Application of High-Performance Size-Exclusion Chromatography to Study the Autoxidation of Unsaturated Triacylglycerols

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A combination of solid-phase extraction (SPE) and high-performance size-exclusion chromatography (HPSEC) was used to study the autoxidation of triacylglycerol (TAG) mixtures separated from low-erucic acid rapeseed oil and butter oil. The samples were autoxidized in the dark at 40°C for four weeks. The polar compounds of the autoxidated samples were separated by SPE (NH_2 stationary phase), and the polar fraction was further characterized by HPSEC with a series of three size-exclusion columns and an evaporative light-scattering detector. The polar fraction contained TAG polymers, polar TAG monomers (PTAG) and diacylglycerols. Peroxide values and anisidine values of the samples were also measured. By using three different types of TAG mixtures, it could be demonstrated that the PTAG content of the TAGs increases during autoxidation. A slight increase was also detected in polymer content. The correlation between PTAG content and the comparative measurements was considered significant. The results indicate that the measurement of PTAG and polymeric material content by HPSEC analysis can be used when studying the autoxidation level of edible oils and in characterizing the autoxidation products of different molecular sizes.

KEY WORDS: Autoxidation, edible oils, high-performance size-exclusion chromatography, solid-phase extraction.

High-performance size-exclusion chromatography (HPSEC) is one of the high-performance liquid chromatography (HPLC) methods introduced in the field of autoxidation analysis. A wide range of HPSEC applications has been published in recent years. HPSEC has been used to analyze the polymerization level of frying oils and fish oils in particular, although applications in the analysis of other alteration products have also been presented.

Dimeric and oligomeric compounds have been analyzed as fatty acid methyl ester derivatives with HPSEC by several research groups (1–4). With this type of analysis, Márquez-Ruíz and co-workers (4) separated four groups of degradation products in frying oils. These were nonpolar fatty acid dimers, oxidized fatty acid monomers, polar fatty acid dimers and fatty acid polymers.

Several published HPSEC methods are suitable to analyze the polymerization level of oil samples without pretreatment (5–12). A collaborative study and a standardized method for this type of analysis have been presented by Wolff *et al.* (13). The authors concluded that this procedure facilitated the rapid determination of polymer content equal to or higher than 3% (w/w) of the total amount of fat.

By combining adsorption chromatography and HPSEC analysis, it is possible to evaluate the quality of oils by quantitating the different groups of polar alteration products. Previous reports have been published on the quantitation of alteration products, such as polymerized material, oxidized triacylglycerols (TAGs), diacylglycerols (DAGs) and free fatty acids. The method has been applied to both frying oils (14) and nonheated samples (14,15). The polar fraction of the samples is separated by the official International Union of Pure and Applied Chemistry (IUPAC) method (6,14,15) or with solid-phase extraction (SPE) cartridges (16).

Although several types of HPSEC methods have been presented, their application in the study of autoxidation at low temperatures has been limited. Moreover, there is limited comparative data for these and other analytical methods used for measuring the autoxidation level of oils and fats. In this study, we examined the possibility of using a combination of SPE and HPSEC analyses when evaluating the autoxidation of edible oils. The method was tested with chromatographically purified TAG mixtures from low-erucic acid rapeseed (RSO) and butter oils (BO) as different types of test materials. Our interest was focused on the contents of the minor glyceridic compounds formed during the autoxidation of TAGs at low temperature (40°C). Anisidine value (AnV) and peroxide value (PV) were used as comparative measurements.

EXPERIMENTAL PROCEDURES

Preparation of TAG mixtures. The TAG fractions were purified from low-erucic acid RSO (received from the Raisio Group, Raisio, Finland) and BO (received from Valio, The Finnish Cooperative Dairies Association, Helsinki, Finland) by means of the chromatographic method described by Lampi *et al.* (17). A pure RSO TAG fraction and two different types of RSO–BO TAG mixtures (75:25 and 50:50) were used as test materials. The chromatographic separation was performed separately for each of the experiments. Trace amounts of γ-tocopherol (10–40 μg/g) were left in the RSO TAG mixtures after chromatographic purification. Moreover, being nonpolar compounds, the sterol esters could not be removed with the multilayer chromatographic method applied.

Autoxidation of TAGs. Eight separate experiments were performed. The autoxidation of the RSO TAG fractions was studied in four experiments, and two autoxidation experiments were carried out for both RSO–BO TAG mixtures. The TAG mixtures were autoxidized in ampules, each containing 5.0 g of the mixture. The ampules were placed in closed Erlenmeyer flasks and kept in the dark at 40°C for up to four weeks. Samples for PV, AnV and HPSEC analysis were taken at regular intervals.

PV and AnV analyses. PV (PV: British Standards Institution BS 684:1976) and AnV (AnV: ISO 6885:1981) were used as comparative measurements.

SPE. The SPE procedure was performed as described in a previous study (18). The SPE columns used were NH_2 columns (Bond Elut 500 mg, Analytichem International,

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Harbor City, CA), with 50–60 mg of sample per SPE column. Separation of the samples was done in duplicate and checked by thin-layer chromatography (Kieselgel 60; Merck, Darmstadt, Germany). If nonpolar TAGs were detected in the polar fraction after SPE separation, the samples were evaporated, dissolved in 2.5 mL of hexane/diethyl ether (90:10), and the SPE separation was repeated with only 7.5 mL of the nonpolar solvent. The lower level of nonpolar eluent was enough to remove the traces of nonpolar material.

HPSEC analysis. The HPLC system was a Waters 6000A HPLC pump (Waters, Milford, MA) equipped with a Rheodyne injector (Model 7125) with a 50-μL loop. A light-scattering detector (Cunow DDL21, Cunow Department DMS, Gergy, St. Christophe, France) was used. The temperature of the evaporation funnel was 54°C, and the pressure of the filtered compressed air was 1.0 bar. The size-exclusion column series included one 100 Å and two 50 Å columns (PLGEL, 30 × 0.8 cm i.d.; Polymer Laboratories Inc., Amherst, MA). The mobile phase was HPLC-grade tetrahydrofuran (Rathburn Chemicals Limited, Walkerburn, Scotland, United Kingdom). Butylated hydroxytoluene (0.025%) was used as a stabilizer, and the solvent was kept in an atmosphere of helium to avoid peroxide formation. The flow rate was 0.6 mL/min at ambient temperature. The integrator was a Hewlett-Packard model 3390A (Palo Alto, CA). The polymeric material, polar TAG (PTAG) and DAG contents were determined. The quantitation of the HPSEC fractions was performed with triolein (polymers and PTAG) and diolein (DAG) as external standards. The optimization of the detector parameters and the HPSEC analysis have been described previously (18).

RESULTS AND DISCUSSION

HPSEC analysis of the autoxidized TAGs. Typical examples of the HPSEC chromatograms obtained in the experiments are shown in Figure 1 and include the PV and AnV. Chromatogram 1 represents the polar-lipid fraction of an RSO sample before chromatographic purification, marking the baseline separation of the polymeric material, PTAGs and DAGs. In our earlier study (18), the HPSEC chromatography of different lipid standards was presented, and demonstrated that the major nonsaponifiable lipid classes are separated from the alteration products of interest. A small amount of polymeric material was found in the RSO, and some PTAGs and DAGs were also present. Immediately after purification (chromatogram 2), the polymeric compounds and traces of DAGs are still present in the polar fraction of the RSO TAG mixture. Chromatographic purification removed the PTAGs, most of the DAGs and free sterols. The PV decreased from 0.2 to 0 and the AnV from 2.9 to 0. As nonpolar compounds, the sterol esters remained, but were not registered in this analysis as they were contained in the nonpolar SPE fraction. Chromatogram 3 represents the polar lipids of an RSO TAG mixture after ten days of autoxidation. The amount of PTAGs has increased, as well as the PV and AnV. After four weeks' autoxidation (Chromatogram 4; PV 65 and AnV 10), a significant increase in PTAG content is seen, as well as a slight increase in polymer content.

Quantitation of the HPSEC fractions. The HPSEC fractions of the autoxidized lipid material are heterogeneous mixtures of partly unknown composition. Therefore, quantitation is problematic due to the differences between the response factors of the compounds studied. The evaporative light-scattering detector (ELSD) response is reported to be universal and is thus more reliable for quantitative work than refractive index detector (19,20). Stolyhwo (19) concluded that ELSD response per mass

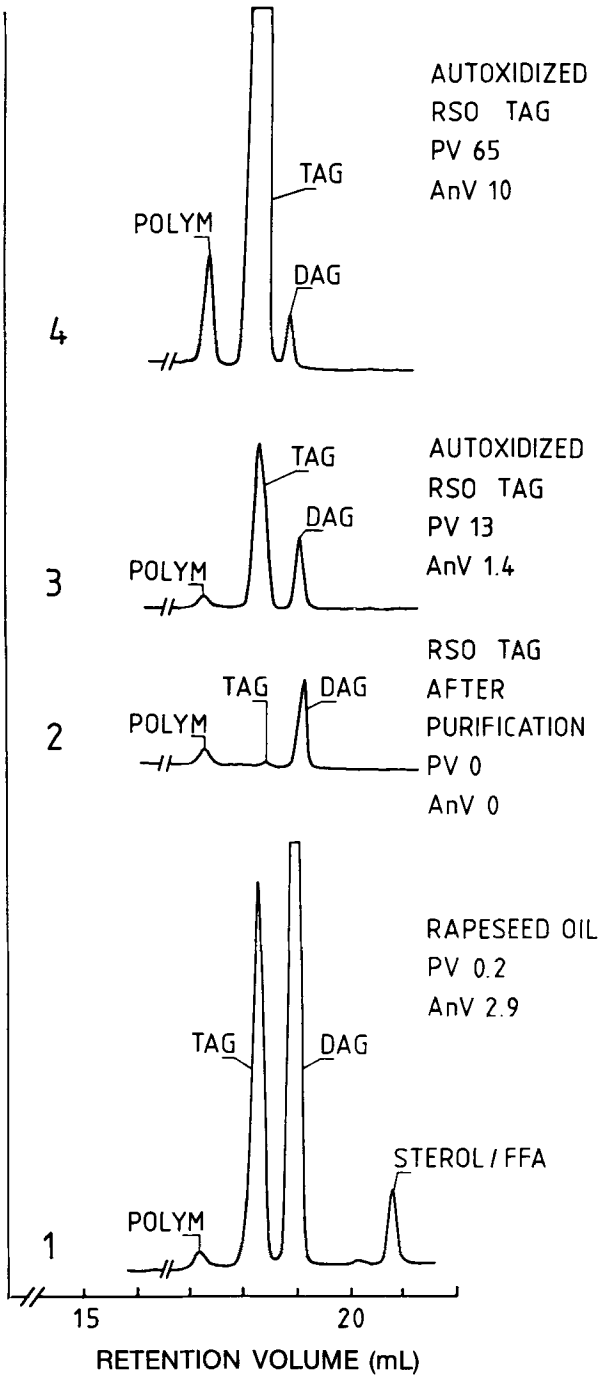

FIG. 1. High-performance size-exclusion chromatography chromatograms of the polar solid-phase (SPE) fractions. 1, Rapeseed oil (RSO); 2, RSO triacylglycerol (TAG) fraction before autoxidation; 3, RSO TAG after ten days of autoxidation; 4, RSO TAG after four weeks of autoxidation. Initial sample size before SPE separation, 50–60 mg/5 mL. Injection volume, 10–40 μL; PV, peroxide value; AnV, anisidine value; DAG, diacylglycerol; Polym, polymer.

unit is approximately constant for compounds that belong to a given chemical group and that condense as liquids. In our previous study (18), we reported that the relative standard deviation of the TAGs tested was 9%, that of DAGs, 13% and that of monoacylglycerols, 11%. In this study triolein was used as external standard for quantitation of the PTAG and TAG polymeric fractions, and diolein for the DAG fraction. Due to the heterogeneity of the HPSEC fractions, a standard deviation of 9–13% is to be expected for the quantitative results.

Autoxidation of RSO TAG. Figure 2 shows the results of the four RSO TAG experiments. The PV, AnV, PTAG contents and polymer content are shown. The results are mean values of two analyses, where the relative standard deviation of PV, AnV and PTAG results are below 5% and that of polymer analysis below 10%. After four weeks' autoxidation, the PTAG content of the samples varied from 14 to 30 mg/g (1.4–3.0 wt%), and the polymer content from 0.5 to 4 mg/g (0.05–0.4 wt%). The interesting thing about the figures is that the PTAG contents of the four experiments clearly follows the pattern of the PV and AnV values. The higher the PV and AnV values, the higher the PTAG content of the samples. The autoxidation rates of the different RSO experiments varied slightly. The effect of the trace amounts of γ-tocopherol was marked and has been reported earlier (21).

Autoxidation of RSO-BO TAG mixtures. The results of the RSO-BO mixtures (75:25 and 50:50) are shown in Figures 3 and 4. They are similar to those of the RSO experiments—the samples with the highest PV and AnV values also had the highest PTAG contents. The total autoxidation rate was decreased compared to that of the pure RSO TAGs. The effect of the low levels of γ-tocopherol was also obvious in these experiments: the higher the γ-tocopherol content left in the material, the lower the total autoxidation rate.

Comparison of the measurements. Figure 5 compares the different measurement data. All eight experiments are included in the calculations. The figure shows PTAG content *vs.* PV, the PTAG content *vs.* AnV, the polymer content *vs.* PV and, finally, the polymer content *vs.* AnV.

For the material studied, the positive correlation between PTAG content and PV and between PTAG content and AnV is markedly strong, the correlation coefficients being 0.96 and 0.97, respectively. The correlation between the measurements is even better when the three materials are focused separately. The correlation coefficients for the three different test materials are shown in Table 1.

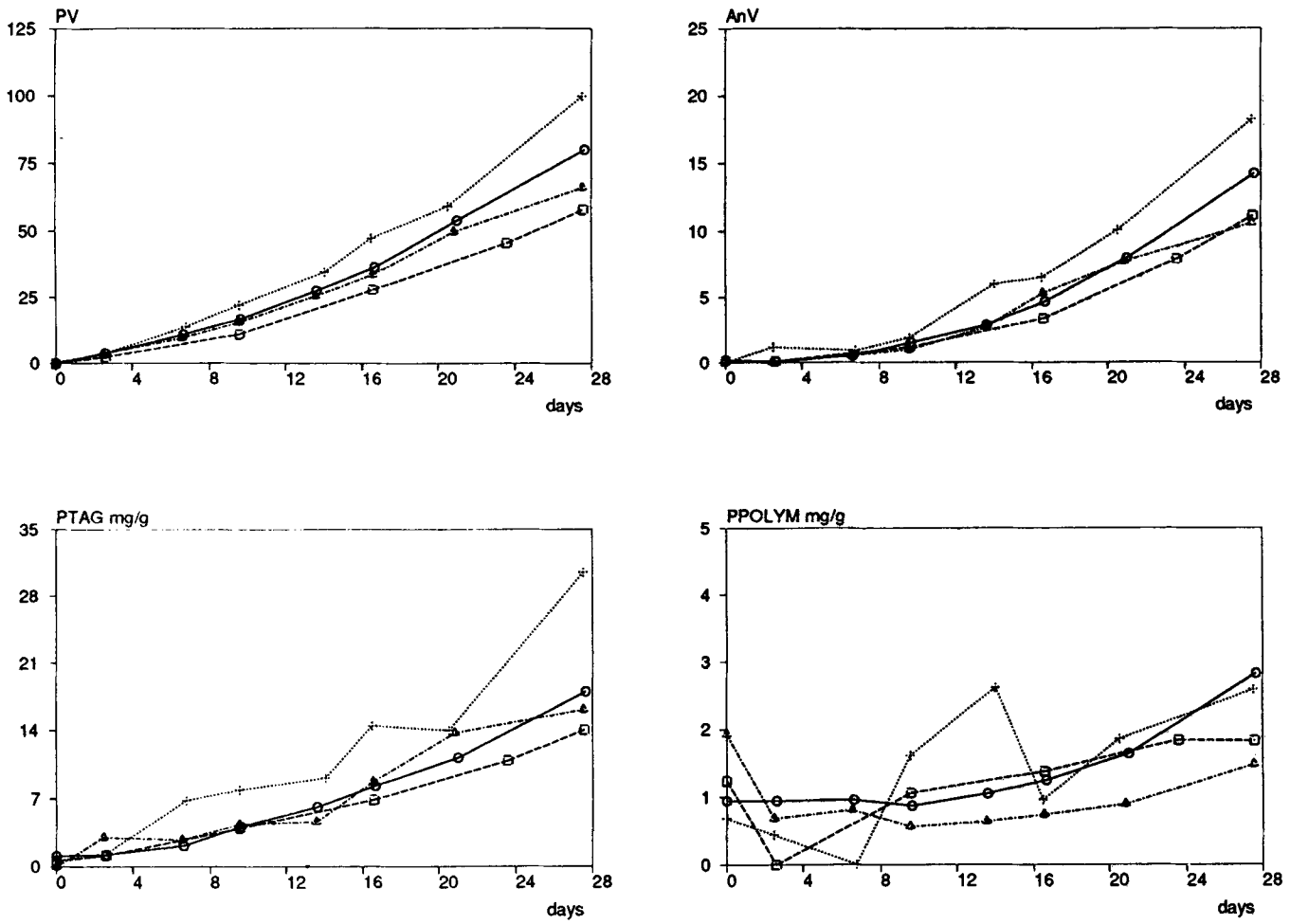

FIG. 2. Autoxidation of RSO TAG experiments. Abbreviations as in Figure 1. PPOLYM, polar polymeric material; PTAG, polar triacylglycerols. + = Experiment 1, □ = Experiment 2, ○ = Experiment 3, △ = Experiment 4.

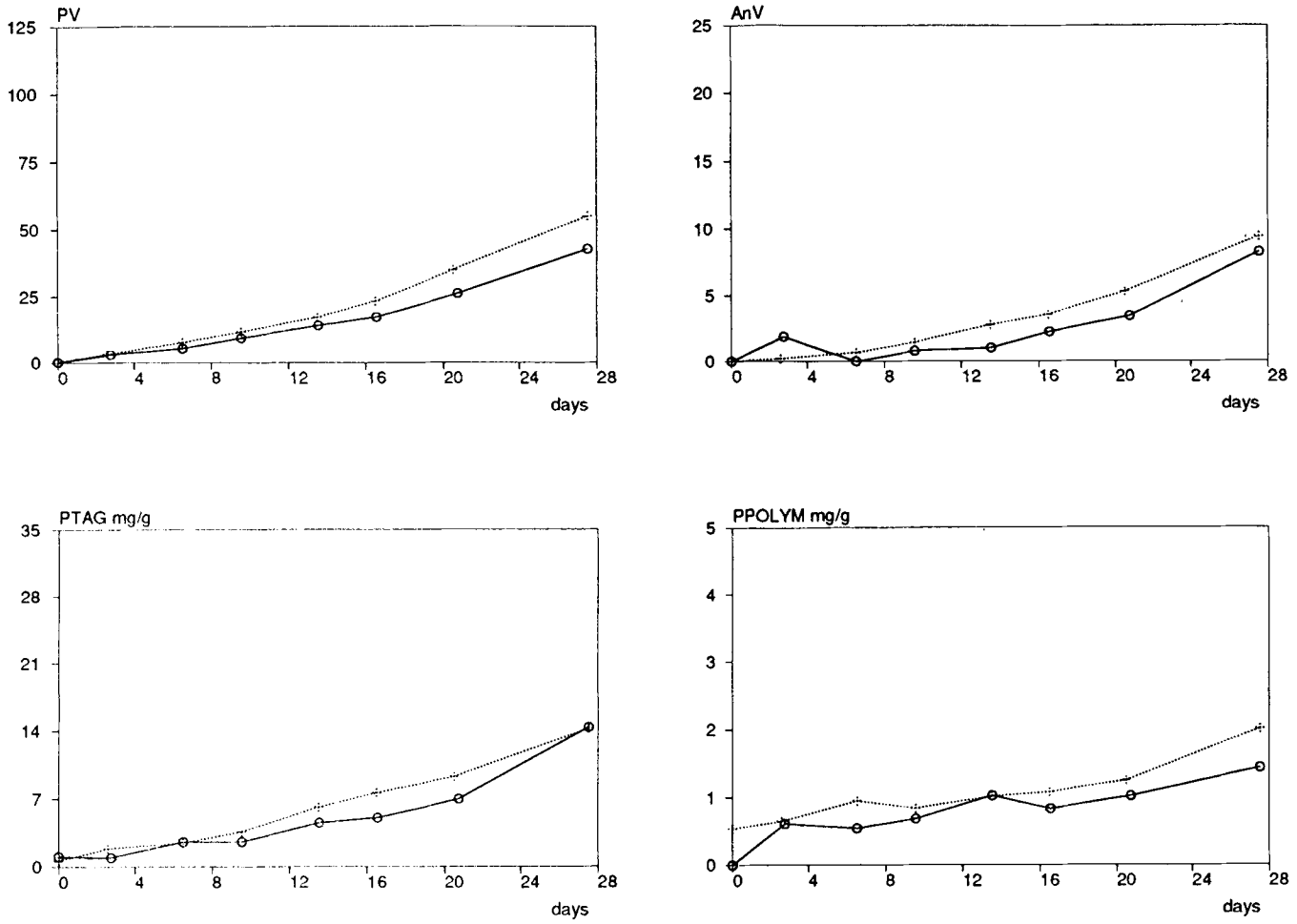

FIG. 3. Autoxidation of RSO–butter oil (75:25) TAG experiments. Abbreviations as in Figures 1 and 2.

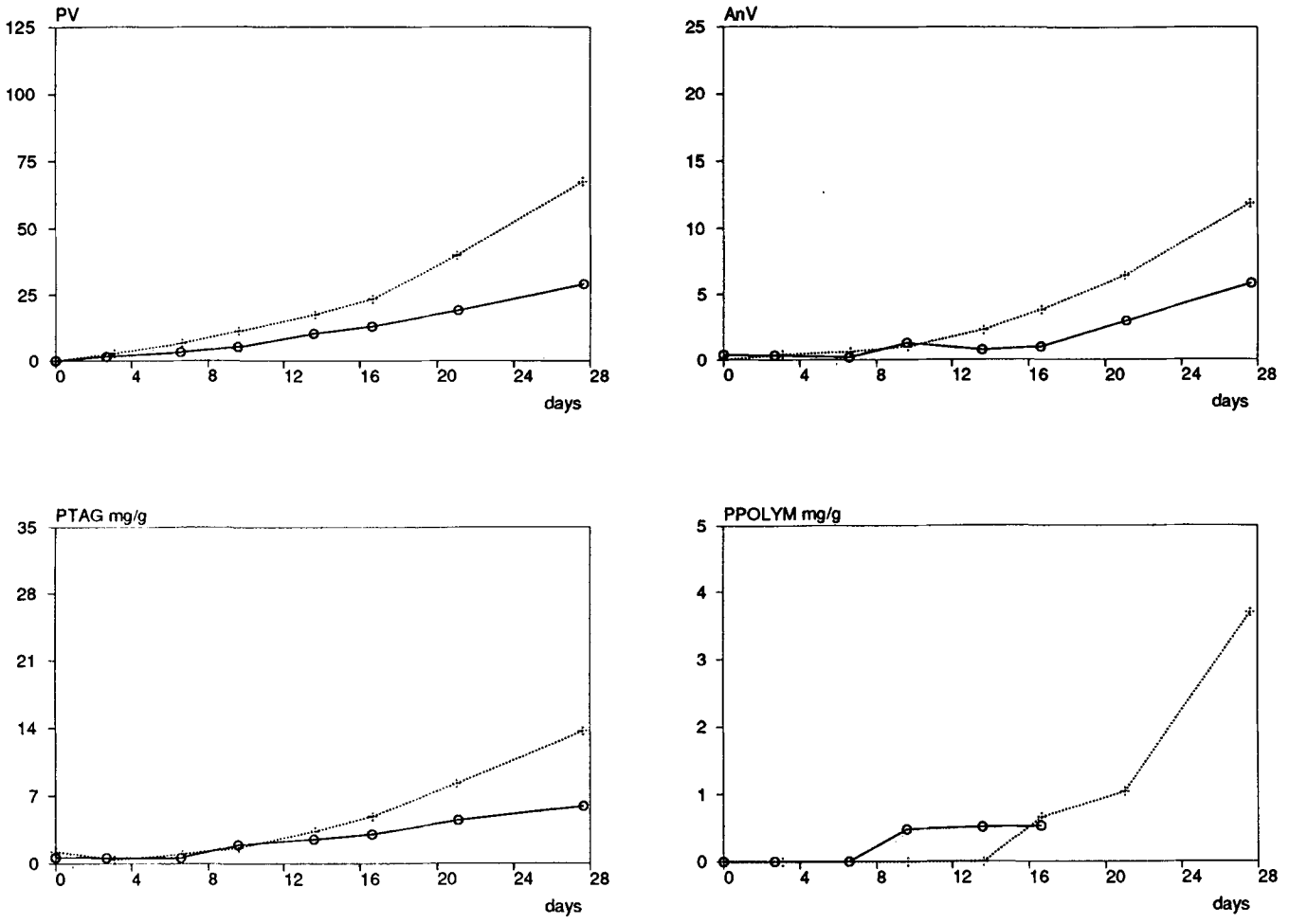

FIG. 4. Autoxidation of RSO-butter oil (50:50) TAG experiments. Abbreviations as in Figures 1 and 2.

Evaluation of the HPSEC analysis. In this study, the HPSEC method was applied in the analysis of minor glyceridic compounds that form during autoxidation. The autoxidation of the test materials can be monitored by measuring the PTAGs and polymer contents of the samples. There was a strong correlation between the PTAG content and the PV/AnV measurements for three different types of TAG mixtures as test materials. The results indicate that HPSEC analysis can be used to monitor autoxidation levels and to characterize autoxidation products of different molecular sizes.

The PTAG fraction contains the oxidized TAGs in the sample. These compounds are formed during autoxidation *via* the reaction of unsaturated lipids and atmospheric oxygen, which increases the polarity of the TAG molecule. Dobarganes *et al.* (15) measured the oxidized TAGs in crude and in refined oils and reported that the level of oxidized TAGs in the samples is an indicator of the total oxidation compounds. The level of oxidized TAGs is of special interest because they remain in edible oils after they are refined (14,15).

HPSEC analysis of polymerized TAGs has been described previously by many researchers (1-16). The fractions eluting before the TAG monomer peak in the HPSEC chromatogram have been identified as total polymerized material or as dimeric, trimeric and higher

TABLE 1

Correlation Coefficients of the Analytical Methods with Three Different Test Materials[a]

Test material	Number of experiments	Samples/experiment	Correlation coefficients			
			PTAG/PV	PTAG/AnV	POLYM/PV	POLYM/AnV
RSO TAG	4	8	0.9707	0.9608	0.6785	0.7209
RSO-BO TAG (75:25)	2	8	0.9817	0.9670	0.9220	0.8839
RSO-BO TAG (50:50)	2	8	0.9914	0.9832	0.9233	0.9420
All experiments	8	8	0.9678	0.9546	0.7457	0.7587

[a]PTAG, polar triacylglycerols; PV, peroxide value; AnV, anisidine value; POLYM, polymer; RSO, rapeseed oil; TAG, triacylglycerols, BO, butter oil.

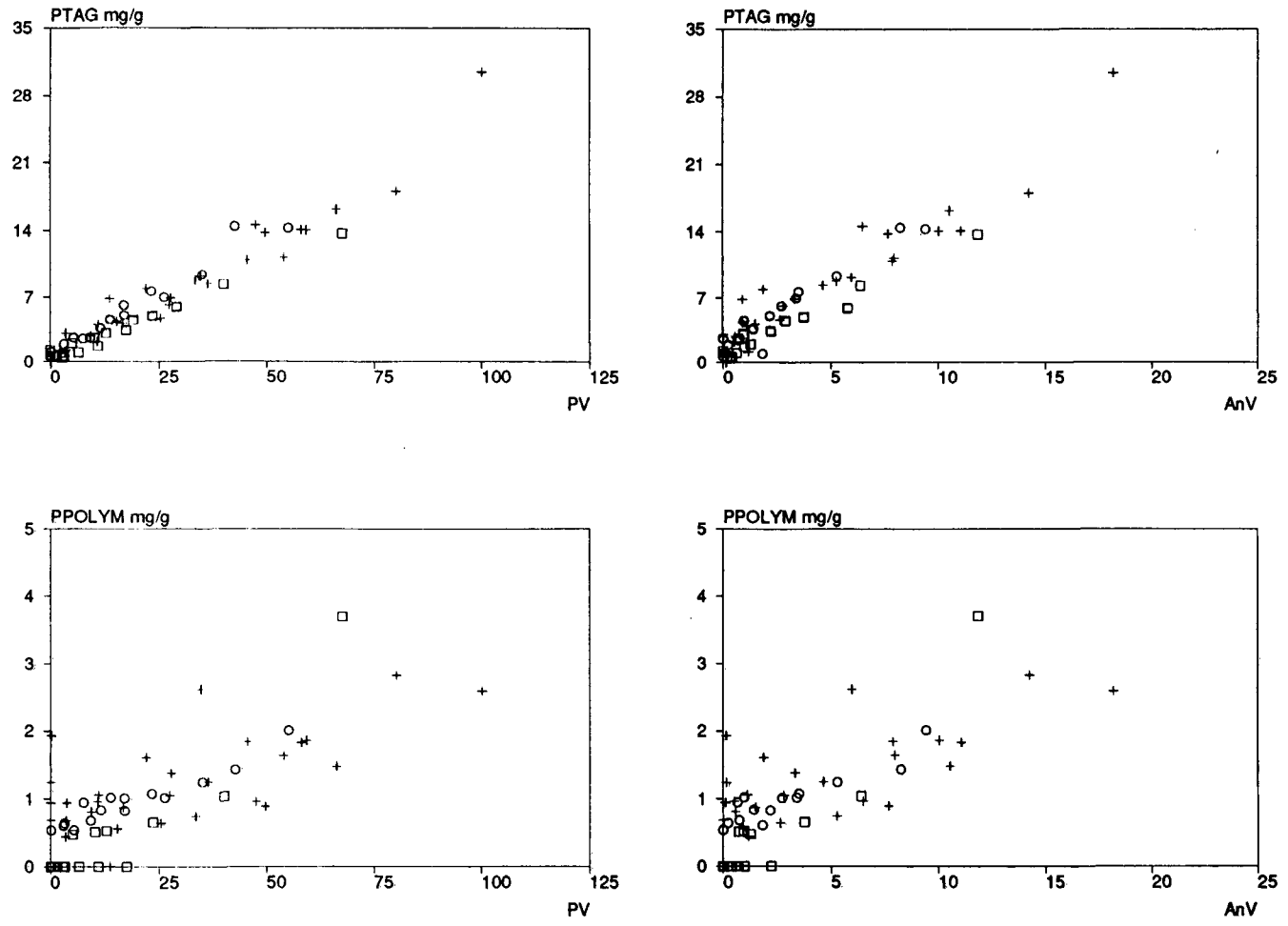

FIG. 5. Comparison of the measurements. + = RSO TAG mixtures, ○ = RSO-butter oil (BO) (75:25) TAG mixtures, □ = RSO-BO (50:50) TAG mixtures. Abbreviations as in Figures 1 and 2. PTAG, TAG monomers.

oligomeric materials if several peaks are distinguishable before the TAG monomer peak.

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[Received November 23, 1992; accepted May 9, 1993]