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Journal of Chromatography A, 1036 (2004) 197–203
JOURNAL OF CHROMATOGRAPHY A
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Interlaboratory evaluation of injection techniques for triglyceride analysis of cocoa butter by capillary gas chromatography

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Received 2 September 2003; received in revised form 5 March 2004; accepted 8 March 2004

Abstract

As part of two international collaborative studies, in which 14 laboratories applied capillary GLC to determine the triglyceride (TG) profile of cocoa butter, the performance of different sample introduction techniques, i.e. cold on-column injection (OCI), split injection and programmed-temperature vapouriser (PTV) injection, was compared. In both studies, the participants did not apply a uniform GLC procedure. Synthetic mixtures of triglycerides were chosen to permit an accurate determination of detector response factors. No statistically significant difference was found between the mean values obtained by different injection modes. The OCI, generally recommended as best practice, did not give superior results than the PTV or the split injection techniques.
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Keywords: Interlaboratory studies; Food analysis; Injection methods; Triglycerides

1. Introduction

Capillary GLC on polarisable liquid phases of high-temperature stability, separating natural triglycerides (TGs) on the basis of molecular mass and degree of unsaturation, is an extremely versatile technique to study the molecular composition of fats and oils. However, critics of the technique may advocate the view that discrimination during sample introduction may occur and highly unsaturated components are lost to a varying degree [1,2]. The injection technique has been considered as being most critical for obtaining high accuracy and precision, since at this stage discrimination against less volatile high molecular mass compounds can occur while the sample is being transferred from the syringe to the column (syringe discrimination) [3,4]. Capillary injection techniques can in principle be divided into two groups, i.e. direct on-column injection (OCI) and injection into an externally heated vapouriser (split/splitless injector). Both techniques can be used in several modifications, differing in the construction of the injector and in the temperature at the time of sample introduction. Temperature programming of the external vapouriser, starting at a temperature below the solvent boiling-point to a temperature high enough to transfer the analyte(s) to the column, can be regarded as an effort to combine the advantages of the traditional on-column and split/splitless injection techniques [programmed-temperature vapouriser (PTV) injection] [5].

Many authors [6–12] published studies concerning the effect of the injection technique on the recovery of TGs. Most of them accentuated the superior use of OCI for TG analysis by capillary GLC. The use of the classical split injection technique for TG analysis by GLC was mostly mentioned in connection with qualitative analysis, while the majority of the authors advised not to use this injection mode for quantitative purposes.

Profiling of the TG composition by GLC is increasingly used in the food industry to confirm authenticity, detect adulteration, and to define the composition of fat or oil blends. For identity control purposes of milk fat, and speciality fats such as virgin olive oil and cocoa butter the knowledge of the TG profile of fats and oils has become an indispensable tool. Those fats do not contain large amounts of polyunsaturated TG species as do other oils of vegetable origin (e.g. soybean, sunflower, rapeseed oil). Cocoa butter contains only a few TG species of comparable molecular mass and unsaturation. Therefore, discrimination during sample injection may not play a prominent role. As a consequence of this and given the availability of high quality inert

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0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.03.011

capillary columns, we speculated that capillary GLC should be a robust technique for the determination of the TG profile of cocoa butter. Positive proof of our assumption would facilitate the implementation of legislation regarding purity of cocoa butter and addition of cocoa butter equivalents to confectionery as laid down in the European Chocolate Directive [13].

In this paper, we detail results of two intercomparison studies for TG analysis by GLC, where the performance of different sample introduction techniques, i.e. OCI, split injection and PTV injection, was compared. Experimentally determined flame-ionisation detection (FID) response factors (RFs) of synthetic TG mixtures formed the basis of the assessment.

Fourteen competent laboratories representing industry, food control authorities and research organisations were invited to participate in this method comparison study, where the participants were free to choose a suitable GLC method.

2. Experimental

2.1. Materials

In the first intercomparison study, a calibration mixture consisting of 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), 1,2-dioleoyl-3-palmitoyl-glycerol (POO), 1,3-distearoyl-2-oleoyl-glycerol (SOS) and 1,2-dioleoyl-3-stearoyl-glycerol (SOO) present in proportions as normally found in cocoa butter had to be individually prepared by each participant. Therefore, TG reference substances (POP, POS, POO, SOS, SOO) with a purity of at least 99% were obtained by the participating laboratories either from Sigma–Aldrich (St. Louis, MO, USA) or Larodan (Malmö, Sweden). An example chromatogram of a calibration mixture is given in Fig. 1.

In the second intercomparison study, the participating laboratories were provided with a common calibration sample distributed by the co-ordinating laboratory of the ring test. For the preparation of the common calibration sample, individual TG standards were obtained from Sigma–Aldrich (Vienna, Austria) and Larodan (Malmö, Sweden). In contrast to the first intercomparison study, the common test mixture contained equal amounts of POP, POS, POO, SOS and SOO. Homogeneity of the common TG calibration standard was checked by GLC. Analysis of variance of the homogeneity data confirmed that the mean values of the content of individual TGs did not differ significantly ($P > 0.05$).

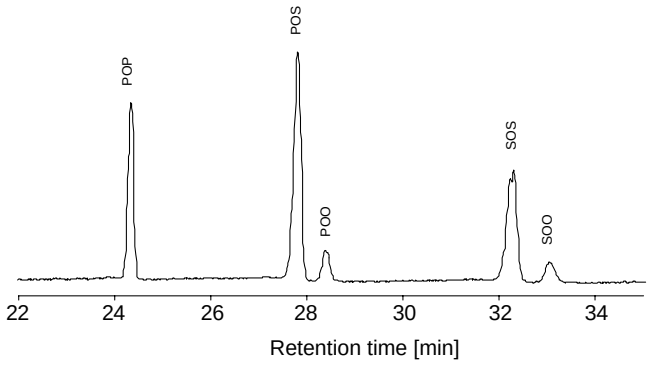

Fig. 1. GLC separation of a calibration mixture used in the 1st intercomparison study by a 30 m × 0.25 mm CB-TAP capillary column (TG amounts composed as present normally in cocoa butter).

2.2. Study protocol

Participants received written instructions detailing the design of the study and electronic reporting forms (MS Excel format). They were requested to make duplicate injection of the test samples on each of four separate working days (in total eight injections). Therefore, injections were not made under repeatability conditions (replicates made by the same experimenter using identical instrumentation, etc., in the shortest time possible) but under intermediate-precision conditions (separate working days). Consequently, the precision data we report are very conservative estimates of repeatability, since other sources of error (day-to-day variation) have been taken into account.

2.3. Methods

Tables 1 and 2 inform about the GLC methods used by the individual laboratories in the two intercomparison studies. All collaborators employed FID for detection purposes. The columns used in the ring test were either from J&W Scientific (30 m × 0.25 mm, 0.15 μm DB-17-HT), from Varian-Chrompack (25 m × 0.25 mm, 0.1 μm CB-TAP) or from Restek (30 m × 0.25 mm, 0.1 μm Rtx-65TG). Different types of sample injection techniques, i.e. OCI, split injection and PTV injection, were applied. Further controllable parameters, different in the individual methods, were the type of carrier gas, the carrier-gas flow rate and/or the inlet pressure and the temperature programming.

Comparison of FID response factors obtained by different injection methods was done by *t*-test, one-way analysis of variance (ANOVA) and Fisher's least significant difference procedure using the Statgraphics Ver. 2.0 computer package (Manugistics, USA).

3. Results

3.1. First intercomparison study

Thirteen laboratories highly experienced in TG analysis by GLC participated in the first method intercomparison study. Details of the applied methods as reported by the collaborators are given in Table 1. All laboratories used narrow bore (0.25 mm i.d.) fused silica columns coated with medium-polarity stationary phases containing 50–65% phenyl groups. Four laboratories used a DB17-HT, seven

Table 1
Chromatographic conditions used by the participants in the 1st intercomparison study

	Laboratory code												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Column characteristics													
Phase	DB-17HT	RTx-65TG	CB-TAP	RTx-65TG	CB-TAP	CB-TAP	CB-TAP	DB-17HT	Ultimetal	CB-TAP	CB-TAP	DB-17HT	DB-17HT
Length (m)	30	30	25	30	25	25	25	30	25	25	25	30	30
i.d. (mm)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Film thickness (μm)	0.15	0.1	0.1	0.1	0.1	0.1	0.1	0.15	0.1	0.1	0.1	0.15	0.15
Temperature mode													
Injector temperature (°C)	Oven track	390	370	360	360	60–350	360	50	–	100/1	200/2	Oven track	50–350
Detector temperature (°C)	360	370	370	360	360	350	360	360	370	360	360	360	350
Injection mode	OCI	Split	Split	Split	Split	PTV	Split	OCI	OCI	OCI	Hot OCI	OCI	PTV
Carrier gas													
Type	H_2	H_2	H_2	He	He	H_2	N_2	H_2	He	H_2	H_2	H_2	He
Constant pressure (kPa)	–	–	100	–	150	140	300	–	90	150	105	–	–
Constant flow (ml/min)	0.8	1.3	–	0.8	–	–	–	2.5	–	–	–	1	3
Sample													
Concentration (mg/ml)	0.3	50	12.5	10	1.98	0.22	8.3	0.5	1–4	0.2	0.65	0.6	3
Volume injected (μl)	0.5	0.2	0.6	1	1.5	0.2	2	0.5	0.1	0.5	0.3	0.5	0.5

Table 2
Chromatographic conditions used by the participants in the 2nd intercomparison study

	Laboratory code										
	1	2	3	4	5	7	8	9	10	11	14
Column characteristics											
Phase	DB-17HT	RTx-65TG	CB-TAP	RTx-65TG	CB-TAP	CB-TAP	DB-17HT	CB-TAP	CB-TAP	CB-TAP	RTx-65TG
Length (m)	30	30	25	30	25	25	30	25	25	25	30
i.d. (mm)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Film thickness (μm)	0.15	0.1	0.1	0.1	0.1	0.1	0.15	0.1	0.1	0.1	0.1
Temperature mode											
Oven											
Injection temperature (°C)/hold time (min)	80/2	340/1	280/0	100/0.5	340/0	100/0.1	50/2	200/2	100/1	200/2	200/0
Programme rate 1 (°C/min)	50	1	10	50	1	70	50	20	30	12	15
Temperature (°C)/hold time (min)	300/0	–	320/0	330/2	–	–	300/1	320/0	300/2	–	360/0
Programme rate 2 (°C/min)	30	–	2	1	–	–	10	1	30	–	1
Temperature (°C)/hold time (min)	–	–	–	–	–	–	340/2	–	–	–	–
Programme rate 3 (°C/min)	–	–	–	–	–	–	0.5	–	–	–	–
Final temperature (°C)/hold time (min)	350/30	360/3	360/6	350/5	360/10	350/21	345/26	360/10	340/35	350/10	370
Injector temperature (°C)	Oven track	390	370	Oven track	360	Oven track	50	–	100	–	390
Detector temperature (°C)	360	370	370	355	360	360	360	370	360	360	390
Injection mode	OCI	Split	Split	OCI	Split	OCI	OCI	OCI	OCI	Hot OCI	Split
Carrier gas											
Type	H_2	H_2	H_2	He	He	H_2	H_2	He	H_2	H_2	H_2
Constant pressure (kPa)	–	120	100	–	150	–	120	90	150	–	150
Constant flow (ml/min)	0.8	–	–	0.8	–	1	–	–	–	2.4	–
Sample											
Concentration (mg/ml)	0.3	50	12.5	0.3	–	15	0.5	1–2	0.5	0.65	10
Volume injected (μl)	0.5	0.1	0.6	0.5	1	0.5	0.5	0.1	0.4	0.3	0.5

collaborators employed a CB-TAP and two participants selected an Rtx-65TG. All collaborators succeeded in separating the critical pairs POS/POO and SOS/SOO with a resolution of at least 1.0. In a previous study [14], the equivalent performance of the CB-TAP and DB-17HT columns for GLC of cocoa butter TGs was already demonstrated.

With respect to precision and accuracy in GLC, no other parameter has received more attention than the sample injection technique. Six laboratories used OCI, five laboratories the split injection and two employed a PTV for sample introduction.

The precision of the applied methods was checked by analysing the in-house prepared calibration mixture by duplicate injection on four separate working days (in total eight replicates). For each laboratory, the relative standard deviation (R.S.D.) of the RF values was determined as a measure of repeatability. Most of the laboratories reported R.S.D. for all TGs of less than 2.0%. Laboratory 9, using OCI for sample introduction, determined R.S.D. values for all TGs close to 5% while laboratories 2 and 7, applying split injection, reported R.S.D. figures >5% for POO and SOO. Laboratory 2 attributed the poorer repeatability to the use of an outdated instrument. The poorer performance of laboratory 7 may be due to the use of nitrogen as carrier gas. Hydrogen and helium are widely used and recommended for TG analysis by GLC. Laboratory 9 could not come up with a reason for the poorer quality of their results. The results of laboratory 6 were not considered in the study, since RF values for POO and SOO were lacking.

Fig. 2 shows the laboratory mean values for the RF of individual TGs obtained by different injection techniques as reported by the remaining 12 laboratories.

The RFs obtained by laboratories using OCI injection varied between 0.78 and 1.31. Collaborators using the split injection reported equivalent results. The lowest response factor was determined for POP (0.78), while for SOO the highest value was 1.32. Only one laboratory submitted results obtained by PTV injection. The RF values ranged from 0.92 to 1.18, which were in accordance with individual results obtained by OCI and split injection.

Table 3
Experimentally determined FID response of standard triglycerides obtained by different injection modes (1st intercomparison study)

Injection mode	POP	POS	POO	SOS	SOO
OCI ($n = 6$)	0.85	0.97	0.93	1.12	1.16
Split ($n = 5$)	0.84	0.98	0.97	1.15	1.22
PTV ($n = 1$)	0.92	0.97	1.09	1.08	1.18
P value	0.22	0.82	0.27	0.55	0.67

ANOVA was used to compare the overall RF mean values obtained by the different injection modes (Table 3). No statistically significant difference between the RF mean values at the 95.0% confidence level was observed ($P > 0.05$).

3.2. Second intercomparison study

Eleven laboratories participated in the second intercomparison study. Laboratories 6, 12 and 13 from the first study did not participate anymore in the second round, while laboratory 14 participated for the first time. The methods employed by the collaborators in the second intercomparison study are listed in Table 2. In comparison to the first study, more details related to the temperature mode were requested from the participants. The participants used the same columns as in the first study. Laboratory 14 selected an RTx-65TG column. With respect to the injection technique, seven laboratories used OCI and four participants split injection. Contrary to the first study, FID response values were determined by using the common calibrant distributed by the co-ordinating laboratory. The resulting R.S.D. figures for the RF values obtained by OCI ranged from 0.1 to 1.1%; for split injection closely agreeing values were found (0.2–1.3%) (Table 4). The RF mean values of individual TGs obtained by different injection in the second intercomparison study are depicted in Fig. 3.

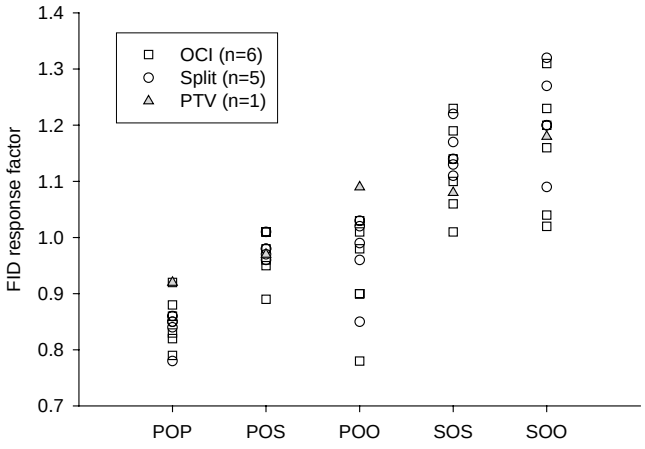

Fig. 2. FID response factors of synthetic triglycerides (1st intercomparison).

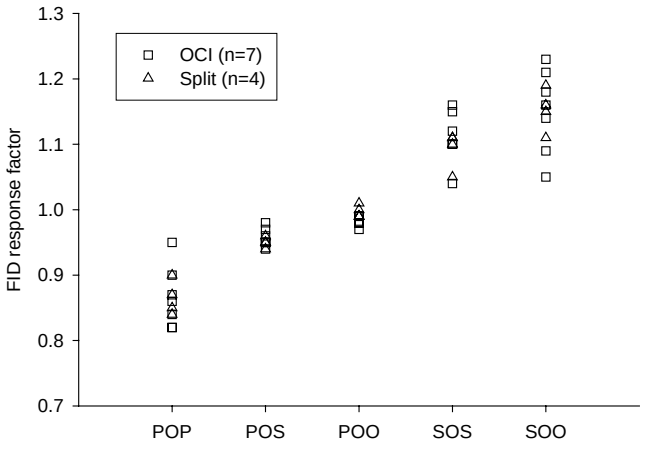

Fig. 3. FID response factors of synthetic triglycerides (2nd intercomparison).

Table 4
Repeatability expressed as relative standard deviation of the FID response factor determination of a triglyceride reference mixture (2nd intercomparison study)

	Injection technique										
	OCI	Split	Split	OCI	Split	OCI	OCI	OCI	OCI	OCI	Split
Laboratory code	1	2	3	4	5	7	8	9	10	11	14
POP	0.28	0.44	1.31	0.20	0.48	0.91	0.67	0.18	0.25	0.59	0.88
POS	0.31	0.64	0.58	0.13	0.23	0.73	0.35	0.28	0.25	0.47	0.59
POO	0.34	0.31	0.63	0.27	0.31	0.42	0.28	0.19	0.20	0.47	0.27
SOS	0.28	0.44	0.59	0.25	0.47	0.73	0.74	0.29	0.46	0.89	0.69
SOO	0.15	0.66	0.87	0.23	0.41	0.39	1.11	0.25	0.25	0.53	0.27

Table 5
Experimentally determined FID response of standard triglycerides obtained by different injection modes (2nd intercomparison study)

Injection mode	POP	POS	POO	SOS	SOO
OCI ($n = 7$)	0.87	0.96	0.98	1.11	1.15
Split ($n = 4$)	0.87	0.95	0.99	1.09	1.15
P value	0.98	0.38	0.12	0.46	0.98

Furthermore, for both OCI and the split injection technique the same spread of individual RF values was observed, i.e. 0.82–1.23 and 0.89–1.19, respectively. The overall RF mean values obtained by OCI and split injection did not indicate any statistically significant difference between both methods (Table 5). For all individual TGs, the resulting P values were >0.05 (t-test). The slight improvement towards a better agreement of the RF values for POO and SOO in comparison to the first study was attributed to the use of the common calibrant.

4. Discussion

The determination of TGs of various edible fats and oils by GLC on medium-polarity stationary phases have been described in several publications [8–12,15–17]. Most modern GLC instruments appear to be suitable, but it is evident that the nature of the injection system can be of crucial importance.

Grob [6] published a detailed study of the effect of the injection technique on TG recovery. Split injection was found to be inappropriate for the analyses of TGs, since recovery, accuracy and precision were poor. Split injections of a test sample composed of trisaturated TGs with acyl-C-numbers (CNs) 30, 36, 42, 48 and 54 resulted in standard deviation figures for the normalised peak areas of 15–30%. Precision of on-column injections was much better; the standard deviation varied only between 1.7 and 3.2%. Recovery of TG in relation to a hydrocarbon (n-pentadecane) was quite uniform for OCI (ca. 80%), while for split injections a strong dependence on the molecular mass of the test compounds was observed. Tricaprin was lost to 20%, while for tristearin the loss amounted to 50%. Highly unsaturated TG species such as trilinolein and particularly trilinolenin have been reported to be lost even to a greater extent on capillary columns coated with a polarisable stationary phase [18,19].

Hinshaw and Seferovic [9,10] demonstrated that a PTV injector is an equivalent alternative to an OCI for TG analysis by capillary GLC, while the classical hot split/splitless injection is by far the least suitable technique, producing strong discrimination effects and decomposition. Geeraert and Sandra [20] reported that excellent reproducibility and accuracy was obtained for cocoa butter and chocolate fats applying a movable OCI.

Contrary to those findings, the results of the two method intercomparison studies suggest that all injection techniques considered were equivalent in terms of repeatability and accuracy, as judged by the magnitude of the obtained FID response factors. OCI, which is generally thought of as a non-discriminatory sample introduction technique and recommended as best practice for TG analysis, did not give results superior than the PTV or the split injection techniques. In the second intercomparison study, all laboratories reported relative standard deviations for POP, POS and SOS of less than 1.0%, independent of the injector type. Moreover, the results obtained by different injection devices were in close agreement to the results published by Geeraert et al. [8], who analysed the TG profile of a cocoa butter sample 10 times by using a non-discriminatory, movable on-column injector.

The main reason for the closely agreeing results is most probably the narrow range of the molecular masses and the degree of unsaturation of the TG species studied. However, this does not restrict the usefulness of the method, since it was our specific aim to optimise it for use on cocoa butter and similar fats.

Even though Geeraert and Sandra [20] stated that the RF values for TGs, except for highly unsaturated substances, are almost equal to one by using OCI and a polarisable capillary column and that no special attention needs to be paid to this subject, none of the laboratories in the present study obtained unity response factors for TGs normally present in cocoa butter. FID response factors deviated to a considerable degree and were in the range of 0.8–1.3. With all chromatographic systems used, a linear increase of the RF values with retention time was observed (coefficient of correlation > 0.93). This is in line with reports detailing the effect of the

carrier gas velocity and the column temperature programme on the RF values of TG [1,18,19]. According to this, the lower the elution temperature of a TG species, which in turn is largely governed by the aforementioned factors, the higher the detector response will be. For highly unsaturated TGs, a deviation from this behaviour has been described [21], which was attributed to a "mass discrimination by stationary phase response quenching", an effect particularly observed for late eluting TGs on polarisable stationary liquids [11]. Even so, as our model TG mixture did not contain highly unsaturated species, the extent of this effect was only moderate. Although we expected that parameter combinations that allowed a rapid elution of TGs should exert some influence on the magnitude of the RF values, no consistent and meaningful pattern was seen. This might be interpreted either as a sign of the robustness of the GLC approach, or that the design of the intercomparison study did not allow to unravel the multifaceted interactions between the parameters governing the quantitative response of the GC systems to a full extent.

The results of our study should not be interpreted in a way that the absolute recovery of TGs, as, e.g. determined by comparison to a hydrocarbon, is independent of the sample introduction technique. However, it can be concluded that using commercially available instrumentation and an optimisation of operating parameters allows a nearly non-discriminatory sample introduction, which can reduce losses of individual TGs but generally cannot eliminate them. Comparability of results between laboratories can only be guaranteed by carefully determining RF values.

The outcome of the two method intercomparison studies indicates that neither the type of capillary column employed nor the sample injection techniques or other chromatographic conditions had a profound effect on the analytical results obtained. Therefore, we conclude that GLC is a robust and reliable method for the analysis of the TG pattern of cocoa butter offering a high degree of procedural flexibility to the analyst.

Acknowledgements

The co-operation of the participants in the collaborative study is gratefully acknowledged: N. Wachter, ADM Noblee & Thörl (D), H. Bernaert, Barry Callebaut (B), R.E. Timms, Britannia Food Ingredients (UK), C. Crews, Central Science Laboratory (UK), European Commission, JRC Ispra (I), H. Zijderfeld, Gerkens Cacao (NL), A. Lomnitz, Karlshamns (S), R. Matissek, Lebensmittelchemisches Institut des Bundesverbandes der Deutschen Süßwarenindustrie (D), I. T'Zand, Loders Croklaan (NL), F. Dionisi, Nestle Research Center (CH), University of Agricultural Sciences, Vienna (A), M.T. Rodriguez-Estrada, Università degli Studi di Bologna (I), B. Cleenewerck, Fuji Oil Europe (B), P. Klagge, Walter Rau Neusser Öl und Fett AG (D).

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