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QUANTIFICATION OF 3-MONOCHLOROPROPANE-DIOL FATTY ACID ESTERS BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY METHOD WITH THEIR SUBSEQUENT REDUCTION IN PROCESSED FOODS

Naveed Ahmad[1], Muhammad Asim Shabbir[1,*], Masood Sadiq Butt[1], Muhammad Shahid[2] and Thomas H. Robert[3]

[1]National Institute of Food Science and Technology, Faculty of Food, Nutrition and Home Sciences, University of Agriculture, Faisalabad, Pakistan; [2]Department of Biochemistry, Faculty of Sciences, University of Agriculture, Faisalabad, Pakistan; [3]Plant Breeding Institute, Sydney Institute of Agriculture, University of Sydney, Sydney, NSW 2006, Australia

[*]Corresponding author's e-mail: asim-shabbir@live.com

Chloroesters of 3-monochloropropane-1,2-diol (3-MCPD) esters are potentially harmful heat-induced contaminants produced during processing. It was aimed to quantify 3-MCPD esters in processed foods and to test different approaches to reduce their abundance. Lipids from bread, UHT milk and chicken nuggets, each of three brands, were extracted and analyzed for their ester-linked 3-MCPD levels using liquid chromatography-mass spectrometry (LC-MS/MS), a direct method. Predominant fatty acids in vegetable oils/ fats were targeted for quantification. Samples were purified using a two-step solid-phase extraction (SPE) via silica and C_{18} cartridges. LC-MS/MS analysis revealed a quantifiable concentration of targeted esters in all nine samples with ≥83% recovery from the SPE. The R^2 value for solvent-constructed linear calibration curves for all selected 3-MCPD esters was ≥0.9984. Our method revealed a quantifiable level of all the targeted esters in all samples. Regarding the mitigation of these esters, enzyme-catalyzed inter-esterification reduced 3-MCPD esters to an undetectable level (100% reduction), while addition of activated bleaching earth had a little effect (9.2% average reduction). Among solvents, ethanol treatment was more efficient in reducing the targeted 3-MCPD esters in comparison to *n*-hexane (61.9% v/s 43.9%) on an average basis in all samples. Our LC-MS/MS method was reliable in quantifying ester-linked 3-MCDPs. Enzyme-catalyzed inter-esterification is the most effective mitigation approach for potentially harmfull 3-MCPD esters.

Keywords: 3-MCPD esters, LC-MS/MS, process-borne contaminant, direct analysis, toxicity, mitigation

INTRODUCTION

Processed foods have been identified to contain the chloroesters of 3-monochloropropane-1,2-diol (3-MCPD) as a contaminant produced during processing (Wohrlin *et al*., 2015). 3-MCPD belongs to a class of chemical compounds known as the chloropropanols and was identified in its free form 40 years ago during the production of acid-hydrolyzed vegetable proteins (A-HVP_S) (Arisseto *et al*., 2017). Recent studies have also shown the presence of 3-MCPD in its free form in many processed foods including soy sauce, meat-based smoked foods, infant formulas, cereal-derived foods, fried and malted products, vegetable soups, mayonnaise, as well as refined edible oils and fats and several other food-grade ingredients (Wohrlin *et al*., 2015).

The prevalence of chloroesters, especially 3-MCPD, in foods poses a public health concern. 3-MCPD has toxic impacts including kidney impairment accompanying tubular hyperplasia, adenomas, and nephropathy (FAO/WHO, 2006). Studies on rat models revealed a potential for induction of male infertility and suppression of immune function (Cho *et al*., 2008). Compelling evidence of Leydig-cell tumors and increased frequency of kidney renal carcinomas has also been reported (Cho *et al*., 2008). The International Agency for Research on Cancer (IARC) has declared 3-MCPD a carcinogen of category 2B; however, with respect to the significant *in vivo* toxicity of 3-MCPD, a convincing demonstration is still lacking (Onami *et al*., 2014; IARC, 2018). The current tolerable daily intake (TDI) of 3-MCPD is 2 $\mu g.kg^{-1}$ bw day^{-1} (WHO, 2006) while more recently the European Food Safety Authority (EFSA) established an even lower intake level of 0.82 $\mu g.kg^{-1}$ bw day^{-1} (EFSA, 2016). European Commission Regulation (EC) prescribed a maximum level of 3-MCPD of 0.02 mg/kg (20 µg/kg) for HVP and soy sauce (on 40% dry matter basis).

The formation of 3-MCPD and its esters in edible oils appears to be due to refining processes. Heat applied during the deodorization step significantly increases the concentration of 3-MCPD esters (Arisseto *et al*., 2017). The formation of ester-linked 3-MCPD is accredited to the presence of glycerol, chlorinated co-reactants, organic/inorganic salt(s) fraction and oil type (Ermacora and Hrncirík, 2014). A reaction between the lipid components and the chloride ions occurs, which produces the esters of 3-MCPD fatty acids in acid-

HVP-based and other heat-treated foods. 3-MCPD may also be produced in foods from food contact coatings treated with epichlorohydrin, which is used to add glaze and fineness to food contact materials. In the smoking process, 3-MCPD is formed by a reaction between 3-hydroxyacetone and chloride ions while enzymatic hydrolysis of 3-MCPD esters releases 3-MCPD (Christova-Bagdassarian *et al*., 2013).

Approaches to reduce the production and abundance of chloroesters in foods have been recommended by several researchers. These involve optimization of system pH, temperature manipulation of refining steps, enzyme-catalyzed conversion of chloroesters to non-toxic glycerol, water degumming of edible oils, washing of raw material (oil-bearing) with ethanol, use of inorganic adsorbents and, more recently, the use of synthetic anti-oxidants (Craft et al., 2012; Bornscheuer, 2014; Li *et al*., 2015).

For the analytical detection of esterified 3-MCPD, a sensitive GC/MS technique with a derivatization step to enhance volatility has been widely adopted. This indirect approach principally measures free 3-MCPD obtained from alkaline, acidic or enzymatic hydrolysis of 3-MCPD esters (Blumhorst *et al*., 2013). Though the reliability and performance of indirect methods for analysis of esterified 3-MCPD have been improved over the years, direct methods are gaining acceptance (Blumhorst *et al*., 2013). For 3-MCPD esters, the formation of analytical artifacts is much less likely during sample make-up for a direct method of analysis than an indirect method. Moreover, results published on 3-MCPD esters in extracted lipids from various foods are limited. In the current study, the abundant fatty acids present in vegetable oils—making up more than 80–90% of the fatty acid composition—were selected to be directly quantified. The study was devised to quantify 3-MCPD esters in processed foods from Pakistan by liquid chromatography-mass spectrometry (LC/MS-MS) with an effort to reduce the abundance of these esters in foods with different approaches.

MATERIALS AND METHODS

***Standards*:** Esterified 3-MCPD standards (purity ≥98%) were supplied by Toronto Research Chemicals, ON, Canada and purchased through PM separations (Qld, Australia). Standards included two homo-diesters, 1,2-dioleoyl-3-chloropropanediol (3-MCPD-OO; CAS # 69161-73-5) and 1,2-dilinoleoyl-3-chloropropanediol (3-MCPD-LL; CAS # 74875-96-0), and one hetero-diester, 1-palmitoyl-2-oleoyl-3-chloropropanediol (3-MCPD-PO; CAS # 1363153-60-9). The internal standard (IS) used was 1,2-dilinoleoyl-3-chloropropanediol-d_5 (3-MCPD-LL-d_5; CAS # N/A). All the ester standards were racemic mixtures, with the purity established by the supplier through MS and ^{1}H-NMR ($CDCl_3$).

***Reagents*:** Liquid chromatography-grade analytical reagents, namely methyl *tert*-butyl ether (MTBE), ethyl acetate, isopropanol (IPA), methanol, ethanol, petroleum ether and acetonitrile, were procured from Merck (KGaA, Darmstadt, Germany) while hexane was purchased from Honeywell (Australia). For the solid phase extraction (SPE) and sample clean-up, the Waters Sep-Pak® Vac C_{18} and Sep-Pak® Vac silica cartridges containing 1000 mg sorbent material were purchased from Waters (Australia). The enzymes halohydrin dehalogenase (HHD), epoxide hydrolase (EH) and lipase were purchased from Novozymes Australia. Activated bleaching earth (ABE) was procured from Sigma-Aldrich (Australia).

***Reconstitution of Standards*:** 3-MCPD-OO, 3-MCPD-LL, 3-MCPD-PO and 3-MCPD-LL-d_5 (the deuterated form) were reconstituted in 1 mL of IPA to prepare stock solutions of concentrations 10, 25, 10, and 2.5 mg.mL^{-1}, respectively. The working solutions of these standards were prepared in IPA at 500 ng mL^{-1}. A standard curve was constructed in IPA by serially diluting the stock solutions to give concentrations of 0, 7.81, 15.62, 31.25, 62.50, 125, 250 and 500 ng mL^{-1} spiked with 5 µL of IS from 100 ng mL^{-1}. Each of the linear constructed curves had an R^2 value of ≥0.998.

***Sample Preparation*:** Bread, UHT milk and chicken nuggets, each of three different brands, were purchased from a local market in Faisalabad, Pakistan. A digital tray dryer (Model # R-5A) was used to dry the bread and chicken nuggets at 60ºC for 3–4 h followed by size reduction using a lab mill. Oil/fat from these products was extracted over 6 h using Soxhlet with petroleum ether as the extracting solvent (AOAC, 2006). The lipid fraction from UHT milk was extracted with a separating funnel. Oil/fat extraction from the processed foods was carried out in the Edible Oils and Fats Laboratory, National Institute of Food Science and Technology, University of Agriculture, Faisalabad. Sample preparation and LC/MS-MS analysis were performed at the Bosch Mass Spectrometry Facility (BMSF), University of Sydney, Australia.

A 1-g sample of extracted fat/oil sample was dissolved in a 10-mL mixture of methyl *tert*-butyl ether (MTBE) and ethyl acetate (4:1 v/v), which was subsequently spiked with 100 µL of IS from 100 ng mL^{-1}. A 100-µL aliquot was removed from the solution above and charged onto a 100-mg Waters Sep-Pak® Vac silica cartridge previously preconditioned with 5 mL hexane. Elution of the targeted compounds was carried out using 5 mL hexane: ethyl acetate (95:5 v/v). The eluate was dried and re-dissolved in 100 µL of MTBE: ethyl acetate (4:1 v/v) and then in 1 mL of IPA and methanol mixture (1:1 v/v). The reconstituted residue was charged onto a 1000-mg Waters Sep-Pak® C_{18} cartridge, which was previously preconditioned with 5 mL methanol. The residue vessel was rinsed with 1 mL IPA: methanol (1:1 v/v), which was also charged onto the C_{18} cartridge. The targeted analytes from the C_{18} cartridge were eluted with a 50-mL mixture of methanol: acetonitrile: ethanol (65:30:5 v/v). The extract was evaporated to dryness and re-dissolved in 1 mL of IPA. The reconstituted eluate was transferred to 1-ml Eppendorf tubes

and centrifuged at 13,200 RPM for 15 min in an Eppendorf microcentrifuge (5414 R). The supernatant was collected in HPLC vials for LC/MS-MS analysis.

***LC/MS-MS Method*:** Liquid chromatography (Agilent 1290 Infinity series) with a tandem mass spectrometer (Agilent QQQ 6460) was used to analyze the 3-MCPD esters. Electrospray ionization (ESI) in a positive ion mode was selected for the analytes. Multiple reaction monitoring (MRM/MS2 scan) was applied for collecting the mass spectra of the fragmented ions. Chamber settings: desolvation gas (nitrogen) at 350°C with a mobile phase flow rate of 200 µL min^{-1}. The flow rate of the drying gas, nebulizer pressure, corona current (nA), and capillary voltage (Vcap) were 5 L min^{-1}, 60 psi, 20,000 and 3500 V, respectively. Vapor temperature in the ESI was 300°C. The Ecplise Plus C_{18} column used for chromatographic separation at 24°C had a particle size, ID and length of 1.8 µm and 2.1 mm x 50 mm, respectively. Mobile phase buffers used for gradient elution: buffer A, 20% IPA: 20% water: 20% acetonitrile containing 0.1% formic acid and 5 mM ammonium carbonate; buffer B, 40% acetonitrile: 60% IPA containing 0.1% formic acid and 5 mM ammonium carbonate. A 15-min elution was programmed as 30% of buffer A and 70% of buffer B for 0 to 0.2 min, followed by an increase to 98% buffer B up to 14 min. At 14.5 min, buffer B was decreased to 70%. The operating parameters for method response and development were optimized using Mass Hunter Optimizer software. A mixed standard solution was run with an unscheduled MRM mode to determine the retention times for the targeted compounds.

***Linearity Response and Detection Limits*:** We tested our analysis method with cold-pressed extra virgin olive oil to validate its reliability and to bypass the need of matrix-matched standards. Double SPE cleanup allowed rigorous analysis of various matrices. An 8-point standard curve was constructed in IPA and recoveries for each standard (100–500 $ng.mL^{-1}$) and IS (100 $ng.mL^{-1}$) were validated for quantification. In cases where a suitable IS was unavailable, the peak areas were used for a direct quantification of esters. Repeatability tests showed standard deviations of 6.1 to 23.3%, demonstrating the success of this method.

***Application and Effectiveness of Reduction Treatments*:** Three different treatments, namely enzyme-catalyzed interesterification, activated bleaching earth (ABE), and solvents (*n*-hexane and ethanol) were tested for their effectiveness in reducing the abundance of chloroesters from extracted lipid samples.

***Enzyme-catalyzed Inter-esterification*:**
Separate preparations of halohydrin dehalogenase (HHD) and epoxide hydrolase (EH) were filtered with buffer before addition to the sample. Enzymatic hydrolysis was performed in glass vials using a thermomixer at 30°C with a reaction volume of 200 µL in the presence of sodium phosphate buffer (0.01 M, pH 5.0±0.02). For both enzymes, a similar amount (10 µg/mL) was used for 24±1 h (Bornscheuer, 2014).

***Activated Bleaching Earth (ABE) Treatment*:** Approximately 5 g of fat/oil was weighed accurately in a round bottom flask with subsequent heating. ABE (20 mg) was added, and a vacuum was applied, with the mixture treated at 110°C for 20 min. The oil was then cooled, separated and filtered. The adsorbed oil was recovered in warm ethanol (Shimizu *et al*., 2012).

***Treatment with Solvents*:** *n*-hexane and ethanol were used to extract ester-liked 3-MCPD from fractionated lipid samples after storing them at -35°C. A fat/oil sample (5 g) was separately incubated with 5 mL of each solvent after mixing at 90–100°C. Freezing imparted crystallization and the liquid fraction of the low-melting-point esters was removed along with solvents.

***Quantification of 3-MCPD Following Reduction Treatments*:** Subsequent 3-MCPD quantification was carried out according to the method discussed in previous section 'LC/MS-MS method'.

RESULTS

***Selection of Standards and Method Validation*:** The optimized LC-MS/MS parameters to acquire and monitor ester-linked 3-MCPD are listed in Table 1. While the running time for all the samples on the column was 15 min, all the diesters were separated within the first 5 min of the run (Table 1). The accuracy of the ester-linked 3-MCPD present

Table 1. Optimized parameters for *m/z* ion monitoring and data acquisition

Compound name	Formula	MW[a]	*m/z* precursor	CE (eV)[b]	*m/z* fragment ion(s)	RT (min)[c]	Polarity
1,2-Dilinoleoyl-3-chloro-1,2-propanediol-d_5 (3-MCPD-LL-d_5)	$C_{39}H_{62}D_5ClO_4$	640.43	657.3	10, 12	263.4, 360.1	3.275	+ve
1,2-Dioleoyl-3-chloropropanediol (3-MCPD-OO)	$C_{39}H_{71}ClO_4$	639.43	656.4	19, 15	265.2, 357.3	3.278	+ve
1,2-Dilinoleoyl-3-chloropropanediol (3-MCPD-LL)	$C_{39}H_{67}ClO_4$	635.40	652.4	9, 15	263.3, 355.3	3.273	+ve
1-Palmitoyl-2-oleoyl-3-chloropropanediol (3-MCPD-PO)	$C_{37}H_{69}ClO_4$	613.39	630.4	13, 13	331.2, 357.2	3.481	+ve

[a]According to the manufacturer's specifications, [b]Collision energy, [c]Retention time

in foods as quantified by a direct method depends on the analytical standards used; therefore, the most abundant fatty acids present in edible fat/oil were selected for the standards. Use of a comprehensive set of standards was prohibitively expensive. The proportion of bound 3-MCPD monoesters in edible oils accounts for <15% in commonly consumed oils (MacMahon *et al*., 2013).

Bound 3-MCPD esters produce a protonated precursor ion in the mass spectrometer, which is difficult to detect. Mostly, the 3-MCPD esters are detected by forming Na^+ or NH_4^+ adduct ions (Dubois *et al*., 2012). In the detection of 3-MCPD esters, a defragmented ion (cleavage of a fatty acid from a *sn*-1 position with intact chlorine) is used for reliable quantification from the base of the peak. Representative mass-spectra of fragmented ions of 3-MCPD-OO, 3-MCPD-LL, 3-MCPD-PO, and 3-MCPD-LL-d_5 are shown in Figure 1.

***Quantification of 3-MCPD Esters by LC-MS/MS*:** The concentrations of 3-MCPD esters in the extracted fat of the

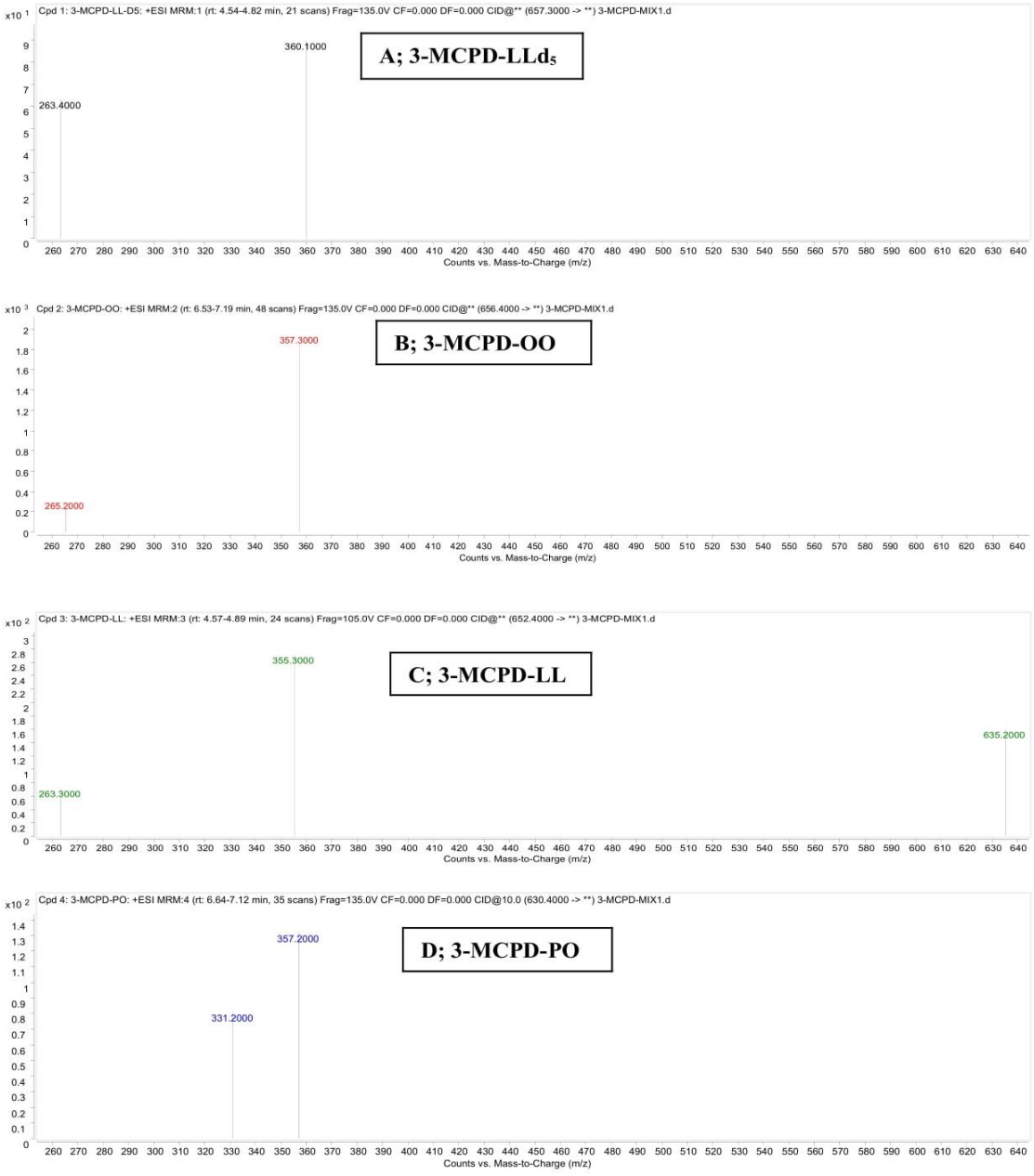

Figure 1. LC-MS/MS mass spectra of fragmented ions of selected 3-MCPD esters namely; A; 3-MCPD-LL-D5, B; 3-MCPD-OO, C; 3-MCPD-LL and D; 3-MCPD-PO

Table 2. Quantified 3-MCPD esters (µg kg^{-1})[a] in extracted oil/fat samples from bread, UHT milk, and chicken nuggets.

Compounds[b]		3-MCPD-OO[c]			3-MCPD-LL[d]			3-MCPD-PO[e]			Total[f]		
Products	Brands	1	2	3	1	2	3	1	2	3	1	2	3
	Bread	6.40	6.64	2.97	7.55	9.51	7.25	5.23	3.42	4.83	19.18	19.56	15.05
	UHT milk	8.75	8.62	2.84	9.25	12.47	17.74	4.36	2.51	3.51	22.36	23.26	24.09
	Nuggets	8.95	0.88	2.20	5.47	10.85	7.74	4.58	3.97	10.40	19.00	15.71	20.34

[a]Average of a duplicate run, [b]Internal standard used for quantification was 3-MCPD-LLd$_5$, [c]1,2-Dioleoyl-3-chloropropanediol, [d]1,2-Dilinoleoyl-3-chloropropanediol, [e]1-Palmitoyl-2-oleoyl-3-chloropropanediol, [f]Sum of all esters in brands 1, 2 and 3 of the product(s), respectively

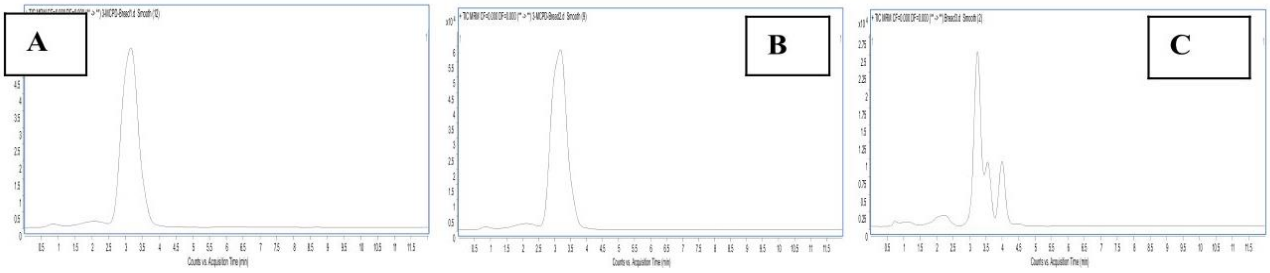

Figure 2A. Chromatograms, A-C represent total ion concentration (TIC) of targeted 3- MCPD esters for bread; brand 1, 2 and 3

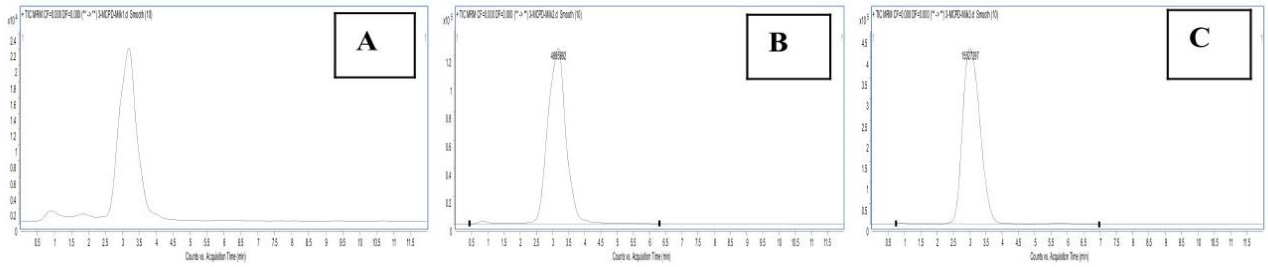

Figure 2B. Chromatograms, A-C represent total ion concentration (TIC) of targeted 3- MCPD esters for UHT milk; brand 1, 2 and 3

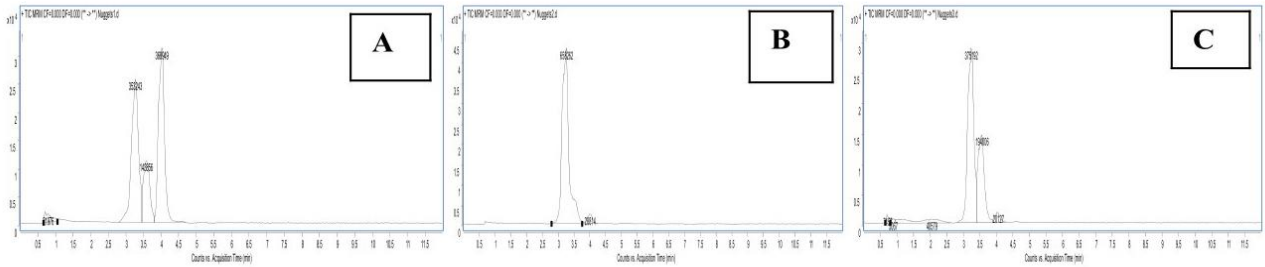

Figure 2C. Chromatograms, A-C represent total ion concentration (TIC) of targeted 3- MCPD esters for chicken nuggets; brand 1, 2 and 3

processed foods tested are given in Table 2, while chromatograms representing the total ion concentration (TIC) and quantitative ions of all brands of the products are shown in Figure 2 (panels A-C).

All the selected brands of the processed foods had 3-MCPD esters levels beyond the established limits of 2 µg kg^{-1} bw day^{-1} set by the Joint WHO/FAO Expert Committee on Food Additives (JECFA). The three brands of bread were found to contain 19.18, 19.56, and 15.05 µg kg^{-1} of the targeted 3-MCPD esters, respectively. For the UHT milk, the highest abundance of the targeted 3-MCPD esters was 24.09 µg kg^{-1}. In the chicken nuggets, the highest abundance of the targeted 3-MCPD esters was 20.34 µg kg^{-1}. For individual diester concentrations in all the brands of the selected foods, 3-

MCPD-LL had the highest relative abundance of the extracted lipid samples while 3-MCPD-PO was the least abundant, a trend compatible with earlier findings (Yamazaki *et al*., 2013). The 3-MCPD-OO was found in the lowest levels in extracted oil/fat samples in one of the chicken nuggets samples (0.88 µg kg^{-1}). While in practice, three diesters were quantified in this study, theoretically, there are four in total including the isomeric form of 3-MCPD-PO, which is 3-MCPD-OP. It was not possible to separate these two esters using our method, even using mass selectivity via MS-TOF, due to the identical calculated masses for these isomers (Yamazaki *et al*., 2013).

DISCUSSION

Research has emphasized chlorine and partial acylglycerols as the potential precursors prior to the refining step (Li *et al*., 2016). The solvent-extracted crude oil, which was degummed and bleached, showed to detectable amounts of 3-MCPD esters (Li *et al*., 2016). Significantly higher 3-MCPD concentrations were detected in deodorized oil (220–226ºC) in comparison to hot-squeezed crude oils, which have very low concentrations of 3-MCPD. Partial acylglycerol seemed to be a less effective precursor in the formation of 3-MCPD esters in comparison to chlorine (Li *et al*., 2016).

Predominant components with potential to favour the generation of the fatty acid esters of 3-MCPD in edible oils include acylglycerols, organic/inorganic chlorine-containing co-reactants and ferric ion (Fe^{3+}) (Ermacora and Hrncirík, 2014). Research has confirmed that 3-MCPD esters are generated during the deodorization of edible oils in the presence of organic and/or inorganic chlorine-containing co-reactants (Li *et al*., 2016). It has also been observed that the rate of 3-MCPD ester formation increases exponentially above 200ºC. Organochlorines and hydrogen chloride (HCl) gas react with acylglycerols by protonation, thus eliminating a fatty acid residue. The proposed formation mechanism is based on a two-step generation of 3-MCPD esters involving an intermediate acyloxonium ion in a regio-selective manner at *sn*-1 and/or *sn*-3 positions of the glycerol backbone.

It has been focused that the tendency for formation of 3-MCPD diesters is far greater than for 3-MCPD monoesters or free 3-MCPD (Yamazaki *et al*., 2013). In most cases, the 3-MCPD esters generated during edible oil refining are carried through to processed foods; however, some studies revealed that these may also be produced during the processing of foods. It has been reported that glycerol and chloride ions are responsible for generating 3-MCPD in bread during baking, accounting for a maximum of 68% of the total 3-MCPD formation in commercial doughs (Baer *et al*., 2010). The glycerol in this system is mainly from yeast, wheat flour or flour improvers. Bread crust undergoes the greatest exposure to high baking temperatures, promoting reactions to form 3-MCPD and its esters. One study analyzed bread ingredients including fat, emulsifiers, baking agent, sugar, salt and sourdough for their potential of the 3-MCPD formation (Breitling-Utzmann *et al*., 2005). It was found that commercial bread improvers used in baking items typically containing soy flour, ascorbic acid and a source of monoacylglycerol may promote formation of 3-MCPD in the dough.

The inclusion of 1% peanut fat increased 3-MCPD levels due to extreme-temperature hydrolysis of triglycerides, resulting in the formation of glycerol, a 3-MCPD precursor (Breitling-Utzmann *et al*., 2005). The commercial baking agent had the greatest influence on the formation of 3-MCPD in bread. Mono- and diacylglycerols added as emulsifiers in the recipe increased 3-MCPD levels in bread. Sugars in the recipe also increased 3-MCPD levels in comparison to doughs containing no sugar (Breitling-Utzmann *et al*., 2005). To our knowledge, generation of 3-MCPD or its esters from glucose or sucrose as precursors has not been reported in the literature (Breitling-Utzmann *et al*., 2005). Bread prepared from previously stored dough had significant higher 3-MCPD levels compared to bread prepared from a fresh dough (Reece, 2005). It has also been concluded that the level of 3-MCPD may reach up to 679 to 716 µg kg^{-1} in different types of bread (Hamlet and Sadd, 2009; Vicente *et al*., 2015). The bread prepared from a pre-fermented dough was associated with higher levels of the contaminant, possibly due to increased glycerol and organic acid release with a reduced pH due to yeast metabolism (Baer *et al*., 2010). The difference in the levels of 3-MCPD or its esters quantified in our study in comparison to other studies may be due to differences in food production methods, recipe ingredients and the analytical method used.

For meat products, higher 3-MCPD levels have been reported when the salt and fat concentrations were higher (Calta *et al*., 2004). Model systems, which closely relate to foods with the potential to generate 3-MCPD during processing, were investigated with water content, glycerol, salt, triolein and lecithin as potential factors promoting 3-MCPD formation. The highest 3-MCPD level of 7.7 mg kg^{-1} was found in the model representing salami production, with 30% water content and glycerol. This amount was three times the level produced from lecithin and from triolein (1.8 and 2.2 mg 3-MCPD kg^{-1} precursor, respectively). It has been suggested that the presence of 3-MCPD in meat products may be accredited to the addition of sauce as an ingredient, as sauces may contain a tremendous amount of 3-MCPD (Calta *et al*., 2004; Lee and Khor, 2015; Vicente *et al*., 2015). Here again, it must be considered that the sauces prepared from natural fermentation had little or no contaminant in comparison to the sauces prepared from chemical hydrolysis (Velisek, 2009). The levels of 3-MCPD in naturally fermented and chemically hydrolyzed sauces ranged from undetectable to 643 µg kg^{-1}, and 144 to 4405 µg kg^{-1}, respectively (Vicente *et al*., 2015).

To our knowledge, our study is the first report of the presence of 3-MCPD in UHT milk. However, milk is said to be a food

that may be naturally contaminated by 3-MCPD; e.g., goat milk (Cerbulis *et al*., 1984). Infant formula and human breast milk have also been reported to contain a significant amount of 3-MCPD. Chloropropanol contamination of natural foodstuffs may be credited to the dietary intake of chlorolipids, which undergo metabolism in a stereo-retentive pathway, ultimately depositing 3-MCPD in mammary glands, where it is excreted in milk after re-esterification. Although the literature lacks data on the formation mechanism for this contaminant, it may be postulated that these esters are formed during UHT treatment due to precursors naturally present. Enzymatic reactions in the system may also be relevant here. The presence of 3-MCPD in infant formula or powdered milk has been accredited to the content of vegetable fat included in these foods (Jędrkiewicz *et al*., 2016). There is now a compelling reason for researchers to analyze pasteurized milk for the presence of chloroesters so that reduction strategies could be explored.

The concentration of quantified 3-MCPD esters following the application of reduction treatments is presented in Tables 3-5. It was observed that enzyme treatment effectively reduced the esters to an undetectable limit in all samples. Among solvents, ethanol treatment was more efficient in reducing the targeted 3-MCPD esters in comparison to *n*-hexane (61.9% v/s 43.9%) on an average basis in the three brands of all the products (Tables 4-5). ABE treatment was ineffective in reducing the abundance of 3-MCPD esters in treated samples (9.2% average reduction; Table 3).

Halohydrin dehalogenase (HHD) (AD2) converted 3-MCPD into glycidol in an aqueous system. Consequently, epoxide hydrolase (EH) converted glycidol into non-toxic glycerol. The reduction of 3-MCPD esters using ABE during the bleaching process of edible oils has been reported but knowledge of the exact mechanism(s) associated with GE_S or 3-MCPD ester reduction after bleaching earth treatment is still lacking (Franke *et al*., 2009). The subsequent quantification of 3-MCPD esters after ABE treatment revealed that this strategy did not remove a significant amount of the targeted esters. The main reason may be that this approach is more suitable to eliminate glycidol esters (GE_S) from triacyl- and diacylglycerol oils rather than 3-MCPD esters exclusively (Shimizu *et al*., 2012). These same authors

Table 3. Quantified 3-MCPD esters (µg kg^{-1})[a] in extracted oil/fat samples from bread, UHT milk, and chicken nuggets after ABE[b] treatment.

Compounds[c]		3-MCPD-OO[d]			3-MCPD-LL[e]			3-MCPD-PO[f]			Total[g]		
Products	Brands	1	2	3	1	2	3	1	2	3	1	2	3
	Bread	5.12	5.98	2.17	6.55	8.41	6.43	5.03	2.42	4.21	16.68	16.81	12.81
	UHT milk	8.15	8.12	2.14	9.21	11.97	16.94	4.06	2.15	3.13	21.42	22.24	22.21
	Nuggets	8.11	0.86	2.07	5.17	10.58	7.47	3.98	3.79	8.61	17.26	15.23	18.15

[a]Average of a duplicate run, [b]Activated bleaching earth, [c]Internal standard used for quantification was 3-MCPD-LLd$_5$, [d]1,2-Dioleoyl-3-chloropropanediol, [e]1,2-Dilinoleoyl-3-chloropropanediol, [f]1-Palmitoyl-2-oleoyl-3-chloropropanediol, [g]Sum of all esters in brands 1, 2 and 3 of the product(s), respectively

Table 4. Quantified 3-MCPD esters (µg kg^{-1})[a] in extracted oil/fat samples from bread, UHT milk, and chicken nuggets after ethanol treatment.

Compounds[b]		3-MCPD-OO[c]			3-MCPD-LL[d]			3-MCPD-PO[e]			Total[f]		
Products	Brands	1	2	3	1	2	3	1	2	3	1	2	3
	Bread	2.80	1.94	1.17	3.59	3.53	2.95	1.01	1.11	1.83	7.49	6.58	5.95
	UHT milk	2.98	3.09	1.11	3.54	4.63	6.00	1.99	0.99	1.49	8.51	8.71	8.60
	Nuggets	4.51	0.48	1.02	1.79	4.44	3.13	1.62	1.17	3.89	7.92	6.09	8.04

[a]Average of a duplicate run, [b]Internal standard used for quantification was 3-MCPD-LLd$_5$, [c]1,2-Dioleoyl-3-chloropropanediol, [d]1,2-Dilinoleoyl-3-chloropropanediol, [e]1-Palmitoyl-2-oleoyl-3-chloropropanediol, [f]Sum of all esters in brands 1, 2 and 3 of the product(s), respectively

Table 5. Quantified 3-MCPD esters (µg kg^{-1})[a] in extracted oil/fat samples from bread, milk, and chicken nuggets after *n*-hexane treatment

Compounds[b]		3-MCPD-OO[c]			3-MCPD-LL[d]			3-MCPD-PO[e]			Total[f]		
Products	Brands	1	2	3	1	2	3	1	2	3	1	2	3
	Bread	4.17	5.13	2.01	5..01	4.15	3.11	2.94	1.11	2.03	12.12	10.39	7.15
	UHT milk	3.91	4.44	2.02	5.98	7.07	10.10	1.99	1.61	1.77	11.88	13.12	13.89
	Nuggets	5.09	0.23	1.87	2.88	6.17	4.99	2.97	2.09	5.77	10.94	8.49	12.63

[a]Average of a duplicate run, [b]Internal standard used for quantification was 3-MCPD-LLd$_5$, [c]1,2-Dioleoyl-3-chloropropanediol, [d]1,2-Dilinoleoyl-3-chloropropanediol, [e]1-Palmitoyl-2-oleoyl-3-chloropropanediol, [f]Sum of all esters in brands 1, 2 and 3 of the product(s), respectively

quantified the GE_S with GC-FID and revealed almost complete elimination from ABE-absorbed oils. It was further observed that the absorption was achieved due to modification by a ring-opening mechanism and reaction with the water in the ABE followed by interesterification in bulk oil. Considering the health concerns associated with ABE, this needs to be eliminated from the system after treatment. Similar results were reported while eliminating 3-MCPD esters by adsorbent materials (Sitrijowski *et al*., 2011).

There is no published data available with respect to 3-MCPD or reduction of its esters exclusively from refined edible oils using solvents. Thus, it is difficult to provide an explanation or mechanism involved here. A partial 3-MCPD reduction may be accredited to solubility in the polar or non-polar solvents used. Researchers have postulated that chloropropanols tend to be more polar as the processing of crude oil proceeds (Craft and Nagy, 2012).

Conclusion: The presence of quantifiable 3-MCPD fatty acid esters in the extracted lipid fraction of all samples is reported. A very reliable instrumental response to give the concentrations of 3-MCPD esters was achieved here. Among mitigation strategies tested so far, a 100% reduction in 3-MCPD esters was achieved through the enzymatic inter-esterification. Regarding *n*-hexane and ethanol treatment, the later had a more pronounced effect in reducing the abundance of harmful 3-MCPD esters. Activated bleach had the least impact on 3-MCPD esters' reduction. For a complete 3-MCPD reduction in refined edible oils, enzymatic interesterification in bulk oils is suggested. Furthermore, our work highlights the need to analyze all heat-treated processed foods for their 3-MCPD fatty acid esters. In additional, risk assessment should also be carried out by analyzing locally consumed foods, including infant foods.

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REFERENCES

Arisseto, A.P., W.C. Silva, G.R. Scaranelo and E. Vicente. 2017. 3-MCPD and glycidyl esters in infant formulas from the Brazilian market: Occurrence and risk assessment. Food Control 77:76-81.

AOAC. 2006. Official Methods of Analysis (18th ed.). Arlington, USA: The Association of Official Analytical Chemists International.

Blumhorst, M.R., M.W. Collison, R., Cantrill, H. Shiro, Y. Masukawa and S. Kawai. 2013. Collaborative study for the analysis of glycidyl fatty acid esters in edible oils using LC–MS. J. Am. Oil Chem. Soc. 90:493-500.

Bornscheuer, U.T. 2014. Enzymes in lipid modification: Past achievements and current trends. Eur. J. Lipid Sci. Technol. 116:1322-1331.

Breitling-Utzmann, C.M., H. Hrenn, N.U. Haase and G.M. Unbehend. 2005. Influence of dough ingredients on 3-chloropropane-1, 2-diol (3-MCPD) formation in toast. Food Addit. Contam. 22:97-103.

Baer, I., B. de la Calle and P. Taylor. 2010. 3-MCPD in food other than soy sauce or hydrolysed vegetable protein (HVP). Anal. Bioanal. Chem. 396:443-456.

Craft, B.D., and K. Nagy. 2012. Mitigation of MCPD-ester and glycidyl-ester levels during the production of refined palm oil. Lipid Technol. 24:155-157.

Craft, B.D., Nagy, K., Sandoz, L. and Destaillats, F., 2012. Factors impacting the formation of monochloropropanediol (MCPD) fatty acid diesters during palm (*Elaeis guineensis*) oil production. Food Addit Contam A 29:354-361.

Cho, W.S., B.S. Han, K.T. Nam, K. Park, M. Choi and S.H. Kim. 2008. Carcinogenicity study of 3-monochloropropane-1,2-diol in Sprague-Dawley rats. Food Chem. Toxicol. 46:3172–3177.

Calta, P., J. Velíšek, M. Doležal, S. Hasnip, C. Crews and Z. Réblová 2004. Formation of 3-chloropropane-1, 2-diol in systems simulating processed foods. Eur. Food Res. Technol. 218:501–506.

Cerbulis, J., O. Parks, R. Liu, G. Piotrowski and H. Farrell. 1984. Occurrence of diesters of 3-chloro-1,2-propanediol in the neutral lipid fraction of goats' milk. J. Agric. Food Chem. 32:474–476.

Christova-Bagdassarian, V., J.A. Tishkova and T.M. Vrabcheva. 2013. 3-Monochloro-1,2- propandiol (3-MCPD) in soy sauce from the Bulgarian market. Food Addit. Contam. Part B. 6:163–167.

Dubois, M., A. Tarres, T. Goldmann, A.M. Empl, A. Donaubauer and W. Seefelder. 2012. Comparison of indirect and direct quantification of esters of monochloropropanediol in vegetable oil. J. Chrom. *A*. 1236:189-201.

EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain). 2016. Scientific opinion on the risks for human health related to the presence of 3- and 2-monochloropropanediol (MCPD), and their fatty acid esters, and glycidyl fatty acid esters in food. Efsa J. 14:159.

Ermacora, A. and K. Hrncirík. 2014. Influence of oil composition on the formation of fatty acid esters of 2-chloropropane-1,3-diol (2-MCPD) and 3-chloropropane-1,2-diol (3-MCPD) under conditions simulating oil refining. Food Chem. 161:383–389.

Franke, K., U. Strijowski, G. Fleck and F. Pudel. 2009. Influence of chemical refining process and oil type on bound 3-chloro-1, 2-propanediol contents in palm oil and rapeseed oil. LWT-Food Sci. Technol. 42:1751–1754.

FAO/WHO. 2006. Discussion paper on acid HVP containing products and other products containing chloropropanols. In: Proc Session 38 of Codex Committee on Food Additives and Contaminants, The Hague, The Netherlands, 24–28 Apr 2006

Hamlet, C.G. and P.A. Sadd. 2009. Chloropropanols and chloroesters, Process-induced food toxicants: Occurrence, formation, mitigation, and health risks, pp. 175–214). Hoboken, United States: John Wiley & Sons Inc. In R.H. Stadler, & D.R. Lineback (Eds.)

International Agency for Research on Cancer (IARC). 2012. 3- Monochloro-1,2-propanediol. IARC monographs on the evaluation of carcinogenc risks to humans. [citied 2015, Jan 26];101:349–374. Available from: http://monographs.iarc.fr/ ENG/Monographs/vol101/mono101-010.pdf.

Jędrkiewicz, R., M. Kupska, A. Głowacz, J. Gromadzka and J. Namieśnik. 2016. 3-MCPD: A worldwide problem of food chemistry. Crit. Rev. Food Sci. Nutr. 56:2268-2277.

Li, C., L. Li, H. Jia, Y. Wang, M. Shen, S. Nie and M. Xie. 2016. Formation and reduction of 3-monochloropropane-1, 2-diol esters in peanut oil during physical refining. Food Chem. 199:605-611.

Li, C., H. Jia, M. Shen, Y. Wang, S. Nie, Y. Chen and M. Xie. 2015. Antioxidants inhibit formation of 3-monochloropropane-1, 2-diol esters in model reactions. J. Agr. Food Chem. 63:9850–9854.

Lee, B.Q, and S.M. Khor. 2015. 3-Chloropropane-1, 2-diol (3-MCPD) in soy sauce: A review on the formation, reduction, and detection of this potential carcinogen. Compr. Rev. Food Sci. F. 14:48-66.

MacMahon, S., E. Mazzola, T.H. Begley and G.W. Diachenko. 2013. Analysis of processing contaminants in edible oils. Part 1. Liquid chromatography–tandem mass spectrometry method for the direct detection of 3-monochloropropanediol monoesters and glycidyl esters. J. Agr. Food Chem. 61:4737–4747.

Onami, S., Y.M. Cho, T. Toyoda, K. Horibata, Y. Ishii, T. Umemura and K. Ogawa. 2014. Absence of *in vivo* genotoxicity of 3-monochloropropane-1, 2-diol and associated fatty acid esters in a 4-week comprehensive toxicity study using F344 gpt delta rats. Mutagenesis 29:295-302.

Reece, P. 2005. The origin and formation of 3-MCPD in foods and food ingredients (final project report). Food Standards Agency, London

Shimizu, M., J. Moriwaki, D. Shiiba, H. Nohara, N. Kudo and Y. Katsuragi. 2012. Elimination of glycidyl palmitate in diolein by treatment with activated bleaching earth. J. Oleo Sci. 61:23-28.

Strijowski, U., V. Heinz and K. Franke. 2011. Removal of 3-MCPD esters and related substances after refining by adsorbent material. Eur. J. Lipid Sci. Technol. 113:387–392.

Velisek, J. 2009. Chloropropanols. Process-induced food toxicants: Occurrence, formation, mitigation, and health risks, pp. 539–562. Hoboken, United States: JohnWiley & Sons Inc. In R.H. Stadler, & D.R. Lineback (Eds.).

Vicente, E., A.P. Arisseto, R.P. Furlani, V. Monteiro, L.M. Gonçalves, A.L.D. Pereira and M.C.F. Toledo. 2015. Levels of 3-monochloropropane-1, 2-diol (3-MCPD) in selected processed foods from the Brazilian market. Food Res. Int. 77:310-314.

Wohrlin, F., H. Fry, M. Lahrssen-Wiederholt and A. Preiß-Weigert. 2015. Occurrence of fatty acid esters of 3-MCPD, 2-MCPD and glycidol in infant formula. Food Add. Cont. A. 32:1810–1822.

Yamazaki, K., M. Ogiso, S. Isagawa, T. Urushiyama, T. Ukena and N. Kibune. 2013. A new, direct analytical method using LC-MS/MS for fatty acid esters of 3-chloro-1, 2-propanediol (3-MCPD esters) in edible oils. Food Add. Cont. A. 30:52–68.

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