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Postcolumn chemiluminescence, ultraviolet and evaporative light-scattering detectors in high-performance liquid chromatographic determination of triacylglycerol oxidation products

Marjukka Mäkinen*, Vieno Piironen, Anu Hopia

Department of Applied Chemistry and Microbiology, Food Chemistry Division, P.O. Box 27, FIN-00014 University of Helsinki, Helsinki, Finland

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Abstract

Postcolumn chemiluminescence (CL), ultraviolet (UV) and evaporative light-scattering (ELS) detectors were compared in the HPLC of triacylglycerol (TAG) oxidation products. Polar lipid fractions from autoxidized TAG samples were first separated from non-polar material by solid-phase extraction and then analysed by RP-HPLC in combination with UV–CL and UV–ELS detectors. TAG hydroperoxides were detected with a CL detector, oxidation products containing conjugated diene structures with a UV detector and all non-volatile oxidation products with an ELS detector. More accurate characterization of the oxidation products was possible by combining the information from different detectors. For samples at early stages of oxidation, the CL and UV detectors were more suitable because of their better sensitivity and selectivity compared with the ELS detector.

Keywords: Detection; LC; Chemiluminescence; Triacylglycerols; Lipids; Hydroperoxides

1. Introduction

The oxidation of unsaturated lipids has been extensively studied in the fields of both food science and the biological and medical sciences. A complex mixture of oxidation products is formed during lipid oxidation. Lipid hydroperoxides are primary products of oxidation which react further forming heterogeneous mixtures of volatile, monomeric and polymeric products. In addition to hydroperoxides, oxidation products containing, among others, carbonyl, hydroxy and epoxy functional groups are thus formed [1,2].

Methods commonly applied for the measurement of lipid oxidation are the peroxide value (PV) for lipid hydroperoxides and a variety of methods for secondary oxidation products, e.g., anisidine, carbonyl and thiobarbituric acid values. Recently, several chromatographic methods have been developed for the determination of lipid oxidation products. In addition to quantita-

* Corresponding author.

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tive data, these methods provide information on the molecular structure of the oxidation products. HPLC has gained popularity especially in the analysis of high-molecular-mass oxidation products; however, owing to the complex nature of lipid oxidation, no single HPLC detector is available to characterize oxidized lipids fully. Detection of oxidation products is based either on the presence of functional groups formed during oxidation or on the use of mass detectors which allow the detection of all non-volatile oxidation products.

The UV detector is an indirect means of detecting the hydroperoxides of polyunsaturated fatty acids. At a wavelength of 234 nm it detects compounds containing conjugated diene structures [3]. Lipid hydroperoxides are also detected selectively by a variety of postcolumn methods. Yamamoto et al. [4] and Miyazawa et al. [5] introduced a method of determining lipid hydroperoxides by postcolumn chemiluminescence (CL) detection. CL detection is based on the reaction of hydroperoxides with heme compounds, producing radical species which oxidize luminol or isoluminol accompanied by reactions leading to the emission of light. Other applications of postcolumn detection of lipid hydroperoxides include the fluorometric diphenyl-1-pyrenylphosphine method [6] and the colorimetric iron(III) thiocyanate method [7].

An evaporative light-scattering (ELS) detector is a mass detector and thus responds to all primary and secondary oxidation products that are sufficiently non-volatile not to evaporate in the detector chamber. Analytical applications of the ELS detector include the determination of lipid oxidation products by high-performance size-exclusion chromatography [8,9] and the determination of cholesterol oxidation products by HPLC [10]. Few applications have been published, however, in which the ELS detector is used in combination with HPLC of triacylglycerol (TAG) oxidation products [11].

The aim of this study was to compare the qualitative and quantitative properties of postcolumn CL, UV and ELS detectors in the RP-HPLC determination of TAG autoxidation products. Special attention was focused on the CL detector to determine the effects of various detection parameters on the yield of the CL reaction.

2. Experimental

2.1. Materials

Methyl linoleate (MeLo), 1,3-dioleate (1,3-DO), trilinoleate (TL) and trioleate (TO) were purchased from Nu-Chek-Prep (Elysian, MN, USA), rapeseed oil (RSO) from Van den Bergh Foods (Helsinki, Finland), *tert.*-butyl hydroperoxide (t-BOOH), isoluminol and microperoxidase (MP 11, from equine heart cytochrome *c*) from Sigma (St. Louis, MO, USA) and sodium borohydride from BDH (Poole, UK). All the solvents were of HPLC grade (Rathburn, Walkerburn, UK).

2.2. Sample preparation

2.2.1. MeLoOOH standard

MeLo was autoxidized in an open 10-ml test-tube in the dark at 40°C for 19 h. MeLo hydroperoxide (MeLoOOH) was separated from non-oxidized MeLo using solid-phase extraction (SPE) (NH_2 columns, Bond Elut 500 mg; Analytichem International, Harbor City, CA, USA) as described by Hopia et al. [12]. The purity of the MeLoOOH fraction was checked using TLC on plates coated with Kieselgel 60 (Merck, Darmstadt, Germany) with hexane–diethyl ether–acetic acid (80:20:2, v/v) as the eluent. The concentration of MeLoOOH was determined by UV spectrophotometry at 234 nm (Lambda Bio; Perkin-Elmer, Überlingen, Germany) using the average molar absorptivity of *cis,trans* and *trans,trans* isomers (27 000 l mol^{-1} cm^{-1}) [13].

2.2.2. Oxidation of TAGs

A natural mixture of RSO-TAGs was prepared with a column chromatographic method [14]. TL, TO and RSO-TAG samples (500 mg) were autoxidized in open 10-ml test-tubes in the dark at 40°C for up to 17 days. Sample aliquots

were taken for PV and HPLC determinations at regular intervals. The PVs were determined with the iron(III) thiocyanate method [15,16]. The polar fraction containing TAG oxidation products was separated from non-polar material by SPE; the purity of the SPE fractions was checked by TLC [12]. The recovery of the TAG oxidation products in SPE was calculated from samples analysed by HPLC–UV and HPLC–ELS before and after SPE.

2.2.3. Reduction of hydroperoxides

The TAG hydroperoxides were reduced to the corresponding hydroxy compounds with sodium borohydride [17].

2.3. HPLC instrumentation

The HPLC instrumentation included two Model 501 pumps equipped with a Model 700 WISP autosampler and a Model 510 pump for postcolumn reagent (Waters, Milford, MA, USA). CL light was detected with an LB 506 C-1 HPLC radioactivity monitor (Berthold, Germany) equipped with a 200-μl measuring cell. The UV detector was a Waters Model 486 tunable absorbance detector and the ELS detector was a DDL21 (Cunow, Cergy St. Christopher, France). A Berthold LB 506 C-1 HPLC program was used for data processing.

2.4. Chromatographic separation of oxidation products

TAG oxidation products were separated using either a Nova-Pak C_{18} cartridge column (150 mm × 3.9 mm I.D., 4 μm; Waters) or a Spherisorb S5 ODS 2 column (250 × 4.6 mm I.D.; Phase Separation, Deeside, UK). The mobile phases for these columns were methanol–2-propanol (90:10, v/v) and methanol–2-propanol–dichloromethane (80:10:10, v/v), respectively, at a flow-rate of 1.0 ml min^{-1}.

2.5. Detection of oxidation products

The postcolumn CL reagent [4] contained isoluminol and microperoxidase in borate buffer (pH 10)–methanol (30:70, v/v). The effects of isoluminol and microperoxidase concentrations and the flow-rate of the CL reagent on the detector response were studied. The concentrations of isoluminol and microperoxidase varied from 0.1 to 1.0 m*M* and from 0.1 to 1.6 mg l^{-1}, respectively, and the flow-rates from 0.3 to 1.1 ml min^{-1}. The CL reagent was mixed with the column eluent in a T-piece at 25°C and the light generated in the CL reaction was monitored by a radioactivity monitor. The length of the capillary between the T-piece and the radioactivity monitor was 3 cm. The UV detector was set at a wavelength of 234 nm. The ELS detector parameters were optimized according to Hopia et al. [8] and included evaporation temperature 90°C, working pressure 1 bar and make-up gas flow off. The standard compounds used in the optimization of the detector parameters and in the quantification of TAG oxidation products were t-BOOH and MeLoOOH, MeLoOOH and 1,3-DO with the CL, UV and ELS detectors, respectively.

3. Results and discussion

3.1. Sample preparation

Since oxidized and non-oxidized material co-eluted, SPE prior to HPLC analysis was essential for TAG samples, especially when the ELS detector was used. The recovery of oxidized lipids in SPE was 85–99% (Table 1); previously, as tested with lipid standards, the recovery of

Table 1
Recovery of TAG oxidation products in SPE determined by HPLC–UV and HPLC–ELS

Sample PV (mequiv. kg^{-1})	Recovery (%)	
	UV detector[a]	ELS detector
TL 22.4	90.0 ± 2.6	85.3 ± 1.3
TL 35.3	90.2 ± 1.3	87.2 ± 1.5
TO 9.5	–	99.5 ± 2.0

[a] Means ± S.D. of triplicate analyses.

polar lipids with the same SPE method was 91–110% [8].

During SPE, minor hydrolysis of TAGs occurred. Diacylglycerols (DAGs) formed through hydrolysis were seen as artificial peaks in the chromatograms of TAG oxidation products observed with the ELS detector (see Fig. 4A and B). The retention times of dilinoleate and dioleate were 3.6 and 6.1 min, respectively. The amount of DAGs varied from 0.05 to 0.15% of the total fat sample. This decreased the sensitivity of the ELS detector in detecting oxidative changes in TAG samples. Hydrolysis of TAGs during the column chromatographic purification of lipid samples has been reported previously with silicic acid columns and commercially available silica gel columns [18,19].

3.2. Optimization of CL detection

The microperoxidase concentration had a marked effect on the CL detector response. The higher the concentration over the range 0.1–1.6 mg/l, the higher was the detector response. The signal-to-noise ratio was highest at 0.4 mg/l; however, the calibration graph for MeLoOOH was not linear at this concentration (Fig. 1). Reduction of the microperoxidase concentration to 0.1 mg/l improved the linearity of the detector response at the expense of a slightly lower minimum detectable concentration of MeLoOOH, hence 0.1 mg/l was selected for further work. The smaller amount of microperoxidase in comparison with a previous study [4] markedly decreased the reagent consumption. In contrast, modification of the isoluminol concentration over the range 0.1–1.0 m*M* had no effect on the detector response or the signal-to-noise ratio of the CL detector. A concentration of 0.5 m*M* was selected for the CL reagent. In our experience, no significant improvement in the sensitivity of the CL detector could be obtained if isoluminol and microperoxidase were replaced with luminol and cytochrome *c* [5].

An increase in the CL reagent flow-rate up to 0.7 ml/min increased the signal-to-noise ratio of the CL detector, and this was selected for further work. The CL reaction was apparently rapid since longer reaction times obtained with longer reaction coils between the T-piece and the radioactivity monitor did not increase the detector response.

The CL detector response was not as stable as that of the other detectors. The increase in the peak area of the MeLoOOH standard by 1.2–1.5-fold within a day was probably due to the increase in the background CL. Between days the variation in the detector response was due to differences in the CL reagent solutions and the freshness of the CL reagent. For quantification, the peak areas were corrected on the basis of the MeLoOOH standard introduced after every fifth injection.

3.3. Detection limits, linearity and repeatability

Detection limits of the standard compounds were calculated on the basis of a signal-to-noise ratio of 3. The detection limits of MeLoOOH were 0.8 ng (2.5 pmol) and 2 ng (6.1 pmol) with the CL and UV detectors, respectively, and that of 1,3-DO with the ELS detector was 142 ng (229 pmol). In the analysis of TAG oxidation products the sample amount needed for the ELS detector was approximately tenfold compared with that for the CL and UV detectors.

Table 2 presents the linear regression equations of the calibration graphs for each detector. The UV detector response was linear over the range 2.2–264 ng (6.7–810 pmol) of MeLoOOH whereas the CL detector response was only approximately linear over the same area. Our finding that the microperoxidase concentration affected the linearity of the CL detector response (Fig. 1) may partially explain the contradictory results in the literature concerning the linearity of the CL detector response. Linear relationships have been reported between the detector response and linoleic acid hydroperoxide content (10–80 pmol) [4] and phospholipid hydroperoxide content (10–1000 pmol) [20]. On the other hand, Holley and Slater [21] reported a non-linear calibration graph for hydroperoxides of cholesterol and eicosatetraenoic acids over the range 0–100 pmol. The ELS detector gave the best correlation coefficients with linear curve

Table 2
Linear regression equations of calibration graphs for CL, UV and ELS detectors

Detector	Range of linearity (ng)	Slope, b	y-Intercept, a	r^2
CL[a]	2.2–264	26 856	977 144	0.9942
UV[a]	2.2–264	360	18 861	0.9999
ELS[b]	142–5700	307	189 890	0.9964

[a] Sample MeLoOOH, injection at eight levels, results calculated on the basis of duplicate injections.
[b] Sample 1,3-DO, injection at nine levels, results calculated on the basis of duplicate injections.

fitting over the range 142–5700 ng (229–9194 pmol) of 1,3-DO. The response of the ELS detector has previously been described variously as linear, sigmoidal or exponential [22]. In addition to the chromatographic conditions and detector parameters, the chemical structure of the sample also affects the detector response [22,23].

The repeatability of the detector responses was evaluated by seven subsequent injections of standard compounds. The R.S.D.s for MeLoOOH at three injection levels (4.2, 42.2 and 126.5 ng) were 1.2–3.8% with the CL detector and 0.4–4.7% with the UV detector. The R.S.D.s of 1,3-DO at two injection levels (285 and 950 ng) were 7.7–11.3% with the ELS detector.

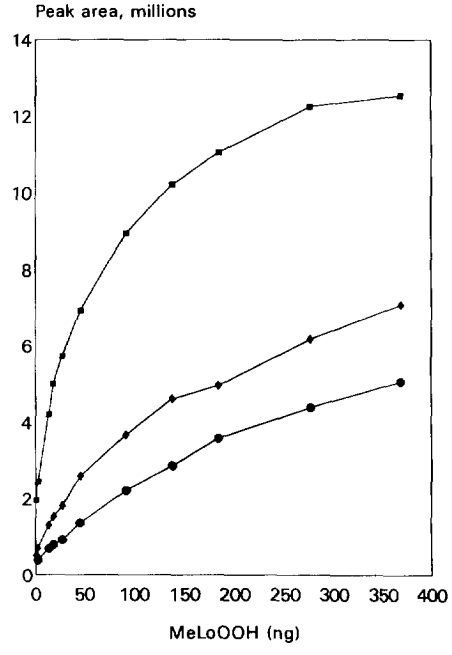

Fig. 1. Effect of microperoxidase concentration of CL reagent on linearity of CL detector response: (■) 0.4; (◆) 0.2; (●) 0.1 mg l^{-1}. Sample, MeLoOOH; concentration of isoluminol in CL reagent, 0.5 mM; flow-rate, 0.7 ml $min.^{-1}$.

3.4. Monitoring of TAG oxidation products

Figs. 2–4 show typical examples of the RP-HPLC of TAG oxidation products obtained with the CL, UV and ELS detectors. The CL and UV detectors monitored the formation of TAG hydroperoxides and thus the primary stage of oxidation. The UV detector at a wavelength of 234 nm did not allow the detection of TO hydroperoxides owing to the lack of conjugated diene structures (Fig. 3B). In RSO-TAG samples, this difference between the UV and CL detectors was not observed since the oxidation of polyunsaturated fatty acids dominated (Figs. 2C and 3C). The detection of oleate oxidation products is important, however, if samples with high levels of oleic acid are to be analysed. The other difference between the CL and UV detectors was a negative peak in the RSO-TAG chromatograms at a retention time of ca. 3 min. detected with the CL detector (Fig. 2C). The peak was due to traces of γ-tocopherol left in the RSO-TAG during chromatographic purification [14]. When the autoxidation proceeded, the γ-tocopherol was consumed in the samples and the peak disappeared. According to Frei et al. [24], negative peaks provide only qualitative information of antioxidative compounds since the response is dependent on the quenching of the background CL of the HPLC system. The selectivity of CL hydroperoxide detection was con-

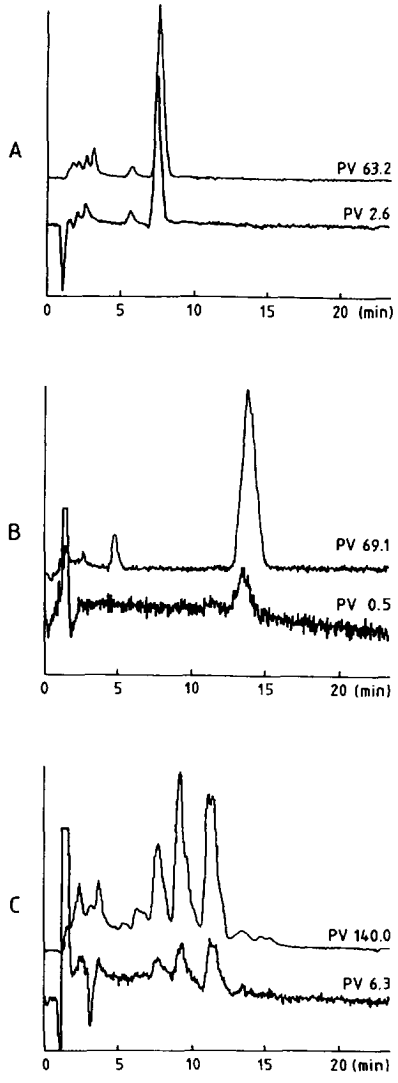

Fig. 2. HPLC–CL of TAG oxidation products at two oxidation levels (PV, mequiv. kg^{-1}). (A) TL; (B) TO; (C) RSO-TAG. Injection volume, 10–50 μl, representing 28–611 μg of original sample before SPE.

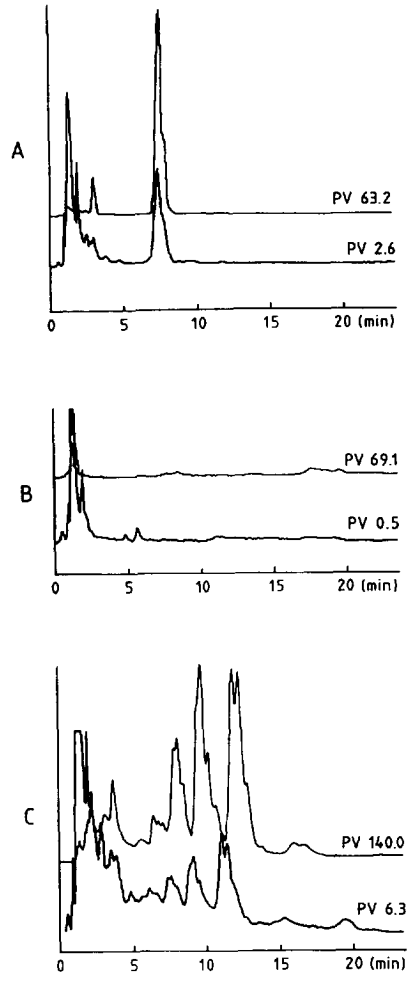

Fig. 3. HPLC–UV of TAG oxidation products at two oxidation levels. Abbreviations and injection volume as in Fig. 2.

firmed by the disappearance of the CL signal after reduction of TAG hydroperoxides to the corresponding hydroxy compounds with sodium borohydride. Hydroxides containing the conjugated diene structures were still observed with the UV detector (chromatograms not shown).

When hydroperoxides react further, secondary oxidation products without hydroperoxide groups or conjugated diene structures are formed. The ELS detector is a mass detector and therefore these decomposition products, which are not observed with the CL or UV detectors, can be detected. In the present study, the chromatographic profiles obtained with the ELS detector were similar to those with the CL detector, indicating that most of the oxidation products still occurred as hydroperoxides and that decomposition had not occurred to a large extent.

The TAG oxidation products were separated by RP-HPLC according to their fatty acid com-

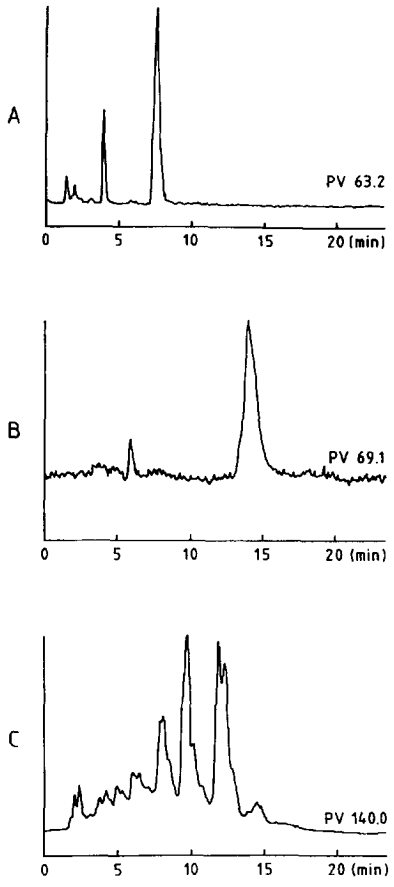

Fig. 4. HPLC–ELS of TAG oxidation products. Abbreviations as in Fig. 2. Injection volume, 10–50 μl, representing 174–2800 μg of original sample before SPE.

position and functional groups found in the lipid structure [11,25]. The first oxidation products were already detectable at PVs below 3 mequiv. kg^{-1}. Peak identification was based on the literature on TAG oxidation products [25–27]. In the TL and TO chromatograms, the main peaks initially seen were apparently mixtures of TL and TO monohydroperoxide isomers. In RSO-TAG chromatograms, the peaks represented monohydroperoxides and hydroperoxy epidioxides of heteroacylglycerols. When oxidation proceeded, these peaks increased and smaller peaks representing more polar oxidation products appeared in the chromatograms. Figs. 5A–C show the relationship between the PVs of the samples and

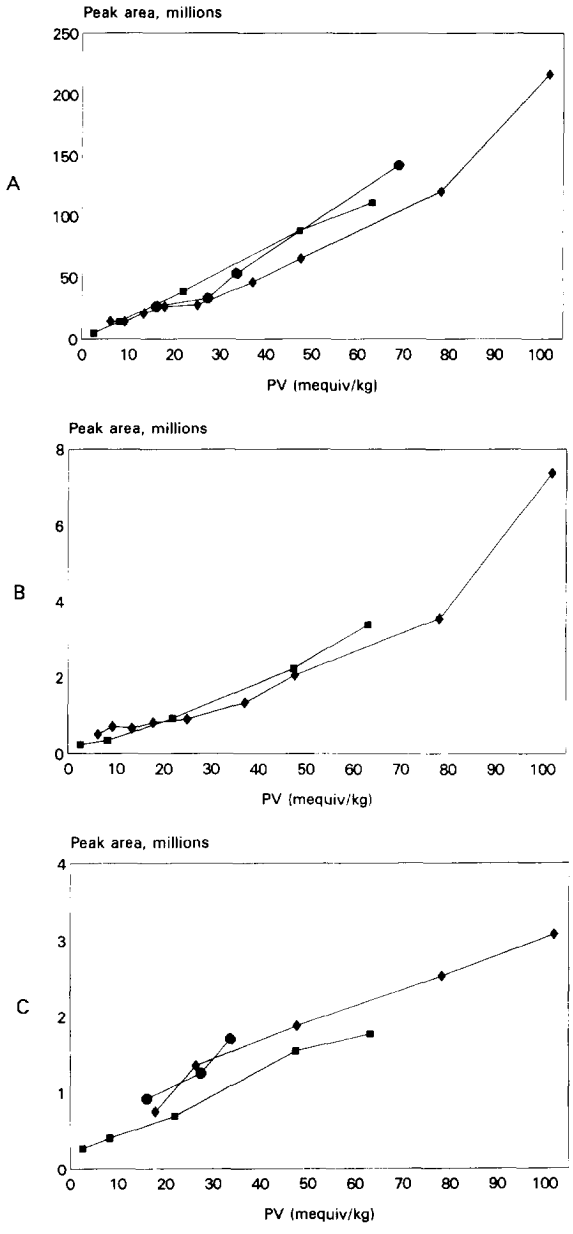

Fig. 5. PVs of TAG samples vs. total TAG oxidation products determined by HPLC with (A) CL, (B) UV and (C) ELS detectors. The total peak areas from HPLC chromatograms were calculated to 1 mg of original sample before SPE. (■) TL; (●) TO; (◆) RSO-TAG.

the total peak areas of the CL, UV and ELS chromatograms. The total peak areas followed PV patterns at the levels of oxidation studied.

These results suggest that determination of the TAG sample oxidation levels is possible with each detector.

To test the quantitative properties of the detectors, the TAG oxidation products were quantified in samples at different oxidation levels. Quantification of these heterogeneous mixtures was performed using MeLoOOH as a standard with the UV and CL detectors and 1,3-DO with the ELS detector. Table 3 gives the amount of TL monohydroperoxides (Figs. 2A–4A, peak at retention time ca. 8 min) at different oxidation levels. The variation in the results between the detectors indicates the difficulty in finding suitable external standard for each detector type. The use of MeLoOOH as a standard compound is considered reliable with the UV detector since the molar absorptivities for all monohydroperoxides with conjugated diene structures are approximately identical [13,25,26]. The ELS detector responses of different lipids are reported to be slightly dependent on molecular structure [22,23]. Recently, Hopia and Ollilainen [23] reported that a standard deviation of at least 13% is to be expected when quantifying heterogeneous mixtures of unknown compounds with the ELS detector. The smaller amounts of TL monohydroperoxides measured with the CL detector may be due to the variations in the relative CL responses of different hydroperoxides [4,21]. To confirm quantification with the CL detector, the response factors of different hydroperoxides should be studied more closely.

4. Conclusions

CL, UV and ELS detectors are suitable for use in connection with RP chromatography of TAG oxidation products. A more accurate characterization of the TAG oxidation products was possible by combining the information from different detectors, even though the exact structures of the oxidation products could not be established. For samples at early stages of oxidation, the CL and UV detectors were more suitable owing to their better sensitivity and selectivity compared with the ELS detector. Determination of the oxidation levels of TAG samples was possible with each detector; however, since the TAG oxidation products were mixtures of several compounds with compositions which were partly unknown, variation between the detector responses is expected and care must be taken in interpreting the quantitative results.

Acknowledgements

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Table 3
Quantification of TL monohydroperoxides at different oxidation levels

PV (mequiv. kg^{-1})	Concentration (mg g^{-1})		
	CL detector[a,b]	UV detector[a,b]	ELS detector[a,c]
2.6	0.10 ± 0.02	0.55 ± 0.13	0.55 ± 0.11
8.3	0.32 ± 0.01	0.74 ± 0.07	1.00 ± 0.15
22.0	0.93 ± 0.02	1.82 ± 0.23	1.75 ± 0.10
47.4	2.04 ± 0.09	4.38 ± 0.08	3.50 ± 0.10
63.2	2.42 ± 0.04	6.39 ± 0.05	4.46 ± 0.34

[a] Means ±S.D. of four analyses.
[b] MeLoOOH was used as an external standard in quantification.
[c] 1,3-DO was used as an external standard in quantification.

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