Critical factors of indirect determination of 3-chloropropane-1,2-diol esters

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Several indirect methods have been developed for the determination of 3-chloropropane-1,2-diol (3-MCPD) esters in fat matrices, but the variation between these methods leads to an uncertainty about the comparison of the results. All the indirect methods are based on the conversion of 3-MCPD esters into free 3-MCPD upon transesterification (either in acid or alkaline solution), its purification, derivatisation and instrumental analysis. In this study, major critical factors of the methodology, particularly transesterification, salting out and the choice of internal standard, were evaluated.

3-MCPD showed low stability under the conditions of alkaline transesterification (only around 40% recovery) affecting the sensitivity of the method. Furthermore, the results obtained within the first 1–2 min were 10–20% higher than those obtained at longer (5–10 min) transesterification time. A comparison of results obtained by using two different internal standards (free or esterified form of deuterated 3-MCPD) revealed a slight overestimation (7–15%) when free deuterated 3-MCPD was used, which may be possibly explained by the different behaviour of the both forms during alkaline transesterification. The use of different salts and pH values of the reaction medium during salting out step had a major impact on the results due to the formation of additional 3-MCPD. Other compounds, like glycidyl esters, present in oil samples may be converted to 3-MCPD during this step, however, the conversion was not complete and varied substantially (12–93%) in dependence on the pH value of the medium. The method based on acid transesterification showed better selectivity and robustness.

Keywords: Analysis / Chloropropanediols / Edible oils / 3-MCPD ester / Transesterification

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1 Introduction

3-Chloropropane-1,2-diol (3-MCPD) esters represent a class of food-borne contaminants that are mainly formed during high-temperature processing of fat-based matrices. Traces of 3-MCPD esters were first detected by Velíšek et al. in 1980 [1] in acid-hydrolysed vegetable proteins together with other chloropropanols, but they were not of general concern till 2004, when their occurrence was reported in processed food [2].

Resulting from the review of toxicology, mutagenicity and carcinogenicity data in short- and long-term toxicity studies by the Joint FAO/WHO Expert Committee on Food Additives, a provisional maximum tolerable daily intake of 2 µg 3-MCPD/kg body weight was assigned [3]. The level of free 3-MCPD in soy sauce and hydrolysed vegetable protein has been regulated by setting up a limit in these products [4], assuming these are the main contributors to the dietary intake. In vitro studies have demonstrated that 3-MCPD mono- and diesters of fatty acids are hydrolysed by the intestinal lipase to release 3-MCPD [5]. Due to the lack of in vivo data the risk assessment of 3-MCPD esters is currently carried out under the assumption that 3-MCPD is completely cleaved from its esters.

Despite the development of a few analytical methods for the determination of free 3-MCPD in various matrices, no standard method has been established. The authorities only defined performance criteria that a method must fulfil in order to provide officially acceptable results [6].
Within last few years several approaches have been reported for the analysis of 3-MCPD esters (also referred as ‘bound 3-MCPD’). They are mostly based on indirect methods that involve the release of 3-MCPD from its esters by transesterification in acid [7] or alkaline medium [8], or enzymatically [9], followed by the purification, derivatisation and quantification of the free form. Modifications of each individual step by different laboratories lead to a number of in-house methods and, consequently, to a wide variation in results (Karasek, T. Wenzl, F. Ulberth: Proficiency test on the determination of 3-MCPD esters in edible oil. JRC Scientific and Technical Reports, 2010, EUR 24356 EN. http://irmm.jrc.ec.europa.eu/html/interlaboratory_comparisons/3_MCPD/index.htm).

There is an urgent need for a reliable validated method, which would fulfil the basic requirements, namely sensitivity, specificity, trueness and robustness.

The aim of this study was to identify and evaluate the critical factors affecting the indirect determination of 3-MCPD esters, and particularly to compare the two basic analytical approaches (i.e. alkaline and acid transesterification) with respect to their impact on the results.

2 Materials and methods

2.1 Reagents and chemicals

1,2-Dipalmitoyl-3-chloropropane (PP-3-MCPD, purity 99.8%) and deuterated 1,2-dipalmitoyl-3-chloropropane (PP-3-MCPD-d5, purity 99.0%) were synthesised according to Kraft et al. [10] and purified on silica gel column. 3-MCPD (purity ≥ 98%), sodium sulphate (purity ≥ 99.0%), ammonium sulphate (purity ≥ 99.5%) and sodium chloride (purity ≥ 99.5%) were purchased from Merck (Darmstadt, Germany), deuterated 3-chloropropane-1,2-diol (3-MCPD-d5, purity ≥ 98%) from CDN Isotopes (Pointe-Claire, QC, Canada), glycidol (purity 96%) from Sigma–Aldrich (Belleville, PA, USA), phenylboronic acid (PBA, purity ≥ 97%), sodium methoxide (saturated, aqueous) and sulphuric acid (25% v/v). Besides ammonium sulphate, sodium hydroxide was also tested as salting out agent. FAME were extracted from the sample by repeated liquid–liquid extraction with isohexane (2 × 1 mL). Free 3-MCPD was extracted by subsequent liquid–liquid extraction with ethylacetate (2 × 0.6 mL) and derivatised by 100 μL of PBA solution (saturated, in diethylether); the reaction was facilitated by sonication for 2–3 min. The sample was evaporated to dryness under a nitrogen stream and the residue was dissolved in 200–500 μL of acetone prior to GC-MS analysis.

2.2 Samples

Physically refined palm oil samples (containing significant levels of 3-MCPD esters), as well as crude (degummed) rapeseed oil (3-MCPD esters not detected) were obtained from local suppliers. Crude rapeseed oil was spiked with PP-3-MCPD (4.98 mg/kg, expressed as bound 3-MCPD) or with glycidol (0.74–6.00 mg/kg).

2.3 Methods

Indirect analysis of 3-MCPD esters in oils was performed using two different procedures. Previously developed methods [11, 12] were adopted and modified as described in Sections 2.3.1 and 2.3.2, respectively.

2.3.1 Method A (involving alkaline transesterification)

One hundred milligram (±5 mg) of oil was weighed in a glass tube and dissolved in 500 μL of tert-butylmethylether. Twenty microlitre of internal standard solution (3-MCPD-d5 or PP-3-MCPD-d5) was added. The cleavage of 3-MCPD esters was achieved by addition of 200 μL of sodium methoxide in methanol (0.5 mol/L). The mixture was allowed to stand at room temperature for 0.5–10 min. The reaction was stopped by addition of 0.6 mL of acidic ammonium sulphate solution (37.7% w/v ammonium sulphate aqueous/25% sulphuric acid, 50:3 v/v). Besides ammonium sulphate, sodium chloride was also tested as salting out agent. FAME were extracted from the sample by repeated liquid–liquid extraction with isohexane (2 × 1 mL). Free 3-MCPD was extracted by subsequent liquid–liquid extraction with ethylacetate (2 × 0.6 mL) and derivatised by 100 μL of PBA solution (saturated, in diethylether); the reaction was facilitated by sonication for 2–3 min. The sample was evaporated to dryness under a nitrogen stream and the residue was dissolved in 200–500 μL of acetone prior to GC-MS analysis.

2.3.2 Method B (involving acid transesterification)

One hundred milligram (±5 mg) of oil was weighed in a glass tube and dissolved in 1 mL of tetrahydrofuran containing the internal standard (3-MCPD-d5 or PP-3-MCPD-d5). 1.8 mL of sulphuric acid solution in methanol (1.8% v/v) was added to the sample and the mixture was incubated at 40°C for 16 h. The reaction was stopped by addition of 0.5 mL sodium hydrogencarbonate solution (saturated, aqueous) and the organic solvents were evaporated under a nitrogen stream. FAME were separated from the sample by addition of 2 mL of aqueous salt solution (20% w/v; sodium sulphate was used in the standard procedure; sodium chloride was tested as an alternative) followed by liquid–liquid extraction with hexane (2 × 2 mL). Two hundred and fifty microlitre of PBA solution (25% w/v, acetonewater, 19:1 v/v) was added to the reaction mixture, which was incubated at 80°C for 20 min. The 3-MCPD-PBA derivative was extracted by 1 mL hexane and analysed by GC-MS.

2.3.3 GC-MS analysis

GC-MS analysis of 3-MCPD derivatised by PBA was carried out on an Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a quadrupole mass selective detector Agilent 5975C MSD (70 eV) and data processing system MSD Productivity ChemStation. GC was performed on a bonded, polydimethylsiloxane) capillary column (Supelco Equity-1,
30 m length × 0.25 mm id × 1.0 μm film thickness). The injector temperature was set to 250 °C. The GC oven temperature was initially kept at 80 °C for 1 min and then raised to 170 °C at 10 °C/min gradient, then to 200 °C at 3 °C/min, then to 280 °C at 15 °C/min and held for 15 min at the final temperature. Helium (flow rate 0.8 mL/min) was used as carrier gas. One microlitre of sample was injected (pulsed splitless mode). For quantification purposes, ions at m/z 147 (3-MCPD) and m/z 150 (3-MCPD-d5) were chosen for single ion monitoring. Ions at m/z 196, 198 (3-MCPD) and m/z 201, 203 (3-MCPD-d5) were used as qualifiers.

3 Results and discussion

The basic principle of indirect determination of bound 3-MCPD is the conversion of a number of individual 3-MCPD esters of fatty acids into a single compound, 3-MCPD, that is quantified. Currently a large number of analytical methods used by different laboratories exist. The analytical protocol of all these methods comprises a uniform sequence of steps: addition of an internal standard to the sample (either free or esterified form of 3-MCPD-d5), transesterification (performed either in acid or alkaline medium at different reaction time), followed by neutralisation of the reaction mixture and salting out (using different neutralising reagents and salts), derivatisation of the extracted 3-MCPD (mostly by PBA or heptfluorobutyrylimidazol) and GC-MS analysis. There are variations within each individual analytical step that may have a significant impact on the specificity, repeatability, reproducibility, trueness and other parameters of the method. A recent proficiency test demonstrated an impact of these variations on the results (Karasek, T. Wenzl, F. Ulberth: Proficiency test on the determination of 3-MCPD esters in edible oil. \textit{JRC Scientific and Technical Reports}, 2010, EUR 24356 EN. http://irmm.jrc.ec.europa.eu/html/interlaboratory_comparisons/3_MCPD/index.htm). Using the in-house methods for the analysis of ‘naturally contaminated’ palm oil, only 19 out of 34 participating laboratories (which included commercial, industrial and official control laboratories) provided results which were considered satisfactory. There is a need for closer investigation of the individual steps and their optimisation in order to achieve overall harmonisation of the analytical methodology.

3.1 Decomposition of 3-MCPD during transesterification

The first step of the analytical protocol in the indirect 3-MCPD determination is the transesterification in the presence of methanol (methanolation), which results in the conversion of TAGs and partial acylglycerols into FAME and glycerol. Simultaneously, 3-MCPD esters are converted to free 3-MCPD. The transesterification step has been proposed both in acid [7] and alkaline [8] media. It is has been shown that 3-MCPD is unstable in alkaline solutions [13, 14] giving rise to glycerol (Fig. 1).

This work was therefore focused on studying the stability of 3-MCPD esters under the conditions of alkaline transesterification employing method A (see Section 2.3.1 for details). The addition of the internal standard (3-MCPD-d5) after transesterification of the sample enabled us to quantify the recovery of 3-MCPD released from its native esters. An initial experiment performed in a model system indicated that 73% of 3-MCPD (released from a standard solution of PP-3-MCPD) was decomposed within 10 min of transesterification. Next, the recovery was evaluated by using two oil samples – crude rapeseed oil spiked by PP-3-MCPD and refined palm oil. The samples were analysed after 1, 10 and 30 min of transesterification (Table 1).

![Figure 1. Mechanism of 3-MCPD esters transformation under conditions of alkaline transesterification](http://example.com/figure1.png)
The results showed a considerable decrease of 3-MCPD within the first 10 min of alkaline transesterification. The recoveries were slightly higher, though comparable, to those found in the model system. After 30 min of alkaline transesterification 3-MCPD was not detected. The small difference (12%) in recovery levels between both samples found after 1 min of transesterification could be due to the fact that the refined palm oil sample contains a mixture of 3-MCPD fatty acid esters, presumably both mono- and diesters in a typical ratio (15:85) as reported by Seefelder et al. \[5\], whereas the spiked rapeseed oil sample contains only PP-3-MCPD. It is reasonable to expect that the conversion of 3-MCPD monoesters into 3-MCPD proceeds more rapidly than the one of diesters and that 1 min reaction time may not be sufficient to complete the transesterification, which possibly results in the higher recovery of 3-MCPD in the sample containing monoesters in the initial phase of the reaction. The analysis of both samples was also carried out using the acid transesterification (method B, Section 2.3.2). In this case the degradation of 3-MCPD was not observed.

Although an advantage of the alkaline transesterification is its short duration, it appears that the transesterification time has a major impact on the sensitivity of the method due to the degradation of 3-MCPD in alkaline medium. It can be expected that the reaction times prescribed by the methods developed, i.e. 5–10 min \[15\] and 10 min \[11\], affect the sensitivity of these methods significantly (based on the data obtained, the sensitivity may be reduced approximately two-times within this range). Thus there may be scope to optimise the method to achieve maximum recovery by minimising the transesterification time.

### 3.2 Effect of transesterification time and internal standard

Assuming that the decomposition of 3-MCPD (released from its native esters) and the deuterated analogue (used as internal standard) occurs at the same rate during alkaline transesterification, the transesterification time should not have an impact on the trueness of the analytical method.

In order to check the validity of this assumption, a sample of refined palm oil was repeatedly analysed according to the method A using variable time of transesterification (from 0.5 to 10 min). PP-3-MCPD-d$_5$, added at the beginning of the transesterification (as routinely performed), was used as internal standard in order to minimise the difference between the analyte (in refined oils 3-MCPD occurs mainly in the form of diesters) and the internal standard.

The 3-MCPD levels measured within the first 1–2 min were repeatedly 10–20% higher than those obtained at longer (5–10 min) transesterification times (see Fig. 2 as an example). This surprising finding suggests different behaviour of the analyte and the internal standard at the beginning of the reaction. This may be due to different conversion rates of the individual 3-MCPD esters (e.g. monoesters being converted faster as proposed in Section 3.1). Nevertheless, at transesterification times longer than 3–5 min the measured level were constant.

### Table 1. Recovery of 3-MCPD found at different periods of alkaline transesterification

<table>
<thead>
<tr>
<th>Method$^a$)</th>
<th>Alkaline transesterification (method A)</th>
<th>Acid transesterification (method B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min</td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>3-MCPD (mg/kg)</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>Spiked rapeseed oil$^b$)</td>
<td>4.13 ± 0.10</td>
<td>82.9</td>
</tr>
<tr>
<td>Refined palm oil$^d$)</td>
<td>3.99 ± 0.32</td>
<td>95.0</td>
</tr>
<tr>
<td>Spiked rapeseed oil$^b$)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$) For the details of the methods see sections 2.3.1 and 2.3.2.

$^b$) Sample spiked at the level of 4.98 mg/kg bound 3-MCPD.

$^c$) Limit of detection: 0.08 mg/kg.

$^d$) Recovery calculated from the mean value of the repeated analysis ($N > 10$) by method B.

Figure 2. Effect of transesterification time (0.5–10 min) on the content of 3-MCPD esters in refined palm oil analysed by alkaline transesterification-based method (method A)
If it is the case that individual 3-MCPD species (free 3-MCPD, mono- and diesters of fatty acid) behave differently, it may be expected that the choice of the internal standard will affect the results. Different laboratories use deuterated forms of either free or esterified 3-MCPD (with PP-3-MCPD-d5 being probably the most common). Free 3-MCPD-d5 is used as an internal standard in validated methods [11, 15] most likely because it was the only suitable compound commercially available at the time of the validation work.

Both internal standards (3-MCPD-d5 and PP-3-MCPD-d5) were tested in both methods (A and B) performed under standard conditions (transesterification 10 min and 16 h, respectively). The results showed that the application of 3-MCPD-d5 led to higher results (7–15%) of 3-MCPD esters measured by method A. This suggests, again, different behaviour (stability and/or transesterification rate) of free and esterified form of 3-MCPD during alkaline transesterification. Taking into account the difference found and the fact that in oils and fats 3-MCPD is predominantly present in its bound forms (mainly as fatty acid diesters) we incline towards using PP-3-MCPD-d5 rather than the free form, which occurs only in trace amounts in fat-based matrices.

3.3 Effect of salting out

Another critical step that requires particular attention is salting out. This step follows the transesterification (performed either in alkaline or acid media) and facilitates the extraction of FAME (produced during the transesterification) from the reaction mixture. The most common salting out agents used in the analysis of 3-MCPD are sodium chloride [7, 8] or sulphate salts [11]. These are added to the reaction mixture either after or during the neutralisation.

In this study two salts, sodium chloride and ammonium sulphate, were alternately used as salting out agents in both methods, which were carried out under standard conditions. The choice of these salts was determined by the concern that the introduction of a source of chloride (as NaCl) in the transesterified mixture could lead to the formation of 3-MCPD ex novo (for more details see Section 3.4). The 3-MCPD levels found in spiked rapeseed oil and refined palm oil samples are given in Table 2.

The results obtained by the method A show dependence on the type of salt, whereas no significant differences were found when the method B was used. This is particularly obvious in the case of refined palm oil. Such an outcome suggests that the reaction mixture of the alkaline transesterification contains compounds that react with chloride ions to give rise to additional 3-MCPD, which leads to the overestimation of the results.

In order to understand the reactivity of chloride ion with 3-MCPD precursors during sample preparation, the pH value of the reaction mixture was checked after transesterification and neutralisation. Contrary to expectations, the methods employing alkaline transesterification had considerable variation in the pH value of neutralised mixture, 1.5–2 (method A) and 6–7 [15], while the pH for the acid transesterification-based method (method B) was around 7. To verify whether the additional formation of 3-MCPD from its precursors (a plausible explanation of the result variation reported in Table 2) is affected by different conditions of alkaline transesterification/neutralisation, a sample of refined palm oil was subjected to the analysis by method A, which was modified in such a way that the pH value in the salting out step was adjusted within the range 1.5–8.5. Both salting out agents – sodium chloride or ammonium sulphate – were used and the levels found were plotted against the pH value (Fig. 3).

In accordance with the previous results the data indicate the yield of 3-MCPD is higher (in comparison to ammonium sulphate) by approximately 20% at pH above 3 when sodium chloride was used for salting out. Moreover, the conversion of its precursors to 3-MCPD seems to be pH-dependent: the level of 3-MCPD found in the palm oil sample almost doubled when the pH value of the reaction mixture was below 3, which indicates considerably higher conversion rate of glycidol into 3-MCPD in the acid environment.

3.4 Method specificity

In 2008, the specificity of the newly developed method based on alkaline transesterification and salting out by sodium chloride [8] was questioned by demonstrating that the content of 3-MCPD esters is overestimated in some samples

| Table 2. Effect of salting out on the content of 3-MCPD esters in refined palm oil |
|---------------------------------|-----------------|-----------------|
| **Method** | **Method A** | **Method B** |
| Salting out agent | 3-MCPD (mg/kg) | 3-MCPD (mg/kg) | 3-MCPD (mg/kg) | 3-MCPD (mg/kg) |
| Sodium chloride | | | | |
| Spiked rapeseed oil | 5.14 ± 0.26 | 4.46 ± 0.06 | 4.93 ± 0.05 | 4.65 ± 0.03 |
| Refined palm oil | 7.03 ± 0.85 | 3.68 ± 0.02 | 3.92 ± 0.02 | 3.89 ± 0.01 |

a) For the details of the methods see Sections 2.3.1 and 2.3.2.
b) Sample spiked at the level of 4.98 mg/kg bound 3-MCPD.
due to the additional 3-MCPD formed de novo during the sample preparation [16]. It was suggested [16], and later confirmed [17], that the compound leading to overestimated levels is glycidol (2,3-epoxy-1-propanol), which is present in refined oils as esters of fatty acids. This finding was later utilised in the development of a methodology for the indirect estimation of the level of glycidyl esters in oils. Its principle is the calculation of the difference between two independent determinations: one (X) that is based on the quantification of the sum of 3-MCPD and glycidyl esters (after their conversion to 3-MCPD) and the other one (Y) that is specific to 3-MCPD esters. Such an approach will provide meaningful results only if the former determination (X) ensures a complete conversion of glycidol into 3-MCPD, whilst in the specific method (Y) any such glycidol conversion during the sample preparation is avoided. The latter can be achieved either by replacing sodium chloride by other salt during salting out or by the deliberate decomposition of glycidyl esters prior to transesterification [15].

The conversion of glycidol under the conditions of salting out was studied in rapeseed oil spiked with glycidol at three different levels. Following the transesterification according to the method A, the neutralisation was performed in such a manner that the final pH value of the mixture was either 2 or 7. Sodium chloride was used for salting out. From the obtained analytical results, the conversion of glycidol into 3-MCPD was calculated (Table 3).

The glycidol conversion in the neutral solution was limited, but was considerably higher at pH 2. This could provide an explanation of the results obtained by the analysis of refined palm oil (which supposedly contains significant level of glycidyl esters) at different pH (Fig. 3). The results also suggest a concentration dependence of the conversion: at low glycidol concentration higher conversion rate was found.

In summary, the conditions of the salting out step (particularly pH value) seem to have a significant impact on the conversion of glycidol. The pH value of the ‘neutralised’ mixture varies between different methods substantially, and although a complete conversion of glycidol is assumed, it is very probable that this will not be always the case and that the results will depend on the method used. Until recently, the indirect differential determination has been the only option to quantify the content of glycidyl esters in oils. It was applied in several works studying the oil refining process, e.g. [17, 18]. In view of the current findings, the results obtained by indirect differential quantification of glycidyl esters should be interpreted carefully and taken as estimation rather than exact levels. It would be desirable to compare the differential approach for glycidyl ester determination with the direct method that has been developed recently [19].

4 Conclusions

Indirect methods for the determination of 3-MCPD esters require a sequence of steps, in which the native esters are transformed into the 3-MCPD derivative. In this study, we focused on the two initial steps, transesterification and salting out. It was found that the time and other factors (e.g. pH value) of these steps may have a considerable impact on the recovery, specificity and trueness of the method used. The method based on alkaline transesterification is particularly prone to condition variation, whilst transesterification in acid medium avoids the problem with selectivity because of the irreversible degradation of glycidyl esters that cannot be

![Figure 3. Effect of pH value and different salts on the content of 3-MCPD esters in refined palm oil analysed by alkaline transesterification-based method (method A); ‘◊’ symbol represents sodium chloride, ‘−’ symbol represents ammonium sulphate](image)

Table 3. Effect of pH value on the conversion of glycidol to 3-MCPD in spiked crude rapeseed oil measured by alkaline transesterification-based method

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glycidol level, spiked (mg/kg)</th>
<th>3-MCPD level (mg/kg)/method A&lt;sup&gt;a)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 2</td>
</tr>
<tr>
<td>Spiked rapeseed oil</td>
<td>0.74</td>
<td>1.03 ± 0.07</td>
</tr>
<tr>
<td>Spiked rapeseed oil</td>
<td>3.26</td>
<td>3.26 ± 0.00</td>
</tr>
<tr>
<td>Spiked rapeseed oil</td>
<td>6.00</td>
<td>5.90 ± 0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Transesterification time 10 min.; for further details of the methods see Section 2.3.1.

<sup>b</sup> The factor used for the conversion of glycidol conc. to 3-MCPD conc. was 1.49.
subsequently converted to 3-MCPD (during salting out) and hence interfere with the analysis. Due to the greater robustness shown, the acid transesterification approach seems to be more advantageous that the one based on alkaline transesterification, although this does not necessarily imply a poor repeatability and trueness of the latter one, provided the analysis is carried out strictly under the correct analytical protocol. It is recommendable to use PP-3-MCPD-d5 (rather than 3-MCPD-d5), as internal standard as it follows the same transesterification/degradation pattern of native 3-MCPD diesters. Further review and critical comparison of current methods for the determination of 3-MCPD esters would be very beneficial. The progress in the development of reliable methodology will hopefully enable the elucidation of the mechanism of formation of 3-MCPD esters in oils and testing different mitigation strategies.

The authors have declared no conflict of interest.

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