# Analysis of Triacylglyceride Hydroperoxides in Vegetable Oils by Nonaqueous Reversed-Phase High-Performance Liquid Chromatography with Ultraviolet Detection

**Christina Bauer-Plank* and Lisette Steenhorst-Slikkerveer**

Unilever Research Vlaardingen, 3130 AC Vlaardingen, The Netherlands

**ABSTRACT:** Triacylglyceride hydroperoxides (HPO-TAG), the primary autoxidation products of triacylglycerides (TAG), have been analyzed in polyunsaturated vegetable oils by means of nonaqueous reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet detection. Using a retention time model based on equivalent carbon numbers, mono- and bishydroperoxy TAG and hydroxy TAG could be identified. The correlation between the peroxide value (POV) determined by iodometric titration and quantitative HPLC results for HPO-TAG was established for sunflower oil samples with POV between 0.5 and 50 meq/kg. The recovery of HPO-TAG in the HPLC procedure was found to be close to 100% in the POV range of 4 to 71 meq/kg. Absolute quantitative results for HPO-TAG in sunflower oil samples could not be obtained accurately, as molar extinction coefficients of HPO-TAG occurring in natural oils deviate from those of available HPO-TAG reference compounds.

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**KEY WORDS:** Autoxidation, nonaqueous reversed-phase HPLC, sunflower oil, triacylglyceride hydroperoxides, triacylglycerols.

The oxidation of polyunsaturated lipids has received great attention due to increased consumption of vegetable oils with high unsaturation levels. Polyunsaturated lipids are oxidatively more labile than saturated and monounsaturated lipids and undergo autoxidation and photooxidation easily in the presence of initiators. Following a free radical chain mechanism, triacylglyceride hydroperoxides (HPO-TAG) are formed as primary products of triacylglyceride (TAG) oxidation. It is well established that HPO-TAG readily decompose into a wide range of aldehydes, ketones, hydrocarbons, and other volatile compounds which contribute to the flavor deterioration of lipid-containing food.

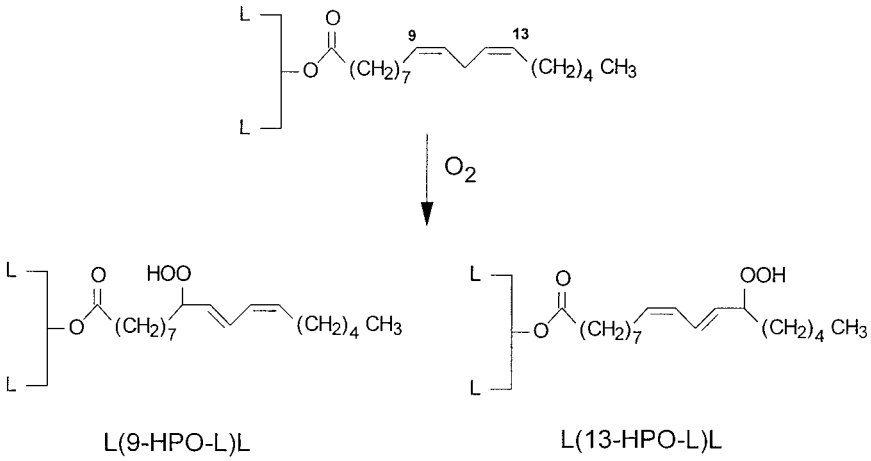
Extensive oxidation experiments with subsequent analysis of the oxidation products using pure fatty acids and TAG as well as real oils have been carried out in order to elucidate the mechanism of lipid oxidation. For oxidation studies of vegetable oil TAG, triolein, trilinolein (LLL), and trilinolenin were frequently used as model systems. The main autoxidation products of triolein and LLL were identified as mono-, bis-, and trishydroperoxides, which are formed by sequential oxygen addition (1,2). The monohydroperoxides of triolein are composed of 8-, 9-, 10-, and 11-isomers (3). Autoxidation of LLL yields a mixture of 9- and 13-hydroperoxides as primary products (2,3; Scheme 1). LLL produces, in addition to mono-, bis-, and trishydroperoxides, substantial amounts of hydroperoxy epidioxides. The monohydroperoxides are composed of 9-, 12-, 13-, and 16-hydroperoxides (3,4). The cyclic peroxides are composed of 9- and 16-hydroperoxy epidioxides (4).

Numerous attempts have been made to analyze hydroperoxides with respect to the position of the hydroperoxy group on the fatty acid chain, the *sn*-position in the TAG molecule, and the number of hydroperoxy groups per TAG molecule or per fatty acid chain (1–5). Park *et al*. (5) compared normal-phase high-performance liquid chromatography (NP-HPLC) and reversed-phase (RP) HPLC for the analysis of autoxidized LLL and some vegetable oils. NP-HPLC of oxidized LLL revealed the geometrical configurations of the 9- or 13-HPO (*c*,*t*) and 9- or 13-HPO (*t*,*t*) HPO-TAG isomers (2,5). NP-HPLC also has been used to determine mono-, bis-, and trishydroperoxides which can be separated according to their polarity (6). RP-HPLC gives much less detailed information about geometrical and positional isomers, but gives a separation of HPO-TAG according to the partition number of the parent TAG (7).

Ultraviolet (UV) absorption is the most frequently employed detection method for HPO-TAG (2,4–6). An absorption band around 235 nm corresponding to the conjugated double bonds, formed by autoxidation of linoleic or linolenic acid, can be used for specific and sensitive detection. However, HPO-TAG deriving from oleic acid cannot be detected at this specific wavelength. Nonspecific and less sensitive detectors, such as a refractive index (RI) detector (2) or an evaporative light-scattering detector (ELSD), can be used instead (7). Song *et al*. (8) employed electrochemical detection with a glassy carbon electrode to monitor HPO-TAG, Miyazawa *et al*. (9) used chemiluminescence for detection of mono-, bis-, and tris-HPO-TAG.

This paper describes the application of a nonaqueous RP-

*To whom correspondence should be addressed at Unilever Research Vlaardingen, Olivier van Noortlaan 120, P.O. Box 114, 3130 AC Vlaardingen, The Netherlands. E-mail: christina.bauer-plank@unilever.com




**SCHEME 1**

HPLC (NARP-HPLC) method for the analysis of HPO-TAG in polyunsaturated vegetable oils. Most of the HPO-TAG occurring in oxidized sunflower oil are identified using a retention time model based on equivalent chain lengths. The correlation of the peroxide value (POV) and the quantitative results obtained by HPLC–UV analysis is established for sunflower oil in the POV range of 0.5 to 50 meq/kg. The recovery of HPO-TAG and the accuracy of absolute quantitative results are investigated.

## EXPERIMENTAL PROCEDURES

LLL, dilinoleoyl-oleoyl-glycerol (LLO), and linoleoyl-dioleoyl-glycerol (LOO) were purchased from Sigma Chemical Co. (St. Louis, MO). The hydroperoxides 1-palmitoyl-2-(13-hydroperoxy-linoleoyl)-3-stearoyl-glycerol [P(13-HPO-L)S] and 1-palmitoyl-2-stearoyl-3-(13-hydroperoxy-linoleoyl)-glycerol [PS(13-HPO-L)] were synthesized in-house. The HPO-TAG were made *via* lipoxygenase from linoleic acid. The linoleic acid was converted into 13-HPO-linoleic acid, which was then protected for the coupling reaction. During the coupling reaction of the 13-HPO-linoleic acid and the desired diacylglyceride, the HPO-TAG was formed. After the synthesis the HPO was deprotected. The purity of these compounds was between 95 and 99% as determined by $^1$H nuclear magnetic resonance (10). Medium-chain TAG (MCT) oil (Ceres Mct, Diät-Speiseöl) was obtained from Union Deutsche Lebensmittel GmbH (Kleve, Germany). Gradient grade methyl *tert*-butyl ether (MTBE), toluene, and *n*-hexane and analytical grade acetonitrile were from Merck (Amsterdam, The Netherlands).

*Isolation of HPO-TAG by solid-phase extraction (SPE).* HPO-TAG were isolated from the TAG matrix by means of SPE on 500 mg amino-modified silica (International Sorbent Technology, Mid Glamorgan, United Kingdom). One milliliter of a solution containing *ca*. 4 mg/mL oxidized triglycerides or 0.5 mg/mL HPO-TAG reference compound was applied to a SPE cartridge preconditioned with 3 mL *n*-hexane. The TAG were eluted with 6 mL of a *n*-hexane/toluene (1:1, vol/vol) mixture. The HPO-TAG were then eluted with 3 mL acetonitrile/MTBE (87.5:12.5, vol/vol). Solvent was removed from the HPO-TAG fraction by a stream of nitrogen at ambient temperature. The HPO-TAG were redissolved in 3 mL of acetonitrile/MTBE (95:5, vol/vol) and subjected to HPLC analysis immediately.

*HPLC analysis of HPO-TAG.* For the HPLC analysis a binary high-pressure gradient HPLC system consisting of a Gilson 305 master pump, a Gilson 306 slave pump, a Gilson 805S manometric module, and a Gilson 811B mixer (Gilson, Villier-le-Bel, France), a Spark Holland Marathon autosampler (Spark Holland, Emmen, The Netherlands), a dual-wavelength UV/VIS detector (UV 2000; Thermo Separation Products, Breda, The Netherlands), an ELSD (Mark II; VAREX Corporation, Rockville, MD) and a chromatographic data aquisition system (Turbochrom 4; PerkinElmer, Gouda, The Netherlands) was used. Gradient elution with acetonitrile (solvent A) and MTBE (solvent B) under the following gradient conditions was employed: 5 min 5% solvent B, in 53 min to 40% solvent B, 1 min 40% solvent B, in 1 min to 5% solvent B, 20 min 5% solvent B. Of the HPO-TAG fraction, 100 µL was injected onto a Sperisorb S5W ODS2 column (200 × 3 mm, dp = 5 µm; Chrompack, Middelburg, The Netherlands) which was operated at a flow rate of 0.4 mL/min. UV detection wavelengths were 210 and 246 nm. The ELSD was operated at a nitrogen pressure of 70 mm and a temperature of 130°C.

*Determination of the POV.* The POV were determined according to the AOCS official method Cd 8b-90 (11).

## RESULTS AND DISCUSSION

*Detection of HPO-TAG.* In order to investigate which detection method was suitable and sensitive enough for the detection of low levels of HPO-TAG in vegetable oils, an HPO-TAG reference compound [P(13-HPO-L)S], oxidized reference TAG, and oxidized sunflower oil were analyzed by

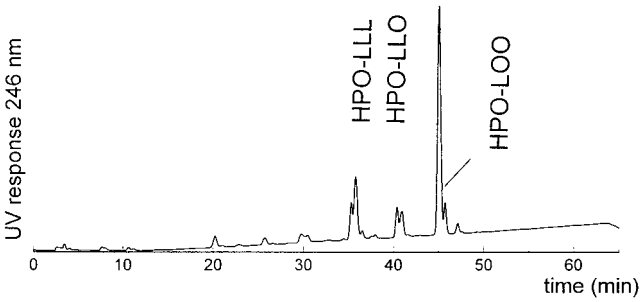



**FIG. 1.** Partition number separation of triacylglyceride (TAG) hydroperoxides obtained by nonaqueous reversed-phase (NARP) high-performance liquid chromatography (HPLC) ultraviolet (UV): HPO-LLL, hydroperoxy-trilinoleoyl-glycerol; HPO-LLO, hydroperoxy-dilinoleoyl-oleoyl-glycerol; HPO-LOO, hydroperoxy-linoleoyl-dioleoyl-glycerol.

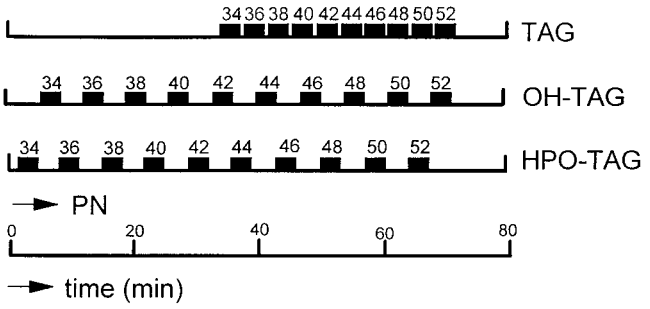



**FIG. 2.** Elution pattern of TAG and oxidized TAG with different partition numbers (PN): TAG, triacylglycerides; OH-TAG, hydroxytriacylglycerides; HPO-TAG, hydroperoxy triacylglycerides.

HPLC with UV detection at 210 and 246 nm and with ELSD. A UV optimum of 246 nm for the detection of HPO-TAG was determined from a full UV spectrum. At this wavelength, HPO-TAG with conjugated double bonds as obtained by autoxidation of linoleic and linolenic acids can be detected at maximal sensitivity. This absorption maximum slightly deviates from the absorption maximum of conjugated dienes as described in (2,4–6), which can be explained by a solvent effect. HPO-TAG derived from monounsaturated fatty acids or lacking conjugated double bonds are not detected at this specific wavelength. UV detection at 210 nm and ELSD detection are unspecific and detect also TAG and HPO-TAG lacking conjugated double bonds at low sensitivity. The sensitive UV detection at the wavelength specific for the analytes (246 nm) was chosen for the analysis of HPO-TAG in slightly autoxidized vegetable oils such as sunflower oil, soybean oil, and safflower oil (Fig. 1).

*Retention behavior of HPO-TAG*. NARP-HPLC was chosen above NP-HPLC, as the well-known elution pattern of TAG-related species in NARP systems allows easy interpretation of the chromatograms. This is of importance for the analysis of real oils, as HPO-TAG reference compounds are not commercially available and only a few types could be synthesized for peak identification and quantification studies.

In NARP-HPLC, the elution sequence of TAG is determined by their partition number (PN), which is defined as the carbon number (CN) minus two times the number of double bonds (DB) (12; Fig. 2). For the TAG LLL, LLO, and LOO with the PN 42, 44, and 46, a difference in PN of 2 resulted in a retention time difference of 7 min under the experimental conditions used.

HPO-TAG differ from TAG in having an additional polar functional group, the hydroperoxy group, which causes a lower retention time in the NARP system. Still, HPO-TAG elute in a sequence following the PN concept with an additional contribution from the HPO group (Fig. 2). The retention time of an HPO-TAG is determined by its equivalent carbon number (ECN), which is defined in Equation 1.

$$ECN = CN - (2 \times DB) - (n \times HPO) \quad [1]$$

where CN represents carbon number, DB the number of double bonds, and ($n \times$ HPO) is the contribution of the HPO group to the polarity of the analyte.

In Figure 1 a NARP separation of the HPO-TAG isolated from oxidized LLL, LLO, and LOO is shown. A schematic elution pattern of TAG and oxidized TAG with different PN is given in Figure 2. This scheme is based on experimentally determined retention times of HPO-TAG isolated from oxidized reference TAG. The term $n \times$ HPO in Equation 1, describing the contribution of the hydroperoxy group to the ECN value, was found to be a function of the PN of the parent TAG. The $n \times$ HPO increases with decreasing PN of the parent TAG (for PN = 48, $n \times$ HPO = 6; for PN = 42, $n \times$ HPO = 10). The contribution of functional groups to the ECN values of the corresponding acylglycerol species increases in the order oxo < OH < HPO.

The analysis of a 1(3)- and a 2-positional isomer of HPO-palmitoyl-linoleoyl-stearoyl glycerol revealed that P(13-HPO-L)S and PS(13-HPO-L) are separated by NARP-HPLC with the 2-isomer [P(13-HPO-L)S] eluting first. A chromatogram of a mixture of the positional isomers is shown in Fig-

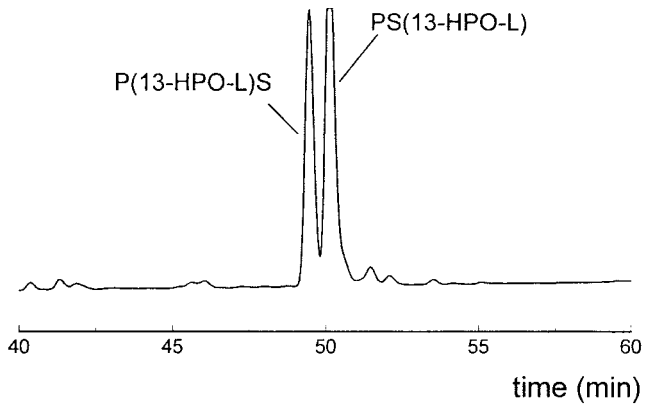



**FIG. 3.** NARP-HPLC separation of positional isomers of triacylglyceride hydroperoxides: P(13-HPO-L)S, 1-palmitoyl-2-(13-hydroperoxy-linoleoyl)-3-stearoyl-glycerol; PS(13-HPO-L), 1-palmitoyl-2-stearoyl-3-(13-hydroperoxy-linoleoyl)-glycerol. See Figure 1 for other abbreviations.

ure 3. The separation of positional isomers results in the fact that for HPO-TAG originating from the same parent TAG, multiple peaks are obtained (Fig. 1).

*Hydroxy TAG and bis-HPO-TAG.* In the HPO-TAG fraction isolated from a mixture of LLL, LLO and LOO, unknown peaks were observed in the chromatogram next to the HPO-LLL, HPO-LLO, and HPO-LOO peaks (Fig. 4). Each HPO-TAG peak group is succeeded by a peak group of much lower intensity which was tentatively assigned as hydroxy TAG (OH-TAG). OH-TAG are secondary products of TAG oxidation and originate from the corresponding HPO-TAG as their precursors. The assignment of the OH-TAG peaks was confirmed by reduction of HPO-TAG to OH-TAG with triphenylphosphine (13).

Prior to the three peak groups of the HPO-TAG in Figure 4, three additional peak groups of much lower intensity can be observed. Assuming that a second hydroperoxy group has a comparable effect on decreasing retention time of the HPO-TAG, one would expect that the retention time will be reduced by 15 min from the parent species. The three peak groups can be assigned as bishydroperoxy-TAG (bis-HPO-TAG).

It can be concluded that with the SPE procedure not only mono-HPO-TAG are isolated from the TAG matrix, but also OH-TAG and higher oxidized TAG, such as bis-HPO-TAG.

*Analysis of sunflower oil samples.* Oxidized sunflower oil samples with POV ranging from 13 to 45 have been analyzed by the procedure outlined. Corresponding chromatograms are shown in Figure 5.

Three groups of HPO-TAG can be observed with retention times corresponding to PN 42, 44, and 46. From the TAG composition of sunflower oil, it can be deduced that these groups correspond to HPO-LLL (PN 42), HPO-LLO, or HPO-LLP (PN = 44), and HPO-LOO or HPO-LOP (PN = 46). The fine structure within the individual groups can be attributed to (i) small differences in retention time of different HPO-TAG of the same PN (e.g., HPO-LLO, HPO-LLP) or (ii) *sn*-positional isomers [e.g., (HPO-L)LL and L(HPO-L)L]. From the relative intensities of the three groups (PN 42 > PN 44 > PN 46), no direct conclusion about the quantitative ratios of the different HPO-TAG can be drawn, as different HPO-TAG can have different response factors.

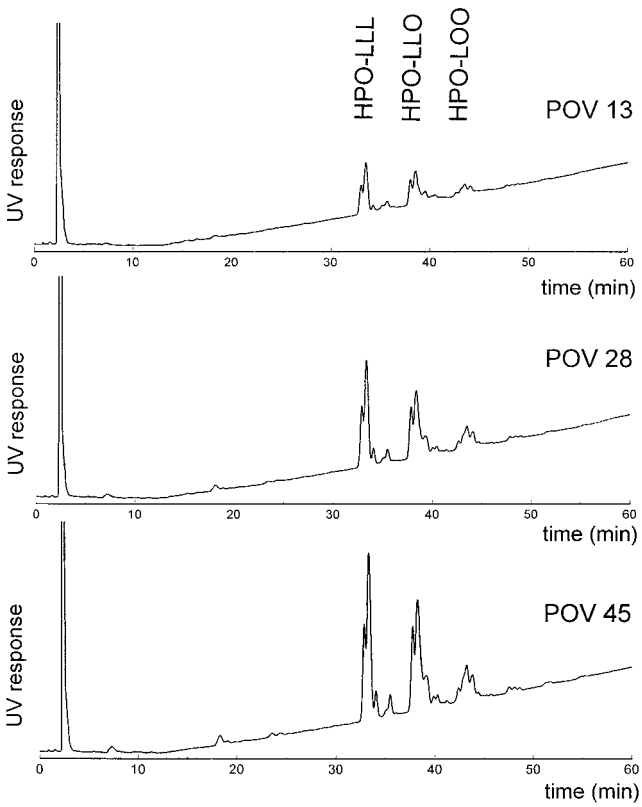



**FIG. 5.** High-performance liquid chromatograms of TAG hydroperoxides from sunflower oils with peroxide values (POV) ranging from 13 to 45. See Figures 1 and 4 for other abbreviations.

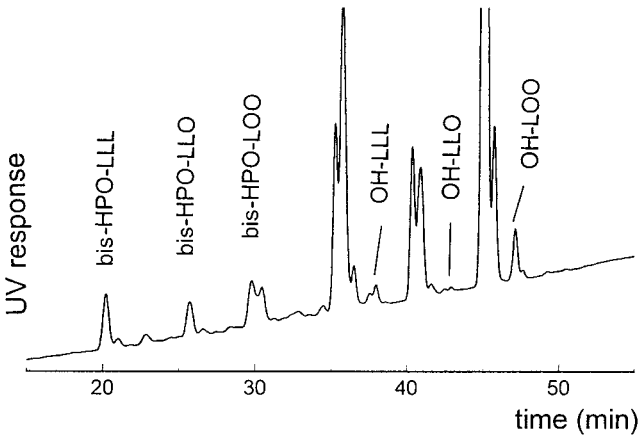



**FIG. 4.** NARP-HPLC of TAG autoxidation products: bis-HPO-LLL, hydroperoxy linoleic-hydroperoxy linoleic-linoleic; bis-HPO-LLO, hydroperoxy linoleic-hydroperoxy linoleic-oleic; bis-HPO-LOO, hydroperoxy linoleic-hydroperoxy oleic-oleic; OH-LLL, hydroxy linoleic-linoleic-linoleic; OH-LLO, hydroxy linoleic-linoleic-oleic; OH-LOO, hydroxy linoleic-oleic-oleic.

*Quantitative analysis.* Quantitative analysis of HPO-TAG was completed using a calibration with the reference compound P(13-HPO-L)S. Solutions with concentrations ranging from 0.003 to 0.08 mg/mL HPO-TAG have been analyzed directly by HPLC-UV without SPE preseparation. The calibration function was found to be linear over the whole tested concentration range [concentration of HPO-TAG (mg/mL) = $3.18 \times 10^{-8}$ × peak area of HPO-TAG (μV × s); $R = 0.999$].

The recovery of the complete analytical procedure (including SPE and HPLC analysis) for HPO-TAG has been checked by analyzing samples of MCT oil spiked with known amounts of P(13-HPO-L)S. The recovery values were calculated from the HPO-TAG concentrations found by HPLC-UV analysis using the calibration function for P(13-HPO-L)S and the actual concentrations. The recovery for P(13-HPO-L)S was found to be close to 100% in the POV range from 4 to 71 meq/kg (Table 1). This means that no HPO-TAG are lost or degraded in the course of the preconcentration steps. The repeatability, which was determined by analyzing sunflower oil

**TABLE 1**
**Recovery of HPO-TAG from the SPE–HPLC–UV Procedure**

| HPO-TAG[a] concentration (%, w/w) | $POV_{calc.}$[b] (meq/kg) | Recovery[c] (%) |
|---|---|---|
| 0.18 | 4.0 | 105.4 ± 14.3 |
| 0.34 | 7.6 | 100.5 ± 0.3 |
| 0.86 | 19.3 | 102.0 ± 1.9 |
| 1.65 | 37.1 | 102.4 ± 0.4 |
| 3.15 | 70.8 | 97.8 ± 0.1 |

[a]HPO-TAG refers to triacylglyceride hydroperoxides.
[b]$POV_{calc.}$ refers to the peroxide value which was calculated from the molar concentration of the TAG hydroperoxides.
[c]The recovery was determined by duplo measurements and is expressed as mean value ± standard deviation. SPE–HPLC–UV, solid-phase extraction–high-performance liquid chromatography–ultraviolet.

seed samples with POV ranging from 2 to 8 meq/kg, was found to be between 1.4 and 4.1% depending on the POV ($n$ = 5). The within-laboratory reproducibility was determined by analyzing an sunflower oil seed sample with a POV of 4.9 meq/kg and found to be 3.5% ($n$ = 5).

*Correlation of HPLC results with POV.* The POV, defined as the number of milliequivalents of peroxide per kilogram of oil, is generally determined by iodometric titration. For oils with low POV it can be assumed that these hydroperoxy groups mainly correspond to mono-HPO-TAG. For oils with high POV, bis- and tris-HPO-TAG contribute to the POV. It can be expected that for a certain POV range a good correlation exists between the POV determined by iodometric titration and the HPLC results for mono-HPO-TAG.

In order to establish this correlation, samples of sunflower oil with POV ranging from 0.5 to 50 have been analyzed by both methods. HPO-TAG peak areas obtained by HPLC-UV analysis are plotted against POV obtained by iodometric titration in samples with low POV and high POV (Figs. 6 and 7). From both graphs, it can be concluded that a good linear correlation exists over a large POV range between the POV determined by iodometric titration and the HPLC-UV results ($R$ = 0.999 for Fig. 6, $R$ = 0.996 for Fig. 7). This confirms the

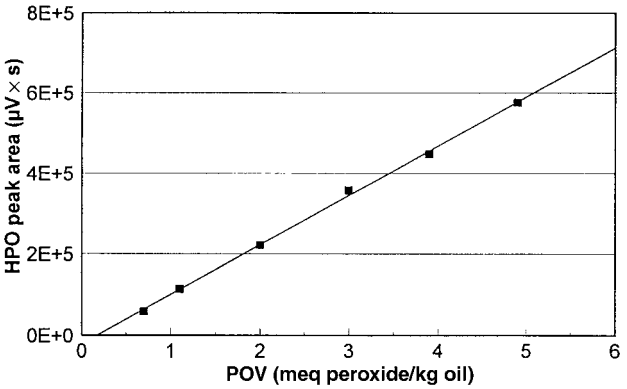



**FIG. 6.** Peak areas of TAG hydroperoxides obtained by HPLC–UV analysis of sunflower oils with POV between 0 and 5 plotted against POV determined by iodometric titration (linear correlation coefficient $R$ = 0.999). See Figures 1 and 5 for abbreviations.

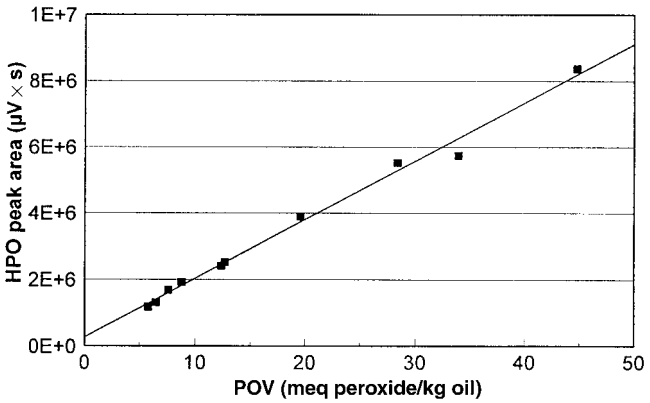



**FIG. 7.** Peak areas of TAG hydroperoxides obtained by HPLC-UV analysis of sunflower oils with POV between 5 and 50 plotted against POV determined by iodometric titration (linear correlation coefficient $R$ = 0.996). See Figures 1 and 5 for abbreviations.

assumption that mainly mono-HPO-TAG contribute to the POV for polyunsaturated fatty acid oils up to a POV of 50. Even the corresponding HPLC chromatograms showed mainly peaks of mono-HPO-TAG.

The molar HPO-TAG concentrations (mmol HPO-TAG/kg oil), which can be calculated from the HPO-TAG peak areas obtained by HPLC analysis and the calibration function determined for P(13-HPO-L)S, can be used to obtain the corresponding POV. The calculated POV are compared to the actual POV as determined by iodometric titration (Table 2). The calculated POV do not correspond to the actual ones, but the calculated POV are on average a factor 5.5 higher than those determined by iodometric titration. This deviation can be explained by differences in response factors of P(13-HPO-L)S and HPO-TAG occurring in sunflower oil, which can be caused by a solvent effect due to the changing gradient composition. As a consequence, quantitative results for vegetable oils cannot be expressed as absolute HPO-TAG concentrations. For many problems, such as the comparison of samples of different oxidative status or monitoring of oxidation experiments with or without antioxidants in time, however, relative quantitative results are sufficient, as only changes in the HPO-TAG concentrations are relevant and not the absolute

**TABLE 2**
**Comparison of Actual POV Determined by Iodometric Titration with POV Calculated from HPLC Results**

| $POV_{actual}$[a] | $POV_{HPLC}$[b] | $POV_{HPLC}/POV_{actual}$[c] |
|---|---|---|
| 0.7 | 3.2 | 4.6 |
| 1.1 | 5.9 | 5.4 |
| 2.0 | 11.0 | 5.5 |
| 3.0 | 17.5 | 5.8 |
| 3.9 | 21.8 | 5.6 |
| 4.9 | 27.9 | 5.7 |

[a]$POV_{actual}$ refers to the POV determined by iodometric titration.
[b]$POV_{HPLC}$ refers to the POV calculated from HPLC results.
[c]$POV_{HPLC}/POV_{actual}$ refers to the POV determined by iodometric titration divided by the POV calculated from HPLC results. See Table 1 for other abbreviations.

concentrations. If absolute HPO-TAG concentrations need to be determined in vegetable oils with low to medium POV, alternative detection methods such as electrochemical detection or chemoluminescence detection must be used. These detection methods respond selectively and sensitively to the hydroperoxy group independent of the structure of the parent TAG.

HPLC-UV is not to be considered as an alternative for the determination of the POV by iodometric titration. If the POV gives sufficient information about the oxidative status of an oil or fat, iodometric titration should be preferred, as it is much more time- and cost-efficient. For oil and fat samples, however, where the POV does not give sufficient information, the analysis of HPO-TAG by HPLC-UV can reveal the nature of the HPO-TAG species. The identity of the parent TAG (e.g., LLL, LLO, LOO) and the number of hydroperoxy groups per TAG molecule (mono-, bis-HPO-TAG) as well as the presence of other oxidized species which do not contribute to the POV can be shown.

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