

Supporting information for

Quantification of glycerol-bound ω -oxo fatty acids in deep-frying oils from restaurants

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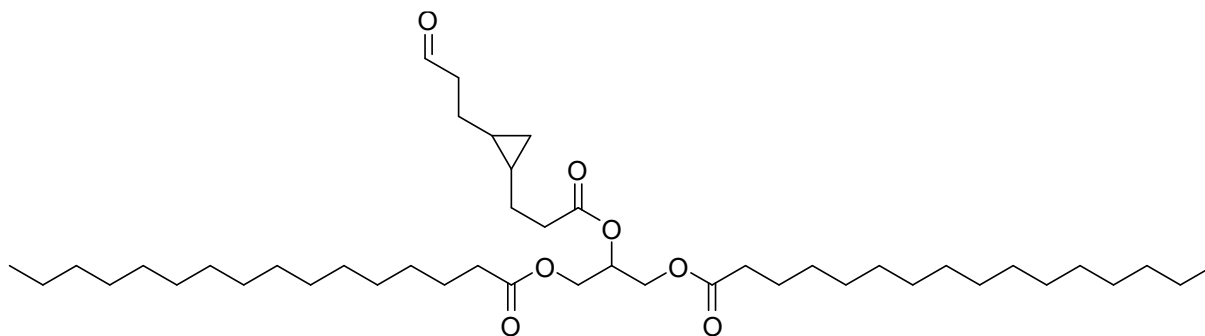
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1. Preparation of standard substances

Synthesis of IS-TG 2-(8-oxo-4,5-cy3-8:0)-1,3-dipalmitate



SI Fig 1 Molecular structure of IS-TG 2-(8-oxo-4,5-cy3-8:0)-1,3-dipalmitate

The synthesis of the internal standard 8-oxo-4,5-cy3-8:0-EE was described in detail previously [1]. For the synthesis of a triacylglycerol containing the internal standard (IS-TG, SI Fig 1) as one fatty acid chain, the aldehyde functional group was protected as acetal. Appr. 15 mg of 8-oxo-4,5-methylene-8:0-EE (75.66 μmol) were transferred into a tube and 1 mL 1,3-propane diol with 0.1% sulfuric acid (w%) were added and stirred for 2 h at 60 °C. After the sample reached room temperature, 2 mL of demin. water and 2 mL of *n*-hexane were added, vortexed and the organic phase isolated. The saponification was performed by a modified protocol described previously [2]. The solvent was removed using a gentle nitrogen stream and 0.5 mL isopropanol, 50 μL demineralized water, and 100 μL 0.6 M KOH in a mixture of methanol/demineralized water: 75/25 (v/v) were added to the residue. The solution was incubated at 60 °C for one hour, acidified to pH 3 with 0.5 M hydrochloric acid. After the sample reached room temperature, 1 mL demineralized water, 5 drops of saturated sodium chloride solution, and 2 mL methyl tert-butyl ether were added. After vortexing, the organic phase was collected, washed with demineralized water, vortexed again, and centrifuged at 3000 rpm for one minute. The organic layer was dried over sodium sulfate, and the solvent was evaporated under a gentle stream of nitrogen.

Esterification was performed after a common protocol [3] with some minor modifications, as adaptation to a smaller scale. The residue (10 mg of 8-dioxane-4,5-cy3-8:0, 44 μmol) was dissolved in 1 mL dichloromethane; 50 mg 1,3-dipalmitoyl glycerol (88 μmol , 2 eq.), 9 mg *N,N'*-dicyclohexylcarbodiimide (44 μmol , 1 eq.), and 2.3 mg 4-dimethylaminopyridine (19 μmol , 0.43 eq.) were added. The mixture was agitated for 4 h at room temperature. The solvent was removed under a gentle stream of nitrogen. A glass cartridge of 0.6 cm diameter was filled with 0.7 g activated silica gel and conditioned with *n*-hexane. The sample was dissolved in the starting eluent and transferred to the column. Fractions were eluted with 6 mL of *n*-hexane/ethyl acetate 90/10, 85/15 and 70/30 (v/v), respectively, and the solvent of the second fraction was evaporated using a gentle stream of nitrogen. To remove the protection group, 5 mL of 0.15 M *p*-toluenesulfonic acid in acetone were added to the triacylglycerol and agitated for 24 h. The solvent was evaporated using a gentle nitrogen stream and the sample was extracted twice with *n*-hexane and demin. water. After solvent removal, the product was purified by semi-preparative HPLC using an Agilent 1200 Series system (Agilent Technologies, Santa Clara, USA), equipped with a G1226A Prep ALS autosampler, G1365D MWD detector, G1364B Prep FC fraction collector, G7161A Prep Bin Pump, and a G1330B Thermostat for temperature control. An Agilent Pursuit 5 C₁₈ column (150 mm $\times$ 10 mm, 5 μm) and Mobile Phases A water/acetonitrile (40:60, v/v) and B acetonitrile/isopropanol (10:90, v/v) were used.

The elution started with 30 % B, and linearly increased to 90 % within 15 min, which was kept constant for 5 min. Then Eluent B was increased to 95 % within 1 min and kept constant for 2 min. Finally, the composition was readjusted to 30 % Eluent B within 2 min followed by a 3 min re-equilibration. Fractions were collected every 30 s from 18 to 21 min retention time and the fractions containing the isolated product were merged.

The product identity and purity were confirmed by LC–MS and GC–MS after alkaline transesterification to fatty acid methyl esters.

Synthesis of reference TG standards

The esterification was carried out analogously to the procedure described above. Three separate mixtures were prepared by adding 10 mg of 1,2-dipalmitoyl glycerol (18 μ mol, $\sim$ 0.5 eq.) to 10 mg of palmitic acid (39 μ mol), oleic acid (35 μ mol), or linoleic acid (36 μ mol), respectively. To each mixture, 7.5 mg N,N'-dicyclohexylcarbodiimide (36 μ mol, $\sim$ 1 eq.) and 5.5 mg 4-(dimethylamino)-pyridine (45 μ mol, $\sim$ 1.2 eq.) dissolved in 1 mL DCM were added. The mixture was shaken for 4 h. After evaporation of the solvent, solid-phase extraction (SPE) was performed as described above, and the TGs were eluted with *n*-hexane/ethyl acetate 95/5 (v/v). The product identity and purity were confirmed by LC–MS and GC–MS after alkaline transesterification to fatty acid methyl esters.

2. Methods

Fatty acid distribution by GC-MS

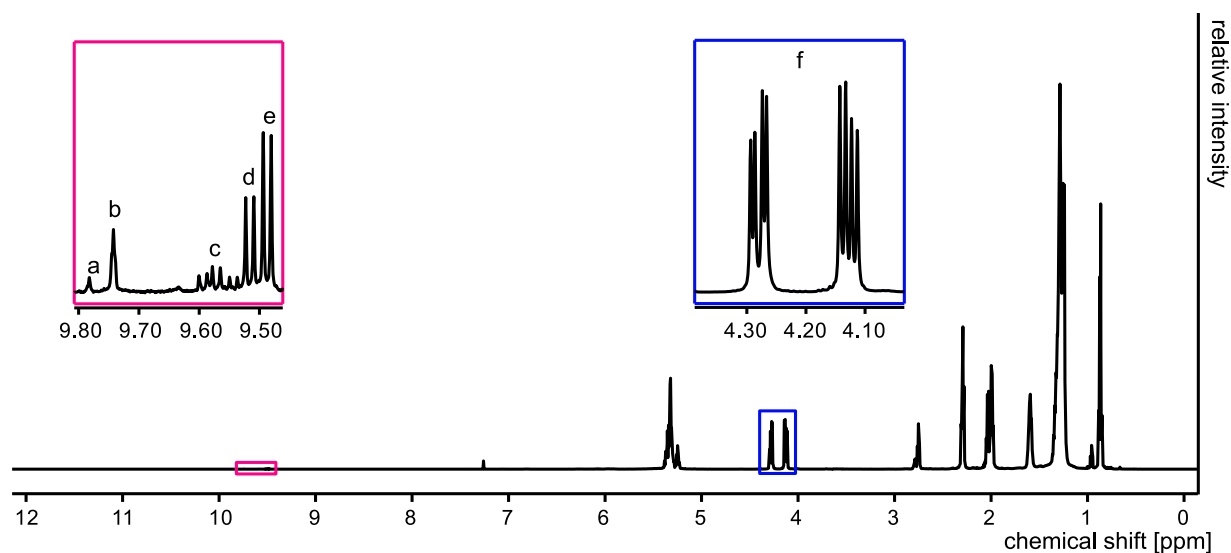
Lipid samples were transesterified to fatty acid methyl esters (FAMES) as previously described by Christie [4] and Berdeaux et al. [5]. Approximately 10 mg of each sample was dissolved in 1 mL of *n*-hexane. An aliquot equivalent to 25 μ g total lipid was transferred into a screw-cap reaction tube and the solvent was evaporated by a gentle nitrogen stream. Subsequently, 1 mL of tert-butyl methyl ether and 2 mL of 0.5 M sodium methanolate in methanol were added. After thorough mixing, the reaction mixture was incubated at 50 °C for 15 minutes. The reaction was then quenched by adding 150 μ L of acetic acid, followed by 10 μ g of ethyl palmitate (16:0-EE) and 2 mL of ultrapure water. FAMES were extracted twice with 2 mL of *n*-hexane. The combined organic phases were evaporated to dryness and the residue was re-dissolved in 1 mL of *n*-hexane for GC-MS analysis. Quantification of the major fatty acids - palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and α -linolenic acid (18:3) - was performed using external calibration. Each sample was processed and analyzed in a single replicate

Blank value determination

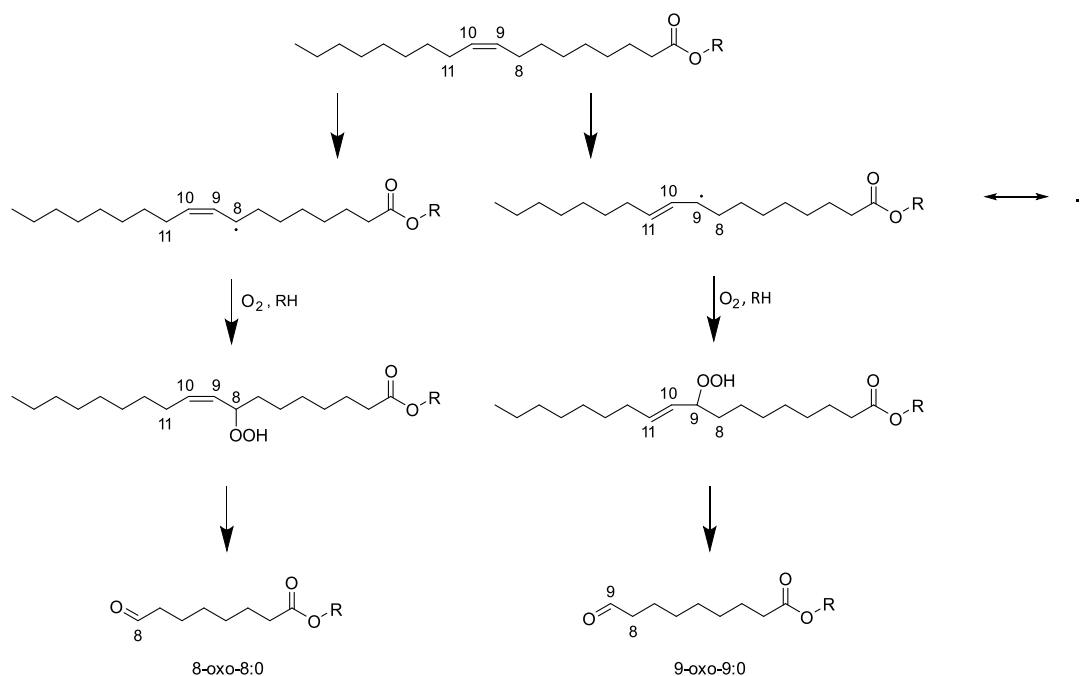
Approximately 1 mg each of tripalmitoyl-glycerol, 1,2-dipalmitoyl-3-oleoyl-glycerol or 1,2-dipalmitoyl-3-linoleoyl-glycerol were spiked with 0.2 μ g of internal standard (IS-TG). The TG standard substances were processed in triplicate as described for the deep-frying oils above (2.4.2), and the blank values of the ω -oxo FA were determined for the respective standard compounds. Subsequently, the ω -oxo FA contents obtained from the TG processing were converted to the ω -oxo FA contents of the corresponding fatty acids of the TGs, allowing for the calculation of blank values for saturated, monounsaturated and PUFA (SI Table 2). Using these values and the fatty acid composition of the samples (SI Table 1), the ω -oxo FA blank values were estimated for each sample (SI Table 2). Due to the blank values, a relatively large number of measurements from only mildly oxidized samples theoretically would have yielded negative values for the ω -oxo FA-contents. Additionally, the different fatty acids of the TG standards did in comparison not show the expected blank values. Nevertheless, across the samples clear patterns are evident in the ω -oxo FA distributions. Therefore, the blank value

was not subtracted from the respective analytes in the sample, but it was marked, when the value of an analyte in the sample was <150% of the blank value.

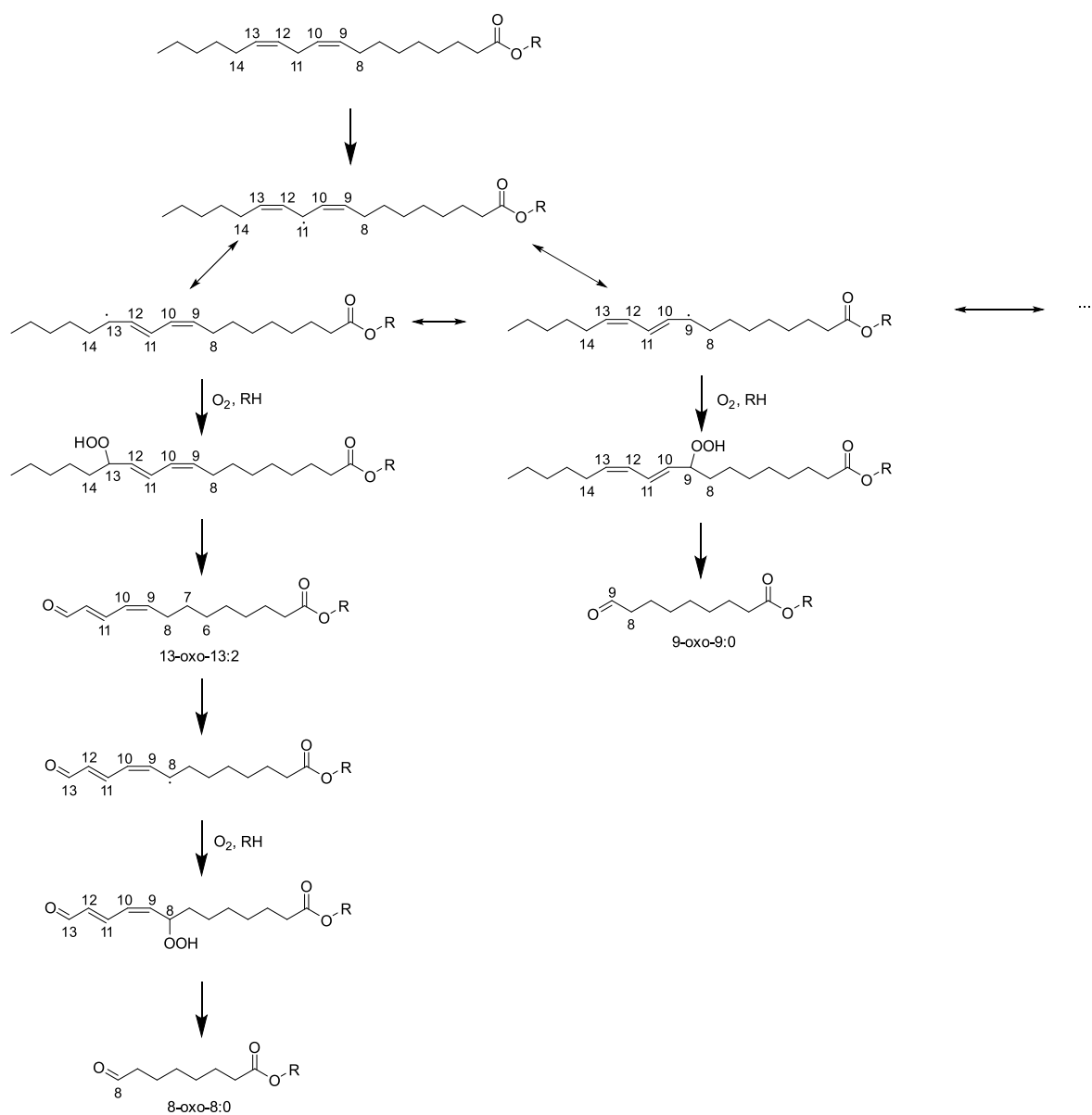
3. Figures and Tables



SI Fig 2 ^1H NMR spectrum of heated deep-frying oil (180 °C, 20 h). The box on the left (magenta) shows the magnified aldehyde region with a: n-alkanals < C5, b: n-alkanals > C5, c: hydroxy-trans-2-alkenals/hydroperoxy-trans-2-alkenals, d: trans,trans-2,4-alkadienals/4,5-epoxy-trans-2-alkenals, e: trans-2-alkenals [6]. The box on the right (blue) shows the signal f of the methylene groups in sn1- and sn3 position of the glycerol



SI Fig 3 Formation mechanisms of selected ω -oxo FA from oleate. "... "represent other possible reactions [7, 8]



SI Fig 4 Formation mechanisms of selected ω -oxo FA from linoleic acid. "... "represent other possible reactions [7, 8]

Table SI 1 Fatty acid composition of in frying fats and vegetable oils from restaurants (r), fast food restaurants (f), canteens after different frying processes (c) and heated plant oils: plant oil mixture labelled as deep-frying oil (FO), peanut oil (PO), rape seed oil (RO) and high oleic sunflower oil (SO) tempered at 40°C for 24 d (LT) and 170°C for 15 h (HT). A single determination was performed to assess the fatty acid distribution. n.d. = not detected

Sample	Content of FAs [mg/g]				
	16:0	18:0	18:1	18:2	18:3
r1	60.6	21.3	231	285	3.67
r2	37.7	9.48	402	113	60.7
r3	153	24.7	309	126	n.d.
r4	125	12.5	359	113	56.6
r5	128	24.0	328	80.3	26.1
r6	128	20.3	288	144	15.0
f1	36.5	6.83	415	135	87.0
f2	43.0	19.7	328	212	19.7
f3	38.0	6.77	375	121	82.4
f4	31.8	3.46	381	123	89.0
f5	111	9.97	339	88.4	44.3
f6	134	21.7	391	59.5	6.7
f7	35.0	28.0	402	130	48.0
f8	58.6	11.5	367	124	67.6
f9	36.6	9.18	452	117	63.1
f10	54.0	25.1	177	292	n.d.
f11	35.5	7.43	371	116	68.4
c1	43.1	12.6	377	138	57.3
c2	37.8	12.6	360	139	55.7
c3	46.3	13.7	353	135	53.0
c4	49.9	14.0	362	134	49.4
c5	58.0	15.2	370	136	49.0
FO HT	35.9	15.0	330	196	31.5
FO LT	32.9	13.9	313	214	42.7
PO HT	60.8	24.1	461	52.7	n.d.
PO LT	53.9	21.5	429	86.9	n.d.
RO HT	33.1	9.54	403	132	49.1
RO LT	31.9	10.2	396	161	69.2
SO HT	37.4	24.7	547	46.2	n.d.
SO LT	34.0	23.9	532	66.2	n.d.

Table SI 2 Estimated blank values for the GC-MS-based quantification of ω -oxo FA, derived from triglycerides with saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA). These blanks reflect background ω -oxo FA formation during derivatization and workup. For each sample from restaurants (r), fast food restaurants (f), canteens after different frying processes (c), blank contributions for 8-A, 9-A, and 10-A were estimated based on its fatty acid composition. No blank correction was applied for 7-A, as its blank value was below the limit of quantification (LOQ)

Standard	Blank value of ω -oxo FA [ng/mg]			
	7-A	8-A	9-A	10-A
SFA	<LOQ	4.1	23	5.9
MUFA	<LOQ	12	57	14
PUFA	<LOQ	2.0	23	3.2

Sample	Calculated ω -oxo FA blank values [ng/mg]			
	7-A	8-A	9-A	10-A
r1	-	3.8	22	4.7
r2	-	5.6	28	6.6
r3	-	4.8	25	5.9
r4	-	5.4	28	6.5
r5	-	4.9	25	6.0
r6	-	4.5	24	5.5
f1	-	5.8	30	7.0
f2	-	4.8	26	5.8
f3	-	5.3	27	6.3
f4	-	5.3	28	6.4
f5	-	5.0	25	6.0
f6	-	5.7	27	6.8
f7	-	5.6	29	6.7
f8	-	5.3	27	6.3
f9	-	6.2	31	7.4
f10	-	3.1	19	4.0
f11	-	5.2	26	6.2
c1	-	5.3	27	6.4
c2	-	5.1	26	6.1
c3	-	5.0	26	6.0
c4	-	5.2	26	6.2
c5	-	5.3	27	6.4
FO HT	-	4.8	25	5.8
FO LT	-	4.6	25	5.6
PO HT	-	6.2	30	7.3
PO LT	-	5.8	28	6.9
RO HT	-	5.6	28	6.6
RO LT	-	5.6	29	6.7
SO HT	-	4.8	25	5.8
SO LT	-	4.6	25	5.6

Table SI 3 Contents of different ω -oxo FA (7-A, 8-A, 9-A, 10-A and their sum (Σ)) in frying oils from restaurants (r), fast-food restaurants (f) and one canteen after subsequent frying processes (c) as well as in heated plant oils (hp) (plant oil mixture labelled as deep frying oil (FO), peanut oil (PO), rape seed oil (RO) and high oleic sunflower oil (SO)) tempered at 40°C for 24 d (LT) and 170°C for 15 h (HT). Values are mean $\pm$ absolute deviation of mean, sample preparation was performed as duplicate. For the canteen samples the Total Polar Compounds (TPC) are displayed.

Sample	Content of ω -oxo FA [ng/mg]				Σ	TPC [%]
	7-A	8-A	9-A	10-A		
r1	<LOQ	6.19 $\pm$ 0.16	74.1 $\pm$ 3.1	<LOQ	80.3 $\pm$ 3.6	-
r2	2.12 $\pm$ 0.13	13.3 $\pm$ 0.7	105 $\pm$ 4	<LOQ	120 $\pm$ 6	-
r3	2.7 $\pm$ 0.05	17.0 $\pm$ 0.2	153 $\pm$ 2	<LOQ	173 $\pm$ 2	-
r4	4.08 $\pm$ 0.06	28.2 $\pm$ 1.7	208 $\pm$ 4	9.68 $\pm$ 0.4	249 $\pm$ 6	-
r5	14.0 $\pm$ 0.1	92.1 $\pm$ 0.8	467 $\pm$ 25	24.9 $\pm$ 4.6	600 $\pm$ 14	-
r6	26.4 $\pm$ 0.8	139 $\pm$ 8	691 $\pm$ 21	33.0 $\pm$ 2.1	889 $\pm$ 33	-
f1	<LOQ	5.81 $\pm$ 0.26	47.1 $\pm$ 1.5	<LOQ	53.0 $\pm$ 2.2	-
f2	<LOQ	<LOQ	67.4 $\pm$ 4.5	<LOQ	67.4 $\pm$ 5.3	-
f3	2.11 $\pm$ 0.23	12.8 $\pm$ 0.9	110 $\pm$ 3	<LOQ	125 $\pm$ 5	-
f4	4.90 $\pm$ 0.76	22.3 $\pm$ 0.1	149 $\pm$ 9	10.2 $\pm$ 1.0a	186 $\pm$ 10	-
f5	4.43 $\pm$ 0.30	24.3 $\pm$ 2.1	177 $\pm$ 12	<LOQ	205 $\pm$ 14	-
f6	6.54 $\pm$ 0.55	28.7 $\pm$ 3.0	175 $\pm$ 10	<LOQ	210 $\pm$ 14	-
f7	6.27 $\pm$ 0.13	37.0 $\pm$ 0.3	288 $\pm$ 1	14.6 $\pm$ 0.2	345 $\pm$ 1	-
f8	6.30 $\pm$ 0.01	39.8 $\pm$ 0.3	293 $\pm$ 4	15.0 $\pm$ 1.0	354 $\pm$ 5	-
f9	8.98 $\pm$ 0.26	52.6 $\pm$ 1.2	372 $\pm$ 2	18.2 $\pm$ 1.0	452 $\pm$ 5	-
f10	6.39 $\pm$ 2.03	64.7 $\pm$ 2.6	439 $\pm$ 1	19.3 $\pm$ 0.8	529 $\pm$ 6	-
f11	10.7 $\pm$ 0.1	67.9 $\pm$ 0.8	436 $\pm$ 1	24.5 $\pm$ 0.1	539 $\pm$ 1	-
c1	2.35 $\pm$ 0.11	14.8 $\pm$ 0.1	148 $\pm$ 1	<LOQ	165 $\pm$ 2	6.0
c2	6.40 $\pm$ 1.20	24.3 $\pm$ 1.5	203 $\pm$ 6	10.1 $\pm$ 0.7 a	244 $\pm$ 9	8.5
c3	8.81 $\pm$ 0.18	46.0 $\pm$ 1.6	281 $\pm$ 3	18.1 $\pm$ 0.2	354 $\pm$ 5	11.5
c4	11.4 $\pm$ 0.1	68.3 $\pm$ 2.1	370 $\pm$ 5	15.9 $\pm$ 0.3	466 $\pm$ 8	16.5
c5	16.0 $\pm$ 0.2	80.3 $\pm$ 0.5	482 $\pm$ 12	21.4 $\pm$ 0.9	598 $\pm$ 31	17.5
FO HT	20.8 $\pm$ 0.9	142 $\pm$ 11	750 $\pm$ 75	27.9 $\pm$ 11.8	941 $\pm$ 98	-
FO LT	0.339 $\pm$ 0.070	3.51 $\pm$ 0.47 ^a	112 $\pm$ 8	3.13 $\pm$ 0.15 ^a	118 $\pm$ 9	-
PO HT	33.6 $\pm$ 1.3	154 $\pm$ 14	601 $\pm$ 83	19.2 $\pm$ 14.3	808 $\pm$ 113	-
PO LT	0.579 $\pm$ 0.170	3.66 $\pm$ 0.92 ^a	25.8 $\pm$ 5.2 ^a	0.758 $\pm$ 0.160 ^a	30.8 $\pm$ 6.5	-
RO HT	27.6 $\pm$ 8.7	147 $\pm$ 41	630 $\pm$ 59	29.7 $\pm$ 19.0	835 $\pm$ 128	-
RO LT	0.490 $\pm$ 0.070	2.44 $\pm$ 0.68 ^a	39.9 $\pm$ 2.9	1.72 $\pm$ 0.31 ^a	44.5 $\pm$ 4.0	-
SO HT	34.7 $\pm$ 3.1	136 $\pm$ 16	538 $\pm$ 10	13.4 $\pm$ 10.9	722 $\pm$ 40	-
SO LT	0.130 $\pm$ 0.010	1.06 $\pm$ 0.06 ^a	17.9 $\pm$ 4.3 ^a	0.544 $\pm$ 0.185 ^a	19.6 $\pm$ 4.5	-

^a For contents, the determined concentration of the ω -oxo FA is less than 150% of the measured blank value (more information on blank value above in SI)

Table SI 4 ¹H NMR spectroscopy was used to quantify *n*-alkanals in used frying fats and vegetable oils from restaurants (r), fast food outlets (f), and canteens (c), as well as in heated plant oils (hp) (plant oil mixture labelled as deep frying oil (FO), peanut oil (PO), rape seed oil (RO) and high oleic sunflower oil (SO)) tempered at 40°C for 24 d (LT) and 170°C for 15 h (HT). The obtained values in mmol/mol TG were subsequently converted to ng/mg TG by calculating the average mass of the ω -oxo FAs (182.8 g/mol; estimated by the average distribution of 7- to 10-A of all samples) and the TGs (875.9 g/mol; estimated by the average fatty acid distribution of all samples). Analyses were based on single measurements

Sample	Content of <i>n</i> -alkanals [mmol/mol]	Estimated content of ω -oxo FA [ng/mg]
r1	<LOQ	<LOQ
r2	<LOQ	<LOQ
r3	0.87	182
r4	0.87	182
r5	3.06	639
r6	3.81	795
f1	<LOQ	<LOQ
f2	<LOQ	<LOQ
f3	<LOQ	<LOQ
f4	0.83	173
f5	<LOQ	<LOQ
f6	1.14	238
f7	1.68	351
f8	1.44	301
f9	1.65	344
f10	2.18	455
f11	2.53	528
c1	<LOQ	<LOQ
c2	0.90	188
c3	1.18	246
c4	2.22	463
c5	2.39	499
FO HT	3.52	735
FO LT	<LOQ	<LOQ
PO HT	3.08	643
PO LT	<LOQ	<LOQ
RO HT	3.86	806
RO LT	<LOQ	<LOQ
SO HT	3.05	637
SO LT	<LOQ	<LOQ

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