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Assessments on the digestibility of oxidized compounds from [1-^{14}C]linoleic acid using a combination of chromatographic techniques

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Abstract

The aim of this study was to evaluate the digestibility coefficients of different groups of oxidized fatty acids by applying a methodology based on chromatographic techniques. After thermoxidative treatment, oxidized labelled linoleic acid was isolated and included at 1% in the experimental diets. Male 250–300 g Wistar rats were fed ad lib for seven days. Lipids extracted from diets and faeces were analyzed using a combination of chromatographic methods and radioactivity measurements to determine the specific digestibilities of four groups of altered fatty acids: oxidized monomers, non-polar dimers, oxidized dimers and polymers. Mean digestibility coefficients of oxidized monomers, dimers and polymers were 91.0, 74.5 and 69.8%, respectively. In contrast, non-polar dimers were poorly absorbed. The presence of unaltered labelled fatty acids in faeces indicated that structural modifications may have been taken place prior to absorption, and oxidized fatty acids seem to be the main compounds affected.

Keywords: Linoleic acid; Fatty acids; Lipids

1. Introduction

The nutritional value of oils and fatty foods subjected to thermal oxidation, as in frying, can be impaired as a result of the complex mixture of oxidized and polymerized products produced [1–6]. In this context, the absorbability of heated and thermally oxidized oils is one of the primary and most necessary points to look for further evidence of metabolic interference, and this has been approached through lymph cannulation [7–9] or digestibility studies [10–15]. Overall, reports on digestibility have indicated significantly lower values for thermally oxidized oils as compared with fresh oils, and this fact has been attributed to the presence of the nondistillable urea-nonadductable fraction, that is, the polymeric fraction [10,12,13]. In our precedent studies, we applied a combined chromatographic analysis to determine the digestibilities of fatty acid monomers, dimers and polymers in rats fed thermoxidized olive oils and found high values

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for oxidized monomers and higher apparent absorbability for polymers than expected [15]. Further experiments showed the marked reduction in the in vitro hydrolysis rate of thermoxidized oils due to the difficulties involved in the pancreatic lipase action on polymeric glycerides [16], also confirmed through quantitation of free and esterified levels of unaltered fatty acids in faecal lipids [17]. Hence, the digestibility of polymers was found to be in great part dependent on previous hydrolytic action by pancreatic lipase.

Alternatively, studies on labelled model compounds, often linoleic acid or methyl linoleate, have provided a valuable picture of the absorption and distribution of fractions enriched in secondary products [18] or in polymers [19] through oral administration of such isolated fractions, although specific data on the digestibilities of the different groups of compounds could not be collected because of the limitations of the separation methods used.

In the present study, our objective was to apply various chromatographic techniques and radioactivity measurements to evaluate the absorbability of different groups of oxidized and polymeric compounds in rats fed diets with thermoxidized labelled linoleic acid added. A free fatty acid was used as the model compound to avoid any difficulty associated with the hydrolytic step by pancreatic lipase.

2. Experimental

2.1. Chemicals and reagents

[1-^{14}C]Linoleic acid was purchased from Sigma (St. Louis, MO, USA) and methyl linoleate was obtained from Nu-Check-Prep (Elysian, MN, USA). Tetrahydrofuran of HPLC grade was purchased from Romil (Cambridge, UK), while hexane and diethyl ether for analysis and HCl were from Panreac (Barcelona, Spain). Silica gel 60 (particle size 0.063–0.200 mm) for column chromatography and silica gel 60 G for thin-layer chromatography were obtained from Merck (Darmstadt, Germany).

2.2. Preparation of thermoxidized [1-^{14}C]linoleic acid

A 10-g amount of [1-^{14}C]linoleic acid (527.25 kBq/g) was thermally oxidized at 180°C in the presence of air for 100 h to favour the formation of oxidative dimers and polymers. Ten grams of the same compound, [1-^{14}C]linoleic acid, were subjected to 200°C under a stream of nitrogen for 200 h in order to obtain substantial amounts of non-polar dimers. Samples were mixed and separated into three fractions of different polarity by means of silica column chromatography, using hexane–diethyl ether (1:1), hexane–diethyl ether (2:3) and diethyl ether–methanol (1:1), respectively. The first fraction was predominantly comprised of unaltered linoleic acid. The second fraction still had unaltered linoleic acid and was discarded, while the third fraction was entirely comprised of altered compounds (14.5% oxidized monomers, 6.9% non-polar dimers, 27.1% oxidized dimers and 51.5% polymers) and was used in the preparation of diets for rats fed thermoxidized [1-^{14}C]linoleic acid.

2.3. Animals and diets

Six male 250–300 g Wistar rats were fed diets containing 9% commercial virgin olive oil and 1% thermoxidized [1-^{14}C]linoleic acid (482.85 kBq/g). A content of 1% thermoxidized fatty acids was selected because it corresponds to 10% when referring to the total dietary fat, which in turn is approximately the amount of altered fatty acids present in frying fats at the limit of rejection (25% polar compounds) [20]. The composition of the basal fat-free semipurified diet was 10% water, 9.8% protein, 57.6% carbohydrates, 5.4% cellulose, 6.2% mineral mixture and 1.0% vitamin mixture. A control group of six rats received diets containing 1% unaltered [1-^{14}C]linoleic acid (527.25 kBq/g). Animals were fed ad lib for seven days. Food intake was recorded, and faeces were collected daily.

2.4. Extraction of faecal lipids

The combined faeces from the experimental period were dried under vacuum to constant

weight, pulverized and extracted in a Soxhlet extractor using diethyl ether for 8 h [21]. The residue was dried, mixed with 3 *M* HCl and re-extracted with diethyl ether, to obtain the total lipids excreted. Faecal lipids thus obtained were dried under vacuum at 60°C to constant weight.

2.5. In vitro experiments

Methyl linoleate was thermally oxidized in the presence of air for 6 h at 180°C. Upon elimination of the unaltered fraction by silica column chromatography, four samples of 150 mg each were suspended in 50 ml of 0.05 *M* HCl in 80% aqueous ethanol (pH 1.8) and heated at 37°C in a stirring bath. After 20 h, the reaction mixture was extracted thoroughly with diethyl ether.

2.6. Quantitative analysis

Radioactivity measurements

Total radioactivity was measured by liquid scintillation counter LKB 1215 Rackbeta II (LKB, Bromma, Sweden). Lipid classes were separated by thin-layer chromatography (TLC) using silica gel 60 G plates activated at 120°C for 3 h and developed with hexane–diethyl ether–acetic acid (70:30:2) to its full length. Then, the percentual radioactivity of lipid classes was determined in an automatic TLC-linear analyzer Berthold LB 2820-1 (Berthold, Wildbad, Germany).

Combination of adsorption and high-performance size-exclusion chromatographies (HPSEC)

Following methylation with diazomethane [22], samples were separated by silica column chromatography. Hexane–diethyl ether (88:12) was used to elute the non-polar fraction, and diethyl ether for the polar fraction, followed by a final elution with methanol to improve recovery of the sample. Separation of non-polar and polar fractions was performed by HPSEC. The samples were analyzed in a Konik 500 A chromatograph (Konik, Barcelona, Spain) with a 10-μl sample loop. A Hewlett-Packard 1037 A refractive index detector (Hewlett-Packard, Pittsburgh, PA, USA) and two 100 Å and 500 Å Ultrastyragel columns (Water, Milford, MA, USA) connected in series operated at 35°C. The columns were 25 cm × 0.77 cm I.D., packed with porous, highly cross-linked styrenedivinylbenzene copolymer. The non-polar fraction, eluted with 88:12 hexane–diethyl ether, contained unaltered linoleic acid methyl esters plus non-polar dimers. The polar fraction, eluted with diethyl ether, contained oxidized monomers, oxidized dimers and polymers. The methodology was described in detail, including calibration and reproducibility data, in an earlier publication [23].

3. Results and discussion

Fig. 1 shows high-performance size-exclusion chromatograms and TLC-linear radioactivity analyzer reports of original (unaltered) and thermoxidized [1-^{14}C]linoleic acid, used in the experimental diets. Direct application of HPSEC is advantageous in that the sample can be analyzed without being subjected to any previous derivatization method which might produce structural modifications. As can be observed, thermoxidized [1-^{14}C]linoleic acid was enriched in dimers and polymers, and devoid of any remaining unaltered linoleic acid, as checked by radioactivity TLC analyzer report. Additionally, thermoxidized [1-^{14}C]linoleic acid was analyzed by HPSEC following methylation with CH_2N_2 and separation by silica column in order to quantitate separately non-polar dimers and oxidized dimers, in the non-polar and polar fractions, respectively (data shown in Table 2). The results obtained for oxidized monomers, total dimers and polymers were very similar for the sample analyzed either directly or after derivatization. A more detailed quantitative evaluation of the compounds originating during thermal oxidation is a difficult task due to the presence of a multitude of individual compounds differing in molecular mass and polarity [24,25]. Total radioactivity data, obtained by scintillation counting, of fat ingested and that recovered from faecal lipids of all the animals, corresponding to the overall experimental period, are listed in Table 1. Digestibilities of unaltered labelled linoleic acid thus evaluated

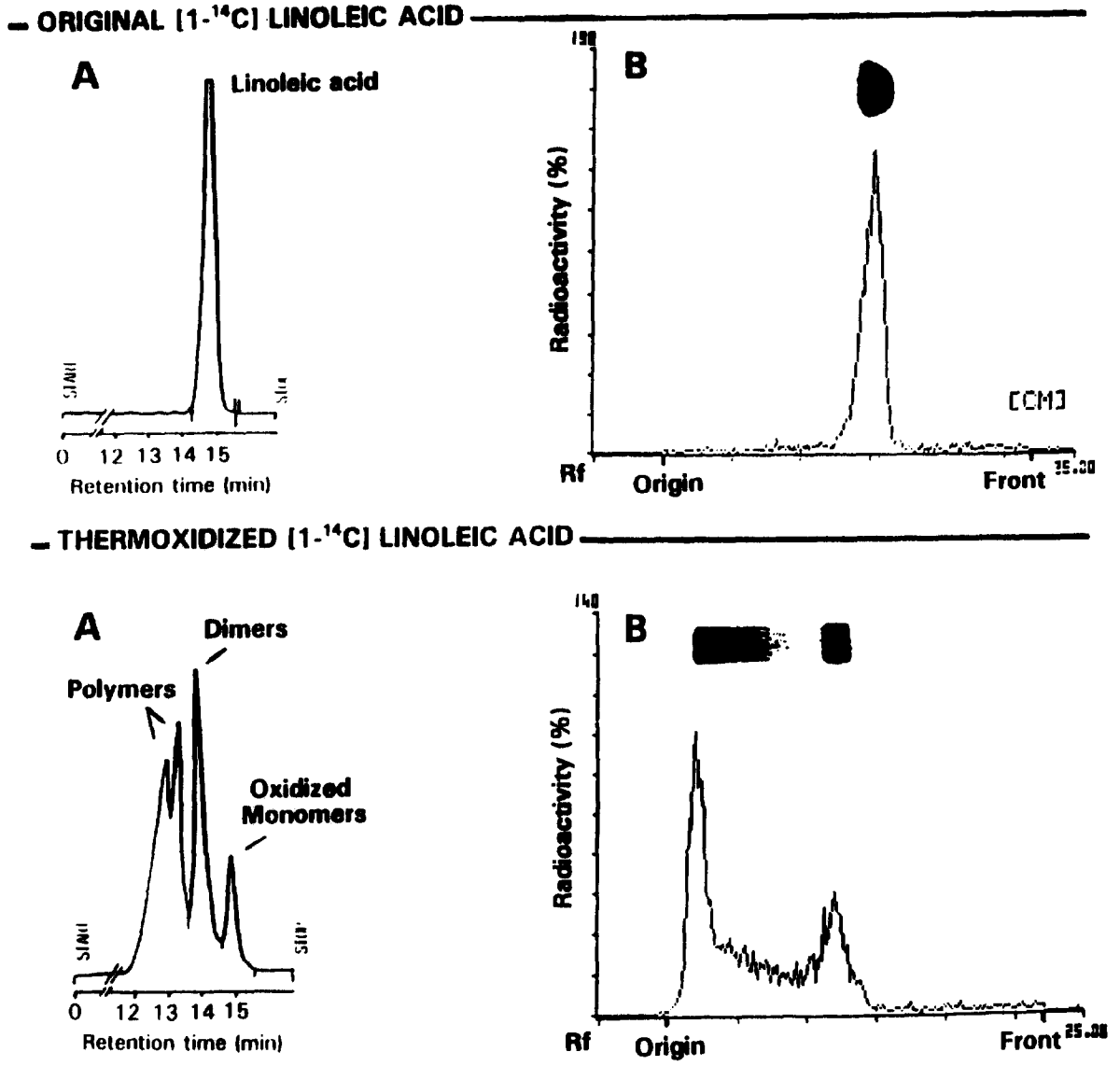

Fig. 1. High-performance size-exclusion chromatograms (A) and TLC-linear radioactivity analyzer reports (B) of original and thermoxidized [1-^{14}C]linoleic acid.

were, as expected, very high (96.7% on average). As to thermoxidized labelled linoleic acid, mean digestibility was 70.3%, which indicated a substantial absorption, considering that the labelled compound administered was entirely comprised of alteration compounds. Interestingly, the lipid fraction obtained by re-extraction of the faecal residue upon adding HCl presented approximately threefold higher activity than that directly extracted, which confirms the need for re-extraction of faeces, at least in the case of lipids of great polarity and complexity, and even if some modifications cannot be excluded. This is clearly illustrated in Fig. 2, which shows the radioactivity TLC analyzer reports of faecal lipids from a rat fed a diet containing thermoxidized [1-

Table 1
Total radioactivity (kBq) ingested and recovered from faeces of rats fed diets containing unaltered and thermoxidized [1-^{14}C]linoleic acid

	Non-altered labelled linoleic acid	Thermoxidized labelled linoleic acid
Ingested	497.3 ± 7.7	467.6 ± 5.3
Excreted in faeces:		
Direct extraction		38.3 ± 1.0
Extraction after hydrolysis		100.4 ± 1.5
Total	14.9 ± 0.5	138.7 ± 2.0
Digestibility (%)	96.7 ± 0.3	70.3 ± 0.6

Means ± S.E.M. of six rats.

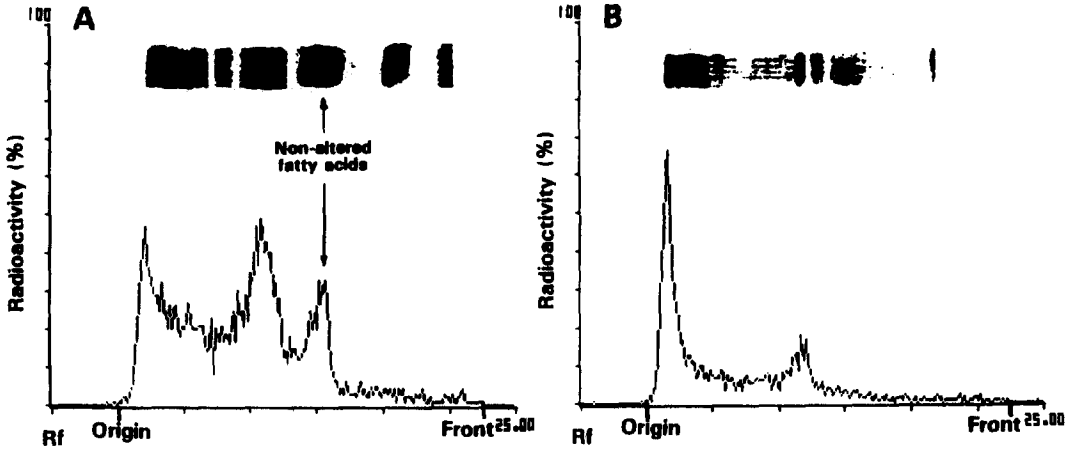

Fig. 2. TLC-linear radioactivity analyzer reports of faecal lipids from a rat fed the diet containing thermoxidized [1-^{14}C]linoleic acid: (A) direct-extracted lipids; (B) re-extracted lipids upon HCl treatment of the faecal residue.

^{14}C]linoleic acid. As can be seen, the re-extracted lipid fraction (B) mostly contained polar material (peak at TLC origin).

On the other hand, it was particularly noteworthy that unaltered labelled fatty acids were found in faecal lipids (fraction A) as they were not present in the diet (Fig. 1). Taking into account that this faecal lipid fraction A was extracted directly from faeces, without any previous treatment, it shows clearly that the ingested thermoxidized linoleic acid was affected by structural modifications somewhere in the gastrointestinal tract.

Table 2 summarizes the specific digestibilities

Table 2
Digestibility of unaltered fatty acids, fatty acid non-polar dimers, oxidized monomers, oxidized dimers and polymers in rats fed diets containing thermoxidized [1-^{14}C]linoleic acid

	Composition of thermoxidized labelled linoleic acid (% on sample[a])	Digestibility[b] (%)
Unaltered	nd	Negative
Oxidized monomers	14.5	91.0 ± 0.9
Non-polar dimers	6.9	25.9 ± 1.1
Oxidized dimers	27.1	74.5 ± 2.3
Polymers	51.5	69.8 ± 2.4

[a] Sample of labelled linoleic acid was included at 1% in diets.
[b] Means ± S.E.M. of six rats.

of fatty acid non-polar dimers, oxidized monomers, oxidized dimers and polymers in rats fed diets containing 1% thermoxidized [1-^{14}C]linoleic acid. The first column lists the percent composition of thermoxidized [1-^{14}C]linoleic acid. Quantitation of these groups of altered fatty acids in dietary and faecal samples was attainable by combination of radioactivity measurements and chromatographic techniques. On one hand, the use of tracer compounds permitted the monitoring of samples without interference due to endogenous fat or the dietary oil included in diets (virgin olive oil), while on the other, application of combined adsorption and size-exclusion chromatographies to the methyl ester derivatives was of great utility to separate and quantitate different groups of altered compounds. Thus, unaltered fatty acids, oxidized fatty acid monomers, non-polar fatty acid dimers, oxidized fatty acid dimers and fatty acid polymers could be satisfactorily differentiated. Preliminary studies showed that endogenous lipids did not interfere with HPSEC determination of either dimers or polymers [15], and on the other hand, the dietary oil, virgin olive oil, is characterized by the absence of dimeric and polymeric compounds [26]. Hence, direct quantitation of labelled non-polar dimers, oxidized dimers and polymers could be attained from their HPSEC chromatographic peak areas in their corresponding fractions separated by silica column (non-polar or polar). However, unaltered monomers include a certain proportion derived from the dietary olive oil, and both unaltered monomers and oxidized monomers could be affected by interference with endogenous lipids. Therefore, evaluation of these latter groups of compounds required the combined use of radioactivity measurements. Amounts of unaltered monomers and oxidized monomers were finally calculated from the total radioactivity data of non-polar and polar fractions, respectively, and the results previously obtained for the rest of compounds. On the other hand, lipids from faeces of the control group, fed diets containing unaltered [1-^{14}C]linoleic acid, were not analyzed by the complex procedure of adsorption and size-exclusion chromatography as they were evaluated by TLC-linear analyzer, and no radioactivity was found at any other R_F than that corresponding to unaltered fatty acids.

In general, oxidized monomers showed very high digestibilities, and these observations were in good agreement with the results reported for global absorption of oxidized monomer fractions isolated from thermoxidized oils and evaluated by lymph collection or digestibility coefficients [7–9,15]. Also, the results obtained here indicated that only minor modifications could have taken place during re-extraction of faeces. Otherwise, increased amounts of oxidized monomers would have been found and hence digestibility underestimated, whereas high values were obtained. Oxidized monomers refer here to non-volatile compounds including a large variety of monomeric fatty acids with at least one oxygenated function, e.g., hydroxy, hydroperoxy, epoxy, keto, which are major oxidation compounds at low temperature, during storage of oils and fatty foods, and also present in appreciable levels in frying fats [27]. In this context, special emphasis has been placed on the metabolic and toxicological effects of specific oxidized compounds administered individually, such as hydroxy fatty acids, oxo fatty acids or α,β-unsaturated ketones [28–31]. Unfortunately, the techniques available at present do not allow quantitative evaluation and hence determination of specific digestibility values of the multitude of individual compounds present in thermally oxidized fats.

Particularly surprising were the digestibility values obtained for oxidized dimers and polymers, as high as 69.8 and 74.5%, respectively, and in contrast to non-polar dimers, which presented very low values. In the case of dimers, the analytical procedure used in this study allowed comparisons between oxidized and non-polar groups, and the differences found gave evidence of the influence that polarity may have on absorption. In this regard, the literature is confusing since dimers have been reported to show very variable apparent digestibility, between 15% and 70% [32–35], although it should be kept in mind that experimental conditions for the preparation of samples and methods used for the

isolation of dimer fractions varied widely. On the other hand, it is important to remark that the finding of unaltered labelled linoleic acid in faeces suggested that chemical modifications may have occurred prior to absorption, leading to less complex products and hence contributing to increased digestibility of oxidized dimers and polymers. A further interesting step would be the administration of different groups of compounds, i.e., oxidized monomers, dimers, and polymers, in separate diets, but this would require overcoming the limitations of HPLC for isolation of sufficient quantities of each fraction for the preparation of diets.

As an additional indication of the absorbability and fate of the originally oxidized compounds, it is worth commenting that the radioactivity recovered in liver lipids was ca. 3% of the level administered to rats fed thermoxidized [1-^{14}C]linoleic acid, from which around 60% was associated with the unaltered fatty acid fraction. The radioactivity found in mesenteric and perirenal adipose pads was sixfold lower in rats fed thermoxidized [1-^{14}C]linoleic acid than in those fed unaltered [1-^{14}C]linoleic acid, but the bulk of radioactivity, ranging from 82.3% to 92.2%, was likewise recovered in the non-polar triglyceride fraction.

In order to gain some insight into the modifications that dimeric and polymeric structures may undergo prior to absorption, in vitro experiments were conducted to evaluate depolymerization effects. Thus, Fig. 3 illustrates the chemical modifications undergone by thermoxidized methyl linoleate upon treatment with HCl under simulated gastric conditions [36]. Oxidized monomer levels changed from 58.3% in the initial samples, to 70.1%, 72.2%, 69.2% and 75.4%, after the four assays carried out. Overall, depolymerization effects were clearly shown in that polymers decreased 31% while monomers increased 21%, on average. These results indicate that the acidic conditions applied here directly to lipids had much stronger effects than those used for re-extraction of faecal residues with the aim of changing the pH of the faeces.

The importance of these observations is that similar reactions could also occur partly under physiological conditions, prior to absorption. Non-polar dimers are compounds with no extra oxygen apart from that of the carboxylic groups, while oxidized dimers contain extra oxygen either in the dimeric linkage or in the fatty acid chains. Whereas carbon–carbon dimeric linkages are very stable, depolymerization by hydrochloric acid of carbon-to-oxygen linkages is very well known [37,38] and might explain both the presence of non-polar fatty acids in faeces and the high digestibility of oxidized dimers and polymers. Clearly, more research is needed on this point in order to obtain knowledge on the complex digestion and absorption processes of oxidized and polymeric compounds, with the aid of novel methodology directed to improve separation and quantitation of such alteration compounds in dietary sources and biological samples.

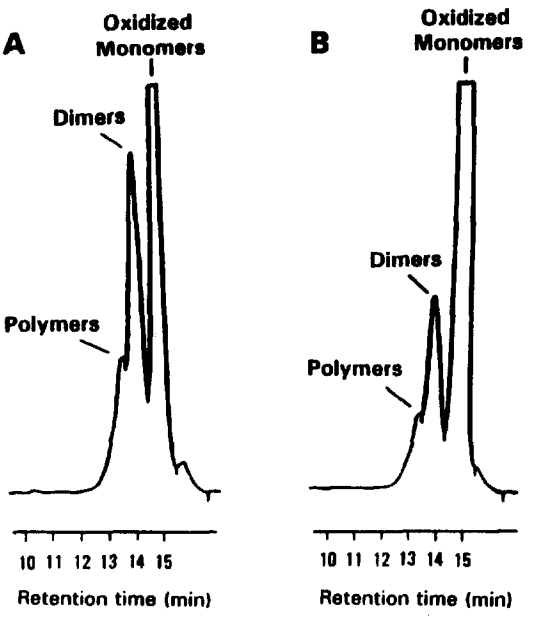

Fig. 3. High-performance size exclusion chromatograms of methyl linoleate before (A) and after (B) depolymerization with HCl.

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