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Determination of 2-alkylcyclobutanones in ultraviolet light-irradiated fatty acids, triglycerides, corn oil, and pork samples: Identifying a new source of 2-alkylcyclobutanones

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ABSTRACT

Previous studies have established that 2-alkylcyclobutanones (2-ACBs) are unique radiolytic products in lipid-containing foods that could only be formed through exposure to ionizing radiation, but not by any other means of physical/heat treatment methods. Therefore, 2-ACBs are currently the marker molecules required by the European Committee for Standardization to be used to identify foods irradiated with ionizing irradiation. Using a spectrum of state-of-the-art analytical instruments, we present in this study for the first time that the generation of 2-ACBs was also possible when fatty acids and triglycerides are exposed to a non-ionizing, short-wavelength ultraviolet (UV-C) light source. An irradiation dosage-dependent formation of 2-ACBs was also observed in UV-C irradiated fatty acids, triglycerides, corn oil, and pork samples. With UV-C irradiation becoming an increasingly common food treatment procedure, it is anticipated that the results from this study will alert food scientists and regulatory officials to a potential new source for 2-ACBs.

1. Introduction

2-Alkylcyclobutanones, 2-ACBs, are among the products formed when fatty acids and triglycerides in lipid-containing foods are exposed to ionizing radiation (Fig. 1) (Horvatovich, Miesch, Hasselmann, Delincée, & Marchioni, 2005; Nawar, Zhu, & Yoo, 1990). There is accumulated evidence to suggest that only ionizing radiation alone, including gamma-radiation, X-rays, or accelerated electron beams could generate 2-ACBs in food; but other food preservation/cooking methods such as UV-irradiation, microwave/oven heating, steaming and roasting do not generate such molecules (Crews, Driffield, & Thomas, 2012; Driffield et al., 2014; Ndiaye, Jamet, Miesch, Hasselmann, & Marchioni, 1999; Song et al., 2014). Therefore, 2-ACBs are considered as unique radiolytic products and have been used as marker molecules for identifying lipid-containing foods that have been treated with ionizing radiation (Chan, Ye, & Leung, 2014; Horvatovich, Miesch, Hasselmann, & Marchioni, 2000; Stevenson & Crone, 1990). Currently, 2-dodecylcyclobutanone, **1**, and 2-tetradecylcyclobutanone, **2**, from the radiolysis of palmitic and stearic acids, respectively (Fig. 1), were required by the European Committee for Standardization (CEN method EN1785:2003) to be used as the indicator molecules for identifying irradiated food (Anon., 2003).

A study by Variyar et al. reported the detection of compounds **1** and **2** in non-irradiated cashew nut and nutmeg samples (Variyar, Chatterjee, Sajilata, Singhal, & Sharma, 2008). The study was later proved to be faulty, due to a poorly designed experiment (Breidbach & Ulberth, 2016; Chen et al., 2012; Leung, Tang, Ye, & Chan, 2013). Nevertheless it has aroused worldwide concern on the natural existence of 2-ACBs and the supposed uniqueness of 2-ACBs as markers for irradiated food identification. Therefore, analytical methods of enhanced sensitivity are highly sought by food chemists and regulatory officials to re-evaluate the claim that 2-ACBs are uniquely found in irradiated food products (Horvatovich et al., 2006; Ye, Liu, Horvatovich, & Chan, 2013).

By combining hydroxylamine (HA) derivatization and liquid chromatography-tandem mass spectrometry (LC–MS/MS) detection, we recently developed a novel and sensitive analytical method for the determination of 2-ACBs (Ye et al., 2013). Using the developed LC–MS/MS method, we analyzed nutmeg and cashew nut samples collected from local supermarkets in Hong Kong (Leung et al., 2013). Our analysis detected neither compounds **1** nor **2** in any of the collected samples, indicating 2-ACBs did not exist naturally in nutmeg and cashew nut samples.

As part of our continuing effort in re-evaluating the uniqueness of 2-ACBs for irradiated food identification, we investigated in this

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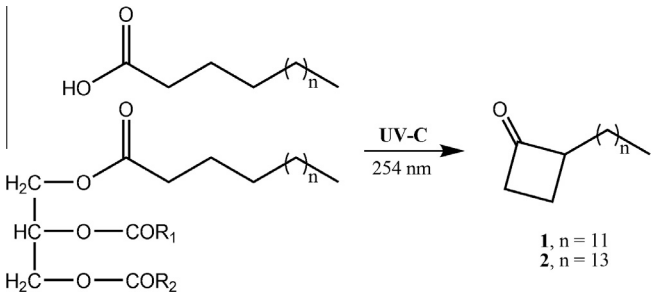

Fig. 1. Formation of 2-dodecylcyclobutanone, **1**, and 2-tetradecylcyclobutanone, **2**, from UV-C irradiation of free palmitic acid (n = 11) stearic acid (n = 13), and their associated triglycerides.

study the potential formation of 2-ACBs in foodstuffs that were processed by common physical treatment/preservation methods that did not involve ionizing radiation. Specifically, we analyzed the presence of 2-ACBs in fatty acids, triglycerides, corn oil, and pork samples that were treated by direct heating, microwave heating, ultrasonication, and ultraviolet (UV) light irradiation (UV-C, 254 nm; and UV-A, 365 nm) methods using LC–MS/MS.

Using the developed LC–MS/MS method, we identified in this study for the first time that 2-ACBs were also produced when fatty acids, triglycerides, corn oil, and pork samples were irradiated with short-wavelength ultraviolet (UV-C) light, which is classified as a non-ionizing radiation (Harm, 1980; Ng, 2003; Sommer et al., 2001). The identification was further verified by the GC–MS-based CEN standard method (EN1785:2003), high accuracy mass spectrometry, and liquid chromatography with fluorescence detection (LC-FLD) (Anon., 2003; Meng, Tong, Wang, Liu, & Chan, 2016). Furthermore, a dose-dependent formation of 2-ACBs was observed in the irradiated samples. With short-wavelength ultraviolet (UV-C) light being increasingly used for food preservation (Begum, Hocking, & Miskelly, 2009; Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000; Lopez-Malo & Palou, 2005), it is expected that results from this study will alert the regulatory agencies to a potential alternative source of 2-ACBs, thus avoiding false positive identification of ionizing radiation-irradiated food.

2. Materials and methods

2.1. Chemicals and reagents

2-Dodecylcyclobutanone, 2-tetradecylcyclobutanone, 2-(2-ethylhexyl)cyclohexanone, palmitic acid, stearic acid, tripalmitin, tristearin, hydroxylamine hydrochloride, and 1-naphthylhydrazine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Corn oil and pork samples were obtained from local supermarket in Hong Kong. HPLC-grade acetonitrile and *n*-hexane were obtained from Tedia (Fairfield, OH). Deionized water that was

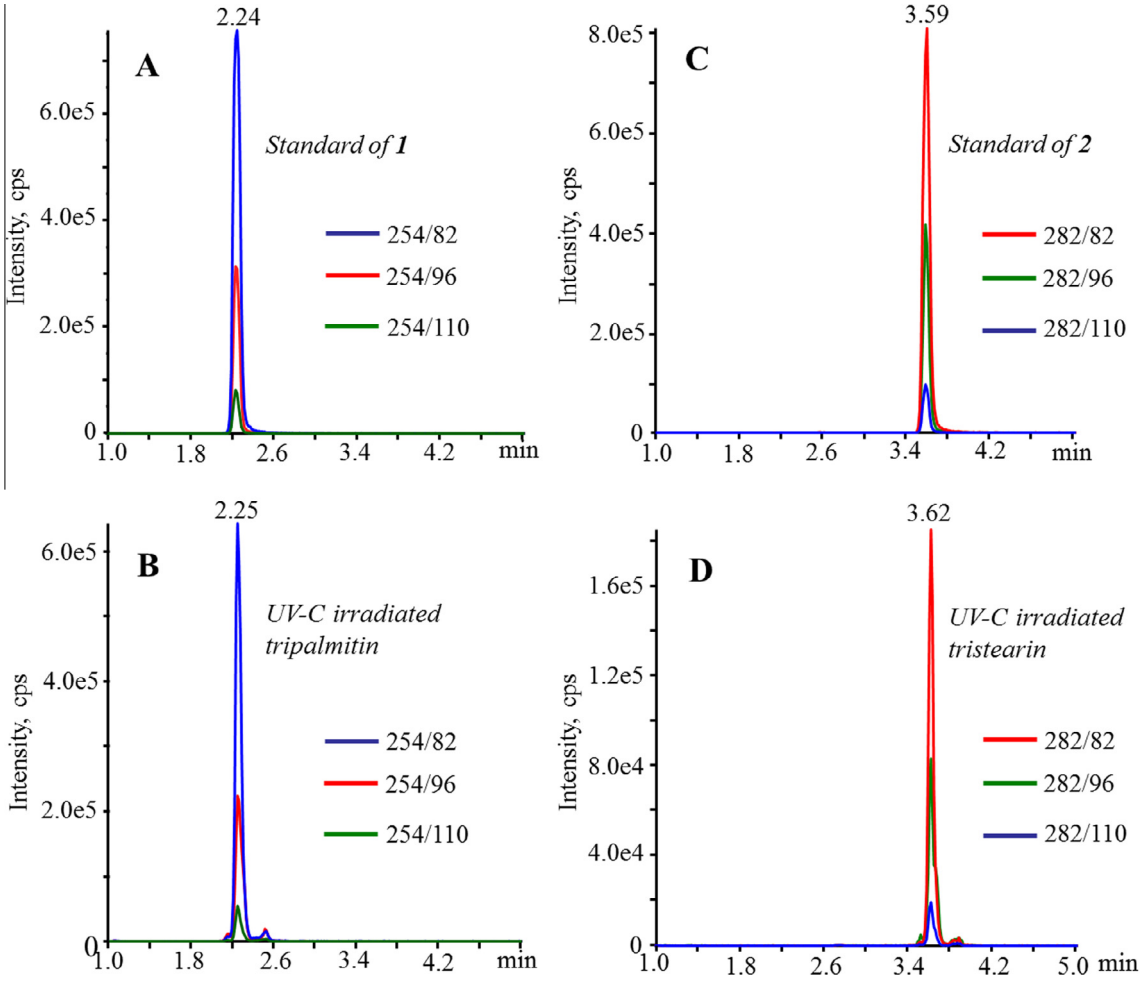

Fig. 2. Typical chromatograms obtained from LC–MS/MS analysis of: A. authentic standard of compound **1**; B. compound **1** in UV-C irradiated tripalmitin; C. authentic standard of compound **2**; and D. compound **2** in UV-C irradiated tristearin. Compounds **1** and **2** were eluted at 2.2 and 3.6 min, respectively.

further purified by a Milli-Q Ultra-Pure water purification system (Millipore, Billerica, MA) was used for the entire study.

2.2. Instrumentation

LC–MS/MS analyses were carried out on an 1100 HPLC system (Agilent, Santa Clara, CA) coupled with a 4000 QTRAP tandem mass spectrometer (Applied Biosystems, Foster City, CA). GC–MS analysis was performed on a 7890B GC system coupled to a 5977A MSD (Agilent, Santa Clara, CA). Ultra-performance liquid chromatography-fluorescence (UPLC–FLD) analysis was conducted on an Agilent 1260 HPLC system equipped with a fluorescence detector. High accuracy MS and MS/MS analyses were performed on a Xevo G2 Q-TOF LC–MS system with a standard electrospray ionization source operating in positive ionization mode (Waters, Milford, MA). UV-C (254 nm) or UV-A light (365 nm) generated by an 8-W UV light tube (Spectronics, Westbury, NY) was used for the sample irradiation. The irradiation dosages were calculated by applying the inverse square law onto the light intensity (UV-C: 450 μW/cm^2 at distance of 15 cm) as suggested by the manufacturer (Jagger, 1961; Okuno, Ojima, & Saito, 2001).

2.3. Physical treatment methods

Palmitic acid, stearic acid, tripalmitin, tristearin (1 mL of 1 mg/mL hexane solution in Eppendorf tube), corn oil and pork fat samples (1 g of sample in cylinder container with 1 cm^2 of surface area) were treated separately by the following procedures as described previously (Driffield et al., 2014; Ndiaye et al., 1999):

- Microwave treatment for 20 min, 800 W;
- Heating in an oven for 30 min at 90 °C;
- Sonication for 20 min, 120 W;
- UV irradiation (254 nm or 365 nm) for 2 h at 25 °C at distance of 3 cm.

While the treated fatty acid and triglyceride samples were directly dried under nitrogen and derivatized with HA using our previously developed method (Ye et al., 2013), the UV-C irradiated corn oil samples and the fat extracted from UV-C treated pork samples were cleaned-up by solid-phase extraction (SPE) prior to chemical derivatization and LC–MS/MS analysis for the presence of 2-ACBs (Ye et al., 2013).

2.4. LC–MS/MS Analyses

LC–MS/MS analysis was performed essentially as described previously (Ye et al., 2013), with minor modifications. In brief, 10 μL of the derivatized sample were injected into a reversed-phase HPLC column for chromatographic analysis. The column used was a 50 mm × 2.1 mm i.d., 2.7 μm, Poroshell 120 EC-C8 column (Agilent) eluted with a combination of 0.4% formic acid in water (**A**) and acetonitrile (**B**). Gradient elution at a constant flow rate of 600 μL/min was adopted for the chromatographic separation, with the proportion of solvent **B** increasing linearly from 50% to 100% in 10 min; held for another 5 min before being re-equilibrated to its initial conditions in 5 min.

The HPLC was coupled to a mass spectrometer equipped with a turbo V ion source operating in positive electrospray ionization

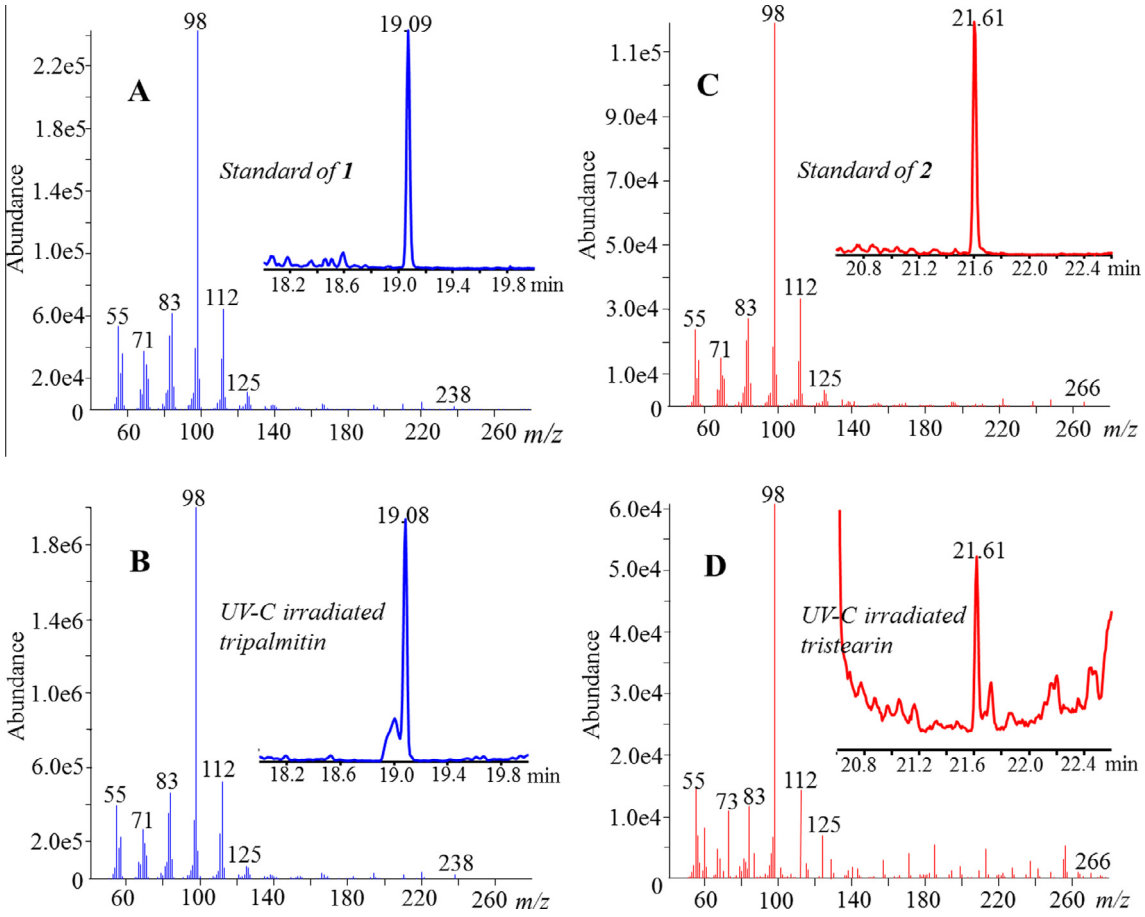

Fig. 3. Typical chromatograms and MS spectra obtained from GC–MS analysis of: A. authentic standard of compound **1**; B. compound **1** in UV-C irradiated tripalmitin; C. authentic standard of compound **2**; and D. compound **2** in UV-C irradiated tristearin. Compounds **1** and **2** were eluted at 19.1 and 21.6 min, respectively.

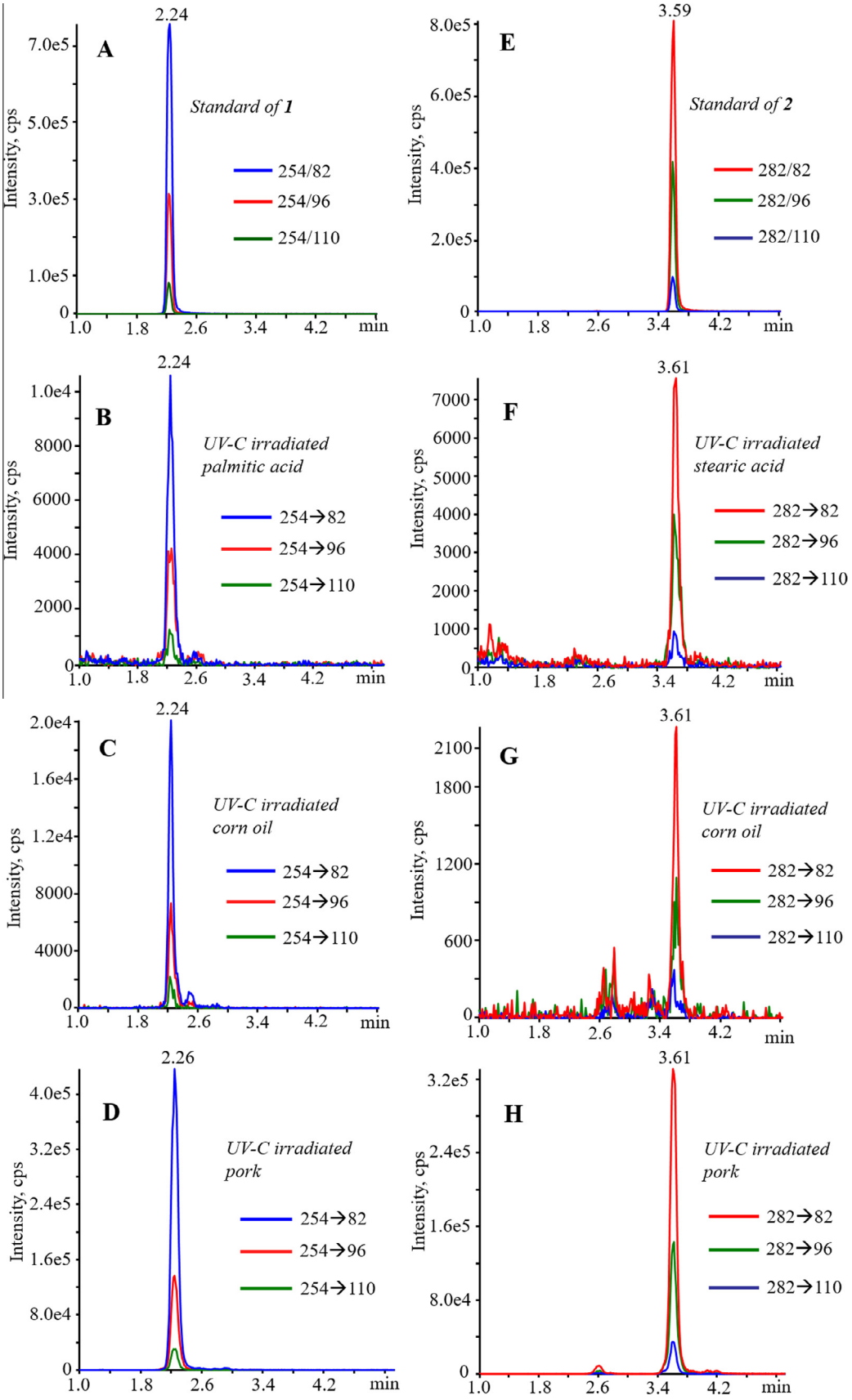

Fig. 4. Typical chromatograms obtained from LC–MS/MS analysis of: A. authentic standard of compound **1**; B. compound **1** in UV-C irradiated palmitic acid; C. compound **1** in UV-C irradiated corn oil; D. compound **1** in UV-C irradiated pork; E. authentic standard of compound **2**; F. compound **2** in in UV-C irradiated stearic acid; G. compound **2** in UV-C irradiated corn oil and H. compound **2** in UV-C irradiated pork; Compounds **1** and **2** were eluted at 2.2 and 3.6 min, respectively.

(ESI) mode. The following ESI parameters were used for the MS/MS analysis: ion spray voltage, 5500 V; declustering potential, 70 V; collision energy, 50; and entrance potential, 8. The ion source gas I (GSI), gas II (GSII), curtain gas (CUR), collision gas (CAD), and the temperature of GSII were set at 40, 20, 20, 5, and 400 °C, respectively. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with the following transitions: **1**: *m*/*z* 254/82, 254/96, and 254/110; **2**: *m*/*z* 282/82, 282/96, and 282/110; 2-2-(2-ethylhexyl)cyclohexanone internal standard: *m*/*z* 226/69. The dwell time for each transition was set at 50 ms. While the MRM transitions at *m*/*z* 254/82 and *m*/*z* 282/82 were used for quantitative analysis of compounds **1** and **2**, respectively, the other transitions (**1**: *m*/*z* 254/96, 254/110; **2**: *m*/*z* 282/96, 282/110) were used as qualifier transitions.

2.5. GC–MS analyses

The column used was a 30 m × 0.25 mm i.d., 0.25 μm film thickness, DB-5 ms column (Agilent) and was coupled with an MSD in electron ionization (EI) mode. The injector temperature was set to 250 °C. The GC oven temperature program was initially held at 60 °C for 1 min, ramped at 8 °C/min to 300 °C, and held for 5 min. A 2-μL aliquot of sample was injected into the instrument under splitless mode and the flow rate of the helium carrier gas was 1 mL/min. Both the transfer line and the ion source were set at 280 °C. MS data were acquired in mass scanning mode (*m*/*z* 40–280).

2.6. UPLC-FLD analysis

UPLC-FLD analysis was performed essentially as described previously (Meng et al., 2016). In brief, 10 μL of 1-NH derivatized samples were injected into the same HPLC column as described above for chromatographic separation. The column was eluted with a binary solvent of water as **A** and acetonitrile as **B**. the solvent gradient program began with 70% **B**, increased linearly to 100% **B** in 5 min, and then held for another 3 min before reconditioning at a constant flow rate of 0.7 mL/min at 50 °C. The detector excitation and emission wavelengths were set to 270 and 380 nm, respectively.

3. Results and discussion

3.1. Identification of 2-ACBs in triglyceride samples treated in different ways

Using GC–MS-based analytical methods, previous studies did not identify any detectable amounts of compounds **1** and **2** in foodstuff that have been heated by an oven or microwave oven, or by grilling or otherwise treated with UV-irradiation or ultrosonication (Driffield et al., 2014; Ndiaye et al., 1999). In line with the reported observations, our study using LC–MS/MS analysis showed no detectable amounts of compounds **1** or **2** in triglyceride samples subjected to the same treatment methods, except for the UV-C light (254 nm) irradiated samples. Specifically, our study using LC–MS/MS analysis revealed for the first time that compounds **1** and **2** were formed during UV-C irradiation of tripalmitin and tristearin samples, respectively. Through retention time matching and by calculating the peak area ratios of the quantifier and qualifier MRM transitions (Fig. 2), (Li, Campbell, Bennett, & Henion, 1996) it was possible to conclusively identify the presence of **1** and **2** in UV-C irradiated triglyceride samples. No 2-ACBs were detected in non-irradiated samples (Fig. S1).

The discrepancy between our current results and those reported previously might be attributed to various differences in experimental conditions. Firstly, the UV-treated triglyceride standard solutions used by Ndiaye et al. (1999) were prepared as an aqueous suspension, whereas hexane was used as the solvent in this study. The insolubility of triglycerides in water may explain the observed differences since chemical reactions are more likely to occur when the reactants are fully dissolved in a homogenous solution. Thus, the solution-phase molecules of triglycerides in this study would be more likely to generate 2-ACBs than the suspended particles of their crystals in water. Furthermore, it was generally believed that the transformation of free fatty acids to 2-ACBs by irradiation was initiated by the formation of a radical cation, followed by hydrogen abstraction and cyclization which lead to 2-ACBs formation (Sin, Wong, & Yao, 2006). However, water is highly reactive towards radicals, which may quench and inhibit the radical-induced formation of 2-ACBs (Chan et al., 2014). Perhaps the most significant improvement was to employ one of the most sensitive detection methods for 2-ACBs analysis, i.e. LC-MS/MS, with a detection limit of 0.18 ng/g fat (Ye et al., 2013), while the EN1785:2003 GC–MS method with 50 ng/g fat was the only approach used to detect 2-ACBs in the previous study (Tsutsumi et al., 2011). Moreover, compared with the 1 h irradiation time applied in the previous study, (Ndiaye et al., 1999) it is reasonable to anticipate that the 2 h irradiation period in our study would generate more 2-ACBs for easier detection. In fact, we have conducted experiments where the triglyceride samples were only exposed to UV irradiation for 1 h, but the results turned out that even our sensitive LC–MS/MS method could hardly distinguish the signal of 2-ACBs from the background. Hence it would be even more difficult for the less sensitive GC–MS employed by Ndiaye et al. (1999) to successfully capture the existence of 2-ACBs in aqueous suspension samples with 1 h irradiation time. Another important factor that may be overlooked is the distance between the positioning of the UV light source and samples. It may be noted that in our experiment the samples were placed 3 cm away from the UV source, whereas this distance was not specified in the study reported in the literature, (Ndiaye et al., 1999) and this may result in variation in radiation intensity affecting the formation of 2-ACBs.

The identification of compounds **1** and **2** in UV-C irradiated triglycerides is further validated by the GC–MS-based CEN standardization method (EN1785) and by using our recently developed UPLC-FLD method (Anon., 2003; Meng et al., 2016). Figs. 3 and S2 (Supplementary material) show typical chromatograms together with the MS spectra obtained from GC–MS and UPLC-FLD analyses of the triglyceride samples after 324 J/cm^2 (8 h) of UV-C irradiation. Confident identification was again achieved by comparing the retention time with that of authentic standards and by comparing the mass spectra. Using a similar approach, compounds **1** and **2** were also confidently identified in UV-C-irradiated fatty acid, corn oil and pork samples (Fig. 4). Fig. S3 (Supplementary material) presents the comparison of typical chromatograms of UV-C irradiated (324 J/cm^2) corn oil sample and untreated oil sample by using LC–MS/MS.

Interestingly, despite both UV-A and UV-C being non-ionizing radiations (Harm, 1980; Ng, 2003; Sommer et al., 2001), our study conclusively revealed the presence of 2-ACBs only in UV-C (254 nm) irradiated samples, but not in samples irradiated with the longer wavelength UV-A light (365 nm). This discrepancy could be attributed to the fact that the 254 nm radiation used in this study was nearer to the UV absorption bands of fatty acids (UV-C range), while UV-A light, e.g., 365 nm, was hardly absorbed (Fraser et al., 2011). Furthermore, it was reported that vegetable oil possessed a transmittance peak at around 260 nm (Anil Kumar & Viswanathan, 2013; Mitchell & Kraybill, 1941). As a result of being better absorbed and getting better transmittance in the corn oil, a higher percent of the shorter wavelength UV light might

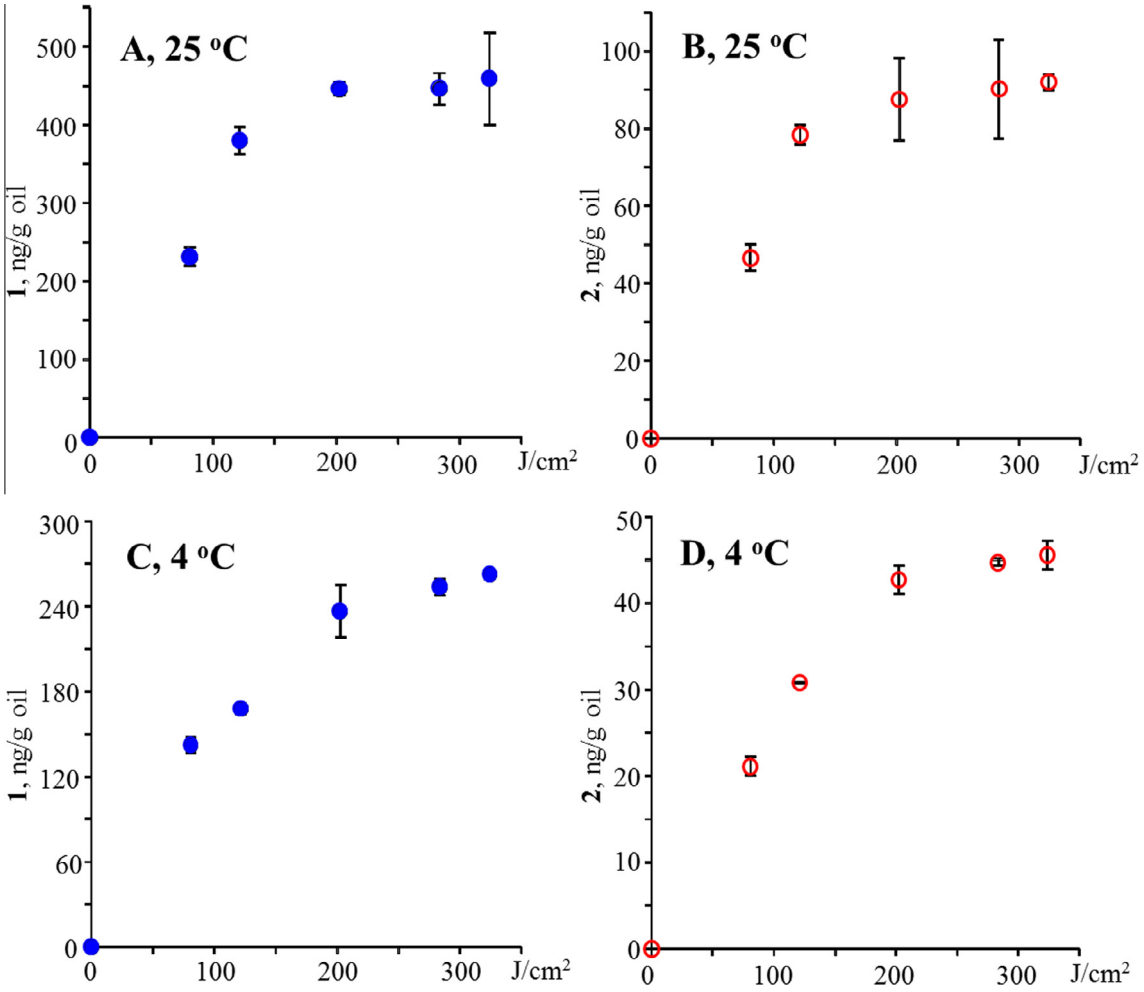

Fig. 5. Dose-dependent formation of compound **1** and **2** in UV-C irradiated corn oil samples. Irradiation performed at 25 °C and 4 °C.

Table 1
Effect of temperature on 2-dodecylcyclobutanone, 2-tetradecylcyclobutanone Formation in UV-C irradiated corn oil samples.

	2-ACBs yields (ng per gram of corn oil per J/cm²)		Molar ratio of **1**:**2**
	1	**2**	
25 °C	2.23	0.45	5.53
4 °C	1.20	0.20	6.56

have reached the fatty acid and triglyceride molecules, and subsequently ionized and converted them to 2-ACBs with the same formation mechanism as under ionizing irradiation (LeTellier & Nawar, 1972).

3.2. Irradiation dosage-dependent formation of 2-ACBs in UV-C irradiated corn oil samples

Having successfully identified compounds **1** and **2** in the UV-C irradiated samples, the study was extended to investigate the effect of irradiation dosage on the formation of compounds **1** and **2**. An UV-C irradiation dosage-dependent formation of **1** and **2** was observed in the LC–MS/MS analysis of corn oil samples that were being irradiated for an extended period from 2 to 8 h, which were subsequently converted into common used UV-radiation unit, J/cm², (Howitz et al., 2003) by multiplying time (h) with radiation intensity (μW/cm²). The result (Fig. 5) showed that varying the exposure dosage to UV-C irradiation induced a dosage dependent formation of compounds **1** and **2** in the lipid samples. Specifically, we observed a molar ratio of 5.53 for **1**:**2** (Table 1), which is in pretty good agreement with the detected molar ration (6.11) of the two precursor fatty acids, palmitic and stearic acids naturally present in the corn oil sample using a method reported by Sommer, Herscovitz, Welty, and Costello (2006), and the reported molar ratio (5.94) (Gunstone & Harwood, 2007; Shen, Duvick, White, & Pollak, 1999). This close relationship was also reported in the literature (Sin et al., 2006). The increasing generation trend of 2-ACBs with UV-C irradiation dosage was observed in fatty acids, triglycerides and pork samples (Fig. 6) as well.

3.3. Effect of temperature on the formation of 2-ACBs

After completing the investigation on the effect of irradiation dosage on the production of 2-ACBs, the study was extended to study the effect of temperature on 2-ACBs formation. To achieve this goal, we cooled the corn oil samples to ~4 °C in an ice bath during the irradiation experiment, the samples were processed and analyzed as described above. Once again, an irradiation dosage-dependent formation of compounds **1** and **2** was observed (Fig. 5). However, their measured levels were roughly 50% lower than those produced at 25 °C (Table 1). This phenomenon is most likely caused by the slowed reaction rate at reduced temperature. A similar observation of reduced production of 2-ACBs has also

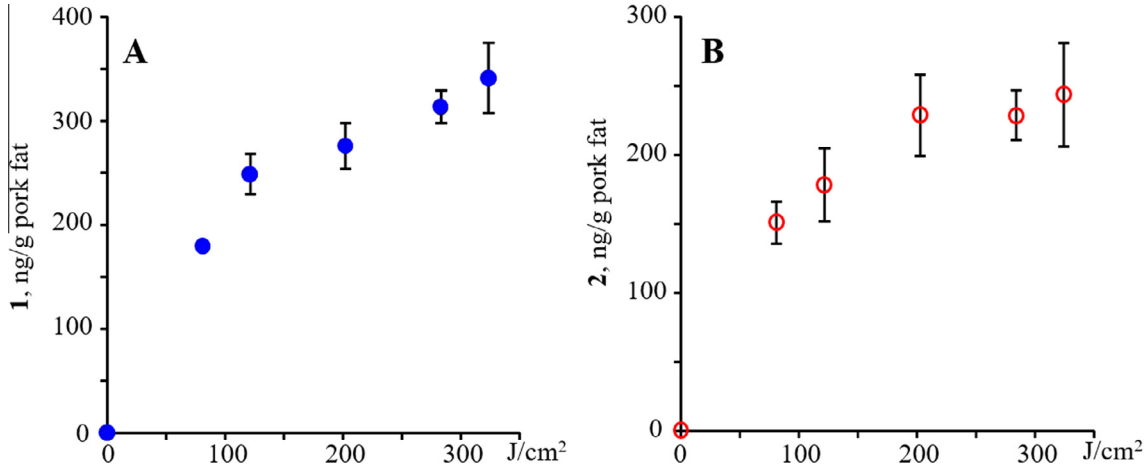

Fig. 6. Dose-dependent formation of compound **1** and **2** in UV-C irradiated pork samples.

been observed in previous studies using ionizing radiation (Obana, Furuta, & Tanaka, 2007; Stewart, Moore, Graham, McRoberts, & Hamilton, 2000).

4. Conclusion

Using a spectrum of state-of-the-art analytical instruments, our study revealed unambiguously for the first time that 2-ACBs were produced by a non-ionizing UV-C irradiation treatment method. Specifically, we demonstrated in this study that compounds **1** and **2** could be generated through exposure to UV-C irradiation, an increasingly common food germicidal procedure (Chun, Kim, Lee, Yu, & Song, 2010; Lyon, Fletcher, & Berrang, 2007). It is anticipated that the results from this study will alert food scientists and regulatory authorities to a potential new source of 2-ACBs in foodstuffs, arising without any exposure to high-energy ionizing radiation.

Notes

The authors declare no competing financial or non-financial interest.

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Appendix A. Supplementary data

Chromatograms from LC–MS/MS analyses of non-irradiated fatty acids, triglycerides, corn oil and pork samples. UPLC–FLD and high-mass accuracy MS characterization of 2-ACBs from UV-C irradiation of triglycerides. Comparison of LC–MS/MS analysis of UV-C-irradiated and control corn oil samples. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.08.127.

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