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Comparison between Solvent Extraction Using Gas Chromatography Mass Spectrometry Triple Quadrupole (GCMS-QQQ) of Cholesterol and Cholesterol Oxidation Derivatives

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Abstract: Cholesterol Oxidation Products (COPS) are well known for their negative biological effects. Till now, there is no study reported on solvent extraction used for COPs. In this study, two solvent extraction techniques, namely chloroform/methanol (2:1, v/v) or Folch method and n-hexane/2-propanol (3:2, v/v) or Hara-Radin method of cholesterol and COPs in beef tallow and lard were compared. Cholesterol and COPs (5 α-cholestane, 7-ketocholesterol and 25-hydroxycholesterol) contents in both fats also analyzed. The analysis passed through four major steps; extraction of lipids, saponification, enrichment of COPs and quantification by gas chromatography mass spectrometry triple quadrupole. All standards showed good linearity with correlation coefficient (r^2) of 0.9998 (5 α-cholestane), 0.9999 (cholesterol), 0.9873 (25-hydroxycholesterol) and 0.9693 (7-ketocholesterol). Data obtained by this method was analyzed based on precision and recovery criteria. Precision measured as standard deviation (SD) was between 0.0040 and 2.2460; and no significant different ($P>0.05$) for the recovery testing using 5 α-cholestane in both method. Recovery by Hara Radin method in lard (27.23%) and beef tallow (8.73%) was higher than the Folch method, 14.25% and 2.97% respectively. This study implies that Hara-Radin method can be an alternative method to avoid the use of harmful solvent such as chloroform in Folch method.

Key words: Cholesterol Oxidation Products · Gas Chromatography Mass Spectrometer Triple Quadrupole · Fat Solvent Extraction

INTRODUCTION

Cholesterol (cholest-5-en-3β-ol) is a steroid alcohol and an essential structural component of biomembranes, which is closely associated with phospholipids. While, cholesterol oxidation products (COPs) are similar to cholesterol, which contain an additional functional group, such as a hydroxyl, ketone or an epoxide group in the sterol nucleus and or on the side chain of the molecule [1, 2].

COPs have been known to be more injurious to arterial cells than pure cholesterol and are more directly connected to the development of atherosclerosis and coronary heart disease [3]. COPs deteriorated the bioavailability of cholesterol by inhibiting cholesterol biosynthesis and dietary uptake of cholesterol [4, 5, 6]. Many studies demonstrated that the amount of COPs in foods could frequently reach 1 % of total cholesterol and occasionally 10 % or more [3].

Thus for health reasons, it is important that cholesterol oxidation products (COPs) in food are identified and quantified accurately [7]. In order to identify the existence of COPs in foods, it is of great importance that the analytical methods are well validated.

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Therefore, a properly performed method validation is crucial for the results to be considered reliable and accurate. The characteristic of method should include evaluations and determination of specificity, precision, accuracy, sensitivity, as well as limitations of the method [8, 9].

Analytical procedures for the determination of cholesterol oxidation in food, especially in heated and/or stored foods with high cholesterol content include capillary gas chromatography (GC) [10, 11], gas chromatography-mass spectrometry (GC-MS) [12, 13] and high performance liquid chromatography (HPLC) [14]. Analysis of COP generally comprise of four major steps; initial extraction of lipids from foods, saponification of extracted lipids and subsequent enrichment of COP and quantification by GC or by HPLC [12, 14].

Due to low levels of cholesterol oxidation products (COPs) from other fat-soluble substances in food matrices such as mono-, di-, triacylglycerols, esterified and free cholesterol, free fatty acids and phospholipids, it is very difficult to isolate COPs. Therefore, many solvents or solvent combination used to extract COPs [15]. Methanol is widely used in extraction of lipids, often together with chloroform, which is considered harmful to our health and environment and it may be wise to avoid using them in routine laboratory work, apart from when there is no other alternative technique [16].

The aim of this research was to compare between the solvent extraction techniques namely n-hexane-isopropanol (3:2, v/v) and chloroform-methanol (2:1, v/v) [17] and of cholesterol and it oxidation derivatives contents in lard and beef tallow using GCMS-QQQ [16, 17].

MATERIALS AND METHODS

Samples and Materials: Lard and beef tallow were purchased from the local shops. Standards cholesterol, 5 α-cholestane, 7-ketocholesterol and 25-hydroxycholesterol were purchased from Sigma-Aldrich (M) Sdn Bhd. Other chemicals used in this study were TMSI + Pyridine, 1:4 (Sylon TP), sodium sulphate anhydrous, chloroform (Merck), methanol, hexane, isopropanol, diethyl ether, acetone and butylated hydroxytoluene (BHT). Cartridge of solid phase extraction (SPE) silica was from Agilent Technologies.

Sample Preparation: Lard and beef tallow were kept in sealed plastic bag and stored in the freezer at temperature -20°C. Both samples were thawed, chopped and then melted using drying oven at 70°C.

Lipid Extraction: Fat samples were extracted according to Hara-Radin and Folch method respectively [16, 17]. The internal standard, 20mg/L 5 α-cholestane was added in each of 1g of melted fat's sample.

Cold Saponification of Cholesterol and Cholesterol Oxidation Products (COPs): Briefly, 10mL of 1N KOH solution in methanol was added to perform a cold saponification at room temperature for 20h, in dark condition and under continuous agitation in an orbital shaker at 100 rpm. A 1ml of BHT (100ngmL^{-1}) was added as antioxidant in order to avoid COPs artifact formation during the saponification. The saponified sample was transferred to a separating funnel with 10mL of distilled water and 10mL of diethyl ether and the organic phase was collected. This extraction procedure was carried out thrice and the collected fractions were pooled together. It was then washed with 10mL of 1N KOH solution in methanol and 10mL distilled water, filtered with anhydrous Na_2SO_4 through filter paper and transferred into a round-bottomed flask. The solvent was evaporated to dryness using rotary evaporator at 30°C and the non-saponifiable extract was redissolved in 5mL of hexane.

Enrichment of Cholesterol and Cholesterol Oxidation Products (COPs) by Solid Phase Extraction (SPE) and Derivatization: Enrichment of cholesterol and COPs was carried out according to method as described by María *et al.* [18]. All samples were derivatized to TMS-ether prior to GC analyses, as described previously [19]. The samples were filtered (0.45µm x 13mm) prior to GCMS-QQQ analyses.

Quantification by GCMS-QQQ: Analysis was performed on an Agilent 7890A GC System combined with a prototype Agilent 7000A Triple Quadrupole GC/MS system or gas chromatography mass spectroctrometry-triple quadrupole (GCMS-QQQ). The analysis was operated with multiple reactions monitoring (MRM) mode.

The data was analyzed by Mass Hunter Work Station Software using Quantitative Version B 03.01. A capillary column DB5: MS UI 30m x 0.25mm x 0.25µm (Agilent Technologies, USA) was used to separate the cholesterol and COPs. The column temperature was set at 250°C and raise to the constant temperature at 280°C for 5 minutes. Then, temperature was raised to 300°C at 50°C/min. The detector was set at 320°C and gas flow was 1.2 mL/min. Volume of injection was 1µL and splitless mode was used. GCMS-QQQ was set-up with the characteristic

Table 1: Characteristic ions of cholesterol and cholesterol oxidation products (COPs)

Retention time, RT	Compounds	Precursor ion	Product ion	Collision energy
3.75	5 α-cholestane	217.1	12.1	7
			93.1	22
7.00	Cholesterol	329.2	109.1	14
			69.0	25
10.5	7-ketocholesterol	367	159.0	22
	25-hydroxycholesterol	271.2	107.2	18
			5.0	15

ions, shown in Table 1. The temperature source was 230°C, electron ionization (EI), while MS1 and MS2 temperature was 150°C.

Statistical Analysis: Data were evaluated by statistical analysis.

RESULTS AND DISCUSSION

The retention times, relative retention times and linearity response of 5 α-cholestane, cholesterol, 25-hydroxycholesterol, 7-ketocholesterol were presented in Table 2. From the results, the orders of elution of standard samples were 5 α-cholestane followed by cholesterol, 25-hydroxycholesterol and 7-ketocholesterol shown in Figure 1.

It can be seen that the retention time of unoxidised cholesterol is more than 2 min longer than for 5 α-cholestane showing a better separation. The total analysis time by GCMS-QQQ is rather moderate, less than 12 min to elute the entire common COPs used in this study. RRT is the normalization of minor peaks to the parent peak (5 α-cholestane) in a chromatogram. The RRT for parent peak, 5 α-cholestane is 1.00. Peak eluting after 5 α-cholestane have RRTs more than 1.00, as the results obtained from this study: 1.38 (cholesterol), 1.94 (25-hydroxycholesterol) and 1.98 (7-ketocholesterol).

Linearity was evaluated by analyzing standard mix solutions containing the cholesterol and COPs at concentration levels of 100ppm, 50ppm, 1ppm, 0.1ppm, 0.01ppm and 0.001ppm for each study compound: 5 α-cholestane, cholesterol, 7-ketocholesterol and 25-hydroxycholesterol. The r for all compounds is between 0.9693 to 0.9999 and is shown in Table 2. Linearity defines the ability of the method to obtain test results proportional to the concentration of analyte [20]. Criteria used to verify linearity was done to obtain the value of r. This value defines how well the data fit to a straight line. For a standard calibration curves, value of r must be as close to +1.00 or -1.00 as possible because this value is a perfect correlation (perfect straight line). This showed that the calibration generated within the mass range for each compound was linear and the instrument used is suitable and fit for this analysis.

The relative precision of two methods was compared by using standard deviation (SD). Table 3 shows SD of the amount of COPs by using two different extraction methods in lard and beef tallow.

Thus, Hara-Radin method has lower data variation and better precision. While, Folch method was found to be more precise with lower SD value than Hara-Radin method in beef tallow sample since Hara-Radin method showed high variation of 5 α-cholestane values.

Cholesterol content in lard sample by Hara-Radin method showed lower SD value compared to Folch method. Therefore, Hara-Radin method has low data variation and more precise than Folch method in quantification of cholesterol in lard. Besides, Folch method was more precise than Hara Radin method in beef tallow, shown by it low SD values. The SD value of 25-hydroxycholesterol in lard for Folch method is lower compared to Hara-Radin method indicate that Folch method is more precise than Hara-Radin method in lard. While, Folch method yielded a low SD value in beef tallow compared to Hara-Radin method that indicate lower variation among data obtained. However all the differences are not significant ($P>0.05$).

There are some factors that may influence the inconsistent values of the COPs compounds in this analysis in order to achieve the good precision value. The samples (lard and beef tallow) stored too long in the freezer may affect the amount of cholesterol and COPs. The fresh foods contain low levels of COPs and the levels go up during processing and storage (time and condition) [2].

In addition, the rate of cholesterol oxidation in pork is greatly accelerated during storage following cooking and appears to follow the same trend as lipid oxidation in general [22]. Unirradiated pork and veal during storage also showed an increase in most COPs, although increases were smaller than with beef [23]. Generally, heat, pH, light, oxygen, water activity and the presence of unsaturated fatty acids are the major factors that influence COPs formation during food processing or storage [1, 2].

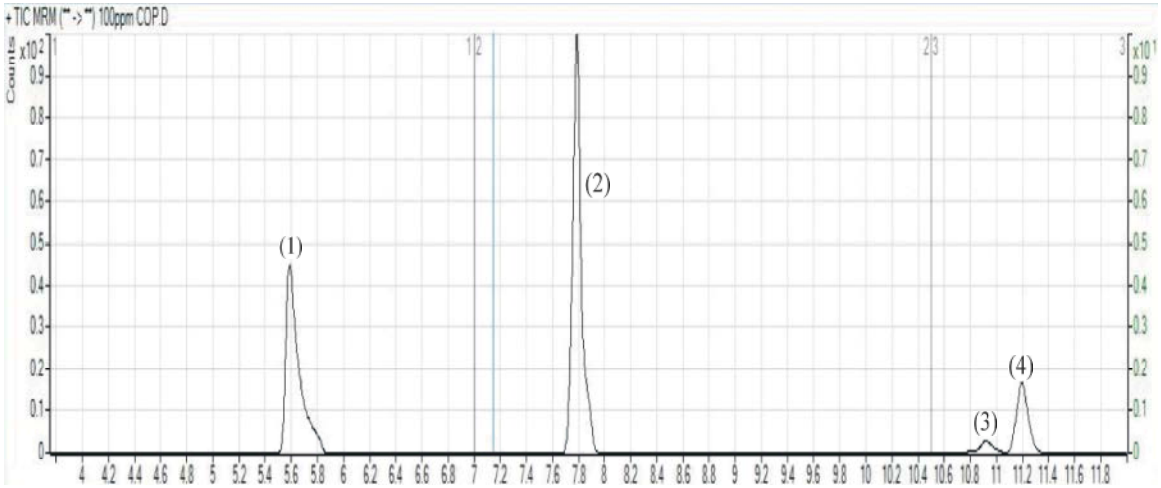

Fig. 1: GCMS-QQQ Chromatogram of the standard mixture of: (1) 5 α-cholestane (internal standard) (2) Cholesterol (3) 25-hydroxycholesterol (4) 7-keto cholesterol

Table 2: Retention time (RT), relative retention time (RRT) and linearity response (r2) of TMS-ether derivatives of standard samples of cholesterol and COPs

Compounds	RT (min)	RRT	r^2
5 α-cholestane	5.58	1.00	0.9998
Cholesterol	7.71	1.38	0.9999
25-hydroxycholesterol	10.85	1.94	0.9873
7-ketocholesterol	11.05	1.98	0.9693

Abbreviations: RT: Retention time; RRT: Relative Retention Time; r: correlation coefficient.

Table 3: SD value of 5 α-cholestane, cholesterol, 7-ketocholesterol and 25-hydroxycholesterol by Folch and Hara-Radin methods in lard and beef tallow

Compounds	Samples	Folch	Hara-Radin
5 α-cholestane	Lard	2.2460^{b}	0.3793^{ab}
	Beef tallow	0.2418^{a}	0.9742^{ab}
Cholesterol	Lard	0.5462^{a}	0.5280^{a}
	Beef tallow	0.2541^{a}	0.9813^{a}
25-hydroxycholesterol	Lard	0.2065^{a}	0.7219^{a}
	Beef tallow	0.3056^{a}	0.4893^{a}
7-ketocholesterol	Lard	0.0040^{a}	0.0139^{a}
	Beef tallow	0.0017^{a}	0.0013^{a}

Table 4: Statistical data on the recovery (in %, n=8) of standard 5α-cholestane

Sample	Folch method	Hara-Radin method
Lard	14.2500^{ab}	27.2313^{b}
Beef tallow	2.9713^{a}	8.7288^{ab}

Note: values are percentage of recovery (n=8); values at different superscript letters indicate the significantly differences (P<0.05); values followed by the same letter do not differ significantly from one another.

The results of the recovery percentages of standard 5 α-cholestane in lard and tallow determined by the two extraction methods are showed in Table 4.

Based on the results, Hara-Radin method showed high recovery percentages of 5 α-cholestane compared with the Folch method in both sample. Hara-Radin extraction method gave the highest percentage of recovery (27.23%) in lard sample and the value was significantly different compared to others. The recoveries that obtained were too low. It may due to errors that occurred during the extraction of COPs.

Recoveries below 100% are commonly reported and could be the results of interactions with the SPE-cartridge or other losses during the analytical procedure [24, 25]. In general, the measurement uncertainty is often affected by factors such as volumes, weights, temperature, storage and treatment and concentration of the sample. All these parameters are important to note when analytical methods are developed and optimized [8].

We were unable to compare our values with other researcher's results because there are no published data from similar experimental conditions. However, published reports have shown that direct method, Maxwell and Folch methods gave comparable results. In addition, Hara-Radin method had shown drastic low COPs recovery, probably due to the non-polar solvents usage in fat extraction [26].

There are many solvents or solvent combinations that can be used to extract COPs and the most important factor to be considered at this point is the complete recovery [15]. These preparation steps are very important due to their possible contribution to the formation of artifacts, COPs recovery, peak resolution, detection efficiency, identification and quantification.

Methanol is widely used in extraction of lipids, often together with chloroform, which is considered harmful to our health and environment [13] and it may be wise to avoid using them in routine laboratory work, apart from when there is no other alternative. Therefore, the use of direct saponification method due to high efficiency, good method precision, minimal artifact formation and easiness in handling to recover COP is suggested [26, 27].

CONCLUSION

There was no significant different between Folch (chloroform-methanol system (2:1, v/v)) and Hara-Radin (n-hexane-isopropanol system (3:2, v/v)) method in analysis of cholesterol and COPs in lard and beef tallow. Hara-Radin method can be an alternative method to avoid the use of more toxic and harmful solvent i.e., chloroform and methanol in Folch method.

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