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A New Analytical Method for the Quantification of Glycidol Fatty Acid Esters in Edible Oils

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Abstract: A novel method to quantify glycidol fatty acid esters (GEs), supposed to present as food processing contaminants in edible oils, has been developed in combination with double solid-phase extractions (SPEs) and LC-MS measurements. The analytes were five species of synthetic GEs: glycidol palmitic, stearic, oleic, linoleic and linolenic acid esters. The use of selected ion monitoring in a positive ion mode of atmospheric chemical ionization-MS with a reversed-phase gradient LC provided a limit of quantification of 0.0045-0.012 μg/mL for the standard GEs, which enables the detection of GEs in μg ranges per gram of edible oil. Using the double SPE procedure first in reversed-phase and then in normal-phase second, allowed large amounts of co-existing acylglycerols in the oils to be removed, which improved the robustness and stability of the method in sequential runs of LC-MS measurements. When the method was used to quantify GEs in three commercial sources of edible oils, the recovery% ranged from 71.3 to 94.6% (average 79.4%) with a relative standard deviation of 2.9-12.1% for the two oils containing triacylglycerols as major components, and ranged from 90.8 to 105.1% (average 97.2%) with a relative standard deviation of 2.1-12.0% for the other, diacylglycerol-rich oil. Although the accuracy and precision of the method may not be yet sufficient, it is useful for determining trace levels of GEs and will be helpful for the quality control of edible oils.

Key words: glycidol fatty acid ester, quantification, LC-MS, solid-phase extraction, edible oil

1 INTRODUCTION

3-Monochloro-1,2-propanediol (3-MCPD), a known food processing contaminant with a maximum tolerable daily intake of 2 μg/kg body weight per day as recommended by the European Scientific Committee on food, is detected in various types of food, such as acid-hydrolyzed vegetable proteins, soy sources, crackers and meat products[1,2]. Since analytical methods for 3-MCPD using GC-MS after acid hydrolysis and derivatization with phenylboronic acid were initially developed, it has been reported that some edible oils contain relatively high levels of 3-MCPD and/or 3-MCPD fatty acid esters[3-5]. The Federal Institute for Risk Assessment (BfR), a scientific agency of the Federal Republic of Germany, recently reported that the Chemical and Veterinary Test Agency (CVUA) Stuttgart detected glycidol fatty acid esters (GEs) in refined vegetable oils, which seems to be one reason why high levels of 3-MCPD and/or 3-MCPD fatty acid esters occur in the oils[6]. However, that report also states that "the analytical method currently available does not permit determination of GEs in the oils", and therefore that "there is an urgent need for the development and validation of a suitable detection method for GEs for reliable risk assessment"[6]. In fact, that report describes only qualitative results for the GEs, and it does not state what analytical methodology was adopted and exactly what analytical conditions were used for the analysis.

In response to this concern, we collected commercial edible oils sold in Japan and qualitatively analyzed them using GC-MS and LC-time of flight (TOF)-MS in a preliminary survey. We found some chromatographic peaks that coincided with standard GEs in terms of retention times and mass spectra both in GC-MS and LC-TOF-MS, and we concluded that GEs, probably food process contaminants, are present in all commercial edible oils tested. Those

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results seemed to agree with the qualitative information obtained by the CVUA Stuttgart[6]. However, the quantitative values remained unknown because there were no reliable methods to quantify GEs in the oils. Only a gas chromatographic method for the analysis of GEs has been reported[7], but that method could not be used to quantify trace GEs in edible oils containing large amounts of acylglycerols. Therefore, we developed and optimized LC-MS measurements to quantify the GEs as well as a double solid-phase extraction (SPE) procedure for the quantitative extraction of GEs from the oils. In this article, we report: 1) a newly developed method for the quantification of GEs in edible oils in combination with double SPE and LC-MS, and 2) the validity of this method that was verified by recovery tests using three commercial sources of edible oils: two containing triacylglycerols (TAGs) as major components and the other being diacylglycerols (DAG)-rich.

2 EXPERIMENTAL

2.1 Reagents

Methanol, acetonitrile, 2-propanol and chloroform, of HPLC grade from Kanto Chemical (Tokyo, Japan), were used to prepare GE-containing extracts from the oils and for the mobile phases in the LC-MS. Ultra-pure water prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA) was used in all procedures. Glycidol (95%, Tokyo Chemical Industry, Tokyo, Japan) was distilled prior to use. Palmitoyl chloride (98%) and linolenic acid (>99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Oleic acid (>99%) and linoleic acid (>97%) were from Wako Pure Chemical Industry (Tokyo, Japan) and from Tokyo Chemical Industry, respectively. All other chemicals used were reagent grade.

2.2 Standard materials

Glycidol stearic acid ester ($C_{18:0}$-GE) was purchased from Tokyo Chemical Industry. Since other GEs, such as glycidol palmitic acid ester ($C_{16:0}$-GE), oleic acid ester ($C_{18:1}$-GE), linoleic acid ester ($C_{18:2}$-GE) and linolenic acid ester ($C_{18:3}$-GE), were not available commercially, they were synthesized in our laboratory. To yield the fatty acid chloride for $C_{18:1}$-GE, $C_{18:2}$-GE and $C_{18:3}$-GE, thionyl chloride (270 mmol = 32.1 g for $G_{18:1}$-GE and $C_{18:2}$-GE, or 22 mmol = 2.6 g for $C_{18:3}$-GE) was added to each fatty acid (180 mmol = 50.8 g and 50.4 g for $C_{18:1}$-GE and $C_{18:2}$-GE, respectively, or 14 mmol = 4.0 g for $C_{18:3}$-GE) cooled at -10℃, by taking the known synthetic method into account [8]. The mixture was stirred at 50℃ for 4 h and then was concentrated under reduced pressure. To synthesize GEs, the fatty acid chloride (40 mmol = 11.0 g obtained as a commercial reagent for $C_{16:0}$-GE, 180 mmol = 57.0 g and 56.6 g synthesized for $C_{18:1}$-GE and $C_{18:2}$-GE, respectively, or 14 mmol = 4.4 g synthesized for $C_{18:3}$-GE) was added to a solution of glycidol (40 mmol = 3.0 g for $C_{16:0}$-GE, 200 mmol = 14.8 g for $C_{18:1}$-GE and $C_{18:2}$-GE, or 16 mmol = 1.2 g for $C_{18:3}$-GE) and pyridine (40 mmol = 3.2 g for $C_{16:0}$-GE, 180 mmol = 14.2 g for $C_{18:1}$-GE and $C_{18:2}$-GE, or 14 mmol = 1.1 g for $C_{18:3}$-GE) in diethyl ether (50 mL for $C_{16:0}$-GE, 100 mL for $C_{18:1}$-GE and $C_{18:2}$-GE, or 20 mL for $C_{18:3}$-GE) cooled at 0℃, and the solution was stirred at room temperature for 3 h. The resulting solution was washed with brine and water, dried with sodium sulfate, filtered, and concentrated to yield each of the standard GEs. The yields were 12.0 g for $C_{16:0}$-GE, 31.5 g for $C_{18:1}$-GE, 43.0 g for $C_{18:2}$-GE, and 4.1 g for $C_{18:3}$-GE. Analyses by ^{1}H-NMR, ^{13}C-NMR and IR confirmed the GE structures in all reaction products. The $C_{18:1}$-GE and $C_{18:2}$-GE products were further purified using silica-gel column chromatography to remove residual fatty acids and the monoacylglycerol (MAG) byproducts. Those unpurified and purified products were used as standard materials. Percentages of purity were obtained using ^{1}H-NMR and GC-FID. ^{1}H-NMR was performed under the following conditions: instrument; Mercury 400 system (Varian Inc., Palo Alto, CA, USA) at 400 MHz, nuclear species; protons, solvent: deuterated chloroform, internal standard; p-dinitrobenzene, spectral width; 6410.3 Hz, data point; 16 K, repetitions; 8, relaxation delay; 5.0 s, and probe temperature; ambient, and GC-FID was under the following conditions: instrument; HP 6890 system (Agilent Technologies, Palo Alto, CA, USA), column; DB-1HT capillary column of 30 m (0.25 mm I.D. and film thickness 0.1 m, carrier gas; helium with 1.0 mL/min constant flow, oven temperature program; initial 60℃ for 3 min, from 60 to 340℃ at 10℃/min, finally 340℃ for 10 min, injection; 1 μL splitless (1:50) under 280℃, and FID; 340℃. The purities were 75.6% for $C_{16:0}$-GE, 94.4% for $C_{18:1}$-GE, 89.8% for $C_{18:2}$-GE and 55.1% for $C_{18:3}$-GE. That the purities of $C_{16:0}$-GE and, especially $C_{18:3}$-GE, were lower was caused by relatively greater contents of the byproduced MAG in $C_{16:0}$-GE and the byproduced MAG and the unreacted fatty acid in $C_{18:3}$-GE. The purity% of the commercially available $C_{18:0}$-GE was 96.2%. By another measurements using GC-FID, all standard materials were confirmed to contain less than 10 μg/g glycidol. Those standard materials were stored under nitrogen at -4℃ in glass bottles. Under such conditions, the standard materials of GEs were stable at least 2 months. The purity% was taken into consideration for the preparation of standard GE solutions. For example, since the $C_{16:0}$-GE standard material had a purity of 75.6%, 132 mg was weighed accurately and was then diluted with 100 mL methanol/2-propanol (1:1 by vol) in order to prepare a 1000 μg/mL $C_{16:0}$-GE solution. Working solutions of standard GEs were obtained by further dilution of the 1000 μg/mL stock solutions with methanol/2-propanol (1:1 vol by vol) prior to use. In other experiments, GEs in the 1000 μg/mL standard solutions were confirmed to be stable for at least 1 month when the solutions were stored under

nitrogen at -4℃ in glass bottles.

2.3 Commercial edible oils

Three commercial edible oils were purchased from Japanese markets, as listed in Table 1, and were used for the quantification of GEs. Sample-A and Sample-B are made up chiefly of TAGs while Sample-C contains DAGs as the major component. Qualitative analyses using GC-MS and LC-TOF-MS detected the presence of the following GEs : Sample-A; $C_{16:0}$-GE, $C_{18:0}$-GE, $C_{18:1}$-GE and $C_{18:2}$-GE, Sample-B; $C_{16:0}$-GE, $C_{18:1}$-GE and $C_{18:2}$-GE, and Sample-C; $C_{16:0}$-GE, $C_{18:1}$-GE, $C_{18:2}$-GE and $C_{18:3}$-GE. Therefore, the analytes for the quantitative method were focused on five species of GEs ($C_{16:0}$-, $C_{18:0}$-, $C_{18:1}$-, $C_{18:2}$- and $C_{18:3}$-GE) in this study.

2.4 Double SPE procedure

A double SPE procedure was optimized as follows: About 0.1 g of each oil was weighed accurately into a centrifuge tube and was then dispersed in 4 mL acetonitrile with stirring for 10 min. After centrifugation of the solution at 3,500 × g for 5 min in a 5417R Centrifuge (Eppendorf, Hamburg, Germany), the supernatant was applied to the first reversed-phase SPE using a Sep-Pak Vac RC C18 cartridge 500mg (Waters, Milford, MA, USA) that had been conditioned with 1 mL methanol and then 2 mL acetonitrile just prior to use. After the supernatant passed through, the cartridge was washed out twice with 2 mL acetonitrile. Those acetonitrile solutions were then combined in a glass tube and were evaporated to dryness using a nitrogen stream. The dried residue was dissolved in 2 mL chloroform, and was then applied to a second normal-phase SPE using a Sep-Pak Vac RC Silica cartridge 500mg (Waters) that had been conditioned with 2 mL chloroform just prior to use. After the solution passed through, the empty tube was washed out twice with 2 mL chloroform, and each of those washes was consecutively applied to the cartridge. The cartridge was then further washed out with 2 mL chloroform. Those chloroform solutions were combined in a glass tube and were dried again using a nitrogen stream. The dried residues (less than 1 mg for Sample-A and Sample-B, and ca. 5 mg for Sample-C) were carefully dissolved in 1 mL methanol/2-propanol (1:1 by vol) in the case of Sample-A and Sample-B, and in 10 mL in the case of Sample-C. The resulting solutions were subjected to LC-MS.

2.5 LC-MS conditions

An Agilent 1200 Series Rapid Resolution LC system connected to an Agilent 6460 Series Triple Quadruple LC/MS system was used together with the provided MassHunter software (Agilent Technologies) and an Acquity UPLC BEH C18 column 2.1 mm I.D. × 100 mm, 1.7 μm (Waters). In the LC-MS instrument, the built-in binary pump was connected to mobile phases A (acetonitrile/methanol/water 17:17:6 by vol) and B (2-propanol), which were consecutively time-programmed as follows: A 98% (B 2%) at the start (0.0 min), a linear gradient of A 98% to 85% (B 2% to 15%) between 0.0 and 15.0 min, an isocratic elution of A 5% (B 95%) from 15.1 to 25.0 min, and finally an isocratic elution of A 98% (B 2%) from 25.1 to 40.0 min (a total run time of 40 min). The injection volume was 5 μl of each solution. The column temperature was maintained at 40℃ and was eluted at a flow rate of 200 μL/min. Atmospheric pressure chemical ionization (APCI) in the mass spectrometer was performed with the following settings: ionization; positive ion mode, vaporizer temperature; 500℃, heater temperature of nitrogen gas; 250℃, flow of heated dry nitrogen gas; 10.0 L/min, nebulizer gas pressure; 0.138 MPa, corona current; 6.0 μA, fragmenter voltage; 100 V. For the selected ion monitoring (SIM) measurement in the positive ion mode of APCI with unit mass resolution, each of the protonated molecular ions $[M + H]^+$ were used: m/z 313 for $C_{16:0}$-GE, m/z 341 for $C_{18:0}$-GE, m/z 339 for $C_{18:1}$-GE, m/z 337 for $C_{18:2}$-GE and m/z 335 for $C_{18:3}$-GE (dwell time 200 ms each).

2.6 Quantitative determination of GEs and recovery test

Calibration lines were generated by the injection of standard GE solutions in the range between 0.05 and 5 μg/mL (0.05, 0.2, 1 and 5 μg/mL) and subsequently by plotting the concentration of standard GEs against their peak areas. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as S/N = 3 and 10, respectively[9], based on 5 μL injection of the standard GE solution at a concentration of 0.01 μg/mL. Each of the three commercial edible oils was analyzed in triplicate. The means ± SD of levels are expressed as microgram weights of GEs to gram

Table 1 Commercial Edible Oils Used in This Study.

Edible oil	Composition[a]	Qualitative results[b]
Sample-A	TAGs 92.6%, DAGs 6.8%, MAGs 0.0%	$C_{16:0}$-GE, $C_{18:0}$-GE, $C_{18:1}$-GE, $C_{18:2}$-GE
Sample-B	TAGs 96.1%, DAGs 3.9%, MAGs 0.0%	$C_{16:0}$-GE, $C_{18:1}$-GE, $C_{18:2}$-GE
Sample-C	TAGs 12.5%, DAGs 87.0%, MAGs 0.5%	$C_{16:0}$-GE, $C_{18:1}$-GE, $C_{18:2}$-GE, $C_{18:3}$-GE

[a]Obtained by GC-FID.

[b]Obtained by GC-MS and LC-TOF-MS.

weight of oil (μg/g). Recovery tests were performed in triplicate by spiking known amounts of the five GE standards to each of the oils. Thus, 20 μL of the 50 μg/mL standard GE solution (= 1 μg of each GE) was transferred to a centrifuge tube in the case of Sample-A and Sample-B, whereas 20 μL of the 500 μg/mL standard GE solution (= 10 μg of each GE) was added in the case of Sample-C. After the solvents were evaporated using a nitrogen stream, 0.1 g of each oil sample was added to the tube with accurate weighing, implying that the amounts of GEs spiked corresponded to 10 μg/g in the oils of Sample-A and Sample-B, and 100 μg/g in the oil of Sample-C. The double SPE procedure was performed as described in Section 2.4, followed by LC-MS measurements as described in Section 2.5. Percentages of recovery were calculated using both quantified levels of the GEs in the spiked oils and those in the non-spiked oils.

3 RESULTS AND DISCUSSION

3.1 LC-MS conditions

The separation of the five standard GEs by LC was studied using a specific column with extremely small packing materials for high separation (Acquity UPLC BEH C18) and a two mobile phase system for a reversed-phase gradient elution. As the mobile phase B, 100% 2-propanol was chosen since it can drive co-existing DAGs and TAGs out of the column after the separation of the GEs. Under reversed-phase conditions, $C_{18:3}$-GE elutes the earliest of all the standards. Acetonitrile/methanol/water (19:19:2 by vol) was tested initially as the mobile phase A, but the retention time of $C_{18:3}$-GE was less than 3 min. To avoid ion suppression/enhancement in the MS detection, the retention time of $C_{18:3}$-GE should be more than 5 min. Further optimization led us to use acetonitrile/methanol/water(17:17:6 by vol) as the mobile phase A, by which the retention time of $C_{18:3}$-GE was about 6 min. The optimized LC conditions allowed the concurrent separation of the five GEs within 17 min (Fig. 1).

To determine optimum conditions for MS detection, electrospray ionization (ESI) and APCI were examined using the positive ion mode, because that mode yielded the protonated molecular ion $[M + H]^+$ as a prominent ion (Fig. 2a) while the negative ion mode provided no molecular-related ions, such as the $[M - H]^-$ ion, but only fragmented ions. When the standard GE solution was injected and its $[M + H]^+$ ion was monitored, the APCI-MS detection was 10 times more sensitive than with the ESI-MS. Therefore, positive APCI-MS was chosen as the optimal ionization for detection of the GEs. On the other hand, upon the tandem MS measurement, no effective product ions were observed in the wide range of collision induced-dissociations tested (Fig. 2 b-d). Therefore, we decided not to use LC-MS/MS which is generally used for sensitive and selective analysis in biological studies[10], and decided that single MS should be used.

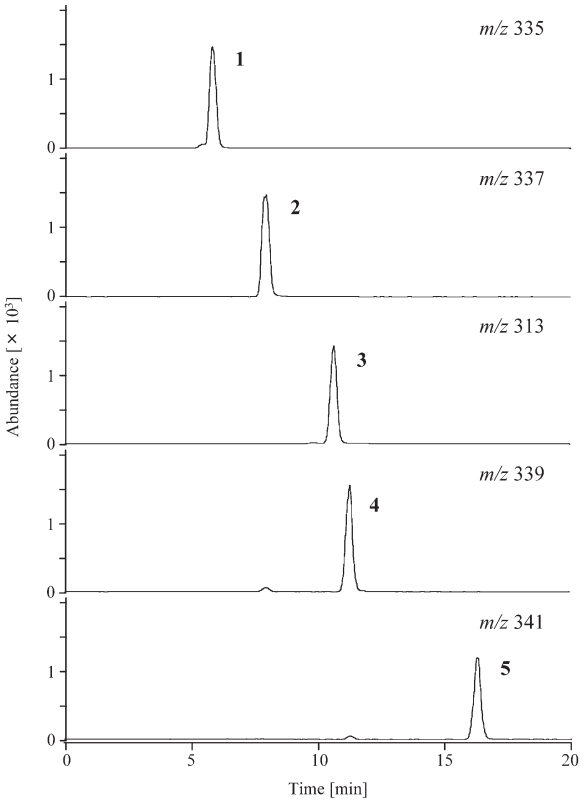

Fig.1 LC-MS SIM Chromatograms of Five Species of GEs at a Concentration of 0.2 μg/mL Each. Peaks: 1 = $C_{18:3}$-GE, 2 = $C_{18:2}$-GE, 3 = $C_{16:0}$-GE, 4 = $C_{18:1}$-GE, 5 = $C_{18:0}$-GE.

The percentages of relative standard deviation (RSD%) for retention times and areas, in six consecutive runs of the standard GE solutions, were in the range of 0.18-0.23% and 4.3-7.0% (Table 2). LOD and LOQ ranged from 0.0014 to 0.0037 μg/mL, and from 0.0045 to 0.012 μg/mL, respectively (Table 2). As for the calibration lines, all R^2 (correlation of determination) were greater than 0.9989 in the range between 0.05 and 5 μg/mL. Based on these results, all data were judged to be acceptable to quantitatively determine levels of GEs in μg ranges per gram of oils.

3.2 Double SPE procedure

One of the oils, Sample-A containing large amounts of TAGs, was dispersed in methanol/2-propanol but without any pretreatment (Procedure I, Table 3), and the supernatant was then subjected to LC-MS under the optimal conditions described above. There were four peaks, assigned as $C_{16:0}$-GE, $C_{18:0}$-GE, $C_{18:1}$-GE and $C_{18:2}$-GE, in the LC-MS SIM chromatograms although the $C_{18:0}$-GE peak

Table 2 Percentage of Relative Standard Deviation (RSD%) during Consecutive Runs, limit of Detection (LOD), Limit of Quantification (LOQ), and Calibration Line Obtained for Standard GEs.

	RSD%[a]		LOD[b]	LOQ[b]	Calibration line[c]	
GE	Retention time	Area	(μg/mL)	(μg/mL)	Equation	R^2
$C_{16:0}$-GE	0.18	4.3	0.0029	0.0096	y = 17344x + 323	0.9998
$C_{18:0}$-GE	0.23	5.0	0.0037	0.012	y = 13104x - 70	0.9999
$C_{18:1}$-GE	0.19	5.1	0.0028	0.0094	y = 18488x - 946	0.9991
$C_{18:2}$-GE	0.21	7.0	0.0023	0.0076	y = 23308x - 143	0.9999
$C_{18:3}$-GE	0.21	5.4	0.0014	0.0045	y = 15120x + 1020	0.9989

aObtained by six consecutive runs of the standard GE solution at a concentration of 0.2 μg/mL.

[b]Defined as S/N = 3 for LOD and 10 for LOQ based on 5 μL injection of the standard GE solution at a concentration of 0.01 μg/mL.

[c]Calculated from the equation y = Ax + B, where x is the injected concentration (μg/mL), y is the peak area, A is the slope, and B is the intercept in the range between 0.05 and 5 μg/mL (0.05, 0.2,1 and 5 μg/mL).

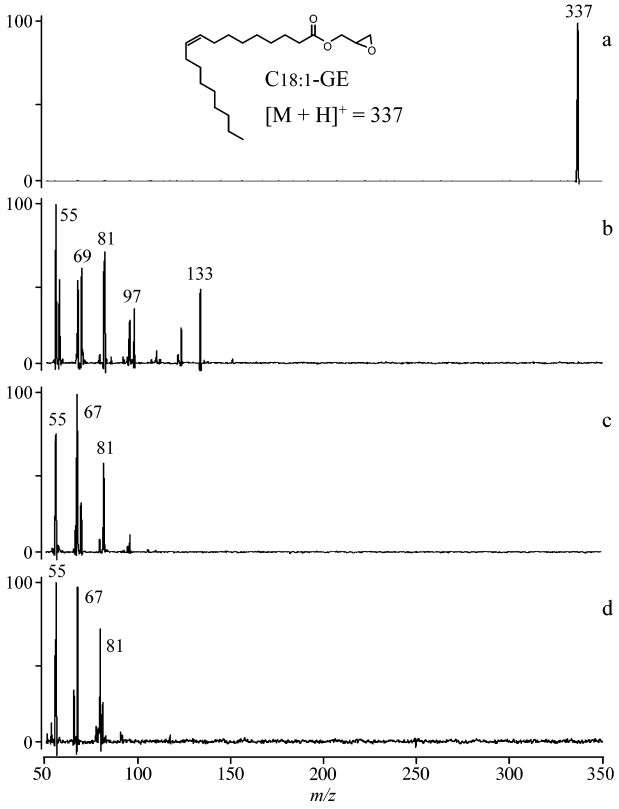

Fig.2 Mass Spectra of Standard $C_{18:1}$-GE under Optimal LC-MS Conditions.
a: mass spectrum in the single MS mode. b-d: product ion spectra in the triple MS mode (b; collision induced-dissociation 20 V, c; 40 V, d; 60 V).

was quite small. When the recovery% of the five standard GEs spiked was calculated, the values were not so bad, as shown in Table 3. However, the LC-MS measurements for oil samples caused some serious problems. For example, the shapes of the peaks were quite broad and were frequently split at the top. The baselines were relatively noisy compared with baselines in the standard GE measurements. Subsequent runs of the oil sample solutions further deteriorated those peak shapes and further increased baseline noises on the chromatograms. Once such problems occurred, much time was required to revitalize the system to the original LC-MS status, because of the necessity of thorough washes of various parts of the LC-MS instruments used. Therefore, those problems had to be overcome to develop a robust and stable method to quantify the GEs in the oils.

As for the tests using Procedure I, we hypothesized that one of the main reasons for the problems originated from the large amounts of acylglycerols (TAGs, DAGs and MAGs) co-existing in the oils, which might adsorb on the inner parts of the LC-MS instruments and might remain there after the runs as contaminants. According to that hypothesis, we tried to remove TAGs, DAGs and MAGs from the oil samples prior to the LC-MS measurements. Since TAGs differ from the GEs due to their hydrophobic characteristics (three acyls in TAGs versus one acyl in GEs), a reversed-phase mode seemed to be the most effective way to separate them. On the other hand, the hydrophilic characteristics of DAGs (one hydroxyl) and MAGs (two hydroxyls) differ from those for GEs (no hydroxyls), so a normal-phase mode would also seem to be effective. Therefore, two procedures using reversed-phase and normal-phase SPEs were studied: one using normal-

Table 3 Percentages of Recovery of Five Standard GEs Spiked into Sample-A in Different Pretreatment Procedures.

GE	Recovery%[a]		
	Procedure I[b]	Procedure II[c]	Procedure III[d]
$C_{16:0}$-GE	61.8 ± 5.7	74.1 ± 10.5	79.8 ± 9.5
$C_{18:0}$-GE	89.3 ± 2.8	67.0 ± 8.8	84.2 ± 4.6
$C_{18:1}$-GE	82.8 ± 7.0	67.4 ± 11.1	92.6 ± 8.6
$C_{18:2}$-GE	76.2 ± 4.5	76.4 ± 12.1	94.6 ± 11.5
$C_{18:3}$-GE	76.8 ± 10.1	75.1 ± 9.8	75.4 ± 2.2

[a]To 0.1 g of Sample-A, 1 μg GEs each were added for the preparation of the spiked samples. Levels of GEs in the spiked and non-spiked samples were determined, and the recovery% were calculated using those quantitative levels.

[b]Dispersed in methanol/2-propanol 1:1, and the supernatant was applied to LC-MS without any SPEs.

[c]Diluted with chloroform, and the solution was applied to normal-phase SPE and subsequently to reversed-phase SPE, followed by application to LC-MS.

[d]Dispersed in acetonitrile, and the supernatant was applied to reversed-phase SPE and subsequently to normal-phase SPE, followed by application to LC-MS.

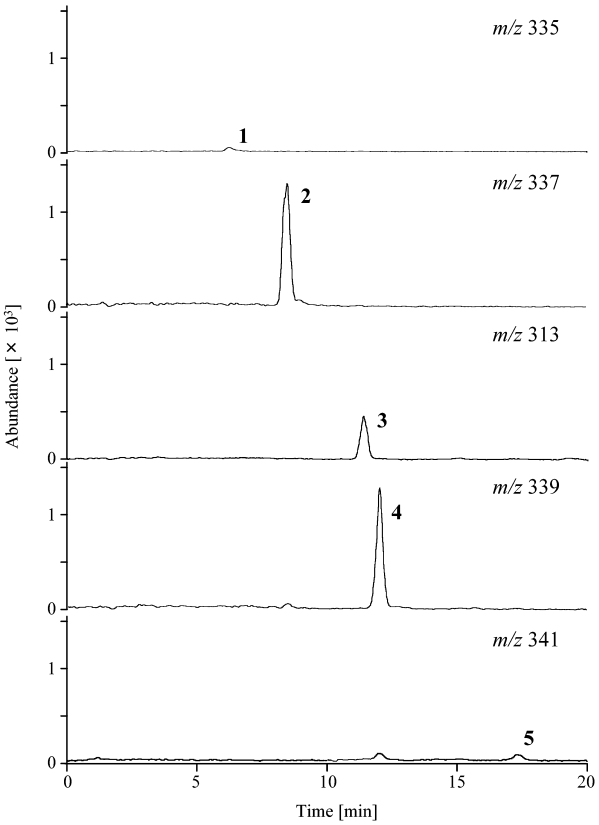

Fig.3 LC-MS SIM Chromatograms of GEs in Sample-A.Peaks: 1 = $C_{18:3}$-GE, 2 = $C_{18:2}$-GE, 3 = $C_{16:0}$-GE, 4 = $C_{18:1}$-GE, 5 = $C_{18:0}$-GE.

phase first and reversed-phase second (Procedure II, Table 3) and the other using reversed-phase first and normal-phase second (Procedure III, Table 3). The LC-MS measurements of oil sample solutions using Procedures II and III provided better shaped-peaks and better baselines (Fig. 3), almost equivalent to those obtained in the standard GE measurements. Procedures II and III did not cause any deterioration even after sequential runs. Therefore, our hypothesis did not seem incorrect, implying that processes to remove TAGs, DAGs and MAGs from the oil samples may be essential to construct the robust and stable method. Considering the recovery%, Procedure III was chosen as the optimal pretreatment because all spiked GEs had high recovery% and reproducibility in case of Procedure III, compared with those values in case of Procedure II (Table 3).

3.3 Quantification of GEs in edible oils

Table 4 shows levels of GEs in the oils determined by the newly developed method reported in this study. Sample-A contained 10.2 μg/g $C_{18:1}$-GE and 9.0 μg/g $C_{18:2}$-GE as major components, and also had $C_{16:0}$-GE and $C_{18:0}$-GE as minor components. Sample-B contained relatively low levels of GEs, 3.4 μg/g $C_{18:1}$-GE as the major component and C16:0-GE and $C_{18:2}$-GE as minor components. Sample-C contained as much as 132 μg/g $C_{18:2}$-GE, 96 μg/g $C_{18:1}$-GE and 32 μg/g $C_{18:3}$-GE, and a minor amount of $C_{16:0}$-GE. Those results are consistent with our preliminary qualitative tests (Tables 1 and 4). The recovery% for the five standard spiked GEs were 75.4-94.6% (average 85.3%) with RSD 2.9-12.1% for Sample-A, 71.3-75.1% (average 73.5%) with RSD 3.0-9.7% for Sample-B, and 90.8-105.1% (average 97.2%) with RSD 2.1-12.0% for Sample-C. The recovery% obtained from Sample-C was relatively higher than the others. This may be because Sample-C is composed chiefly of DAGs, which do not greatly affect the double SPE procedures and/or LC-MS measurements, characteristics that are different from TAGs. Judging from the overall accuracy and precision, this method is appropriate for the quantitative analysis of GEs in the edible oils. However, it should be noted that the accuracy and precision are not ideal, as exemplified in the recovery% being 71.3% for $C_{16:0}$-GE in Sample-B and the recovery RSD% being 12.1% for $C_{18:2}$ in Sample-A (Table 4). Further work to improve the method to a more accurate and precise one is required, referring to our analytical approaches and results. Thus, higher sensitivity may be needed, because if one could further dilute the oil sample solutions in the preparation, the influence of co-existing acylglycerols on the detection of GEs would be reduced. Alternatively, the SPE procedures may be

Table 4 Levels of GEs in Commercial Edible Oils and Recovery Test.

		Levels [μg/g][a]		Recovery%[a]	
Edible oil		Mean±S.D.	RSD%	Mean±S.D.	RSD%
Sample-A	$C_{16:0}$-GE	2.6 ± 0.06	2.2	79.8 ± 9.5	11.9
	$C_{18:0}$-GE	0.5 ± 0.03	6.2	84.2 ± 4.6	5.5
	$C_{18:1}$-GE	10.2 ± 0.49	4.8	92.6 ± 8.6	9.3
	$C_{18:2}$-GE	9.0 ± 0.09	1.0	94.6 ± 11.5	12.1
	$C_{18:3}$-GE	< 0.5[b]	-	75.4 ± 2.2	2.9
Sample-B	$C_{16:0}$-GE	1.2 ± 0.06	5.3	71.3 ± 6.9	9.7
	$C_{18:0}$-GE	< 0.5[b]	-	73.8 ± 5.2	7.0
	$C_{18:1}$-GE	3.4 ± 0.22	6.4	75.1 ± 5.1	6.7
	$C_{18:2}$-GE	1.1 ± 0.09	7.6	75.1 ± 5.9	7.9
	$C_{18:3}$-GE	< 0.5[b]	-	72.4 ± 2.2	3.0
Sample-C	$C_{16:0}$-GE	6 ± 0.4	6.1	90.8 ± 3.5	3.9
	$C_{18:0}$-GE	< 5[b]	-	99.4 ± 2.1	2.1
	$C_{18:1}$-GE	94 ± 5	4.4	99.5 ± 11.9	12.0
	$C_{18:2}$-GE	132 ± 3	2.4	105.1 ± 10.5	10.0
	$C_{18:3}$-GE	32 ± 2	6.7	91.2 ± 8.1	8.9

[a]Analyzed in triplicate.

[b]Determined by the lowest concentration of the standard solutions for calibration and dilution degrees in the oil sample preparation.

changed so that TAGs are more effectively removed, because the recovery% was worse in TAG-rich edible oils.

4 CONCLUSION

A new method has been developed for the quantification of GEs in edible oils, in combination with double SPEs and LC-MS. The method is highly sensitive and can be applied to the quantitative determination of GEs in μg ranges per gram edible oils containing TAGs or DAGs as major components. The method has another advantage regarding the robustness and stability that sequential runs in the LC-MS measurement work well without any complications. On the other hand, it is also a fact that the method is not yet sufficient to accurately and precisely quantify GEs in the oils, as shown in the recovery tests using the three commercial sources of edible oils. This needs to be improved in the future. However, there is no doubt that this study is the first report on a quantitative method for GEs in edible oils. Using our analytical approaches and results, a method for more accurate and precise quantification of the GEs could be developed. This technique will be fundamental for the quality control of GEs, probably food processing contaminants, in edible oils.

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