July 2014

Investigation of the formation of 3-chloropropane-1-2-diol (3-MCPD) from mono- and di-esters of its fatty acids in foods (FS231006, FS231074, FS231075)

Colin G. Hamlet[1]*

Lisa Asuncion[1]

Jan Velíšek[2]

Marek Doležal[2]

Zuzana Zelinková[2]

Colin Crews[3]

Warwick Anderson[3]

Celine Pye[3]

[1] Premier Analytical Services, Lord Rank Centre, Lincoln Road, High Wycombe, Bucks HP12 3QR, UK

[2] Institute of Chemical Technology, Technická 1903, 166 28 Prague 6, Czech Republic

[3] Food and Environment Research Agency, Sand Hutton, York, Y041 1LZ, UK

*Phone: +44 (0)1494 428211

*Fax: +44 (0)1404 428114

*email: colin.g.hamlet@premierfoods.co.uk

1st draft 16th January 2014

2nd draft 17th February 2014

Final Version 21st July 2014

1. Summary

The results of new research into the formation of chloropropanediol (MCPD) esters and related compounds in both model systems and foods are presented covering four main work areas: development and extension of analytical methods for bound forms of MCPD and glycidol (GE); occurrence in target foods; model system studies with pure reference chemicals; a study of the effects of food processing on the relative amounts of GE and free and bound forms of MCPD in target foodstuffs.

1.1 Key findings

1.1.1 Development and extension of analytical methods

- A method was developed to quantify amounts of bound mono- and diesters of MCPD in foods using solid phase extraction to separate mono- and diesters with GC/MS/MS detection.
- A method was developed and validated to quantify individual GE in fats extracted from foods using gel permeation chromatography (GPC) clean up followed by HPLC coupled to either a quadrupole (LC-MS/MS) or Time of Flight mass spectrometer (LC-TOF-MS).
    - Extraction of fats from infant formulae with cold solvents was incomplete.
    - The use of pressurised liquid extraction at effective temperatures, i.e. 125°C, appeared to generate GE, even from virgin olive oil.

1.1.2 Occurrence in target foods

1.1.2.1 Non-cereal foods (cheeses, salami, cooking oils, potato products)

- For all samples, other than the refined fats and oils, amounts of bound GE and 3-MCPD levels in the whole food were low.
- Consumer exposure to GEs and bound 3-MCPD is likely to be dominated by occurrence in refined vegetable oils.

1.1.2.2 Cereal products

- Amounts of bound MCPD measured in a bakery fat and a retail soft dough biscuit were 1285 and 632 µg/kg and consistent with amounts reported previously.
- Application of the developed method for bound mono- and diesters of MCPD showed that mono-esters of MCPD accounted for 15.7% and 9.4% of the total bound MCPD in the bakery fat and biscuit respectively.

1.1.3 Model system studies with pure chemicals

- A model system was developed to simultaneously measure the kinetics of formation and decomposition of 3-MCPD and glycidol esters (GE) from the starting materials tripalmitin, dipalmitin, monopalmitin, 3-MCPD dipalmitate, 3-MCPD monopalmtitate and glycidyl palmitate.
- Triacylglycerol (TAG) did not act as a direct precursor of 3-MCPD esters and glycidyl esters.
    - e.g. tripalmitin must first be hydrolysed to di- and tripalmitin
    - The extent of hydrolysis increased with increasing temperature and led to the formation of 3-MCPD esters and esters of glycidol, but in significantly lower amount than from monopalmitin directly
- In models with tripalmitin, amounts of 3-MCPD esters and GE were not directly influenced by chloride ions in the investigated range of 0.1-1%.
- In models with monopalmitin, the amount of 3-MCPD esters and glycidol esters

formed showed a linear dependence on the concentration of chloride ions.

- In studies with enzymes, both nonspecific and specific lipases did not change the concentration of 3-MCPD esters present in fats under the conditions of enzyme-catalyzed transesterification, i.e. typical of those used in the production of structural fats for the manufacture of margarines and spreads.

1.1.4 Effects of processing

1.1.4.1 Non-cereal foods (cheeses, salami, cooking oils, potato products)

- There was no evidence of substantial changes in GE or bound MCPD concentrations during cooking.

1.1.4.2 Cereal products

- Under conditions simulating the baking of a cereal product (e.g. short dough biscuits) concentrations of added (labelled) and native (present in fats) bound MCPD remained unchanged.
    - under these conditions, some formation and decay of free non-labelled MCPD was observed presumably from precursors present in the recipe ingredients (e.g. glycerol, acylglycerols, salt)
- In cereal dough containing a commercial lipase, free MCPD isomers were readily released from added (labelled) 3-MCPD esters.

1.1.5 Recommendations for future work

1.1.5.1 Processing

- Studies into the formation of GE under mild temperature conditions including the effect of moisture content, pH, time etc.

1.1.5.2 Analysis

- Develop / extend methods (direct and indirect) for the analysis of GE with particular reference to:
    - fats extracted from food products
    - matrices where lipids may bind to proteins, such as cheese and infant formulae.

Contents

FIGURES

TABLES

Terms and abbreviations

3-MCPD	3-chloropropane-1,2-diol
PP-3-BPD	dipalmitate ester of 3-bromopropane-1,2-diol
P-3-MCPD	palmitate ester of 3-MCPD
PP-3-MCPD	dipalmitate ester of 3-MCPD
P-glycidol	palmitate ester of glycidol
DAG	diacylglycerol
FFA	free fatty acid
GE	glycidol ester
HFBI	heptafluorobutyrylimidazole
HPLC	high performance liquid chromatography
IHRM	in-house reference material
MAG	monoacylglycerol
RSD	relative standard deviation
SIM	Selected Ion Monitoring
SPE	solid phase extraction
SD	standard deviation
TAG	triacylglycerol

2. Introduction

2.1 Background

Chloropropanols and their fatty acid esters are contaminants that are formed during the processing and manufacture of certain foods and ingredients. The presence of these compounds in foods is of concern because toxicological studies have shown that they could endanger human health. Although fatty acid esters of chloropropanols are known by-products of the manufacture of acid-hydrolysed vegetable proteins, the occurrence of these compounds in foods is still a relatively recent discovery. These esters are structurally related to the parent chloropropanols, e.g. 3-chloropropane-1,2-diol (3-MCPD) and hence the main toxicological concerns here are the potential release of toxic 3-MCPD from the parent chemical during the processing of foodstuffs, and *in* vivo in the gastrointestinal tract. Because of this, the term "bound" 3-MCPD has been used to describe the amount of 3-MCPD that could be released from the corresponding ester.

In an early opinion on 3-MCPD esters (BFR 2007), the German Bundesinstitut für Risikobewertung (BfR) chose to base its risk assessment on toxicological data for 3-MCPD. This assessment, which was subsequently endorsed by the EFSA Scientific Panel on Contaminants in the Food chain (CONTAM 2008) assumed that 100% of 3-MCPD is released from its esters in-vivo. A tolerable daily intake for 3-MCPD of 2 μg/kg body weight has been set by JECFA (2002).

Early reports showed that the 3-MCPD esters may be widespread in processed foods derived from cereals, coffee, fish, meat, potatoes, nuts and refined oils (Hamlet et al 2004; Hamlet and Sadd 2004; Svejkovská et al 2004; Karšulínová et al 2007; Doležal et al 2005; BFR 2007; Zelinková et al 2008; OKO-TEST 2008). The refined oils (e.g. palm) accounted for some of the highest amounts of 3-MCPD esters and hence commodities using these ingredients in their recipe (e.g. infant formulae) or during cooking/processing (e.g potato products), were shown to contain elevated levels of bound 3-MCPD. Consequently the extent to which 3-MCPD might be released from parent esters during the processing (thermal / enzyme) of foods using oil contaminated with 3-MCPD esters came under scrutiny. However, limited data on the occurrence of 3-MCPD in processed foods would indicate that the occurrence of the free chloropropanol is very relatively low.

The direct analysis of 3-MCPD can be a lengthy and relatively expensive process mainly due to the many species arising from the different fatty acid combinations associated with each chloropropanol moiety, and the high cost of these reference compounds. Because of the potential for 3-MCPD to be released by hydrolysis of the ester, methods to measure this so called "bound" 3-MCPD have found favour. Bound 3-MCPD has been estimated using chemical (acid or alkaline) treatments of isolated 3-MCPD esters to release the free chloropropanol for determination by GC/MS. To date it has been assumed that 3-MCPD esters are associated with plant lipids and methods to extract fats have been employed to isolate these compounds. However, little recovery data for extraction efficiency directly from foods has been published.

Although 3-MCPD esters have previously been identified as contaminants of acid-HVP and their formation mechanisms studied to an extent, far less is known about the formation routes in processed foods and ingredients. Consideration of the chemistry of acylglycerols suggests that the mono- and diacylglycerols (together with a source of chloride ions) could be important precursors of these compounds. While early studies showed that the stability of 3-MCPD esters decreases with increasing temperature, the actual degradation products are not yet known although the corresponding levels of free 3-MCPD did not appear to increase during this process. This may offer scope for minimisation of these contaminants in e.g. oil refining.

The more recent discovery that the related glycidol esters (GE) are present in refined edible fats and oils is also a concern as these chemicals may share a similar fate to 3-MCPD esters with the potential release of toxic glycidol in the GI tract (BFR 2009). The BfR has called for the urgent development of robust analytical methods and biokinetic studies to support reliable risk assessments.

2.2 Project brief and lines of approach

This project was undertaken in response to Food Standards Agency Research Requirement 30 concerning "Investigation of the formation of 3-MMCPD (3-monochloropropane -1,2-diol) from mono and di-esters of its fatty acids in food", a copy of which is given in 9.1 of the Appendix. The overall objective of the project was to determine the extent to which MCPD esters and related chemicals contribute to amounts of non-esterified 3-MCPD in foodstuffs.

The project was undertaken jointly by three project partners, namely: The Institute of Chemical Technology (ICT), Prague, Czech Republic; The Food and Environment Agency (FERA) Laboratories in York, UK; and Premier Analytical Services (PAS) in High Wycombe, UK. The study was divided into four main work areas: development and extension of analytical methods to separate and quantify bound MCPD (PAS) and development of methods to measure the recently discovered glycidol esters (GE) in a range of foods (FERA); occurrence of MCPD and GE esters in cereal (PAS) and non-cereal foods (FERA); development and application of model systems to study the formation of MCPD esters and GE using pure reference chemicals (ICT); and a study of the effects of food processing on the relative amounts of GE and free and bound forms of MCPD in Cereal (PAS) and non-cereal (FERA) foodstuffs.

Key reference chemicals required for the project were prepared by ICT and a survey of the literature was undertaken jointly by all project partners at the outset. For consistency of analysis, a reference material was circulated between project partners for the analysis of 3-MCPD esters (see 9.3 of the Appendix). Progress in the project was followed by means of a Gantt chart, details of which can be found in 9.2 of the Appendix.

3. Literature review (objective 01)

A review document covering aspects of toxicology, chemistry and analysis, occurrence, formation routes and potential mitigation measures was prepared and presented to the Agency early in the project (Hamlet et al 2010). The key findings / knowledge gaps of relevance to this project were as follows:

Toxicokinetics

- Information concerning the extent to which MCPD-esters (mono and di-esters) are hydrolysed in the intestinal enterocytes and the fate of 3-MCPD-*sn*-2-monoesters is not complete
- The fate of glycidol esters in the gut is not known, e.g. do they form MCPD-esters in the stomach by the action of hydrochloric acid?

Analysis

- Indirect methods that utilise alkaline transesterification procedures to measure bound MCPD and glycidol-esters may not be sufficiently robust and can give a significant positive bias in results
- Direct methods to determination MCPD- & glycidol-esters are possible but require expensive / specialised kit and are limited by the requirement for a large number of reference standards (not all are available)
- These methods may facilitate the development / validation of lower cost indirect methods

Occurrence / intake

- Mono- and diesters of MCPD are likely to have different metabolic pathways in vivo, consequently their relative amounts should be determined in foods
- In situ formation of 3-MCPD-esters appears to be significant in thermally treated cereal products and correlates with increases in free 3-MCPD
- Formation of 3-MCPD in non-thermally processed foods may be significant for products utilising or exposed to lipases

Formation routes in foods

- The immediate precursors of MCPD-esters in foods are likely to be the mono and diacylglyerols including possibly the phosphatidylglycerols
    - Partial acylglycerols have the ability to form ‘activated’ cyclic acyloxonium ion intermediates under thermal processes which can efficiently direct the chloride ion substituent
    - It has recently been shown that TAG may also form acyloxonium ion intermediates under hydrophobic conditions and the role of these precursors needs to be considered
- The role of glycidol as a precursor of MCPD-esters is not clear
- Other sources of chloride ions in foods should be considered e.g. covalently bound oil soluble forms
- There may me be other “bound” forms of MCPD in foods

4. Synthesis of reference chemicals

At the outset of the project the following certified reference standards were prepared by ICT and distributed to FERA and PAS (see 9.4.1 for certificates of purity):

COMPOUND	AMOUNT [mg]	PURITY [%]
1,2-dipalmitate ester of 3-chloropropane-1,2-diol-d_5 (3-PP-MCPD-d_5)	1000	98.2
1,2-dipalmitate ester of 3-chloropropane-1,2-diol (3-PP-MCPD)	1000	98.9
1,2-dioleate ester of 3-chloropropane-1,2-diol	549	99.2
oleate ester of glycidol	1020	98.3
palmitate ester of glycidol (P-glycidol)	1000	98.0
glycidylpalmitate -d_5	1070	99.0
1-palmitate ester of 3-chloropropane-1,2-diol-d_5 (3-P-MCPD-d_5)	1090	98.2
1-palmitate ester of 3-chloropropane-1,2-diol (3-P-MCPD)	1130	98.7

5. Development and extension of analytical methods (objective 03)

5.1 Analytical quality assurance

Prior to the onset of analytical investigations requiring the measurement of bound 3-MCPD, a palm oil reference material was prepared by FERA for distribution to project participants. A summary of the results from these analyses given in Table 1 below (and 9.3 of the Appendix) demonstrated that there was reasonable agreement in methods used by the project partners.

Table 1. Inter laboratory analysis of bound 3-MCPD in a palm oil reference material

Round	Test method	ICT result (mg kg^{-1})		PAS result (mg kg^{-1})	
		mean	SD	mean	SD
1 (2010/2011)	GC/MS	0.652	0.016	0.585	0.014
2 (2012)	GC/MS	0.560	0.021	0.626	0.023

5.2 Mono- and di-esters of 3-MCPD (task 03.1, PAS)

A review of the literature had indicated that monoesters and diesters of 3-MCPD are likely to have different metabolism pathways in vivo. Hence the objective of this task was to develop and extend a GC/MS method for bound 3-MCPD to distinguish between mono- and di-esters of 3-MCPD.

5.2.1 Experimental

5.2.1.1 SPE separations

The methods of International Olive Council (2006), Pérez-Camino et al. (1995), Seefelder et al. (2008) and Zelinková et al. (2009) were modified for the preparative separation of mono- and diesters of 3-MCPD according to the scheme given in Figure 1. For the initial evaluation, palm or olive oils (330

mg) were spiked with 3-PP-MCPD and 3-P-MCPD (1 ml of 1000 µg/ml in THF added to oil; solvent removed) and the separation was monitored by means of TLC using 2',7'-dichlorofluorescein indicator to visualise the MMCPD esters under UV light. For the GC/MS evaluation/validation, olive oil (330 mg ± 17 mg) was weighed accurately into a 50 ml glass centrifuge tube and 50 µl each of 3-MCPD-PP-d_5 (80.80 µg/ml) and 3-MCPD-P-d_5 (50.20 µg/ml) were added. For the spiked recovery experiments, 50 µl each of 3-PP-MCPD (80.00 µg/ml) and 3-P-MCPD-d_5 (50.20 µg/ml) were add at this stage. Hexane/diethyl ether (40 ml of 1:1 v/v) was then added, the vial capped and mixed thoroughly (vortex). The organic layer was washed with deionised water (2 x 5 ml, vortex mix) and the aqueous layer discarded. The wash and centrifugation step was repeated and the aqueous layer discarded. The organic layer was filtered through anhydrous sodium sulphate and the solvent was removed (rotary evaporator at 40 ºC). The residue was quantitatively transferred to a 15 ml screw cap vial using hexane and the solvent under nitrogen stream. The sample was reconstituted in hexane (300 µl) prior to SPE separation on either Bond Elut Diol or Amino columns according to the scheme given in Figure 1.

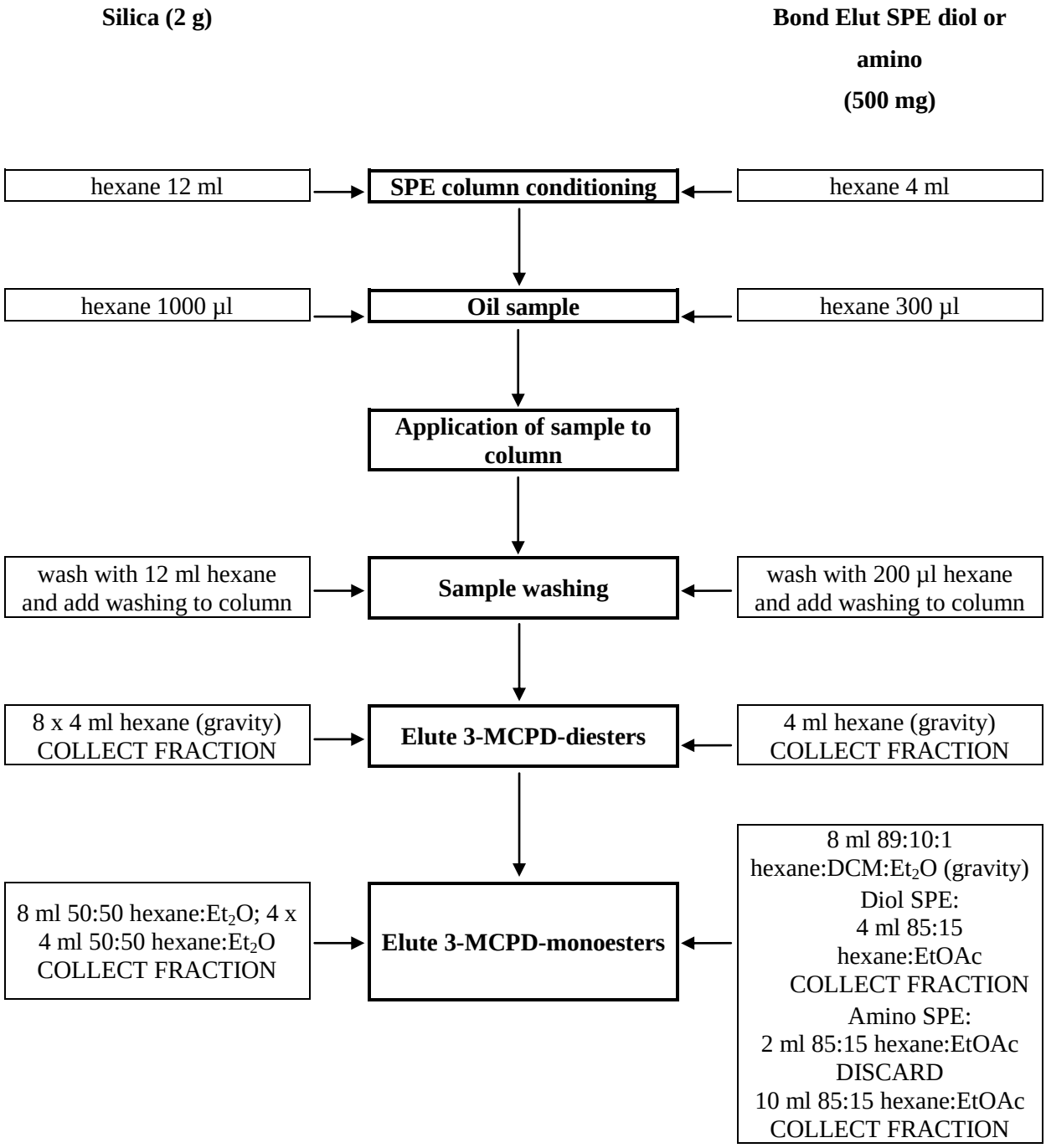


Figure 1 Schematic of SPE system for the separation of 3-MCPD esters

5.2.1.2 **Analysis of bound MCPD in SPE fractions**

The method of Hamlet and Asuncion (2011) based on acid catalysed transesterification, HFBI derivatisation and GC/MS/MS was used to quantify bound 3-MMCPD contained in SPE fractions.

5.2.2 Results and discussion

At the outset, a unique reference standard (dipalmitate ester of 3-bromopropane-1,2-diol, PP-3-BPD) was initially prepared from 3-bromopropane-1,2-diol and palmitoyl chloride according to the procedure of Hamlet and Asuncion (2011) to monitor the separation of mono- and diesters by GC/MS, i.e. following transesterification PP-3-BPD should be absent from the monoester fraction. However PP-3-BPD was only partially recovered after acid-catalysed transesterification indicating that the compound was unstable under the conditions used and hence unsuitable for use as a control standard.

A range of silica, diol and amino SPE chromatographic media (International Olive Council 2006; Pérez-Camino et al. 1995; Seefelder et al. 2008; Zelinková et al. 2009) was then evaluated and the separation of mono- and diesters of 3-MCPD monitored initially by means of TLC. It was found that a clean separation of mono- and diesters of 3-MCPD could not be achieved at any oil loadings (50 – 330 mg, Si:oil ratio range 40-6 w/w) using silica SPE columns based on the procedure of the International Olive Council (2006) developed for the determination of 2-glyceryl monopalmitate. This lack of separation was probably due to the limited oil capacity of the silica column and was consistent with the findings Mathieu et al (2011) who reported that a SPE silica:oil ratio of >75 was required to separate 3-MCPD mono- and diesters. Following optimisation of the elution solvents, both the diol and amino SPE columns were able to separate PP-3-MCPD and P-3-MCPD at the maximum loading of 330 mg oil with the former media providing slightly better resolution of the P-3-MCPD band (by TLC).

Table 2 shows the results obtained from the SPE separation of an olive oil blank in-house reference material (IHRM) with an intrinsically low level of bound 3-MCPD (mean = 49.1 µg/kg, SD = 5.5 µg/kg, n=5) and the high blank value obtained from the diol column. The separated fractions had been prepared by acid catalysed transesterification and analysis by GC/MS according to the procedure of Hamlet and Asuncion (2011). For quantification, PP-3-MCPD-d_5 and P-3-MCPD-d_5 had been added to the oil at the outset. Analysis of procedural blanks (also containing PP-3-MCPD-d_5 and P-3-MCPD-d_5 ISTD) subsequently revealed that much of the over-recovery of bound 3-MCPD from the diol SPE column was obtained from the mono-ester fraction (circa 300 µg/kg expressed on sample basis). Analyses of the individual SPE solvents for bound 3-MCPD could not account for the high blank results indicating that formation of bound 3-MCPD had occurred from an interaction of components of the SPE system, the oil and subsequent trans-esterification. The possibility of one of these components also being present in the internal standards could not be excluded.

Table 2 Bound 3-MCPD values obtained from the SPE separation of an olive oil blank IHRM (total bound 3-MCPD 49.1 µg/kg)

	n	Bound 3-MCPD (µg/kg)					
		Diesters		Monoesters		Total	
		mean	SD	mean	SD	mean	SD
Diol SPE	2	83.0	-	706	-	789	-
Amino SPE	5	36.2	14.21	13.3	5.07	49.5	15.1

Because of the relatively high blank values obtained from the diol SPE columns, the efficiency of the separation of 3-MCPD-esters was subsequently validated by means of spiked recovery experiments using the Bond Elut amino columns. Known amounts of PP-3-MCPD and P-3-MCPD were added to the blank olive oil IHRM and the recovery of each chloroester was compared to the theoretical amount following preparation of the separated fractions by acid catalysed transesterification according to the procedure of Hamlet and Asuncion (2011). The purity of all standards was first confirmed by the analysis of total bound 3-MCPD in a reference palm oil and an olive oil fortified with PP-3-MCPD and P-3-MCPD using a standard method (*ibid*). Table 3 shows that no significant difference could be found in recovery of bound 3-MCPD from the palm oil reference using either labelled ISTD (PP-3-MCPD-d_5 or P-3-MCPD-d_5); the combined recovery of 3-MCPD bound in mono- and diesters of 3-MCPD added to olive oil at circa 2300 µg/kg in each chloroester (total bound 3-MCPD circa 4600 µg/kg) was 97.4% ±7% (95% CI, n=5) using PP-3-MCPD-d_5 ISTD; and these results confirmed the expected purity of the reference standards.

Table 3 Recoveries of bound 3-MCPD from a palm oil reference using either PP-3-MCPD-d_5 or P-3-MCPD-d_5 ISTDs

	Bound 3-MCPD (µg/kg)	
ISTD	3-PP-MCPD-d_5	P-3-MCPD-d_5
Mean	609.2	588
n	9	2
SD	35.8	34
95%CI	27.6	-

The recoveries of bound 3-MCPD from olive oil fortified with PP-3-MCPD and P-3-MCPD using amino SPE columns to separate the mono- and diesters are given in Table 4.

Table 4 Recovery of bound 3-MCPD from samples of olive oil spiked with PP-3-MCPD and P-3-MCPD (circa 2300 µg/kg bound 3-MCPD in each ester) using amino SPE columns

Spiked oil	Bound 3-MCPD recovery (%)	
	PP-3-MCPD	P-3-MCPD
A	90.8	82.4
B	97.8	91.5
C	92.2	86
D	101.5	95.5
E	91.9	-
Mean	94.8	88.9
n	5	4
SD	4.61	5.89
95% CI	5.7	9.2

The SPE method was subsequently used to measure the distribution of 3-MCPD bound in mono- and diesters of a commercial bakery fat containing palm and rape seed oils. The sum of bound 3-MCPD measured by SPE showed good agreement with that obtained by a standard method (see Table 6).

5.3 Glycidol esters (task 03.2, FERA).

Since the commencement of this project several new methods for the determination of GE have been published (Deutsche Gesellschaft 2009; Dubois et al 2011; Kuhlmann 2011; Masukawa et al. 2010; Shimizu et al. 2010) and these were reviewed in Task 01. Hence the older method of Engbersen (1976), identified at the project proposal stage, was dropped in favour of more a recent method based on direct determination using LC/MS.

As it was felt that more useful information could be obtained by measuring the occurrence and fate of individual GE a method based on chromatographic separation of the intact esters (direct method) was preferred to methods that determined the total GE by its cleavage from the acid and conversion to free MCPD (indirect methods). Some direct methods reviewed measured both GE and bound MCPD but as more detailed information on the bound forms of MCPD was to be provided by the other partners we selected a method based on separation of the GE from the fat glycerides and the esterified MCPD. The method had been published by Dubois et al. (2011).

5.3.1 Fat extractions

Glycidyl esters are non-polar compounds associated with the fat component of foods. The esters can be separated from triacylglycerols using gel permeation chromatography (GPC) and detected by gas

chromatography-mass spectrometry (GC-MS) or high performance liquid chromatography-mass spectrometry with mass spectrometry/mass spectrometry (LC-MS/MS) or with Time of Flight mass spectrometry (LC-TOF-MS).

GPC can isolate GEs from up to about 0.8 g fat without breakthrough of high molecular weight materials and a target weight of 0.5 g fat was selected for the GPC step. The majority of the foods of interest (cracker biscuits, pies, chips, cheese, infant formula and salami) contained about 25% fat, requiring extraction from a food sample of approximately 2 g.

Food samples were extracted four to six times and the weight of extracted fat compared with the labelled fat content to provide an approximate recovery.

In the finalised method the samples (2 g) were blended with 30 ml hexane:acetone 4:1 in a 50 ml plastic tube using a homogeniser at high speed for two minutes, and then left to shake for three hours. The sample was centrifuged and the solvent decanted into a pre-weighed vial. The solid residue was shaken for 10 mins with a further 20-30 ml hexane:acetone 4:1 and the centrifugation repeated. The solvent portions were combined and evaporated under nitrogen at 40 °C. The vials were weighed and the fat content calculated by difference. The results are shown in Table 5.

Table 5 GE analysis: recovery of fat from various foods

Food	n	% oil (label)	% recovery (average)	stdev
Infant formula	6	28	11	3.3
Meat pie	4	24	75	2.1
Potato fries (1)	4	11	75	0.5
Potato fries (2)	4	14	84	1.5
Potato waffles	4	10	99	3.1
Processed cheese	6	25	37	1.4
Salami	6	32	93	1.7

5.3.2 Results and discussion

The recovery of fat from meat pies, potato fries, potato waffles and salami was good to very good (75-99% however the extraction efficiency from the infant formula sample by this method was unsatisfactory (11%), and for processed cheese the extraction efficiency was possibly inadequate 37%).

Extraction of fats from the infant formula by simple shaking/solvent procedures is known to give low recoveries on account of lipid binding to proteins, and a similar effect probably accounts for the poor

recovery for cheese. Fats can be recovered in high yield from these materials using acid digestion procedures but these would almost certainly destroy the glycidol-esters linkages.

Pressurised liquid extraction (PLE, accelerated solvent extraction) has been used to determine the quantity of fats in infant formulae and was applied to infant formulae and cheese samples. By using the recommended PLE conditions for infant formulae fat recoveries of over 90% were achieved consistently for both infant formulae and cheeses.

However, when recoveries of added GEs were measured it was found that several hundred mg/kg GEs were generated in the samples. GEs were also produced in extra virgin olive oils under these conditions. This might be due to the formation of GEs from the lipid in the samples at the extraction temperature employed, i.e. 125°C, although GE formation has been reported to require higher temperatures than this (typically over 200°C). However, the use of dry diatomaceous earth support during extraction may have facilitated formation at the lower temperature, as similar materials are used in the refining of vegetable oils, a process which is known to leads GE formation. Extraction at 30°C did not compromise the recovery of GEs from infant formulae and this approach was used for subsequent extractions that showed no GEs in the single sample analysed.

5.3.2 Development and validation of the GE method

During the validation and analysis of food extracts occasional levels of GEs were measured in a number of food samples including cracker biscuits, potato chips, potato crisps fried in sunflower oil, and meat pies but also in non-heated foods including salami and cheeses. When measured by LC-MS/MS all product ion transitions were found to agree with those of the reference standards thereby appearing to confirm the presence of GEs in these samples. The LC-MS/MS chromatograms for the GE reference standards signals for the apparent glycidyl linoleate in feta cheese are shown in Figure 2 and Figure 3.

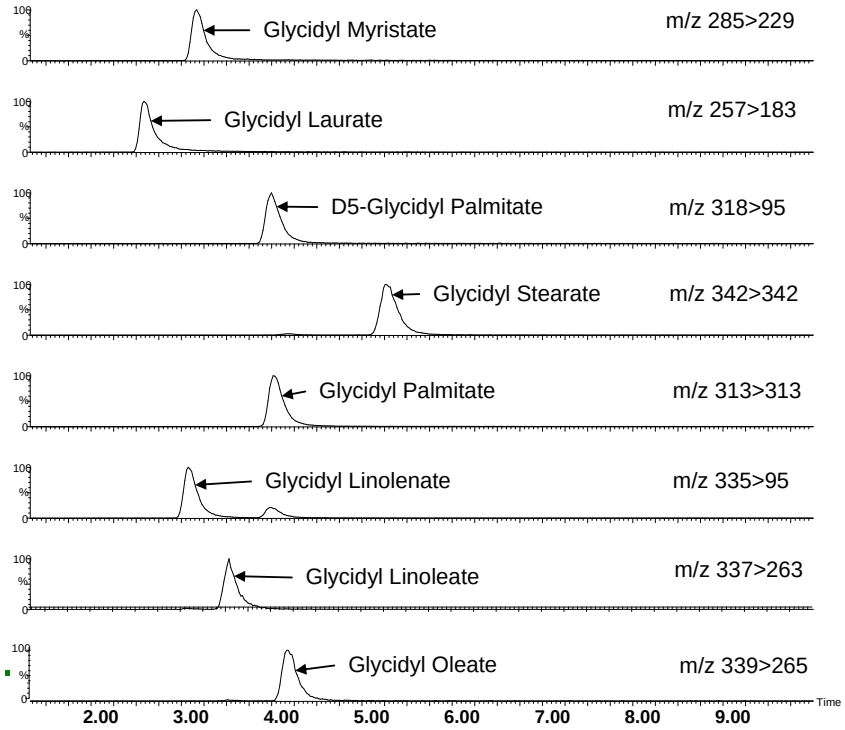


Figure 2 LC-MS/MS Chromatogram for GE standards.

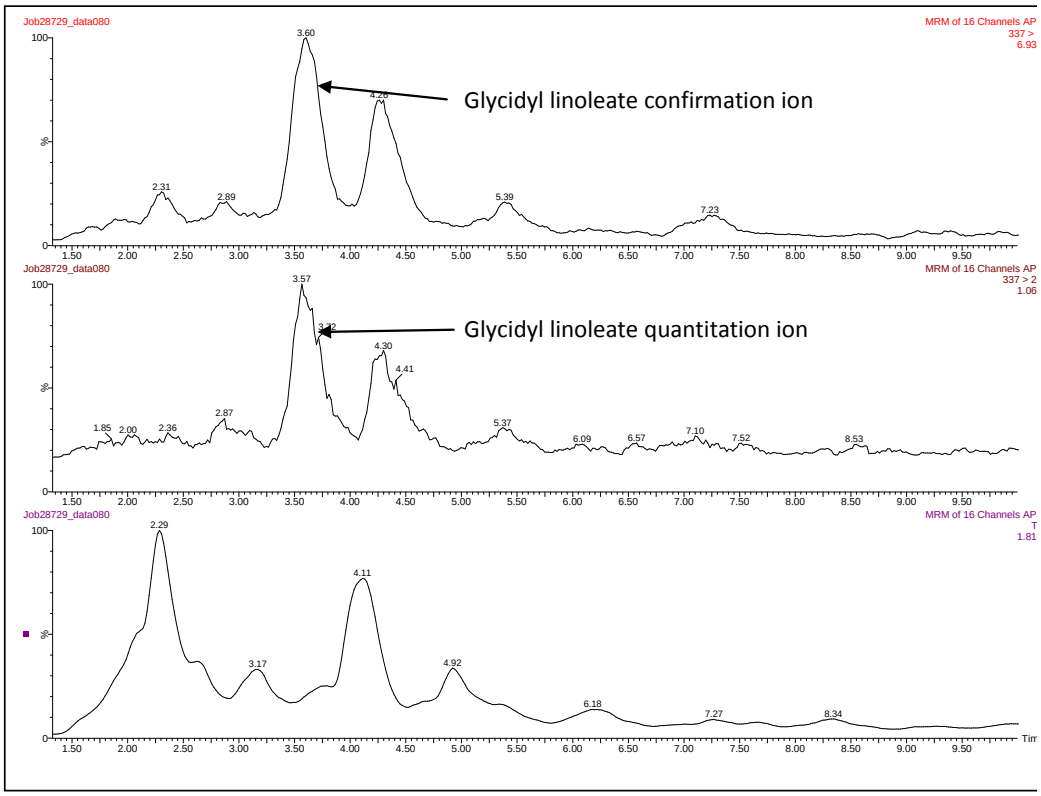


Figure 3 Apparent glycidyl linoleate in feta cheese by LC-MS/MS.

5.3.4 Results and discussion

The fatty acid profiles of the GEs identified in each of these foods did not match the fatty acid profiles of the oils present in the food samples. For example the potato crisps fried in sunflower oil contained high levels of glycidyl linoleate but no other GEs whereas linoleate is not abnormally high in sunflower oil. Consequently it was considered that the GEs measured in these samples were in fact false positives.

GE-related compounds having the same empirical formulae that eluted very closely to GEs were reported in vegetable oils by Destaillats et al. (2012). They were identified as oxopropyl esters of fatty acids. Analysis of reference oxopropyl esters of palmitic and linolenic acids (synthesised and supplied by ICT, Prague) under the same conditions gave different retention times to the false positive GE responses found in the cracker samples etc confirming that the latter were not therefore oxopropyyl esters.

Further analysis using LC-TOF-MS showed that reference GEs formed adduct ions with sodium and ammonium in the LC solvents whereas the false positive GEs measured in the food extracts did not (see Figure 3).

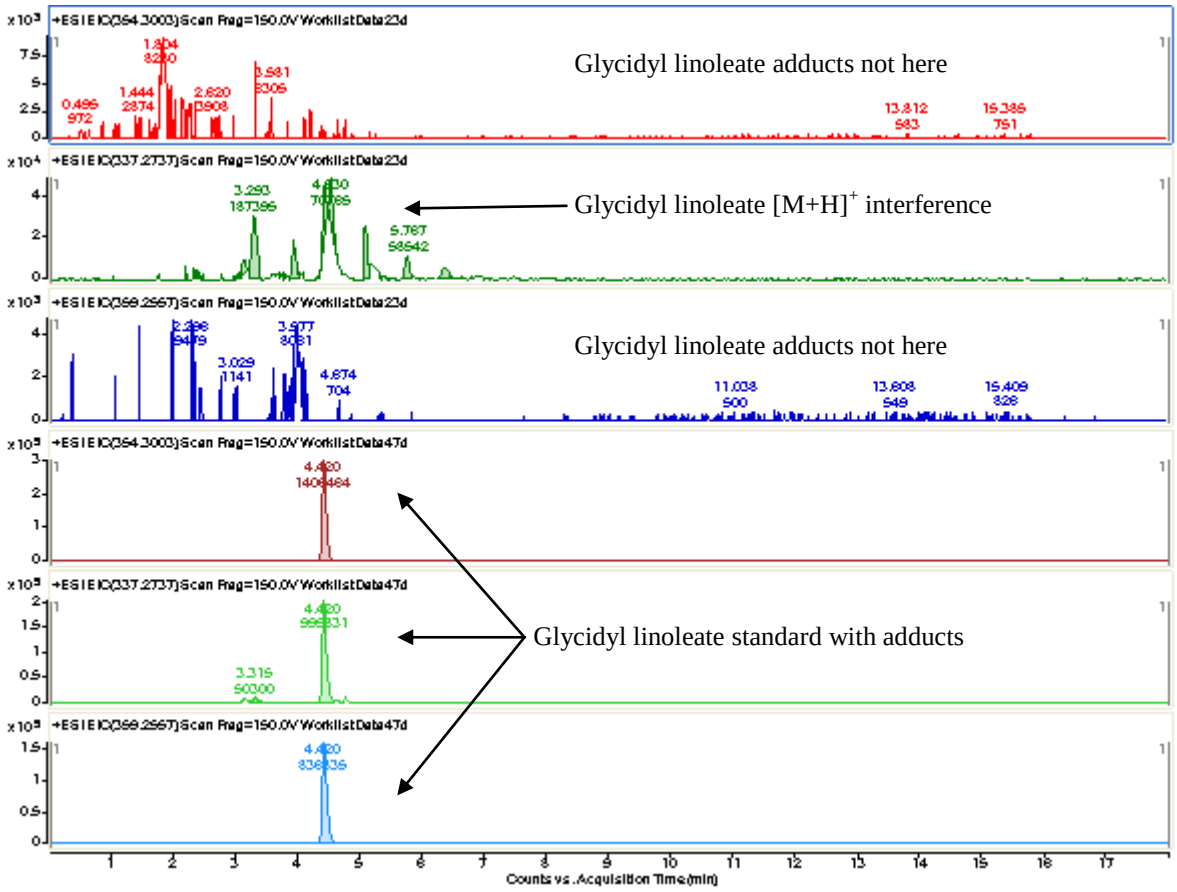


Figure 4 Apparent glycidyl linoleate in feta cheese by LC-TOF-MS: sodium and ammonium ion adduct ion formation and comparison with glycidyl linoleate reference standard

That the interferences existed as a series related to the GEs suggested strongly that they could be fatty acid esters although further investigation ruled out a MAG since this was also chromatographically separated from GEs under the conditions employed.

While analysis of e.g. salami & cheese by LC-TOF-MS could subsequently demonstrate that GEs were absent from these samples, no cleanup methods proved suitable to remove this apparent GE interference until the application of a solid phase extraction (SPE) procedure newly published by Becalski et al. (2012). For this procedure, a 10 mg sample of edible oil or fat was dissolved in acetone and the GEs separated by two-stage SPE using successive C18 and normal silica cartridges with elution solvents of methanol and 5% ethyl acetate in hexane, respectively. A further advantage of the method was that larger (0.5 g) samples of oil could first be concentrated first using a separate silica column. The dried final extract was dissolved in 250 µL of a mixture of methanol/isopropanol (1:1, v/v), 15 µL is injected on the analytical C18 LC column and analytes are eluted with 100% methanol. Detection of target glycidyl fatty acid esters was accomplished by LC-MS/MS using positive ion atmospheric pressure chemical ionization operating in Multiple Reaction Monitoring mode monitoring

2 ion transitions for each analyte. The limit of quantification of individual GE measured in fat by this procedure was found to be 3 to 25 μg kg^{-1}.

6. Occurrence in foods / ingredients to be studied (objective 04)

This work was undertaken to determine the relative amounts of mono- and diesters of 3-MCPD, glycidol esters (if feasible) and any other key components of the foods to be studied under objective 06 to assist with the interpretation of results from the effects of processing.

6.1 Cereal samples (Task 04.1, PAS)

Table 6 shows the distribution of mono- and diesters of 3-MCPD and the lipid content in a typical retail biscuit and a sample of bakery fat. The mono-esters of 3-MCPD were between 9 and 15 % of the total 3-MCPD esters. The total bound 3-MCPD value of 632 μg/kg obtained for the biscuit was comparable to that reported previously for a UK digestive product (614 μg/kg)[1].

Table 6 Bound 3-MCPD and the fat content of cereal products / ingredients for investigation

Sample	n	Bound 3-MCPD (μg/kg, mean and range)			3-MCPD mono-esters (% total)	Lipid content (%)
		diesters	monoesters	Sum of mono- and diesters		
Digestive biscuit	3	573 (530 – 604)	59.7 (58.8 – 60.7)	632	9.4	25.1
Bakery margarine	2	1084 (1052 – 1115)	201.6 (200 – 204)	1285	15.7	N/A

The effects of processing on MCPD esters in a cereal dough and a biscuit model system are discussed in Section 8.2

6.2 Non-cereal products (FERA)

6.2.1 Materials and methods

Samples were purchased in local retail outlets and were analysed for GEs, bound MCPD and free MCPD. Attention was focused on GEs and not all samples were analysed for all contaminants. The sample comprised 6 dairy products, 5 meats, 5 oils and fats and 5 potato products. Details of theses samples including, ingredients and fat content can be found in Table 45 of the Appendix.

Analysis

[1] Survey of process contaminants in UK retail foods 2008 – report on the analysis of 3-monochloropropane-1,2-diol esters (3-MMCPD esters). Available at: http://www.food.gov.uk/multimedia/pdfs/fsis3mMCPDesters.pdf

GEs were measured by the method described in Section 5.3 with a limit of quantification of 3 to 25 μg kg^{-1}. Bound 3-MCPD was determined using the acid hydrolysis method of Hamlet *et al.* (2011) which had a limit of quantification of about 40 μg kg^{-1}. Free 3-MCPD and 2-MCPD were determined using the FERA in-house method as published (Brereton *et al*) which had a limit of quantification of about 4 μg kg^{-1}.

6.2.1.1 Cooking experiments

Oven chips

All oven chips were cooked according to the packet instructions. Portions (500 g) were place on a baking tray in an electric oven preheated to 220 °C. To simulate the effects of over-cooking, selected chips were baked at the manufacturer's recommended (20 minutes) extended cooking times (35 minutes). All chips were allowed to cool at ambient temperature prior to analysis.

Fried chips

Chips were prepared from raw Maris Piper potatoes from a single packet, peeled and cut into chips of approximately 1 cm square in cross section and 5-10 cm in length. They were fried for 9 minutes by immersion in oil previously heated to 180 °C in an electric fryer. The chips were fried in either extra virgin olive oil (known to not contain GEs or MCPD esters) or refined corn oil that did contain GEs.

After frying a first batch of chips for 9 minutes the chips were removed and the oil re-used to fry a second batch. For each oil, four batches of chips were fried this way.

Salamis

Salami slices of about 3 cm diameter and about 0.5 cm thickness were grilled or oven baked in aluminium dishes of 5 cm diameter placed on a baking tray, or fried in a pan without additional oil. The electric grill was set to a temperature of 180 °C and the trays arranged so that the salami was about 10 cm from the element. The samples were grilled for 10 minutes to represent a relatively long consumer cooking.

The electric oven was set to 230 °C. The trays arranged in the centre of the oven. For frying the pan was heated to about 120 °C. The approximate temperature of the salamis immediately after cooking was measured rapidly using an infra-red thermometer.

Cheeses

Slices of cheese about 3 cm diameter and about 0.5 cm thickness were prepared from Emmental, Parmesan and goat's cheese. The slices were grilled or oven baked in aluminium dishes of 5 cm diameter. The grilling and oven baking procedures were the same as those used for salami.

Baking ingredient fats

Cake pastries were made with extra virgin olive oil (EVOO) that contained no GEs before cooking and also with refined baking fat that contained all of the common GEs. Recipes were prepared with and without added water. The recipes comprised (A) flour (250g) + olive oil (50g) ± water (50g), and (B) flour (250g) + baking fat (50g) ± water (50g).

The cake pastries were cooked in an oven at 180 °C for 45 mins.

6.2.1.2 **Storage experiments**

Three samples of cheeses (French goat's, Parmesan and Emmental) and three samples of salami were stored under typical refrigerator conditions (+4°C) for one months and at elevated room temperature (+20°C) for 6 months. Samples were removed for analysis at weekly intervals (+4°C) and at monthly intervals (+20°C) and the GE content compared with control samples stored at -18°C.

6.2.2 Results and discussion

6.2.2.1 **GEs**

The limits of quantification of the method for individual GEs in matrices typical of those analysed are shown below.

GE	LOQ μg kg^{-1}
C12:0	3
C14:0	10
C16:0	10
C18:3	3
C18:2	3
C18:1	25
C18:0	10

The results, expressed as μg kg^{-1} individual GEs in the fat portion of the foods are shown in Table 7.

Recovery of glycidyl palmitate based on the internal standard response ranged from 70 to 88%. The EVOO blank contained less than 3 μg kg^{-1} GEs.

No GEs were detected in any of the dairy or meat products or in the blanks sample of EVOO used throughout. Two potato chip samples contained similar levels and profiles of GEs. Sample 1, made with 11% beef dripping and about 3% sunflower oil contained 30 μg kg^{-1} glycidyl palmitate (C16:0); 63 μg kg^{-1} glycidyl oleate (C18:1) and 195 μg kg^{-1} glycidyl linoleate (C18:3) which matched reasonably the fatty acid profile of sunflower oil, although the unsaturated fatty acid content of beef dripping is low.

Sample 2, made with palm oil contained 20 μg kg^{-1} glycidyl palmitate (C16:0); about 40 μg kg^{-1} glycidyl oleate (C18:1) and about 20 μg kg^{-1} glycidyl linoleate (C18:3) which matched reasonably the fatty acid profile of palm oil.

The refined fat samples all contained GEs at levels similar to those reported elsewhere (Kuhlmann 2011). Although the parent oils were not identified on the labels the use of a high proportion of palm kernel fat is apparent particularly in sample 19.

Table 7 Food sample survey - Glycidyl Esters in fat portion of foods µg kg^{-1}

Sample Name	Class	ID	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
Cheese - Goat's	Dairy	4	nd	nd	nd	nd	nd	nd	nd
Cheese - Parmesan	Dairy	5	nd	nd	nd	nd	nd	nd	nd
Cheese - Emmental	Dairy	9	nd	nd	nd	nd	nd	nd	nd
Cheese - Brie	Dairy	14	nd	nd	nd	nd	nd	nd	nd
Cheese - Roquefort	Dairy	15	nd	nd	nd	nd	nd	nd	nd
Cheese - Brie	Dairy	16	nd	nd	nd	nd	nd	nd	nd
Salami sausage	Meat	3	nd	nd	nd	nd	nd	nd	nd
Salami sausage	Meat	6	nd	nd	nd	nd	nd	nd	nd
Salami sausage	Meat	7	nd	nd	nd	nd	nd	nd	nd
Salami sausage	Meat	8	nd	nd	nd	nd	nd	nd	nd
Salami sausage	Meat	10	nd	nd	nd	nd	nd	nd	nd
Baking fat	Oils and fats	11	6	28	1244	222	2260	753	36
Baking fat	Oils and fats	12	8	11	117	23	644	196	16
Baking fat	Oils and fats	13	18	26	1024	223	764	534	48
Baking fat	Oils and fats	19	697	301	153	28	309	50	nd
Palm oil	Oils and fats	23	nd	nd	1442	185	3438	977	nd
Chips	Potato	1	nd	nd	29	nd	63	195	nd
Chips	Potato	2	nd	nd	21	nd	42	21	nd
Potato product	Potato	17	nd	nd	68	38	231	806	nd
Potato product	Potato	18	nd	nd	53	27	167	536	nd
Oven chips	Potato	20	nd	nd	30	24	118	368	nd

nd = see text

The results showed that the fat incorporated into the selected commercially available composite food samples did not contain detectable GEs with the exception of potato products. The likely explanation for this was that, for cheeses and salami, the fat was of natural origin and not subjected to the refining processes that produce GEs. The bakery fats were refined, and the potato products were all partially fried in oils that can be assumed to have been refined as this is normal industry practice.

6.2.2.2 **MCPD esters**

Table 8 shows the levels of bound 3-MCPD in representative survey samples. In the cheeses bound 3-MCPD was found just at the limit of quantification in the fat portions of goat's and Parmesan samples but at comparatively high level in the fat from processed cheese slices.

Levels in salamis were within the ranges reported elsewhere (Zelinkova´ et al 2006) as were those in the refined vegetable fats intended for baking (Kuhlmann 2011). Moderate levels (0.5 to 1 mg kg^{-1})

were measured in the fat extracted from the coating of partially pre-cooked chips and potato products. For all samples other than the refined fats the bound 3-MCPD levels in the whole food were low.

Bound 3-MCPD has previously been reported at low level in goat's milk (Cerbulis et al. 1984) and it is therefore unsurprising that it was present in the cheese, and its natural presence in Parmesan cheese is therefore also possible. Processed cheeses are likely to have been heated to melting point and to also incorporate emulsifiers made from partial glycerides that might be susceptible to MCPD ester formation.

Table 8 Food sample survey - Bound MCPD mg kg^{-1}

Sample Description	Class	ID	3-MCPD bound	
			fat basis	food basis
Cheese - Goats	Dairy	4	0.04	0.01
Cheese - Parmesan	Dairy	5	0.04	0.01
Cheese - Emmental	Dairy	9	< 0.04	< 0.01
Cheese - Brie	Dairy	14	< 0.04	< 0.01
Cheese - Aged Roquefort	Dairy	15	< 0.04	< 0.01
Cheese - Somerset Brie	Dairy	16	< 0.04	< 0.01
Cheese - Slices	Dairy	21	2.98	0.60
Salami sausage	Meat	3	0.08	0.02
Salami sausage	Meat	6	0.04	< 0.01
Salami sausage	Meat	7	0.06	0.01
Salami sausage	Meat	8	< 0.04	< 0.01
Salami sausage	Meat	10	< 0.04	< 0.01
Vegetable baking fat	Oils and fats	11	1.82	1.82
Vegetable baking fat	Oils and fats	12	2.15	1.99
Vegetable baking fat	Oils and fats	13	1.54	0.91
Vegetable baking fat	Oils and fats	19	< 0.04*	
Chips	Potato	1	0.68	0.02
Frying chips	Potato	2	0.48	0.01
Potato product	Potato	17	1.00	0.06
Potato product	Potato	18	1.08	0.06

* Unlikely result - suspected analysis failure.

6.2.2.3 **Food survey free MCPD**

Table 9 shows the levels of free 3-MCPD in representative survey samples. In the cheeses free 3-MCPD was found just at the limit of quantification. Levels of free 3-MCPD in salamis ranged from 0.011 to 0.085 mg kg^{-1}. No free 3-MCPD was detected in two potato products.

Table 9 Food sample survey - Free MCPD mg kg^{-1}

Sample Description	Class	ID	3-MCPD free	2-MCPD free
Cheese - Slices	Dairy	21	0.007	< 0.005
Cheese - Parmesan	Dairy	5	<0.005	< 0.005
Cheese - Emmental	Dairy	9	<0.005	< 0.005
Cheese - Goats	Dairy	4	0.005	< 0.005
Cheese - Aged Roquefort	Dairy	15	<0.005	< 0.005
Cheese - Somerset Brie	Dairy	16	<0.005	< 0.005
Salami sausage	Meat	3	0.029	< 0.005
Salami sausage	Meat	7	0.011	< 0.005
Salami sausage	Meat	6	0.085	< 0.005
Salami sausage	Meat	8	0.019	< 0.005
Chips	Potato	1	<0.005	< 0.005
Oven chips	Potato	20	<0.005	< 0.005

7. Model system studies (objective 05, ICT Prague)

7.1 Formation and decomposition of 3-MCPD and glycidyl esters in model systems

The aim of this work was to design a model system to study the kinetics and reactivity chloropropanol esters and related compounds (e.g. esters of glycidol with fatty acids). At the outset of this project, knowledge of the mechanisms of formation of these substances was very limited and it was anticipated that these models would also help to confirm expected routes to the formation of MCPD esters in foods and vegetable oils. To accomplish this, it was proposed to monitor the kinetics of formation and decomposition of MCPD-esters from the starting materials tripalmitin, dipalmitin, monopalmitin, 3-MCPD dipalmitate, 3-MCPD monopalmtitate and glycidyl palmitate.

A GC/MS method was subsequently established which permitted the analysis of all expected compounds simultaneously: Deuterium labelled analogues of all the target analytes were used as internal standards; silica was chosen as an inert material to simulate the food matrix. In the kinetic study, the effects of the following parameters on the formation and decomposition of all compounds was followed: chloride ion concentration; water content; change in temperature; and the duration of heating.

7.1.1 Materials and methods

7.1.1.1 Chemicals and materials

1,3-Dipalmitin (Fluka Chemie, CH), 1-monopalmitin (Aldrich, D), palmitic acid-d_{31} (Sigma Aldrich Isotec, USA), silica gel 60 (0,063-0,200 mm, Merck, D), SPE columns (Extract Clean^{TM} Silica 5000 mg, Grace, USA), tetrabutylammonium chloride (Aldrich, D) were purchased.

3-MCPD dipalmitin, 3-MCPD-d_5 dipalmitin, 3-MCPD monopalmitin, 3-MCPD-d_5 monopalmitin, glycidyl palmitin, glycidyl-d_5 palmitin, tripalmitin-d_5, 1,3-dipalmitin-d_5, 1-monopalmitin-d_5 were synthesized at ICT Prague:

7.1.1.2 Model system

Reactions of tripalmitin, monopalmitin and dipalmitin: Acylglycerol (20 mg, i.e. 10 % of the mixture), tetrabutylammonium chloride (7.8 mg, i.e. 0.5 % of chloride ions in the mixture), water (1 mg, i.e. 0.5 % of the mixture) and silica gel (178 mg, i.e. 89 % of the mixture) were placed in a 5-ml reaction vial. The reaction vial was sealed and heated in an oven at 200°C for 1 hour and then cooled to room temperature. The cold vial was opened and 1 ml of the first internal standards mixture (3-MCPD- d_5 dipalmitate, 3-MCPD- d_5 monopalmitate, glycidol palmitate- d_5) was added. The analytes were repeatedly extracted with tetrahydrofuran. Part of sample was analyzed by GC / MS. To the second part of sample was added the second batch of internal standards (tripalmitin-d_5, 1,3-dipalmitin-d_5, 1-monopalmitin-d_5, palmitic acid-d_{31}) and then also analyzed by GC / MS.

The conditions of model experiments were modified as follows:

- the influence of temperature: 110, 140, 170, 200, 230, 260°C
- the influence of heating time: 0.5, 1, 2, 3 hours
- the effect of chloride ions content: 0.1, 0.5, 1% of the mixture
- the effect of water content: 0, 0.5, 1, 5, 10% of the mixture

GC/MS analysis

Chromatographic capillary column DB-1HT

- (100% dimethylpolysiloxane, 15 m x 0.25 mm i.d., 0.1 μm film thickness)

Injection Volume:	1.0 μl
Injection mode:	Pulsed Splitless
Injection temperature:	280ºC
Column flow:	1.0 ml/min
Initial oven temperature:	170ºC
Oven program:	
	Initial oven temperature 140 ºC
	Then 10ºC/min to 300ºC

Then 40ºC/min to 340ºC for 15 min

Transfer line temperature: 300ºC

1st analysis of 3-MCPD esters and glycidyl ester:

Mass spectrometer mode: SIM

1 group (1 min) - m/z 239, 283, 288, 312, 317, 348, 353

2 group (15 min) – m/z 239, 331, 333, 336, 338

Quantification ions: m/z 283 – glycidol palmitate $[M-C_2H_5]^+$

m/z 288 – glycidol-d_5 palmitate $[M-C_2H_5]^+$

m/z 348 - 3-MCPD monopalmitate $[M]^+$

m/z 353 - 3-MCPD-d_5 monopalmitate $[M]^+$

m/z 331 - 3-MCPD dipalmitate $[M-RCO]^+$

m/z 336 - 3-MCPD-d_5 dipalmitate $[M-RCO]^+$

2nd analysis of palmitic acid and acylglycerols with palmitic acid:

Mass spectrometer mode: SIM

1 group (1 min) - m/z 239, 256, 287, 299, 302

2 group (15 min) – m/z 239, 313, 318, 551, 556

Quantification ions: m/z 256 – palmitic acid $[M]^+$

m/z 288 – palmitic acid-d_{31} $[M]^+$

m/z 299 - monopalmitin $[M-CH_2OH]^+$

m/z 302 - monopalmitin-d_5 $[M-CH_2OH]^+$

m/z 313 - dipalmitin $[M-RCO]^+$

m/z 318 - dipalmitin-d_5 $[M-RCO]^+$

m/z 551 - tripalmitin $[M-RCO]^+$

m/z 556 - tripalmitin-d_5 $[M-RCO]^+$

7.1.2 Results and discussion

7.1.2.1 Preparation of the model systems

The model systems were developed to monitor the formation and decomposition of 3-MCPD esters. Palmitic acid was chosen because of its frequent occurrence in oils and hence food, and also because

of its oxidative stability. In addition to monitoring the esters (mono-and diesters) of 3-MCPD it was also necessary to develop a method which permitted the quantification of other degradation products and related compounds, especially glycidyl esters, recently identified in refined oils. It was also considered important to compare the relative reactivity of triacylglycerols (TAG), diacylglycerols (DAG) and monoacylglycerols (MAG) as part of the validation of proposed reaction mechanisms for MCPD and glycidyl esters.

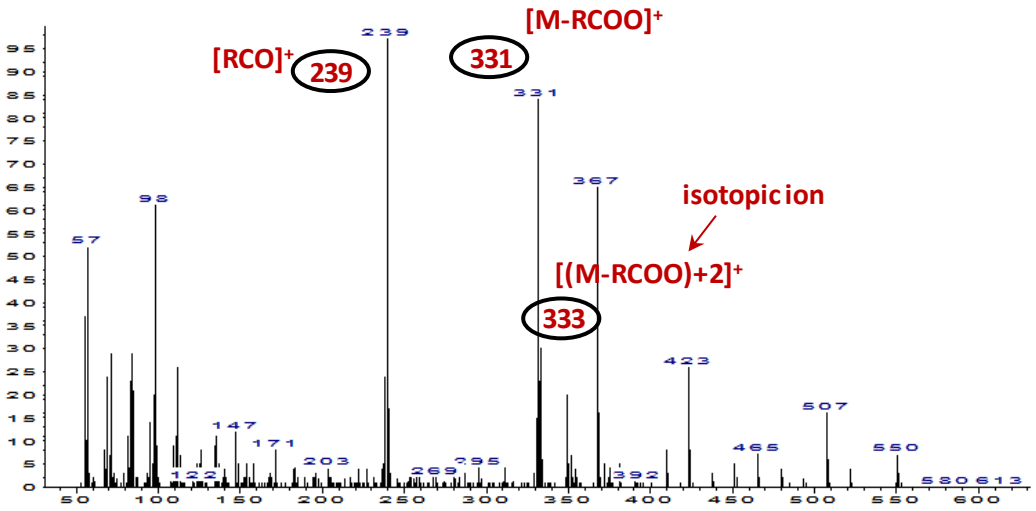
It was first necessary to obtain and characterise reference standards of 3-MCPD esters and glycidyl palmitate for the model system. GC/MS mass spectra were obtained and quantification and confirmation ions containing chlorine atoms in the molecules were preferentially selected (the mass spectrum contains M+2 isotope ion; for one chlorine atom in the molecule is valid $^{35}Cl:^{37}Cl$ = M:M+2 = 3:1). The mass spectra of 3-MCPD dipalmitate, 3-MCPD monopalmitate and glycidol palmitate together with the ions selected for quantification are given in Figure 5. Deuterium labeled analogues were used as internal standards, i.e. 3-MCPD-d_5 dipalmitate, 3-MCPD-d_5 monopalmitate, glycidol-d_5 palmitate.

To quantify acylglycerols prepared with palmitic acid (i.e. monopalmitin, dipalmitin, tripalmitin) and palmitic acid itself, it was also necessary to obtain suitably labelled reference standards. Palmitic acid-d_{31} was purchased, the remaining compounds were synthesized as a mixture (the starting substance was deuterated glycerol-d_5) and then separated by SPE using a solvent mixture of hexane and diethyl ether. As previously, the mass spectra of suitable quantification and confirmation ions were selected.

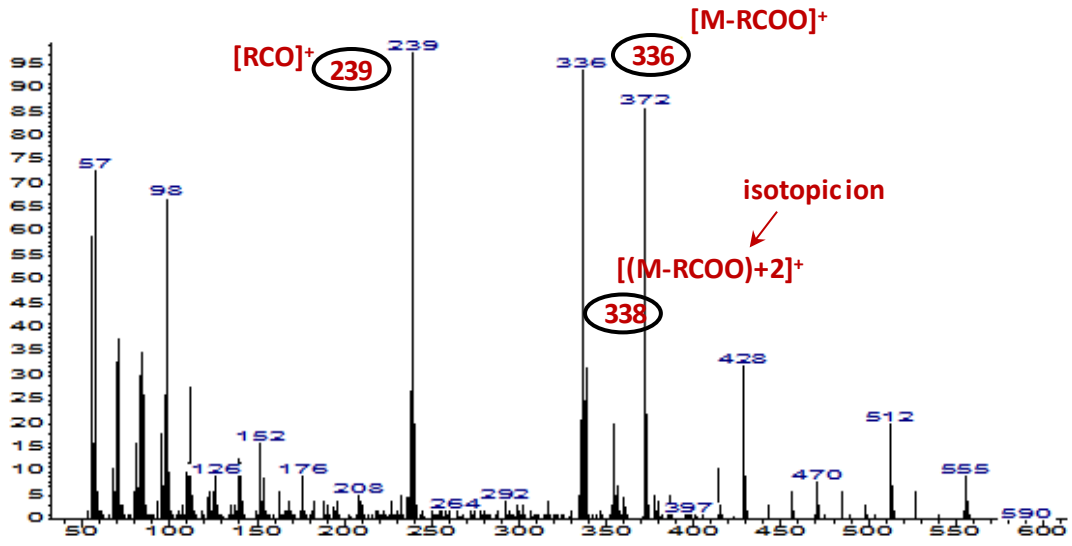
The proposed model system is schematically illustrated in Figure 6. Dried silica gel was chosen as an inert material to simulate the food matrix, to which was quantitatively added acylglycerols (or 3-MCPD esters or glycidyl palmitate) dissolved in tetrahydrofuran and chloride ions (as tetrabutylammonium chloride dissolved in acetone). After evaporation of the solvent by stream of nitrogen, the required amount of water was them added and the reaction vial was sealed and heated. After cooling, the following internal standards were added: 3-MCPD- d_5 dipalmitate, 3-MCPD- d_5 monopalmitate, glycidol palmitate- d_5, and the mixture was repeatedly extracted with tetrahydrofuran. The extract was divided: one fraction was analyzed by GC / MS directly; a second batch of internal standards (tripalmitin-d_5, 1,3-dipalmitin-d_5, 1-monopalmitin-d_5, palmitic acid-d_{31}) were then added to the second fraction which was then analyzed by GC / MS.

The first analysis was therefore the determination of esters of 3-MCPD and glycidol; in the second analysis, palmitic acid and its acylglycerols were measured. These two groups of substances were determined separately using GC/MS because of their very different concentrations in the model system.

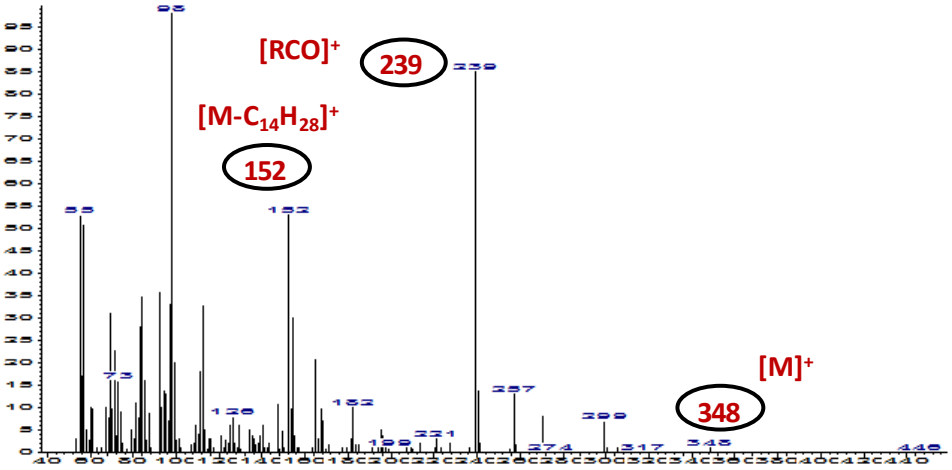
3-MCPD dipalmitate:



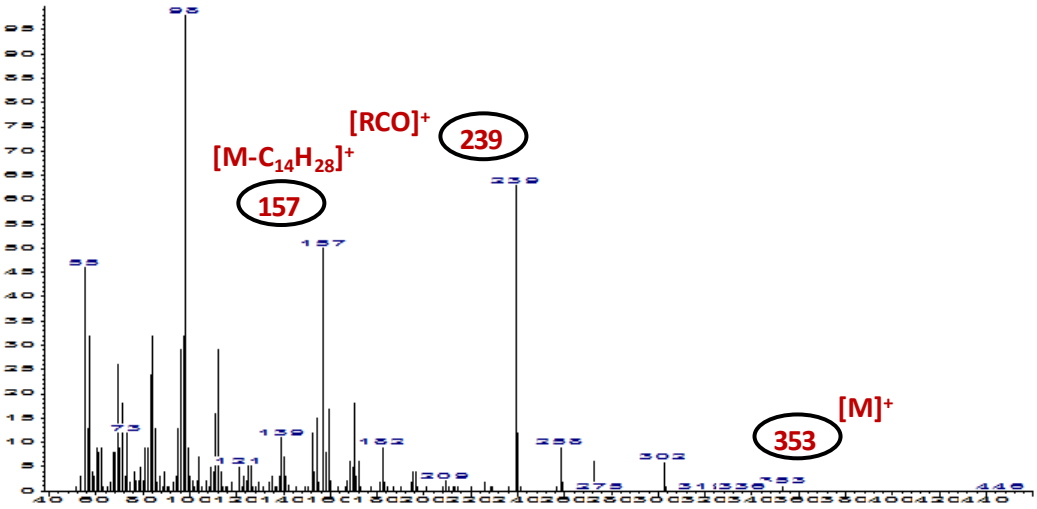
3-MCPD-d_5 dipalmitate:



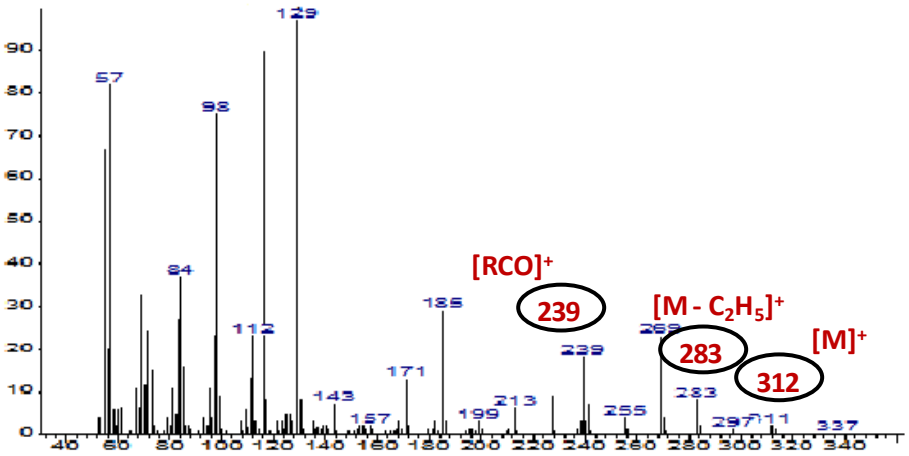
3-MCPD monopalmitate:



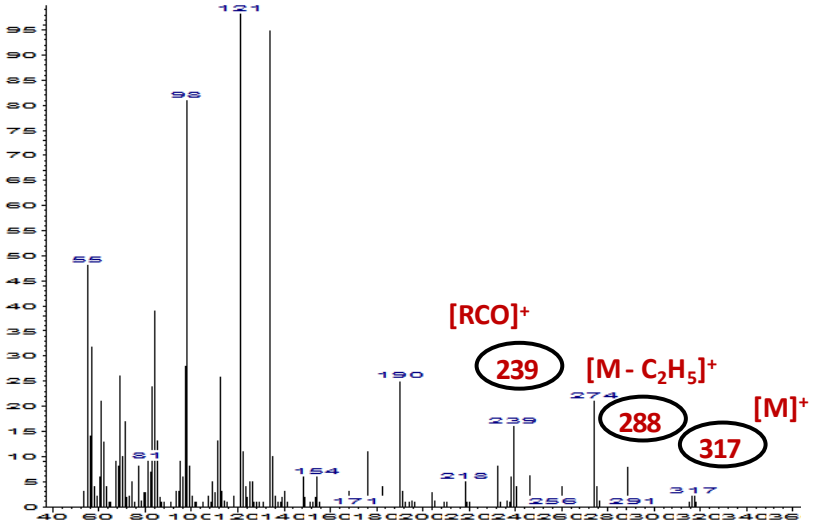
3-MCPD-d_5 monopalmitate:



Glycidyl palmitate:



glycidyl-d_5 palmitate:



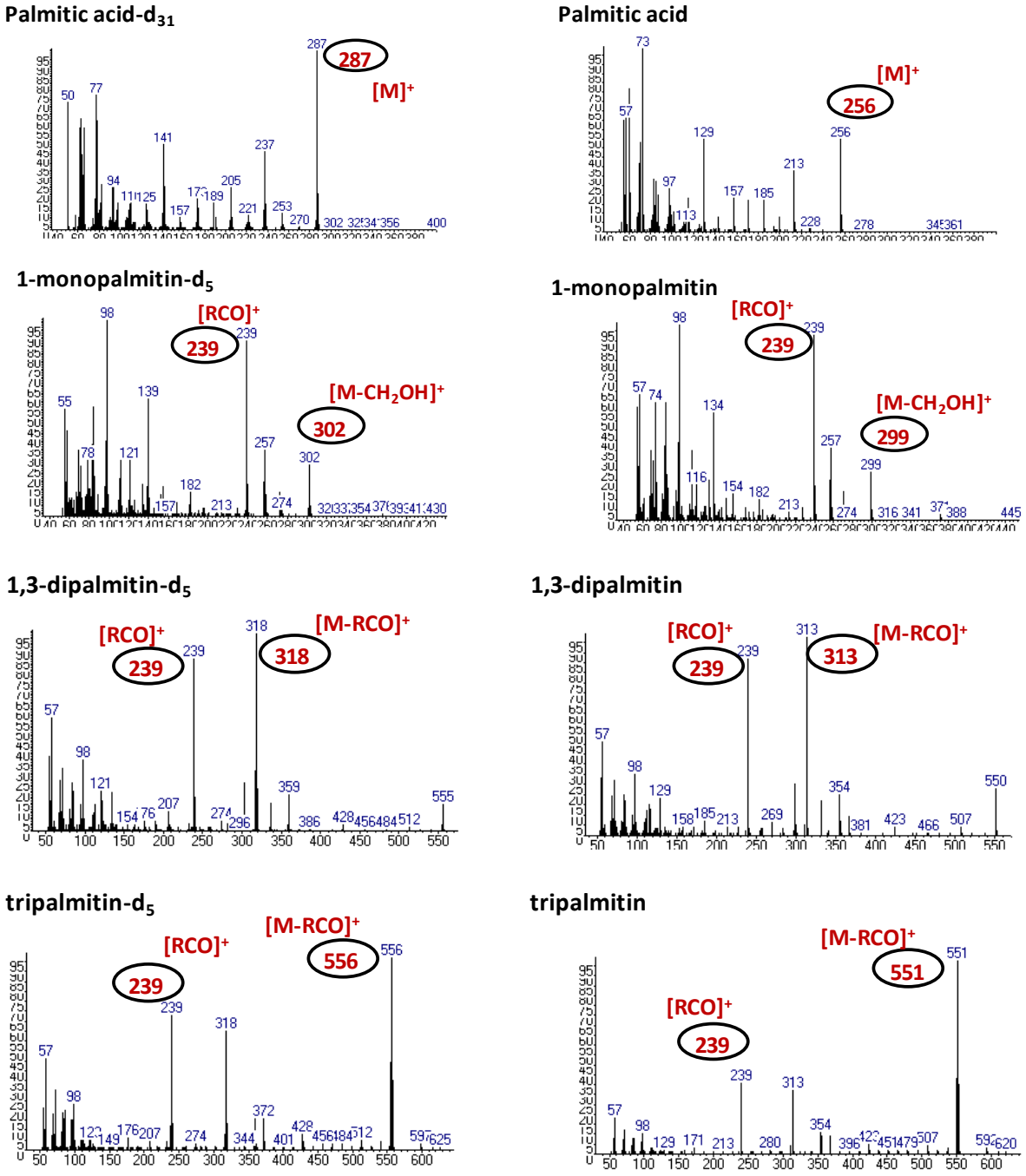


Figure 5. Full scan mass spectra of model system reference chemicals

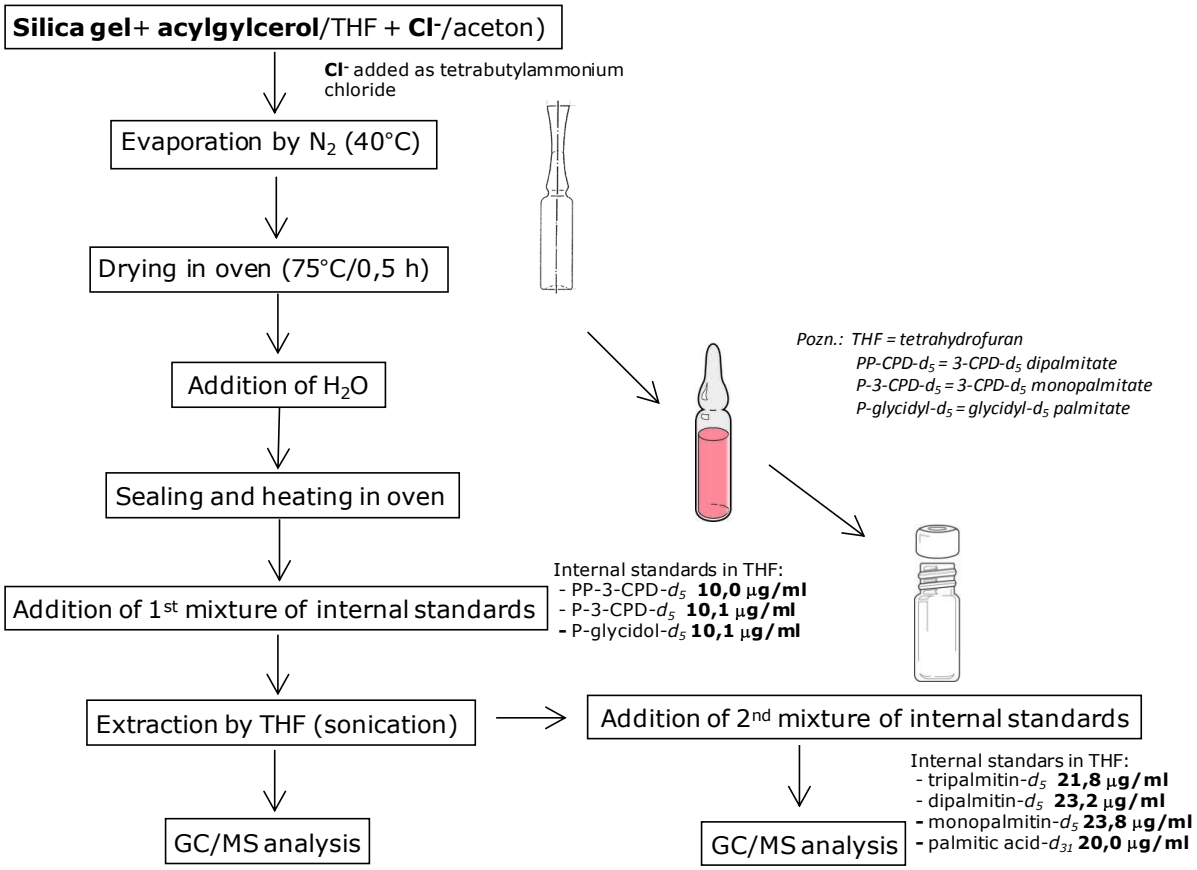


Figure 6. Scheme of the model system experiment

The tabulated results from each experiment are given below while the charted data can be found in 9.4.2 of the appendix.

7.1.2.2 **Formation of MCPD and glycidol esters from tripalmitin**

The formation of 3-MCPD esters and glycidol esters was studied in a model system comprising tripalmitin, water, chloride ions and silica gel.

The influence of temperature was studied over the range 110 to 260°C and the results are shown in Table 10 (and Figure 19). The concentrations of both P-glycidol and P-3-MCPD increased with increasing temperature while that of PP-3-MCPD did not increase significantly until much higher temperature (260°C). The concentration of tripalmitin decreased with increasing temperature while the concentrations of FFA, MAG and DAG all increased, consistent with hydrolysis of hydrolysis of TAG the fatty acid esters.

Table 10. Influence of temperature on tripalmitin model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

Temperature [°C]	P-glycidol	P-3-MCPD [μg/mg]	PP-3-MCPD	MAG [%]	DAG	TAG	FFA
110	0.04	0.01	0.06	8.8	11.5	61.7	18.0
140	0.07	0.01	0.06	7.7	13.4	53.7	25.2
170	0.16	0.09	0.04	27.5	11.0	43.0	18.4
200	0.17	0.11	0.07	11.0	17.6	23.3	48.1
230	0.30	0.20	0.07	10.9	18.2	18.6	52.3
260	0.76	0.43	0.25	21.7	14.8	12.5	51.0

The influence of heating time was studied over 0.5 to 3 hours and the results are shown in Table 11 (and Figure 20). Amounts of P-3-MCPD and PP-3-MCPD reached a maximum at circa 1 h with a slight decrease thereafter, while amounts of P-glycidol continued to increase with increasing heating time. The concentration of tripalmitin decreased with heating time while the concentrations of FFA, MAG and DAG all increased, consistent with hydrolysis of hydrolysis of TAG the fatty acid esters.

Table 11. Influence of heating time on tripalmitin model system (0.5% of water, 0.5% of chloride ions, temperature 200°C)

Time [h]	P-glycidol	P-3-MCPD [μg/mg]	PP-3-MCPD	MAG	DAG	TAG [%]	FFA
0.5	0.07	0.12	0.02	24.3	13.5	39.2	22.9
1	0.17	0.11	0.07	11.0	17.6	23.3	48.1
2	0.31	0.08	0.07	11.8	23.4	27.1	37.7
3	0.38	0.06	0.06	10.2	24.2	19.2	46.4

The effect of chloride ions content was studied over the range 0.1 to 1% and the results are shown in Table 12 (and Figure 21). Overall, changes in chloride ions appeared to have no additional impact on the generation of glycidol and 3-MCPD esters or concentrations of tripalmitin and the corresponding FFA, MAG and DAG.

Table 12. The effect of chloride ions content on tripalmitin model system (0.5% of water, heating time 1 hour, temperature 200°C)

Chloride [%]	P-glycidol	P-3-MCPD [µg/mg]	PP-3-MCPD	MAG	DAG	TAG [%]	FFA
0.1	0.20	0.09	0.02	9.2	19.4	35.9	35.5
0.5	0.17	0.11	0.07	11.0	17.6	23.3	48.1
1	0.16	0.10	0.03	16.4	12.7	42.3	28.6

The effect of water content was studied in the range of 0 and 10%. The results are shown in Table 13 (and Figure 22). Overall changes in water content appeared to have no additional impact on the generation of 3-MCPD esters while some increase in P-glycidol was observed. Concentrations of TAG, MAG, DAG and FFA showed little change.

Table 13. The effect of water content on tripalmitin model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C)

Water [%]	P-glycidol	P-3-MCPD [µg/mg]	PP-3-MCPD	MAG	DAG	TAG [%]	FFA
0	0.14	0.10	0.03	6.4	12.3	29.0	52.3
0.5	0.17	0.11	0.07	11.0	17.6	23.3	48.1
1	0.19	0.18	0.02	20.3	16.8	28.7	34.2
5	0.20	0.09	-	30.4	11.2	19.8	38.6
10	0.36	0.17	0.19	16.0	13.8	10.6	59.5

7.1.2.3 **Formation of MCPD and glycidol esters from monopalmitin**

The formation of 3-MCPD esters and glycidol esters was studied in a model system comprising of monopalmitin, water, chloride ions and silica gel under the same conditions as previous for tripalmitin.

The results for models heated over the range of 110 to 260°C are shown in Table 14 (and Figure 23). Compared to the tripalmitin model, the amount of P-3-MCPD after heating to 260°C under the same conditions was, by a factor of 2, greater indicating that the MAG may be a direct precursor of P-MCPD; a slight increase in the amount of P-glycidol was also observed while the concentrations of PP-3-MCPD were similar. The concentration of MAG showed a slight decrease (and a corresponding increase in FFA); some formation of MAG and TAG was observed.

Table 14. Influence of temperature on monopalmitin model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

Temperature [°C]	P-glycidol	P-3-MCPD [μg/mg]	PP-3-MCPD	MAG	DAG	TAG [%]	FFA
110	0.11	0.07	0.18	73.6	8.7	4.3	13.5
140	0.42	0.12	0.22	62.9	14.6	6.9	15.6
170	0.53	0.29	0.13	54.0	17.4	10.1	18.5
200	0.98	0.26	0.13	53.6	11.6	6.6	28.2
230	1.14	0.87	0.23	47.1	11.1	4.5	37.3
260	1.19	0.86	0.29	47.3	10.9	6.6	35.2

The influence of heating time was studied over the range of 0.5 to 3 hours. The results are shown in Table 15 (and Figure 24). Concentrations of P-glycidol, P-3-MCPD and PP-3-MCPD reached a maximum at circa 1 h of heating: the MCPD esters were not detected after 3 h of heating.

Table 15. Influence of heating time on monopalmitin model system (0.5% of water, 0.5% of chloride ions, temperature 200°C)

Time [h]	P-glycidol	P-3-MCPD [μg/mg]	PP-3-MCPD	MAG	DAG	TAG [%]	FFA
0.5	0.68	0.23	0.15	55.8	4.9	2.4	37.0
1	0.98	0.26	0.13	53.6	11.6	6.6	28.2
2	0.83	0.24	0.14	30.4	9.0	5.3	55.2
3	0.80	0.00	0.03	13.9	23.5	9.8	52.8

The effect of chloride ions content was studied over the range 0.1 to 1% and the results are shown in Table 16 (and Figure 25). The concentrations of P-glycidol and P-MCPD increased with increasing concentrations of chloride ions while the concentration of MAG reduced significantly.

Table 16. The effect of chloride ions content on monopalmitin model system (0.5% of water, heating time 1 hour, temperature 200°C)

Chloride [%]	P-glycidol	P-3-MCPD	PP-3-MCPD	MAG	DAG	TAG	FFA
	[µg/mg]			[%]			
0.1	0.21	0.12	0.06	57.0	3.5	1.7	37.8
0.5	0.98	0.26	0.13	53.6	11.6	6.6	28.2
1	1,81	0.82	0.13	24.8	12.5	7.0	55.6

The effect of water content was studied over the range of 0 to 10% and the results are shown in Table 17 (and Figure 26). The concentrations of P-glycidol, P-MCPD and PP-MCPD all increased with increasing water concent, reaching a maximum at circa 5 %. A corresponding decrease in MAG and increase in FFA was also observed.

Table 17. The effect of water content on monopalmitin model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C)

Water [%]	P-glycidol	P-3-MCPD	PP-3-MCPD	MAG	DAG	TAG	FFA
	[µg/mg]			[%]			
0	0.81	0.18	0.08	49.2	7.5	3.8	39.4
0.5	0.98	0.26	0.13	53.6	11.6	6.6	28.2
1	1.09	0.45	0.16	36.9	18.2	14.0	30.9
5	1.27	1.04	0.56	16.6	5.6	3.5	74.3
10	1.23	0.78	0.14	18.7	11.4	8.3	61.6

7.1.2.4 **Formation of MCPD and glycidol esters from dipalmitin**

The formation of 3-MCPD esters and glycidol esters was studied in a model system comprising dipalmitin, water, chloride ions and silica gel.

The influence of temperature was studied over the range 110 to 260°C. The results are shown in Table 18 (and Figure 27). A rapid increase in the concentration of P-MCPD was observed indicating that dipalmitin may be a direct precursor of PP-MCPD (compared to the formation of P-MCPD from MAG). The concentration of P-glycidol also increased rapidly indication the involvement of MAG in its formation.

Table 18. Influence of temperature on dipalmitate model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

Temperature [°C]	P-glycidol	P-3-MCPD	PP-3-MCPD	MAG	DAG	TAG	FFA
	[μg/mg]			[%]			
110	1.33	0.06	1.45	0.9	59.3	32.1	7.7
140	1.05	0.00	0.50	0.0	51.3	32.6	16.1
170	1.00	0.03	0.07	16.0	43.6	26.1	14.3
200	0.88	0.13	0.03	11.3	40.4	24.9	23.4
230	0.91	0.05	0.19	21.0	17.7	13.9	47.5
260	0.40	0.00	0.05	0.0	18.8	19.3	61.9

The influence of heating time was studied over the range of 0.5 to 3 hours. The results are shown in Table 19 (and Figure 28.) Concentrations of P-MCPD and PP-MCPD showed little dependence on heating time and in keeping with the results for the MAG and TAG, were not detected after heating at 200°C for 3 h: the concentration of P-glycidol however continued to increase with increasing temperature.

Table 19. Influence of heating time on dipalmitate model system (0.5% of water, 0.5% of chloride ions, temperature 200°C)

Time [h]	P-glycidol	P-3-MCPD	PP-3-MCPD	MAG	DAG	TAG	FFA
	[μg/mg]			[%]			
0.5	0.34	0.14	0.02	3.7	20.4	11.8	28.1
1	0.88	0.13	0.03	11.3	40.4	24.9	23.4
2	0.91	0.00	0.13	11.9	39.0	22.6	26.5
3	1.20	0.00	0.00	16.4	32.0	21.3	30.2

The effect of chloride ions content was studied over the range of 0.1 to 1%. The results are shown in Table 20 (and Figure 29). P-glycidol showed a linear dependence with the concentration of chloride ions.

Table 20. The effect of chloride ions content on dipalmitate model system (0.5% of water, heating time 1 hour, temperature 200°C)

Chloride [%]	P-glycidol [μg/mg]	P-3-MCPD	PP-3-MCPD	MAG [%]	DAG	TAG	FFA
0.1	0.50	0.68	0.07	0.0	32.3	28.2	39.5
0.5	0.88	0.13	0.03	11.3	40.4	24.9	23.4
1	1.28	0.47	0.23	11.0	38.4	21.0	29.7

The effect of water content was studied over the range 0 to 10% and the results are shown in Table 21 (and Figure 30). P-3-MCPD and PP-3-MCPD showed little dependence on water content while the concentration of P-glycidol increased steadily indicating the requirement for hydrolysis of DAG in the formation of the latter species..

Table 21. The effect of water content on dipalmitate model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C)

Water [%]	P-glycidol [μg/mg]	P-3-MCPD	PP-3-MCPD	MAG [%]	DAG	TAG	FFA
0	0.70	0.05	0.07	16.1	41.0	20.8	22.1
0.5	0.88	0.13	0.03	11.3	40.4	24.9	23.4
1	2.16	0.08	0.02	9.7	34.2	17.2	38.9
5	3.29	0.11	0.00	22.0	28.6	19.4	29.9
10	3.10	0.12	0.00	17.6	15.7	12.9	53.8

7.1.2.5 **Decomposition reactions of 3-MCPD monopalmitate**

The decomposition of 3-MCPD monopalmitine (P-3-MCPD) was studied in the model system consisting of 3-MCPD monopalmitine, water, chloride ions and silica gel.

The influence of temperature was studied over the range 110 to 260°C and the results are shown in Table 22 (and Figure 31). The concentration of P-3-MCPD decreased with increasing temperature

while the concentration of p-glycidol increased rapidly reaching a maximum at circa 140°C and then decreasing.

Table 22. Influence of temperature on P-3-MCPD model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

Temperature [°C]	P-glycidol [μg/mg]	P-3-MCPD	PP-3-MCPD
110	55.57	192.47	3.42
140	110.24	51.88	5.24
170	13.97	24.92	15.81
200	60.81	37.62	50.16
230	6.46	25.13	16.61
260	1.65	4.93	5.19

The effect of water content was studied over the range 0 to 10% . The results are shown in Table 23 and Figure 32. The concentration of P-3-MCPD decreased with increasing water content while the concentration of P-glycidol reached a maximum at circa 0.5% water before decreasing.

Table 23. The effect of water content on P-3-MCPD model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C)

Water [%]	P-glycidol [μg/mg]	P-3-MCPD	PP-3-MCPD
0	42.83	22.25	41.74
0.5	60.8	37.6	50.2
1	21.70	10.97	13.46
5	0.59	15.44	8.71
10	0.00	2.61	0.58

7.1.2.6 **7.1.2.5 Decomposition reactions of 3-MCPD dipalmitate**

The decomposition of 3-MCPD dipalmitate (PP-3-MCPD) was studied in the model system consisting of 3-MCPD dipalmitate, water, chloride ions and silica gel.

The influence of temperature was studied over the range 110 to 260°C. The results are shown in Table 24 (and Figure 33). As expected, the concentration of PP-3-MCPD decreased rapidly with increasing temperature; some concurrent formation of P-glycidol and P-3-MCPD occurred rapidly and concentrations thereafter decreased with increasing temperature.

Table 24. The effect of temperature on PP-3-MCPD model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour, temperature 200°C)

Temperature [°C]	P-glycidol [µg/mg]	P-3-MCPD	PP-3-MCPD
110	11.83	27.85	165.18
140	11.10	22.59	103.76
170	11.36	4.90	78.27
200	3.66	2.88	38.15
230	9.02	0.70	16.05
260	13.79	4.80	18.14

The effect of water content was studied over the range 0 to 10%. The results are shown in Table 25 and Figure 34. As expected, the concentration of PP-3-MCPD decreased with increasing water content.

Table 25. The effect of water content on PP-3-MCPD model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C)

Water [%]	P-glycidol [µg/mg]	P-3-MCPD	PP-3-MCPD
0	3.66	5.70	80.85
0.5	3.66	2.88	38.15
1	0.29	0.90	34.25
5	1.37	1.69	36.01
10	3.41	0.22	36.18

7.1.2.7 **Decomposition reactions of glycidyl palmitate**

The decomposition of glycidyl palmitate was studied in a model system comprising of glycidyl palmitate, water, chloride ions and silica gel.

The influence of temperature was studied over the range 110 to 260°C and the results are shown in Table 26 (and Figure 35). The concentration of P-glycidol fell quickly with increasing temperature while corresponding concentration of P-3-MCPD increased rapidly (maximum at 110°C), presumably due to formation from P-glycidol and chloride ions under the conditions employed.

Table 26.The effect of temperature on glycidyl palmitate model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour, temperature 200°C)

Temperature [°C]	P-glycidol [μg/mg]	P-3-MCPD	PP-3-MCPD
110	42.28	155.60	6.17
140	11.17	32.10	2.46
170	2.04	2.07	0.04
200	3.55	0.05	0.10
230	2.80	0.33	0.00
260	1.91	0.35	0.00

The effect of water content was studied over the range 0 to 10% . The results shown in Table 27 (and Figure 36) indicate that P-glycidol was little affected by water content as it appeared to be unstable after heating for 1 h at 200°C (0% water).

Table 27. The effect of water content on glycidyl palmitate model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C)

Water [%]	P-glycidol [μg/mg]	P-3-MCPD	PP-3-MCPD
0	2.12	0.00	0.50
0.5	3.55	0.05	0.10
1	6.36	0.00	1.15
5	3.12	0.07	0.00
10	2.06	0.27	0.00

7.1.3 Conclusions from formation and decomposition reactions

A model system was developed to follow the kinetics of formation and decomposition of 3-MCPD esters and esters of glycidol. For quantification, deuterium labelled analogues of all analytes were used as internal standards.

From knowledge of the chemistry and the experimental results, tripalmitin did not act as a direct precursor of 3-MCPD esters and glycidyl esters. The likely reason for this is due to the low electron density found around the acyl oxygen at the sn-2 position of TAG which disables cyclisation, i.e. the formation of a five-membered intramolecular ring intermediate (cyclic ketal, substituted 1,3-dioxolane). It would appear that tripalmitin must first be hydrolysed to a partial acylglycerol (e.g. diacyl- and monoacylglycerols) and the extent of the hydrolysis increases directly with increasing temperature in the presence of water. This subsequently leads to the formation of 3-MCPD esters and esters of glycidol, but in significantly lower amounts than from monopalmitin directly.

The content of 3-MCPD esters and glycidol esters was not directly influenced by chloride ions over the range of 0.1-1% in models with tripalmitin, probably due to the fact that this concentration of chloride ions represents a large excess of reactant compared to the amount of partial acylglycerols formed. On the other hand, in models with monopalmitin, the amount of 3-MCPD esters and glycidol esters showed a linear dependence on the concentration of chloride ions.

7.2 Model system studies with enzymes[2]

The aim of this work was to design a model system to monitor the activities of non-specific and specific lipases leading to the formation of 3-MCPD esters. In light of the sporadic knowledge concerning these enzyme formation routes, the models would also help to confirm the expected mechanisms that lead to the formation of esters of chloropropanols in foods, especially in vegetable oils and during enzyme-catalyzed transesterification processes used for the production of structural fats. These fats are suitable for the manufacture of low SAFA (Saturated Fatty Acid) poly/mono unsaturated margarines and spreads. The kinetics of formation and decomposition was monitored using the following starting materials: high oleic sunflower oil, fully hydrogenated coconut oil and methyl laurate. In the kinetic study the effects of non-specific and specific lipases and the influence of heating time on the formation of 3-MCPD esters were investigated.

7.2.1 Experimental

7.2.1.1 Chemicals and materials

Oliol – high oleic sunflower oil (Palma Group, a.s., SK), coconut oil (MPD plus Rakovník, s.r.o, CZ), lauric acid methyl ester (Fluka Chemie GmbH, CH), tetrabutylammonium chloride (Aldrich, D), molecular sieve 0.3 nm Art-No. 5704 (Merck, D) were purchased. Lipozyme 435 - non-specific lipase (NL) from *Candida antarctica* and Lipozyme RM IM – 1,3 – specific lipase (SL) from *Rhizomucor miehei* were delivered by Novozyme A/S (DK). Coconut oil was fully hydrogenated at Institute of Chemical Technology Prague (ICT Prague). 3-MCPD-d_5 dipalmitin was synthesized at ICT Prague.

7.2.1.2 Model system for optimization of enzymatic transesterification

Model reaction systems were prepared by mixing Oliol and lauric acid methyl ester in a molar ratio of 1:2 for 1,3-specific lipase and 1:3 for nonspecific lipase. The mixture (30 g) was placed in a 250-ml reaction vessel, stirred continuously and dried under vacuum (30-40 hPa) in argon atmosphere at 100-110°C for 60 min. To the mixture, maintained at the reaction temperature (60, 70 and 80°C), were added: molecular sieves (2 g, pore size: 30 nm) to remove residual water; followed by the appropriate enzyme (10% w/w). Enzymatic transesterification reactions were carried out with constant stirring of the mixture under argon for 6 hours. During the reaction, samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6 h and analyzed by GC/MS using pentadecanoic acid methyl ester added as internal standard. The loss of lauric acid methyl ester and increase of oleic acid methyl ester were monitored.

[2] Marek Doležal, Vladimír Filip, Markéta Tesařová, Vojtech Ilko, Jan Velíšek, ICT Prague, Departments of of Food Chemistry and Analysis & Dairy, Fat and Cosmetics, Technická 5, 166 28 Prague 6, Czech Republic.

7.2.1.3 **Model system of enzymatic transesterification**

Model reaction systems were prepared as follows:

- Oliol
- Oliol and fully hydrogenated coconut oil (FHCO) in a molar ratio of unsaturated to saturated fatty acid 1:1
- Oliol and lauric acid methyl ester in a molar ratio 1:2 for 1,3-specific lipase
- Oliol and lauric acid methyl ester in a molar ratio 1:3 for nonspecific lipase

The fatty acid composition of Oliol and fully hydrogenated coconut oil were the basis for the calculation of molar weight of these components. The mixture (30 g) was placed in a 250-ml reaction vessel, stirred continuously and dried under vacuum (30-40 hPa) in argon atmosphere at 100-110°C for 60 min. To the reaction mixture maintained at 60°C for 1,3-specific lipase or 80°C for nonspecific lipase, were added: tetrabutylammonium chloride (TBAC; calculated as 100 mg of chlorides per kg of reaction mixture) as a source of chloride ions; molecular sieves (2 g) to remove residual water; and the appropriate enzyme (10% w/w).

Enzymatic transesterification was carried out with constant stirring of the mixture under argon for 4 h for non-specific lipase and 6 h for 1,3-specific lipase. During the reaction, samples were taken at 0, 0.5, 1, 2, 3, 4 and 6 h and analyzed by GC/MS with 3-MCPD-d_5 dipalmitate as internal standard. The formation of 3-MCPD esters was monitored.

7.2.1.4 **Determination of 3-MCPD esters**

The oil (100 mg) placed in a 10-ml distillation flask was dissolved in tetrahydrofuran (1 ml) and 1ml of 3-MCPD-*d5* dipalmitate (internal standard, 2 μg/ml) was added. To this solution, 1.8 ml sulphuric acid solution (98%, 1.8 ml in 100 ml methanol) was added and the mixture was heated at 40°C for 16 h. The cooled mixture was then neutralised with saturated $NaHCO_3$ solution (0.5 ml) and evaporated to dryness using a vacuum rotary evaporator at 55°C. The residue was dissolved in 20% sodium sulphate (2 ml) and extracted with two portions of hexane (2 ml). After the addition 0.2 ml of derivatisation reagent (3.0 g phenylboronic acid+11.4 ml acetone+0.6 ml water) and 5 min sonication, the analytes were extracted by 1 ml hexane and analyzed by GC/MS. Three parallel determinations of each sample were made. 3-MCPD esters (as a sum of all the respective esters) in oil were expressed as free 3-MCPD equivalent.

7.2.1.5 **GC/MS analysis**

Chromatographic capillary column Equity-1 (Supelco, USA)

- (100% dimethylpolysiloxane, 30 m x 0.25 mm i.d., 1 μm film thickness)

Injection Volume: 1.0 μl

Injection mode:	Pulsed Splitless
Injection temperature:	280ºC
Column flow:	1.0 ml/min
Initial oven temperature:	170ºC
Oven program:	
	Initial oven temperature 140 ºC
	Then 10ºC/min to 300ºC
	Then 40ºC/min to 340ºC for 15 min
Transfer line temperature:	300ºC

For quantification, SIM mode was used to monitor ions at m/z 147 (3-MCPD) and at m/z 150 (3-MCPD-d_5). Ions at m/z 91and 196 (3-MCPD) and at m/z 93 and 201 (3-MCPD-d_5) were used for confirmation of identity.

7.2.1.6 **Determination of fatty acids composition**

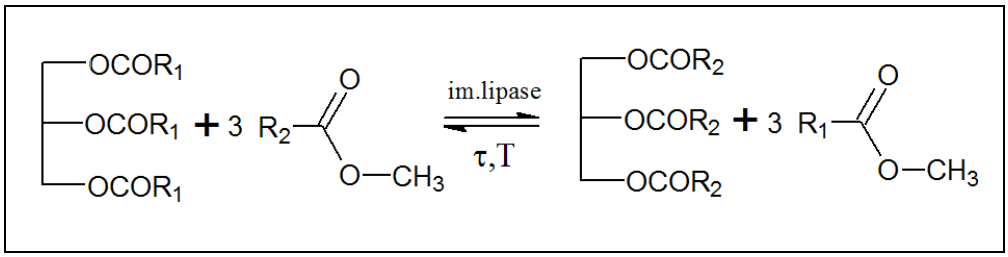
Derivatization of fatty acids was based on the base catalysed reaction using NaOH-methanol as reagent. Fatty acid methyl esters (FAMEs) were then extracted to hexane. FAMEs were analysed by GC using a SP-2560 fused silica capillary column (100 m × 0.25 mm i.d., 20 μm film thickness, Supelco, Bellefonte, USA) in a Agilent 6890 gas chromatograph (Palo Alto, USA) equipped with flame ionizing detector (FID). The oven temperature was held at 175°C for 30 min, then increased by 1°C/min to 220°C and this temperature was maintained for 30 min. Detector and injection port temperatures were 220°C and the nitrogen carrier gas flow was 1 ml/min. For the identification of FAMEs, standard FAME mixtures were analysed. To confirm the identification of some FAMEs, GC/MS analysis were carried out on the GC/MSD system Agilent 5975 (Agilent, Palo Alto, USA) with the same column and temperature conditions as above, except for the helium flow, which was 0.6 ml/min and the transfer-line temperature 250°C.

7.2.2 Results and discussion

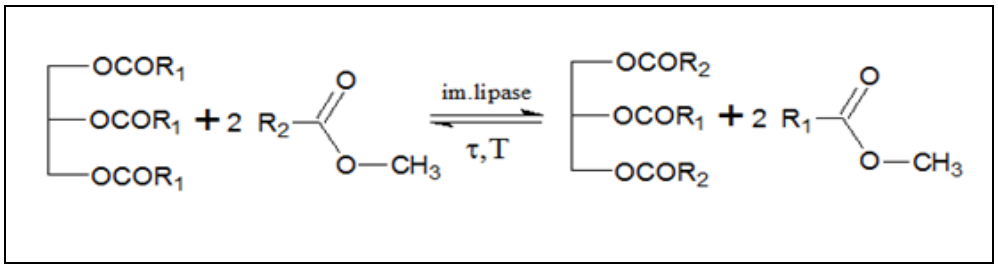
The newly prepared model system was developed to monitor the formation of 3-MCPD esters by the activities of non-specific and specific lipases under conditions typical of enzyme-catalyzed transesterification leading to production of structural fats, which are suitable for the manufacture of low SAFA (Saturated Fatty Acid) poly/mono unsaturated margarines and spreads.

Lipases: commercial immobilized enzymes were tested

Enzymatic transesterification catalyzed by the enzyme unspecific lipase:



Enzymatic transesterification catalyzed by the enzyme *sn*-1,3 specific lipase:



7.2.2.1 **Characterization of raw materials**

The initial parameters of the components of model mixtures were characterized by determining the following: acid number, iodine number and peroxide value (Table 28**);** the fatty acid composition (Table 29); and the amount of 3-MCPD esters (Table 30).

Table 28. Acid number, iodine number and peroxide value of model components

Determination of	Oliol	FHCO	Lauric acid methyl ester
Acid number [mg KOH/g]	0.2	0.6	0.4
Iodine number [g I_2/100 g]	82.68	0.64	-
Peroxide value [meq. act.O/kg]	3.03	0.59	7.32

Table 29. Fatty acid composition of model components (% of total fatty acids)

Fatty acid	Oliol	FHCO	Lauric acid methyl ester
C 4:0	-	-	-
C 6:0	-	0.48	-
C 8:0	-	6.69	-
C 10:0	-	5.6	0.14
C 12:0	-	45.55	99.23
C 12:1	-	-	0.36
C 14:0	-	18.57	0.27
C 15:0	-	-	-
C 16:0	3.49	10.15	-
C 16:1	0.11	-	-
C 17:0	0.02	-	-
C 18:0	2.72	12.7	-
∑ C 18:1	81.44	0.04	-
∑ C 18:2	10.15	-	-
∑ C 18:3	0.2	-	-
C 20:0	0.25	0.17	-
C 20:1	0.33	-	-
C 22:0	0.95	-	-
C 22:1	-	-	-
C 24:0	0.33	-	-

Table 30. The concentration of 3-MCPD esters in model components (expressed as free 3-MCPD equivalent)

Component	Concentration of 3-MCPD (mg/kg fat)
Oliol	0.23
FHCO	< 0.10*
Lauric acid methyl ester	< 0.03**

* no residues exceeding limit of quantification (LOQ) were found in examined sample

** no residues exceeding limit of detection (LOD) were found in examined sample

7.2.2.2 **Optimization of enzymatic transesterification conditions**

Reactions were carried out under an inert argon atmosphere at 60, 70 or 80°C and a reaction time of 6h. The transesterification was monitored by the increase in concentration of oleic acid methyl ester (see Figure 7). Slightly improved conditions for *sn*-1,3 specific lipase were observed at 60°C than at 70°C. The highest yield was obtained for the nonspecific lipase at 80°C after 6 h, but the increase in oleic acid methyl ester after 4 h was negligible.

On the basis of these results, the following conditions of enzymatic catalyzed transesterification were selected: a) 60°C and 6 h using *sn*-1,3 specific lipase; and b) 80°C and 4 h using nonspecific lipase.

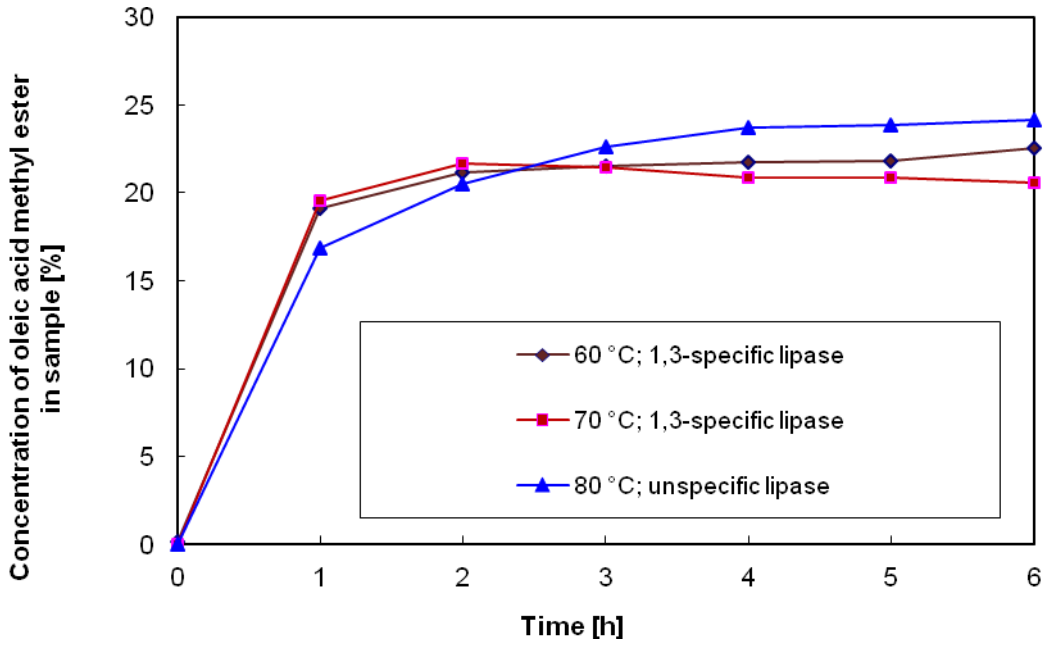


Figure 7. Efficiency of transesterification based on *sn*-1,3 specific lipase and nonspecific lipase

7.2.2.3 **Enzymatic transesterification catalyzed by nonspecific lipase**

The formation of 3-MCPD esters was studied initially in the model system consisting of Oliol (high oleic sunflower oil), tetrabutylammonium chloride as a source of chloride ions and 10% Lipozyme 435 - non-specific lipase from *Candida antarctica* at 80°C. As Table 31 shows, these conditions did not lead to the formation of 3-MCPD esters as their concentration remained static over 4 h. Similar results were obtained for mixture consisting of Oliol and fully hydrogenated coconut oil in a molar ratio of unsaturated to saturated fatty acid 1:1, tetrabutylammonium chloride and 10% Lipozyme 435 - non-specific lipase (Table 32**)** and for mixture consisted of Oliol and Oliol and lauric acid methyl ester in a molar ratio 1:3, tetrabutylammonium chloride and 10% Lipozyme 435 - non-specific lipase (Table 33**)**.

Table 31. The concentration of 3-MCPD esters in Oliol (expressed as free 3-MCPD equivalent) using a non-specific lipase

Time [h]	Concentration of 3-MCPD [mg/kg fat]	RSD [%]
0	0.23	2.9
0.5	0.21	2.3
1	0.22	3.7
2	0.21	1.5
3	0.21	3.2
4	0.21	2.8

Table 32. The concentration of 3-MCPD esters in Oliol and fully hydrogenated coconut oil in a molar ratio of unsaturated to saturated fatty acid 1:1 (expressed as free 3-MCPD equivalent) using a non-specific lipase

Time [h]	Concentration of 3-MCPD [mg/kg fat]	RSD [%]
0	0.17	1.9
0.5	0.17	3.1
1	0.19	3.7
2	0.18	2.6
3	0.17	2.6
4	0.19	2.4

Table 33. The concentration of 3-MCPD esters in Oliol and lauric acid methyl ester in a molar ratio 1:3 (expressed as free 3-MCPD equivalent) using a non-specific lipase

Time [h]	Concentration of 3-MCPD [mg/kg fat]	RSD [%]
0	0.15	3.6
0.5	0.16	3.4
1	0.14	3.3
2	0.15	2.7
3	0.16	2.4
4	0.16	3.8

7.2.2.4 **Enzymatic transesterification catalyzed by specific lipase**

The formation of 3-MCPD esters was studied in a model system consisting of Oliol (high oleic sunflower oil), tetrabutylammonium chloride as a source of chloride ions and 10% Lipozyme RM IM – 1,3 – specific lipase (SL) from *Rhizomucor miehei* at 60°C. These conditions did not lead to formation of 3-MCPD esters, and again the concentration remained the same as shown in Table 34. Similar results were obtained for mixture consisting of Oliol and fully hydrogenated coconut oil in a molar ratio of unsaturated to saturated fatty acid 1:1, tetrabutylammonium chloride and 10% Lipozyme RM IM – 1,3 – specific lipase (Table 35**)** and for a mixture consisting of Oliol and Oliol and lauric acid methyl ester in a molar ratio 1:2, tetrabutylammonium chloride and 10% Lipozyme RM IM – 1,3 – specific lipase (Table 36**)**.

Table 34. The concentration of 3-MCPD esters in Oliol (expressed as free 3-MCPD equivalent) using a specific lipase

Time [h]	Concentration of 3-MCPD [mg/kg fat]	RSD [%]
0	0.23	2.9
0.5	0.23	2.8
1	0.21	3.5
2	0.21	1.3
3	0.21	4.0
4	0.21	2.1
6	0.22	3.5

Table 35. The concentration of 3-MCPD esters in Oliol and fully hydrogenated coconut oil in a molar ratio of unsaturated to saturated fatty acid 1:1 (expressed as free 3-MCPD equivalent) using a specific lipase

Time [h]	Concentration of 3-MCPD [mg/kg fat]	RSD [%]
0	0.17	2.5
0.5	0.17	3.4
1	0.18	4.1
2	0.17	3.0
3	0.16	3.1
4	0.18	3.6
6	0.17	1.8

Table 36. The concentration of 3-MCPD esters in Oliol and lauric acid methyl ester in a molar ratio 1:2 for 1,3-specific lipase (expressed as free 3-MCPD equivalent) using a specific lipase

Time [h]	Concentration of 3-MCPD [mg/kg fat]	RSD [%]
0	0.15	2.9
0.5	0.15	3.8
1	0.14	2.5
2	0.17	2.7
3	0.17	4.0
4	0.18	3.6
6	0.17	3.5

7.2.3 Conclusions from model system studies with enzymes

A model system was developed to follow the activities of non-specific and specific lipases leading to the formation of 3-MCPD esters. The model provided a controlled simulation of an enzyme-catalyzed transesterification process, e.g. typical of that used in the production of structural fats for the manufacture of low SAFA (Saturated Fatty Acid) poly/mono unsaturated margarines and spreads.

The concentrations of 3-MCPD esters remained unchanged in all model system reaction mixtures subjected to enzyme-catalysed transesterification and corresponded to the amount of 3-MCPD esters present in raw material Oliol-high oleic sunflower oil, where they formed during deodorisation, i.e. prior the model system experiments (Figure 8 and Figure 9). Furthermore, the temperature range of 60 - 80°C employed is too low for measurable formation of 3-MCPD esters by the chemical / thermal route.

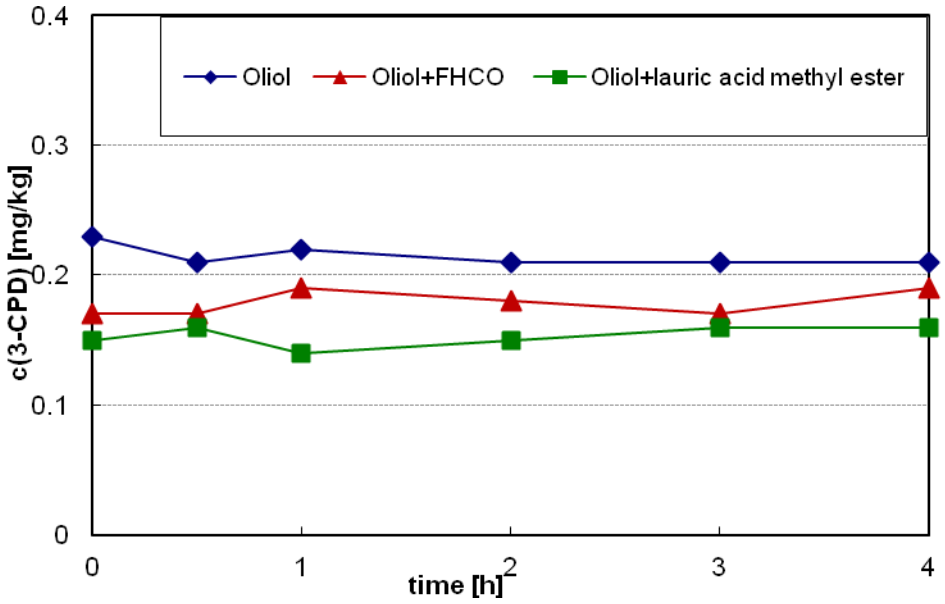


Figure 8. The concentration of 3-MCPD esters under transesterification using nonspecific lipase

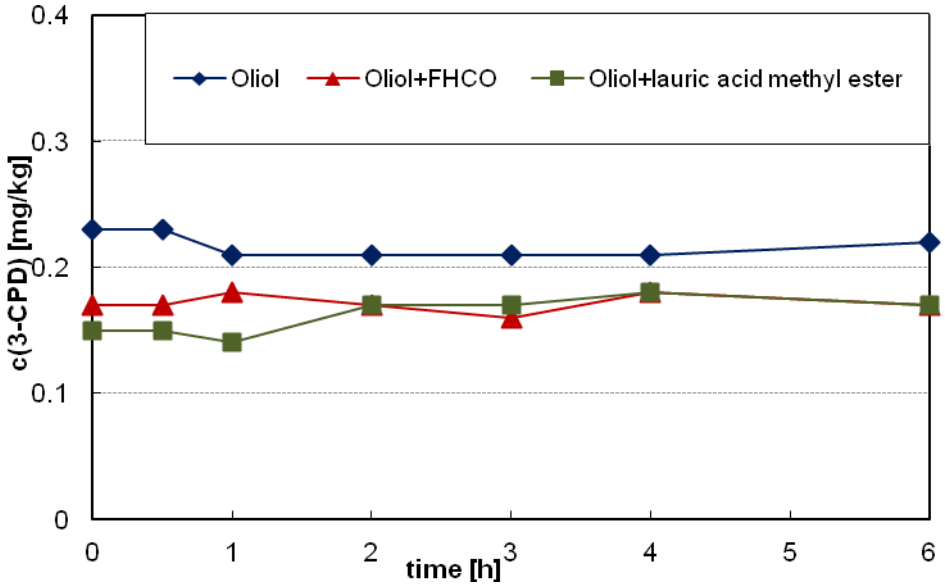


Figure 9. The concentration of 3-MCPD esters under transesterification using 1,3-specific lipase

These model experiments demonstrate that nonspecific or specific lipases are unlikely to contribute to any change in the concentration of 3-MCPD esters during the production of structural fats using of enzyme-catalyzed transesterification.

8. Processing / storage experiments (objective 6)

8.1 Non-cereal products (Task 06.1 FERA, York, UK)

8.1.1 Processing / cooking experiments

8.1.1.1 Cooking experiments

Meat and dairy products

Salami samples (4 samples each in duplicate) grilled at 180 °C for 10 mins; grilled at 80 °C for 20 mins; or fried in a pan at 85 °C for 2 mins.

Cheeses (Emmental, Parmesan and goat's, 1 sample of each type in duplicate) each grilled at 180 °C for 20 mins or oven baked at 180 °C for 20 mins.

Oven chips were baked at 220˚ C at up to 35 minutes according to the packet instructions.

Chipped raw potatoes were fried in corn oil or extra virgin olive oil and sampled at 9, 18, 27 and 36 minutes.

8.1.1.2 Results and discussion

Meat products:

No detectable GE were found in the grilled or fried salami slices.

Dairy products:

Cheeses also formed no detectable GEs on grilling or baking. As expected, interference problems, were encountered with the analysis of the cooked cheese slices (see section 5.3.2.).

Potato products

Table 37 shows the levels of GEs present in the fat portion of oven chips before and after cooking, and in the oil in which raw potatoes were fried in successive batches.

GE levels in the oil coating of oven chips were not clearly affected by domestic cooking at 220 °C over 35 minutes. The content of glycidyl linoleate (C18:2) appeared to increase from 278 to over 400 μg kg^{-1} in the cooked sample but a level of about 370 μg kg^{-1} was measured previously in the uncooked product.

Detectable GEs were not formed in EVOO heated over 36 minutes during the cooking of four batches of chips from raw potatoes. When four batches of raw potatoes were fried in the same portion of refined corn oil over 36 minutes there was a small decrease < 10% in the GE content of all GEs detected (C16 to C18:3).

Table 37 Cooking experiments - Glycidyl Esters in fat portion of chips µg kg^{-1}

GE ug/kg	Heating time min	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
Oven chips	None	nd	nd	28	24	116	278	nd
Oven chips 220°C	20	10	17	54	26	104	421	nd
Oven chips 220°C	35	7	15	52	30	118	411	nd
EVOO	0 to 36	nd	nd	nd	nd	nd	nd	nd
Chips in EVOO	9	nd	nd	nd	nd	nd	16	nd
Chips in EVOO	18	nd	nd	nd	nd	nd	nd	nd
Chips in EVOO	27	nd	nd	nd	nd	nd	nd	nd
Chips in EVOO	36	nd	nd	nd	nd	nd	nd	nd
Corn oil	0	nd	nd	177	28	541	966	28
Corn oil	0	nd	nd	172	34	573	1019	27
Corn oil	9	nd	nd	173	30	542	976	24
Corn oil	18	nd	nd	165	27	488	927	22
Corn oil	27	nd	nd	183	26	477	839	20
Corn oil	36	nd	nd	163	26	466	923	20
Chips in corn oil	9	nd	nd	139	29	449	856	23
Chips in corn oil	18	nd	nd	139	27	388	872	27
Chips in corn oil	27	nd	nd	162	25	374	866	24
Chips in corn oil	36	nd	nd	136	25	381	824	20

Table 38 shows the levels of bound 3-MCPD present in chips, salamis and cheese before and after cooking. Levels of bound 3-MCPD doubled in oven chips on cooking, possibly on account of the relatively high salt content. There was some erratic evidence of increases on cooking salamis and cheeses.

Table 39 shows the levels of free 3-MCPD present in chips, salamis and cheese before and after cooking. No 3-MCPD was formed on frying raw potatoes. Levels of free 3-MCPD in salamis varied erratically with their cooked counterparts. Levels in most cheeses increased a little on grilling.

Table 38 Cooking experiments – Bound MCPD in cooked foods mg/kg

Sample	Type	Code	Method	Temp (measured °C)	Time (min)	3-MCPD	
						Fat	Food
Chips (oven chips)	Potatoes	20	Uncooked		0	0.45	0.01
Chips (oven chips)	Potatoes	20	Oven	220	20	1.01	0.02
Chips (oven chips)	Potatoes	20	Oven	220	35	0.89	0.03
Salami sausage	Meat	6	Uncooked			0.04	<0.01
Salami sausage	Meat	6	Grill	120	10	0.13	0.05
Salami sausage	Meat	6	Grill	120	50	0.09	0.05
Salami sausage	Meat	6	Fry	85	2	0.29	0.11
Cheese - Slices	Dairy	21	Uncooked			2.98	0.60
Cheese - Slices	Dairy	21	Grill	120	5	3.78	0.80
Cheese - Emmental	Dairy	9	Uncooked			<0.04	<0.01
Cheese - Emmental	Dairy	9	Grill	120	5	<0.04	<0.01
Cheese - Goats	Dairy	4	Uncooked			0.04	0.01
Cheese - Goats	Dairy	4	Grill	120	5	0.19	0.05
Cheese - Parmesan	Dairy	5	Uncooked			0.04	0.01
Cheese - Parmesan	Dairy	5	Grill	120	5	0.04	0.01

Table 39 Cooking experiments – Free MCPD in cooked foods mg/kg

Sample	Type	Code	Method	Temp (measured °C)	Time (min)	3-MCPD
Chips (fresh potatoes)	Potatoes		Uncooked		0	<0.005
Chips (fresh potatoes)	Potatoes		Fried	180	20	<0.005
Chips (fresh potatoes)	Potatoes		Fried	180	35	<0.005
Salami sausage	Meat	3	Uncooked			0.029
Salami sausage	Meat	3	Grilled			0.012
Salami sausage	Meat	24	Uncooked			na
Salami sausage	Meat	24	Grilled			0.018
Salami sausage	Meat	6	Uncooked			0.085
Salami sausage	Meat	6	Grilled	120	10	0.010
Salami sausage	Meat	6	Grilled	120	10	0.076
Cheese - Slices	Dairy	21	Uncooked			0.050
Cheese - Slices	Dairy	21	Grilled	120	5	0.109
Cheese - Emmental	Dairy	9	Uncooked			0.008
Cheese - Emmental	Dairy	9	Grilled	120	5	0.006
Cheese - Goats	Dairy	4	Uncooked			<0.005
Cheese - Goats	Dairy	4	Grilled	120	5	0.014
Cheese - Parmesan	Dairy	5	Uncooked			<0.005
Cheese - Parmesan	Dairy	5	Grilled	120	5	0.008

Baking experiments with ingredient oils and fats

Table 40 shows the levels of GEs present in the fat extracted from the cake pastries after cooking.

Table 40 Cooking experiments – cake pastries - Glycidyl Esters in fat portion µg kg^{-1}

		C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
EVOO	no water	0.0	0.0	68.7	0.0	0.0	224.5	20.3
EVOO	no water	0.0	0.0	73.3	0.0	0.0	217.0	17.5
EVOO	with water	0.0	0.0	36.9	0.0	0.0	61.1	5.4
EVOO	with water	0.0	0.0	22.5	0.0	0.0	43.0	0.0
Baking fat	no water	16.5	14.2	666.0	92.2	597.5	913.2	75.1
Baking fat	no water	18.6	13.4	650.5	76.2	665.0	825.4	76.1
Baking fat	with water	11.2	16.1	720.8	92.2	1073.5	630.2	48.9
Baking fat	with water	10.5	12.3	670.9	67.7	1115.2	610.0	50.7

Cake pastries made with EVOO that contained no GEs cooking formed low levels of glycidyl palmitate (~ 70 μg kg^{-1}) and glycidyl linoleate (~ 220 μg kg^{-1}) and glycidyl linolenate (~ 70 μg kg^{-1}). In the pastry mixture that had added water the same GEs were present at much lower levels.

For cake pastries made with refined baking fat that contained all of the common GEs cooking in the presence of water did not affect the glycidyl esters of saturated fatty acids but doubled the quantity of glycidyl oleate while reducing the proportions of glycidyl linoleate and linolenate.

The EVOO experiments suggest that heating the oil produces GEs. However when the GEs were present before heating as in the baking fat, unexplained changes in the relative proportions of the unsaturated C18 esters were seen.

Table 9 shows the levels of bound 3-MCPD present in the cooked cake pastries.

Table 41 Cooking experiments – cake pastries - Bound MCPD in fat portion mg kg^{-1}

Flour	Water	Olive oil	Fat	3-MCPD	2-MCPD
250	0	50	0	0.23	< 0.1
250	20	50	0	0.13	0.13
250	0	0	50	7.66	1.10
250	20	0	50	2.42	0.55

Bound 3-MCPD was produced in the cake mixture containing EVOO. The presence of water halved the quantity of bound 3-MCPD and caused the formation of bound 2-MCPD.

In cake mixtures made from baking fat containing bound 3-MCPD the presence of water caused losses of both bound 3-MCPD and bound 2-MCPD.

8.1.1.2 **Storage experiments**

Sampling was made of stored samples of cheese and salamis (3 of each) to the planned schedule but owing to time constraints only the zero time and final storage time samples were analysed.

No GEs were detected in the stored samples.

Table 42 shows the results of measurements of the levels of 3-MCPD esters in the stored samples. There was no apparent change in the bound 3-MCPD content of dairy foods or a significant change in the levels in salamis.

Table 42 Storage experiments – Bound MCPD in stored foods mg/kg

a) Storage at 4 °C for 4 weeks

Sample		Code	3-MCPD	
			Fat	Food
Cheese - Goats	Dairy	4	0.04	0.01
Cheese - Parmesan	Dairy	5	<0.04	<0.01
Cheese - Emmental	Dairy	9	<0.04	<0.01
Salami sausage	Meat	3	0.18	0.07
Salami sausage	Meat	6	0.04	0.01
Salami sausage	Meat	7	0.06	0.04

b) Storage at 20 °C for 6 months

Sample		Code	3-MCPD	
			Fat	Food
Cheese - Goats	Dairy	4	0.03	<0.01
Cheese - Parmesan	Dairy	5	0.04	0.01
Cheese - Emmental	Dairy	9	0.11	0.02
Salami sausage	Meat	3	0.08	0.03
Salami sausage	Meat	6	0.04	0.01
Salami sausage	Meat	7	0.05	0.03

8.1.2 Conclusions from process studies with non-cereal products

GE levels in the limited number of composite foods investigated (processed cheese, salamis, potato products) were low compared to those found in refined vegetable oils, and in the case of the former foods, these GEs might be derived from refined ingredient vegetable oils used. Cooking fatty foods under grilling or baking conditions did not affect GE formation or loss with the exception of a slight decrease in GEs on repeated use of cooking oils. This is contrary to what was expected, as heating alone is regarded as leading to the formation of GEs. Levels of bound 3-MCPD increased in the fat portion of oven chips on baking but did not clearly do so in cooked salamis and cheeses.

Storage of dairy and meat products at refrigerator or elevated temperature for periods exceeding the likely consumer practice did not alter the levels of GEs or bound 3-MCPD.

Consumer exposure to GEs and bound 3-MCPD is dominated by the occurrence in refined vegetable oils, and there is no evidence of substantial changes in cooking or of the formation or loss of 3-MCPD from bound forms.

8.2 Cereal products (Task 06.2, PAS, High Wycombe, UK)

8.2.1 Model systems

A biscuit model system was developed and optimised to study the effects of thermal processing (temperature and time) on both the formation and decay of bound 3-MCPD in cereal products. The recipe was chosen to be representative of current commercial practices in the UK (Whitely 1971). The chosen system had been used previously to study the effects of processing parameters on acrylamide and was known to have good control of temperature (Hamlet, Sadd, Liang, Jayaratne, Skingle, 2007). Details of the system can be found in 9.6.4.

Lipases are used in some commercial cereal products to modify the properties of doughs in some beneficial way by virtue of their ability to break down triglycerides into constituent mono- and diglycerides and free fatty acids. However, commercial lipases have previously been shown to release free MCPD from the parent chloroesters in the laboratory praxis (Hamlet and Sadd 2004). To study this further, a pasta dough model system was optimised so that the effects of the addition of a commercial lipase on the formation of free 3-MCPD could be followed over time.

8.2.1.1 Preparation of labelled MCPD-esters

To follow the fate of MCPD-esters in each of the model systems a custom synthesis of ^{13}C-3-MCPD-palmitate esters was undertaken so that the fate of MCPD-esters in fortified cereal products could be monitored and distinguished from that present naturally or formed from precursors in the ingredients.

Routes to the preparation of carbon-13 labelled 3-MCPD mono- and dipalmitate reference standards from readily available carbon-13 labelled glycerol were therefore identified at the project outset. While a direct esterification or chlorination of glycerol starting material was feasible for the intermediate production of e.g. mono- and diacylglycerols and chloropropanols, the poor regioselectivity of these reactions and practical limitations (e.g. due to the low solubility of glycerol in the required organic solvents) hampered these approaches. To overcome these limitations, synthetic routes utilising protecting / directing groups on the glycerol moiety were identified and the reaction conditions for key steps, e.g. protection of glycerol, chlorination, were evaluated using non-labelled chemicals (see Figure 10).

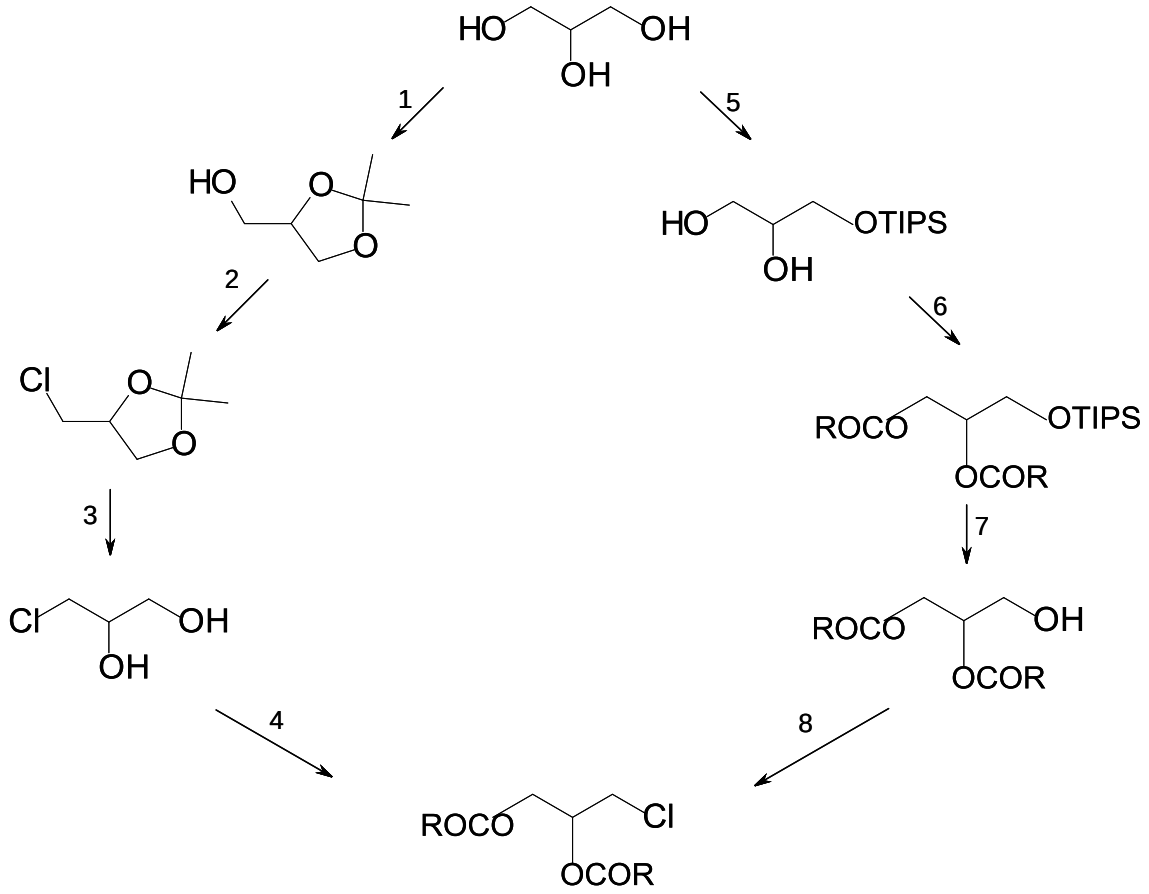


Figure 10. Synthetic routes to the preparation of esters of 3-MCPD from glycerol.

Despite considerable effort, steps 1-3, 4 and 5 either failed to produce the target chemicals or were of insufficient yield to progress to latter steps of the synthetic route (see Figure 10). Step 4, esterification of 3-MCPD, had previously been carried out in our laboratory with good yields (Hamlet and Asuncion 2011). Details of some of the steps evaluated are given in Table 43 below.

Table 43. Reaction conditions evaluated for the preparation of key intermediates in the synthesis of 3-MCPD-esters

Step / strategy	Route / conditions	References
Glycerol protection (diol) / deprotection		
1	PTA / 2,2-DMP / acetone	Vanlaldinpuia and Bez (2011)
Chlorination of protected glycerol / deprotection		
2(a)	$SOCl_2$ / pyridine	
2(b)	TCT / DMF / DCM	Luca, Giacomelli and Porcheddu (2002); Chang, Chang, Chao and Mong (2009)
3	Acidic resin (Amberlyst)	Che, Lee, Chein and Lee (2003)
Alcohol protection / diol esterification		
5(a)	DMF / DIEA / TIPSCl	Yu, Lui and Hu (2000)
5(b)	DMF / TEA / TIPSCl	Adapted from Yu, Lui and Hu (2000)
5(c)	DMF / DIEA / TBDPSCl	Adapted from Yu, Lui and Hu (2000)
6	Palmitic acid / EDU / DCM / DMAP	Han and Razdan (1999)

Key:

2,2-DMP	2,2-dimethoxypropane
DCM	dichloromethane
DIEA	N,N-Diisopropylethylamine
DMAP	dimethylaminopyridine
DMF	dimethylformamide
EDU	ethylenediurea
PTA	phosphotungstic acid
TBDPSCl	tert-Butyl(chloro)diphenylsilane
TCT	2,4,6-trichloro[1,3,5]triazine

To overcome these difficulties, methods used previously in our laboratory to prepare $^{13}C_3$-3-MCPD (Hamlet & Sadd 2003) and 3-MCPD-esters (Hamlet and Asuncion 2011) were chosen for optimisation:

$^{13}C_3$-glycerol -> $^{13}C_3$-3-MCPD -> $^{13}C_3$-3-MCPD-palmitate esters

While the likelihood of a successful synthesis was high, the yield of labelled 3-MCPD was known to be relatively low (circa 35%). Consequently a series of "cold" reactions were undertaken using unlabelled glycerol and the conditions of reaction time, temperature and relative concentrations of chlorination reagent (Rider and Hill 1930) were carefully optimised until the yield of 3-MCPD was sufficient (57%) to proceed with the latter step of the synthesis. Details of the final preparation can be found in 9.6.3 of the Appendix.

8.2.1 Thermal studies

8.2.1.1 Effect of bake time at constant temperature

Biscuit (soft) dough fortified with $^{13}C_3$-3-MCPD-PP was prepared according to the recipe given in 9.6.4 of the Appendix and cooked in batches of three biscuits in the oven of a GC. The preparation of the biscuits and cooking conditions are given in the Appendix.

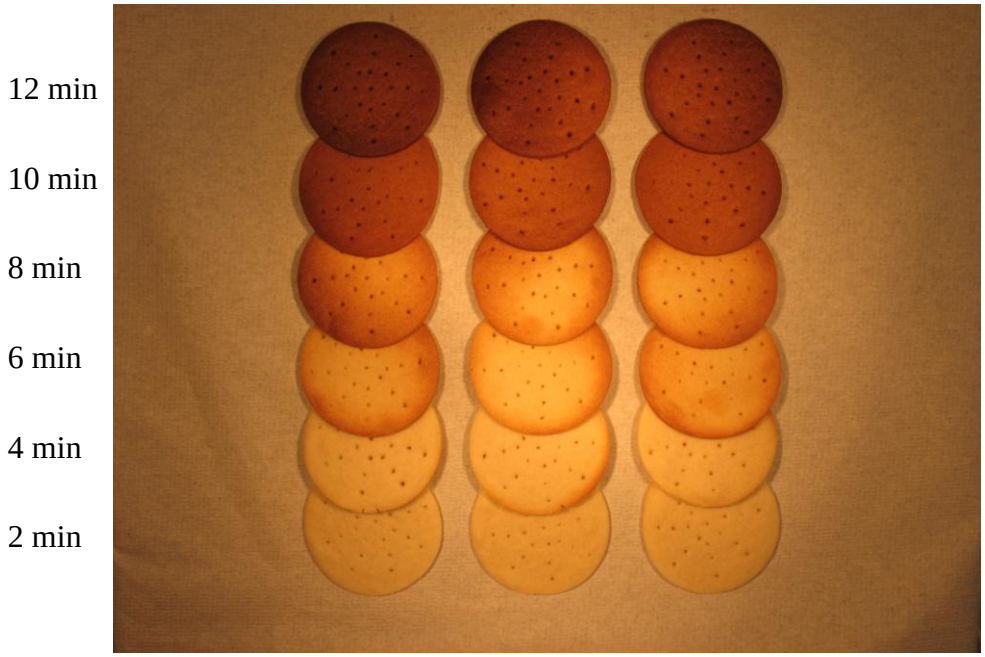


Figure 11 colour change in soft dough biscuits baked at 180°C over 2 – 12 min

Free MCPD. When the biscuits were cooked at 180°C Figure 12 shows that amounts of free ^{12}C-3-MCPD rose quickly, presumably due to formation from precursors such as salt, glycerol and acylglycerols present in the ingredients, and then decayed as baking progressed (Hamlet & Sadd 2004a and b). Significantly, no free ^{13}C-3-MCPD was detected during the course of these experiments indicating that the free chloropropanol was not released from its parent ester under the conditions of baking.

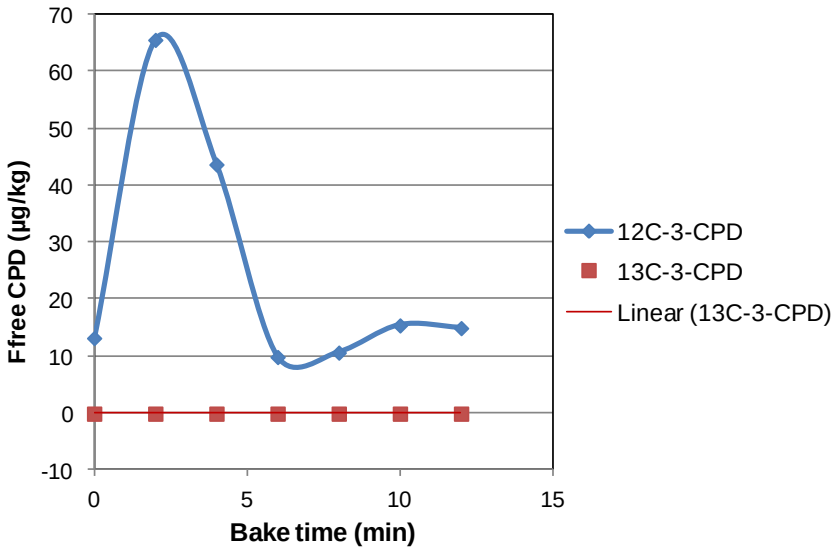
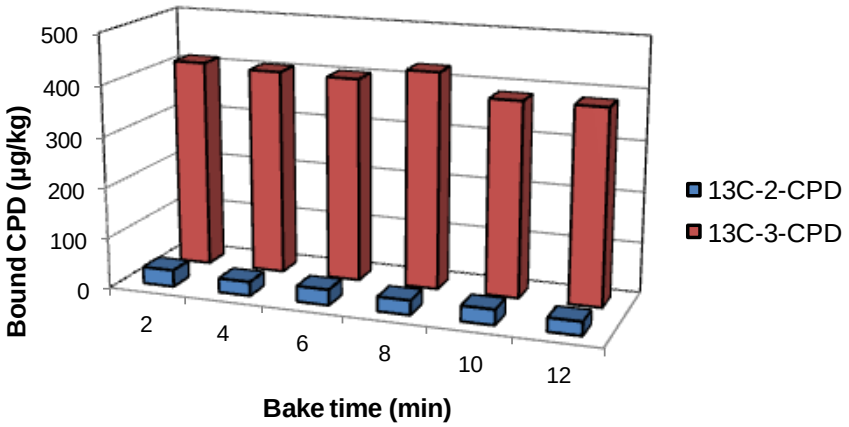


Figure 12 Change in concentrations of free MCPD in biscuit dough with cook time at 180°C: all data normalised to dry weight basis.

Bound MCPD. Figure 13 shows that the measured concentrations of labelled (added) and non-labelled MCPD isomers remained unchanged during the course of baking, even up to 12 min at 180°C (i.e. in excess of typical baking conditions, see Figure 11), indicating that the MCPD esters were relatively stable.

A)



B)

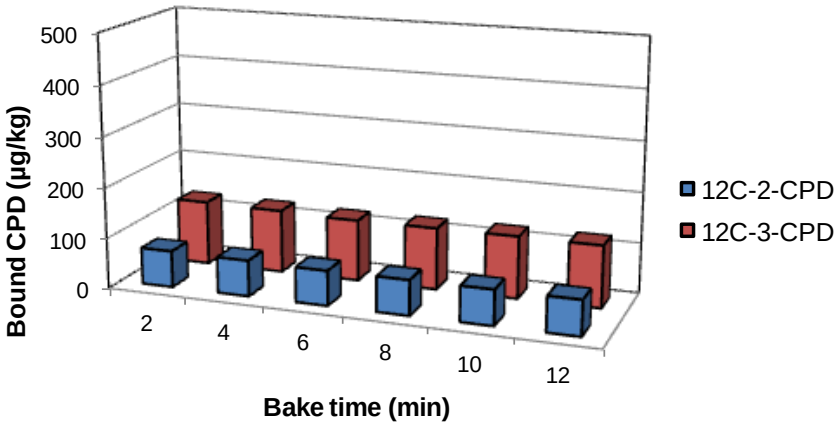


Figure 13 Change in concentrations of bound MCPD in biscuit dough with cook time at 180°C: A) added PP-$^{13}C_3$-3-MCPD (contained low level of 2-MCPD isomer); B) $^{12}C_3$-MCPD esters present in recipe fat. Data normalised to dry weight basis.

8.2.1.2 **Effect of bake temperature at fixed time**

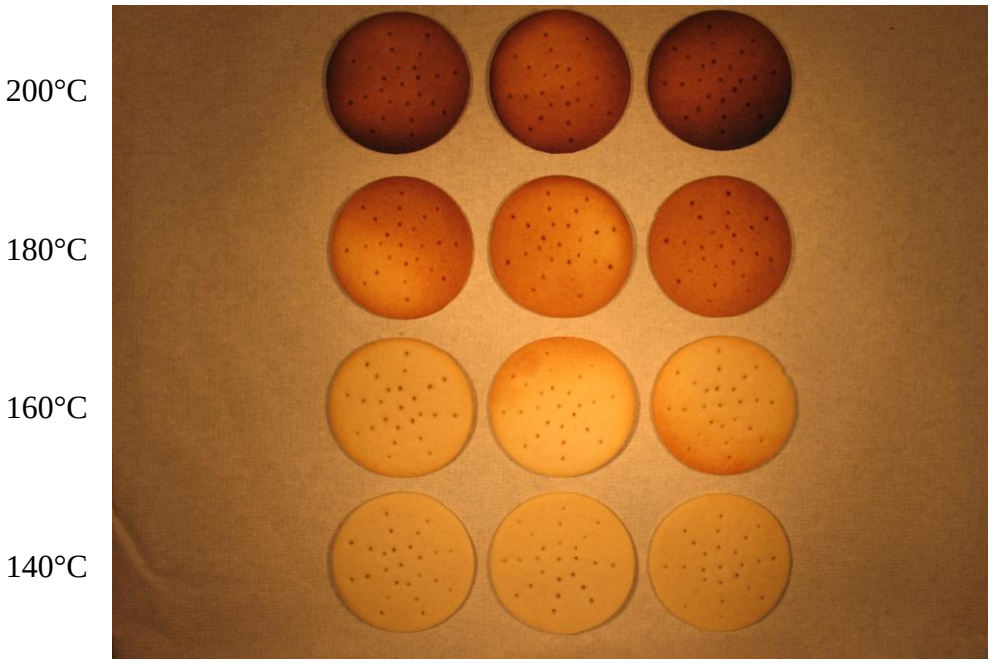


Figure 14 colour change in soft dough biscuits baked for 8 min over 140-200°C

Free MCPD. In keeping with the baking results at 180°C, formation of free 3-MCPD was observed at 140°C and this continued to decay during the course of baking. As expected, no free labelled 3-MCPD was measured.

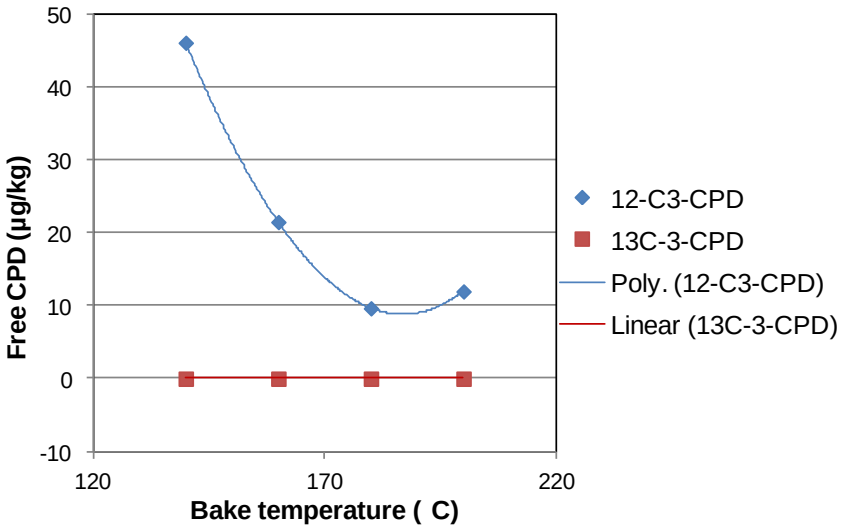
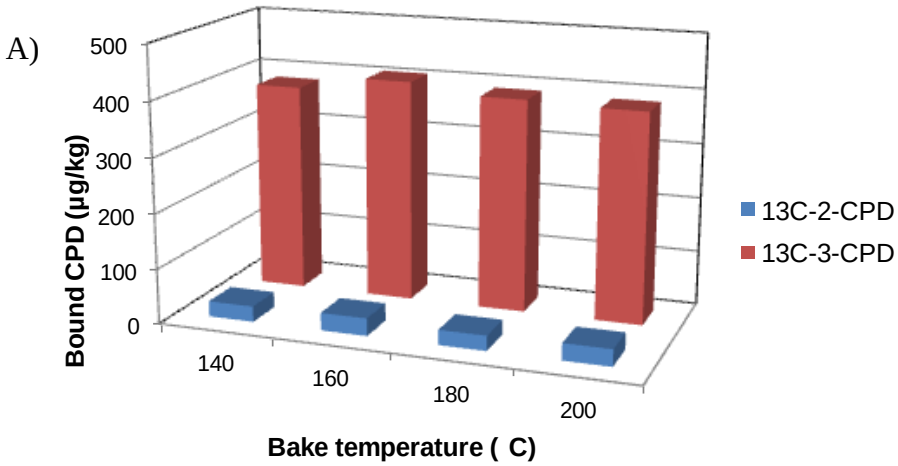


Figure 15. Change in concentrations of free MCPD in biscuit dough with cook temperature at fixed time (8 min): all data normalised to dry weight basis.



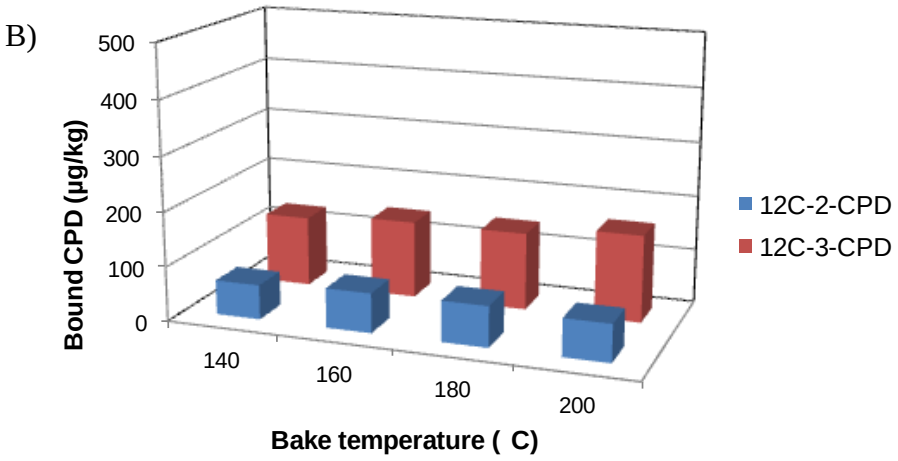


Figure 16 Change in concentrations of bound MCPD in biscuit dough with cook temperature at fixed time (8 min): A) added PP-$^{13}C_3$-3-MCPD (contained low level of 2-MCPD isomer); B) ^{12}C3-MCPD esters present in recipe fat. Data normalised to dry weight basis.

Bound MCPD. As expected, Figure 16 shows that concentration of the added bound $^{13}C_3$-3-MCPD remained unchanged during the course of baking while the concentration of the bound non-labelled MCPD showed a slight increase with increasing temperature.

8.2.2 Ambient studies

Pasta doughs fortified with PP-$^{13}C_3$-3-MCPD (containing circa 10% PP-$^{13}C_3$-2-MCPD) were prepared at concentrations of 0.5 g/100 g and 2.5 g/100 g Lipopan (lipase). The dough was maintained at 21°C and aliquots removed with time for the analysis of free $^{13}C_3$-MCPD. Figure 17 shows that the initial release of free $^{13}C_3$-3-MCPD occurred rapidly at both concentrations of added Lipopan but within the timeframe of the experiment significantly more free $^{13}C_3$-3-MCPD was formed at the higher concentration of lipase. The release of free $^{13}C_3$-2-MCPD on the other hand occurred rapidly and appeared to go to completion at both concentrations of added lipase (see Figure 18). This result was consistent with the known higher affinity of lipases for the *sn*-1 and *sn*-3 positions of the fatty acyl groups on the glycerol moiety and hence the relatively rapid formation of free $^{13}C_3$-2-MCPD from the parent PP-$^{13}C_3$-2-MCPD.

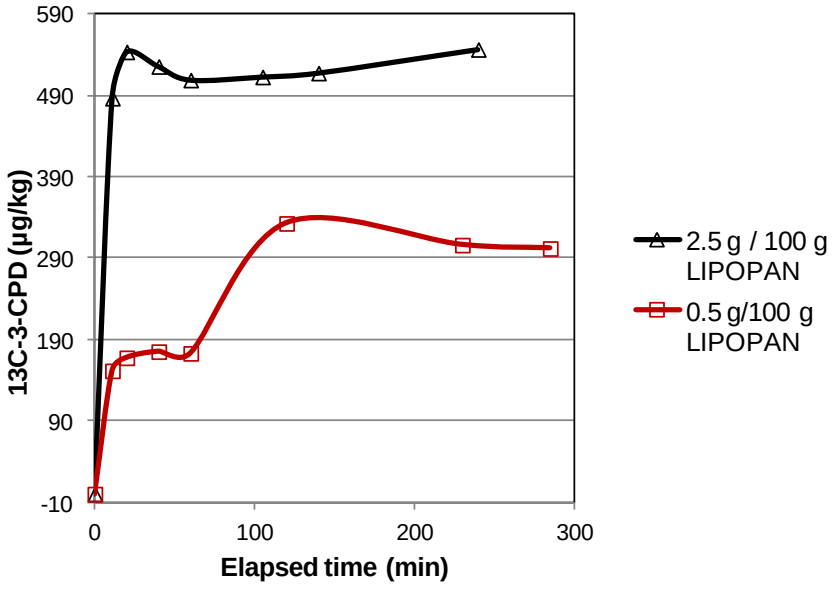


Figure 17. Change in concentrations with time of free $^{13}C_3$-3-MCPD in pasta dough fortified with PP-$^{13}C_3$-3-MCPD after the addition of a commercial lipase at 0.5 g/100 g and 2.5 g/100 g: incubation was carried out at 21°C

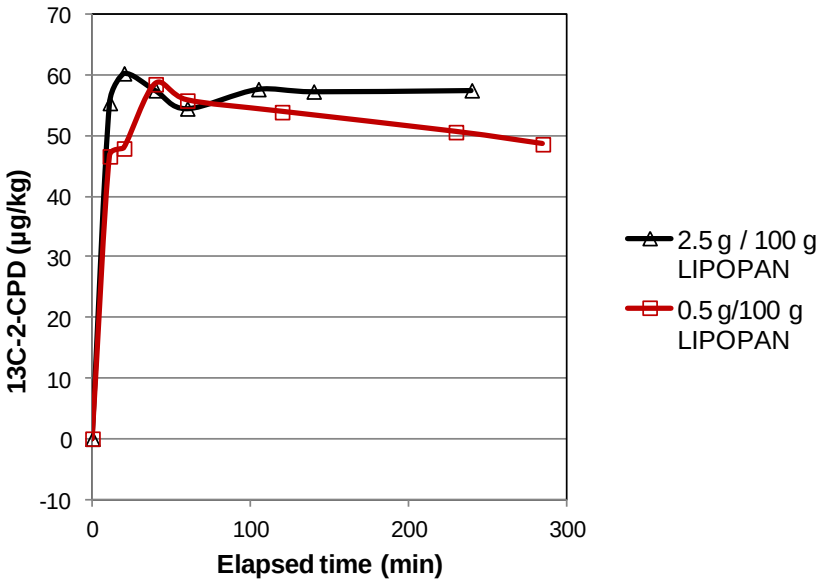


Figure 18. Change in concentrations with time of free $^{13}C_3$-2-MCPD in pasta dough fortified with PP-$^{13}C_3$-2-MCPD after the addition of a commercial lipase at 0.5 g/100 g and 2.5 g/100 g: incubation was carried out at 21°C

8.2.3 Conclusions from process studies of cereal products

Under the conditions simulating the baking of a cereal product (e.g. short dough biscuits) concentrations of added (labelled) and native (present in fats) bound 2- and 3-MCPD remained unchanged. However, under these conditions, some formation and decay of the free non-labelled 2- and 3-MCPD was observed, presumably from precursors present in the recipe ingredients such as glycerol, acylglycerols and salt (Hamlet et al 2004a; 2004b)

In a cereal dough containing under ambient conditions, free 2- and 3-MCPD isomers were readily released from added (labelled) 3-MCPD esters by the action a commercial lipase.

9. APPENDICES

9.1 FSA Research Requirement (RRD 30)

RRD 30: Programme C04 - Process contaminants

Thursday 26 March 2009

Deadline for receipt of applications: 5 June 2009 (1 requirement)

Introduction

Process contaminants are chemical substances which are produced in food during food manufacturing, cooking, packaging and other processing activities. They are absent in the raw unprocessed foods and are only formed when components in food undergo chemical changes during processing via manufacture and home cooking. Processing methods may include fermentation, acid hydrolysis, smoking, drying as well as domestic cooking (grilling, frying and barbecuing). Process contaminants of interest to the Agency include acrylamide, 3-MMCPD (3-monochloropropane -1,2-diol), ethyl carbamate and furan. Experts advise that these compounds have the potential to increase the risk of cancer and therefore it is considered that dietary exposure to these compounds should be reduced to as low as reasonably practicable.

Research Requirement (1 requirement)

Investigation of the formation of 3-MMCPD (3-monochloropropane -1,2-diol) from mono and di-esters of its fatty acids in food

Recent reports indicate that 3-MMCPD esters (mono- and di- fatty acid esters) may be widespread in processed foods, such as bread/toast, coffee, fish, French fries, crisps, meat, nuts and refined oils. Highest levels have been found in refined oils and products where such oils are used as an ingredient. The possible release of 3-MMCPD from its esters is of concern because of it toxicity and the possible contribution to consumer exposure in the diet. Research has indicated that release of 3-MMCPD from its mono and di-esters can occur by the action of lipases, leading to the possibility of 3-MMCPD formation occurring during food storage as a result of residual lipase activity. Furthermore, it is feasible that 3-MMCPD may also be released from these esters via chemical cleavage during thermal processing/cooking. The mechanism and extent of formation of 3-MMCPD from its esters is not yet fully understood.

The proposal is therefore to assess the extent of release of 3-MMCPD from its mono- and di-esters in food, i.e. by thermal processing/cooking and/or the action of lipases during storage as a means of investigating the mechanism of formation.

It is anticipated that the project will involve studies on both real foods and model systems, although real foods should be employed wherever possible. Isotopic approaches (stable isotopes and possibly radio-isotopes) will be used to quantify the percentage conversion of 3-MMCPD esters to 3-MMCPD. The proportion of 3-MMCPD that can thus be attributed to formation via 3-MMCPD esters will be quantified. Studies will be conducted on both labelled mono-esters and di-esters, reflecting the importance of each type. Other parameters that may be examined include cooking temperature and time, re-use of frying oil (where relevant) and effects of treatments known to inhibit lipase activity.

The project will not address the issue of potential in vivo formation of 3-MMCPD.

Findings from the currently proposed research will clarify the extent to which 3-MMCPD esters act as precursors to 3-MMCPD in food. The project will also help in the understanding of the mechanism of such action and will help to facilitate the development of appropriate mitigation measures. The work will also inform future discussions at the EU level.

Proposals are therefore invited to:

Requirement Reference: C04R0010 - Investigate the formation of 3-MMCPD (3-monochloropropane -1,2-diol) from mono and di-esters of its fatty acids in food.

Further Information

Before preparing your proposals please contact the named person below for advice and information on the specific scientific issues or the policy background/objectives:

Mrs Nina Webber, Incident Prevention and Chemical Risk Management,

Tel: 020 7276 8708; Fax: 020 7276 8446;

email: nina.webber@foodstandards.gsi.gov.uk

Proposals should be sent, to be received by **17:00 hrs on Friday 5 June 2009** to:

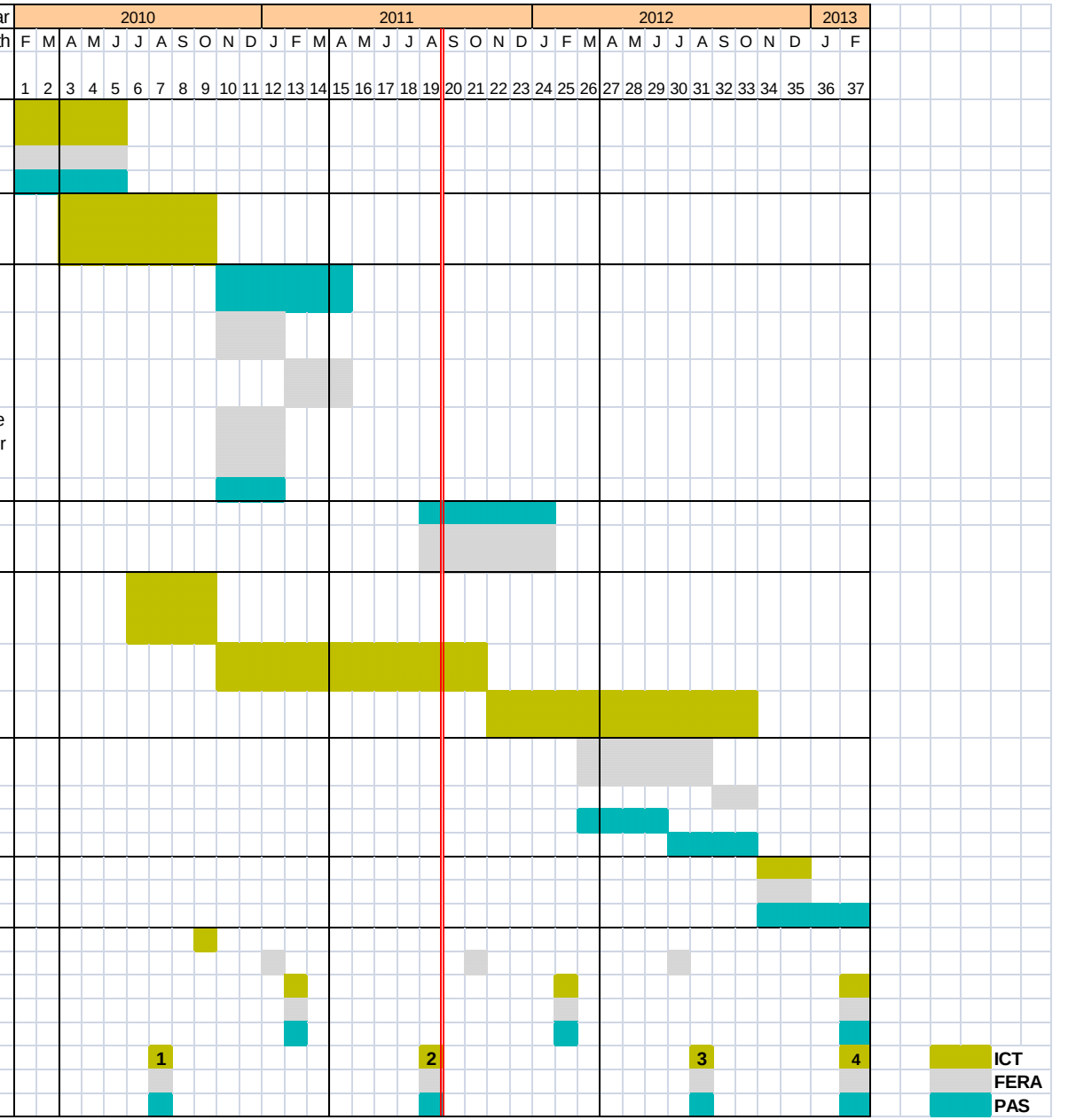
email: FSA_Remind@foodstandards.gsi.gov.uk

and

Post:
Ms Adenike Banjoko
Food Protection Division
Food Standards Agency
4B, Aviation House
125 Kingsway
London WC2B 6NH

9.2 Project Gantt chart

Objective	Description	Task	Description	Sub-task	Description
1	Literature review				
2	Synthesis of reference chemicals				
3	Methods development	3.1	3-MCPD mono- & diesters	3.1.1	Develop / extend lit methods
		3.2	Glycidol esters	3.2.1	Evaluate method of Engerberson et al
				3.2.2	Develop method for foods
		3.3	AQA measurement MCPD esters	3.3.1	Prepare and distribute reference foodstuff for AQA
4	Occurrence	4.1	Cereal products		
		4.2	Non-cereal products		
5	Model system experiments	5.1	Design representative model systems	5.1.1	Model experiments to test formation
				5.1.2	Model experiments on reactions (chemical)
				5.1.3	Model experiments on reactions (enzyme)
6	Processing / storage	6.1	Non-cereal products	6.1.1	Processing/cooking
				6.1.2	Storage experiments
		6.2	Cereal products	6.2.1	Thermal studies
				6.2.2	Ambient studies
7	final report	7.1	ICT report		
		7.2	FERA report		
		7.3	Final report		
Distribution of RMs					
AQA					
Reports					
Meetings					



9.3 Analytical quality assurance

Table 44 Inter laboratory analysis of bound 3-MCPD in a palm oil reference material

Date of test	Test method	Concentration (mg/kg)		n	RSD	FAPAS
		ICT	PAS[a]		(%)	(z-score)
Nov 2010	GC/MS	0.652	-	-	2.4	-
Jan 2011	GC/MS	-	0.585	3	2.4	0.2[b]
Oct 2011	GC/MS	-	0.626	2	3.6	-0.3[c]
Mar 2012	GC/MS	0.560	-	-	3.7	-

[a] Method according to Hamlet & Asuncion (2011); [b] Proficiency test No 2628 February-March 2011; [c] Proficiency test No 2631 September-November 2011

9.4.1 Certified details of prepared reference chemicals.

INSTITUTE OF CHEMICAL TECHNOLOGY, PRAGUE
Faculty of Food and Biochemical Technology
Department of Food Chemistry and Analysis

Dr Colin G Hamlet,
Chemical Food Safety Scientist,
Central Technical,
Premier Foods.

Tel: +44(0)1494 428211
Facsimile: +44(0)1494 428114
Mobile: 07753 897892

Lord Rank Centre, Lincoln Road, High Wycombe, HP12 3QR

List of the chemicals:

COMPOUND	AMOUNT [mg]	PURITY [%]
1,2-dipalmitate ester of 3-chloropropane-1,2-diol-d_5	1000	98.2
1,2-dipalmitate ester of 3-chloropropane-1,2-diol	1000	98.9
1,2-dioleate ester of 3-chloropropane-1,2-diol	549	99.2
oleate ester of glycidol	1020	98.3
palmitate ester of glycidol	1000	98.0

These compounds were synthesised at the Institute of Chemical Technology Prague and are intended for the chemical research.

Prague, 20.7.2010 Assoc. Prof. Marek Doležal, PhD

Institute of Chemical Technology, Prague, public university established by Act No. 111/1998 Coll., in the wording of subsequent regulations, based at Technicka 5, 166 28 Prague 6 - Dejvice, Czech Republic, IČ: 60461373, DIČ: CZ60461373, bank account: ČSOB, 130197294/0300.

INSTITUTE OF CHEMICAL TECHNOLOGY, PRAGUE
Faculty of Food and Biochemical Technology
Department of Food Chemistry and Analysis

Dr Colin G Hamlet,
Chemical Food Safety Scientist,
Central Technical,
Premier Foods.

Tel: +44(0)1494 428211

Lord Rank Centre, Lincoln Road, High Wycombe, HP12 3QR
United Kingdom

List of the standards:

COMPOUND	AMOUNT [mg]	PURITY [%]
1-palmitate ester of 3-chloropropane-1,2-diol-d_5	1090	98.2
1-palmitate ester of 3-chloropropane-1,2-diol	1130	98.7

These standards were synthesized at the Institute of Chemical Technology Prague and are intended for the chemical research.

Standards -for laboratory use only
- are not dangerous substances in term of transport regulations

Prague, 7.1.2011 Assoc. Prof. Marek Doležal, PhD

INSTITUTE OF CHEMICAL TECHNOLOGY, PRAGUE
Faculty of Food and Biochemical Technology

Department of Food Chemistry and Analysis

Dr Colin Crews
The Food and Environment Research Agency
Room 08GA08
Sand Hutton
York
YO41 1LZ
United Kingdom

COMPOUND	AMOUNT [mg]	PURITY [%]
Glycidylpalmitate -d_5	1070	99.0

This standard was synthesized at the Institute of Chemical Technology Prague and is intended for the chemical research.

Standard -for laboratory use only
-is not dangerous substance in term of transport regulations

Prague, 7.1.2011 Assoc. Prof. Marek Doležal, PhD

9.4.2 Charted results from model system studies of section 7.1

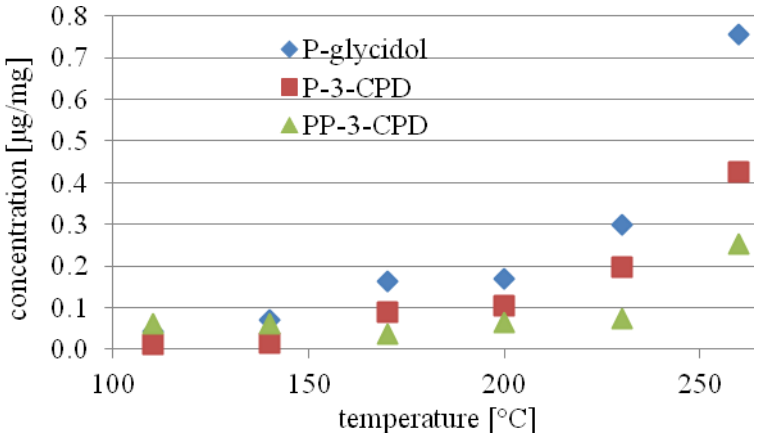


Figure 19. Influence of temperature on tripalmitin model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

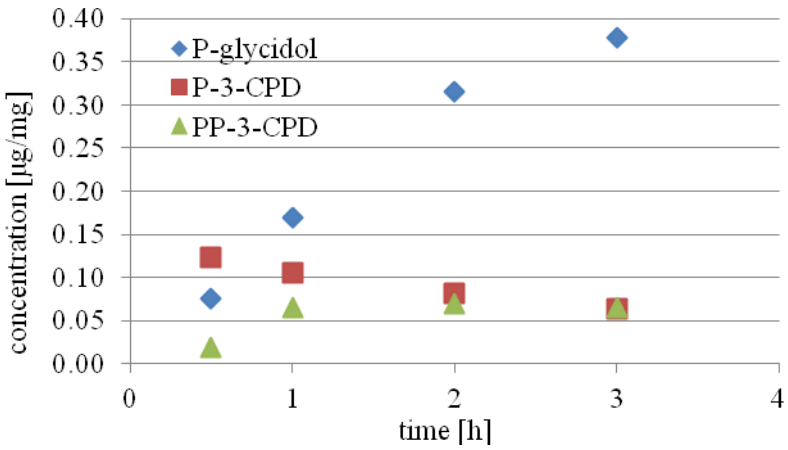


Figure 20. Influence of heating time on tripalmitin model system (0.5% of water, 0.5% of chloride ions, temperature 200°C)

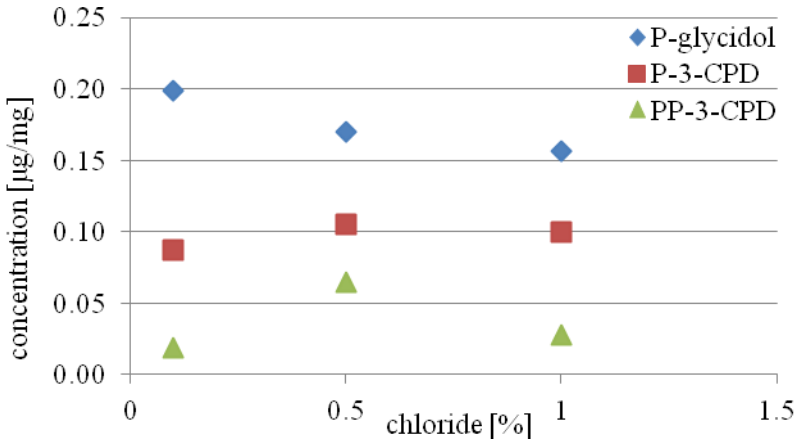


Figure 21. The effect of chloride ions content on tripalmitin model system (0.5% of water, heating time 1 hour, temperature 200°C)

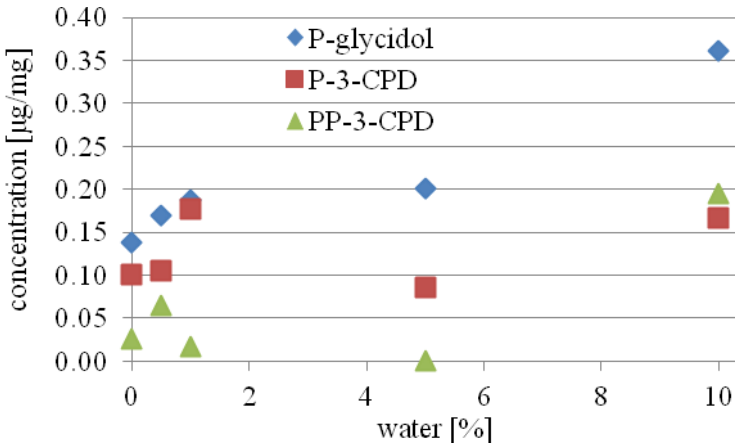


Figure 22. The effect of water content on tripalmitin model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C)

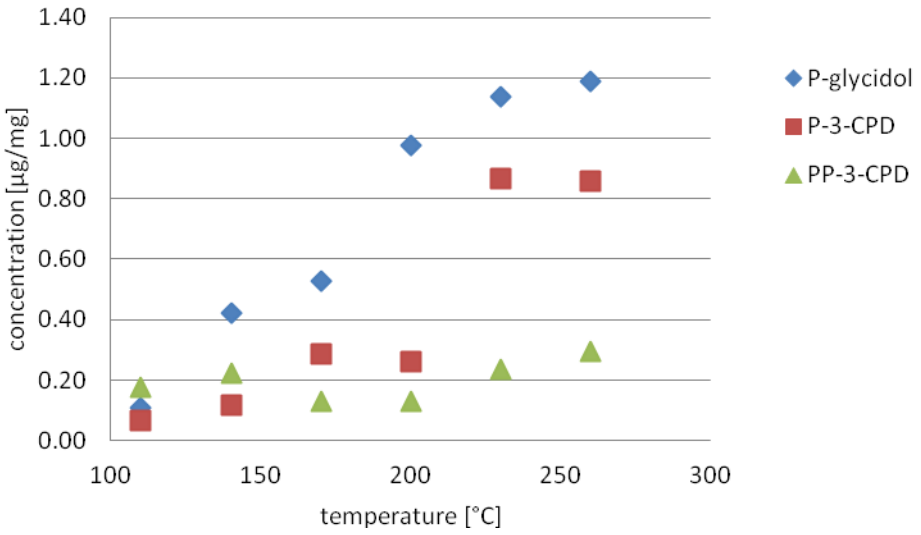


Figure 23. Influence of temperature on monopalmitin model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

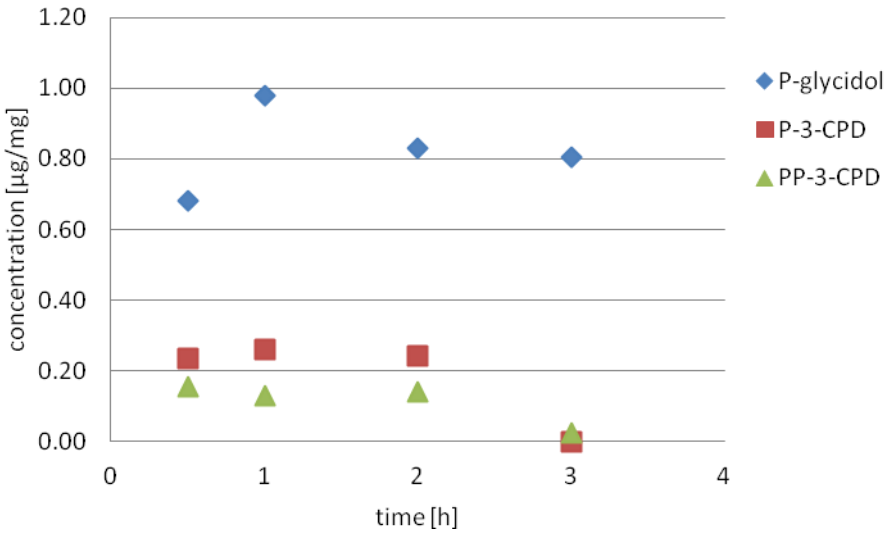


Figure 24. Influence of heating time on monopalmitin model system (0.5% of water, 0.5% of chloride ions, temperature 200°C)

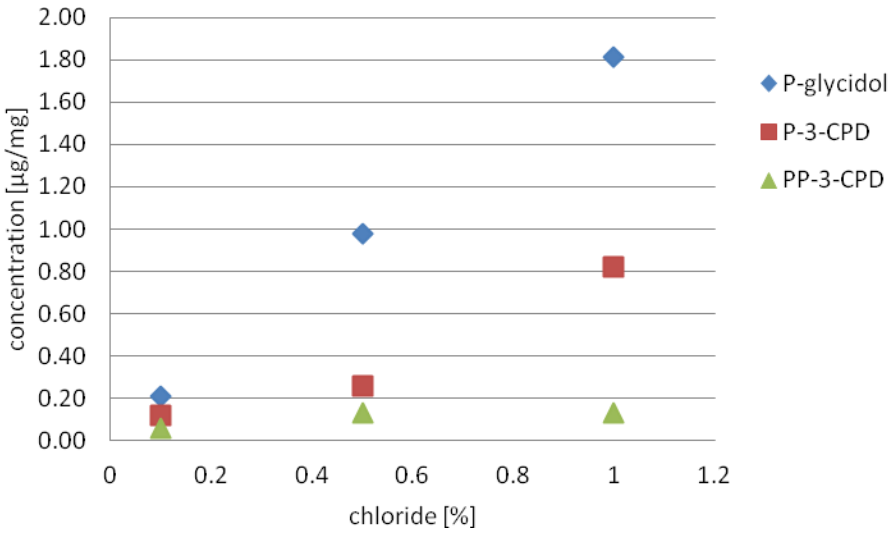


Figure 25. The effect of chloride ions content on monopalmitin model system (0.5% of water, heating time 1 hour, temperature 200°C)

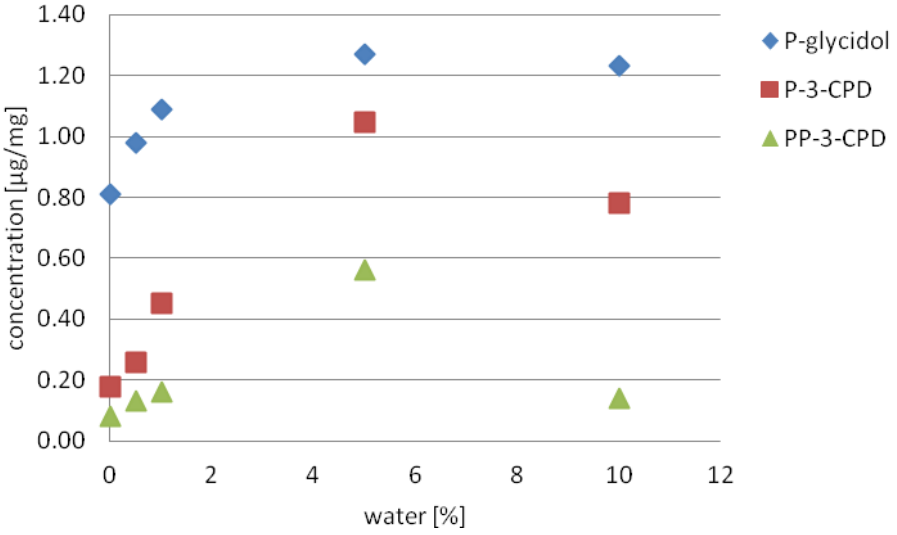


Figure 26. The effect of water content on monopalmitin model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C)

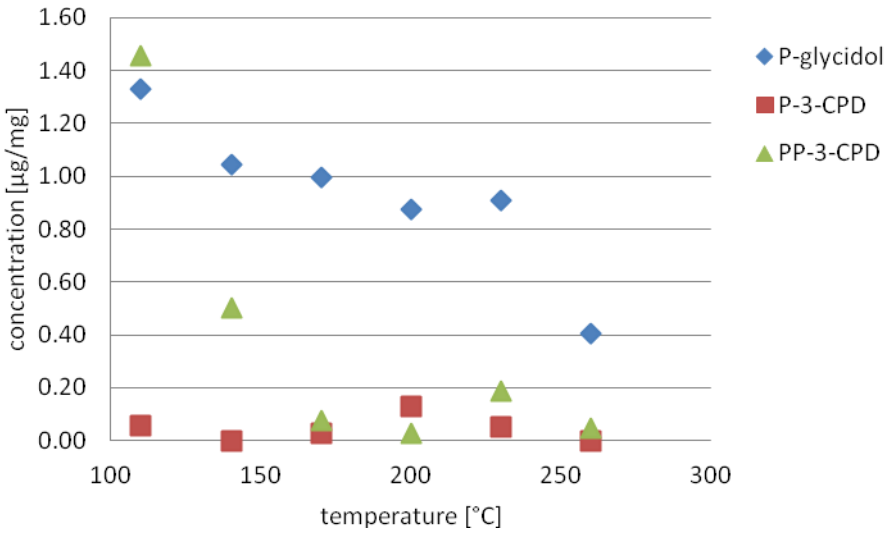


Figure 27. Influence of temperature on dipalmitate model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

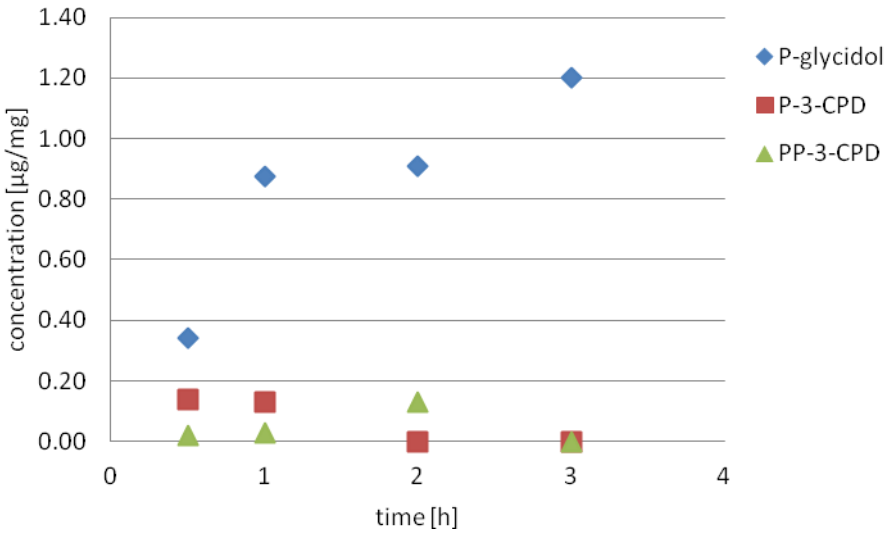


Figure 28. Influence of heating time on dipalmitate model system (0.5% of water, 0.5% of chloride ions, temperature 200°C)

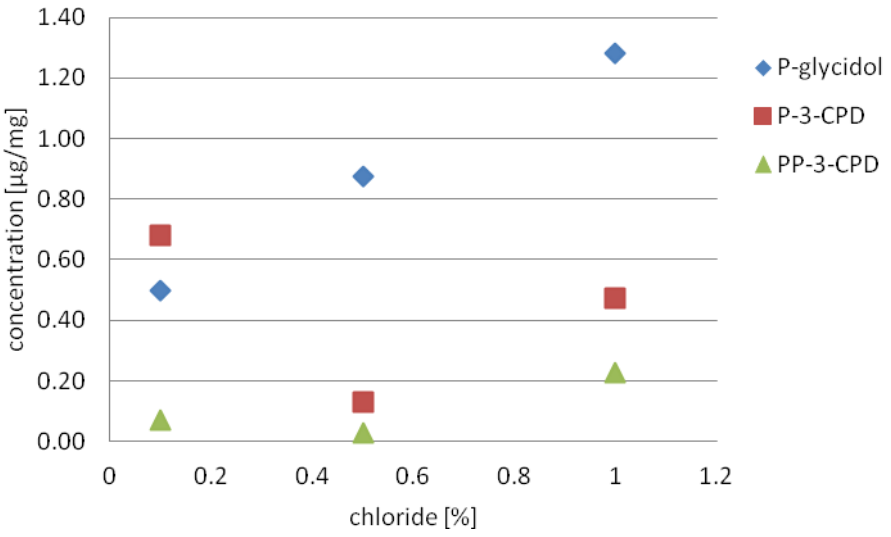


Figure 29. The effect of chloride ions content on dipalmitate model system (0.5% of water, heating time 1 hour, temperature 200°C)

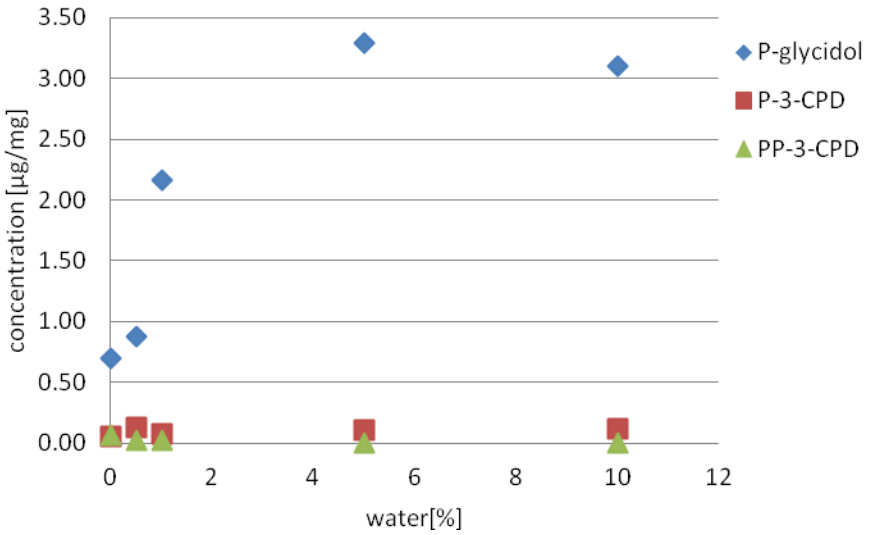


Figure 30. The effect of water content on dipalmitate model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C)

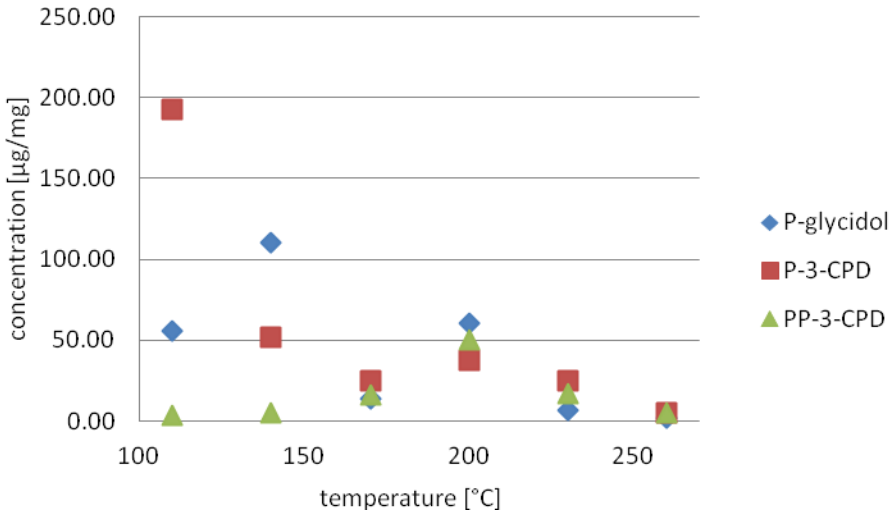


Figure 31. Influence of temperature on P-3-MCPD model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

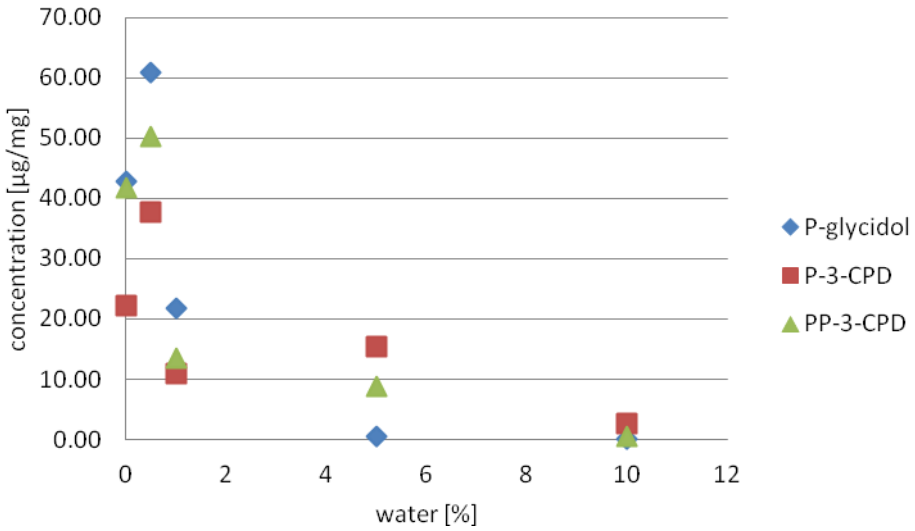


Figure 32. The effect of water content on P-3-MCPD model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C)

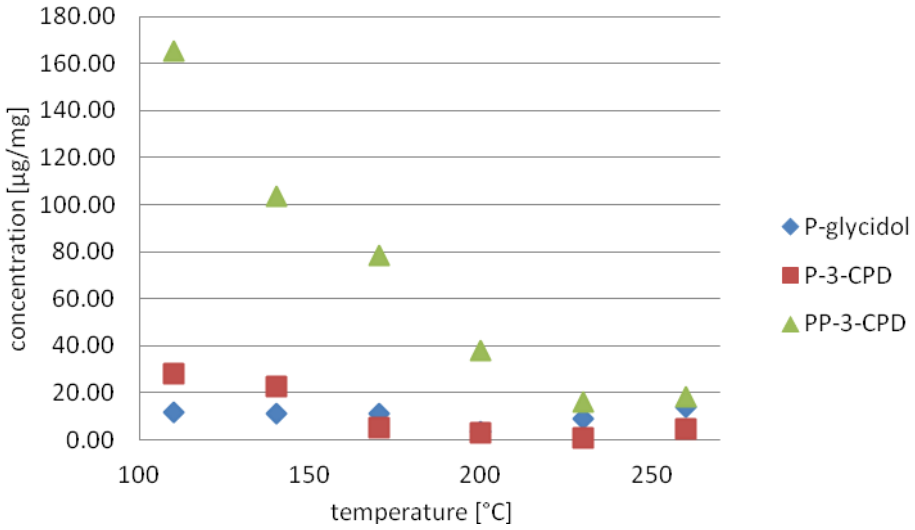


Figure 33. Influence of temperature on PP-3-MCPD model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

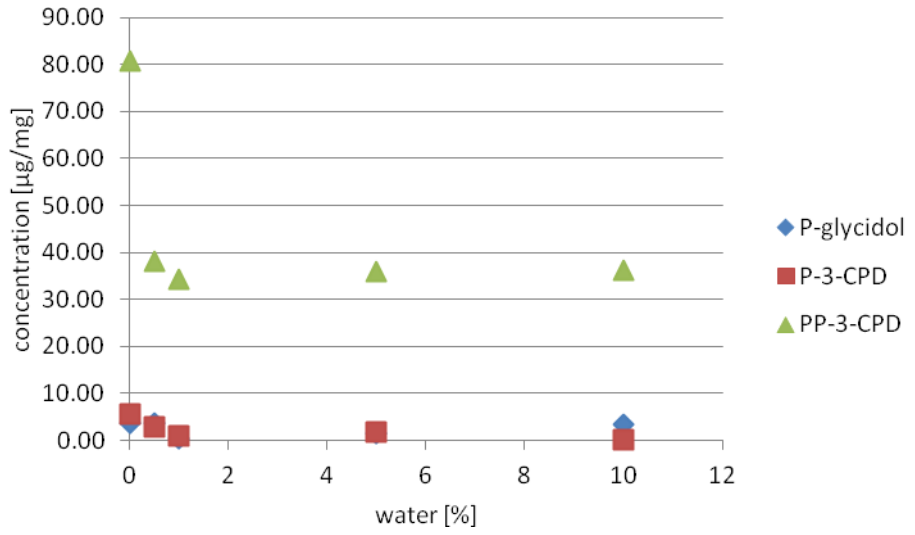


Figure 34. The effect of water content on PP-3-MCPD model system (0.5% of chloride ions, heating time 1 hour, temperature 200°C

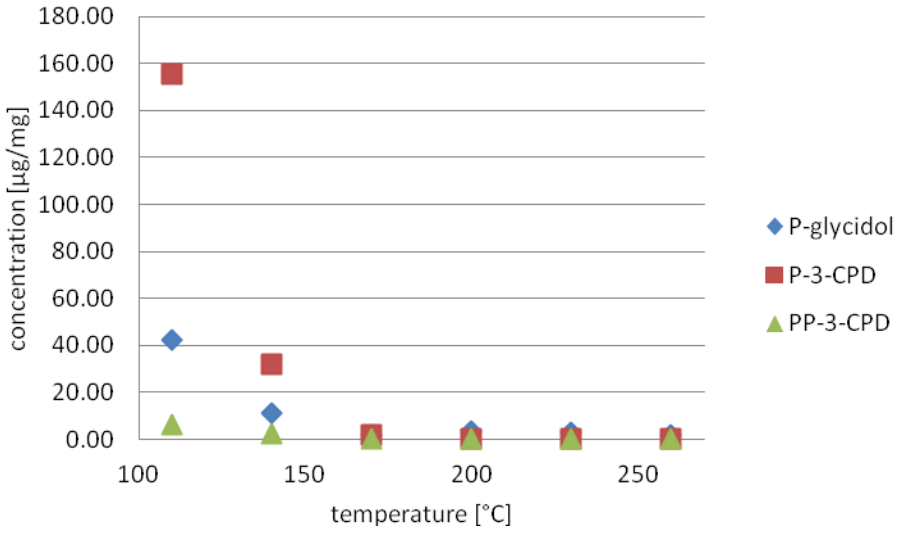


Figure 35. Influence of temperature on glycidyl palmitate model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

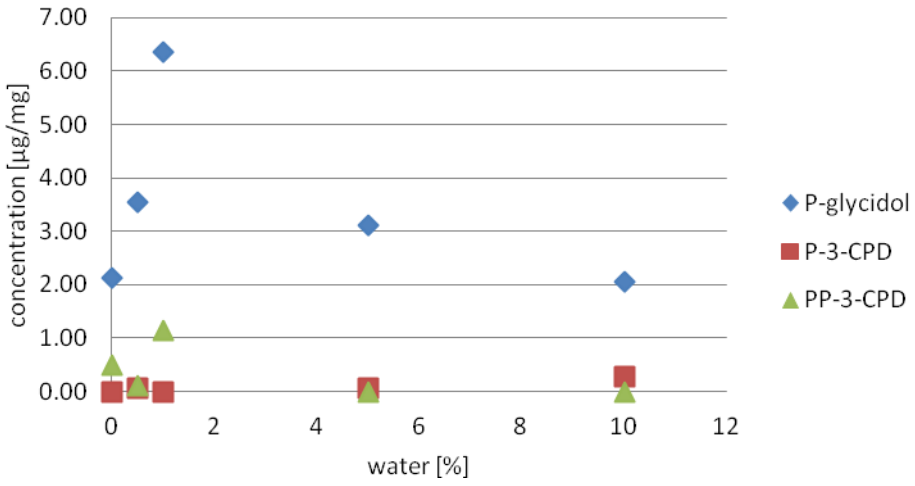


Figure 36. Influence of water content on glycidyl palmitate model system (0.5% of water, 0.5% of chloride ions, heating time 1 hour)

9.5 FERA materials and methods

Table 45 Food Samples

Sample Name	Class	Code	Ingredients	Stated fat content
French goat's cheese	Dairy	4	Protein 20%	Fat 26%, of which saturates 18%
Parmigiano	Dairy	5		Fat 29%
Emmental	Dairy	9	Pasteurized milk, lactic starters, coagulant, salt	
Brie	Dairy	14		Fat 24% of which saturates 14.5%
Roquefort	Dairy	15	Protein 11% carbohydrate 0.5%,	Fat 32%, of which saturates 24%
Brie (Somerset)	Dairy	16	Not stated	
Cheese singles	Dairy	21	Cheese (60%); water, whey powder, butter, sodium polyphosphate, trisodium phosphate, calcium phosphate, milk proteins, carotene, paprika extract.	Fat 21%, saturates 13.9%, monounsaturates 5.1%, polyunsaturates 0.7%.
Chorizo Iberico	Meat	3	Pork, salt, paprika, lactose, dextrose, corn syrup, spices, potassium sorbate, Natamycin.	Fat 14%
Spanish chorizo ring	Meat	6	Pork, salt, paprika, powdered milk, garlic powder, oregano.	Fat 21%.
Hungarian Mangalica sausage	Meat	7	Pork, salt, paprika, caraway seed, garlic, dextrose, paprika extract, sodium nitrite.	Fat 45.3%
Salami Milano	Meat	8	Pork, salt, white wine, corn dextrose, white pepper, black peppercorns, sodium ascorbate, ascorbic acid, potassium nitrate, potassium nitrite, garlic.	
Polish sausage	Meat	10	Not stated	
Saucisson Sec Superior	Meat	24	Pork, salt, spices, dextrose, lactose, sugar, potassium nitrate, fermenting agents.	Fat 34.5%
Chorizo picante	Meat	25	pork, paprika, salt, garlic, rosemary extract.	
Vegetable baking fat	Oils and fats	11		Monounsaturates 40%, polyunsaturates: 14%

Vegetable baking fat	Oils and fats	12		Saturates 47%, monounsaturates 37%, polyunsaturates: 11%
Vegetable baking fat	Oils and fats	13		Fat 75% of which saturates 28%
Bakery fat RBD	Oils and fats	19	Not stated	
EVOO	Oils and fats	22		Fat: 91.4%, of which saturates:13.1%, monounsaturates 66.7%, polyunsaturates 7.5%.
Palm oil	Oils and fats	23	Not stated	
Chips	Potato	1	Potatoes, beef dripping 11%, sunflower oil, salt.	Fat 14.3%, saturates 6.4%.
Chips crinkle cut	Potato	2	Potatoes, palm oil	Fat 10%
Potato crunchies	Potato	17	Potatoes 84%, sunflower oil, potato flake, potato starch, pea fibre, salt, onion, dextrose, white pepper, turmeric, ascorbic acid.	
Potato waffles	Potato	18	Potatoes, vegetable oil, potato granules, potato starch, salt, stabilizer (E464), white pepper.	Fat 8.7%, saturates 0.9%
Oven chips	Potato	20	Potato, sunflower oil	
Potato	Potato	26		

9.6 PAS materials and methods

9.6.1 Bakery ingredients

Bakery fat, flours, castor sugar, biscuit flour, Durum semolina, salt, were obtained from commercial suppliers.

9.6.2 Food samples

Palm oil reference material was supplied by FERA (York). Olive oil and digestive biscuits were obtained from a local retail outlet.

9.6.3 Chemicals and reagents

All solvents were high purity grade (Pestanal®, Sigma-Aldrich), otherwise all other reagents were of analytical grade unless stated: (±)-3-bropropane-1,2-diol (98%), (±)-3-MCPD (purum, >98 %), (±)- 3-MCPD-1,1,2,3,3-D_5 (98 atom % D, 97% chemical purity), anhydrous pyridine (99.8% spectrophotometric grade), glycerol (99.5%), glycerol (1,2,3-$^{13}C_3$, > 99 atom %) and palmitoyl chloride (98%) were from Sigma-Aldrich (Gillingham, UK); Bond Elut SPE columns (Silica 2 g; Diol, 500 mg; Amino, 500 mg), were from Agilent.

$^{13}C_3$-3-MCPD was prepared to 57% yield (GC) according to the basic method of Conant and Quayle (1947) with modifications (to improve yield) by Rider and Hill (1930). Briefly, concentrated HCl (3.18 ml of ≥37%, ACS reagent) was added slowly (dropping funnel) with gently swirling to a mixture of $^{13}C_3$-glycerol (1010 mg warmed gently to aid dispensing / mixing) and glacial acetic acid (96 µl) in a micro-scale 2-neck pear shaped flask (10 ml) fitted with a reflux condenser. The temperature of the reaction mix was raised slowly to 105°C (over 85 min) then maintained at 105°C – 110°C for 10 h. The cooled reaction mixture was then poured into 40 ml of 5M sodium chloride solution in a separating funnel. The apparatus was rinsed repeatedly with a little ethyl acetate and the latter transferred to the separation funnel. The reaction mixture was extracted with a total of 3 x 50 ml ethyl acetate and the combined organic layer was dried and filtered over anhydrous sodium sulphate.

Dipalmitate esters of 3-bromopropane-1,2-diol, (3-BPD-PP) and $^{13}C_3$-3-MCPD ($^{13}C_3$-3-MCPD-PP) were prepared from the corresponding 3-halo-1,2-diol and palmitoyl chloride according to the procedure of Hamlet and Asuncion (2011). 3-PP-MCPD-d_5, 3-PP-MCPD, 3-P-MCPD-d_5 and 3-P-MCPD were supplied by ICT, Prague.

9.6.4 Preparation of soft dough biscuits

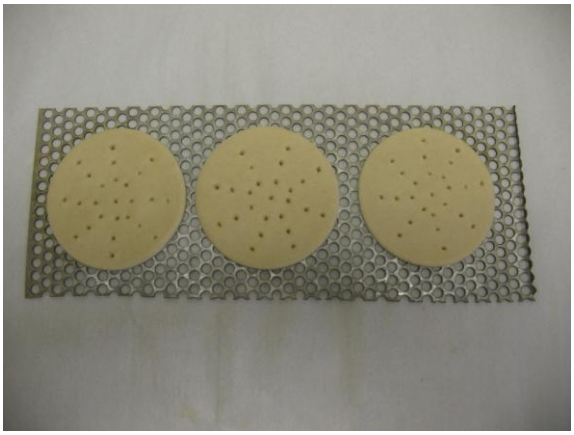
Mixing. Dough samples were prepared on a 200 g flour basis according to the recipe given in

Table 46. Bakery fat was weighed directly into the stainless steel bowl of a food mixer (Kenwood) and 1.70 ml of a solution of $^{13}C_3$-3-MCPD-PP (1.00 mg ml^{-1} in THF) was added directly to the fat. The THF was allowed to evaporate (fume hood) and the

remaining ingredients (excluding flour) were mixed as follows: 10s on slow, 10s on fast then 100s on slow to give a cream. Flour (200 g) was then added and mixed for a further 240s on the slow speed setting. The resultant dough was transferred to a polyethylene bag and left to stand for a minimum of 30 min at 20°C.

Sheeting. Dough (250 g piece) was reduced to a thickness 3 mm (between baking parchment sheets) using two plastic rules as guides. Discs (72 mm diameter) of dough were cut to a target weight of circa 16 g ± 1 g prior to docking (25 holes).

Baking. Discs of experimental and control dough were baked simultaneously on a custom built baking tray fabricated from stainless steel mesh: three discs per row were baked in the oven of a Carlo Erba 900 series GC to specified target temperatures / times. Baked biscuits were then allowed to cool to room temperature prior to



reducing to a fine powder using a domestic food processor. Samples were retained in sealed containers in the dark at -18°C until required for analysis.

Table 46 Recipe used for soft dough biscuits[3]

Ingredient	weight (g)
Biscuit flour	200
Bakery fat	60
Castor sugar	64
Salt	10.8
Water	138

9.6.5 Preparation of pasta dough

Mixing. Dough samples were prepared on a 200 g flour basis according to a commercial recipe given in Table 47. Olive oil was weighed directly into the stainless steel bowl of a food mixer (Kenwood) and 1.50 ml of a solution of $^{13}C_3$-3-MCPD-PP (1.00 mg ml^{-1} in THF) was added directly to the oil. The THF was allowed to evaporate (fume hood) and all the remaining ingredients were then added and mixed on slow for 3600 s. The resultant dough was then transferred to a polyethylene bag on the laboratory bench (21°C) for the time course analysis of $^{13}C_3$-3-MCPD.

Table 47 Recipe used for pasta dough[4]

Ingredient	Dough 1 (g)	Dough 2 (g)
Durum semolina	200	200
Lipopan 50 BG	1.0	5.0
Olive oil	15	15
Salt	2.86	2.86
Water	80.0	80.0

9.6.6 Moisture analysis

The moisture content of samples was determined gravimetrically following heating overnight at 105 °C.

9.6.7 Analysis of labelled ($^{13}C_3$) and unlabelled 2-MCPD and 3-MCPD

The isomeric compounds, 2-MCPD and 3-MCPD, were determined as the heptafluorobutyryl esters by the procedure of Hamlet and Sutton (1997) with modifications for $^{13}C_3$-3-MCPD (Hamlet & Sadd

[3] Derived from Whiteley (1971)

[4] Adapted from MAFF (1995)

(2003) and dry cereal products (Hamlet & Sadd 2005). Method performance (typical): The limits of detection and quantification were <3 and 10 µg/kg respectively; Blank response < 3 µg/kg; precision (RSD) e.g. IHRM (soy sauce) gave 11 µg/kg (RSD 5.6%, n=3); accuracy / bias from spiked recoveries was 96 ± 6% at 25 µg/kg and 101 ± 9% at 100 µg/kg (95% confidence level); the estimated value for the method uncertainty (single determination) was ± 1.3 µg/kg at 11 µg/kg (expanded uncertainty with a coverage factor of 2).

9.6.8 Analysis of bound labelled ($^{13}C_3$) and unlabelled 2-MCPD and 3-MCPD

The method of Hamlet and Asuncion (2011) based on acid catalysed transesterification, HFBI deritisation and GC/MS/MS was used to quantify bound 2- and 3-MCPD. Additional precursor and product ion transitions for $^{13}C_3$-2-MCPD and $^{13}C_3$-3-MCPD were added to published MRM method for the for the qualification of the labelled MCPD esters.

9.6.1 Analytical quality assurance

All analyses were performed by trained staff in a UKAS accredited laboratory operating an internal audit and review process. The methods used for the analysis of 3-MCPD and moisture measurement were all validated in-house and accredited by UKAS (ISO 17025). Method performance was monitored by analysing in-house reference materials and / or spiked reference materials and assessing the test results in accordance with the rules governing Shewhart control charts (BS 1991). The laboratory also participated in the FAPAS performance testing scheme for 3-MCPD.

10. REFERENCES

Becalski A, Feng SY, Lau BP-Y, Zhao T (2012). Glycidyl fatty acid esters in food by LC-MS/MS: method development. Analytical and Bioanalytical Chemistry, 403, 2933–2942.

BfR (2009). Initial evaluation of the assessment of levels of glycidol fatty acid esters detected in vegetable fats. 2009.

Brereton P, Kelly J, Crews C, Honour S, Wood R, Davies A. (2001). Determination of 3-chloro-1,2-propanediol in foods and food ingredients by gas chromatography with mass spectrometric detection: Collaborative study. Journal of AOAC International 84 (2) 455-465.

Cerbulis J, Parks OW, Liu RH, Piotrowski EG, Farrell Jr, HM. (1984). Occurrence of diesters of 3-chloro-1,2-propanediol in the neutral lipid fraction of goats' milk. Journal of Agricultural and Food Chemistry 32 474-476 (1984).

Chang, C-W., Chang, S-S., Chao, C-S., and Mong, K-K. (2009). A mild and general method for preparation of α-glycosyl chlorides. Tetrahedron Letters, 50, 4536-4540.

Conant, J. B., and Quayle, O. R. (1947) Glycerol –monochlorohydrin. Organic Syntheses Collective. 1, 294-296.

CONTAM (2008) Statement of the Scientific Panel on Contaminants in the Food chain (CONTAM) on a request from the European Commission related to 3-MMCPD esters. Available at: http://www.efsa.europa.eu/cs_l/results.jsp [accessed April 2008].

De Luca, L., Giacomelli, G., and Porcheddu, A. (2002). An Efficient route to alkyl chlorides from alcohols using the complex TCT/DMF. Organic Letters, 4, 553-555.

Destaillats, F., Craft, B.D., Dubois, M., Nagy, K. (2012). Glycidyl esters in refined palm (Elaeis guineensis) oil and related fractions. Part I: Formation mechanism. Food Chemistry, 131, 1391–1398

Deutsche Gesellschaft für Fettwissenschaft (DGF). Ester-bound 3-chloropropane-1,2-diol (3-MCPD esters) and glycidol (glycidyl esters). Determination in fats and oils by GC-MS. Stuttgart: Deutsche Gesellschaft für Fettwissenschaft, 2009.

Doležal, M., Chaloupská, M., Divinová, M., Svejkovská, B., Velíšek, J. (2005). Occurrence of 3-chloropropane-1,2-diol and its esters in coffee. European Food Research and Technology, 221, 221-225.

Dubois M, Tarres A, Goldmann T, Loeffelmann G, Donaubauer A, Seefelder W. (2011). Determination of seven glycidyl esters in edible oils by gel permeation chromatography extraction and liquid chromatography coupled to mass spectrometry detection. Journal of Agricultural and Food Chemistry, 59, 12291–12301.

Federal Institute for Risk Assessemnt, BfR (2007), Frequently Asked Questions about 3-monochloropropane-1,2-diol (3-MMCPD), BfR FAQs, 17 December 2007. Available at: http://www.bfr.bund.de/cd/10581 [accessed April 2008].

Federal Institute for Risk Assessemnt, BfR (2009). Initial evaluation of the assessment of levels of glycidol fatty acid esters detected in vegetable fats. 2009.

Hamlet, C. G., and Asuncion, L. (2011). Single-laboratory validation of a method to quantify bound 2-chloropropane-1,3-diol and 3-chloropropane-1,2-diol in foodstuffs using acid catalysed transesterification, HFBI derivatisation and GC/MS detection. European Journal of Lipid Science and Technology, 113, 345–355.

Hamlet, C. G., Asuncion, L., Velíšek, J., Doležal, M., Zelinková, Z., Crews, C., (2010) The occurrence of fatty acid esters of chloropropanediols in foods: a review prepared for the UK Food Standards Agency. PAS Report No C028. High Wycombe: Premier Analytical Services.

Hamlet, C. G., Sadd, P. A., Liang, L., Jayaratne, S. M, Skingle, M. (2007) *Exploiting processing conditions to reduce acrylamide in cereal-based foods, C03052.* Report C021 prepared on behalf of the UK Food Standards Agency. High Wycombe: RHM Technology.

Hamlet, C. G. & Sadd, P. A. (2005) Effects of yeast stress and pH on 3-MCPD producing reactions in model dough systems, Food Additives and Contaminants, 22(7), 616-623.

Hamlet, C. G., Sadd, P. A., & Gray, D. A., (2004), Chloropropanols and their esters in baked cereal products. In: W. Yokoyama, ed. Abstracts of the Division of Agricultural and Food Chemistry 227th annual meeting, Anaheim, California, March 28-April 1, 2004. Anaheim: American Chemical Society.

Hamlet, C. G., & Sadd, P. A. (2004) Chloropropanols and their esters in cereal products. *Czech Journal of Food Sciences*, **22**, 259-262. (Poster presented at CHEMICAL REACTIONS IN FOODS V, 29th September-1st October 2004, Prague, Czech Republic).

Hamlet, C. G., Sadd, P. A., & Gray, D. A. (2004) Generation of monochloropropanediols (MCPDs) in model dough systems. 1. Leavened doughs. *Journal of Agricultural and Food Chemistry*, **52**, 2059-2066.

Hamlet, C. G., Sadd, P. A., & Gray, D. A. (2004) Generation of monochloropropanediols in model dough systems. 2. Unleavened doughs. *Journal of Agricultural and Food Chemistry*, **52**, 2067-2072.

Hamlet, C. G., Sadd, P. A., & Gray, D. A. (2003) Influence of composition, moisture, pH and temperature on the formation and decay kinetics of monochloropropanediols in wheat flour dough. European Food Research and Technology, 216, 122-128.

Han, L., Razdan, R. K., (1999). Total synthesis of 2-Arachidonylglycerol (2-Ara-Gl). Tetrahedron Letters, 40(9), 1631-1634.

Imai, H., Oishi, T., Kikuchi, T., and Hirama, M. (2000). Concise synthesis of 3-*O*-(2-*O*-α-D-Glucopyranosyl-6-*O*-acyl-α-D-glucopyranosyl)-1,2-di-*O*-acyl-*sn*-glycerols. Tetrahedron, 56, 8451-8459.

Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2002, Evaluation of certain food additives and contaminants: fifty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives, Rome, 5-14 June 2001. WHO technical report series; 909. Geneva: WHO.

Karšulínová.L., Folprechtová, B., Doležal, M., Dostálová, J., Velíšek, J. (2007). Analysis of the lipid fractions of coffee creamers, cream aerosols, and bouillon cubes for their health risk associated constituents. Czech Journal of Food Sciences, 25, 257-264.

Kuhlmann, J., 2011, Determination of bound 2,3-epoxy-1-propanol (glycidol) and bound monochloropropanediol (MCPD) in refined oils. European Journal of Lipid Science and Technology, Special Issue: Fatty acid esters of chloropropanols and glycidol, 13(3), 335–344.

MAFF (1995) Authenticity of dried durum wheat pasta, The forty-seventh report of the Steering Group on Chemical Aspects of Food Surveillance, Food Surveillance Paper No. 47 (ISBN 0112430066) London HMSO.

Masukawa, Y., Shiro, H., Nakamura, S., et al. A New Analytical Method for the Quantification of Glycidol Fatty Acid Esters in Edible Oils. Journal of Oleo Science, 2010, 59, 81-8.

OKO-TEST (2008), Frozen potato products. Available at: http://www.oekotest.de/cgi/ot/otgs.cgi?suchtext=3-mMCPD&doc=66893 [accessed April 2008].

Rider, T. H., and Hill, A. J. (1930) Studies of glycidol. I. Preparation from glycerol monochlorohydrin. Journal of the American Chemical Society, 52 (4), 1521–1527.

Shimizu, M., Kudo, N., Shiro, H., Yasunaga, K., Masukawa, Y., Katsuragi, Y., Yasumasu, T., 2011. Comparison of indirect and direct quantification of glycidol fatty acid ester in edible oils. Journal of Oleo Science, 59(10), 535–539.

Svejkovská, B., Novotný, O., Divinová, M., Réblová, Z., Doležal, M., Velíšek, J. (2004). Esters of 3-Chloropropane-1,2-Diol in Foodstuffs. Czech Journal of Food Sciences, 22, 190-196.

Vanlaldinpuia, K., and Bez, G. (2011). Useful methods for the synthesis of isopropylidines and their chemoselective cleavage. Tetrahedron Letters, 52, 3759-3764

Whitely, P. R., (1971), Biscuit manufacture; fundamentals of in-line production. London: Elsevier Publishing Company Ltd.

Yu, C., Lui, B., and Hu, L. (2000). Aconvenient biphasic process for the monosilylation of symmetrical 1,*n*-primary diols. Tetrahedron Letters, 41, 4281-4285.

Zelinková, Z., Doležal, M., Velíšek, J. (2009). Occurrence of 3-chloropropane-1,2-diol fatty acid esters in infant and baby foods. European Food Research and Technology, 228, 571-8.

Zelinková Z., Novotný O., Schůrek J., Velíšek J., Hajšlová J., Doležal M. (2008): Occurrence of 3-MMCPD fatty acid esters in human breast milk. Food Additives & Contaminants: Part A, 25, 669-676.

Zelinkova´, Z., Svejkovska´, B., Velı´sˇek, J., Dolezˇal, M., 2006, Fatty acid esters of 3-chloropropane-1,2-diol in edible oils. Food Additives and Contaminants, 23, 1290–8.