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Epoxidized soy bean oil migrating from the gaskets of lids into food packed in glass jars
Analysis by on-line liquid chromatography–gas chromatography

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Abstract

The migration of epoxidized soy bean oil (ESBO) from the gasket in the lids of glass jars into foods, particularly those rich in edible oil, often far exceeds the legal limit (60 mg/kg). ESBO was determined through a methyl ester isomer of diepoxy linoleic acid. Transesterification occurred directly in the homogenized food. From the extracted methyl esters, the diepoxy components were isolated by normal-phase LC and transferred on-line to gas chromatography with flame ionization detection using the on-column interface in the concurrent solvent evaporation mode. The method involves verification elements to ensure the reliability of the results for every sample analyzed. The detection limit is 2–5 mg/kg, depending on the food. Uncertainty of the procedure is below 10%.
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1. Introduction

Gaskets of the lids for glass jars are commonly made of plastisol, i.e. plasticized poly(vinyl chloride) (PVC). They have a soft, sponge-like structure, sometimes foamed with a blowing agent, in order to provide tightness with a minimum of mechanical force (easy opening). Many gaskets contain 35–45% epoxidized soy bean oil (ESBO), which serves as a plasticizer and at the same time as a scavenger for hydrochloric acid liberated from PVC as a result of heat treatment during manufacturing. The ESBO of the part of the gasket in contact with the food may largely be transferred into the food if the latter contains some oil, easily resulting in a migration into food far exceeding the legal limit of 60 mg/kg (see Part II).

The commonly used method for analyzing ESBO in foods (primarily infant foods) originates from Castle et al. [1,2] and is based on determining the methyl ester of diepoxy linoleic acid, from which the ESBO concentration is calculated. Diepoxy linoleic acid is the analyte of choice, because the saturated fatty acids in ESBO are common to all fats and oils and epoxy oleic acid is an oxidation product present at substantial concentrations in edible oil or fat. The method described here follows this route.

The method of Castle et al. extracts the lipids from food and transesterifies them. Then the cyclopentanone dioxolanes of the epoxy functions are formed, which enables the detection of the diepoxy linoleic acid without preseparation of the methyl esters, exploiting the sensitivity and selectivity of mass spectrometry (MS). The dioxolanes increase the retention, pulling the analyte away from the bulk of the fatty acid methyl esters. This method provided good results in numerous laboratories and has a detection limit in infant foods of around several mg/kg.

The method described here does without extraction of the lipids and without formation of dioxolanes, which not only eliminates most of the sample preparation work, but also has the advantage that with transesterification in the food, fatty acids from ESBO bonded to food components by one of the

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acids are included. The transmethylated fatty acids are pre-separated by high performance liquid chromatography (LC), isolating the diepoxy fatty acids, and transferred on-line to gas chromatography (GC) with flame ionization detection (FID) in fully automated mode [3,4]. The method is suitable for routine analysis of large numbers of samples and the verification elements built in ensure a high reliability of the results.

2. Outline of the method

2.1. Transesterification

The lipids are transesterified to methyl esters directly in the homogenized food under the conditions optimized for the fast determination of the fat content and fatty acid composition in foods [5,6]. The kinetics of the transesterification are optimized such that the presence of water does not disturb: it takes 1 min at ambient temperature, i.e. is stopped before saponification starts being significant. Alkali transesterification does not affect epoxy functions and, as long as it is performed at ambient temperature for 1 min, chlorohydrins are not converted (back) to epoxy compounds [7].

2.2. NPLC preseparation

The transmethylated extract is analyzed by on-line coupled liquid chromatography (LC)–GC. Normal-phase high-performance liquid chromatography (NPLC) serves to isolate the diepoxy fatty acids (the diepoxy linoleic acid and the internal standards). A cyano-derivatized silica phase was preferred to silica gel primarily because of the more stable retention properties. A 2 mm I.D. column was used with a flow rate of 300 μl/min, since this provides sufficient capacity and the flow rate fits the requirements of the transfer to GC. The fraction to be transferred to GC was about 1 min broad, i.e. had a volume of about 300 μl.

In NPLC as well as in GC, the diepoxy acids derived from linoleic acid (analyte) and *cis*-11,14-eicosadienoic acid (internal standard) form two completely separated peaks of diastereomers. The elution order on silica gel is opposite to that from the cyano phase. In fact, the combination of the two columns (10 cm × 2 mm I.D. silica gel and 25 cm × 2 mm I.D. cyano phase, compensating for different retention power) resulted in coelution of the two diastereomers. This option was discarded, however, because of the possibility to exploit the separated diastereomers for verification of the transfer window (see below).

The determination of the LC window by UV detection presupposes the injection of large amounts of solute. To rule out subsequent interference by memory effects, control of NPLC preseparation occurred with a component not analyzed for the samples, i.e. the 1-propyl ester of ESBO. First the retention times of the components of interest and the ESBO propyl esters were determined and interrelated. For later control, only the propyl ester was injected.

2.3. LC–GC transfer

Transfer to GC involves concurrent solvent evaporation [8], i.e. the mobile phase is evaporated at the same rate as it is introduced into the oven-thermostatted precolumn. This enables to use a short precolumn (no eluent accumulates during transfer) and renders adjustment of conditions uncritical. Since no solvent trapping is achieved, volatile components are lost, but this is not relevant for the application.

In the past, concurrent solvent evaporation was commonly performed with the loop-type interface [9]. In the interest of unifying LC–GC transfer techniques with large volume on-column injection, the on-column interface was preferred [10], i.e. the permanently installed transfer line introduces the eluent through an on-column injector into the GC precolumn. The liquid is driven by the LC pump. A solvent vapor exit (SVE) is installed between the precolumn and the separation column to release the vapors at a high flow rate (above 100 ml/min), resulting in a correspondingly high solvent evaporation rate. As precolumn, a short coated capillary is used: there is no significant band broadening in space (short flooded zone), i.e. no retention gap effect is needed to reconcentrate the initial band [10]; the presence of stationary phase renders retention of the solutes of interest during solvent evaporation more robust. A short uncoated precolumn was added as inlet as it appeared that more samples could be run before replacement (around 100).

2.4. Quantitative determination

As the ESBO concentration is calculated from a single fatty acid, the results depend on the content of diepoxy linoleic acid in the ESBO. However, the variation is limited (see below), i.e. for many purposes the results are of sufficient accuracy when an average content is assumed. For a more accurate measurement, the composition of the ESBO in the gasket was determined. The analysis of the extract from the gasket also shows the presence of epoxidized linseed oil (ELO) and some other plasticizers being used instead of or in addition to ESBO.

Concentrations were determined with diepoxy eicosanoic acid as internal standard, prepared by epoxidation of methyl *cis*-11,14-eicosadienoate. Since the purity of the standard and the yield of the epoxidation were considered unknown, the calibration referred to a standard of ESBO.

2.5. Verification

The method is designed with verification elements, i.e. internal standards checking the performance of the critical steps for every analysis. Saponification during transesterification and poor extraction of the methyl esters from the food are detected by an internal standard added after working up

the sample. The most critical step is the cut of the LC window for transfer to GC. As the retention times of the solutes of interest in the sample cannot be verified by the UV detector, they are controlled by GC using the diastereomers which are at the edge of the relevant LC fraction and not used for the quantitative determination.

3. Procedure

3.1. Materials

ESBO (Edenol D82) was a gift from Vernicolor, Grüningen, Switzerland. Methyl *cis*-11,14-eicosadienoate was from Sigma (through Fluka, Buchs, Switzerland). Sodium methoxide (30% in methanol), sodium ethoxide (21% in ethanol), sodium, disodiumhydrogen citrate, 3-chloroperoxybenzoic acid, chloroform and 1,4-dioxane were from Fluka, methanol and ethanol from Merck (Darmstadt, Germany). Pentane and methyl *tert*-butyl ether (MTBE) were of technical grade (Siegfried, Zofingen, Switzerland) and redistilled. At the time of the publication of this paper, diepoxy methyl eicosadienoate and ESBO will be available from Fluka.

For on-line LC–GC, a Dualchrom 3000 (Fisons, today Thermo Electron, Milan, Italy) was used, composed of an autosampler, a syringe pump, a manifold with an injection and a backflush valve, a UV detector, a transfer valve and a GC with on-column injector and SVE. The instrument is fully automated.

3.2. Preparation of standards

The internal standard 1 (IS1), diepoxy methyl eicosanoate, was prepared analogously to Castle et al. [2]. Fifty milligrams of 3-chloroperoxybenzoic acid was dissolved in 2 ml chloroform. Twenty-five milligrams of methyl *cis*-11,14-eicosadienoate was added and this mixture kept at ambient temperature overnight. It was then extracted three times each with 1% sodium sulfite, 10% sodium hydrogencarbonate and water. The chloroform solution was centrifuged to remove residual water and then 1:10 diluted in 20% MTBE/hexane to obtain a solution of about 1 mg/ml. The content was measured mixing this solution 1:1 with a solution of ESBO and comparing the area of the two diastereomers of diepoxy methyl eicosanoate with the sum of all ESBO peaks by GC–FID.

For determining the LC window, ESBO methyl esters were prepared by transesterification as described below for food samples, except that no internal standards were added (10 mg ESBO ending in 10 ml extract, i.e. 1 mg/ml). ESBO ethyl esters, serving as internal standard 2 (IS2), were prepared with 5% ethoxide in ethanol in the same way as the methyl esters, but with a reaction time of 2 min (1 mg/ml). The 1-propyl ester was obtained by reaction at 60 °C for 15 min. Sodium propylate was prepared by adding 50 ml 1-propanol to 0.5 g sodium and stirring at 80 °C until the sodium was dissolved.

3.3. Sample preparation

The content of three to five jars was combined in order to obtain an average result. Food adhering to the lid was removed by a spatula as thoroughly as it might be realistic in normal consumption and combined with the content of the jar. The whole content of the jar(s) was homogenized using a Polytron (Kinematica, Luzern, Switzerland). For products in oil, homogenization was difficult owing to rapid sedimentation. Mostly it was helpful to cool the samples in the refrigerator to increase the viscosity of the oil prior to (short) mixing. In other cases, only the oil was analyzed and the concentration referring to the whole product calculated assuming that the whole ESBO was in the oil.

To 200 mg homogenated food or 100 mg oil in a 50 ml Erlenmeyer with glass stopper, 4 ml dioxane and 10 μl of IS1 solution were admixed, dissolving or suspending the sample and rendering lipids (including ESBO) accessible. For transesterification, 5 ml methoxide/methanol 5% were added. After 1.5 min, the reaction was stopped and the esters extracted by first adding 10 ml 20% MTBE/hexane (short mixing, resulting in a single phase of liquid) and then 10 ml disodium hydrogencitrate 15% (separating two phases). 50 μl IS2 (ESBO ethyl esters) were admixed to the extract before separation of the phases. Clean phase separation may take some minutes.

3.4. NPLC preseparation

Twenty microliters of the transesterified extract was chromatographed on a 250 mm × 2 mm I.D. column packed with the cyano phase Grom-Sil 100 Cyano-2 PR, 5 μm (Grom, Rottenburg-Hailfingen, Germany) using 20% MTBE/pentane at 300 μl/min as mobile phase.

The transfer window was established by UV detection at 225 nm, the first time separately injecting the methyl, ethyl and 1-propyl esters of ESBO and the IS1 at 1 mg/ml. Fig. 1 shows the retention times of these components, with the peak maxima of the two diastereomers interconnected by a horizontal line. The dark gray area is the part of the transfer

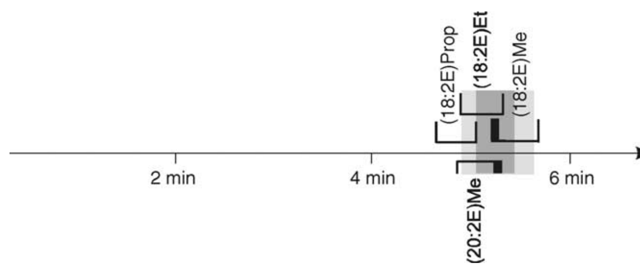

Fig. 1. Elution of the components of interest from the NPLC column: the vertical lines indicate the retention times of the peak maxima. The two diastereomers are interconnected by a horizontal line.

window which served for the measurement of ESBO. The broader light gray zone shows the window selected in practice to verify the correct position.

The diastereomers are referred to as 1 and 2 by the elution order from the cyano LC column and from GC, 1 being approximately of doubled size compared to 2. The diastereomer 1 of diepoxy methyl linoleate, abbreviated as (18:2E)Me1, is the analyte measured for determining ESBO; diastereomer 2 of the diepoxy methyl eicosadienoate (20:2E)Me2, is used as internal standard.

Verification of the transfer window makes use of (18:2E)Me2 and (20:2E)Me1, i.e. the diastereomers not used for quantitation: they must be present (visible in GC) to ensure that (18:2E)Me1 and (20:2E)Me2 are fully included in the fraction. As they were completely separated from the compounds used for measurement, the presence of a small amount was sufficient (see also GC analysis).

The ethyl esters of the diepoxy linoleic acid (from the ethyl esters of ESBO, IS2), are used for the verification of the extraction yield and absence of relevant saponification during the transesterification step. Quantitative transfer of (18:2E)Et2 to GC is ensured if the two compounds at the edge of the fraction, (20:2E)Me1 and (18:2E)Me2, are present in the gas chromatogram. The yield is calculated as the ratio (18:2E)Et2/(20:2E)Me2 corrected by the calibration of the responses and concentrations.

The retention times of the compounds of interest are related to those of the propyl esters in order to enable checking of the LC window without injection of the solutes of interest. For the column used, the transfer window started at the onset of the peak of (18:2E)Prop2. It was 1 min broad. The window could be narrowed in case disturbing interferences had to be removed, but the broader margin renders the method more robust.

3.5. LC–GC transfer and GC analysis

The transfer valve feeds the effluent from the LC detector to waste or to GC. The transfer line, reaching from the transfer valve through the on-column injector about 5 mm deep into the oven-thermostatted GC precolumn, consisted of a 25 cm × 0.17 mm O.D. raw fused silica capillary. After transfer it was backflushed through a 25 cm × 50 μm I.D. fused silica resistance mounted in the transfer valve by the carrier gas entering from the inlet of the GC precolumn.

The GC precolumn consisted of a 25 cm × 0.53 mm I.D. deactivated uncoated precolumn and a 25 cm × 0.53 mm I.D. capillary in the laboratory coated by a 0.2 μm film of immobilized methyl polysiloxane PS-255 (Fluka). The coated precolumn ended in a press-fit Y-piece connecting to the 30 m × 0.25 mm I.D. separation column coated with a 0.2 μm film of PS-255 and the vapor outlet consisting of a 20 cm × 0.53 mm I.D. raw fused silica capillary. The SVE was fully open from the start of transfer to 5 s after its end and then switched to a 20 cm × 50 μm I.D. fused silica restriction.

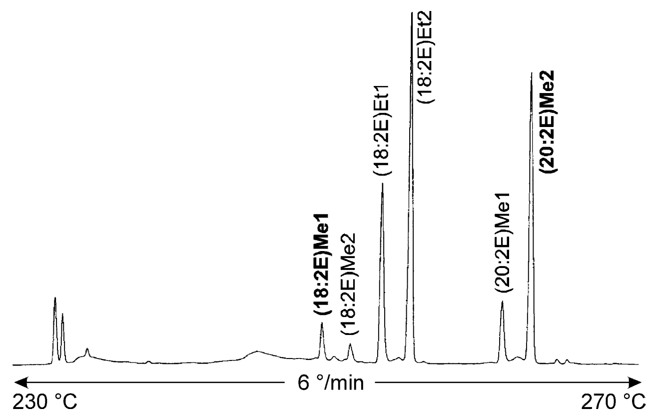

Fig. 2. Relevant section of an LC–GC–FID chromatogram from an infant food, highlighting, firstly, that chromatograms from infant foods usually show hardly any interfering peak and, secondly, that the fraction window was correct (see text).

Transfer occurred at 80 °C oven temperature and with a gas inlet pressure of 90 kPa (hydrogen). The oven temperature was programmed at 30 °C/min to 160 °C, then at 6 °C/min to 260 °C and at 30 °C/min to 330 °C (5 min). After completing transfer to GC, the LC column was backflushed with 1 ml MTBE (introduced into the backflush loop from a pressurized reservoir) during 15 min.

3.6. Gas chromatograms

Fig. 2 shows the relevant section of the gas chromatogram from an infant food (Lasagne) containing 8 mg/kg ESBO. The sequence of elution of the two diastereomers corresponds to that from the cyano column in NPLC, but now the main analyte (18:2E)Me1, is the first peak and the internal standard (20:2E)Me2, is the last. (18:2E)Et2, used to determine the recovery, reached 96% in this case (it was hardly ever below 90%).

The LC window is checked by the diastereomers eluted at the borders of the fraction. The starting cut is checked by (20:2E)Me1 and (18:2E)Et1, which are contained in ESBO at about doubled concentrations compared to the diastereomers 2. They are present in the gas chromatogram as substantially smaller peaks, indicating that the major part was cut away, but owing to the complete separation in LC, even a small amount ensures complete inclusion of the diastereomers 2. The terminating cut of the window is checked by (18:2E)Me2. The peak is about three times smaller than (18:2E)Me1, indicating that it was largely included (it is about half of the size to start with). It is concluded that the analysis performed correctly; the window could have been shifted slightly earlier in order to more evenly use the margin.

Fig. 3 shows a complete chromatogram from a more demanding sample (dried tomato in olive oil) containing 100 mg/kg ESBO, pointing out the interferences often encountered with oily foods. The first quarter of the chromatogram does not show peaks, because the volatile components are lost during concurrent solvent evaporation. The

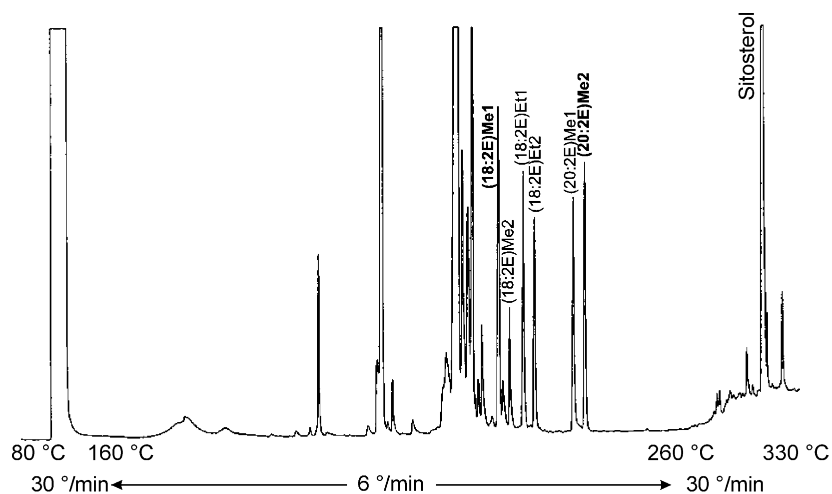

Fig. 3. LC–GC–FID chromatogram from edible oil from an oily product.

compounds of interest are well separated from a group of derivatives of fatty acids not identified so far, but present also in products not in contact with ESBO (probably largely hydroxy fatty acids of 16 and 18 carbon atoms). A frequently observed, potentially interfering compound can be placed between (18:2E)Me1 and (18:2E)Me2 by adjustment of the elution temperature: at rather high elution temperature it may be coeluted with (18:2E)Me1, i.e. the temperature program was slowed to 6 °/min and the inlet pressure increased to 90 kPa.

Sometimes the peaks of interest were disturbingly broadened and distorted when edible oils or extracts from samples rich in oil were analyzed. It turned out to be the result of overloading of the GC column with sterols which are eluted later in the gas chromatogram (Fig. 3). This overloading affects the earlier eluted components by partial solvent trapping and phase soaking [11–13]. In fact, sterol concentrations in edible oils are usually above 1000 mg/kg, i.e. exceed those of the components of interest easily by a factor of 100.

The peak broadening could be reduced or avoided by injecting less sample material, i.e. running the analysis at lower attenuation. More effective, however, was the adjustment of the fraction transferred from NPLC: as the sterols were eluted slightly after the components of interest, a sharp termination of the transfer window strongly reduced the amount of sterols reaching GC. In fact, in Fig. 3, less than half of (18:2E)Me2 is observed and the amount of sitosterol is around 300 mg/kg, i.e. some 80% was removed. If necessary the transfer window could be further restricted, although on cost of the robustness of the analysis.

The sterols can be completely removed by silylation of the sample prior to injection into NPLC: the silyl ethers are eluted before the fraction of interest. Silylation also removes most of the large peaks eluted before the components of interest (the hydroxy fatty acids?) from the window transferred to GC. Using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) without trimethylchlorosilane as catalyst, the epoxy functions of the analytes are not affected.

3.7. Quantitative determination

Calibration occurred with every series of analysis: 50 μl of a 1 mg/ml ESBO solution in 1,4-dioxane and 10 μl IS1 solution were treated as a sample, i.e. transesterified and analyzed by LC–GC–FID. The resulting ratios of peak areas account for the FID response, the true concentration of the IS1 and the content of (18:2E)Me1 in the ESBO are used for calibration. Since the ESBO solution is the reference point, the accurate concentration of the IS1 is uncritical. The proportion of (18:2E)Me1 in the ESBO used for calibration was determined by normalization, analyzing the ESBO methyl esters directly by GC–FID on a 15 m × 0.25 mm I.D. column coated with a methyl polysiloxane.

3.8. Composition of ESBO

From 73 lids the fatty acid composition of ESBO was analyzed. The mean content of (18:2E)Me1 was 33.0%, with a standard deviation of 1.9% (5.8% relative standard deviation). The extreme values were 26.7 and 36.4%. Thus, a (18:2E)Me1 content of 33% can be assumed without analyzing the ESBO composition in the gaskets if an error contribution to the final result corresponding to a relative standard deviation of 5.8% can be tolerated. In this instance, the calibrated factors should be recalculated for an ESBO of 33% (18:2E)Me1.

For a more accurate determination, the concentration of (18:2E)Me1 is determined for the ESBO in the gasket of the lid. The lid was cleaned with water and rinsed with pentane to remove oil or fat (maximum contact time, 30 s). Then dioxane was poured into the lid and the lid covered by a plate. After 1 h extraction time, 1 ml of the extract was transesterified (adding 5 ml methoxide/methanol, etc. as described above). The methyl esters were analyzed by GC–FID using a 15 m × 0.25 mm I.D. PS-255 column and injecting the sample by the on-column technique.

The concentration of (18:2E)Me1 was calculated by normalization on the sum of all ESBO components. Fat or oil transferred from the food into the gasket is relevant for the saturated fatty acids (16:0 and 18:0), as these are the acids in common with the ESBO. It is detected by the presence of non-epoxidized unsaturated acid (primarily 18:1 and 18:2). When these acids amounted to more than 3% of the total methyl esters, the peak areas of methyl palmitate and methyl stearate introduced by the oil or fat were calculated and deduced from the area belonging to ESBO. In the case of oily food, it may be easier to cut some gasket material from the region without food contact, i.e. from outside the zone tightening against the glass, and to extract this.

3.9. Products in oil

For products immersed in oil it may be satisfactory or preferable to analyze the oil alone and to calculate this data to the total product assuming that all ESBO is in the oil phase. This was checked for a sample of peppers in oil from which 36% oil dropped off. The oil contained 240 mg/kg of ESBO, which is calculated as 87 mg/kg when referred to the total jar content. The peppers contained 16 mg/kg ESBO, probably primarily from the adhering oil. Calculated to the total content of the jar, this is 12 mg/kg. In conclusion, if only the oil was analyzed, the result was 14% too low. Analogous tests for dried tomatoes, artichokes and garlic in oil resulted in differences between 5 and 12%.

4. Validation of the method

For infant foods, the detection limit of the method was 2 mg/kg ESBO, the limit for quantitative determination 6 mg/kg. For foods rich in oil or fat, occasional disturbance enhanced the detection limit to about 5 mg/kg.

The transesterified extract from a sample containing some 86 mg/kg ESBO (infant food composed of potato and meat) was analyzed five times by LC–GC. The results had a relative standard deviation of 1.5%. A sample of infant food (potato, broccoli and meat) was carried through the whole analysis six times. The mean result of 47 mg/kg had a relative standard deviation of 2.7%. Four fundamentally different infant foods with low ESBO contents were spiked with 100 mg/kg ESBO. 94–103 mg/kg were recovered. Linearity was checked by spiking a sample of infant food (rice and vegetables) virtually free of ESBO with 20, 50, 80 and 100 mg/kg ESBO. 22, 50, 77 and 99 mg/kg ESBO were found.

These experiments were performed with a given ESBO. As shown above, the content of (18:2E)Me1 in ESBO extracted from lids varied with a relative standard deviation of 5.8%. If the ESBO composition in the lid is not determined and an average content of 33% is introduced into the calculation, this is the predominant contribution to uncertainty and the results can be considered to be accurate to within about 15%. If the ESBO composition is determined, the tests on reproducibility and many duplicate analyses performed in routine indicated an uncertainty of the measurement below 10%.

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