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Effect of Processing Conditions on the Oxidation of Cholesterol in Ghee*

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Abstract: The cholesterol oxidation products (COP) in fat-rich dairy products were identified and quantified. Fresh cream contained no COP whereas fresh butter contained trace levels of 7β-hydroxy- and 5,6α-epoxycholesterol. Low levels of various major COP were present in ghee (clarified butter fat). Intermittent heating and frying of ghee induced severe oxidation of cholesterol and a number of COP were detected by GC and TLC. Most atherogenic COP, viz 25-hydroxycholesterol and cholestantriol, were formed more in intermittently heated ghee (8·1–9·2% of the total COP) than in fried ghee samples (7·1%).

Key words: Cholesterol, oxidation, ghee, processing, quantification.

INTRODUCTION

The tendency of cholesterol to autoxidise spontaneously in air and to peroxidise *in vivo*, and the reported presence of cholesterol oxidation products (COP) in foods, has evoked much interest recently, due largely to the reports implicating them in atherogenic and other adverse effects on human health. In-vivo studies (Imai *et al* 1976, 1980; Peng *et al* 1977; Taylor *et al* 1979; Jacobson *et al* 1985) and *in-vitro* studies (Baranowski *et al* 1982; Peng *et al* 1982) have shown the atherogenic properties of COP. Inhibition of cholesterol biosynthesis due to repression of HMG-CoA reductase activity by COP has also been reported (Sinesky *et al* 1981; Gibbon 1983; Parish *et al* 1986). More than 60 oxidation products of cholesterol are known (Smith 1987). The oxidation products of cholesterol have been identified and quantified in various foodstuffs (Ryan *et al* 1981; Finocchiaro *et al* 1984; Tsai and Hudson 1984; Park and Addis 1985, 1986, 1987; Sugino *et al* 1986; Nourooz-Zadeh and Appelqvist 1987; Bovencamp *et al* 1988; Sander *et al* 1988, 1989a, b). Jacobson (1987) reported the presence of various COP in Indian ghee (clarified butter fat) and hypothesised that ingestion of COP present in ghee might be the causative factor for the high frequency of atherosclerotic complications in the Indian immigrant population.

Ghee is prized as an important article of the diet by Indians and is used for culinary purposes as a deep fat frying medium. A decrease in the content of cholesterol *per se* in ghee heated at high temperature has been reported (Bector and Narayanan 1975; Rai and Narayanan 1986); the decrease might be due to oxidation into various derivatives (Naresh and Singhal 1991). Since no other report is available on the presence of COP in Indian ghee, this study was designed to obtain basic information on the effect of various processing treatments on cholesterol autoxidation and the levels of various COP present in ghee.

MATERIALS AND METHODS

Reagents

The common (used in this text) and systematic names of the COP are: 7α-hydroxycholesterol (5-cholesten-3β,7α-diol), 7β-hydroxycholesterol (5-cholesten-3β,7β-diol), 7-ketocholesterol (5-cholesten-3β-ol-7-one), 5,6α-epoxycholesterol (5α-cholestan-5,6α-epoxy-3β-ol), 25-hydroxycholesterol (5-cholesten-3β,25-diol), 20α-hydroxycholesterol (5-cholesten-3β, 20α-diol), and cholestan-triol (5α-cholestan-3β,5α,6β-triol). The systematic name for cholesterol is 5-cholesten-3β-ol.

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Cholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol, 20α-hydroxycholesterol, 5,6α-epoxycholesterol and 5α-cholestane were obtained from Sigma Chemical Co, St Louis, MO, USA. 7α-Hydroxycholesterol was procured from Steraloid Inc, Wilton, NH, USA. Cholestan-triol was synthesised in the laboratory by the method of Fieser and Rajgopalan (1949) and the purity was checked by IR spectroscopy.

All the solvents and reagents were of analytical grade and were procured from Glaxo India Ltd (Bombay), Loba Chemie (Bombay) and Spectrochem (Bombay). Hexamethyldisilazane and trimethylchlorosilane were procured from Sigma. Solvents were purified by the methods of Vogel (1961).

Fat samples

Fat from cream (obtained from the Experimental Dairy of the Institute) was extracted by the method of Folch *et al* (1957).

Butter fat was obtained by melting butter at 40°C and centrifuging at 300 × *g*.

Ghee samples were prepared by the method of Ganguli and Jain (1973) by clarification at 115–120°C.

Intermittent frying of 'purees' (flour dough) in ghee

The frying operation was conducted in an open iron pan (diameter 35·5 cm, depth 10·2 cm). The ghee used was made in the Experimental Dairy of the Institute by the creamery butter method. Wheat flour dough was prepared by adding 125 ml water to 200 g wheat flour. The dough was then divided into 20 balls of approximately the same size. The balls were pressed into a circular shape to form 'purees' which were fried one by one at an interval of 3 min each in the ghee (about 2 kg) maintained at 185–200°C. The frying process continued for 1 h. The ghee left over in the container was then kept at room temperature (20–25°C) and the frying process was repeated after 24 h storage on the following two days. Samples were drawn before and after each frying and were analysed for chemical changes.

Intermittent heating

Buffalo and cow ghee (about 2 kg each), prepared in the laboratory by the creamery butter method at the clarification temperature of 115–120°C, were each heated for 30 min in an iron pan placed in an electric oven maintained at 225°C. The process was repeated the following two days after 24 h storage at room temperature (20–25°C).

Peroxide value (PV) and free fatty acid (FFA) contents were measured by the IS:3508 (1966) methods.

Cold saponification

Ethanolic KOH (5·0 ml, 300 g litre^{-1}) and 5 ml ethanol were added to about 1 g fat. The mixture was shaken vigorously until it became free from dispersed fat particles and then saponified at 25–30°C for 20–22 h with occasional shaking. The unsaponifiable matter (USM) was then extracted by the method of Park and Addis (1986).

Thin layer chromatography

TLC of the USM to identify individual COP was performed on glass plates (20 × 20 cm or 10 × 20 cm) coated with silica gel G (0·25 mm thick layer) activated at 110°C for 2 h prior to use. The plates were developed in a solvent system consisting of ethyl acetate/heptane (1:1 v). The plates were sprayed with 650 g kg^{-1} H_2SO_4 and heated at 180°C for 1 h to visualise the spots. Standard cholesterol oxides were co-chromatographed for the measurement of R_c values (distance travelled by components relative to cholesterol as unity).

Gas chromatography

A gas chromatograph (AIMIL 5700, Nucon, New Delhi) coupled with FID and a dual column system was used. Glass columns 1·83 m × 3·2 mm (6 ft × $\frac{1}{8}$ in) packed with 30 g kg^{-1} OV-17 on 80–100 mesh Gas Chrom Q were used. Trimethyl silyl (TMS) ethers of the USM were prepared by the method of Miettinen *et al* (1965). The TMS reagent comprised dimethylformamide, hexamethyldisilazane and trimethylchlorosilane (40:40:1 v) and was added at 0·2 ml mg^{-1} USM. The chromatogram of the TMS ethers of COP was recorded on an Omni Scribe Recorder (Nucon). Operating conditions were: oven temperature, 270°C (isothermal); injection port temperature, 290°C; and detector temperature 330°C. The carrier and fuel gases were nitrogen and hydrogen, respectively.

5α-Cholestane (Sigma) was used as an internal standard in the GC analysis of oxides. To 1 g fat was added 0·4 ml of 0·4 mg ml^{-1} 5α-cholestane in heptane, and the solvent was evaporated under vacuum. The mixture was then cold saponified as above. Quantification of COP and cholesterol was as described by Adams *et al* (1986).

RESULTS AND DISCUSSION

Fat-rich dairy products

The levels of COP in fat-rich dairy products, viz cream, butter and ghee, are depicted in Table 1. Fresh cream from cow and buffalo milks contained no detectable cholesterol oxides. The absence of COP in fresh cream has been reported by Finocchiaro *et al* (1984), Kou and

TABLE 1
Levels of cholesterol oxidation products in fat-rich dairy products

Product	*COP (mg kg^{-1})*						
	7α-OH	*7β-OH*	*25-OH*	*20α-OH*	*Epoxy*	*Triol*	*7-Keto*
Cow							
Cream	ND	ND	ND	ND	ND	ND	ND
Butter	ND	Tr	ND	ND	Tr	ND	ND
Ghee	2·1 ± 1·2	4·2 ± 1·8	ND	ND	5·6 ± 1·8	ND	3·1 ± 0·2
Buffalo							
Cream	ND	ND	ND	ND	ND	ND	ND
Butter	ND	Tr	ND	ND	Tr	ND	ND
Ghee	3·6 ± 0·8	5·9 ± 1·0	ND	2·7 ± 1·0	4·6 ± 0·9	ND	4·1 ± 0·9

ND, Not detected; Tr, < 1·0 mg kg^{-1}.
Mean ± SD, $n = 3$.

Holmes (1985) and Jacobson (1987), although sour cream has been reported to contain low levels of 5,6α-epoxycholesterol (Sander *et al* 1988, 1989a).

Butter manufactured by churning fresh cream contained only traces of 7β-hydroxy- and 5,6α-epoxycholesterol (1·0 mg kg^{-1}). Sander *et al* (1988, 1989a) had also reported low levels of 7β-hydroxy- and 5,6α-epoxycholesterol in fresh butter. 7-Ketocholesterol, reported by Nourooz-Zadeh and Appelqvist (1988) and Pie *et al* (1990) in butter, could not be detected in the butter samples used in our study. Luby *et al* (1986a, b) and Jacobson (1987), however, found no detectable COP in fresh butter.

Transformation of butter into ghee at the clarification temperature of 120°C induced the formation of various COP in the ghee samples of both species (cow and buffalo), though no significant differences were observed in the levels of total COP between the two species. Quantitative analysis by GC revealed that 7β-hydroxy- and 5,6α-epoxycholesterol were higher in proportion (4–6 mg kg^{-1}) compared with 7-keto- and 7α-hydroxycholesterol (2–4 g kg^{-1}) (Table 1). Buffalo ghee contained a small amount of 20α-hydroxycholesterol also. The concentration of COP was 0·7–0·9% of that of total cholesterol. Jacobson (1987), however, reported as high as 123 g kg^{-1} COP of the total cholesterol in the ghee samples. Apart from 7α-hydroxy-, 7β-hydroxy-, 5,6α-epoxy- and 7-ketocholesterol, he also observed the presence of 20α-hydroxy- and 25-hydroxycholesterol (about 60 mg kg^{-1}) and trace level of cholestan-triol. However, the quality of ghee samples used by Jacobson was not indicated. TLC analysis of the unsaponifiable matter confirmed the presence of 7β-hydroxy- (R_f 0·53) and 5,6α-epoxy- (R_f 0·73) cholesterol in both cow and buffalo butter and ghee samples and 20α-hydroxycholesterol (R_f 0·91) in buffalo ghee only (Fig 1). A spot with an R_f value of 0·53 corresponding to 7β-hydroxycholesterol (spot 6) appears to be present in buffalo cream by TLC analysis, but this could not be confirmed by GC.

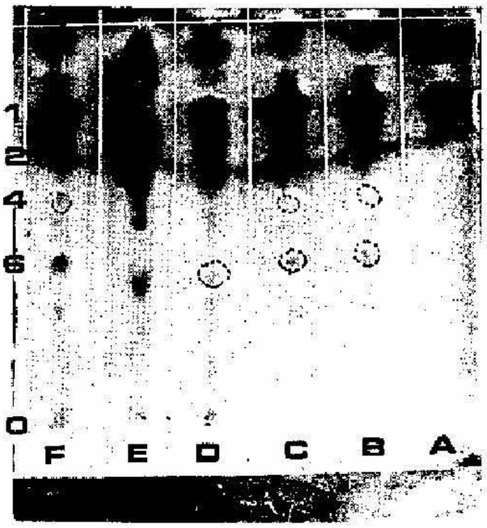

Fig 1. TLC pattern of unsaponifiable matter of fat-rich dairy products. A—cow cream; B—cow butter; C—cow ghee; D—buffalow cream; E—buffalo butter; F—buffalo ghee. 1—Cholesterol; 2—20α-hydroxycholesterol; 4—5,6α-epoxycholesterol; 6—7β-hydroxycholesterol; O—origin.

Intermittent frying

A gradual increase in the FFA content was observed during frying (Fig 2). At the end of the third frying the FFA content increased from 2·5 g kg^{-1} to 5·3 g kg^{-1} which was well below the Agmark standards in India for special-grade ghee (14 g kg^{-1}). However, the PV increased abruptly from 0·07 to 9·1 during the first frying, then decreased to 7·87 during the second frying and

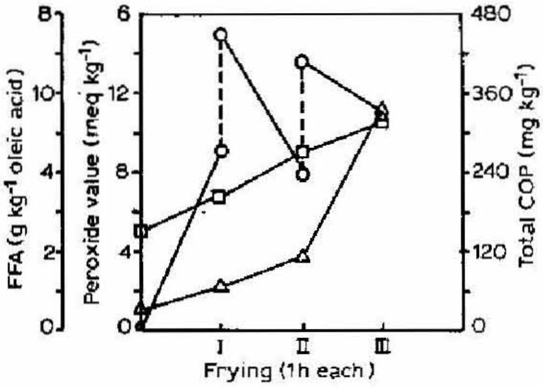

Fig 2. Effect of intermittent frying on the free fatty acid content, peroxide value and total COP in ghee ($n = 3$). —□—□— FFA; —○—○— peroxide value; —△—△— total COP; —— 24 h storage (20–25°C).

increased again to 10·93 during the third. During storage (24 h) of ghee left over from each frying, heat-induced oxidation was pronounced, as indicated by increased PV (dotted lines, Fig 2). The hydroperoxides being thermally unstable decomposed during the second and third frying as was evident from the fall in the peroxide value. Similar observations were made by Rai (1982) during intermittent frying in ghee.

A decrease of about 13·2% in total cholesterol was observed as a result of the three successive fryings (Table 2), and it was almost linear throughout. Rai and Narayanan (1986) reported 28·2 and 49·0% decreases in total cholesterol content in buffalo ghee heated intermittently for 12 h in aluminium and iron containers, respectively.

With the decrease in cholesterol content, increases in the levels of total COP were observed (Table 2). However, the increase in the level of total COP did not tally with the overall decrease in the cholesterol content. The total concentration of COP reached 332 mg kg^{-1}, about 11·6% of the initial cholesterol content as a result of the three fryings. All the major COP increased with frying time. About a sevenfold increase in the levels of 7α-hydroxy- and 7β-hydroxycholesterol was observed after three fryings. The ratio of 7β-hydroxy- to 7α-hydroxycholesterol was 2·6:1. Nourooz-Zadeh and Appelqvist (1988) and Sander *et al* (1989b) reported ratios of 7β-hydroxy- to 7α-hydroxy-cholesterol in butter heated at 200°C for 10 min and butter oil heated at 110°C for 24 days of 2:1 and 1:1, respectively. Sander *et al* (1989b), however, reported a slight decrease in the levels of epimeric 7-hydroxycholesterols after 8 days' heating of butter oil at 110°C.

Thermal decomposition of C-7 hydroperoxides has been reported to give rise to stable autoxidation products, viz 7α-hydroxy- and 7β-hydroxycholesterol and the dehydration product, 7-ketocholesterol (Smith *et al* 1973). A preference for the 7β-epimer over the 7α-epimer, possibly due to the greater thermodynamic stability of the equatorial (7β) over the axial (7α) epimer, has been reported (Kimura *et al* 1979).

5,6α-Epoxycholesterol showed the highest concentration (96 mg kg^{-1}) after three fryings (Table 2) whereas 7-keto- and 7β-hydroxycholesterol were present in the second highest quantity. High levels of 5,6-epoxycholesterol in heated butter or butter oil have been reported by various other workers (Fischer *et al* 1985; Nourooz-Zadeh and Appelqvist 1988; Sander *et al* 1989b). About a 13-fold increase in the levels of 7-ketocholesterol was observed in ghee after three fryings. Formation of 7-ketocholesterol was nearly linear with time up to two fryings but it increased dramatically after three (84 mg kg^{-1}). Csiky (1982) reported a twofold increase in the level of 7-ketocholesterol after heating 1 g butter for 10 min at 180°C in an open glass vessel. Fischer *et al* (1985), on the other hand, could not detect 7-ketocholesterol in butter heated at 170°C for 24 and 72 h; it might have been destroyed during the hot saponification procedure he employed. Sander *et al* (1989b) found that

TABLE 2

Effect of intermittent frying (1 h) on the levels of COP in ghee

Treatment	*Total cholesterol (mg kg^{-1} ghee)*	*COP (mg kg^{-1})*							
		7α-OH	*7β-OH*	*25-OH*	*20α-OH*	*Epoxy*	*Triol*	*7-Keto*	*Total COP*
Control	2853 ± 28	5·2 ± 0·2	12·8 ± 5·4	ND	ND	12·4 ± 3·5	ND	6·5 ± 0·7	35·0 ± 9·0 (1·23)
I Frying	2731 ± 38	7·1 ± 1·0	22·1 ± 3·4	3·1 ± 1·4	Tr	23·8 ± 2·5	Tr	14·0 ± 1·4	71·3 ± 7·0 (2·50)
II Frying	2617 ± 48	13·6 ± 2·2	35·2 ± 4·5	6·2 ± 1·4	3·2 ± 1·1	29·0 ± 1·7	3·1 ± 1·3	23·5 ± 2·6	112·3 ± 0·6 (3·94)
III Frying	2476 ± 21	33·8 ± 6·8	86·9 ± 5·9	16·2 ± 3·9	8·3 ± 1·9	95·6 ± 4·6	7·3 ± 2·6	84·6 ± 3·4	332·5 ± 6·7 (11·65)

ND, Not detected; Tr, < 1·0 mg kg^{-1}.
Mean ± SD, $n = 3$.
Values in parentheses are the COP as percentage of the total initial cholesterol.

the 7-ketocholesterol level increased to about 12 % of the initial cholesterol content in butter oil heated at 110°C for 24 days.

Side chain COP, viz 25-hydroxycholesterol and 20α-hydroxycholesterol, and cholestan-triol, the most atherogenic cholesterol oxide, were detected at low levels (8–16 mg kg^{-1}) after the third frying (Table 2). No such COP were detected by Csiky (1982) and Nourooz-Zadeh and Appelqvist (1988) in butter heated at 180–200°C for 5–10 min. Sander *et al* (1989b), however, reported 37 mg kg^{-1} cholestan-triol (about 1 % of the initial cholesterol) in butter oil heated at 110°C for 24 days.

7-Ketocholesterol (R_c 0·62) and 7α-hydroxycholesterol (R_c 0·45) were visible on the TLC plates after one and two fryings, respectively (Fig 3), whereas 25-hydroxycholesterol (R_c 0·78), 20α-hydroxycholesterol (R_c 0·91) and cholestan-triol (R_c 0·10) were visible after three fryings only.

As cholesterol contains one Δ^5-double bond, formation of any free radical is expected to initiate cholesterol oxidation (Smith 1980). Polyunsaturated fatty acids (PUFA), which are likely to be more susceptible to oxidation than cholesterol, might affect the oxidation of cholesterol as well (Park and Adis 1987). A close relationship between oxidation of cholesterol and peroxide value was observed (Fig 2); the concentration of total COP increased with the increase in peroxide value and FFA content in ghee. A nearly linear increase in the levels of total COP was observed up to two fryings, though peroxide value showed a slight decrease as a result of the second frying. However, total COP increased abruptly with the increase in peroxide value after three fryings. Luby *et al* (1986a, b) reported an increase in the levels of COP with the increase in peroxide value of butter exposed to fluorescent light. Park and Addis (1987) observed an increase in the levels of total COP with increase in TBA value in highly rancid muscle foods. Similar observations were made by Nourooz-Zadeh and Appelqvist (1988) in heated butter.

Intermittent heating

Cow and buffalo ghee samples were heated at 225°C in an open iron pan for 30 min each on three successive days after 24 h storage at room temperature (20–25°C). The increase in the FFA content of cow ghee was greater than that in buffalo ghee after three heatings (Fig 4A, B). However, peroxide value increased more in buffalo ghee than in cow ghee.

Total cholesterol content of cow and buffalo ghee samples decreased by 7·3 and 10·6 %, respectively, after three heatings. A comparison between intermittent frying and heating operations (Tables 2 and 3) revealed that cholesterol oxidised more in the heating process (producing 195·6–209·1 mg kg^{-1} of total COP after three intermittent heatings of 30 min each) than during the frying process (332·5 mg kg^{-1} of total COP after three fryings of 1 h each). This may be due to the steam blanket produced over fat by water volatilising from the food being fried, which helped protect the fat from oxidation as suggested by Yuki (1967) and Artman (1969). The levels of total COP increased throughout the heating period. As with the observations of Fischer *et al* (1985) and Nourooz-Zadeh and Appelqvist (1988), 5,6α-epoxycholesterol was formed in highest concentration (Table 3) in both species (60–70 mg kg^{-1}). 7β-Hydroxy- and 7-ketocholesterol were in second highest concentration (40–50 mg kg^{-1}). The ratio of 7β-hydroxy- to 7α-hydroxycholesterol was 2:1 in both species. Sander *et al* (1989b) reported a 1:1 ratio of 7β-hydroxy- to 7α-hydroxycholesterol in butter oil heated at 110°C for 24 days. 7α-Hydroxycholesterol, however, showed a slight decrease in cow ghee after first heating. The total concentration of 25-hydroxy-, 20α-hydroxycholesterol and cholestan-triol was significantly high in both species compared with the concentration of other COP. Csiky (1982) and Nourooz-Zadeh and Appelqvist (1988), however, reported no such COP in butter heated at 180–200°C for a short period of 5–10 min.

A comparison of the data of intermittent frying and heating operations suggests that direct heating induced more oxidation of cholesterol and resulted in higher levels of most atherogenic COP, viz 25-hydroxychol-

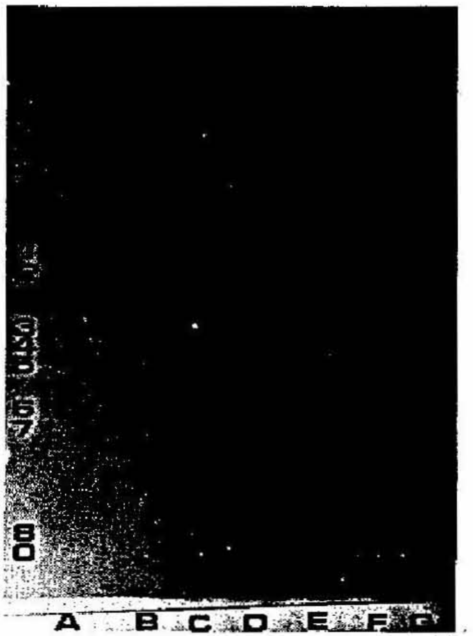

Fig 3. TLC pattern of unsaponifiable matter of ghee used for intermittent frying. A—Control; B—one frying; C—two fryings; D—three fryings; E—oxidised cholesterol; F—25-hydroxycholesterol; G—20α-hydroxycholesterol. 1—Cholesterol; 2—20α-hydroxycholesterol; 3—25-hydroxycholesterol; 4—5,6α-epoxycholesterol; 5—7-ketocholesterol; 6—7β-hydroxycholesterol; 7—7α-hydroxycholesterol; 8—cholestan-triol; O—origin.

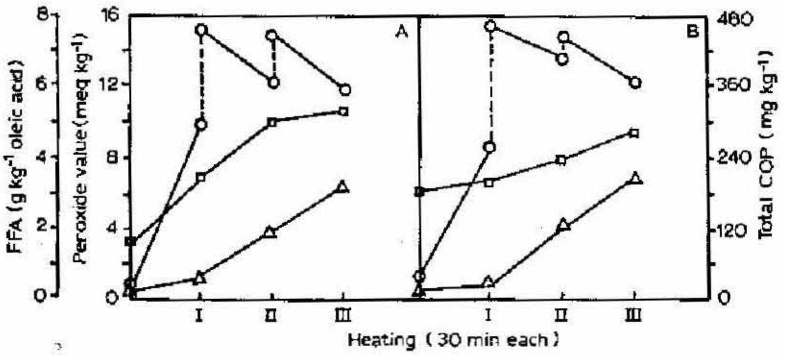

Fig 4. Effect of intermittent heating (225°C) on free fatty acid content, peroxide value and total COP in ghee ($n = 3$). A = Cow, B = buffalo. —□—□— FFA; —○—○ total COP; —△—△— peroxide value; ----- 24 h storage (20–25°C).

TABLE 3

Effect of intermittent heating (225°C for 30 min each) on the levels of COP in ghee

Treatment	*Total cholesterol ($mg\ kg^{-1}$ ghee)*	*COP ($mg\ kg^{-1}$)*							
		7α-OH	*7β-OH*	*25-OH*	*20α-OH*	*Epoxy*	*Triol*	*7-Keto*	*Total COP*
Cow									
Control	2992±56	3·7±1·4	6·4±1·8	ND	ND	7·6±1·4	ND	4·1±1·6	21·9±5·9 (0·73)
1 Heating	2946±68	2·9±0·2	7·8±0·9	2·2±0·6	Tr	13·1±2·3	Tr	7·9±0·9	33·9±3·6 (1·13)
2 Heatings	2899±32	6·8±0·9	27·1±3·8	7·4±0·7	2·3±0·5	37·7±4·8	4·0±0·8	35·3±4·8	120·4±4·8 (4·02)
3 Heatings	2774±19	22·3±3·7	42·4±5·2	10·2±0·4	7·7±1·5	60·1±4·7	7·6±0·9	45·3±3·6	195·6±3·8 (6·54)
Buffalo									
Control	2259±18	3·1±1·1	5·5±1·0	ND	1·6±0·7	7·5±0·8	ND	3·3±0·7	19·4±3·6 (0·86)
1 Heating	2179±38	4·0±0·6	10·7±1·5	2·3±0·6	Tr	14·2±1·5	Tr	11·3±2·5	42·4±2·8 (1·88)
2 Heatings	2085±31	12·2±2·1	34·8±5·0	6·4±0·9	2·7±0·4	42·1±2·7	3·3±1·0	26·7±4·3	128·3±5·1 (5·68)
3 Heatings	2019±48	22·9±1·6	45·9±3·8	10·8±1·1	7·3±1·6	68·8±1·6	6·1±0·2	47·4±2·1	209·1±3·7 (9·26)

ND, Not detected; Tr, $< 1·0$ mg kg^{-1}.
Values in parentheses are COP as percentage of total initial cholesterol.

esterol and cholestan-triol (8·1–9·2% of the total COP) compared with the frying operation (7·1%).

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