Precision of Low *trans* Fatty Acid Level Determination in Refined Oils. Results of a Collaborative Capillary Gas–Liquid Chromatography Study

P.C. van Bruggen[a,*], G.S.M.J.E. Duchateau[b], M.M.W. Mooren[a], and H.J. van Oosten[a]

[a]Unilever Research Vlaardingen, 3133 AT Vlaardingen, The Netherlands, and [b]Unilever Research US, Edgewater, New Jersey 07020

ABSTRACT: The results of a collaborative study by 38 laboratories were analyzed statistically to calculate the precision of a novel capillary gas–liquid chromatography (GLC) method for the determination of low levels of *trans* fatty acids (TFA) in edible oils. The participants came from 17 countries, mainly European, and were spread evenly between Unilever companies and external laboratories. All participants used the same GLC method, including a temperature optimization step, which is suitable for the determination of a large range of TFA levels in refined oils and fats and for the determination of total saturated fatty acid, *cis* mono- and *cis-cis* methylene-interrupted polyunsaturated fatty acid isomers. For TFA levels down to 0.5%, the collaborative study yielded values for R_{within} that ranged from 0.08 to 0.13% (absolute values) and for $R_{between}$ from 0.2 to 0.4%, depending on the isomer distribution in a particular edible oil. The proposed GLC method allows reliable TFA analysis at low levels that is suitable for monitoring oil processing practices and intake control.

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KEY WORDS: Collaborative study, GLC analysis, interlaboratory study, physical refining, precision, $R_{between}$, reproducibility, R_{within}, *trans* fatty acids.

Analytical procedures for accurate and precise determination of *trans* fatty acid (TFA) levels in edible oils and fats have been discussed in many papers (1–6). Methods proposed differ in scope with regard to the expected level of TFA or in the analytical techniques used. Almost all methods were developed to determine TFA levels that are usually found in animal or hydrogenated vegetable fats in a range of 5 to 60%. Techniques used include gas–liquid chromatography (GLC) (1–3), infrared (IR) spectroscopy (4), or the combination of both (5). The most suitable methods are incorporated in method libraries of normative bodies, such as American Oil Chemists' Society (AOCS) or Association of Analytical Chemists (7–9). The discussions on TFA levels and their possible role in human health have mainly focused on the higher levels of TFA found in partially hydrogenated material or in animal fats (6,10). Nutritionally, the minor amounts of TFA in refined oils and dairy products are of little significance. However, because these methods were developed to analyze TFA levels in oils and fats above 5%, or with some care down to 2%, only a few papers describe methodology to be used in the determination of low TFA levels (4,11). Levels below 2% are generally found in nonhydrogenated refined oils. The typical TFA formed during the refining step to remove free fatty acids were described in the early 1970s by Ackman *et al.* (12). These low levels of TFA isomers are formed during the refining step in which the double bonds (DB) isomerize from *cis* to *trans* and vice versa. Only geometrical isomers are formed, with no shifts of the DB position. This process is time- and temperature-dependent (13,14). The rate of conversion also depends on the level of unsaturation of the fatty acid (FA) molecule: trienoic FA isomerize more easily than dienoic FA. The lowest conversion rate is observed for the monoenoic FA. This effect, together with the natural FA distribution, results in typical TFA levels in specific refined oils.

Owing to the still-increasing attention on TFA levels in edible oils and products that contain edible oils, TFA levels in these refined oils have to be monitored. This has to be viewed against a trend in which levels of TFA in consumer products decrease (15). Secondly, because these specific TFA isomers are formed by a combination of time and heat stress, the levels observed in the oils are an important criterion in determining good oil processing practices (10). At these low levels of TFA, in the range of 0–2%, the precision of the analytical technique must be extremely high if the TFA values obtained are to be used for setting specifications, for monitoring oil processing practices, or for rejecting batches. An earlier organized internal collaborative study (November 1994; van Bruggen, P.C., H.J. van Oosten, and M.M.W. Mooren, unpublished results) showed that results obtained for low TFA levels required further refinement of the GLC procedures.

Recently, we have demonstrated that GLC procedures can be optimized to measure both the TFA isomers found in hydrogenated material and the TFA isomers found in refined oils and fats (16). Our method does not rely on a fixed combination of stationary phase and temperature conditions but recommends minor adjustments of the temperature to meet care-

*To whom correspondence should be addressed at Unilever Research Vlaardingen, Olivier van Noortlaan 120, 3133 AT Vlaardingen, The Netherlands. E-mail: peter-van.bruggen@unilever.com.

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fully described separation criteria for the different *cis* and *trans* isomers. The method is suitable for reliable determination of a wide range of TFA levels in refined oils and fats as well as for determination of total saturated fatty acid (SAFA), *cis* mono- and *cis-cis* methylene-interrupted polyunsaturated fatty acid isomers (*cis*-MUFA and *cis-cis*-PUFA).

For high levels of TFA, as found in hydrogenated oils and fats, accurate alternative methods exist for reference. GLC procedures can be checked with IR methods, with reference materials, or by participating in existing proficiency schemes, such as the AOCS Smalley program. Finally, by a suitable combination of GLC with argentation chromatography, the level of isolated *cis* and *trans* isomers can be measured, which is especially useful if lack of resolution prevents correct TFA determination (17).

For refined oils and fats, no accurate reference methods exist nor are reference materials available to check the accuracy of the method at low TFA levels. Because of the emphasis on the presence of TFA at low levels in edible oils and products that contain edible oils, a collaborative study was organized to obtain information on method performance for these low TFA levels.

EXPERIMENTAL PROCEDURES

Collaborative study. Approximately 100 potential participants were invited to participate in this interlaboratory test. Candidates were selected from the authors' affiliation, main oil suppliers, and already existing contacts with contract laboratories. A number of competitors participated at their own request. The participants received a first announcement and the analytical protocol (see below) in July 1995, followed by the samples in November 1995.

The double split-level design of the study was based on international standard norms for interlaboratory tests (18,19). Only small modifications of these procedures were required to accommodate our specific needs.

Samples and sampling. Suitable samples were selected from the commercially most important vegetable oils, namely sunflowerseed, soybean, and rapeseed oils. At Unilever Research Vlaardingen (The Netherlands), the selected batches were screened for their levels, of TFA by using the analytical protocol (see below). Batches of the same type of oil, but with different TFA levels, were mixed to obtain the desired levels for the double split-level design, i.e., for each oil type, two TFA levels close to each other (target TFA levels sunflowerseed oil, 0.35 and 0.45%; soybean oil, 0.80 and 0.90%; rapeseed oil, 1.10 and 1.20%). The homogeneity was checked before and during dispensing by TFA analyses. Each participant received 12 uniquely coded sample bottles: two blind duplicates for the two levels for each of these three oils. All samples had to be analyzed only once. Oil oxidation was minimized by minimizing the head-space in the bottles. The oil type was not disclosed on the bottle labels. Transport of the samples was organized by courier service to ensure fast delivery. Details on sample storage between receipt and actual analysis were mentioned in the study protocol.

Study protocol. The study protocol stated how to handle the samples and results and included a copy of the analytical protocol (see below). Participants were allowed to report the results only on preprinted forms, specifying the *trans* 18:1, 18:2, and 18:3 levels and the sum of TFA per sample. A sum-check on the 12 results per participant was used for data entry control. Chromatograms of all samples had to be returned to the organizer of the test as well as details of the equipment and settings used.

Analytical protocol. The analytical method prescribed in the protocol is based on the paper from Duchateau *et al.* (16). In short, the method is as follows: From the oil samples, methylated fatty acids (FAME) are prepared with the AOCS BF_3-methanol procedure (Ce 2-66). The FAME are analyzed for the individual FA isomers by a capillary GC method with high-polarity phases. The participants could select SP-2340 (Supelco, Bellefonte, PA), CP™-Sil 88 (Chrompack, Middleburg, The Netherlands), or BPX70 (SGE Inc., Austin, TX) as the column. For each of the columns, a recommended isothermal temperature is given, resulting in separation of the FA isomers of interest (see Ref. 16 for illustrations of the required separations). To optimize the separation and to correct for small instrument and column batch differences, fine-tuning instructions were provided. Detection was based on flame-ionization detector, and quantitation was based on area percentages. The FA isomers were identified from example chromatograms for each oil type. Original FA identification was based on equivalent chainlength values and standards.

Statistical evaluation. The data were screened based on normative procedures (18,19) with SAS software (SAS Institute Inc., Cary, NC) to detect outliers. Outliers were defined as data that lay outside the expected distribution with a confidence level of 99% or more. Two types of outlier tests were used: the Cochran Maximum Variance Test which detects participants with a larger variance of the analytical results than other participants, and the Dixon Test, which detects participants with a larger or smaller mean value than other participants. After deletion of possible outliers, the within-laboratory reproducibility (R_{within}) and the between-laboratory reproducibility ($R_{between}$) were calculated. The difference between two measurements on the same sample should not exceed the value of R_{within} or $R_{between}$ when performed by one laboratory or by two laboratories, respectively (95% confidence interval).

RESULTS AND DISCUSSION

Acceptance of data. Sixty out of the 100 notified laboratories actually participated. Because the intention of this collaborative study was to obtain information about the newly developed GLC method for low TFA levels in refined oils, all participants were urged to adhere strictly to the protocol. Major deviations to that protocol could not be allowed, and prior to the statistical analysis, the data set was screened and some

had to be removed for several reasons: (i) Although an optimal isothermal GLC oven temperature was explicitly indicated in the protocol, 15 participants measured the TFA content in the samples with a temperature gradient. Data of these participants were not accepted. (ii) The data of one participant were unacceptable because they were largely incomplete. (iii) The chromatograms of all samples of all participants were inspected in detail. Nine participants (out of the 60) showed chromatograms in which the *cis*-11 20:1 peak coeluted with the *tcc* 18:3 peak. These participants incorrectly labeled the *trans* isomer as *cis*-11 20:1, resulting in a much lower total TFA content. Because the analytical protocol clearly described the necessary peak separation (examples were included) and procedures as to how this separation could be achieved, it is clear that these participants did not adhere to the protocol. For this reason, their results were not accepted (more information will be given in the Instrumentation section).

Among the 38 remaining participants, there were still a few with small deviations from the analytical protocol: Three participants used a different stationary phase, i.e., SP-2560, which has a different selectivity from that of the prescribed CP™-Sil 88 or SP-2340 columns and requires probably a different optimal isothermal temperature. Three other participants used nitrogen as the carrier gas, although helium or hydrogen was recommended. However, after optimizing, SP-2560 is expected to give the same performance as the other stationary phases mentioned in the protocol, and the nitrogen carrier gas will be of minor influence. Therefore, these data were accepted.

Reproducibilities. The data of the 38 remaining participants (about half Unilever companies and half non-Unilever companies, divided over 17 countries of which 11 were European) were subjected to the outlier tests and calculations of reproducibilities. The TFA results of all accepted data can be graphically presented in bar graphs for each sample type and level. An example is given in Figure 1. This figure shows not only that the total TFA levels (total bar height) show some variability but also that the individual *trans* isomer distributions vary as well.

By using the Cochran and Dixon Outlier Tests, results of 10 duplicates (of seven different participants), out of the available 228 duplicates (38 participants, three oil types, two levels), were detected as outliers and were subsequently removed from the data set. Most of the outliers were detected by the Cochran Outlier Test. Other data from these seven participants were accepted. After removal of the outliers, the reproducibilities were as given in Table 1.

The results in Table 1 could suggest a linear relation between the mean of the total TFA level and $R_{between}$. However,

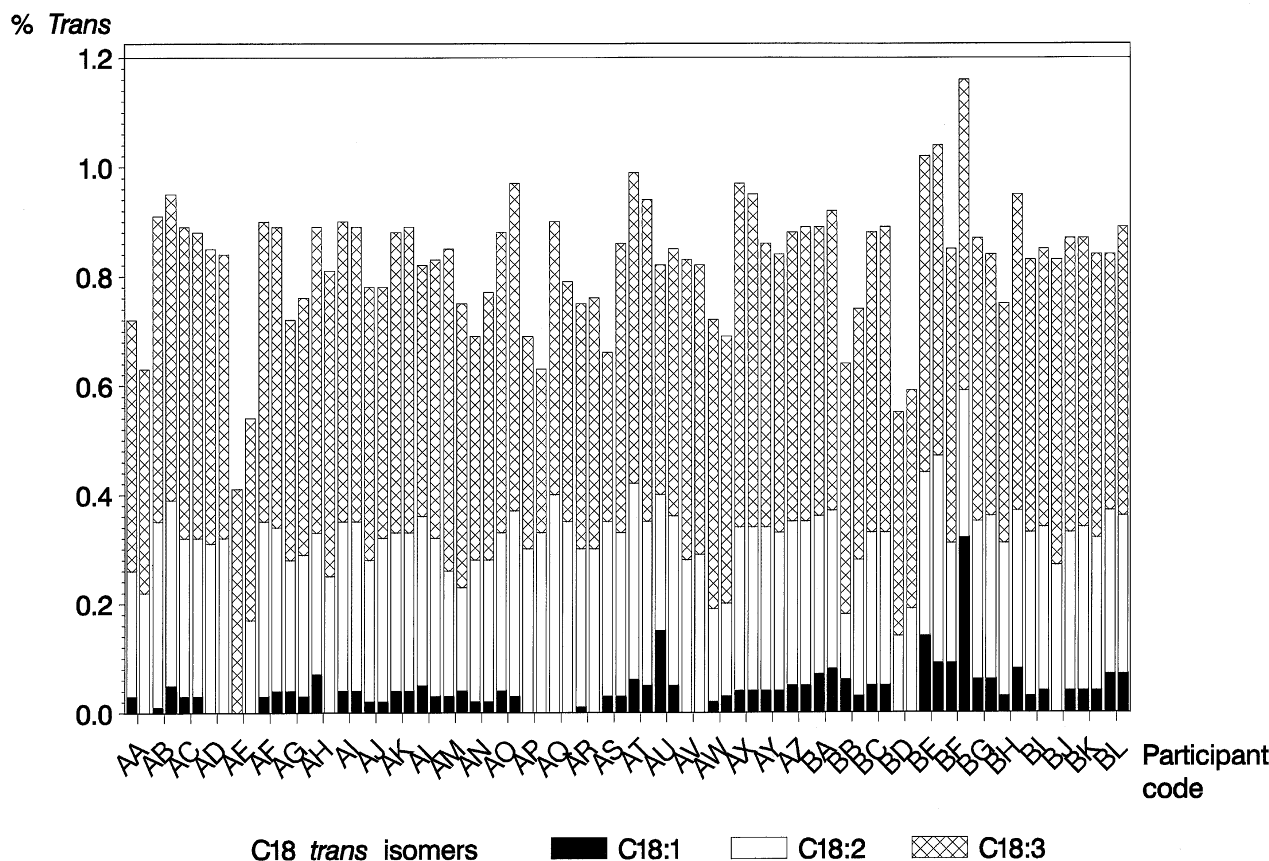

FIG. 1. Representative bar graph of the accepted data for one batch of soybean oil [target *trans* fatty acid (TFA) level 0.90%]. For each of the 38 participants, the blind duplicate results are given in adjacent bars.

TABLE 1
Mean Level, Within-Laboratory Reproducibility (R_{within}) and Between-Laboratory Reproducibility ($R_{between}$) of the Total *trans* Fatty Acid (TFA) Level After Removal of the Outliers

Type of oil	Mean total TFA level (%)	R_{within}	$R_{between}$
Sunflowerseed	0.34	0.08	0.21
Soybean	0.78	0.13	0.31
Rapeseed	1.09	0.13	0.40

TABLE 2
Results of the First Collaborative Study on Low TFA Levels Held in November 1994[a,b]

Type of oil	Mean total TFA level (%)	$R_{between}$
Sunflowerseed	0.52	0.9
Soybean	0.93	1.4
Rapeseed	1.44	1.7

[a]Source: van Bruggen, P.C., H.J. van Oosten, and M.M.W. Mooren, unpublished results.
[b]R_{within} not available because samples were not shipped in duplicate. See Table 1 for abbreviations.

it can be argued that the variability largely depends on the presence of the relative amounts of the individual *trans* isomers. The distribution of the FA 18:1, 18:2, and 18:3 in the starting oils is quite different. In natural sunflowerseed oil, for example, a high level of 18:2 is present, and 18:3 is almost absent. In soybean oil and in rapeseed oil, relatively large amounts of 18:3 are present, but they differ strongly in the 18:2 and 18:1 levels. Consequently, the amounts of detectable *trans* isomers, formed during refining, are different.

Moreover, the number of individual *trans* peaks varies strongly for the various *trans* isomer groups, e.g., one peak for 18:1, a maximum of three peaks for 18:2, and seven (of which five are mostly detected) for 18:3. So, it can easily be understood that the different FA compositions of the three types of oil strongly influence the differences in the $R_{between}$.

In November 1994, a first collaborative study on the determination of low TFA levels was organized. In this test, of which the results were made available only to the 32 participants, the GLC protocol for the determination of the TFA levels was based on an international standard (7) that prescribes a condition for each particular stationary phase. Although these standards mention that other conditions might be required to obtain a better separation (guidelines are, however, not given), most of the participants (about 75%) did not alter the conditions.

The results of this test, compiled in Table 2 (mean TFA level and $R_{between}$ only), clearly show that the variability of the total TFA level was unacceptably high. The $R_{between}$ values are even larger than the mean total TFA levels. Upon closer analysis, several participants of the first test reported unrealistically high TFA levels. The chromatograms of half of the participants showed major shortcomings, which could be related to a poor technical setup. In the current test, this was reduced to about 15% of the accepted participants (see also the chromatographic quality section).

Comparison of the values of the $R_{between}$ in Tables 1 and 2 shows a significant improvement in the performance of the participants. The spread of the TFA values was much less with the new protocol, fewer extreme values were found, and the general quality of the chromatograms was improved. This improvement is, in our opinion, mainly caused by describing a set of minimal separation requirements for the monoenoic, dienoic, and trienoic FA.

Chromatographic quality. A detailed study of the chromatograms returned revealed a number of deviations. The most serious deviation is the coelution of the *tcc* 18:3 peak with the *cis*-11 20:1 peak, resulting in a much lower TFA level. The results of the participants with this deviation in their chromatograms were removed from the data set prior to statistical evaluation. In Table 3, the other deviations are given with their estimated impact on the total TFA level. Also, the number of these deviations as found in the accepted data is given.

Five of the 38 participants showed combinations of these deviations in such a way that the general quality of their chromatograms cannot be rated as sufficient for reporting reliable total TFA results. The most important deviations on which we based this decision are detection and separation problems, i.e., deviations numbered (i), (ii), and (iii) in Table 3. The influence of the results of these five participants on the reproducibilities is presented in Table 4 and shows a clear effect on $R_{between}$.

The reproducibilities in Table 1 represent the performance averaged over the accepted participants. If, however, all participants had scrutinized their GLC equipment and operational practices for most potential pitfalls, this would probably have resulted in the lower reproducibilities of Table 4, which can be considered to be the best achievable for analysis of low TFA levels by capillary GLC.

TABLE 3
Deviations in the Chromatograms and Their Impact on the Total TFA Level[a]

Number	Quantitative effect	Maximal impact (% TFA)	Number of participants
i	No separation or incomplete separation of *tc* 18:2 and *cc* 18:2.	–0.2	13
ii	Small peaks (partly) not detected, not integrated, or not recognized.	–0.1 to –0.2	18
iii	Poor separation of *tcc* 18:3 and *ccc* 18:3 or *cis* 20:1.	–0.2 to –0.3	6
iv	Coelution of *cis* 20:1 and *ccc* 18:3.	0 to –0.1	3
v	Instable and/or noisy baseline.	–0.1	5
vi	Retention of *cis* 20:1 and 18:3 deviating from expected	0 to –0.1	12

[a]See Table 1 for abbreviations.

TABLE 4
Comparison of the Reproducibilities of Total TFA Level with and without Removal of Participants Who Reported Chromatograms of Insufficient Quality[a]

Type of oil	All participants (from Table 1) (*n* = 38)		Excluding participants reporting chromatograms rated as of insufficient quality (*n* = 33)	
	R_{within}	$R_{between}$	R_{within}	$R_{between}$
Sunflowerseed	0.08	0.21	0.08	0.14
Soybean	0.13	0.31	0.11	0.21
Rapeseed	0.13	0.40	0.13	0.33

[a]See Table 1 for abbreviations.

It is not expected that further improvement of the variability (i.e., lower values for the reproducibilities) can be achieved for this type of analysis. A higher precision would probably require a major modification of the GLC analysis and quantitation method.

Oven temperature. As previously stated, the chromatograms of nine of the 60 participants showed coelution of the *cis*-11 20:1 peak with the *tcc* 18:3 peak. They incorrectly identified this *trans* isomer as *cis*-11 20:1, and the results of these participants were not accepted for this collaborative study. Six of these nine participants used isothermal conditions exactly at the temperatures indicated in the analytical protocol for their specific stationary phase type (175°C for CP™-Sil 88, 192°C for SP-2340, and 198°C for BPX70); the other three participants used a temperature gradient. Comparing the separation obtained with the example chromatograms would have made clear that the separation was not yet optimal. This again stresses the need of checking the optimal temperature for this type of analysis, by varying the temperature a few degrees around the advised starting temperature. Of the 38 accepted participants, 24 used an isothermal temperature that was slightly different from the advised initial temperature for their specific stationary phase, as a result of the optimization steps as outlined in the method. The remaining participants could simply have taken the advised initial temperature or found by coincidence exactly that temperature after optimization.

Column stationary phase type. Three participants used SP-2560 as stationary phase, which was not recommended in the analytical protocol, but this phase behaves almost identically to the SP-2340 and the CP™-Sil 88 phases, as far as the retention order of the various peaks is concerned. An effect of the use of this column type on the reproducibilities could not be established. The BPX70 column type was used only by a limited number of participants (six). The specific properties of BPX70 cause the *cis*-11 20:1 isomer to elute after *ccc* 18:3 (linolenic acid). With the other columns, the position of the *cis*-11 20:1 isomer is before *ccc* 18:3 and may interfere with the *tcc* 18:3 isomers. Incorrect labeling of *tcc* 18:3 as *cis*-11 20:1 cannot occur with the BPX70 stationary phase. However, for analysis of PUFA or hydrogenated material, this column type is less appropriate (16).

Carrier gas. Only three participants used nitrogen as carrier gas. Although, from a chromatographic point of view, this gas is less suitable than helium or hydrogen for this type of analysis, the number of participants using nitrogen was too low to find an effect.

Data processing. Most of the participants (28 out of the 38) had chromatographic software packages available for data processing, while the other 10 participants used electronic integrators. One could expect that users of an electronic integrator could miss the very small peaks [deviation (ii) in Table 3, i.e., small peaks not detected/integrated]. Indeed, six of these 10 participants showed deviation (ii) in their chromatograms. The other four indicated, however, that good results could be obtained with an integrator.

The results of this interlaboratory study clearly show that accurate TFA analyses of refined oils at low levels are possible when a capillary GLC method, based on the use of a polar stationary phase, is used to describe the separation of the individual *cis* and *trans* fatty acids isomers in terms of separation requirements. The precision and reproducibility obtained with this new method at low *trans* levels are markedly better than those achieved earlier with a current international reference method that also uses capillary GLC. Within a laboratory, the difference between two duplicate TFA determinations at low TFA levels will be, in most cases, no more than about 0.1% TFA. However, in intercompany trade, the difference between two TFA measurements on the same sample can range from 0.2% TFA maximum for sunflowerseed oil to 0.4% TFA for rapeseed oil.

Although the analytical protocol clearly stated the necessary separation of the individual *trans* and *cis* isomers and the number of expected peaks, there were still some participants with erroneous results due to major separation and integration problems. When all participants scrutinize their GLC equipment and operational practices for most potential pitfalls, the variability of the method may be lowered to a $R_{between}$ of about 0.15% TFA maximum for sunflowerseed oil, 0.2% TFA for soybean oil, and about 0.3% TFA for rapeseed oil. These are probably the best results that can be achieved with straightforward GLC. Correct peak assignment, sufficient resolution, good instrument settings, and low noise and drift are decisive factors to achieve optimal precision and reproducibility.

The results also show that the analytical results are robust for several adjustments of the GLC apparatus around the optimal values, such as column type (other columns than the proposed, e.g., SP-2560) and carrier gas type. The most important factor here is the oven temperature because it largely defines the quality of the separation in the chromatograms and, therefore, the accuracy of the TFA level. If the predefined separation is not obtained, small adjustments in the isothermal temperature should be made.

It seems to be important that the analyst knows what the maximal performance is of the system and how the system reacts to certain changes of the instrumental settings. Next to that, knowledge about the expected composition of the sam-

ples investigated is important. Sufficient time for a thorough interpretation of the chromatograms seems to be indispensable. The data of those participants who followed the analytical protocol, including temperature optimization and correct peak labeling and quantitation, show that it is possible to determine low TFA levels with a high precision.

We conclude that the tested GLC procedure is universally suitable for monitoring oil processing and testing against strict TFA specifications.

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