RESEARCH ARTICLE

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Oxidation of Cyclo-Lino Peptides in Linseed Oils during Storage

Ludger Brühl, Anja Bonte, Katharina N'Diaye, and Bertrand Matthäus*

Linseed oil is appreciated for its high level of linolenic acid. In order to avoid oxidation, the precious storage lipids are protected in the seeds by lignans, tocopherols, and cyclo-lino peptides. Oxidation of specific cyclo-lino peptides can be attributed to the formation of a bitter off-taste in cold pressed linseed oils. A complete degradation of tocopherols and of intact cyclo-lino peptides CLO, -M, -N, -L, and -B to oxidized forms is observed with intermediate oxidation products of the peptides containing methionine oxidized to methionine sulfoxide and further to methionine sulfone during a storage period of 25 d at 60 °C in the dark and of 14 weeks at day light and room temperature. The peroxide value increases to 255 meq O_2 kg^{-1} at the same time. However, during storage at room temperature in the dark only a very limited decrease of the initial tocopherols content of 400 mg/100 g is observed contrasted by an increase of the peroxide value to about 50 meq O_2 kg^{-1} and a more or less complete degradation of cyclo-lino peptides. While at 6 °C in the dark all oxidation parameters keep stable. The tocopherols and cyclo-lino peptides show different and independent degradation during these storage conditions.

***Practical Application*: This work elucidates the role of cyclo-lino peptides and tocopherols in the complex reactions taking place during oxidation of linseed oil and enables a first evaluation of the antioxidative power of cyclo-lino peptides at different storage conditions.**

1. Introduction

Linseed oil is highly appreciated for human consumption due to its outstanding high level of linolenic acid at a range of up to over 50%. Linolenic acid (ALA) is a fatty acid belonging to the group of omega-3 fatty acids and many studies examined effects of linseed oil on diseases like cardiovascular disorders, osteoporosis, obesity, and cancer.[1,2] However, linolenic acid is also prone to oxidation due to the high number of double bonds.[3–5] Therefore, linseed protects the precious storage lipids against oxidation by several means such as the lignans in the coating of the seeds and in addition tocopherols and hydrophobic cyclic peptides, which can be found in the oil.[6–8]

In parallel to the oxidation of linseed oil, a bitter off-taste is formed with the storage time, which can be attributed to the formation of cyclo-lino peptide E (CLE), which is one species of a group of cyclic peptides found and identified in linseed oil.[9,10] A first cyclo-lino peptide (CLP) was already identified in 1959 by Kaufmann and Tobschirbel in linseed oil and especially in the foots from the degumming of linseed oil.[11] Up to now a number of additional CLPs were isolated and identified.[12–16] These CLPs are cyclic peptides of eight or nine amino acids. The amino acids are linked by peptide bonds and comprise proline, phenylalanine, leucine, iso-leucine, valine, tryptophan, and methionine in different combinations. The latter methionine was also detected in its mono- and di-oxidized form as methionine sulfoxide and methionine sulfone as a result of oxidation reactions in the oil. In the freshly extracted oil, these oxidation products are only found in small amounts. The bioactivities of CLPs have been reviewed by Dahiya et al.,[17] while the nomenclature was reviewed by Shim et al.[18] as different systems were used by authors in the past. A summary of the nomenclature of the CLPs and the acronyms used in this study are given in **Table 1**.

Storage stability of CLPs was studied by Aladedunye et al.[19] in linseed oil and meal. They detected a decrease for the methionine containing CLPs and an increase of methionine sulfoxide containing CLPs in the oil, whereas there were no significant changes in the meal. Jadhav et al.[15] searched for the presence of methionine sulfone containing CLPs and detected them in oxidized ground linseed heated at 100 °C for more than 4 h. As very harsh conditions were needed for their formation, their detection could serve as a proof of severe oxidation. The stabilizing properties of CLPs were examined by Sharav et al.[7] They observed a reduced stability of linseed oil after a complete removal of CLPs by adsorption on acidified silica. In another work, Zou et al.[20] proposed to use the content of methionine sulfone containing CLPs as indicators to evaluate the oxidation process of linseed oils. They showed that methionine containing CLPs significantly oxidized before γ-tocopherol, while the decrease of γ-tocopherol

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Table 1. Summary of the nomenclature of cyclo-lino peptides with names according to ref. [18], acronym used in this work, amino acid sequence, chemical formula, mass obtained by LC-MS-Q-TOF as [M+H] [g mol^{-1}], peak numbers as assigned in Figure 6 and retention times [min].

Name	Acronym	Amino acid sequence	Chemical formula	Mass [M+H]	Peak no.	RT [min]
[1-9-NαC]-CLA	CLA	ILVPPFFLI	$C_{57}H_{85}N_9O_9$	1040.6540	17	15.0
[1-9-NαC]-CLB	CLB	MLIPPFFVI	$C_{56}H_{83}N_9O_9S$	1058.6090	16	13.9
[1-9-NαC],[1-Met-O]-CLB	CLC	MsoLIPPFFVI	$C_{56}H_{83}N_9O_{10}S$	1074.6050	5	5.7
[1-9-NαC],[1-Met-O_2]-CLB	CLK	MsnLIPPFFVI	$C_{56}H_{83}N_9O_{11}S$	1090.5940	8	9.2
[1-8-NαC]-CLD	CLM	MLLPFFWI	$C_{57}H_{77}N_9O_8S$	1048.5680	21	19.4
[1-8-NαC],[1-Met-O]-CLD	CLD	MsoLLPFFWI	$C_{57}H_{77}N_9O_9S$	1064.5640	12	11.3
[1-8-NαC],[1-Met-O_2]-CLD	ND	MsnLLPFFWI	$C_{57}H_{77}N_9O_{10}S$	ND	ND	ND
[1-8-NαC]-CLE	CLL	MLVFPLFI	$C_{51}H_{76}N_8O_8S$	961.5570	18	15.4
[1-8-NαC],[1-Met-O]-CLE	CLE	MsoLVFPLFI	$C_{51}H_{76}N_8O_9S$	977.5520	6	8.1
[1-8-NαC],[1-Met-O_2]-CLE	CLJ	MsnLVFPLFI	$C_{51}H_{76}N_8O_{10}S$	993.5300	13	11.4
[1-8-NαC]-CLF	CLN	MLMPFFWV	$C_{55}H_{73}N_9O_8S_2$	1052.5080	19	17.0
[1-8-NαC],[1-Met-O]-CLF	CLI + CLI[a)]	MsoLMPFFWV	$C_{55}H_{73}N_9O_9S_2$	1068.5030	10 + 11	10.5 + 11.1
[1-8-NαC],[3-Met-O]-CLF	CLT	MLMsoPFFWV	$C_{55}H_{73}N_9O_9S_2$	1068.5030	7	9.0
[1-8-NαC],[1,3-Met-O]-CLF	CLF + CLF[a)]	MsoLMsoPFFWV	$C_{55}H_{73}N_9O_{10}S_2$	1084.5010	1 + 2	3.9 + 4.1
[1-8-NαC]-CLG	CLO	MLMPFFWI	$C_{56}H_{75}N_9O_8S_2$	1066.5230	20	18.4
[1-8-NαC],[1-Met-O]-CLG	CLP + CLP°	MsoLMPFFWI	$C_{56}H_{75}N_9O_9S_2$	1082.5200	14 + 15	12.1 + 12.7
[1-8-NαC],[3-Met-O]-CLG	CLH	MLMsoPFFWI	$C_{56}H_{75}N_9O_9S_2$	1082.5200	9	10.1
[1-8-NαC],[1,3-Met-O]-CLG	CLG + G[a)]	MsoLMsoPFFWI	$C_{56}H_{75}N_9O_{10}S_2$	1098.5140	3 + 4	4.9 + 5.1
[1-9-NαC]-CLQ	ND	MLKPFFWI	$C_{66}H_{87}N_{11}O_9S$	ND	ND	ND
[1-9-NαC],[1-Met-O]-CLQ	ND	MsoLKPFFWI	$C_{66}H_{87}N_{11}O_{10}S$	ND	ND	ND
[1-9-NαC]-CLR	ND	GIPPFWLWL	$C_{54}H_{76}N_{10}O_{10}$	ND	ND	ND

[a)] Split peaks; ND: not detected; F: phenylalanine, G: glycine, I: isoleucine, K: lysine, L: leucine, M: methionine, Mso: methionine sulfoxide, Msn: methionine sulfone, P: proline, V: valine, W: tryptophane.

was accompanied by an increase of the acid, peroxide, and anisidine values.

The aim of this work was to follow up the degradation of the different CLPs during storage to classify their antioxidant activity. We used different storage scenarios in the dark with temperatures at 6 °C, room temperature (RT), and 60 °C and at ambient light at room temperature. The different storage conditions were chosen to reflect storage of linseed oil at accelerated storage conditions (60 °C, dark), production and transport (RT, dark), commercialization (RT, light), and household (6 °C, dark). In addition, we analyzed the peroxide value (POV) and tocopherol content to examine the effect of these antioxidants in comparison to the fate of the CLPs. The results will give an insight into the dependencies of the different antioxidative effects of the individual CLPs and whether there is any relationship to the degradation of tocopherols.

2. Experimental Section

2.1. Linseed Oil

About 3 kg of linseed from a local supplier were pressed by screw press (IGB Monforts, Germany) with a rotation frequency of 32 min^{-1} and a nozzle of 6 mm ID. The oil obtained (about 1000 mL) was filtered under vacuum through a paper filter (Machery & Nagel, Germany) in order to remove fine sediments from the seeds and to obtain a clear, yellow oil. Care was taken to avoid excessive foaming or squirting by allowing the oil to drain out with a constant contact to a surface. Bottles were blanketed with nitrogen and headspace was avoided, where possible.

2.2. Reagents

Isolation of CLPs: Methanol, ultragradient grade for HPLC, was purchased from Fisher Scientific (Schwerte, Germany), acetic acid ethyl ester (analysis grade > 99%) and acetonitrile, ultra LS-MS, from Roth (Karlsruhe, Germany), diethyl ether was freshly redistilled in the laboratory, petroleum ether (boiling range 40–60 °C), *n*-heptane (analysis grade), silica gel (particle size from 0.063 to 0.200 mm) all from Merck (Darmstadt, Germany), and water was obtained from a Milli-Q water purification system. The silica gel was dried overnight in an oven at 105 °C and stored in a desiccator to cool to room temperature. An amount of 3 g of water per 100 g of silica gel was added and the silica gel was equilibrated after homogenization overnight before use. SPE glass cartridges of 6 mL volume were filled with a filter disc and covered by a layer of 0.5 g of silica gel with a water content of 3 g/100 g. On top of the settled silica gel a second filter disc was placed for easy solvent addition. A set of four solvent mixtures were prepared for elution: solvent mixture 1 consisted of 5 mL acetic acid ethyl ester in 95 mL of *n*-heptane, mixture 2 of 10 mL acetic acid ethyl ester in 90 mL of *n*-heptane, mixture 3 of 50 mL acetic acid ethyl ester in 50 mL of *n*-heptane, and mixture 4 of 6 mL diethyl ether in 34 mL of petroleum ether.

2.3. Storage Experiments

Linseed oil was stored in bottles of 100 mL at 6 °C in the dark (12 months), at room temperature in the dark (26 weeks) as well as at daylight (30 d) and at 60 °C in an oven (30 d). Samples of about 25 g were taken from the beginning and in regular intervals from the linseed oil, sealed and stored at −18 °C until analysis.

2.4. Sample Preparation of Linseed Oil for CLP Analysis

Sample preparation was done following in principle the isolation procedure described in ref. [13] with modifications according to the following protocol. Linseed oil (1.5 g) was solved in 4 mL of solvent mixture 1 and purred onto a silica SPE column prepared according to Section 2.2, which was preconditioned by 6 mL of solvent mixture 1. Interfering substances were removed by elution with each 5 mL aliquots of solvent mixtures 2–4. Afterward, CLPs were eluted by elution with 9 mL of pure methanol. The solvent was evaporated in a stream of nitrogen and the residue was dissolved in 1 mL of methanol. The solution was passed through a 0.45 µm syringe filter and used for HPLC injection.

2.5. Separation and Determination of the Relative Composition of CLPs

Separation and determination were done following in principle the separation conditions as described in ref. [13] with some modifications on a Lachrom HPLC system consisting of a HPLC gradient pump, column oven, autosampler with 10 µL sample volume, UV detector at 207 nm, and RP-18e separation column from Merck (Darmstadt, Germany). Elution started with 1 mL min^{-1} and a mixture of 60% water and 40% acetonitrile which was changed to 35% water and 65% acetonitrile in 20 min, then to 25% water and 75% acetonitrile in 5 min and further to 100% acetonitrile in 2 min. After 10 min of isocratic elution the system was reconditioned in 8 min to the initial composition. Figure S1 (Supporting Information) shows an example of the separation. As no reference standard substances were available no quantitative results were obtained, but the signal areas of the different CLPs were compared without any response factors.

2.6. Tocopherol Analysis

Tocopherol analysis was carried out according to method DGF-F-II 4a (00). In brief, 150 mg of linseed oil were dissolved in 1 mL of *n*-heptane. After two filtrations with a syringe filter 1.0 µm and another with 0.45 µm the sample was used for the HPLC without further treatment. The HPLC system consisted of a Merck-Hitachi low-pressure gradient system, fitted with a L-6000 pump (Merck-Hitachi, Darmstadt, Germany), a Merck-Hitachi F-1000 fluorescence spectrophotometer (Darmstadt, Germany; detector wavelengths for excitation 295 nm, for emission 330 nm), and a ChemStation integration system (Agilent Technologies Deutschland GmbH, Böblingen, Germany). The samples were injected in amounts of 20 µL by a Spark Holland Basic Marathon autosampler (Spark-Holland Emmen, The Netherlands) onto a Diol phase HPLC column 25 cm × 4.6 mm ID (Merck, Darmstadt, Germany). The flow rate of the mobile phase (*n*-heptane/*tert*-butyl methyl ether; 489:11 v/v) was adjusted to 1.3 mL min^{-1}.

2.7. Oxidative Stability

Oxidative stability was done according to method DGF-C-VI 6 f (06) (DGF, 2021) using a *Rancimat* 743 (Metrohm, Herisau, Switzerland). In brief, 3.6 g of linseed oil was weight into a reaction tube which was placed into the heating block kept at 120 °C with an airflow of 20 L h^{-1}. The volatile compounds formed and released during the oxidation process, together with the air, were passed into a flask containing distilled water and an electrode for measuring the conductivity. At the end of the induction period the conductivity started to increase rapidly, and the system automatically calculated the induction period by using the maximum of the second derivative of the curve.

2.8. Peroxide Value

Peroxide value was determined according to the modified method ISO 3976:2006 (E) – Milk fat – Determination of peroxide value.[21] In brief, an appropriate part of linseed oil (0.1 g or an aliquot) was dissolved in 9.60 mL of a mixture of methanol/1-decanol/*n*-hexane in ratio 3:2:1 (v:v:v). Then 0.05 mL of ammonium thiocyanate solution (300 mg mL^{-1}) were added and mixed before 0.05 mL of iron (II) chloride solution (1 mg mL^{-1}) were given to the mixture. After 10 min reaction time in the photometer cell and equilibration in the photometer the measurement was done at 500 nm against methanol/1-decanol/*n*-hexane mixture (E_2). In addition, E_0 (test blank) and E_1 (reagent blank) were also measured against methanol/1-decanol/*n*-hexane mixture. The corrected extinction E was calculated as $E = E_2 - (E_0 + E_1)$. The mass (m_c) of Fe_3^+, expressed in micrograms, was calculated by using the following equation $M_c = E/b$ with b as numerical value of the extinction coefficient of the red iron (III) complex. From M_c the peroxide value of the linseed oil (PV), expressed as millimoles of oxygen per kilogram linseed oil is calculated: $PV = 0.5\ m_c/55.84m$, with m as mass, in gram, of the test portion.

2.9. Identification and Confirmation of CLPs

2.9.1. Solvents and Solutions

Acetonitrile (≥99.9%, Avantor Performance Materials, Gliwice, Poland), ESI-L low concentration mix (Agilent, Santa Clara, CA), formic acid eluent additive for LC-MS (>97.5%, Sigma Aldrich, St. Louis, MO), isopropanol (Chromasolv LC-MS ≥ 99.9%, Honeywell Riedel-de Haën, Seelze, Germany), methanol (Optima LC-MS ≥ 99.9%, Fisher Scientific, Loughborough, UK), and water (LC-MS Optigrade, LCG Promochem GmbH, Wesel, Germany) were used to run the HPLC-Q-TOF (on-line coupled mass detectors with quadrupole and time-of-flight technique).

2.9.2. Instrument Settings

Analysis was performed on an Ultimate 3000 UHPLC (Thermo Fisher Scientific, Dreieich, Germany), combined with a Maxis

Table 2. UHPLC gradient for separation with LC-Q-TOF.

Time[min]	Eluent B[%]	Flow[mL min^{-1}]
0	40	0.3
5	40	0.3
25	65	0.3
26	75	0.3
32	99	0.6
33	99	0.6
36	40	0.3

Impact HR-Q-TOF (Bruker Daltonics, Bremen, Germany), and separation of CLPs was achieved on a Kinetex core–shell phenyl-hexyl column with 100 mm × 2.1 mm × 2.6 µm parameter (Phenomenex, Aschaffenburg, Germany). The flow rate was set to 0.3 mL min^{-1} at 30 °C column temperature. Eluent A contained 95% water, 5% acetonitrile, and 0.1% formic acid, whereas eluent B consisted of 95% acetonitrile, 5% water, and 0.1% formic acid. UHPLC gradient is shown in **Table 2**. The mass spectrometry was done with an ESI source in positive mode and a scan interval from 50 to 2200 m/z. The capillary voltage was set at 4500 V and the end plate offset at −500 V. The nebulizer was operated at 3 bars and the dry heater maintained at 180 °C with a dry gas current of 10 L min^{-1} and a total transfer time of 120 µs. The quadrupole was run with an ion energy of 5.0 eV and an isolation mass of 200 m/z. Internal mass calibration was performed at high precision calibration (HPC) with ESI-L low concentration mix.

Extracts from Section 2.4 were diluted 1:10 with 50% acetonitrile and 1 µL were injected into an UHPLC-Q-TOF system and data obtained were compared with ref. [22]. Data analysis was conducted with the Data analysis 4.2 software (Bruker Daltonics, Bremen, Germany). Mass spectra were recalibrated internally and peaks were integrated on extracted ion chromatograms (EIC) of CLP masses as shown in Table 1, with a mass tolerance of ±0.05.

3. Results and Discussion

3.1. Separation of CLPs

For the analysis of the CLP composition two different systems were used. While the CLPs were separated for all storage experiments on a common endcapped reversed phase column, some extracts were analyzed in addition on a second system with a core–shell phenyl-hexyl column and Q-TOF identification to clarify the composition of any overlapping peaks. The reversed phase column achieved acceptable resolution for most CLPs as can be seen in Figure S1 (Supporting Information). In both separation systems some overlapping of signals occurred. A combined signal was observed for CLP° and CLJ as well as for CLE and CLK and a split peak was observed for CLF, CLG, CLP/CLP°, and CLI /CLI* using the reversed phase column as already described by ref. [22]. The core–shell phenyl-hexyl column used for Q-TOF identification also failed in resolving all peaks. However, due to the exact mass assignments all CLPs described in literature were identified as shown in Figure S2 (Supporting Information). For verification of the identification of individual CLP fractions were collected from the RP-18 HPLC system containing single species or mixtures of CLPs with nonresolved overlapping signals and reinjected them after reconcentration into the Q-TOF system.

3.2. Increase of Peroxide Value during Storage of Linseed Oils

Linseed oils were stored at four different storage conditions: in the dark at 6 °C (A), RT (B), and 60 °C (C) as well as in ambient light at RT (D). The peroxide value was monitored in regular intervals and **Figure 1** shows the effect of the different storage conditions from A to D. No significant increase of the POV was observed at 6 °C up to about 12 months with a final maximum level at 2 meq O_2 kg^{-1}. During storage at RT in the dark an increase was recognized after an induction period of six weeks reaching a final maximum level at 45 meq O_2 kg^{-1}. At 60 °C an increase was noticed after 5 d of induction period with a final maximum level already reached after 24 d at about 200 meq O_2 kg^{-1}. However, during storage at daylight no stable initial period can be observed, but a steady increase right from the beginning and a final maximum level of more than 200 meq O_2 kg^{-1} was reached after 14 weeks. This is in line with literature as light enables the formation of singlet oxygen, which is a powerful oxidant and no lag-time can be observed due to availability of increased activation energy.[23]

3.3. Tocopherol and Tocotrienol Contents of Linseed Oils during Storage

Tocopherols and tocotrienols protect unsaturated fatty acids for oxidation by interrupting the oxidative radical chain reaction.[24,25] The linseed oil contained γ-tocopherol as the major tocopherol and plastochromanol-8, a tocotrienol, at a level of 295 and 119 mg kg^{-1}, respectively. In addition, small amounts of 7 mg kg^{-1} α-tocopherol and 3 mg kg^{-1} δ-tocopherol were detected. These results are in good agreement to literature.[26–30] However, other studies only reported the presence of γ-tocopherol.[20,31] **Figure 2** shows the reduction of the contents of γ-tocopherol and plastochromanol-8 in the course of the storage experiment. For storage at 6 °C and RT in the dark no significant decrease of both substances was observed, while at 60 °C their initial contents were depleted during a period of 20 d and in daylight during 12 weeks. The degradation at 60 °C in the dark was very comparable to the findings of Zou,[20] who also reported a complete diminishing in 20–24 d depending on variety. Tanska et al.[28] reported in their study that plastochromanol-8 showed the highest effect to improve oil stability at 4 ± 2 °C in a refrigerator during three months of storage, while in our study both substances degraded in parallel. Wagner et al.[32] also examined the effects of tocopherols on the oxidative stability of heated linseed oils and observed extended induction periods after addition of γ-tocopherol up to a level of 100 mg/100 g in addition to the initial content of linseed oils stored at 120 °C. However, oxidation reactions at higher temperatures like 120 °C differ significantly from results

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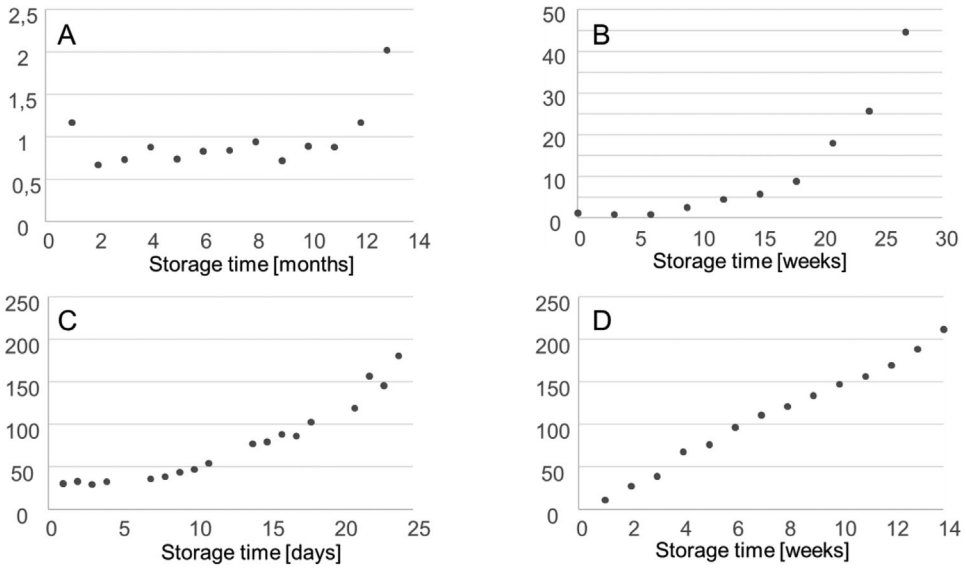

Figure 1. Peroxide value [meq O_2 kg^{-1}] of linseed oils stored in the dark at A) 6 °C, B) RT, and C) 60 °C as well as D) in daylight at RT.

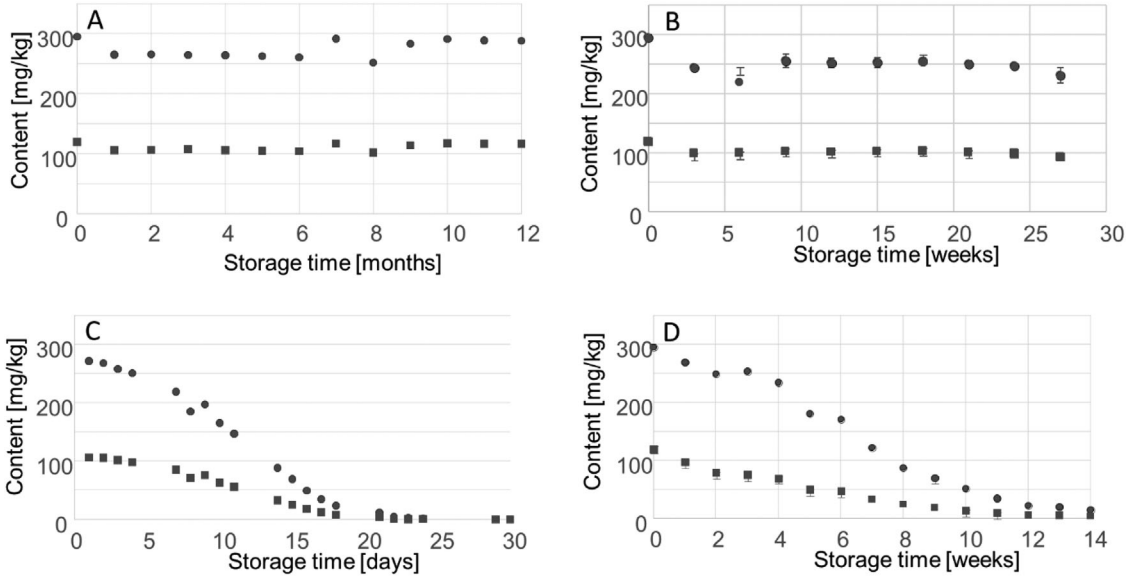

Figure 2. γ-Tocopherol (●) and plastochromanol-8 (■) content [mg kg^{-1}] in linseed oils stored in the dark at A) 6 °C, B) RT, and C) 60 °C as well as D) in daylight at RT.

obtained at moderate temperatures up to 60 °C as intermediate stable hydroperoxides will degrade rapidly.[33]

3.4. Oxidation of Cyclo-Lino Peptides at Room Temperature in the Dark

Figure 3 shows the intensities of selected CLP peaks as area counts in order to follow up the oxidation of CLO during the storage experiment at room temperature in the dark for 26 weeks. The results obtained for CLO, CLP°, CLP, CLH, and CLG were combined to reflect the oxidation pathway of individual CLPs as schematically described in Figure S1 (Supporting Information). Since CLO contains two methionine molecules in the amino acid sequence (MLMPFFWI, see Table 1 for abbreviations) both of them could be oxidized individually. In a first step this led to the formation of CLH (MsoLMPFFWI) and CLP (MLMsoPFFWI), where one of these two methionines were oxidized to methionine sulfoxide. In a second step the second methionine was also oxidized and led to the formation of CLG (MsoLMsoPFFWI) containing two methionine sulfoxides in its sequence. The content of CLO decreased from initial 0.5 E^6 to not detectable in a time interval of about 13 weeks, while CLH showed a very small maximum at three weeks and its signal was overlapped by the

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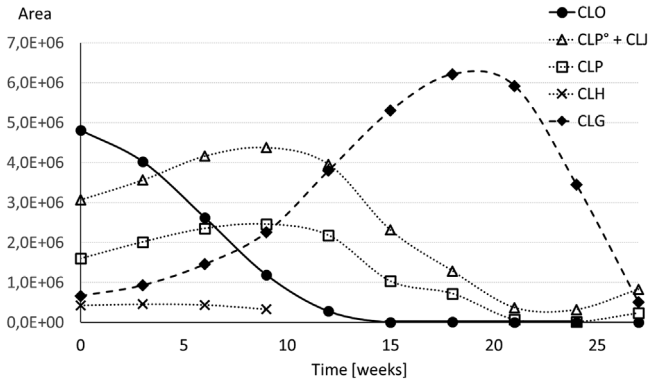

Figure 3. Signal area of cyclo-lino peptides CLO, CLP°, CLP, CLH, and CLG during storage of linseed oil for 26 weeks at room temperature in the dark. For abbreviation of individual cyclo-lino peptides, see Table 1.

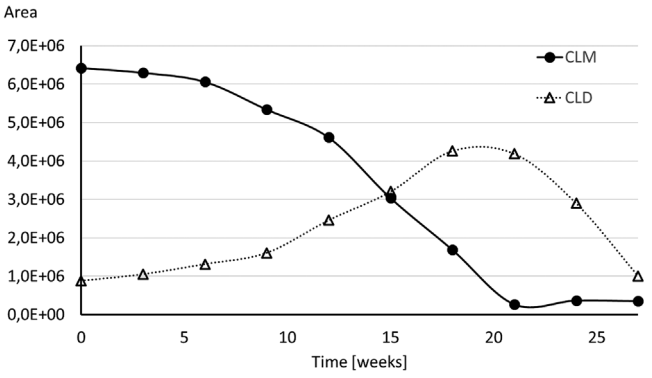

Figure 4. Signal area of cyclo-lino peptides CLM and CLD during storage of linseed oil for 26 weeks at room temperature in the dark. For abbreviation of individual cyclo-lino peptides, see Table 1.

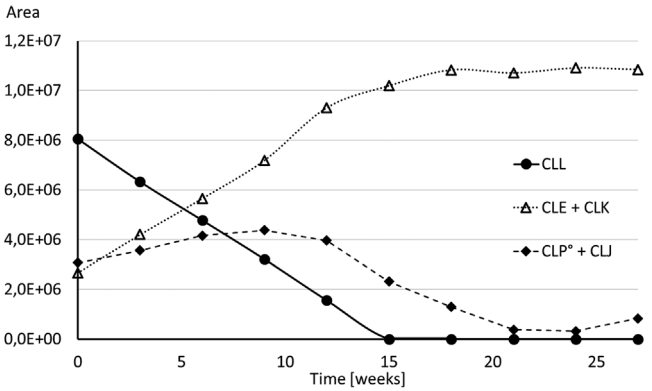

Figure 5. Signal area of cyclo-lino peptides CLL, CLE (+CLK), and CLJ (+CLP°) during storage of linseed oil for 26 weeks at room temperature in the dark. For abbreviation of individual cyclo-lino peptides, see Table 1.

increasing signal of CLE after a time interval of nine weeks and therefore was not evaluated any more. The initial level of CLH at 0.05 E^6 was much lower compared to the signals for CLP° and CLP, which started at about 0.15 E^6 and 0.3 E^6, passed their maximum at nine weeks of storage and decreased until the signal of CLP° was also overlapped in the final measurement by CLJ. A reason for the split peak of CLP° and CLP showing exact the same masses was not recognized, but was also observed by ref. [22]. The signal for the double oxidized CLG rose from a very low content to about 0.6 E^6 and passed its maximum at 20 weeks with a subsequent decreasing below the starting level. This decrease might be attributable to the formation of a more oxidized form containing one or even two methionine sulfones instead of methionine sulfoxide. However, although corresponding masses were monitored in the data set of the whole chromatogram using LC-MS Q-TOF no signal in the provisioned mass interval could be detected. This was in contrast to the results obtained for CLB and CLL (see below), where we could identify CLK and CLJ, respectively, as CLPs containing a methionine sulfone instead of methionine sulfoxide. This might be due to an increased polarity or further reactions taking place, which lead to a dimerization, ring opening or other chemical changes of such further oxidized products that these substances were not recovered any more by the sample clean-up procedure.

Figure 4 shows the intensities of the signals of CLM and CLD as its first oxidation product. CLM degraded much slower than CLO in a time interval of about 20 weeks. For CLD no further oxidation product was identified searching for the provisioned molar mass of 1080.54 for $[M+H]^+$ ion and a postulated CLP with the amino acid sequence of MsnLLPFFWI although the signal of CLD declined to the initial level. Nevertheless, these findings were in accordance to ref. [22].

Figure 5 shows the intensities of the signals of CLL, CLE, and CLJ. In this case the two steps of oxidation from methionine to methionine sulfoxide and methionine sulfone could be observed. However, the signal of CLJ was severely overlapped with the signal of CLP°. Therefore, the first part of the data set is more or less dominated by CLP° up to week 23 and only the last increase of this signal might be attributed to the formation of CLJ. This was confirmed by LC-MS-Q-TOF for the storage experiments at 60 °C in the dark (see Figures S17–S21, Supporting Information).

During the storage at room temperature in the dark a high level of CLE was apparently maintained during the time interval of week 20–26. However, the signal of CLE was overlapped by the signal of CLK, which was assumed to be formed as the second oxidation product from CLB. Therefore, the stability of CLE in this storage experiment at room temperature is in line compared to the results obtained by ref. [20], who carried out their storage experiment at 60 °C for 24 d.

Figure 6 shows the intensities of the signals of CLB, CLC, and CLK (+CLE). Also, for CLB both oxidation steps could be observed. However, the signal of CLK was overlapped by the signal of CLE. CLB oxidized in a comparable time interval as CLM. **Figure 7** shows the intensities of the signals of CLN, CLI*, CLI, CLT, and CLF. The decreasing signal of CLN overlapped with the signal of CLS cyclo-lino peptide A–S, respectively (CLA) starting from week 10. Oxidation of CLN was completed at week 15, while intermediate oxidation products CLT, CLI* and CLI passed their maximum at week 3, 9, and 10, respectively and the double oxidation product at week 19. The signal of CLF decreased after its maximum to the initial level and the formation of further oxidation products could be expected. Evaluation of the data set of the LC-MS-Q-TOF revealed no substances with matching masses in the range of CLPs with an amino acid sequence of MsnLMsoPFFWV, MsoLMsnPFFWV or MsnLMsnPFFWV, with provisioned

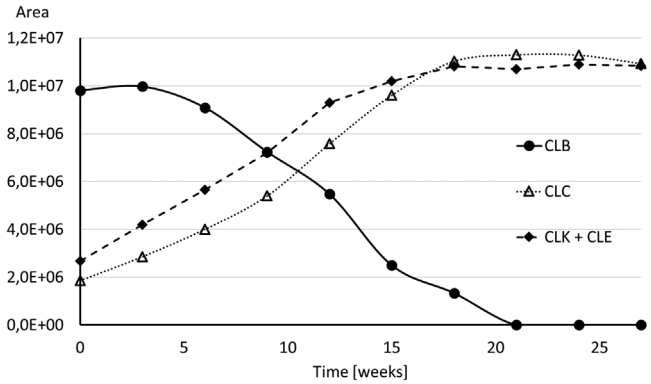

Figure 6. Signal area of cyclo-lino peptides CLB, CLC, and CLK (+CLE) during storage of linseed oil for 26 weeks at room temperature in the dark. For abbreviation of individual cyclo-lino peptides, see Table 1.

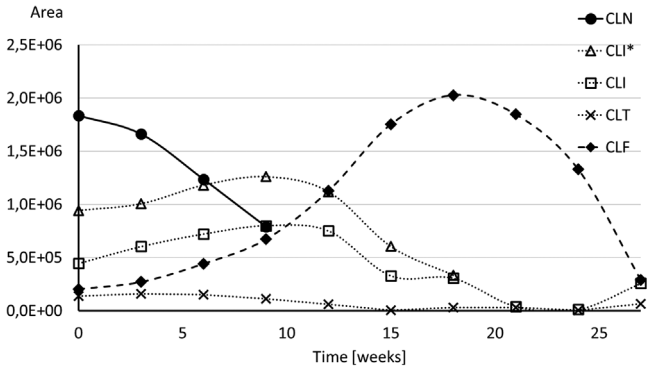

Figure 7. Cyclo-lino peptides CLN, CLI*, CLI, CLT, and CLF during storage of linseed oil for 26 weeks at room temperature in the dark. For abbreviation of individual cyclo-lino peptides, see Table 1.

molar masses of 1100.48 or 1116.48 for $[M+H]^+$ ions, respectively. CLQ, CLR, and CLS as described by ref. [34] were not detected by LC-Q-TOF.

3.5. Oxidation at Room Temperature in Daylight

The reaction at room temperature at ambient daylight proceeded much faster in a time interval of about six weeks showing the same reaction process (see Figures S3–S7, Supporting Information). CLO was completely oxidized in about two weeks, while oxidation in the dark took 15 weeks. However, no final oxidation product could be identified by Q-TOF. Oxidation of CLM was completed during three weeks at daylight compared to 21 weeks in the dark and CLL was completed in even one week compared to 15 weeks, respectively. Also for CLN and CLB a much faster oxidation at daylight in one and two weeks was observed, respectively.

3.6. Oxidation at 6 °C in the Dark

Changes of the CLP compositions are shown in Figures S8–S12 (Supporting Information). During this set of experiments the oxidation was only limited and observed during one year. The stability of the observed CLPs could be sorted in the order of CLM > CLB > CLO > CLL > CLN under these conditions.

3.7. Oxidation at 60 °C in the Dark

In this experiment oxidation proceeded very rapidly and most reactions were finished during a time interval of 30 d (see Figures S13–S17, Supporting Information). The signal of CLO was completely diminished during three days. The final observable oxidation product CLG passed its maximum at day 4. However, the signal of the intermediate oxidation product CLP° was overlapped by the signal of CLJ, which is the final observable oxidation product of CLL. This was the most probable explanation for a second increase of this combined signal of CLP° and CLJ, which reached a maximum at the end of the observation period. The oxidation speed of CLP and CLP° were assumed to be equal.

Oxidation of CLL was also completed at the third day and the intermediate oxidation product CLE reached a high level at the fourth day. Interestingly, this high level was maintained during almost the whole time interval with a small decrease beginning at about the 25th day. However, the signal of CLE was overlapped by the signal of CLK, which was the final observable oxidation product of CLB. Therefore, a maximum for CLE and subsequent degradation was not observed as described in ref. [20]. This might be due to a simultaneous formation of CLK during the degradation of CLE and due to the overlapping of the two signals we see only a shifting ratio of CLE and CLK responsible for the stable combined signal. A decrease of CLE could affect the sensorial bitterness of oxidized linseed oils. In former sensorial tests no decrease of the bitter off-taste was observed during storage of linseed oil for 150 d at room temperature.[35] This was of special importance as in two studies[9,10] CLE was identified as the most important bitter tasting compound in stored bitter linseed oil. Possibly, also CLJ as the oxidized form of CLE or another oxidized CLP might be also responsible for the bitter off-taste of stored linseed oil. This has to be clarified in further studies.

Figure S16 (Supporting Information) shows the oxidation reaction of CLB, which was nearly completed on the third day. The intermediate oxidation product CLC passed its maximum after the seventh day and decreased steadily until the end of the time interval of 30 d. As the signal of CLK was overlapped by CLE we could conclude that an increasing proportion of the combined signal of CLE and CLK had to be assigned to CLK.

The oxidation of CLM and CLN was also monitored and shown in Figures S14 and S17 (Supporting Information), respectively. While no final oxidation product was detectable for CLM, oxidation of CLN was completed after the third day and intermediate oxidation products passed their maximum on second and third day. The final observable oxidation product CLF passed its maximum on the fifth day and decreased very rapidly until the 15th day.

3.8. Comparison of Tocopherol and CLP Oxidation at Different Storage Conditions

Sometimes synergies or dependencies might be observed during oxidation. In this study, we examined the oxidation of tocopherols

and CLP during storage in the dark at 6 °C (A), RT (B), 60 °C (C), and in ambient daylight at RT (D). While tocopherols maintained stable at storage conditions A and POV increased only at the end of the storage period to a level of 2 meq O_2 kg^{-1}, a limited decrease was observed for CLO, CLL > CLN > CLB > CLM.

At RT in the dark the tocopherol contents were also very stable during 26 weeks of storage in contrast to a POV rising after five weeks to reach a final maximum at 45 meq O_2 kg^{-1} accompanied with a complete degradation of the non-oxidized CLPs in a time interval of 15 weeks (CLO, CLL) to 20 weeks (CLM, CLB, CLN). Therefore, the oxidation of CLPs was observed earlier than the oxidation of the tocopherols at RT in the dark. From this observation it can be assumed that the oxidation of CLPs protected the oxidation of the tocopherols at the expense of the nonoxidized CLPs.

This finding was in contrast to the results obtained during storage at 60 °C in the dark, with an increase of the POV after five days to reach a final maximum at about 200 meq O_2 kg^{-1} after 30 d, while tocopherol contents were depleted until the 20th day and CLPs showed a phased oxidation with CLO, CLL, CLN, and CLB completed after 3 d and CLM after 7 d as well as a maximum for intermediate oxidation products from two to four days and maximum contents of complete oxidized CLPs from 3 to 30 d (CLJ and CLK). In this case, tocopherols were degraded together with CLPs in the course of the storage experiment. But it is also worth to mention that the initial CLPs were again oxidized within the first five days while the fastest decrease of γ-tocopherol and plastochromanol-8 started from about day 5. This leads to the hypothesis that for these conditions the nonoxidized CLPs showed some potential to protect tocopherols, while the intermediate CLP oxidation products seemed to have only limited protective effects on tocopherol oxidation.

Storage with daylight yielded an instant and constant rise of the POV during the 14 weeks of storage, while tocopherols were completely depleted in the same time interval, but all CLPs oxidized very rapidly in the first two days. So, in this case the oxidation of CLPs was observed well before oxidation for tocopherols and some protective effects could be attributed to the presence of CLPs.

4. Conclusion

In this study, the oxidation of the different CLPs was examined at 6 °C in the dark, room temperature in the dark as well as in ambient daylight and at 60 °C in the dark. While only limited oxidation of CLPs and tocopherols occurred at 6 °C in the dark this reaction increased significantly at room temperature for CLPs. Also the peroxide value increased already to about 50 meq O_2 kg^{-1}, while tocopherols maintained on a stable initial level. However, a complete degradation of CLPs and tocopherols was observed during a storage period of 25 d at 60 °C in the dark and of 14 weeks at ambient day light at RT. The peroxide value increased to 255 meq O_2 kg^{-1} in these periods with a small induction period at 60 °C of 5 d, but none in ambient light at RT. Therefore, tocopherols and CLPs showed independent degradation patterns during these storage conditions. Degradation of the CLPs more rapidly responded to the increase of the peroxide value and complete degradation of most CLPs can be observed reaching a level of 50 meq O_2 kg^{-1}. It becomes also obvious that oxidation of initial unoxidized CLPs took place earlier and faster than the oxidation of γ-tocopherol and plastochromanol-8 leading to the assumption that CLPs protect the vitamin-E-active compounds to some extent. Consequently, CLPs are important ingredients for retarding the oxidation of linseed oil, which is very susceptible to oxidation due to its high content of linolenic acid.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

L.B.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, supervision, validation, visualization, writing – original draft, writing – review & editing. A.B.: formal analysis, investigation, methodology, writing – original draft. K.N.: formal analysis, investigation, methodology, writing – original draft. B.M.: conceptualization, data curation, formal analysis, methodology, resources, supervision, validation, visualization, writing – original draft.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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bitter taste, cyclo-lino peptides, linseed oil, oxidation

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