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Analytical Methods

Detection of virgin coconut oil adulteration with animal fats using quantitative cholesterol by GC × GC–TOF/MS analysis

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ABSTRACT

A new method based on the cholesterol level was developed to detect the presence of animal fats in virgin coconut oil (VCO). In this study, the sterols in VCO and animal fats was separated using conventional one-dimensional gas chromatography (1D GC) and comprehensive two-dimensional gas chromatography (GC × GC). Compared with 1D GC, the GC × GC system could obtain a complete baseline separation of the sterol trimethylsilyl ethers derived from cholesterol and cholestanol, so that the cholesterol content in pure VCO and false VCO adulterated with animal fats could be accurately determined. Cholesterol, a main sterol found in animal fats, represented less than 5 mg/kg of VCO. The study demonstrated that the determination of the cholesterol level in VCO could be used for reliable detection of the presence of lard, chicken fat, mutton tallow, beef tallow, or their mixture in VCO at a level as little as 0.25%.

1. Introduction

As a relative newcomer in the oil markets, virgin coconut oil (VCO) is rapidly becoming a type of oil as valuable as olive oil. VCO is obtained from fresh endosperm of mature coconut by wet processing (Hamid, Sarmidi, Mokhtar, Sulaiman, & Aziz, 2011). Unlike commercial coconut oil, VCO is unrefined and therefore not processed by deodorizing and bleaching. In this way, the natural volatile and chemical components in VCO are preserved, including the functional components like vitamin E, pro-vitamin A, and polyphenols (Dayrit et al., 2011). Several researches demonstrated that VCO was beneficial to human health, such as preventing nicotine dependence and relapse (Anggadiredja, Barlian, Pinang, & Anggraeny, 2011) and reducing the liver damage caused by administration of paracetamol (Zakaria et al., 2011). It was also found that VCO had the ability to increase high density lipoprotein (HDL) cholesterol and to decrease the levels of lipids, total cholesterol, triglycerides, phospholipids, low density lipoprotein (LDL) cholesterol, and very low density lipoprotein (VLDL) cholesterol in the serum and tissues (Nevin & Rajamohan, 2004). In addition, Nevin and Rajamohan (2006) also reported that VCO had the ability to increase the levels of antioxidant enzymes and prevent the peroxidation of lipids both *in vitro* and *in vivo* conditions due to vitamin E and polyphenol fractions in VCO. Owing to the pleasant flavor and beneficial biological activities, VCO currently commands a higher price than common vegetable oils and fats. Therefore, VCO has become a target for adulteration with lower-price oils and fats. In China, a lot of by-products from animal or poultry slaughterhouses, such as waste fat tissue, reticular connective tissue, and valueless skin, etc, were produced every year. All of these waste fat tissue were collected by unscrupulous merchants at a very low price. And then through rendering and refining, the low-cost animal fats were usually adulterated to edible oils, such as VCO, peanut oil, etc. In addition to animal fat adulteration, recycled cooking oil was also illegally adulterated into edible oils after re-refining. Possessing some similarities with VCO such as transparency to creamy white color and solid state at room temperature,

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the junk animal fats and re-refined deep-frying palm oil (usually contained a higher cholesterol level compared with normal plant oils) can be potential candidates to be used as oil adulterant in VCO. The practice of VCO adulteration with lard is not only a commercial fraud but a serious religious issue. From a religious perspective, Muslims were not allowed to consume any food containing lard. Therefore, there is a great demand for highly sensitive and reliable method for the detection of VCO adulteration with junk animal fats or recycled cooking oil.

In recent years, analytical methods have been reported for detection and quantification of adulterants in VCO, such as phosphorus-31 nuclear magnetic resonance (^{31}P NMR) (Dayrit, Buenafe, Chainani, & de Vera, 2008), electronic sensing (Marina, Che Man, & Amin, 2010), differential scanning calorimetry (DSC) (Mansor, Che Man, & Shuhaimi, 2012), and Fourier transform infrared (FTIR) spectroscopy (Rohman & Che Man, 2011; Rohman, Che Man, Ismail, & Hashim, 2010; Tengku Mansor, Che Man, & Rohman, 2011). Authentication methods applied to oils are generally classified as physical (non-separative) or chemical (separative) methods. The former techniques such as NMR, FTIR spectroscopy, DSC, and surface acoustic wave (SAW) sensor electronic nose, require a combination of measurements carried out on samples whereas the latter techniques focus simply on the presence or absence of a specific constituent. However, the physical techniques are usually low sensitive and not suitable for comprehensive practical applications. Hence, we attempt to develop a reliable, highly-sensitive, and accurate method to detect and quantify animal fats in VCO based on chromatography separation and quantification of specific constituents. According to the relevant reports (Abu-Tarboush & Dawood, 1993; Chizzolini, Zanardi, Dorigoni, & Ghidini, 1999; Russo, De Leonardis, & Macciola, 2005; Stajić, Živković, Perunović, Šobajić, & Vranić, 2011) and our previous study, cholesterol, which was a sterol found in high concentration in animal fats, was only present as traces in VCO. Therefore, cholesterol can be used as a marker to detect VCO adulteration with junk animal fats.

Due to its low concentration and the complexity of matrices, the separation and accurate quantification of the traced cholesterol in VCO were challenged. Cholesterol and phytosterols in fats and oils are usually analyzed by gas chromatography coupled with flame ionization detector (GC–FID) or GC–mass spectrometer (GC–MS) after they have been extracted and derived (Abidi, 2001). However, GC–FID is always suffered from the presence of co-elution of naturally occurring matrix-interfering compounds and may be insufficiently sensitive for cholesterol in trace levels. GC–MS, while being able to determine the presence of cholesterol from co-eluting compounds, cannot always accurately quantify its amounts. Increased resolving power is desirable, but conventional one-dimensional gas chromatography (1D GC) is unlikely to offer satisfactory separation of closely-eluting compounds. With the further development of chromatography techniques, comprehensive two-dimensional gas chromatography (GC × GC) has become a powerful analytical tool in unraveling the composition of complex samples (Dallüge, Beens, & Brinkman, 2003). It has been proved to be advantageous over conventional 1D GC in terms of peak capacity (separation power), signal-to-noise ratio, sensitivity, resolving power, and it could also offer unique structured chromatograms when structurally-related classes of substances (analogs, congeners, and isomers) were analyzed (Adahchour, Beens, & Brinkman, 2008).

In this paper, we have performed the separation of sterol trimethylsilyl ethers derived from cholesterol and cholestanol in VCO samples, using GC × GC coupled with time-of-flight mass spectrometry (TOF/MS). The purpose of this study is to separate and quantify the cholesterol in VCO at a specific level to evaluate the VCO purity.

2. Materials and methods

2.1. VCO preparation

VCO was prepared according to the method reported by Hamid et al. (2011) with slight modification. The fresh endosperm of mature coconut was grated and made into viscous slurry and then squeezed using a cheese cloth to obtain coconut milk. The coconut milk was chilled to 10 °C to break the emulsion and then centrifuged at 830*g* for 5 min to obtain raw coconut cream, skim milk, and some proteins or solid components. The coconut cream was kept at 4 °C for 24 h and then subjected to mild heating (65 °C) in a thermostat oven until the cream temperature reached 65 °C, followed by centrifugation (2306*g* for 10 min) to separate non-oil fractions from the oil phase. Finally, the raw oil was filtered through Whatman filter paper to obtain VCO. The obtained VCO was labeled VCO-1 and used for the adulteration study.

The other five VCO samples were purchased from Wuhan local markets (Wuhan, China) and labeled VCO-2 (Production place: Hainan province, China), VCO-3 (Manufacturer: Peter Paul Philippine Corporation), VCO-4 (Original production place: Sri Lanka), VCO-5 (Manufacturer: Earth Born Co., Ltd. in Thailand), and VCO-6 (Brand: Nature's Way; Production place: USA), respectively.

2.2. Preparation of animal fats

Animal fats, namely lard (LD), chicken fat (CF), beef tallow (BF), and mutton tallow (MT), were prepared according to the method described by Rohman and Che Man (2009) with slight modification. The adipose tissues collected from various parts of the corresponding animals were rendered in the oven at 115 °C for 2 h. The melted fat was strained with a triple-folded muslin cloth, dried by addition of anhydrous sodium sulfate (Na_2SO_4), and then centrifuged at 2306*g* for 10 min. The fat layer was decanted and filtered through Whatman filter paper. The filtered samples were used for further analysis. The VCO and animal fats were stored in darkness at 4 °C and melted at 60 °C prior to use.

2.3. Preparation of blends

Binary mixtures of the VCO and LD (CF, BT, or MT) were prepared according to the percentage of the LD (CF, BT, or MT) in VCO, that is, 0.1%, 0.25%, 0.5%, 1%, 2%, and 4% (w/w) of the LD in binary mixtures. To ensure homogeneous state of binary mixture, 0.5 g of VCO, LD, CF, BT, and MT were dissolved in 10 mL n-hexane, and vortexed for 3 min at 2000 rpm, respectively. Then 4.995 mL of VCO and 5 μL of LD (CF, BT, or MT) solutions were mixed together, and vortexed for 3 min at 2000 rpm. As a result, 0.1% LD (CF, BT, or MT) binary mixture solution was obtained, whose total concentration was 0.05 g/mL. In the same way, the other concentration binary mixtures could also be prepared. Multiple mixtures of the VCO and animal fats were prepared according to the same percentage of the total animal fats in VCO, namely, 0.1%, 0.25%, 0.5%, 1%, 2%, and 4% (w/w) of the animal fats in multiple mixtures. Considering the actual production and consumption of LD, CF, BT, and MT in China, the used animal fats were the mixture of LD, CF, BT, and MT at a ratio of 5:3:2:1 according to their weights. To ensure homogeneous state of the mixed animal fats, the mixture was melted at 60 °C, and vortexed for 3 min at 3000 rpm. The subsequent procedures for the preparation of the multiple mixtures were the same as that of the binary mixtures.

2.4. Reagents and solvents

Cholesterol (3β-cholest-5-en-3-ol, with purity of 99%) was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), and cholesta-

nol (5α-cholestan-3β-ol, of the analytical standard) and N-Methyl-N-trimethylsilylheptafluorobutyramide (MSHFBA) were purchased from Sigma-Aldrich, Chemie Gmbh (Steinheim, Germany). 1-methylimidazole was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Normal hexane (HPLC grade), diethyl ether (analytical grade), and anhydrous sodium sulfate (analytical grade) were purchased from Merck (Darmstadt, Germany), and the Sep-Pak cartridges (0.5 g Silica) were obtained from Dikma Technologies Inc. (Beijing, China).

2.5. Sterol purification by solid-phase extraction (SPE)

According to the Dikma's instructions, about 1.5 g anhydrous Na_2SO_4 was loaded onto the Sep-Pak cartridge. Fifty milligrams of VCO (spiked VCO, animal fats, binary admixtures, or multiple admixtures described above) had cholestanol used as an internal standard by adding 1 μg cholestanol to VCO, spiked VCO, binary admixtures, and multiple admixtures samples and adding 50 μg cholestanol to animal fat samples. The VCO (spiked VCO, animal fats, binary admixtures, or multiple admixtures) were dissolved in 5 mL n-hexane and then loaded onto the cartridge, which was first equilibrated with 10 mL n-hexane at the flow speed of 1.5 mL/min, with the effluent discarded. Triglycerides on the cartridge were washed off with 10 mL mixture of n-hexane: ethyl ether (95:5, v/v), at the flow speed of 1.5 mL/min. Finally, cholesterol and cholestanol were eluted with 25 mL n-hexane: ethyl ether (90:10, v/v), at the flow speed of 1.5 mL/min.

2.6. Derivatization procedures

Trimethylsilyl ether derivatives of sterols were prepared according to ISO 12228: 1999. The final eluted fractions (25 mL) containing cholesterol and cholestanol were rotary-evaporated to approximately 1 mL under vacuum at 50 °C, and then the concentrated solution was transferred to a reaction vial, which was dried by a gentle nitrogen flow, then added with 100 μL N-Methyl-N-trimethylsilylheptafluorobutyramide: 1-methylimidazole (95:5, v/v). After that, the vial was sealed and heated at 105 °C for 15 minutes, and then cooled to room temperature for GC analysis.

2.7. Analytical conditions

2.7.1. GC–FID condition

1D-GC analysis was performed on an Agilent 7890A GC coupled with a FID (Agilent Technologies, USA). A 30 m DB-5 column (0.25 mm I.D.; 0.25 μm film thickness, (5%-Phenyl)-methylpolysiloxane; Agilent Technologies) was used with a temperature program of the initial 200 °C, which was held for 1 min, ramped at 15 °C/min to 315 °C, and finally held for 25 min. The injection volume was 2 μL in splitless mode for silylated sterols obtained from VCO, spiked VCO, binary mixture, and multiple mixture samples, while the injection volume for animal fat samples was 1 μL in split mode, at a ratio of 50:1. Nitrogen was used as the carrier gas at a flow-rate of 0.7 mL/min, and the temperature of the injector and detector was kept at 320 °C.

2.7.2. GC–MSD condition

1D-GC analysis was also performed on an Agilent 7890A GC equipped with an Agilent 5975C mass selective detector (MSD). The analytes were separated using a DB-5 ms column (30 m × 0.25 mm I.D. × 0.25 μm film thickness, Phenyl Arylene polymer; Agilent Technologies). The temperature program was that the initial 200 °C was held for 1 min, increased to 315 °C by a ramp of 15 °C/min, and finally held for 25 min. The injection volume and mode were the same as that described in Section 2.7.1. Helium was used as the carrier gas at a flow-rate of 0.7 mL/min. The temperature of the injector, transfer line, and ion source was set to 320 °C, 300 °C, and 250 °C, respectively. The mass scan range was 50−550 *m/z*.

2.7.3. GC × GC–TOF/MS conditions

As GC × GC–TOF/MS, a LECO Corporation Pegasus 4D instrument (LECO Corp., St. Joseph, MI, USA) equipped with an Agilent 7890A GC, which contained a primary oven and a separate secondary oven (Agilent Technologies, Santa Clara, CA, USA) was used in 2D-GC analysis. The column-set for the GC × GC analysis consisted of two columns. The 1D column was a 30 m DB-5 ms (0.25 mm I.D. × 0.25 μm film thickness, Phenyl Arylene polymer), and the 2D column was a DB-17ht with dimensions of 1.5 m × 0.10 mm I.D. × 0.15 μm film thickness, (50%-Phenyl)-methylpolysiloxane (Agilent Technologies). The injection volume and mode were the same as that described in 2.7.1, with the injector temperature being 320 °C. Helium was used as the carrier gas at a constant flow rate of 0.7 mL/min. The primary oven temperature program was that 200 °C held for 1 min and then increased to 315 °C at a rate of 15 °C/min, with the final temperature held for 25 min; and the secondary oven followed the primary oven with a lead of 5 °C. The modulator temperature offset and transfer line temperature were 35 °C and 300 °C, respectively. The modulation period was 2 s, and the hot pulse time and cool time between stages were set to 0.8 s and 0.2 s, respectively. The mass spectrometer was operated at an acquisition rate of 100 spectra/s and scanned from 50 *m/z* to 550 *m/z*. No mass spectrum was collected during the solvent delay for the first 10 min of each run. The detector voltage was set to 1750 V, and the electron energy was −70 V. The ion source temperature was kept at 250 °C. For quantification of the peak area, the quantitative masses were 247 + 329 + 368 + 458 for cholesterol trimethylsilyl ether (Che-TME), and 215 + 306 + 445 + 460 for cholestanol trimethylsilyl ether (Cha-TME). The data was processed using LECO Corp's Chromatography TOF software version 4.43.3.0 optimized for Pegasus 4D.

2.8. Statistical analysis

The experiment was carried out in quintuplicate and expressed as mean ± standard deviation. The obtained results were subjected to statistical analysis using the program Statistical Package for Social Science (SPSS) ver.13.0 for Windows (LEADTOOLS, LEAD Technologies, Inc., 2004). Data calculated and determined for cholesterol of the samples used in the present study were analyzed by a paired samples *t*-test.

3. Results and discussion

3.1. Comparison of 1D GC and GC × GC

In this study, we performed the separation of sterol trimethylsilyl ethers, aiming to successfully separate Che-TME and Cha-TME, so that the content of cholesterol in edible oils and fats could be determined accurately. The mixed standard of Che-TME and Cha-TME, both of 1 μg/100 μL, was used for 1D GC and GC × GC analyses. The injection volume was 2 μL in splitless mode, and other analytical conditions were described in 2.7 of this text. Fig. 1 presents the partial gas chromatograms of the standard Che-TME and Cha-TME by using 1D GC–FID, 1D GC–MSD, and GC × GC–TOF/MS. According to the chromatograms in Fig. 1(A and B), we can see that two major peaks dominate both chromatograms. It shows that the separation of Che-TME and Cha-TME was partially achieved, but complete baseline separation could not be achieved. Fig. 1(C) shows the chromatogram obtained by GC × GC–TOF/MS using a 2 s modulation period for the cryogenic trap and 0.8 s for hot pulses, indicating complete separation between Che-TME and Cha-TME. The same result can also be demonstrated

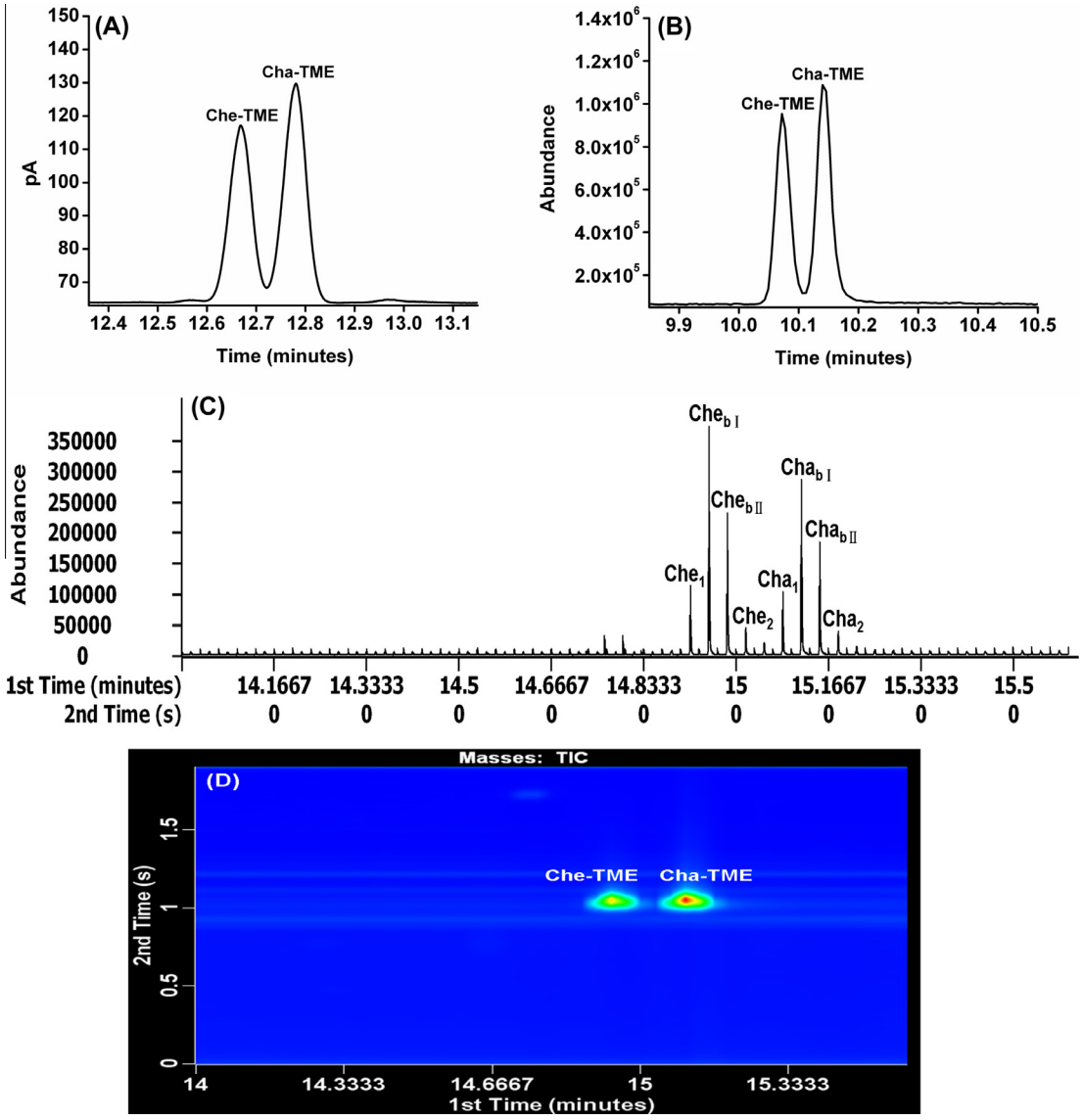

Fig. 1. Chromatograms of the Che-TME and Cha-TME standards by GC–FID (A), GC–MSD (B), and GC × GC–TOF/MS (C and D). The peaks Che_{bI} and Che_{bII} are base peaks for Che-TME, and Cha_{bI} and Cha_{bII} for Cha-TME; the peaks Che_1 and Che_2 are sub-peaks for Che-TME, and Cha_1 and Cha_2 for Cha-TME.

by Fig. 1(D), with the contour plot obtained by the GC × GC system. Using GC × GC–TOF/MS, Che-TME and Cha-TME were separated into 4 peaks: CHe_1, CHe_{bI}, CHe_{bII}, and CHe_2 for Che-TME, and Cha_1, CHa_{bI}, CHa_{bII}, and CHa_2 for Cha-TME, respectively.

The structures of Che-TME and Cha-TME are very similar, so the complete baseline separation of the two compounds cannot be achieved by 1D GC. As the chromatographic conditions were essentially the same for 1D separation and ^1D in the GC × GC system, it could be reasoned that partial peak co-elution occurred in the first column in GC × GC, and the second ^2D weak-polar column resolved the clustering of the two compounds. Therefore, GC × GC–TOF/MS was selected to analyze and quantify the cholesterol content in VCO and animal fat samples. The four peaks (CHe_1, CHe_{bI}, CHe_{bII}, and CHe2 for Che-TME, and Cha_1, CHa_{bI}, CHa_{bII}, and CHa_2 for Cha-TME) obtained by GC × GC separation were selected for the quantitative analysis of cholesterol.

3.2. Repeatability and recovery test

In order to evaluate the precision and accuracy of the analytical method for the determination of the cholesterol content in oils and fats, a repeatability and recovery test ($n = 5$) was carried out. According to the relevant literature and our previous study, the cholesterol concentration in the VCO samples was less than 5 mg/kg, and the level of cholesterol in animal fats, such as LD, CF, BT, or MT, ranged from 300 mg/kg to 2000 mg/kg (Abu-Tarboush & Dawood, 1993; Chizzolini et al., 1999; Russo et al., 2005; Stajić et al., 2011). In our study, the adulteration percentage of animal fats in VCO was set to 0.1% to 4%, so the cholesterol content in adulterated VCO samples could change in gradient, varying approximately from 3.3 mg/kg to 84.8 mg/kg. Therefore, we spiked the VCO samples with standard cholesterol at the levels ranging from 2 mg/kg to 64 mg/kg, and the repeatability and recovery test on cholesterol ($n = 5$) was performed to verify precision and accuracy of the GC × GC–TOF/MS method. The GC × GC system was calibrated by injecting six silylated standard cholesterol solutions at the concentration of 0.1, 0.2, 0.4, 0.8, 1.6, and 3.5 μg/100 μL in silylating agents, and each silylated standard cholesterol solution contained 1 μg silylated standard cholestanol. A linear relationship was obtained between the silylated cholesterol concentration and the value calculated from the peak area of the silylated cholesterol divided by the peak area of the silylated cholestanol over the

entire concentration range. The correlation coefficient was 0.99990.

The cholesterol contents in non-spiked and spiked VCO samples were determined and the results were listed in Table 1. The relative standard deviation (RSD) of the repeatability for the determination of the cholesterol content by GC × GC–TOF/MS ranges from 2.49% to 7.14%, which indicates that the precision of the analytical method is acceptable over the whole concentration range. It also shows the mean recovery rate, ranging from 89.97% to 100.92% with a standard deviation less than 10.96%, which demonstrates that the accuracy of the GC × GC–TOF/MS method is reliable. Therefore, the comprehensive analytical method could be suitable for the detection of VCO adulteration with animal fats using quantitative cholesterol.

3.3. Determination of the cholesterol content in VCO and animal fats

Animal fats have high cholesterol content, while in plant oils the levels of cholesterol are very low. To be able to validate the method of adulteration detection, the cholesterol content in VCO and animal fats used in the study must be accurately determined using GC × GC–TOF/MS. Because of high level of cholesterol in animal fats, the GC × GC system was recalibrated by injecting five silylated standard cholesterol solutions at the concentration of 10, 20, 40, 60, and 80 μg/100 μL in silylating agents, and the added cholestanol amount was 50 μg. In GC × GC–TOF/MS analysis, the volume of 1 μL was injected in split mode, at a ratio of 50:1, and other analysis conditions were according to Section 2.7.3 in this text. In this way, a new calibration equation was obtained with the correlation coefficient being 0.99949.

Fig. 2 shows the partial chromatograms of Che-TME and Cha-TME obtained by a conventional GC–FID, GC–MSD, and GC × GC–TOF/MS. It was found that Che-TME and Cha-TME were not completely separated from VCO and LD using 1D GC, as represented by the chromatogram data in Fig. 2(A and B) and (D and E), respectively. Similar results were obtained in CF, MT, and BT samples. This result in Fig. 2(A and B) indicated that the peak profiles of Che-TME and Cha-TME in VCO could be discriminated by their shapes, with the peak of Che-TME showing a smoother shape. It implied that the quantitative determination of cholesterol in the VCO samples by 1D GC was difficult to achieve due to the relatively poor separation. Although in Fig. 2(D and E), the peak profiles of Che-TME and Cha-TME in LD in the same region were sharp, dominating the chromatograms, but the complete baseline separation of the two compounds could not be achieved. However, Fig. 2(C and F) showed that the baseline separation of Che-TME and Cha-TME obtained from VCO and LD could be achieved by GC × GC–TOF/MS, as the intensity of the individual peaks was different due to the different concentration of the two compounds. In this case, the cholesterol content in LD and other animal fats could be determined by using 1D GC. However, to obtain complete separation of the two compounds by GC × GC–TOF/MS, the latter method was chosen in our study for more accurate quantitative results of cholesterol.

The levels of cholesterol in VCO and animal fats used in this study have been determined by GC × GC–TOF/MS, as shown in Table 2. The concentration of cholesterol in VCO, ranging from 3.48 (± 0.22) mg/kg to 4.19 (± 0.13) mg/kg, was very low compared with that in animal fat samples, and the slight difference in the cholesterol content among different VCO samples may be caused by the difference of tree species, growth environment, and maturity level of coconut. To date, the cholesterol content in VCO reported by other authors was very little. On the other hand, the four possible adulterated fats were rich in cholesterol. Specially, BT had the highest absolute content of cholesterol, reaching 1721.22 (± 39.72) mg/kg, and the levels of cholesterol in LD, CF, and MT were 652.48 (± 17.06) mg/kg, 701.42 (± 15.67) mg/kg, and 714.60 (± 23.07) mg/kg, respectively. As far as the cholesterol content in animal fats concerned, there are more related literatures. Russo et al. (2005) reported that the cholesterol content in LD (commercial sample) was 830–860 mg/kg, which was higher than that found in our samples. Other data of cholesterol in pork fats was mainly about the cholesterol distribution and its content in different adipose tissues. For example, the data reported by Lan, Mckeith, and Novakofski (1993) revealed that the cholesterol contents in subcutaneous, intermuscular, and internal fats were around 1000 mg/kg, similar to 1160 mg/kg (mean value) in the dewlap reported by Dorado, Martín Gómez, Jiménez-Colmenero, and Masoud (1999), while the values in back fat tissues ranging from 355 mg/kg to 472 mg/kg were recently observed by Stajić et al. (2011). Although the cholesterol contents in different adipose tissues could not be directly compared with that in our LD sample, the internal correlation between them was strong, because LD used in the study was rendered from the adipose tissues mixed with various parts of pork. Overall, the cholesterol content in our LD sample is within a reasonable range. Compared with pork and poultry, beef fats have a higher level of cholesterol accumulated in the fat tissues, which has been confirmed by our research data (Table 2). A similar conclusion was achieved by Abu-Tarboush and Dawood (1993), who found a mean cholesterol content (about 2080 mg/kg) from various beef fats (rendered from different adipose tissues), higher than that found in our BT sample. In addition, our result of the cholesterol content in CF is also comparable to that reported by Chizzolini et al. (1999), which ranged from 614 mg/kg to 940 mg/kg. But as for the cholesterol content in MT, the data from different literatures has a significant difference,

Table 1
The repeatability and recovery rates of the GC × GC–TOF/MS method for the detection of cholesterol in VCO samples.

Sample	Spiking value (mg/kg of VCO)	Determined value range[a] (mg/kg of VCO)	Mean[b] ± SD[c] (mg/kg of VCO)	RSD[d] (%)	Recovery[e] range (%)	Mean recovery ± SD (%)
VCO-1	0	3.66–4.28	4.08 ± 0.24	5.88		
	2	5.69–6.24	5.88 ± 0.22	3.73	80.58–108.18	89.97 ± 10.96
	4	7.60–8.14	7.93 ± 0.20	2.49	88.19–101.49	96.34 ± 4.94
	8	11.25–12.97	11.85 ± 0.73	6.15	89.65–111.19	97.16 ± 9.11
	16	19.02–22.15	20.22 ± 1.44	7.14	93.37–112.95	100.92 ± 9.02
	32	34.63–36.85	35.55 ± 0.93	2.62	95.49–102.41	98.35 ± 2.91
	64	61.66–72.04	66.69 ± 4.08	6.11	89.97–106.20	97.84 ± 6.37

[a] Values represent the cholesterol level found in VCO samples, only show the minimal and maximum levels of the five independent analyses.
[b] Values given are the average of five replicate analyses.
[c] SD = standard deviation.
[d] RSD = relative standard deviation.
[e] Recovery rates were calculated by dividing the difference of cholesterol level between spiked and the pure VCO-1 samples by the corresponding addition of standard cholesterol; the mean cholesterol level of VCO-1 was used as the background value in all calculation.

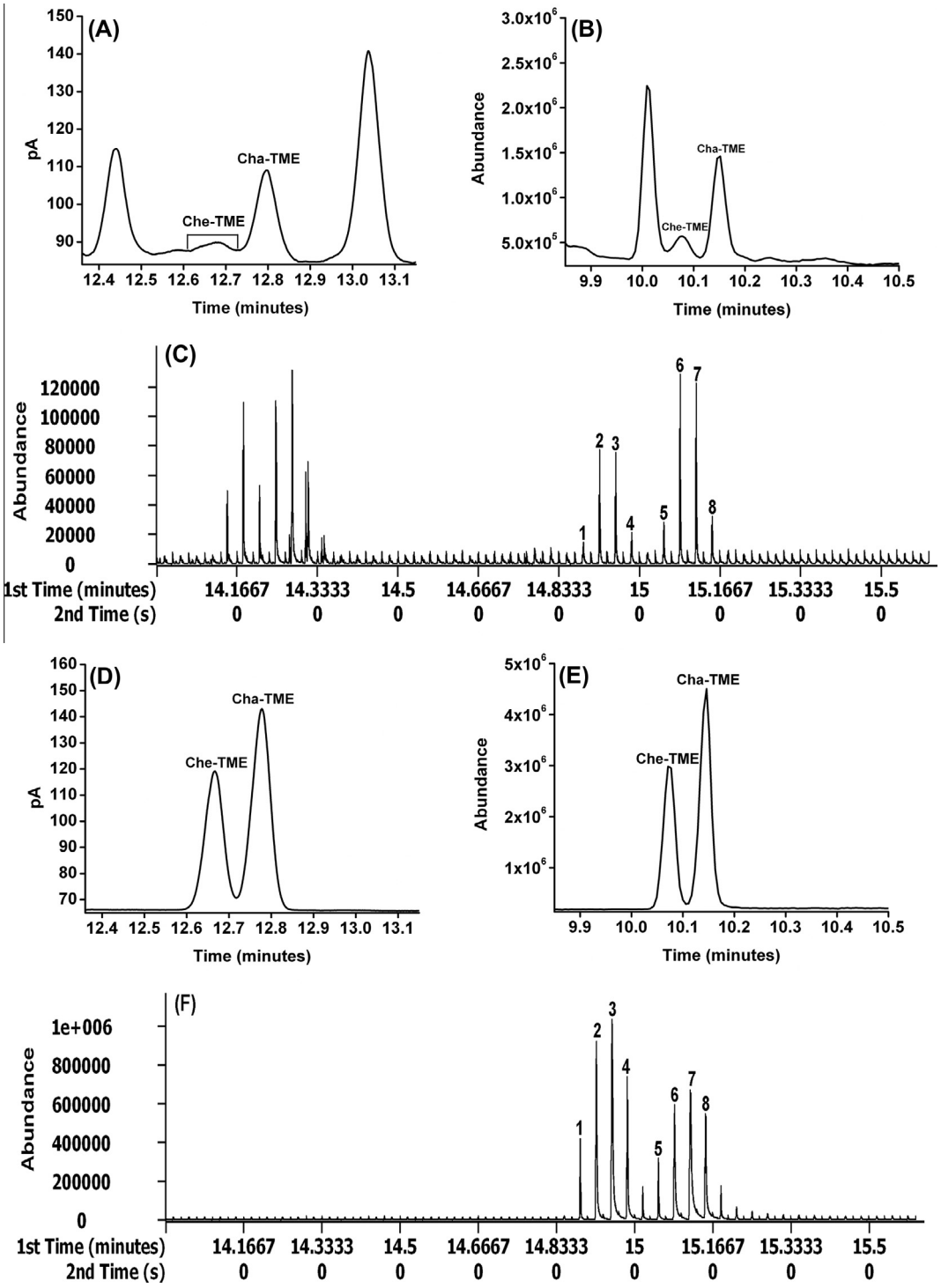

Fig. 2. Partial chromatograms of the Che-TME and Cha-TME in VCO and LD samples. (A) VCO by GC–FID, (B) VCO by GC–MSD, (C) VCO by GC × GC–TOF/MS, (D) LD by GC–FID, (E) LD by GC–MSD, (F) LD by GC × GC–TOF/MS. 1: Che_1, 2: CHe_{bI}, 3: CHe_{bII}, 4: CHe_2, 5: Cha_1, 6: CHa_{bI}, 7: CHa_{bII}, 8: CHa_2.

ranged from 750 mg/kg (in intermuscular fats) to 1471 mg/kg (in mixed adipose derived form shoulders, ribs, loin, and legs) (Abu-Tarboush & Dawood, 1993; Chizzolini et al., 1999), which were higher than our research data. The difference of the cholesterol content between our results and the data reported by other authors may be caused by the difference of the animal breed, age, sex, and the sampling position of fats. In Table 2, it also clearly shows the RSD values of the repeatability by GC × GC–TOF/MS ranging from 2.23% to 7.22%, which indicates that the analytical method is accurate for the determination of the cholesterol in VCO and animal fat samples.

3.4. Detection of adulteration in VCO with animal fats

To validate the adulteration detection method, the cholesterol level of all studied oil and fat samples was determined using GC × GC–TOF/MS. Since the false VCO samples were deliberately "adulterated" by the animal fats of a known cholesterol level, the

Table 2
Ranges of the cholesterol content in studied oils and animal fats.

Oil	Cholesterol level (mg/kg of oil or fat)		
	Range of cholesterol content[a]	Mean[b] ± SD	RSD (%)
VCO-1	3.66–4.28	4.08 ± 0.24	5.88
VCO-2	3.82–4.37	4.04 ± 0.21	5.28
VCO-3	3.23–3.70	3.48 ± 0.22	6.48
VCO-4	4.00–4.30	4.19 ± 0.13	3.14
VCO-5	3.46–4.12	3.85 ± 0.28	7.22
VCO-6	3.89–4.54	4.15 ± 0.26	6.35
LD	632.87–672.09	652.48 ± 17.06	2.61
CF	680.43–715.72	701.42 ± 15.67	2.23
MT	687.98–737.01	714.60 ± 23.07	3.23
BT	1673.16–1758.21	1721.22 ± 39.72	2.31

[a] Values represent the cholesterol level found in the VCO and animal fat samples used in the present study, only show the minimal and maximum levels of the five independent analyses.
[b] Values given are the average of five replicate analyses.

expected level of cholesterol in the mixture could be calculated to certify the accuracy of the analytical technique. Table 3 lists the cholesterol levels (determined and calculated) for the VCO blends with 0.1%, 0.25%, 0.5%, 1%, 2%, and 4% of the adulterated fats.

In this study, a paired samples t-test was conducted to compare the levels of cholesterol in determined and calculated conditions, as shown in Table 3. There was no significant difference between the determined group ($M = 16.53$, SD = 15.21) and the calculated group ($M = 16.43$, SD = 15.13); t (29) = 0.613, $p = 0.544$, $\alpha = 0.05$. The results indicated that the GC × GC–TOF/MS was a reliable and accurate method for determining both low cholesterol levels in pure VCO and adulterated VCO and high cholesterol levels in pure animal fats. Specifically, the results indicated that this method could be used to detect VCO adulteration with animal fats accurately by quantitative analysis of cholesterol. According to the relevant literature and our study, the maximum level of cholesterol in VCO was 4.19 (± 0.13) mg/kg (VCO-4), so we considered that 5 mg/kg was the highest acceptable level of cholesterol in pure VCO. For the blending carried out using BT, the cholesterol level above 5 mg/kg was detected at the adulteration level of 0.1% as anticipated from the calculated cholesterol value. For low levels of cholesterol containing fats relative to BT, the method allowed establishment of a 99.75% purity label in the case of LD, CF, and MT. Similar results were obtained from the mixture with these four types of animal fats at a specific mixing ratio. Therefore, our study suggested that the cholesterol level is a valid parameter that could be used to detect the adulteration of VCO with animal fats at a level as low as 0.25%. The detection limit of LD adulterated in VCO samples was lower than that obtained by a fast GC–SAW detector system, whose detection limit was 1% (Tengku Mansor et al., 2011).

The linear calibration curves obtained by plotting the percentages of the added animal fats versus the cholesterol contents (Fig. 3) show the possible rough estimation on the extent of VCO adulteration with the four types of animal fats and their mixture (LD, CF, BT, and MT at a ratio of 5:3:2:1 according to their weight) at levels equal to or higher than 0.25% by their regression equations, which can be represented as follows:

$$\text{Added fat } (\%) = (\text{cholesterol level} - a)/b$$

where a and b are the regression equation constants, and the unit of the cholesterol level is mg/kg. These constants are different according to the linear calibration curves of the added fats. As a result, the added LD, CF, MT, BT, and a mixture of the four animal fats can be calculated from their regression equations as follows:

$$\text{LD } (\%) = (\text{cholesterol level} - 3.9397)/7.0385 \qquad R^2 = 0.9998$$

$$\text{CF } (\%) = (\text{cholesterol level} - 4.087)/7.1912 \qquad R^2 = 0.9971$$

$$\text{MT } (\%) = (\text{cholesterol level} - 4.3582)/6.836 \qquad R^2 = 0.9984$$

$$\text{BT } (\%) = (\text{cholesterol level} - 3.7558)/17.089 \qquad R^2 = 0.9979$$

$$\text{Mixture } (\%) = (\text{cholesterol level} - 3.7459)/9.8903 \qquad R^2 = 0.9981$$

Table 3
Determined[a] (dete.) and calculated[b] (calc.) contents of cholesterol in mixtures[c] of VCO-1 and studied animal fats.

VCO-1 (w/w, %)	Cholesterol level (mg/kg of oil)					
	LD		CF		BT	
	Dete.	Calc.	Dete.	Calc.	Dete.	Calc.
100	4.08 ± 0.24		4.08 ± 0.24		4.08 ± 0.24	
99.9	4.55 ± 0.43	4.72	4.38 ± 0.20	4.77	5.68 ± 0.49	5.79
99.75	5.76 ± 0.30	5.70	5.96 ± 0.33	5.82	9.19 ± 0.23	8.37
99.5	7.30 ± 0.30	7.32	7.70 ± 0.50	7.56	12.68 ± 0.56	12.66
99	11.12 ± 0.68	10.56	12.28 ± 0.65	11.05	19.30 ± 0.90	21.25
98	17.87 ± 0.71	17.04	17.64 ± 1.23	18.02	36.34 ± 2.82	38.42
96	32.15 ± 2.04	30.01	33.02 ± 1.44	31.97	73.17 ± 1.65	72.76
0	652.48 ± 17.06		701.42 ± 15.67		1721.22 ± 39.72	

	MT		Mixture of LD:CF:BT:MT (5:3:2:1)	
	Dete.	Calc.	Dete.	Calc.
100	4.08 ± 0.24		4.08 ± 0.24	
99.9	4.78 ± 0.35	4.79	4.88 ± 0.39	5.02
99.75	6.26 ± 0.28	5.85	6.67 ± 0.39	6.45
99.5	7.52 ± 0.56	7.63	8.91 ± 0.43	8.82
99	12.01 ± 0.50	11.18	12.80 ± 0.40	13.56
98	17.96 ± 0.78	18.29	22.60 ± 0.72	23.04
96	31.56 ± 2.02	32.50	43.92 ± 2.95	42.01
0	714.60 ± 23.07		952.52 ± 28.55	
t-test[d]	$M_1 = 16.53$, $SD_1 = 15.21$, $M_2 = 16.43$, $SD_2 = 15.13$; t (29) = 0.6, $p = 0.54$, $\alpha = 0.05$			

[a] Values given are the average of five independent analyses ± SD.
[b] Values given are calculated according to the percentage of studied animal fats adulterated in VCO-1.
[c] Mixtures of VCO-1 and studied animal fat at concentrations of 99.9%, 99.75%, 99.5%, 99, 98%, and 96% (VCO vs. studied animal fats).
[d] A paired samples t-test, M_1 and SD_1 are the mean cholesterol level and statistical standard deviation for group-Dete. ($n = 30$), while M_2 and SD_2 for group-Calc. ($n = 30$).

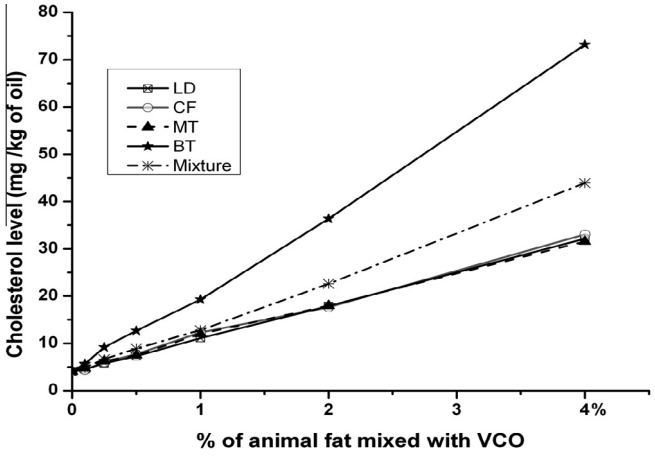

Fig. 3. Changes in the cholesterol levels due to the mixing of animal fats with VCO.

4. Conclusion

The Che-TME and Cha-TME obtained from VCO, LD, CF, MT, and BT was separated using 1D-GC and GC × GC system. As a result, a baseline separation of the two compounds could be achieved by using GC × GC–TOF/MS, and the cholesterol level in pure VCO, animal fats, or false VCO adulterated with animal fats could be determined accurately. The analytical method based on GC × GC–TOF/MS is a precise, reliable, and easy method that can be used to detect the adulteration of VCO with animal fats by determining only the cholesterol level in VCO samples. In this study, the detection limit of LD, CF, MT, BT, or their mixture in VCO was 0.25%.

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