Analytica Chimica Acta 1239 (2023) 340712

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Development of a QuEChERS method for simultaneous analysis of 3-Monochloropropane-1,2-diol monoesters and Glycidyl esters in edible oils and margarine by LC-APCI-MS/MS

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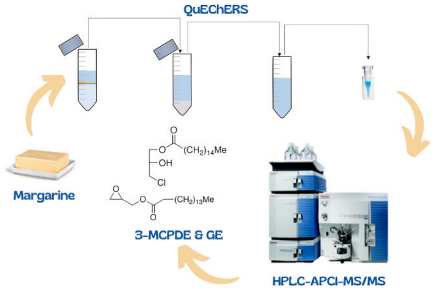
HIGHLIGHTS

- Direct determination using atmospheric-pressure chemical ionization (APCI) source.
- Comparison of APCI and HESI-II sources in the analysis of 3-MCPDE and GE.
- Cost-effective approach with simpler mobile phases and no matrix effect.
- Novel QuEChERS approach to extract of 3-MCPDE and GE from margarines.
- The presence of glycidyl oleate was confirmed in all analyzed samples.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
APCI
Food contaminants
Glycidyl
HPLC method
3-MCPD
QuEChERS

ABSTRACT

A simple, fast and effective direct method based on HPLC-APCI-QqQ-MS/MS has been developed to simultaneously determine four 3-monochloropropane-1,2-diol monoesters (3-MCPDE) esterified with palmitic, linoleic, stearic, and oleic acid, and two glycidyl esters (GE) with palmitic and oleic acid in margarine and olive oil using a QuEChERS approach. Factors affecting the efficiency of the extraction process were assessed, including type and amount of salt, extraction solvent, test portion amount, and clean-up sorbent. The analytical method was validated according to Food and Drug Administration (FDA) guidelines using matrix-matched calibration with internal standards and showed good results in terms of linearity ($r^2 > 0.9992$), accuracy (80<Recovery<120%), and precision (RSD<15%). The method was successfully applied for the first time to 11 margarine samples for simultaneous analysis of 3-MCPDE and GE.

1. Introduction

Vegetable oils are one of the most-used foods in the food industry, so

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https://doi.org/10.1016/j.aca.2022.340712
Received 7 November 2022; Received in revised form 5 December 2022; Accepted 6 December 2022
Available online 7 December 2022

Abbreviations

3-MCPD	3-monochloropropane-1,2-diol
ACN	acetonitrile
APCI	Atmospheric-pressure chemical ionization
CONTAM	Contaminants in the Food Chain
dSPE	dispersive solid phase extraction
EFSA	European Food Safety Authority
ESI	electrospray ionization
EVO	extra virgin olive oil
FDA	USA Food and Drug Administration
GE	glycidyl ester
IARC	International Agency for Research on Cancer
LC-MS/MS	liquid chromatography tandem mass spectrometry
Li	1-linoleoyl-3-chloropropanediol
LLOQ	lower limit of quantification
MTBE	methyl *t*-butyl ether
OL	1-oleoyl-3-chloropropanediol
OL-d5	1-oleoyl-3-chloropropanediol-d5
OLGE	glycidyl oleate
OLGE-d5	glycidyl oleate-d5
PA	1-palmitoyl-3-chloropropanediol
PA-d5	1-Palmitoyl-3-chloropropanediol-d5
PAGE	glycidyl palmitate
PAGE-d31	glycidyl palmitate-d31
PLLE	partitioned liquid-liquid extraction
PSA	primary secondary amine
QCs	quality control substances
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
ST	1-stearoyl-3-chloropropanediol
ST-d5	1-stearoyl-3-chloropropanediol-d5
TDI	tolerable daily intake
ULOQ	upper limit of quantification

it is essential to ensure their quality and safety for human consumption. During the oil refining process, mainly in the deodorization step carried out at low pressures and high temperatures (180–220 °C), food processing contaminants, including fatty acid esters of 3-monochloropropane-1,2-diol (3-MCPDE) and glycidyl ester (GE), can be formed [1,2]. Additionally, GE can be formed from monoacylglycerols or diacylglycerols by dehydration at high temperatures or from 3-MCPDE by elimination of HCl in refined edible oils [2]. Lipases can hydrolyze 3-MCPDE in the gastrointestinal system to release free 3-MCPD, which can be absorbed by the body and cause health risks. Toxicological studies have shown the ability of 3-MCPD to induce the formation of tumors in various organs, especially the kidney, and produce infertility [3].

The International Agency for Research on Cancer (IARC) classifies 3-MCPD as *"Possibly carcinogenic to humans''* (group 2B) and GE as *"Probably carcinogenic to humans"* (group 2A) [4]. The concentration of these compounds varies according to the type of oil, but they are especially abundant in palm oils. 3-MCPDE and GE levels are important indexes to evaluate food safety [1,2].

These potentially toxic compounds decrease the nutritional value of foods, harm consumers' health, and represent a constant concern of organizations such as the USA Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) [3]. Establishing a maximum level for 3-MCPD esters and GE in foods such as edible oils is appropriate to ensure a high level of human health protection. Institutional concern of these compounds began in the early 2000's, but solid guidance has not been fully established. The Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) established a tolerable daily intake (TDI) of 2 μg/kg body weight per day for 3-MCPD and 3-MCPDE. However, even though GE are genotoxic carcinogens, no TDI has been established for these compounds [3,5]. The latest Commission Regulation (EU) No. 2020/1322 sets a maximum level of 20 μg/kg for 3-MCPD in soy sauce and hydrolyzed vegetable protein and 1250 μg/kg and 2500 μg/kg for the sum of 3-MCPD and its esters in vegetable oils and fats, expressed as 3-MCPD. It also sets a maximum level of 1000 μg/kg for GE as glycidol in vegetable fats and oils [5].

To quantify these contaminants, direct and indirect analytical methods were reported based on liquid and gas chromatographic and mass spectrometry techniques. Indirect methods require the hydrolysis of the 3-MPCD esters and GE to their corresponding free forms, followed by their derivatization and subsequent GC-MS analysis [6–8]. Direct methods are based on determining individual esters by LC-MS, with neither modifications nor derivatization needed [9–16]. However, the nature and characteristics of the target compounds interfere with their identification and determination, as in the case of GE and 3-MCPDE monoesters of the same fatty acid.

An electrospray ionization (ESI) source is often used to simultaneously determine 3-MCPDE and GE with fatty acids in edible oils and oil-based food [11–16,19–25]. However, other sources have been proposed, including heated electrospray ionization (HESI) in the 3-MCPDE analysis [9,10] and atmospheric pressure chemical ionization (APCI) in the GE analysis [16,26,27]. Thermally-focused HESI has been described as a modification of a traditional ESI source that uses electrospray at high temperatures during the nebulization [28]. HESI source applies additional sheath gas to assist the transformation of the ions in the solution into the gas phase [28,29]. Lately, HESI sources, in their different available designs, have gained popularity over conventional ESI sources [30,31]. Recent studies suggest a significant improvement in ionization efficiency when using a HESI source, while other studies report a higher propensity for matrix effect [28–31].

On the other hand, many authors have observed similar performances between APCI and ESI sources regarding different analytical features [28]. Additionally, a significant reduction in matrix effects has been demonstrated when using APCI sources [31]. Concerning the mobile phase, the general compositions do not vary greatly when using ESI, HESI, or APCI sources, except in their acidity, which is necessary to stimulate ionization in the ESI and HESI sources [32]. At the same time, its absence or presence is not necessarily influential in APCI ionization [31,32]. For this work, Thermo Fisher Scientific's HESI-II and APCI sources were assessed for their performance in determining fatty acid monoesters of 3-MCPD and GE.

Based on most abundant fatty acids in oil-based foods (including margarine), monoesters of 3-MCPD and GE with oleic (OL and OLGE), palmitic (PA and PAGE), linoleic (LI), and stearic (ST) acids were selected for this study. The structural similarity of the products causes the MS detector to report similar intensities for both precursor ions and transitions. For this, a chromatographic column capable of separating these analytes and providing complementary selectivity for their determination is needed.

The sample preparation step of complex matrices like oils, fats, and oil-based foodstuff is often complicated, requiring large volumes of organic solvents. Previous studies have reported a double SPE procedure using an SPE C18 cartridge first and then an SPE silica cartridge with solvents of different polarities [11–16]. Several approaches were recently proposed and tested to improve sample preparation using other lipid-removal sorbents. Graziani et al. (2017) proposed the extraction of esters using acetonitrile-2-propanol (1:1 v/v), followed by clean-up with a mixture of PSA and C18 powder using vortex agitation. The supernatant was evaporated to dryness under a nitrogen stream, and the dry extract was reconstituted with acetonitrile-2-propanol (1:1 v/v) [9]. In a

previous study applied to 3-MCPDE diesters, we developed an approach that includes partitioned liquid-liquid extraction (PLLE) with 20% EtOAc/MTBE followed by a solvent exchange after evaporation with nitrogen, using 2% Et_2O/hexane. Then, two stages of dispersive solid phase extraction (dSPE) were used for clean-up, the first using Si-SAX and PSA and the second using Z-Sep+ and PSA. The final extract was evaporated to dryness and rebuilt in 75% MPA/25%MPB (the mobile phase solution) [10].

In response to the need to simplify sample preparation, a direct method for the simultaneous determination of 3-MPCD monoesters and GE in oils was developed and validated. The analysis was carried out by LC-APCI-MS/MS using a 5 μm Luna OMEGA PS C18 chromatographic column taking only 4 min. This column improves labor efficiency by having comprehensive polar and non-polar retention. For sample preparation, a simplified but effective procedure of QuEChERS is proposed that uses a 15 mg sample, 3 mL of acetonitrile, 3 mL of water, and 150 mg of EMR-lipid sorbent. This novel method was used to assess 11 margarine samples in triplicate for the presence of PA, OL, LI, ST, PAGE, and OLGE.

2. Materials and methods

2.1. Chemicals and materials

Acetonitrile (ACN), 2-propanol (IPA), chloroform ($CHCl_3$), methanol (MeOH), acetone (ACO), n-hexane, ethyl acetate (EtOAc) and diethyl ether (Et_2O) were all LC-MS grade with a purity of ≥99% and purchased from Merck (Darmstadt, Germany). Ammonium sulfate (purity ≥99%), ammonium formate, and ammonium acetate (purity ≥99.99%) from Merck. Ultrapure water was obtained using a Millipore purification system (Millipore, Billerica, MA, USA). SPE sorbents: Bond Elut EMR-Lipid (EMR-Lipid), C18, primary secondary amine (PSA), and Silica Strong Anion Exchange (Si-SAX) were supplied from Agilent® (Santa Clara, CA, USA), OASIS HLB was purchased from Waters® (Milford, MA, USA) and Supel™ QuE Z-Sep + bulk (Z-Sep+) from SUPELCO (Bellefonte, USA). The standards used were 1-oleoyl-3-chloropropanediol (**OL**, CAS No. 10311-82-7), 1-palmitoyl-3-chloropropanediol (**PA**, CAS No. 30557-04-1), 1-stearoyl-3-chloropropanediol (**ST**, CAS No. 22094-20-8), 1-linoleoyl-3-chloropropanediol (**LI**, CAS No. 1246833-46-4), glycidyl palmitate (**PAGE**, CAS No. 7501-44-2), glycidyl oleate (**OLGE**, CAS No. 5431-33-4), 1-oleoyl-3-chloropropanediol-d5 (**OL-d5**, CAS No. 1639207-37-6), 1-palmitoyl-3-chloropropanediol-d5 (**PA-d5**, CAS No. 63326-63-6), 1-stearoyl-3-chloropropanediol-d5 (**ST-d5**, CAS No. 1795785-84-0), glycidyl palmitate-d31 (**PAGE-d31**, CAS No. 1246819-24-8), glycidyl oleate-d5 (**OLGE-d5**, CAS No. 1426395-63-2), all purchased from Toronto Research Chemical (Toronto, Ontario, Canada). Individual and mixed working standard solutions were prepared in methanol and stored at −18 °C. Calibration solutions were prepared at six concentration levels between 100 and 1000 ng/g from the mixed solution by appropriate dilution; the internal standard (IS) containing the deuterated species was added at 250 ng/g.

Instruments and devices used were a vortex mixer from Instruments GmbH & Co (Schwabach, Germany), a Centromix II-BL centrifuge from J. P. Selecta (Barcelona, Spain), an AS 82/220.R2 Analytical Balance from RADWAG (Radom, Poland), a EC-1V-130 heater, a sample concentrator from VLM GmbH (Bielefeld, Germany), a HPLC-MS/MS Thermo Scientific™ equipped with a triple quadrupole mass spectrometer TSQ Quantum Access MAX from Thermo Fisher Scientific, (San Jose, CA, USA) provided with an atmospheric pressure chemical ionization APCI source, a heated electrospray ionization source HESI-II, an Accela 1250 pump fitted with a degasser and an autosampler.

2.2. Samples

An extra virgin olive oil (EVO) sample was used to develop and validate the proposed method. A palm oil-free margarine sample was used for the matrix extension study. A total of 11 margarine samples composed of mixtures of various vegetable oils, including olive, sunflower, linseed, soybean, corn, rapeseed, and palm oil, in different proportions, were analyzed for 3-MPCD monoesters and GE. All samples were purchased from local supermarkets in Santiago de Compostela, Spain, and stored in their original packet at 4 °C until analysis. Once opened, samples were stored in the freezer at −20 °C.

2.3. Sample preparation

In a conical-bottom glass tube, a 15 mg test portion was accurately weighed, followed by the addition of the internal standard solution, 3 mL of ultrapure water, 3 mL of acetonitrile, and 0.9 g of ammonium sulfate. The mixture was vortex-mixed for 2 min and centrifuged at 2601 rcf for 5 min to separate it into two phases. The upper phase was pipetted into another conical-bottom glass tube containing 150 mg of EMR-Lipid, vortex-mixed for 2 min for clean-up, and centrifuged for 5 min at 2601 rcf. After this, a 2 mL aliquot of the supernatant was evaporated under a nitrogen stream to dryness at 70 °C. The extract was reconstituted in 0.25 mL acetonitrile to be analyzed by LC-MS/MS. A scheme of the entire process is presented in Fig. 1.

2.4. Instrumental conditions

Analysis was carried out using a Luna OMEGA PS C18 column (5 μm, 100 Å, 50 × 2.1 mm) equipped with a Security Guard pre-column (4mm × 2 mm i.d.) from Phenomenex (Torrance, USA). The flow rate was set to 400 μL/min, the column temperature was 30 °C, and the sample injection volume was 10 μL. The mobile phase composition was ACN (A), and water (B), programmed to 85% A at 0 min, increased linearly to 90% An until 5 min, and returned to 85% A at 6 min and kept until 10 min for stabilization. However, the analytes were separated in less than 5 min. Additionally, the performance of the ACQUITY UPLC BEH Shield RP18 Column (1.7 μm, 130 Å, 100 × 2.1 mm) from Waters (Milford, MA, USA) on the separation of 3-MCPDE and GE was investigated in this paper. Both Luna OMEGA PS C18 and ACQUITY UPLC BEH Shield RP18 columns were tested under the same conditions to compare their separation capabilities for target compounds. Initially, the determination of the compounds by tandem mass spectrometry (QqQ-MS/MS) was carried out with a HESI-II source in positive mode with the operational conditions described in the supplementary material (Table S1). Subsequently, APCI source, also operated in positive mode, were used with the ionization and transfer temperatures set at 350 and 340 °C. Nitrogen (>99.98%) was selected as sheath and auxiliary gas set at a pressure of 35 and 10 arbitrary TSQ Quantum units, respectively. Detection was performed in the Multiple Reaction Monitoring mode (MRM) using Argon as collision-induced-dissociation (CID) gas at 18 bar. The monitored range was 200–700 *m/z*.

2.5. Method validation

The FDA guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products [17] and Bioanalytical Method Validation Guidance for Industry [18] were used for the analytical validation of this method. EVO was used as a blank sample to assess the method's selectivity, linearity, detection limits, precision, and accuracy.

The specificity of the method was assessed using ten different EVO samples and based on the selection of a quantifier transition and two qualifier transitions for each analyte and IS. Sensitivity of the method involve setting a limit of detection (LOD), and the lower and upper limit of quantification (LLOQ, ULOQ). The lowest and highest amount of each analyte that can be quantitatively determined with acceptable precision and accuracy in this method falls between the respective LLOQ and ULOQ. LOD was calculated for analytes concentration at which signal-to-noise ratio was 3, and 10 for LLOQ, and the ULOQ was set at 1000

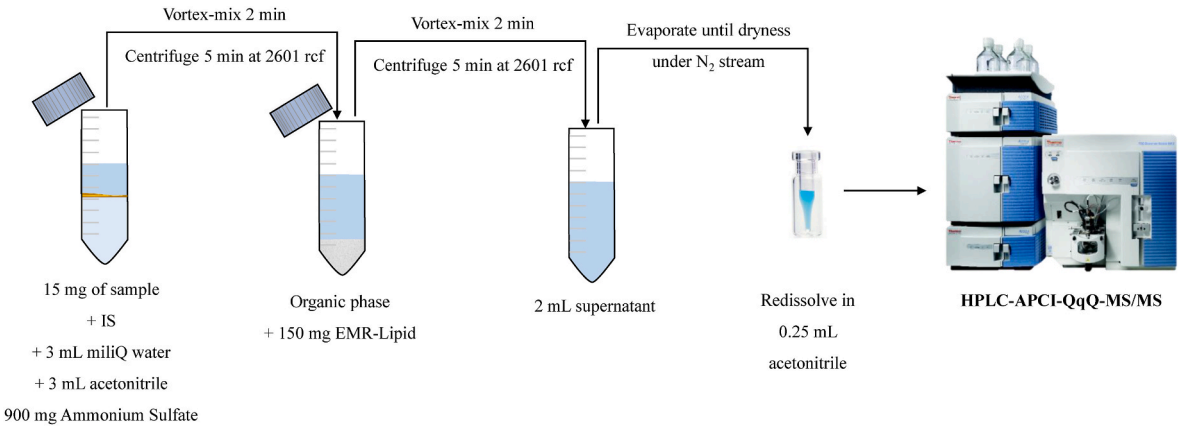

Fig. 1. Scheme of the QuEChERS protocol for simultaneous extraction of four monoesters of 3-monochloropropane-1,2-diol and two glycidyl esters from oil-based foodstuff.

ng/g.

Sensitivity of the method was also assessed at the LLOQ in terms of relative standard deviation (%RSD) for each analyte in triplicate. Accuracy of the optimized process was assessed using quality control substances (QCs) at three levels of concentration within the linear range (100, 250 and 500 ng/g). According to FDA guidelines, acceptable criteria for accuracy are recoveries between 80% and 120% of the nominal value. The precision of the method was evaluated by intraday and interday assays in terms of %RSD in quintuplicate. A Matrix extension study was performed using QCs at the same three levels of concentration in a palm oil-free margarine sample to confirm the applicability of the method to this group of samples.

3. Results and discussion

3.1. Optimization of the HPLC-APCI-QqQ-MS/MS method

No problems were observed using the HESI-II source for PAGE, OLGE, PAGEd31, and OLGEd5 determinations. Different fragments were observed for each GE, including $[M+H]^+$ and $[M+NH_4]^+$, allowing the selection of a quantifier and two qualifier transitions for these analytes. However, significant signal suppression was observed when infusing PA, OL, PAd5, and Old5, so the low intensity of the observed ions made it impossible to select the transitions necessary for their determination. Said signal suppression has been previously reported when using this source type [9]. It is associated with the fact that thermal focusing causes rapid desolvation, increasing the spray's concentration while the excess surface charge remains constant. This situation encourages competition for this extra charge, so the matrix effect on the analyte signal is compounded [29,32].

All analytes were successfully determined using the APCI source, archiving ions with enough intensity to set quantitative and qualitative transitions for PA, PAGE, OL, OLGE, Li, ST, PAd5, PAGEd31, Old5, OLGEd5, and STd5 (Table 1). Nevertheless, an interconversion of the 3-MCPD monoesters with their GE analog was observed in this work when using the APCI source. Epoxidation of 3-MCPD monoesters has been described before [33], not as a problem but as a resource to determine 3-MCPD monoesters, so it was decided to use the APCI source in this work. However, to simultaneously determine 3-MCPD monoesters and GE using APCI sources, a proper chromatographic separation must be done before epoxidation during MS determination. Luna OMEGA PS C18 and ACQUITY UPLC BEH Shield RP18 Column were tested for 3-MCPD monoesters and GE separation. ACQUITY UPLC BEH C18 column particles incorporate a series of trifunctional ligands that stabilize the stationary phase over a wide pH range [34]. The structure of the 1.7 μm BEH particles also uses novel final protection processes, improving the shape of the peak for basic analytes, which increases the sensitivity of

Table 1
Retention time, quantifier and qualifier transitions for 3-MCPD monoesters and GE.

Analyte	RT	Quantifier transition (*m/z*)		Qualifier transition (*m/z*)		IS
	min	Q1	Q3 (CE)	Q3 (CE)	Q3 (CE)	
LI	0.50	371.1	95.1 (26 V)	81.1 (23 V)	67.2 (28 V)	PAd5
PA	1.21	313.3	57.3 (21 V)	71.3 (18 V)	95.2 (17 V)	PAd5
OL	1.19	339.3	95.2 (18 V)	93.2 (28 V)	69.3 (24 V)	OLd5
ST	1.18	341.1	95.10 (37 V)	105.0 (40 V)	91.1 (24 V)	STd5
PAGE	1.42	313.3	66.4 (24 V)	82.3 (20 V)	98.3 (18 V)	PAGEd31
OLGE	1.48	339.2	69.3 (24 V)	95.2 (18 V)	83.2 (20 V)	OLGEd5
PAd5	1.39	318.3	57.3 (23 V)	71.3 (19 V)	95.2 (17 V)	–
OLd5	1.24	344.3	95.2 (20 V)	69.3 (25 V)	81.2 (21 V)	–
PAGEd31	1.17	344.5	66.4 (24 V)	82.3 (20 V)	98.3 (18 V)	–
OLGEd5	1.49	344.3	95.2 (19 V)	69.3 (26 V)	83.2 (19 V)	–
STd5	1.80	346.1	57.2 (26 V)	71.2 (21 V)	95.0 (20 V)	–

3-MCPD, 3-monochloropropane-1,2-diol; GE, glycidyl esters; IS, internal standard; CE, collision energy; LI, linoleoyl-3-MCPD; PA, palmitoyl-3-MCPD; OL, oleoyl-3-MCPD; ST, steareoyl-3-MCPD; PAGE, palmitoyl glycidyl ester; OLGE, Oleoyl glycidyl ester.

LC-MS analysis and simplifies sample preparation [34]. On the other hand, the 5 μm Luna Omega PS C18 column contains different ligands that give it a broad polar and non-polar retention [35]. However, the exact composition of the stationary phase of this column is not available as it is patented information. According to the manufacturer, it has a positive charge, possibly from some amino group incorporated at the end of a short alkyl chain bonded to the silica surface. This positive surface provides a retention factor for acidic compounds through ionic interactions, while for the reversed-phase separation, C18 carries out retention. In this way, the mixed selectivity of this column allows greater separation between compounds with functional diversity. The positive surface of its particles increases the retention of acidic compounds and repel strong alkaline species. In contrast, the C18 ligand increase the resolution of the analytes of interest by stimulating hydrophobic retention.

The separation of 3-MCDP monoesters and GE is challenging due to

their structural similarities that lead to similar physicochemical properties. Not enough separation of GE and analog 3-MCPD monoesters was achieved when using ACQUITY UPLC BEH C18 with different ratios of aqueous-organic mobile phases (Fig. S1). However, when using OMEGA PS C18 as a stationary phase, different retention times were observed between PA and PAGE, OL and OLGE, and OLd5 and OLGEd5. The best separation was achieved using up to 15% water in the mobile phase. ACN and MeOH were compared, and a better resolution in terms of peak shape was achieved with ACN, so it was chosen as an organic solvent in the mobile phase.

Moreover, the addition of formic acid was tested as an attempt to improve the ionization of the analytes. No significant difference was observed between the addition of 0.01% formic acid versus the absence of it. At the same time, a decrease in the ionization of the analytes was observed using 0.02% formic acid, so it was decided to use un-acidified ACN-water as the mobile phase. Although isocratic 15% water-ACN was enough to elute all the analytes, a gradient program described in section 2.4 was used to ensure the complete elution of all other matrix components.

3.2. Development and optimization of QuEChERS procedure

All experiments in this study were performed in triplicate.

Different organic solvents were tested for oil dilution and the extraction of the analytes, including MeOH, $CHCl_3$, ACN, IPA, ACO, n-hexane, Et_2O, and EtOAc. Only $CHCl_3$, ACN, and IPA proved to sufficiently extract the analytes, even though other matrix components were coextracted. $CHCl_3$ was discarded due to its toxicity, and IPA, ACN, and a 1:1 (v/v) mixture were tested in terms of recovery. Results (Fig. 2A) show that better recoveries were achieved using ACN. This solvent is also used in the mobile phase, so it was decided to use ACN as the extraction solvent. Aqueous to ACN ratios in the liquid-liquid partition were assessed at three levels, 1:3, 1; 1, and 3:1 (v/v) in terms of recovery, achieving better results with 1:1 (v/v) for most analytes (Fig. 2B).

The test portion amount was tested at two levels (15 and 150 mg), achieving similar results in terms of recovery for all the analytes, so the smallest test portion should be used. A salting-out effect is an essential tool in QuEChERS since it increases the ion strength of the aqueous phase, enhancing the mass transfer phenomenon into the organic phase and the aqueous-organic phase separation. Ammonium salts have proved to be good salting-out agents and are source-friendly [36], so three different ammonium salts were assessed at different concentrations. Fig. 3 shows that the higher chromatographic peak areas were achieved when using ammonium sulfate compared to ammonium, formate, and acetate. Moreover, the effect of the ammonium sulfate was tested at 5, 10, 15 and 25% w/w in triplicate and in terms of recovery. It was observed that the efficiency of the method varies linearly with the addition of said salt for the analytes. Since there is no significant difference between 15% and 25%, it was decided to use 15% w/w ammonium sulfate as the salting-out agent.

A clean-up step using silica-based sorbents is included in QuEChERS to remove other matrix eluents. In this work, six sorbents were tested, including Bond Elut EMR-Lipid®, Oasis ® HLB, Z-sep+, primary secondary amine, C-18, and Strong Anion Xchange silica sorbents. Most of these sorbents were purchased in bulk except for EMR-Lipid and Oasis HLB sorbents, which were purchased as SPE cartridges and cut in the lab to get the sorbent. Fig. 4 shows that the best results in terms of recovery were achieved using EMR-Lipid at 150 mg. The specific absorbability of this sorbent of C5 and long-chain hydrocarbons, as well as glyceride and waxes, promotes stronger selective adsorption for lipids compared with other conventional sorbents reported in previously published analysis [37–39].

3.3. Analytical features

Validation of the proposed method was assessed following FDA guidelines for analytical method validation [17,18]. The selectivity and specificity of the process (Table 1) are based on the selection of one quantifier and two qualifier transitions from a parent ion to a product ion at a specific collision energy. Moreover, as discussed before, 3-MCPD monoesters and GE of the same fatty acid shared the same parent ion, so the chromatographic separation of the analyte is a crucial factor in this work. Successfully, different retention times are given for these analytes using Luna OMEGA PS C18 (Table 1). No matrix interference was observed in the retention times of the analytes or the IS (Fig. 5). Chromatographic signals of 3-MCPD seem to be 10 times smaller than those of the GE analog, perhaps because of the transformation of 3-MCPD into GE during ionization, nonetheless, sensibility was sufficient for simultaneous determination in a wide range of concentrations.

Table 2 presents analytical features, including limits, linearity, accuracy, and precision. LOD and LLOQ were 3 and 10 ng/g for GE and 30 and 100 ng/g for 3-MCPD monoesters. All the analytes achieved excellent determination coefficients ($r^2 \geq 0.9992$) within the linear range (LLOQ to 1000 ng/g). Accuracy assessed using QCs in triplicate resulted in recoveries spanning 84.8%–118.7%. Excellent precision is confirmed by intraday and interday assays, both in quintuplicate with a coefficient of variation (%RSD) lower than 12.4%. Even when the APCI source is less susceptible to matrix effect when compared to other sources [31], a matrix-matched calibration with internal standard in a margarine sample was used to avoid possible matrix interferences due the matrix complexity. The matrix extension study supports the applicability of the developed method in the analysis of 3MCP monoesters and GE in margarine samples (Table S2).

Previously published articles (Table S3) on the simultaneous determination of GE and 3-MCPD monoesters reported quantification limits

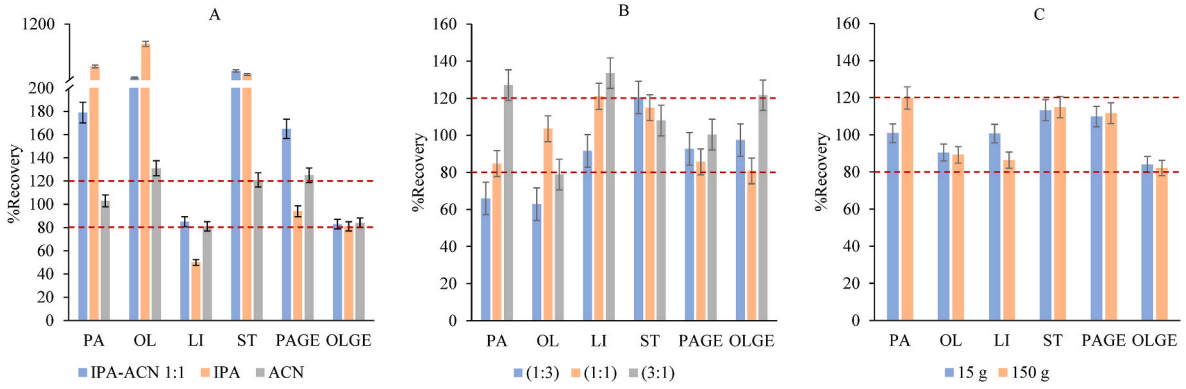

Fig. 2. Selection of extraction solvent (A), aqueous-organic phases ratio (B) and test portion (C). IPA, isopropanol; ACN, acetonitrile. In triplicate using an EVO sample spiked at 0.5 μg/g.

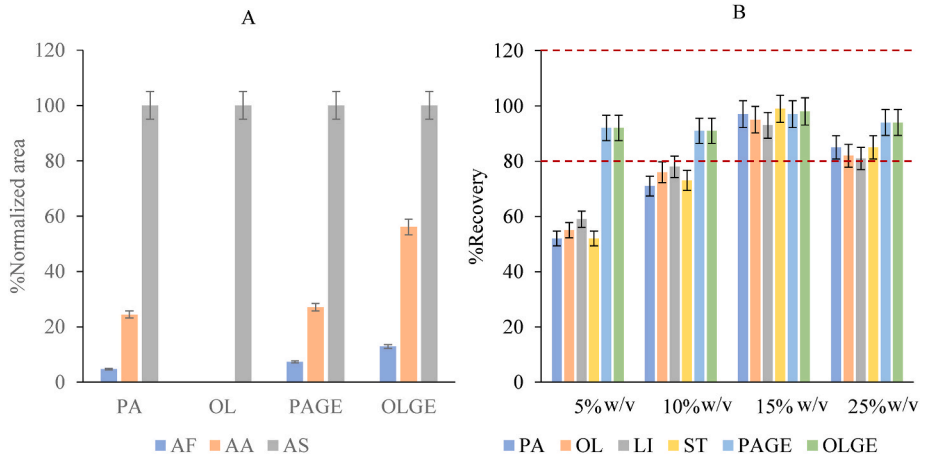

Fig. 3. Study of type of salt (A) and amount of ammonium sulfate (B). AF, ammonium formate; AA, ammonium acetate; AS, ammonium sulfate. In triplicate using an EVO sample spiked at 0.5 μg/g.

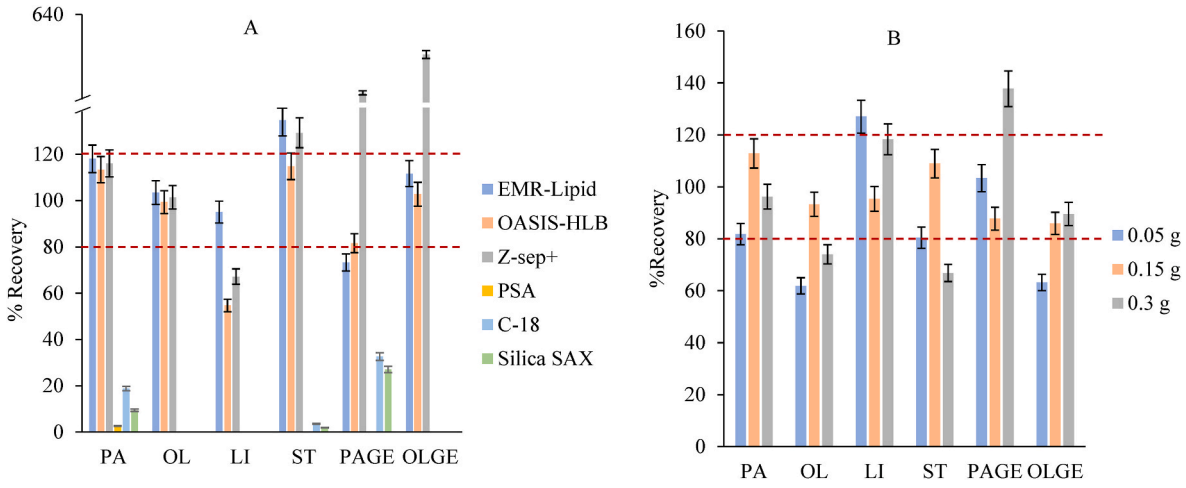

Fig. 4. Study of type of clean-up sorbent (A) and amount of EMR-lipid (B). In triplicate using an EVO sample spiked at 0.5 μg/g.

similar to or higher than those presented here [11,12,16,20–27]. Hidalgo-Ruiz et al. (2021) achieved lower LOQ for most of the 3-MCPD monoesters by using a much larger test portion size (more than sixty times) than that of this work, but reporting similar LLOQs for LI and GE [21]. Other works focused on the determination of 3-MCPD monoesters [21–24], often alongside 3-MCPD diesters, and also reported equal or higher limits than those of this work, except for Chai et al. (2016) for the determination of 3-MCPD mono- and di-esters in beef flavoring samples [20]. Chai et al. also accomplished lower LOQs by increasing the test portion amount to 1g [20]. However, despite the potential improvement in method sensitivity with larger test portion sizes, its miniaturization is preferred as it follows the trends of green analytical chemistry while allowing volume reduction of solvents, adsorbents, and waste. Moreover, most methods developed for GE determination reported limits much higher than those reported here [16,26]. Blumhorst et al. (2013) presented lower limits in their collaborative study on edible oils [27]; however, they are instrumental limits calculated in the absence of a sample. So, they are related to the instrument response and not to the application of the entire analytical method. The work presented in this paper is more accurate and precise than those reported before in terms of % recovery and variation coefficients ≤12.4% [10,11,15,19–22,24,25].

3.4. Analysis of margarine samples

This new QuEChERS protocol was used to simultaneously determine and quantify two GE and four 3-MCPD monoesters with fatty acids in 11 margarine samples by HPLC-APCI-QqQ-MS/MS. The results are presented in Table 3 and Fig. S2. All samples were positive for at least one GE or 3-MCPDE. PA and PAGE were below the LLOQ in all samples. This could be due to the current trend to promote healthy nutrition, which would imply avoiding or reducing the use of palm oil in processed foods such as in the blend of oils used in margarine production. LI and OL were only quantifiable in two samples at levels between 0.24 and 0.28 μg/g, and 0.14–0.16 μg/g, respectively. ST was present in all samples and quantified in nine of them at concentrations among 0.10–0.54 μg/g. ST was also the analyte with the highest amounts found in the analyzed samples. Regarding GE, OLGE was quantifiable in all samples at levels from 0.03 to 0.22 μg/g. These values are in accordance with those previously reported by Hidalgo-Ruiz et al. [21]. Since the analysis of 3-MCPDE and GE in margarines has been mostly performed using indirect methods [6,7], an estimation of the equimolar contribution of these analytes to the presence of free 3-MCPD and GE were evaluated using equations (1) and (2). The results are present in Table 3.

$$Equivalent\ 3MCPD = \sum_{n=1}^{i} \frac{[3MCPDE_i]}{Molecular\ weight\ 3MCPDE_i} * 110.539\ g/mol \cdots \quad (1)$$

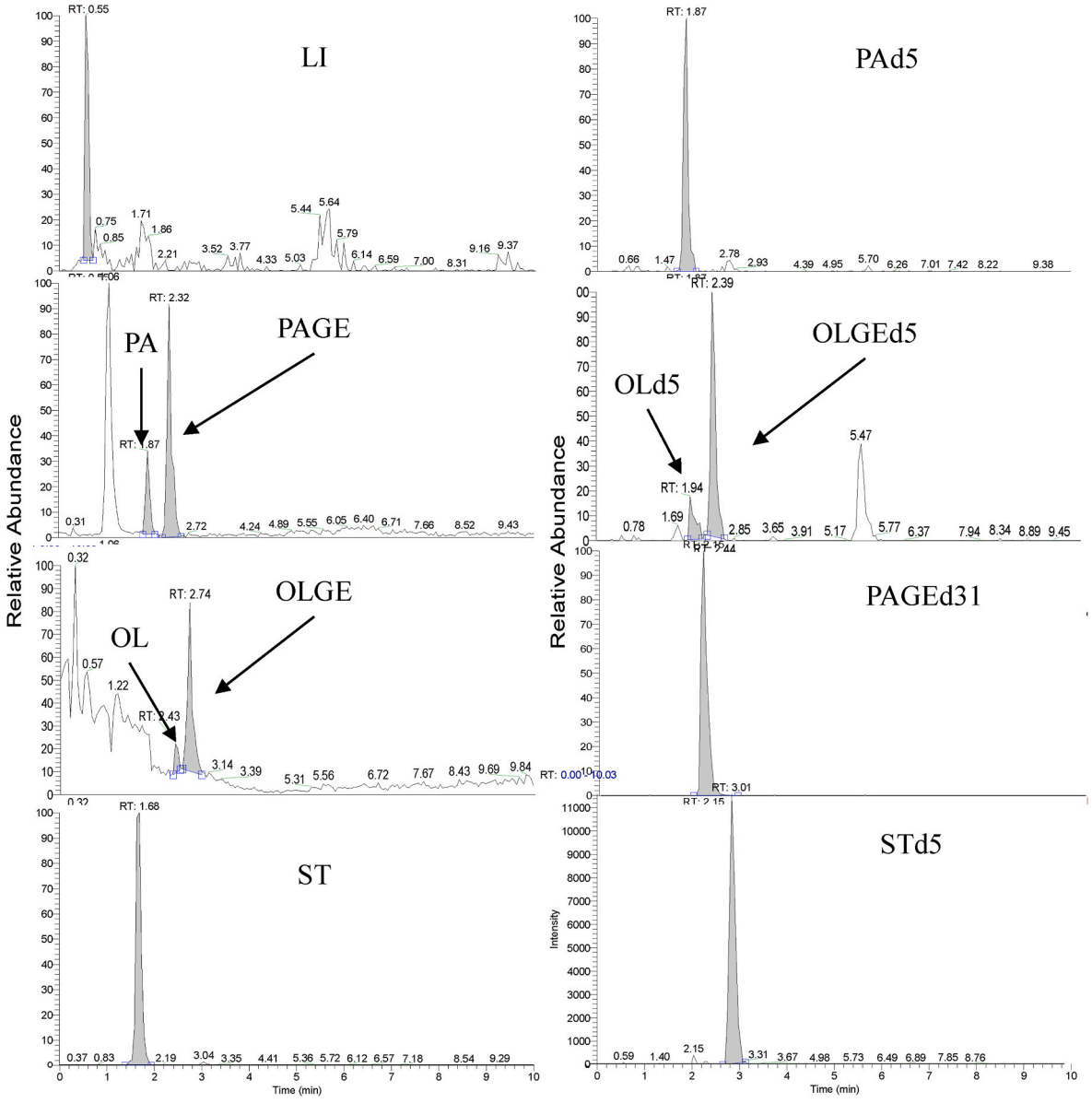

Fig. 5. Chromatogram of four monoesters of 3-MCPd and two GE in a margarine sample enriched at 0.5 μg/g.

Table 2
Analytical method validation parameters of QuEChERS-HPLC-APCI-QqQ-MS/MS method for analysis of four monoesters of 3-MCPD and two GE in oil-based foodstuff.

Analyte	LLOQ	r^2[a]	Accuracy			Precision					
			(n = 3)% recovery			Intraday (n = 5) % RSD			Interday (n = 5)% RSD		
	ng g^{-1}		L-Qc	M-QC	H-QC	L-Qc	M-QC	H-QC	L-Qc	M-QC	H-QC
LI	100	0.9996	111	95	98	7	5	5	9	4	6
PA	100	0.9999	110	112	104	1	4	7	11	8	8
OL	100	0.9993	91	115	103	10	10	3	5	12	8
ST	100	0.9992	107	109	119	2	7	5	6	4	4
PAGE	10	0.9995	109	100	93	12	10	7	9	4	12
OLGE	10	0.9998	85	87	104	11	2	3	12	12	5

3-MCPD, 3-monochloropropane-1,2-diol; GE, glycidyl esters; CV, coefficient of variation; L-QC; low quality control at 100 ng/g; M-QC; middle quality control at 250 ng/g; high quality control at 500 ng/g.
[a] Linear range from LLOQ to 1000 ng/g.

$$Equivalent\ Glycidol = \sum_{n=1}^{i} \frac{[3MCPDE_i]}{Molecular\ weight\ 3MCPDE_i} * 74.08\ g/mol \cdots \quad (2)$$

The results obtained in this work for the individual and simultaneous quantification of PA, OL, LI, ST, PAGE and OLGE are in accordance with those reported by Hidalgo-Ruiz et al. [21] for the analysis of 3-MCPDE and GE in fatty matrices (including margarines). Equivalent 3-MCPD and glycidol ranged from 0.07 to 0.16 μg/g and 0.01–0.05 μg/g. These

Table 3
Content of 3-MCPD monoesters and GE in margarine samples.

Sample	LI		PA		OL		ST		PAGE		OLGE		3-MCPD*	Glycidol*
	ng/g	±SD	ng/g	±SD	ng/g	±SD	ng/g	±SD	ng/g	±SD	ng/g	±SD	ng/g	ng/g
M1	ND	–	ND	–	ND	–	203.8	17.2	ND	–	43.4	20.4	59.7	9.5
M2	ND	–	ND	–	ND	–	284.3	20.2	ND	–	27.8	5.9	83.4	6.1
M3	ND	–	ND	–	ND	–	538.2	26.9	ND	–	100.4	22.1	157.8	22.0
M4	ND	–	ND	–	ND	–	542.7	26.0	ND	–	54.9	3.2	159.1	12.0
M5	ND	–	ND	–	157.4	16.3	309.1	52.1	ND	–	70.3	57.7	137.0	15.4
M6	ND	–	ND	–	ND	–	438.8	9.0	ND	–	82.3	56.0	128.7	18.0
M7	ND	–	ND	–	143.6	19.9	96.8	19.8	ND	–	216.5	13.9	70.7	47.4
M8	ND	–	ND	–	ND	–	346.0	5.5	ND	–	43.4	59.7	101.4	9.5
M9	242.9	10.4	ND	–	ND	–	ND	–	ND	–	78.9	14.1	72.0	17.3
M10	ND	–	ND	–	ND	–	ND	–	ND	–	157.9	47.8	–	34.5
M11	276.5	16.9	ND	–	38.6	7.5	149.7	6.0	ND	–	191.0	31.4	125.8	41.8

3-MCPD, 3-monochloropropane-1,2-diol; GE, glycidyl esters; LI, linoleoyl-3MCPD; PA, palmitoyl-3MCPD; OL, oleoyl-3-MCPD; ST, steareoyl-3-MCPD; PAGE, glycidyl palmitate; OLGE, oleoyl palmitate. *Estimated from their esterified forms. ND, not determined.

estimations agree with the 2016 EFSA report [2] and those reported by previous works [21]. Nevertheless, since most of these articles are based on the indirect determination, the levels of 3-MCPD reported include 3-MCPD released from both mono- and diesters, so levels of total 3-MCPD similar and higher than those reported here have been found [6,7]. This could be related to the contribution of 3-MCPD diesters to total 3-MCPD.

Based on the last annual report on food consumption in Spain [40], the per capita domestic consumption of margarine was 0.55 kg per person. Since the composition of this margarine is not specified in this report, the risk of exposure was estimated based on the highest results of the 3-MCPD equivalent obtained in this study. The consumption of this margarine would not imply a concern in intake, which would be well below the established TDI of 2 μg/kg body weight per day.

However, this margarine sample would be within the maximum limits set by the European Regulation Commission [5]. Since margarine is a product used in preparing other foods or culinary activities, it could represent a risk of increased consumption of 3-MCPD and GE. And so, it will be crucial to study the increase in the occurrence of these contaminants in margarines when cooked.

4. Conclusions

A novel method for simultaneously determining 3-MCPD monoesters and glycidyl esters in margarine has been successfully developed and validated. The APCI source allows this analysis to be performed using a much simpler mobile phase without acidification. The miniaturization of the test portion size in the proposed QuEChERS method follows the trends of green analytical chemistry. The use of decreased volumes of extraction solvents and clean-up sorbents compared to other sample preparation methods currently used significantly impacts the profitability of this method. This method showed excellent performance in terms of limits of determination, linearity, accuracy, precision, and suitability for analyzing the 3-MCPDE and GE levels in margarine samples, according to FDA validation guidelines.

Determining food contaminants such as glycidyl esters and 3-MCPD with fatty acids in margarine is crucial in ensuring food quality. Beyond their domestic consumption, margarines are widely used in the food industry as raw material in the production of other products such as cakes and pastries due to their lower price than fats of animal origin (butter) or oils of vegetable origin such as extra virgin olive oil. So, it will be imperative to monitor the potentially increased exposure to these contaminants after cooking. Here, the simultaneous determination of four 3-MCPDE and two GE in margarine is performed in less than 40 min from sampling to data analysis. All samples were positive for OLGE. To the best of our knowledge, this is the first simultaneous study of 3-MCPD monoesters and GE esters in margarine samples.

Funding

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

CRediT authorship contribution statement

Jorge A. Custodio-Mendoza: Investigation, Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Raquel Sendón:** Methodology, Writing – review & editing, Supervision. **Ana Rodríguez-Bernaldo de Quirós:** Methodology, Writing – review & editing, Supervision. **Rosa A. Lorenzo:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Antonia M. Carro:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A. M. Carro reports financial support was provided by Spanish Ministry of Science, Innovation, and Universities.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the Spanish Ministry of Science, Innovation, and Universities (Project RTI2018-096450-B-I00) and FEDER funds.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2022.340712.

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