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Short Communication

Isolation and quantification of polymers from autoxidized fish oils by high-performance size-exclusion chromatography with an evaporative mass detector

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ABSTRACT

Triacylglycerol polymers from autoxidized fish oils were isolated and quantified by size-exclusion chromatography with an evaporative mass detector. Optimum chromatographic conditions were obtained using dichloromethane as the mobile phase and three styrene–divinylbenzene columns coupled in series. The results showed a lack of correlation between polymer content and standard methods for oxidative rancidity assessment. The polymer content was *ca.* 1% in the starting oils, but the value rapidly increased during autoxidation. Depending on the level of oxidation, a mixture of dimer, trimer and higher polymeric triacylglycerols are formed, the dimers predominating when the total polymer content is less than 10%.

INTRODUCTION

Recently there has been increased interest in the polymeric triacylglycerols formed during the autoxidation of highly unsaturated fats and oils and their influence on the quality of marine dietary lipids [1–3]. However, it is not yet known to what extent high-molecular-weight compounds contribute to the possible deleterious effects of oxidized lipids in nutrition. This is partly due to the difficulties encountered during the analysis of these polymers, caused by the complexity of the triacylglycerols present in marine oils.

Among various methods used for the determination of oligomeric and polymeric materials from oxidized lipids, size-exclusion chromatography (SEC) seems to be the most promising. The most successful approach is to use high-performance liquid chromatographic (HPLC) methods with columns packed with macroporous styrene–divinylbenzene copolymers, and such techniques have been used for the sep-

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aration of high-molecular-weight compounds from oxidized fatty acids [4–7] and vegetable oils [8–11].

We have previously reported a simple method for the analysis of polymeric triacylglycerols from autoxidized fish oils [12]. The method is based on high-performance SEC with a chromatographic system consisting of a single pump, a 500 Å styrene–divinylbenzene column and an evaporative mass detector. In this study, a more advanced method using SEC columns with styrene–divinylbenzene copolymers of different pore sizes coupled in series was developed. The method is useful for the separation of oligomeric and polymeric triacylglycerols from autoxidized oils and can also be applied to the separation of partial triacylglycerols and non-esterified fatty acids from triacylglycerols.

EXPERIMENTAL

Cod liver oil (CLO) was obtained from Peter Møller (Oslo, Norway), capelin oil (CO) from J. C. Martens (Bergen, Norway) and CPL (chromatographically purified lipid) Fish Oil 30 from Karlshamns (Karlshamn, Sweden). Antioxidants were not added to the oils. All chemicals used were of analytical-reagent grade and were supplied by Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany), except the solvents, which were of HPLC grade from Rathburn Chemicals (Walkerburn, U.K.). Polystyrene standards (MW 2510, 4080 and 7030) were supplied by Millipore (Milford, MA, U.S.A.).

Analysis of polymers was carried out using an HPLC system consisting of a Waters Model 501 pump with a U6K injector and an Applied Chromatography Systems Model 750/14 evaporative mass (light-scattering) detector. The mass detector was operated with the internal air pressure adjusted to 24 p.s.i. and the evaporator set at 20 (arbitrary figure). The analyses were performed using Waters Ultrastyragel 500 Å and 1000 Å SEC columns (both 30 cm × 7.8 mm I.D., particle size 10 μm). Dichloromethane (0.8 ml/min) was employed as the mobile phase. The sample concentration was 10–50 mg/ml in the mobile phase and the injection volume was 40 μl. Glycerol was used as an internal standard and peak integration was carried out using a Shimadzu C-R6A integrator.

The method for the analysis of methyl esters of fatty acids has been described previously [12]. Peroxide values (PV) were measured by iodometric titration [13], thiobarbituric acid (TBA) values were determined as described by Ke and Woyewoda [14] and anisidine values (AV) were measured according to IUPAC method 2.504 [15].

The rancification experiments were performed at 35°C with irradiation from an artificial daylight fluorescent tube (1.1×10^{19} Q $m^{-2}s^{-1}$). The oils (40 g) were oxidized in open beakers (4.8 cm I.D.) with stirring.

RESULTS AND DISCUSSION

In order to determine the optimum chromatographic conditions for polymer separation, a number of exploratory experiments were carried out using cod liver oils (CLOs) of different oxidative rancidity as test materials. The separation of polymeric triacylglycerols was hardly affected by replacing the 500 Å (effective molecular weight

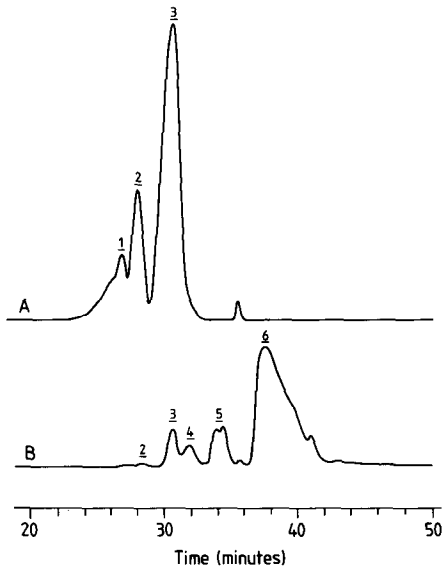

Fig. 1. SEC analyses of cod liver oil using the 500 Å + 500 Å + 1000 Å column system with dichloromethane (0.8 ml/min) as mobile phase. (A) Cod liver oil containing 25% polymers; (B) hydrolysed cod liver oil. Peaks: 1 = trimers and higher polymers; 2 = dimers; 3 = triacylglycerols; 4 = diacylglycerols; 5 = monoacylglycerols; 6 = fatty acids.

range 100–10 000) with the 1000 Å styrene–divinylbenzene column (effective molecular weight range 200–30 000). However, the separation was greatly improved by coupling columns in series, and the results clearly demonstrate that three columns in series (500 Å + 500 Å + 1000 Å) are necessary for baseline separation of oligomeric and polymeric material from triacylglycerols in highly oxidized samples (Fig. 1). The total time of analysis varied from 15 to 45 min depending on the number of columns used.

Although the mobile phase normally affects separation by SEC to only a minor extent, we found that a good separation was dependent on the solvent chosen. Dichloromethane, chloroform, tetrahydrofuran and toluene were tested, all having different solvent polarity indices and solvent strength parameters [16]. Toluene was completely unsuitable as a solvent whereas the others, giving slightly different separations, could be used, indicating the need for a mobile phase of medium polarity (Fig. 2). Dichloromethane was finally chosen as the solvent when the separation between triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids was also taken into consideration.

Although the most widely used detector in SEC is the refractive index detector, the evaporative mass detector was chosen owing to its slightly superior response for different polymers [17]. In order to achieve reliable results, optimization of the mass detector with respect to aerosol flow and temperature is very critical [12,18,19]. The temperature setting is the most important operating parameter, but unfortunately affects the different compounds to different extents. An increase in the evaporator temperature was followed by an enhanced polymer response whereas the response of

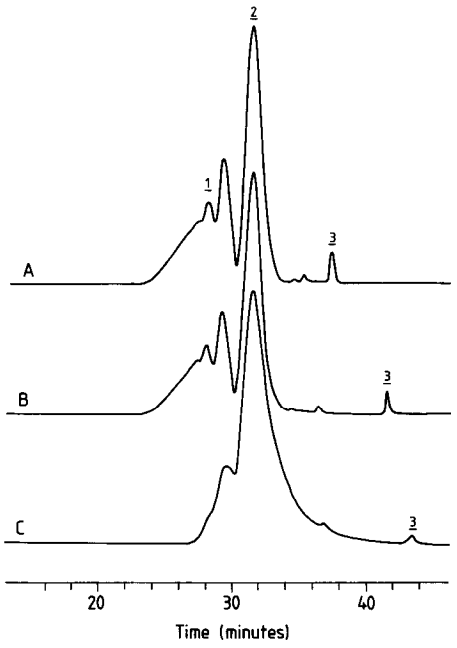

Fig. 2. SEC analysis of cod liver oil containing 18% polymers using the 500 Å + 500 Å + 1000 Å column system and different mobile phases at 0.8 ml/min. (A) Tetrahydrofuran; (B) dichloromethane; (C) toluene. Peak: 1 = polymers; 2 = triacylglycerols; 3 = glycerol.

the internal standard (glycerol) decreased. The triacylglycerol response was not influenced by the temperature setting in the normal working range.

Injection of 2.6–20.8 μg of the polymeric material gave a 20-fold instead of the expected 8-fold area increase. In addition, no linear response relationship between glycerol and polymeric material was observed. Quantification was therefore performed using a constant amount of internal standard added to the samples, and the ratio observed was then corrected according to a curved calibration graph giving the response–weight relationship between the polymers and internal standard [12]. There

TABLE I

CONTENTS OF POLYUNSATURATED FATTY ACIDS (PUFA), PEROXIDE VALUE (PV), ANISIDINE VALUE (AV), THIOBARBITURIC ACID (TBA) VALUE AND THE AMOUNT OF POLYMERS IN STARTING OILS

Oil[a]	PUFA (%)	PV (mequiv./kg)	AV	TBA (μmol/g)	Polymers (%)
CLO	33	4.3	23	0.7	1.3
CO	22	4.1	10	0.4	0.9
CPL	38	3.0	2	0.3	1.0

[a] CLO = Cod liver oil; CO = capelin oil; CPL = chromatographically purified fish oil.

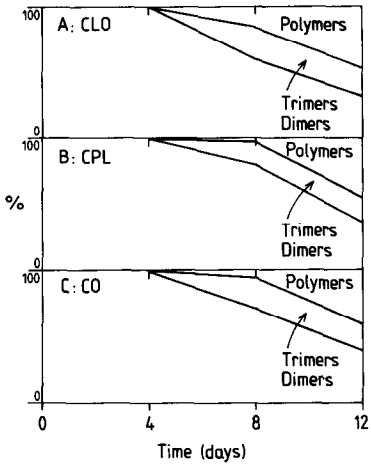

Fig. 3. Distribution of dimers, trimers and higher polymers in autoxidized fish oils. (A) Cod liver oil; (B) chromatographically purified fish oil; (C) capelin oil.

may be several reasons for this non-linear behaviour, including aerosol particle size, refractive index and opacity of the particles [20,21]. A further complication was the finding that different calibration graphs had to be prepared, depending on the number of columns connected in series and the mobile phase used.

The amount of polymers in CLO, capelin oil (CO) and chromatographically purified fish oil (CPL) was found to be *ca.* 1% (Table I). For comparison, the amount of polyunsaturated fatty acids (PUFA) and the standard quality assessment parameters peroxide value (PV), anisidine value (AV) and thiobarbituric acid (TBA) value are also shown. The results show a lack of correlation between polymer content and standard methods for oxidative rancidity assessment. This is an important finding as the polymers seems to be the main oxidation product in autoxidized marine oils [12]. The polymer content increase rapidly during autoxidation, with values as high as 43–48% after 12 days at 35°C. Depending on the level of oxidation, a mixture of dimer, trimer and higher polymeric triacylglycerols is formed (Fig. 3), the dimers predominating when the total polymer content is less than 10%. Peak identification of the triacylglycerols, diacylglycerols, monoacylglycerols and fatty acids was based on authentic samples. The molecular weight determination of the polymers was based on polystyrene standards, and can only be regarded as approximate as the exclusion process is a function of the size and shape of the molecules.

As the past history of the oils was not known, no final conclusions can be drawn regarding the relative stability of the different oils towards autoxidation.

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