Applications of Chromatographic Techniques to Evaluate Enzymatic Hydrolysis of Oxidized and Polymeric Triglycerides by Pancreatic Lipase *in vitro*

G. Márquez-Ruiz*, G. Guevel, and M.C. Dobarganes

Instituto de la Grasa (CSIC), 41012 Sevilla, Spain

ABSTRACT: With the aim of studying the susceptibility to enzymatic hydrolysis of oxidized and polymeric triglycerides (TG) that are formed during frying, various chromatographic techniques were applied in combination, i.e., adsorption chromatography, high-performance size-exclusion chromatography (HPSEC), and thin-layer chromatography–flame ionization detection (TLC–FID). Polar fractions, isolated by adsorption chromatography from thermoxidized trilinolein as model system, and real used frying fats and oils, were analyzed by HPSEC before and after incubation with pancreatic lipase *in vitro*. Also, the influence of degradation level of used frying oils on hydrolysis of intact TG was investigated with the additional aid of TLC–FID. Results showed the high hydrolysis rate of oxidized TG monomers in contrast to the significant discrimination of pancreatic lipase against TG dimers and, particularly, TG polymers. On the other hand, hydrolysis of intact TG can be affected by the presence of dimers and polymers in abused frying oils. *JAOCS 75*, 119–126 (1998).

KEY WORDS: Adsorption chromatography, enzymatic hydrolysis, frying fats and oils, high-performance size-exclusion chromatography, oxidized triglycerides, pancreatic lipase, polar compounds, thin-layer chromatography–flame ionization detection, triglyceride dimers, triglyceride polymers.

There is much current interest in the physiological effects of dietary fried foods, the consumption of which has increased considerably over the last decades, in great part owing to the notable expansion of fast-food restaurants. Among the numerous aspects related to the nutritional value of fried products, digestion of lipid degradation compounds that are formed during frying is of undoubted relevance as a discriminatory process in the overall assimilation of such compounds. However, few studies have been reported on lipolysis of heated and frying oils (1–3) and, in particular, little is known about the selectivity of pancreatic lipase toward the oxidized and polymeric compounds present in used frying fats and oils.

Thermal oxidation of fats during frying is mainly associated with the process of autoxidation, which proceeds *via* a free-radical mechanism. Ultimately, a wide variety of volatile and nonvolatile compounds will be formed at frying temperatures. Of greatest nutritional significance are the nonvolatile alteration compounds because they are retained in the fried products and hence ingested. They are basically comprised of oxidized triglyceride (TG) monomers, dimers, and polymers (4). "Oxidized TG monomers" is the generic term for monomeric TG that contain at least one oxygenated function, thus referring to a complex mixture of compounds, e.g., epoxides, ketones, alcohols, as well as polyoxygenated compounds (5). Furthermore, the high temperatures used in frying contribute greatly to the formation of dimers and polymers of complex structures, mainly with C-C and C-O-C linkages (6). Determination of polar compounds provides a good measurement for the global amount of oxidized and polymeric products, and it is currently the method most widely used to control frying-fat alteration for human consumption (7). Reports from different countries have shown that a considerable number of samples often surpass the limit that is generally established for frying-fat alteration, i.e., 25% polar compounds (8–10). These findings clearly support the need for further investigation on the nutritional effects of such polar compounds.

In a preceding study from our laboratory, we made use of high-performance size-exclusion chromatography (HPSEC) to analyze thermoxidized oils subjected to *in vitro* hydrolysis by pancreatic lipase and showed the difficulties involved in the enzymatic action on complex glycerides, i.e., TG dimers and polymers (3). Later, a similar methodology was applied in studies on air-oxidized fish oils to show that TG polymers can be degraded by pancreatic lipase *in vitro* although less efficiently in fish oils that contain high levels of polymers compared to those with moderate or low levels (11).

In agreement with observations from *in vitro* experiments were the results we later obtained from feeding trials, which showed that rats fed thermoxidized oils excreted considerable amounts of nonhydrolyzed polymers. Moreover, we found that fecal levels of esterified nonoxidized fatty acids were significantly higher compared to those obtained for rats fed unaltered oils, and this might have been in part due to incomplete hydrolysis of the unoxidized TG (12).

In view of these results, a combination of chromatographic

*To whom correspondence should be addressed at Instituto de la Grasa (CSIC), Avda. Padre García Tejero, 4, 41012 Sevilla, Spain.
E-mail: GMARQUEZ@CICA.ES.

techniques was applied in the present study to further investigate, *in vitro*, two points of relevance: (i) The comparative susceptibility to hydrolysis of altered compounds present in used frying oils was studied through assays on fractions that were comprised of oxidized and polymeric TG, isolated from used frying oils and thermoxidized trilinolein (LLL). Toward this end, adsorption chromatography and HPSEC were used, and (ii) The influence of the degradation level of used frying fats on hydrolysis of unoxidized TG was determined in real used frying oils. For this purpose, thin-layer chromatography–flame ionization detection (TLC–FID) was additionally introduced as a complementary method.

EXPERIMENTAL PROCEDURES

Materials. Samples of LLL (Nu-Chek-Prep, Elysian, MN) were placed in glass tubes and heated in a hot plate at 180°C for different periods of time. Used frying oils from a number of restaurants and fried-food outlets in the south of Spain were supplied by Food Inspection Services. Thirteen samples were selected for the present work to cover a wide range of degradation level, from 3 to over 50% polar compounds. Fractions of polar compounds were separated from the LLL samples and used frying oils by silica column chromatography (13), with hexane/diethyl ether 90:10 to elute the first fraction, constituting nonoxidized TG, and diethyl ether to obtain the polar fraction.

Enzymatic hydrolysis. Samples subjected to enzymatic hydrolysis included used frying oils plus the polar fractions separated from thermoxidized LLL and used frying oils. Samples (100 ± 0.1 mg) were weighed in centrifuge tubes. Tris(hydroxymethyl)-aminomethane buffer (0.2 mL of 1 M, pH = 8.0), 2 mL of 22% $CaCl_2$, 0.5 mL of 0.1% sodium cholate, and 20 mg pancreatic lipase were added. The tubes were stirred in an electric shaker at 37°C. After the digestion period, 1 mL of 6 M HCl was added to the reaction mixtures. The acidified mixtures were transferred to a separatory funnel and extracted four times with four portions of 10 mL diethyl ether. The combined extracts were washed with distilled water to remove the HCl. Solvent was evaporated under vacuum.

Purified porcine pancreatic lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3) was prepared in our laboratory, and its activity, as checked by the IUPAC method, was 1.2 lipase unit per mg (14).

HPSEC. Fractions of polar compounds, obtained by adsorption chromatography on silica columns (14), and used frying oils were analyzed by HPSEC, before and after hydrolysis, with a Konik 500A chromatograph (Konik S.A., Barcelona, Spain) with a 10-µL loop. A refractive index detector (Hewlett-Packard, Pittsburgh, PA) and 100- and 500-Å Ultrastyragel columns (Waters Associates, Milford, MA), connected in series and operated at 35°C, were used. The columns were 25 × 0.77 cm i.d., packed with a porous highly cross-linked styrenedivinylbenzene copolymer (<10 µm). High-performance liquid chromatography-grade tetrahydrofuran served as the mobile phase at a flow of 1 mL/min, and sample concentration was between 15 and 20 mg/mL in tetrahydrofuran. Thus, oxidized TG monomers (oxTGM), TG dimers (TGD), and TG polymers (TGP) were quantitated in polar fractions from thermoxidized LLL before hydrolysis, together with diglycerides (DG), monoglycerides (MG) and free fatty acids for used frying oils and hydrolyzed samples. This methodology was described in detail in an earlier publication (15).

TLC–FID. Hydrolyzed used frying oil samples were dissolved in a diisopropyl ether solution of squalane (5 mg/mL), so that 1 µL of sample solution that contained 10 µg of hydrolysis products and 5 µg squalane, used as internal standard, was spotted on Chromarod S-III quartz rods with a coating of silica gel (Iatron Labs, Tokyo, Japan). All analyses were carried out in triplicate. The rods were developed with hexane/diethyl ether/acetic acid (98:2:1, vol/vol/vol) for 40 min, dried and scanned in an Iatroscan MK-5 TLC–FID analyzer (Iatron Laboratories). The Iatroscan was operated under the following conditions: flow rate of hydrogen, 150 mL/min; flow rate of air, 1500 mL/min; and scanning speed, 0.33 cm/s. Nonhydrolyzed nonoxidized TG were determined by the internal standard method. No response factor was necessary to be applied because the relative response of squalane with respect to LLL and triolein was approximately 1 (0.97 and 1.01, respectively). Details on calibration data were published in a previous paper (16).

Titration of free fatty acids. Hydrolyzed samples were analyzed for total free fatty acid content by titration with 0.02 N KOH in ethanol.

RESULTS AND DISCUSSION

Figure 1 presents HPSEC chromatograms of a used frying oil, before and after lipolysis, and shows the increase in complexity of the sample after partial glycerides and free fatty acids are released. Useful information can be obtained, even though, after extensive hydrolysis, some hydrolytic products coelute with other compounds on account of their similar molecular weight (MW). For example, this occurs for the fatty acid trimers and fatty acid dimers coeluted with TG monomers and DG, respectively. Nevertheless, the quantitated compounds can be distributed into three groups according to their MW. First, at retention times (R_t) longer than 14 min are compounds produced exclusively by hydrolysis, MG and fatty acid monomers, so their global level indicates the minimum of hydrolytic products formed. On the other hand, the group of high MW (>1800), at R_t below 12 min, provides a measurement for the amount of complex glycerides that remain after hydrolysis. Finally, in the 12–14 min region are included both partially hydrolyzed compounds and complex fatty acids (fatty acid dimers and trimers).

Table 1 shows repeatability data from HPSEC analyses of samples that were hydrolyzed in quadruplicate for two representative assays of the conditions used in this study, i.e., a

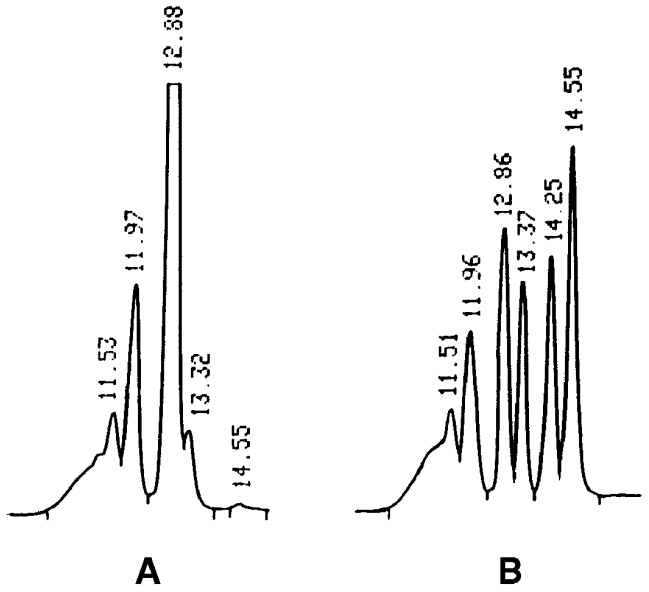

FIG. 1. High-performance size-exclusion chromatograms of used frying oil before (A) and after (B) lipolysis. Retention times (min): 11.5, triglyceride polymers; 11.9, triglyceride dimers; 12.8, triglyceride monomers plus fatty acid polymers; 13.3, diglycerides plus fatty acid dimers; 14.2, monoglycerides; and 14.5, fatty acid monomers.

polar fraction, comprised of oxidized and polymeric TG after incubation for 20 min, and a used frying oil subjected to lipolysis for 2 min. The main difference between the two samples was the preliminary elimination of nonoxidized TG in the first sample; hence, the peak corresponding to TG monomers here accounts for oxidized molecules exclusively. In the used frying oil, however, TG monomers include both the nonoxidized and oxidized types. The relative standard deviations found for the different groups of compounds were between 5–17%. These values were highly satisfactory, considering the reproducibility generally associated with this kind of enzymatic assay.

TABLE 1
Precision of Analysis of Hydrolyzates by HPSEC and Titration

	Polar fraction C[a] (20 min hydrolysis)		Used frying oil O-7 (2 min hydrolysis)	
	Mean (wt%)	RSD%[b]	Mean (wt%)	RSD%
TGP	39.8	7.0	6.7	6.6
TGD	10.9	13.8	6.1	17.0
TGM[c]	4.7	12.4	12.9	6.6
DG	10.0	11.2	14.4	11.6
MG	12.7	10.8	20.6	6.6
FAM	21.0	9.6	40.0	5.0
TFA[d]	39.7	6.4	39.9	9.6

[a]Polar fraction from thermoxidized trilinolein.
[b]RSD% = (SD/mean) × 100, $n = 4$.
[c]Including both nonoxidized and oxidized triglyceride monomers (oxTGM) in the used frying oil, and exclusively oxTGM in the polar fraction.
[d]Determined by titration. Abbreviations: TGP, triglyceride polymers; TGD, triglyceride dimers; TGM, triglyceride monomers; DG, diglycerides, MG, monoglycerides; FAM, fatty acid monomers; TFA, total fatty acids; HPSEC, high-performance size-exclusion chromatography.

To compare the rates of hydrolysis of oxidized TG monomers, dimers and polymers, a pool of polar fractions, isolated from a large number of used frying fats and oils, was incubated with lipase, and disappearance of the components was followed by HPSEC (Fig. 2). Under the conditions used in this study, the proportion of oxTGM decreased rapidly over the initial 2 min, while a similar extent of hydrolysis was observed for TGD after 20 min; TGP showed the slowest rate of hydrolysis. The pattern observed here was useful to decide the most appropriate times of incubation for further assays. Thus, 20 min was selected as a suitable reaction time for testing fractions that contained TGP, TGD, and oxTGM and for establishing comparisons between these compounds when they are all hydrolyzed to a measurable degree. In turn, at a short time of hydrolysis, 2 min, fractions enriched in oxTGM could be readily hydrolyzed with the advantage that small amounts of hydrolytic products were released from TGP and TGD and hence did not interfere in quantitation of other compounds by HPSEC.

First, LLL was used as a model monoacid TG to examine the action of lipase, avoiding any possible influence of other compounds present in used frying fat, such as DG, MG, free fatty acids, and other minor constituents. Tables 2 and 3 show the results obtained for fractions isolated by silica column chromatography from LLL samples that were subjected to thermoxidative conditions at 180°C. By testing, under identical conditions, fractions enriched in either oxidized monomers or polymeric compounds and devoid of nonoxidized TG, it was possible to compare the hydrolysis rate of the different compounds formed.

Table 2 corresponds to samples that contained high levels

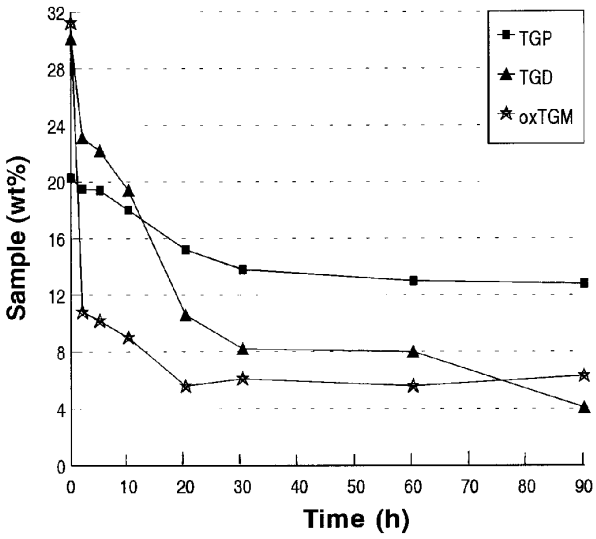

FIG. 2. Time course of hydrolysis of oxidized triglyceride monomers (oxTGM), dimers (TGD), and polymers (TGP) in a pool of polar fractions separated from used frying fats and oils.

of polymers and dimers and are listed according to decreasing proportion of TGP. Clearly, oxTGM are the type of altered compounds that are more extensively hydrolyzed, and they show high hydrolysis percentages, between 80–89%, even though these values were underestimated due to the concomitant quantitation, in the oxTGM peak, of hydrolytic products from TGP of similar MW, such as fatty acid polymers. Quite in contrast, TGP, which were quantitated without interference of any other coeluting compounds, showed low hydrolysis values, particularly in those samples that contained initially the highest proportion of polymers. On the other hand, the hydrolysis values obtained for TGD were consistently higher and lower than those of TGP and oxTGM, respectively, in all samples analyzed. Differences between samples were also reflected in the data obtained for MG and free fatty acid monomers (FAM), as quantitated by HPSEC, and total free fatty acids (TFA) as determined by titration (last column). As expected, TFA values were always higher than FAM owing to the presence of high-MW acids that came from polymeric glycerides.

Pancreatic lipase shows fatty acyl specificities for chain-length, position and geometry of double bonds, generally suggested to be due to steric hindrance for formation of the activated substrate–enzyme complex (17,18). This could also explain the results obtained here for high-MW TG molecules, although the influence of other factors involved in the hydrolytic process, such as availability of the molecules in the water–oil interface and hydrophilic properties, should not be ruled out. Nevertheless, no apparent differences were found between the samples used in terms of emulsion formation.

Table 3 lists data for hydrolysis after 2 min of the same fractions tested above at 20 min (A to F), along with other fractions from thermoxidized LLL enriched in oxTGM (G to M). Under these conditions, it was evident that oxTGM were by far the most readily hydrolyzed altered compounds. Conversely, TGP and TGD remained practically nonhydrolyzed, and these results were consistent with the similar values obtained for TFA and FAM, which indicates that the amounts of complex fatty acids released by lipase were low. The extent of hydrolysis of oxTGM increased when the polymer content was lower, and reached values as high as 90% in sample M. For comparison, incubation of intact LLL with lipase gave 97.7% hydrolysis after 2 min reaction time. High hydrolysis rates have also been reported for TG monohydroperoxides, at similar degrees as their original TG, and this finding has been explained by their similar molecular structure and hydrophilic properties (19). Under the thermoxidation conditions used in these experiments, however, oxTGM are not hydroperoxides, which are rather unstable at high temperature, but are largely made up of other oxygenated compounds, containing functions such as hydroxy, keto, or epoxy groups (5).

Compositions of used frying fats and oils, as quantitated by HPSEC after separation of nonoxidized TG by silica column chromatography, are shown in Table 4. Samples were provided by Food Inspection Services (Junta de Andalucia, Spain) and had been collected from restaurants and fried-food outlets in the south of Spain. They represented different types of fats or oils, frying conditions and fried products, and they were selected to test variable degradation levels, which ranged from only 3.1 to 61.4% polar compounds. Also, an unused sunflower oil was included as reference oil in the last row. Although sample O-1 was among the used frying oils supplied by Food Inspection Services, it showed a polar compound level and distribution typical of an unused oil, with oxTGM and TGD contents below those for the unused sunflower oil included for comparison. On the other hand, it was not unexpected to find different patterns of compound distribution among samples of similar percentages of polar compounds because these oils might have undergone different treatments. In general, oxTGM constituted a major fraction for oils of low degradation level, while highly degraded samples contained predominantly dimers and polymers (20).

Table 5 lists the results from polar fractions of the above oils that were isolated by silica column chromatography, before and after hydrolysis for 20 min. In spite of the differences between LLL samples and used frying oils (the latter were of

TABLE 2
HPSEC Quantitation of Groups of Compounds (wt% on sample) in Fractions Enriched in Triglyceride Dimers and Polymers from Thermoxidized Trilinolein, Before and After Hydrolysis (for 20 min)

Sample	Hydrolysis time (min)	TGP	TGD	oxTGM[a]	DG[a]	MG	FAM	TFA[b]
A	0	52.1	23.5	24.4				
	20	46.6	9.2	4.9	9.9	12.7	16.7	25.9
B	0	50.8	21.7	27.5				
	20	43.1	10.6	5.9	8.9	13.1	18.4	31.2
C	0	48.3	24.4	27.3				
	20	39.8	10.9	4.7	10.0	12.7	21.0	39.7
D	0	31.7	25.6	42.7				
	20	23.0	10.1	6.0	10.5	24.0	26.4	38.3
E	0	28.2	28.3	43.5				
	20	16.5	9.0	7.0	12.5	22.3	32.7	44.9
F	0	27.7	27.6	44.7				
	20	18.3	10.2	4.9	11.8	21.5	33.3	41.0

[a]Peaks may include hydrolytic products. For abbreviations, see Table 1.
[b]Expressed as percentage oleic acid, as determined by titration.

TABLE 3
HPSEC Quantitation of Groups of Compounds (wt% on sample) in Fractions from Thermoxidized Trilinolein, Before and After Hydrolysis (for 2 min)

Sample	Hydrolysis time (min)	TGP	TGD	oxTGM	DG	MG	FAM	TFA[a]
A	0	52.1	23.5	24.4				
	2	45.8	18.8	12.0	7.2	6.1	10.1	15.0
B	0	50.8	21.7	27.5				
	2	43.2	14.6	11.8	7.1	8.4	14.9	20.9
C	0	48.3	24.4	27.3				
	2	44.5	18.6	12.1	6.9	6.0	10.9	17.4
D	0	31.7	25.6	42.7				
	2	27.0	18.4	19.9	11.6	9.2	13.9	20.2
E	0	28.2	28.3	43.5				
	2	22.9	21.4	18.6	12.3	9.5	15.3	23.5
F	0	27.7	27.6	44.7				
	2	23.2	21.2	20.0	11.7	8.6	15.3	19.1
G	0	11.7	11.0	77.3				
	2	10.1	9.7	35.4	12.9	17.4	14.5	20.9
H	0	10.1	12.5	77.4				
	2	9.0	7.3	34.0	15.6	20.1	14.0	16.9
I	0	5.1	13.5	81.4				
	2	4.5	8.9	30.0	15.9	23.5	17.2	24.7
J	0	4.8	12.9	82.3				
	2	4.5	8.8	28.8	14.7	23.9	19.3	21.1
K	0	1.1	6.1	92.8				
	2	1.0	4.7	23.1	21.2	21.1	28.9	31.3
L	0	0.7	5.5	93.8				
	2	0.5	4.2	18.9	20.1	26.2	30.1	35.0
M	0	—	3.5	96.5				
	2	—	3.0	8.7	20.2	27.8	40.3	42.3

[a]Expressed as percentage oleic acid, as determined by titration. For abbreviations, see Table 1.

greater complexity in terms of fatty acid composition and presence of additional polar compounds and minor constituents), similar results were obtained in terms of the relative hydrolysis rates of TGP, TGD, and oxTGM. The poorest substrate for pancreatic lipase was again TGP, and the lowest hydrolysis degrees were found for the samples with the highest amounts of TGP.

Additionally, used frying oils were subjected to enzymatic hydrolysis without any preliminary separation of nonoxidized TG to attain comparative results on the susceptibility to hydrolysis of intact frying oil samples. Nonoxidized and oxidized TG monomers were not quantitated separately because the quantities available of hydrolyzed samples were not sufficient for separation by column chromatography. After 2 min reaction time (Table 6), it was clear that the extent of hydrolysis decreased when alteration of the samples was greater. Thus, the most degraded used frying oils showed a significant reduction in the level of total hydrolytic products (DG, MG,

TABLE 4
Composition of Used Frying Oils (wt% on oil)[a]

		Polar compounds					
Oils	Nonoxidized TG	Total	TGP	TGD	oxTGM	DG	FAM
O-1	96.9	3.1	—	0.1	0.8	1.5	0.6
O-2	92.5	7.5	0.3	1.5	3.2	2.1	0.4
O-3	82.8	17.2	1.8	5.2	8.0	1.7	0.5
O-4	79.7	20.3	4.9	6.1	4.4	4.2	0.7
O-5	77.8	22.2	4.1	9.4	6.2	1.8	0.7
O-6	75.7	24.3	7.6	8.6	4.6	2.9	0.6
O-7	71.1	28.9	8.2	8.9	5.9	5.0	0.9
O-8	70.5	29.5	9.1	9.1	7.1	4.2	—
O-9	58.1	41.9	14.3	13.7	10.6	2.1	1.2
O-10	57.3	42.7	15.7	14.2	8.9	2.9	1.0
O-11	57.2	42.8	11.3	13.3	9.3	8.9	—
O-12	43.7	56.3	22.6	17.0	16.4	—	0.3
O-13	38.6	61.4	32.8	14.8	9.7	3.6	0.5
Unused oil	97.0	3.0	—	0.6	0.9	0.9	0.6

[a]TG, triglycerides. For other abbreviations, see Table 1.

TABLE 5
HPSEC Quantitation of Groups of Compounds (wt% on sample) in Fractions from Used Frying Oils (before and after hydrolysis for 20 min)

Sample	Hydrolysis time (min)	TGP	TGD	oxTGM[a]	DG[a]	MG	FAM
F-2	0	3.5	20.0	42.4	28.6	—	5.5
	20	1.5	2.7	4.4	10.4	26.4	54.6
F-3	0	10.6	30.2	46.6	9.8	—	2.8
	20	5.1	15.0	10.1	14.9	18.6	36.3
F-4	0	24.2	30.1	21.6	20.8	—	3.3
	20	13.6	5.9	7.7	12.8	25.2	34.8
F-5	0	18.3	42.3	27.8	8.3	—	3.3
	20	8.8	16.9	4.3	13.3	21.0	35.7
F-6	0	31.5	35.6	18.8	11.7	—	2.4
	20	19.5	8.1	5.0	13.6	15.9	37.9
F-7	0	28.4	30.7	20.4	17.5	—	3.0
	20	20.6	13.3	5.3	11.7	15.7	33.4
F-8	0	31.0	30.9	23.8	14.3	—	—
	20	21.0	13.1	4.5	10.8	17.3	33.3
F-9	0	34.2	32.6	25.3	5.0	—	2.9
	20	24.0	17.9	4.2	10.3	17.3	26.3
F-10	0	36.8	33.0	20.9	6.9	—	2.4
	20	26.7	12.9	3.6	11.6	14.6	30.6
F-11	0	26.4	31.2	21.7	20.7	—	—
	20	18.2	13.3	5.1	13.4	17.1	32.9
F-12	0	40.2	30.3	29.0	—	—	0.5
	20	29.7	15.1	5.6	10.2	12.5	26.9
F-13	0	53.5	24.1	15.8	5.8	—	0.8
	20	42.6	10.2	5.0	10.7	10.8	20.7

[a]Peaks may include hydrolytic products. For other abbreviations, see Table 1.

and FAM). Moreover, increasing amounts of polymers and dimers, even though such compounds were scarcely hydrolyzed, affected remarkably the hydrolysis rate of monomeric TG, as indicated by the slower disappearance of these compounds in samples of higher concentration in dimers and polymers. Similar observations were made upon hydrolysis of oxTGM (Table 3).

With the aim of examining specifically the influence of the content in polymers and dimers on hydrolysis of the nonoxidized TG in used frying samples, a different analytical approach was used. A procedure based on TLC–FID was developed to quantitate the levels of nonoxidized TG remaining after lipolysis in microsamples. Figure 3 illustrates the good separation achieved between the internal standard (squalane), the peak that contains fatty acid monomers, the peak that corresponds to nonoxidized TG, and the rest of the compounds, which remain practically at the origin of the rod because of their higher polarity. The coefficients of variation of the values obtained for nonhydrolyzed nonoxidized TG in two used frying oils that are representative of quite different alteration levels, O-11 and O-13, hydrolyzed in duplicate and spotted in triplicate, were 4.6 and 7.5%, respectively, which indicated good reproducibility.

Our results show that hydrolysis of nonoxidized TG by pancreatic lipase can be affected by the presence of TGP and TGD. Thus, an excellent correlation was obtained between percentage of nonhydrolyzed nonoxidized TG and the global percentage of dimeric and polymeric compounds (Fig. 4), regardless of the different types of used frying oils tested. The most altered oil (O-13), with a polar compound content of 61.4% and a level of dimers plus polymers of 47.6%, showed a percentage of hydrolysis of nonoxidized TG as low as 52% whereas it was 95% for an oil of low degradation level (7.5% polar compounds and 1.8% dimers plus polymers). Even those samples around the limit of rejection, established in frying-fat regulations for human consumption at 25% polar compounds (7), showed reduced hydrolysis of nonoxidized TG.

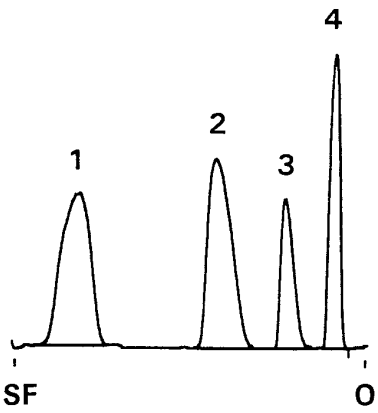

FIG. 3. Thin-layer chromatography–flame-ionization detection trace of a used frying oil hydrolyzed for 2 min. Peaks: 1, squalane (internal standard); 2, fatty acid monomers; 3, nonoxidized triglycerides; and 4, other compounds of high polarity (oxidized triglyceride monomers, dimers and polymers, partial glycerides, and complex fatty acids). O = origin, SF = solvent front.

TABLE 6
HPSEC Quantitation of Groups of Compounds (wt% on sample) in Used Frying Oils (before and after hydrolysis for 2 min)

Sample	Hydrolysis time (min)	TGP	TGD	TGM[a]	DG	MG	FAM	TFA[b]
O-1	0	—	0.1	97.7	1.5	—	0.6	
	2	—	—	3.8	13.8	33.5	48.9	47.2
O-2	0	0.3	1.5	95.7	2.1	—	0.4	
	2	0.1	1.6	6.1	11.6	29.9	50.7	48.9
O-3	0	1.8	5.2	90.8	1.7	—	0.5	
	2	0.9	2.9	17.6	15.2	20.7	42.7	45.8
O-4	0	4.9	6.1	84.1	4.2	—	0.7	
	2	3.7	2.6	7.4	12.4	32.4	41.5	39.0
O-5	0	4.1	9.4	84.0	1.8	—	0.7	
	2	3.1	5.8	14.7	13.9	20.2	42.3	43.8
O-6	0	7.6	8.6	80.4	2.8	—	0.6	
	2	5.1	4.7	15.6	13.4	22.6	38.6	36.1
O-7	0	8.2	8.9	77.0	5.0	—	0.9	
	2	6.7	6.1	12.9	14.4	20.6	40.0	39.9
O-8	0	9.1	9.1	77.6	4.2	—	—	
	2	8.1	7.0	19.4	16.8	14.8	33.9	37.1
O-9	0	14.3	13.7	68.7	2.1	—	1.2	
	2	12.8	11.0	18.3	14.4	13.5	30.0	33.5
O-10	0	15.7	14.1	66.2	2.9	—	1.1	
	2	13.4	12.5	25.2	11.6	14.5	30.6	34.8
O-11	0	11.3	13.3	66.5	8.9	—	—	
	2	9.6	9.8	25.1	14.5	14.3	26.7	30.4
O-12	0	22.6	17.0	60.0	—	—	0.4	
	2	22.2	15.9	32.5	11.9	13.9	13.6	15.8
O-13	0	32.8	14.8	48.3	3.6	—	0.5	
	2	30.2	9.7	15.0	10.5	11.0	23.6	26.1
Unused oil	0	—	0.6	97.9	0.9	—	0.6	—
	2	—	0.3	4.0	9.2	34.8	51.7	45.1

[a]TGM, triglyceride monomers (nonoxidized plus oxidized). For abbreviations, see Table 1.
[b]Expressed as percentage oleic acid, as determined by titration.

For example, around 20% of nonoxidized TG remained unhydrolyzed in samples O-6, O-7 and O-8, which contained between 16–18% dimers plus polymers and total polar compound levels between 24–29%.

However, under rapid and extensive hydrolysis in the lumen, the extension of lipolysis should be significantly higher because, under physiological conditions, pancreatic lipase activity is much greater than it is under *in vitro* conditions. In fact, it has been estimated to be 100–1000-fold in excess of that needed for complete hydrolysis of TG in the small intestine (21). Yet, in our preceding studies, HPSEC analyses of fecal lipids from rats that were fed thermoxidized oils showed that a considerable fraction of the high-MW compounds ingested remained nonhydrolyzed and, moreover, fecal levels of esterified nonoxidized fatty acids were significantly higher than those obtained for rats that were fed unaltered oils (12). The maldigestion effects observed here *in vitro* for intact TG in the bulk of abused frying oils should be further studied under physiological conditions and could be of special interest in relation to patients who are affected by pancreatic disorders (22).

In conclusion, the combination of chromatographic techniques selected for this study enabled us to gain useful information on the hydrolysis of complex TG that are formed dur-

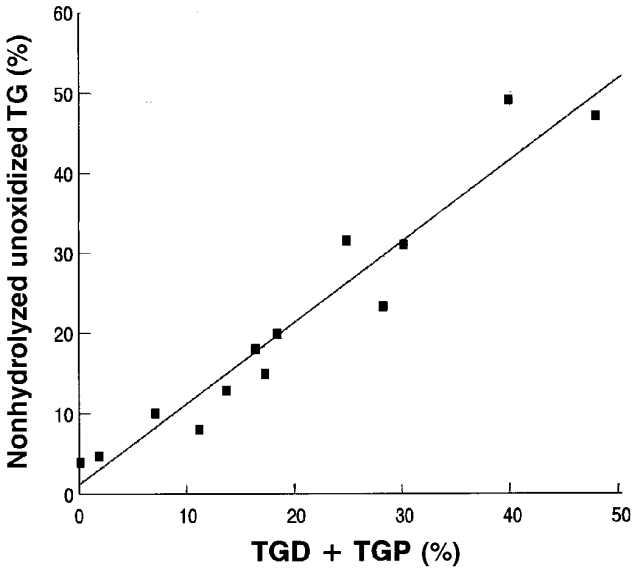

FIG. 4. Correlation between content of triglyceride dimers and polymers (TGD + TGP), and nonoxidized triglycerides (TG) remaining after hydrolysis of used frying fats and oils for 2 min. $Y = 1.0489X + 0.1017$, $r = 0.9648$, $P < 0.0005$ ($n = 13$).

ing thermoxidation and frying. Our results showed that there was a significant discrimination of pancreatic lipase against dimers and particularly polymers. The relative ease of hydrolysis remained constant at different times of exposure to lipolysis, in the following order: oxidized monomers >> dimers > polymers, which gives evidence of the important influence of MW on the action of pancreatic lipase. Of particular relevance was the high hydrolysis rate of oxidized TG monomers because the oxidized fatty acids yielded therefrom can be found at levels as high as 6.6% in real used frying oils (20), are readily absorbed (23), and are of great concern because of their potential physiological implications (24–27). On the other hand, it was observed that hydrolysis of TG monomers, either intact or oxidized, was impaired by the presence of dimers and polymers in abused frying fats.

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