Cholesterol oxidation products – analytical methods and levels in sweets containing heated butter oil

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Abstract

Various methods are used to analyse cholesterol oxidation products (COP) due to the unavailability of a standard method. In order to select a suitable method for the enrichment of COP, three methods of saponication (A – C),and transesterication (D) of tallow with three levels (5,10 and 20 μg) of spiked COP, were evaluated. Further enrichment of COP was done by solid phase extraction, quantied by GC, and confirmed by GC–MS. The in-house method A, and method D, showed the best results among the four methods evaluated. The recoveries at all levels of spiked COP were generally higher than 60% in method A. The recoveries of all spiked COP at 5 μg level were consistently lower in method D compared with method A. From the results of this study it can be concluded that method A may be more suitable for the analysis of very low levels of COP in foods.

Ghee (clarified butter oil), a major ingredient in Indian sweets, is an important source of saturated fatty acids, cholesterol and COP that are considered as risk factors for atherosclerosis. The high frequency of atherosclerotic complications reported among the Indian immigrants in England prompted determination of lipids and lipid oxidation status of a ghee sample and fifteen Indian sweets that are available in London supermarkets. The fatty acid profile of the samples shows saturated fats (about 73%), mainly composed of myristic, palmitic and stearic acids, except in two samples, which had levels of oleic acid in excess of 60%. TBARS values ranging from 19 to 260 μg/100 g might be due to the different oxidative status of different sweets samples. Cholesterol (approximately 0.22%) was the main sterol in all samples, except two where unspecified oils were used instead. Variation of total COP was from 0.94 to 38.41 μg/g sample. Some of these sweets may be a source of considerable amounts of saturated fatty acids, cholesterol and COP in the diet that possibly contribute to atherosclerosis.

Contents

1. Introduction

1.1 Cholesterol

Cholesterol (cholest-5-en-3β-ol) is a steroid alcohol and an essential structural component of biomembranes, which is closely associated with phospholipids. The cholesterol molecule inserts itself in the membrane with the same orientation as the phospholipid molecules (Figure 1). It's an important part of an animal body because it is acting as an important determinant of membrane fluidity, nerve fiber insulation, and hormones and is needed for other functions such as production of vitamin D and synthesis of various steroids. Biosynthesis and diet are the two methods that organisms obtain cholesterol. Cholesterol biosynthesis, a highly regulated process occurs in all animal tissues but in higher mammals this machinery is significant in liver, adrenal glands, ovaries and testis. Bile is the major excretion route of cholesterol from the body, as unesterified cholesterol. In adult human, approximately 400 mg of cholesterol per day are converted to bile acids and only approximately 50 mg are converted to hormones. Cholesterol that is manufactured in the liver is transported to cell membranes by lipoprotein. Excess cholesterol collects in fatty insoluble deposits called plaques along artery walls narrowing and clogging them restricting the blood flow, a major feature of coronary artery disease. (Vlenzuela, et al., 2003).

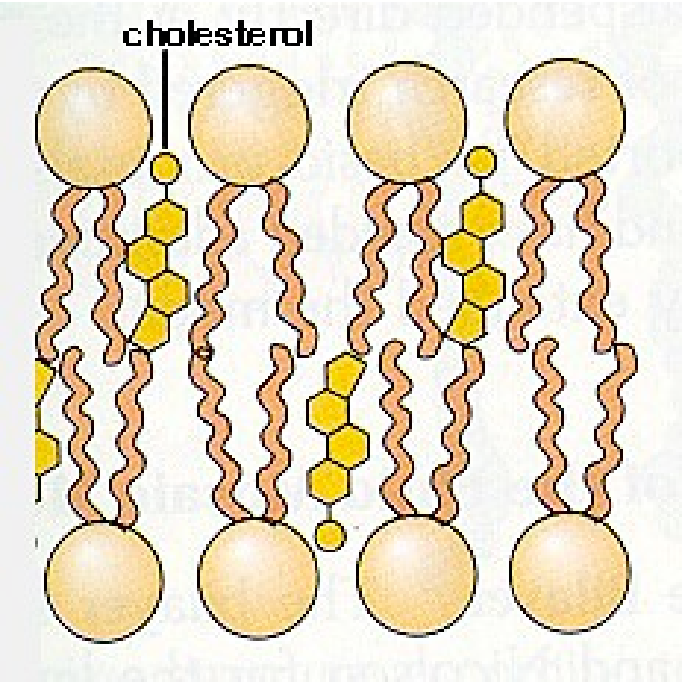

Figure 1. Position of cholesterol among phospholipid molecules in cell membrane

1.2 Formation of COP

Cholesterol present in animal origin foods undergoes autoxidation during processing as well as during storage yielding toxic products. Cholesterol oxidation products (COP) 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.

Oxidation of lipids and sterol (cholesterol) follows the same oxidation patterns such as autoxidation, photoxidation and enzymic oxidation, producing relevant hydroperoxides. It is believed that the hyroperoxides derive from oxidation of unsaturated fatty acids play a significant role to facilitate cholesterol oxidation at Δ-5 double bond, which is more sensitive to oxidation (Lercker et al., 2002).

Autoxidation of cholesterol

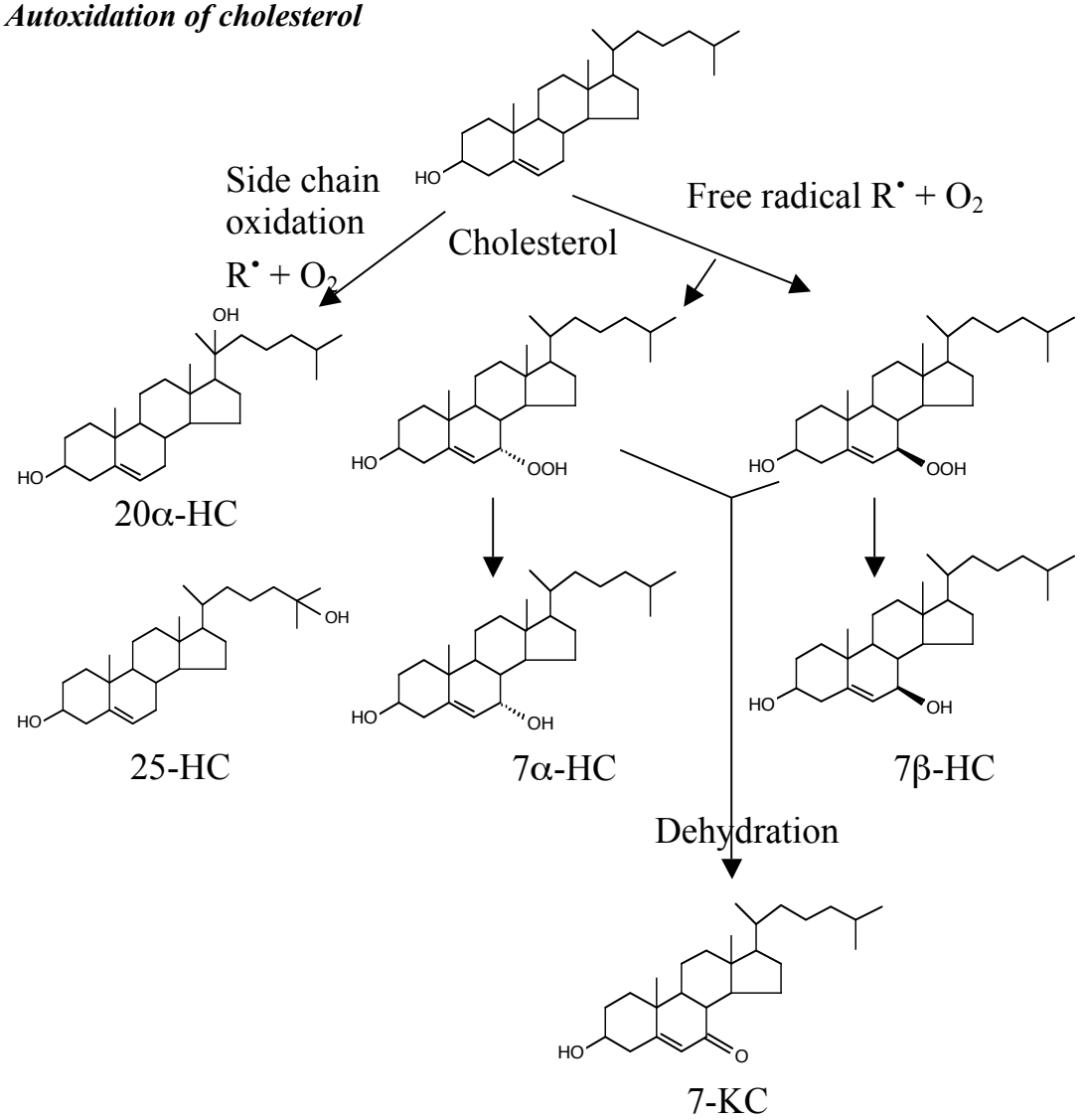

Figure 2. Autoxidation pathways of cholesterol

Autoxidation, the main reaction involved in oxidative deterioration of cholesterol, is a self-catalytic reaction with molecular oxygen. Cholesterol autoxidation usually starts at C-7 by abstraction of hydrogen following the addition of an oxygen molecule forming primary COP, isomers of 7-hydroperoxycholesterol (Figure 2). These 7-hydroperoxycholesterols can further convert into 7α-hydroxycholesterol and 7β-hydroxycholesterol, which are commonly found in food (Lercker et al., 2002).

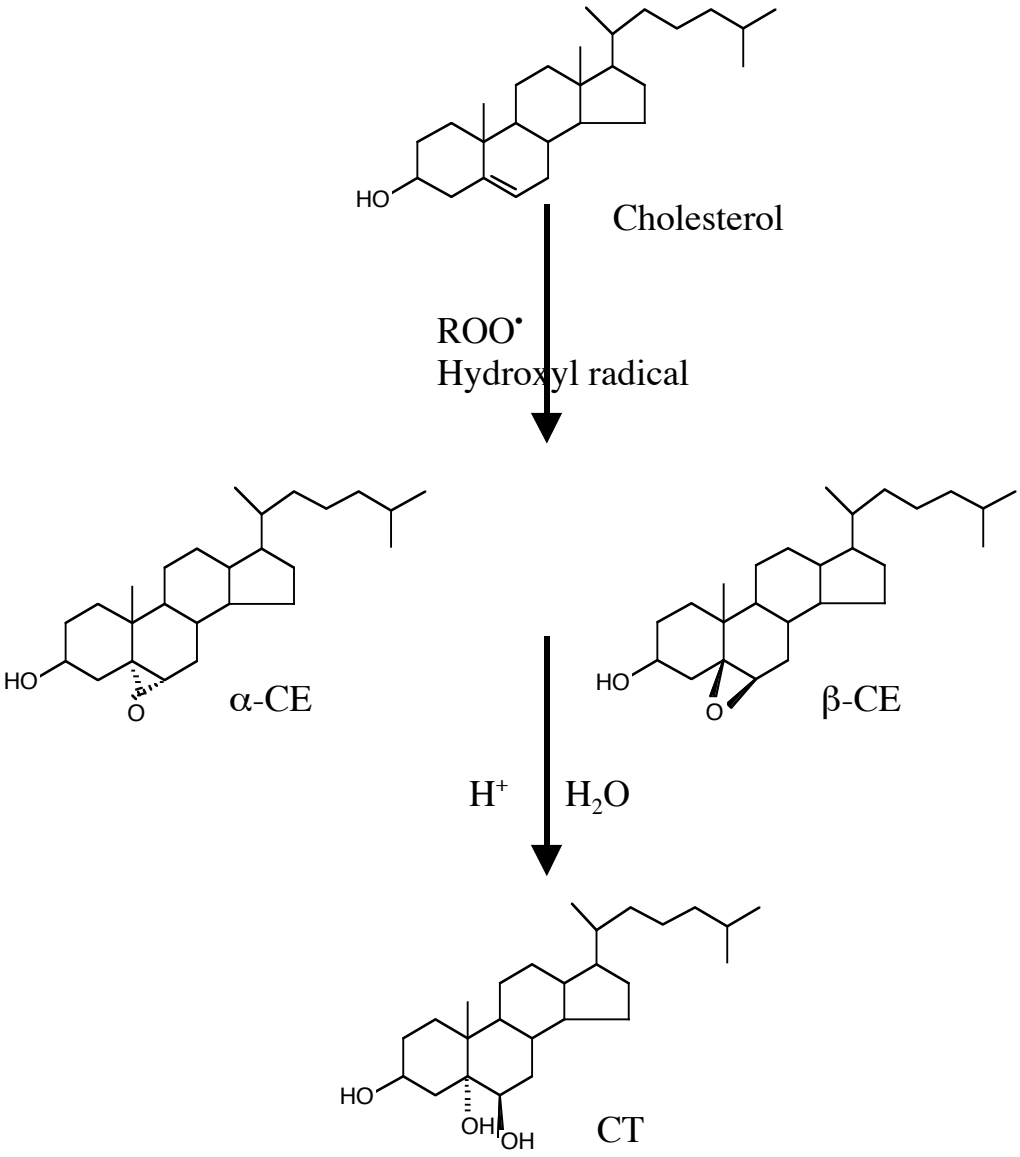

Figure 3. Formation of cholesterol epoxides

Both isomeric 7-hydroperoxycholesterol can also undergo dehydration during heating forming 7-ketocholesterol. In addition, 7-ketocholesterol can be formed by dehydration of isomeric 7-hydroxycholesterol in the presence of radicals. 7-ketocholesterol is also considered as a major COP in food matrix. Formation of

isomeric epoxy cholesterols occurs due to interaction between cholesterol and hydroxy radical (Figure 3) and these epoxy compounds can be hydrolyzed in acidic medium converting them into most toxic triols. The side chain oxidation occurs at C-20 and C-25 positions resulting in the production of relevant hydroperoxide, 20-hydroperoxide and 25-hydroperoxide respectively, which can further reduce into 20α-hydroxycholesterol and 25-hydroxy cholesterol (Figure 2) (Lercker et al., 2002).

Photoxidation of cholesterol

In photoxidation of cholesterol, single oxygen is formed from triplet oxygen by light in the presence of an active sensitizer (natural pigment or synthetic colorant). Cholesterol can react with singlet oxygen in the presence of photo-sensitizer, forming dominant hydroperoxide at C-5.

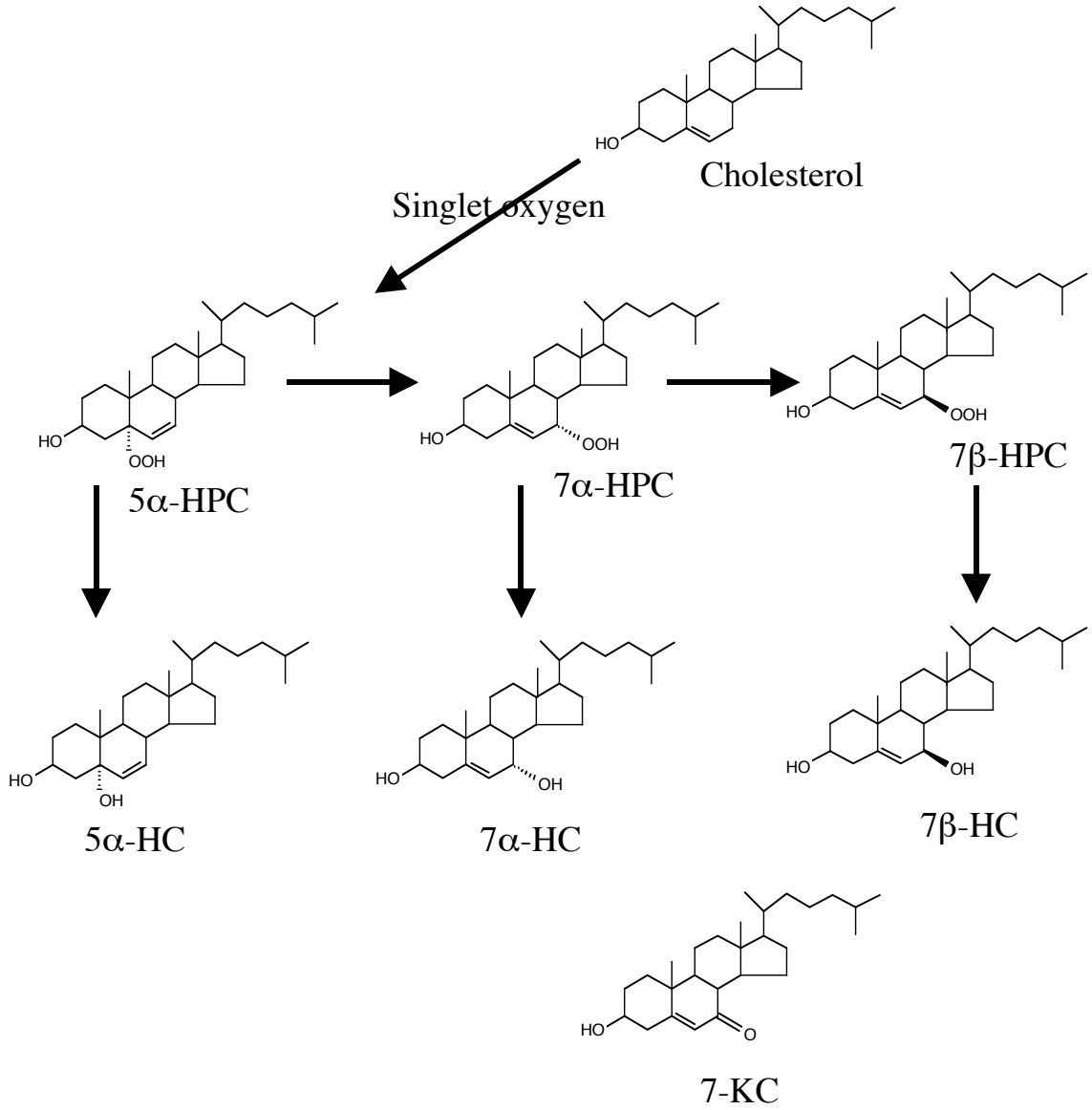

Figure 4. Photoxidation pathways of cholesterol

A part of this hydroperoxide converts into 5α-hydroxycholesterol and the other part of the 5-hydroperoxide is further converted into stable 7-hydroxiperoxide and 6-hydroperoxide that are present in minor amounts. 7-hydroperoxides can be converted into isomeric 7-hydroxycholesterol and into 7-ketocholesterol at the same time 5-hydroxycholesterol can be formed (Figure 4) (Lercker et al., 2002).

Enzymic-oxidation of cholesterol

Some enzymes in food oxidize cholesterol. Available reports show that the conversion of 5α- hydroperoxides (5α-HPC) into 7α-HPC, epimerization of 7α-HPC into 7β-HPC, and formation of 7-HC epimers from the corresponding hydroperoxides occur by enzymatic reactions. But this has to be studied to a further extent due to the fact that these products can be formed by the usual non-enzymatic reactions (Lercker et al., 2002). Monooxygenase, dioxygenase, dehydrogenase and oxidases are the main enzymes that can oxidize cholesterol. The COP, 7α-hydroxycholesterol, 25-hydroxycholesterol, 20α- hydroxycholesterol, (25R)-26-hydroxycholesterol, 22R-hydroxycholesterol are produced by enzymic oxidation of cholesterol (Lercker et al., 2002).

These oxides are produced endogenously in human tissues during conversion of cholesterol to bile acids and steroid hormones. Non-enzymatic or autoxidation of cholesterol also occurs in vivo. Cholesterol can be oxidized effectively in the presence of human gastric fluid and it acts as a better medium for cholesterol oxidation and produce COP *in vivo* (Dobarganes et al, 2003). Cholesterol shows antioxidant effects against some biological oxidant *in vitro* and on liposomes. Due to this property, COP can arise from reactions with endogenous oxidants. In diet, cholesterol can act as an antioxidant due to lack of other antioxidants, leading to higher levels of COP (Garcia-Cruset et al., 2002).

These enzymic and non-enzymic reactions can occur separately or simultaneously in food production, processing, distribution and storage (Ubhayasekera et al., 2004; Dobarganes & Marquez-Ruiz, 2003).

1.3 Cholesterol oxidation products and biological effects

The major products of the reaction are 5-cholesten-3β, 7α-diol (7α-HC), 5-cholesten-3β, 7β-diol (7β-HC), 5-cholesten-3β-ol-7-one (7-KC), 5-cholestan-5α, 6α-epoxy-3β-ol (α-CE), 5-cholestan-5β, 6β-epoxy-3β-ol (β-CE), 5-cholesten-3β,20α-diol (20α-HC), 5-cholesten-3β, 25-diol (25-HC) and 5-cholestane-3β, 5α,6β-triol (CT). These eight COP are usually found in processed foods. COP are present in our diet, especially in foods with high cholesterol contents (egg, whole milk, meat, sea food, etc). But the fresh foods contain low levels of COP and the levels go up during processing and storage (time and condition) (Paniangvait et al., 1995). COP formation is also incorporated with storage condition and time. Generally, heat, pH, light, oxygen, water activity and the presence of unsaturated fatty acids are the major

factors that influence COP formation during food processing or storage (Larkeson et al., 2000; Paniangvait et al., 1995).

COP have been shown to have a variety of potentially atherogenic, cytotoxic, mutagenic and possibly carcinogenic affects in both in vivo and in vitro studies. COP also cause cell membrane damage and inhibition of cholesterol biosynthesis. Several investigations have demonstrated that 25-HC cholesterol and CT are toxic agents causing atherosclerosis. Also α-CE and β-CE have been reported to be carcinogenic (Osada, 2002; Dobarganes & Marquez-Ruiz, 2003).

The consumption of pre-cooked foods of animal origin is becoming more popular in the world due to the low cooking time. Therefore, the intake of cholesterol oxide containing food has been increased. In view of the health implications of the oxidation products of cholesterol in foods, it is important to identify and quantify these products in foods in the open market (Savage et al., 2002; Dutta & Savage, 2002).

1.4 Analysis of cholesterol oxidation products

The COP levels reported for similar food, sometimes not lie in the same range and sometimes a considerable difference could be observed. These differences are due to the differences in manufacturing technology and analytical procedure. This brings up a very important need of accurate quantification (McCluskey & Devery, 1993; Guardiola, et al., 2004).

The quantification of COP in food is difficult because there are interruptions by large mounts of interfering cholesterol, triglycerides, phospholipids and other lipids (Ulberth & Buchgraber, 2002). On the other hand technical difficulties associated with cholesterol oxide analysis play a major part due to similar chemical structures and presence of cholesterol oxides at trace levels (parts per million). Therefore, the extraction, purification, and detection methods that use to identify and quantify these products play a major role (Ulberth & Buchgraber, 2002).

The analytical procedure should be designed in a way to guarantee efficient recovery of COP from food matrix or biological fluids and minimization of the generation of artifacts during sample clean-up and work-up steps. Most common analytical protocol used for COP analysis, has more or less the same route: (i) extraction of COP from food matrix or biological system; (ii) purification of the sample extract; (iii) derivatization to suitable compounds and (iv) quantification with a suitable chromatographic method (Ulberth & Buchgraber, 2002; Guardiola, et al., 2004).

1.4.1 Extraction, purification and enrichment

COP are fat-soluble compounds. It is very difficult to isolate COP due to low levels from the other fat-soluble substances in food matrices, such as mono, di, tri-acylglycerols, esterified and free cholesterol, free fatty acids and phospholipids. Many solvents or solvent combinations can be used to extract COP and the most important factor to be considered at this point is the complete recovery. These preparation steps are very important due to their possible contribution to the formation of artifacts, COP recovery, peak resolution, detection efficiency, identification and quantification (Rodriguez-Estrada & Caboni, 2002).

Choose of a solvent or a solvent system play a significant role as the solvent system must be capable of disrupting the associative forces binding the cholesterol and lipids to non-lipid material in biological matrix. Therefore no single solvent is capable of complete cholesterol extraction whereas a mixture of solvents is used (Ulberth & Buchgraber, 2002). The most commonly reported methods for cholesterol extraction are

1. Methods involving a preliminary fat extraction followed by a saponification
    a. Chloroform/methanol (2:1, v/v); (Folch et al., 1957)
    b. n-hexane/2-propanol (3:2, v/v); (Hara & Radin, 1978)
    c. Dichloromethane/methanol (9:1, vol/vol); (Maxwell, 1986) / Dry column method (Higley et al., 1986; Zubillaga & Maerker, 1991).
2. Methods that directly treat the sample
    a. Direct saponification or transesterification (Dionisi et al., 1998)

Apart from these methods, reports of usage of single solvents like chloroform and Soxhlet extractions using solvents such as tert-butylmethyl ether and dichloromethane are also available (Ulberth & Buchgraber, 2002).

Very few reports are available on the comparison of different extraction methods. Dionisi et al. (1998) have compared commonly used four lipid extraction methods, direct saponification, modified Maxwell's method, modified Folch's method and modified Radin's method by extracting lipid followed by saponification, solid phase extraction (SPE) column purification and GC-MS (gas chromatography coupled to mass spectrometric detector) quantification.

Direct method, Maxwell's and Folch's methods gave comparable results. But Radin's method had shown drastic low COP recovery, probably due to the apolar solvents usage in fat extraction. Interestingly, Radin's method has shown that spiking gives better recovery. Comparing all the aspects, Dionisi et al. have suggested use of direct saponification method due to high efficiency, good method precision, minimal artifact formation and easiness in handling to recover COP (Ulberth & Buchgraber, 2002).

Accelerated solvent extraction (ASE) is well known for high efficiency in lipid extraction but not many available reports on COP analysis. In a total lipid extract

triglycerides and/or phospholipids are major compounds whereas sterols are present in minor levels and COP are in trace levels. To analyze with less interference and for clear detection these COP need to be enriched. There are two enrichment methods such as saponification and transesterification (Ulberth & Buchgraber, 2002).

Saponification is employed to remove triglycerides, free fatty acids and water-soluble impurities during extraction of COP by converting them to water-soluble derivatives using methanoic or ethanoic NaOH or KOH. COP extraction to a suitable organic solvent is done after adding water to the saponified mixture. The unsaponifiable fraction contains COP. Two saponification procedures, cold and hot saponification are often used. Cold saponification at 25°C for 18-22 hours has shown high recovery (Tai et al., 1999) and low artifact formation. Use of hot saponification at 60°C for 45-60 minutes has reduced the saponification time. Hot saponification leads to artifact formation by degrading 7-keto and isomeric epoxides. To overcome this problem purification of COP in silica gel- or C_{18} cartridges have been used (Ulberth & Buchgraber, 2002; Guardiola et al., 2002).

Apart from the disadvantages of possible artifact formation, saponification also has some practical drawbacks. The saponified triglycerides form a soap solution giving bad separation of the evolved emulsions and micelle formation leads to loss of compounds of interest. (Schmarr et al., 1996)

The other method used to free the sterol and its oxides from the bulk of the accompanying lipids is transesterification. The transesterification is a method with mild conditions converting esterified oxysterols and triglycerides into fatty acid methyl esters. After esterification, the lipid primarily consists of acid methyl ester, free cholesterol and its oxidation products and some minor apolar and polar compounds. This mixture could be separated by a solid phase extraction column and stepwise elution with solvent of increasing polarity. No artifact formation is detected with this method (Schmarr et al., 1996).

Solid phase extraction (SPE) column uses the polarity differences in the matrix component to separate a mixture of compounds with different polarity. Phospolipids, cholesterol and its oxidation products and cholesteryl esters are most, medium and least polar respectively. Therefore, SPE method helps to separate COP with minimum contamination with coeluted cholesterol and phytosterol, if present. The advantages of using a SPE column are as follows: (i) no destruction of sensitive COP as no strong alkali is used (ii) artifact formation is minimized due to the removal of free cholesterol. The factors such as pressure of air, light, peroxides in solvents, heat and contact with reactive groups (e.g. silicic acid) leads to artifact formation (Ulberth & Buchgraber, 2002).

There are several different matrices used in SPE columns whereas the COP analysis has been reported with silica (Si-), Florisil, aminopropyl-modified silica (NH_2-) and octadecyl-modified silica (ODS-) (Ulberth & Buchgraber, 2002).

1.4.2 Thin-layer chromatography (TLC)

TLC is an efficient, rapid and simple technique used to separate, isolate and identify COP in crystalline cholesterol, food and biological samples. Use of TLC is limited to the separation of COP but not for accurate quantification. TLC is capable of separating hydroxycholesterols but not cholesterol hydroperoxides (Lebovics, 2002).

TLC is useful in confirming the identity of COP based on the color development after spraying sulfuric acid and observation under UV (Tai et al., 1999). There are reports of separation of COP such as 7α-HC, 7β-HC, and 5,6-CE with TLC method and purification of COP (Nourooz-zadeh & Appelqvist, 1988).

The major drawbacks of TLC analysis are incapability of 5,6-EP isomer separation, tedious method, inaccuracy of quantification of COP based on the spot area, poor resolution, instability, time consuming procedure. There are many advantages of TLC such as parallel analysis of many samples, possibility of fast screening, ability to guess the amount of COP, purification to a certain level and low cost (Lebovics, 2002).

1.4.3 High Performance Liquid Chromatography (HPLC)

COP are present in small quantities in food. Therefore, detection and quantification are challenging. COP have different characteristics such as diverse polarity and chemical properties due to the different functional groups in their chemical structure. On the other hand, isomers of some COP show similar chemical, spectrometric and fragmentation characteristics. All these reasons stress the requirement of high sensitive methods and systems to identify and quantify COP. The choice of analytical tool is governed by, the type of matrix and the scope of the analysis (Rodriguez-Estrada & Caboni, 2002).

HPLC is a better method to analyze COP due to the possibility of separation, detection and quantification, especially the thermo-labile molecules (e.g. cholesterol hydroperoxides) as it is a non-destructive technique. This is a versatile analytical system due to its ability to couple into many detector systems, and to run in normal or reverse phase modes using different column types and dimensions and mobile phase compositions, leading to different separation, identification and quantification. HPLC coupled to MS detectors has become a very powerful tool over the past years due to the increment of the sensitivity (Rodriguez-Estrada & Caboni, 2002).

Detectors connected to HPLC can give different results. In an HPLC analysis with a spectrometric detection, it has been reported the inability in detection of some human harmful COP due to poor UV absorption. (Careri et al, 1998).

1.4.4 Gas Chromatography (GC)

The gas chromatography is the common method of COP determination using derivatized COP as trimethylsilyl ether (TMSE) derivatives. This derivatization method avoids peak tailing and it improves the thermal stability of some hydroxy cholesterols. There are few factors affecting COP response in GC such as injection technique and conditions, reagents, and conditions use to get derivatives (Guardiola, et al., 2002).

GC gives an accurate quantification of COP with a flame ionization detector (FID), or with a mass selective detector (MS/MSD). It has been observed a clear separation of COP with a good resolution when a silica capillary (dimethyl poly siloxane) column connected to GC (Tai et al, 1999). The best combination of techniques to identify COP rapidly and sensitively is GC-MS with a selected ion monitoring mode (SIM) and a capillary column. The outcome of this, the mass spectrum can be compared with the chemical library to identify COP with high accuracy (Tai et al, 1999; Guardiola, et al., 2002).

The following strategies are useful to increase the sensitivity of the method.

1. Increase of analyte introduced into column
    a. reduction of split ration
    b. increase the volume injected
    c. concentration of the silanized extract
    d. avoid use of split injection and instead use splitless, on-column or PTV (programmable temperature vaporizing) injection
2. Increase of selectivity and the reduction of baseline noise
    a. Use of GC-MS operated in SIM mode instead of GC-FID

Columns with different polarity such as medium, low and non-polar have been used for COP determination (Guardiola, 50, book chapter 3). There are observations on better resolution in medium polar column (65% phenyl + 35% methyl silicone) than non-polar column (100% methyl silicone) (Rodgues-Estrada, et al, 1998) whereas Dutta (1999) observed poor resolution in a medium polar column (14% cyanopropyl phenyl + 86% methyl silicone) than a low polar column (5% phenyl + 95% methyl silicone). Medium polar columns are designed to have high thermostability than low and non-polar columns (Guardiola, et al., 2002).

The literature shows that the use of capillary column coated with phases of 100% methyl silicone (DB-1) or 5% phenyl + 95% methyl silicone (DB-5). Dutta at al (1999) have reported that DB-5 gave better resolution than DB-1. The elution patterns of COP in DB-1 and DB-5 columns are different (figure 5) (Guardiola, et al., 2002).

Frequently, quantification of COP is carried out by internal standard technique (IS) whereas the methods need powerful purification systems to make sure the appropriate selectivity and sensitivity (Guardiola, et al., 2002).

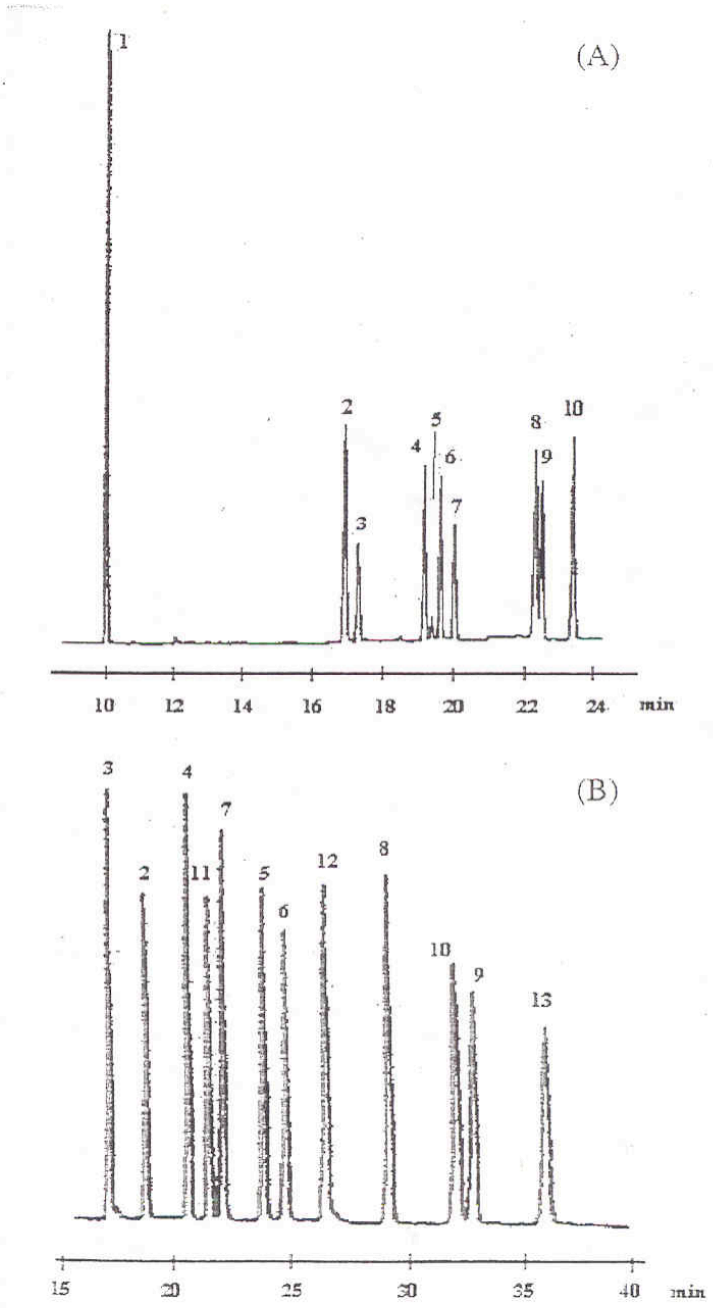

Figure 5. Separation on different polarity columns of the trimethylsilyl ether of the most common COP in foods (A) 100% methyl silicone (B) 5% phenyl + 95% methyl silicone. (1, 5α-cholestane; 2, cholesterol; 3, 7α-hydroxy cholesterol; 4, 19-hydroxy cholesterol; 5, cholesterol-5β, 6β epoxide; 6, cholesterol-5α, 6α epoxide; 7, β-hydroxy cholesterol; 8, cholestanetriol; 9, 7-keto cholesterol; 10, 25-hydroxy cholesterol; 11, cholesta-3,5-dien-7-one; 12, 20α-hydroxy cholesterol; 13, (25R)-26-hydroxy cholesterol+(25S)- 26-hydroxy cholesterol

1.4.5 Harmonization

Analysis of COP in food is a multi-step task that is very difficult in obtaining the same results even for the same food. The different approaches taken by different analyst in this multi-step analytical procedure have lead to concern about the reliability of different COP levels published for the same food. Harmonization of methodology among laboratories was needed urgently because of these different results. Therefore, few attempts have been taken to harmonize methods. The first attempt was the round-robin analysis in 1995, which lead to unsatisfactory results. After the second attempt comparing both trials to harmonize methodology, it was concluded that to develop and optimize a common procedure to measure COP efficiently in various food matrices involves much detailed work. Clear identification of critical steps in the analytical procedure is needed to reduce error in harmonization of the methodology (Dutta & Savage, 2002).

Validation is the formal and systematic proof that a method compiles with the requirements for testing a product while observing defined procedure. It is the process from sample preparation, analysis up to result evaluation. When chromatographic methods are validated in food analysis, all the performance characteristics have to be taken into account; e.g. specificity, precision, accuracy, sensitivity and limitations (McCluskey & Devery, 1993).

1.5 Ghee and cholesterol oxidation products

Ghee, the Sanskrit word meaning 'bright' originated long time ago in India, is a clarified butter from cow or buffalo, and is the most common milk product in the Indian sub-continent. It is used as a cooking, shallow and deep frying medium, sweet additive (toffees, burfi etc.) and is also consumed directly with rice or chapatis, apart from that it is used in traditional medicines (Sserunjogi *et al*., 1998). Home made ghee is prepared by heating and stirring sufficient amount of traditional unsalted butter or cream that made by hand churning whole milk at room temperature, in a suitable open vessel in a low-flame (at about 103°C) to dry out all moisture. When the residue has settled down on cooling, the clear fat is called ghee (Sserunjogi *et al*., 1998). Typical composition of ghee is milk fat (99-99.5%), moisture (less than 0.5%), protein (0.1%), lactose (0.0%) and ash (0.0%). Ghee fat contains saturated fatty acids (53.9- 66.8%), unsaturated fatty acids (22.8 - 38%), free fatty acids (1-3%) and cholesterol (0.15 to 0.30%). Oxidation of cholesterol in ghee has been studied previously and it has been shown that 12% of cholesterol could be oxidized (Jacobson, 1987; Sserunjogi *et al*., 1998).

It is known that the immigrants from Indian subcontinent in London have higher than expected mortality rate from atherosclerosis, without showing the commonly known risk factors (Jacobson, 1987). The author observed that dietary intake of COP from ghee may offer a logical explanation for the high frequency of atherosclerotic complications in the Indian populations.

1.6 Aim of the Study

The main objective of the study I is to evaluate our in-house method by comparing it with a number of commonly used methods for analysis of COP. In order to achieve a better, the methods of purification and enrichment of COP by various saponification methods and transesterification of lipids with spiked samples of standard COP in tallow were investigated.

There is no literature available on ghee containing Indian sweets for lipid oxidation status particularly on the level of COP. The major aim of study II was to investigate lipid composition, status of lipid oxidation by measuring TBARS, and the content of COP in Indian sweets that are available in London Supermarkets.

2.0 Materials and methods

2.1 Materials

Crude tallow fat was donated by Baeten (Overmere, Belgium). Standard samples of 7α-HC cholesterol, Cholest-5-en-3β, 19α diol, 7β-HC, cholesterol, α-CE, β-CE, 20α-HC, CT, 25-HC and 7-KC were obtained from Steraloids (Wilton, NH, USA); 5α-cholestane was from Sigma-Aldrich (Stockholm, Sweden); Tri-Sil reagent was purchased from Pierce (Rockford, IL, USA). Ethanol and methanol were from Kemetyl (Haninge, Sweden). All other chemicals and solvents were of analytical grade and were purchased from Merck Eurolab AB (Stockholm, Sweden), unless otherwise stated.

Indian sweets samples and a pure ghee sample that are available in London supermarkets were supplied by SPK consultancy services (No. 14 Holmemoor Drive, Sonning, Reading, Berkshire RG4 6TE, The UK). Names of the samples are listed below (Table 1).

Table 1
List of Indian sweet samples and a sample of butter oil (ghee)

Referred in this paper	Name
S1	BURFI (R) (type I)
S2	BURFI (R) (type II)
S3	BURFI (R) (type III)
S4	BURFI (R) (type IV)
S5	BURFI (I) (type A)
S6	BURFI (I) (type B)
S7	BURFI (I) (type C)
S8	BURFI (I) (type D)
S9	LADOO (R)
S10	LADOO (A)
S11	Carrot Halva (I)
S12	Carrot Halva (A)
S13	PATISA (A)
S14	Carrot Halva
S15	Barbi
S16	Butter Oil (ghee)

2.2 Methodology

Combination of all methods

To calculate the linearity response and relative response factors, various amounts (1, 5 ,10, 15 and 20 μg) of standard samples of COP were mixed with a fixed amount of 5α cholestane (3.06 μg) and 19 hydroxy cholesterol (2.69 μg) as the internal standards. Then the trimethylsilyl (TMS)-ether derivatives of the above mixtures were analyzed by GC as described below. Response factor for each oxysterol was calculated using the area ratio versus weight ratio of each compound by constructing a multilevel calibration curve (Lee, Yang & Bartle, 1984).

2.2.2.1 Analysis of COP using methods A-D

A) ***Cold saponification*** *with* 1M *KOH/95% EtOH*: Tallow with or without spiking with standard samples of COP (5 μg, 10 μg, 20 μg) was saponified. Approximately 200 mg of tallow was dissolved in 3 ml of dichloromethane in a glass tube with stopper and mixed well by vortexing with 7 ml KOH (1M) in 95% EtOH. This mixture was mixed well and left overnight (18-20 hours) in a dark place in room temperature following previously published methods after some modifications (Park & Addis,1986; Larkeson et al., 2000). Then 10 ml dichloromethane and 10 ml water were added and the mixture was shaken vigorously. Water phase was removed and the organic phase was washed once with 5 ml of 0.5 M KOH in water and few drops of saturated NaCl to facilitate phase separation. After that, the organic phase was washed repeatedly with water until the solution became clear. The solvent was dried under nitrogen, and the unsaponifiables were dissolved in 1ml hexane/diethyl ether (75:25, v/v). The COP fraction was enriched further by solid phase extraction (SPE) as described below.

B) ***Hot saponification*** *with KOH/95% EtOH*: As procedure A, except that the sample was incubated in a 60° C water bath for 1 hour. The reaction was stopped by cooling the sample tubes under running water. The method followed was in accordance with a published method, with minor modifications (Park, Guardiola, Park & Addis, 1996).

C) ***Cold saponification*** *with KOH/MeOH* : As procedure A, except that 95% EtOH was replaced by analytical grade methanol (Park & Addis,1986).

Enrichment of COP by solid phase extraction (SPE) for methods A-C

Enrichment of COP was done essentially following a published method (Larkeson et al., 2000) with slight modifications in above procedures (A), (B) and (C). The unsaponifiable fraction was dissolved in 1 ml of n-hexane / diethyl ether (75:25, v/v) and applied to a silica cartridge (International Sorbent Technology Ltd., Mid Glamorgan, UK) pre-equilibrated with 3 ml of n-hexane. Then the cartridge was

eluted with a solvent series of two. The first solvent mixture was 3 ml n-hexane / diethyl ether (75:25, v/v) and the second was 3 ml acetone / methanol (60:40, v/v). After the second elution, the eluent was collected due to the presence of COP in this fraction. This fraction was dried under nitrogen and derivatised to TMS-ether prior to GC and GC-MS analyses as described below.

D) ***Transesterification*** of tallow was done in accordance with a published method (Schmarr, Gross & Shibamoto,1996). For this purpose ca. 200 mg tallow was weighed and 5μg of 5α-cholestane dissolved in MTBE was added as an internal standard. For transesterification, 2ml of 10% sodium methylate in methanol, diluted with *tert*-butyl methyl ether (MTBE) (4:6, v/v) was added and mixed by vortex for 1 minute. The mixture was allowed to stand for 1 hour at room temperature with additional mixing after ca. 30 minutes. Then, 2 ml of water and 5 ml of chloroform were added and the vial was closed with a tight cap. The sample was shaken for 1 minute to extract the organic material, followed by 5 minutes centrifugation at 2000 rpm to facilitate phase separation. The upper (aqueous) phase was removed. Neutralization of excessive alkali was accomplished by adding 2 ml of 1% citric acid in water, shaking, centrifuging and again disposing of the aqueous phase. The residual chloroform extract was then evaporated under a stream of nitrogen. The COP fraction was enriched further by SPE as described below.

Enrichment of COP by SPE for method D

A small amount of anhydrous sodium sulfate was placed on top of 500 mg of a bonded amino-phase SPE cartridge (International Sorbent Technology Ltd., Mid Glamorgan, UK) for binding traces of moisture. Then the column was conditioned with 5 ml of hexane. Transesterified lipid was dissolved in 250 μl of chloroform, then transferred onto the cartridge and then washed again with 2 x 2.5 ml of hexane. Apolar material and free cholesterol were then eluted with another 5 ml of hexane, 5 ml of hexane/MTBE (5:1, v/v), and 5 ml of hexane/MTBE (3:1, v/v). A polar fraction containing the components of interest was eluted with 7 ml of acetone and collected. Elution proceeded under gravity (Schmarr et al., 1996).

Analytical thin layer chromatography (TLC)

The unsaponifiables (methods A-C) and transesterified samples (method D) were analysed by analytical precoated TLC plates (silica gel 60, 20 X 20 cm, 0.25 mm thickness were used , Merck, Eurolab AB, Stockholm, Sweden) to check visually the completeness of hydrolysis of cholesterol esters. The unsaponifiables, and the transesterified samples along with a TLC-reference standard 18-4A (Nu-Chek-Prep Inc., Elysian, Minnesota, USA), were spotted onto a TLC plate and developed in the solvent system hexane : diethyl ether : acetic acid (85 : 15 : 1, v/v/v). The TLC plate was dried briefly in air and then sprayed with 10 % phosphomolybdic acid in diethyl ether : ethanol (50:50, v/v). The plate was placed in an oven at 120°C for 15 min for color development (Dutta & Appelqvist, 1997).

The enriched samples of COP after SPE from methods A-D were also checked by analytical TLC. For this purpose, high performance thin-layer chromatography plates (HPTLC), silica gel 60 F_{254}, 10 X 10 cm, 0.1 mm thickness (Merck, Darmstadt, Germany) were used. Along with the standard samples of COP, the enriched COP samples were spotted onto the HPTLC plate. The plate was developed in diethyl ether : cyclohexane (9:1, v/v) (13), up to the top of the plate. After a brief drying of the plate in the air, a reagent prepared by dissolving 1g each of phosphomolybdic acid and cerium sulfate in 5.4 ml of sulfuric acid was sprayed and the plate was heated in an oven at 120°C for 15 min for color development (Dutta & Appelqvist, 1997).

Preparation of trimethylsilyl ether (TMS-ether) derivatives of COP

Prior to GC and GC-MS analyses all samples were derivatised to TMS-ethers as described previously (Dutta & Appelqvist, 1997). In brief, after drying the solvent, 100 ml of Tri-Sil reagent (Pierce, IL, USA) were added and the tubes were kept at 60°C for 45 min. The solvent was evaporated under a stream of nitrogen and the TMS-ether derivatives were dissolved in 100 ml hexane. The tubes were sonicated in an ultrasonic bath for 1 min and were centrifuged for 3 min and the tubes were stored at -20°C for subsequent analyses by GC and GC-MS within one week after derivatisation.

Analysis of COP by GC

Analysis of COP as their TMS-ether derivatives was carried out by GC using a Varian 3700 instrument (Palo Alto, CA) fitted with falling needle injector and flame ionization detector. A capillary column DB-5MS 30m x 0.25 mm, 0.50 μm (J&W Scientific, Folsom, CA, USA) was used to separate the COP. The column temperature was set at 290°C for 20 min and raised to 300°C at 0.5°C / min. The detector was set at 320°C. Helium was used as the carrier gas at a pressure of 14 psig and nitrogen was used as the make-up gas at a flow rate of 30 ml / min. A HP 3396 integrator (Hewlett-Packard company, Avondale, PA) was used for recording chromatographic data. Identification and purity percentages were performed using TMS ether derivatives of COP standards. For quantification of COP, response factors (Table 2), as calculated prior to analysis of spiked samples, were used. The indigenous content of COP in the tallow was deducted from all spiked samples when calculating recovery percentages. All samples were determined in duplicates.

GC-MS analysis

A GC 8000 Top Series gas chromatograph (ThermoQuest Italia S. p. A., Rodano, Italy) coupled to a Voyager mass spectrometer with MassLab data system version 1.4V (Finnigan, Manchester, England) was used for this purpose. The COP were separated on the same column used in the GC analysis. Helium was used as carrier gas at an inlet pressure of 80 kpa. The injector temperature was 250°C and the samples were injected in a splitless mode of injection. Oven temperature was at 60°C for 0.5 min and then raised to 290°C at a rate of 50°C/min, and finally the

temperature was raised to 300°C at a rate of 0.5°C/min. The mass spectra were recorded at an electron energy of 70 eV and the ion source temperature was at 200°C.

Determination of Thio Barbituric Acid Reacting Substances (TBARS)

Determination of TBARS values was performed according to the method by Miller (1998). In brief, 0.5 ml of 0.2 mg/ml BHT (Butylated hydroxytoluene) was added to approximately 2.5 g of samples. The volume was made to 25 ml by adding 24.5 ml TCA(Trichloroacetic acid) /H_3PO_4. These samples were blended 30 seconds, filtered through Whatman no. 1 paper, and filtrates were collected. Two aliquots of each filtrate were put into screw caped test tubes followed by adding 2.5 ml TBA and 2.5 ml distilled H_2O to prepare samples and sample blanks respectively. Reagent blank was prepared by pipetting 2.5 ml of extracted solution and 2.5 ml of TBA reagent. Then, the content of these tubes were mixed by inverting them 3 times and kept in dark cabinet for 20 hours. Absorbances were read at 530 nm against distilled H_2O. Reagent blank absorbances were subtracted from all samples and standard readings. Sample blank absorbances were subtracted from only sample readings. Standard curve (Absorbance = 0.3919 x MDA (malondialdehyde) concentration, micro gram per ml + 0.0022) of absorbance versus concentration of MDA (as nmol MDA/ml) was plotted. Then the MDA recovery and TBARS were calculated.

Lipid extraction

A slightly modified version of Hara and Radin method for lipid extraction was used. Approximately 10 g of each sweet sample were homogenized (3 x 30 seconds in a 250 ml Erlenmeyer flask with 150 ml of HIP (hexane/isopropanol, 3:2 vol/vol) using an Ultra-Turrax T25 homogenizer (Jankel & Kunkel GmbH, Staufen, Germany). The homogenized samples were filtered and 85 ml 6.67% Na_2SO_4 in water was added. The upper organic layers were separated after well mixing and settling. These organic layers were dried with anhydrous Na_2SO_4. The organic layers were transferred to 100 ml volumetric flasks and the volumes were adjusted to 100 ml with hexane (Hara & Radin, 1978). Total fat content was determined gravimetrically on a Mettler UMT2 microbalance (Mettler-Toledo AG, Greifensee, Switzerland). Then the extracted fat samples were stored in –20°C until further analysis.

Analysis of fatty acid methyl esters (FAME)

FAME were prepared with ca. 10 mg of extracted lipids in 0.5 ml hexane in a test tube. 2 ml NaOH in dry methanol was added, and the tubes were incubated in a water bath at 60°C for 10 minutes. Thereafter, 3 ml of BF_3 (Boron Trifluoride) was added, followed by another incubation for 10 minutes in the same way. The tubes were cooled under running tap water. 2 ml 20%NaCl and 1 ml hexane was added, the tubes were shaken vigorously, and then allowed to stand to separate into two layers. The hexane layer containing the FAME was transferred to a smaller tube and stored at -20°C for subsequent analysis on GC (Kaijser *et al*., 2000).

Analytical thin layer chromatography (TLC) for control of the FAME

An analytical TLC was employed to check the methanolysis reaction on pre-coated TLC plate (silica gel 60, 20x20 cm, Merck, Darmstadt, Germany). A FAME reference mixture 18-4 A (Nu-Chek-prep, Elysian, USA) along with samples were spotted on the TLC plate, and the plate was developed in the mobile phase hexane: diethyl ether: acetic acid (85:15:1, v/v/v). The TLC plate was air dried for a short time and then sprayed with the molybdate phosphoric acid 10% in ethanol: ether (1:1, v/v). For colour development, the TLC plate was dried at 120°C for 10 minutes.

Analysis of the FAME by GC

Analysis of the FAME was performed on a Chrompack CP 9001 gas chromatograph (Chromapack, Middelburg, Netherlands) equipped with a flame ionization detector and split/splitless injector. Separation of FAME was achieved on a fused silica capillary column BPx70 (SGE, Austin, Texas, USA) length 50 m, id. 0.22 mm, 0.25 μm film thickness. The samples were injected using an auto sampler CP 9050, at split mode of injection and split ratio was 1:30. The GC oven was programmed at 150°C for 7 minutes and increased to 220°C at the rate of 2°C/min. Detector and injector temperatures were 250°C and 230°C, respectively. Helium was used as the carrier gas at 150 kPa and nitrogen as the make up gas at the flow rate of 30 ml/min. FAME were identified comparing their retention times with those of standard FAME mixtures F07 and ME62 (Larodan Fine Chemicals AB, Malmö, Sweden). The peak areas had been integrated using Mastro version 2.4 (Chromapack, Middelburg, Netherlands).

Analysis of sterols

About 20 mg extracted lipids and 20 μg of 5α-Cholestane (Sigma Chemical Co. St. Louis, USA) as an internal standard was saponified with 2 ml 2M KOH in 95% ethanol in a glass tube at 60°C for 45 minutes in a water bath. The reaction was stopped by cooling the tubes and then 1 ml water, 2 ml hexane, and 0.1 ml ethanol were added. The tubes were shaken vigorously and centrifuged at 3000 rpm for 3 minutes. The upper hexane layer was transferred to a smaller glass tube with a ground glass stopper. The solvent was evaporated under nitrogen stream, derivatised to trimethylsilyl (TMS) ethers for subsequent analysis by GC according to (Larkeson *et al*., 2000).

Preparation of TMS ether derivatives for sterols

The TMS ether derivatives of sterols were prepared by adding 100 μl Tri-Sil reagent (Pierce Chemical Co, Rockford, USA) to the saponified samples. The tubes were incubated at 60°C for 45 minutes. The solvent was evaporated under nitrogen stream, and the TMS ether derivatives were dissolved in 100 μl hexane. The samples were centrifuged at 3000 rpm for 3 minutes and kept at –20°C until further analysis by GC.

Analysis of Sterols by GC

A non polar capillary column, DB-5MS (J & W Scientific, Folsom, Ca, USA), 30 m x 0.25 mm x 0.50 μm film thickness was connected to a GC instrument Varian Star 3400 Cx (Palo Alto, CA, USA). The detector was a flame ionization detector and was set at 310°C. Helium was used as carried gas at the pressure of 80 kPa. Nitrogen had been used as make up gas at the rate of 30 ml/min. The oven temperature was at 295°C. TMS ether derivatives of sterols dissolved in hexane, were injected by a falling needle injector. Cholesterol and phytosterols were identified, by comparing the retention times, with those of standard samples and 5α-cholestane was used as internal standard for quantification. The peaks were integrated using a HP 3396 integrator (Hewlett-Packard, Avondale, PA, USA).

3.0 Combined results and discussion

Method evaluation

Baseline separations among the standard samples of 5α-cholestane (internal standard, IS), 7α-HC, 19-HC (IS), 7β-HC, β-CE, α-CE, 20α-HC, CT, 25-HC and 7-KC were achieved with the DB-5 capillary column used in this investigation (see Materials and Methods). The resolution and elution pattern of COP are consistent in this column compared with published results where same column type, apart from higher film thickness, was used (Pie & Seillan, 1992; Regueiro & Maraschiello, 1997).

The retention times, and relative retention times by using both 5α-cholestane and 19-HC as internal standards, linearity response, and response factors of each COP are presented in Table 2. From the results it can be seen that the retention time of unoxidized cholesterol is more than 2 minutes longer than for 7α-OH showing a better separation.

The linearity of response of all COP is almost 1.0 showing that the signals generated for each COP was linear within the mass ranges (1-20 g) in this study confirming a minimal selective loss of COP.

Table 2
Retention times (t_R), relative retention times (RRT) and linearity response (r) of the TMS-ether derivatives of standard samples of cholesterol oxidation products (COP).

COP	t_R (min)	RRT[a]	RRT[b]	r[c]	B[d]
5a-cholestane	13.98	1.00	0.61	-	1.00
7a-HC	19.47	1.39	0.85	0.99	1.02
Cholesterol	21.74	1.56	0.95	ND	ND
19-HC	22.91	1.64	1.00	ND	ND
7b-HC	24.49	1.75	1.07	0.99	0.99
b-CE	27.02	1.93	1.18	0.98	1.54
a-CE	27.84	1.99	1.22	0.97	1.27
20a-HC	28.56	2.04	1.25	0.98	0.82
CT	30.73	2.20	1.34	0.95	1.48
25-HC	33.51	2.40	1.46	0.97	1.35
7-KC	35.11	2.51	1.53	0.97	1.24

[a]Retention time relative to 5α-cholestane, [b]Retention time relative to 19-HC; [c]Linearity response; [d]Response factor was determined by constructing a multi-level calibration curve (area ratio versus mass ratio of internal standard and COP). For details, see Materials and methods section.

The calculated RRF (relative response factor) values in this study are listed in Table 2, where generally the RRF values are higher than 1, except 20-OH. The differences

of the response factors may be influenced by the different functional groups in different COP, nature of internal standard, the nature of the column, GC conditions, etc.

The results of the recovery percentages, all of the COP followed high recovery percentages in method A compared with the other methods (C-D)(Figure 6). The recovery percentages ranged from 68% to 88%, except for 7αa-OH and CT, which had comparatively lower recoveries of 58% and 57%, respectively. The recovery of CT was lowest (13%) in method B. The recovery of β-CE was low in methods C (18%) and D (16%), compared with the highest recovery in method A (69%). No comparable results on method A have been published previously, but published recovery percentages of various COP at 5 g level by method D were more than 86% for all COP analysed (Schmarr et al., 1996), which could not be reproduced in this study. Since methods B and C consistently showed lower recovery of COP (Figs. 6a to 6c).

The results of the recovery percentages at a level of 10μg of each COP are shown in Figure 6b. Method A produced the highest recovery percentages for β-CE (75%), α-CE (78%), 20α-HC (60%) and 7-KC (81%). Method D had the highest recoveries for 7α-HC (75%), 7β-HC (78%) and CT (55%). At this level, method A showed generally higher recoveries than method D, except slightly lower recoveries for 7αa-OH, 7βb-OH, and CT.

The results of the recovery percentages at a level of 20μg of each COP are shown in Figure 6c. Method A had higher recovery percentages for β-CE (78%), α-CE (88%), 20α-OH (66%), and 25-HC (97%), compared with method D. Although the other COP showed higher recoveries with method D, however, only in the case of CT the recovery was considerably higher than in method A.

Comparative statistical data on the results of recoveries of COP by methods A and D are presented in Table 3. The CV % of the recoveries of standard COP spiked at various levels in tallow showed consistently lower values given by method D compared with method A, although recovery percentages were generally higher in method A. Results of t-statistics show that most of the recovery percentages were not significantly different between methods A and D, except for α-CE ($P \leq 0.001$), β-CE ($P \leq 0.01$), and CT ($P \leq 0.01$) at 5 g level (Table 3).

For 7α-HC, 7β-HC, β-CE and 7-KC in 5 μg spiked and cold saponified samples, the CV% were considerably higher in method A compared with method D, although in all these cases considerably higher recovery percentages were recorded in method A (Table 3).

20 μg spiked samples gave more than 10% of CV in 20α-HC, CT and 7-KC after cold saponification with ethanol. For10 μg spiked samples; this value is less than 10% for all COP except CT, 25-HC and 7-KC. But in transesterification CV% is

lower than 10% in all the samples except β-CE (5 μg spikes) and 20α-OH (5 μg and 20 μg spiked).

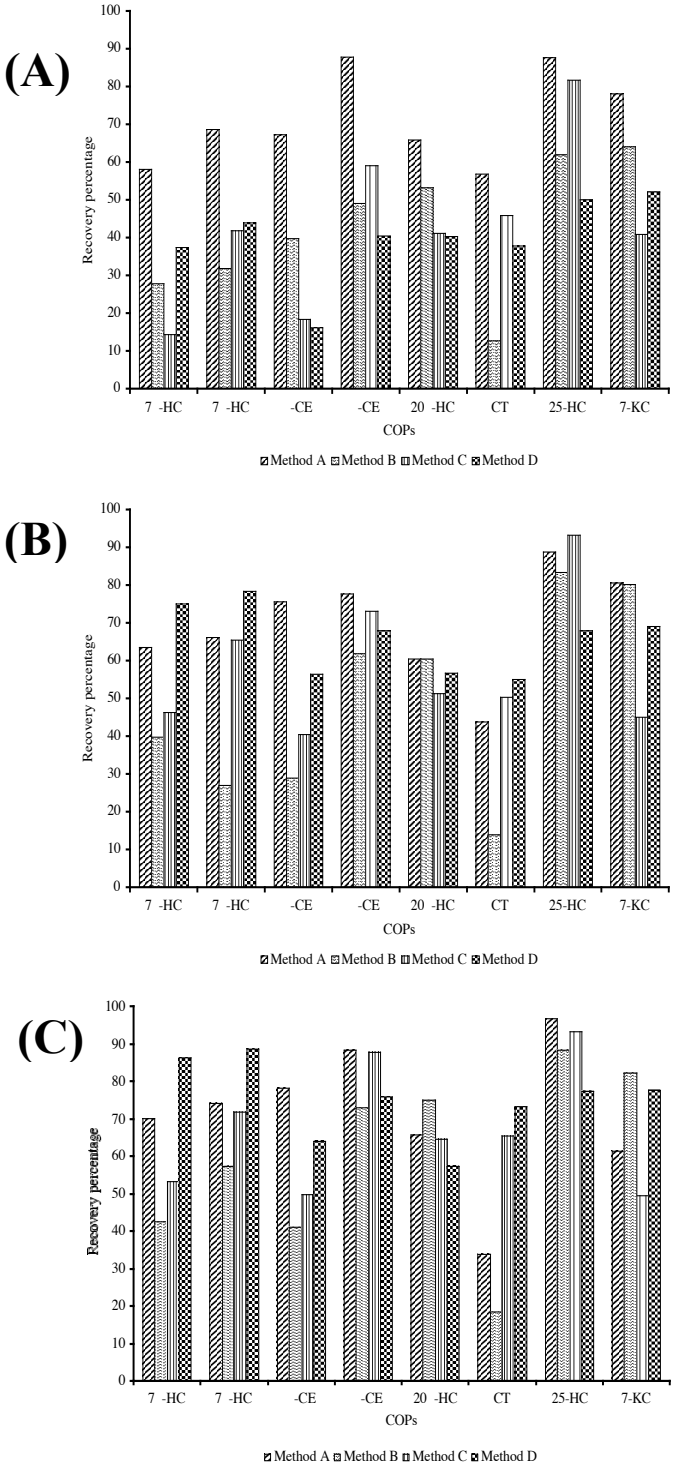

Figure 6. Recovery percentages of COP spiked with (A) 5μg (B) 10 μg (C) 20 μg standard samples of each component; Method A- cold saponification with 2M KOH in 95% ethanol in water; Method B – cold saponification with 2M KOH in methanol; Method C – hot saponification with 2M KOH in 95% ETOH in water; Method D – Transesterification.

Table 3

Statistical data on the recovery (%) of cholesterol oxidation products (COPs) at different levels of spiking by methods of cold saponification (A), and transesterification (D).

COPs	Amount Spiked (μg)	Cold saponification (A) (95% ethanol)		Transesterification (D)		
COP		Mean % (± SEM)	CV %	Mean % (± SEM)	CV %	t value df = 2
7α-HC	5	58.1 (21.0)	36.1	37.3 (2.5)	6.8	0,98
	10	63.6 (1.2)	1.8	75.1 (3.0)	3.9	3.62
	20	70.2 (3.2)	4.6	86.5 (7.3)	8.4	2.05
7β-HC	5	68.6 (11.7)	17.0	43.9 (1.3)	2.9	2.11
	10	66.1 (1.9)	2.9	78.4 (2.6)	3.6	3.77
	20	74.1 (0.4)	0.5	88.7(5.9)	6.7	2.47
β-CE	5	67.3 (20.3)	30.2	16.2(2.9)	18.1	2.49
	10	75.6 (5.9)	7.8	56.5(3.0)	5.2	2.98
	20	78.1 (5.0)	6.4	64.1(0.4)	0.7	2.81
α-CE	5	87.8 (1.2)	1.4	40.5 (0.2)	0.5	37.59
	10	77.7 (4.6)	6.0	67.9 (2.3)	3.4	1.89
	20	88.4 (2.9)	3.3	76.1 (3.1)	4.1	2.90
20α-HC	5	65.8 (1.4)	2.1	40.3 (5.2)	12.6	4.86
	10	60.5 (5.8)	9.7	56.7 (2.8)	4.9	0.59
	20	65.9 (9.3)	14.2	57.4 (8.7)	15.2	0.67
CT	5	56.8 (0.6)	1.1	37.8 (1.2)	3.2	13.93
	10	43.8 (5.1)	11.6	55.0 (3.7)	6.8	1.77
	20	34.1 (7.7)	22.6	73.4 (1.9)	2.5	4.97
25-HC	5	87.7 (1.8)	2.1	50.1 (0.4)	0.7	20.44
	10	88.7 (9.8)	11.0	67.9 (2.7)	4.0	2.05
	20	96.9 (8.3)	8.5	77.5 (1.2)	1.5	2.33
7-KC	5	78.0 (12.0)	15.3	52.2 (1.8)	3.5	2.14
	10	80.6 (8.7)	10.8	69.1 (2.8)	4.0	1.26
	20	61.6 (9.5)	15.4	77.6 (3.3)	4.2	1.59

Abbreviations: SEM, standard error of mean (n = 2); CV %, coefficient of variation; t, value of t-statistic; df, degrees of freedom.

Conclusion of method evaluation

Both cold saponification (95% EtOH) and transesterification gave comparable results, where cold saponification (95% EtOH) gave better results at low levels of COP.

Trasnesterification is a quicker method but needs to be optimized at enrichment step by SPE

Indian sweet analysis

Fat percentages in the samples analyzed in this study had large variation ranging from 10 to 24% (Table 5). Probably the main reason of variation in fat content is that all the samples were different products, composed of different ingredients, generally made in small-scale production equipments. To our knowledge no literature values are available to compare with our results.

Presence of COP in foods, have evoked much interest due to health concerns (Dobarganes & Marquez-Ruiz, 2003). The content of the seven common polar COP found in the samples are presented in Table 4. Two samples (S5 & S6) contained mainly phytosterols and no effort was made to determine the individual phytosterol oxidation products (POP) in these samples, and only the total amount of POP is presented in the Table 4. Generally, the amount of total COP varies from 0.94 to 38.41 μg/g sample in all the samples. Only trace amounts of COP were found in the sample of ghee (S16), and no cholestanetriol and 25-hydroxycholesterol were detectable in this sample. The reasons for this difference could be heating or frying during sweet manufacturing, storage (time and condition), presence of prooxidants and the degree of unsaturation of fatty acids in the lipids etc.

The levels of COP in this study generally lie within the range of previous published results (Kumar et al., 1992; Kumar *et al*., 1999) with few exceptions. Jacobson (1987) have reported that the total amount of COP in ghee was 12.3% of total cholesterol but no COP was found in butter. It has also been supported that the formation of COP occurred only after autoxidation to such an extent that ghee becomes unacceptable for consumption (Nath *et al*., 1988). It was shown in another study that when ghee was used as a frying medium for short frying periods COP could be detected only after 15 minutes heating (Nath *et al*., 1996). However, it has also been shown qualitatively by TLC that homemade ghee contained cholesterol epoxides and cholestanetriol, but not in commercial ghee (Prasad *et al*., 1992).

Kumar *et al*. (1992) observed that fresh milk or cream from cow or buffalo contained no detectable COP, but fresh butter had traces of 7β - hydroxy- and 5,6α-epoxycholesterol. The authors also reported that the production of ghee from butter at 120°C induced the formation of various COP with no significant differences between cow and buffalo ghee, in which the levels of COP were 0.7 and 0.9% of total sterol, respectively. Further it was demonstrated that intermittent heating of ghee at 225°C and frying of "purees" in ghee at 185-200°C, induced increased peroxide value and increased the levels of total COP. The authors showed that intermittent heating generated higher levels of atherogenic COP 25-hydroxycholesterol and cholestanetriol in ghee (8-9% of total COP) than that of frying of "puree" in ghee (7.1%). Similar effects of heating on the generation of COP in ghee have been observed, where the level of total COP increased from 1.32% to 17.6% of total cholesterol, after heating of ghee at 120°C for 40 hours (Kumar *et al*., 1999).

Table 4
Contents of cholesterol oxidation products (COP) (μg/g sample) in the lipids of Indian sweet samples and a sample of butter oil (mean of triplicate analysis)

	COP								
Sample	7α-HC	7β-HC	β-CE	α-CE	CT	25-HC	7-KC	TUC [a]	TIC [b]
S1	1.34	0.75	0.38	0.25	1.15	0.15	0.54	8.6 (3)	4.55
S2	0.82	0.62	0.24	0.19	0.88	0.11	0.20	7.3 (4)	3.06
S3	1.57	1.05	0.16	0.16	1.45	0.10	0.30	10.7 (4)	4.78
S4	1.52	0.51	0.26	0.23	1.60	0.10	0.31	9.1 (4)	4.53
S5	-	-	-	-	-	-	-	-	6.91[c]
S6	-	-	-	-	-	-	-	-	18.79[c]
S7	0.15	0.17	0.06	0.14	0.40	0.16	1.00	3.4 (3)	2.03
S8	0.24	0.17	0.18	0.11	0.05	0.06	0.12	-	0.94
S9	2.32	3.19	1.87	1.25	0.33	0.27	1.02	-	10.23
S10	1.73	2.10	1.22	0.85	0.27	0.13	0.73	3.1 (1)	7.03
S11	2.00	0.67	0.63	0.24	3.27	0.18	0.64	17.2 (3)	7.60
S12	1.37	0.22	0.27	0.32	1.87	0.40	0.74	21.3 (4)	5.18
S13	6.85	2.00	2.17	3.00	3.97	0.73	1.75	18.1 (3)	20.31
S14	1.24	0.21	0.82	0.91	0.91	1.10	0.85	6.9 (3)	5.98
S15	1.41	0.70	0.38	0.27	1.41	0.4	0.21	7.2 (3)	4.80
S16	1.10	1.36	1.77	1.20	-	-	1.10	-	6.46

7α-HC, 7α-Hydroxycholesterol; 7β-HC, 7β-Hydroxycholesterol; β-CE, Cholesterol-5β, 6β-epoxide; α-CE, Cholesterol-5α, 6α-epoxide; CT, Cholestanetriol; 25-HC, 25-Hydroxycholesterol; 7-KC, 7-Ketocholesterol
[a]TUC, Number of total unidentified COP; [b]TIC, Total amounts of COP were calculated only for the seven identified COP; [c]Presented as total phytosterol oxidation products (POP); -, Not detected.

The major COP in the ghee containing samples in this study were 7α-hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol. According to the mechanism of cholesterol autoxidation, stable autoxidation products such as 7α-hydroxy- and 7β-hydroxycholesterol and the dehydration product 7-Ketocholesterol forms due to thermal decomposition of C-7 hydroperoxides (Lercker and Rodriguez-Estrada, 2002). The total COP in samples S9, S11 and S13 have considerably higher (Table 4). It is to be noted that these samples had higher contents of linoleic acid and linolenic acids compared with other sweet samples (see Table 5), which may facilitate cholesterol oxidation (Larkeson *et al*., 2000).

Presence of short chain fatty acids, caproic acid and caprylic acid (6:0, 8:0), a characteristic feature of milk fats, in the samples except two confirms that the butter oil was a main ingredient in the sweets. The dominating saturated fatty acids were myristic acid (14:0) ranged from 7 to 11%, palmitic acid (16:0) ranged from 9 to 33%, and stearic acid (18:0) ranged from 9 to 13%, in all samples, except S1, S5 and S6. The major mono unsaturated fatty acid was oleic acid (18:1) ranging from 16 to

25% except samples S5 and S6 (Table 5). Linoleic (18:2) acid ranged from 1-5% in all samples except S5 and S6. Minor amount of linolenic acid (18:3) (<1%) was found in all samples.

Table 5
Contents of fat, Thio Barbituric Acid Reacting Substances (TBARS), and composition of fatty acids in the extracted lipids of Indian sweet samples and a butter oil sample (mean of duplicate analysis)

	Fat %	TBARS µg/ 100g	Fatty acids %										
			6:0	8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3	Others
S1	23.7	80	0.7	3.0	4.0	19.0	13.1	24.2	8.5	15.8	1.6	0.3	9.8
S2	19.6	149.8	0.9	1.1	2.7	3.4	10.6	32.3	11.4	20.6	1.7	0.3	15.0
S3	13.4	218.9	0.8	1.0	2.9	4.0	11.3	32.9	11.8	19.4	1.8	0.3	13.8
S4	15.2	82.4	0.7	1.0	2.5	3.5	9.5	29.4	10.1	24.9	5.2	0.4	12.8
S5	17.6	140.7	-	-	-	-	0.1	9.4	9.3	60.6	18.1	0.2	2.3
S6	13.4	256.9	-	-	-	-	0.1	8.7	8.0	63.3	17.6	0.2	2.1
S7	10.7	21.5	0.8	0.8	1.7	2.2	10.8	33.3	11.7	20.0	1.2	0.3	17.2
S8	13.6	19.1	0.9	0.6	1.4	1.8	8.8	32.0	11.6	21.4	2.1	0.3	19.1
S9	19.9	167.8	1.1	1.3	3.0	3.4	10.4	27.6	10.8	20.6	3.3	0.7	17.8
S10	22.4	155.9	1.0	1.1	2.3	3.6	10.5	27.5	11.2	21.7	3.8	0.7	16.6
S11	9.8	106.3	0.6	1.3	1.5	6.0	7.1	27.7	8.5	22.3	5.3	0.4	19.3
S12	13.4	209.5	1.1	1.2	2.8	3.6	10.6	28.0	11.6	21.7	1.6	0.6	17.2
S13	19.1	64.5	1.2	1.1	2.4	3.0	9.6	27.0	11.4	22.8	4.1	0.7	16.7
S14	20.5	82.1	1.1	1.2	3.0	3.7	10.7	29.3	10.5	21.6	2.6	0.6	15.7
S15	12.3	48.7	1.0	1.1	2.6	3.2	10.2	28.2	12.1	23.1	1.4	0.6	16.5
S16	100.0	-	1.2	1.4	3.4	3.7	11.1	27.3	12.9	17.5	1.0	1.0	19.5

-, Not detected; Others, include 4:0, 20:0, 20:1, 14:1, 16:1, 18:1$^{\Delta}$9*trans* and some unidentified fatty acids

Fatty acids presented under "Others" (Table 5), would contain butanoic acid (4:0), arachidic acid (20:0), gadoleic acid (20:1) tentatively identified as myristoleic (14:1), palmitoleic acid (16:1) and elaidic acid (18:1$^{\Delta}$9*trans*) and some unidentified fatty acids. None of these fatty acids were more than 0.05%. Literature values of fatty acid composition of cow ghee are within the range found in this study (Al-Khalifah & Al-Kahtani, 1993; Kumar *et al*., 1999).

The fatty acid profile of sample S1 shows that apart from milk fat, possibly palmkernel or coconut oil has been used in the preparation of this sweet. Because this sample had higher levels of lauric acid (12:0), myristic and palmatic acids, compared with other ghee containing samples (Padley *et al*., 1994). Sample S5 and S6 are remarkably different than the other samples. The short-chain saturated fatty acids were not present in these samples. Palmatic and stearic acids were relatively low, but oleic (63%) and linoleic (18%) acids were the highest in these two samples. This fatty acid profile gives enough proof to believe that both these sweets were not prepared with ghee. From the fatty acid table it is apparent that the oil used was not known for samples of vegetable origin and to check it, hydrogenated fat was used (Nawar, 1996). We also analysed these samples for *trans* fatty acids but only trace amounts of elaidic acid (<0.05%) could be found (Results are not shown separately in Table 5). It is possible that these two samples (S5 and S6) contained mixture of unspecified vegetable oils.

The TBARS test determines the contents of malonaldehyde (MDA) and other 2-thio barbituric acid reactive substances (TBARS) in the samples. TBARS values ranged from 19 to 260 μg/ 100g (Table 5). The TBARS of ghee sample did not show any positive value when measured at 530 nm. Kosugi *et al*. (1991) have shown that oxidized fats and oils release only small amounts of malonaldehyde and considerable amounts of other aldehydes, e.g. alkenals and dienals which also react with TBA. The major limitation of TBARS analysis is the reactivity of the TBA with other aldehydic substances in the sample. There also can be other interfering compounds, which react with TBA to yield the characteristic red pigment such as sugars, ascorbic acid and nonenzymatic browning products. Malonaldehyde often bound to proteins lowering the TBA values (Nawar, 1996). In spite of these limitations, TBARS test is still used to determine the level of lipid oxidation (Dobarganes & Marquez-Ruiz, 2003).

There are reports on the possible association of ingestion of lipid peroxidation products changing the blood low-density lipoproteins leading to chronic non-transmittable diseases such as formation of atherosclerotic plaques and coronary artery disease. Malonaldehyde is known to be mutagenic and carcinogenic. But there is no available literature on a standard value that is in low risk region that has been proved (Ferrari & Torres, 2002) and no literature data are available on TBARS in such samples we analysed in this study.

In order to check if there was any relationship between TBARS and total unsaturated fatty acids, a simple statistical analysis was done. The correlation coefficient between TBARS and poly-unsaturated fatty acids (18:2 + 18:3) was 0.420. This weak but positive relationship shows that the increased amounts of unsaturated fats that gives more lipid oxidation (MDA). A rather similar but negative correlation (-0.412) was observed with TBARS and total saturated fatty acids, thus showing the expected opposite relationship of that of poly-unsaturated fatty acids and TBARS.

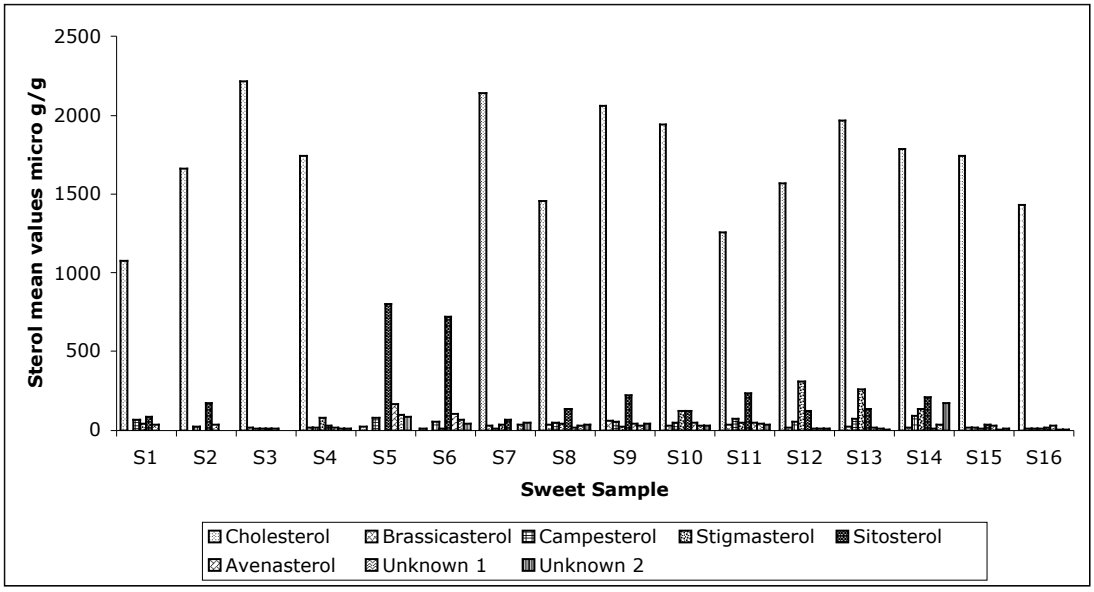

Figure 7.
Contents of total sterol (μg/g lipids) in the lipids of Indian sweet samples and in a sample of butter oil (mean of duplicate analysis).

The levels of the identified sterols in the lipids of the samples are shown in figure 7. The major sterol was cholesterol in all samples except S5 and S6 ranging from 1077 to 2217 μg/g lipids. There were minor amounts of phytosterols such as campesterol, stigmasterol, sitosterol and Δ5-avenasterol in all samples. It is worthy to note that brassicasterol could not be traced in the samples S1, S2, S5 and S6. The presence of small amounts of brassicasterol may indicate that rapeseed/mustard oil was partially used in the preparation of the sweets. The values for cholesterol amounts in ghee agree with the literature values (Kumar *et al*., 1999). Sitosterol, the main sterol in all most all kinds of vegetable oils was the main sterol in the samples S5 and S6, ranged from 720-803 μg/g lipid (Piironen and Lampi, 2004). Sterol composition of these two samples supports our conclusion that unspecified mixture of vegetable oil was used in preparation of sweets and devoid of ghee. All the other samples contained traces of phytosterols varied from 2-311 μg/g lipid, which originate from the flour used in the preparation of the sweets.

Conclusion of Indian sweet analysis

From the analysis of fatty acids and sterols it was confirmed that both the samples S5 and S6 were made of mixed vegetable oils, and ghee was a main ingredient in all other sweet samples. This study shows that Indian sweets available in London supermarkets contain considerable amount of saturated fat (53 – 73% of total fat), cholesterol (0.11-0.22% of total fat) COP (0.94 – 38.41 μg/g sample). It may be concluded that regular consumption of these sweets may result in increased intake of saturated fats, unspecified lipid and non-lipid oxidation products, cholesterol and COP, which may contribute to atherosclerosis.

The total amount of COP found in this study is lower than the results of Jacobson (1987). In Jacobson's study the author has identified COP from unsaponifiable fraction of ghee by TLC and has quantified these COP by densitometry.

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Appendix

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