Fast and Accurate Method for Total 4-Desmethyl Sterol(s) Content in Spreads, Fat-Blends, and Raw Materials

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ABSTRACT: Plant sterols are added as their FA esters to vegetable oil table spreads at levels of approximately 8% as a means to reduce blood cholesterol levels. A new chromatographic method was designed to quantify quickly the level of plant sterol FA esters in incoming (raw) materials and to monitor their processing and final product quality with respect to total sterol level. The method shows a significant improvement in elapsed time and thus labor cost over the classical methods for sterols published in normative references. This improvement was obtained together with high performance characteristics, as shown by the internal method validation for recovery and repeatability. Its validity and robustness were further tested and confirmed in an international collaborative test. The method allows monitoring of sterol content of raw materials, fat-blends, and consumer products at the target level, with a range of 10% or less around this target. The calculated within- and between-laboratory reproducibility were 0.680 and 1.194 w/w%, respectively, for sterol-containing spreads. The results afforded by this method can be used for setting tight product specifications or to monitor trade between companies. We propose to add this new and fast method for total 4-desmethyl sterol(s) to analytical method collections as an adjunct to methods already listed for more detailed sterol analysis.

Paper no. J10044 in *JAOCS 79,* 273–278 (March 2002).

KEY WORDS: Capillary gas chromatography, functional food, phytosterol esters, phytosterols, plant sterol esters, plant sterols, quality control.

Plant sterols (PS) and plant sterol FA esters (PSE) are recognized for their therapeutic efficacy in lowering blood cholesterol levels. Recent reviews on the pharmacological properties of PS and their fully saturated isomers (stanols) focus on this cholesterol-lowering effect (1,2). The blood cholesterol-lowering (BCL) effect of sterols, in particular the 4-desmethyl sterol(s) such as sitosterol, has been known since the 1950s. With the use of the FA esters instead of the free PS, a wider range of formulation options exists. The new interest and formulation options have resulted in a range of consumer products, such as vegetable-oil table spreads, designed to lower total blood cholesterol and LDL-cholesterol (3). The normal daily intake of about 20 g of a vegetable fat spread containing 8% PS, equivalent to 13–14% PSE, reduces the LDL-cholesterol by about 10% (4). With the use of PS and PSE at these elevated levels, these natural edible oil constituents have become functional food ingredients. Sterols used for the BCL effect are mixtures of mainly 4-desmethyl sterol(s) (sitosterol, campesterol, stigmasterol, and brassicasterol).

To support quality control of raw materials, processing, and final consumer product control at these levels, a new and fast total sterol analysis for the content of mainly 4-desmethyl sterol(s) has been developed, validated, and collaboratively tested. The method is designed for a total content of these 4-desmethyl sterol(s) in a range of approximately 15–20% for vegetable oil blends, 8% in consumer products such as spreads, and approximately 60% in PSE concentrates. A new method was necessary because current analytical methods for PS and PSE, found in several normative method collections, did not meet our requirements for ease of use, time involved per analysis, and concentration range. Normative methods have focused on the relatively low levels of PS and PSE that occur naturally in edible oils and fats (typically below 1%) (5,6). These methods are generally time-consuming and are based on outdated sample cleanup steps such as TLC.

The described method is based on whole-sample saponification, one-step liquid/liquid extraction, GC determination without derivatization, and quantification using an internal standard (IST). The IST causes no interference with any known PS or cholesterol under the GC conditions chosen. The simplicity of the method (no sample cleanup and no derivatization) contributes to a fast analysis. The steps followed to ensure the validity and robustness of data produced with this new method for total 4-desmethyl sterol(s) levels as found in raw materials, in vegetable oil blends, and consumer spreads are described in this paper.

MATERIALS AND METHODS

Sample preparation. An appropriate amount of sample (~50 mg) was saponified with ethanolic potassium hydroxide solution (1 mL) at 70°C for 50 min. The internal standard (IST, β-cholestanol = 5α-cholestane-3β-ol; Sigma, St. Louis, MO) was added to the sample before saponification. The IST amount was adjusted depending on the expected total sterol content of the sample. It is recommended to test and correct for the IST purity before actual use. The unsaponifiable part

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was extracted using liquid/liquid partitioning into heptane. The extract contained the sum of free and esterified PS, but now all present as free sterols. The IST was selected based on its GLC properties, i.e., its lack of interference with known PS or with low levels of cholesterol present in samples. Reference standards of cholesterol, stigmasterol, campesterol, and β-sitosterol were also obtained from Sigma. Samples used for recovery tests and to check the initial within-laboratory repeatability were prepared from commercial PSE concentrates and typical vegetable oil-blends. All chemicals used were analytical grade or better.

Gas chromatography. The samples were analyzed by means of GLC using a nonpolar stationary phase capillary column and cool-on-column injection. The gas chromatograph (Carl-Erba/Fisons GC8000; Interscience, Breda, The Netherlands) was equipped with a Fisons AS800 autosampler and cool-on-column injector set to deliver 0.5 µL injections, a suitable deactivated precolumn (1 m × 0.53 µm) and a capillary column with a CP-Sil-5CB stationary phase, dimensions 10 m × 0.32 mm internal diameter and 0.12 µm film thickness (Chrompack, Middelburg, The Netherlands). The precolumn was connected to the analytical column by means of a glass press-fit connector. The carrier, helium, was set to a linear velocity of 45 cm/s. The GLC oven program was as follows: start at 60°C, hold for 1 min and then heat to 300°C at 20°C/min, final hold 3 min. Data collection for the validation test and collaborative test sample analysis was done with PerkinElmer Nelson 2700 TurboChrom software running on a local network (PerkinElmer, Norwalk, CT).

Samples, participants, collaborative test design, and statistics. Participants in the collaborative test were provided with a training set of samples and the analytical protocol, well in advance of the actual collaborative test, held by the end of the year 2000. This allowed each participant to get acquainted with the new method. In particular, setting up the cool-on-column injection technique requires ample analyst experience. For the actual collaborative test, the participants were obliged to follow exactly the analytical protocol and to report details on instrument and settings used. The test follows a uniform level design with duplicates for every concentration level provided. In total, eight samples from four different products and batches were shipped: one batch of vegetable oil spread (target level ~8% PS), two different batches of vegetable fat blends (target ~12 and 20%), and one batch of sterol esters (~60%). The samples were sent to 10 participating quality assurance/quality control laboratories distributed within our parent company and one non-Unilever participant. They were located in Australia, Brazil, Canada, France, Germany, Japan, the United Kingdom (2), the United States, and The Netherlands (two, of which one was not associated with Unilever). All participants in this interlaboratory test responded with a complete set of results.

Statistical analysis was performed as described in the normative reference ISO 5725 (7). Only minor modifications of this procedure were required to accommodate our specific needs. Two types of tests to detect outliers and suspected values were used: (i) *Cochran tests* on duplicate differences and internal precision, and (ii) *Dixon tests* for the determination of outliers in batch means and laboratory means. Outliers were defined as data outside the expected data distribution derived from all received data, with a confidence level of 99% or more. Suspected values were defined as data outside the expected data distribution with a confidence level between 95 and 99%. The statistician decided whether an outlier should be removed from the data set. Suspected values remained in the data set. After the detected outliers, if any, had been removed from the data set, the following statistical parameters were calculated: (i) mean value, (ii) the within-laboratory SD ($s_{R\text{-within}}$), the within-laboratory reproducibility (R_{within}), the between-laboratory SD ($s_{R\text{-between}}$), and the between-laboratory reproducibility ($R_{between}$).

RESULTS AND DISCUSSION

Chromatography. A representative chromatogram obtained with this new method is shown in Figure 1. The peaks were identified by comparison with peak profiles originating from typical sterol methods such as reviewed by Goad and Akihisa (8). Further identification was obtained on the basis of the electron impact mass spectra in GC–MS. Details on the GC–MS method fall outside the scope of this paper, but one example to show the perfect match between chromatographic performance in the GLC method and GC–MS and the obtained experimental and National Institute of Standards and Technology/National Bureau of Standards library spectra is presented in Figure 2. To assess the elution window of the sterols of interest for the purpose of sample quantification, samples were first analyzed by a classical sterol GLC method showing individual sterols, and subsequently by the method discussed in this paper. Second, the overall peak profile was

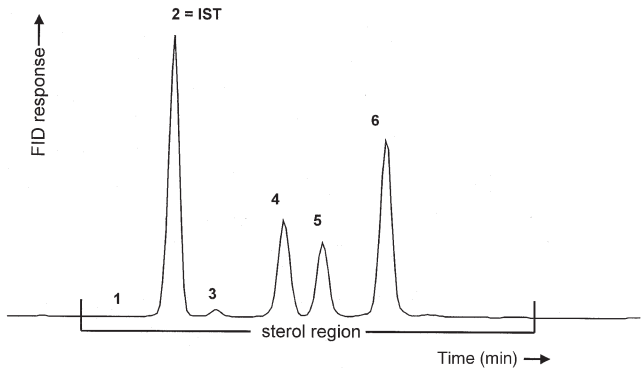

FIG. 1. Representative chromatogram of the method for total 4-desmethyl sterol(s), showing the retention window of interest (real time frame shown is from ~6.7 to ~8.9 min). Peak numbers indicate 1 = cholesterol, 2 = internal standard (IST) β-cholestanol, 3 = brassicasterol, 4 = campesterol, 5 = stigmasterol, and 6 = β-sitosterol. The relative retention times (RRt) are reported with respect to the RRt of IST = 1.00, and are as follows: cholesterol, 0.98; β-cholestanol, 1.00; brassicasterol, 1.02; 24-Me-cholesterol, 1.04; campesterol, 1.04; campestanol, 1.04; stigmasterol, 1.06; Δ7-campesterol, 1.08; clerosterol, 1.08; β-sitosterol, 1.08; sitostanol, 1.08; Δ5-avenasterol, 1.10; Δ7-stigmasterol, 1.10; Δ7-avenasterol, 1.13. Peaks 4 and 6 might include small amounts of campestanol and sitostanol, respectively.

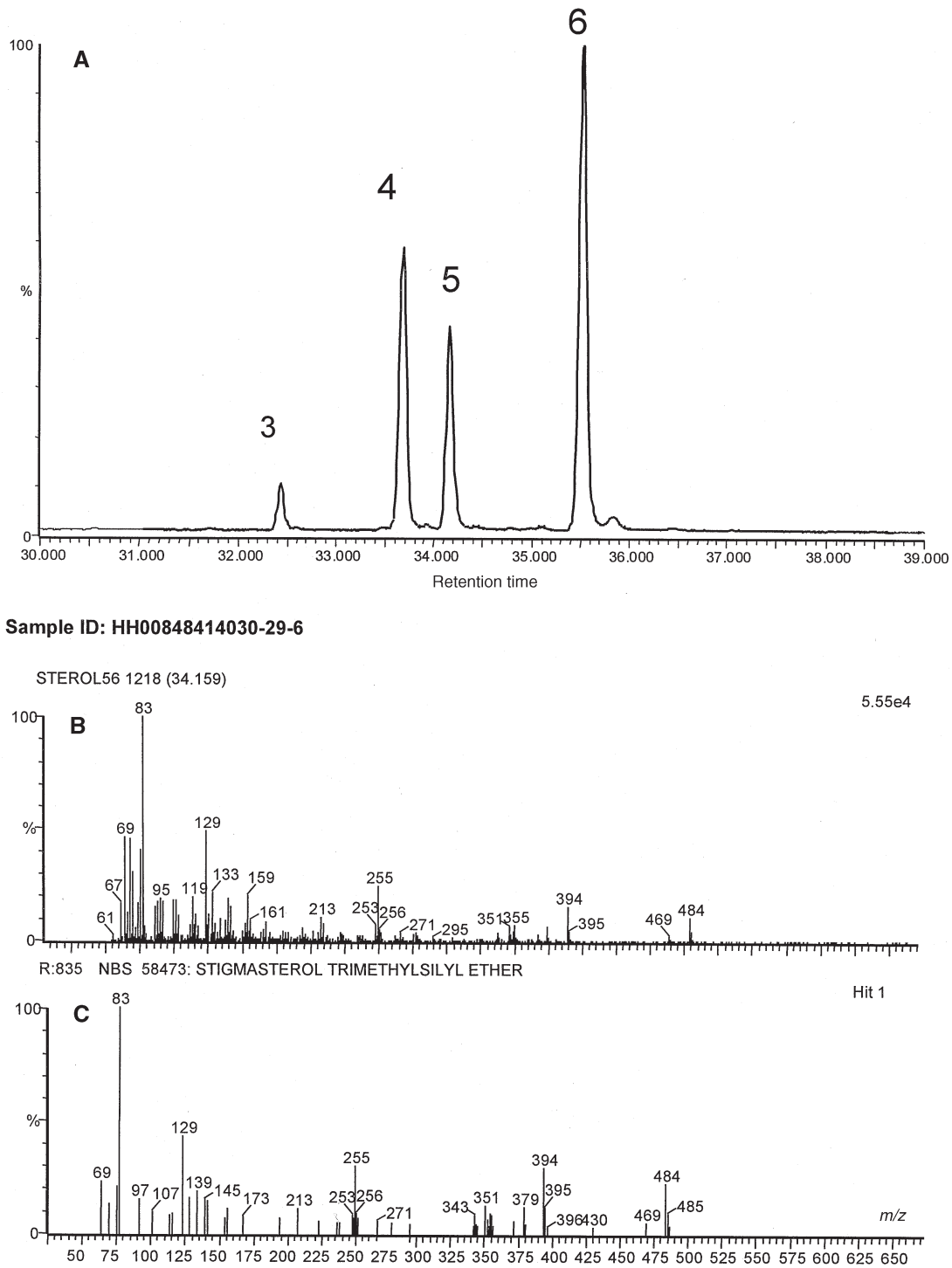

FIG. 2. (A) GC–MS chromatogram of a derivatized [*N,O*-bis(trimethylsilyl)trifluoroacetamide] sterol sample similar to the one shown in Figure 1. Peak labels are identical. (B,C) GC–MS electron-impact spectra of peak no. 5 (A); sample spectrum (B) and (C) from the NIST/NBS library spectrum of stigmasterol.

mapped out by injection of individual reference standards. By careful comparison of all the data (literature, data from pure standards, GC–MS) the sterol peaks could be labeled. In addition, calculation of relative retention times (relative to the IST) was carried out (see Fig. 1). Vegetable oil sterols have been extensively analyzed by GLC, and their characteristic peak pattern has been described in the open scientific literature. The relative retention order of the sterols in this method is quite similar to the order reported for (derivatized) sterols in several other publications (6,8,9). It should be noted that this method is designed for speed, and the PS elute in a relatively short retention time window with partial overlap between certain PS or stanols. The method is therefore not recommended for the identification of unknown sterols.

TABLE 1
Results[a] of the Repeatability Test with Different Products Containing PSE Over the Range 7–60%

	Total PS content (%, ww)			
Repetition no.	35% fat spread	70% fat spread	Fat blend	PSE concentrate
1	7.31	10.27	12.67	61.31
2	7.58	10.30	12.68	60.95
3	7.58	10.30	12.57	61.33
4	7.69	10.25	12.57	61.45
5	No value	10.30	12.60	61.35
Mean ± SD_{n-1}	7.54 ± 0.14	10.28 ± 0.02	12.62 ± 0.05	61.28 ± 0.19
RSD (%)	1.86	0.22	0.42	0.30

[a]RSD, relative SD; PSE, plant sterol esters; PS, plant sterols.

Validation. Prior to the collaborative test we validated the new method for recovery, accuracy, and repeatability. A more definite answer regarding method performance and robustness would be available only after the actual collaborative test. For determination of the accuracy, two samples of medium-chain TAG oil (MCT) were spiked with 15.17 and 15.08 (w/w) of a typical PSE concentrate. The composition of the PSE concentrate was as follows (w/w%): PS 9.1; PSE 90.9. The total level of PS is thus (w/w%) 9.1 + (90.9 × 0.608) = 64.4. The correction factor 0.608 is based on the weight average M.W. of soybean-derived PS (410) and the weight average M.W. of the sunflower-derived FA esters (674). The theoretical levels are thus 9.77 and 9.71%. The analytical results for those MCT spiked samples were 9.78 and 9.66%, respectively, corresponding to 100.1 and 99.5% recovery.

The repeatability was tested with samples of two different spreads (70% fat and 35% fat), a vegetable oil blend, and a PSE concentrate. Each sample was analyzed five times (four times for 35% fat spread) on the same day. The results are presented in Table 1.

To monitor the long-term stability of the total procedure, a control sample (vegetable oil blend with emulsifiers and other ingredients added, equivalent to material used in the production of sterol-enriched vegetable spreads) was selected and analyzed at least once during each run (different days). The sample was dispensed in small vials for individual usage and stored at −20°C. From the initial 20 individual sample runs, the target, warning, and control limits were determined. The mean (or target) value for this particular sample was calculated at 13.04 % and the SD_{n-1} was 0.34% (relative SD 2.61%).

Collaborative test results. All invited participants returned results and technical information, which were screened for deviations from the protocol or for outliers based on the indicated statistical method. One participant (No. 11) used another protocol since this laboratory was unable to carry out the analysis with the cold-on-column injection device. As a result more than half of the data of participant 11 were found to be outliers. This laboratory also was an outlier on laboratory mean in a first screening of the data. The most likely cause for this was the use of a nonprescribed injection method. Split injection of high-boiling components results in discrimination or thermal degradation in the hot GLC injector (10). It was therefore decided to remove all the results submitted by participant 11 from the data set. Upon GLC specialist technical judgment, all data were deemed acceptable for further statistical analysis. Now, participant 10 was found to be outlying based on the laboratory mean. Further investigation of this particular participant's results revealed that although precision was good, the results were always lower than average. All results of participant 10 were removed from the data set. The technical details supplied did not reveal a cause for the outlying results. For participant 8, one outlying duplicate was removed from the data set.

All the data are presented in Figure 3. After removal of the one outlying duplicate from participant 8 and the results of participants 10 and 11 from the data set, the statistical parameters were calculated. Table 2 presents the overall results of the interlaboratory test.

Although this new method is not intended for sterol identification, for most PS the peak identity has been confirmed by GC–MS and accepted library spectra. Moreover, the typical chromatograms obtained with this method are compatible with those described by others or in normative references.

The precision and accuracy as determined by repeated analyses of (spiked) samples are well within typical specification ranges for products, generally ±10% around the target level. The relative SD from the precision tests, the collaborative test data, and the control chart values indicate that such a specification window can be monitored with the current method based on single-sample analysis. However, the method requires attention from the analyst with respect to quality of the IST used, the correct use of the cold-on-column injector, and GC instrument performance.

With respect to the limits to which standard analytical equipment can be pushed; we encountered limitations in the

TABLE 2
Summary Results of the Interlaboratory Test for Total 4-Desmethyl Sterol(s) Analysis[a]

Sample type	Sample number	Number of data	Target level (w/w%)	Overall mean (w/w%)	$s_{R\text{-within}}$	R_{within}	$s_{R\text{-between}}$	$R_{between}$
Spread	SPR (1,2)	16	~8	8.30	0.242	0.680	0.426	1.194
Fat blend								
(low concentration)	FB (3,4)	16	~12	11.83	0.235	0.658	0.345	0.966
(high concentration)	FB (5,6)	16	~20	19.63	0.162	0.454	0.650	1.819
Sterol ester concentrate	SE (7,8)	14	~60	59.95	1.069	2.994	1.084	3.036

[a]$s_{R\text{-within}}$, within-laboratory SD; R_{within}, within-laboratory reproducibility; $s_{R\text{-between}}$, between-laboratory SD; and $R_{between}$, between-laboratory reproducibility.

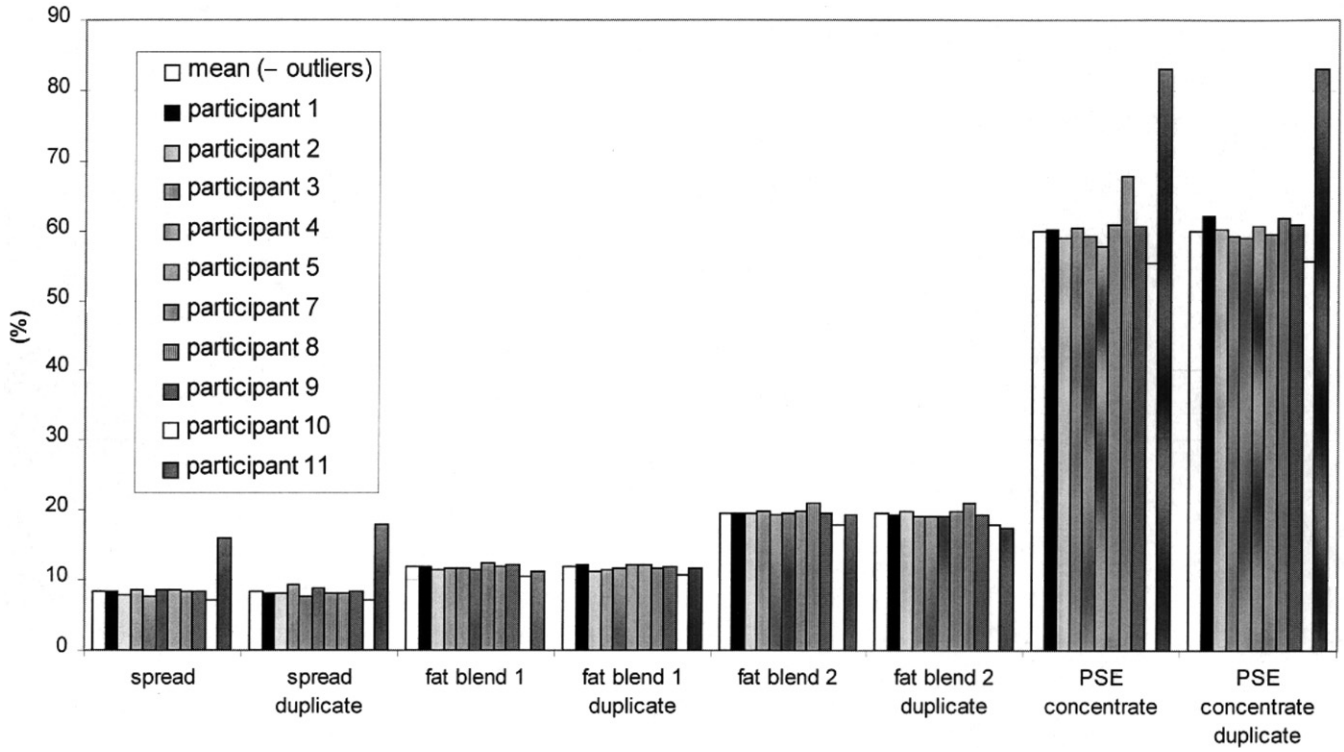

FIG. 3. Graphical presentation of all the received data of the collaborative test for 4-desmethyl sterol(s) content in eight different samples. (x-Axis is sample type; y-axis is percentage of total 4-desmethyl sterol content reported.) PSE, plant sterol FA esters. The far left bar of each group represents mean minus outliers; remaining bars in each group represent participants 1–5, 7–11.

heating rate of older GC instruments in developing the fast GC oven program, thus limiting the method speed. Care should be taken in applying fast heating rates, which are sometimes accepted as instrument keyboard entry but are actually not reached by older instruments at higher oven temperatures. This might result in chromatographic performance

TABLE 3
Instrumental Details from Collaborative Test Participants[a]

Participant	1	2	4	5	7	8	9	10	11
GC brand, type	HP 6890	Shimadzu GC-14B	PerkinElmer Autosystem XL	HP 5890 II	HP5890 II	Fisons, Trace 2000	IS 8000	HP5890 II	STANG2000 (Perichrom)
GC precolumn	1 m × 0.53 mm	2.5 m × 0.53 mm	1 m × 0.53 mm		2.5 m × 0.25 mm CP8007	1 m × 0.53 mm	0.5 m × 0.53 mm	1 m × 0.53 mm	—
Analytical column	CP-Sil5-CB	CP-Sil5-CB	HP1	SP-1	CP-Sil8CB	CP-Sil5CB	CP-Sil5 CB	J&W DB-1	CP-Sil8 CB
Length	10 m	10 m	10 m	10 m	25 m	10 m	10 m	10 m	30 m
Internal diameter	0.32 mm	0.32 mm	0.32 mm	0.32 mm	0.25 mm	0.32 mm	0.32 mm	0.32 mm	0.25 mm
Film thickness	0.12 µm	0.12 µm	0.17 µm	0.12 µm	0.25 µm	0.12 µm	0.12 µm	0.12 µm	0.25 µm
Injector type	COC	Shimadzu OCI-14	OC	COC	HP7673	COC AS2000	AS800	HP7673	Split
Injection volume	0.5 µL	0.5 µL	1.0 µL	1.0 µL	0.5 µL	0.5 µL	0.5 µL	0.5 µL	1.5 µL
Carrier linear velocity	2.2 mL/min	2.2 mL/min	2.2 mL/min	4.99 mL/min	0.600 mL/min	2.2 mL/min (He)	2.2 mL/min	He	He
Temp. program	60°C (1 min) 40°C/min 300°C (3 min)	60°C (1 min) 15°C/min 300°C (3 min)	60°C (1 min) 20°C/min 300°C (3 min)	180°C (0.5 min) 17°C/min 244°C 20°C/min 320°C	60°C (1 min) 35°C/min 150°C 25°C/min 200°C 20°C/min 270°C (15 min)	60°C (1 min) 20°C/min 300°C (3 min)	60°C (1 min) 20°C/min 300°C (3 min)	60°C (1 min) 20°C/min 300°C (3 min)	255°C
FID temperature	320°C	320°C	320°C	320°C	320°C	320°C	320°C	320°C	290°C

[a]No instrument data were received from participant 3. (C)OC = (cool)-on-column.

differences between different instruments. The more modest heating rate of 20°C/min in the final protocol, however, still resulted in a significant decrease in run time. We have shown before that fast GLC runs are possible for typical oil and fats analysis, without compromising the performance, using standard GLC equipment (11).

Participants made several minor changes or modifications in the analytical protocol despite the request to follow the procedure as closely as possible. The changes were based on difficulties in obtaining the required materials, separation with the Unilever Research Vlaardingen GLC oven temperature program (see above), or for other practical reasons (Table 3). To what extent these changes negatively influenced the results remains unknown.

If one looks in more detail into the $R_{between}$ values for the fat-blends and sterol ester raw materials (PSE concentrate), one will notice an increase in $R_{between}$ with increasing level of the sample PSE content. We speculated that the larger $R_{between}$ values are caused by incomplete saponification of the esters. Sterol esters hydrolyze at a slower rate than TAG; hence, the saponification time is set to 50 min at 70°C. Based on the applied chromatographic method, the PS should be present only as PS but not as their FA esters (PSE). Care should thus be taken to ensure a full hydrolysis.

The developed method meets the requirement for fast and accurate total 4-desmethyl sterol(s) analysis for use in quality control and processing of PSE over a wide range of concentrations. The sample preparation was reduced to a minimum without compromising recoveries or precision. The GLC run time improved significantly (about 15 min including cool-down). On top of that we could shorten the sample preparation time significantly compared to the classical approaches based on TLC sample cleanup steps followed by GC analyses of (derivatized) PS.

The internal method validation for recovery and repeatability, and an international collaborative test, confirmed the high performance characteristics of the method.

We suggest that this method be considered by relevant bodies maintaining analytical method collections as a new and improved total 4-desmethyl sterol(s) analysis to meet the new use and concentration range of PS/PSE in consumer products.

ACKNOWLEDGMENTS

Marnix P. van Amerongen, Arjan A. de Groot, Hubert A. Engels, and Edwin B.W. Hendriks were very instrumental in providing us with samples, raw materials, and in providing data on the long-term stability of the method.

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[Received July 24, 2001; accepted December 28, 2001]