Thermally oxidised sunflower-seed oil increases liver and serum peroxidation and modifies lipoprotein composition in rats

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Peroxidation of LDL and other lipoproteins is thought to play a central role in atherogenesis. Dietary thermally oxidised oils may increase atherogenic risk in consumers by increasing their oxidative status. The present paper compares the effects of two diets containing unused sunflower-seed oil (US) or sunflower-seed oil repeatedly used in frying (FS) (both 15 g/100 g diet) on weight gain, food efficiency ratio, serum lipid levels and lipoprotein composition, and the content of thiobarbituric acid-reactive substances (TBARS) in the liver, serum, and lipoproteins in growing Wistar rats. After sixty potato fryings the FS contained 27·7 g polar material/100 g oil and 16·6 g oligomers/100 g oil. The FS-fed rats had a significantly lower weight gain and food efficiency ratio. Liver-TBARS increased due to the consumption of the highly altered oil and showed a significant linear relationship (all $r > 0.68$; $P < 0.002$) with the ingestion of thermally oxidised compounds. Serum-, VLDL-, LDL- and HDL-TBARS were significantly higher in the FS-fed rats (all $P < 0.001$). Concentrations of serum total and non-esterified cholesterol and phospholipids were significantly higher in the FS-fed rats ($P < 0.05$, $P < 0.05$, and $P < 0.001$, respectively). Serum triacylglycerol concentrations did not vary between the two dietary groups. Total and esterified cholesterol and phospholipid levels increased significantly in the HDL fraction ($P < 0.05$, $P < 0.05$, and $P < 0.001$, respectively) of the FS-fed rats. HDL-cholesterol and HDL-phospholipids were significantly correlated with liver-TBARS ($r > 0.747$; $P < 0.0001$), VLDL-TBARS ($r > 0.642$; $P < 0.003$), LDL-TBARS ($r > 0.475$; $P < 0.04$), and HDL-TBARS ($r > 0.787$; $P < 0.0001$). The data suggest that the rat increases HDL as a protecting mechanism against the peroxidative stress induced by the consumption of a diet containing the thermally oxidised oil.

Deep-fat frying: Serum and lipoprotein peroxidation: Serum and lipoprotein lipids: Liver peroxidation

Diets of North Americans and inhabitants of the European Union countries contain substantial quantities of unsaturated fat that have been subjected to various degrees of processing and heat treatment. In addition, the majority of fast foods fried in oil contain oxidised lipids (Frankel *et al.* 1984) and the amount of oxidised lipids significantly increases when frying is performed following a slow or null replenishment with unused oil (Romero *et al.* 2000; Bastida & Sánchez-Muniz, 2002). Romero *et al.* (2000) found that frozen pre-cooked food in sunflower-seed oil also contains a relatively high content of altered fatty acids. Pancreatic lipase hydrolyses oxidised triacylglycerols *in vitro* (Sánchez-Muniz *et al.* 2000) and *in vivo* (Sánchez-Muniz *et al.* 1999), and the altered compounds are actively absorbed (Sánchez-Muniz *et al.* 1999).

Thus, diet may be the source of oxidised lipoproteins found in the intestinal lymph of rodents (Stapräns *et al.* 1993*a*). A direct relationship between the levels of oxidised lipids in the diet and the amount of oxidised lipids in the mesenteric lymph chylomicrons has been observed (Stapräns *et al.* 1993*a*,*b*). This finding suggests that, in rats, dietary oxidised lipids are absorbed by the small intestine, packaged in chylomicrons and transported to the bloodstream. Furthermore, these studies (Stapräns *et al.* 1993*a*,*b*) have shown that the metabolism of oxidised chylomicrons is similar to that of normal chylomicrons; a substantial proportion of oxidised lipids is delivered to the liver, where the lipids can be repackaged and secreted into VLDL. In a previous study, we found that liver-thiobarbituric acid-reactive substances (TBARS) were related to dietary fat alteration, suggesting that the liver actively takes up oxidised lipoprotein components (Sánchez-Muniz *et al.* 1998). Izaki *et al.* (1984) reported that liver-TBARS were increased significantly in rats receiving four levels of thermally oxidised rapeseed oils in comparison with their counterparts fed fresh oil.

The effect of heated oils on plasma lipids has been previously reviewed (Sánchez-Muniz & Cuesta, 1998). However, information on the effect of the consumption of oils frequently used for frying on lipoprotein lipid and lipoprotein peroxidation is limited (Stapräns *et al.* 1993*a,b*). Because of the increased consumption of food fried in oils rich in PUFA, the present study aimed to assess the effect of consuming a diet containing an abused sunflower-seed oil used repeatedly for frying (FS) on (i) acceptability and growth in young rats; (ii) liver size and liver-TBARS concentration; (iii) plasma lipids and lipid composition and TBARS concentration of VLDL, LDL and HDL.

Materials and methods

Animals and diets

The study was approved by the Spanish Science and Technology Advisory Committee (Comisión Asesora de Ciencia y Tecnología; CICYT) and by an ethics committee of the Facultad de Farmacia of the Universidad Complutense de Madrid (Spain). Rats were handled according to the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

Eighteen growing male Wistar rats weighing approximately 65 g were obtained from the Instituto de Nutrición y Bromatología (CSIC, Madrid, Spain). After an adaptation period of 4 d, they were transferred to the experimental diets (Table 1). The animals were randomly divided into two groups of nine rats each and housed individually in metabolism cells in a temperature-controlled room (21–24°C) with a 12 h light–12 h dark cycle. They had free access to food and water during the 27 d experimental period.

The energy densities of the diets were 1793 kJ/100 g DM for the unused sunflower-seed oil (US) diet and 1787 kJ/ 100 g DM for the FS diet.

Frying procedure

Domestic deep-fat fryers with 3-litre vessels were used for the repeated potato-frying procedure. A total of sixty fryings, at a rate of ten fryings/d, was carried out. The potato: frying oil ratio was kept at 500 g/3 litres. The potatoes (sliced about 2 mm thick) absorbed a considerable amount of oil and it was necessary to replenish the fryer baths with oil from the other fryers. The initial number of fryers was seven, but one fryer was eliminated after each ten frying operations, emptying its contents to make up the volume of the other fryers to 3 litres. More details about the frying procedure have been described previously (Sánchez-Muniz *et al.* 1993).

Fatty acid and tocopherol analyses

Samples of the oils were saponified with 0·5 M-sodium hydroxide and then methylated following the method of Metcalfe *et al.* (1966). The non-altered and altered fatty acid methyl esters (Me-FA) of the sunflower-seed oils were separated on silica gel columns (E Merck AG, Darmstadt, Germany), using hexane–diethyl ether for the unaltered Me-FA and diethyl ether for the altered Me-FA fraction as previously described (Cuesta *et al.* 1991). Non-altered Me-FA were analysed by GC, as previously described (Sánchez-Muniz *et al.* 1998).

Contents of α- and γ-tocopherol were determined in the oils by HPLC and fluorescence detection (International Union of Pure & Applied Chemistry, 1992). The results

Table 1. Composition of the semi-synthetic diets containing unused sunflower-seed oil or sunflower-seed oil used repeatedly for frying (g/kg dry matter)

	Amount in diet	
Ingredient	Unused sunflower-seed oil	Used sunflower-seed oil
Protein*	140	140
Fat†	150	150
Wheat starch‡	327·4	327·4
Sucrose‡	300·0	300·0
Microcrystalline cellulose‡	50·0	50·0
Mineral premix§‖	30·0	30·0
Vitamin premix§¶	1·6	1·6
Butylated hydroxytoluene§	0·5	0·5
Butylated hydroxyanisole§	0·5	0·5

* Acid casein (99·8 %; Central Ibérica de Drogas, SA, Madrid, Spain) plus 0·2 % DL-methionine (E Merck AG, Darmstadt, Germany).
† Sunflower-seed oil (KOIPE SA, Andújar, Jaén, Spain).
‡ Central Ibérica de Drogas, SA, Madrid, Spain.
§ E Merck AG, Darmstadt, Germany.
‖ Mineral mix composition (mg/kg diet): KI, 0·21; Na_2SeO_3, 0·24; $Na_2CrO_4.4H_2O$, 1·58; NaF, 2·43; $CuSO_4.5H_2O$, 24·72; $ZnCO_3$, 25·50; $MnSO_4.H_2O$, 169·20; $FeSO_4.7H_2O$, 199·00; $MgCO_3$, 769·70; NaCl, 906·30; $MgSO_4.7H_2O$, 2250; $Na_2H_2PO_4.2H_2O$, 2930; $KHCO_3$, 6100; $CaHPO_4.2H_2O$, 8590; KH_2PO_4, 8200; $CaCO_3$, 10000 (National Research Council, 1995).
¶ Vitamin mix composition (mg/kg diet): choline, 1111·11; folic acid, 1·11; niacin, 22·22; calcium pantothenate, 8·88; riboflavin, 3·33; thiamin, 4·44; pyridoxine hydrochloride, 6·66; menadione, 0·055; α-tocopheryl acetate, 33·33; cholecalciferol, 0·028; retinyl acetate, 0·0015. Because oils contain vitamin E, the amount of this vitamin in the unused sunflower-seed-oil-diet can be estimated at about 110 mg tocopherol equivalents/kg and in the used sunflower-seed-oil-diet at about 40 mg tocopherol equivalents/kg (National Research Council, 1995).

were expressed as tocopherol equivalents (Food & Nutrition Board, 1989).

Separation of polar and non-polar materials

The amount of polar material from the FS and the US was determined using silica gel columns (Romero *et al.* 1995). The purity of each fraction was then checked by TLC with Merck (Darmstadt, Germany) plates (60_{250}, 0·5 mm thickness, using a mixture of hexane–diethyl ether–acetic acid (80:20:1, by vol.) as a solvent system).

High-performance size-exclusion chromatography

The polar material obtained by column chromatography on silica gel was analysed by high-performance size-exclusion chromatography (Dobarganes *et al.* 1988) to investigate the polar compounds (polymers and dimers of triacylglycerols, oxidised triacylglycerols, diacylglycerols and non-esterified fatty acids). These compounds were determined in a Konic 500 A chromatograph (Barcelona, Spain) with a 10 μl sample loop. A Hewlett-Packard 1037 A refractive index detector (Palo Alto, CA, USA) and two Polymer Laboratories gel (PL-gel) columns of 0·05 μm and 0·01 μm (Hewlett-Packard, Madrid, Spain), connected in series, were operated at 40°C. HPLC-grade tetrahydrofuran (E Merck AG, Darmstadt, Germany) served as the mobile phase, with a flow rate of 1 ml/min and a sample concentration of 10–15 mg/ml in tetrahydrofuran. All eluents, as well as samples, were pre-cleaned by passing them through a 2 μm filter. The quantity of each compound was calculated as previously described (Sánchez-Muniz *et al.* 1993).

Lipoprotein isolation

At the end of the experimental period the rats were anaesthetised with an intraperitoneal injection of sodium pentobarbital (45 mg/kg body weight) and killed by extracting blood via carotid puncture. Serum was separated from whole blood within 30 min of collection by centrifugation at 1200 *g* for 20 min at 4°C. VLDL, LDL and HDL fractions were separated from serum by gradient-density ultracentrifugation for 7 h at 232 000 *g* and 8°C in a Beckman L8-70M ultracentrifuge, SW-50·1 rotor (Palo Alto, CA, USA) following the method of Terpstra *et al.* (1981). Lipoproteins were separated by tube slicing at the density range of VLDL ($\rho^{20} < 1{\cdot}0063$ g/ml), LDL ($1{\cdot}0063 < \rho^{20} < 1{\cdot}057$ g/ml) and HDL ($\rho^{20} > 1{\cdot}057$ g/ml).

Serum lipid and lipoprotein lipid analyses

Total and non-esterified cholesterol, triacylglycerol and phospholipid contents were determined in serum, VLDL, LDL and HDL fractions by standard enzymic analysis (tests 172626, 310328, 701904 and 691844, respectively; Boehringer Mannheim, Darmstadt, Germany). The intraassay CV was <5·5 %.

Liver, serum, and lipoprotein thiobarbituric acid-reactive substances

Liver samples were homogenised (50 mg/ml) in 1 % phosphate buffer. TBARS levels in liver and in serum and lipoprotein fractions were measured at 532 nm on a Philips PU 8620/UV/Vis/NIR spectrophotometer (Cambridge, UK), following the method of Yagi (1993).

Statistical analysis

Data were analysed using Student's *t* test. Pearson productmoment correlations were also used. Results were considered significant at $P < 0{\cdot}05$.

Results

Fatty acid composition, polar material, tocopherol, oligomers and thermal oxidised compounds in sunflowerseed oil

Polar material increased ($P < 0{\cdot}001$) in the FS compared with the US. Moreover, compounds related to thermoxidative alteration, such as triacylglycerol polymers, triacylglycerol dimers and oxidised triacylglycerols also increased ($P < 0{\cdot}001$) in the FS. After sixty fryings, the oil became highly altered. More than 25 % of this oil consisted of polar material and more than 16 % of oligomers (Table 2). Because of the formation of polar material and polymeric triacylglycerols, the level of linoleic and oleic acids decreased significantly ($P < 0{\cdot}05$). The amount of α- and γ-tocopherols, and thus the tocopherol equivalents, also decreased ($P < 0{\cdot}001$) after the sixty fryings of potatoes (Table 2). Taking into account the tocopherol equivalents of the diets (vitamin mix plus the oils) and the linoleic acid content of the oils, the tocopherol equivalents:linoleic acid ratio was 1·44 mg/g in the US diet, and 0·68 mg/g in the FS diet.

Food intake and body-weight gain

Food and fat intakes were similar in both groups, but the FS-fed rats consumed significantly lower amounts of oleic and linoleic acids ($P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively) than those given the US diet. On the other hand, the intake of polar material, polymers, dimers, oligomers, oxidised triacylglycerols and thermoxidised compounds was significantly higher in the FS-fed rats than in the US-fed rats (all $P < 0{\cdot}001$) (Table 2). Weight gain was significantly lower ($P < 0{\cdot}05$) in the FS-fed rats (Table 3).

Faecal excretion

Faecal excretion tended to decrease in the FS-fed rats (Table 4). However, faeces of the FS-fed rats contained proportionally more fat and moisture than those of the US-fed rats.

Liver size and composition

FS consumption did not significantly affect liver size, hepatosomatic index or liver fat content. However, livers

Table 2. Polar material, thermal oxidation and hydrolysis compounds, altered methyl esters and tocopherol content in unused sunflower-seed oil or sunflower-seed oil used repeatedly for frying†

(Mean values and standard deviations of three measurements)

	Unused oil		Used oil	
	Mean	SD	Mean	SD
Polar material (g/100 g oil)	4·0	0·2	27·7***	0·3
Triacylglycerol polymers (g/100 g oil)	0·1	0·0	5·6***	0·2
Triacylglycerol dimers (g/100 g oil)	0·5	0·0	11·0***	0·2
Non-oxidised triacylglycerols (g/100 g oil)	96·0	0·2	72·3***	0·3
Oxidised triacylglycerols (g/100 g oil)	1·9	0·1	8·9***	0·2
Diacylglycerols (g/100 g oil)	1·0	0·1	1·7*	0·1
Monoacylglycerols (g/100 g oil)	ND	ND	ND	ND
Non-esterified fatty acids (g/100 g oil)	0·5	0·0	0·5	0·1
Non-altered fatty acid methyl esters (g/100 g oil)	98·4	0·5	84·1***	0·8
Altered fatty acid methyl esters (g/100 g oil)	1·6	0·5	15·9***	0·8
Oleic acid (g/100 g oil)	32·5	0·06	30·5***	0·04
Linoleic acid (g/100 g oil)	55·6	0·09	44·1***	0·05
Tocopherol equivalents (g/kg oil)*‡	530	11·9	49***	1·2
α-Tocopherols (g/kg oil)	478	10·5	44***	1·2
γ-Tocopherols (g/kg oil)	51·2	1·5	7·2***	0·4

ND, not detected.
Mean value was significantly different from that for the unused oil: *$P < 0.05$, ***$P < 0.01$ (Student's t test).
† For details of procedures, see pp. 258–259.
‡ Calculated according to the Food and Nutrition Board (1989).

of the FS-fed rats had a significantly higher TBARS content ($P < 0.01$) than those of the US-fed rats (Table 3). Liver-TBARS concentrations showed a significant linear relationship ($P < 0.01$) with the ingestion of all thermoxidised compounds (data not shown).

Serum lipids

Serum total and non-esterified cholesterol concentrations were significantly higher ($P < 0.05$) in the FS-fed rats (Table 5). Serum phospholipids were also higher ($P < 0.001$) in the FS-fed rats, whereas serum triacylglycerol concentrations did not vary between the two groups.

Lipoprotein lipids

Concentrations of VLDL-lipids and VLDL-esterification index were not significantly different in both groups (Table 5). LDL of the FS-fed rats showed higher total and non-esterified cholesterol content ($P < 0.01$ and

Table 3. Weight gain, food and fat intake, and food efficiency ratio in rats fed semi-synthetic diets, containing unused sunflower-seed oil or sunflower-seed oil used repeatedly for frying, for 27 d†

(Mean values and standard deviations)

	Unused sunflower-seed-oil diet (n 9)		Used sunflower-seed-oil diet (n 9)	
	Mean	SD	Mean	SD
Initial body weight (g)	73·8	6·5	72·3	2·9
Final body weight (g)	180·9	14·1	144·1***	14·4
Weight gain (g)	107·1	6·3	71·8***	4·0
Food intake (g/d)	12·2	0·8	11·7	0·6
Fat intake (g/d)	1·77	0·11	1·84	0·09
Linoleic acid intake (g/d)	0·95	0·03	0·78**	0·03
Oleic acid intake (g/d)	0·61	0·03	0·54*	0·03
Polar material intake (g/d)	0·08	0·01	0·50***	0·02
Triacylglycerol polymer intake (g/d)	0·001	0·000	0·10***	0·01
Triacylglycerol dimer intake (g/d)	0·01	0·00	0·20***	0·01
Triacylglycerol oligomer intake‡ (g/d)	0·01	0·00	0·30***	0·01
Oxidised triacylglycerol intake (g/d)	0·04	0·00	0·16***	0·01
Thermoxidised compound intake§ (g/d)	0·05	0·00	0·46***	0·02
Food efficiency ratio‖	0·33	0·03	0·23*	0·05

Mean value was significantly different from that for the unused oil diet: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's t test).
† For details of diets and procedures, see Tables 1 and 2 and pp. 258–259.
‡ Triacylglycerol polymers plus triacylglycerol dimers.
§ Triacylglycerol polymers and dimers plus oxidised triacylglycerols.
‖ Weight gain (g)/food intake (g DM/27 d).

Table 4. Faecal weight, moisture and fat of rats fed semi-synthetic diets, containing unused sunflower-seed oil or sunflower-seed oil used repeatedly for frying, for 27 d†

(Mean values and standard deviations)

	Unused sunflower-seed-oil diet (*n* 9)		Used sunflower-seed-oil diet (*n* 9)	
	Mean	SD	Mean	SD
Faeces				
Weight (g/d)	7·10	1·41	6·75	1·32
Moisture (%)	5·39	0·96	5·79	0·99
Fat (%)	4·19	1·02	5·05	1·56
Liver				
Weight (g)	5·86	1·26	5·11	0·63
Moisture (%)	72·3	2·25	73·6	2·97
Fat (%)	3·98	0·78	4·19	0·72
TBARS (mmol/g)	0·89	0·22	1·41**	0·32
Hepatosomatic index (g/100 g body weight)	3·59	0·48	3·98	0·48

TBARS, thiobarbituric acid-reactive substances.

Mean value was significantly different from that for the unused oil diet: ** $P < 0.01$ (Student's *t* test).

† For details of diets and procedures, see Tables 1 and 2 and pp. 258–259.

$P < 0.001$, respectively) but the other lipids and the esterification index were not significantly affected. Total cholesterol and the esterified cholesterol concentrations in HDL were significantly higher ($P < 0.05$) in the FS-fed rats. Similarly, HDL-phospholipid concentrations were higher ($P < 0.001$) in the FS-fed rats. The VLDL-lipid mass tended to be lower whereas the LDL-lipid mass and the HDL-lipid mass tended to be higher in the FS-fed rats.

Serum- and lipoprotein-thiobarbituric acid-reactive substances

The FS-fed rats had higher TBARS contents in serum and in all lipoproteins than their US-fed counterparts ($P < 0.001$) (Table 5). The HDL fraction was the major TBARS carrier. However, the lipid:TBARS ratio denotes that VLDL was more affected than the other lipoproteins. Serum-TBARS and HDL-TBARS showed a high and significant correlation with liver-TBARS (Figs. 1 and 2), HDL-phospholipids ($r > 0.6996$; $P < 0.001$), and HDL-cholesterol ($r > 0.7148$; $P < 0.001$).

Discussion

Oil alteration

Present legislation in many European countries rules that oil should be discarded when its total polar material content is higher than 25 % (Firestone, 1996). However, other European countries indicate that oils with a 10 % oligomer (polymers plus dimers of triacylglycerols) content should be discarded (Firestone, 1996). Nonetheless, in The Netherlands and other countries the limit for oil discarding has been set at 16 % oligomer level (Firestone, 1996). In the present study, the FS surpassed the 16 % oligomer content and the total polar material value of 25 mg/100 mg oil. Thus, according to current legislation this oil should not be used for frying or other alimentary purposes. However, in the routine study of oil samples from snack bars and restaurants, oils displaying alterations similar to those seen in the present study were found (Dobarganes

& Márquez-Ruiz, 1998). According to Quiles *et al.* (2002) tocopherol equivalents decreased after frying, suggesting that vitamin E is being used to protect the oil against the thermal damage. All these findings prompted us to study the effect of a diet containing this abused oil on food consumption, growth, lipoprotein profile and liver, serum and lipoprotein peroxidation.

Diet consumption

Food intake is primarily influenced by factors such as taste, smell and texture (Naim *et al.* 1977). Lipid oxidation has a great influence on flavour, and the oxidation and hydrolysis of fat affect food palatability, spoiling its taste. Nevertheless, the consumption of FS in the present study did not have any significant effect on food intake. It has been reported that the smell and taste of fats that contain more than 25 % polar material was still acceptable, but that fat was considered deteriorated and unacceptable when polar material content exceeded 30 % (Billek, 1985). Oxidised compounds affect fat palatability, whereas polymer compounds related to thermal oxidation do not (Clark & Serbia, 1991). In a parallel study, our group (Sánchez-Muniz *et al.* 1998) found that a diet including oil with less polar material (18 g/100 g oil) was well accepted by growing rats. In the present study, the FS had an oligomer content that was thirty times higher than that of the fresh sunflower-seed oil but had an oxidised triacylglycerol content that was only four times higher. These findings may explain, at least in part, the lack of variation in food intake between the two groups.

Final body-weight gain and the food efficiency ratio were lower in the FS-fed rats than in the US-fed rats. Rats fed a high amount of polar material over a 1-year period exhibited growth retardation (Billek, 1985). However, no significant effect on growth was observed when rats were fed used sunflower-seed oil with an oligomer content below 10 % (Sánchez-Muniz *et al.* 1998). Thus, the 10 % limit suggested for oil discarding seems to be a good initial cut-off point to avoid harmful effects on growing young animals. The presence of lipid peroxides in food

Table 5. Lipid concentration and different indexes of serum VLDL, LDL and HDL of rats fed semi-synthetic diets, containing unused sunflower-seed oil or sunflower-seed oil used repeatedly in frying, for 27 d†

(Mean values and standard deviations)

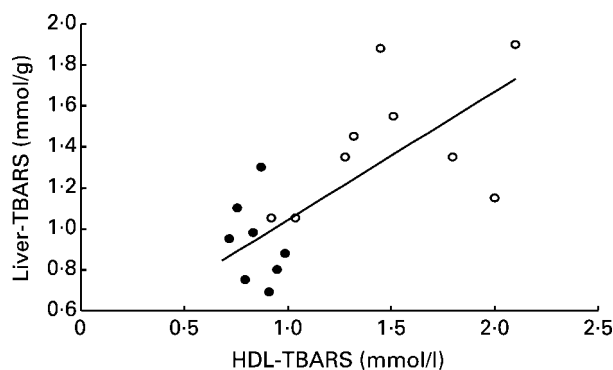|	Unused sunflower-seed-oil-diet (n 9)		Used sunflower-seed-oil-diet (n 9)	
	Mean	SD	Mean	SD
Total cholesterol (mmol/l)				
Serum	1·64	0·07	2·04*	0·11
VLDL	0·03	0·01	0·03	0·02
LDL	0·15	0·06	0·22**	0·03
HDL	1·38	0·23	1·69*	0·23
Non-esterified cholesterol (mmol/l)				
Serum	0·27	0·12	0·42*	0·03
VLDL	0·02	0·01	0·02	0·03
LDL	0·05	0·02	0·10***	0·03
HDL	0·19	0·07	0·28	0·11
Esterified cholesterol (mmol/l)				
Serum	1·26	0·06	1·63***	0·09
VLDL	0·01	0·00	0·01	0·01
LDL	0·10	0·06	0·13	0·03
HDL	1·09	0·40	1·41*	0·21
Triacylglycerols (mmol/l)				
Serum	0·34	0·18	0·30	0·06
VLDL	0·13	0·06	0·11	0·09
LDL	0·06	0·04	0·05	0·01
HDL	0·13	0·04	0·13	0·04
Phospholipids (mmol/l)				
Serum	1·38	0·04	1·81***	0·05
VLDL	0·04	0·01	0·02	0·01
LDL	0·04	0·02	0·06	0·02
HDL	1·23	0·18	1·64***	0·19
Esterification index‡ × 100				
VLDL	75·3	8·5	65·6	9·9
LDL	61·7	6·7	57·3	4·7
HDL	86·0	1·8	84·2	2·1
Total lipid mass (mmol/l)				
VLDL	0·20	0·06	0·16	0·06
LDL	0·25	0·12	0·34	0·20
HDL	2·64	0·60	3·46	0·67
TBARS (mmol/l)				
Serum	1·27	0·14	2·22***	0·48
VLDL	0·20	0·07	0·35***	0·10
LDL	0·16	0·05	0·34***	0·17
HDL	0·85	0·12	1·49***	0·41
Lipid:TBARS ratio				
VLDL	1·00	0·12	0·46***	0·09
LDL	1·56	0·63	1·00	0·12
HDL	3·10	0·66	2·32*	0·29

TBARS, thiobarbituric acid-reactive substances.
Mean value was significantly different from that for the unused oil diet: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
† For details of diets and procedures, see Tables 1 and 2 and pp. 258–259.
‡ Esterified cholesterol/total cholesterol.

is undesirable because nutritional value decreases with the destruction of unsaturated fatty acids and other essential food constituents that possess an unsaturated lipid structure (Kahl & Hildebrandt, 1986). This effect was also found in the present study, as the amount of linoleic acid ingested by the FS-fed rats was significantly lower than that of the control group, because a considerable amount of linoleic acid has been oxidised or converted into altered compounds.

Fig. 1. Relationship between HDL-thiobarbituric acid reactive substances (TBARS) and liver-TBARS in rats fed semi-synthetic diets containing unused sunflower-seed oil (●) or sunflower-seed oil used repeatedly for frying (○). The relationship is described by $y = 0.6262x + 0.4174$, where y is liver-TBARS and x is HDL-TBARS (r 0·7217; $P < 0.001$).

Liver composition and peroxidation

In agreement with Izaki *et al.* (1984), the present results on liver-TBARS appeared highly influenced by the intake of thermoxidised compounds. The peroxide content of chylomicrons in rats is determined by the lipid peroxide content of the diet (Stapräns *et al.* 1993a). Remnants of these oxidised chylomicrons are recognised and cleared by the liver. Following 6 h of intraduodenal infusion of peroxidised oil emulsion the rat liver displays a considerable level of lipid hydroperoxides (TBARS) (Ty *et al.* 1992). Thus, it should be accepted that the higher TBARS level in the liver of the rats fed the FS diet is a consequence of the higher alteration of the oil. The thermoxidised compounds that most affected liver-TBARS levels were polymers, because the regression line describing the correlation between these two parameters had the steepest slope. In turn, liver-TBARS values seemed to significantly affect the levels of VLDL-TBARS and those of the other lipoproteins, suggesting that a substantial percentage of the oxidised lipids delivered to the liver were repackaged and secreted into VLDL.
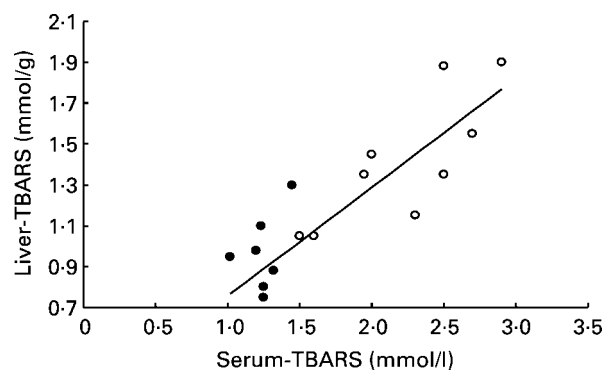
Fig. 2. Relationship between serum-thiobarbituric acid reactive substances (TBARS) and liver-TBARS in rats fed semi-synthetic diets containing unused sunflower-seed oil (●) or sunflower-seed oil used repeatedly for frying (○). The relationship is described by $y = 0.5335x + 0.2222$, where y is liver-TBARS and x is serum-TBARS (r 0·8393; $P < 0.001$).

Effects on serum lipids and lipoproteins

PUFA, primarily linoleic acid, are known to be hypocholesterolaemic (Grundy & Denke, 1990; Mensink & Katan, 1992). A high intake of linoleic acid has been shown to decrease both LDL- and HDL-cholesterol levels (Grundy & Denke, 1990; Mensink & Katan, 1992). Thus, one explanation for the rise of serum total cholesterol and LDL- and HDL-cholesterol levels following the ingestion of the FS diet may be the lower intake of linoleic acid by the rats of this group. However, although diets with a high linoleic acid content reduce serum LDL-cholesterol, linoleic acid appears to be a major source of lipid oxidation in atherogenesis (Esterbauer *et al.* 1992). Some time ago, our research group (Cuesta *et al.* 1987) reported higher levels of total and esterified cholesterol in FS-fed rats than in their US-fed counterparts. In that study the concentration of linoleic acid in the olive oil decreased from 7·89 to 3·76 % after the oil was used thirty times to fry potatoes. Our group further found that rats fed fried sunflower-seed oil with 18 % polar material exhibited higher total serum HDL- and LDL-cholesterol levels than their respective controls (Sánchez-Muniz *et al.* 1998).

The diets in the present study contained the antioxidant vitamin E, butylated hydroxytoluene and butylated hydroxyanisole but these did not appear to be completely effective in blocking the peroxidative stress in the FS-fed rats. In fact, serum-TBARS appeared to be increased in the FS-fed rats. Several authors have found that vitamin E decreases during the frying of oil (Márquez-Ruiz *et al.* 1999; Andrikopoulos *et al.* 2002). Compared with the control diet, the diet containing the altered oil had a lower amount of tocopherol equivalents. However, the amount of tocopherol equivalents in both diets seemed enough and adequate to prevent further linoleic acid oxidation, because an α-tocopherol:linoleic acid ratio of about 0·6 mg/g has been suggested as adequate to avoid oxidative damage (Arbolés *et al.* 2003).

We also evaluated the effects of the FS and US diets on serum lipoprotein levels and composition. The consumption of FS had non-significant effects on the composition of the VLDL. However, the LDL and HDL fraction of the FS-fed rats was enriched in total and non-esterified cholesterol, possibly because these rats consumed less linoleic acid, known to exert a hypocholesterolaemic effect on the LDL and HDL fractions (Grundy & Denke, 1990; Mensink & Katan, 1992).

Lecithin cholesterol acyltransferase, an enzyme that acts on HDL and whose substrate is non-esterified cholesterol, is a key component of reverse cholesterol transport (Steinberg, 1987). In murine lipid metabolism, HDL accepts non-esterified cholesterol from the peripheral tissues and the non-esterified cholesterol is converted to the ester form by the action of lecithin cholesterol acyltransferase. Subsequently, the cholesteryl esters are delivered to the liver. In the rat, a great hepatic uptake of cholesteryl ester from HDL has been observed (Glass *et al.* 1983; Steinberg, 1987). Thus, higher levels of HDL may enhance reverse cholesterol transport from the peripheral tissues to the liver.

Furthermore, HDL is known to carry a large amount of cholesteryl ester hydroperoxides via reverse transport (Mackness & Durrington, 1995). In addition, HDL limit the oxidative modifications of LDL (Mackness & Durrington, 1995), decreasing the formation of lipid peroxides and related molecules. Thus, increased HDL-cholesterol and HDL-phospholipid levels in FS-fed rats may be a protective mechanism against the higher intake of thermoxidised compounds and a mechanism to avoid oxidative change in other lipoproteins such as LDL.

In addition, HDL particles are associated with paraoxonase 1 activity. This enzyme exerts an important role in protecting LDL and HDL from oxidation in man and other animals (Canales & Sánchez-Muniz, 2003) and is very active in rats (Kudchodkar *et al.* 2000). Of all serum lipids, serum phospholipids and very-high-density lipoprotein-phospholipids were strongly related to paraoxonase 1 (Kudchodkar *et al.* 2000). Plasma paraoxonase 1 mass depends on the number of HDL particles (Nevin *et al.* 1996). In the present paper, the HDL lipid mass tended to increase following the consumption of the FS diet. Moreover, HDL-phospholipids contributed 95·3 % of total serum phospholipids in the FS-fed rats while this contribution was 93·9 % in their US-fed counterparts. Thus, it can be hypothesised that the increase in HDL and HDL-phospholipid levels may be a mechanism to keep as low as possible the oxidative alteration in serum and tissues induced by the consumption of diets containing oxidised lipids. The results also suggest the need for future studies on the long-term effects of modified lipid intake on lipoprotein profile and atheroma development. Meanwhile, the consumption of altered oils should be markedly restricted because of the deleterious implications for health of the toxic compounds they contain (López-Varela *et al.* 1995).

Conclusions

The present study documents that dietary polymerised and thermoxidised sunflower-seed oil increases the levels of cholesterol and phospholipid in plasma and HDL and the levels of TBARS in liver and plasma lipoproteins. These insights have human dietary implications since there is evidence that diets of developed countries contain substantial quantities of PUFA that have been subjected to various degrees of processing and heat treatment, particularly deep-fat frying, which is know to cause lipid oxidation.

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