to a previously published protocol [39] and using a FAEE/urea/methanol ratio of 1/6/20 (w/w/v).

Third, we carried out Flash-LC relying on the same conditions as described above and still using FAEE.

2.5. Assessment of fatty acid composition by GC-MS

Gas chromatography-Mass spectrometry (GC-MS) was used for the control of composition and purity at each step of the chemical synthesis and purification. FAEE were analysed with an Agilent 7890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a bonded fused silica capillary column (BPX-90; 100 m×0.25 mm, 0.25 µm thickness; SGE Analytical Science, Melbourne, Australia). Helium was used as the carrier gas. Mass spectra were recorded with an Agilent 5975C MSD (Agilent Technologies). The mass spectrometer was operated under electron impact ionization conditions (electron energy 70 eV, source temperature 230°C). Data were obtained in full-scan mode with a mass range of m/z 50–550 amu.

Identification of fatty acids was based on (1) retention times compared with that of available pure standards and (2) mass spectrum of FAEE peaks. Peak integration was accomplished with MassHunter Workstation Qualitative Analysis B.07.00 software for Windows (Agilent Technologies).

2.6. Characterization of the final purified TPA by Nuclear Magnetic Resonance (NMR)

¹H and ¹³C NMR spectra were recorded on Bruker Avance III (400 MHz) and Avance III HD (500 MHz) spectrometers at 25 °C using CDCl₃ as the solvent, as previously described [40].

3. RESULTS

3.1. Dietary sources of CPA

Macadamia nut oil and Provinal[®] greatly differ comparing first their CPA content. CPA represents about 13% of total fatty acids (analyzed here as FAEE) in macadamia nut oil and almost 50% in Provinal[®], respectively (**Table 1**). Furthermore, Provinal[®] is characterized by C16:2 and C16:3 fatty acids in elevated amounts. This result is consistent with the fact that Provinal[®] stems from a first purification step from Peruvian Anchovy oil. Conversely, macadamia nut oil has a fatty acid

composition typical of that of vegetable oils, deprived of atypical fatty acids. Therefore, relying on the purity criterion, Provinal[®] should be considered as the best dietary source of CPA.

As regards to the C16:1 profiles, they also differ between the two sources (**Table 1**). First, macadamia nut oil is mainly characterized by CPA, and one should not expect to find high amounts of positional C16:1 isomers in such a vegetable oil. Accordingly, other *cis*-C16:1 positional isomers accounted for 0.78% of total *cis*-C16:1 fatty acids in macadamia nut oil. Second, Provinal[®] had some C16:1 isomers, both *trans* and *cis*: this is consistent with the raw material from which Provinal[®] stems from [41]. Other *cis* C16:1 positional isomers accounted for 3.8% of total *cis*-C16:1 fatty acids in Provinal[®]. Overall, considering specifically the C16:1 profiles between both dietary sources, macadamia nut oil would yield a 5-time more purified CPA than Provinal[®].

After analysing the fatty acid profile of the considered dietary sources, we cannot conclude which one is the best. The amount criterion is perhaps the most convenient one, and it is tempting to choose Provinal[®] as the best dietary source. However, meeting the purity criterion is crucial when it comes to nutritional studies, and from this point of view, macadamia nut oil is better than Provinal[®]. Consequently, it is mandatory to compare the easiness of removal of other fatty acids in the flash-LC step to assess the best dietary source.

3.2. Purification of CPA by flash LC

Purification of CPA by flash LC of either macadamia nut oil or Provinal[®] is shown in **Figure 1**. Concerning macadamia nut oil, a co-elution is noticed with linoleic acid, while oleic acid elutes way further. We hypothesized here that LA would undergo the isomerization step like CPA. Thus, it was crucial to remove as much LA as possible, but of course at the expense of the collected amount of CPA.

C16:2 and C16:3 fatty acids of Provinal[®] can be viewed on the chromatogram, eluting earlier than any other fatty acid. CPA eluted later, making its separation from poly-unsaturated fatty acids easy. Importantly, myristic acid (C14:0) co-eluted with CPA, thus the fraction containing CPA also contained some myristic acid. As regards to the subsequent isomerization step, myristic acid is not an issue since it will not be impacted.

Because the removal of LA was challenging, high levels of LA is a drawback when it comes to the isolation of CPA. We cannot rule out the fact that LA will be isomerized during the isomerization step, yielding *trans*-C18:2 and perhaps conjugated linoleic acid isomers that may hinder the physiological effects of TPA. Therefore, at this stage, it is now possible to choose Provinal[®] as a better dietary source of CPA than macadamia nut oil. Flash LC with multiple injections was therefore applied to Provinal[®] to isolate as much CPA as possible (**Supplementary Figure 2**).

3.3. *cis/trans* isomerization, and large-scale *cis/trans* fractionation

The isomerisation of CPA yielded a mix of CPA and TPA, according to a 36/64 ratio. Both the excess of reactants and the co-products were removed by Flash LC with the same conditions as described above (data not shown).

After low-temperature solvent fractionation, crystals looking like “snow eggs” were obtained (**Figure 2** and **Supplementary figure 3**). The crystals contained mostly TPA, but also C14:0 that remained after CPA purification by flash LC. On the contrary, neither urea complexation nor flash LC was as efficient as low-temperature solvent fractionation for large-scale removal of CPA (**Table 2**). Of note, these methods were more burdensome than low-temperature solvent fractionation. Thus, we used the latter method for large-scale *cis/trans* fractionation by repeating freezing of FAEE/methanol solutions (**Figure 3**).

3.4. Characterization of the final purified TPA

Figure 4 shows the GC-MS profile of fatty acid ethyl esters obtained after the whole purification process. The mass spectrum of the main peak, suspected to be the TPA ethyl ester (retention time = 25.5 min), was characterized by a molecular ion at m/z 282 amu (data not shown), which confirmed that it possessed a monounsaturated C16 ethyl ester structure. Since MS data on ethyl esters are not informative on *cis/trans* double bond position and configuration, we additionally performed ^1H and ^{13}C NMR on the final purified product.

Figure 5 shows the ^1H NMR spectrum of the final purified TPA ethyl ester. The determination of the coupling constant of the methylene protons ($^3J = 15\text{Hz}$) undoubtedly demonstrated the *trans* configuration of the single double bond of the analyzed molecule. In addition, by combining the results obtained in the hydrogen and carbon resonance spectra, signal positions corresponding to the 9 and 10 carbons carrying the double bond could be assigned (**Supplementary Figure 4**). The respective $-\text{CH}_3$ terminal methyl group of carbons 1 and 18 of the fatty acid carbon chain and ethyl group, could also be assigned, as well as carbon 1 (carbonyl function) and specific $\beta\text{-CH}_2$ of carbons 2 and 17.

Coming back now to **Figure 4**, it finally shows that, considering only the CPA and TPA ethyl esters, the final TPA product had a purity greater than 99%. Taking into account myristic acid, the overall purity was at 85%.

4. DISCUSSION

In this study, our target was to synthesize pure TPA in enough amounts, to carry out nutritional studies and unravel the physiological effects of dietary TPA. For that purpose, we first isolated CPA from Provinal[®], a fish-oil based dietary supplement. Despite not having the purest CPA compared with macadamia nut oil, the content in CPA in Provinal[®] was higher. In addition, isolating CPA by flash-LC from Provinal[®] was way easier than from macadamia nut oil. Therefore, Provinal[®] was considered as the best dietary source of CPA. After isolating CPA, we relied on a known workflow in

the field of chemical synthesis of *trans* monounsaturated fatty acids: CPA was isomerized, then removed from TPA by large-scaled *cis/trans* fractionation. We finally obtained TPA with an 85%-purity, the main impurity being myristic acid.

Wittig reaction is commonly used for *cis* monounsaturated fatty acid synthesis. Such a method is indeed reliable since the location of the ethylenic bond is accurate and certain, without generating positional isomers [27]. As a result, previous research relied on Wittig reaction to get CVA ready for isomerisation to TVA [21–28]. Translating to *cis*-C16:1 fatty acids and assuming that convenient reagents are commercially available, Wittig reaction can be performed to get high amounts of pure CPA. However, CPA is a dietary fatty acid that does occur in several foods in significant amounts [33]. Therefore, we assessed in our study a new means of getting high amounts of pure CPA ready for isomerisation.

Regarding the main target of our study, it is important to highlight here how particular the case of CPA is. For instance, getting high amounts of pure CVA could not be based on food sources even if CVA does occur in a wide range of oils. Indeed, oleic acid (*cis*-9 C18:1, or C18:1 n-9 *cis*) is present as well. Unless large-scale separation between CVA and oleic acid is applied, both CVA and oleic acid would isomerize yielding TVA and elaidic acid (*trans*-9 C18:1, or C18:1 n-9 *trans*), respectively. None of this occurs in the case of CPA because it is by far the major C16:1 fatty acid in both macadamia nut oil and Provinal[®]. Again, we took advantage of this fact in our study rather than considering the Wittig reaction.

Flash LC is not widely used when it comes to purification of fatty acids. Instead, preparative LC is a more popular technique of purification because the resolution offered is rather good and is convenient for purification/fractionation targets [42]. Flash LC goes beyond preparative LC, allowing higher amounts but at the expense of resolution. To the best of our knowledge, our research group is the first to report fractionation of a mix of fatty acids (*i.e.*, saturated, mono-unsaturated and poly-unsaturated) employing flash LC, clearly being a novelty of this article. Despite the amount of FAEE injected (approx. 20 mL), the resolution was good enough to ensure a convenient final product-purity. This outcome is encouraging concerning the physiological impacts of dietary fatty acids on human health. Flash LC might have a strong role to play when it comes to settling nutritional studies about minor dietary fatty acids that are not commercially available. This holds for TPA, which this study deals with, but also for CPA that was first purified in our experiment. Further research is therefore needed to assess the ability for flash LC to be a reliable means of fractionation for other minor dietary fatty acids. Importantly, it also implies the knowledge of sources of particular fatty acids that may be pre-purified (*e.g.*, Provinal[®]), and can be brought to a higher purity. It should also be underlined that we did not investigate the ability of urea complexation to fractionate CPA from other fatty acids in both food sources.

Based on the far lower content in linoleic acid, Provinal[®] was found to be a better source of CPA. However, we assumed in our study that linoleic acid would undergo the further isomerization step like CPA: we did not investigate any further on this topic. Should linoleic acid not be isomerized during this step, removing as much linoleic acid as possible would not be crucial. Independently of the fate of linoleic acid, the higher CPA content in Provinal[®] than in macadamia nut oil makes it a better source of CPA. Of note, we did not assess other well-known sources of CPA such as sea buckthorn. Likewise, we did not try purification of CPA through urea complexation.

LC flash not being efficient at separating CPA from TPA was unexpected. Indeed, we previously succeeded in doing the same between CVA and TVA, reaching a 5/95 purity ratio [29–30]. As a result, we hypothesized that LC flash would work as well for C16:1 fatty acids. The negative outcomes following urea complexation were however more in line with our hypothesis: to our knowledge, this widespread technique has never been used for *cis/trans* fractionation. That said, we did not investigate whether the *cis/trans* fractionation with urea complexation worked better using FFA, given that several guidelines rely on FFA [43]. Second, the FAEE/urea/methanol ratio might play a strong role when large-scale purification of fatty acids takes place. Given that such ratio has a wide range of values depending on the fatty acid of interest and the studies, we believe that some optimization is necessary as regards to a *cis/trans* monounsaturated fatty acids fractionation by urea complexation.

Instead, *cis/trans* palmitoleic acid fractionation was performed by low-temperature crystallization in methanol. For decades, such a procedure has been widely used as an excellent means to separate *cis/trans* mono-unsaturated fatty acids [44], especially oleic and elaidic acids [45–47]. Here, we were able to translate all this knowledge to comply with the field of *trans*-C16:1 fatty acids, which is another new kind of information brought by this article. Like previous studies [48], we performed large-scaled *cis/trans* fractionation directly on fatty acid esters rather than on FFA [21]. It enables working all along with FAEE, which are easily analysed by GC-MS, so that purity can be assessed at each step without additional effort. Should work had been performed with FFA, a derivatization step would have been mandatory to assess the purity of each fraction, which is undoubtedly time-consuming. Thus, keeping working on FAEE is a strength of our study. Furthermore, FAEE are suitable for supplementation studies not only on rodent models [49] but also on humans [32]: no side effects were reported with such derivatives, and they are equally digested compared with triglycerides.

The major impurity that remains with our method is myristic acid (C14:0), accounting for almost 15% in the final product. Accordingly, this is a weakness of our study and may represent a strong bias when it comes to supplementation studies. Our strategy was to collect as much CPA as possible: due to the co-elution with C14:0, our choice was surely done at the expense of the purity of CPA. At the *cis/trans* fractionation step, considering both the chain length and the number of double bonds, myristic acid theoretically behaves almost like TPA [50]. Therefore, this step was designed to remove as much CPA as possible, rather than focusing on myristic acid. Still, our main purpose was to settle supplementation studies at least on rodents since the physiological impacts of TPA remain unknown. In such a case, it is possible to adjust the fatty acid composition of foods and pellets given to rodents, therefore deleting the bias of myristic acid. Otherwise, in the case of clinical trials, adjusting fatty acid composition is much more burdensome and the risk of bias with our final product is high. Thus, our final product is suitable for studies carried out on rodents, but maybe not for clinical trials. Future research should, therefore, focus on removing myristic acid to significantly improve the purity of our final product, and perhaps exploring other strategies.

Great attention was paid at the remaining amount of CPA in the final TPA product. On the one hand, CPA is thought to have beneficial physiological impacts, especially on inflammation and insulin sensitivity. On the other hand, high levels of circulating TPA are associated with higher insulin sensitivity as well [9–10]. It was even hypothesized that CPA and TPA have the same physiological

impacts [10]. Consequently, whatever the way of getting a large amount of pure CPA, the *cis/trans* fractionation is of the highest importance.

5. CONCLUSION

While relying in part on previously described techniques, we report for the first time a method to get pure TPA in large amounts. For that purpose, we relied on Provinal[®] as a dietary source of CPA, the latter being subsequently isolated by flash-LC and submitted to an isomerization step. Large-scale *cis/trans* fractionation was afterwards applied to isolate TPA. In light of our target to carry nutritional studies out, the remaining amount of myristic acid represents an issue, especially regarding clinical trials. Nonetheless, it is possible to adjust the amount of myristic acid between groups, tackling this potential bias. Last but not least, we successfully minimized other *trans*-C16:1 positional isomers that could have interfered with TPA in terms of physiological impacts. Overall, our method has to be improved, especially as regards to the presence of myristic acid in the final product. We emphasize the fact that such a method was developed at the scale of a research laboratory, making it easily reproducible. Meanwhile, our method enables further *in vivo* research to unravel the physiological impacts of dietary TPA.

ACKNOWLEDGEMENTS

EG acknowledges a CIFRE (Industrial Agreement of Training through Research) PhD fellowship from both the French Dairy Interbranch Organization (CNIEL) and the National Association for Research and Technology (ANRT) (CIFRE fellowship number 2015/1195).

FUNDING

The present research was financially supported by (1) the French Dairy Interbranch Organization (CNIEL) (PALMITO project) (2) the Lactalis Group, and (3) the Lipids and Nutrition Group (GLN).

CONFLICTS OF INTERESTS

EG is employed by the French Dairy Interbranch Organization (CNIEL).

AUTHOR CONTRIBUTIONS

Identification and assessment of CPA sources: EG, GD, DC. Purification of CPA: EG, GD, DC. Isomerisation of CPA: DC. Large scale separation of CPA and TPA: EG, DC. Manuscript: EG, GD, DC, PL, VR.

REFERENCES

1. E. Guillocheau, P. Legrand, & V. Rioux, Trans-palmitoleic acid (trans-9-C16:1, or trans-C16:1 n-7): Nutritional impacts, metabolism, origin, compositional data, analytical methods and chemical synthesis. A review. *Biochimie*, **169** (2020) 144–160. <https://doi.org/10.1016/j.biochi.2019.12.004>.
2. E. Guillocheau, C. Penhoat, G. Drouin, A. Godet, D. Catheline, P. Legrand, & V. Rioux, Current intakes of trans-palmitoleic (trans-C16:1 n-7) and trans-vaccenic (trans-C18:1 n-7) acids in France are exclusively ensured by ruminant milk and ruminant meat: A market basket investigation. *Food Chemistry: X*, **5** (2020) 100081. <https://doi.org/10.1016/j.fochx.2020.100081>.
3. F. Imamura, A. M. Fretts, M. Marklund, A. V. A. Korat, W.-S. Yang, M. Lankinen, W. Qureshi, C. Helmer, T.-A. Chen, K. Wong, J. K. Bassett, R. Murphy, N. Tintle, C. I. Yu, I. A. Brouwer, K.-L. Chien, A. C. Frazier-Wood, L. C. del Gobbo, L. Djoussé, J. M. Geleijnse, G. G. Giles, J. de Goede, V. Gudnason, W. S. Harris, A. M. Hodge, F. B. Hu, I. Consortium, A. Koulman, M. Laakso, L. Lind, H.-J. Lin, B. McKnight, K. Rajaobelina, U. Risérus, J. G. Robinson, C. Samieri, D. S. Siscovick, S. S. Soedamah-Muthu, Nona Sotoodehnia, Q. Sun, M. Y. Tsai, M. Uusitupa, L. E. Wagenknecht, N. J. Wareham, J. H. Y. Wu, R. Micha, N. G. Forouhi, R. N. Lemaitre, & D. Mozaffarian, Fatty acid biomarkers of dairy fat consumption and incidence of type 2 diabetes: a pooled analysis of prospective cohort studies. *PLoS Medicine*, **15** (2018). <https://doi.org/10.1371/journal.pmed.1002670>.
4. W. Yoo, D. Gjuka, H. L. Stevenson, X. Song, H. Shen, S. Y. Yoo, J. Wang, M. Fallon, G. N. Ioannou, S. A. Harrison, & L. Beretta, Fatty acids in non-alcoholic steatohepatitis: Focus on pentadecanoic acid. *PLoS One*, (2017) 1–15. <https://doi.org/10.1371/journal.pone.0189965>.
5. M. Y. Yakoob, P. Shi, W. C. Willett, K. M. Rexrode, H. Campos, E. John Orav, F. B. Hu, & D. Mozaffarian, Circulating Biomarkers of Dairy Fat and Risk of Incident Diabetes Mellitus Among US Men and Women in Two Large Prospective Cohorts. *Circulation*, **133** (2016). <https://doi.org/10.1161/CIRCULATIONAHA.115.018410>.
6. M. S. Da Silva, P. Julien, L. Pérusse, M.-C. Vohl, & I. Rudkowska, Natural Rumen-Derived trans Fatty Acids Are Associated with Metabolic Markers of Cardiac Health. *Lipids*, **50** (2015) 873–882. <https://doi.org/10.1007/s11745-015-4055-3>.
7. R. J. de Souza, A. Mente, A. Maroleanu, A. I. Cozma, V. Ha, T. Kishibe, E. Uleryk, P. Budyłowski, H. Schünemann, J. Beyene, & S. S. Anand, Intake of saturated and trans unsaturated fatty acids and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: systematic review and meta-analysis of observational studies. *BMJ*, **351** (2015) 1–16. <https://doi.org/10.1136/bmj.h3978>.
8. M. Kratz, S. Marcovina, J. E. Nelson, M. M. Yeh, K. V. Kowdley, H. S. Callahan, X. Song, C. Di, & K. M. Utzschneider, Dairy fat intake is associated with glucose tolerance, hepatic and systemic insulin sensitivity, and liver fat but not β -cell function in humans. *The American Journal of Clinical Nutrition (AJCN)*, **99** (2014) 1385–1396. <https://doi.org/10.3945/ajcn.113.075457.1>.
9. D. Mozaffarian, M. C. de Oliveira Otto, R. N. Lemaitre, A. M. Fretts, G. S. Hotamisligil, M. Y. Tsai, D. S. Siscovick, & J. A. Nettleton, trans-Palmitoleic acid, other dairy fat biomarkers, and incident diabetes: the Multi-Ethnic Study of Atherosclerosis (MESA). *The American Journal of Clinical Nutrition (AJCN)*, **97** (2013) 854–61. <https://doi.org/10.3945/ajcn.112.045468>.
10. D. Mozaffarian, H. Cao, I. B. King, R. N. Lemaitre, X. Song, D. S. Siscovick, & G. S. Hotamisligil,

- Trans-palmitoleic acid, metabolic risk factors, and new-onset diabetes in U.S. adults. *Annals of Internal Medicine*, **153** (2010) 790–9. <https://doi.org/10.7326/0003-4819-153-12-201012210-00005>.
11. J. Molkentin & D. Precht, Occurrence of trans-C16:1 acids in bovine milkfats and partially hydrogenated edible fats. *Milchwissenschaft*, **52** (1997) 380–385.
 12. F. Destailats, R. L. Wolff, D. Precht, & J. Molkentin, Study of individual trans- and cis-16:1 isomers in cow, goat, and ewe cheese fats by gas-liquid chromatography with emphasis on the trans- $\Delta 3$ isomer. *Lipids*, **35** (2000) 1027–1032. <https://doi.org/10.1007/s11745-000-0614-y>.
 13. C. J. Field, H. F. H. Blewett, S. D. Proctor, & D. F. Vine, Human health benefits of vaccenic acid. *Applied Physiology, Nutrition and Metabolism*, **34** (2009) 979–999. <https://doi.org/10.1139/H09-079>.
 14. J.-M. Chardigny, C. Malpuech-Brugère, F. Dionisi, D. E. Bauman, B. German, R. P. Mensink, N. A. Combe, P. Chaumont, D. M. Barbano, F. Enjalbert, J.-B. Bezelgues, I. Cristiani, J. Moulin, Y. Boirie, P. A. Golay, F. Giuffrida, J.-L. Sébédio, & F. Destailats, Rationale and design of the TRANSFACT project phase I: A study to assess the effect of the two different dietary sources of trans fatty acids on cardiovascular risk factors in humans. *Contemporary Clinical Trials*, **27** (2006) 364–373. <https://doi.org/10.1016/j.cct.2006.03.003>.
 15. I. Laake, J. I. Pedersen, R. Selmer, B. Kirkhus, A. S. Lindman, A. Tverdal, & M. B. Veierød, A prospective study of intake of trans-fatty acids from ruminant fat, partially hydrogenated vegetable oils, and marine oils and mortality from CVD. *British Journal of Nutrition (BJN)*, **108** (2012) 743–754. <https://doi.org/10.1017/S0007114511005897>.
 16. M. C. de Oliveira Otto, J. A. Nettleton, R. N. Lemaitre, L. M. Steffen, D. Kromhout, S. S. Rich, M. Y. Tsai, D. R. Jacobs, & D. Mozaffarian, Biomarkers of Dairy Fatty Acids and Risk of Cardiovascular Disease in the Multi-Ethnic Study of Atherosclerosis. *Journal of the American Heart Association*, **2** (2013) e000092. <https://doi.org/10.1161/JAHA.113.000092>.
 17. D. Precht & J. Molkentin, Recent trends in the fatty acid composition of German sunflower margarines, shortenings and cooking fats with emphasis on individual C16:1, C18:1, C18:2, C18:3 and C20:1 trans isomers. *Nahrung*, **16** (2000) 0–6. [https://doi.org/10.1002/1521-3803\(20000701\)44:4<222::AID-FOOD222>3.0.CO;2-9](https://doi.org/10.1002/1521-3803(20000701)44:4<222::AID-FOOD222>3.0.CO;2-9).
 18. FDA, Final determination regarding partially hydrogenated oils. *Federal Register*, **83** (2018) 23358–23359.
 19. N. K. Huang, N. R. Matthan, J. M. Galluccio, P. Shi, A. H. Lichtenstein, & D. Mozaffarian, Supplementation with Seabuckthorn Oil Augmented in 16:1 n-7 t Increases Serum Trans - Palmitoleic Acid in Metabolically Healthy Adults: A Randomized Crossover Dose-Escalation Study. *The Journal of Nutrition*, (2020). <https://doi.org/10.1093/jn/nxaa060>.
 20. S. K. Gebauer, F. Destailats, F. Dionisi, R. M. Krauss, & D. J. Baer, Vaccenic acid and trans fatty acid isomers from partially hydrogenated oil both adversely affect LDL cholesterol: a double-blind, randomized controlled trial. *The American Journal of Clinical Nutrition (AJCN)*, **102** (2015) 1339–46. <https://doi.org/10.3945/ajcn.115.116129>.
 21. S. K. Gebauer, F. Destailats, Z. Mouloungui, L. Candy, J.-B. Bezelgues, F. Dionisi, & D. J. Baer, Effect of trans fatty acid isomers from ruminant sources on risk factors of cardiovascular disease: study design and rationale. *Contemporary Clinical Trials*, **32** (2011) 569–76.

- <https://doi.org/10.1016/j.cct.2011.03.012>.
22. Y. Wang, M. M. Jacome-Sosa, M. R. Ruth, S. D. Goruk, M. J. Reaney, D. R. Glimm, D. C. Wright, D. F. Vine, C. J. Field, & S. D. Proctor, Trans-11 vaccenic acid reduces hepatic lipogenesis and chylomicron secretion in JCR:LA-cp rats. *The Journal of Nutrition*, **139** (2009) 2049–54. <https://doi.org/10.3945/jn.109.109488>.
 23. M. M. Jacome-Sosa, J. Lu, Y. Wang, M. R. Ruth, D. C. Wright, M. J. Reaney, J. Shen, C. J. Field, D. F. Vine, & S. D. Proctor, Increased hypolipidemic benefits of cis-9, trans-11 conjugated linoleic acid in combination with trans-11 vaccenic acid in a rodent model of the metabolic syndrome, the JCR:LA-cp rat. *Nutrition & Metabolism*, **7** (2010) 1–10. <https://doi.org/10.1186/1743-7075-7-60>.
 24. M. M. Jacome-Sosa, F. Borthwick, R. Mangat, R. Uwiera, M. J. Reaney, J. Shen, A. D. Quiroga, R. L. Jacobs, R. Lehner, & S. D. Proctor, Diets enriched in trans-11 vaccenic acid alleviate ectopic lipid accumulation in a rat model of NAFLD and metabolic syndrome. *The Journal of Nutritional Biochemistry (JNB)*, **25** (2014) 692–701. <https://doi.org/10.1016/j.jnutbio.2014.02.011>.
 25. X. Wang, J. Gupta, M. Kerslake, G. Rayat, S. D. Proctor, & C. B. Chan, Trans-11 vaccenic acid improves insulin secretion in models of type 2 diabetes in vivo and in vitro. *Molecular Nutrition & Food Research (MNFR)*, **60** (2016) 846–857. <https://doi.org/10.1002/mnfr.201500783>.
 26. M. M. Jacome-Sosa, C. Vacca, R. Mangat, A. Diane, R. C. Nelson, M. J. Reaney, J. Shen, J. M. Curtis, D. F. Vine, C. J. Field, M. Igarashi, D. Piomelli, S. Banni, & S. D. Proctor, Vaccenic acid suppresses intestinal inflammation by increasing anandamide and related N-acylethanolamines in the JCR:LA-cp rat. *Journal of Lipid Research (JLR)*, **57** (2016) 638–649. <https://doi.org/10.1194/jlr.M066308>.
 27. Z. Mouloungui & L. Candy, Chemical synthesis of monounsaturated trans fatty acids. In F. Destailats, J.-L. Sébédio, F. Dionisi, & J.-M. Chardigny, eds., *Trans Fat. acids Hum. Nutr.*, Second, (Oily Press, 2009), pp. 77–103. <https://doi.org/10.1533/9780857097873.77>.
 28. P. E. Duffy, S. M. Quinn, H. M. Roche, & P. Evans, Synthesis of trans-vaccenic acid and cis-9-trans-11-conjugated linoleic acid. *Tetrahedron*, **62** (2006) 4838–4843. <https://doi.org/10.1016/j.tet.2006.03.006>.
 29. C. Garcia, E. Guillocheau, L. Richard, G. Drouin, D. Catheline, P. Legrand, & V. Rioux, Conversion of dietary trans-vaccenic acid to trans11,cis13-conjugated linoleic acid in the rat lactating mammary gland by Fatty Acid Desaturase 3-catalyzed methyl-end Δ 13-desaturation. *Biochemical and Biophysical Research Communications*, **505** (2018) 385–391. <https://doi.org/10.1016/j.bbrc.2018.09.132>.
 30. E. Guillocheau, C. Garcia, G. Drouin, L. Richard, D. Catheline, P. Legrand, & V. Rioux, Retroconversion of dietary trans-vaccenic (trans-C18:1 n-7) acid to trans-palmitoleic acid (trans-C16:1 n-7): proof of concept and quantification in both cultured rat hepatocytes and pregnant rats. *The Journal of Nutritional Biochemistry (JNB)*, **63** (2019) 19–26. <https://doi.org/10.1016/j.jnutbio.2018.09.010>.
 31. K. Ahmad, F. M. Bumpus, & F. M. Strong, Synthesis of cis-11-Octadecenoic and trans-11-Octadecenoic (Vaccenic) Acids. *Journal of the American Chemical Society*, **70** (1948) 3391–3394. <https://doi.org/10.1021/ja01190a051>.

32. A. M. Bernstein, M. F. Roizen, & L. Martinez, Purified palmitoleic acid for the reduction of high-sensitivity C-reactive protein and serum lipids: a double-blinded, randomized, placebo controlled study. *Journal of Clinical Lipidology*, **8** (2014) 612–617. <https://doi.org/10.1016/j.jacl.2014.08.001>.
33. L. Hodson & F. Karpe, Is there something special about palmitoleate? *Current Opinion in Clinical Nutrition and Metabolic Care*, **16** (2013) 225–31. <https://doi.org/10.1097/MCO.0b013e32835d2edf>.
34. Voshuiles, Voshuiles. (n.d.). <https://www.voshuiles.com/>.
35. C. O. de Souza, G. K. Vannice, J. C. R. Neto, & P. C. Calder, Is Palmitoleic Acid a Plausible Non-Pharmacological Strategy to Prevent or Control Chronic Metabolic and Inflammatory Disorders? *Molecular Nutrition & Food Research (MNFR)*, **1700504** (2017) 1700504. <https://doi.org/10.1002/mnfr.201700504>.
36. H. Cao, K. Gerhold, J. R. Mayers, M. M. Wiest, S. M. Watkins, & G. S. Hotamisligil, Identification of a Lipokine, a Lipid Hormone Linking Adipose Tissue to Systemic Metabolism. *Cell*, **134** (2008) 933–944. <https://doi.org/10.1016/j.cell.2008.07.048>.
37. LifeExtension, LifeExtension. (n.d.). <https://www.lifeextension.com/>.
38. A. Hara & N. S. Radin, Lipid extraction of tissues with a low toxicity solvent. *Analytical Biochemistry*, **90** (1978) 420–426. [https://doi.org/10.1016/0003-2697\(78\)90046-5](https://doi.org/10.1016/0003-2697(78)90046-5).
39. M. Schröder & W. Vetter, Detection of 430 fatty acid methyl esters from a transesterified butter sample. *Journal of the American Oil Chemists' Society (JAOCS)*, **90** (2013) 771–790. <https://doi.org/10.1007/s11746-013-2218-z>.
40. G. Barouti, S. S. Liow, Q. Dou, H. Ye, C. Orione, S. M. Guillaume, & X. J. Loh, New Linear and Star-Shaped Thermogelling Poly([R]-3-hydroxybutyrate) Copolymers. *Chemistry - A European Journal*, **22** (2016) 10501–10512. <https://doi.org/10.1002/chem.201601404>.
41. A.-R. Fardin-Kia, P. Delmonte, J. K. G. Kramer, G. Jahreis, K. Kuhnt, V. Santercole, & J. I. Rader, Separation of the fatty acids in menhaden oil as methyl esters with a highly polar ionic liquid gas chromatographic column and identification by time of flight mass spectrometry. *Lipids*, **48** (2013) 1279–1295. <https://doi.org/10.1007/s11745-013-3830-2>.
42. P. Van der Meeren & J. Vanderdeelen, Preparative high-performance liquid chromatography of lipids. In W.W. Christie, ed., *Adv. Lipid Methodol. - Four* (The Oily Press, 1993).
43. D. G. Hayes, Free fatty acid fractionation via urea inclusion compounds. *International News on Fats, Oils and Related Materials (INFORM)*, **13** (2002) 832–834.
44. S. A. Fusari, K. W. Greenlee, & J. B. Brown, Syntheses of Cis- and Trans-7- and 8-Octadecenoic Acids : Comparison of the Properties of Cis- and Trans-6-, 7-, 8-, 9-, and 11-Octadecenoic Acids. *Journal of the American Oil Chemists' Society (JAOCS)*, **28** (1951) 416–420. <https://doi.org/10.1007/BF02589677>.
45. J. B. Brown, Low-temperature crystallization of the fatty acids and glycerides. *Chemical Reviews*, **29** (1941) 333–354. <https://doi.org/10.1021/cr60093a009>.
46. J. B. Brown, Fractional Solvent Crystallization. *Journal of the American Oil Chemists' Society (JAOCS)*, **32** (1955) 646–652. <https://doi.org/10.1007/BF02637558>.

47. J. C. Smith, Higher aliphatic compounds. Part VIII. Purification of oleic and elaidic acids. Binary systems from oleic, elaidic, palmitic and stearic acids. The technique of low-temperature crystallisation. *Journal of the Chemical Society*, (1939) 974–980.
48. D. L. Cramer & J. B. Brown, The component fatty acids of human depot fat. *Journal of Biological Chemistry (JBC)*, **151** (1943) 427–438.
49. G. Drouin, E. Guillocheau, D. Catheline, C. Baudry, P. Le Ruyet, V. Rioux, & P. Legrand, Impact of n-3 Docosapentaenoic Acid Supplementation on Fatty Acid Composition in Rat Differs Depending upon Tissues and Is Influenced by the Presence of Dairy Lipids in the Diet. *Journal of Agricultural and Food Chemistry (JAFC)*, **66** (2018) 9976–9988. <https://doi.org/10.1021/acs.jafc.8b03069>.
50. J. B. Brown & D. K. Kolb, Applications of Low Temperature Crystallization in the Separation of the Fatty Acids and Their Compounds. In R.T. Holman,ed., *Prog. Chem. Fats Other Lipids* (1955).

TABLE 1

Fatty acid composition of macadamia nut oil and Provinal[®], expressed as a % of total fatty acids.

Fatty acid	Macadamia nut oil (%)	Provinal [®] (%)
C14:0	1.5	2.6
C15:0 iso	ND	1.4
C15:0 anteiso	ND	0.4
C15:0	ND	0.8
C16:0	25.6	1.6
C16:1 <i>trans</i> (total)	ND	0.4
C17:0 iso	ND	0.7
C16:1 n-7 <i>cis</i> (CPA)	12.8	47.4
C16:1 <i>cis</i> (other than CPA)	0.1	1.8
C17:1	ND	0.8
C16:2 (total)	ND	11.5
C16:3 (total)	ND	9.4
C18:0	9.8	ND
C18:1 n-9	37.2	3.2
C18:1 n-7	2.9	1.6
C16:4	ND	10.5
C18:2 n-6	1.2	ND
C20:0	6.4	ND
C18:3 n-3	1.4	1.0
C18:4	ND	3.8
C22:0	0.9	ND
C20:5 n-3	ND	1.0
C22:6 n-3	ND	0.3

ND: not detected.

TABLE 2

Comparisons of three methods for large-scale fractionation of CPA and TPA.

	CPA	TPA
Initial mix	36	64
Low-temperature solvent crystallization	20	80
Urea complexation	35	65
Flash LC	29	71

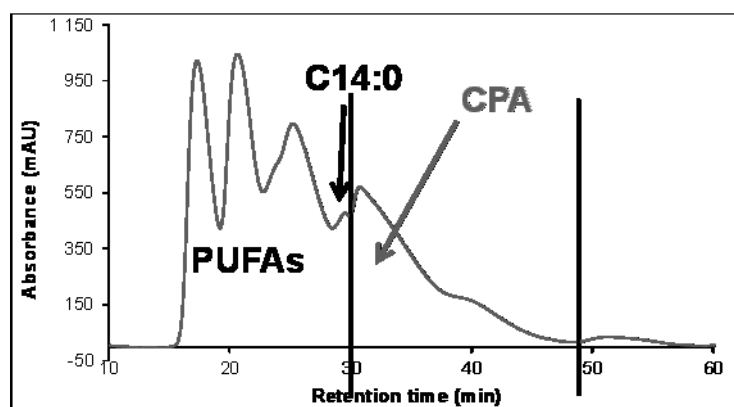
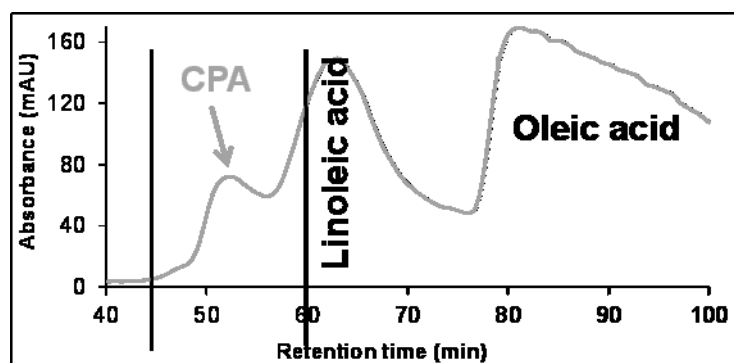
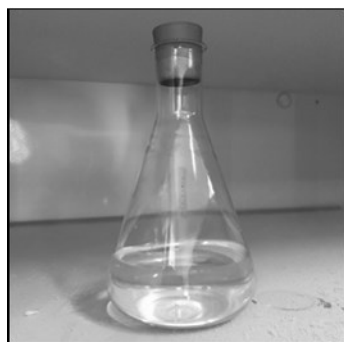
FIGURE 1*Colour for the online version*

FIGURE 2



Initial state



Final state: t + 2h

Colour for the online version

FIGURE 3

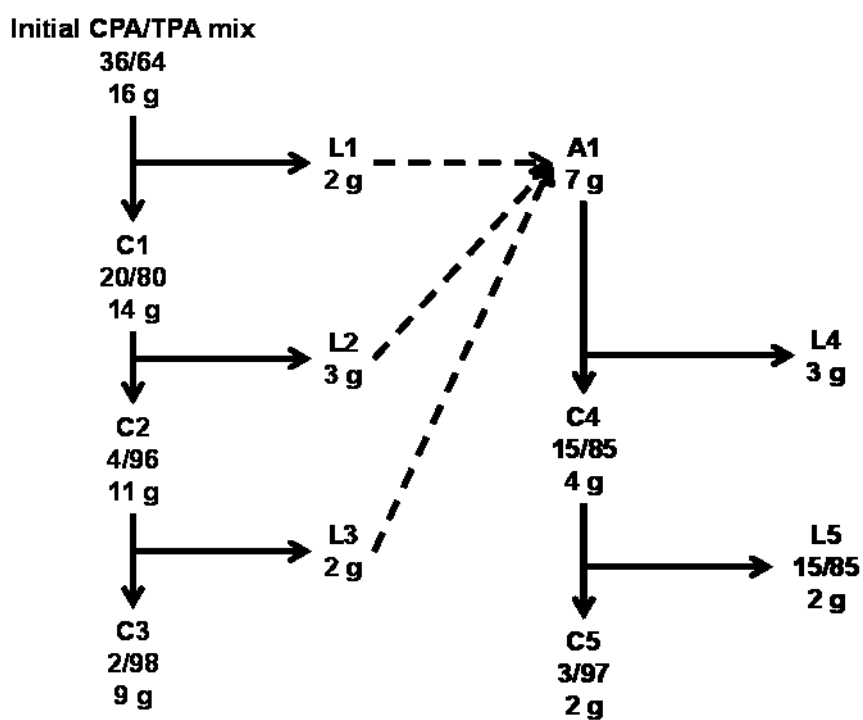


FIGURE 4

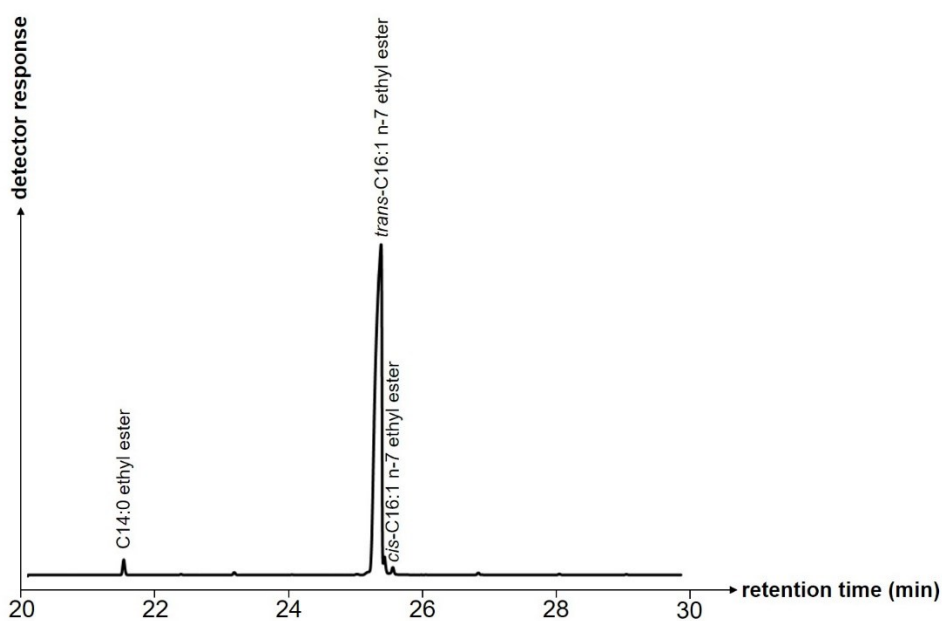


FIGURE 5

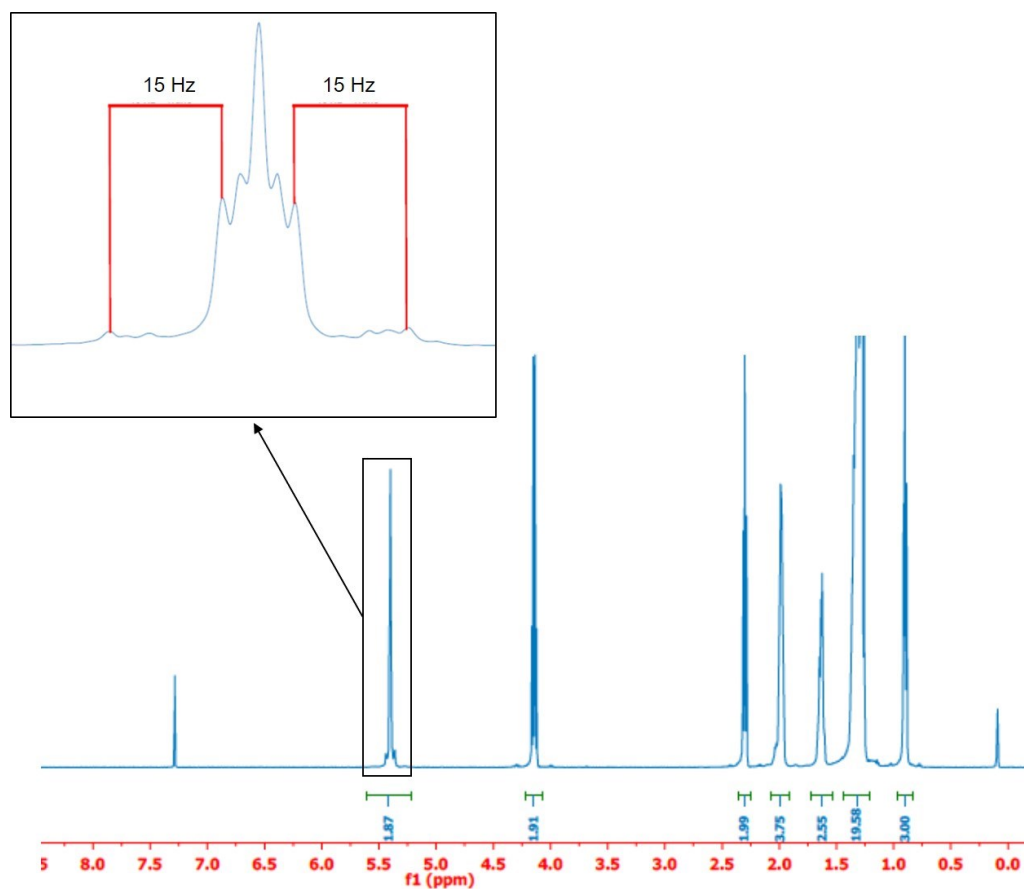


FIGURE LEGENDS

FIGURE 1

Flash LC separation of (A) macadamia nut oil FAEE and (B) Provinal FAEE. Comparison of the fatty acid composition of macadamia nut oil and Provinal®: emphasis on the C16:1 fatty acid zone.

FIGURE 2

Crystals looking like “snow eggs” enriched with TPA ethyl ester obtained after low-temperature solvent fractionation, while CPA ethyl ester mainly remained soluble in the solvent liquid.

FIGURE 3

Schematic description of the successive stages used during fractional crystallization.

FIGURE 4

GC-MS profile of fatty acid ethyl esters obtained after the whole purification process. The identity of each important fatty acid is indicated above its respective peak.

FIGURE 5

^1H NMR (500 MHz, CDCl_3 , 25 °C) spectrum of the final purified TPA ethyl ester. The determination of the coupling constant ($^3J = 15\text{Hz}$) of the methylene protons around 5.5 ppm undoubtedly demonstrated the *trans* configuration of the single double bond of the monounsaturated fatty acid.