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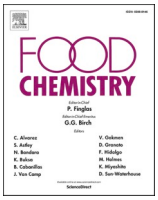

Ultrasound-assisted enzymatic indirect determination of total 3-monochloropropane-1,2-diol esters in canned fish oil fraction

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rac 1–2-Bispalmitoyl-3-chloropropanediol (PubChem CID: 171090)
rac 1-Oleoyl-3-chloropropanediol (PubChem CID: 6441321)
rac 1-Palmitoyl-3-chloropropanediol (PubChem CID: 147357)
rac-1,2-Dilinoleoyl-3-chloropropanediol (PubChem CID: 129850365)
rac-1-Linoleoyl-3-chloropropanediol (PubChem CID: 71749801)
rac-1-Stearoyl-3-chloropropanediol (PubChem CID: 45040418).

ABSTRACT

A novel, fast, and cost-effective indirect enzymatic method was successfully developed to assess the total 3-monochloropropane-1,2-diol (3-MCPD) in canned food's oil fraction by the action of Burkholderia cepacia lipase. The total 3-MCPD were derivatized with *n*-Heptafluorobutyrylimidazole (HFBI) for GC–MS analysis during dispersive liquid–liquid microextraction (DLLME). An asymmetrical $2^2 1^3 // 8$ screening design was used to study the influence of critical factors on the method's effectiveness. The analytical features of the proposed method were assessed following Food and Drug Administration (FDA) guidelines using extra virgin olive oil (EVOO) as a blank sample. Outstanding results were achieved in terms of linearity ($r^2 = 0.9995$), sensitivity, precision (2.1 % to 10.4 % RSD), and accuracy (98.7 % $\leq$ recovery $\leq$ 101.9 %). Method efficacy was tested by comparing the results of 10 edible oils for total 3-MCPD with those reported in previous works. A total of 41 samples were analyzed. The lowest 3-MCPD content was found in samples of albacore canned in EVOO oil, while the highest amounts were found in albacore, mackerel, and Atlantic saury samples, all preserved in refined sunflower oil.

1. Introduction

In recent years, 3-monochloropropanol esters (3-MCDPE) have been reported mainly in edible oils, oil-based foodstuffs, and foods with high lipid content (Custodio-Mendoza et al., 2019; Gao, Li, Huang, & Yu, 2019; MacMahon & Beekman, 2019). 3-MCPDE are fatty acid esters of 3-monochloropropane-1,2-diol (3-MCPD), a food pollutant classified as "possibly carcinogenic to humans" (Category 2B) by the International Agency for Research in Cancer (International Agency for Research on Cancer (IARC), 2014) based on toxicological reports.

The health concerns about 3-MCPDE lie in 3-MCPD released during human digestion, which increase exposure to this contaminant

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(Custodio-Mendoza et al., 2019; Gao et al., 2019; MacMahon and Beekman, 2019). Commission Regulation (EC) No. 2020/1322 sets maximum levels for the sum of 3-MCPD and its esters in vegetable oils and fats (1250 μg kg-1 and 2500 μg kg-1). In 2018 the European Food Safety Authority (EFSA) set a tolerable daily intake (TDI) at 2 μg kg^{-1} of body weight per day for 3-MCPD and its esters (EFSA Panel on Contaminants in the Food Chain (CONTAM), Knutsen, et al., 2018).

Analytical methods to determine 3-MCPDE are based on either LC–MS or GC–MS (Gao et al., 2019; MacMahon and Beekman, 2019). LC-MS approaches (direct methods) identify and quantify 3-MCPDE individually, without any derivatization needed (Custodio-Mendoza et al., 2019; MacMahon, Begley, & Diachenko, 2013a; MacMahon, Mazzola, Begley & Diachenko, 2013). However, direct methods usually have higher determination limits, and the analysis of total (free and bound) 3-MCPD is not possible. Alternatively, indirect methods use hydrolysis to release free 3-MCPD from its esters and a derivatization reaction to make 3-MCPD suitable for GC–MS (Becalski, Zhao, Feng, & Lau, 2015; Jędrkiewicz, Kupska, Głowacz, Gromadzka, & Namieśnik, 2016; Kuhlmann, 2019; Küsters, Bimber, Reeser, Gallitzendörfer, & Gerhartz, 2011; Sadowska-Rociek, 2020; Zheng et al., 2021). The hydrolysis may be carried out in acid or alkaline media but reactions often require strong conditions and long reaction times (Becalski et al., 2015; Jędrkiewicz et al., 2016; Kuhlmann, 2019; Küsters et al., 2011; Sadowska-Rociek, 2020; Zheng et al., 2021) enabling a greener and cheaper process. Lately, enzymatic hydrolysis has been used for 3-MCPDE determination in edible oils, fish oils, and oil-based foodstuffs in milder conditions (Chung & Chan, 2012; Chung, Chan, Chung, Xiao, & Ho, 2013; Koyama et al., 2016; Miyazaki & Koyama, 2016, 2017). Lipases from Candida Rugosa, Penicillium Camemberti, Burkholderia Cepacia, Pseudomonas Fluorescens, and Candida Antarctica have been assessed, achieving various yields of 3-MCPDE transesterification (Chung & Chan, 2012; Chung et al., 2013; Miyazaki & Koyama, 2017). Ultrasound agitation can accelerate this process, but this is limited to a reduced system volume and process time to prevent enzyme denaturation (Islam, Zhang, & Adhikari, 2014; Lerin et al., 2014). Based on American Oil Chemists' Society's official methods (AOCS, 2017a; AOCS, 2017b; AOCS, 2017c), phenylboronic acid (PBA) is used for 3-MCPD derivatization, but other reagents have been used with similar effects, such as *N*-Heptafluorobutyrylimidazole (HFBI); N, O-Bistrifluoroacetamida (BSTFA) or Heptaflurobutyric anhydride (HFBA) (Carro, Gonzalez & Lorenzo, 2013; Nemati et al., 2021; Xu, Jin, Yang, Rao, & Chen, 2020). Derivatization reactions also require high temperatures (above 90 °C) for extended periods (0.5–1 h) and typically involve a solvent exchange to a more suitable organic one before GC–MS.

Microextraction techniques, such as solid-phase microextraction (SPME) or dispersive liquid–liquid microextraction (DLLME), stand as a greener, lower-cost, and more effective alternative to conventional approaches (Carro et al., 2013; Nemati et al., 2021; Xu et al., 2020). Moreover, these techniques quickly and simultaneously perform solvent exchange and derivatization (Carro et al., 2013; Xu et al., 2020).

Canned foods stand as a reliable option to quickly access different food products with an extended shelf life (Aubourg, 2001; Fukuda, 2015; Vergara-Balderas, 2016). According to the Spanish Ministry of Agriculture, Fisheries and Food (MAPA, 2021 canned fish purchases grew 10 % in 2020, with a total per capita consumption of 4.85 Kg. Food products are submitted to heat during canning to sanitize their contents (Aubourg, 2001; Vergara-Balderas, 2016). However, heat-induced pollutants, such as 3-MCPDE, are generated at those conditions (Custodio-Mendoza et al., 2019; MacMahon and Beekman, 2019). The assessment of 3-MCPDE in canned foods is limited and usually focuses on the meat fraction (Crews et al., 2002; Ostermeyer, Merkle, Karl, & Fritsche, 2021).

This study presents an ultrasound-accelerated enzymatic hydrolysis approach for the GC–MS determination of total 3-MCPD in the oil fraction of canned fish. This method releases the 3-MCPD from its esters and, using HFBI, derivatizes total 3-MCPD during DLLME. To the best of our knowledge, this is the very first use of ultrasound to enhance enzymatic transesterification in the indirect analysis of 3-MCPDE, and the first occurrence study of total 3-MCPD in the oil fraction of canned fish.

2. Materials and methods

2.1. Chemicals and materials

Acetonitrile (ACN), chloroform, citric acid monohydrate, ethyl acetate (EtOAc), isooctane (IOA), methanol (MeOH), *n*-Heptafluorobutyrylimidazole (≥98.5 % for GC-derivatization), *n*-hexane, sodium chloride (≥99.5 %), sodium phosphate dibasic (anhydrous, ≥99.99 %) were analytical grade and purchased from Merck (Darmstadt, Germany). Amano lipase G from *Penicillium camemberti* (PCL, ≥50,000 U/g), Amano lipase PS from *Burkholderia cepacia* (BCL, ≥30,000 U/g), esterase Pseudomonas fluorescens recombinant from *Escherichia coli* (ECE, 35 U/mg), lipase from *Candida rugosa* (CRL, 700 U/mg) were supplied by Merck. Ultrapure water type I was obtained from Wasserlab, water purification system (Barbatáin, Spain). McIlvaine buffer was prepared by mixing the precise amounts of Na_2HPO_4 0.2 M and citric acid 0.1 M solutions to obtain the desired pH. An AS 82/220. R2 Analytical Balance from RADWAG (Radom, Poland), a Centromix II-BL Centrifuge from J. P. Selecta (Barcelona, Spain), a micro-syringe from Hamilton Company (100 μL, Nevada, USA), a Crison pH meter (Crison Instruments, Barcelona, Spain, model Basic 20), a 2510EMTH ultrasonic bath from Branson Ultrasonics (Danbury, USA), and a Reax top vortex mixer from Instruments GmbH & Co (Schwabach, Germany) were used in this work.

2.2. Standard solution and QC samples

Individual stock solution of *rac*-1-Linoleoyl-3-chloropropanediol (LI, CAS No. 74875–98-2), *rac*-1,2-Dilinoleoyl-3-chloropropanediol (LILI, CAS No. 7487–96-0), rac 1-Oleoyl-3-chloropropanediol (OL, CAS No. 10311–82-7), rac 1-Oleoyl-3-chloropropanediol-d5 (OL-d5, CAS No. 1246834–03-6), rac 1-Oleoyl-2linoleoyl-3-chloropropanediol (OLLI, CAS No. 1336935–03-5), rac 1,2-Dioleoyl-3-chloropropanediol (OLOL, CAS No. 69161.73–5), rac 1,2-Dioleoyl-3-chloropropanediol-d5 (OLOL-d5, CAS No. 1246833–00-0), rac 1-Oleoyl-2-stearoyl-3-chloropropanediol (OLST, CAS No. 1336935–05-7), rac 1-Oleoyl-2-stearoyl-3-chloropropanediol-d5 (OLST-d5, CAS No. 1336935–05-7), rac 1-Palmitoyl-3-chloropropanediol (PA, CAS. No. 30557–04-1), rac 1-Palmitoyl-3-chloropropanediol-d5 (PA-d5, CAS. No. 1346599–60-7), rac 1-Palmitoyl-2-linoleoyl-3-chloropropanediol (PALI, CAS No. 1246833–87-3), rac 1-Palmitoyl-2-oleoyl-3-chloropropanediol (PAOL, CAS No. 1363153–60-9), rac 1–2-Bispalmitoyl-3-chloropropanediol (PAPA, CAS No. 51930–97-3), rac 1–2-Bispalmitoyl-3-chloropropanediol-d5 (PAPA-d5, CAS No. 1185057–55-9), *rac*-1-Stearoyl-3-chloropropanediol (ST, CAS No. 22094–20-8), *rac*-1-Stearoyl-3-chloropropanediol-d5 (ST-d5, CAS No. 1795785–84-0) were supplied by Toronto Research Chemical (Toronto, Ontario, Canada) and prepared either in MeOH or EtOAc in accordance to manufacturer's recommendations. 3-chloro-1,2-propanediol (3-MCPD, CAS No. 96–24-2) and 3-chloro-1,2-propane-1,1,2,3,3-d5-diol (3-MCPD-d5, CAS No. 342611–01-2) were purchased from Merck (Darmstadt, Germany).

The relative distribution of fatty acids predetermines the presence of 3-MCPDE. Virgin olive oil, refined olive oil, and sunflower oil are the most common edible oils used in canned fish. Since these oils are rich in linoleic, oleic, stearic, and palmitic acids, 3-MCPD monoesters LI, OL, ST, PA, and diesters LILI, OLLI, OLOL, OLST, PALI, PAOL, and PAPA were selected as precursors of released 3-MCPD to provide accurate analytical scope for assessment of this contaminant in the oil fraction canned fish. The development of this method includes all four monoesters and seven diesters and the use of isotopically labeled compounds

as internal standards (IS). The ideal situation would use the deuterated species of each analyte, but only those that were available with labeled 3-MCPD-d5 were added. These are OL-d5, PA-d5, ST-d5 monoesters, and OLOL-d5, OLST-d5, PAPA-d5. No problems were observed for any of these analytes in terms of linearity and analytical suitability.

Starting from each individual stock solution, an analyte solution containing 40 μg g^{-1} of each mono and diester was prepared in MeOH, as well as an IS solution containing deuterated species at 10 μg g^{-1} each. Quality control (QC) samples spiked with a mixture of standards at 20 ng g^{-1} (L-QC), 125 ng g^{-1} (M−QC) and 400 ng g^{-1} (H-QC) were used to assess analytical performance and precision. QC were freshly prepared in EVOO and stored at 4 °C for not longer than a week.

2.3. Samples

A total of 10 samples, including EVOO, virgin linen oil (VLO), refined olive oil (ROO), refined sunflower oil (RSO), refined palm oil (RPO), and pomace olive oil (POO), were used to test the developed method. Then, total 3-MCPD was assessed in 41 samples of canned fish' oil fraction including albacore (n = 15), Atlantic saury (n = 2), mackerel (n = 5), melva (n = 1), sardine (n = 14), and tuna (n = 4). All samples were purchased from local supermarkets in Santiago de Compostela, Spain, and stored in their original containers at room temperature until analysis.

2.4. Sample preparation

The oil fraction of the canned fish samples was separated by gravity filtration. A 0.1 g portion of the oil was accurately weighed in a 15 mL conical-bottom tube, adding the corresponding IS solution at 120 ng g^{-1} and 0.5 mL of isooctane. After vortexing for 1 min, a 3 mL aliquot of Burkholderia cepacia lipase solution (BCL 250 U mL^{-1} in McIlvaine buffer pH 7.0) was added, and the entire solution was incubated at 50 °C for 5 min in an ultrasound bath. To avoid the conversion of glycidol or other matrix derivatives present in the system to 3-MCPD, as discussed by Cheng, Liu, Wang, and Liu (2017), 1 mL of 70 % w v^{-1} NaBr solution was added and vortexed for 1 min. The enzyme's activity was stopped by putting the tube in a water bath at 80 °C for 10 min, followed by cooling the sample in an ice bath for 5 min until room temperature. Once the total 3-MCPD was released, the aqueous extract was washed by a double LLE with 3 mL hexane each, discarding the organic phase each time.

Simultaneous DLLME and HFBI derivatization of 3-MCPD was then performed according to the procedure reported by Carro, González, and Lorenzo (2013) with slight modifications. Briefly, 2 mL of the previously-cleaned aqueous phase was then transferred into another 15 mL conical-bottom tube, then was diluted with ultra-pure water to 10 mL. 1.8 g of NaCl were added, and the solution was vortexed for 1 min. In a chromatography vial, 0.9 mL of ACN, 60 μL of chloroform, and 50 μL of HFBI were mixed and rapidly injected into the aqueous phase to enhance the formation of the dispersion phase using a micropipette. The mixture was sonicated at 40 °C for 5 min and centrifuged at 4000 rpm for another 5 min. Finally, the extract drop (60 μL) containing the 3-

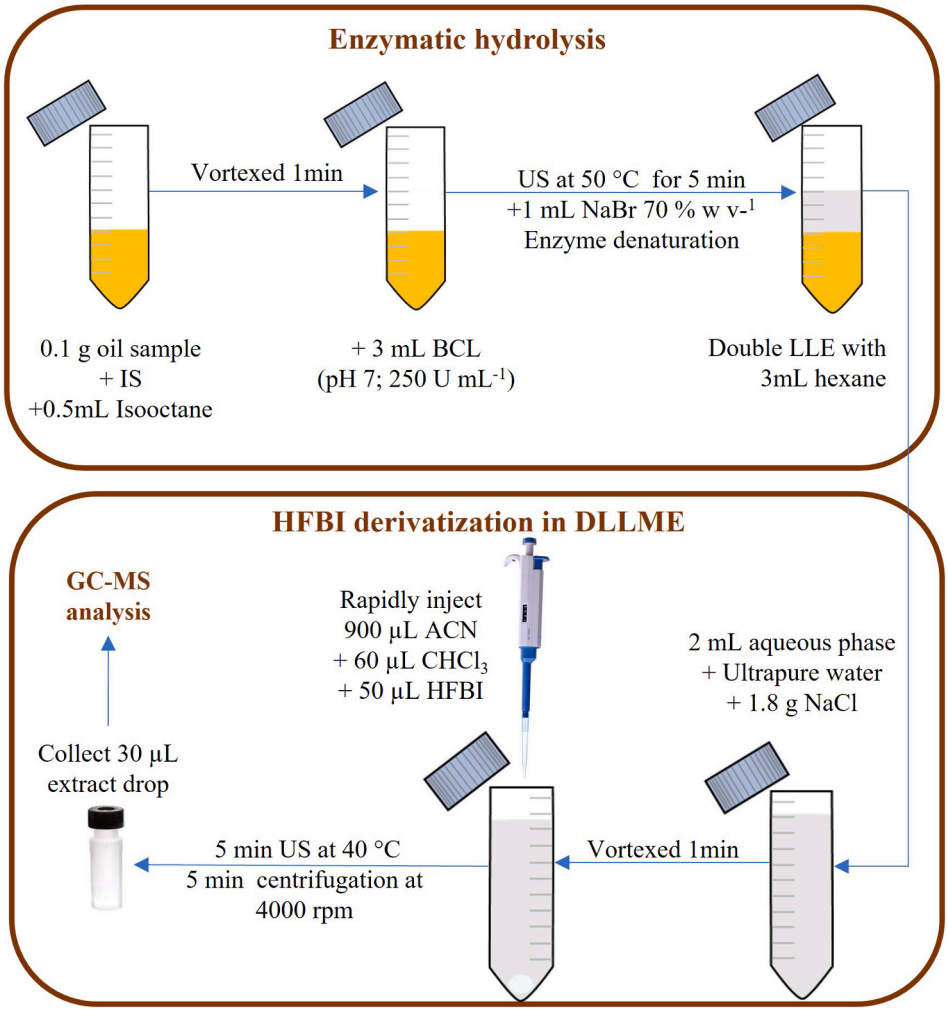

Fig. 1. Scheme of the ultrasound accelerated enzymatic transesterification follow by HFBI derivatization in DLLME of total 3-MCPD from edible oil samples.

MCPD-HFBI and 3-MCPD-d5-HFBI adducts was collected using a 100 μL microsyringe and put into a chromatography vial with a 0.2 mL inert glass insert for GC–MS analysis. A scheme of the method is in Fig. 1.

2.5. Instrumental conditions

The analysis was performed by a GC–MS system (7890B-5977B-MSD, Agilent Technologies, CA, USA) equipped with an automatic liquid sampler (model 7650A, Agilent Technologies, CA, USA). A 1 μL of extract was injected in the splitless mode in an ultra-inert double taper liner (model 5190–3983, Agilent Technologies, CA, USA) at an inlet temperature of 280 °C with a flow of 24.2 mL min^{-1}. Separation was carried out by a J&W HP-5MS column (30 m × 0.25 mm iD × 0.25 μm; Agilent Technologies, CA, USA). The oven temperature was initially set at 50 °C, held for 2 min, then increased to 100 °C at a rate of 3 °C min^{-1}, and finally increased to 280 °C at a rate of 40 °C min^{-1} and held for 10 min. The analysis of 3-MCPD and its labeled analog was obtained around 15 min. The GC system was coupled to a single quadrupole MS analyzer with an electron impact (EI) ionization source at 230 °C. The transfer line temperature was set at 280 °C and the quadrupole temperature at 150 °C. Ions 253 *m/z* (quantifier), 289, and 453 *m/z* (qualifiers) were chosen in selected ion monitoring (SIM) for the 3-MCPD-HFBI adduct. Similarly, 257 *m/z* (quantifier), 294, and 456 *m/z* (qualifiers) were selected for the 3-MCPD-d5-HFBI adduct. System was operated by Agilent Mass Hunter Workstation Software (version B.07.00).

2.6. Method validation

The analytical features of this method were assessed in terms of selectivity, linearity, detection limits, precision, and accuracy based on The Food and Drug Administration (FDA) guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products (Food and Drug Administration, U. S., 2019) and Bioanalytical Method Validation Guidance for Industry (Food and Drug Administration, U. S., 2018). A Matrix extension study was performed by selecting EVOO, ROO, and SO as the blank samples.

No matrix interference was observed in the retention time of the analyte nor the IS in the blank sample. The selection of a quantifier ion and two qualifier ions for both analyte and IS ensures the method's selectivity. Standard addition calibration curves were used to avoid the matrix effect due to the complexity of samples, and to assess the limit of detection (LOD), the lower limit of quantification (LLOQ), the linearity from LLOQ to the upper limit of quantification (ULOQ) at 600 ng g^{-1}, and the matrix effect. LLOQ and ULOQ are defined as the lower and the highest amounts of an analyte that can be quantitatively determined with acceptable precision and accuracy, respectively. LOD and LLOQ were calculated as:

$$y_{LOD/LLOD} = yblack + 3.3/10{*}SD \quad (1)$$

The method's sensitivity at LLOQ was evaluated in triplicate in terms of relative standard deviation (%RSD), which must be below 20 %. The accuracy of the method is assessed in terms of recovery using QCs at three levels of concentration and must be in the range of 80–120 %. The precision of the method is evaluated in terms of %RSD using QCs at three levels of concentration in intraday (n = 5) and interday assays (n = 5) and must be below 15 %.

2.7. Statistics

Simultaneous assessment of several factors at two or more levels in a reduced number of experiments is possible by experimental design. In this work, NemrodW ® statistical software (LPRAI, Marseille, France) was used for experimental design generation and evaluation and plotting the results (Mathieu, Nony, & Phan-Tan-Luu, 2000). Some experimental conditions of enzymatic hydrolysis were achieved using an asymmetrical screening design ($2^2 1^3 // 8$) to evaluate the statistical effect of three variables at different levels in 8 experiments.

The statistical study of the analyzed sample was performed by XLSTAT ® software. Box and whisker plots were used to report the content of the total 3-MCPD of the canned fish oil fraction. The middle line of the box represents the median, and the x in the box represents the mean. The median divides the data set into a bottom and a top half. The bottom and top lines of the box represent the first and the third quartiles, respectively. The vertical lines (whiskers) extend from the ends of the box to the minimum and maximum values. A result is considered an outlier if it exceeds 1.5 times the interquartile range.

3. Results and discussion

3.1. Development of ultrasound-assisted enzymatic hydrolysis

PCL, BCL, ECE, and CRL were tested for 3-MCPDE transesterification efficacy. All lipases were prepared at 250U mL^{-1} in McIlvaine buffer, and a 1 h lipolysis was performed at the optimal pH, temperature and concentration proposed by the manufacturer in triplicate. All four lipases were capable of 3-MCPD release from 3-MCPDE, but the best chromatographic results for 3-MCPD were achieved using BCL (Fig. 2.a). Parameters such as lipolysis time, temperature, pH, and agitation mode were studied individually for BCL. Lipolysis time was assessed at 5, 10, and 15 min in triplicate and by means of recovery, achieving acceptable results at all three levels. 5 min was adopted for practical purposes (Fig. 2.b).

Temperature and pH were studied in triplicate spanning the optimal range proposed by the manufacturer ($5 \leq pH \leq 7$). Significant differences were found between pH 5 and pH 7. Both solutions containing BCL were prepared in McIlvaine buffer solution at the exact pH studied. Highest 3-MCPD signals were achieved using pH 7 at 50 °C (Fig. 2. c). The effect of ultrasound agitation was tested over mechanical agitation by vortex (Fig. 2. d). Lipolytic activity is improved with ultrasound treatment. Other authors have reported positive effects of ultrasound on lipase activity due to cavitation, but this is limited to small volumes and reduced reaction times to prevent enzyme denaturation by heat (Islam et al., 2014; Lerin et al., 2014). For this reason, ultrasound agitation was selected for further experiments.

An asymmetrical screening design $2^2 3^1 // 8$ was used to study-two factors at two levels (b_1: oil dilution solvent and b_2: enzyme denaturation approach) and one factor at three levels (B_3: enzyme concentration) in 8 experiments, performed in random order to avoid systematic errors.

Fig. 3 shows the total effects plot for 3-MCPD released from 3-MCPDE. The length of each bar is proportional to the influence of each factor on the effectiveness of hydrolysis. Notably, the factor with the greatest influence is denaturation, while a significant difference is not observable for the type of solvent and the amount of enzyme. Thus, the following parameters were selected for this process: isooctane to dissolve the oil samples due to its lower toxicity, a rapid temperature change for enzyme denaturation and an intermediate concentration of enzyme at 250 U mL^{-1} to favor the cost-effectiveness of the process.

Enzyme catalytic activity is tightly related to temperature and pH. When one of these factors is out of the optimal range, the enzyme may suffer a denaturation: A loss of the proteinic structure of the enzyme and hence a loss of its activity (Sharma, Gupta, Ahmad, Mansoor, & Kaur, 2021). An increase of temperature was used to stop the reaction in this work. The system was placed in a water bath at 80 °C for 10 min to rapidly be immersed in an ice bath to reach room temperature.

The released 3-MCPD must be derivatized to make it suitable for GC–MS determination. Published enzymatic methods use PBA as a derivatization reagent based on the AOCS Cd 29a official method (AOCS, 2017a). But these protocols require reaction times from 5 min up to 1 h at temperatures ranging from room temperature to 90 °C. In this work HFBI derivatization follows a DLLME procedure proposed by Carro

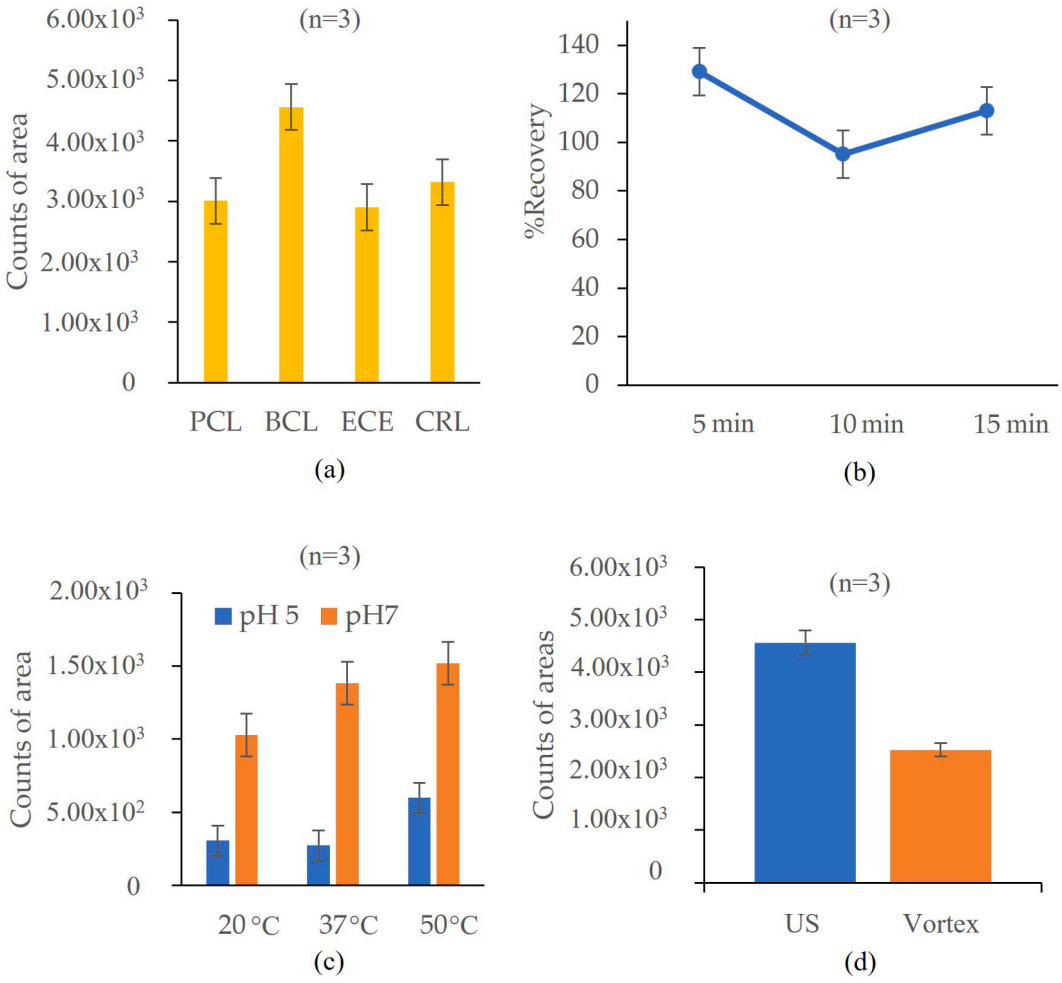

Fig. 2. Optimization of sample preparation protocol: (a): Effectiveness of the lipase used for 3-MCPDE esterification results in terms of 3-MCPD chromatographic signal. PCL, *Penicillium camemberti*; BCL, *Burkholderia cepacia*; ECE, *Escherichia coli*; CRL, *Candida rugosa*; (b) Effect of lipolysis time expressed in terms of recovery; (c) Effect of pH and Temperature in lipolysis; (d) Ultrasound effect in lipolysis (10 min stirring at room T).

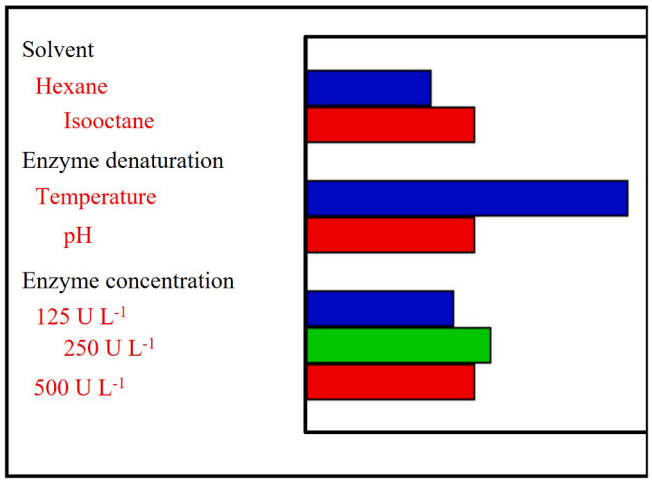

Fig. 3. Total effect chart for asymmetrical $2^2 1^3 // 8$ screening design.

et al. (2013) with some modifications. The use of DLLME allows simultaneous derivatization of the released 3-MCPD in the cleaned aqueous phase and extract the 3-MCPD-HFBI adduct into a suitable solvent for GC–MS. Importantly, adding salt in DLLME is critical since it increases ionic strength, enhancing phase separation and analyte extraction from the sample into extractive solvent (Fu, Li, Sun, & Xie, 2021). It was experimentally confirmed that the chromatographic signal is improved when 18 %w v^{-1} of NaCl is added to the aqueous solution. Although NaBr was earlier added, subsequent 1:5 dilution results in a lack of ionic strength exerted by the NaBr. Hence, the addition of a complementary salt solves this. The protocol followed is comprehensive in section 2.4 of this work.

3.2. Analytical method validation

The proposed method was validated following FDA guidelines for analytical method validation (Food and Drug Administration, U. S. 2018; Food and Drug Administration, U. S. 2019) and are summarized in Table 1. The selectivity and specificity of the method were evaluated by replicating the analyses using EVOO as a blank and spiking EVOO at various concentration levels. These samples were submitted to the entire analytical method. The total 3-MCPD concentration released from the 3-MCPDE standards was calculated using eq. (2).

$$\sum_{i=1}^{n} \frac{3MCPDE_i \text{ concentration} \times 3MCPD \text{ molecular weight}}{3\ MCPDE_i \text{ molecular weight}} \quad (2)$$

The analogous equation was used for 3-MCPDd5 concentration released from 3-MCPDEd5. At instrumental conditions described before, 3-MCPD and 3-MCPDd5 eluted at 15.10 and 15.88 min, respectively, one quantification and two qualification ions were selected (Table 1),

Table 1
Analytical features of Ultrasound-assisted enzymatic indirect method for the analysis of total 3-MCPD in canned food's oil fraction.

Analyte	RT	Quantification ion	Qualification ions		LOD	LLOQ	r^2	Sensitivity % RSD
	min	m z^{-1}	m z^{-1}	m z^{-1}	ng g^{-1}	ng g^{-1}		LLOQ
3-MCPD	15.10	253	289	453	1.8	6.1	0.9995	2.8
3-MCPDd5	15.88	257	294	456	–	–		–

Precision %RSD						**Accuracy % Recovery**		
Intraday (n = 5)			**Interday (n = 5)**			**(n = 3)**		
L-QC	M−QC	H-Qc	L-QC	M−QC	H-Qc	L-QC	M−QC	H-Qc
2.1	1.5	2.2	10.4	8.0	9.2	98.7	100.0	101.9

Linearity was assessed from LLOQ-ULOQ (600 ng g^{-1}); L-QC, quality control substance in blank sample at 20 ng g^{-1}; M−QC, quality control substance in blank sample at 125 ng g^{-1}; H-QC, quality control substance in blank sample at 400 ng g^{-1}.

leading to a specific ion identification of both analytes. The limit of detection (LOD) and lower limit of quantification (LLOQ) were calculated using a standard addition calibration for the EVOO blank sample and set at 1.8 ng g^{-1} and 6.1 ng g^{-1}, respectively. The ULOQ was set at 600 ng g^{-1}. Furthermore, a standard addition calibration line was built from LLOQ to ULOQ within six levels and three replicates per level. Regression analysis showed an excellent determination coefficient (r^2 = 0.9995). Sensitivity was studied in triplicate at LLOQ and expressed as % RSD was 2.8 %. The precision of the method, assessed in the intraday and interday assays, was verified both in quintuplicate by using QCs which were prepared at three levels of concentration: Low (L-QC) at 20 ng g^{-1}, medium (M−QC) at 125 ng g^{-1} and high (H-QC) at 400 ng g^{-1} in EVOO. The intraday precision ranged from 1.5 % to 2.2 %, while the interday precision ranged from 8.0 % to 10.4 %. The method's accuracy was evaluated using QCs, comparing the STD/IS ratio to that obtained with 3-MCPD and 3-MCPDd5 standards in triplicate, resulting in recoveries between 98.7 % and 101.9 % for the three levels.

QCs at the same three concentrations in ROO and SO were used for a matrix extension study: a validation of the method's performance ensuring that this will continue to produce accurate and reliable results with a new matrix. It is assessed in terms of accuracy and intraday and interday precisions (Table 1S). The precision ranged from 4.0 to 11.0 % and 3.3 to12.8 % for ROO and SO, respectively. The accuracy ranged from 93.5 to 116.1 % and from 82.0 to 114.9 % for ROO and SO, respectively. The matrix effect was evaluated by comparing the slope of the addition calibration curves in ROO and SO, resulting in no matrix effect for 3-MCPD. Nevertheless, addition calibration curves were used in the analysis, due to the highly complex nature of canned fish.

The limits of determination reported in this work are similar to or lower than those reported by Chung and Chan (2012), Chung et al. (2013), Koyama et al. (2016), Miyazaki and Koyama (2016), and Miyazaki and Koyama (2017) for the indirect determination of 3-MCPD by enzymatic hydrolysis in edible oils, fish oils, or/and various oil-based foodstuffs. The accuracy of the method reported, expressed in terms of recovery, is closer to 100 % than those reported before (Chung and Chan (2012), Chung et al. (2013), Koyama et al. (2016), Miyazaki and Koyama (2016) and Miyazaki and Koyama (2017)). The interday precision is similar to the results reported by Chung et al. (2013) for the analysis of various oil-based foodstuffs; while the intraday precision is lower than those reported by Chung et al. (2013) and similar to those reported by Koyama et al. (2016) and by Miyazaki and Koyama (2016) in the analysis of edible oils.

Compared with other non-enzymatic indirect methods, detection limits reported here mostly remain similar to (Sadowska-Rociek, 2020; Zheng et al., 2021) or lower than previously published articles (Küsters et al., 2011, Ostermeyer, Merkle, Karl, & Fritsche, 2021, Xu et al. 2020). Nemati et al. (2021) achieved a lower limit of detection and quantification than those reported here by using an Air-assisted liquid–liquid microextraction to analyze total 3-MPCD in edible oils. However, this could be since they used a sample size 20x larger.

Recoveries reported here remain closer to 100 % than those reported by Küsters et al. (2011), Nemati et al. (2021), Ostermeyer et al. (2021), Sadowska-Rociek (2020), Xu et al., (2020), and Zheng et al. (2021). On the other hand, this method's precision is similar to those reported in such indirect methods. Xu et al. (2020) reported lower precision when using SPME in the analysis of 3-MCPD esters through released 3-MCPD in edible oils.

3.3. Total 3-MCPD occurrence in canned fish lipid fraction

This new ultrasound-assisted enzymatic indirect protocol was used to assess the presence of free and bound 3-MCPD in the oil fraction of 41 canned fish. All samples were analyzed in triplicate using QCs as acceptance criteria for the daily sequences. All samples were positive for total 3-MCPD, as seen in the chromatogram (Fig. 4). The results are plotted in Fig. 5 and summarized in Table 2S.

Concerning the type of oil used (Fig. 5.a), the lowest levels of 3-MCPD were found in two samples canned in EVOO, albacore, and sardine samples contained 0.2 and 0.2 μg g^{-1}, respectively. The foodstuffs canned in ROO presented levels of 3-MCPD ranging from 0.5 to 2.9 μg g^{-1}. These values were found in two albacore samples with different oil and salt content, so 3-MCPD could vary according to the amount of salt and oil among similar fishes. The highest amounts of free and bound 3-MCPD were found in fish canned in sunflower oil in values from 3.1 to 22.2 μg g^{-1}. Vegetable oils used in canning may contain 3-MCPDE derived from its processing, as reported before (Custodio-Mendoza et al. (2019), Gao et al. (2019), MacMahon et al. (2013a), MacMahon, Begley, & Diachenko (2013b) and MacMahon, Mazzola, Begley, and Diachenko (2013), Nemati et al., 2021). On the other hand, the formation of 3-MCPDE is related to the presence of chloride ions and fatty acids (Crews et al., 2002, EFSA et al., 2018; Gao et al., 2019), so, as these results suggest, the degree of formation of 3-MCPDE in canned fish is correlated to the degree of processing of the edible oil and the amount of salt used in its manufacture.

Among samples canned in refined olive oil (Fig. 5.b), the amount of 3-MCPD found in albacore varies in the range of 0.5 to 2.9 μg g^{-1}, followed by sardine with values from 1.1 to 2.3 μg g^{-1}. Mackerel present values in the range of 0.5–1.8 μg g^{-1} while the Atlantic saury canned in ROO present an average amount of total 3-MCPD of 2.3 μg g^{-1}.

Regarding fish canned in SO (Fig. 5.c), albacore shows 3-MCPD contents from 5.0 to 20.5 μg g^{-1}. The highest amount found in this group corresponds to a Mackerel sample (22.2 μg g^{-1}), and the lowest to a Melva sample (3.1 μg g^{-1}). Tuna presented values of total 3-MCPD from 0.5 to 2.9 μg g^{-1}.

Both free and bound 3-MCPD in canned foods have only been reported by Ostermeyer et al. (2021) for canned fish in olive oil and sunflower oil at 0.065 and 0.109 μg g^{-1}. Free 3-MCPD was also determined in anchovy filets preserved in olive oil by Crews et al. (2002) at 0.081 μg g^{-1}. Karl, Merkle, Kuhlmann, and Fritsche (2016) reported that no 3-MCPD nor 3-MCPDE were detected in untreated raw fish (14

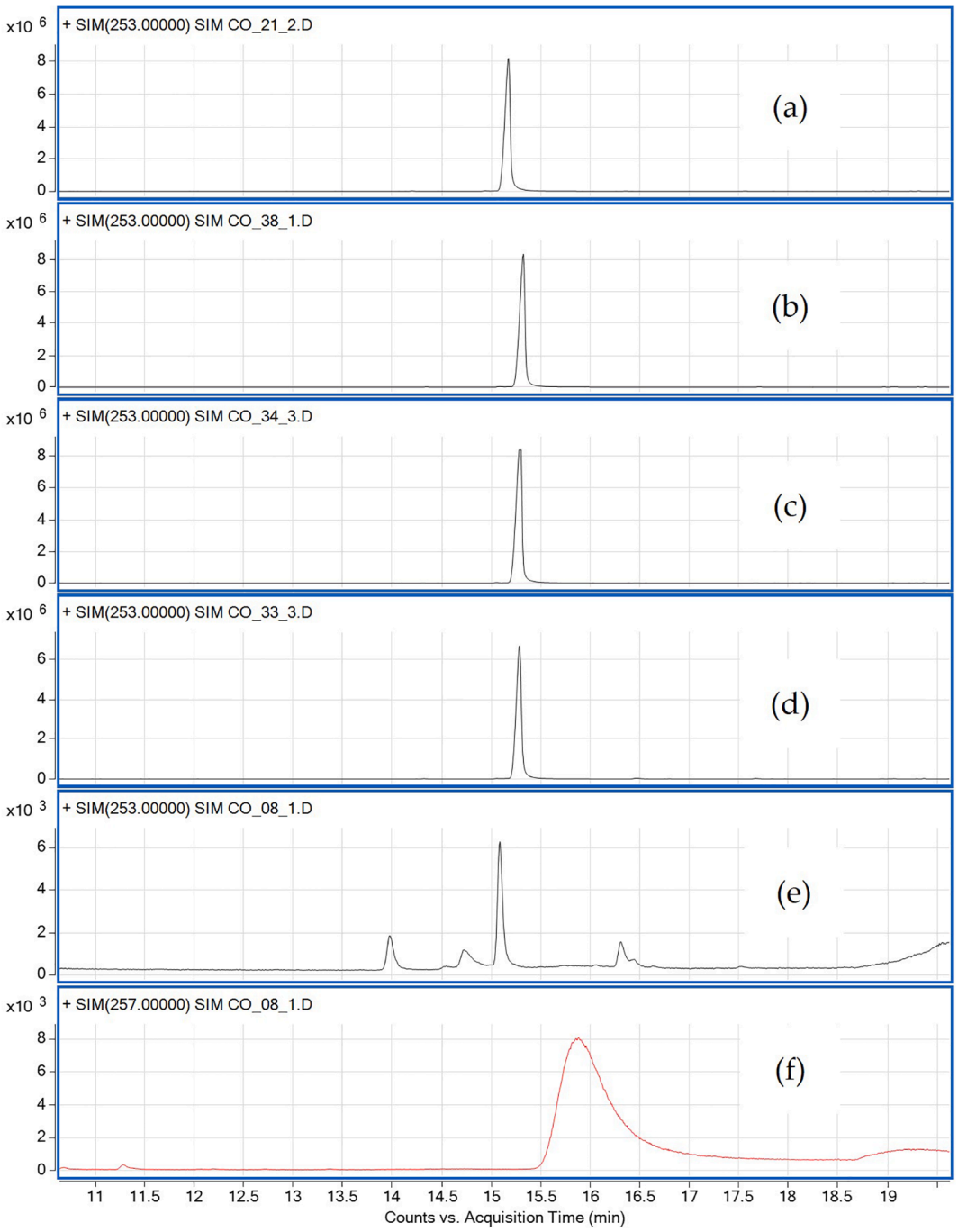

Fig. 4. Chromatogram of total 3-MCPD at 15.17 min founded in (a) canned oil samples of Albacore in RSO; (b) Atlantic saury in RSO; (c) Mackerel in RSO; (d) Sardine in RSO; (e) and Albacore in EVOO; (f) chromatogram of 3-MCPD-d5 at 15.88 min used as ISTD in the albacore in EVOO sample.

samples of the edible part without skin), however these contaminants were quantified in different fishery products, which suggests that their formation took place during processing or cooking. Nevertheless, this data is not comparable to those reported here since they studied only the meat portion of the canned food samples. However, there is a higher amount of 3-MCPD in those samples preserved in highly refined and processed oils.

The levels of total 3-MCPD found in these samples were higher than those previously reported by Custodio-Mendoza et al. (2019), Gao et al. (2019), MacMahon et al. (2013a), MacMahon et al. (2013b). and MacMahon, Mazzola et al. (2013). for the corresponding vegetable oil types. This excess of 3-MCPD could be related to the differences in high-temperature processing, to which fish and oil are both submitted during the canning process and enhanced by the chloride ions present in fish tissue (Aubourg, 2001).

A study of 10 edible oil samples was carried out and compared to those previously reported by other authors to support the reliability of the developed method. Results are summarized in Table 3S in the supplementary material section. As expected, and reported before no 3-MCPD was found in EVOO, nor in virgin olive oil (Custodio-Mendoza et al., 2019; Koyama et al., 2016; MacMahon et al., 2013a; MacMahon et al., 2013b; Xu et al., 2020). Refined olive oil presented an amount of total 3-MCPD in the range of 0.8–1.0 μg g^{-1}, while pomace olive oil presented the highest amount of total 3-MCPD ranging from 3.6 to 4.2 μg g^{-1}. Refined sunflower oil has a level of 3-MCPD similar to those reported by MacMahon et al. (2013b). Palm oil also showed higher

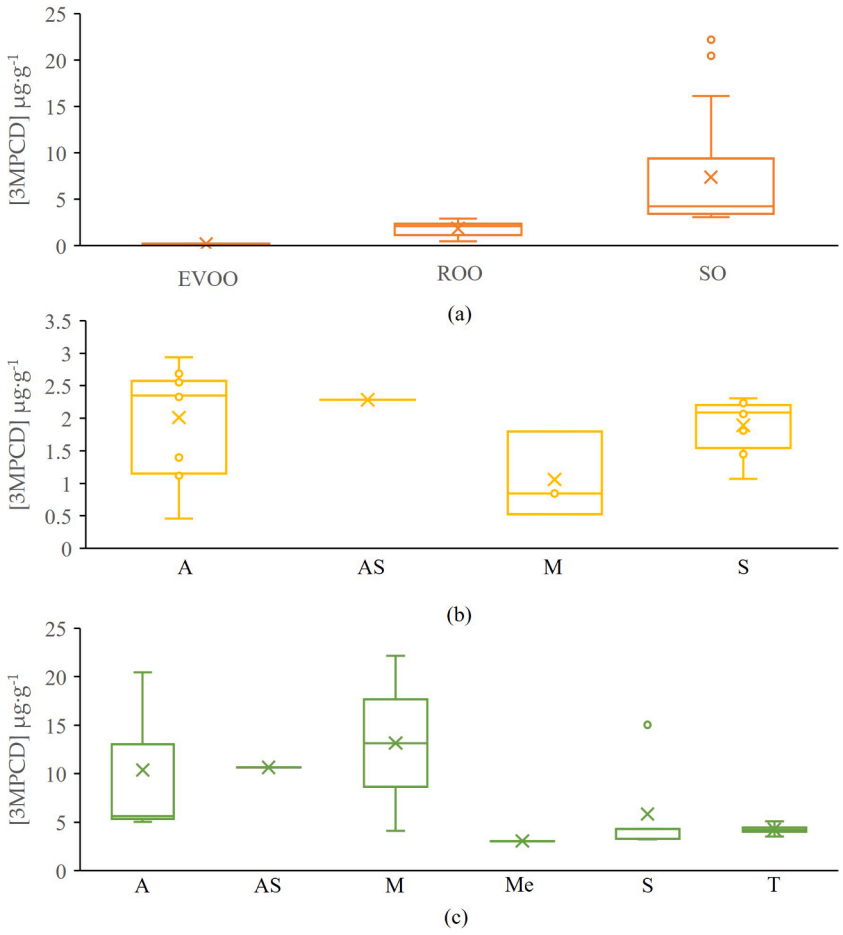

Fig. 5. Box and whisker chart of the analyze samples: a) Occurrence by typo of oil; b) occurrence in olive oil categorized by canned food; c) occurrence in sunflower oil categorized by canned food; EVOO, extra virgin olive oil; ROO, refined olive oil; SO, sunflower oil; A, Albacore; AS, Atlantic saury; M, Mackerel; Me, Melva; S, Sardine; T, tuna.

amounts of 3-MCPD (0.8–2.0 µg g^{-1}), as reported by Koyama et al. (2016).

4. Conclusions

A new, fast, effective, and economical protocol for the analysis of total 3-MCPD in the oil fraction of canned foods has been successfully developed and validated. This method is based on the ultrasound-accelerated enzymatic hydrolysis of 3-MCPDE using *Burkholderia cepacia* lipase and HFBI derivatization for the indirect determination of free and bound 3-MCPD, achieving excellent results in terms of limits of determination, linearity, accuracy, precision, and suitability for the analysis of the oil fraction of food canned in EVOO, ROO and SO. In this process, 3-MCPD release from 3-MCPDE is performed in an ultrasound bath at 50 °C in only 5 min. HFBI derivatization is carried out simultaneously during extraction by DLLME in only 5 min at 40 °C, which significantly reduces the total analysis time, from sample collecting to data analysis, to under 60 min. A total of 41 canned fish samples were analyzed, all positive for total 3-MCPD. This is the first analysis of the lipid fraction of canned fish for total 3-MCPD. Canned food is a growing industry in which northern Spain is a leading producer of canned fish. Analytical information concerning the content of contaminants derived from their processing, such as 3-MCPD esters, is essential in ensuring the food safety of these products. Results suggest a correlation between total 3-MCPD content and three variables: The type of oil used in canning, the degree of processing of the vegetable oil, and the amount of salt used in canning. More research is needed to investigate the influence of these parameters on resulting 3-MCPD content.

CRediT authorship contribution statement

Jorge Antonio Custodio-Mendoza: Investigation, Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Andrea Ramos:** Investigation, Validation. **Rosa Antonia 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 that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134423.

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[AOCS] American Oil Chemists' Society. (2017) b. AOCS official method Cd 29b-13 2- and 3- MCPD fatty acid esters and glycidol fatty acid esters in edible oils and fats by alkaline transesterification and GC/MS. Official methods and rec-ommended practices of the AOCS. Available online: https://www.aocs.org/attain-lab-services/methods/methods/search-results?method=118273&SSO=True (Accessed May 19th 2021st).

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