In Pursuit Of Healthier Palm Oil

Reducing 2- & 3-MCPD esters, and glycidyl esters in organic palm oil via intricate mitigation strategies

Sergio B. Oey

Propositions

1. Close collaboration between the refinery, milling plant and a plant geneticist is the key to achieve lower 2-, 3-MCPDE, and glycidyl esters concentrations in palm oil and other vegetable oils.
   (This thesis)

2. A solid analytical procedure is the foundation of chemistry and biology-related research.
   (This thesis)

3. The wooden skewer method is the best ancient and scientifically preferred method to check the readiness of baked cakes.

4. The Dutch cardiovascular risk management guidelines for general practitioners are not adequate to treat patients under the age of 40.

5. Playing videogames with children has beneficial effects on their general development.

6. The commercially available 'satésaus' in The Netherlands dishonours the traditional Indonesian satay-dish.

Propositions belonging to the thesis, entitled
In pursuit of healthier palm oil: Reducing 2- & 3-MCPD esters, and glycidyl esters in organic palm oil via intricate mitigation strategies.

Sergio B. Oey

Wageningen, 15 September 2023

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Thesis

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Theory will take you only so far.
J. Robert Oppenheimer (Oppenheimer, 2023)

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Chapter 1

Introduction

1.1 Oil refining

Palm oil is globally the most produced vegetable oil (FAO, 2022). Palm oil can be found in many food products, such as cookies, cakes, soups, and many more. Despite being surrounded by food products containing palm oil, many consumers often do not get to see palm oil in its crude nor its refined form. Crude palm oil has a very vivid red-orange hue with a quite pungent odour and a quite distinct taste, while refined palm oil has a more white-yellow or even broken-white hue combined with neutral odour and taste. Oil refining is predominantly done to remove colour, neutralize odour and taste, and to remove free fatty acids making the oil more stable with increased shelf-life (Gharby, 2022; Wen et al., 2023). In contrast, olive oil or extra virgin olive oil, to be more specific, is a crude oil type that consumers are quite familiar with. Because people are accustomed to the taste and odour of extra virgin olive oil, it does not require further processing. Briefly, there are two oil refining methods: physical refining and chemical refining. Physical refining usually consists of a degumming step, a bleaching step, and a deodorization step. Common additives that often are used in physical refining are a type of acid, such as malic acid, phosphoric acid or citric acid to degum the oil, bleaching earth to reduce the oil's hue, and activated carbon to adsorb unwanted odour and flavour components as well as small organic molecules or heavy metals. Free fatty acids and other volatile molecules are distilled out of the oil during the deodorization step. This process is typically performed at high temperatures and under a deep vacuum. Chemical refining is slightly different than physical refining. Between the degumming and bleaching steps, the oil is neutralized with a lye. This induces a reaction called saponification where fatty acid esters are hydrolyzed by the lye. The created soapstock is then removed by washing the oil with water. This process is continued by the same bleaching process as is usually done in physical refining.

1.2 Food Safety

Within the food industry, there are many processes involved which often are not immediately visible to the general consumers. One of those aspects lies within the area of food safety. In the past, there have been several occurrences where food safety was at risk. For instance, in 1999, the Dioxin/polychlorinated biphenyl (PCB) incident affected poultry and pork products and in 2004, high levels of Polychlorinated dibenzo-p-dioxins (PCCDs) in milk from two farms in The Netherlands was found (Bernard & Fierens, 2002; Hoogenboom et al., 2010). After such incidents, that sector was hit hard not only by public concern, e.g. people tend to avoid buying associated food products, but also from potential import bans instilled by other countries (Buzby & Chandran, 2003). An economical model study on the financial consequences based on the 2004

PCCD-in-milk incident have shown that the financial impact of a single dioxin incident can go as high as €141.2 million in the worst-case scenario (Lascano Alcoser et al., 2011). Those incidents, in combination with some previous incidents in Europe, led to the introduction of the General Food Law (GFL) and the setting up of the European Food Safety Authority (EFSA). The role of EFSA is to perform risk assessments. The European Commission (EC) is the risk management organization and deals with the implementation of legal limits, such as Maximum Levels (ML) for the presence of substances in food, and other risk management measures to control and improve food safety in Europe. At the national level, authorities are responsible for assessing if food manufacturers and suppliers comply with the regulations. Food producing companies have to comply with GFL; They are responsible for the safety of the products that they produce and introduce into the market.

1.3 The process contaminants 2-MCPDE, 3-MCPDE, and GE and its toxicology

While this thesis focuses mainly on 2-monochloropropane-1,3-diol (2-MCPDE), 3-monochloropropane-1,2-diol (3-MCPDE), and glycidyl ester (GE), as shown in Figure1.1, the fatty acid ester form of 2-MCPD, 3-MCPD, and glycidol, those ester bound chloropropanols is not the core reason why EFSA performed a risk assessment of the potential health risks upon exposure to 3-MCPDE and GE. Historically, it all starts with the toxic, unbound 3-MCPD (Figure 1.2). The unbound 3-MCPD were predominantly found as process contaminant in acid-hydrolyzed vegetable protein produced soy sauce (Lee & Khor, 2015). Under certain chemical conditions, which will be explained in-depth later, 2-MCPD can be created next to 3-MCPD from glycidol and vice versa.

In 2001, the Scientific Committee on Food adopted an opinion regarding the toxicity of unbound 3-MCPD (Scientific Committee on Food, 2001). In the years to come, more toxicological data became available confirming the toxicity of 3-MCPD even more (Andres et al., 2013; National Toxicology Program, 2007). Glycidol, due to its epoxide, is more reactive than 3-MCPD and is labelled as genotoxic carcinogen (Bakhiya et al., 2011; MELNICK, 2006). Recent research suggests that co-exposure of 3-MCPD and glycidol might have a synergistic effect on their nephrotoxicity (Liu et al., 2021).

The biological key aspect is that bound chloropropanols and GE can be transformed into their toxic unbound counterpart in the gastrointestinal tract during the digestion process (Seefelder et al., 2008). This is the core reason why 2-MCPDE, 3-MCPDE, and GE also receive the same amount of attention as unbound 3-MCPD and glycidol. As it turns out, 3-MCPDE and GE are predominantly found in refined vegetable fats and oils which

can further contaminate other food products which uses contaminated vegetable fats and oils (EFSA, 2013; MacMahon et al., 2013; Weißhaar, 2011; Zelinková et al., 2009).

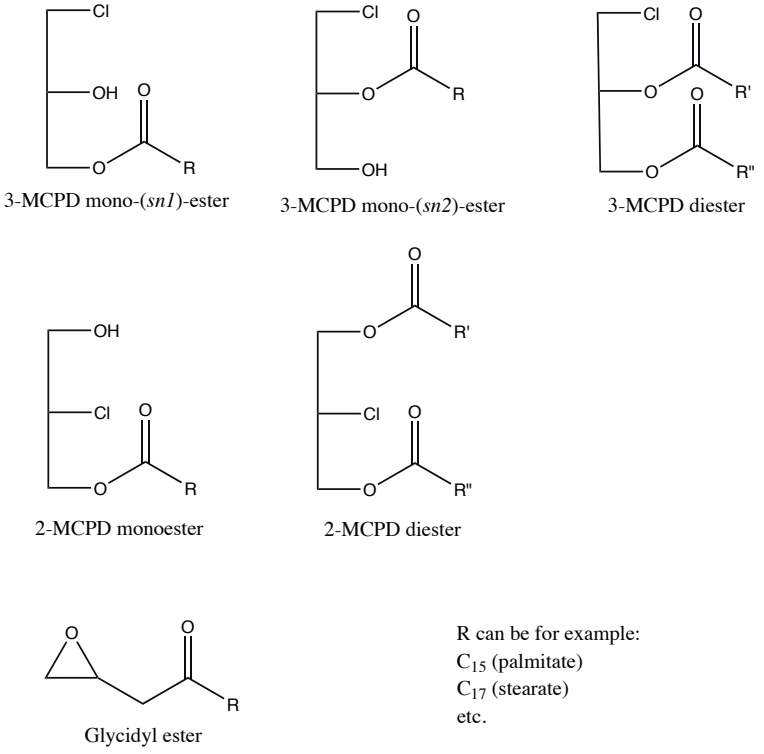

Figure1.1 Structure formula of bound chloropropanols and glycidyl ester.

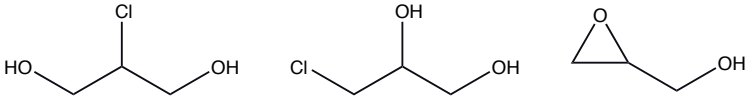

Figure1.2 Structure formula of unbound chloropropanols and glycidol.

1.4 History behind the current regulations for 3-MCPDE and GE

With the knowledge that 2-MCPDE, 3-MCPDE, and GE forms a potential health risk, EFSA published an opinion on the toxicology of 2-MCPDE, 3-MCPDE, and GE in 2016; Establishing a tolerable daily intake (TDI) of 0.8 µg/kg body weight per day for 3-MCPDE and a margin of exposure (MoE) of larger than 25.000 was considered relatively safe (EFSA CONTAM Panel, 2016). Due to the genotoxic nature of GE, the approach EFSA took was to try to minimize exposure as reasonably possible, or also known as the "as low as reasonably achievable" (ALARA) principle. One year later in 2017, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) published a provisional maximum tolerable daily intake (PMTDI) of 4 µg/kg body weight per day for 3-MCPDE (JECFA, 2017) . This discrepancy between the two TDI led to a reinvestigation by EFSA, who released a revised TDI of 2 µg/kg body weight per day for 3-MCPDE in 2018 (EFSA CONTAM Panel, 2016; Knutsen et al., 2018).

All of this has led to the implementation of MLs for 3-MCPDE and GE as of 2021 (European Commission, 2020). The most current regulations are invoked earlier this year, April 2023, and can be found in the Commission Regulation 2023/915 (European Commission, 2023). To date, no MLs has been established for 2-MCPDE nor 2-MCPD. Initially, toxicological data for 2-MCPD and 2-MCPDE were very limited, and no concrete conclusion could have been drawn on its toxicology. Based on more recent occurrence data it can be observed that the concentration of 2-MCPDE is lower than 3-MCPDE in vegetable oils and fats (Becalski et al., 2015; Kamikata et al., 2019). Therefore, an immediate ML for 2-MCPDE in vegetable oils and fats might not be needed after all. Nonetheless, many vegetable oil refineries were put up for a challenge in order to bring down the 3-MCPDE and GE concentration in their refined oil products within a relatively short period of time. This was one of the many reasons why this collaboration between our project partners, SPACK B.V., Care Naturkost GmBH, SRC B.V., and the Wageningen University were formed; Supported by the Dutch ministry of Agriculture, Nature and Food Quality.

1.5 Formation mechanism of 2-MCPDE, 3-MCPDE, and GE

Reaction mechanisms for the formation of 2-, 3-MCPDE, and GE have been proposed previously. The suggested reaction mechanisms involve the formation of an acyloxonium intermediate (Figure 1.3) (Destaillats, Craft, Sandoz, et al., 2012; Hamlet et al., 2011; Šmidrkal et al., 2016; Yao et al., 2019). GE is supposedly being formed via a slightly different mechanism. A brief summary is given below. However, these proposed

reaction mechanisms give only insight into how these esters are being formed, the addition of chlorine into the glycerol backbone, during the processing of vegetable oils.

As it is now generally known, the formation of MCPDE involves a chlorine source. It is equally important to Figure out where the majority of the chlorine comes from to get a hold of a complete picture of the formation of the MCPDE along with the formation of GE. Tiong et al. (2018) have shown that organochlorine present in crude oils can be seen as the main chlorine precursors with sphingolipid organochlorines being the major culprit. As these sphingolipids are endogenously formed by the plant itself, it might be difficult to prevent them from being formed with only chemical mitigation strategies. Nonetheless, Nagy et al. (2019) published their findings showing a lab-scale executed method for the removal of these endogenous organochlorines from crude oils, which will be discussed in more depth in this thesis.

Even though the works of Tiong et al. (2018), Šmidrkal et al. (2016), Destaillats et al. (2012), Hamlet et al. (2011), and Yao et al. (2019) have significantly helped in deducing the formation pathways of 2- and 3-MCPDE, it is still not clear how the sphingolipids organochlorine precursor reacts during oil refining. Would it be possible that the sphingolipids themselves might also act as the fat-based precursor like the proposed 1,2-DAG by Yao et al. (2019). Furthermore, it is also not clear how each oil refining process will affect the sphingolipids. The experimental approach followed in this thesis might help to better understand those processes.

In the proposed mechanism of Šmidrkal et al. (2016), sodium chloride (NaCl) contributes as the major chlorine source by first creating equilibrium between the dissociated fatty acids and the sodium chloride itself. After the dissociation of the acid, hydrochloric acid is formed which plays an important role in the protonation of the oxygen atom of the glycerol-oxo-group. The available oxygen of the hydroxyl group (or the ester) on the neighboring branch of the glycerol backbone is now able to close the 1,3-dioxolan cycle, while water is being eliminated. Finally, 3-MCPDE can be formed when the chlorine anion performs a nucleophilic substitution on the less sterically hindered carbon atom in its open Configuration. The formation of 2-MCPDE is expected to be more difficult than 3-MCPDE. For 2-MCPDE to be formed, the chlorine anion has to attack the more sterically hindered carbon atom (in the open Configuration). Despite the less favorable route, 2-MCPDE is nevertheless being formed together with 3-MCPDE out of monoacylglycerol (MAG), diacylglycerol (DAG), and triacylglycerol (TAG).

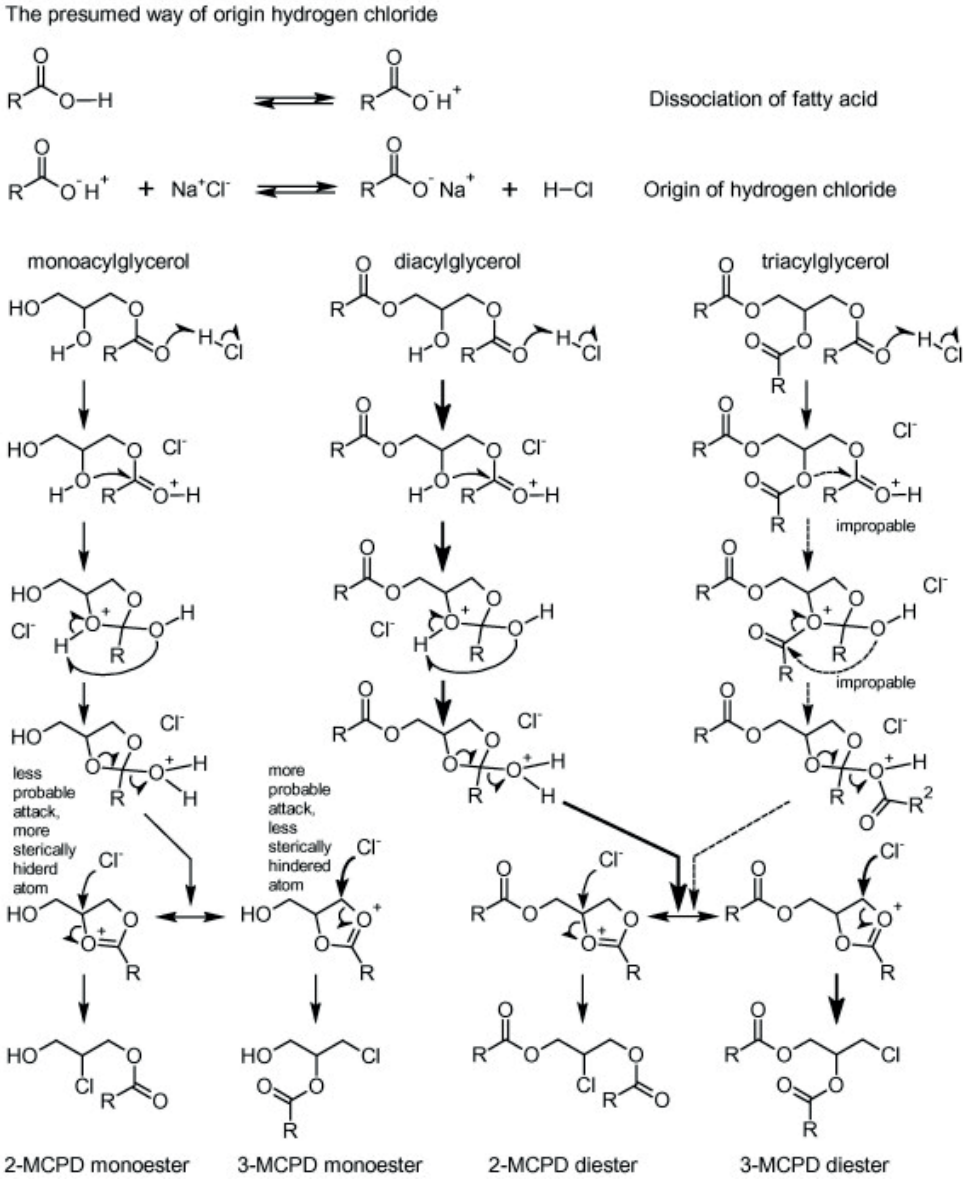

Figure 1.3 Possible formation mechanism of 2-MCPDE and 3-MCPDE (the latter part of the figure) in the presence of HCl with NaCl as its possible origin (first part of the figure). The formation of 3-MCPDE is more favorable because of the less sterically hindered carbonyl atom (next to the positively charged oxygen) on which a chlorine anion can perform a nucleophilic substitution. Figure reproduced from Šmidrkal et al., 2016.

Recently, four proposed formation mechanisms of GE were reviewed by Cheng et al. (2017). 1,3-diacylglycerols (1,3-DAGs), 1,2-diacylglycerols (1,2-DAGs), 1-monoacylglycerols (1-MAGs), and 2-monoacylglycerols (2-MAGs) are proposed to play a key role in the formation of GE as its precursor molecule. On their turn, DAGs and MAGs can be formed when triacylglycerol undergoes hydrolysis or (thermal)decomposition (Figure. 1.4B). 1,2-DAGs and 1,3-DAGs are proposed to form GE via a direct intramolecular rearrangement, resulting in the formation of an Acyloxonium ion, followed by the elimination of fatty acid. This pathway is supported by early studies performed by Velisek et al. (2002) and Destaillats et al. (2012) (Figure 1.4A path b+b'). Furthermore, 1,2-DAGs can follow a different pathway to form GEs via the formation of a cyclic acyloxonium ion (Figure 1.4A, path d). This occur when 1,2-DAGs undergo deacidification (elimination of a fatty acid). This cyclic acyloxonium can also be formed from 1-MAGs and 2-MAGs via a dehydration reaction (Figure 1.4A, path c+c'). Recent detection and confirmation of the cyclic acyloxonium ion by Cheng et al. (2016) provide evidence for the formation of GEs via the formation of a cyclic acyloxonium intermediate. The last pathway considers the formation of GEs from 1-MAGs via a similar reaction as applied for the formation of acyloxonium ions from 1,2-DAGs and 1,3-DAGs. Instead of the elimination of fatty acid after a direct intramolecular rearrangement, water is eliminated, resulting in the formation of an Oxonium ion. Hamlet et al. (2011) has proposed this pathway in their publication. Recently, Yao et al. (2019) have proposed five different reaction pathway (Figure 1.5) for the formation of 3-MCPDE that may occur during processing. As 1,3-diacylglycerols are found to be less common in vegetable oils, 1,2-diacylglycerols are assumed to play a major role as a fat-based precursor where 3-MCPDE might originate from. The five pathways proposed by Yao et al. (2019) all starts with 1,2-DAG as precursor.

These formation pathways are part of the fundamental knowledge that is required to develop mitigation strategies to reduce the concentration of 2-MCPDE, 3-MCPDE, and GE. As these formation pathways focuses more on the aspects of the addition of chlorine into the glycerol backbone of the mono- and di-esters, this thesis will contribute and expand the current established knowledge of the formation of 2-MCPDE and 3-MCPDE by exploring the origin of the required chlorine source; More specifically the organochlorine-containing precursors. As part of the bigger picture, mitigation strategies to reduce 2-MCPDE, 3-MCPDE, and GE concentration in vegetable oils and fats should not only be limited at the refinery level, but expanded towards the source itself, the palm tree itself.

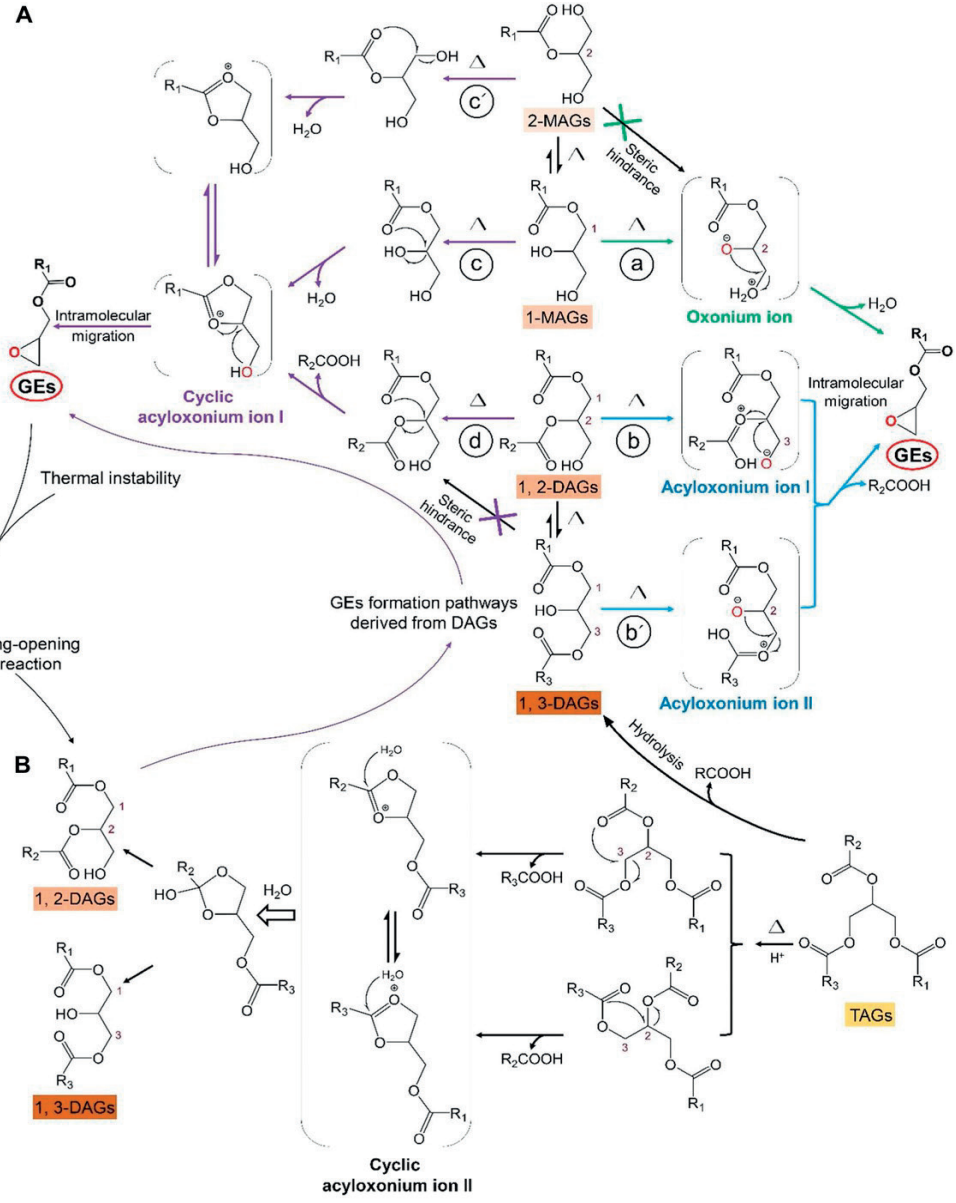

Figure 1.4 (A) Schematic overview of the four proposed pathways (a, b+b′, c+c′, and d) for the formation of GEs and (B) high temperature decomposition of triacylglycerols leading to the formation of DAGs and MAGs. R1, R2, and R3 are fatty acyl groups. Figure reproduced from Cheng et al., 2017.

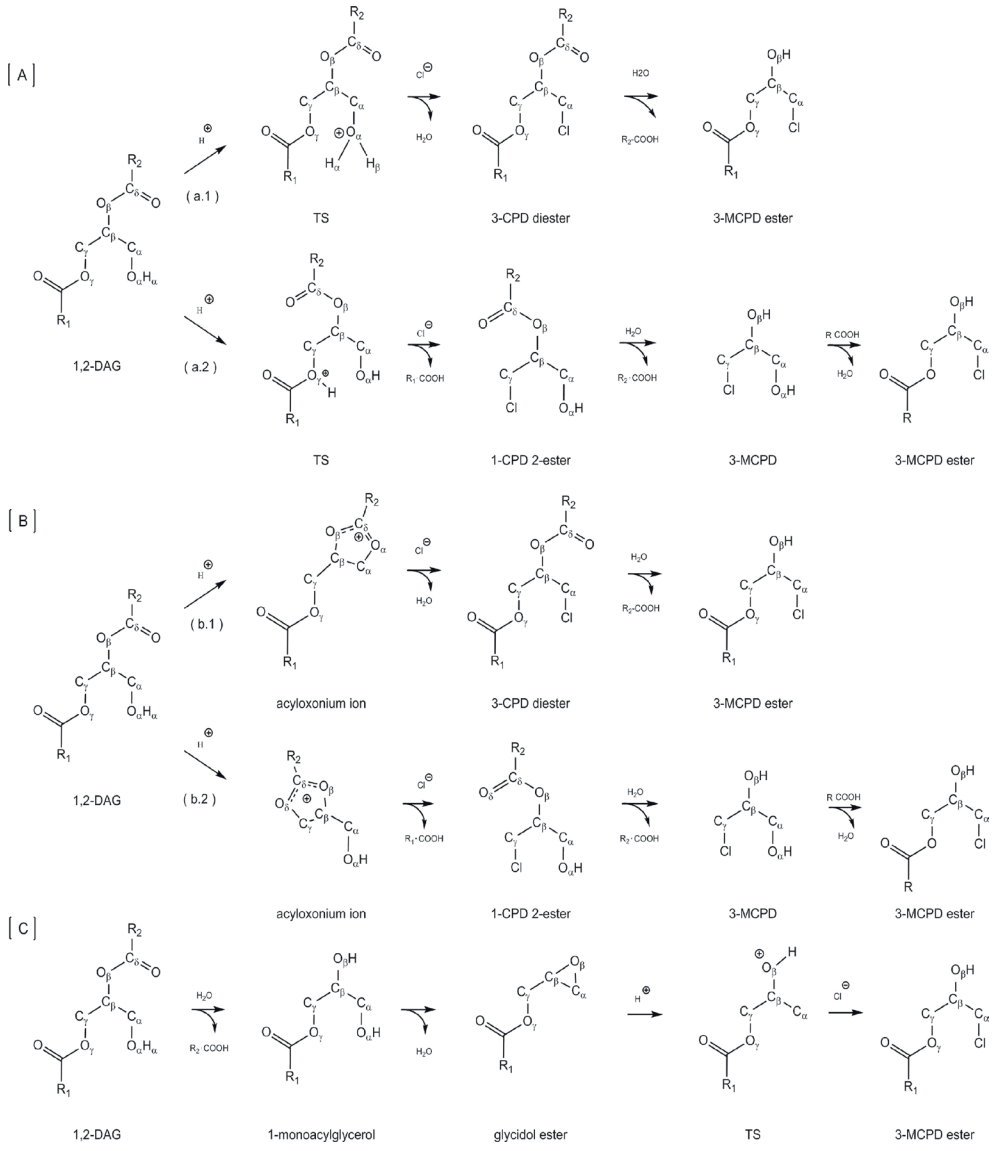

Figure 1.5 Five possible reaction pathways of the formation of 3-MCPDE from 1,2-diacylglycerol in vegetable oils as proposed by Yao et al. (2019). Figure reproduced from Yao et al., 2019.

1.6 Outline of this thesis

Considering that 2-MCPDE, 3-MCPDE, and GE are process contaminants and the implemented MLs for the industry, there is a large demand for mitigation strategies in order to bring the concentration of these process contaminants under control in vegetable oils and fats. This thesis focuses on the development of mitigation strategies which are tested on organic palm oil. Figure 1.6 shows a schematic representation of the main aim of the individual chapters within this thesis. Chapter 2 provides a comprehensive review of available, peer-reviewed, mitigation strategies to reduce the concentration of 2-MCPDE, 3-MCPDE, and GE in vegetable oils and addresses the knowledge gap in the field. Chapter 3 covers the initial attempts and development of effective mitigation strategies based on the principles of physical oil refining. Several treatments were compared against each other to gain a better understanding of the effect of certain parameter changes during oil refining. The effect of temperature at the different oil refining stages were explored. In continuation, Chapter 4 exhibits the development and results of a series of mitigation strategies based on the chemical refining principles or also known as alkali refining. Wash cycles of the crude palm oil at different stadia were compared and evaluated to see whether chlorine salts and hydrophilic chlorine containing molecules could be removed prior to refining. Finally, the effect that the deodorization temperature has on the formation of 2-MCPDE, 3-MCPDE, and GE during the alkali refining was monitored closely. Both the experiments described in Chapters 3 & 4 were performed using a pilot plant for a greater simulation of the physio-chemical behavior of the oil. Chapter 5 expands the current known formation mechanism of 2-MCPDE and 3-MCPDE, as described here before, by observing the kinetical behavior of endogenous organochlorine-containing molecules which may act as potential precursors and a chlorine donor. Palm oil refining was simulated on a small-scale in the laboratory, allowing for the collection of a massive number of samples over time during the various pre-treatment steps. Chapter 6 contains a general review and overarching summary of the work presented in this thesis. Future perspectives aiming towards an even lower concentration of 2-MCPDE, 3-MCPDE, and GE in vegetable oils, in combination with the ability to develop mitigation strategies that can be universally applied on a multitude of vegetable oils and fats are discussed as well.

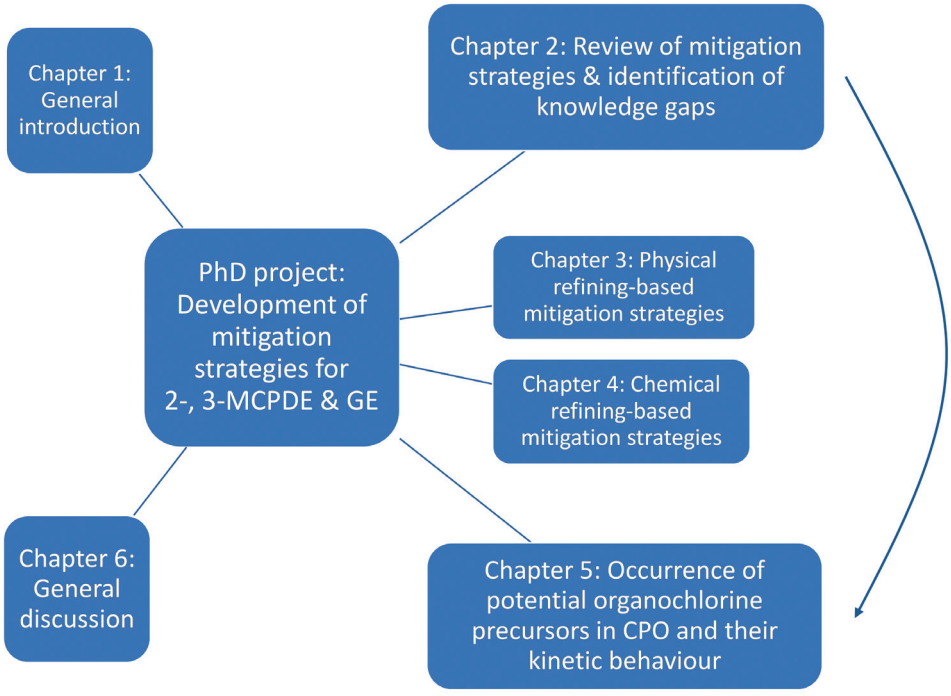

Figure 1.6 Schematic representation of the PhD project with the main aim of the individual chapters.

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Chapter 2

Mitigation Strategies for the Reduction of 2- and3-MCPD Esters and Glycidyl Esters in the Vegetable Oil Processing Industry

Adapted from:
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Abstract

The refining of vegetable oils leads to the formation of 2- and 3-monochloropropane-1,2-diol esters (2- and 3-MCPDE), and glycidyl esters (GE). A literature review was performed aiming to provide up-to-date knowledge on mitigation strategies during oil refining that can reduce the formation of these three processing contaminants. The review used the database Scopus and covered the period from 2009 to 2017. Most of the 18 papers dealt with palm oil and two papers with vegetable oil. Most studies focused on 3-MCPDE, some on GE, and none on 2-MCPDE.Water degumming was able to reduce the concentrations of 3-MCPDE by 84% and GE by 26%. Neutralization of the oil reduced concentrations of 3-MCPDE by 81% and GE by 84%. Bleaching with synthetic magnesium silicate reduced the 3-MCPDE concentration by 67%. For the deodorization step, several mitigation strategies, such as double-deodorization, the addition of various antioxidants, or a longer deodorization time, can reduce the formations of 3-MCPDE by 82% and GE by 78%. Post refining mitigation, including the use of absorbents, enzymes, or rebleaching of the oil, has also been reported to produce desirable contaminant reduction. Post refining treatment with calcinated zeolite was able to reduce the 3-MCPDE concentration by 19% and the GE concentration by 77%. Applying combined mitigation strategies to multiple steps of oil refining is likely crucial in order to adequately reduce levels of3-MCPDE and GE.

Keywords: 3-monochloropropane-1,2-diol esters, glycidyl esters, oil refining, processing contaminants, vegetable oil

2.1. Introduction

Mounting evidence regarding the toxicity of 2- and 3-monochloropropane-1,2-diol esters (2- and 3-MCPDE) and glycidyl esters (GE) in food products suggests the urgency to limit the formation of these process contaminants in the edible oil industry. Their reduction has become crucial given the maximum limits (MLs) for GE in vegetable oils recently set by the European Commission (European Commission, 2018). The ML for GE in vegetable oils and fats used for the consumer market or as ingredients in food preparation has been set at 1000 µg/kg and as low as 500 µg/kg when used for the production of baby food and processed cereal-based food for infants and young children. Unbound 3-MCPD and glycidol both are marked as carcinogenic compounds by the World Health Organization–Intl. Agency for Research on Cancer (World Health Organization, 2000, 2013). Studies with rats have shown that 3-MCPD is able to induce nephrotoxicity (Bakhiya, Abraham, Gürtler, Appel, & Lampen, 2011). Glycidol is known for its carcinogenicity because it has been shown to induce tumor growth in various organs of rodents (Habermeyer, Guth, & Eisenbrand, 2011). Currently, there is insufficient data available on toxicity to draw conclusions on the toxicity of 2-MCPD. These three process contaminants are most abundant in refined oils, including palm oil and refined olive oil. (Hrncirik & van Duijn, 2011; Jedrkiewicz, Głowacz, Gromadzka,& Namie, 2016; Weißhaar & Perz, 2010). Important factors leading to the formation of these compounds in oils include prolonged exposure to high temperatures during the refining process such as during deodorization, and also the chloride content, and the acidity of the oil (Destaillats, Craft, Dubois, & Nagy, 2012; Franke,Strijowski, Fleck, & Pudel, 2009; Smidrkal et al., 2016). Destaillats, Craft, Sandoz, and Nagy (2012), Smidrkal et al. (2016), and Hamlet et al. (2011) proposed a possible reaction mechanism for the formation of 2-, 3-MCPDE, and GE (Destaillats et al., 2012; Hamlet et al., 2011; Smidrkal et al.,2016). The proposed mechanism involves the incorporation of chlorine in the glycerol backbone of mono- and diacylglycerols via formation of an acyloxonium intermediate. Triacylglycerols are known to be indirectly involved in the formation of 2- and 3-MCPDE because they can be converted into diacylglycerols and monoacylglycerols by chemical conversion (under acidic or basic condition), enzymatic conversion, or heat decomposition. Like 2- and 3-MCPDE, GE can be formed from diacylglycerols and monoacylglycerols (Cheng, Liu, Wang, & Liu, 2017). However, GE can also be formed from both 2- and 3-MCPDE under certain temperature, time, and pH conditions, and this pathway of GE formation is reversible. Cheng et al. (2017) and Shimizu et al. (2012) have explained this reversible conversion of 2- and3-MCPDE into GE in more detail. Various food products containing refined oils were shown to have 2-, 3-MCPDE, and/or GE concentrations of 1 mg/kg oil or more (European Food Safety Authority, 2013; Hamlet et al.,2011). Several studies have shown that 3-MCPDE and GE concentrations in infant formula can reach ≥1mg/kg fat, and that 2-MCPDE concentrations can reach half of that amount (Leigh & MacMahon,

2017; Wöhrlin, Fry, Lahrssen-Wiederholt, & Preiß-Weigert, 2015). The application of different mitigation strategies at the various steps of vegetable oil and fat production to limit the formation of these process contaminants is therefore a high priority of many research groups and the food industry. The strategies explored so far have focused on different (sub)processes of oil production, from the palm plantation, through novel oil refining methods, up to methods designed specifically to break down 2-,3-MCPDE, and GE after the refining process (Matthaus & Pudel, 2013). Although the mitigation strategies developed to date show promising results, current knowledge remains limited and scattered, as discussed below. In the last five years, several reviews of GE and 3-MCPD have been published, of which two either focused on GE esters or (unbound) 3-MCPD, and two on both of these compounds. All four of these reviews covered the subject from a broad perspective and did not provide in-depth knowledge about the effectiveness of mitigation strategies to limit the formation of 2-, 3-MCPDE, and/or GE. Additionally, the discussed mitigation strategies are mostly limited to either 3-MCPDE or GE. However, mitigation strategies should consider all three contaminants at the same time, because formation of the three compounds are related to each other, and the three compounds often are found together in refined oils. Data on mitigation strategies of 2-MCPD are very limited. This lack of research data on 2-MCPD and its esters is reflected in the aforementioned reviews and in the European Commission's regulations, in which no MLs for 2-MCPD and/or2-MCPDE have been established to date. This review aims to provide up-to-date insights into the mitigation strategies for the formation of 2-, 3-MCPDE, and GE during vegetable oil processing, based on scientific literature. The current state-of-the-art knowledge on potential prevention and control strategies during oil production is provided, as well as gaps in the data. The results presented will facilitate further research on the development of an optimal mitigation strategy. Due to the narrow and precise scope of this paper, the mechanisms underlying formation, toxicity, and occurrence of the three process contaminants are not covered.

2.2. Materials and Methods

A literature review was conducted using the online database Scopus. Articles from 2009 to 2017 were included in the selection. The search terms were are as follows: TITLE-ABS-KEY(monochloro∗OR MCPD∗OR glycid∗lAND oil OR∗olein AND mitigat∗OR reduc∗OR remov∗OR degumm∗OR neutrali∗OR wash∗OR bleach∗OR deodori∗OR refin∗) These search terms were assembled in order to cover all three process contaminants (2-MCPD, 3-MCPD, and glycidol in both the esterified and unbound forms) at once. The second part of the search was aimed at publications about mitigation strategies during oil refining, including those for individual refining stages. Additional subject area selection was performed, as the search terms included articles from irrelevant subject areas

such as dentistry, nursing, immunology, or microbiology. The following subject areas were excluded from the selection: environmental science, pharmacology, toxicology and pharmaceutics, energy, dentistry, immunology and microbiology, physics and astronomy, nursing, social sciences, business, management and accounting, computer science, mathematics, and psychology. This resulted in 116 hits. Applying more filters or making the search terms more specific could result in unwanted elimination of relevant publications. For this reason, the 116 hits were manually selected for their relevance to this review by screening their titles and abstracts. For example, publications about analytical techniques to determine the concentration of only GE or 3-MCPDE, publications about risk assessment, or publications about a completely different subject were excluded from the final selection.

2.3. Results and Discussion

2.3.1. General review results

In total, 18 relevant papers were obtained. Seventeen papers focused on mitigation strategies for one or more of the three contaminants considered during one or multiple stages of vegetable oil refining. From these, 16 papers dealt with palm oil, and the remaining two did not refer to palm oil but more generally to vegetable or edible oils. This preference towards palm oils is because they contain the highest concentration of 3-MCPDE and related compounds, thus there is an acute need for reliable mitigation strategies for this type of oil. Mitigation strategies for the degumming step were covered in six papers; for the neutralization step in four papers; for the bleaching step in five papers; and for the deodorization step in eight papers. Five additional papers provided alternative insights into the ways of reducing contaminants by means of post refining removal. Finally, one of the five post refining papers described a model that tested the combination of multiple mitigation strategies. The total sum of the articles dis-cussed in the specific section regarding refining stages is greater than the 18 cited because multiple articles covered more than one mitigation strategy. Therefore, such articles are discussed twice in the corresponding and relevant sections. In the current review, mitigation strategies designed for any other vegetable oils, except palm oils, are specifically addressed. The level of detail presented in the methods and results sections varied among the papers selected. To present all results in a comparable and uniform way, it was occasionally necessary to perform additional calculations based on the original data in some papers. If contaminant reduction was presented only as concentration, we expressed the associated relative reduction as a per-centage. In another case, data were presented in bar graphs, and we estimated the percentage of contaminant reduction from these graphs.

2.3.2. Degumming

Gums (hydratable and nonhydratable phospholipids) and other phosphorus-containing compounds are removed during degumming. Oils high in gum concentration, including sunflower seed oil and rapeseed oil, are usually treated with a water degumming method to remove the water-soluble gums. Gibon, De Greyt, and Kellens (2007) reported that palm oil is usually low in phospholipid and phosphorus contents (10 to 20 ppm) (Gibon et al., 2007). Therefore, palm oils are usually degummed with either phosphoric acid or citric acid. This so-called "dry degumming" results in lower oil losses as compared to water washing. However, water degumming may be more beneficial than dry degumming in terms of lowering the 2- and 3-MCPDE concentrations. As mentioned by Destaillats et al. (2012), Smidrkal et al. (2016), and Hamlet et al. (2011), the formation of 2- and 3-MCPDE requires a chlorine source that easily could dissociate to create the required chloride ions. Water degumming can be seen as a washing step with water. During this step, chlorine containing polar precursors of 2- and3-MCPDE can be removed from the oil. Therefore, the elimination of chlorine sources can be an effective method to reduce the formation of 2- and 3-MCPDE. The results of mitigation strategies involving various degumming methods are presented in Table 2.1.

Out of the five studies, only one analysed GE and proposed a mitigation strategy for this compound during degumming. Matthäus, Pudel, Fehling, Vosmann, and Freudenstein (2011) reported a strategy to reduce 3-MCPDE and GE in palm oil. In this case, the crude palm oil (CPO) was washed with water prior to a simulated deodorization step. They achieved a reduction of 25% (from 2.8 to 2.1 mg/kg) for 3-MCPDE, and 16% (from 3.5 to3.0 mg/kg) for GE in comparison with unwashed CPO. Improved results were obtained by washing the CPO with 75% ethanol; in this case, 3-MCPDE was reduced by 36%, from 2.8 to1.8 mg/kg, and GE was reduced by 26%, from 3.5 to 2.6 mg/kg (Matthäus et al., 2011). However, 2 years later, Matthäus and Pudel (2013) reported a reduction of 38% of 3-MCPDE by a water wash step. In another study by Pudel et al. (2011), the water degumming step was compared to various water degumming methods using different water percentages, such as water degumming followed by a 0.2% phosphoric acid or 0.3% citric acid treatment. The best reduction was achieved when the oil was washed with 5% deionized water only, reducing the combined concentration of 3-MCPDE and related compounds (mainly GE) by 64% (from 5.5 to 2.0 mg/kg) compared with unwashed raw palm oil (Pudel et al., 2011). Ramli et al. (2011) noted a marked reduction in 3-MCPD concentration between degumming with (plain) water compared with 0.02% phosphoric acid solution. When 0.02%phosphoric acid was used, 2.1 mg/kg 3-MCPDE was found in the oil, while using water with no added acids reduced the 3-MCPDE concentration to 0.75 mg/kg (64% reduction) (Ramli et al., 2011).

Table 2.1 Effectivity of various degumming conditions for the mitigation of 2-, 3-MCPD esters, and Glycidyl esters.

Degumming conditions	Reduction (%) [actual concentrations in mg/kg]			References
	3-MCPD esters	2-MCPD esters	Glycidyl esters	
Water wash (ND[1])	38% [ND]	ND	ND	Matthäus and Pudel (2013)
Deionized water wash (2% w/w); 80-85 °C; 15 min.	64% [2.1 -> 0.75]	ND	ND	Ramli et al. (2011)
Water wash (5% w/w)	80% [1.0 -> 0.2]	ND	ND	Zulkurnain et al. (2013)
Water wash (1% w/w); 70 °C; 20 min.	84% [9.79 -> 1.55]	ND	ND	Zulkurnain et al. (2012)
Water wash (ND)	25% [2.8 -> 2.1]	ND	16% [3.5 -> 3.0]	Matthäus et al. (2011)
75% Ethanol wash	36% [2.8 -> 1.8]	ND	26% [3.5 -> 2.6]	Matthäus et al. (2011)
Deionized water wash (5 % w/w)	64% [5.5 -> 2.0] Incl. related compounds[2]	ND	*Combined with 3-MCPDE*[2]	Pudel et al. (2011)

1. ND: not defined
2. The concentration of glycidyl esters has been summed together with the 3-MCPD esters

Zulkurnain et al. (2012) reported an even larger re-duction of 3-MCPDE using water degumming with 1% water at 70 °C for 20 min. The 3-MCPDE concentrations were reduced from 9.8 to 1.6 mg/kg (reduction of 84%) in comparison with acid degumming with 0.05% phosphoric acid. This might explain the large difference in 3-MCPD ester reduction, since bleaching can also lower the 3-MCPDE concentration (as discussed below). Zulkurnain, Lai, Tan, Abdul Latip, and Tan (2013) published a physical refining process for the reduction of 3-MCPDE. They reported a reduction of 80% when using 5% water to degum the oil without the addition of any acids (from 1.0 mg/kg for 0 %water to 0.2 mg/kg for 5% water) (Zulkurnain et al., 2013). This can be explained by the removal of 3-MCPDE precursors by water. Based on the presented data, water degumming was shown to be effective in reducing the concentrations of 3-MCPDE and GE during the degumming process. However, Zulkurnain et al. (2012) and Ramli et al. (2011) noted that the quality of the oil, as defined by the terms refined, bleached, and deodorized (RBD), might be also affected by water degumming. The colour of the oil[1] is one of the quality parameters that are monitored by re-fineries and suppliers. Ramli et al. (2011) observed strong red colouring (3.5 Red on the RYBN colour scale) of their final

1 A standardized colour chart or a series of colour filter combinations can be used to help identify and compare the colour components. One of the most popular colour scales used to compare oils and fats is the LovibondR© RYBN colour scale (The Tintometer Ltd, 2016). RYBN stands for red, yellow, blue, and neutral, with a corresponding range of 0 to 70 for red, 0 to 70 for yellow, 0 to 40 for blue, and 0 to 3.9 for neutral. Increasing numbers on the scale correlate with darker and more intense tints of that particular colour.

product, together with a less efficient removal of phosphatides and iron in comparison with their standard procedure. Zulkurnain et al. (2012) reported a stronger red colour in the range of 4.0 to 5.9 in RBD palm oil (Zulkurnain et al., 2012). Gibon et al. (2007) mentioned that incomplete removal of phosphatides during the degumming step may result in oil darkening during deodorization, which negatively impacts the oil flavour (Gibon et al., 2007). Interestingly, a reduction of red colouring (approximately 2.0 Red) can be achieved by using a larger amount of water (from 1% to 5% water) to degum the oil (Zulkurnain et al., 2013). In conclusion, water degumming can effectively reduce GE and 3-MCPDE concentrations, with 3-MCPDE concentrations reduced to <1 mg/kg. Unfortunately, not all the papers mentioned sufficient details of the applied washing conditions (that is, amount of water or time and temperature of the treatment) for a full comparison of the investigated methods.

2.3.3. Neutralization

In physical refining, FFAs are removed via strip distillation during deodorization. This is possible because FFAs are more volatile than triacylglycerols. During chemical refining, the FFAs are removed earlier in the process during alkali neutralization. This is a necessary step and is unique to chemical oil refining, and it is an effective way to neutralize most of the FFAs present in the oil. During chemical refining, FFAs are removed by converting them into soap under alkaline conditions. The produced soap stock can then be separated from the oil, removing not only the FFAs but also the residual phospholipids, oxidized products, and metal ions that were not completely removed during degumming. Even though most of the FFAs are removed during the neutralization step in chemical refining, a final deodorization step is still necessary to remove any remaining FFAs, fragrant molecules, and other volatile compounds.

Table 2.2 represents the results of mitigation strategies during neutralization. Ramli et al. (2011) used 0.2% calcium oxide to neutralize the 0.02% phosphoric acid. This resulted in a 3-MCPDE concentration of 1.4 mg/kg in the neutralized oil compared with a concentration of 2.2 mg/kg in the non-neutralized oil, which is a reduction of 36% (Ramli et al., 2011). Matthäus and Pudel (2013) reported similar findings. They observed a reduction of 45% and 35%, respectively, when potassium hydroxide (KOH) or sodium hydroxide (NaOH) was used for neutralization (Pudel et al., 2011). Lastly, Freudenstein, Weking, and Matthäus (2013) reported the effect of neutralization with either sodium carbonate (Na_2CO_3) or sodium hydrogen carbonate ($NaHCO_3$) in model oil that was spiked with 16 mg NaCl/g to promote the formation of 3-MCPDE and related compounds (for example, GE). Results showed that $NaHCO_3$ was more effective thanNa_2CO_3 in reducing concentrations of 3-MCPDE and related compounds. At a concentration of 5 mmol of Na_2CO_3/kg sample, a maximum reduction of 53% (from 5.9 to 2.8 mg/kg) for 3-MCPDE and 69% (from 7.0 to 2.2 mg/kg) for 3-MCPDE and related compounds (combined)

was achieved. $NaHCO_3$ needed a concentration of only 1 mmol/kg sample in order to achieve an 81% reduction in the concentration of 3-MCPDE (from 5.9 to1.1 mg/kg) and an 84% reduction for 3-MCPDE combined with related compounds (7.0 to 1.1 mg/kg) (Freudenstein et al., 2013).The highest reduction in 3-MCPDE and GE concentrations was obtained when $NaHCO_3$ was used to neutralize the oil after degumming. However, the observed reduction was obtained in a model oil spiked with chlorine to facilitate the formation of 2- and 3-MCPDE. The effect of KOH or NaOH addition has been tested in palm oils and, even though the observed reduction was lower compared to using $NaHCO_3$, the reduction was still considerable. Such levels of reduction can be relevant when combinations of mitigation strategies are needed to achieve the desired reduction of esters. Although an alkali neutralization step is not required when physical refining is applied, it is still useful to have it in the refining process. Any acids present in the oil, either naturally or introduced during the degumming step, must be neutralized prior to deodorization, as neutral oils tend to develop less 3-MCPDE.

Table 2.2 Effectivity of various neutralization conditions for the mitigation of 2-, 3-MCPD esters, and Glycidyl esters

Neutralization conditions	Reduction (%) [actual concentrations in mg/kg]			References
	3-MCPD esters	2-MCPD esters	Glycidyl esters	
45% KOH	45% [ND[1]]	ND	ND	Matthäus and Pudel (2013)
35% KOH	35% [ND]	ND	ND	Matthäus and Pudel (2013)
1 mmol/kg $NaHCO_3$	81% [5.9 -> 1.1]	ND	84% [7.0 -> 1.1] (3-MCPDE and related compounds)	Freudenstein et al. (2013)
5 mmol/kg Na_2CO_3	53% [5.9 -> 2.8]	ND	69% [7.0 -> 2.2] (3-MCPDE and related compounds)	Freudenstein et al. (2013)
ND% KOH	45% [5.5 -> 3.0] Incl. related compounds[2]	ND	*Combined with 3-MCPDE*[2]	Pudel et al. (2011)
ND% NaOH	35% [5.5 -> 3.5]	ND	ND	Pudel et al. (2011)
0.2% CaO	36% [2.2 -> 1.4]	ND	ND	Ramli et al. (2011)

1. ND: not defined
2. The concentration of glycidyl esters has been summed together with the 3-MCPD esters

2.3.4. Bleaching

Treatment of oil with bleaching clay is used, in addition to thermal degradation, to remove coloured pigments such as carotenes. Bleaching clay is porous, and colour pigments are physically entrapped into the clay. Pigments can also be adsorbed onto bleaching clay by van der Waals forces and can be chemically bound via ionic or covalent bonds (Gibon et al., 2007; Silva et al., 2014). In addition to the removal or reduction of pigments from oil, bleaching also removes other impurities such as lipid oxidation products and metals (Gibon et al., 2007). Heat treatment and bleaching are used in combination because the bleaching step alone is not able to remove colour pigments completely. Most pigments are not heat-stable and further removal can be achieved by degradation during the subsequent deodorization step. The results of the mitigation strategies during bleaching are presented in Table 2.3.

Table 2.3 Effectivity of various bleaching conditions for the mitigation of 2-, 3-MCPD esters, and Glycidyl esters

Bleaching conditions	Reduction (%) [actual concentrations in mg/kg]			References
	3-MCPD esters	2-MCPD esters	Glycidyl esters	
Pre-refined palm oil; Tonsil optimum 214 FF (1%); 10 mbar; 90 °C; 20 min.	59% [6.06 -> 2.48]	ND[1]	ND	Franke et al. (2009)
Crude palm oil; Tonsil optimum 214 FF (1%); 10 mbar; 90 °C; 20 min.	4% [1.04 -> 1.00]	ND	ND	Franke et al. (2009)
Acid degum; neutral clay (1%); 50 mbar; 105 - 110 °C; 30 min.	22% [2.82 -> 2.21]	ND	ND	Ramli et al. (2011)
Water degum; neutral clay (1%); 50 mbar; 105 - 110 °C; 30 min.	46% [0.91 -> 0.49]	ND	ND	Ramli et al. (2011)
Wet bleaching: Taiko Supreme 1B activated clay (0.5%) & Magnesol R60 slurry (20% w/w); 90 °C; 30 min.	41% [0.39 -> 0.23]	ND	ND	Zulkurnain et al. (2013)
Acid activated clay (1%); under vacuum; 95 °C; 30 min.	11% [1.75 -> 1.55]	ND	ND	Zulkurnain et al. (2012)
Magnesol R60 (1%); under vacuum; 95 °C; 30 min.	67% [1.55 -> 0.51]	ND	ND	Zulkurnain et al. (2012)
Tonsil 4191 FF (1%); 60 °C	45% [5.5 -> 3.0] Incl. related compounds[2]	ND	*Combined with 3-MCPDE*[2]	Pudel et al. (2011)

1. ND: not defined
2. The concentration of glycidyl esters has been summed together with the 3-MCPD esters

Franke et al. (2009) focused on 3-MCPDE concentrations in crude and prerefined palm oil and in crude rapeseed oil. Before and after every refining process (including degumming, neutralization, washing, drying, and bleaching), they analyzed the 3-MCPDEconcentration in each of the three oil samples. Most notable was the decrease in 3-MCPDE concentration after the bleaching process in the prerefined oil. Right before the bleaching step, the prerefined palm oil had a 3-MCPDE concentration of 6.06 mg/kg, while it was 2.48 mg/kg after bleaching, which is a decrease of 59% (Franke et al., 2009). In the CPO, the decrease was negligible. Right before the bleaching step, the oil contained a 3-MCPDE concentration of 1.04 mg/kg, which was reduced to 1.00 mg/kg after the bleaching process (Franke et al., 2009). The 3-MCPDE concentration in the rapeseed oil was below the detection limit (0.4 mg/kg) throughout the entire refining process, except for the final product (after deodorization), which had a concentration of 1.04 mg/kg (Franke et al., 2009). As a bleaching agent, Franke et al. (2009) used 1% Tonsil optimum 214 FF bleaching earth, which is a commercially available as acid-activated calcium bentonite. It can not only adsorb polar compounds, including peroxides and phospholipids, but also carotenoids, which makes it suitable for refining vegetable oils. The suffix "FF" stands for fast filtration, indicating a uniform grain-size distribution. Similar to Franke et al. (2009), Pudel et al. observed a marked reduction of3-MCPDE concentration by 45% (5.5 to 3.0 mg/kg) when the oil was bleached with 1% Tonsil 4191 FF bleaching earth (Pudel et al., 2011). Ramli et al. (2011) compared acid-activated bleaching clay and natural bleaching clay in combination with water degumming. In their control, natural bleaching clay resulted in an average concentration of 2.2 mg/kg 3-MCPDE, while acid-activated clay resulted in a concentration of 2.8 mg/kg. In combination with water degumming, the acid-activated bleaching clay was able to further reduce the 3-MCPDE concentration to 0.91 mg/kg (68% less than the control). The use of neutral bleaching clay, on the other hand, resulted in a greater reduction of the 3-MCPDE concentration to 0.49 mg/kg (78% less than the control) (Ramli et al., 2011). The positive effect of the neutral clay, in comparison with the acid-activated bleaching clay, was also greater in the water-degummed oil (22% reduction in the control compared with 46% reduction in the water-degummed oils). The observed differences between the acid-activated and neutral clay originated from the clay being activated by the addition of hydrochloric acid or phosphoric acid in order to increase its surface area (Ramli et al., 2011; Taylor, 2009). Residual acids, especially hydrochloric acid, in the bleaching clay may act as an external chloride source, promoting the formation of 3-MCPDE (and possibly 2-MCPDE) (Destaillats et al., 2012; Matthäus et al., 2011; Smidrkal et al., 2016). Therefore, activation of bleaching clay by hydrochloric acid should be avoided. Zulkurnain et al. (2012) also tested the usage of neutral clay, but with different results. They observed that neutral clay resulted in a slightly higher 3-MCPDE concentration than acid-activated clay. When neutral clay was used, the 3-MCPDE concentration peaked at 1.75 mg/kg, as compared to 1.55 mg/kg for use of acid-activated clay (Zulkurnain et al., 2012). This result is

the opposite of those reported in a study by Ramli et al. (2011), in which neutral clay performed better than acid-activated clay. However, Ramli et al. (2011) used a different type of clay. Thus, no conclusions regarding the use of neutral compared with acid-activated bleaching clays can be made. Zulkurnain et al. (2012) used magnesium silicate (MagnesolR60) as a bleaching agent because it has a large surface area and the most active basic sites compared to alumina, silica, and activated carbon (Zulkurnain et al., 2012). Using 1% MagnesolR60 as bleaching agent resulted in a reduction of 3-MCPDE concentration to 1.55 mg/kg, while acid-activated clay reduced3-MCPDE concentration to 0.51 mg/kg. This is a further improvement of 67%. Zulkurnain et al. (2013) also explored effects of several combinations of bleaching methods using magnesium silicate, which acts as an auxiliary adsorbent for chloroester precursors. During the wet bleaching experiments, they combined1% of a 20% w/w magnesium silicate (Magnesol R60) slurry alone, and in combination with 0.5% Taiko Supreme 1B-activated clay (added before and after the magnesium slurry). The setup of the experiments for wet bleaching was similar to the setup for the dry-bleaching experiments, but they also used a mixture of magnesium silicate and activated clay. Wet bleaching initiated with activated clay, followed by use of magnesium silicate, resulted in the lowest3-MCPDE concentration (0.23 mg/kg) (Zulkurnain et al.,2013). However, for all four dry-bleaching conditions the FFA concentration observed was the highest (0.10% FFA). However, this elevated FFA value did not exceed the maximum FFA limit (also 0.10%) set by the Palm Oil Refiners Association of Malaysia (PORAM) to be qualified as "good" RBD/neutralized, bleached, and deodorized palm oil (Malaysian Palm Oil Board, 2000). Finally, although the 3-MCPDE concentrations were higher in the dry-bleached palm oils, Zulkurnain et al. (2013) suggested dry bleaching with a mixture of magnesium silicate and activated clay as the preferred method because it resulted in a relatively low 3-MCPDE concentration in combination with satisfactory colour removal.

2.3.5. Deodorization

2.3.5.1. Temperature and duration effects.

The deodorization step is the most critical for the formation of GE, which is temperature dependent; GE concentration increases almost exponentially when oils are exposed to temperatures >230 °C to 250 °C for a prolonged period (>1 hour) (Craft, Nagy, Seefelder, Dubois, & Destaillats, 2012; Hrncirik & van Duijn,2011; Pudel et al., 2011). Note that 3-MCPDE is formed rapidly at lower temperatures, as low as 175 °C, while itis maintained at the same level with increasing temperatures. Deodorization conditions vary among refining plants and according to the quality of the crude oil. The most important deodorization parameters are usually reduced pressure (1 to 5 torr), duration (0.5 to 3 hr), and temperature (200 °C to 240 °C). The application of mild deodorization conditions is a valuable mitigation strategy. The results of mitigation strategies during deodorization are shown in Table 2.4.

Table 2.4 Effectivity of various deodorization conditions for the mitigation of 2-, 3-MCPD esters, and Glycidyl esters

Deodorization conditions	Reference conditions	Reduction (%) [actual concentrations in mg/kg]			References
		3-MCPD esters	2-MCPD esters	Glycidyl esters	
Chemical refining (neutralized), deodorized at 180 °C (5 hr)	180 °C (1 hr)	15% [4.8 -> 4.1]	ND[1]	0% [0.4 -> 0.4]	Hrncirik and van Duijn (2011)
290 °C (4 hr)	290 °C (2 hr)	100% [5 -> 0]	ND	83% [48 -> 8]	Pudel et al. (2011)
290 °C (6 hr)	290 °C (2 hr)	80% [5 ->1]	ND	94% [48 -> 3]	Pudel et al. (2011)
Double deodorization, 180 °C (4 hr) followed by 240 °C (1 hr)	240 °C (4 hr)	0% [1.5]	ND	17% [120 -> 100]	Shimizu et al. (2013)
Double deodorization, 240 °C (1 hr) followed by 180 °C (4 hr)	240 °C (4 hr)	0% [1.5]	ND	58% [120 -> 50]	Shimizu et al. (2013)
Double Deodorization, 200 °C (2 hr) followed by 250 °C (5 min)	250 °C (1.5 hr)	65% [2 -> 0.7]	ND	35% [2 -> 1.3]	Matthäus and Pudel (2013)
Double Deodorization, 200 °C (2 hr) followed by 270 °C (5 min)	270 °C (1.5 hr)	75% [2 -> 0.5]	ND	78% [16 -> 3.5]	Matthäus and Pudel (2013)
Addition of either ethanol or glycerol before DEO, 0.5-2.5% v/v	ND	30% [ND]	ND	ND	Matthäus and Pudel (2013)
Addition of $NaHCO_3$ or $KHCO_3$ before DEO, 1-5 mmol/kg	ND	66% [ND]	ND	66% [ND]	Matthäus and Pudel (2013)
Addition of diacetin during DEO, before evaporation, amount ND	ND	50% [ND]	ND	ND	Matthäus and Pudel (2013)
6% w/w Rosemary extracts; 240 °C (1 hr); 0.5 kPa; nitrogen strip-gas	No additives; 240 °C (1 hr); 0.5 kPa; nitrogen strip-gas	82% [2.44 -> 0.43]	ND	ND	Zhang et al. (2016)
6% w/w lipophilic tea polyphenols; 240 °C (1 hr); 0.5 kPa; nitrogen strip-gas	No additives; 240 °C (1 hr); 0.5 kPa; nitrogen strip-gas	75% [2.44 -> 0.61]	ND	ND	Zhang et al. (2016)
1.8 mg/g TBHQ in palm oil; 4% *sn*-1,2-dipalmitin; 200 °C (1 hr)	4% sn-1,2-dipalmitin; 200 °C (1 hr)	ND	ND	53% [1.7 -> 0.8]	Cheng et al. (2017)

Table 2.4 Continued.

Deodorization conditions	Reference conditions	Reduction (%) [actual concentrations in mg/kg]			References
		3-MCPD esters	2-MCPD esters	Glycidyl esters	
nitrogen atmosphere; 200 °C (1 hr)	Regular air; 200 °C (1 hr)	ND	ND	41% [1.7 -> 1]	Cheng et al. (2017)
Short-path distillation; 0.001 mbar; condenser temp 60 °C; evaporator temp 170 °C; stirrer speed 100 rpm; pump frequency 20 Hz; additional post-distillation deodorization at 180 °C (2 hr)	Regular deodorization; 260 °C (1.5 hr); 2-3 mbar	90% [3.0 -> 0.3]	ND	98% [6.4 -> *(LOD < 0.1)*]	Pudel et al. (2016)

1. ND: not defined

An example of a typical temperature-time profile of a deodorization process is shown in Figure2.1(A). Hrncirik and van Duijn (2011) reported a 3-MCPDE concentration of approximately 4.8 mg/kg in chemically refined palm oil, which had been deodorized at 180 °C for 1hr (Hrncirik & van Duijn, 2011). Extending the deodorization time from 1 to 5 hr decreased the 3-MCPDE concentration to 4.1 mg/kg in chemically refined palm oil. The GE concentration remained unchanged at 0.4 mg/kg (Hrncirik & van Duijn, 2011). Craft, Nagy, Sandoz, and Destaillats (2012) reported a considerable increase in GE concentration when they deodorized a commercially available bleached palm oil at 240 °C in comparison with both 200 °C and 220 °C. At both 200 °C and 220 °C, the concentration was 0.4 mg/kg GE, while it was 1.7 mg/kg GE at 240 °C (an increase of 76%) (Craft et al., 2012). Similar results were reported by Hrncirik and van Duijn (2011). GE concentration was increased by 76% (from 0.4 to 1.7 mg/kg) in a physically refined palm oil, which has been deodorized for 3 hr at 230 °C (Hrncirik & van Duijn, 2011). These results showed the temperature dependency of the formation of GE during deodorization. Pudel et al. (2011) performed a laboratory experiment in which palm oil was deodorized in a con-trolled environment (Pudel et al., 2011). Samples were collected at different combinations of temperatures and deodorization times, varying from 200 °C to 290 °C and from 90 to 360 min, respectively. They found approximately 2.5 mg/kg GE (expressed as 3-MCPDE and related compounds) and no 3-MCPDE in the samples that were deodorized at 240 °C for 240 and 360min (Pudel et al., 2011). The highest GE concentration was found in a sample that had been deodorized at 290 °C for 120 min (53 mg/kg 3-MCPDE and related compounds, equalling 48 mg/kg GE). The highest 3-MCPDE concentration(6 mg/kg 3-MCPDE) was found in a sample that was deodorized at 250 °C for 90 min. Notably, in the oil sample deodorized at 290 °C, but for a prolonged period of 240 and 360 min, the GE concentration decreased to 8 mg/kg and

3 mg/kg, respectively (a reduction of >83%) (Pudel et al., 2011). It was suggested that possibly some precursors had been degraded or distilled at such high temperatures.

Several authors tested double-deodorization strategies in which oil was deodorized twice, but with different temperature-time combinations, to minimize the formations of MCPDE and GE. Figures 2.1(B) and (C) illustrate the possible implementation of this approach in a typical refining process. Shimizu, Weitkamp, Vosmann, and Matthäus (2013) performed double-heating experiments with diolein oil prepared from oleic acid and glycerol, in which 10 mg/kg chloride per kg oil (as t-butyl ammonium chloride) was added (Shimizu et al., 2013). As a control, the oil was heated at 240 °C for 4 hr, which resulted in a 3-MCPDE concentration of 1.6 mg/kg and a GE concentration of 120 mg/kg. Applying one of the two double-heating strategies (first at 180 °C for 4 hr, followed by 240 °C for 1 hour) resulted in a decrease of 3-MCPDE (1.6 to 1.5 mg/kg) and a 17% decrease (120 to 100 mg/kg) of GE. Reversing the temperature profile from low-high to high-low with similar durations resulted in a small decrease of 3-MCPDE. However, using the high-low temperature profile (240 °C for 1 hr, followed by 180 °C for 4 hr), the GE concentration was reduced from 120 mg/kg to 50 mg/kg, which corresponds to a reduction of 58% compared with the low-high profile. These results showed that 3-MCPDE formation was not affected by the deodorization temperature and that GE was not stable under heating conditions. There is a fine balance between the rate of formation and the rate of degradation of GE under high temperatures. 3-MCPDE is very heat stable as shown by the unchanged concentration throughout the heating experiments and a stability study conducted by these authors. The authors suggested that the reduction of GE could be caused by distillation of GE and transformation at low temperatures. An important side note is that Shimizu et al. (2013) performed this double-deodorization experiment in a laboratory environment with a very specific diolein oil and an artificial chloride source. Matthäus and Pudel (2013) studied two double-deodorization strategies and compared those to two standard single-deodorization conditions (Table 2.4). In the first double-deodorization strategy, oil was first deodorized at 200 °C for 120 min, followed by a short temperature boost at 250 °C for 5 min. A decrease of 65% was observed for 3-MCPDE (from 2 mg/kg to 0.7 mg/kg) and 35% for GE (from 2 mg/kg to 1.3mg/kg) (Matthäus & Pudel, 2013). In the second strategy, oil was first deodorized at 200 °C for 120 min followed by a second deodorization at 270 °C for 5 min. A reduction of 78% was observed for GE (from 16 mg/kg to 3.5 mg/kg) and 3-MCPDE, which was reduced by approximately 75% (from 2 mg/kg to 0.5 mg/kg) (Matthäus & Pudel, 2013). These results were the opposite with respect to those by Shimizu et al. (2013). A direct comparison between the two studies cannot be made because of too many differences between the experimental designs, including the temperatures, starting concentration in the oils, heating durations, and oil compositions. Nonetheless, both studies showed that double-deodorization might be an effective mitigation strategy. Matthäus and

Pudel (2013) performed their double-deodorization experiments under more realistic conditions than Shimizu et al. (2013) did, but additional confirmatory studies need to be performed with true crude vegetable oil in an industrial refining setting. Lower deodorization temperature can be beneficial for the final concentrations of 3-MCPDE and GE.

2.3.5.2. Effects of additives during deodorization

Another mitigating approach is the use of additives in the oil. The effect of using additives has been tested mostly for 3-MCPDE and to a limited extent for GE. No studies have dealt with 2-MCPDE. Matthäus and Pudel (2013) mentioned three additives (ethanol, glycerol, and diacetin) that led to a reduction of3-MCPDE and/or GE, and their data were reported as per-centages of reduction (Matthäus & Pudel, 2013). The addition of 0.5% to 2.5% of either ethanol or glycerol into bleached oil prior to deodorization reduced the 3-MCPDE concentration by 30% Similar results were obtained by Craft et al. (2012). The supposed mechanism consists of the alcohol acting as a chloride scavenger and forming volatile chlorinated adducts that can be stripped away during deodorization. Adding 1 to 5 mmol/kg carbonates (either potassium hydrogen carbonate or sodium bicarbonate) helps to prevent the formation of 3-MCPDE and GE, resulting in a 66% decrease of the concentrations (Matthäus & Pudel, 2013). The third additive that Matthäus and Pudel mentioned is diacetin. Diacetin is a short-chained diacylglycerol (DAG) which can compete with other DAGs for the available chlorine. Since diacetin is more volatile than other DAGs, it can be completely removed during deodorization. A 50% reduction of 3-MCPDE was observed after the addition of diacetin (Matthäus & Pudel, 2013). Zhang et al. (2016) used four different antioxidants (α-tocopherol, rosemary extract, lipophilic tea polyphenols, and L-ascorbyl palmitate) as additives to bleached palm oil. With this mitigation strategy, they also proposed the involvement of free radicals in the formation of 3-MCPDE and GE.

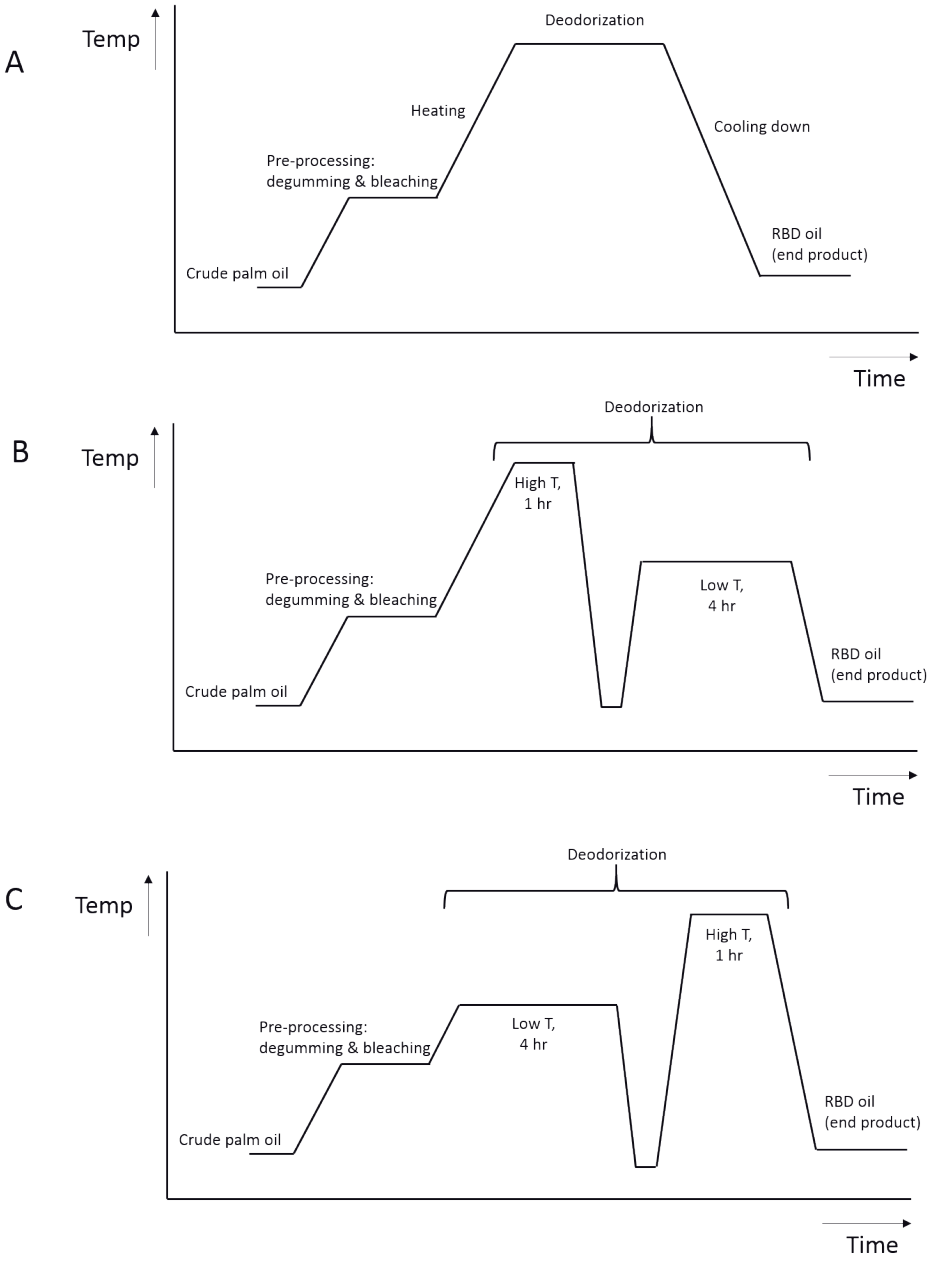

Figure 2.1 Schematic temperature-time profile of the typical oil refining process. (A) shows the normal process, (B) shows a high-low temperature profile during deodorization, (C) shows a low-high temperature profile during deodorization

The antioxidants can act as free radical scavengers and prevent the formation of contaminants. Rosemary extract at 6% w/w (carnosic acidor rosmarinic acid) significantly reduced 3-MCPDE concentration (82.4%, from 2.44 mg/kg in the control to 0.43 mg/kg) (Zhang et al., 2016). However, rosemary extract can have a strong odour, which may add unwanted fragrance to the oil. Lipophilictea polyphenols (6%, w/w of (-)-epigallocatechin gallate) were able to reduce the concentration of 3-MCPDE by 75% (from 2.44 mg/kg to 0.61 mg/kg). More recently, Cheng, Liu, and Liu (2017) investigated the effect of the artificial antioxidant tert-butyl hydroquinone (TBHQ) on the formation of GE in palm oil (prepared in the laboratory), camellia oil, soybean oil, and linseed oil. Under laboratory conditions, the addition of an increasing amount of TBHQ resulted in an increasing reduction of GE. With the addition of1.8 mg/g TBHQ to palm oil, a reduction of GE concentration of approximately 53% was achieved, from 1.7 mg/kg GE in the control compared with 0.8 mg/kg in palm oil with 1.8 mg/g TBHQ (Cheng et al., 2017). The results observed for the other three oils were comparable to the results with palm oil. The combination of the results of Zhang et al. (2016) and Cheng et al. (2017) suggested the involvement of a free radical-mediated formation of 3-MCPDE and GE. This can be viewed as a possible additional pathway to those already known. Cheng et al. (2017) also compared the difference between deodorization under nitrogen or regular air. Since nitrogen is nonreactive, it should prevent any oxygen-based lipid oxidation from occurring. GE concentration was markedly reduced by 41% when all four types of oils were heated under nitrogen (from 1.7 mg/kg under air to 1.0 mg/kg under nitrogen). No significant differences were observed between the four oil types. Effects of alternative equipment geometry. Temperature and additives as mitigation parameters could be easily implemented in existing refining protocols. Adjusting the design of the deodorizer is a more challenging mitigation strategy. Pudel, Benecke, Vosmann, and Matthäus (2016) suggested the use of the short-path distillation process as a replacement for the conventional deodorization process (Pudel et al., 2016). Short-path distillation allows for a gentler removal of volatile compounds without the need to heat the oil at high temperatures. The vacuum applied during short-path distillation is approximately 10−3mbar (compared with 2 to 4 mbar in the conventional process), which reduces the boiling point of the volatile compounds. The main difference with a conventional deodorizer is its distillation chamber, which is a double-walled glass cylinder. By letting the oil flow down the inner wall, the temperature can be regulated more carefully. The thin layer of flowing oil formed increases the surface area of the oil remarkably for an improved distillation of volatile compounds. Under optimal short-path distillation conditions, a reduction of 90% and 98% in 3-MCPDEand GE concentrations, respectively, was observed. Under standard deodorization conditions, 3.0 mg/kg of 3-MCPDE and 6.4 mg/kg of GE were formed, while 3-MCPDE and GE were reported to be <0.1 mg/kg when short-path distillation was applied (Pudel et al., 2016). However, Pudel et al. (2016) mentioned that the taste and odour were negatively influenced by

short-path distillation. Furthermore, the colour remained orange-red, which may not be desirable for applications in some food products, al-though red colour indicates the oil is still rich in carotenes, tocopherols, and phytosterols. An extra post distillation deodorization step under mild conditions (180 °C, 120 min) was able to resolve the taste and odour issue. 3-MCPDE concentration after deodorization increased to 0.3 mg/kg, while no increase in GE was detected. In conclusion, several mitigation strategies during deodorization, such as double deodorization, addition of various antioxidants, or use of lower deodorization temperatures for an extended period, have been shown to reduce the formation of 3-MCPDE and GE.

2.3.6. Post refining removal strategies

The strategies described in this section are designed to remove the process contaminants from already refined oil. Details of the experimental conditions and concentrations reported in the literature are presented in Table 2.5. Adsorbing contaminants onto adsorbents, such as bleaching clays or activated carbon, is a common strategy. This strategy for removing contaminants is used with bleaching during oil refining but can also be applied to refined oil. Since most of the undesirable components in crude oil are already removed during the refining process, the addition of adsorbents to refined oils might improve the removal of process contaminants. In this way, the newly added adsorbents are more readily available to adhere to 2-, 3-MCPDE, and GE. Strijowski, Heinz, and Franke (2011) tested several adsorbents such as amorphous magnesium silicate, zeolite, and synthetic magnesium silicate (Strijowski et al., 2011). The adsorbent was added to the refined palm oil at 80 °C and left to react for 30 min. The adsorbent was then separated from the oil by means of centrifugation. Interestingly, five out of the nine tested adsorbents increased the concentrations of GE to >1 mg/kg, while 3-MCPDE seems to be less negatively affected (data not shown). Two adsorbents, calcinated zeolite and synthetic magnesium silicate (65% silicon oxide and 15% magnesium oxide), gave promising results. Use of 10% synthetic magnesium silicate resulted in an insignificant reduction (5%) of the 3-MCPDE concentration (from 4.3 to 4.1 mg/kg), while the GE concentration was markedly reduced by 41% (from 2.2 to 0.9 mg/kg). Both3-MCPDE and GE concentrations were markedly reduced when 10% calcinated zeolite was used. A 19% reduction of 3-MCPDE (from 4.3 to 3.5 mg/kg) was observed in comparison to an untreated palm oil control. GE concentration was reduced by 77% (from 2.2 mg/kg to 0.5 mg/kg). Strijowski et al. (2011) also conducted sensory and quality tests (oxidation stability) on the treated oils. The use of calcinated zeolite improved the sensory quality of the oil without leading to a worsening of the oxidation stability. On the other hand, the synthetic magnesium silicate resulted in oil with poor sensory quality, but improved oxidation stability.

Table 2.5 Effectivity of post-refining processes for the mitigation of 2-, 3-MCPD esters, and Glycidyl esters

Post-refining conditions	Reduction (%) [actual concentrations in mg/kg]			References
	3-MCPD esters	2-MCPD esters	Glycidyl esters	
10% calcinated zeolite (<1% water)	19% [4.3 -> 3.5]	ND[1]	77% [2.2 -> 0.5]	Strijowski et al. (2011)
10% Synthetic MgSi (65% silicon oxide, 15% magnesium oxide)	5% [4.3 -> 4.1]	ND	41% [2.2 -> 0.9]	Strijowski et al. (2011)
Candida antarctica lipase A, halohydrin dehalogenase from Arthobacter sp. AD2 & epoxide hydrolase from Agrobacterium radiobacter AD1	100 % [10 mM -> 0 mM]	ND	ND	Bornscheuer and Hesseler (2010)
30 mg/100 mL acid washed OPAC	ND	ND	95% (3.75 -> 0.2)	Cheng et al. (2017)
1% acid activated bleaching earth V2R	ND	ND	99% [10.3 -> LOQ (<0.1)]	Shimizu et al. (2012)
Combinations: 3.5% water degumming, 0.08% phosphoric acid, 60 °C degum temp., 0.3% bleaching clay, 260 °C deo temp.	87.2% [2.95 -> 0.37]	ND	ND	Zulkurnain et al. (2013)

1. ND: not defined

Another adsorbent that is capable of efficiently removing GE is acid-washed oil palm wood-based activated carbon (OPAC). OPAC is an activated carbon made from oil palm wood. The activated carbon is washed with acid to achieve high mesoporosity and an acidic environment on its surface. Cheng et al. (2017) showed GE removal efficacy of acid washed OPAC as part of akinetic and mechanistic study of the adsorptive capacity of OPAC (Cheng, Liu, Wang, & Han, 2017). At an acid washed OPAC concentration of 30 mg/100 mL, a 95% reduction of the level of GE was observed (3.75 to 0.2 mg/kg). This adsorbent showed the best reduction of GE in comparison with other adsorbents including alkaline cellulase, activated clay, and non-acid washed OPAC. Equilibrium was attained after 40 min and the maximum adsorption capacity was found to be 36.2 mg GE/g acid washed OPAC. Cheng et al. (2017) mentioned that the mode of GE removal was due to both adsorption on the acid washed OPAC and degradation of GE at activated sites with acidic character. No difference in oil quality was observed between before and aftertreatments with acid washed OPAC, making it a suitable adsorbent to adopt in mitigation strategies. However, since the effect of acid-washed OPAC on the 2- and 3-MCPDE concentrations was not tested, more experiments need to be performed before it can be used as an adsorbent for 2-, 3-MCPDE, and GE. Shimizu et al. (2012) used acid-activated bleaching clay to remove GE from different types of vegetable oils including palm oils and rice bran oils (Shimizu et al., 2012). In all tested oils, the concentration

of GE was reduced by 99% or more when using 1% acid-activated bleaching clay. As an example, the sum of all measured GE concentrations (which consisted of palmitate, stearate, oleate, linoleate, and linolenate esters) was reduced from an average of 10.3 mg/kg to below the LOQ of 0.1 mg/kg (Shimizu et al., 2012). Those authors noted that glycidyl palmitate was modified instead of being absorbed by the activated bleaching clay. An epoxide ring-opening reaction of glycidyl palmitate with water can occur prior to the interesterification reaction be-tween monopalmitate and glycerol dioleate. The acidic condition of acid-activated bleaching clay can play a key role in initiating the reaction. The end products of the reactions were reported to be simple acylglycerols and glycerol. A comparable approach to reduce contaminants is the break-down of targeted contaminants with enzymes, as shown by Shimizu et al. (2012) for converting GE to acylglycerols and glycerols. The main principle is the conversion of un-bound 3-MCPD into glycidol by halohydrin dehalogenase, followed by hydrolysing glycidol into glycerol by epoxide hydrolase (Bornscheuer & Hesseler, 2010). 3-MCPDE can also be con-verted into glycerol by the same enzymes, but it requires unbound 3-MCPD to initiate the reaction. A lipase was used to hydrolyze3-MCPDE into unbound 3-MCPD. In a biphasic system at 5% v/v water-in-oil ratio, the lipase was able to convert 100% of 3-MCPD oleate esters into 3-MCPD. In a separate experiment using 5% v/v water-in-oil ratio, the halohydrin dehalogenase was able to convert 100% of the 3-MCPD into glycidol, followed by the complete conversion (100%) of glycidol into glycerol by epoxide hydrolase. However, in an experiment by Bornscheuer and Hesseler (2010) the concentration of the formed glycidol (7.7 mM) did not correspond with the starting concentration of 3-MCPD (10 mM). The mechanism of the enzymatic conversion could not completely be explained by the observed data. Nevertheless, no 3-MCPD could be detected after incubation with halohydrin dehalogenase, nor after incubation with epoxide hydrolase. It is important to note that the experiments of Bornscheuer and Hesseler (2010) were performed under laboratory conditions. The use of enzymes to remove 3-MCPD from vegetable oil is promising, but it requires more research before the processed oil can be regarded as safe for consumption. The safety of degradation products due to enzymatic treatment and the remnants of the enzyme itself must be investigated. In conclusion, several post refining removal strategies are available, including the use of absorbents and enzymes, and have been shown to be effective in removing pro-cess contaminants from fully refined vegetable oils. 3-MCPDE concentrations were reduced up to 19% when refined oil was treated with an adsorbent (calcinated zeolite). This method was more effective for GE. Finally, post refining treatment with 1% activated bleaching clay (V2R) was most efficient for GE, with a reduction of 99%.

2.3.7. Combining strategies

Oil refining is a complex process with multiple possibilities for mitigation strategies to be applied during the process, as dis-cussed above. Therefore, a combined mitigation strategy may be promising for limiting the final concentrations of 2-, 3-MCPDE, and GE in refined oil. For example, Zulkurnain et al. (2013) applied a modified refining process that was optimized using response surface methodology (RSM) in addition to their previously discussed optimization steps (Zulkurnain et al., 2013). For their RSM optimization, five refining parameters were combined. These five parameters are water dosage, phosphoric acid dosage, degumming temperature, activated clay dosage, and deodorization temperature. Their optimized degumming conditions were 3.5% water dosage, 0.08% phosphoric acid, and 60 °C degumming temperature. Combined with 0.3% of bleaching clay and 260 °C as the deodorization temperature, they were able to reduce the 3-MCPDE concentration by 87.2%, from 2.95 to 0.37 mg/kg (Zulkurnain et al., 2013). Some parameters showed contradictory effects, something that can also be seen from several previously discussed strategies. It is important to find the balance between reducing the process contaminants as much as possible (3-MCPDE in this particular case) while maintaining the oil quality.

2.3.8. General aspects

Designing an efficient mitigation strategy requires more than one modification in the entire refining process. Many papers have focused on a single parameter or a single refining stage, which may be sufficient for high-quality starting oils, but could still be inadequate for initial low-quality oils. Degumming, neutralization, bleaching, and deodorization are potential targets for the development of a multifaceted mitigation strategy. Post refining removal could be utilized when dealing with low-grade oils in which residual process contaminants are most likely to still be present. With the recent MLs set by the European Commission for GE, and possible future regulations for 3-MCPDE, it is worthwhile for oil refineries to invest in the development of sound mitigation strategies over the entire refining process. Providing a clear, ready-made solution in terms of combining mitigation strategies is subject to several limitations. Crude oil quality plays a significant role, but also the design of the individual refining plant could limit the possibilities. Minor hardware changes or small changes in standard operating procedures are usually not problematic. However, addition of a water wash step, varying the amount of acid or bleaching clay, performing double deodorization, or even double refining is more difficult to achieve; nonetheless, it is still manageable. When these changes are optimized, they could significantly decrease contaminants. The availability of resources may also be a limitation for implementation. Although time and financial considerations have not been discussed in this review, they are often the major limitations. Optimizing a single parameter and validating a new method can be temporally and financially cumbersome for oil refining companies. Most mitigation

tests are performed in the laboratory and would require a scale-up of research and operation costs for implementation in a production plant. The mitigation strategies presented show reductions ranging from 4% up to 94% for 3-MCPDE, and 16% up to 100% for GE. Unfortunately, none of the 20 papers reported data regarding2-MCPDE. This clearly shows that there is a significant gap in the data and knowledge concerning the mitigation of 2-MCPDE. It is important to also include 2-MCPD in future studies in order to prevent reduction of one compound while increasing the formation of another. When, for example, a certain mitigation strategy is able to successfully reduce GE (or one of the other process contaminants) with a certain processing condition, it might be difficult to determine whether GE is being physically removed from the oil or if it is being converted or degraded into something else. Finally, we observed that GE concentrations are not always being reported in the reviewed publications. It is known that high temperature is the main cause for the formation of GE (Destaillats et al., 2012). GE formation occurs at a much higher temperature (>220 °C) than the temperatures at which 2- and 3-MCPDE can start to form (Matthäus & Pudel, 2013; Shimizuet al., 2013) Notably, the temperature applied during degumming, neutralization, and bleaching processes are usually well below 180 °C. Therefore, we assume that the missing GE concentrations for the degumming, neutralization, and bleaching processes are not crucial, because it is unlikely that a large amount of GE would have already been formed during the mentioned processes prior to the deodorization process. However, this assumption can only be confirmed if GE concentrations become available from these stages of the refining process. This review focused on an in-depth analysis of mitigation strategies reported in peer reviewed publications. Most of these studies are reported by academic and applied research organizations. From these studies, it is not clear to what degree these strategies were implemented by industry and if they are routinely applied.

2.4. Conclusion

The current review shows that degumming, neutralization, bleaching, and deodorization are the main processing steps in oil refining with the potential to mitigate the formation of 3-MCPDE and GE. Water degumming was able to reduce 3-MCPDE concentrations by 84% and by 26% for GE. Neutralization of oil with a base such as $NaHCO_3$ or KOH was able to reduce the 3-MCPDE concentration up to 81% and up to 84% for GE. Bleaching of oil with neutral clay after a water degumming treatment was able to reduce the 3-MCPDE concentration by 46%. Magnesol R60 (magnesium silicate) reduced 3-MCPDE by 67%. Deodorizing oil at high temperatures for prolonged periods (for example, >230 °C to 250 °C for >1 hr) increased GE concentration, while 2- and 3-MCPDE concentrations were more dependent on the amount of available chlorine. Other less common deodorization methods, including double-deodorization or deodorization with certain additives, are

very promising mitigation strategies that can reduce 3-MCPDE concentration up to 82% and GE concentration up to 78%. However, there is no effective "one-size-fits-all" single point solution for oil refining. It is advisable to develop a mitigation strategy combining multiple mitigation steps due to the nature of the different reaction mechanisms of formation. Combination of water washing in order to remove chlorine precursors, pH neutralization prior to any extensive heat treatments, and double-deodorization with or without additives to adsorb remaining contaminants can be very effective in reducing 2-, 3-MCPDE, and/or GE in vegetable oils. The combined strengths and specificities of each mitigation strategy can result in good-quality oil with very low concentration of process contaminants. Noticeably, none of the discussed papers provided data on 2-MCPDE. This lack of data cannot be ignored when new regulations and/or mitigation strategies are being developed.

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Author Contributions

S.B. Oey conducted the literature search, compiled data in tables and graphs, drafted the manuscript, and corrected comments. H.J. van der Fels-Klerx, V. Fogliano, and S.P.J. van Leeuwen contributed to scoping the study, reviewing draft manuscript versions, and providing editorial corrections.

Author Disclosures

All authors have read and approved the manuscript. Authors declare to not have any conflict of interest.

Appendix

Chemical and Physical Refining Oil refining can be performed via a chemical refining or physical refining process. There are several principal differences between chemical and physical refining with related advantages and disadvantages. The major difference is that during the chemical refining process, free fatty acids (FFAs) are removed chemically by saponification with an alkali solution, which results in the formation of soap. The separated soap stock can be sold for the making of soap or biodiesel (Piloto-Rodriguez, Melo, Goyos-Perez, & Verhelst,2014). Physical refining removes the FFAs in a later stage during deodorization by means of vacuum steam distillation. Figure 2.2 shows a schematic illustration of both processes.

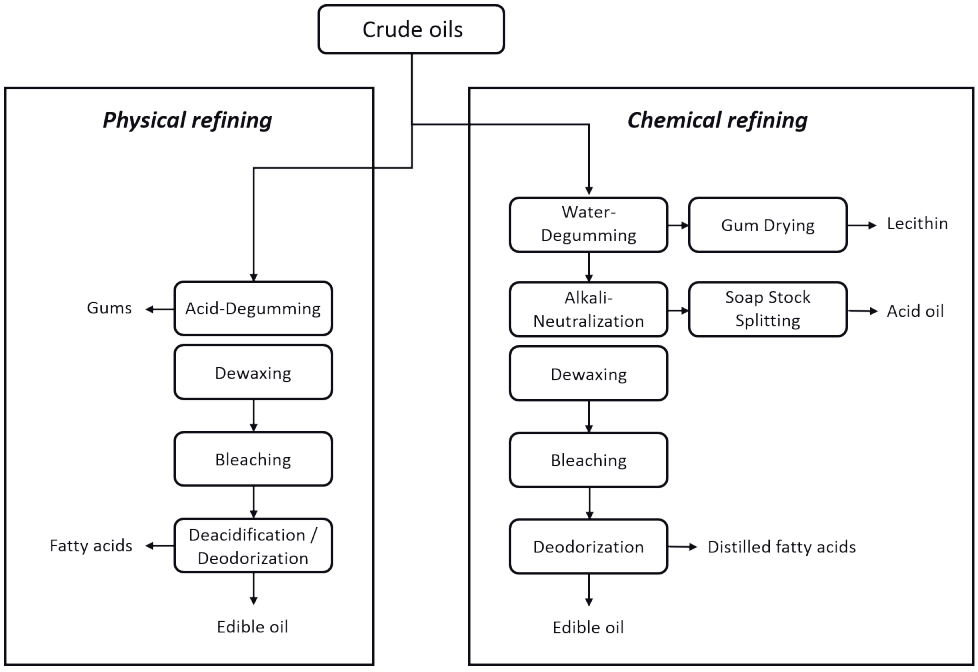

Figure 2.2 Schematic representation of physical and chemical oil refining. (R.S. Zeldenrust, AOCS Lipid Library, reproduced from lipidlibrary.aocs.org accessed on 15-02-2018

The choice between the two refining processes depends on the type and quality of the crude oil that needs to be refined. Unwanted products are easier to remove during the alkali neutralization step in chemical refining. However, physical refining can be applied more widely to various types of oils and fats than chemical refining (Gibon et al., 2007). In the case of physical refining, the crude oil is not washed or neutralized before it is deodorized. It is there-fore preferable to start with a higher quality of oil with limited levels of unwanted compounds such as FFAs, phospholipids, metals, and

soaps. Optimized degumming and bleaching steps during physical refining helps to increase the general oil quality before deodorization. It is advantageous to utilize physical refining because the final oil yield is often higher than that of chemical refining. Furthermore, less chemical treatment is needed and there is no soap stock formed that needs to be disposed or processed properly, providing a better environmental profile. The downside is that more bleaching clay is needed to purify the oil prior to the deodorization step. Also, with physical refining the complete deodorization process, often performed at temperatures up to 270 °C, can destroy natural antioxidants such as tocopherols and tocotrienols. Careful monitoring of temperature and time is therefore important if such minor components are needed to be preserved. Although Gibon et al. (2007) reported oil losses ranging between 20% and 30% during chemical refining, it remains the better solution in terms of removal of unwanted products. The alkali neutralization applied during chemical refining is better at removing unwanted products than the acid-degumming step performed during physical refining. Oils high in phospholipids, such as sunflower oil and rapeseed oil, also benefit more from water degumming applied during chemical refining, as water degumming is able to efficiently remove these phospholipids. As the majority of the FFAs are already removed during the alkali-neutralization step, a less vigorous deodorization step can be applied, and therefore preserve the previously mentioned minor components. In terms of strategies to mitigate 2-, 3-MCPDE, GE, both the physical and chemical refining should be considered. The focus of the discussed mitigation strategies is on the degumming, neutralization (chemical refining only), bleaching, and deodorization steps. These steps in either physical or chemical refining may have the potential to contribute to lowering the final concentration of the mentioned contaminants.

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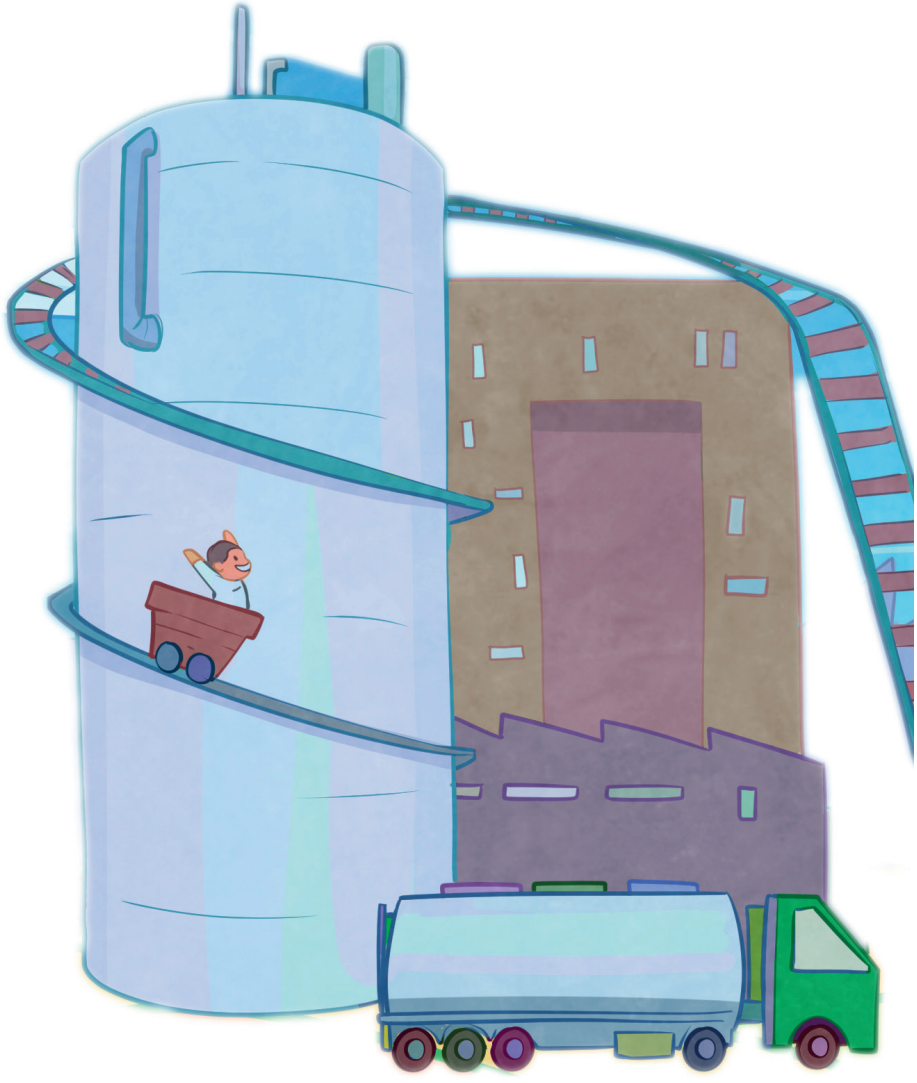

Chapter 3

Effective physical refining for the mitigation of processing contaminants in palm oil at pilot scale

Adapted from:
Sergio B. Oey, H.J. van der Fels-Klerx, Vincenzo Fogliano, and Stefan P.J. van Leeuwen, Effective physical refining for the mitigation of processing contaminants in palm oil at pilot scale. Food Research International, 2020, 138, part A, 109748.

Abstract

This study aimed to develop a mitigation strategy for the formation of 2-monochloropropane-1,3-diol esters (2-MCPDE), 3-monochloropropane-1,2-diol esters (3-MCPDE), and glycidol fatty acid esters (GE) during palm oil refining. Single physical refining was the starting point (the control) for this study. Experimental treatments including a double refining repeating the entire single refining process (T1), double refining with a high-low deodorization temperature (T2), and double deodorization (T3) with similar temperature settings as T2 were performed. Compared with the control experiment, T2 successfully reduced the formation of GE by 87%; in particular, the second degumming and bleaching were crucial for eliminating GE. Both 2- and 3-MCPDE were formed prior to the deodorization process in all treatments. MCPDE concentrations remained stable throughout the refining process and, hence, they require a different mitigation approach as compared to GE. These results provide useful insights which can directly be implemented by the oil industry.

Keywords: 3-monochloropropanediol; Glycidyl esters; Refined edible oils; Pilot plant refining; Mitigation strategies; Elimination methods

3.1. Introduction

2-monochloropropane-1,3-diol fatty acid esters (2-MCPDE), 3-monochloropropane-1,2-diol fatty acid esters (3-MCPDE), and glycidol fatty acid esters (GE) are process contaminants that can be found in refined vegetable oils and fats. Occurrence data showed that palm oil samples can have high concentration of these contaminants compared to other oil types and consequently 2-, 3-MCPDE, and GE were also found in food products containing palm oil as ingredient (Becalski et al., 2015, Cheng et al., 2017, Kuhlmann, 2016, MacMahon et al., 2013). In the last decade, several mitigation strategies for the formation of 3-MCPDE and GE have been published, as recently reviewed by Oey et al. (2019). The source, the quality of the crude oil, and potential enzymatic hydrolysis of acylglycerols occurring between harvesting and processing play a role in the potential formation of 2-, 3-MCPDE, and GE during oil refinery (Matthäus & Pudel, 2013). These parameters need to be well regulated to achieve high quality vegetable oils with low 2-, 3-MCPDE and GE contents. However, full control over all the previously mentioned parameters is difficult to achieve. Optimizing the refining conditions would therefore be a more accessible strategy.

2-, 3-MCPDE and GE are mainly formed during processing of the crude oils in refineries where the oils are treated at elevated temperatures for an extended amount of time. On one hand, reaction mechanisms proposed by Šmidrkal et al., 2016, Destaillats et al., 2012, and Hamlet et al. (2011) show the important role of chlorine as precursor element and cyclic acyloxonium ion reaction intermediates in the formation of 3-MCPDE and 2-MCPDE. On the other hand, GE is formed at high temperatures via an intramolecular rearrangement that does not involve chlorine or chlorine precursors (Cheng et al., 2016, Cheng et al., 2017, Destaillats et al., 2012).

In recent years, 2-, 3-MCPDE and GE received great attention after the report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) meeting in 2016, the scientific opinion of the European Food Safety Authority (EFSA) in 2016 and the updated scientific opinion of the latter in 2018 (EFSA Contam Panel, 2016, EFSA Contam Panel, 2018, JECFA, 2017). In 2018, a maximum limit (ML) for the presence of GE in vegetable oils and infant formulae has been established by the European Commission (European Commission, 2018). This ML was set at 1000 µg/kg for vegetable oils and fats, either for direct consumption or as ingredient. For vegetable oils and fats that are going to be used for the production of baby food or cereal-based food for young children, the ML for GE was set at 500 µg/kg (European Commission, 2018) pushing the research toward development of effective mitigation strategies. Currently, there are no maximum levels in place for 2- and 3-MCPDE in the EU. However, the European Commission is currently

discussing new maximum limits regarding 3-MCPDE in various types of oils (European Commission, 2020).

Oey et al. (2019) concluded that most of the mitigation strategies do not act at multiple refining steps, which could potentially be the most effective strategy. Ramli et al., 2011, Zulkurnain et al., 2012, have published mitigation strategies focusing on the degumming or bleaching process, respectively. Ramli et al. (2011) achieved a reduction of 64% MCPDE, while Zulkurnain et al. (2012) achieved a 67% reduction for 3-MCPDE. More recently, Sim et al. (2018) reported the successful combined effect of phosphoric acid and acid-activated bleaching earth with acidic pH for the reduction of GE in palm oil (<0.20 mg/kg), but 3-MCPDE mitigation required other conditions. New insights reporting that each type of bleaching earth has its own optimal dosage for the mitigation of 3-MCPDE and GE was provided by Hew et al. (2020). To date, mitigation strategies for 2-MCPDE have not been reported yet.

The aim of this study is to investigate the effects of various time- and temperature profiles in physical refining through pilot plant oil refining experiments, to reduce the formation of 2-, 3-MCPDE, and GE simultaneously. Three treatments, double refining, double refining with a high-low deodorization temperature, and single refining with double deodorization are compared against a single refining control. Pilot plant scale experiments are considered to mimic more realistically oil refining conditions in practice than laboratory scale experiments. Moreover, the implementation of the mitigation strategies to a full-scale refinery can be made with less effort due to the smaller scale differences.

3.2. Materials & methods

3.2.1. Materials

All chemicals used for the experiments and analysis are described in the supplemental material as well as the materials used for the pilot plant experiment. In all pilot plant treatments, the following materials and quantities were used. 0.5% w/w of a 25% v/v citric acid solution was used for the degumming process. 1.0% w/w Pure-Flo® B80 natural bleaching earth, 0.1% w/w Cabot Norit® SA 4 PAH-HF activated carbon, and 0.2% w/w Dicalite® 478 perlite filter aid was used during the bleaching process. An Eaton CLEARGAF polyester needlefelt filter bag was used to physically remove all solid, insoluble additives prior to the deodorization process. Each treatment used 100 kg crude palm oil (CPO) from a single batch as starting each material. 30 kg of the same CPO batch was used to flush the entire pilot plant with 'fresh' CPO prior to each experiment.

3.2.2. The pilot plant

An experiment on physical refining of CPO was performed at the pilot plant located in the refining factory of Special Refining Company (SRC) in Zaandam, The Netherlands. Figure 3.1 shows the schematic drawing of the pilot plant. The pilot plant consists of two main reaction tanks which each can hold 100 kg of oil for the pre-treatment (degumming and bleaching) and for the deodorization. Fully digital time- and temperature control and monitoring are available. The built-in sample collection valves in both tanks allow for sample collection at any moment for any duration of time and/or volume. More details of the pilot plant's design and functionalities are described in the supplemental materials.

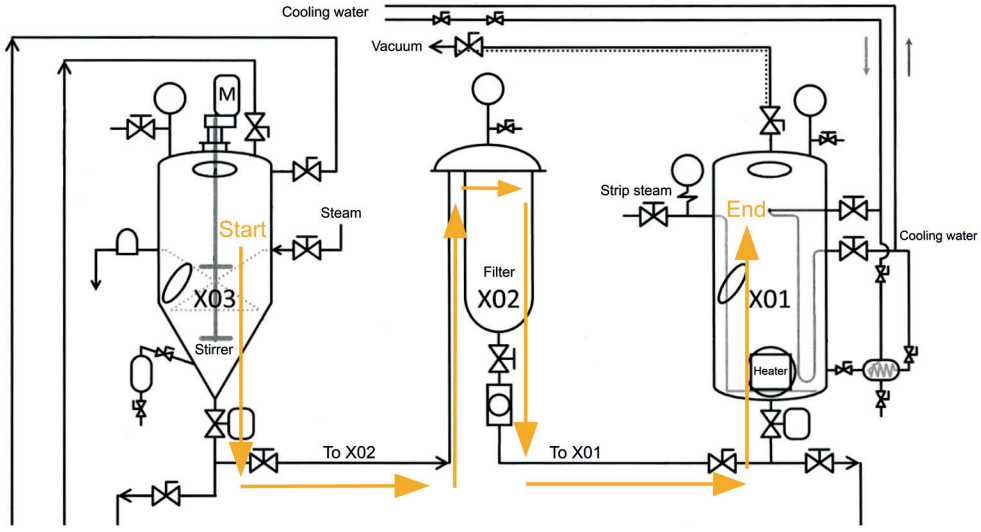

Figure 3.1 Schematic overview of the major elements of the pilot plant. The thick orange arrows indicate the flow direction of the oil during refining. Tank 'X03' is the pre-treatment tank for the degumming and bleaching of the oil. Tank 'X01' is the deodorization tank. 'X02' is the filtration unit. The experiment starts after loading the CPO in tank X03 and the refined product is obtained after deodorization in tank X01 ('end').

3.2.3. Experimental design

To investigate the temperature–time effect and the impact of the individual refining processes, three physical refining treatments were conducted. The control was a single physical refining to simulate a regular palm oil refining at full scale. A second, identical refining cycle was performed after the control treatment. Immediately after the control treatment, the oil was pumped back into the first tank (Tank X03) and was refined for the second time creating the T1 treatment. After performing this second identical cycle of physical refining, the produced oil was called a double physical refined oil. The

temperature–time diagrams of all treatments can be seen in Figure 3.2; More specific Figure 3.2A shows both the control and T1. Note that the control experiment was the same as the first refining step of T1 (left side of the dashed line in Figure 3.2A), and therefore combined in the same experiment. All samples collected during the second refining process (combined with the samples of the control treatment) were used to study the effect of the T1 treatment.

The second treatment (T2) was similar to T1, except that the second deodorization was at a lower temperature (Figure 3.2B). The choice to perform the second deodorization at 220 °C originates from previous in-house research performed by our industrial project partner. That temperature is the proverbial border where the formation of GE during the second deodorization can still be kept at a minimum, without compromising other processing parameters such as the vacuum, the strip-steam flow, and reaction times (data not shown). The first refining process of T2 can be directly compared to the control. After the first refining process, the oil was pumped back to Tank X03. This was immediately followed by the start of the second refining process. The same types and amounts of additives used in the control was also used in T2. The design of the experiments considered that whenever food-grade oil has come in contact with bleaching earth or other non-sterile additives, it must undergo a secondary heat treatment to re-sterilize the oil. Therefore, the secondary heat treatment does not need to be executed at the same temperature as the first deodorization temperature.

The third treatment (T3) was a double deodorization experiment (Figure 3.2C). In this experiment, the degumming, bleaching, and filtering processes were performed only once, followed by a deodorization at 265 °C for 60 min. Immediately after 60 min, the oil was cooled down from 265 °C to 220 °C. The deodorization was then continued for another 60 min. Each treatment and the control were refined once.

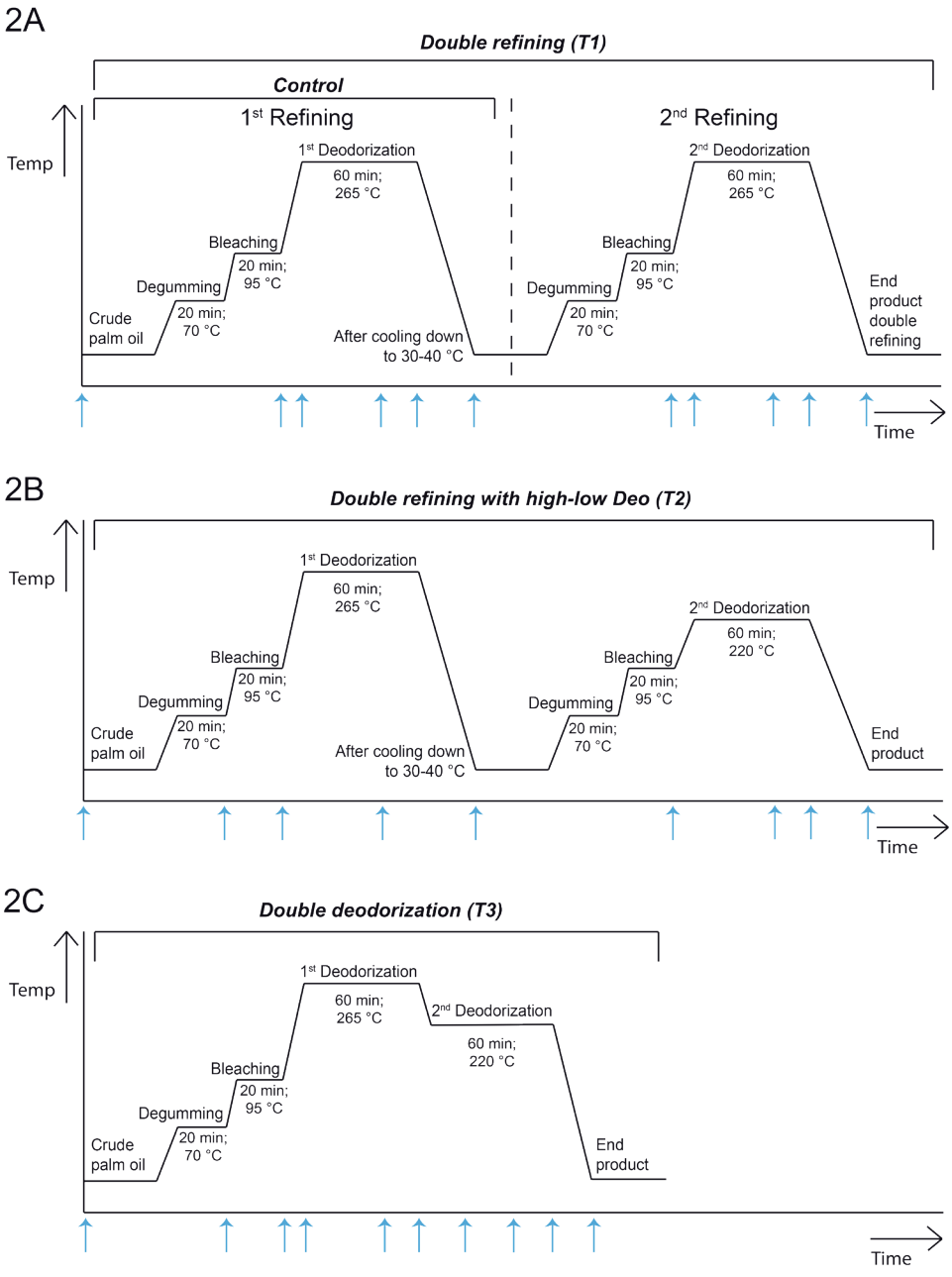

Figure 3.2 Time-temperature profile and sampling moments (indicated with blue arrows) of the control experiment (Control, 2A-left), regular double refining (T1, 2A-right), double refining with high-low deodorization temperatures (T2, 2B), and double deodorization experiment (T3, 2C).

3.2.4. Analytical determinations

Analysis of the oil samples for the concentrations of 2-, 3-MCPDE, and GE were performed using a modified AOCS Cd29a-13 method (Ermacora & Hrncirik, 2013). Some modifications to the AOCS Cd29a-13 method were made to improve the accuracy and sensitivity of the method. The modifications concerned the addition of a separate, third internal standard for 2-MCPDE (1,3-dipalmitoyl-2-chloropropanediol-d5; PP-2-MCPD-d5; CAS: 1426395–62-1) and the implementation of a multiple reaction monitoring (MRM) MS-mode for the phenylboronic acid (PBA) derivatives of 2-MCPD, 3-MCPD, 2-monobromopropane-1,3-diol (2-MBPD), and 3-monobromopropane-1,2-diol (3-MBPD). Analysis was performed on an Agilent 7010B Triple Quadrupole GC–MS system (Agilent, USA) with an Agilent DB-35MS UI GC column (30 m × 0.250 mm × 0.25 µm) (Agilent, USA). A complete description of the analytical method can be found in the supplemental material together with the in-house validation summary. In general, 100–110 mg of each sample was weighed individually as the starting amount. Internal standards are added and tetrahydrofuran (THF) was used as the main solvent. Conversion of GE into 3-MBPD monoester was performed with an acid aqueous solution of 3 mg/mL sodium bromide and 5% v/v sulfuric acid. After neutralization and clean-up, transesterification of all esters was performed with 1.8% v/v sulfuric acid in methanol at 40 °C for 16 h. Prior to derivatization with PBA, the mixture was neutralized and cleaned up by a series of liquid–liquid extractions to remove the fatty acid methyl esters. Sample analysis for the quantification of the MCPDE and GE were performed in singlets after thorough method validation.

The analytical method validation was performed according to the Dutch NEN 7777 standard. Linearity validation was performed on 8 different days with independent calibration curves. The method accuracy was determined on different days with a set of 8 different samples of vegetable oils (palm and olive oils, three replicates) with known levels of the contaminants. The deviations (e.g. RSD%) were determined by analysis of 8 different oil samples with levels between the limit of detection (LOD) and 3.5 mg/kg. Each sample was analyzed in duplicate on a single day, and another (single) measurement was done on another day. Finally, the LOD and limit of quantification (LOQ) were determined by analyzing 8 spiked oil samples between 0.2 and 0.3 mg/kg. Our quantification method was proven to be linear for the used calibration range for all three contaminants (>0.990 r2; intercept < 0.009). The average accuracy for 2- and 3-MCPDE, and GE were 99%, 98%, and 105% respectively. The limit of quantification (LOQ) for 2- and 3-MCPDE, and GE were 0.07, 0.10, and 0.07 mg/kg respectively. Additionally, the limit of detection (LOD) for 2- and 3-MCPDE, and GE were 0.04, 0.05, and 0.03 mg/kg respectively. Z-scores from recent proficiency test (FAPAS 2657 in 2019) were −0.2, 0.0, and 0.1 for 2- and 3-MCPDE, and GE respectively. Additionally, the free fatty acid (FFA) concentration and the color of the oil were analyzed. These analyses were performed on

site at SRC. The FFA concentration was determined by titration and was expressed as '% oleic'. The color of the oil samples was determined manually with a visual colorimeter and a 5¼" cuvette. Since the color of the oil is only important for the final samples of each refining, only the last three samples of each treatment were analyzed. The color of the oil samples is expressed in the Lovibond® Red, Yellow, Blue, and Neutral color scale. Sliders with various filters can be arranged in all kinds of configuration until the best color match with the tint of the sample is obtained.

3.3. Results and discussion

3.3.1. Control & regular double refining (T1)

The results of the control treatment and T1 (unmodified double physical refining) are shown in Figure 3.3A. At the end of the control treatment, 2-MCPDE concentration was 0.82 mg/kg, while 3-MCPDE concentration was 1.62 mg/kg. GE concentration was 3.73 mg/kg. After the second full refining process, the final samples of the T1 treatment showed a 2-MCPDE and 3-MCPDE concentrations which are respectively 15% and 17% higher. This is a minor increase compared to the final sample of the control. However, the GE concentration increased by 126%. The vast majority of 2- and 3-MCPDE was already formed before the start of the deodorization (at 265 °C) and remained stable at that level. This finding is similar to the deodorization experimental results of Hrncirik and van Duijn (2011). They have reported that 3-MCPDE are already being formed at temperature as low as 180 °C for 1 h and were not much affected by further temperature increase up to 230 °C. More recently, Li et al. (2016) showed that samples heated up to 130 °C did not show significant differences in their 3-MCPDE concentrations and that the largest increase in 3-MCPDE concentration was observed between 150 and 180 °C.

GE on the other hand continues to increase during the entire deodorization process until the oil is actively cooled down after 60 min of deodorization. This indicates that MCPD Esters have a lower formation temperature than GE. The formed 2- and 3-MCPDE cannot be removed by a single or double refining process. It seems that 2- and 3-MCPDE are thermally stable and that they remain stable after the addition of 0.5% w/v citric acid. These results confirmed that 2- and 3-MCPDE have a different mechanism of formation than GE. GE concentration was reduced by 51% during the second pre-treatment (after the second degumming and bleaching process) to 1.82 mg/kg, and further decreased until the start of the second deodorization step, followed by large increase upon exposure to high temperatures during the second deodorization. The formation of GE during the first refining was not surprising since GE is formed at high temperatures. Destaillats et al., 2012, Hrncirik and van Duijn, 2011, and Pudel et al. (2011) performed

various experiments on the effect of temperature on the formation of GE during palm oil refining. They reported GE formation temperatures between 200 and 230 °C depending on the reaction time. However, the increased formation rate (from 0.19 to 8.59 mg/kg) observed during a second deodorization under the same temperature and duration as the first deodorization has never been reported before.

One of the requirements for the formation of GE is to have either mono- or diglycerides as starting components (Cheng et al., 2016, Cheng et al., 2017). Triglycerides in oils can be hydrolyzed under several conditions such as high or low pH environments (Frankel, 2012). A second exposure to citric acid during the second degumming process can cause the hydrolysis of a small part of the triglycerides, forming mono- and/or diglycerides. On its turn, the mono- and/or diglycerides can be converted into GE during the second deodorization process. In a lab scale testing, Cheng et al. (2016) reported a GE concentration of approximately 3.4 mg/kg after deodorization at 260 °C for 1 h. That GE concentration is quite similar to what we have found after our first deodorization at 265 °C for 1 h in the control. As a double refining strategy has not been reported before, there is not much reference for the observations after the second deodorization.

The FFA concentrations in the refined palm oil samples of both the control and the regular double refining experiment (T1) are shown in Table S3.1 of the supplemental material. The FFA concentration in the final sample after the control treatment and the second refining (T1) are respectively 0.73% and 0.25%. The color of the final sample of the control was 3.1 Red(R)/31.0 Yellow(Y) and the color of the final sample of T1 was 2.8 R/27.0 Y. For large-scale refining, the industry usually strives for a maximum FFA concentration of 0.10% and a color with a maximum of 3.0 R for neutralized/refined, bleached, and deodorized palm oil (Malaysia Department of Standards, 2007). However, as this is still a pilot plant experiment, we do not implement those limits to our results, but we are aware of its existence. The focus of this study was put on the comparison of the different treatments in an almost ideally simulated refining condition.

3.3.2. Double refining with high-low deodorization (T2)

Like the first experiments above, the majority of the 2- and 3-MCPDE were formed after the filtration step and remained stable throughout the remaining refining process until the end of the second refining process (Figure 3.3B). The final concentrations for 2-MCPDE and 3-MCPDE are similar compared to the control treatment. After the second pre-treatment process (degumming & bleaching), GE was reduced by 93% compared to the 4.00 mg/kg observed after one full cycle of physical refining. This very low GE concentration was maintained throughout the second deodorization. This resulted in an 87% reduction compared to the single refined oil from the control experiment. As this reduction happened before the second deodorization process, the degradation or

removal of GE must be correlated to the secondary pre-treatment processes (degumming & bleaching). As GE has a higher formation temperature than 2- and 3-MCPDE, a lower deodorization temperature of 220 °C prevented additional formation of GE. The final sample of T2 has an FFA concentration of 0.39% and a color of 1.9 R/19.0 Y. Full list of FFA concentration and oil color results of T2 can be found in Table S3.2. Epoxides, as can be found in GE, are unstable in low or high pH environment and high temperatures (Cheng et al., 2016). Exploiting the instability of epoxide under certain conditions could potentially be used to degrade GE.

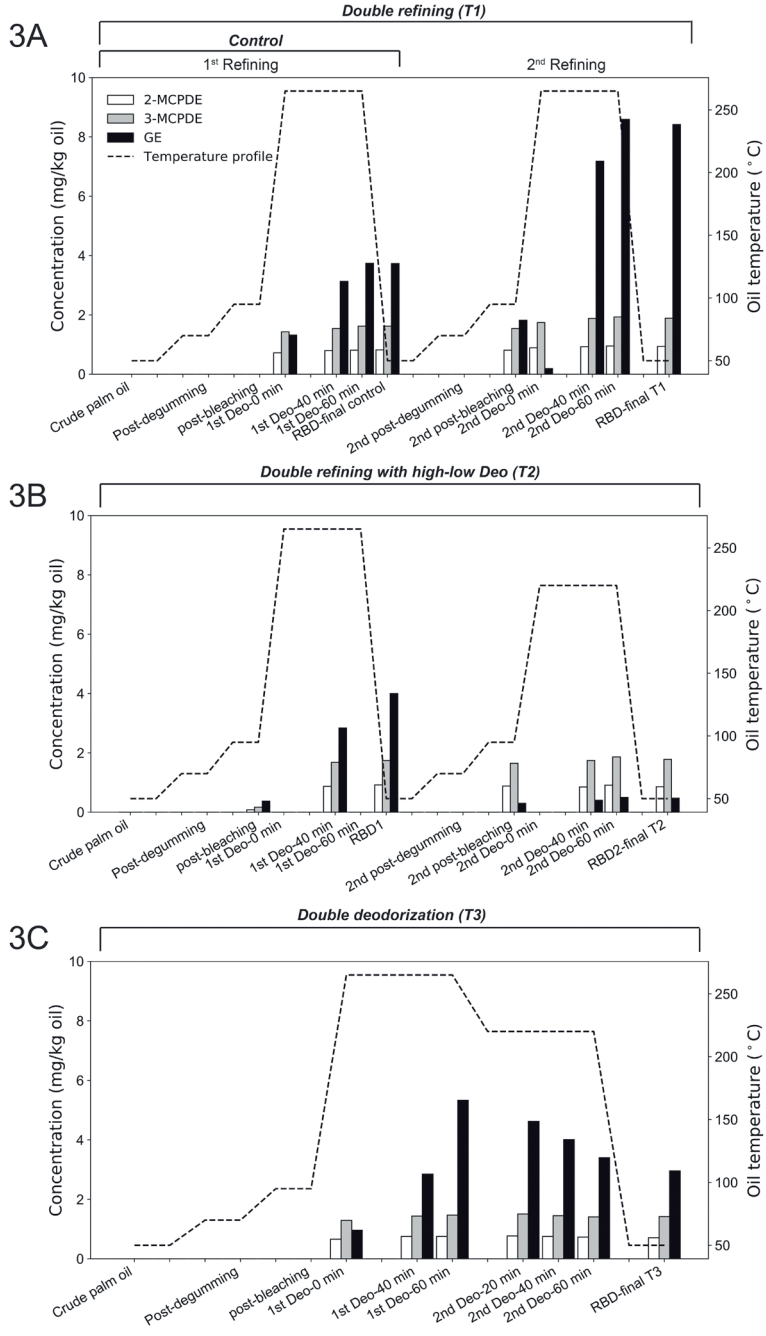

Figure 3.3 Results of the control experiment (Control; 3A-left), the regular double refining experiment (T1; 3A-right), the double refining experiment with high-low deodorization temperature (T2; 3B), and the double deodorization experiment (T3; 3C). The oil temperature profile for all experiments are shown as a reference (dashed line). The samples were collected and displayed chronologically. These graphs correspond respectively with Table S3.1–S3.3 in the supplemental material.

3.3.3. Double deodorization (T3)

The double deodorization treatment (T3) results provide insights into the effects of the secondary pre-treatment used during T2, as well as the isolated (dual) temperature effect during deodorization. Figure 3.3C shows the concentrations of the three contaminants in the collected samples during experiment T3. The concentration of 2-MCPDE after 60 min deodorization was 0.75 mg/kg and the concentration of 3-MCPDE was 1.47 mg/kg, and these remain stable throughout the experiment, like both T1 and T2 experiments. In terms of mitigating 2- and 3-MCPDE, it is essential to prevent formation in the first place. However, from a practical point of view this can be challenging because there are still many uncertainties regarding the precursors and perhaps even physical limitations of certain refineries to implement a good mitigation strategy. The GE concentration after 60 min of deodorization was 5.33 mg/kg. After the second deodorization process, the GE concentration in the final sample was 2.96 mg/kg. This is a 21% reduction against the final sample of the control, which is less than the reduction achieved in the T2 experiment. This result once again shows the importance of the second degumming and/or the bleaching process in the mitigation of GE, which was part of T2, but not included in T3 (see Figure 3.2). Although a substantial GE reduction was achieved in T3, it is clear double deodorization with a high-low temperature scheme is not sufficient to produce refined palm oil that meets the GE maximum limits.

Two publications have reported results of double deodorizations (Matthäus and Pudel, 2013, Shimizu et al., 2013). Matthäus and Pudel (2013) reported that a double deodorization performed at 200 °C for 120 min, followed by a brief 5 min at 250 °C was able to achieve the lowest amount of GE and 3-MCPDE, both at approximately 1 mg/kg. This was a reduction of approximately 50% for both GE and 3-MCPDE. When the second high-temperature phase was increased to 270 °C, the final GE concentration was approximately 3 mg/kg. In contrast to the findings of Matthäus and Pudel (2013), the 3-MCPDE concentrations in our experiments remained stable once being formed. Shimizu et al. (2013) performed a two-step heating test (240 °C for 60 min followed by 180 °C for 240 min) with artificially added chloride into their oil and reported a reduction of approximately 67% for GE (from 105 mg/kg to 35 mg/kg). Similar to Shimizu et al. (2013), we were able to observe a reduction in GE concentration with the high-low double deodorization temperature approach. However, a direct comparison between the results of previous studies and our study is not possible due to the many differences in refining methodology.

The experimental design of the control, first refining of T2, and up to the first deodorization (at 265 °C) of T3 had identical temperature–time settings and same amounts and batch of chemicals, crude oil and other additives. However, our data still show a certain degree of variation for all three contaminants. In average, 3-MCPDE varied with ±8.4%, 2-MCPDE

with ±10.5%, and GE with ±24.5%. Unlike the control and the T2 treatment, T3 was able to achieve a low FFA concentration and a less red-colored oil. The low FFA concentration of 0.08% in the final sample may be explained by a prolonged deodorization process in which more FFA are distilled and/or degraded without the addition of additives that might induce formation of FFA. However, the exact reason for the variation of observed FFA concentrations amongst all the experiments remains unclarified. Nonetheless, as mentioned before, upscaling the pilot plant refining methods into full-scale resulted in a refined palm oil with good color and FFA concentration. A recent study on the type and amount of bleaching earth usage by Hew et al. (2020) did not show any obvious correlation between the type or amount of bleaching earth and the FFA concentration. Full list of FFA concentration and oil color results of T3 can be found in Table S3.3.

Due to the current regulatory EC maximum limits for GE in vegetable fats and oils, refineries must focus on meeting those limits. These mitigation strategies will lead to increased total costs per weight refined palm oil than for example a traditional single physical refining. However, exact financial statements and calculations of the costs of the proposed refining scheme are beyond the aim of this study.

3.4. Conclusions

Double refining with a high-low deodorization temperature strategy (T2) showed great potential to mitigate GE formation during oil refining at pilot plant scale experiments; it resulted in an 87% reduction as compared to single physical refining while double deodorization (T3) was less effective. Further contaminant reductions may be achieved by varying refining other oil refining parameters, that now have been kept the same, such as the type of acid and/or BE and their respective concentrations. From the double refining with a high-low deodorization temperature treatment, bleaching and degumming appeared to be the key steps to achieve the GE reduction. Both the double refining with a high-low deodorization temperature and the double deodorization treatments were not able to reduce the presence of 2- and 3-MCPDE. Once these contaminants are formed, they remain in the oil at a constant concentration. Therefore, their formation should be prevented as much as possible before the physical refining.

CRediT authorship contribution statement

Sergio B. Oey: Methodology, Investigation, Writing - original draft, Visualization. H.J. Fels-Klerx: Conceptualization, Writing - review & editing, Supervision, Funding acquisition. Vincenzo Fogliano: Conceptualization, Writing - review & editing, Supervision. Stefan P.J. Leeuwen: Conceptualization, Validation, Writing - review & editing, Supervision, Project administration.

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Author contributions

S.B. Oey proposed the experimental design, analyzed the samples, compiled data in tables and graphs, drafted the manuscript, and corrected comments. H.J. van der Fels-Klerx, V. Fogliano, and S.P.J. van Leeuwen contributed to scoping the study, maturing the experimental design, reviewing draft manuscript versions, and providing editorial corrections.

Author disclosures

All authors have read and approved the manuscript. Authors declare to not have any conflict of interest.

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Supplemental materials

Table S3.1. results of the control treatment and the normal double refining experiment (T1)

Treatment	Sample name	2-MCPDE (mg/kg)	2-MCPDE difference against 'RBD-final control' sample (%)	3-MCPDE (mg/kg)	3-MCPDE difference against 'RBD-final control' sample (%)	GE (mg/kg)	GE difference against 'RBD-final control' sample (%)	FFA (%)	Color (Lovibond®)
Control	Crude palm oil	< LOQ	-	< LOQ	-	< LOQ	-	4.13	N.A.
	Post-degumming	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	post-bleaching	< LOQ	-	< LOQ	-	< LOQ	-	4.12	N.A.
	1st Deo-0 min	0.72	-12.2	1.43	-11.7	1.32	-64.6	1.97	N.A.
	1st Deo-40 min	0.8	-2.4	1.54	-4.9	3.13	-16.1	0.92	5.1 R / 60.0 Y
	1st Deo-60 min	0.81	-1.2	1.62	0.0	3.74	0.3	0.74	4.1 R / 40.0 Y
	RBD-final control	0.82	0.0	1.62	0.0	3.73	0.0	0.73	3.1 R / 31.0 Y
T1	2nd post-degumming	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	2nd post-bleaching	0.81	-1.2	1.54	-4.9	1.82	-51.2	0.86	N.A.
	2nd Deo-0 min	0.89	8.5	1.74	7.4	0.19	-94.9	0.85	N.A.
	2nd Deo-40 min	0.93	13.4	1.88	16.0	7.18	92.5	0.27	2.7 R / 27.0 Y
	2nd Deo-60 min	0.95	15.9	1.93	19.1	8.59	130.3	0.24	2.7 R / 27.0 Y
	RBD-final T1	0.94	14.6	1.89	16.7	8.42	125.7	0.25	2.8 R / 27.0 Y

FFA - free fatty acid; N.A. - not available; R - red; Y - yellow.
LOQ 2-MCPDE: 0.07 mg/kg; LOQ 3-MCPDE: 0.10 mg/kg; LOQ GE: 0.07 mg/kg.

Table S3.2. results of the double refining experiment with high-low deodorization temperatures (T2)

Treatment	Sample name	2-MCPDE (mg/kg)	2-MCPDE difference against 'RBD-final control' sample (%)	3-MCPDE (mg/kg)	3-MCPDE difference against 'RBD-final control' sample (%)	GE (mg/kg)	GE difference against 'RBD-final control' sample (%)	FFA (%)	Color (Lovibond®)
T2	Crude palm oil	< LOQ	-	< LOQ	-	0.10	-97.3	4.32	N.A.
	Post-degumming	< LOQ	-	< LOQ	-	< LOQ	-	4.29	N.A.
	Post-bleaching	0.08	-90.2	0.17	-89.5	0.37	-90.1	4.28	N.A.
	1st DEO-0 min	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	1st Deo-40 min	0.87	6.1	1.68	3.7	2.84	-23.9	1.04	4.8 R / 48.0 Y
	1st Deo-60 min	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	RBD1	0.92	12.2	1.74	7.4	4.00	7.2	0.40	4.7 R / 47.0 Y
	2nd post-degumming	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	2nd post-bleaching	0.88	7.3	1.65	1.9	0.30	-92.0	0.51	N.A.
	2nd Deo-0 min	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	2nd Deo-40 min	0.85	3.7	1.74	7.4	0.40	-89.3	0.48	2.5 R / 25.0 Y
	2nd Deo-60 min	0.91	11.0	1.86	14.8	0.50	-86.6	0.38	2.0 R / 20.0 Y
	RBD2-final T2	0.86	4.9	1.78	9.9	0.47	-87.4	0.39	1.9 R / 19.0 Y

FFA - Free fatty acid; N.A. - Not available; R - Red; Y - Yellow

LOQ 2-MCPDE: 0.07 mg/kg; LOQ 3-MCPDE: 0.10 mg/kg; LOQ GE: 0.07 mg/kg

Table S3.3. results of the double deodorization experiment (T3)

Treatment	Sample name	2-MCPDE (mg/kg)	2-MCPDE difference against 'RBD-final control' sample (%)	3-MCPDE (mg/kg)	3-MCPDE difference against 'RBD-final control' sample (%)	GE (mg/kg)	GE difference against 'RBD-final control' sample (%)	FFA (%)	Color (Lovibond®)
T3	Crude palm oil	< LOQ	-	< LOQ	-	< LOQ	-	N.A.	N.A.
	Post-degumming	< LOQ	-	< LOQ	-	< LOQ	-	4.21	N.A.
	Post-bleaching	< LOQ	-	< LOQ	-	< LOQ	-	4.07	N.A.
	1st DEO-0 min	0.66	-19.5	1.29	-20.4	0.96	-74.3	2.47	N.A.
	1st Deo-40 min	0.75	-8.5	1.44	-11.1	2.85	-23.6	0.50	4.3 R / 43.0 Y
	1st Deo-60 min	0.75	-8.5	1.47	-9.3	5.33	42.9	0.16	3.8 R / 38.0 Y
	2nd Deo-20 min	0.77	-6.1	1.51	-6.8	4.62	23.9	0.14	3.5 R / 35.0 Y
	2nd Deo-40 min	0.75	-8.5	1.45	-10.5	4.01	7.5	0.11	3.2 R / 32.0 Y
	2nd Deo-60 min	0.73	-11.0	1.41	-13.0	3.40	-8.8	0.08	3.0 R / 30.0 Y
	RBD-final T3	0.71	-13.4	1.42	-12.3	2.96	-20.6	0.08	3.0 R / 30.0 Y

FFA - free fatty acid; N.A. - not available; R - red; Y - yellow.
LOQ 2-MCPDE: 0.07 mg/kg; LOQ 3-MCPDE: 0.10 mg/kg; LOQ GE: 0.07 mg/kg.

Supplemental materials

Additional pilot plant information

Citric acid and Dicalite® perlite 478 filter aid were purchased via Univar Solutions (Rotterdam, The Netherlands). Norit® SA 4 PAH-HF activated carbon was purchased from Cabot Norit Nederland B.V. (Amersfoort, The Netherlands). Pure-Flo® B80 natural bleaching earth was purchased from Oil-Dri (Ripley, Mississippi, USA). The crude organic palm oil (CPO) originating from Ivory Coast were supplied by CARE Naturkost GmbH Co & KG. These materials were used for the pilot plant experiment. The two tanks of the pilot plant can be put under vacuum (which can reach as low as ±3 mbar) to accommodate the set experimental requirements, while the vacuum aids in the relocation of the oil between the tanks. The pre-treatment tank (tank X03, Figure 3.1) is heated by a closed loop of hollow metal coil-shaped tube powered by steam. This hollow metal coil-shaped tube has solid tube walls and does not allow steam to come into contact with the oil. The deodorization tank (tank X01, Figure 3.1) is heated with electric heating elements located at the bottom of the tank. The deodorization tank is also fitted with an additional hollow ring at the bottom, allowing strip steam to enter the tank and permeate through the oil. Strip steam is often used to help agitate the oil during a deodorization process and it helps with the removal of the free fatty acids (FFA) from the oil by evaporation. Rapid cooling of the oil after a deodorization process is achieved by a cold-water flow in a loop of hollow metal coil with solid walls inside the deodorization tank without the possibility of the cold water encountering the oil. The pilot plant had been optimized as close as possible to simulate the standard oil refining process as applied in the regular refining plant. Oil quality parameters such as the deterioration of bleachability index (DOBI) for palm oils, color, and FFA concentration are monitored closely to ensure the desired product quality. The DOBI is checked prior to the refining while only the deodorized oil samples are tested for their color. The FFA content was determined for every collected sample.

Analysis of 2-, 3-MCPDE, and GE

The sample preparation was performed following the AOCS Cd29a-13 method with minor adjustments. The adjustments were made to improve the accuracy, sensitivity, and selectivity of the analytical method. By implementing a calibration line with lower concentration, we have improved the sensitivity in the low concentration range. The calibration line used for the analysis of the samples taken during the experiments can be found in Table S4 The addition of the PP-2-MCPD-d5 as internal standard for the 2-MCPDE improves its quantification accuracy and, finally, operating the MS in multiple-reaction monitoring mode improves the selectivity of the measurements. The following chemicals were used for the analysis of the palm oil samples resulting from the pilot

plant experiments. 3-Chloro-1,2-propanediol-dipalmitate (PP-3-MCPD), 3-chloro-1,2-propanediol-dipalmitate-d5 (PP-3-MCPD-d5), 2-chloro-1,3-propanediol-dipalmitate (PP-2-MCPD), 2-chloro-1,3-propanediol-dipalmitate-d5 (PP-2-MCPD-d5), glycidyl palmitate (Gly-P), and glycidyl palmitate-d5 (Gly-P-d5) were bought in ampules of 1000 µg/mL in toluene from Campro Scientific (Veenendaal, The Netherlands), an official retail partner of Chiron AS (Trondheim, Norway). Phenylboronic acid (PBA), sodium bromide (NaBr), anhydrous tetrahydrofuran (THF) containing 250 ppm BHT as inhibitor were purchased from Sigma-Aldrich (Schnelldorf, Germany). Sodium sulfate (Na_2SO_4), sodium hydrogen carbonate ($NaHCO_3$), and sulfuric acid (H_2SO_4) were purchased from Merck (Darmstadt, Germany). Acetone, iso-octane, methanol, and toluene were purchased from Actu-All Chemicals (Oss, The Netherlands). n-Heptane was purchased from VWR International (Fontenay-sous-Bois, France). PP-2-MCPD, PP-3-MCPD, Gly-P, PP-2-MCPD-d5, PP-3-MCPD-d5, Gly-P-d5 standards, H_2SO_4 in methanol solution, acidified NaBr solution, two $NaHCO_3$ solutions, Na_2SO_4 solution, H_2SO_4 solution, and PBA in acetone solution were prepared prior to the sample analysis.

Table S3.4 Preparation of calibration line

			Theoretical amount of equivalent µg of unbound 3-MCPD	**Theoretical amount of equivalent µg of unbound 2-MCPD**	**Theoretical amount of equivalent µg of unbound glycidol**
Cal 0	-	0	0	0	0
Cal 1	-	5	0.005	0.005	0.007
Cal 2	-	10	0.01	0.01	0.013
Cal 3	-	25	0.026	0.026	0.033
Cal 4	-	50	0.052	0.052	0.065
Cal 5	-	100	0.104	0.104	0.13
Cal 6	20	-	0.21	0.21	0.26
Cal 7	40	-	0.41	0.41	0.52
Cal 8	60	-	0.62	0.62	0.78

Calibration mix 1 had a concentration of 55 µg/mL. Calibration mix 2 was prepared by diluting calibration mix 1 by ten-fold (5.5 µg/mL). Calibration line samples Cal 0 - Cal 8 were prepared from either calibration mix 1 and calibration mix 2 containing equal concentration of PP-2-MCPD, PP-3-MCPD, Gly-P. Both calibration mixes were prepared in toluene.

Next to the calibration mixtures, an internal standard mixture was prepared in toluene containing equal concentration of PP-2-MCPD-d5, PP-2-MCPD-d5, and Gly-P-d5 (40µg/mL). Each sample, including the calibration samples contains the equivalent of 0.39 µg of unbound 2-, 3-MCPD-d5 and 0.50 µg of unbound glycidol-d5.

In addition to the calibration mixtures and internal standard mixtures, several other reagents are required as well to perform the sample preparation of the oil samples. An acid aqueous solution of sodium bromide containing 3 mg/mL sodium bromide and 5% v/v sulfuric acid was prepared to convert glycidyl esters into 3-monobromopropane-1,2-diol (3-MBPD) esters. For the transesterification of the esters into unbound 2-, 3-MCPD and 3-MBPD (representing the GE concentration), a 1.8 % v/v sulfuric acid in methanol solution was prepared. To neutralize the acid after the GE conversion and transesterification, two sodium hydrogen carbonate solutions were prepared with a concentration of respectively 0.6% w/v and 9% w/v. As the liquid-liquid extraction clean-up uses n-heptane as organic phase, a 20% w/v sodium sulfate solution was used to salt-out the water phase and promote the migration of the unwanted fatty acid methyl esters (FAMEs) into the heptane phase. A 0.25 g/mL of phenylboronic acid in a 19:1 v/v aceton:water solution was used to perform the derivatization of the unbound 2-, 3-MCPD and 3-MBPD. Finally, to monitor the analytical performance of the GC-MS, a 1 µg/mL 1,2,3,4-tetrachloronaphthalene (TCN) solution was added as syringe standard. TCN is a non-interfering and very stable molecule under various conditions which dissolves well in iso-octane, the final solvent used to make the samples ready for injection in the Agilent 7010B Triple Quadrupole GC/MS system (Agilent, USA). Table S5 shows the used MRM transitions and the collision-induced dissociation voltages. The MS was set up with an electron impact ionizer operating at 70 eV. Gas-chromatographical separation were performed on an Agilent DB-35MS UI column (30 m x 0.250 mm x 0.25 µm) (Agilent, USA) with helium as carrier gas at a constant flow rate of 1.0 mL/min. Temperature gradient of the GC oven is shown in Table S6 and the total run time was just short of 20 min per sample.

Table S3.5 *MRM mass transitions as used during the analysis*

Components	Precursor (m/z)	Product ion 1 - Quantifier (m/z) [Collision energy in eV]	Product ion 2 - Qualifier (m/z) [Collision energy in eV]
3-MCPD	196.0	147.0 [8]	91.1 [25]
2-MCPD	196.1	104.1 [10]	91.2 [5]
3-MBPD	240.1	147.0 [10]	91.1 [14]
3-MCPD-d5	201.1	150.1 [20]	106.1 [40]
2-MCPD-d5	201.1	107.1 [10]	104.1 [35]
3-MBPD-d5	245.2	150.0 [10]	93.1 [30]
1,2,3,4-TCN	266.0	229.3 [15]	194.0 [10]

The precursor and product ions correspond with the derivatized MCPD and MBPD molecules. 3-MBPD represents GE after the sample preparation.

Table S3.6 *GC oven temperature gradient as used during the analysis*

	Rate (°C/min)	Set oven temperature (°C)	Hold (min)	Total (min)
Start	-	80	1	1
Ramp 1	40	140	0	2.5
Ramp 2	6	160	0	5.8
Ramp 3	15	240	0	11.2
Ramp 4	50	340	0.5	13.7
Post run	-	340	5	18.7

Injector mode: pulsed splitless; Injection temperature: 250 °C; Injection volume: 2 µL; Transferline temperature: 280 °C.

Step-by-step sample preparation procedure

1. 100 – 110 mg of each sample are weighed individually in a glass reaction tube with a screw cap. A locally store-bought and tested extra virgin olive oil was used as blank matrix for the calibration samples.
2. 50 µL of the internal standard mix are added into all samples.
3. 2 mL of anhydrous tetrahydrofuran (THF) are added in all samples and vortexed vigorously to completely dissolve all sample.
4. 30 µL of the acid aqueous sodium bromide solution are added into each sample to convert the GE into 3-MBPDE. The samples are kept at 50 °C for 15 min.
5. After the samples have cooled down to room temperature, the acid is neutralized with 3 mL of 0.6% w/v sodium hydrogen carbonate solution.
6. The 2-, 3-MCPDE, and 3-MBPDE are extracted with 2 mL n-heptane into a new glass reaction tube. To help the layer separate after vortexing, the samples are centrifuged for 5 min at 2862 xG.
7. After all n-heptane has been evaporated under a gentle nitrogen stream for 20 min at 40 °C, the residue is re-dissolved in 1 mL THF.
8. The transesterification is performed with 1.8 mL of the sulfuric acid solution in methanol for 16 h at 40 °C.
9. After transesterification, the acid is neutralized with 0.5 mL of 9% w/v hydrogen carbonate solution.
10. The volatile organic solvents in the glass tubes are evaporated with a gentle nitrogen stream for 20 min at 40 °C prior to the removal of the FAMEs.
11. 2 mL of the sodium sulfate solution is added into the remaining aqueous phase, followed by 2 mL of n-heptane. To help the layer separate after vortexing, the samples are centrifuged for 5 min at 2862 xG.
12. The n-heptane layer containing the unwanted FAMEs are carefully removed, while making sure to leave as least of n-heptane as possible. This extraction process is performed twice.
13. To derivatize the unbound 2-, 3-MCPD, and 3-MBPD, 250 µL of the PBA solution is added to each sample and the reaction takes place with the help of an ultrasonic bath for 5 min at room temperature.

14. As a final clean-up step, the PBA derivatives of 2-, 3-MCPD, and 3-MBPD are extracted out of the solution with 1 mL of n-heptane and transferred into a new glass tube.
15. To make sure that the injected solvent is as compatible with the GC as possible, the solvent is changed from n-heptane into iso-octane by means of evaporation until dry with a gentle nitrogen stream (20 min at 40 °C).
16. 400 µL of iso-octane together with 25 µL of TCN are used to re-dissolve the residue. Vigorous vortexing is required to cover all the inner surface of the glass tube.
17. The entire iso-octane solution is transferred into a 1.5 mL polypropylene Eppendorf® tube, followed by centrifugation for 10 min at 16,000 xG to remove undissolved particles and remaining PBA crystals. The supernatant can be transferred into a suitable GC vial.

Analytical method validation

The method was developed according to the AOCS Cd29a-13 method, which is similar to the later introduced standard NEN-ISO18363-3. These standards define criteria for e.g. linearity, accuracy, precision, etc. The results of the validation report here below meet all of those criteria, meaning that the method is fit for purpose.

The validation was performed according to the Dutch standard NEN7777 (Environment and food - Performance characteristics of measurement methods). This approach allows the validation of methods using real-life samples, thereby guaranteeing that the validation is performed in a concentration range that is encountered in everyday practice. In this case, this means at levels relevant for the mitigation experiments reported here. Below, the validation results are discussed per item.

Linearity

Throughout the validation experiments, performed on 8 different days, independent calibration curves were prepared. In the tables below, the average calibration curves are shown.

Table S3.7 Linearity results

Component	Correlation r^2	Intercept
3-MCPD	1.000	0.009
2-MCPD	1.000	0.007
Glycidol (3-MBDP)	0.999	0.006
Criterium* 3-MCPD / glycidol	> 0.990	< 0.020 abs
Criterium* 2-MCPD	> 0.990	< 0.050 abs
Meets criterium?	Yes	Yes

*Criterium according to the AOCS Cd29a-13 method

Accuracy

The accuracy was determined by analysis of vegetable oils with known levels of the contaminants (either by spiking or previous analysis). The accuracy analysis was determined on different days, under reproducibility conditions.

Table S3.8 Accuracy of 3-MCDP and 2-MCPD

			3-MCPD			2-MCPD		
#	**Monster-ID**	**n**	**Reference (mg/kg)**	**Results (mg/kg)**	**Accuracy (%)**	**Reference (mg/kg)**	**Results (mg/kg)**	**Accuracy (%)**
1	Olive oil	3	$\bar{x}$ 0.972	0.95	98	$\bar{x}$ 0.972	0.97	100
2	Palm oil	3	1.63	1.51	93	0.81	0.78	96
3	Palm oil	3	1.34	1.24	93	0.33	0.33	100
4	Palm oil	3	1.30	1.23	95	0.60	0.62	103
5	Palm oil	3	$\bar{x}$ 1.69	1.72	102	$\bar{x}$ 1.00	1.03	103
6	Olive oil	3	$\bar{x}$ 0.24	0.21	88	$\bar{x}$ 0.24	0.24	99
8	Palm oil	3	6.32	6.22	98	3.11	3.20	103
13	Palm oil	3	1.46	1.56	107	1.46	1.53	105
	Criterium*				75-120			75-120
	Average	24			98			99
	RSD (%)	24			5			6
	Meets criterium?	24			Yes			Yes

*Criterium according to the AOCS Cd29a-13 method

Table S3.9 Accuracy of glycidol

			Glycidol (3-MBPD)		
#	**Sample-ID**	**n**	**Reference (mg/kg)**	**Result (mg/kg)**	**Accuracy (%)**
1	Olive oil	3	1.22	1.18	96
5	Palm oil	3	$\bar{x}$ 1.26	1.32	105
6	Olive oil	3	$\bar{x}$ 0.30	0.25	83
9	Palm oil	3	0.49	0.49	101
10	Palm oil	3	0.96	0.99	103
11	Palm oil	3	1.94	2.03	104
12	Palm oil	3	3.44	3.67	107
13	Palm oil	3	1.45	1.52	105
	Criterium*				75-120
	Average	24			105
	RSD (%)	24			5
	Meets criterium?	24			Ja

*Criterium according to the AOCS Cd29a-13 method

Repeatability and reproducibility

The repeatability and reproducibility were determined by analysis of 8 different oil samples were taken all at relevant MCPDE and GE levels (between LOD and 3.5 mg/kg). Each sample was analyzed in duplicate on a single day, and another (single) measurement was done on another day. All measurements on different days were performed under reproducibility conditions. This approach allows the calculation of repeatability and reproducibility.

Table S3.10 Repeatability and reproducibility

		Repeatability			n	Reproducibility		
Component	**n**	**Concentration (mg/kg)**	**RSD_r (%)**	**(%)**		**Absolute (mg/kg)**	**RSD_{RL} (%)**	**(%)**
3MCPD	8	0.10	2	6	8	0.14	5	14
2MCPD	8	0.08	3	8	8	0.12	11	31
Glycidol (3MBDP)	8	0.15	4	11	8	0.14	11	32
Criterium*			8	22			16	45
Meets criterium?			Yes	Yes			Yes	Yes

*Criterium according to the AOCS Cd29a-13 method

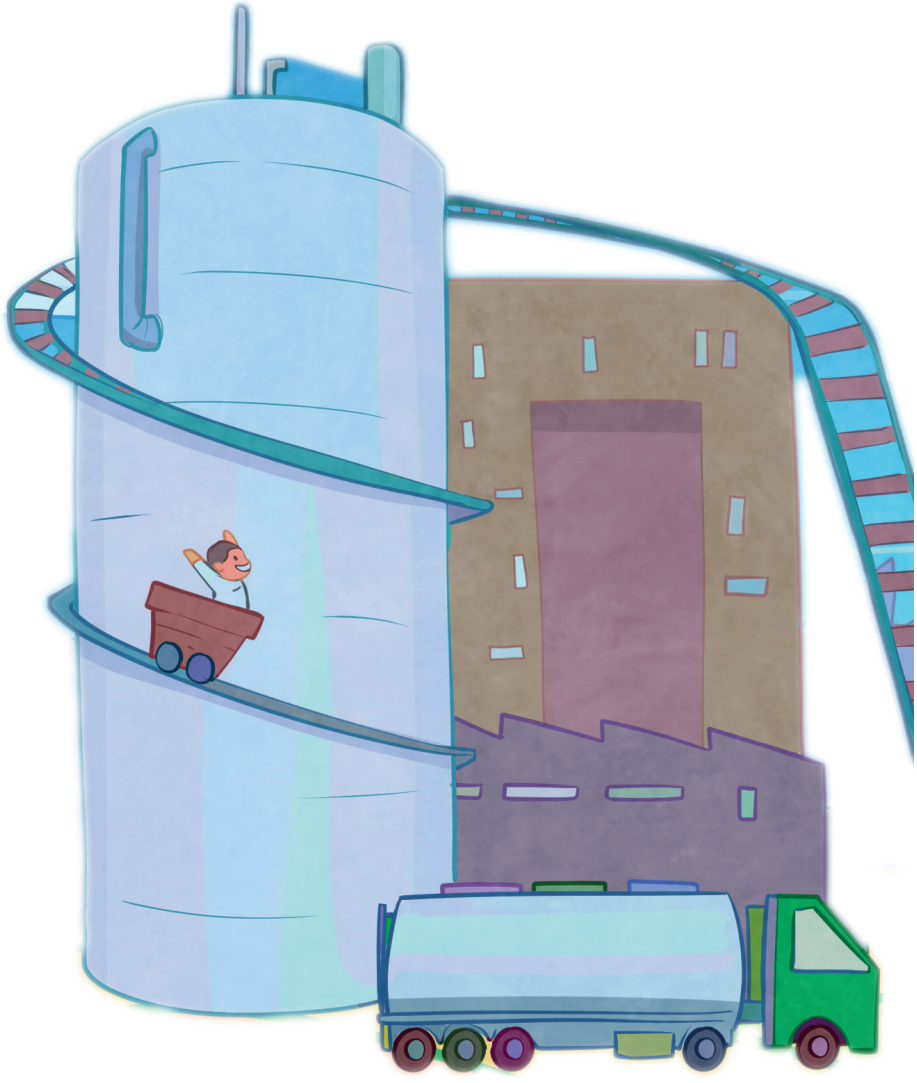
Limit of detection and limit of quantification

The LOD and LOQ were determined by analysis of 8 spiked olive oil samples at the level of 0.2-0.3 mg/kg.

Table S3.11 LOD and LOQ validation results

Component	LOD (mg/kg)	LOQ (mg/kg)
3-MCPD	0.05	0.10
2-MCPD	0.04	0.07
Glycidol (3-MBDP)	0.03	0.07
Criterium* MCPDs	< 0.21	< 0.21
Criterium* glycidol	< 0.26	< 0.26
Meets criterium?	Ja	Ja

*Criterium according to the AOCS Cd29a-13 method



Chapter 4

Chemical refining methods effectively mitigate 2-MCPD esters, 3-MCPD esters, and glycidyl esters formation in refined vegetable oils

Adapted from:
Sergio B. Oey, H.J. van der Fels-Klerx, Vincenzo Fogliano, and Stefan P.J. van Leeuwen, Chemical refining methods effectively mitigate 2-MCPD esters, 3-MCPD esters, and glycidyl esters formation in refined vegetable oils. Food Research International, 2022, 156, 111137

Abstract

Esters of 3-monochloro-1,2-propanediol (3-MCPDE), 2-monochloro-1,3-propanediol (2-MCPDE), and glycidyl esters (GE) are processing contaminants that can be found in refined edible fats and oils. Recently, the European Commission has implemented maximum limits for the presence of free and bound 3-MCPDE in vegetable fats and oils and in marine and fish oils. This boosted the necessity of oil producers to develop refining methods to limit the concentration of both 3-MCPDE and GE in their final products. Physical refining may lack the potential to mitigate the formation of 2- and 3-MCPDE. Therefore, in this study, chemical refining method were explored to provide a viable mitigation strategy aimed at industrial application. Several pilot plant treatments with organic palm oil were performed. The investigated refining methods included a neutralization, a water washing process, reduced deodorization temperature, and a combination of them. The best performing chemical refining treatment achieved a final concentration of 0.42 (−49%), 0.78 (−52%), and 0.99 (−73%) mg/kg for 2-MCPDE, 3-MCPDE, and GE in organic palm oil, respectively. Results thus showed chemical refining has great potential for the simultaneous mitigation of 2-, 3-MCPDE, and GE.

Keywords: 3-Monochloropropanediol; Glycidol; Processing Contaminants; Refined Edible Oils; Pilot Plant Refining; Mitigation Strategies

4.1. Introduction

In recent years, many studies about mitigating the formation of 3-monochloropropane-1,2-diol fatty acid esters (3-MCPDE), and glycidol fatty acid esters (GE) in refined vegetable oils have been conducted. Together with 2-monochloropropane-1,3-diol fatty acid esters (2-MCPDE), 3-MCPDE and GE are potentially carcinogenic compounds which can particularly be abundant in refined palm oil. In 2018, the European Commission has defined a maximum limit (ML) for the concentration of GEs in vegetable fats and oils (European Commission, 2018). In continuation, the European Commission published an Annex (D066084/02) regarding an amendment on the Regulation (EC) 1881/2006 in 2020, in which a ML has been established for the sum of the presence of 3-monochloropropane-1,2-diol (3-MCPD) and 3-MCPDE in vegetable oils, fish oils, and oils from other marine organisms next to the existing ML for GE (European Commission, 2020).

Physical oil refining method in the food industry is widely used. Studies on physical refining methodologies to reduce the formation of 3-MCPDE and GE in vegetable oils have been published, often solely tested in a lab environment, as recently reviewed by Gao et al. (2019) and Oey et al., (2019). Gao et al. (2019) investigated 18 publications about mitigation approaches for 3-MCPDE and concluded that diacylglycerols (DAGs), monoacylglycerols (MAGs), free fatty acids (FFAs), and chlorine can be seen as the precursors for 3-MCPDE, but many papers are still contradictive. Gao et al. (2019) also summarize several potential mitigation strategies including controlling the deodorization temperature, adding chelating agents, changing CPO processing conditions, and many more. However, these potential mitigation strategies are often the results of lab experiments, which has to overcome its own challenges when it is going to be upscaled to a full-scale refining. Oey et al. (2019) primarily shows the lack of data and mitigation strategies for 2-MCPDE, but also highlighted that the majority of the published strategies are often tailored for one of the three process contaminants. Furthermore, physical refining strategies were most successful in mitigating GE, but has less impact on 3-MCPDE. Interestingly, only few papers have reported the occurrence of unbound 3-MCPD and/or unbound glycidol and, at the moment of writing, there are no peer-reviewed publication available regarding mitigation strategies aimed at unbound 2-, 3-MCPD, and glycidol (MacMahon et al., 2013; Zelinková et al., 2006). With the addition of unbound 3-MCPD to the EU regulations, more data is requested to have a better understanding of the relevance of unbound MCPDs in the refined oil products.

The chemical refining route has been investigated infrequently because of increased product losses as oil is being neutralized (e.g. saponification) (Chumsantea et al., 2012; Dijkstra, 2016). Several publications have mentioned the advantages of chemical

refining in comparison to physical refining, including the effective removal of gums and FFAs in oil types that cannot be refined well via the physical approach and that chemical refining removes less desired components such as tocopherols, phenols, and sterols. However, compared to physical refining, chemical refining is often related with higher neutral oil loss, larger waste product, and higher chemical usage (Pal, Patra, Sahoo, Bakhara, & Panda, 2015; Zhu et al., 2015; Gotor & Rhazi, 2016).

When extrapolating mitigation methods investigated at the lab-scale, difficulties might be encountered during upscaling to an industrial-scale refining method. Not only is the physical geometry of the lab-scale set-up often very different from a full-scale refinery tower, but other physio-chemical attributes such as surface area of the oil or the thermal capacity are different as well after upscaling the method.

According to several proposed formation pathways, the formation of 2- and 3-MCPDE requires a certain chlorine source (Destaillats et al., 2012; Šmidrkal et al., 2016; Yao et al., 2019). It can be expected that small amounts of chlorine are present in the crude palm oil. Zhao et al. (2016) showed the potential of various organic and inorganic chlorides, such as lindane, KCl, NaCl, and $FeCl_3$, reacting with monoglycerides to form 3-MCPDE and GE. Furthermore, Svejkovská et al. (2006) have reported that other glyceride compounds, such as 1-monopalmitin or tripalmitin may also be seen as precursors for 3-MCPDE. Based on that knowledge, removal of the inorganic chlorine by washing the crude palm oil with deionized water prior to refining can be one of many plausible mitigation strategies. In addition, only a limited number of published mitigation strategies have included 2-MCPDE; most of them focused on 3-MCPDE.

Lampen (2022) have summarized the toxicology of 2-MCPDE and concluded that more toxicological data about 2-MCPDE is required to gain better understanding of its toxicity, but also reported that 2-MCPDE's toxicity may trigger different mode of action in vivo than 3-MCPDE. Furthermore, Buhrke et al. (2015) reported that upon hydrolysis in the gut, free 2-MCPD is cytotoxic at concentrations above 1 mM and that the used Caco-2 cells showed reduced cellular viability when subjected to 2- and 3-MCPDE concentrations above 10 µM. Finally, 2-MCPDE toxicity in rat kidney, liver, and heart has also previously been reported (Frenzel et al., 2018; Schultrich et al., 2017). Therefore, mitigation strategies should not neglect 2-MCPDE as its toxicity has been shown in several studies.

Chemical refining involves a neutralization step of the oil with a base (usually a lye), followed by the removal of FFAs by water washing. Whenever unbound or inorganic chlorine is still present after the neutralization process, it can be expected that they will be removed together with the FFAs during the water washing process. The potential

of the neutralization process in the development of a good mitigation strategy has been noted. Ramli et al. (2011) succeeded to lower the 3-MCPDE concentration from 2.2 to 1.4 mg/kg with 0.2% CaO, and Freudenstein et al. (2013) achieved a similar end concentration of 1.1 mg/kg 3-MCPDE with 1 mmol/kg $NaHCO_3$. A water washing step can also be implemented prior to the actual start of the oil refining. Crude oil can be mixed with deionized water to remove water soluble precursors. Ramli et al. (2011) and Zulkurnain et al. (2013) reported final 3-MCPDE concentrations of 0.75 mg/kg and 0.2 mg/kg, respectively, with a pre-refining water wash process. In chemical refining, the neutralization of the acid during the degumming process is a key factor, together with either a pre-refining or post-neutralization water wash. As most of the FFAs are saponified during neutralization and removed during the post-neutralization water wash, there is no need for high-temperature steam distillation during the deodorization phase. Hrncirik and van Duijn (2011) used neutralization and a 5-hour deodorization process at 180 °C and observed a minimal reduction in 3-MCPDE from 4.8 to 4.1 mg/kg and a notably low GE concentration of 0.4 mg/kg in comparison of their reference conditions (180 °C for 1 h). Altogether, only a few studies explored mitigation strategies based on chemical refining, leaving room for improvement and further explorations. None of those studies have significantly explored the potential benefits of combining multiple strategies. Combining several mitigation strategies can result in a good all-round chemical refining mitigation strategy which addresses all the contaminants (2-, 3-MCPDE, and GE) at once. The difficulties lie in the fact that 2- and 3-MCPDE have different mechanism of formation than GE and thus requires a different mitigation approach (Cheng et al., 2017; Destaillats et al., 2012; Šmidrkal et al., 2016; Yao et al., 2019). Furthermore, 2- and 3-MCPDE are less thermolabile than GE, therefore a strategy to prevent their formation instead of removing them with a heat treatment, like a deodorization process, might be more successful (Shimizu et al., 2013).

The aim of this study was to investigate the effectiveness of various chemical refining strategies for the mitigating of the formation of 2-, 3-MCPDE, and GE during vegetable oil refining at the industrial scale. Several treatments including water washing were conducted to explore the crucial steps of chemical oil refining.

4.2. Materials & methods

All materials used for the pilot plant treatments were of food grade quality. The organic crude palm oil (CPO) from Ivory Coast were supplied by Spack B.V. (Nieuwe-Tonge, The Netherlands) and were certificied as organic by Skal Biocontrole (NL-BIO-01) on the basis of Article 29 (1) of Regulation (EC) No. 834/2007 and Regulation (EC) No. 889/2008. Pure-Flo® B80 natural bleaching earth was purchased from Oil-Dri (Ripley,

Mississippi, USA). Citric acid, Dicalite® Perlite 478 filter aid, and the sodium hydroxide were purchased from Univar Solutions (Rotterdam, The Netherlands). Norit® SA 4 PAH-HF activated carbon was purchased from Cabot Norit Nederlands B.V. (Amersfoort, The Netherlands). One hundred kg of organic CPO was used as starting material for each pilot plant treatment. Separately, 30 kg of the same organic CPO was used to pre-flush the pilot plant prior to each treatment. 0.5% w/w-oil of a 25% citric acid solution was used during the degumming process. 10 % w/w of pre-heated reverse osmosis (RO) water and 2L sodium hydroxide (33% w/v solution) was used when the treatments required them. Bleaching process was applied for all treatments. This was performed with 1.0% w/w Pure-Flo® B80 natural bleaching earth. A filter bag made from polyester (Eaton CLEARGAF™ polyester needlefelt) was used for the filtration of the oil prior to deodorization. Detailed explanation of the pilot plant, stationed in an oil refinery in Zaandam, and a schematic illustration are described previously (Oey et al., 2020). In essence, the pilot plant, with a capacity of 100 kg oil, consists of a treatment tank, a filtration set-up, and a deodorization tank. Both tanks are equipped with electric heating elements, but the deodorization tank is also equipped with strip-steam capabilities and vacuum can be applied to it. Prior to usage, the pilot plant has been extensively tested by our project partner SRC B.V., validating its performance in the oil refining process.

4.2.1. Experimental design

The experiment covered the effects of several treatments, including thermal treatment, water washing, and oil neutralization prior to deodorization, on the formation of the 2-, 3-MCPDE, and GE. The control and the four treatments were all conducted in a pilot plant. Schematic overviews of the temperature–time profile of the control and all treatments are presented in Figure 4.1, Figure 4.2. Details of the experimental conditions are reported in Table 4.1. The control (Figure 4.1A) was designed as two individual physical refining treatments, performed consecutively. This allows for the other treatments to be compared against a normal single physical refining, which is commonly used for the refining of CPO, and against a worst-case scenario in which the refined palm oil was processed for the second time – using similar conditions as the first refining. The four treatments studied in this experiment are: a pre-refining water wash treatment (Treatment A, Figure 4.1B), a post-degumming water wash treatment (Treatment B, Figure 4.1C), alkali neutralization of the oil with sodium hydroxide after the degumming process plus a water wash thereafter (Treatment C, Figure 4.2A), and finally a 'full' chemical refining including neutralization, post-degumming water wash, and a lower deodorization temperature which is only required to sterilize the oil (Treatment D, Figure 4.2B). These four treatments were selected so that the effect of each individual chemical refining steps on the formation of 2-, 3-MCPDE, and GE can be isolated. This allows for better observation and comparison.

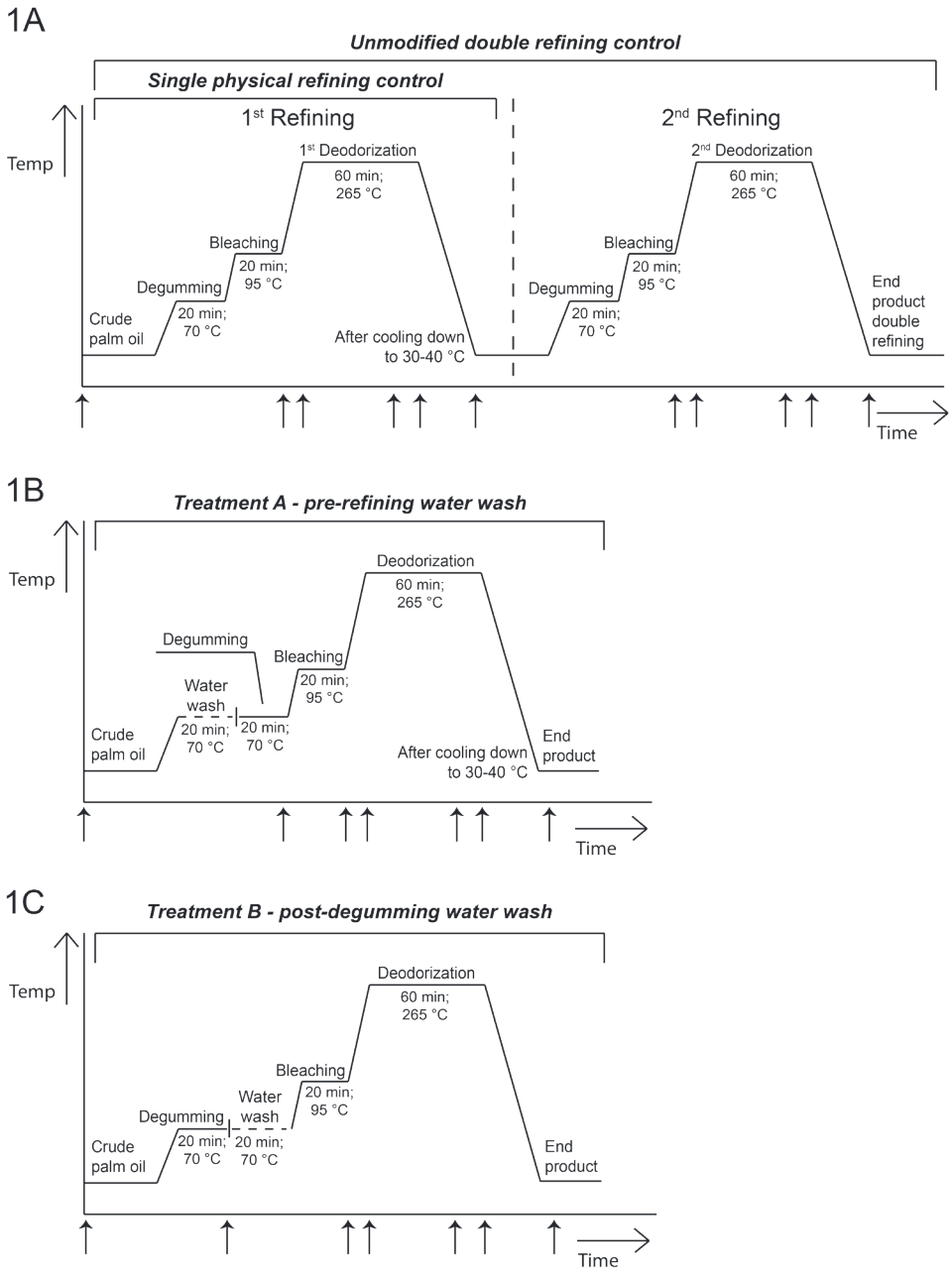


Figure 4.1 Schematic depiction of the temperature-time profile of the control treatment (1A), pre-refining water wash (1B), and post-degumming water wash (1C). The vertical arrows underneath the graphs indicate the sampling moments.

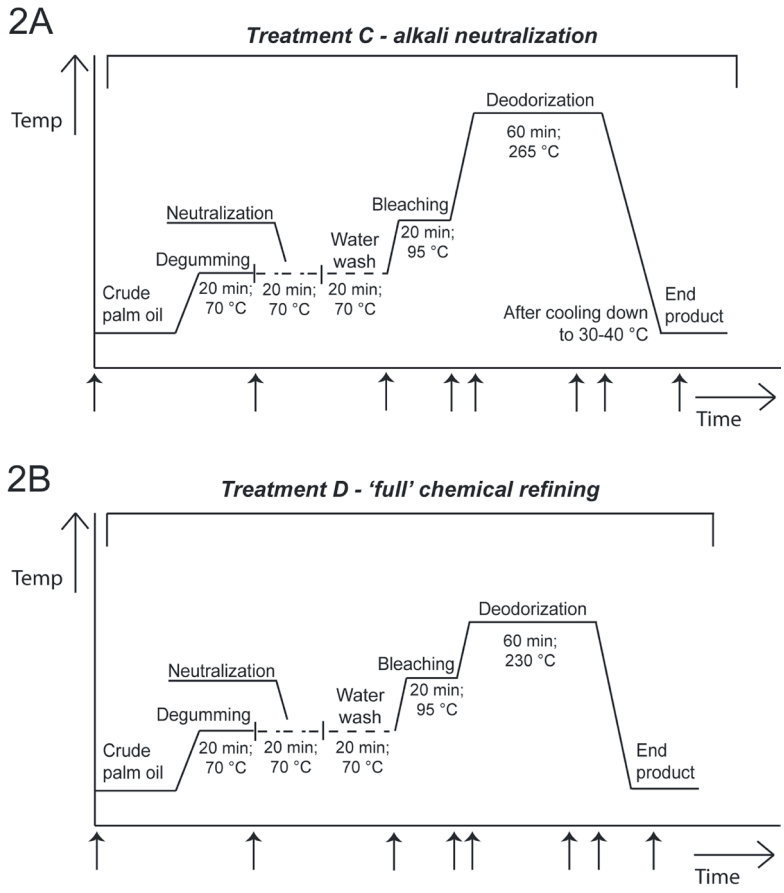


Figure 4.2 Schematic depiction of the temperature-time profile of Treatment C - alkali neutralization treatment with a water wash (2A) and Treatment C - 'full' chemical refining treatment (2B) which has a lower deodorization temperature. The vertical arrows underneath the graphs indicate the sampling moments.

Table 4.1 Experimental conditions of the four chemical refining treatments (Treatment A - D) and one control treatment (a double physical refining) which were refined in the pilot plant.

	Water wash of CPO	Degumming	Neutra-lization	Water wash	Bleaching	Deodorization
Control Treatment (single & double physical refining)	–	20 min., 70 °C, 0.5 % citric acid (from a 25 % citric acid solution)	–	–	20 min., 95 °C, 1.0 % Pure-Flo® B80, 0.1 % Norit®, 0.2 % Dicalite® 478	60 min., 265 °C, strip-steam, 3 mbar vacuum, rapid cooling down after 60 min.
Treatment A – pre-refining water wash	20 min., 70 °C, 10 % DI-water (water pre-heated to 70 °C)	20 min., 70 °C, 0.5 % citric acid (from a 25 % citric acid solution)	–	–	20 min., 95 °C, 1.0 % Pure-Flo® B80, 0.1 % Norit®, 0.2 % Dicalite® 478	60 min., 265 °C, strip-steam, 3 mbar vacuum, rapid cooling down after 60 min.
Treatment B – post-degumming water wash	–	20 min., 70 °C, 0.5 % citric acid (from a 25 % citric acid solution)	–	20 min., 70 °C, 10 % DI-water (water pre-heated to 70 °C)	20 min., 95 °C, 1.0 % Pure-Flo® B80, 0.1 % Norit®, 0.2 % Dicalite® 478	60 min., 265 °C, strip-steam, 3 mbar vacuum, rapid cooling down after 60 min.
Treatment C – alkali neutralization	–	20 min., 70 °C, 0.5 % citric acid (from a 25 % citric acid solution)	20 min., 70 °C, 2 L of 33 % NaOH solution	20 min., 70 °C, 10 % DI-water (water pre-heated to 70 °C)	20 min., 95 °C, 1.0 % Pure-Flo® B80, 0.1 % Norit®, 0.2 % Dicalite® 478	60 min., 265 °C, strip-steam, 3 mbar vacuum, rapid cooling down after 60 min.
Treatment D – 'full' chemical refining	–	20 min., 70 °C, 0.5 % citric acid (from a 25 % citric acid solution)	20 min., 70 °C, 2 L of 33 % NaOH solution	20 min., 70 °C, 10 % DI-water (water pre-heated to 70 °C)	20 min., 95 °C, 1.0 % Pure-Flo® B80, 0.1 % Norit®, 0.2 % Dicalite® 478	60 min., 230 °C, strip-steam, 3 mbar vacuum, rapid cooling down after 60 min.

4.2.2. Sample collection

Sample collection during each treatment was performed manually at the pilot plant. The pilot plant allows for on-line sample collection at any given moment during a refining process. The moment of sample collection during the control and the four experimental treatments are indicated with black arrows in Figure 4.1 and Figure 4.2. Each of the collected sample was approximately 300 mL in size and were stored in the freezer at −18 °C prior to analysis. The sample that was indicated with 'DEO-0 min' was taken at the beginning of the deodorization process when the oil had reached the set temperature of 265 °C. The 'DEO-40 min' and the 'DEO-60 min' samples were taken after 40 and 60 min of deodorization.

4.2.3. Analytical determination

The analyses of 2-, 3-MCPDE, and GE in all oil samples were performed with an in-house validated method, at Wageningen Food Safety Research, according the Dutch NEN 7777 standard. Detailed materials and methods used for the in-house validation and analyses of 2-, 3-MCPDE, and GE are previously described (Oey et al., 2020). The core concept of this method was based on the AOCS Official Method Cd 29a-13. We expanded the selectivity of this method by introducing a third penta-deuterated internal standard to quantify 2-MCPDE and by performing additional multiple-reaction-monitoring (MRM) measurements in the MS for the phenyl boronic acid (PBA) derivates of 2-, 3-MCPDE, and GE. Essentially, GE was first converted into primarily 3-monobromopropane-1,2-diol esters (3-MBPDE). After the conversion, a mild acid-mediated transesterification of the 2-, 3-MCPDE, and 3-MBPDE was performed. The samples were then cleaned-up using liquid–liquid extraction methods with heptane and, finally, a derivatization step was performed with PBA.

The samples were analyzed using an Agilent 7010B Triple Quadrupole GC–MS/MS system (Agilent, USA). Separation occurred in an Agilent DB-35MS UI GC column (30 m × 0.250 mm × 0.25 µm) (Agilent, USA). A nine-point calibration line in a blank extra virgin olive oil matrix was used for the quantification, including a blank calibration point. The highest calibration point was equivalent to 0.62 µg unbound 3-MCPD, 0.62 µg unbound 2-MCPD, and 0.78 µg unbound glycidol. The results of the in-house validation can be found in the supplemental materials. The average accuracy (i.e. being able to detect the true concentration) for 2-, 3-MCPDE, and GE were determined during the validation of the method, as well as the linearity, limit of quantification (LOQ), and limit of detection (LOD). The method was linear for the used calibration range. The average accuracies were 99%, 98%, and 105%, respectively, for 2-, 3-MCPDE, and GE. The LOQ for 2-, 3-MCPDE, and GE were 0.07, 0.10, and 0.07 mg/kg, respectively. The LOD for 2-, 3-MCPDE, and GE were 0.04, 0.05, and 0.03 mg/kg, respectively. The repeatability and reproducibility of the method were determined by analyzing eight different oil samples

in duplicates with varying concentration between the LOD and 3.5 mg/kg. The relative standard deviations (RSD) for the repeatability of 2-MCPDE, 3-MCPDE, and GE are 3%, 2%, and 4%, showing good precision (i.e. has a low amount of spread in the results). Furthermore, the real-life performance of this method has been proven and guaranteed by participating with multiple proficiency tests organized by FAPAS and the European Reference Laboratory for Process Contaminants with satisfactory Z-scores. The extensive in-house validation and the proficiency tests showed that the method is fit for purpose and provides accurate results.

4.2.4. Physicochemical quality parameters of the oil samples

The water content in the individual oil samples were determined by coulometric Karl Fischer titration. A Metrohm 917 coulometer was used with Hydranal™-coulomat CG as the catholyte solution and Hydranal™-coulomat oil as the anolyte solution. In this study, the water content in the oil samples is expressed in percentages. The FFA concentration, expressed as '% oleic', and the color of the oil were analyzed as well. The FFA was determined by potentiometric titration using sodium hydroxide. Oil color determination was performed manually using a visual colorimeter and a 5¼-inch cuvette on the Lovibond® scale (Red, Yellow, Blue, and Neutral color scale).

4.3. Results & discussion

4.3.1. Single & double physical refining control

Results of the control treatment are shown in Figure 4.3A. The single physical refining control, which is the first half of the control treatment, resulted in a GE concentration in the end product of 3.73 mg/kg, whereas the concentrations of 2-MCPDE and 3-MCPDE were 0.82 and 1.62 mg/kg, respectively. However, by the end of the second refining step GE concentration was elevated to 8.42 mg/kg. After the formation of 2- and 3-MCPDE in the single refining control, the levels of these two compounds remained stable throughout the entire second physical refining. As a side note, our analytical method has been thoroughly validated in-house and the results' variances caused by the analytical method have been deemed very low. A complete set of the water content results of this control treatment and the other four treatments are presented in Appendix A (Figure 4.5 – Figure 4.9). Results of the FFA and oil color can be found in the Supplementary material section, Table S4.1 – S4.5.

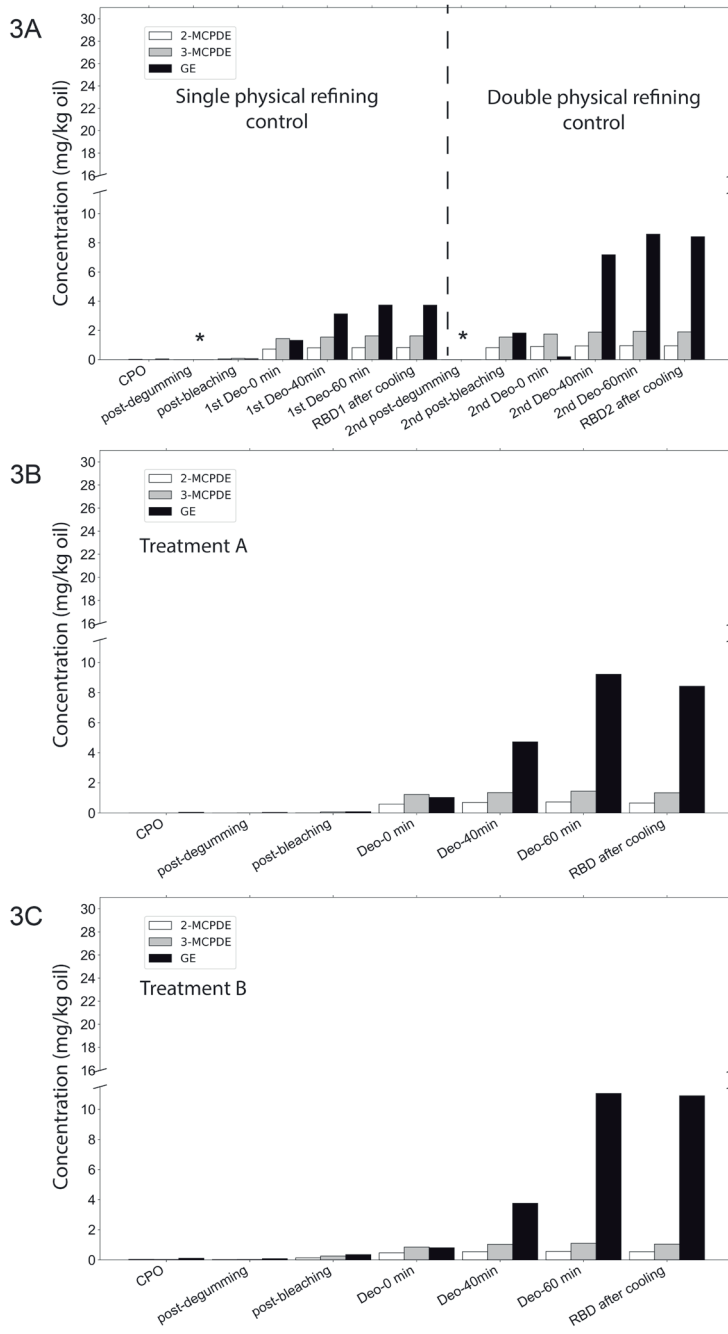


Figure 4.3 Results of the two-part control treatment (3A), pre-refining water wash (3B), and the post-degumming water wash (3C). The vertical dashed bar in 3A shows the two separate refining processes that were used as single physical refining control and a 'worst-case scenario' control of an unmodified double refining. The asterisk (*) shows samples that were not collected during refining.

The control treatment was designed to obtain results of both a single physical refining, which is commonly used in the oil industry, and a double physical refining without any modifications. The unmodified double physical refining method does not provide any practical benefits, but it shows the effect of a worst-case scenario in which a refined oil would be subjected twice to high deodorization temperatures.

The observed stability of 2- and 3-MCPDE during the second refining procedure is in line with the proposed mechanisms of the formation of 2- and 3-MCPDE. The formation of 2- and 3-MCPDE requires a chlorine source such as sodium chloride or hydrochloric acid that can be incorporated into a MAGs and/or DAGs via multiple pathways which involves nucleophilic substitution and the formation of an intermediate (Destaillats et al., 2012; Hamlet et al., 2011; Rahn and Yaylayan, 2011; Šmidrkal et al., 2016). Once the chlorine atom has been incorporated, it forms a stable covalent bond. Ermacora and Hrncirik (2014) showed that the formation of 2- and 3-MCPDE is positively correlated with the concentration of available chlorine in the oil. When the available chlorine sources were depleted during the first refining in our control, no additional formation of 2- and 3-MCPDE occurred during the second refining. This is in line with earlier observations in another study on physical refining of CPO (Oey et al., 2020).

Interestingly, a drop in the GE concentration after the second bleaching process was observed. GE is known to be less chemically stable than 2- and 3-MCPDE due to its epoxide. Cheng et al., (2020) showed that GEs can indeed be degraded via an acid-induced ring-opening reaction. Furthermore, Shimizu et al., (2012) previously has reported the usage of activated bleaching earth to eliminate GE. They have reported that glycidyl palmitate was transformed into glycerol monopalmitate, glycerol palmitate oleate, and glycerol dipalmitate rather than absorption of GE into the bleaching earth. As acid and bleaching earth was re-introduced to the oil for the second time during the secondary refining process in our control experiment, this change of pH in combination with the bleaching earth might have degraded GE. The increase of GE during the second deodorization is expected due to oil degradation as it is being exposed to high temperature for a second time. Triacylglycerols (TAGs) can degrade into DAGs or MAGs which are precursors for MCPDEs.

4.3.2. Treatment A – Pre-refining water washing

The first treatment (Treatment A) examined the effect of a pre-refining wash of the CPO with reverse osmosis (RO) water. Results are reported in Fig. 3B. As observed in the single physical refining control, 2-, 3-MCPDE, and GE were formed after the bleaching process. The concentration of 2- and 3-MCPDE started to increase at the beginning of the deodorization process and showed a minor increase in concentration for the remaining duration of the deodorization process. The final concentration of 2- and 3-MCPDE were

0.67 and 1.34 mg/kg, respectively (see Figure 4.3B). The concentration of 2-MCPDE were decreased by 18% and 3-MCPDE was decreased by 17% in comparison to the single physical refining control (Figure 4.3A).

The levels of GE increased during the deodorization process. After 40 min of deodorization during the secondary physical refining, GE reached a concentration of 8.42 mg/kg. In this treatment, the levels of GE were higher than in the control treatment (Figure 4.3A). The exact cause of this increase in GE concentration remains inconclusive, but some speculations can be made. Temperature and duration can be ruled out as the source of the large increase, because those refining parameters are the same as in the physical refining control. Having said that, the water wash process may have caused the increase in GE concentration by hydrolyzing the TAGs into DAGs. Silva et al. (2019) performed a series of experiments in which bleached palm oil (BPO) was washed with different solvents. When BPO was washed a single time with water, they observed a 21% increase in the GE concentration, which is less than what was observed at the end of Treatment A. Furthermore, DAGs concentration was approximately increased by 72%. Previous studies have indicated that GE can be formed from both MAGs and DAGs without the need of a chlorine source via intramolecular rearrangement (Cheng et al., 2016; Cheng et al. 2017; Destaillats et al., 2012). Increased concentrations of MAGs or DAGs might therefore lead to higher concentrations of GE.

The relatively high water content of 0.46% (see Figure 4.6 in Appendix A) in the 'post-degumming' sample was expected because the separation of water and oil was done only by gravitational separation as the pilot plant did not have a centrifuge to efficiently remove the water. After the 'post-bleaching' sample, the water content decreased to below 0.1%. It can be expected that most of the water has evaporated by the end of the bleaching process as the oil temperature was set at 95 °C. However, it is possible that this small percentage of water could have indirectly caused the large increase in GE concentration. The residual water might have unfavorably altered the pH or have reacted with the TAGs, acid and/or bleaching earth. An increase of the presence of MAGs and DAGs due to hydrolysis is plausible and cannot be ruled out.

Recently, Ramli et al. (2020) washed 900 Tonne (900,000 kg) of CPO at the palm oil mill using 5–10% hot, softened water (90–95 °C, total chlorine of 5 mg/kg). Two refineries have processed the oil in almost similar manner to our double physical refining control except for the left-out secondary degumming step and the lower second deodorization temperature. The details of the refining conditions were unfortunately not reported. After a single refining process without post-refining processing, the washed CPO sample had a 2-, 3-MCPDE, and GE concentration of 0.49 ± 0.12, 1.37 ± 0.33, and 3.84 ± 0.42 mg/kg, respectively (Ramli et al., 2020). With post-refining bleaching and deodorization, they

found a final 3-MCPDE and GE concentrations around 1.4 and 1.2 mg/kg, respectively. Post-refining 2-MCPDE concentration was not reported. The 3-MCPDE concentrations observed by Ramli et al. (2020) were similar to the concentration in our experimental treatment with pre-refining water washing. However, their GE concentrations, with and without post-refining processing, were both lower than those concentrations in our treatment.

4.3.3. Treatment B – Post-degumming water wash

The second treatment (Treatment B) examined the effect of a water wash treatment performed after a degumming process. More specifically, the wash treatment occurred between the degumming process and the bleaching process. The results of this experimental treatment are shown in Fig. 3C. Similar to the pre-refining water washing, the formation of 2-, 3-MCPDE, and GE started after the bleaching process. Again, using the post-degumming water wash, the majority of the 2- and 3-MCPDE were formed during the pre-heating stage prior to the start of the deodorization process. However, in this case (Treatment B) the final concentrations of 2- and 3-MCPDE (0.54 and 1.04 mg/kg, respectively) were, respectively, 19% and 22% lower than the final concentrations observed in Treatment A. Compared to the single physical refining control treatment, this treatment resulted in a reduction of 34% for the 2-MCPDE concentration and 36% for the 3-MCPDE concentration. A wash process after the degumming process resulted in the removal of citric acid that has been used during the degumming step. According to Destaillats, Craft, Sandoz, et al. (2012), acidic environments promote the formation of 3-MCPDE. A wash process beneficially reduces the potential catalytic role of citric acid or any other acids in the formation of 2-, 3-MCPDE, and GE. Therefore, the observed results were in line with the prior expectations that this treatment (Treatment B) results in a lower 2- and 3-MCPDE concentration as compared to the single physical refining control treatment and the pre-refining water wash treatment (Treatment A).

Several similarities and differences were observed comparing the results of this post-degumming water wash with the control treatment. As observed in the control treatment, 2- and 3-MCPDE were formed prior to the start of the deodorization step. Once 2- and 3-MCPDE have been formed, their concentrations remained stable throughout the entire deodorization process. The results were different for the concentration of GE: its formation started during the deodorization process but GE concentration in this treatment rose to 11.05 mg/kg after 60 min of deodorization and the final oil sample after the cool down had a concentration of 10.90 mg/kg. This final GE concentration was higher than the GE concentrations of both the single and double physical refining controls (3.73 and 8.42 mg/kg, respectively). The increased GE concentration in Treatment B might share a similar cause as what was discussed previously in Treatment A. Water that was still present in the oil after the wash process might indirectly promote the hydrolysis

of TAGs into MAGs and DAGs and. Furthermore, compared to Treatment A, Treatment B showed a greater GE increase. This can be explained by the deliberate increase of oil temperature right after the washing process in Treatment B to accommodate the bleaching condition. This higher oil temperature after a wash treatment may lead to an increase of reactivity resulting in more MAGs and DAGs formation.

Recently, Hew et al. (2021) optimized a modified refining process in laboratory-scale using a process similar to our post-degumming water washing treatment (Treatment B). The main differences are the volume of the CPO, the adoption of phosphoric acid instead of citric acid, the usage of less water during the water degumming or in our case the post-degumming water wash process (1.0% instead of 10%), and a slightly different temperature–time profile of the deodorization condition (90 min at 250 °C instead of 60 min at 265 °C). With this refining method, Hew et al. (2021) achieved a final 3-MCPDE concentration of 0.71 mg/kg which is slightly lower than ours at the end of this treatment (1.04 mg/kg, Treatment B). Interestingly, their GE concentration of 0.32 mg/kg is lower than at the end of our Treatment B (10.90 mg/kg). Unfortunately, it is almost impossible to compare the quality of the CPO used by Hew et al. (2021) and our CPO. If they have used a higher quality CPO, it might explain why the observed concentrations in their experiments are lower than ours. Furthermore, the difference in the physical experimental set-up makes a direct comparison very challenging. Nonetheless, our results and the results of Hew et al. (2021) indicate the potential of this treatment to mitigate 2-, 3-MCPDE, and GE.

4.3.4. Treatment C – Alkali neutralization

The key factor differentiating this alkali neutralization treatment from the other investigated treatments is the chemical neutralization process. Therefore, the experimental design choice was selected to exclusively monitor the effect of the alkaline neutralization with sodium hydroxide. The addition of sodium hydroxide is useful not only to neutralize the citric acid used for the degumming, but also to remove the majority of the FFAs by turning them into soap stock. As the FFAs are no longer present during the deodorization process, lower deodorization temperatures can be use during chemical refining. Whereas in principle, the deodorization temperature can be lowered after alkali neutralization, the deodorization temperature during this treatment was kept unchanged for the sake of equal comparison with the control and especially with the post-degumming washing treatment (Treatment B).

Looking at the results reported in Figure 4.4A, the final concentrations of 2- and 3-MCPDE was 0.31 and 0.59 mg/kg, respectively. This resulted in a 62% and 64% reduction for the 2- and 3-MCPDE, respectively, compared to the single physical refining control. Furthermore, the final 2- and 3-MCPDE concentrations of this neutralization

treatment were 43% lower compared to the results of the post-degumming washing process. The main difference between this treatment and Treatment B is the addition of sodium hydroxide in this treatment. With this design we were able to isolate the effect of adding a lye to neutralize the oil. As reported by Šmidrkal et al. (2016), 2- and 3-MCPDE formation is favorable in an acidic environment. The added sodium hydroxide neutralizes the pH of the oil. This results in an inhibition of 2- and 3-MCPDE formation due to the less favorable condition.

The three contaminants (2-, 3-MCPDE, and GE) are formed before the start of the deodorization process. This is similar to what has been observed in the control and previous two treatments. These results showed that the creation of a basic environment is beneficial for the reduction of the formation of 2- and 3-MCPDE. The post-degumming water washing treatment (Treatment B) was able to slightly reduce the 2- and 3-MCPDE formation, however the effect is minimal when compared to the effect of sodium hydroxide in this treatment (Treatment C).

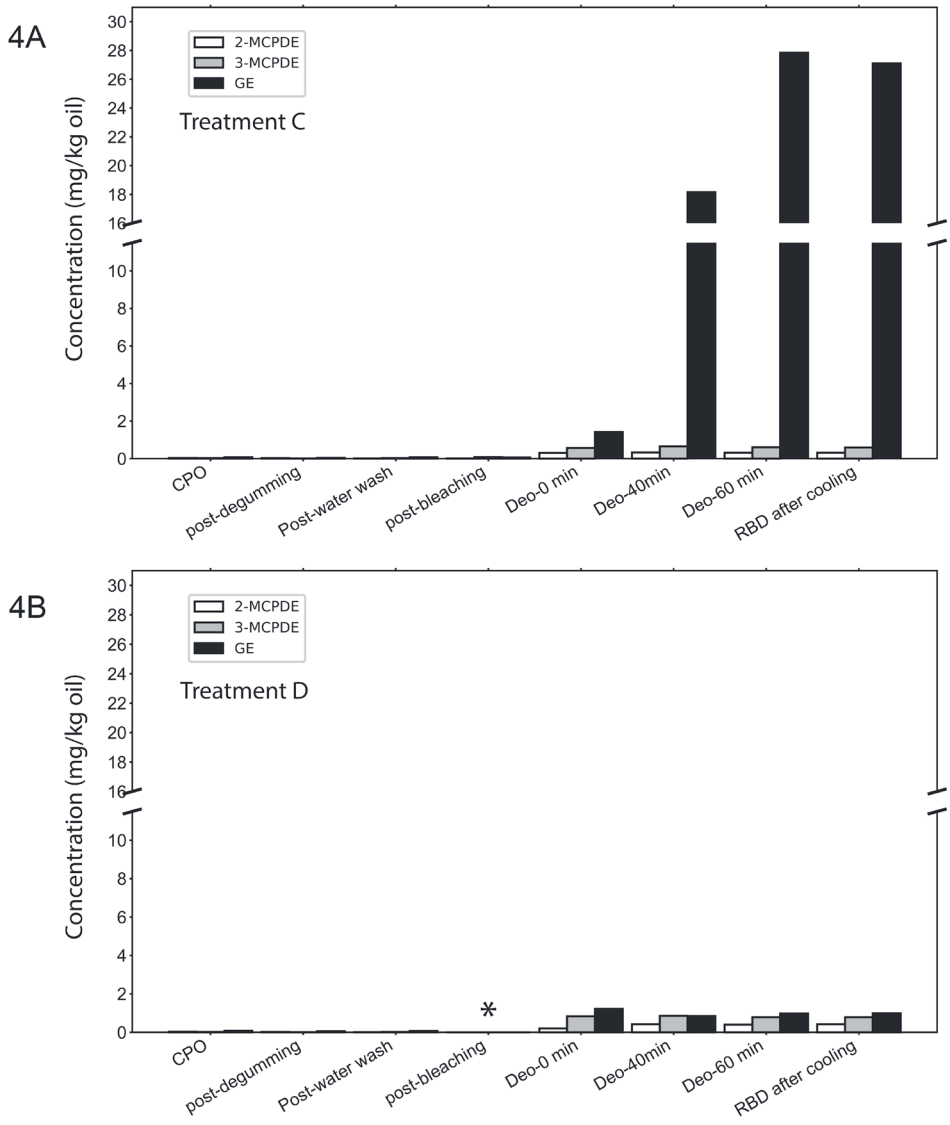


Figure 4.4 Results of Treatment C – alkali neutralization (4A) and Treatment D – 'full' chemical refining (4B). The only difference between these two treatments is the lower deodorization temperature in Treatment D (230 instead of 265 °C). The asterisk (*) shows samples that were not collected during refining.

The final GE concentration in this treatment was 29.4 mg/kg, making it the highest observable GE concentration in the experimental treatments of our study. Furthermore, the water content in the 'post-degumming' sample was 2.46% which was also the highest among all other samples. Based on the physical–chemical properties of FFAs, it is possible that it might have created an emulsion. This is especially the case when FFAs reacts with a

lye and create a soap stock via a reaction called saponification (Chew et al., 2017; Dumont and Narine, 2008) This might explain the relatively high water content which could not effectively be removed due to limitations of the experimental set-up. The water content in the final oil sample of this treatment was 0.04%. This particular refining condition, i.e. addition of sodium hydroxide and high deodorization temperatures, could also have lead to higher formation of MAGs and/or DAGs via hydrolysis of TAGs. Cheng et al., (2016) have reported that the combination of temperatures over 200 °C and the presence of MAGs and DAGs would lead to an increase in GE concentration. However, an extremely high amount of MAGs and DAGs must be present to explain our measured GE concentration in treatment C. According to Zelinková et al. (2017), analytical artefacts leading to biased GE results might be introduced when large amounts of MAGs are present in food samples. These MAG in food samples are often emulsifiers that have artificially been added into the food products. As our samples consists of pure palm oil, we do not expect extreme concentrations of MAGs that could have caused such analytical artefacts. Additionally, our analytical method includes a liquid–liquid extraction step where FFAs are removed post-transesterification. Large amount of FFAs from MAGs would, therefore, be removed during the sample preparation. Therefore, with a slight note of cautiousness, we consider the observed GE concentration to be an undesired effect from this treatment. Further investigation is required to confirm this adverse effect.

As a side note, the 'post-degumming' sample was taken right after the addition of the sodium hydroxide when the degumming process had finished. The soap formation, which commonly occurred after the addition of sodium hydroxide, caused two challenges. First, the separation of water and oil took twice as long (>1.5 hr) in comparison to what was needed during Treatments A and B. As the formed soap stock acted as surfactant, an emulsion was easily created. This emulsion was more stable than when only oil and water would have been mixed, as was the case in Treatment A and B. Therefore, it was more difficult to achieve a good water and oil separation. Beside this, the soap phase might help with the removal of polar chlorine containing molecules, metallic-, and inorganic chlorides.

The combination of observations from Treatments A and B with the observation from the current treatment leads to hypothesis that sodium hydroxide, the relatively high water content after the degumming process, possible increase in DAGs and MAGs formation, and the unadjusted high deodorization temperatures resulted in abundant formation of GE. Secondly, the usage of lye to neutralize and remove FFAs is known for its increased oil losses. The compromise is incorporated in our main aim which is the development of a practical way to simultaneously reduce the amount of 2-, 3-MCPDE, and GE. Especially because the European Commission has implemented new MLs for unbound 3-MCPD and bound 3-MCPDE. Successive research can focus on making this chemical refining method more efficient.

4.3.5. Treatment D – Chemical refining

The results of Treatment D are shown in Figure 4.4B. Similar to the previous three experimental treatments and the control, 2- and 3-MCPDE started to form after the bleaching process, prior to the start of the deodorization. Continuing the mild deodorization process (230 °C, 1 h), GE concentration stabilizes at 0.99 mg/kg, while the final 2- and 3-MCPDE concentration reached 0.42 and 0.78 mg/kg, respectively. Lower deodorization temperature can be utilized in chemical refining as the majority of the FFAs were already removed during the neutralization process. Compared to the single physical refining control, 2- and 3-MCPDE were reduced by 49% and 52%, respectively, while GE was reduced by 73%. Upon comparison with other physical refining methods, one can notice that our GE concentration might not be as low as the reported GE concentrations in physical refined oil (Sim et al., 2020). However, our primary goal was to simultaneously mitigate the concentration of 2-, 3-MCPDE, and GE. Furthermore, the main advantage of our method and experiment is the scale of experimentation and that all our experiments have been performed in a pilot plant. With sample sizes of 100 kg for each treatment, our pilot plant offers a better simulation of full-scale industrial refining. Additionally, upscaling a lab-scale experiment is no easy feat as demonstrated by Sim et al. (2020). With our pilot plant experiments, we were able to simulate an on-line degumming, neutralization, water wash, bleaching, and deodorization process. Finally, citric acid has been used specifically in all treatments and control experiment. Mitigation approaches demonstrated by Ramli et al. (2020), Sim et al. (2020), and others often use phosphoric acid to degum palm oil. Every country has their own regulations and a list of additives that can be used to produce organic food products. In The Netherlands, phosphoric acid is not allowed to be used in the production or organic food products. Therefore, citric acid is often used if Dutch refineries want to produce organic certified vegetable oils. Refineries in The Netherlands, but not limited to, can adopt our method of chemical refining when the production of an organic vegetable oil is desired.

The main difference between this treatment (Treatment D) and the alkali neutralization treatment (treatment C) is the deodorization temperature. Due to the lower deodorization temperature, GE formation can be kept to a minimum. Additionally, the alkali neutralization step neutralized the oil acidity which created a less favorable formation condition for 2- and 3-MCPDE to be formed as this theory was demonstrated by Šmidrkal et al. (2016). The subsequent washing step also helps with the removal of potential polar precursor elements such as chlorine salts. More investigation is however required to lower the concentrations of 2-, 3-MCPDE, and GE even more and to pinpoint other important precursors. Furthermore, GE concentration spikes were not observed during the entire refining process. Previous treatments, especially when lye was used, had a fair amount of trapped water in the oil and resulted in high GE concentrations with the combination of high deodorization temperatures. The final GE concentration in Treatment D remained

low (0.99 mg/kg), even though the water content in the 'post neutralization' and 'wash' sample reached 0.72% which is higher than what was observed in post-degumming water wash (Treatment B), but lower than those with alkali neutralization (Treatment C).

Looking at the FFA concentration and oil color, chemical refining can produce oil with low FFA (0.48 %) and 6.0 R / 61.0 Y in color. Compared to the single physical refining control (0.73 % FFA & 3.1 R / 31.0 Y color), the chemically refined oil has almost twice as low FFA and slightly more orange color to it, but this color difference is marginal. With these FFA and oil color results, it can be concluded that chemical refining is able to perform similarly as physical refining. Furthermore, there were no perceptible differences in the odor of the final chemically refined oil when compared to the final product of the physically refined oil. These results were only surpassed by Treatment B with 0.04 % FFA and 4.1 R / 40.0 Y color. However, the high GE concentration is undesirable and does not outweigh the benefits of having a lower FFA concentration.

Using the chemical refining process, the final GE concentration was just below the European Commission's ML of 1.00 mg/kg. Concerning 3-MCPDE, the observed final level of 0.78 mg/kg was well below the new ML in the EU. These new limits were recently changed (over the course of these treatments) and now also include unbound 3-MCPD. But as we focused primarily on the mitigation of bound 2-, 3-MCPDE, and GE in our study, future research should be conducted to evaluate (i) if unbound 3-MCPD is present in CPO at the start, (ii) if during the treatments new unbound 3-MCPD is generated and if this can be mitigated, and (iii) how the oil loss can be reduced.

The combination of lower deodorization temperature and a neutralization step seems to be the key to reduce 2-, 3-MCPDE, and GE simultaneously. Therefore, chemical refining could also potentially mitigate the formation of GE that requires higher formation temperatures than 2- and 3-MCPD esters.

4.4. Conclusions

Mitigating 2-, 3-MCPDE, and GE simultaneously would be a cost and time efficient solution for oil refineries. However, differences in the formation pathway of 2- and 3-MCPDE and that of GE hinder the development of effective mitigation strategies for these three contaminants at the same time. The aim of our study with its four experimental treatments was to develop a mitigation strategy which addresses simultaneously the mitigation of 2-, 3-MCPDE, and GE. The intrinsic effects of water washing at different moments during the refining process, acid degumming with and without neutralization with a lye, and the effect of the deodorization temperature were explored. The most successful treatment

was the chemical refining, where a combination of acid degumming, lye neutralization, water washing, and mild deodorization temperatures were able to significantly mitigate the formation of 2-, 3-MCPDE, and GE simultaneously. However, we acknowledge that not all the phenomena that were observed in the pilot plan treatments can be underpinned by the existing literature. Nevertheless, this approach holds good promise for a procedure able to meet the stricter EU safety regulation now in place.

Author contributions

S.B. Oey proposed the experimental design, analyzed the samples, compiled data in tables and graphs, drafted the manuscript, and corrected comments. H.J. van der Fels-Klerx, V. Fogliano, and S.P.J. van Leeuwen contributed to scoping the study, maturing the experimental design, reviewing draft manuscript versions, and providing editorial corrections.

CRediT authorship contribution statement

Sergio B. Oey: Methodology, Investigation, Writing – original draft, Visualization. H.J. van der Fels-Klerx: Conceptualization, Writing – review & editing, Supervision, Funding acquisition. Vincenzo Fogliano: Conceptualization, Writing – review & editing, Supervision. Stefan P.J. van Leeuwen: Conceptualization, Validation, Writing – review & editing, Supervision, Project administration.

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.

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Appendix A

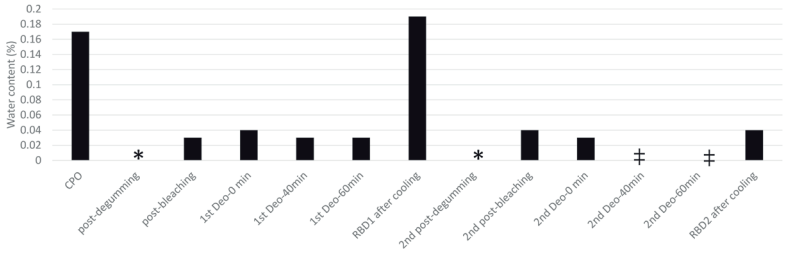

Figure 4.5 Results of the water content in the collected samples during the Control Treatment. The water content in each sample was determined with the Karl-Fisher method and are expressed in percentage. The water content of the samples marked with (‡) were not determined. The samples marked with (*) were not collected.

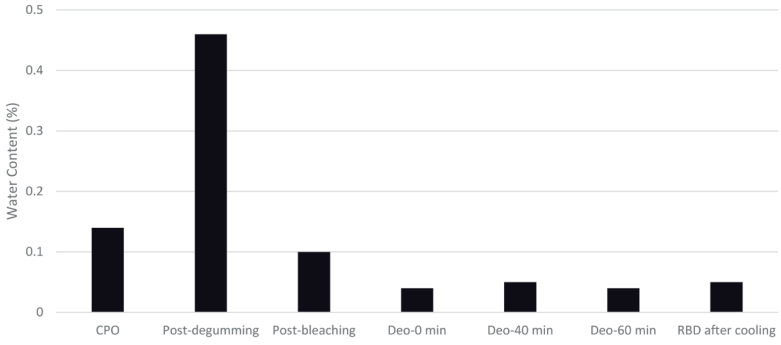

Figure 4.6 Results of the water content in the collected samples during Treatment A – pre-refining water wash. The water content in each sample was determined with the Karl-Fisher method and are expressed in percentage.

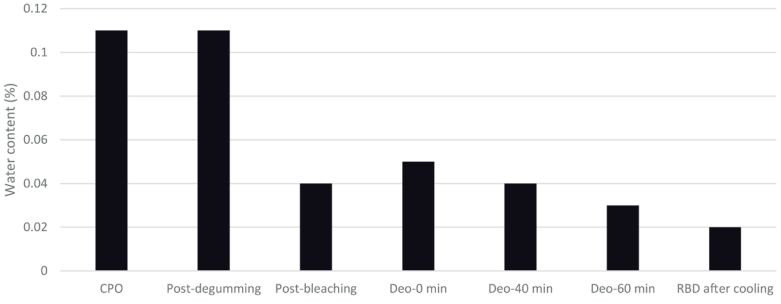

Figure 4.7 Results of the water content in the collected samples during Treatment B – post-degumming water wash. The water content in each sample was determined with the Karl-Fisher method and are expressed in percentage.

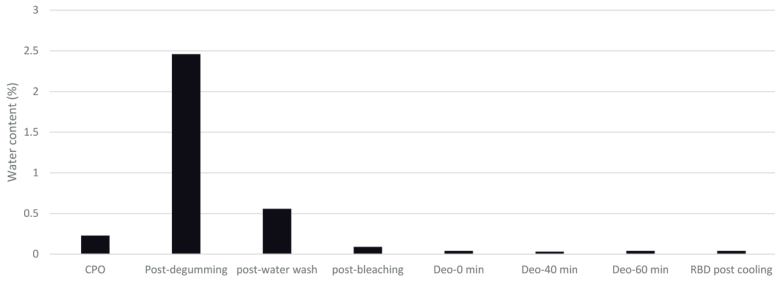

Figure 4.8 Results of the water content in the collected samples during Treatment C – alkali neutralization. The water content in each sample was determined with the Karl-Fisher method and are expressed in percentage.

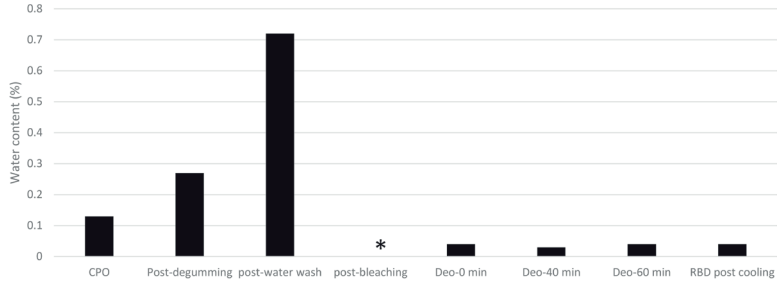

Figure 4.9 Results of the water content in the collected samples during Treatment D – 'full' chemical refining. The water content in each sample was determined with the Karl-Fisher method and is expressed in percentage. The samples marked with (*) were not collected.

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Supplementary material

1. Additional FFA data and oil color

Table S4.1 Results of the single and double physical refining control depicting the individual concentrations of 3-MCPDE, 2-MCPDE, and GE expressed in mg/kg oil. Deo: deodorization; RBD: refined, bleached, deodorized.

Sample No.	Reference code	3-MCPDE (mg/kg)	2-MCPDE (mg/kg)	GE (mg/kg)	FFA (% oleic)	Color (R-Red/Y-yellow)
534907	CPO	0.00	0.02	0.05	4.13	- (*)
- (†)	post-degumming	-	-	--	-	-
534908	post-bleaching	0.08	0.05	0.06	4.12	-
534909	1st Deo-0min	1.43	0.72	1.32	1.97	-
534910	1st Deo-40min	1.54	0.80	3.13	0.92	5.1 R / 60.0 Y
534911	1st Deo-60min	1.62	0.81	3.74	0.74	4.1 R / 40.0 Y
534912	RBD1 after cooling	1.62	0.82	3.73	0.73	3.1 R / 31.0 Y
- (†)	2nd post-degumming	-	-	-	-	-
534914	2nd post-bleaching	1.54	0.81	1.82	0.86	-
534913	2nd Deo-0min	1.74	0.89	0.19	0.85	-
534915	2nd Deo-40min	1.88	0.93	7.18	0.27	2.7 R / 27.0 Y
534916	2nd Deo-60min	1.93	0.95	8.59	0.24	2.7 R / 27.0 Y
534917	RBD2 after cooling	1.89	0.94	8.42	0.25	2.8 R / 27.0 Y

* Color not analyzed in samples indicated with a dash (-).
† Samples not collected

Table S4.2 Results of Treatment A - pre-refining water wash depicting the individual concentrations of 3-MCPDE, 2-MCPDE, and GE expressed in mg/kg oil. Deo: deodorization; RBD: refined, bleached, deodorized.

Sample No.	Reference code	3-MCPDE (mg/kg)	2-MCPDE (mg/kg)	GE (mg/kg)	FFA (% oleic)	Color (R-Red/Y-yellow)
535948	CPO	0.00	0.00	0.05	4.52	- (*)
535949	post-degumming	0.01	0.00	0.04	4.45	-
535950	post-bleaching	0.07	0.01	0.09	4.39	-
535951	Deo-0min	1.23	0.59	1.03	2.07	-
535952	Deo-40min	1.35	0.70	4.73	0.18	4.7 R / 47.0 Y
535953	Deo-60min	1.45	0.73	9.21	0.09	4.1 R / 41.0 Y
535954	RBD after cooling	1.34	0.67	8.42	- (*)	-

* Color and FFA not analyzed in samples indicated with a dash (-).

Table S4.3 Results of Treatment B – post-degumming water wash depicting the individual concentrations of 3-MCPDE, 2-MCPDE, and GE expressed in mg/kg oil. Deo: deodorization; RBD: refined, bleached, deodorized.

Sample No.	Reference code	3-MCPDE (mg/kg)	2-MCPDE (mg/kg)	GE (mg/kg)	FFA (% oleic)	Color (R-Red/Y-yellow)
535982	CPO	0.02	0.03	0.10	4.43	- (*)
535983	post-degumming	0.02	0.01	0.08	4.69	-
535984	post-bleaching	0.25	0.13	0.35	3.15	-
535985	DEO-0 min	0.85	0.46	0.81	2.13	-
535986	DEO-40 min	1.03	0.54	3.76	0.08	5.1 R / 51.0 Y
535987_1	DEO-60 min	1.10	0.56	11.05	0.05	4.3 R / 43.0 Y
535988_1	RBD post cooling	1.04	0.54	10.90	0.04	4.1 R / 40.0 Y

* Color not analyzed in samples indicated with a dash (-).

Table S4.4 Results of Treatment C – alkali neutralization depicting the individual concentrations of 3MCPDE, 2-MCPDE, and GE expressed in mg/kg oil. Deo: deodorization; RBD: refined, bleached, deodorized.

Sample No.	Reference code	3-MCPDE (mg/kg)	2-MCPDE (mg/kg)	GE (mg/kg)	FFA (% oleic)	Color (R-Red/Y-yellow)
535955	CPO	0.02	0.03	0.07	4.27	- (*)
535956	post-degumming	0.01	0.02	0.04	4.56	-
535958	post-water wash	0.02	0.01	0.07	3.56	-
535965	post-bleaching	0.05	0.04	0.30	3.32	-
535968	DEO-0 min	0.57	0.30	1.42	1.56	-
535969	DEO-40 min	0.65	0.33	18.15	0.18	7.0 R / 70.0 Y
535970	DEO-60 min	0.60	0.31	27.85	0.10	7.0 R / 70.0 Y
535972	RBD post cooling	0.59	0.31	27.11	0.10	7.0 R / 70.0 Y

* Color not analyzed in samples indicated with a dash (-).

Table S4.5 Results of Treatment D - 'full' chemical refining depicting the individual concentrations of 3-MCPDE, 2-MCPDE, and GE expressed in mg/kg oil. Deo: deodorization; RBD: refined, bleached, deodorized.

Sample No.	Reference code	3-MCPDE (mg/kg)	2-MCPDE (mg/kg)	GE (mg/kg)	FFA (% oleic)	Color (R-Red/Y-yellow)
535974	CPO	0.02	0.03	0.08	4.51	- (*)
535975	post-degumming	0.01	0.02	0.05	4.71	-
535976	post-water wash	0.02	0.01	0.07	2.1	-
- (†)	post-bleaching	-	-	-	-	-
535978	DEO-0 min	0.83	0.20	1.22	1.72	-
535979	Deo-40 min	0.86	0.42	0.84	0.94	7.0 R / 70.0 Y
535980	Deo-60 min	0.78	0.40	0.98	0.52	6.2 R / 62.0 Y
535981	RBD post cooling	0.78	0.42	0.99	0.48	6.0 R / 61.0 Y

* Color not analyzed in samples indicated with a dash (-).
† Sample not collected

Supplementary material

1. Analytical method in-house validation

The method was developed according to the AOCS Official Method Cd29a-13: 2- and 3-MCPD Fatty Acid Esters and Glycidol Fatty Acid Esters in Edible Oils and Fats by Acid transesterification and GC/MS, which is similar to the later introduced standard NENISO18363-3: Animal and vegetable fats and oils - Determination of fatty-acid-bound chloropropanediols (MCPDs) and glycidol by GC/MS - Part 3: Method using acid transesterification and measurement for 2-MCPD, 3-MCPD and glycidol. These standards define criteria for e.g. linearity, accuracy, precision, etc. The results of the validation report meet those criteria, meaning that the method is fit for purpose.

The validation was performed on 8 different days under reproducibility conditions, using a variety of oil types (palm oil, olive oil). A wide range of concentration levels was included in validation, by working with previously analyzed samples and samples to which the contaminants were spiked at the desired levels.

Linearity

Throughout the validation experiments, independent calibration curves were prepared. In the tables below, the average calibration curves are shown.

Table S4.6 Linearity results

Component	Correlation r^2	Intercept
3-MCPD	1.000	0.009
2-MCPD	1.000	0.007
Glycidol (3-MBDP)	0.999	0.006
Criterium* 3-MCPD / glycidol	> 0.990	< 0.020 abs
Criterium* 2-MCPD	> 0.990	< 0.050 abs
Meets criterium?	Yes	Yes

*Criterium according to the AOCS Official method Cd29a-13

Accuracy

The accuracy was determined on different days, under reproducibility conditions.

Table S4.7 Accuracy of 3-MCDP and 2-MCPD

	n	Accuracy 3-MCPD (%)	Accuracy 2-MCPDE (%)
Criterium*		75-120	75-120
Average	24	98	99
RSD (%)	24	5	6
Meets criterium?	24	Yes	Yes

*Criterium according to the AOCS Official method Cd29a-13

Table S4.8 Accuracy of glycidol

Sample-ID	n	Accuracy Glycidol (3-MBPD) %
Criterium*		75-120
Average	24	105
RSD (%)	24	5
Meets criterium?	24	Yes

*Criterium according to the AOCS Official method Cd29a-13

Repeatability and reproducibility

The repeatability and reproducibility were determined by analysis of 8 different oil samples. Each sample was analyzed in duplicate on a single day, and another (single) measurement was done on another day. This approach allows the calculation of repeatability and reproducibility.

Table S4.9 Repeatability and reproducibility

	Repeatability				n	Reproducibility		
Component	n	Concentration (mg/kg)	RSD_r (%)	(%)		Absolute (mg/kg)	RSD_{RL} (%)	(%)
3MCPD	8	0.10	2	6	8	0.14	5	14
2MCPD	8	0.08	3	8	8	0.12	11	31
Glycidol (3MBDP)	8	0.15	4	11	8	0.14	11	32
Criterium*			8	22			16	45
Meets criterium?			Yes	Yes			Yes	Yes

*Criterium according to the AOCS Official method Cd29a-13

Limit of detection and limit of quantification

The LOD and LOQ were determined by analysis of 8 spiked olive oil samples at the level of 0.2-0.3 mg/kg.

Table S4.10 LOD and LOQ validation results

Component	LOD (mg/kg)	LOQ (mg/kg)
3-MCPD	0.05	0.10
2-MCPD	0.04	0.07
Glycidol (3-MBDP)	0.03	0.07
Criterium* MCPDs	< 0.21	< 0.21
Criterium* glycidol	< 0.26	< 0.26
Meets criterium?	Yes	Yes

*Criterium according to the AOCS Official method Cd29a-13

Chapter 5

The dynamics of organochlorines modification during palm oil refining suggest a plethora of potential precursors of 2- and 3-MCPD esters

Manuscript in finalization

Abstract

The interactions and role of precursors in the formation of fatty acid esters of 2-monochloro-1,3-propanediol (2-MCPDE) and 3-monochloro-1,2-propanediol (3-MCPDE) are not fully elucidated. This study aimed to investigate the effect of the palm oil refining process on the formation and elimination dynamics of potential endogenous, nonpolar organochlorine precursors. Water soluble chlorides did not fully account for the formation of 2- and 3-MCPDE as water washing of the crude oil did not result in a complete prevention of the formation. Therefore, it can be speculated that other endogenous, nonpolar organochlorines are involved in the formation of 2- and 3-MCPDE. Three different batches of crude palm oil were refined in the laboratory mimicking conventional industrial refining process. Samples were taken every 3 minutes during refining and analyzed with a LC-high resolution orbitrap-MS. Using the specific chlorine isotopic ratio 258 organochlorine compounds were detected. These compounds were further categorized into four different kinetic patterns during the refining: upward, downward, intermediates, and unchanged. Sixty-two compounds showed a downward kinetic trend, implying they are being converted into other molecules during the refining process. It is speculated that chlorine can be released during such conversions, fulfilling the role of chlorine donor for the formation of 2- and 3-MCPDE. Current study results add to the growing knowledge of precursors in the formation of 2- and 3-MCPDE. Further research should reveal the identity and role of these molecules, in particular the down-regulated molecules, in the reaction pathway of the MCPDEs.

Keywords: 3-monochloropropanediol; glycidol; processing contaminant; high resolution mass spectrometry; kinetics; oil refining

5.1. Introduction

Fatty acid esters of 2-monochloro-1,3-propanediol (2-MCPDE), 3-monochloro-1,2-propanediol (3-MCPDE), and glycidol (also known as glycidyl esters, GE) are process contaminants that can be found in processed vegetable and marine fats and oils (Beekmann et al., 2022). High concentrations of 2-MCPDE, 3-MCPDE and GE can especially be found in palm oils (Arris et al., 2020; Pudel et al., 2011). When contaminated oils are used as ingredients in food products, those food products become contaminated as well.

Upon consumption of oils and oil-containing products, these esters are cleaved into their unbound state (i.e. the free non-esterified forms) when digested in the human gastrointestinal tract, and it's the free forms of the esters that exhibit toxic effects (Arisseto et al., 2018). In their unbound state, glycidol is classified as a genotoxic carcinogen, while 3-MCPD is a non-genotoxic carcinogen. In addition, recent toxicity studies have shown that 2-MCPD toxicity should not be neglected (Buhrke et al., 2015; Zhang et al., 2019).

In order to protect human health, the European Commission (EC) regulates the presence of these contaminants in fats and oils destined for consumption in Europe. As of April 2023, the EC has instated an update on the existing MLs, which include, but not limited to, MLs for various types of vegetable oils, and fish and other marine organisms (European Commission, 2023). Vegetable oils destined for the production of infant food and infant formula have a stricter ML than for "regular" vegetable oils. Up to date, there is no ML established for 2-MCPDE. These newly implemented ML cause oil refineries with European customers to face new mitigation challenges.

Previous research has identified refining methods to mitigate 2- and 3-MCPDE, as reviewed by Oey et al. (2019). Mitigation options during refining include a pre-refining water wash to remove residual chlorine, double refining, or chemical refining. However, application of most of these proposed mitigation strategies does not lead to meeting the ML of 1250 µg/kg for 3-MCPDE in oils, while mitigation strategies for 2-MCPDE are often not considered (Oey et al., 2019).

Chlorine sources, such as chlorine-containing salts or hydrochloric acid, monoacylglycerols (MAG), and diacylglycerols (DAG), are known to play a key role in the formation of 2-, and 3-MCPDE (Destaillats et al., 2012; Šmidrkal et al., 2016; Zhao et al., 2016). This process happens readily at temperatures above 100 °C becoming prominent at the temperature adopted for oil refinery (above 200 °C) (Li et al., 2016; Zulkurnain et al., 2012).

A viable strategy that can reduce MCPDEs is to eliminate the chlorine source. If polar chlorine sources can easily be removed in the pre-refining wash step, it is expected that 2- and 3-MCPDE will be mitigated. However, water wash experiments, as performed by Sim et al. (2018) and Ramli et al. (2020), showed that a certain amount of 2- and 3-MCPDE is still formed during the refining process. This may indicate the presence of lipophilic and/or high molecular weight chlorine sources in the crude oils, which cannot be removed by water washing.

Few papers dealt with organochlorines as precursors for 2- and 3-MCPDE in vegetable oils. Tiong et al. (2018) have reported a possible source of natural organochlorines in crude palm oil (CPO). They reported that organochlorines are constituents of wax esters, fatty acids, diacylglycerols, and sphingolipids. These organochlorines are present in various vegetable oils, including palm oil. As these organochlorines are endogenous, have high molecular weights, and are mostly lipophilic, they cannot be removed from the oil by a simple wash step. Nagy, Redeuil, Lahrichi & Nicolas (2019) proposed a method to remove similar-like organochlorine precursors with a trapping agent such as monopalmitin, monostearin, or a mixture of monopalmitin/-olein/-linolein. As organochlorines have physical-chemical properties similar to the trapping agent, they diffuse towards the trapping agent. The organochlorines can be removed from the oil after separation and crystallization of the trapping agent. However, this experiment was only performed on a small scale and upscaling this method might be economically unattractive due to the large amounts of required trapping agents and/or unforeseen neutral oil losses. Furthermore, exact molecular structures of the precursors have not been identified yet, nor the kinetics during refining. This makes it challenging to utilize a more targeted mitigation approach.

The aim of this research is to investigate the effect of the palm oil refining process on the formation and elimination dynamics of potential endogenous, nonpolar organochlorine precursors. Previous study has shown that water washing of the oil did not prevent the formation of 2- and 3-MCPDE completely (Oey et al., 2022). 2- and 3-MCPDE are formed during refining thanks to the incorporation of a chlorine atom into a mono- or di-acyl glycerols. The source of these chlorine atoms can be endogenous organochlorine molecules and here we adopted a mass spectrometry strategy based on chlorine isotopic pattern to test these possibilities.

5.2. Materials & methods

5.2.1. Lab-refining experiments

Each of three CPO (SRC B.V., Zaandam, The Netherlands) were refined in the lab on separate days, following the same refining procedure. The experiment consisted of three replications, each performed with a different CPO with varying free fatty acid concentration between 3.46% and 5.13% and a deterioration of bleachability index (DOBI) for palm oil between 2.2 and 2.6. The solid CPO was stored in the refrigerator before refining to ensure that it stayed as fresh as possible. Each CPO batch was melted overnight at a maximum temperature of 40 °C prior to each experimental treatment. A schematic representation of the lab-refining equipment set-up is shown in Figure 5.1. Approximately 800 mL of the melted CPO was poured into a 2L three-neck glass round bottom flask. A 2000-watt variable electric heating mantle (Fisherbrand™ Silver Heating Mantle 2L, Fisher Scientific, Merelbeke, Belgium) was used to heat the oil in the 2L three-neck glass round-bottom flask and an electric overhead stirrer (Digital Overhead Stirrer LLG-uniSTIRRER OH2, Boom B.V., Meppel, The Netherlands) was used to continuously mix the oil. The exact amount of required citric acid (Univar Solutions, Rotterdam, The Netherlands), bleaching earth (Pure-Flo® B80 natural bleaching earth, Oil-Dri, Ripley, Mississippi, USA), activated carbon (Norit® SA 4 PAH-HF, Cabot Norit Nederlands B.V., Amersfoort, The Netherlands), and filter aid (Dicalite® Perlite 478, Univar Solutions, Rotterdam, The Netherlands) was calculated based on the net oil weight in the round-bottom flask. Oil samples (± 10 mL each) were collected from the 2L three-neck round bottom flask every 3 minutes throughout the experimental treatment. All collected samples were immediately put on ice to rapidly cool them and to cease any thermal-dependent reaction. The oil temperature was recorded throughout the entire lab refining process to ensure that critical moments of the refining happen under the right condition.

The experimental treatments started with the degumming process by adding 0.5% w/w of a 25% v/v citric acid solution. This was performed at 70 °C for 20 minutes, after which the oil was heated to 95 °C. Once the oil reached 95 °C, pre-mixed bleaching earth, 1.0% w/w Pure-Flo® B80 natural bleaching earth, 0.1% w/w Cabot Norit® SA 4 PAH-HF activated carbon, and 0.2% w/w Dicalite® 478 perlite filter aid was added to the oil. After 20 minutes of bleaching, the oil was directly filtered once through a Büchner-vacuum filtration while the oil was still hot. A combination of one Whatman® 113 ('wet strengthened', 150 mm diameter) filter paper and a 150 mm diameter circular cut-out from an industrial size polyester filter bag (Eaton Cleargaf 5 µm polyester needlefelt) were stacked on top of each other in the Büchner funnel to filter the oil. A vacuum pump was used to aid the filtration rate. Afterwards, the filtered oil was poured into

a clean 2L three-neck round-bottom flask. The entire flask containing the filtered oil was then heated to 265 °C within 40 minutes to mimic the temperature ramp of the deodorization phase. As mentioned above, during the entire experiment, oil samples were taken every 3 minutes. The last sample was taken when the oil had reached 265 °C. A schematic temperature-time diagram of the oil refining process is shown in Figure 5.2. During the first treatment, a total of 43 oil samples were collected, 39 oil samples were collected during the second refining treatment, and 40 oil samples were collected during the third refining treatment.

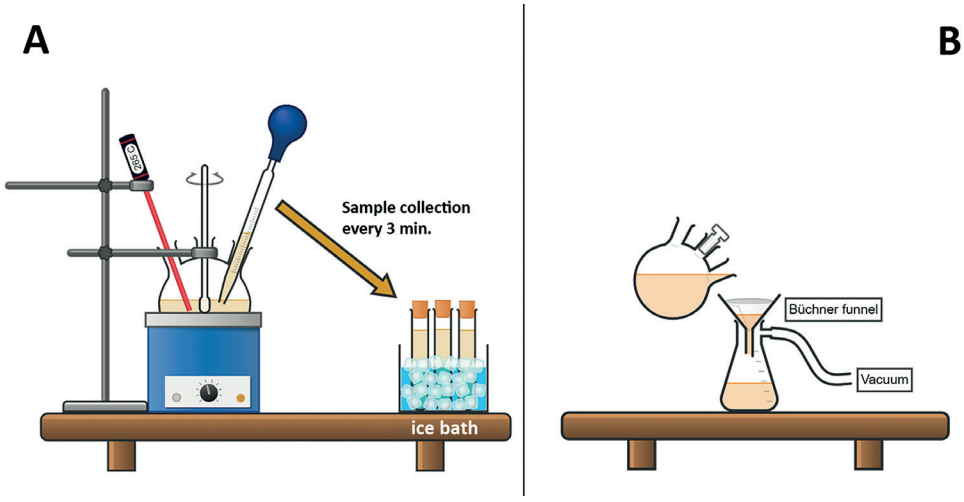

Figure 5.1 Schematic representation of the lab-refining experimental set-up (A) and the post-bleaching vacuum filtration set-up (B) with a Whatman® 113 filter and a custom size Eaton Cleargaf 5 µm polyester needlefelt filter.

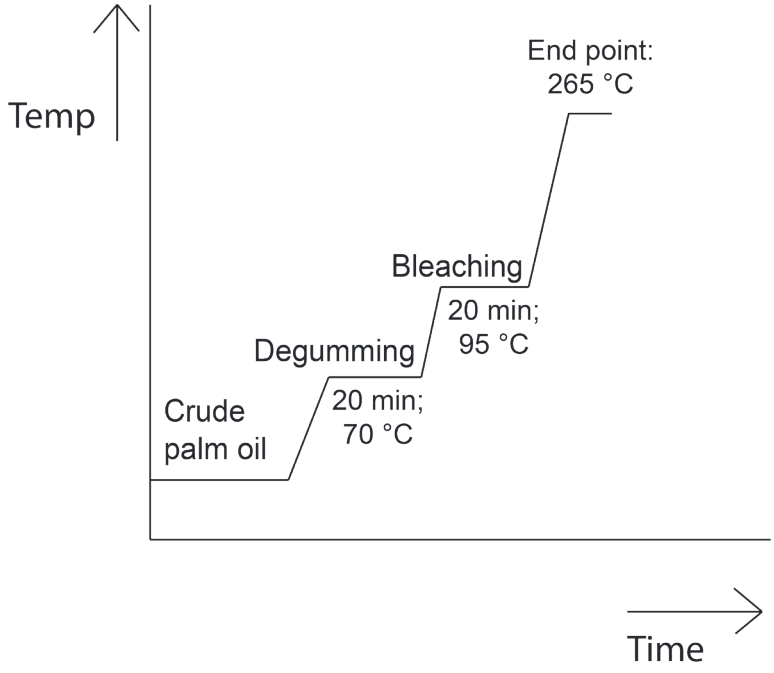

Figure 5.2 Temperature-time diagram of lab oil refining

5.2.2. Chemical analysis

5.2.2.1. Quantification of 2- and 3-MCPDE

The quantification of 2- and 3-MCPDE was performed according to a previously published method (Oey et al., 2020). Five oil samples from one of the three CPO batches were analysed for 2-, 3-MCPDE concentrations. This analytical method allows for the simultaneous quantification of 2-, 3-MCPDE, and GE. However, as the experiments in this publication focus on 2- and 3-MCPDE, the GE results are not reported. The analysis was based on acid transesterification of the MCPD esters followed by a derivatization reaction with phenyl boronic acid (PBA). The 2-MCPD-PBA and 3-MCPD-PBA complex were then quantified in the temperature programmed GC-triple quad-MS with the help of a calibration line from freshly made calibration mixtures. Stability of the samples and analytes were guaranteed by the addition of internal standard mixtures into each sample prior to sample preparation which consists of penta-deuterated 2-MCPD-di-palmitate and 3-MCPD-di-palmitate.

5.2.2.2. Analysis of organochlorine precursors by LC-orbitrap MS

The collected oil samples were minimally cleaned up prior to analysis to prevent the premature removal of the organochlorine precursors. The organochlorines were analyzed in all samples of the 3 different oil batches that were obtained from the lab-scale refining experiments. All oil samples were mildly heated to 45 °C to achieve a homogeneous melted sample. One hundred ±1.0 mg of each oil sample was used for the analysis. An equivalent of 10 ng Fluazinam (HPC Standards GmbH, Cunnersdorf, Germany) was added to each sample as internal standard and the total volume of each sample was brought to 1000 µL with a solution of 60:40 (v/v) acetonitrile:isopropanol. A small magnetic stir bar was used in each glass tube to agitate and mix the sample with the solvent. The samples were vigorously stirred for 30 min. in a water bath set at 40 °C on top of a submersible, multi-channel magnetic stirring plate (MIXdrive 60 HT, 2mag AG, Munich, Germany). The samples were then centrifuged for 15 min. at 15 °C at 2862 x ***g*** to centrifuge the bulk of undissolved triglycerides (TAG). Finally, the supernatant was filtered through a PTFE syringe filter (13mm, 0.45 µm, Rezist, Whatman®) and transferred into LC vials.

Analysis was performed by a liquid chromatography-high resolution orbitrap mass spectrometry (LC-HRMS) system (Dionex Ultimate 3000, Thermo Scientific™ coupled to a Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer, Thermo Scientific™). The analysis method was based on Tiong et al. (2018). Separation was performed on an Acquity BEH C8 UPLC column (2.1 mm x 100 mm x 1.7 µm, Waters™, Milford MA, U.S.A.) with 40:60 (v/v) acetonitrile:water as mobile phase A and 90:10 (v/v) isopropanol:acetonitrile as mobile phase B. Both mobile phases contain 10 mM ammonium acetate as buffer. The used mobile phase gradient is shown in Table 5.1.

Table 5.1 Liquid chromatography gradient used during the 2- & 3-MCPDE precursor experiment.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (µL/min)
0	68	32	260
1.5	68	32	260
5	48	52	260
18	25	75	260
21	3	97	260
25	3	97	260
25.1	68	32	260
30	68	32	260

The ESI parameters were automatically optimized by the software for a flow of 260 µL/min and set to negative mode analysis. The spray and capillary voltage were respectively 2.5 kV and -0.4 V. The column was kept in the column heater at 55 °C, while the sample

tray was set at 15 °C. Nitrogen was used as the auxiliary and sheath gas. Prior to each measurement, the system was calibrated using the official Thermo Fisher calibration mix (Pierce™ ESI Negative Ion Calibration Solution, Thermo Scientific™, Rockford IL, U.S.A.) and calibrated at least once every three days. The MS was set to perform a full scan-ddMS/MS intermitted with All Ion Fragmentation (AIF). The ddMS/MS was used to obtain spectra of the top 5 highest intensity molecules immediately after the full scan. A dynamic exclusion window for the ddMS/MS was set at 10.0 s with an intensity threshold of 1.6e5. The resolution for the full scan was set at 140,000, ddMS/MS was set at 35,000, and the AIF was set at 17,000. The full scan was performed with a mass range between 100 – 1500 m/z and the ddMS/MS and AIF were performed with a mass range between 200 – 2000 m/z. A procedural blank which consists out of solely 60:40 (v/v) acetonitrile:isopropanol and an academic spiked sample, containing an equivalent of 10 ng fluazinam in 60:40 (v/v) acetonitrile:isopropanol, were used as quality control samples to monitor the performance of the system and to correct for trace sources of artificially introduced contamination. All oil samples were analyzed once due to the sheer amount of collected samples from all three oil batches. The procedural blank and academic spiked sample were alternately injected after every 7-9 oil samples.

5.2.3. Data processing

Data processing was divided into two parts. The first part aimed to identify known potential precursors that have been reported in the literature. The second part aimed at identifying additional, not earlier reported organochlorines that may serve as potential precursors.

Data processing started with Compound Discoverer 3.1 (Thermo Scientific, Waltham, MA, USA), which was used to process the HRMS data. A workflow (Figure S1) was designed to process the data according to certain parameters which are defined in the "nodes". A full list of the workflow parameters for each node is shown in the supplementary materials (S1). The goal of this level of data processing is to identify organochlorines, both known and unknown, that are present in the samples. The two "Create Pattern Trace" nodes analyses the raw spectrum for a specific isotope pattern. In our case that would be the isotope pattern of Cl and Cl2. This is an important step to narrow down the amount of potential precursor hits as we are focused on precursors containing at least a chlorine atom. After initial filtering and grouping, the relevant compounds are matched against an online mass spectral database of mzCloud, a matching based on the exact masses on chemspider, and a composed local mass list (Table 5.2) which consists of mol masses that have been reported previously by Nagy et al. (2011, 2019) and Tiong et al. (2018). These reported mol masses from the literature are potential precursors of 2- and/or 3-MCPDE and provide initial lead to the kinds of compounds that could be expected. Any hit from ChemSpider or mzCloud helps with the identification of those compounds. The stabil-

ity of the fluazinam internal standard in each sample was monitored as well to ensure the overall stability of the analysis. The second part of data analysis covered manually charting the detected molecules from the full-scan MS data. In order to evaluate if peak areas increased or decreased during every stage of the experiment, each unique mass reported from the Compound Discoverer software was manually evaluated. In this step, the relative ratios of both the previously reported masses of potential organochlorine precursors and new, unknown organochlorine precursors were mapped. This method of data visualization allows to arrange the potential precursors into four preliminary kinetic-profiles: up-regulated, down-regulated, intermediates, and unchanged.

Table 5.2 A list of 30 previously published $[M-H]^-$ m/z-values of potential organochlorines precursors

RT (min)	m/z [M-H]-	Type	Source
5.57	291.20932	Unknown	Tiong et al., 2019, JAFC 66(4)
4.54	313.19397	Unknown	Tiong et al., 2019, JAFC 66(4)
5.16	315.20917	Unknown	Tiong et al., 2019, JAFC 66(4)
5.94	317.22479	Unknown	Tiong et al., 2019, JAFC 66(4)
6.62	319.24072	Unknown	Tiong et al., 2019, JAFC 66(4)
4.62	334.25143	Unknown	Tiong et al., 2019, JAFC 66(4)
3.99	337.21497	Unknown	Tiong et al., 2019, JAFC 66(4)
6.77	345.25616	Unknown	Tiong et al., 2019, JAFC 66(4)
2.64	347.19901	Fatty Acid	Tiong et al., 2019, JAFC 66(4)
4.15	358.25165	Unknown	Tiong et al., 2019, JAFC 66(4)
4.97	360.26712	Unknown	Tiong et al., 2019, JAFC 66(4)
5.05	365.24591	Unknown	Tiong et al., 2019, JAFC 66(4)
4.54	389.24622	Wax Esters	Tiong et al., 2019, JAFC 66(4)
5.33	391.26184	Wax Esters	Tiong et al., 2019, JAFC 66(4)
6.17	393.27731	Unknown	Tiong et al., 2019, JAFC 66(4)
3.95	600.38947	Sphingolipid	Tiong et al., 2019, JAFC 66(4)
10.36	614.48956	Sphingolipid	Tiong et al., 2019, JAFC 66(4)
10.14	649.45978	Unknown	Tiong et al., 2019, JAFC 66(4)
11.53	653.49274	Unknown	Tiong et al., 2019, JAFC 66(4)
8.56	659.46552	DAG	Tiong et al., 2019, JAFC 66(4)
8.17	683.46582	DAG	Tiong et al., 2019, JAFC 66(4)
13.35	700.60114	Sphingolipid	Tiong et al., 2019, JAFC 66(4)
N.D.	700.6028	phytosphingosines	Nagy et al. 2011, FAC-A 28(11); Nagy et al. 2019,FAC-A 36(5)
N.D.	702.61807	phytosphingosines	Nagy et al. 2011, FAC-A 28(11); Nagy et al. 2019,FAC-A 36(5)
N.D.	716.59723	phytosphingosines	Nagy et al. 2011, FAC-A 28(11); Nagy et al. 2019,FAC-A 36(5)
12.83	718.61255	Sphingolipid	Tiong et al., 2019, JAFC 66(4)
N.D.	718.61284	phytosphingosines	Nagy et al. 2011, FAC-A 28(11)
N.D.	718.61357	phytosphingosines	Nagy et al. 2011, FAC-A 28(11); Nagy et al. 2019,FAC-A 36(5)
N.D.	734.60809	phytosphingosines	Nagy et al. 2011, FAC-A 28(11); Nagy et al. 2019,FAC-A 36(5)
9.82	776.54437	Sphingolipid	Tiong et al., 2019, JAFC 66(4)

N.D. - not defined

The first assessment made on the LC-HRMS dataset of the 3 oil batches was by screening 30 exact masses of potential precursors. These 30 target masses originate from research papers that previously has been published (Nagy et al., 2011, 2019; Tiong et al., 2018). Compound Discoverer was set to report hits from the target list with 30 known m/z-values within a 5-ppm error margin.

5.3. Results & Discussion

5.3.1. Concentrations of 2- & 3-MCPDE during lab-refining conditions

The results of the analysis are shown in Table 5.3. As the formation of 2- and 3-MCPDE occurs at temperatures well below the 265 °C, the deodorization starting temperature, the lab-refining experiment were designed to not include the actual deodorization part (Hrncirik & van Duijn, 2011; Oey et al., 2020). The formed 2- and 3-MCPDE during this experiment shows concentrations that are comparable to the earlier pilot plant experiments (Oey et al., 2020). No substantial 2- and 3-MCPDE formation occurred prior to the beginning stage of the deodorization process (i.e. levels in CPO were low).

Table 5.3 Results of 2, 3-MCPDE, and GE analysis in 5 oil samples from one of the three refining treatments.

	3-MCPDE (mg/kg)	2-MCPDE (mg/kg)	GE (mg/kg)
T0 – CPO	0.05	0.02	0.19
T48 min – Start Bleaching	0.05	0.02	0.24
T72 min – End Bleaching	0.05	0.02	0.12
T105 min – Mid pre-heating deodorization at 145 °C	0.13	0.03	0.15
T126 min – Theoretical start of Deodorization at 265 °C	1.17	0.71	1.02

5.3.2. Detection of Organochlorine compounds during lab-refining conditions

The experiments were identical to the ones described by Oey et al. (2020) except for the final deodorization process. The deodorization process was deliberately not performed during this experiment as 2- and 3-MCPDE are formed particularly during the temperature increase in the early stage of the deodorization, well before the desired high temperature of deodorization is reached. Therefore, the lab-refining experiment did not have to follow the full refining procedure and focused only on the initial stages of the refining, including the temperature ramping.

In the collected samples, in total, over 250 unique organochlorines could be resolved from the data (represented by m/z and retention time), containing one or multiple chlorines. The feature selection was based on a minimum target peak area of at least 1e06 to allow a detected organochlorine to be included in the analysis process. Figure 5.3 shows the spread of the fluazinam internal standard that has been used to ensure the overall stability of the analysis. The difference between the highest and lowest internal standard area was 1.95e7, close to a 2.2 times difference. For an uncorrected raw area this was considered stable.

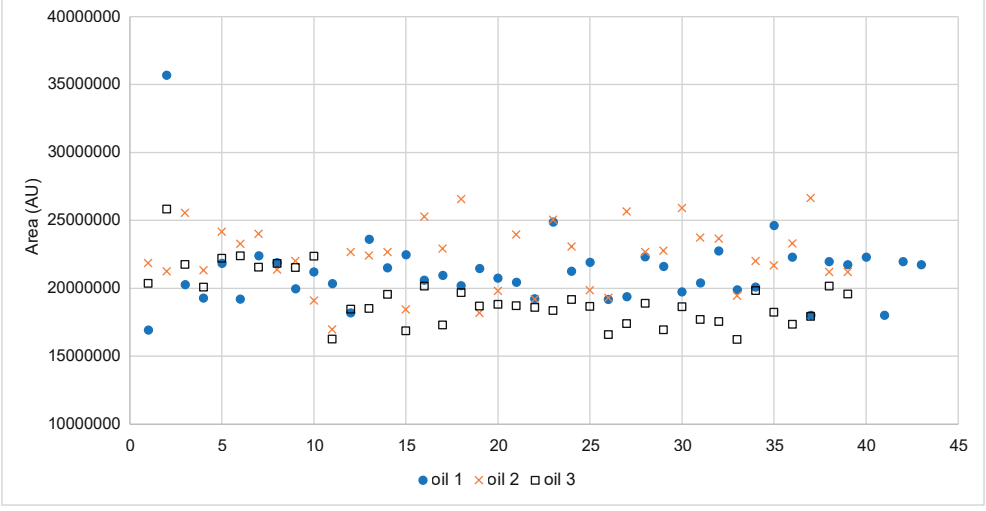

Figure 5.3 The spread of the fluazinam internal standard in each oil sample. The values shown are the uncorrected chromatographical area under the curve.

The semi-automated data processing with the Compound Discoverer software was set to detect both single and double chlorinated molecules. However, there were no hits reported of double chlorinated molecules. The large number of samples taken over time during the entire refining process on its own is unique and it allows to evaluate the trend of individual molecules for the first time in details. In this study, four different trends were observed. These trends of relative areas were categorized as up-regulated, downregulated, intermediates, and unchanged. An example of the four categories is shown in Figure 5.4.

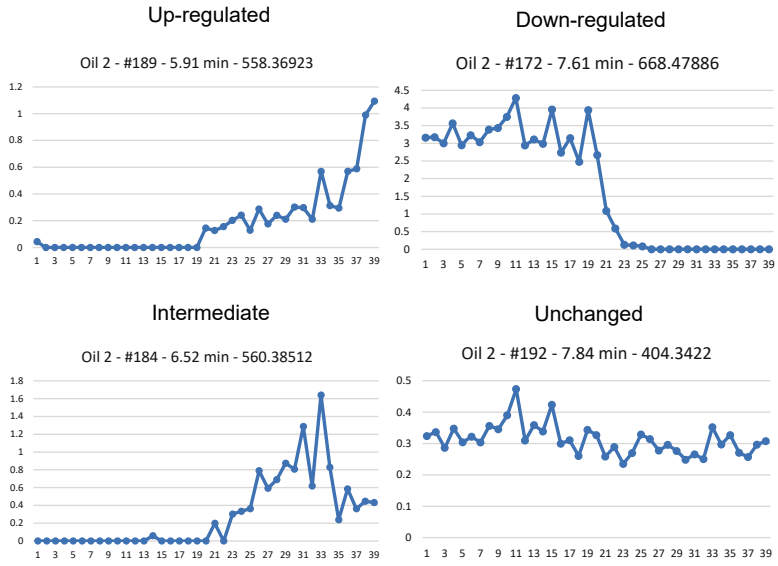

Figure 5.4 Example of overall trends of certain organochlorine. The relative ratio is plotted on the Y-axis (peak area of the exact mass relative to the internal standard peak area). Each data point originates from consecutive individual samples taken during the entire refining process with an interval of 3 minutes. Data points that had a raw peak area lower than 1e6 are displayed as zero. Compound oil 2-#172 was predicted to be C40 H65 Cl N4 O2, oil2-#184 was C32 H53 Cl N4 O2, oil2-#189 was C32 H51 Cl N4 O2, and oil2-#192 was C24 H49 Cl O2.

It should be noted that the amounts (or concentrations) of all assessed molecules at this point cannot be quantified because it was not possible to establish the exact identity of the compounds in this study. Although the exact mass was obtained, the corresponding molecular formula and structure could not be established based on the obtained data. In relation to that, the pure compound as a reference standard was not available for all these molecules. It is therefore not possible to provide quantitative data on the concentrations of these potential precursors in the oils. The assessments in this study therefore have a qualitative nature, focusing on trends. Variability in the data, related to LC-HRMS instrumental variabilities as well as sample preparation, has been ruled out using an internal standard.

The 'down-regulated' trend describes the scenario that a potential precursor is present in the CPO. This precursor starts at a certain concentration, and during the refining of the oil, it will show a downward trend caused by, for example, degradation or conversion into not chlorinated compounds. It is during this degradation or conversion phase that the chlorine atom can potentially become available for the formation of 2- and 3-MCPDE.

Another interesting observed trend is that of the molecules with an 'intermediate trend', i.e. starting with zero or a low concentration, followed by a rise in concentration in the midst of the oil refining experiment, and finally dropping down again to a lower concentration and thus displaying a" bell-shaped" trend. The intermediate pattern shows that OCs can be generated, but at a later stage (i.e. higher temperature), their concentrations decrease again. Possibly, these intermediates also play a role in the molecular pathway of creating an available chlorine atom. Intermediates can be formed from other precursors, e.g. from the ones showing a downward trend. After such conversion, a "new" molecule, a reaction intermediate, can arise midst oil refining. This reaction intermediate can also be converted into another molecule or can degrade completely at a later stage of the oil refining, explaining the "mountain-shaped" trend. Moreover, molecules with an increasing trend, up regulated, can be the result of such a conversion reaction similar to the intermediates, except that it is not being degraded or converted into another molecule. Molecules that show an unchanged trend can be assumed to be stable or unreactive during a physical refining setting. These molecules with unchanged concentrations during the entire oil refining have a low probability to be part in the formation of 2- and 3-MCPDE. We therefore focus on those molecules that showed a down-ward trend and the intermediates as these two groups of compounds have the highest probability to be the chlorine precursor for the formation of 2- and 3-MCPDE. A list with an overview of the up-regulated and unchanged molecules can be found in the supplementary materials.

5.3.3. Screening of previously reported precursors

The first screening of the LC-HRMS data resulted in 36 unique hits from the 30 earlier reported exact masses. If two reported masses have a peak-to-peak retention time difference larger than 0.4 minute and showed clearly a different kinetic trend, then the two masses are considered to be different molecules. Several of these 36 unique hits might share the same mass but show a different kinetic trend and/or retention time. This then may result into a larger number of hits than what was enlisted in the mass list. All previously reported molecules from the literature, as shown in Table 5.2, were detected in the 3 different oil batches in this study: some of the molecules were found in one batch, some in two, and others were found in all three batches. The 36 hits are shown in Table 5.4. Ten of these 36 hits were present in all three oils batches and had consistent trends (6 were unchanged, 3 were downregulated, and 1 was upregulated). Among all 36 hits, twelve showed a downregulated trend and 2 molecules showed an intermediate trend. If two reported masses have a peak-to-peak retention time difference larger than 0.4 minute and showed clearly a different kinetic trend, then the two masses are considered to be different molecules. Based on this additional information next to the molecular masses, it is possible to make better assumptions whether two or more peaks belong to the same molecule or not. The molecule formula

shown in Table 5.4-5.6 and the two other tables in the supplemental information (up regulated & unchanged kinetic trends) were estimated by the data processing software which also took isotopic information into account together with the ion fragments. It should be noted that the software provides its best estimate of a formula, but the actual formula may be different (yet unknown). However, clearly, a chlorine atom is present in the molecule. Unfortunately, this is where the confidence halts in terms of molecule identification. With the current experimental design and obtained analytical data it is only possible to claim a Schymanski-confidence level of level 5 for all reported masses (Schymanski et al., 2014).

Seven out of the 12 down regulated organochlorine precursors had a mass lower than 650 Da, while 5 had a mass higher than 650 Da with 777.5531 Da as the largest organochlorine precursor from the target list (Table 2). It is speculated that molecules with masses larger than 600-650 Da are most likely intact TAGs which were able to dissolve in the injected solution after sample clean-up. This assumption was made based on a case where two palmitic acids and one linoleic acid are part of a TAG, as the composition of CPO consists mostly out of TAG containing palmitic acid (Cheong et al., 2014). 14 out of the 36 hits remained unchanged throughout the entire oil refining, from the start (T0-CPO) to the oil heated to 265°C. Therefore, it is unlikely that these molecules act as potential organochlorine precursors of 2- and 3-MCPDE. This might indicate that a part of the organochlorines present in the CPO are chemically- and thermally stable (up to a brief exposure of 265 °C).

A more detailed look at the molecule structure of the detected organochlorines might help establish connection between the observed trends, mass and molecule structure. With these results, we are able to show that our lab refining, and analysis method is effective as we were able to detect a large portion of the exact masses that have been reported before (as exact m/z-values). Nagy et al. (2019), reported m/z-values of potential precursors that were observed in other oil types such as soybean oil, coconut oil, or olive oil. Those reported m/z-values that were mainly seen in other oil types were included in our assessment. As expected, we were not able to detect them in palm oil suggesting the specificity of those potential precursors in particular oil types. Finally, the fact that detected molecules with identical m/z-values also showed similar trends in two or even in all three refined oils provide us with additional information to rule-out isomers and that those molecules are most likely identical.

5.3.4. Newly discovered potential precursors

To expand the current knowledge of other organochlorine precursors that may potentially act as precursors, we observed additional organochlorines in the samples from the 3 oil batches. It resulted in 62 downregulated molecules ranging from 208 Da

to 963 Da. One out of these 62 downregulated molecules showed a similar trend in all three oil batches, while eleven out of the 62 molecules showed a similar downward trend in two out of the three oil batches. Table 5.5 shows an overview of the 62 molecules. The accompanying mass spectra of these 62 molecules can be found in the supplemental information. Most of the downregulated organochlorine precursors have a mass of over 600 Da, indicating the presence of many chlorine-containing TAGs or other large molecules such as chlorine-containing phospholipids or glycolipids that have not been reported before. As for comparison, 65 organochlorines have been categorized with an up-regulated trend and 100 molecules with an unchanged trend. Combined with the 14 unchanged molecules which have been reported before, these 100 new, unlisted molecules indicate that a more sophisticated approach is required to obtain more insights into the precursors for 2- and 3-MCPDE formation during to oil refining, (which could be used for screening the palm oil prior to refining). Our data show that organochloride precursors with MW over 600 can breakdown in chlorine containing compounds with MW around 300. Unfortunately, their identity is unclear but this it at least a clear trend that was observed in the earlier discussed targeted approach. One could argue that the compounds causing the upward trend may correspond to the chlorinated DAGs and/or MAGs which ultimately are the MCPD esters. However, the theoretical masses of these esters do not correspond to the masses observed in this study. It is therefore more likely that the observed organochlorines are either intermediates or other unknown stable organochlorines.

Only 31 molecules were categorized as intermediates. These molecules are listed in Table 5.6. Out of the 31 molecules, 13 have a mass lower than 600 Da which might be an indication that most of the detected intermediates might have undergone a form of intra-molecular rearrangement instead of a major degradation. It could be expected that a major degradation of a e.g. TAG into a DAG or MAG resulted in a much larger molecular mass loss and should have resulted in smaller molecules. From other biological reactions, we know that fast-reacting intermediates do exist, and those fast-acting intermediates could have undergone other conversion or degradation under the 3 minutes sampling windows in this experiment. It is likely that the temporal resolution of 3 minutes is still too long and that those fast-reacting intermediates would have appeared and disappeared rapidly. The current study design did not allow their detection.

The plethora of organochlorines which were present in the CPO and the oil samples in this study (the number of downregulated molecules combined with the number of molecules with an unchanged trend) shows that there are large numbers of molecules that potentially act as chlorine donor for the formation of 2- and 3-MCPDE. Furthermore, comparing the numbers of downregulated molecules with the unchanged molecules shows the complexity of predicting which compounds in the CPO will react as chlorine precursors for 2- and 3-MCPDE. Nevertheless, for future progress to mitigate 2- and 3-MCPD levels, it is important to further elucidate the relevance of the molecules found

in this study, to determine their exact identity, and to evaluate their role in the generation of the undesirable contaminants. This will support the future development of effective mitigation strategies. Unfortunately, our current methodology was not suitable to reveal the molecular structure of these organochlorines. Additional experiments such as optimization of the detection and quantification with a triple quad-MS or collecting fraction after LC-column separation for further analysis with an NMR could potentially provide further insights into the organochlorines that act predominantly as precursors and help in unravelling their structure.

Table 5.4 36 Detected organochlorines from a list of previously published research papers (ref), detected by LC-HRMS. * Molecular formula as suggested by Compound Discoverer.

Unique reference number	Molecule formula*	Exact mass	RT (min)	trend		
				oil 1	oil 2	oil 3
1	C18 H31 Cl O2	314.2012	2.67	x	x	unchanged
2	C18 H33 Cl O2	316.2170	3.59	unchanged	x	unchanged
3	C18 H35 Cl O2	318.2325	4.37	unchanged	x	unchanged
4	C18 H37 Cl O2	320.2482	5.02	x	x	up
5	C18 H31 Cl O3	330.1963	1.57	x	up	up
6	C18 H31 Cl O3	330.1964	1.84	x	down	down
7	C18 H33 Cl O3	332.2119	1.62	x	down	down
8	C18 H33 Cl O3	332.2119	2.12	up	x	x
9	C18 H35 Cl O3	334.2275	1.79	x	x	up
10	C18 H35 Cl O3	334.2276	2.00	unchanged	unchanged	unchanged
11	C18 H37 Cl O3	336.2432	2.01	unchanged	unchanged	unchanged
12	C20 H39 Cl O2	346.2635	5.48	unchanged	x	x
13	C18 H33 Cl O4	348.2068	1.72	unchanged	x	x
14	C18 H33 Cl O4	348.2069	1.30	x	inter	inter
15	C18 H33 Cl O4	348.2069	1.65	x	down	down
16	C18 H35 Cl O4	350.2226	1.32	x	up	up
17	C18 H37 Cl O4	352.2381	1.51	up	up	up
18	C20 H40 Cl N O2	361.2748	4.83	down	x	x
19	C19 H39 Cl O4	366.2537	4.61	unchanged	unchanged	unchanged
20	C21 H39 Cl O4	390.2537	4.06	x	x	inter
21	C21 H39 Cl O4	390.2538	4.16	x	down	x
22	C21 H39 Cl O4	390.2539	3.89	x	unchanged	x
23	C21 H41 Cl O4	392.2695	4.79	unchanged	unchanged	unchanged
24	C21 H43 Cl O4	394.2852	5.65	unchanged	unchanged	x
25	C30 H56 Cl N5 O5	601.3976	3.88	down	x	x
26	C36 H70 Cl N O4	615.4997	9.76	down	down	down
27	C39 H67 Cl O5	650.4683	9.44	unchanged	unchanged	unchanged

Table 5.4 Continued.

Unique reference number	Molecule formula*	Exact mass	RT (min)	trend		
				oil 1	oil 2	oil 3
28	C39 H71 Cl O5	654.4995	10.91	unchanged	unchanged	unchanged
29	C40 H65 Cl N4 O3	684.4738	7.53	down	down	down
30	C40 H65 Cl N4 O3	684.4740	7.25	up	x	x
31	C40 H65 Cl N4 O3	684.4740	6.57	x	x	unchanged
32	C40 H65 Cl N4 O3	684.4741	6.83	x	x	down
33	C42 H84 Cl N O4	701.6100	13.34	up	x	x
34	C42 H86 Cl N O4	703.6250	13.23	down	x	x
35	C43 H82 Cl N5 O2	735.6153	11.75	down	down	down
36	C43 H76 Cl N5 O5	777.5531	9.72	down	x	x

The molecules are ordered by its exact mass and the trends of each molecule are shown for each of the three CPO. The trends are color coded with green corresponding with up-regulation, red with down-regulation, yellow with intermediates, and white / colorless with unchanged. Undetected masses in a particular oil are depicted with an "x". The retention time (RT) is shown in minutes.

Table 5.5 List of all detected downregulated organochlorines

Suggested formula	mol mass	RT (min)	oil 1	oil 2	oil 3
C10 H6 Cl F N2	208.0193	1.121		x	*
C12 H12 Cl N O4	269.0447	1.245		x	
C10 H11 Cl N4 O4	286.0477	1.064		x	
C18 H31 Cl O3	330.1962	2.131	x		
C15 H22 Br Cl O	332.0533	0.978		x	
C15 H10 Cl2 O5	339.9906	1.011		x	x
C18 H31 Cl O4	346.1912	1.387		x	
C15 H9 Cl3 O5	373.9516	1.619	x		
C16 H5 Cl3 N4 O	373.9517	1.009		x	
C23 H47 Cl O2	390.3267	7.329		x	
C23 H31 Cl O4	406.1911	2.415	x		
C22 H35 Cl N4 O	406.2486	1.972		x	
C24 H49 Cl O3	420.3362	6.032			x
C23 H47 Cl O4	422.3165	6.551		x	x
C29 H55 Cl O5	518.3741	7.756		x	
C34 H70 Cl N O3	575.5048	10.419	x		
C29 H26 Cl F N4 O4 S	580.1374	1.692	x		
C36 H49 Cl O4	580.3325	6.730		x	
C30 H56 Cl N5 O5	601.3976	3.880	x		
C36 H55 Cl N4 O2	610.4004	6.452	x		
C30 H62 Cl2 N4 O4	612.4161	6.261		x	x
C36 H57 Cl N4 O2	612.4162	6.678	x		
C36 H61 Cl N4 O2	616.4476	7.259		x	

Table 5.5 Continued.

Suggested formula	mol mass	RT (min)	oil 1	oil 2	oil 3
C36 H67 Cl N2 O4	626.4769	6.953		x	
C35 H66 Cl2 N2 O3	632.4428	7.202		x	
C38 H69 Cl O5	640.4835	10.343			x
C37 H69 Cl N2 O4	640.4921	7.676	x		
C32 H68 Cl2 N4 O4	642.4632	7.548		x	
C38 H65 Cl N4 O2	644.4788	8.057		x	x
C38 H65 Cl N4 O2	644.4788	8.622	x	x	
C38 H65 Cl N4 O2	644.4789	9.087	x		
C38 H67 Cl N4 O2	646.4945	9.148		x	x
C34 H70 Cl2 N2 O5	656.4635	6.954		x	
C40 H63 Cl N4 O2	666.4633	7.115		x	x
C40 H65 Cl N4 O2	668.4789	7.612		x	x
C40 H65 Cl N4 O2	668.4791	8.053	x		
C40 H67 Cl N4 O2	670.4945	8.240		x	x
C40 H67 Cl N4 O2	670.4948	8.683	x		
C40 H69 Cl N4 O2	672.5104	9.296		x	
C38 H67 Cl N4 O4	678.4844	6.108		x	x
C38 H67 Cl N4 O4	678.4845	6.110			
C40 H65 Cl N4 O3	684.4738	7.972	x		
C41 H79 Cl O5	686.5622	12.871			x
C40 H67 Cl N4 O4	702.4844	8.509		x	
C40 H67 Cl N4 O4	702.4845	8.969	x		
C42 H86 Cl N O4	703.6250	13.226	x		
C42 H69 Cl N4 O4	728.5000	8.681		x	
C42 H69 Cl N4 O4	728.5002	9.144	x		
C43 H88 Cl N O5	733.6358	13.313	x		
C43 H82 Cl N5 O2	735.6151	12.215	x		
C44 H90 Cl N O5	747.6517	13.737	x		
C43 H76 Cl N5 O5	777.5531	9.722	x		
C51 H73 Cl N2 O4	812.5219	8.405			x
C56 H85 Cl N2 O	836.6308	12.288	x	x	
C54 H89 Cl N4 O3	876.6622	12.292			x
C54 H95 Cl N4 O3	882.7090	13.719		x	x
C55 H101 Cl N2 O4	888.7426	13.717		x	
C56 H95 Cl N4 O3	906.7090	13.336	x	x	x
C56 H97 Cl N4 O3	908.7251	14.390	x		
C56 H99 Cl N4 O3	910.7412	14.456		x	
C57 H105 Cl N2 O4	916.7749	14.430			x
C65 H103 Cl N2 O	962.7796	14.949	x		

Note: in all blank fields, the molecular mass was not detected.

Table 5.6 List of organochlorines with 'intermediate' trend

Suggested formula	mol mass	RT (min)	oil 1	oil 2	oil 3
C7 H5 Cl2 N3 O5	280.9595	1.237	x		
C6 H5 Cl3 N2 O5	289.9254	1.256	x		
C18 H33 Cl O4	348.2068	1.942	x		
C22 H37 Cl N4 O2	424.2595	1.540		x	
C26 H49 Cl O4	460.3325	6.811		x	x
C28 H51 Cl O4	486.3481	6.996		x	x
C30 H51 Cl N4 O2	534.3692	6.231			x
C30 H51 Cl N4 O2	534.3693	6.201		x	
C31 H61 Cl O5	548.4214	9.317			x
C32 H53 Cl N4 O2	560.3848	6.534			x
C32 H53 Cl N4 O2	560.3851	6.516		x	x
C33 H61 Cl O5	572.4212	8.776		x	
C33 H61 Cl O5	572.4213	8.787			x
C34 H55 Cl N4 O3	602.3964	5.391		x	
C42 H10 Cl2 O3	632.0012	0.923		x	x
C38 H63 Cl N4 O2	642.4635	8.035		x	x
C34 H66 Cl2 N4 O3	648.4528	8.785		x	
C39 H65 Cl O5	648.4528	8.799			x
C38 H68 Cl2 N2 O2	654.4658	7.725		x	
C40 H63 Cl N4 O2	666.4635	7.548		x	
C38 H63 Cl N4 O4	674.4533	6.508		x	
C38 H65 Cl N4 O4	676.4689	6.205			x
C40 H63 Cl N4 O3	682.4581	7.237	x		
C40 H67 Cl N4 O4	702.4851	6.329		x	x
C37 H73 Cl2 N3 O5	709.4906	8.850		x	
C43 H85 Cl O5	716.6096	14.377		x	
C45 H86 Cl N5 O2	763.6466	13.038	x		
C49 H85 Cl N4 O5	844.6206	10.489		x	
C54 H95 Cl N4 O5	914.6992	11.753		x	x
C54 H97 Cl N4 O5	916.7155	12.171	x		
C71 H101 Cl N4 O2	1076.7683	14.274	x		

Note: in all blank fields, the molecular mass was not detected.

5.4. Conclusion

Three batches of CPO were refined under laboratory conditions and analysed for organochlorines. Molecules which are down-regulated in our experiment are the ones which should have priority for further investigation, as it is likely that these molecules are instable, and may therefore act as chlorine-donors in the MCPD formation process. In our study we have extended the list of potential precursors and we have studied the kinetics of these potential precursors during the refining process. We have recorded 258 organochlorines, which substantially increases the database on potential precursors. We have classified them according to four trend categories: up-regulated (n=65), down-regulated (n=62), intermediates (n=31), and unchanged (n=100). Future research should reveal the identity and role of these molecules, in particular the down-regulated molecules, in the reaction pathway of the MCPDEs.

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Supplementary materials S1

Compound Discoverer workflow settings:

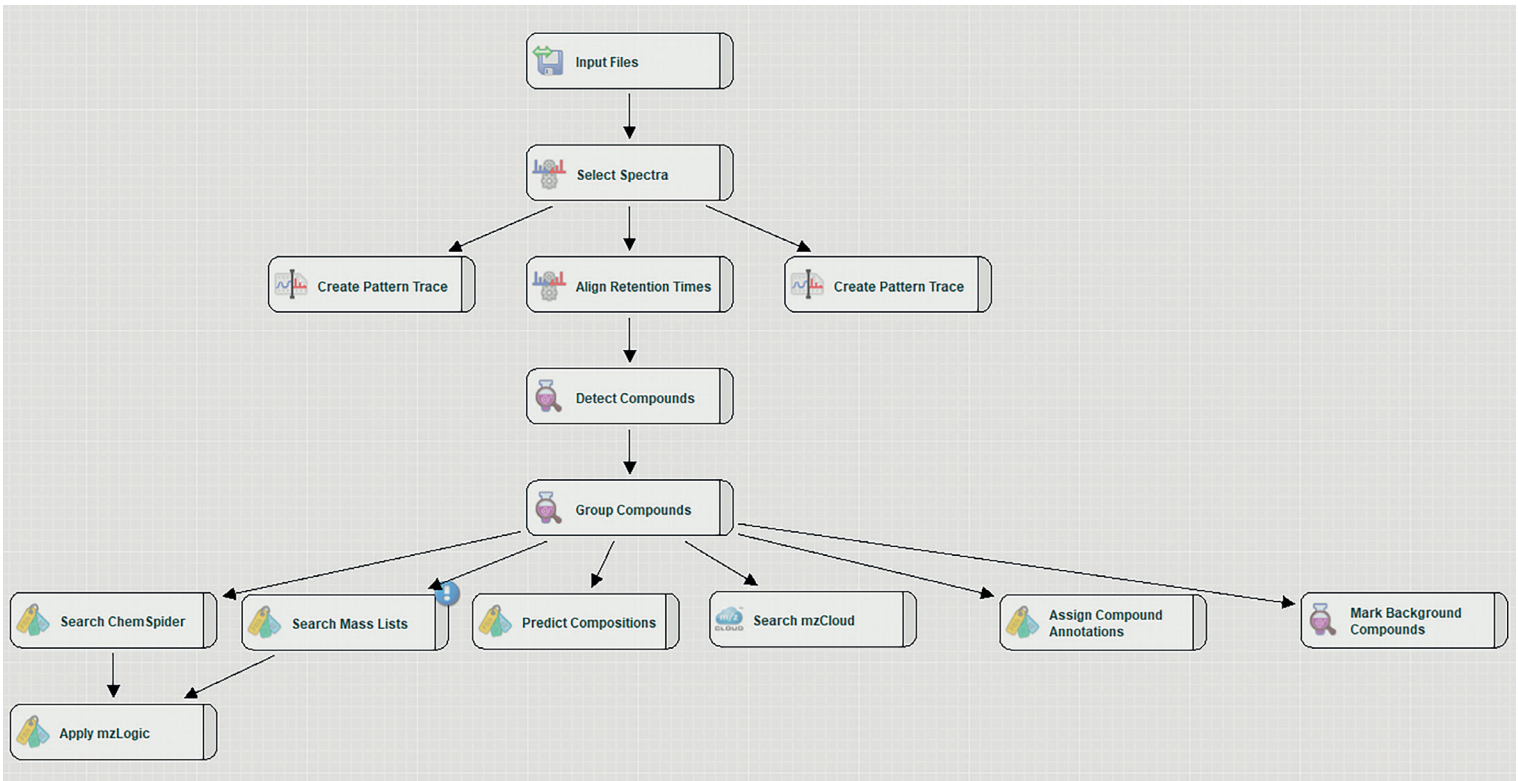


Figure S5.1 Compound Discoverer workflow for data processing

A summary of the processing node parameters settings used in Compound Discoverer during data processing.

1. General Settings:
- Precursor Selection: Use MS(n - 1) Precursor
- Use Isotope Pattern in Precursor Reevaluation: True
- Provide Profile Spectra: Automatic
- Store Chromatograms: False

2. Spectrum Properties Filter:
- Lower RT Limit: 0
- Upper RT Limit: 0
- First Scan: 0
- Last Scan: 0
- Ignore Specified Scans: (not specified)
- Lowest Charge State: 0
- Highest Charge State: 0
- Min. Precursor Mass: 0 Da
- Max. Precursor Mass: 5000 Da
- Total Intensity Threshold: 0
- Minimum Peak Count: 1

3. Scan Event Filters:
- Mass Analyzer: (not specified)
- MS Order: Any
- Activation Type: (not specified)
- Min. Collision Energy: 0
- Max. Collision Energy: 1000
- Scan Type: Any
- Polarity Mode: (not specified)

4. Peak Filters:
- S/N Threshold (FT-only): 1.5

5. Replacements for Unrecognized Properties:
- Unrecognized Charge Replacements: 1
- Unrecognized Mass Analyzer Replacements: ITMS
- Unrecognized MS Order Replacements: MS2
- Unrecognized Activation Type Replacements: CID
- Unrecognized Polarity Replacements: -

- Unrecognized MS Resolution@200 Replacements: 60000
- Unrecognized MSn Resolution@200 Replacements: 30000

Processing node 7: Align Retention Times

---

1. General Settings:
- Alignment Model: Linear
- Alignment Fallback: Use Linear Model
- Maximum Shift [min]: 2
- Shift Reference File: True
- Mass Tolerance: 5 ppm
- Remove Outlier: True

Processing node 12: Detect Compounds

---

1. General Settings:
- Mass Tolerance [ppm]: 5 ppm
- Intensity Tolerance [%]: 30
- S/N Threshold: 3
- Min. Peak Intensity: 100000
- Ions: [M-H]-1
- Base Ions: [M+H]+1; [M-H]-1
- Min. Element Counts: C H O
- Max. Element Counts: C90 H190 Cl5 N5 O5

2. Peak Detection:
- Filter Peaks: True
- Max. Peak Width [min]: 0.5
- Remove Singlets: True
- Min. # Scans per Peak: 5
- Min. # Isotopes: 2

Processing node 13: Group Compounds

---

1. Compound Consolidation:
- Mass Tolerance: 5 ppm
- RT Tolerance [min]: 0.2

2. Fragment Data Selection:
- Preferred Ions: [M-H]-1

Processing node 21: Assign Compound Annotations

---

1. General Settings:
- Mass Tolerance: 5 ppm

2. Data Sources:
- Data Source #1: MassList Search
- Data Source #2: mzCloud Search
- Data Source #3: ChemSpider Search
- Data Source #4: Predicted Compositions
- Data Source #5: (not specified)
- Data Source #6: (not specified)
- Data Source #7: (not specified)

3. Scoring Rules:
- Use mzLogic: True
- Use Spectral Distance: True
- SFit Threshold: 20
- SFit Range: 20

Processing node 16: Mark Background Compounds

---

1. General Settings:
- Max. Sample/Blank: 5
- Max. Blank/Sample: 0
- Hide Background: True

Processing node 18: Search mzCloud

---

1. General Settings:
- Compound Classes: All
- Precursor Mass Tolerance: 10 ppm
- FT Fragment Mass Tolerance: 10 ppm

- IT Fragment Mass Tolerance: 0.4 Da
- Library: Autoprocessed; Reference
- Post Processing: Recalibrated
- Max. # Results: 10
- Annotate Matching Fragments: False

2. DDA Search:
- Identity Search: HighChem HighRes
- Match Activation Type: True
- Match Activation Energy: Match with Tolerance
- Activation Energy Tolerance: 20
- Apply Intensity Threshold: True
- Similarity Search: None
- Match Factor Threshold: 60

3. DIA Search:
- Use DIA Scans for Search: False
- Max. Isolation Width [Da]: 500
- Match Activation Type: True
- Match Activation Energy: Match with Tolerance
- Activation Energy Tolerance: 20
- Apply Intensity Threshold: True
- Match Factor Threshold: 20

Processing node 19: Search ChemSpider

---

1. Search Settings:
- Database(s):
    KEGG
    LipidMAPS
    MassBank
    Pesticide Common Names
    PlantCyc
    Toxin, Toxin-Target Database
- Search Mode: By Formula or Mass
- Mass Tolerance: 5 ppm
- Max. # of results per compound: 20
- Max. # of Predicted Compositions to be searched per Compound: 3
- Result Order (for Max. # of results per compound): Order By Reference Count (DESC)

2. Predicted Composition Annotation:
- Check All Predicted Compositions: False

Processing node 26: Apply mzLogic

---

1. Search Settings:
- FT Fragment Mass Tolerance: 10 ppm
- IT Fragment Mass Tolerance: 0.4 Da
- Max. # Compounds: 0
- Max. # mzCloud Similarity Results to consider per Compound: 10
- Match Factor Threshold: 30

Processing node 20: Search Mass Lists

---

1. Search Settings:
- Mass Lists: mass list mcpd precursor_aangepast.massList
- Mass Tolerance: 5 ppm
- Use Retention Time: True
- RT Tolerance [min]: 2

Processing node 17: Predict Compositions

---

1. Prediction Settings:
- Mass Tolerance: 5 ppm
- Min. Element Counts: C H O
- Max. Element Counts: C90 H190 Cl5 N5 O5
- Min. RDBE: 0
- Max. RDBE: 40
- Min. H/C: 0.1
- Max. H/C: 3.5
- Max. # Candidates: 10
- Max. # Internal Candidates: 200

2. Pattern Matching:
- Intensity Tolerance [%]: 30
- Intensity Threshold [%]: 0.1
- S/N Threshold: 3

- Min. Spectral Fit [%]: 30
- Min. Pattern Cov. [%]: 90
- Use Dynamic Recalibration: True

3. Fragments Matching:
- Use Fragments Matching: True
- Mass Tolerance: 5 ppm
- S/N Threshold: 3

Processing node 24: Create Pattern Trace

---

1. General Settings:
- Isotope Ratios: Cl
- Mass Tolerance: 2 ppm
- Intensity Tolerance [%]: 30
- MS Order: MS1
- Polarity: -
- Custom Label: Cl1

Processing node 25: Create Pattern Trace

---

1. General Settings:
- Isotope Ratios: Cl2
- Mass Tolerance: 2 ppm
- Intensity Tolerance [%]: 30
- MS Order: MS1
- Polarity: -
- Custom Label: Cl2

Processing node 22: Differential Analysis

---

1. General Settings:
- Log10 Transform Values: True

Processing node 23: Descriptive Statistics

No parameters

Table S5.1 List of up-regulated organochlorines.

Suggested formula	mol mass	RT (min)	oil 1	oil 2	oil 3
C18 H31 Cl O3	330.1962	1.939	x		
C18 H33 Cl O3	332.2119	2.121	x		
C18 H33 Cl O4	348.2068	1.721	x		
C18 H37 Cl O4	352.2381	1.837	x		
C22 H31 Cl N2 O	374.2124	2.677			x
C22 H45 Cl O2	376.3108	7.264	x		
C21 H39 Cl O4	390.2537	4.290	x		
C24 H49 Cl O2	404.3421	8.284	x		
C24 H49 Cl O3	420.3365	6.494	x		
C23 H40 Cl N O4	429.2645	4.057			x
C22 H49 Cl N4 O3	452.3479	8.281	x		
C26 H49 Cl O4	460.3324	7.252	x		
C26 H53 Cl O4	464.3636	8.409	x		
C28 H35 Cl N4 O	478.2520	1.308		x	
C28 H37 Cl N4 O	480.2677	1.836	x		
C28 H37 Cl N4 O	480.2679	1.454		x	
C28 H49 Cl O4	484.3325	6.496		x	
C28 H49 Cl N4 O2	508.3535	5.271		x	
C28 H55 Cl N4 O2	514.4025	7.537	x		
C29 H57 Cl O5	520.3897	8.823	x		
C30 H49 Cl N4 O2	532.3535	5.690		x	
C29 H56 Cl2 N2 O2	534.3693	5.460		x	
C31 H51 Cl N4 O2	546.3692	5.943		x	x
C31 H53 Cl N4 O2	548.3850	6.552		x	
C31 H61 Cl O5	548.4213	9.781	x		
C31 H61 Cl O5	548.4214	9.308		x	
C32 H51 Cl N4 O2	558.3692	5.910		x	x
C32 H53 Cl N4 O2	560.3848	6.955	x		
C33 H53 Cl N4 O2	572.3850	6.256		x	x
C33 H55 Cl N4 O2	574.4008	6.763		x	
C32 H53 Cl N4 O3	576.3799	5.202		x	
C33 H53 Cl N4 O3	588.3800	5.667		x	
C34 H67 Cl O5	590.4683	7.536		x	
C38 H63 Cl N4 O2	642.4633	8.423	x		
C35 H67 Cl N4 O4	642.4845	8.642	x		
C38 H65 Cl N4 O2	644.4790	9.570	x		
C38 H69 Cl N4 O2	648.5105	8.890		x	x
C38 H63 Cl N4 O3	658.4586	6.506		x	
C40 H65 Cl N4 O2	668.4791	8.681	x		
C40 H65 Cl N4 O2	668.4794	8.222		x	x
C38 H65 Cl N4 O4	676.4688	6.617	x		
C38 H65 Cl N4 O4	676.4689	6.182		x	
C40 H65 Cl N4 O3	684.4740	7.251	x		
C41 H81 Cl O5	688.5778	14.034			
C42 H84 Cl N O4	701.6100	13.339	x		

Table S5.1 Continued.

Suggested formula	mol mass	RT (min)	oil 1	oil 2	oil 3
C40 H71 Cl N4 O4	706.5158	9.323	x		
C43 H83 Cl O5	714.5936	14.211	x		
C43 H83 Cl O5	714.5937	13.698		x	
C39 H77 Cl3 N2 O3	726.5030	9.220	x		
C42 H73 Cl N4 O4	732.5317	9.505	x		
C42 H73 Cl N4 O4	732.5319	9.038		x	
C45 H85 Cl O5	740.6095	14.451	x		
C45 H85 Cl O5	740.6100	13.933		x	
C45 H87 Cl O5	742.6253	14.509			x
C48 H83 Cl N4 O4	814.6099	10.941		x	
C50 H83 Cl N4 O4	838.6101	10.427		x	
C49 H85 Cl N4 O5	844.6199	10.947	x		
C56 H99 Cl N4 O3	910.7408	14.708	x		
C54 H95 Cl N4 O5	914.6991	12.241	x		
C58 H102 Cl2 O3	916.7163	11.709		x	
C65 H98 Cl N O	943.7342	12.184	x		
C62 H100 Cl2 N2 O	958.7250	13.233	x		
C63 H101 Cl N2 O4	984.7410	13.366	x		
C63 H103 Cl N2 O4	986.7572	12.767		x	
C69 H103 Cl N2 O5	1074.7534	13.215		x	

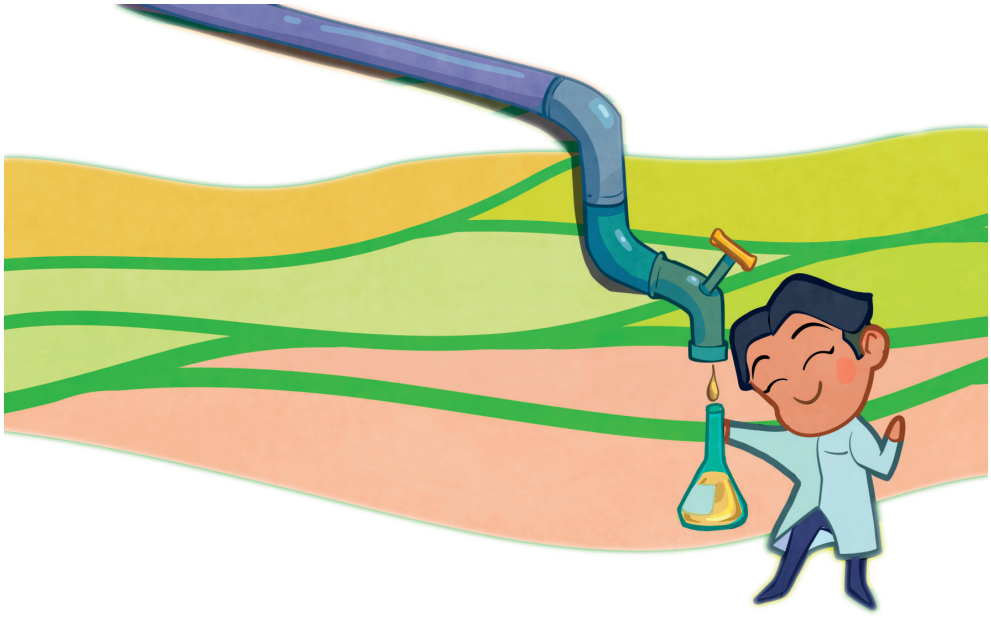
Table S5.2 List of organochlorines with 'unchanged' trend.

Suggested formula	mol mass	RT (min)	oil 1	oil 2	oil 3
C13 H19 Cl N2 O	254.1184	2.870		x	
C18 H33 Cl O2	316.2170	3.588	x		
C18 H35 Cl O3	334.2275	2.381	x		
C18 H37 Cl O3	336.2431	2.380	x		
C18 H31 Cl O4	346.1911	1.768	x		
C20 H39 Cl O2	346.2635	5.479	x		
C20 H39 Cl O2	346.2636	5.201			x
C20 H41 Cl O2	348.2793	6.334	x		
C20 H41 Cl O2	348.2795	5.925		x	
C20 H41 Cl O2	348.2795	6.042			x
C17 H33 Cl O5	352.2017	2.873		x	
C20 H40 Cl N O2	361.2748	4.834	x		
C19 H39 Cl O4	366.2536	4.999	x		
C15 H9 Cl3 O5	373.9517	1.305			x
C22 H45 Cl O2	376.3109	6.841		x	
C21 H37 Cl O4	388.2381	3.725	x		
C21 H37 Cl O4	388.2382	3.340		x	
C21 H39 Cl O4	390.2537	4.526	x		
C21 H41 Cl O4	392.2694	5.273	x		
C21 H43 Cl O4	394.2851	6.080	x		

Table S5.2 Continued.

Suggested formula	mol mass	RT (min)	oil 1	oil 2	oil 3
C18 H41 Cl N4 O3	396.2851	6.330	x		
C18 H41 Cl N4 O3	396.2852	5.924		x	
C23 H29 Cl O4	404.1755	1.867	x		
C24 H49 Cl O2	404.3422	7.841		x	
C23 H31 Cl O4	406.1911	2.075	x		
C24 H49 Cl O3	420.3360	5.986		x	
C23 H47 Cl O4	422.3165	6.981	x		
C23 H47 Cl O4	422.3165	6.551		x	
C23 H33 Cl O5	424.2020	1.469		x	
C21 H47 Cl N4 O3	438.3322	5.583	x		
C26 H47 Cl O3	442.3190	6.496	x		
C28 H43 Cl O2	446.2954	6.543		x	
C28 H43 Cl O2	446.2954	6.968	x		
C28 H43 Cl O3	462.2904	6.576		x	
C28 H43 Cl O3	462.2904	6.996	x		
C20 H15 Cl3 N4 O3	464.0195	3.850	x		
C26 H9 Cl N2 O5	464.0196	3.451		x	
C29 H45 Cl O3	476.3058	7.242	x		
C23 H51 Cl N4 O4	482.3587	6.908	x		
C29 H59 Cl O4	506.4106	9.981	x		
C29 H59 Cl O4	506.4107	9.500		x	
C29 H57 Cl O5	520.3897	8.368		x	x
C33 H61 Cl O5	572.4212	9.243	x		
C33 H63 Cl O5	574.4368	9.995	x		
C33 H63 Cl O5	574.4369	9.523		x	x
C33 H65 Cl O5	576.4524	10.723	x		
C33 H65 Cl O5	576.4525	10.254		x	
C33 H65 Cl O5	576.4525	10.248			x
C36 H49 Cl O4	580.3326	7.162	x		
C35 H67 Cl O5	602.4681	10.891	x		
C35 H67 Cl O5	602.4682	10.416		x	x
C35 H69 Cl O5	604.4837	11.591	x		
C35 H69 Cl O5	604.4838	11.102		x	
C36 H70 Cl N O4	615.4998	10.251	x		
C37 H67 Cl O5	626.4681	10.429	x		
C37 H67 Cl O5	626.4682	9.949		x	x
C37 H74 Cl N3 O2	627.5442	10.682	x		
C37 H69 Cl O5	628.4837	11.056	x		
C37 H69 Cl O5	628.4838	10.558		x	
C37 H71 Cl O5	630.4994	11.773	x		
C37 H71 Cl O5	630.4996	11.338		x	
C37 H73 Cl O5	632.5150	12.423	x		
C37 H73 Cl O5	632.5151	11.937		x	x

C38 H69 Cl O5	640.4835	10.342		x		
C38 H69 Cl O5	640.4835	10.820	x			
C35 H67 Cl N4 O4	642.4842	8.545		x		
C38 H71 Cl O5	642.4993	11.496	x			
C38 H71 Cl O5	642.4995	11.014		x		x
C38 H73 Cl O5	644.5149	12.176	x			
C38 H73 Cl O5	644.5151	11.697		x		x
C40 H61 Cl N4 O	648.4527	9.251	x			
C39 H67 Cl O5	650.4682	9.911	x			
C39 H69 Cl O5	652.4837	10.614	x			
C39 H69 Cl O5	652.4839	10.165		x		
C39 H71 Cl O5	654.4994	11.369	x			
C39 H73 Cl O5	656.5149	12.078	x			
C39 H73 Cl O5	656.5151	11.622		x		
C39 H75 Cl O5	658.5308	12.611	x			
C39 H75 Cl O5	658.5310	12.156		x		
C35 H69 Cl N4 O5	660.4949	7.008		x		
C39 H77 Cl O5	660.5466	13.211	x			
C39 H77 Cl O5	660.5468	12.703		x		x
C41 H79 Cl O5	686.5620	13.382	x			
C41 H79 Cl O5	686.5621	12.888		x		
C42 H77 Cl N4 O	688.5779	13.527		x		
C40 H71 Cl N4 O4	706.5158	8.858		x		
C45 H87 Cl O5	742.6251	15.064	x			
C45 H87 Cl O5	742.6253	14.541		x		
C48 H92 Cl2 N4 O5	874.6461	12.216	x			
C54 H89 Cl N4 O3	876.6620	12.794	x			
C56 H73 Cl N2 O5	888.5239	7.676		x		
C56 H101 Cl N4 O3	912.7565	15.172	x			
C67 H101 Cl N2 O5	1048.7362	13.048		x		
C67 H101 Cl N2 O5	1048.7364	13.536	x			
C67 H105 Cl N2 O5	1052.7675	14.659	x			
C67 H105 Cl N2 O5	1052.7676	14.149		x		
C67 H107 Cl N2 O5	1054.7831	15.259	x			
C67 H107 Cl N2 O5	1054.7833	14.736		x		
C67 H109 Cl N2 O5	1056.7987	15.858	x			
C69 H109 Cl N2 O5	1080.7988	15.434	x			



Chapter 6

General Discussion

Oil refining is performed to produce ready-to-use vegetable oil from crude vegetable oils. Colors, odors, impurities (e.g. pollutants) and FFA are removed during an oil refining process, and the oil is sterilized. FFA can oxidize the oil and the removal of FFA in particular stabilizes the oil. This enhances the thermostability of the oil and extents its shelf life. Unfortunately, the refining conditions may lead to the formation of the process contaminants 2-MCPDE, 3-MCPDE, and GE. The urge to get these process contaminants under control is stimulated by enforcements of MLs regarding these contaminants in the European Union in vegetable oils. This thesis explored different mitigation strategies to reduce the presence of potential carcinogenic contaminants in palm oil from a chemical and physical oil refining point of view. The main aim of this thesis was to develop mitigation strategies to reduce or eliminate the process contaminants 2-, 3-MCPDE, and GE in palm oil. While the development of mitigation strategies in palm oil might sound straight forward from a chemical or industrial point-of-view, palm oil itself is shrouded in many controversies. In the last decade, consumers have become more judicial and have often taken an opinion whether we should or should not consume food containing palm oil. Independent from the ethical considerations related to the consumption of this oil, it is important to improve its safety. In this chapter, the main themes of formation of the three contaminants, chemical and physical oil refining, quantification of the contaminants and detection and identification of their potential precursors are revisited. Furthermore, the limitation, potential, and future recommendation regarding mitigation strategies to reduce 2-, 3-MCPD esters, and glycidyl esters are discussed. Finally, environmental and socio-economical impacts of palm oil and its cultivation, but also other possible applications of the developed mitigation strategies within the vegetable oil refining industry will be touched upon briefly.

6.1. The necessities of mitigation strategies in oil refining

Palm oil is globally the most used vegetable oil and is often found in many common food items such as baked cookies, soups, ready-to-eat meals, ice-cream, etc. (Dian et al., 2018; Tan et al., 2021). Due to its physio-chemical properties, palm oil is often used as shortening agents in baked goods, as frying mediums, it can be used to achieve a desired texture, and, in certain cases, palm oil might be able to stabilize the shelf life of food products (Sulaiman et al., 2022; Wroniak et al., 2021). During the last decades, certain processing contaminants have been reported to be present in refined vegetable oil, namely 2-, 3-MCPDE, and GE. Refined palm oil has the potential to contain the highest concentration of those contaminants among other vegetable oils. In Europe, the European Food Safety Authority (EFSA) has produced an opinion on the toxicity of 3-MCPD and glycidol in 2016 (EFSA CONTAM Panel, 2016). The tolerable daily intake (TDI) for 3-MCPD was set at 0.8 µg/kg body weight. However, in 2018 a revised TDI was

published and was set at 2.0 µg/kg body weight (Knutsen et al., 2018). As glycidol is a genotoxic compound, a margin of exposure (MoE) approach was used for its risk characterization. A MoE of 25,000 or more was found to be relatively safe. This risk assessment was adopted by the European Commission (EC) who have set maximum limits (MLs) for 3-MCPDE and GE. As of 25 April 2023, the most up to date MLs can be found in the Commission Regulation 2023/915 (European Commission, 2023). Europe is so far the only region where these MLs were implemented on 3-MCPDE and GE in a variety of vegetable oils and infant formula. Other food safety authorities in other countries outside of Europe have not (yet) adopted such limits, but research about the toxicology of 3-MCPDE and GE has been acknowledged by authorities outside the EU (Fattore et al., 2023; U.S. Food & Drug Administration, 2022).

Because of the enforced MLs, vegetable oil refineries in Europe and vegetable oil suppliers with Europe as sales market must comply with these regulations. Driven by the carcinogenic nature of these process contaminants and the ensuing MLs, a mitigation strategy is needed in order to eliminate or at least minimize the concentrations of those process contaminants in the various vegetable oils and fats. Chapter 2 exhibited several examples of mitigation strategies that act on different levels prior, during, and/or after the refining process of vegetable oils.

In general, mitigation strategies that have been published in peer reviewed journals target one or two parameters that play an important role in the oil refining process. Furthermore, the majority of the current mitigation strategies reviewed in Chapter 2 were based on lab-scale experiments. As far as we know, there is one other peer-reviewed publication reporting on mitigation strategies results done in large scale (Ramli et al., 2020). While lab-scale experiments have their advantages, such as being cost effective, the ability of having finer controls of all parameters, and less challenging to perform experimental duplicates or, a lab-scale experiment is not capable of representing the complexity of full-scale production and daily practice of an oil refinery. A lab-scale experiment does not have to be a limitation on its own, but upscaling a lab-scale experiment to a full refining process still requires many steps that each have to be carried out carefully. The results of several mitigation strategies that have been developed with the use of a pilot plant are shown in Chapter 3 & 4. These experiments were conducted to investigate physical and chemical refining methods in order to mitigate the formation of 2-, 3-MCPDE, and GE.

6.2. Pilot plant experiments

Experiments can be conducted with different order of magnitudes. Lab experiments are very common to start an investigation with. In essence, lab experiments try to mimic reality by means of deconstructing the real-life environment and using a magnifying glass to focus on a specific target within those deconstructed reality. The strength of lab experiments lies in their size and simplicity. It is relatively small in its extent and therefore relatively cost effective. At the same time, the design of lab experiments can be tailored to accommodate all kinds of strategies and visions from scientists. However, the condition in which lab experiments are performed in, have to be designed and monitored well as it can have a large impact on the outcome of the experiments. One of the major downfalls of lab experiments is that replicating real life conditions, whether or not miniaturized, is often very hard. One simple example is the time and condition that it is required to heat up a certain volume of oil with comparable amount of energy. Pilot-plants are a step-up beyond lab experimentations, and they can be used to identify critical points during the upscaling phase of certain processes going from lab-scale to full-scale. Pilot plants are designed to simulate the full-scale production capacity, but on a manageable scale. In our case, the pilot plant can hold 100 kg of oil and is capable of simulating the heating, mixing, and vacuum distillation of a full-size refining tower. In case something goes sideways in a pilot plant, the loss is much more economically manageable.

6.3. Our findings

Mitigation of these undesired effects was aimed at in the current studies. The major outcomes from this thesis are summarized in Table 6.1. Chapter 3 and Chapter 4 in this thesis shows the results of mitigation strategies based on two major oil refining approaches: physical refining and chemical refining. Table 6.2 shows the most successful mitigation strategies. The key difference between the two approaches is the way FFA is removed from the oil. Physical oil refining generally uses steam distillation to remove FFA from the oil while chemical refining uses a base, like potassium hydroxide to neutralize the FFA (saponification). Chapter 3 showed the effect of the refining temperature and the combined effect of post-refining treatment with bleaching earth and phosphoric acid. Briefly, exposing palm oil to high temperatures, above ±180 °C, increases the amount of GE in the oil. The amount of 2- and 3-MCPDE increases as well, but as they are chlorine dependent, it reaches a certain limit where no more 2- and 3-MCPDE are formed. Post refining treatments, i.e. treating the refined oil for the second time with phosphoric acid and bleaching earth, in combination with a lower second deodorization temperature proves to result in the lowest concentration of GE when physical refining is the chosen

method (2.96 mg/kg). Chapter 4 showed the effect of water-washing of the crude oil and FFA removal by means of saponification. This so-called chemical refining method does not subject the oil to high temperatures as is the case during physical refining. Chemical refining, where both water-washing and neutralization of the FFA with potassium hydroxide are combined, resulted in the overall lowest amount of 2-MCPDE, 3-MCPDE, and GE with 0.42, 0.78, and 0.99 mg/kg, respectively.

Table 6.1. A summary of the primary results reported in this thesis.

Objectives	Main findings
Chapter 2 To identify knowledge gaps and data gaps regarding the mitigation of 2-, 3-MCPDE and GE in the vegetable oil refining.	The following knowledge gaps and data gaps were identified: I) None of the reviewed mitigation strategies included 2-MCPDE in their research. II) There was no mitigation strategies available focusing on the role of organochlorine containing precursors or other "large-molecule" potential precursors. III) Several published mitigation strategies provided very limited relevant information in such a way that experimental replication by peers would have been impossible to do. IV) There was barely any mitigation strategy which specifically investigated the bleaching process and its mitigating effect on GE. V) The majority of the reviewed mitigation strategies data who reported GE concentration did not manage to reduce GE concentration to below the 1000 µg/kg ML. VI) The majority of the reviewed mitigation strategies were conducted on lab-scale. While this does not have to be an issue, upscaling a lab-scale-based methodology to full-industrial-scale is very challenging.
Chapter 3 To fill the identified knowledge gap I, III, IV & VI and to investigate potential mitigation strategies for the contaminants based on the physical refining approach.	The following knowledge gaps and primary results are clarified: I) In this chapter, several experiments were performed to explore the effect of the different processes used in physical refining. The reported, successful mitigation strategies focused also on 2-MCPDE. III) This chapter also provided a detailed breakdown of the experimental design together with the analytical procedure to quantify 2-MCPDE, 3-MCPDE, and GE. IV) The bleaching process was part of the reported mitigation strategies and GE concentration was reported for all experiments including the control treatment. Furthermore, a post-refining degumming and bleaching treatment in combination with a low second deodorization process appears to be a very effective strategy to reduce GE concentration. VI) All experiments reported in this chapter, including the control, were performed in a pilot-plant which can hold 100 kg of organic CPO as starting material.

Table 6.1. Continued.

Objectives	Main findings
Chapter 4 To fill the identified knowledge gap I, III - VI and to investigate potential mitigation strategies for the contaminants based on the chemical refining approach.	The following knowledge gaps and primary results are clarified: I) In this chapter, several experiments were performed to explore the effect of the different processes used in chemical refining. The reported, successful mitigation strategies included 2-MCPDE data as well. III) This chapter also provided a detailed breakdown of the experimental design. The analytical procedure used to quantify 2-MCPDE, 3-MCPDE, and GE is identical to what was previously reported in Chapter 3. IV) The bleaching process was part of the reported mitigation strategies and GE concentration was reported for all experiments including the control treatment. A combination of post-degumming neutralization followed by water washing of the oil, and a relatively low deodorization temperature appears highly effective. V) The aforementioned refining condition here above (point IV) succeeded in producing palm oil which is able to meet the set MLs for 3-MCPDE and GE. VII) All experiments reported in this chapter, including the control, were performed in the same pilot-plant used for the experiments described in Chapter 3. This allows for a close comparison between all reported mitigation strategies in both chapters.
Chapter 5 To address knowledge gap II by investigating the occurrence of potential organochlorine precursors that may play a role in formation of 2- and 3-MCPDE	The following knowledge gap and primary results are clarified: II) The mitigation strategies which were reviewed in Chapter 2 and the ones reported in Chapter 3 & 4 were all developed to act at the refining level. Furthermore, those mitigation strategies were based on the formation mechanism theory where readily available chloride ions are present as precursors. This chapter explored the occurrence of potential endogenous organochlorine precursors in CPO and expanded the current knowledge of the origins of the chloride ions. Lab-scale refining pre-treatment experiments were successfully conducted to gain information about the kinetic behavior of those potential organochlorine precursors during oil refining.

Table 6.2. A summary of the major pilot plant mitigation studies. The amount of achieved reduction is compared against the control.

	2-MCPDE (mg/kg)	3-MCPDE (mg/kg)	GE (mg/kg)
Control – Single physical refining	0.82	1.62	3.73
Double physical refining with adjusted temperatures	0.71	1.42	2.96
Chemical refining including alkali neutralization & post-neutralization water wash	0.42	0.78	0.99

6.4. Advantages and disadvantages of chemical and physical refining

Vegetable oil is prone to oxidation almost immediately after the seed or fruit is crushed during extraction (Machado et al., 2022). The purpose of oil refining is done to improve the overall quality and stability of the oil. While these are the beneficial aspects of refining, refining is also known to remove many compounds that are often desired such as sterols and tocopherols, and polyunsaturated fatty acids (Hashempour-Baltork et al., 2022). Table 6.3 presents a summary of the of the major advantages and drawbacks of both physical and chemical refining. One example of an oil type that specifically is left unprocessed is cold pressed extra virgin olive oil. Olive oil has a desirable taste, odor, and is known for its positive health effects due to its composition (González-Acedo et al., 2023; Heras et al., 2023). Fortunately, chemical refining is able to preserve more of the desirable components in comparison to physical refining (Pal et al., 2015). During physical refining, high deodorization temperature is required to remove the majority of FFA. With such high temperatures, many desirable components can undergo decomposition or are extracted together with the FFA during deodorization in combination with vacuum distillation. Furthermore, the requirements of high temperatures and deep vacuum is intrinsically linked to a larger energy requirement. This is where a small paradox arises. While it seems as if physical refining, compared to chemical refining, is the one with the larger energy consumption, chemical refining involves more steps to execute correctly. Chemical refining actually introduces an additional processing step of neutralization, which exposes the oil to water or a lye solution to remove FFAs, on top of the, often still required, bleaching and deodorization process. After neutralizing the oil, it must be dried, and the refinery also have to deal with additional waste in the form of soap stock. Due to the formation of soap stock, the net yield will be lower as part of the oil undergoes saponification. Physical refining, in comparison, does not suffer from this oil losses. This ongoing dilemma of which refining method should be chosen is not easily solved as the choice depends on many factors. Chemical refining, for instance, can offer better refining results when refining oil with high phospholipid content (Zeldenrust, n.d.). When the chemical refining process has been tuned in carefully, it can produce good, refined oil whilst maintaining a low level of 2- and 3-MCPDE concentration. There are also attempts made in order to reduce the natural oil loss due to chemical refining (Kozyuk & Reimers, 2016). Based on our experience, the process of physical refining is less complicated to perform and often requires less time in total than chemical refining. However, when mitigating the concentration of 2-MCPDE, 3-MCPDE, and GE, perhaps a combination of certain processes derived from both physical and chemical refining would provide the best result.

Table 6.3. Comparison of the advantages and disadvantages between physical and chemical refining

	Advantages	Disadvantages
Physical refining	Less processes required	Removal of desirable oil components
	Higher product yield	More energy is required to reach high temperature
	Fewer chemical required	Potential formation of chloropropanols, GE, or other heat related by-products
Chemical refining	Overall lower formation of chloropropanols and GE	Higher product loss
	Potentially smaller loss of desirable oil components	Formation of more chemical waste
	More chemicals required	Process is more elaborate
	Lower deodorization temperature is more energy efficient	Drying of the oil is crucial prior to deodorization

6.5. One 3-MCPDE and GE mitigation strategy applicable for all types of vegetable oils

Hypothetically speaking, one might want to combine certain processes from the chemical refining method with the physical refining method to make use of the strengths of the two refining methods. This could be an efficient way for refineries as this approach would allow them to set-up their refinery equipment in such a way that it is suitable to produce all kinds of vegetable oils while keeping 3-MCPDE and GE concentration in check. As MCPDEs are known to be chlorine dependent, a pre-refining water washing process can for example be equipped to remove water soluble chlorine sources, while the post-refining treatment from our double physical refining experiment takes care of the post-refining GE removal. This theory, combining chemical refining processes with physical refining processes, can be further explored and fined tuned to establish a 'universal' refining method that can be used for many different types of crude vegetable oils. This theory originates and can be build upon the similarities that are found in the core principles of the many refining methods used to refine different types of crude vegetable oils. The exact temperature for a certain type of vegetable oil, the amount of required additives, reaction time, etc. might differ for specific type of vegetable oil, but in the end, physical refining is just physical refining consisting out of degumming, bleaching, deodorization as its core principles. Therefore, in principle one could come up with a 'universal' refining method to produce good quality oil that fulfils the requirements of can pass the strict MLs for 3-MCPDE and GE. To go even further, it would be possible to fine tune the exact recipe of required additives, temperature, and

processing duration per individual oil batch. A refinery could perform a quick analysis of the CPO prior to refining and check the levels of certain chemical markers in the CPO. The final recipe can be adjusted based on the concentrations of those chemical markers. This process requires a prediction 'tool' where good understanding of the reaction pathways and the precursors of MCPDEs and GEs is embedded in said tool.

Another motivation to aim for a 'universal' mitigation strategy is to make sure that the refining method not only can be applied to many various vegetable oils, but also on organic oils. In Europe, food products are allowed to be called organic if certain protocols have been followed during the entire cultivation and production process. This applies to vegetable oils too. As an example, certain additives, such as certain types of acids for the degumming process or certain types of bleaching agents, are not allowed to be used during the production of organic vegetable oils. This is another challenge that must be taken into consideration when designing a 'universal' mitigation strategy. On the other hand, more research should be performed to proof whether there are significant differences in terms of the reduction of MCPDE and GE between a conventional refining method compared to a refining method meant to produce organic oils. The mitigation strategies investigated in this thesis should also be tested with other types of vegetable oils in order to assess their universality. Nonetheless, with the ever-growing market share of organic vegetable oils, this side of the industry must not be neglected. The mitigation strategies shown in this thesis, that were developed in the pilot plant, were therefore designed with that thought in mind and can now be applied to produce organic palm oils.

6.6. The complexity of MCPDE precursors

There is a Dutch saying: 'voorkomen is beter dan genezen' which translates into 'prevention is better than cure'. This philosophy is applicable in many situations, but it is exceptionally true in mitigating process contaminants such as MCPDE and GE. Thanks to research that has been done to unravel the formation mechanisms of MCPDE and GE, we now know that the formation of GE is temperature dependent while 2- and 3-MCPDE are chlorine dependent. The mitigation strategies presented in this thesis have carried elements of both prevention and removal of MCPDE and GE. However, from literature and our own observations, it became apparent that once 2- and 3-MCPDE are formed, they are rather difficult to remove from the oil. Ermacora and Hrncirik (2014) and Zhao et al. (2016) showed the thermal stability of 3-MCPD di-esters. According to Ermacora and Hrncirik (2014) 3-MCPDE can degrade under prolonged exposure of high temperature (50% reduction after 8 h at 260 °C). However, such prolonged exposure at high temperature has a negative impact on the quality of the refined oil. Therefore,

strategies aimed at eliminating the precursors of MCPDE were adopted in order to prevent the formation of MCPDE as much as possible. This resulted in a reduction in 2- and 3-MCPDE concentrations, but unfortunately, it seems as if a persistent small amount of 2- and 3-MCPDE are still present. Chapter 5 investigated the use of alternate chlorine sources besides the most obvious ones such as HCl, NaCl, and KCl; In particular, endogenous apolar organochlorine-containing molecules. Endogenous chlorine-containing precursors has been previously reported. According to Nagy et al. (2011) the observed potential chlorine donor has similar structure to phytosphingosines while Tiong et al. (2018) have classified a part of the detected chlorine-containing precursors to be constituents of wax esters, fatty acids, and sphingolipids. Despite their valuable classifications of these chlorine containing precursors, many remained unclassified. In contrast, the large amount of unique potential precursors that has been observed in the CPO during our lab-refining experiments is an indication that the current understanding of chlorine-containing precursors, namely that a handful of molecules has been detected and identified as potential organochlorine containing precursors, has only revealed the tip of the iceberg. It can be argued that those molecules or mol masses that has been reported previously are the ones with the highest concentration in their samples. However, the observed potential chlorine-containing molecules from our lab-refining experiments showed divergent masses that exceed the previously reported categories or classes of chlorine-containing precursors. This implies that the issue with these chlorine-containing precursor is potentially much larger at hand than what was initially assumed. As far as we know, each of the reported potential precursor with a descending-type of kinetic pattern has the potential to be involved in becoming a true chlorine-source which on its turn can partake in the formation of MCPDEs.

As this theory is still in its infancy, there are several limitations involved in this study. To eliminate these organochlorine-containing precursors or stop them in their tracks in becoming MCPDE, it is crucial to know what the molecular structures are of the observed precursors. Otherwise, it would be impossible to design mitigation strategies if the physical-chemical properties of those potential precursors are unknown. Whilst molecule identification often starts with discovering the masses of the unknown molecules, further analysis, whether or not in combination with specific extractions, isolation, and clean-up, is required to fully unravel the molecular structures. Further research on this matter may involve but is not limited to MSn experiments or 13C / 1H Nuclear Magnetic Resonance (NMR). MSn experiments can be set-up in such a way that the target molecule is reduced in size step-by-step by repetitive collision with atoms from the collision gas in for example an ion-trap. When the target molecule is analyzed over-and-over again, hence MSn experiments, after each collision, it is possible to gather enough data to perform calculations to elucidate the original structure formula. NMR experiments has a more challenging approach because NMR analysis requires a rather

clean sample, preferably but technically not limited to, containing one single molecule. This would be possible if fractionation and pre-concentration of the sample can be applied in order to collect multiple fractions of one original sample, each containing one molecule ideally speaking. Another limitation of this study finds it origins in the study design of the experiment. As the lab-refining was designed to simulate the physical refining conditions, based on the previous pilot-plant experiments, it is currently impossible to distinguish observed potential chlorine-containing precursors that are true endogenous from the ones that might be exogenous. One speculative source of an exogenous precursor may be persistent organic pollutants (POP). This can be chlorine-containing pesticides that were used directly or indirectly at the plantation or other POPs like chlorinated paraffins which can be introduced into crude oils during the harvesting or pressing process (Shen et al., 2023). However, at the moment, it is unclear whether POPs can be reactive enough to readily dissociate their bound chlorine atoms under the chemical condition that are present during refining in order for the released chloride ions to act as potential chlorine donor in the formation of 2-MCPDE and 3-MCPDE. In the case of future experiments with non-organic CPOs, where pesticides or worse, chlorine-containing pesticides are allowed to be used, data processing methodologies for the identification of chlorine-containing molecules should be carefully designed to filter out known chlorine-containing contaminants as much as possible from the data. Nevertheless, with the current MLs for 3-MCPDE in mind, the potential of introduction of new MLs in certain food products, or perhaps an even stricter limit of the current MLs in the future, the industry must eventually come up with more advanced mitigation strategies. These organochlorine-containing precursors have the potential to become the next core target for future mitigation strategies. Nevertheless, the initial steps forward should be aimed towards the analytical chemistry, focusing on identification and quantification of these chlorine-containing precursors; Followed by understanding their specific reaction kinetics and interactions to be able to discriminate which ones are the true chlorine-containing precursors. Once the identification of the culprits is clear, mitigation strategies can be developed, perhaps more from the agronomic perspective. Additionally, the knowledge that arise from future research in this specific field may contribute to the development of a prediction tool that can be used to finetune refining recipes and processes to achieve the lowest amount of MCPDE and GE whilst maintaining the quality of the product.

6.7. Societal impact

As mentioned at the beginning of this chapter, the topic palm oil and its usage are shrouded in several controversies that have led to a dichotomy. It is safe to say that the stories of illegal deforestation to cultivate palm oil trees are globally well-known as one

of the major arguments why palm oil usage should be brought down. Exact numbers are relatively hard to come by, but public debate related to palm oil cultivation and its usage has been quite fierce in numerous countries. In my opinion, the discussions in The Netherlands have not yet reached the same polarizing powers as the ongoing debates between vegetarian or vegan food consumption versus dietary meat consumption or the COVID-19 vaccination program, but it has the potential to grow into one. So, the moral question that I often encountered during my research from outsiders was: why would anyone commit 4-5 years of their lives performing research on a topic which is shrouded in many controversies? The short and unsatisfying answer would be that I started working on this topic while I was doing my internship for my analytical chemistry master's degree where I have helped with the initialization and implementation of the analytical method to quantify 2-, 3-MCPDE, and GE. The longer, philosophical, and more nuanced answer would start by observing the current situation of our food consumption and the food industry. According to the United Nations (UN) the global human population has reached 8.0 billion in November 2022 (United Nations, 2022). Many people unfortunately still rely on basic resources such as unprocessed corn, wheat, and flour as their primary food source, but I think that we are allowed to praise ourselves lucky, in the most modest and humbled way, to live in a world where modern food industry is very efficient in producing large quantities of food to keep up with the ever-growing demand.

In Europe, it is quite common to use sunflower oil, peanut oil, or olive oil in our day-to-day life for our meal preparations. The latter one is especially expensive and could even be classified as luxury goods. However, in other countries in continental Africa, Asia, and South-East Asia, palm oil can be found more readily in people's homes. In those countries, people tend to use palm oil in similar ways Europeans or North Americans would use sunflower oil or peanut oil. One reason, based on an educated guess, would be the fact that palm oil is much more economically affordable, and it has been used for decades in those countries from a food-heritage point of view. Furthermore, per square hectare of farmland, the palm oil tree is capable of producing the highest amount of vegetable oil (Nomanbhay et al., 2017). Returning to the exorbitant large number of people who need to be fed, the food industry requires large amounts of ingredients to keep up with the global demand. Vegetable oil is one of the key ingredients in many types of food. Therefore, it is a relatively straightforward conclusion that large quantities of vegetable oils are required by the food industry. An immediate follow-up question would usually be: "But can't the industry not just switch to a different type of vegetable oil?" Hypothetically speaking, yes, the food industry can stop using palm oil and switch to a different type of vegetable oil, for instance high oleic sunflower oil is now largely used in bakery products and oleogels made from other types of vegetable oils are being developed (Ansorena et al., 2022; Giacomozzi et al., 2018; Schubert et al.,

2022; M. Zhao et al., 2020). The reality, however, is that it would be quite a challenge to produce sunflower, peanut, and/or rapeseed oil in the same quantities as what currently is possible with palm oil. A sudden transition from palm oil into another type of vegetable is not without risks. Imagine that all palm oil trees are successfully switched for sunflower or rapeseed crops. Due to the lower oil production capacities, the same amount of farmland is now not enough anymore. This in its turn could lead to the same problems that the palm oil farmland faced in the early days: deforestation, whether legal or not. Furthermore, palm oil or palm fat has certain physical-chemical properties that often are ideal in food processing. Transitioning into a different type of vegetable oil is not impossible, but it takes a lot of effort from the food manufacturers. Imagine your favorite cupcake recipe that usually requires some butter and suddenly you have to switch it with a different type of oil. The recipe must be altered accordingly to achieve similar cupcake quality. Not only will such a transition require time, but also a lot of money, which eventually will be charged to the customers.

The EFSA and the EC work together and monitor food safety in Europe. 3-MCPDE and GE have been marked as carcinogens and therefore MLs has been set based on risk assessments. As mentioned here above, transitioning away from palm oil will take a lot of time and in the meantime, palm oil is still going to be the most used and consumed vegetable oil. From the food safety perspective, it is not ethical to do nothing just because of all the controversies around palm oil. while it is still being consumed. This research has directly contributed to making palm oil safer, and potentially other types of vegetable oils as well, by developing new mitigation strategies to produce palm oil, specifically, organic palm oil. Finally, this research and the collaboration with our partners from the industry have shown that the academia with their scientists are able to work together with the industry, instead of against each other. New risk assessments and potential MLs that might be derived from it will always be an obstacle to the food industry, but independent risk assessments must be performed for the sake of food safety, human health, and the scientific integrity of those independent researchers. Everyone would be better off if the two parties would work together, instead of pointing their fingers to blame the culprit in search of a scapegoat. In the end, there are still many known and unknown knowledge gaps and pitfalls, such as the circular bioeconomy of the palm oil industry or its social sustainability, that should be tackled by everyone who is part of this sector and definitely not limited to the industry, government, and academia (Cheah et al., 2023; Ngan et al., 2022).

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Chapter 7

Summary

Summary

Palm oil belongs to the most used and produced vegetable oils globally. Due to its physio-chemical characteristics, it has been used in a wide variety of food applications. Furthermore, palm oil crops produce the most oil in comparison to other vegetable oils such as olive oil or sunflower oil. In the past decade, it became apparent that the process contaminants 2-MCPDE, 3-MCPDE, and GE are present in refined vegetable fats and oils, but most predominantly in refined palm oil. These process contaminants are formed during refining. GE appears to be temperature dependent, while 2-MCPDE and 3-MCPDE are limited by the amount of chlorine that is present in the oil. The European Commission has implemented regulations which put a limit on the maximum concentration of 3-MCPDE and GE. As there was not enough toxicological data, no conclusion could be made during risk assessment by the EFSA regarding the toxicity of 2-MCPDE in humans. This thesis focuses on the development of mitigation strategies in organic palm oil to reduce the concentration of 2-MCPDE, 3-MCPDE, and GE in the refined product. Furthermore, this thesis expands the current understanding about precursors of 2-MCPDE and 3-MCPDE. **Chapter 2** exhibits mitigation strategies for limiting the formation of 2-MCPDE, 3-MCPDE, and GE during oil refining that have been published in peer-reviewed scientific literature and identifies knowledge gaps. These were: the limited reducing effects of available mitigation strategies, the lack of mitigation strategies which included 2-MCPDE (in addition to MCPDE and GE), and the niche and limited application condition of the published strategies. **Chapter 3 and 4** present the results of the mitigation strategies based on either physical refining or chemical refining, respectively. Post-refining processing or "double-refining" resulted into the lowest 2-MCPDE, 3-MCPDE, and GE concentration when the physical refining approach is preferred. From a chemical refining point of view, the "full" chemical refining, which includes alkali neutralization and post-neutralization water washing, turned out to result into the greatest overall reduction of 2-MCPDE, 3-MCPDE, and GE. With the development of these effective mitigation strategies, the next knowledge gap that needs to be investigated are the precursors for the formation of 2-MCPDE, 3-MCPDE, and GE. **Chapter 5** explores the impact of potential chlorine donors, which is the key element for the formation of 2-MCPDE and 3-MCPDE. Those chlorine-containing molecules are endogenous in crude palm oil and were marked as potential precursors based on their kinetic behaviour during oil refining. These precursors share a similar trait in the way that they all have a covalently bonded chlorine atom. Furthermore, these potential precursors are identified to have a wide variety of masses. This discovery might play a key role in future developments of more effective mitigation strategies that transcends the oil refining part itself and ventures off towards mitigation strategies in the agronomics sector. Overall, this thesis presents useful insights into effective mitigation strategy development to mitigate the formation of the process contaminants 2-MCPDE, 3-MCPDE, and GE in organic palm oil.

Addendum

Acknowledgements
About the author
List of Publications
Overview of completed training activities

Acknowledgements

The last 5.5 years have been a true rollercoaster ride and a very challenging chapter of my life. It was a period of my life where I met my true self and where I have made the largest growth about me as a person. It was a period where I met so many great, admirable, and talented colleagues and researchers. It was a period full of excitement when our papers got accepted, when I got to use state-of-the-art high-resolution LC-MS systems, and when the experiments in the lab finally went as planned. However, as with everything in life, the forces must be kept in balance. Both of my feet were put straight back on the ground upon reading a rejection e-mail from editors, because of failed experiments, and we have not even started talking about the lockdowns due to COVID-19. Yes, those experiences can hurt, but you can either run from it or learn from it. The latter one has brought me to where I am right now.

A PhD does not come out of the blue. The last 5.5 years and all our achievements would not have been made possible without the support and guidance from **Stefan**, **Ine**, and **Vincenzo**. With the countless meetings and discussion sessions, the three of you have shared your knowledge with me and guided me through all the ups and downs. You have shown me your visions on what it takes and how to be an independent researcher. I am very much aware that you have fulfilled a quite challenging task with me as your PhD candidate; Pumping out papers is just not my cup of tea, which made it even more appreciable. I am truly thankful for the opportunities that you have given me, for all the necessary motivational pushes, and for your approachable attitude. I could not have wished for a better team of supervisors. Thank you!

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About the author

Sergio Bernardi Oey, born on the 11th of June 1990 in Jakarta, Indonesia. Since his 10th age, he grew up in Delft, The Netherlands. He did his bachelor's degree in Bio-Pharmaceutical Sciences at the Leiden University. As he was fond of the field of analytical chemistry, he continued his education in Amsterdam where he achieved his MSc degree in chemistry with specialization in analytical sciences at the University of Amsterdam. His master's internship focuses on the development and implementation of an analytical method to simultaneously quantify 2-MCPD esters, 3-MCPD esters, and glycidyl esters in vegetable oils and fats on a GC-triple quad MS. Thanks to this internship he became familiar with the application of analytical chemistry in the food, feed, and food safety sector. It was not long before he received the opportunity to pursue a PhD in vegetable oil processing in 2019 at the Wageningen University, more specific at RIKILT, which is now known as Wageningen Food Safety Research. During his PhD research, he developed mitigation strategies to reduce the process contaminants 2-MCPD esters, 3-MCPD esters, and glycidyl esters in organic palm oil. The results obtained between 2019 – 2022 are presented in this thesis. Sergio's thirst and desire to continuously explore new topics that will expand his horizon shall guide him like a compass in his future career.

List of publications

Oey, S. B., van der Fels-Klerx, H. J., Fogliano, V., & van Leeuwen, S. P. J. (2022). Chemical refining methods effectively mitigate 2-MCPD esters, 3-MCPD esters, and glycidyl esters formation in refined vegetable oils. *Food Research International, 156*. https://doi.org/10.1016/j.foodres.2022.111137

Oey, S. B., van der Fels-Klerx, H. J., Fogliano, V., & van Leeuwen, S. P. J. (2020). Effective physical refining for the mitigation of processing contaminants in palm oil at pilot scale. *Food Research International, 138*(109748), 1–7. https://doi.org/10.1016/j.foodres.2020.109748

Oey, S. B., van der Fels-Klerx, H. J., Fogliano, V., & van Leeuwen, S. P. J. (2019). Mitigation Strategies for the Reduction of 2- and 3-MCPD Esters and Glycidyl Esters in the Vegetable Oil Processing Industry. *Comprehensive Reviews in Food Science and Food Safety, 18*(2), 349–361. https://doi.org/10.1111/1541-4337.12415

Oey, S. B., van der Fels-Klerx, H. J., Fogliano, V., & van Leeuwen, S. P. J. The dynamics of organochlorines in palm oil suggest a plethora of potential precursors of 2- and 3-MCPD esters. *Manuscript in preparation*

Publications not included in this thesis

Beekmann, K., Sloot, S. J., **Oey, S. B.**, & van Leeuwen, S. P. J. (2022). MCPD esters and glycidyl esters in food supplements of fish oils, algae oils, and krill oils. *Food Control, 136*. https://doi.org/10.1016/j.foodcont.2022.108865

Yan, J., **Oey, S. B.**, van Leeuwen, S. P. J., & van Ruth, S. M. (2018). Discrimination of processing grades of olive oil and other vegetable oils by monochloropropanediol esters and glycidyl esters. *Food Chemistry, 248*, 93–100. https://doi.org/10.1016/j.foodchem.2017.12.025

Overview of completed training activities

Discipline specific activities	Organizer	Place	Year
Reaction Kinetics in Food Science 10th edition	VLAG	Wageningen	2019
Advanced Food Analysis ‡	VLAG	Wageningen	2019
Symposium on MCPD Esters and Glycidyl Esters: Analytics, Toxicology, Risk Assessment, Mitigation - Where we are today?	Deutsche Gesellschaft für Fettwissenschaft e.V. (DGF)	Berlin	2017
MVO Mini-symposium on 3-MCPD esters *	MVO - de ketenorganisatie voor oliën en vetten	Amsterdam	2018
MCPD esters and Glycidyl esters - Symposium 2019: New developments in Toxicology, Legislation, Analytics and Mitigation ‡	Deutsche Gesellschaft für Fettwissenschaft e.V. (DGF)	Berlin	2019
CHRONECT Competence Day-MOSH/MOAH and MCPD *	Axel Semrau GmbH & Co. KG	Wageningen	2019
Smart Tech for Food Conference	Food Smart Phone EU – The Netherlands; RAFA associated event	Online	2020
AOCS Annual Meeting 2021 (finalist poster presentation processing division) ‡	AOCS – USA	Online	2021

General courses	Organizer	Place	Year
VLAG PhD week, 39th ed	VLAG	Baarlo	2017
Interpersonal Communication for PhD students	WGS	Wageningen	2017
Competence Assessment	WGS/Maas	Wageningen	2017
Project and time management	WGS	Wageningen	2019
Scientific Writing	WGS	Wageningen	2020
Career orientation	WGS/hertz	Wageningen	2021
Adobe InDesign – from Dissertation Layout to Poster Design	WUR Library	Wageningen	2021

Other activities	Organizer	Place	Year
Preparation of research proposal	VLAG	Wageningen	2017
Business Unit meetings at RIKILT, lunch poster presentations, Presentations given within the team of organic contaminants, meeting with external project partners	RIKILT/WFSR	Various	2017-2021
FQD PhD meetings/ FQD lunch lectures / colloquia	FQD	Wageningen	2017-2021

Reviewing scientific articles:	Supervisor	Year
Optimization and validation of in-situ derivatization and headspace solid-phase microextraction for gas chromatography-mass spectrometry analysis of 3-MCPD, 2-MCPD and glycidyl esters in edible oils via central composite design; Ms.Ref.No.: FOODCHEM-D-19-03516	dr.ing. S.P.J. van Leeuwen	2019
Effects of neutralization combined with steam distillation on the formation of monochloropropanediol esters and glycidyl esters in palm oil under laboratory-scale conditions; Ms.Ref. No.: LWT-D-20-05099	dr.ing. S.P.J. van Leeuwen	2020

* represents oral presentation

‡ represents poster presentation

Colophon

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