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A comprehensive two-dimensional liquid chromatography method for the simultaneous separation of lipid species and their oxidation products

Eleni Lazaridi[a,c], Hans-Gerd Janssen[b,c,d], Jean-Paul Vincken[a], Bob Pirok[c], Marie Hennebelle[a,*]

[a] Wageningen University and Research, Laboratory of Food Chemistry, Wageningen, the Netherlands
[b] Wageningen University and Research, Laboratory of Organic Chemistry, Wageningen, the Netherlands
[c] University of Amsterdam, Analytical-Chemistry Group, Amsterdam, the Netherlands
[d] Unilever Food Innovation Center, Wageningen, the Netherlands

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ABSTRACT

Lipid oxidation is one of the major causes of food spoilage for lipid-rich foods. In particular, oil-in-water emulsions, like mayonnaises and spreads, are prone to oxidation due to the increased interfacial area that facilitates contact between the lipids and hydrophilic pro-oxidants present in the water phase. Polar, amphiphilic lipid species present at the oil/water interface, like the mono- (MAGs) and di-acylglycerols (DAGs), act as oxidation starters that initiate subsequent oxidation reactions of the non-polar lipids in the oil droplets. A comprehensive two-dimensional liquid chromatography (LC×LC) method with evaporative light-scattering detection (ELSD) was set up to study the composition of the complex mixture of oxidized polar and non-polar lipids. The LC×LC-ELSD method employs size exclusion chromatography (SEC) in the ^{1}D (1^{st} dimension) to separate the various lipid species according to size. In the ^{2}D (2^{nd} dimension), normal-phase liquid chromatography (NPLC) is used to separate the fractions according to their degree of oxidation. The coupling of SEC with NPLC yields a good separation of the oxidized triacylglycerols (TAGs) from the large excess of non-oxidized TAGs. In addition, it allows the isolation of non-oxidized DAGs and MAGs that usually interfere with the detection of a variety of oxidized products that have similar polarities. This method facilitates elucidating how lipid composition affects oxidation kinetics in emulsified foods and will aid in the development of more oxidation-stable products.

1. Introduction

Lipid oxidation in food products is a crucial problem that causes undesirable changes in a food's flavor, texture, nutritional value and consequently reduces its shelf life. Even though lipid oxidation has been studied extensively, the governing processes in more complex food systems like emulsified foods are not fully understood. Oil-in-water emulsions, such as mayonnaises, salad dressings and infant formulas are among the most widely consumed lipid-rich foods [1,2]. In these oil-in-water emulsions, lipid droplets are dispersed in a continuous water phase, and stabilized by emulsifiers such as free fatty acids and mono- and di-acylglycerols (MAGs and DAGs), proteins and phospholipids. In such food products, lipid oxidation generally proceeds from the exterior of the oil droplet (interface) to the interior, making it important to understand how the compounds present at the interface impact oxidation kinetics [3]. Hence, analysis of the various lipid classes and their oxidation products is key.

High-performance liquid chromatography (HPLC) is the most versatile analytical method available to study lipid oxidation due to the variety of separation modes available. Normal-phase HPLC (NPLC) separates lipid classes based on their polarity resulting from hydroxy groups and double bonds or other functional groups and neglects mostly the non-polar lipid chain. Non-aqueous reversed-phase HPLC (NARP-HPLC) is widely used for the separation of TAGs according to their non-polar moiety [4]. Even though

* Corresponding author: Phone: (+31) 317 482 533
E-mail address: marie.hennebelle@wur.nl (M. Hennebelle).

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size-exclusion chromatography (SEC) has not been widely used in lipid analysis, SEC methods for the rapid separation of low molecular weight lipid species from TAGs or for the quantification of polymerized TAGs in e.g. frying oils have been described [5,6].

In oxidized lipids, a large variety of species is present, covering a wide range of molecular weights and polarities. Small volatile species are present besides polymeric structures and in terms of polarity, the entire spectrum from non-polar alkanes and TAGs to heavily oxidized species is covered. Previously published studies on lipid oxidation products mostly focused on oxidized TAGs [7–9]. Zeb for example used a NARP-HPLC method to characterize the TAG composition of camellia oil before and after auto-oxidation and identified three main TAG autoxidation products: epoxy-hydroperoxides, epoxy-epidioxides and mono-epoxides [8]. Kato *et al.* utilized NARP-HPLC to investigate the oxidation mechanisms and TAG-hydroperoxides found in canola oil [9]. Steenhorst-Slikkerveer and colleagues finally applied NPLC-MS for the identification and quantitation of non-volatile TAG oxidation products (e.g., mono and di-hydroperoxy-TAG, epoxy-TAG, oxo-TAG, mono- and di-hydroxy-TAG) in rapeseed and linseed oils [10].

Despite the wide range of advanced HPLC methods developed for studying lipid oxidation, there is no single chromatographic technique that provides the level of detail required for building a true understanding of the complex processes of lipid oxidation in emulsified foods. One of the main limitations is that non-oxidized DAGs and MAGs interfere with the detection of a variety of oxidized TAG products of similar polarity. Multidimensional chromatography set-ups use a combination of different chromatographic techniques and separation modes to achieve a much higher resolving power and peak capacity than one-dimensional chromatography. Several multidimensional platforms for lipid analysis have been reported. Comprehensive two-dimensional liquid chromatography (LC×LC) has been used successfully to improve TAG analysis in a variety of oils by coupling silver ion chromatography (Ag-HPLC) with NARP-HPLC, but none of these specifically focus on oxidized food lipids [11–13]. Since current food lipidomic platforms cannot deal with the sheer complexity of lipid oxidation in emulsified foods, a novel approach is needed. The combination of two independent separation steps, where lipids will first be separated at their lipid class level (MAG, DAG, and TAG) followed by a subsequent separation based on the degree of oxidation should allow monitoring the oxidative fate of the different lipid classes in emulsified foods down to the molecular level. Clearly, the chromatographic method will present several challenges such as a reduced sensitivity because of the additional dilution step upon transfer from the first to the second dimension and the risk of mobile phase incompatibility, two key difficulties to be taken into consideration during method development in LC×LC [14].

The current contribution focusses on the development of an on-line comprehensive LC×LC method that enables the study of the oxidative fate of the different lipid classes present in emulsified foods. The method specifically focusses on the non-volatile oxidation products (NVOPs). SEC is used as the first-dimension separation mode to separate the different lipid classes according to size. In the second dimension, each band of size-separated species is subsequently separated according to polarity, *i.e.* degree of oxidation, by NPLC. The efficiency of the separation modes selected for each dimension is first evaluated off-line and afterwards the method is validated on-line. To develop the method, DAG and MAG standards are used and rapeseed oil is selected as a representative oil sample used in emulsified food products. The applicability of the final LC×LC method is tested by the analysis of samples obtained from an accelerated aging study.

2. Materials and Methods

2.1. Chemicals and Materials

2.1.1. Chemicals

Tetrahydrofuran (THF, >99.9%), toluene (ACS, Reag. Ph. Eur. grade) and *n*-hexane were purchased from VWR chemicals (Amsterdam, The Netherlands). Methanol (MeOH, UPLC/MS-CC/SFC grade) was purchased from Biosolve (Valkenswaard, The Netherlands). Chloroform ($CHCl_3$, stabilized with 0.5% ethanol) was obtained from Rathburn (Walkerburn, UK).

2.1.2. Standards

1,3-dilinoleoyl-glycerol (C18:2/OH/C18:2) and 1-linoleoyl-glycerol (C18:2/OH/OH) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Tristearin (C18:0/C18:0/C18:0), glyceryl-1,2-dipalmitate (C16:0/C16:0/OH) and 1-stearoyl-glycerol (C18:0/OH/OH) were obtained from Larodan (Solna, Sweden).

2.1.3. Oil Samples

Unilever Research (Wageningen, The Netherlands) provided oxidized and non-oxidized rapeseed oils isolated from fresh and aged mayonnaise, as well as a mixture of aged frying oil spiked with free fatty acids (FFAs). Here it should be emphasized that even at an advanced stage of lipid oxidation, the concentration of ox-TAGs is significantly lower than that of the non-oxidized TAGs. For method development, a highly oxidized rapeseed oil sample was produced using an accelerated aging protocol. A thin layer of oil isolated from fresh mayonnaise was put on a glass petri dish and was incubated at 70 °C for a week followed by 5 h at 150 °C. A highly oxidized frying oil was used for the optimization of the individual dimensions, whereas the oxidized rapeseed oil isolated from aged mayonnaise was employed during the optimization of the on-line LC×LC method. Finally, the highly oxidized rapeseed oil sample was used for testing the applicability of the finalized method. Furthermore, since the concentrations of DAGs and MAGs in the oil samples are generally very low, oil samples were spiked with DAG and MAG standards to facilitate method optimization. Glyceryl-1,2-dipalmitate and 1-stearoyl-glycerol standards were used for spiking in the off-line proof of concept experiments, whereas 1,3-dilinoleoyl-glycerol and 1-linoleoyl-glycerol were used to spike the oil samples used during the on-line validation. Concentrations varying between 6 mg/mL and 50 mg/mL were used.

2.2. Instrumentation and chromatographic conditions

2.2.1. Individual optimization of ^1D and ^2D

2.2.1.1. Size-exclusion chromatography. Size-exclusion chromatography (SEC) experiments were performed on a Shimadzu HPLC system consisting of an LC-20AT isocratic pump equipped with a CBM-20Alite controller, a SIL-20AC autosampler, a CT0-10ACVP column oven and an RID-10A reflective-index detector (Shimadzu, Den Bosch, The Netherlands). Two serially connected 300×7.5 mm, 5 μm, PLgel polystyrene-divinylbenzene SEC columns (Agilent, Amstelveen, The Netherlands), one packed with particles of 500 Å and the second featuring a pore size of 100 Å, were used for the separation. The compounds were separated using THF as the eluent, at 0.8 mL/min flow rate for 30 min. The column oven temperature was 30 °C and the injection volume 20 µL. All samples and standards were diluted in *n*-hexane/$CHCl_3$ (1:1) prior injection. LabSolutions software (Shimadzu) was used for data acquisition and data processing.

2.2.1.2. Normal-phase liquid chromatography. NPLC experiments were performed on a Shimadzu HPLC system consisting of an LC-10AT binary pump equipped with a SIL-20AC autosampler and

a CT0-10ACVP column oven, connected to an evaporative light-scattering detector 1260 Infinity II ELSD (Agilent). A custom-made 150×4.6 mm, 100 Å, 2.6 μm particle size core-shell silica column from Phenomenex (Torrance, CA, USA) was used for the separations. The eluent composition was adapted from the method developed by Olsson *et al.*, who used *n*-hexane as solvent A and toluene/MeOH containing acetic acid and trimethylamine (60:40:0.2:0.1) as solvent B [15]. The compounds were separated using an isocratic mixture of solvent A and solvent B at a ratio of 90:10 (v/v), at 1 mL/min flow rate. The composition of solvent B was optimized by testing MeOH percentages ranging from 10 to 40%. The injected volume was 10 μL. The total run time was 30 min. The optimized parameters for the ELSD were 80 °C for the evaporation temperature, 60 °C for the nebulizing temperature and 0.9 L/min for the nebulizer gas flow. All samples and standards were diluted in *n*-hexane/$CHCl_3$ (1:1) prior to injection. LabSolutions software (Shimadzu) was used for data acquisition and processing.

2.2.1.3. Direct mass spectrometry.

2.2.1.3.1. Preparation of fractions for mass spectrometric analysis. Direct-inlet MS analysis was performed on fractions collected post-column and prior to the ELSD from the NPLC analyses to evaluate the NPLC performance regarding the separation of non-oxidized and oxidized compounds. The collected fractions were initially diluted in the eluent used for NP analysis (*n*-hexane/toluene/MeOH (90:8.5:1.5)), but, because of the low solvent polarity, this resulted in poor ionization. For this reason, all fractions were evaporated to dryness under a flow of nitrogen gas and were then re-dissolved in $CHCl_3$/MeOH (2:1) prior to MS analysis.

2.2.1.3.2. Direct electrospray ionization mass spectrometry (ESI/MS) parameters. Direct-inlet MS was carried out on a Bruker micro TOF-Q ESI mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. The sample was introduced into the ESI source using a syringe pump and a 250 μL Hamilton glass syringe, at a flow rate of 2.0 μL/min. The mass spectrometer was operated in positive ESI mode with the mass scan range set from *m/z* 200 to 1500. Typical experimental conditions were as follows: drying gas flow rate 5 L/min at 200°C, capillary voltage 4500 V, collision energy 10 eV, collision RF 600 Vpp, transfer energy 140 μs, and pre-pulse storage 10 μs. Acquisition of the MS data was performed using DataAnalysis 4.3 software.

2.2.2. On-line analysis

2.2.2.1. Comprehensive two-dimensional liquid chromatography. The instrument used in this study was an Agilent Infinity 2D-LC system (Agilent, Waldbronn, Germany). The system included an autosampler (G1313A), a capillary pump (G1376A), a binary pump (G7120A) with V35 Jet Weaver mixers (G4220-60006), a 2-pos/8-port valve (5067-4214) fitted with two 50 μL loops and an ELSD (G4260B). The experimental conditions optimised during the off-line proof of concept experiments were initially used in the on-line LC×LC analyses and were then further optimized.

The system was controlled by Agilent OpenLAB CDS Chemstation Edition A02.02 software. Data were collected using Agilent OpenLAB CDS ChemStation Edition for LC & LC/MS Systems, Version C.01.07 with Agilent 1290 Infinity LC×LC Software, Version A.01.02. Data were processed using MOREPEAKS software (previously called PIOTR) developed by Pirok *et al.* [16].

3. Results and discussion

As outlined in the introduction, the large number of compounds formed during the oxidation of edible oils and fats results in a sample complexity that cannot be resolved by any single dimensional LC set-up. LC×LC with its much higher peak capacity might offer the required separation power to achieve this. The separation system envisaged here would separate the sample according to the different size classes of lipids present in the first dimension (^{1}D) and subsequently separate the various oxidation products within each size group in the second dimension (^{2}D). The two key requirements for the ^{2}D separation in LC×LC are (i) that it provides an orthogonal separation and (ii) that the separation is sufficiently fast [17,18]. SEC and NPLC present a satisfactory degree of orthogonality, since SEC separates the sample molecules according to size with little or no contribution of polarity, whereas NPLC separates according to polarity with just a limited size influence [19]. Regarding the second consideration, the ^{2}D separation in an LC×LC method needs to provide a separation that is sufficiently fast to ensure that all compounds present in a particular fraction have eluted before the subsequent fraction enters the ^{2}D column. There are several ways to increase the speed of analysis, such as the use of shorter columns, columns packed with smaller particles, the use of higher flow rates or the use of fast gradient conditions. Prior to setting up a fully automated LC×LC method, the separation characteristics of the individual dimensions were first evaluated and optimized using an off-line setup.

3.1. Individual optimization of ^{1}D and ^{2}D

3.1.1. ^{1}D separation: SEC

The selection of SEC as the separation mode for the ^{1}D was logical since TAGs, DAGs and MAGs differ considerably in size. Moreover, SEC would also allow separation of TAG from the polymerized lipid species that are formed as secondary oxidation products [20]. To allow efficient separation over the entire molecular weight range from mono-glycerides to oligomerized TAG two serially connected columns with different pore sizes were used. A 6 mg/mL sample of aged oxidized frying oil, spiked with a DAG and a MAG at 6 mg/mL each, was used for method optimization. The resulting separation is shown in Fig. 1a. From the chromatogram, it can be seen that the method successfully separated the sample into the three lipid classes of decreasing molecular weight with TAGs eluting first (16 min), DAGs second (16.8 min) and MAGs last (17.5 min). The small peaks eluting before the TAG peak might be polymerised species, yet these were not of interest for the current study. Consequently, the relevant elution range started at approximately 15.0 min. No FFAs were detected in the sample. If these would be present in a sample, they would elute after the MAG peak.

3.1.2. ^{2}D separation: NPLC

After demonstrating that SEC could be successfully employed as the ^{1}D separation mode to separate the three lipid classes of interest (TAGs, DAGs and MAGs), the potential of NPLC to further separate these size classes according to the degree of oxidation based on the polarity of the oxidized species was tested. The oxidation products formed can range from rather non-polar (e.g. with just one epoxide group in the structure) to relatively polar molecules (e.g. with three or more hydroxy groups in a heavily oxidized molecule).

Due to the lack of oxidized-TAG (ox-TAG) standards and their low concentration in oxidized oil compared to non-oxidized TAGs, the method development was initially conducted using DAG standards, since these compounds feature a polarity comparable to some ox-TAGs [21], and can be spiked to the level required for easy detection. This experiment was performed using a 2 mg/mL solution of aged frying oil spiked with 2 mg/mL DAG. Since speed of separation was relevant, an isocratic method was first attempted, as this would eliminate the need for re-equilibration required with

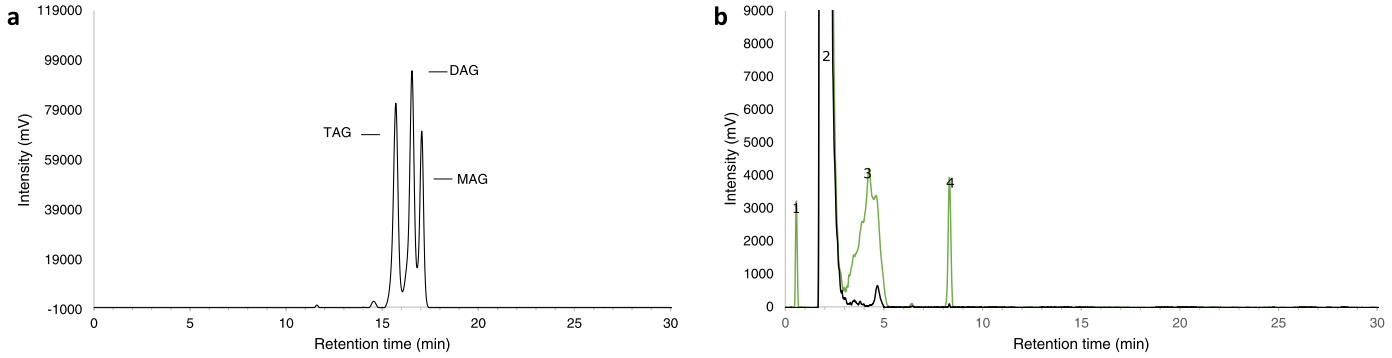

Fig. 1. One dimensional chromatograms of the individual optimization of ^{1}D and ^{2}D a) Lipid class separation of 6 mg/mL mixture of aged frying oil spiked with 6 mg/mL diacylglycerol (DAG) and monoacylglycerol (MAG) by SEC. Two PLgel columns (300×7.5 mm, 5 μm) of 500 Å and 100 Å pore size connected in series were used for the separation. b) Overlaid chromatograms of a 40 mg/mL oxidized oil sample (green chromatogram) and a 44 mg/mL non-oxidized oil sample (black chromatogram) analyzed by normal phase chromatography using a custom-made core-shell silica column (150×4.6 mm, 2.6 μm, 100 Å).

gradient elution. The eluent tested consisted of 90% *n*-hexane as solvent A and 10% toluene/MeOH as solvent B. Five different concentrations of MeOH in toluene (10, 15, 20, 30 and 40%) were tested, but only 10, 15 and 20% MeOH presented sufficient resolution between non-oxidized TAG and DAG peaks (Supplementary data). The best resolution between TAG and DAG was obtained when using 15% of MeOH; hence, this MeOH concentration was chosen to pursue further method optimization.

A concentrated oxidized oil sample (40 mg/mL) was prepared and analysed along with a non-oxidized oil sample (44 mg/mL) using the same NPLC method. The resulting chromatograms are shown in Fig. 1b. Four peaks can be seen to elute in the oxidized oil sample (Fig. 1b). Peak 2 was present in both oil samples. In combination with its high intensity and very short retention time, it was therefore identified as the non-oxidized TAGs. Peak 1, which is not present in the non-oxidised oil sample is hardly retained by the silica stationary phase, so it most likely corresponds to very nonpolar oxidation products. The remaining two peaks (peak 3 and 4) that increased significantly in the oxidized oil (Fig. 1b) are likely to correspond to oxidation products. To confirm this, three fractions were collected for further assessment (peaks 2, 3 and 4 in Fig. 1b). A two-step verification process was performed using first SEC to verify the size of the compounds, and then a direct MS analysis.

SEC analysis was used to estimate the molecular weight of the compounds in the different fractions collected. Fractions 1, 2 and 3 showed peaks that elute at the same retention time (around 16 min) meaning that all of them consisted of molecules of similar size as that of TAGs (Supplementary data). Consequently, it was concluded that all thus originated from TAGs. Minor size differences due to the addition of e.g. a hydroperoxy- or epoxy- group in the molecule during oxidation would not be detected with the current SEC column set. A direct MS analysis was then performed to verify the presence of oxidized TAG species in these three fractions.

The mass spectra obtained for fractions 1 and 2 are presented in Fig. 2. The analysis of fraction 3 was unsuccessful, most likely due to low concentration or use of incompatible ionisation method and will not be further discussed. When focusing on the typical *m/z* range for TAGs (900-1000), clusters at *m/z* 899.7-910.7 (cluster I), *m/z* 915.7-923.7 (cluster II) and *m/z* 929.7-940.7 (cluster III) appeared in different intensities in the two fractions. In fraction 1, cluster I showed a higher response than the other two clusters that were barely visible. Oppositely, in fraction 2, the clusters II and III were more abundant than cluster I. TAGs are the main components (up to 97%) of rapeseed oil and they mostly consist of oleic, linoleic and stearic acid [22]. This yields a variety of TAGs of similar masses since all these fatty acids are C18 fatty acids with just slight differences in molecular mass due to the different degrees of saturation (Table 1). Cluster of ions I corresponded to the sodiated adducts ($[M+Na]^+$) of non-oxidized TAGs with different degrees of saturation (Fig. 2a). A difference of *m/z* 14 was observed between the non-oxidized TAGs and the cluster of ions II, whereas a difference of *m/z* 30 was found between non-oxidized TAGs and cluster III (Fig. 2b). These indicate that the aforementioned unidentified clusters of ions could belong to ox-TAGs with a ketone group and a keto-epoxide or an epidioxide, respectively. These are indeed some of the typical oxidation species also found by Zeb [8] and Ahern *et al.* [23]. Clusters II and III differed by approximately *m/z* 16, *i.e.* the introduction of an oxygen.

Altogether, the results obtained from the SEC and the direct MS analysis showed that fraction 1 mainly contained non-oxidized TAGs while fraction 2 corresponded to the oxidized ones. This confirmed that the developed NPLC method was suitable to separate the non-oxidized TAGs from their oxidation products.

3.2. On-line SEC×NP-ELSD

The results from the individual separation systems suggested that the combination of SEC and NPLC could be used to separate an oil sample according to the (largely independent) dimensions of size and polarity. This motivated the development of a fully automated on-line comprehensive LC set up.

3.2.1. Separation speed optimization of ^{2}D

As mentioned earlier, in order for an on-line LC×LC method to be efficient and allow an acceptable run time, the ^{2}D separation must be fast to ensure that the ^{2}D analysis of a fraction is

Table 1
Main triacylglycerols (TAGs) present in rapeseed oil, their molecular formula, monoisotopic mass, protonated $[M+H]^+$ and sodiated $[M+Na]^+$ adducts.

TAG		Molecular Formula	Monoisotopic	$[M+H]^+$	$[M+Na]^+$
oleic-oleic-oleic	C18:1/C18:1/C18:1	$C_{57}H_{104}O_6$	884.78	885.79	907.77
oleic-oleic-linoleic	C18:1/C18:1/C18:2	$C_{57}H_{102}O_6$	882.76	883.77	905.76
oleic-oleic-linolenic	C18:1/C18:1/C18:3	$C_{57}H_{100}O_6$	880.75	881.76	903.74
stearic-oleic-oleic	C18:0/C18:1/C18:1	$C_{57}H_{106}O_6$	886.79	887.81	909.79

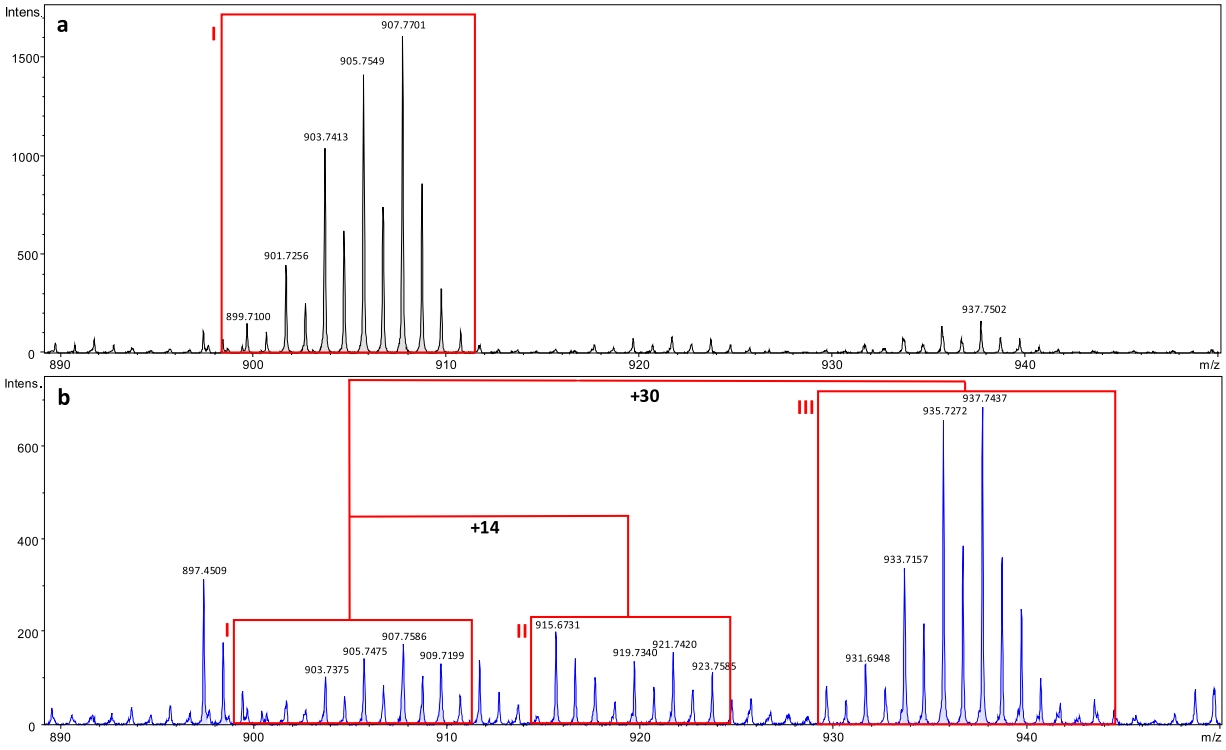

Fig. 2. Direct-inlet mass spectrometric analysis of fractions 1 (a) and 2 (b) collected from normal phase liquid chromatography. The clusters of ions tentatively assigned to compounds of interest are surrounded by a red box. a) Cluster I (m/z 899.7 to 909.7) contains the sodiated adducts ($[M+Na]^+$) of non-oxidized TAGs with different degrees of unsaturation. b) Based on the m/z difference with the non-oxidized TAGs the other two clusters of ions could be assigned. Cluster II (m/z 915.7-923.7) (i.e., + 14 m/z) was assigned to ox-TAGs with a ketone group whereas cluster III (m/z 931.7-940.7) (i.e., + 30 m/z) was assigned to ox-TAGs with either a keto-epoxide or an endoperoxide functionality.

Table 2
Gradients tested during the speed optimization of normal phase liquid chromatography (NPLC) for the second dimension (^{2}D). Peak capacity was estimated based on gradient time divided by the average peak width.

Gradient	Maximum %B	Hold time at min %B (min)	Hold time at max %B (min)	Gradient steepness (%B/min)	Flow rate (mL/min)	MAG retention time (min)	Separation TAG & DAG (min)	Peak capacity
A	50	4	1	40	1	11.5	8.5	13.5
B	70	4	1	60	1	10.5	7.5	13.5
C	90	4	1	80	1	9.5	5	13
D	50	3	0.1	40	1	13	8.5	13
E	50	2	0	20	1	8.5	4	9
F	50	2	1	20	1	8.5	4	9
G	50	1	1	40	1	7	2.5	10.5
H	50	1	1	40	2	4.5	2	12
I	50	1	1	40	3	4	2	10
J	50	1	1	40	4	2.5	0.5	9.5

completed before the subsequent fraction is transferred onto the ^{2}D column. Our aim was to achieve a ^{2}D run time below 5 min as a compromise between ^{2}D resolution and total analysis time.

Oxidized rapeseed oil samples isolated from aged mayonnaise (0.3 mg/mL) spiked with 0.25 mg/mL DAG and MAG standards were used in the experiments for separation speed optimisation. When applying the isocratic conditions from the off-line NPLC method (90%A and 10%B), the MAG peak eluted at 18.5 min, which was clearly unacceptable. Gradient operation was studied to reduce the ^{2}D run time. The quality of the separation obtained was assessed based on run time and the estimated peak capacity. Several settings were optimized: maximum %B reached during the gradient, the steepness of the gradient, hold time at minimum and maximum %B and the flow rate (Table 2). The starting %B was 10% to avoid re-equilibration all the way to 0% polar modifier (MeOH), which would result in an excessive column reconditioning time.

By going from isocratic conditions to gradient elution, the total run time of the method was reduced from 30 to 15 min. As the impact of the maximum %B on the retention time of MAG was limited (Table 2, gradients A-C), 50%B was chosen as the maximum limit for this gradient (i.e. gradient A in Table 2). The optimisation of the steepness of the gradient and hold time at minimum and maximum %B (Table 2, gradients D-G) showed that a steeper gradient in combination with a 1 min hold at maximum %B led to a shorter retention of MAG (~7 min) (i.e. gradient G). Finally, the flow rate of the ^{2}D separation (^{2}F) was optimized (Table 2, gradients H-J). Employing a high ^{2}F (4 mL/min) combined with the adjusted gradient program (Table 2, gradient J) allowed the reduction of the ^{2}D run gradient time of the ^{2}D to 3 min resulting in a total cycle time including re-equilibration of 4 min. Even though peak resolution decreased when optimising the fast separation in the ^{2}D, the present conditions still retained a satisfactory degree of separation.

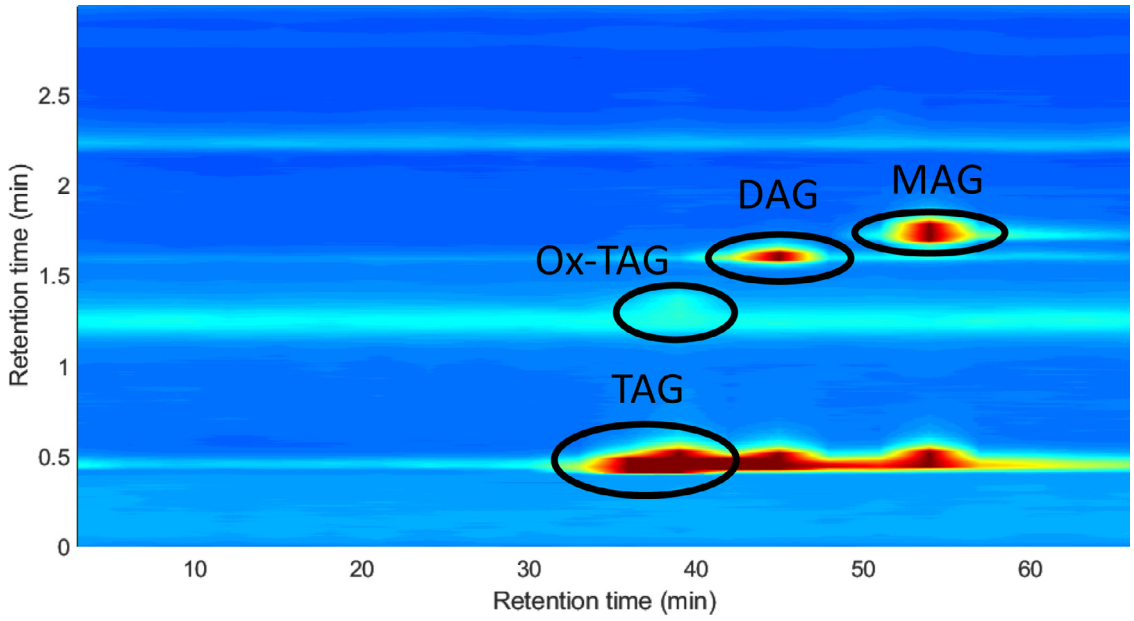

Fig. 3. Comprehensive two-dimensional liquid chromatogram of a 6 mg/mL oxidized oil sample spiked with 6 mg/mL diacylglycerol (DAG) and 6 mg/mL monoacylglycerol (MAG). Size exclusion chromatography was used as ^{1}D and normal phase liquid chromatography for the ^{2}D. First dimension flow rate (^{1}F) from 0 to 13.99 min was 0.8 mL/min and from 14 to 70 min 80 μL/min. Modulation time was 3 min and two 240 μL nominal volume loops were used. MAG, DAG, TAG (and ox-TAG) were clearly separated, but two peaks that were unretained in the ^{2}D (45/0.5 and 55/0.5 min) were noticed.

3.2.2. On-line optimization of LC×LC separation

The transfer of the optimized off-line method to the on-line LC×LC system required additional fine-tuning in the parameters of the ^{1}D separation. In the ^{1}D SEC separation, a flow gradient was employed to rapidly elute the first, empty part of the chromatogram to waste. As a result, only fractions in the separation window of the SEC column were transferred to the ^{2}D. The first-dimension flow rate (^{1}F) started at 0.8 mL/min from 0 to 13.99 min and was then reduced to 80 μL/min.

Fig. 3 shows the separation of a 6 mg/mL oxidized rapeseed oil sample isolated from aged mayonnaise and spiked with 6 mg/mL DAG and MAG. Retention times of the peaks are here reported as ^{1}D/^{2}D, e.g. as x min/y min. In the figure, a good orthogonality between the ^{1}D SEC separation and the ^{2}D NPLC is apparent. The low intensity peak at 38 min/1.3 min most likely belongs to ox-TAGs and is nicely separated from the non-oxidized TAG at 30-42 min/0.5 min. The peak eluting at 45 min/1.6 min represents spiked DAG standard compounds and the last eluting peak (55 min/1.8 min) is the MAG standard. Even though the separation in the ^{2}D is acceptable, two unidentified and unresolved peaks appeared in the lower part of the 2D chromatogram (at 45 min/0.5 min and 55 min/0.5 min, respectively). Peaks at this position would represent compounds with the size of a DAG or MAG but without hydroxy or other polar groups. Since such compounds are not formed in lipid oxidation [20], the bands here must be artifacts possibly caused by non-optimal parameter settings. To investigate their origin, a series of experiments was conducted. The most likely causes for the unretained bands were believed to be severe column overload in the ^{1}D and/or sample breakthrough with the solvent plug in the ^{2}D. The ability to distinguish between these two mechanisms is essential in order to resolve the issue.

Column overload can manifest itself by broad peaks, with signs of fronting and tailing. Large injection volumes and/or of highly concentrated samples are its main causes. Sample breakthrough in the ^{2}D run, on the other hand, is the result of insufficient mixing of the ^{1}D eluent with the ^{2}D solvent. This can result in two separate peaks, one representing the analytes that remain dissolved in the transferred ^{1}D eluent plug and the other for the compounds that are being retained. This sample breakthrough is frequently seen if strong solvents and large fraction volumes are transferred from the ^{1}D to the ^{2}D [24]. In our experiments, neither reducing sample concentration (from 3 to 1 mg/mL) nor decreasing the ^{1}D injection volume (from 20 to 2.5 μL) resolved the issue of the peak splitting into two peaks (Supplementary data). This suggested that the aforementioned peak splitting and the broad band of species eluting at the ^{2}D void time was not due to column overload and hence must be due to sample breakthrough with the solvent plug in the ^{2}D column.

There are a few options for resolving sample breakthrough in the ^{2}D of an LC×LC analysis. The first option is to use a weaker eluent in the ^{1}D. Unfortunately, the use of *n*-hexane as the ^{1}D eluent instead of THF did not improve the chromatogram (results not shown). The second solution is to reduce the fraction volume transferred from the ^{1}D to the ^{2}D column by choosing transfer loops of lower volume. When changing the volume of the sample loops connecting the ^{1}D and ^{2}D, it is important to adjust ^{1}F and the ^{2}D run time to ensure the complete transfer and analysis of sufficient fractions. A 240 μL (experimentally determined volume 235 μL) loop was employed in our initial experiment (Fig. 3). Three different smaller loop volumes were tested, i.e., 157 μL, 50 μL and 50 μL partially filled (30 μL collected from ^{1}D and the rest filled with ^{2}D eluent) (Fig. 4). A clear improvement in the size of the breakthrough peak was observed for the MAG peak when reducing the loop size (Fig. 4 a-c), while the DAG peaks remained unchanged. Based on the results above, the 50 μL loop was selected for the subsequent experiments.

To further study the origin of DAG peak splitting, a series of injections was performed using only the NPLC second dimension of the LC×LC system. A DAG standard (0.1 mg/mL in THF) was tested with four injection volumes, i.e., 50, 20, 10 and 5 μL. The results are shown in Fig. 5. The peak eluting at 2 min was the DAG, the one at 0.5 min was suspected to be due to breakthrough and the one at 2.5 min was a system peak. The characterization of the last peak was not pursued since it was eluting after the compounds of interest, not interfering with the separation and was present in blanks too. Reducing the injection volume gradually decreased the

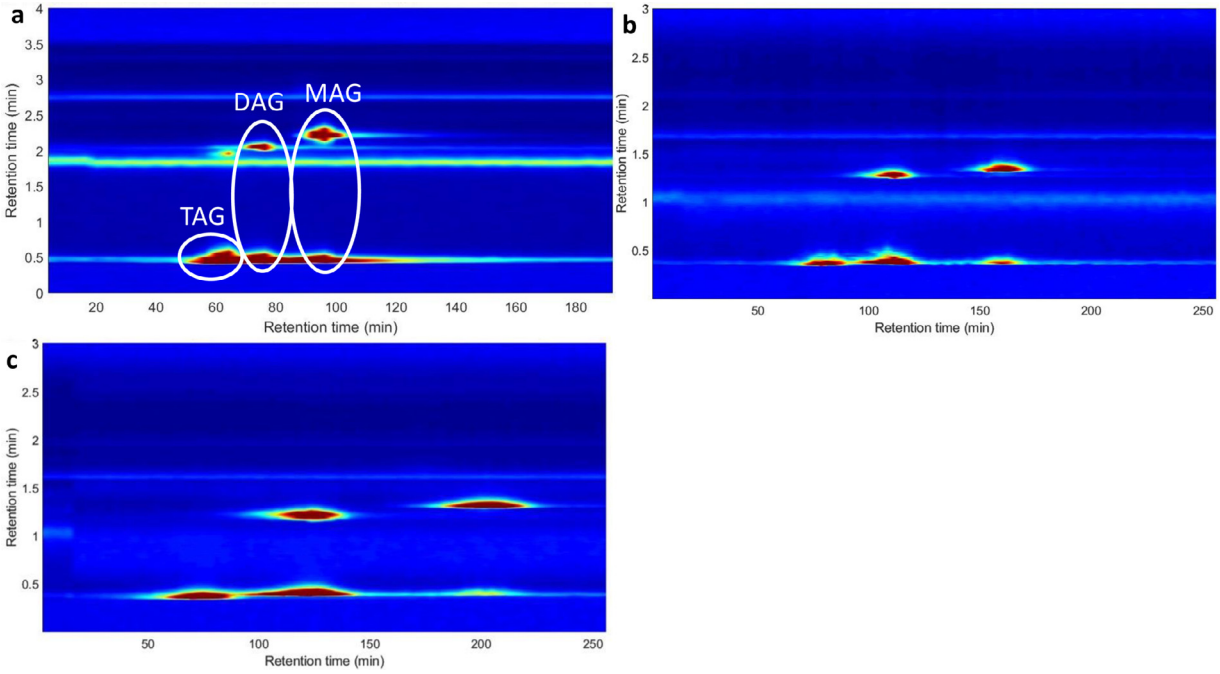

Fig. 4. Comprehensive two-dimensional LC×LC chromatograms of a 1.3 mg/mL non-oxidized rapeseed oil sample spiked with 2.3 mg/mL diacylglycerol (DAG) and 2.3 mg/mL monoacylglycerol (MAG). Three different transfer volumes were used (V_{loop}). Size exclusion chromatography was used for ^{1}D and normal phase liquid chromatography for the ^{2}D. To ensure that the whole loop would be transferred from ^{1}D to ^{2}D, some other settings (e.g., ^{1}F, modulation time) were adjusted. The different run times are caused by the different ^{1}F flows applied. a) V_{loop}= 157 μL, ^{1}F = 40 μL/min, 4 min modulation time and 200 min total run time. b) V_{loop}= 50 μL, ^{1}F = 16.6 μL/min, 3 min modulation time and 260 min total run time. c) V_{loop}= 50 μL partial loop (30 μL effluent from ^{1}D and the rest ^{2}D eluent, ^{1}F = 10 μL/min, 3 min modulation time and 260 min total run time.

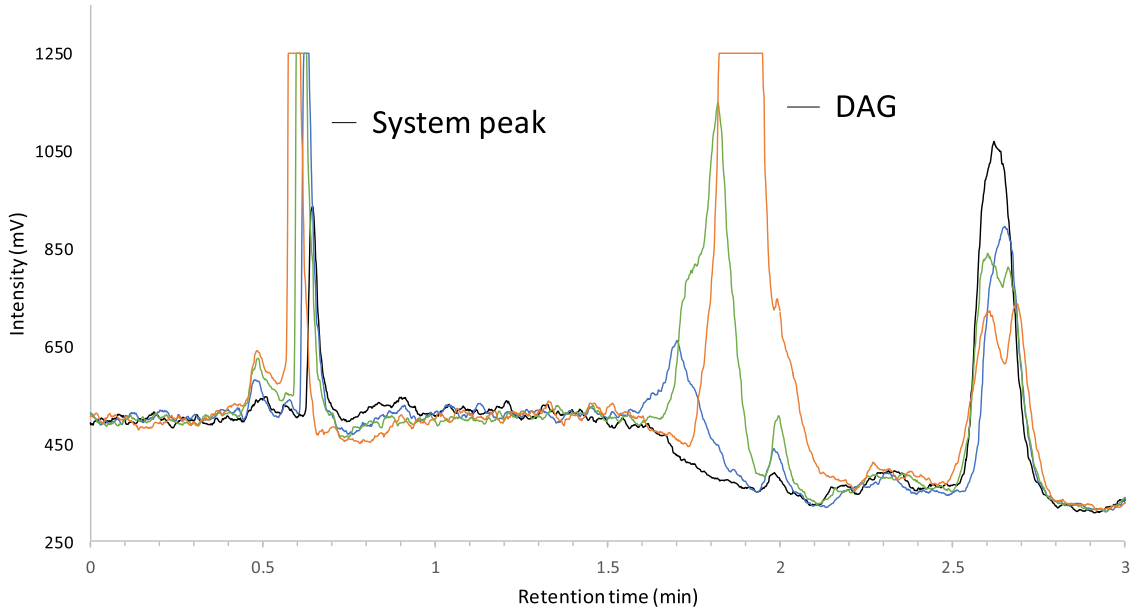

Fig. 5. Overlaid one-dimensional chromatograms of 0.1 mg/mL diacylglycerol (DAG) in THF acquired by normal phase chromatography using a custom-made core-shell silica column (150×4.6 mm, 2.6 μm, 100 Å). Four different injection volumes were tested: 50 μL (orange chromatogram), 20 μL (green chromatogram), 10 μL (blue chromatogram), 5 μL (black chromatogram).

intensity of the DAG peak, but the peak at 0.5 min did not respond in the same way and only started decreasing when the smallest volume was injected and the DAG peak was barely detectable. This suggested that this peak was also a system peak and was not caused by sample breakthrough, but by another distortion mechanism, called peak or solvent displacement [25]. This phenomenon can occur in all forms of chromatography but is most frequently seen in NPLC [26]. It results from displacement of the mobile-phase components adsorbed onto the stationary phase when the sample compounds adsorb. Solvent displacement generates a system peak that elutes unretained. With the universal ELSD detector employed here, there is no solution to this issue.

The applicability of the optimized method was tested by comparing a non-oxidized rapeseed oil to an oxidized rapeseed oil isolated from mayonnaise produced under accelerated aging conditions (both at approx. 50 mg/mL). The latter sample was analysed

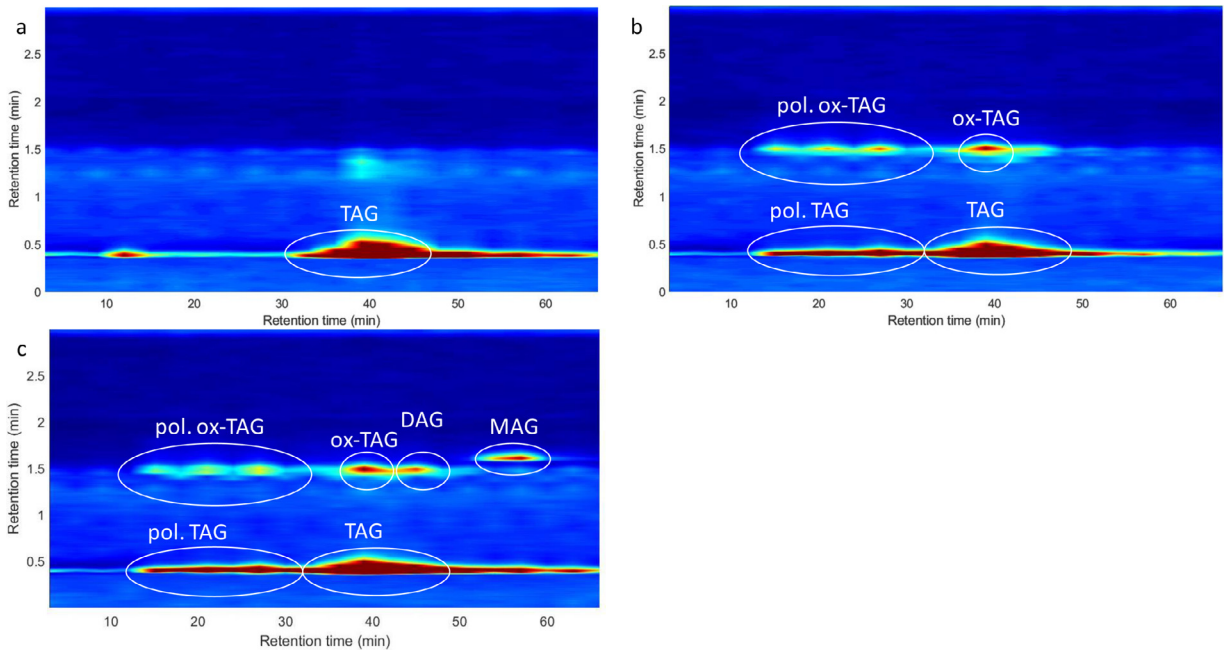

Fig. 6. Comprehensive two-dimensional liquid chromatography separation (LC×LC) of a) 48.5 mg/mL non-oxidized rapeseed oil b) 49.6 mg/mL oxidized oil sample (from the accelerated aging test) c) 49.6 mg/mL oxidized oil sample spiked with 2.5 mg/mL diacylglycerol (DAG) and 2.5 mg/mL monoacylglycerol (MAG). Size exclusion chromatography was used as ^{1}D and normal phase liquid chromatography for the ^{2}D. The optimized parameters were: V_{inj}=35 μL, V_{loop}= 50 μL, ^{1}F from 0 to 14 min 1 mL/min and from 14 to 70 min 40 μL/min, modulation time 3 min and total run time 70 min.

either as such or spiked with DAG and MAG (at 2.5 mg/mL each) to facilitate peak identification. In order to separate, detect and identify the oxidized compounds in the aged oils, the sample concentration, injection volume, and ^{1}F were adjusted. The final parameters were: sample concentration around 50 mg/mL, 35 μL injection volume, ^{1}F started at 1 mL/min from 0 to 13.99 min and was then reduced to 40 μL/min, 50 μL transfer loop volume, ^{2}F at 4 mL/min and 3 min modulation time. Although based on the optimal conditions of the LC×LC method each ^{1}D volume fraction was 120 μL, transfer loops of 50 μL were preferred, because larger transfer volumes resulted in a significant volume overloading and resolution loss in the ^{2}D. The results are shown in Fig. 6. The non-oxidized oil sample (Fig. 6a) showed a clear peak of non-oxidized TAG at 40 min/0.5 min; the small, very light peak appearing around 40 min/1.5 min suggested that the oil was already slightly oxidized. The oxidized oil sample (Fig 6b) presented four main groups of peaks, i.e. non-oxidized TAGs eluting between 40 min/0.5 min, ox-TAGs that elute around 40 min/1.5 min and two more groups of peaks eluting at 12-30 min/ 0.5 min and 12-30 min/1.5 min. These two groups of peaks most likely correspond to polymerized TAG, non-oxidized and oxidized, respectively. Polymerization products are readily formed from radicals [20]. Their rapid formation is enhanced during early stages of oxidation when heating is applied. The use of 150 °C for 5 h during the accelerated aging test promoted the formation of these higher molecular weight compounds.

The analysis of an oxidized oil sample spiked with DAG and MAG (Fig. 6c) confirmed that the optimized method successfully separates at the same time all lipid species present in the sample (polymerized TAG, TAG, DAG and MAG), as well as ox-TAG from non-oxidized TAG, in one chromatographic run. Further optimization, such as the use of a shorter ^{2}D column, could improve the performance of the method in terms of e.g. coverage of the 2D separation space or sensitivity even further. This work has shown that it is possible to separate the compounds of interest into groups of similar size and polarity. This already provides a good insight in the identity of the oxidation products formed. If identification to the molecular level or a better sensitivity is needed, MS detection can be employed. Clearly, the novel method provides an enhanced level of detail in the analysis of oxidized lipid species. In particular, it also solves the issue of the interference between non-oxidized MAGs and DAGs and low levels of oxidized TAGs.

4. Conclusions

In this work, a novel on-line comprehensive LC×LC-ELSD method was developed for the separation of lipid classes and their oxidation products. The combination of SEC and NPLC, as the ^{1}D and ^{2}D respectively, successfully achieved the simultaneous separation of all compounds of interest (polymerized TAG, TAG, DAG, MAG, ox-TAG and polymerized ox-TAG) in one chromatographic run. Moreover, the final total run time of 70 min is considered relatively short for an on-line comprehensive LC×LC analysis. Sources for peak distortion problems were diagnosed and were solved when possible. Solvent displacement in the NPLC dimension is a particular concern that cannot be avoided. Despite that, this method can facilitate the elucidation of lipid oxidation pathways in emulsified foods and aids in the development of more oxidation-stable products.

Declaration of Competing Interest

Hans-Gerd Janssen is employed by Unilever, a multi-national company in the field of foods and home and personal care products.

CRediT authorship contribution statement

Eleni Lazaridi: Investigation, Methodology, Visualization, Writing - original draft. **Hans-Gerd Janssen:** Conceptualization, Resources, Supervision, Writing - review & editing. **Jean-Paul Vincken:** Supervision, Writing - review & editing. **Bob Pirok:** Methodology, Resources. **Marie Hennebelle:** Conceptualization, Supervision, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462106.

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