JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Determination of Seven Glycidyl Esters in Edible Oils by Gel Permeation Chromatography Extraction and Liquid Chromatography Coupled to Mass Spectrometry Detection

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ABSTRACT: A method based on a gel permeation chromatography (GPC) extraction procedure combined with an additional cleanup by solid-phase extraction (SPE) on silica gel and liquid chromatography−mass spectrometry (LC-MS) detection has been validated for the analysis of seven glycidyl esters (GEs) including glycidyl laurate, myristate, palmitate, stearate, oleate, linoleate, and linolenate in various edible oils. This method was conjointly developed and validated by two different laboratories, using two different detection systems, a LC time of flight MS (LC-ToF-MS) and a LC triple-quadrupole MS (LC-MS/MS). The extraction procedure allowed targeting low contamination levels due to a highly efficient matrix removal from the 400 mg oil sample loaded on the GPC column and is suitable for routine analysis as 24 samples can be extracted in an automated and reproducible way every 12 h. GPC extraction combined with SPE cleanup and LC-MS/MS detection leads to a limit of quantification in oil samples between 50 and 100 μg/kg depending on the type of glycidyl ester. Recoveries ranged from 68 to 111% (average = 93%). Quantification was performed by automated standard addition on extracts to compensate matrix effects artifacts. To control recoveries of each sample four isotopically labeled GEs ($^{13}C_3$ or $^{13}C_4$) were included in the method.

KEYWORDS: edible oils, glycidyl esters, palm oil, liquid chromatography−tandem mass spectrometry, LC-MS/MS, LC-ToF

INTRODUCTION

Fatty acid esters of glycidol (glycidyl esters, GEs) have recently been detected[1,2] in the frame of fatty acid esters of 3-chloropropane-1,2-diol (MEs) analysis in refined vegetable oils. The presence of GEs in food has raised safety concerns due to a potential release of glycidol by means of enzymes in the gut. The toxicity of glycidol has been studied in several animal species. Glycidol is generally considered to be a genotoxic carcinogen and therefore no health-based guidance value has been established.[3−6] In its initial safety evaluation, the German Federal Institute for Risk Assessment (BfR) indicated the necessity to develop and validate suitable detection methods for GEs to support risk assessment.[1]

Due to structural similarity between the GEs and MEs, the first method for the determination of GEs was close to that used for MEs and 3-monochloropropane-1,2-diol (3-MCPD) determination, which have also been found in refined oils.[7−9] The different methods published for 3-MCPD ester determination are mainly based on transesterification of the esters to release free 3-MCPD, analysis of which benefit from more than 20 years of expertise.[7,10−12] These approaches rely on the formation of a stable volatile derivative further characterized mainly by the use of gas chromatography coupled to mass spectrometry (GC-MS). The first critical step involves a transesterification commonly conducted under acidic or alkaline conditions, and discrepancies in generated results depending on these transesterification conditions have been already reported in 2008.[13,14] Thus, amounts of 3-MCPD esters were overestimated when an alkaline treatment was combined with the use of a derivatization solution containing inorganic chloride, which led Kuhlmann and Weißhaar[13] to postulate the presence of GEs in oil samples, later confirmed in 2009 by Weißhaar and Perz.[2]

Until today three types of quantification methods have been proposed for GE determination. The first one estimates GEs by calculating the difference between total 3-MCPD and true 3-MCPD. It postulates that under alkaline transesterification followed by an acidic treatment in the presence of inorganic chlorine, GEs are quantatively transformed into 2- and 3-MCPDs. True 3-MCPDs being not affected by this treatment, the sum of true 3-MCPDs and newly formed 3-MCPDs from GEs gives a total 3-MCPDs.[15] This approach has allowed evaluation for the first time of levels of GEs in edible oils but is still subject to high measurement uncertainty due to cumulative error in subtracting two independent results.

The second type of method addressing GE determination is based also on transesterification under smooth alkaline conditions, followed by glycidol transformation into monobromopropanediol (MBPD). MBPD as well as 2- and 3-MCPDs are then derivatized and analyzed in a single GC-MS acquisition.[14] Although this approach allows determination of GEs and MEs, the high reactivity and instability of glycidol generated as well as possible transformation of MCPD into glycidol during alkaline transesterification have driven a third type of method based on direct determination to be considered.

Received: July 15, 2011
Revised: October 20, 2011
Accepted: October 22, 2011
Published: October 22, 2011

Table 1. Chemical Formula of GEs and Their Respective Retention Times Used for Data Treatment

GE	chemical formula	RT (min)	monoisotopic mass (Da)
glycidyl laurate	$C_{15}H_{28}O_3$	1.58	256.2040
glycidyl myristate	$C_{17}H_{32}O_3$	2.30	284.2351
glycidyl stearate	$C_{21}H_{40}O_3$	3.82	340.2977
glycidyl palmitate	$C_{19}H_{36}O_3$	3.07	312.2664
glycidyl oleate	$C_{21}H_{38}O_3$	3.27	338.2821
glycidyl linoleate	$C_{21}H_{36}O_3$	2.81	336.2664
glycidyl linolenate	$C_{21}H_{34}O_3$	2.42	334.2508
glycidyl palmitate IS	$^{13}C_4C_{15}H_{36}O_3$	3.07	316.2799
glycidyl oleate IS	$^{13}C_4C_{17}H_{38}O_3$	3.27	342.2955
glycidyl linoleate IS	$^{13}C_3C_{18}H_{36}O_3$	2.81	339.2765
glycidyl linolenate IS	$^{13}C_3C_{18}H_{34}O_3$	2.42	337.2609

Since 2010, several methods have been published for intact GE determination by liquid chromatography coupled to mass spectrometry (LC-MS) without any chemical transformation of analytes. One proposed by Masukawa et al.[16−19] is based on GE extraction by two orthogonal solid-phase extraction (SPE) and LC-MS detection by single ion monitoring, whereas a second one proposed by Haines et al.[20] is free of any sample preparation and allows detection of MEs and GEs by simple dilution of the oil sample and injection on a high-resolution mass spectrometer (time of flight (ToF)-MS) coupled to liquid chromatography. Another development of direct method was recently proposed by Granvogl and Schieberle and is based on silica gel extraction in combination with stable isotope dilution analysis.[21] Whereas evidence for the presence of GEs in edible oils is now plentiful, reliable and accurate methods for their quantification applicable in routine environments for a large range of edible oils are still not available. In a mitigation perspective supported by governmental organization, in which levels of GEs in oils are expected to be decreased in the future, an efficient monitoring should rely on a sensitive and robust method able to accurately quantify concentrations over 3−4 orders of magnitude.

This paper presents an integrated approach, from the choice of standards regarding the different types of oils to be monitored, to validation data to illustrate method performances, until quantification approaches. The direct method presented here uses GE extraction by gel permeation chromatography (GPC) as proposed in 2009 by Weißhaar,[2] following a procedure described in an official method for pesticide residue analysis (DFG S19[22]) and in method G of document EN 1528-3:1996. In addition, a cleanup by SPE was added for matrices having high monoacylglycerol (MAG) and diacylglycerol (DAG) contents. It was combined with a LC-MS detection system, either with LC-TOF-MS mainly for method development or with triple quadrupole (LC-MS/MS) mainly for method validation and routine analysis.

MATERIALS AND METHODS

Chemicals and Reagents. HPLC grade LiChrosolv water, 2-propanol, and formic acid 98−100% were from Merck (Darmstadt, Germany). Methanol LC-MS grade was from Fisher (Waltham, MA). HPLC grade acetone, cyclohexane, and dichloromethane as well as ammonium formate were supplied by Sigma-Aldrich (St. Louis, MO). Ethyl acetate was from Carlo Erba Reactifs SDS (Val de Reuil, France). Bakerbond 500 mg silica cartridges, 3 mL, were provided by Aventor (Phillipsburg, NJ). Glycidyl oleate (purity > 99%, GC), glycidyl palmitate (purity > 99%, GC), glycidyl linolenate (purity = 86%, GC), and glycidyl linoleate (purity > 94%, GC) were from Wako (Osaka, Japan). Glycidyl laurate, glycidyl myristate, and glycidyl stearate were from Toronto Research Chemicals TRC (Ontario, Canada). Four isotopically labeled chemical standards have been custom synthesized by Atlanchim Pharma (Nantes, France) and included glycidyl palmitate and glycidyl oleate labeled with four ^{13}C (three ^{13}C on glycidol backbone and one ^{13}C on carboxyl group) and glycidyl linoleate and glycidyl linolenate, each labeled with three ^{13}C (on glycidol backbone).

Standard Solutions. All stock solutions were prepared in dichloromethane. Individual stock standard solutions (labeled and unlabeled) were prepared at a 1 mg/mL concentration (minimum weighed amount = 20 mg). An unlabeled GE composite stock solution comprising glycidyl laurate, myristate, palmitate, stearate, oleate, linoleate, and linolenate, each at 50 μg/mL, was subsequently prepared from individual stock solutions and further stored at 4 °C. An isotopically labeled GE mix solution (IS-GE mix solution), containing $^{13}C_4$-glycidyl palmitate, $^{13}C_4$-glycidyl oleate, $^{13}C_3$-glycidyl linoleate, and $^{13}C_3$-glycidyl linolenate was prepared similarly. Both composited standard solutions were stored at 4 °C and allowed to warm at room temperature before use. For quantification purposes by external calibration on LC-ToF-MS instrument, a calibration curve was generated by diluting in acetone the unlabeled GE mix solution at concentrations of 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0 μg/mL, each containing the isotopically labeled glycidyl ester standards at a constant concentration of 0.2 μg/mL. For quantification by the matrix-matched standard addition procedure, two standard solutions used for spiking of the sample extracts (before LC-MS injection) were prepared by diluting the GE mix solution and IS-GE solution at 0.2 μg/mL (spike 1) and 0.8 μg/mL (spike 2) in acetone.

Samples. A total of 70 edible oil samples were considered for analysis and included palm oil (21), palm olein (8), palm kernel oil (6), coconut oil (5), rapeseed oil (3), sunflower oil (6), blended oils (17), and soy oil (1). Considering oils with different fatty acid compositions was necessary to ensure a broad application scope of the method. All samples were kept at 4 °C and protected from light in airtight containers until analysis. Solid oil samples were melted in an oven at 60 °C (maximum 1 h, depending on sample size) and thoroughly homogenized prior to analysis.

Sample Preparation. One gram was accurately weighed into a 10 mL volumetric flask, followed by the addition of 10 μL of the IS-GE mix solution at 50 μg/mL (corresponding to a 0.5 μg/g equivalent in sample concentration) and further dissolved in cyclohexane/ethyl acetate (1:1, v/v) up to the 10 mL mark. The whole solution was then transferred into a GPC tube. A 4 mL aliquot was injected on an automated GPC cleanup system J2 Scientific AccuPrep MPS as described by Weißhaar and Perz.[2] GE extraction was made on a glass column (380 mm length, 25 mm i.d.) packed with Bio-Beads S-X3 (55 g, 200−400 mesh, Bio-Rad Laboratories) using cyclohexane/ethyl acetate (1:1, v/v) in isocratic mode (5 mL/min). Total GPC run time was 30 min per sample (150 mL elution volume). The 18−26 min fraction was collected (about 40 mL) in a round-bottom flask and evaporated to a small volume (ca. 1 mL) under vacuum (200 mbar at 40 °C), quantitatively transferred into a 7 mL amber tarred vial, and then dried under a gentle stream of nitrogen and finally weighed. The residue was reconstituted either in 1 mL of acetone prior to LC-MS analysis or in 500 μL of dichloromethane for an additional cleanup, as described later under Additional SPE Cleanup.

In the frame of method development, GPC elution profiles of GEs as well as matrix constituents were beforehand determined by spiking 1 g of palm oil sample with 100 μL of GE mix solution containing the seven GE standards, to have each GE at a final concentration above 5 μg/g in oil.

The sample was extracted as described previously (diluted in a 10 mL volumetric flask with GPC solvent followed by 4 mL injection), but collecting 24 fractions of 5 mL from elution volume of 30−150 mL. Each fraction was then evaporated under a stream of nitrogen, weighed in a beforehand tarred vial, reconstituted in 1 mL of acetone and further diluted 5 times for fractions exceeding 20 mg, and finally analyzed by LC-ToF-MS as described under LC-ESI-ToF-MS.

Table 2. Transition Reactions Monitored by LC-MS/MS for the Analysis of GEs with Corresponding Collision Energy (CE)

analyte	RT (min)	fragmentor (V)	precursor ion $(M + H)^+$	product ion[a]	CE (V)
glycidyl laurate	5.89	100	257.3	Q: 57.1	20
				C: 71.1	15
				C: 95.0	10
glycidyl myristate	7.60	130	285.3	Q: 57.1	30
				C: 71.2	15
				C: 95.1	15
glycidyl palmitate	8.93	130	313.3	Q: 57.0	25
				C: 71.0	20
				C: 85.2	15
glycidyl stearate	10.15	150	341.3	Q: 57.1	30
				C: 71.2	25
				C: 85.1	20
glycidyl oleate	9.22	150	339.3	Q: 57.0	25
				C: 69.1	25
				C: 83.2	25
glycidyl linoleate	8.39	150	337.3	Q: 57.0	25
				C: 67.1	30
				C: 81.1	15
glycidyl linolenate	7.61	150	335.3	Q: 57.0	25
				C: 67.1	30
				C: 81.1	20

[a] Q is the transition reaction used for quantification, and C is the transition reaction used for confirmation.

Additional SPE Cleanup for Oil Containing High DAG and MAG Contents. A 500 mg silica cartridge was first conditioned with 10 mL of dichloromethane. The GPC extract (500 μL) obtained as described above was quantitatively loaded onto the column by further rinsing twice the initial vial with 1 mL of dichloromethane. Elution was then performed with 8 mL of dichloromethane (total elution volume = 10 mL). Extract was then dried under a stream of nitrogen and finally reconstituted in 1 mL of acetone prior to LC-MS analysis.

LC-MS Analyses. *LC-ToF-MS* was used for method development purposes such as sample preparation and liquid chromatography optimization (screening capabilities and mass accuracy of this instrument), as well as for collision-induced dissociation (CID) GE ion identification. LC-ToF-MS was used to a lesser extent for quantitative analysis of GEs in oil samples. The LC-ToF-MS instrument comprised a 1290 Infinity UHPLC (Agilent Technologies, Santa Clara, CA) coupled to a 6540 ultrahigh definition Q-TOF MS analyzer (Agilent Technologies). The chromatography column used was a 50 × 2.1 mm i.d., 1.8 μm, Acquity UPLC HSS T3, with a 5 × 2.1 mm i.d. Acquity UPLC HSS T3 Van Guard column (Waters, Milford, MA). Mobile phase A consisted of methanol/water (75:25, v/v) with 0.1% formic acid, and mobile phase B consisted of 2-propanol with 0.1% formic acid. The column temperature was maintained at 60 °C. The injection volume was 2 μL, and a gradient program was applied at a 600 μL/min flow rate as follows: linear gradient from 0 to 95% B from 0 to 12 min, then kept at 95% B for 3 min, and equilibrated from 12 to 15 min at 100% A. An injector program was used to perform standard addition on extracts. Three additional vials were placed in the autosampler: the first vial containing acetone and the second and third vials containing a mix of all GEs and isotopically labeled GEs (standard solution section, spike 1 and spike 2 solutions). When spike 1 and spike 2 solutions were mixed with an equal volume of sample extract, standards added were equivalent to 0.5 and 2 μg/g of oil. Each sample extract was divided in two portions, one being diluted 5 times in acetone, leading to two vials for LC-MS injection, a diluted and a nondiluted sample extract. For each of these two sample extracts, three runs were performed as follow: the needle withdrew 1 μL of the extract and 1 μL of one of the three vials (acetone, spike 1, and spike 2), mix in the syringe and inject (total of six injections per oil sample). Ionization

Table 3. Fatty Acid Distribution (Weight Percentage) in Various Oils (Those above 40% Are Shown in Bold) and GE Standard Availability[a]

	caprylic acid	capric acid	lauric acid	myristic acid	palmitic acid	stearic acid	oleic acid	linoleic acid	linolenic acid
canola oil				0.1	5	2	**60**	22	10
coconut oil	8	6	**48**	18	9	3	6	2	0.1
corn oil					12	2	28	**56**	1
cottonseed oil				1	24	2	17	**53**	0.2
flaxseed oil					6	3	18	17	**55**
grapeseed oil					4	2	18	**65**	0.6
olive oil					12	3	**72**	9	0.6
palm oil			0.4	1	**44**	4	39	10	0.3
palm olein			0.2	1	38	4	**44**	11	0.2
palm kernel oil	4	4	**46**	18	8	2	16.4	3	
safflower oil					7	2	13	**78**	0.2
soybean oil				0.1	11	4	24	**52**	8
GE availability as standards	no	no	yes	yes	yes	yes	yes	yes	yes

[a] Some minor fatty acids were not included, leading to totals slightly lower than 100%.

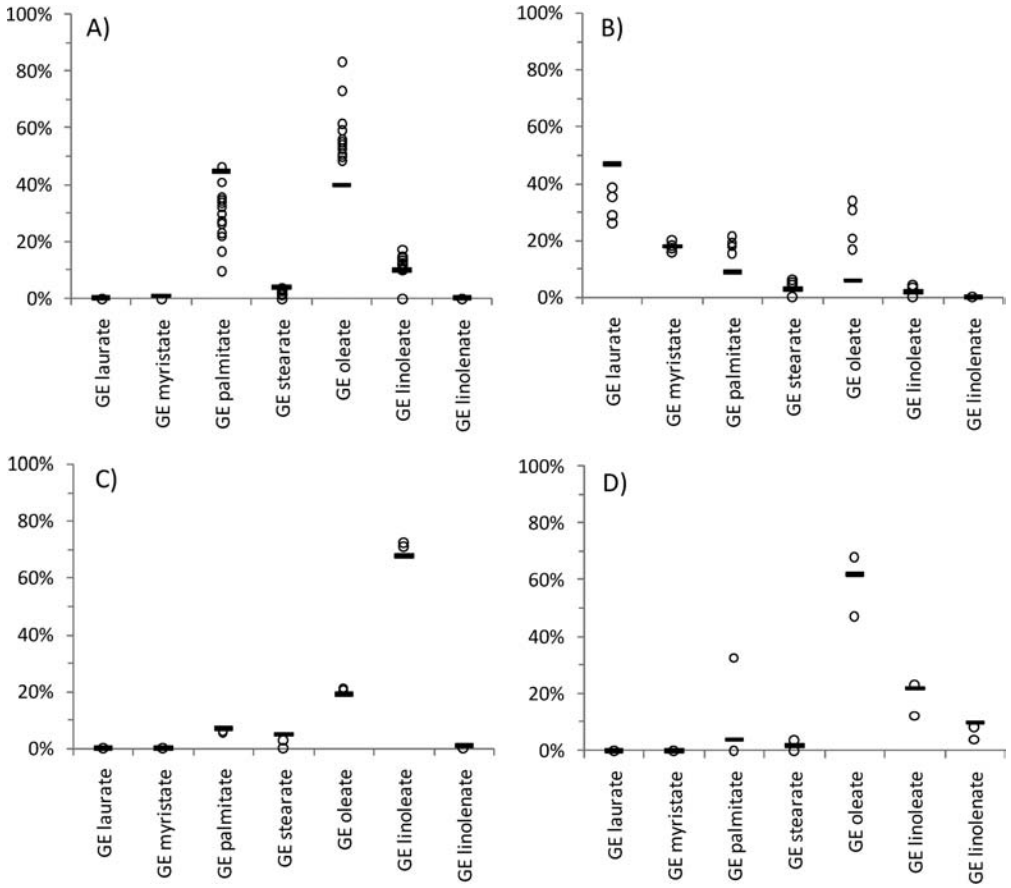

Figure 1. Fatty acid distribution in palm oil (—, values from Table 3) and GE distribution (○, as percentage of total GEs weight) measured in 13 palm oil samples (A), in 4 coconut oil samples (B), in 2 sunflower oil samples (C), and in 2 canola oil samples (D).

and detection of GEs were performed with an electrospray ionization source (Jet Stream) operated in the positive mode, using the following operation parameters: capillary voltage, 3500 V; nebulizer pressure, 50 psig; drying gas flow rate, 9 L/min; gas temperature, 350 °C; skimmer voltage, 60 V; octapole dc 1, 37.5 V; octapole rf, 250 V; fragmentor voltage (in-source CID fragmentation), 150 V. The extended dynamic range at 2 GHz was used, which allowed a mass resolution from 12000 at m/z 200 to 25000 at m/z 1500 with an acquisition range from m/z 100 to 1600 (2 scans/s). Ion source parameters were optimized for all glycidyl esters. Accurate mass measurement was achieved thanks to an automated calibrant delivery system for mass spectra correction. A dual-nebulizer electrospray source introduces the outlet of the chromatography at the same time as the calibrant solution containing purine ($C_5H_4N_4$, m/z 121.050873) and HP-0921 (hexakis-(1*H*,1*H*,3*H*-tetrafluoropentoxy)phosphazene, $C_{18}H_{18}O_6N_3P_3F_{24}$, m/z 922.009798). The full scan spectra data were processed with Agilent Mass Hunter Qual software

LC-ToF-MS Data Treatment. Because ToF-MS analyses provide exact mass measurement with <2 ppm error, the identification of GEs was performed through both their exact mass measurement and their retention time (Table 1). These data were gathered in a database, which was then converted into a csv Excel file to be used by Agilent Mass Hunter software for data treatment. When using the searching compounds by molecular formula option in the Qualitative Mass Hunter software, each compound in the database was searched from the raw data. Even if the proton adduct was the most abundant, and as no major interference on the other adducts was found, the four adducts were searched and extracted (H^+, NH_4^+, Na^+, and K^+). The identification criteria were set at ±10 ppm for accurate mass tolerance and at ±0.1 min for retention time tolerance. The extraction window for extracted ion chromatogram generation was set at 10 ppm. Retention times were determined by injection of analytical standards.

LC-MS/MS was used for acquisition of validation data and for quantitative analysis of GEs in oil samples on a routine basis. The LC-MS/MS instrument comprised a 1200 HPLC (Agilent Technologies) coupled to a 6410 triple-quadrupole MS analyzer (Agilent Technologies). The chromatography column used was a 50 × 3 mm i.d., 3 μm, Luna C18(2). Mobile phase A consisted of methanol/water (75:25, v/v) with 0.5% formic acid, and mobile phase B consisted of 2-propanol with 0.5% formic acid. The column temperature was maintained at 30 °C. Total injection volume was 20 μL comprising 8 μL of sample extract and 12 μL of GE standard solutions for quantification by means of standard addition on extracts. A gradient program was applied at a 500 μL/min flow rate as follows: linear gradient from 0 to 50% B from 0 to 8 min, and from 50 to 95% B from 8 to 10 min, then kept at 95% B for 10 min, and equilibrated from 20 to 25 min at 100% A. An injector program was used to perform standard addition on extracts. Ionization and detection of GEs were performed with an electrospray ionization source operated in the positive mode, using the following operation parameters: capillary voltage, 3500 V; nebulizer pressure, 50 psig; drying gas flow rate, 10 L/min; gas temperature, 350 °C; and dwell time, 50 ms. Fragmentor voltage (in-source CID fragmentation) was optimized for each GE as summarized in Table 2 and collision energy (CE) for each transition. The data were processed with Agilent Mass Hunter Quant software.

Method Validation. Linearity was checked on LC-MS/MS with the seven GEs in solvent in triplicate at 0.175, 0.3, 0.5, 0.75, 1, 1.4, 2, 3, and 3.5 ng/μL levels.

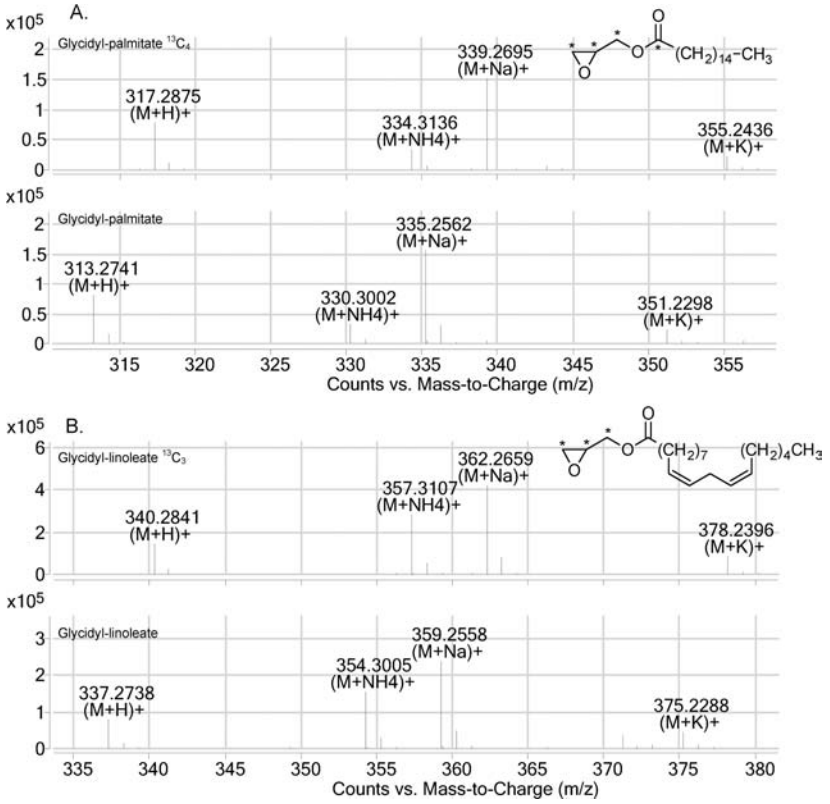

Figure 2. Mass spectra of (A) glycidyl palmitate and glycidyl palmitate isotopically labeled with $^{13}C_4$ and (B) glycidyl linoleate and glycidyl linoleate isotopically labeled with $^{13}C_3$. Injection of 1 ng was used for LC-ToF-MS. Position of ^{13}C is indicated by am asterisk (∗) on the chemical formula.

Absolute recoveries were determined on blank safflower oil. These experiments were conducted under intermediate reproducibility (iR) conditions[23] for glycidyl palmitate, stearate, oleate, linoleate, and linolenate. Thus, blank safflower oil was spiked before workup at nine fortification levels (0.1, 0.2, 0.3, 1, 2, 2.5, 3, 5, and 7.5 mg/kg) and analyzed in triplicate over 6 days. A total of $n = 18$ separate experiments per fortification level were thus obtained over $k = 6$ different days. Quantification was performed by means of standard addition on extracts. *Within-day* (RSD_r) and *between-day precision* (RSD_{iR}) data were calculated from these trials.

Absolute recoveries were determined on safflower oil under repeatability conditions for glycidyl laurate and myristate due to their late inclusion in the method, at the same levels as the other glycidyl esters. Thus, they were analyzed in triplicate over 1 day.

Decision limits ($CC\alpha, \alpha = 1\%$), detection capabilities ($CC\beta, \beta = 5\%$), and limits of quantification leading to reporting limit were calculated according to ISO 11843 described in ref 23.

RESULTS AND DISCUSSION

Choice of Analytical Standards. To cover a broad range of oils with different fatty acid compositions, the following approach for the selection of standards had been taken: Assumption was made that the relative GE abundances should follow the fatty acid composition of the individual oils. Table 3 illustrates these fatty acid compositions for the surveyed oils (data from ref 24 for palm olein and from ref 25 for other vegetable oils). Focusing on the seven GE chemical standards currently commercially available (glycidyl laurate, glycidyl myristate, glycidyl palmitate, glycidyl stearate, glycidyl oleate, glycidyl linoleate, and glycidyl linolenate) allows most of the oils to be efficiently analyzed (Table 3). This theoretical approach should be taken with caution because it does not take into account the natural preferential positions of fatty acids on the glycerol backbone, which could lead to different proportions of GEs when these latter compounds are formed. Moreover, fatty acid composition of oils depends also on the plant cultivar used for oil manufacturing as well as the geographic origin, which lead to variability of the fatty acid profile. However, trends of GE proportions have been confirmed with analytical data of different types of oil. Figure 1 shows a correlation between fatty acids of the glycidyl esters and the fatty acid composition of the individual oil, respectively. The variability observed, for example, from one palm oil to another palm oil in the GE distribution can be explained by the variability of the natural fatty acid composition within a specific type of oil. Due to these external factors, a perfect fit between theoretical and observed values was not expected. Interestingly, glycidyl laurate and glycidyl myristate were not mentioned in the first published direct methods[16−20] and were included only recently.[21,26] Indeed, these two compounds are among the main GEs encountered in coconut oil and palm kernel oil and, therefore, should not be omitted when pure or blended oils containing coconut and palm kernel oils are subjected to analysis. It should be mentioned as well that GEs with caprylic acid and capric acid as esters are not yet available commercially. This affects mainly the analysis of coconut and palm kernel oils; these two fatty acids represent 14% of total fatty acids in coconut oil and 8% in palm kernel.

Internal Standards (IS). Improving the performance of the method by controlling the extraction process was made feasible by the use of four isotopically labeled GE standards ($^{13}C_4$-glycidyl palmitate, $^{13}C_4$-glycidyl oleate, $^{13}C_3$-glycidyl linoleate, and $^{13}C_3$-glycidyl linolenate). Compared to the unlabeled species, a

Table 4. TAG and DAG Molecular Species Detected in Palm Oil Fractions Collected after Gel Permeation Chromatography[a]

ACN:DB[b]	chemical formula	RT (min)	monoisotopic mass (Da)
TAG			
38:0	$C_{41}H_{78}O_6$	7.10	666.5798
42:1	$C_{45}H_{84}O_6$	7.46	720.6268
48:0	$C_{51}H_{98}O_6$	8.15	806.7363
48:1	$C_{51}H_{96}O_6$	8.16	804.7207
48:2	$C_{51}H_{94}O_6$	8.02	802.7050
50:1	$C_{53}H_{100}O_6$	8.35	832.7520
50:2	$C_{53}H_{98}O_6$	8.21	830.7363
50:3	$C_{53}H_{96}O_6$	8.08	828.7207
52:1	$C_{55}H_{104}O_6$	8.55	860.7833
52:2	$C_{55}H_{102}O_6$	8.40	858.7676
52:3	$C_{55}H_{100}O_6$	8.27	856.7520
52:4	$C_{55}H_{98}O_6$	8.12	854.7363
52:5	$C_{55}H_{96}O_6$	8.00	852.7207
54:2	$C_{57}H_{106}O_6$	8.60	886.7989
54:3	$C_{57}H_{104}O_6$	8.46	884.7833
54:4	$C_{57}H_{102}O_6$	8.31	882.7676
54:5	$C_{57}H_{100}O_6$	8.17	880.7520
54:6	$C_{57}H_{98}O_6$	8.03	878.7363
DAG			
32:2	$C_{35}H_{64}O_5$	5.56	564.4754
34:1	$C_{37}H_{70}O_5$	6.20	594.5223
34:2	$C_{37}H_{68}O_5$	5.95	592.5067
36:1	$C_{39}H_{74}O_5$	6.56	622.5536
36:2	$C_{39}H_{72}O_5$	6.30	620.5380
36:3	$C_{39}H_{70}O_5$	6.05	618.5223
36:4	$C_{39}H_{68}O_5$	5.80	616.5067
38:1	$C_{41}H_{78}O_5$	6.90	650.5849
38:2	$C_{41}H_{76}O_5$	6.67	648.5693

[a] Retention time and exact mass were used for detection by LC-ToF-MS. [b] Number of acyl group carbons: number of double bonds.

mass shift higher than 3 avoided the problematic isotope contribution (Figure 2A for glycidyl palmitate and Figure 2B for glycidyl linoleate). In the case of glycidyl palmitate, the isotopic ion (M + 3) has an abundance of 0.26% of the monoisotopic mass and the isotopic ion (M + 4) has an abundance of 0.02%, which ensures no contribution of the unlabeled to the labeled one. Conversely, Figure 2A exhibits two ions having very close m/z values: m/z 335.3164 and 335.2562 corresponding respectively to the (M + 1) isotopic ion of the ammonium adduct of $^{13}C_4$-glycidyl palmitate and the monoisotopic ion of glycidyl palmitate sodium adduct. The difference between these two ions is 180 ppm, which is far above the identification criteria and the extraction window both set at 10 ppm for LC-ToF-MS data treatment, ensuring selectivity of detection. The case is simpler for analysis by triple-quadrupole instruments as the ion selected for fragmentation is the proton adduct, free of any interferences. So far, only deuterated GEs can be purchased from suppliers, but these compounds are known to be prone to proton exchange and to have a shift in retention time compared to unlabeled compounds when analyzed by liquid chromatography. For these reasons, GE IS labeled with ^{13}C were preferred even if they had to be obtained through custom synthesis.

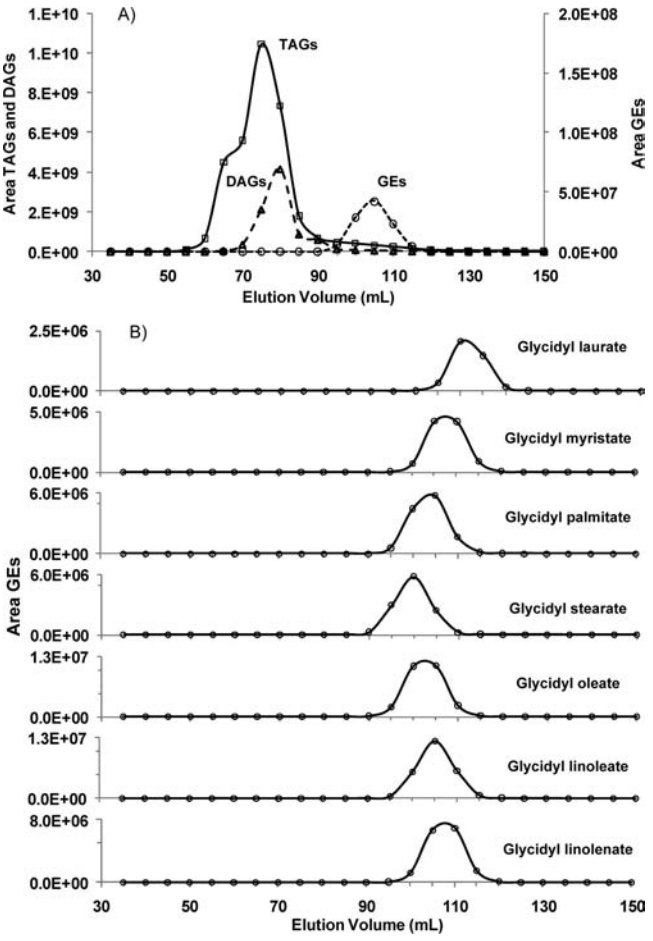

Figure 3. GPC elution of a palm oil sample spiked at 5 μg/g with seven GEs, obtained by 5 mL fraction collection from 30 to 150 mL elution volume, analyzed by LC-ToF-MS: (A) TAG (□), DAG (△), and GE (○) elution; (B) individual GE elution.

Optimization of Extraction Methods. Elution profiles of GEs as well as matrix constituents by GPC were determined in a palm oil sample spiked with a GE mix solution at 5 μg/g level. The 24 collected fractions of 5 mL from elution volume of 30−150 mL were reconstituted in 1 mL of acetone after solvent evaporation, and fractions 7−10 were further diluted 5 times (corresponding to elution between 60 and 80 mL) to avoid saturation of ToF detector as these fractions contained >20 mg of matrix. Even though the global elution profile of matrix was given by the weight of the different fractions, this approach cannot distinguish TAGs, DAGs, and individual GEs. The main TAGs and DAGs, previously observed in a palm oil sample as summarized in Table 4, were then analyzed in each fraction by LC-ToF-MS using their exact mass as identification criteria. The extracted ion chromatogram of the individual compounds was integrated, and areas of compounds belonging to the same class were summed to provide an elution profile of TAGs, DAGs, and GEs as illustrated in Figure 3A. The seven GEs included in the method eluted between 90 and 130 mL (Figure 3B), without major interference of matrix components that mainly elute between 55 and 90 mL for TAG and between 70 and 90 mL

for DAG. The main drawback of this extraction procedure is the solvent consumption, that is, 150 mL of cyclohexane:ethyl acetate (1:1, v/v) per sample. However, this extraction procedure is efficient in removing triacylglycerides (TAGs) and most of the diacylglacerides (DAGs) as only a few milligrams (generally 0–5 mg) is recovered with most of the oils analyzed, which means that >98% of the matrix is removed. GPC extraction presents also good performances (see Performance of Method) for the GEs tested, and it has the advantage of automation; for example, the GPC instrument used was equipped with an autosampler, allowing the extraction of 24 samples in one batch, making the extraction process fit for routine analysis. Compared to other published approaches using SPE chromatography,[16,17,19] GPC is the most versatile approach as 400 mg of oil is currently loaded on the column in the present method (compared to 10 mg for SPE), with possibilities to scale up to 800 mg with the GPC column used. The sequential aspect of sample preparation (one sample at once, one sample every 30 min) could appear to be a time-consuming step, but the automation of extraction by GPC is clearly an asset as it enhances reproducibility.

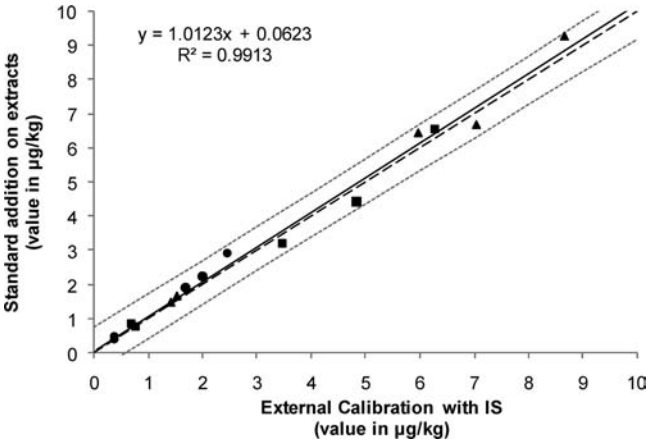

Figure 4. Comparison of quantification approach for five different palm oil samples. Glycidyl palmitate (■), oleate (▲), and linoleate (●) values obtained by standard addition on extracts (*y*-axis values) were compared to those obtained by means of external calibration with IS (*x*-axis values). Regression line (plain line) is comparable to $Y = X$ curve (dashed line). All points of data set are included within the 95% confidence interval (dotted line).

In some rare cases, a higher amount (>10 mg) of matrix is recovered after this first extraction by GPC. This is linked to the presence of short-chain TAGs and short-chain DAGs containing myristic and lauric acid. In the case of coconut and palm kernel oils, characterized by a higher amount of short fatty acid chains (lauric acid and myristic acid), elution of TAGs and DAGs overlapped with the GE collection window as DAGs and TAGs containing myristic and lauric acid have a lower molecular weight compared to those encountered in other oils. The presence of TAGs and DAGs has a limited impact on LC-MS quantification as TAGs and DAGs do not interfere (different retention time on LC column) with GEs. The risk of injecting high amounts of DAGs and TAGs is to overload the chromatographic column and to soil the ion source of the mass spectrometer (decrease in system stability, increase in maintenance frequency). High amounts of matrix recovery after GPC can also occur when monoacylglycerides (MAGs) and free fatty acids (FFAs) are present in oil at an

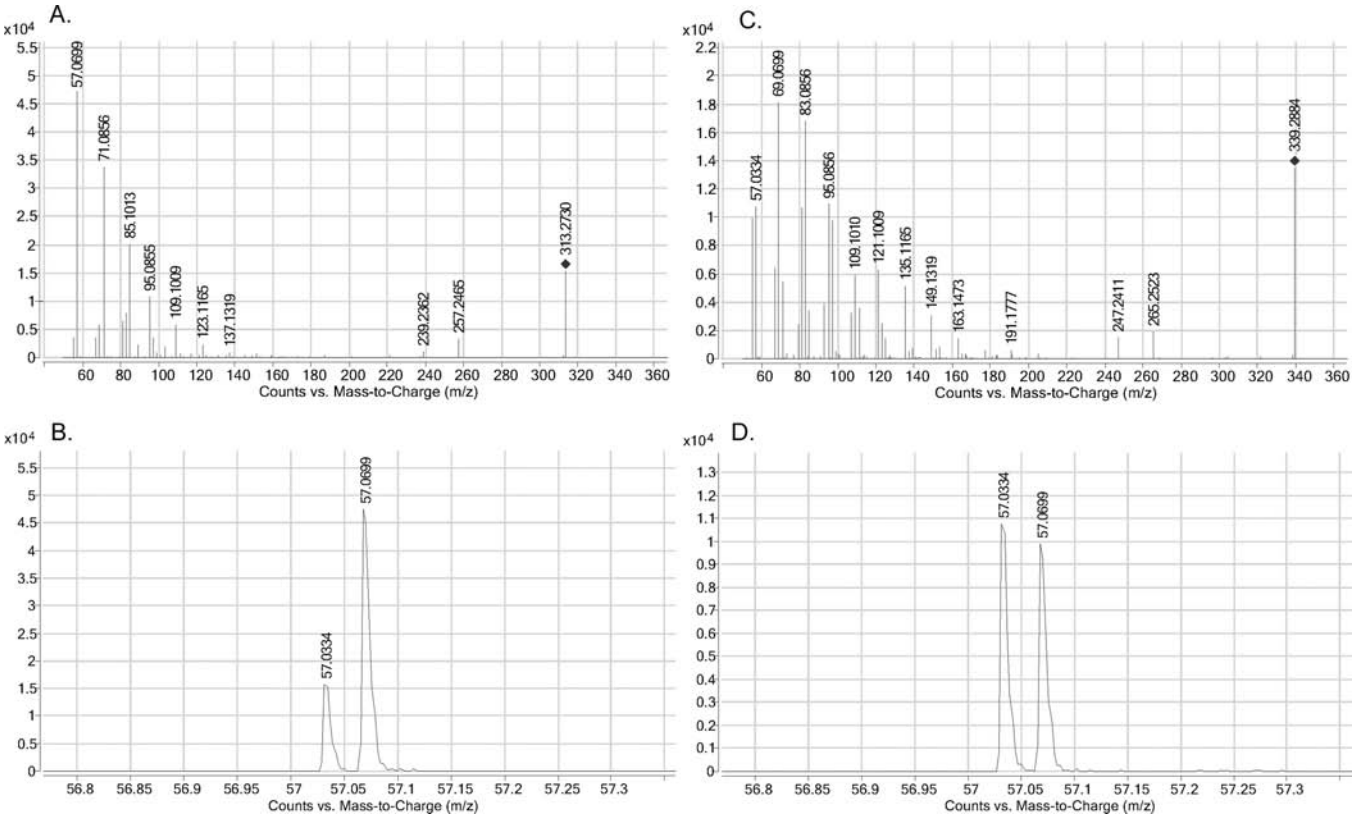

Figure 5. Fragmentation spectra of (A, B) glycidyl palmitate (m/z 313.2730) and (C, D) glycidyl oleate (m/z 339.2884) on QToF instrument. Expansion of the m/z 57 region (spectra B and D) exhibits two different product ions not resolved by triple-quadrupole instruments.

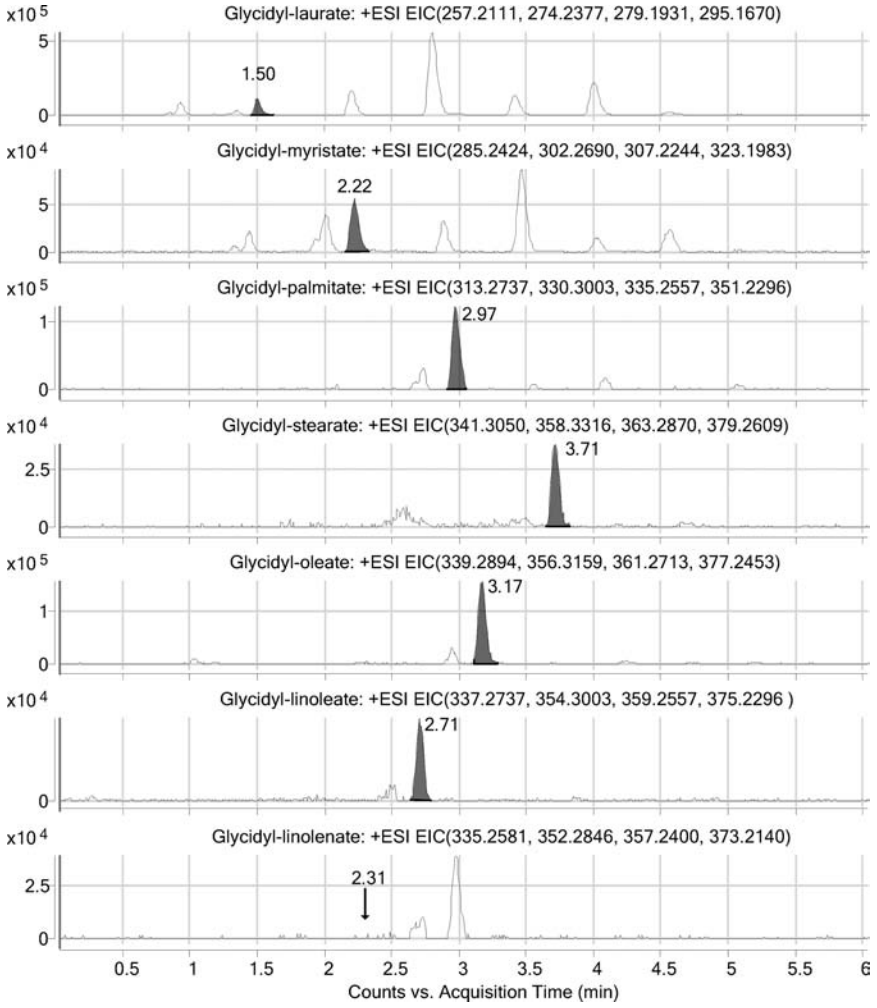

Figure 6. Extracted ion chromatogram of GEs in a coconut oil sample after GPC extraction and LC-ToF-MS analysis. Measured levels were 0.6 μg/g glycidyl laurate, 0.4 μg/g glycidyl myristate, 0.6 μg/g glycidyl palmitate, 0.2 μg/g glycidyl stearate, 0.9 μg/g glycidyl oleate, and 0.2 μg/g glycidyl linoleate.

abnormally high level. MAGs and FFAs have molecular weights close to those of GEs and are thus not separated by GPC, which affects extract solubility in injection solvent, as the extract can no longer be reconstituted in acetone (can be replaced efficiently by dichloromethane without any effect on chromatography when the injection volume is kept low, e.g., 2 μL). Furthermore it affects also detection as MAGs are eluting in the GE retention time window by liquid chromatography, generating a strong matrix effect and thus decreasing method performances (repeatability and sensitivity). To reduce DAGs, MAGs, and FFAs in extracts when a high amount (>10 mg) of matrix was recovered after the GPC step, an additional SPE cleanup has been developed to increase sensitivity. The cleanup is based on silica as stationary phase and dichloromethane as eluting solvent. In such conditions, TAGs elute first, then GEs, followed by DAGs and MAGs. With an elution volume of 10 mL of dichloromethane, GEs are eluted, whereas DAGs and MAGs remain on the column. It should be noted that this cleanup does not allow removing TAGs, which is not an issue due to the efficiency of the GPC extraction for most oils.

Quantification Approach. Seven GEs are targeted by the current approach, and four isotopically labeled GEs (IS) have been used. However, for three of the GEs that do not have a corresponding IS, an external calibration (standards in solvent) cannot correct efficiently matrix effects, which appeared to be highly variable related to the different types of oils. Considering the good performances of the extraction method, a quantification approach by standard addition to the final extracts was tested. This was done in an automated way using the LC autosampler capabilities. The two sample extracts (the first one diluted, the second one non-diluted) were injected three times each, together with a solution of standards at three different concentrations using the injector program detailed under LC-ToF-MS. This customized injection took 1 min compared to 20 s for a conventional one, and six injections per sample (and thus six analyses, 1.5 h per sample) could be performed. As a wide range of contamination of GEs is expected (mainly from 0 to 10 μg/g), the standard addition performed on diluted extracts allows targeting the high contamination levels (up to 10 μg/g), whereas the non-diluted one allows targeting low contamination levels (up to 2 μg/g). Besides, oil samples are always spiked with isotopically labeled internal standards at the 0.5 μg/g level before extraction. GE IS were also quantified by standard addition as described before, at the same time as the other GEs contained in the oil. The isotopically labeled compounds have the same physicochemical properties as unlabeled GEs, allowing absolute recoveries assessment

Table 5. Recovery and Precision Data Obtained by the GPC Combined with SPE Method, on Spiked Safflower Oil at Nine Fortification Levels[a]

	absolute recovery (under repeatability conditions, $n^b = 3$, $k^c = 1$)		absolute recovery ± RSD_{IR} (under intermediate reproducibility conditions, $n^b = 18$, $k^c = 6$)				
spike level (mg/kg)	glycidyl laurate	glycidyl myristate	glycidyl palmitate	glycidyl stearate	glycidyl oleate	glycidyl linoleate	glycidyl linolenate
0.1	99	102	90 ± 8	97 ± 14	100 ± 23	105 ± 13	104 ± 13
0.2	92	79	88 ± 7	90 ± 5	95 ± 21	97 ± 9	98 ± 10
0.3	111	110	89 ± 8	95 ± 10	100 ± 11	100 ± 7	97 ± 10
1	**68**	71	89 ± 32	93 ± 36	91 ± 30	101 ± 22	111 ± 20
2	81	82	90 ± 19	94 ± 22	91 ± 20	102 ± 12	111 ± 8
3	82	87	84 ± 15	87 ± 21	87 ± 13	100 ± 24	108 ± 20
2.5	80	82	95 ± 12	93 ± 21	98 ± 18	103 ± 20	99 ± 21
5	75	75	94 ± 7	93 ± 10	98 ± 9	102 ± 5	98 ± 7
7.5	80	83	95 ± 7	92 ± 9	97 ± 11	101 ± 8	103 ± 15

[a] Absolute recovery values significantly outside the 70-120% range are written in bold. [b] n = number of replicates per level. [c] k = number of days.

at a given level, whatever the contamination level of GEs in the oils are, and this, for each sample (usually between 80 and 120%, data not shown) without extra sample preparations. Furthermore, the isotopically labeled GEs allowed assessment of the reliability of the standard addition approach, by comparing its results with the ones obtained using external calibration with IS. Five palm oil samples containing glycidyl palmitate, oleate, and linoleate were quantified by the two approaches, and the 15 values obtained by standard additions were compared with the 15 values obtained by external calibration curve with IS. As depicted in Figure 4, both approaches gave similar results, with an average difference of 6% (maximum of 22% difference) and a slope of 1.012. Data sets analyzed using an in-house statistical tool have shown neither a proportional bias nor a systematic bias. Due to the diversity of oils analyzed leading to different matrix effects and the absence of internal standards for three of the GEs targeted, external calibration could not be used and the standard addition approach was preferred.

With regard to the LC-MS detection aspect, development was performed with a ToF mass spectrometer, whereas validation and routine analyses were performed with a triple-quadrupole instrument. When two different columns, a Luna C18 50 × 3 mm with a particle size of 3 μm from Phenomenex and an Acquity UPLC HSS T3 2.1 × 50 mm with a particle size of 1.8 μm equipped with an Acquity UPLC HSS T3 Van Guard column (both from Waters, Milford, MA), were compared, the latter gave the most efficient chromatographic separation. On the mass spectrometry side, GEs are well ionized with electrospray ionization (ESI), but not prone to a good fragmentation pathway (no major product ion) as many product ions are generated by CID as shown in Figure 5 spectra obtained by LC-ToF-MS. Most product ions are from the fragmentation of the fatty acid chains, identified on the mass spectrum by a m/z difference between product ions of 14.0151 (CH_2) corresponding to loss of alkanes. Product ion at m/z 239.2465 for glycidyl palmitate (Figure 5A) and 265.2523 for glycidyl oleate (Figure 5C) can be attributed to acylium ion, that is, $C_{16}H_{31}O^+$ and $C_{18}H_{33}O^+$, respectively, for palmitate and oleate fatty acids, measured with an m/z error of 1 ppm compared to theoretical values. The protonated fatty acid $[R{-}COOH_2]^+$ was mainly observed with saturated fatty acid esters of glycidol (e.g., m/z 257.2465 for glycidyl palmitate in Figure 5A). It should also be noted that a product ion shared by all GEs at the unitary m/z 57 can be actually resolved in two different product ions using high-resolution mass spectrometry as shown in Figure 5B, D. The first one measured at m/z 57.0334 (theoretical m/z 57.03349) is a glycidol product ion $[C_3H_5O]^+$, whereas the second one at m/z 57.0699 (theoretical m/z 57.06988) is a product ion $[C_4H_9]^+$ from alkane fragmentation, and the relative abundance of these two product ions is compound dependent. The absence of a preferential fragmentation pathway makes the choice of a commonly accepted transition difficult for triple-quadrupole detection and may also decrease sensitivity. Besides, single MS detection using ToF instrument allows the detection of intact analytes without fragmentation, which is an advantage for such molecules with no preferential product ion, specificity being ensured by high mass resolution of ToF instruments (R > 10 000). However, the sensitivity of ToF instruments is known to be poorer than that of triple-quadrupole instruments in MS/MS mode and finally, both instruments used (LC-ToF-MS for method development and LC-MS/MS for routine analysis) led to comparable sensitivity. As an example, extracted chromatograms of the seven GEs in a coconut oil sample obtained by LC-ToF-MS after GPC extraction are shown in Figure 6. GEs were detected in this sample at 0.6 μg/g (glycidyl laurate), 0.4 μg/g (glycidyl myristate), 0.6 μg/g (glycidyl palmitate), 0.2 μg/g (glycidyl stearate), 0.9 μg/g (glycidyl oleate), and 0.2 μg/g (glycidyl linoleate).

Performance of the Method. Absolute recovery values assessed for the nine spiking levels in safflower oil are presented in Table 5. These values were obtained by GPC extraction and subsequent SPE cleanup, followed by a quantification by LC-MS/MS. Recoveries values fall within the 70−120% range (as recommended in pesticide residues analysis[27]) with one exception, that is glycidyl laurate (around 68%) at the 1 mg/kg spiking level.

For glycidyl laurate as well as for glycidyl myristate, recovery values have been obtained in repeatability conditions as these compounds were later included in the analytical scope. For these two compounds, median recoveries are, respectively, 81 and 82% and above 90% for the other GEs. More validation data are under acquisition for the seven GEs in the frame of ongoing performance verification, as it is suggested to evaluate additional analytes in document SANCO/10684/2009,[27] where recovery of analytes is measured with each batch of analyses. The reporting limits for glycidyl myristate, palmitate, stearate, and linoleate were set at 50 μg/kg and at 100 μg/kg for laurate, oleate, and linolenate according to the LOQ obtained. On the basis of these experiments, one would conclude that the extraction efficiencies achieved were high and consistent despite few validation data for the two shortest forms of GEs.

Applicability in Routine. The proposed method has demonstrated a high extraction efficiency with high loading capacity on a GPC column. In a mitigation process by which contamination levels are expected to decrease to low amounts, the versatility and sensitivity of methods are highly required. By loading 400 mg on the GPC column, most matrix constituents are discarded, whereas GEs are quantitatively recovered and can then further be concentrated. None of the methods making use of the conventional SPE on C_{18} media affords such a sample test portion for extraction associated with such efficiency in matrix removal. Up to 24 samples can be loaded on the GPC autosampler, which were overnight extracted in 12 h. Solvent consumption could appear as the major drawback, as 150 mL per sample is required. Some investigations could undoubtedly lead to a scale down of the extraction by GPC, as the capacity of the column currently used is 800 mg of fat, and only 400 mg is actually loaded. In the same way, the extract is reconstituted in 1 mL of acetone, whereas only 6×1 μL (6 μL) is enough for LC-MS analysis. Therefore, using a smaller preparative GPC column could allow reduced flow rate and elution volume, without affecting sample extraction efficiency.

When many different oil samples have to be analyzed with matrix effects varying within and between oil types, the standard addition approach undoubtedly constitutes the best option, combining good sample throughput with reliable quantitative results. Indeed, the systematic use of the seven isotopically labeled GE standards seems today not easily feasible for all laboratories due to cost implications.

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