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The occurrence of fatty acid esters of chloropropanediols in foods: a review prepared for the UK Food Standards Agency

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1. Summary

Although chloropropanols have historically been associated with the savoury food ingredient hydrolysed vegetable protein, prepared by acid hydrolysis, the discovery of significant amounts of 3-chloropropandiol (3-CPD) “bound” as fatty acid esters in foodstuffs (3-CPD-esters), especially in refined edible oils, is still a relatively recent discovery. The toxicological significance of these 3-CPD-esters and their contribution to dietary intakes of e.g. non-esterified 3-CPD is not yet known and this review considers their likely metabolic fate *in vivo*. The latest developments in methods of analysis are presented while occurrence routes, mechanisms of formation and potential measures of control are discussed.

2. Contents

FIGURES

TABLES

3. Terms and abbreviations

BfR	Bundesinstitut für Risikobewertung (German Institute for Risk Assessment)
3-CPD	3-chloropropane-1,2-diol
3-CPD-P	Palimtoyl ester of 3-CPD
3-CPD-PP	1,2-dipalmitoyl esters of 3-CPD
3-CPD-D_5-OO	1,2-dioleate ester of 3-CPD-D_5
CONTAM	EFSA scientific panel on contaminants in the food chain
DAG	diacylglycerol(s)
DCP	dichloropropanol
EFSA	European Food Safety Authority
ESI	electrospray ionisation
FAME	fatty acid methyl ester
FFA	free fatty acid
GE	glycidol esters
GI	gastrointestinal
GPL	glycerophospholipid
HFBI	heptafluorobutyrylimidazole
MAG	monoacylglycerol(s)
PBA	phenylboronic acid
TAG	triacylglycerol(s)

4. Introduction

Fatty acid esters of chloropropanediols (CPD-esters) are contaminants that have now been isolated from various foodstuffs in numerous laboratories. Davídek *et al*. [1] and Velíšek *et al*. were the first to report the discovery of CPD-esters together with other chloropropanols such as 3-chloropropane-1,2-diol (3-CPD) and 1,3-dichloropropan-2-ol (1,3-DCP) as reaction products of hydrochloric acid with triacylglycerols (TAG) [2-4] and in acid-hydrolysed vegetable proteins (HVP) [5]. Gardener *et al*. [6] subsequently isolated CPD-esters from toxic Spanish rapeseed oil that had been adulterated with aniline and treated with hydrochloric acid. The first reported occurrence of CPD-esters in a natural unprocessed food was by Cerbulis *et al*. [7] who identified a small but significant quantity (< 1% of total neutral lipids) of diesters of 3-CPD in raw milk from several herds of goats. Subsequent investigations [8, 9] appeared to exclude the biosynthesis of these chloroesters *in-vivo*, however it was not clear whether these compounds were formed from dietary substances, e.g. 3-CPD or its esters, passed through the organism or from anthropogenic chlorine containing compounds (such as chlorine based sanitizers used in dairy operations) post secretion. While these contaminants can now be controlled in HVP by good manufacturing practices, the presence of CPD-esters in other foods has only recently been observed, most notably in refined edible oils and products using these oils as ingredients.

CPD-esters are the esterified form of the parent chloropropanediols such as 3-CPD. In reality, 3-CPD is a chiral compound, which occurs in foods in racemic mixtures of its two enantiomers [4], (2*R*)- and (2*S*)-3-chloropropane-1,2-diol. The structures of these enantiomers and the corresponding chiral 3-CPD fatty acid esters are shown in Figure 1.

CPD-esters have previously been evaluated for their anti-tumor properties [10] and are widely used as substrates for the chemical synthesis of e.g. biologically active chemicals [11], otherwise they appear to have no direct industrial application.

Studies have linked 3-CPD with infertility in rats, suppression of the immune function and possible carcinogenicity. In 2001, the EC Scientific Committee on Food established a Tolerable Daily Intake of 2 µg/kg body weight for 3-CPD [12] and a maximum concentration level of 20 µg/kg has been specified in EU legislation for 3-CPD in hydrolysed vegetable proteins and soy sauce [13] (for full reviews of 3-CPD in foods and ingredients see references [14-17]). Hence a toxicological concern here is the potential contribution to the dietary intake of 3-CPD from the hydrolysis of 3-CPD-esters *in vivo* during digestion, e.g. by lipases in the gastrointestinal tract[1]. Because of this, the term "bound" 3-CPD has been used to describe the amount of 3-CPD that could be released from the corresponding fatty

[1] The human fat-digestive enzymes include TAG- and phospholipases [18]. TAG lipase catalyzes the hydrolysis of TAG to free fatty acid, mono- and diacylglycerol. The human lipases include the pre-duodenal lingual and gastric lipases (EC 3.1.1.3), extra-duodenal pancreatic and hepatic lipases, endothelial lipase (EC 3.1.1.3), and lipoprotein lipase (EC 3.1.1.34).

acid ester. Based on the opinion of the German Institute for Risk Assessment (BfR) on CPD-esters [19], the EFSA COMTAM panel has assumed a 100% release of 3-CPD from its esters in the gut [20].

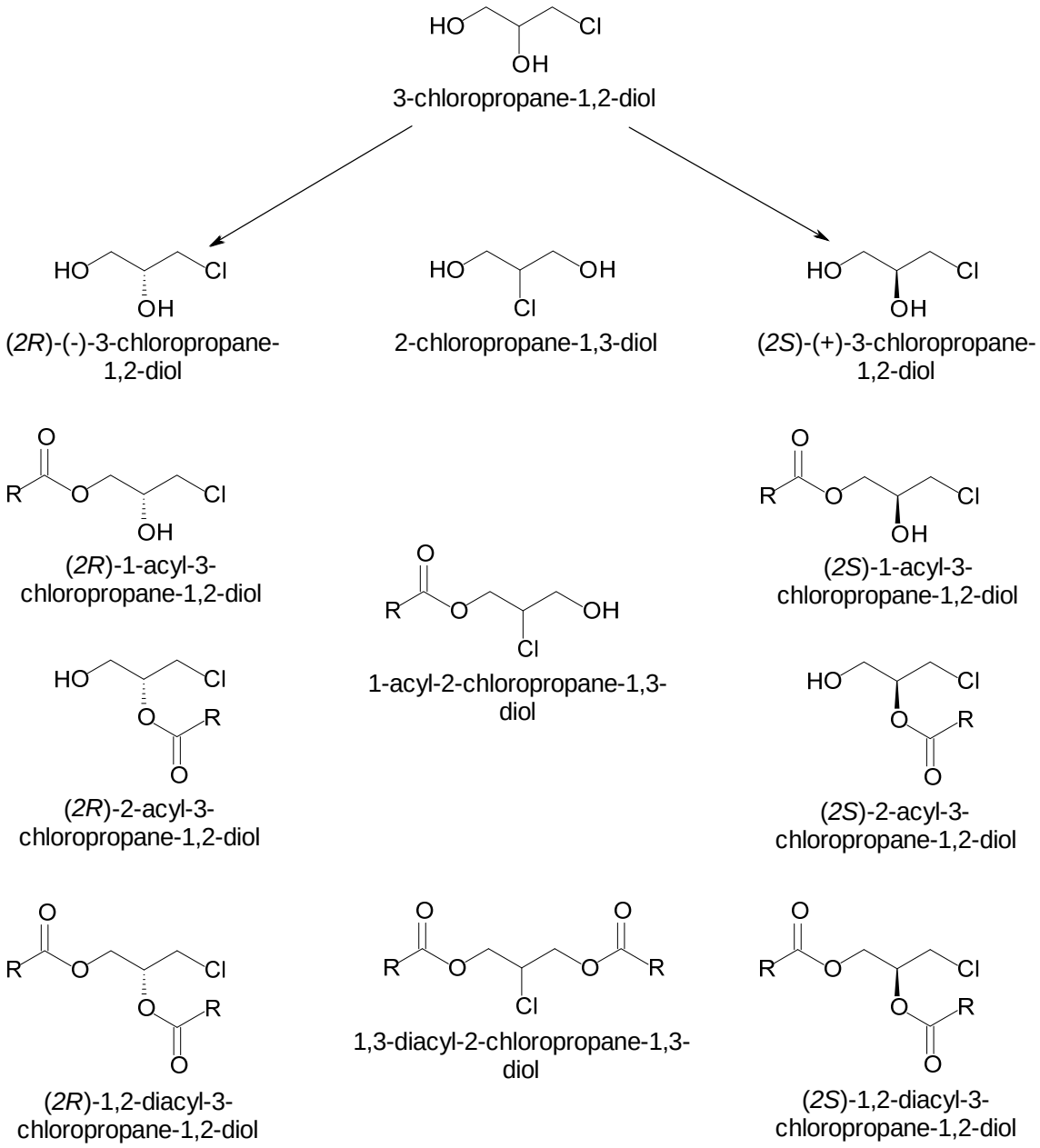


Figure 1. Structures of mono- and diesters of 2- and 3-CPD (R= alkyl)

5. Toxicological aspects

While there have been a number of toxicological animal studies on 3-CPD (for previous reviews see [21-24]), equivalent studies with 3-CPD-esters have yet to be undertaken.

The toxicological concern associated with exposures to 3-CPD-esters is likely to vary according to their metabolic fate in the human gut. Limited preliminary data from *in vitro* biokinetic studies [25, 26] indicate that like TAG, 3-CPD-esters may become substrates of lipases, releasing 3-CPD, and that

this “free” 3-CPD may also be rapidly absorbed in the intestine. In model systems with pancreatic lipases, the efficiency of this enzymatic reaction was found to be higher toward *sn*-1(3)-monoesters than toward diesters [26]. In addition, the release of 3-CPD was significantly reduced when the esters were provided in an oil matrix. Altogether, these data suggest that a limited hydrolysis of 3-CPD-esters in the gut is probable, although a complete release of bound 3-CPD is unlikely. Therefore, to address the health significance of exposure to 3-CPD-esters, it is required to consider both the esters *per se*, and the potentially released 3-CPD from this source.

The systemic effects of 3-CPD-esters will depend on their rate of absorption. Because of their structural similarity and sensitivity to pancreatic lipase (EC 3.1.1.3), the mechanisms of TAG absorption [27] may provide relevant information for 3-CPD-esters. Ingested TAG are not absorbed as such. In the stomach and small intestine, lipases with higher affinity for positions *sn*-1 and *sn*-3 release free fatty acids, and 2-monoacyl-*sn*-glycerol (2-MA-*sn*-G) from the TAG. This 2-MA-*sn*-G produced by hydrolysis of dietary fats by lipases is readily taken up by the enterocytes, re-esterified by the action of cellular 2-acylglycerol *O*-acyltransferase (EC 2.3.1.22) (see Figure 2) to 1,2-diacyl-*sn*-glycerols and further to TAG employing diacylglycerol (DAG) *O*-acyltransferase (EC 2.3.1.20), then incorporated into TAG-rich lipoprotein particles (i.e. chylomicrons). Chylomicrons are secreted into the lymph, enter the circulation through the thoracic duct, and supply tissues and organs with TAG. Excess of fats is stored in adipose tissue.

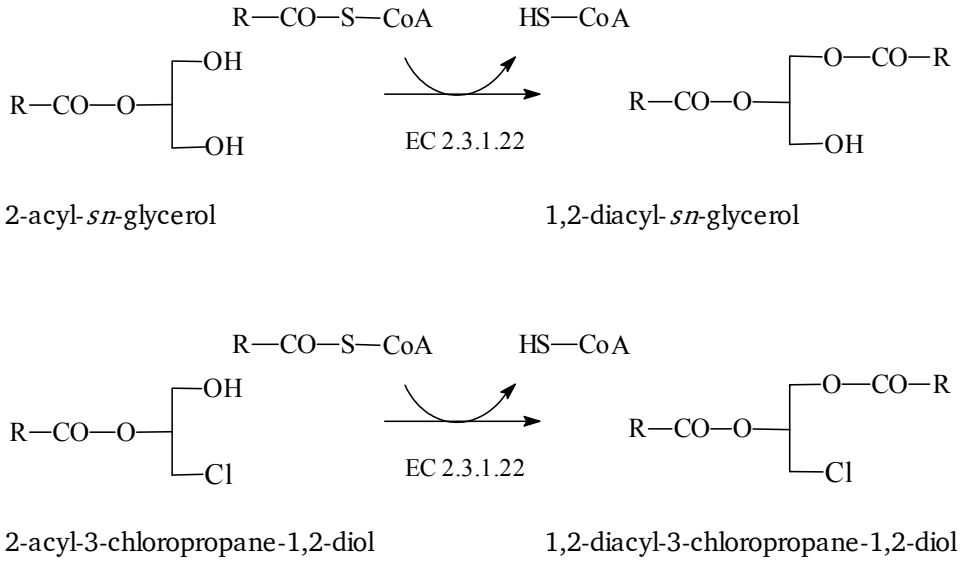


Figure 2. Formation of 1,2-DAG and 1,2-diacyl-3-CPD from 2-MAG and 2-monoacyl-3-CPD

In all cells TAG are mostly biosynthesised from glycerol, which predominates in liver and adipose tissue [28] (a MAG pathway proceeds in the intestines). The glycerol backbone is activated by esterification (phosphorylation) at the C-3 position by glycerol kinase (EC 2.7.1.30) and forms prochiral glycerol 3-phosphate. TAG are produced from *sn*-glycerol 3-phosphate, by esterification

with fatty acyl-CoA residues. The first step, catalyzed by glycerol 3-phosphate *O*-acyltransferase (EC 2.3.1.15), yields 1-acylglycerol 3-phosphoric acid, also known as lysophosphatidic acid. This compound is then esterified at the C-2 hydroxyl by 1-acylglycerol 3-phosphate *O*-acyltransferase (EC 2.3.1.51) giving rise to 1,2-DAG 3-phosphoric acid (commonly identified as phosphatidic acid). The phosphate group is then removed by phosphatidate phosphatase (EC 3.1.3.4) prior to the last esterification of 1,2-DAG to TAG, which is catalyzed by DAG *O*-acyltransferase (EC 2.3.1.20). 3-CPD cannot be phosphorylated by glycerol kinase [29], therefore the only possibility of 3-CPD esters incorporation into the body lipids is the ingested 3-CPD esters. Assuming a similar metabolism for 3-CPD-esters, the de-esterification of diesters in position *sn*-1 would be favoured by pancreatic lipases. Hence 3-CPD, and 3-CPD-2-monoesters would therefore be released respectively from the 1-monoesters, and the diesters potentially present in food.

The 2-monoesters of 3-CPD resemble 2-MAG (see Figure 2), would be absorbed, re-esterified in the enterocytes by 2-acylglycerol *O*-acyltransferase (EC 2.3.1.22), bound in the chylomicrons, then possibly incorporated in cellular membranes and/or adipose tissue and hydrolysed by lipoprotein lipase to monoesters in the target tissues (e.g. mammary gland) [8]. Therefore, it seems important to distinguish between monoesters and diesters because they have different metabolism pathways. The biological significance of such a fate for 3-CPD-esters is unknown. It may however explain the relatively high concentrations of 3-CPD-esters reported in some samples of mammalian milks [7], including human breast milk [30].

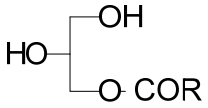
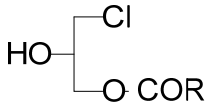
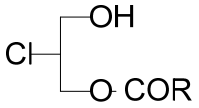
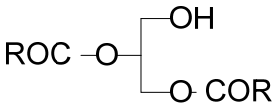
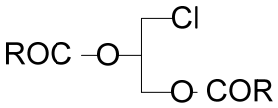
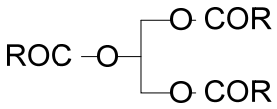
6. Chemistry and Analysis

6.1 Chemistry in general

6.1.1 Physical properties.

CPD-esters are likely to have similar physical and chemical properties to the naturally occurring acyl-glycerols with which they are probably associated in foods. Most CPD-esters are solids at room temperature and the replacement of -OH by -Cl on the acyl-glycerol backbone results in a small decrease in melting point (see Table 1), and as expected the increasing alkyl chain length results in a progressive increase in melting point. Although the gas chromatographic (GC) separation of CPD-esters has been reported in the literature [5, 30, 31], the relatively large molecular weight of many CPD-esters may place limitations on the wholesale use of this technique. In the laboratory, reference CPD-esters exhibit similar solubility characteristics to naturally occurring acyl-glycerols and solvents such as diethyl ether [31-33], hexane/acetone [34], and ethyl acetate [35, 36] have been used for the extraction of CPD-esters from foodstuffs. However, the efficiency of these solvents is not known as few recovery data for the extraction of CPD-esters from foods is available.

Table 1. Melting points of synthetic CPD-esters and the corresponding acyl-glycerols (data from reference [37])

R	MP (°C)		
1-acyl-*sn*- glycerols			
$C_{11}H_{23}$	54-55	-[a]	-
$C_{15}H_{31}$	71-72	36-37	34-35
$C_{17}H_{35}$	75-75	43-44	-
$C_{21}H_{43}$	-	54-56	-
1,2-diacyl-*sn*- glycerols			-
$C_{15}H_{31}$	68-69	-	-
$C_{17}H_{35}$	74-76	46-47	-
1,2,3-triacyl-sn-glycerols		-	-
$C_{15}H_{31}$	66.4	-	-
$C_{17}H_{35}$	73.5	-	-

[a] oil at 0°C

6.1.2 Chemical properties.

CPD-esters can represent a wide range of chemical functionality which includes the chemistry of alkyl halides, aliphatic carboxylic acid esters and in the case of CPD-monoesters, the aliphatic alcohols. Like their mono-and diacyl-glycerol counterparts, CPD-monoesters are also likely to exhibit acyl-migration to the thermodynamically favoured *sn*-1(3) positions on the glycerol backbone (see Figure 3) as influenced by temperature, pH and solvent [38-40]. This diverse chemical functionality has implications for the analyst and extreme caution must be exercised with analytical strategies that seek to determine isomeric distribution and/or modify the chemistry of 3-CPD-esters for detection. Some of the more relevant chemical reactions of 3-CPD-monoesters are illustrated in Figure 3.

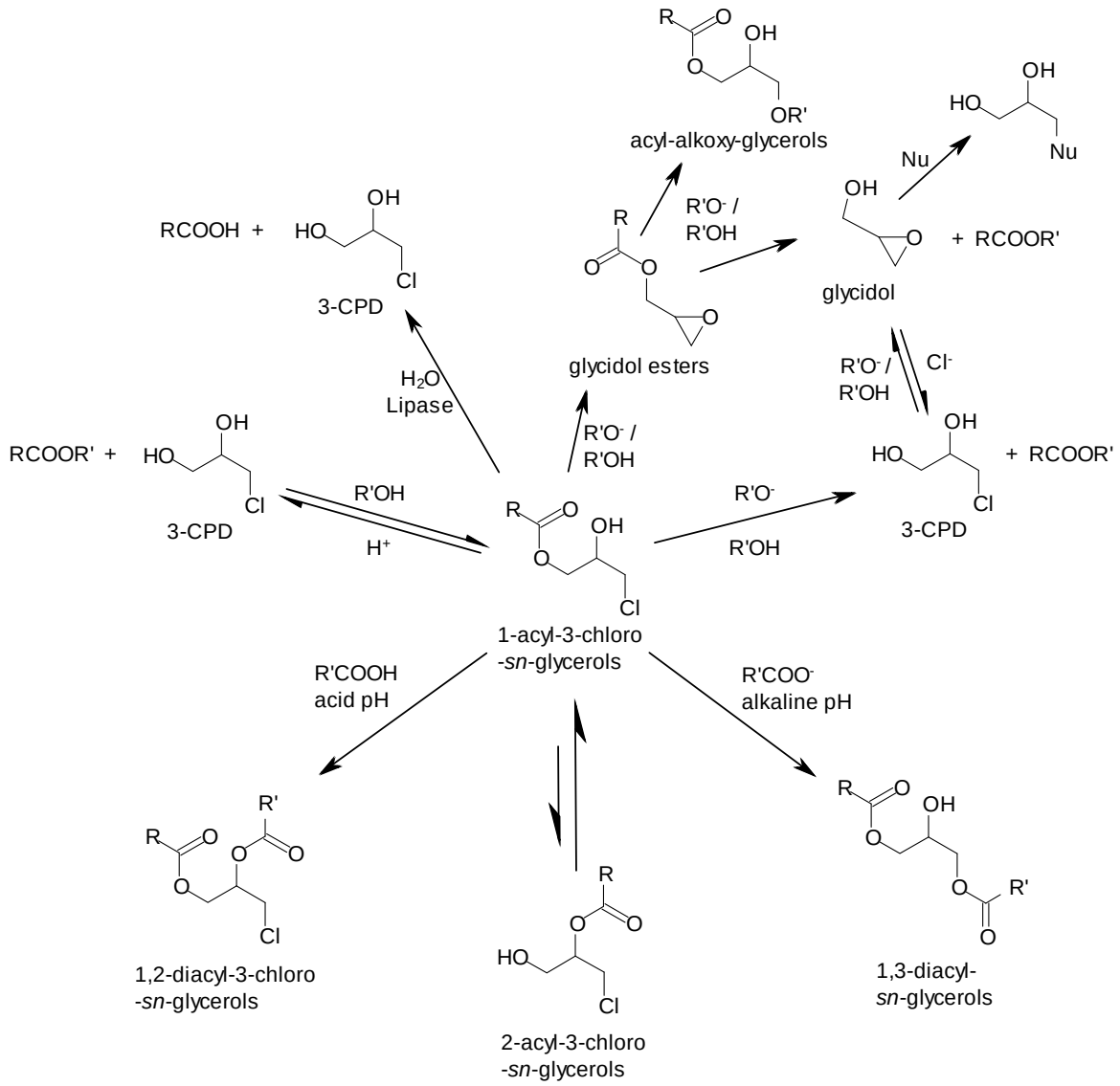


Figure 3. Some key chemical reactions of 1-acyl-3-chloro-*sn*-glycerols

6.1.3 Strategies for the analysis of CPD-esters.

A summary of the strategies employed for the analysis of CPD-esters in edible oils or fat extracted from foods is given in Figure 4. For direct determination using GC/MS, the CPD-esters were first separated from co-extracted lipids (e.g. TAG) into fractions containing mono- and diesters of CPD using preparative chromatographic techniques such as thin layer or column chromatography. The individual CPD-esters were then separated using capillary GC and identified by GC/MS from reference mass spectral data (see 6.1.5). Until recently, these methods have provided only a partial quantification of individual CPD-esters due to the limited availability of reference CPD-esters (see

6.1.4). However, a recent method utilising multiple reference CPD-esters and combining chromatographic separation and detection based LC/MS looks very promising [41].

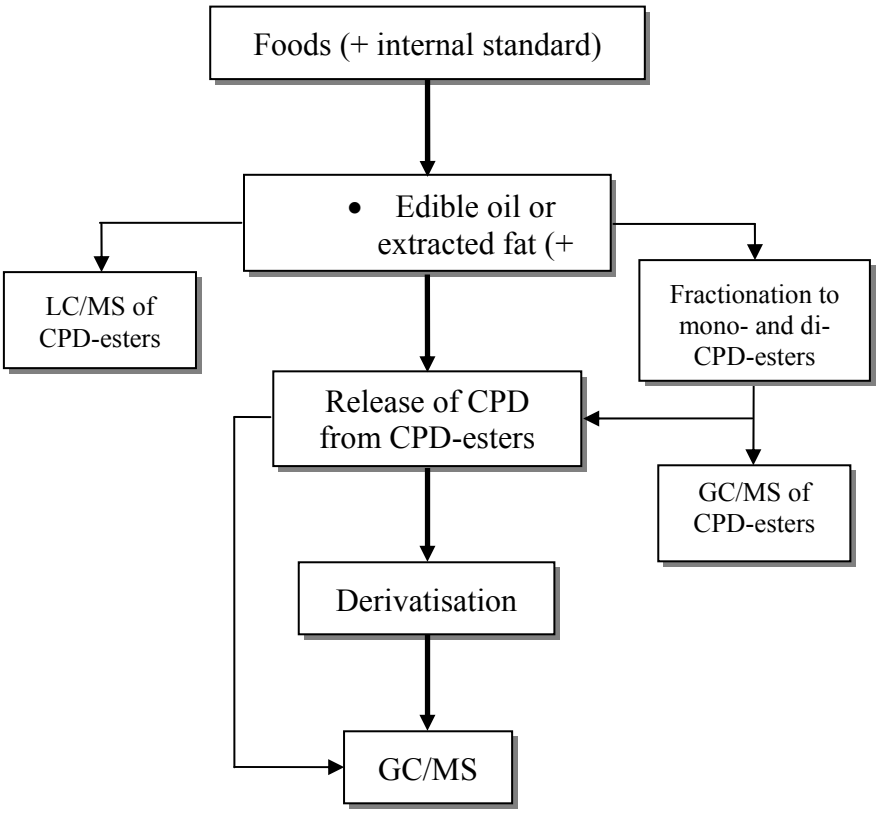


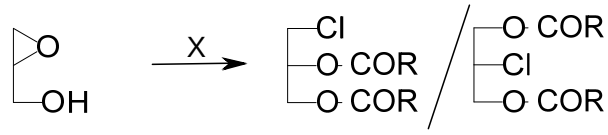
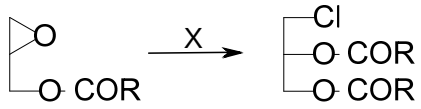
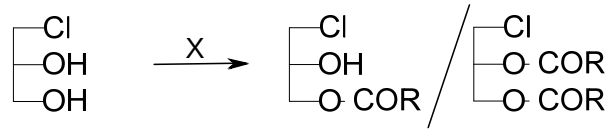
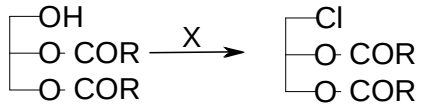
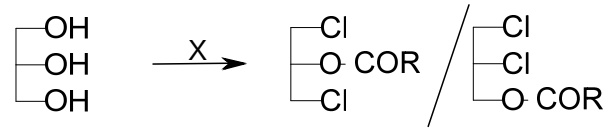
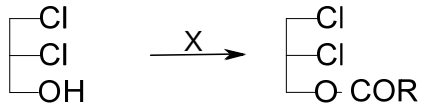
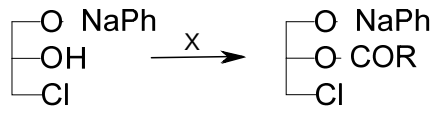
Figure 4. Strategies used to determine 3-CPD-esters in edible oils and fats extracted from foods

Many of the current methods for CPD-esters analysis actually measure the amount of CPD released from its esters using, for example, enzyme (lipases) or chemical treatment. The CPD moiety released in this way is then typically derivatised for detection using established GC/MS procedures that are documented elsewhere [16]. While these methods can not provide information concerning the nature of individual CPD-esters, prior fractionation of the oil/extracted fat does permit a quantification of total mono- and total diester species.

6.1.4 Synthesis of reference chemicals.

Until recently, limited availability of CPD-ester reference chemicals has necessitated custom syntheses. Some of the methods used to prepare CPD-esters together with other general routes to chlorohydrin esters are summarised in Table 2.

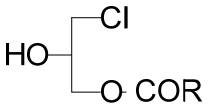
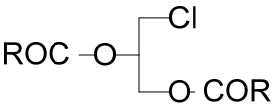
Table 2. Synthetic routes to reference CPD-esters and related compounds

Reaction route to:	R	X	Reference
esters of 2- and 3-CPD			
	CH_3	RCOCl	[42, 43]
	$C_{15}H_{31}$, $C_{17}H_{35}$	Bu_4NCl, $(RCO)_2O$, TMSCl	[11]
	$C_{15}H_{31}$, $C_{17}H_{35}$, $C_{17}H_{33}$	RCOCl (DMF / pyridine)	[1, 37, 44]
	$C_{15}H_{31}$, C_7H_{15}	$SOCl_2$	[45]
other chlorohydrin esters			
	$C_{15}H_{31}$, C_7H_{15}	RCO_2H, TMSCl	[46]
	C_3H_7	*Candida cylindracea* on Chromosorb®, tributyrin	[47, 48]
	-	Lipase PS (from *Pseudomonas sp.*), hexane / $CHCl_3$, $(RCOO)_2$	[49]

6.1.5 Mass spectra (EI 70eV).

The 70-eV electron ionisation (EI) mass spectra of synthetic CPDs esterified with C_{16} and C_{18} fatty acids is given in Table 3. Both the mono- and diesters give weak molecular ions but they are readily characterised by relatively intense even-electron ions corresponding to $[RCO]^+$. The diesters of CPD give additional diagnostic ions corresponding to loss of $[RCO_2]^+$. For a detailed review of the mass spectral behaviour of CPD-esters see Kraft *et al*. [37] and Davídek *et al*. [1].

Table 3. EI (70ev) mass spectra of mono- and diesters of 3-CPD (data adapted from references [1, 37])

Ion	*m/z* (%)					
						
R	$C_{15}H_{31}$	$C_{17}H_{35}$	$C_{17}H_{33}$	$C_{15}H_{31}$	$C_{17}H_{35}$	$C_{17}H_{33}$
Fatty acid	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$
$[M]^{+\cdot}$	348 (1)	376 (1)	374 (1)	586 (1)	642 (1)	638 (2)
$[M\text{-}C_2H_4\text{-}(CH_2)_n]^{+\cdot}$ (McL series)	-	-	-	390[a] (2)	418[b] (2)	-
$[M\text{-}OH]^+$	331 (2)	359 (0.4)	357 (0.4)	-	-	-
$[M\text{-}Cl]^+$	313 (0.5)	341 (0.5)	339 (0.3)	-	-	-
$[M\text{-}CH_2Cl]^+$	299 (2)	327 (2)	325 (3)	537 (1)	593 (1)	589 (3)
$[M\text{-}RCO\text{-}2H]^+$	-	-	-	349 (8)	377 (3)	375 (1)
$[M\text{-}RCO_2]^+$	-	-	-	331 (29)	359 (10)	357 (10)
$[M\text{-}RCO_2H]^{+\cdot}$	92 (32)	92 (24)	92 (7)	-	-	-
$[RCO_2CH_3]^{+\cdot}$	286 (2)	314 (2)	312 (0.5)	-	-	-
$[RCO_2H_2]^+$	257 (6)	285 (4)	283 (1)	-	-	-
$[RCO]^+$	239 (43)	267 (27)	265 (24)	239 (47)	267 (17)	265 (23)
$[RCO\text{-}H]^{+\cdot}$	-	-	264 (37)	-	-	264 (36)
$[C_4H_7]^+$ / $[C_2H_3CO]^+$	55 (51)	55 (59)	55 (100)	55 (54)	55 (59)	55 (100)
$[C_3H_7]^+$ / $[CH_3CO]^+$	43 (100	43 (100)	43 (85)	43 (100	43 (100)	43 (43)

[a] n=12; [b] n=14

6.2 Review of recent methods.

Table 4 gives a summary of analytical methods for CPD-esters published since 2004. GC/MS has been widely used for the direct analysis of CPD-esters in studies where detailed information concerning the types of fatty acids was required. Silica gel [50, 51] columns were used for the chromatographic isolation and fractionation of CPD-mono- and diesters using e.g. petroleum ether / diethyl as elution solvents. In all cases the CPD-esters were identified from reference mass spectral data (see Table 3) and quantified as 3-CPD-PP using either external or internal standard (3-CPD-D_5-PP added to fat / oil) methods.

Transesterification of CPD-esters in extracted fats and oils followed by GC/MS of the CPD-moiety has been widely used to provide a relatively rapid measure of total CPD-esters or total mono- and diesters (using column fractionation described previously). Transesterification has been carried out under both alkalic and acidic conditions and the method typically comprises a number of steps outlined in Figure 5.

Table 4. Summary of recent methods for the analysis of 3-CPD-esters in foodstuffs (since 2004)

Method principle	CPD-esters quantified	Matrix	Detection	Quantification	Reference
Direct determination	Individual C 16 and C18 mono- and diesters	Fat from salami	GC/MS	3-CPD-PP (external standard)	[31]
	Individual C 16, and C18 diesters	Edible oils / fat from cheese, infant and baby foods	GC/MS	3-CPD-D_5-PP, 3-CPD-D_5-P	[52, 53]
	Individual C 12 – C 18 diesters	Human breast milk	GC/MS	3-CPD-D_5-PP	[30]
	Individual C 16 and C18 mono- (5) and diesters (15)	Edible oils	LC/TOF-MS	3-CPD-D_5-OO	[41]
Lipase catalysed hydrolysis	total	Bread, roasted cereals	GC/MS of HFBI derivative	3-CPD-D_5-PP	[35, 36, 54]
Transesterification					
Acidic	Total	Edible oils / fat from foods	GC/MS of PBA derivative	3-CPD-D_5	[32]
Acidic	Total	Foods and edible oils	GC/MS/MS of HFBI derivative	3-CPD-D_5-PP	[55]
Acidic	Mono- and diesters	Edible oils / fat from foods	GC/MS (underivatised)	3-CPD-D_5-PP, 3-CPD-D_5-P	[52]
Acidic	Mono- and diesters	Edible oils / fat from foods	GC/MS of PBA derivative	3-CPD-D_5-PP, 3-CPD-D_5-P	[51]
Acidic	Mono- and diesters	Edible oils	GC/MS of HFBI derivative	3-CPD-D_5-PP, 3-CPD-D_5-P	[26]
Alkalic	Total	Edible oils	GC/MS of PBA derivative	3-CPD-D_5	[56]
Alkalic	Total	Edible oils	GC/MS of PBA derivative	3-CPD-D_5	[57]
Alkalic	Total	Edible oils	GC/TOF-MS of PBA derivative	3-CPD-D_5	[58]
Alkalic	Total	Foodstuffs	GC/MS of PBA derivative	3-CPD-D_5-ester	[59]

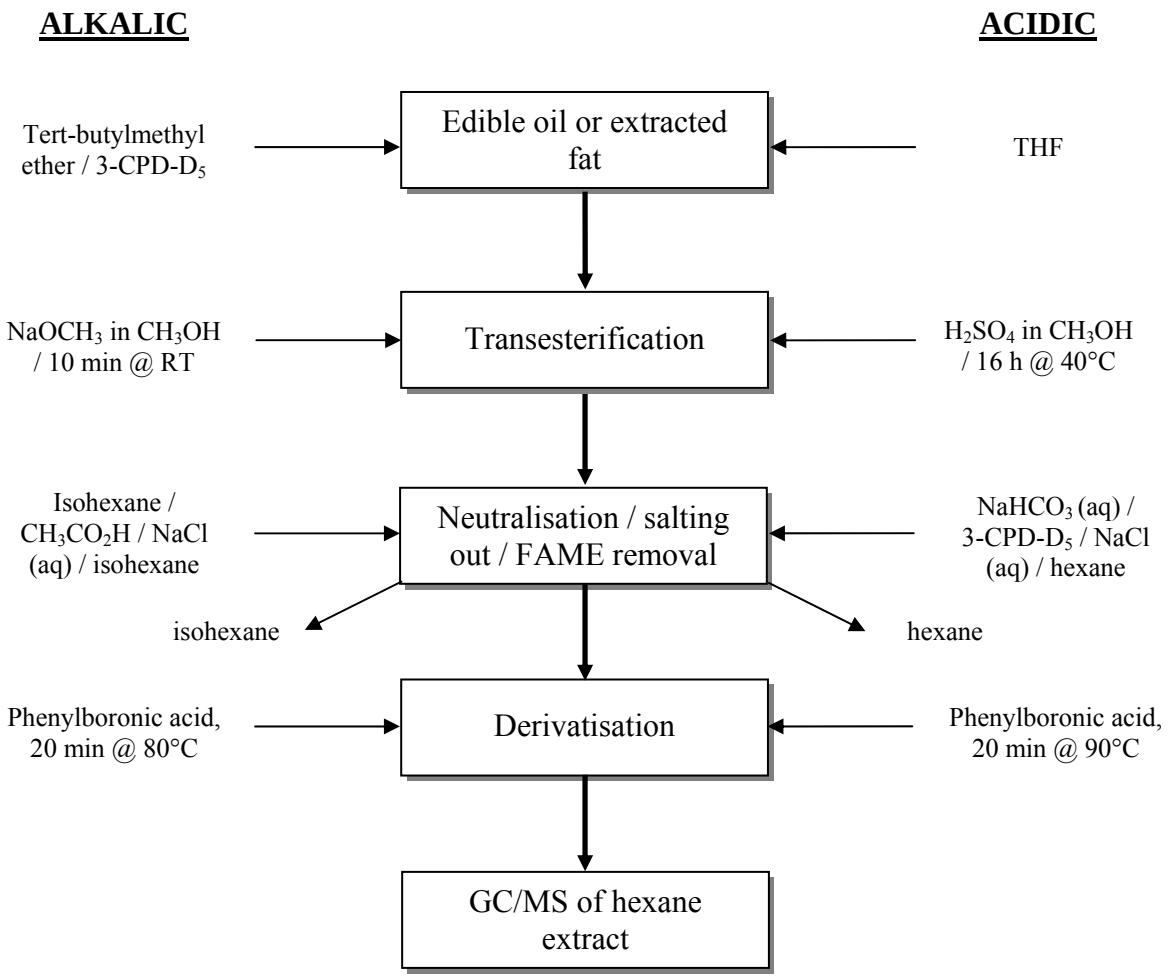


Figure 5. Sequence of steps required to release 3-CPD from its esters using either alkali or acid catalysed transesterification

Divinová *et al*. [32] were the first to develop a transesterification method for CPD-esters in fats/oils based on the acid-catalysed methanolysis procedure of Cerbulis *et al*. [7] used to confirm total CPD-esters in goat milk. Extracted fat / oil (100 mg) dissolved in a small quantity of THF was transesterified using sulphuric acid in methanol. After neutralisation of the acid with sodium hydrogen carbonate and removal of FAME, the released 3-CPD was derivatised with phenylboronic acid (PBA) for detection using GC/MS. In keeping with the observations of Cerbulis et al. [7], these conditions of acid catalysed transesterification did not reveal any additional 3-MCPD formation, e.g. from chloride ion and lipid precursors present in the matrix. Subsequent results obtained from the analysis of a wide variety of foods, fats and oils that may contain chloride ions have also shown good agreement with results obtained from the analysis of separated 3-CPD diester and monoester fractions using silica gel chromatography, i.e. fractions devoid of potential 3-CPD precursors [34]. Divinová *et al*. [32] added 3-CPD-D_5 after the neutralisation step while Zelinková *et al* [51] and Seefelder *et al*. [26] later used

labelled 3-CPD-esters added to extracted fat, prior to column fractionation and transesterification, for improved quantification. Using these adaptations, method detection limits of circa 300 – 1000 µg/kg have been achieved on a fat / oil basis. Seefelder *at al*. and Hamlet and Asuncion [55] used heptafluorobutyrylimidazole HFBI to derivatise 3-CPD released by transesterification and added labelled 3-CPD-D_5-dipalmitate (3-CPD-D_5-PP) directly to foodstuffs to provide recovery information. With these modifications, the mean recovery of 3-CPD-PP added to a range of cereal, oil and potato products over the concentration range 9-5673 µg/kg was 102.9±5.6% with limits of detection of 3-11 µg/kg for foods and 60 µg/kg for oils.

Weißhaar [56] demonstrated that additional 3-CPD could be generated during acid catalysed transesterification if chloride ions are present, presumably from the reaction of chloride ions with residual lipids. The author demonstrated that no additional formation of CPD occurred when transesterification was carried out under alkalic conditions using sodium methoxide in methanol according to the steps given in Figure 5. However, 3-CPD was found to be relatively unstable under alkalic conditions and the author observed 20 – 60% losses of 3-CPD-D_5 during transesterification necessitating the addition of a labelled internal standard to compensate for these losses. Under these conditions a limit of detection of circa 100 µg/kg could be attained for total 3-CPD obtained from its esters in oils. Later, variable recoveries of derivatised CPD were attributed to the presence of glycidol (from glycidol esters in fats / oils) from which additional 3-CPD was believed to have formed during the addition of acetic acid / saturated sodium chloride. The method was subsequently modified to include a glycidol ester removal step via pre-treatment of samples with sulphuric acid in propanol, and the difference in CPD measured with and without this pre-treatment step was proposed as a measure of glycidol esters in oils (expressed as 3-CPD) [57]. Küsters *et al*. [59] later added a labelled 3-CPD-ester internal standard and reported recoveries of better than 84% for 3-CPD-esters added to a range of foodstuffs including bakery, meat, fish, soups and seasonings in the concentration range 50-2000 µg/kg.

The conditions of transmethylation using sodium methoxide can be considered relatively harsh. In a recent proficiency trial [60] a strong positive bias was observed for laboratories using alkalic transesterification and a critical appraisal of the chemistry of CPD and CPD-esters under these conditions may provide additional interpretation. Sodium methoxide is a powerful base and under the conditions of alkalic transmethylation 3-CPD will decompose rapidly via the intermediate glycidol [61, 62] (see Figure 3), and the rate of this dehydrochlorination is likely to be greater than the rate of transesterification of 3-CPD-esters [63]. Hence 3-CPD-D_5 internal standard added under these conditions will decompose to glycidol-D_5 and this is consistent with the observation that 3-bromopropane-1,2-diol-D_5 is formed (from glycidol-D_5) when sodium chloride is substituted for sodium bromide post alkalic transesterification [41]. Hence the calculated recovery of 3-CPD by these methods will be dependent not only on the decomposition of labelled and non labelled 3-CPD during transesterification but also their subsequent reformation from glycidol during the acidification /

chloride ion addition step. Furthermore, it cannot be assumed that glycidol esters will remain for measurement under the conditions of alkalic transesterification. It is well known that the epoxide ring can opened under both alkalic (e.g. by methoxide ion) and acidic conditions although the rate of ring opening under the former conditions may be slower. Recently, Collison *et al*. [41] reported a method for the direct determination of individual 3-CPD-ester species in edible oils using LC/TOF-MS without the requirement for a lengthy column chromatographic and cleanup. Based on a method developed for DAG [64], the authors reported an optimised and rapid quantification of CPD-esters in oils using positive ion electrospray ionisation (ESI) with a low concentration of sodium acetate in the mobile phase to generate sodiated ions. Using this approach the limit of detection for the sum of 15 diesters and five monoesters of CPD was 500 µg/kg. Comparison of this method with that based on alkalic transesterification [57] showed that LC/MS gave significantly lower concentrations of 3-CPD in commercial oils (see Table 5). Furthermore, the method could also determine five glycidol esters at a total limit of detection of 100 µg/kg.

A review of methods for the analysis of glycidol esters can be found in appendix 11.1.

Table 5. Comparison of total 3-CPD concentrations measured in oils by LC/TOF-MS and alkalic transesterification (data from reference [41])

Sample	Total 3-CPD (µg/kg)	
	by alkalic transesterification[a]	3-CPD equivalent by LC/TOF-MS
Palm oil A	7740	1200
Palm oil B	2850	<100[b]
Commercial oil blend A	3640	<100[b]
Commercial oil blend C	1830	<100[b]

[a] limit of detection 100 µg/kg; [b] inferred limit of detection from that given for total 3-CPD esters

7. Occurrence

7.1 Food ingredients

Published data on 3-CPD-ester concentrations in food ingredients are given in Table 6. The highest concentrations of ester-bound 3-CPD have been found in the refined oils whereas virgin and non-refined oils have contents below or slightly above the detection limits [31, 65].

7.1.1 Refined oils

There are two methods used to process crude vegetable oil into edible or refined oil. These are termed "physical" and "chemical" and differ in the processes by which the free fatty acids (FFA) are removed from the oil (see Figure 6). In the physical process, the FFA are removed by distillation and in the

chemical process they are neutralised by alkali to give soaps which can then be removed by phase separation.

Table 6. Concentrations of 3-CPD-esters measured in food ingredients (expressed as total 3-CPD bound in esters on commodity weight basis)

Foodstuff	No samples	mean ($\mu g\ kg^{-1}$)	range ($\mu g\ kg^{-1}$)	reference
DATEM	1	-	66	[35, 36]
Malt				
Caramel	1	-	6.9	[66]
light	1	-	11.0	[66]
dark	1	-	10.5	[66]
Crystal	4	13.8	7.3-29.6	[33]
Czech	1	-	6.5	[66]
Dark	1	-	580	[67]
Karapils	1	-	21.7	[33]
Munich	2	4.4	4.0-4.8	[33]
Pilsner	2	8.2	5.2-11.1	[33]
Roasted	4	537.5	463-650	[33]
Wheat	1	-	6.7	[33]
Oils and fats				
Refined coconut oils	2	1556	1418-1694	[68]
Refined olive oils	5	1464	<300-2462	[31]
Refined palm kernel oils	3	1168	850-1400	[68]
Refined palm oils	4	2821	1390-4170	[68]
Refined seed oils[a]	5	524	<300-1234	[31]
Refined vegetable fats / oils	126	-[b]	<200-21500	[65]
Refined vegetable fats / oils[c]	11	1535 (155[d])	897-2435 (61-299[d])	[26]
Unrefined animal fats[e]	25	-[b]	<100-140	[65]
Unrefined vegetable fats / oils	122	-[b]	<100-310	[65]
Virgin germ oils	2	100	<100-<300	[31]
Virgin olive oils	4	75	<100-<300	[31]
Virgin seed oils	8	63	<100-<300	[31]
roasted	1	-	337	[31]
Potato flake	1	-	17.9	[69]
Roasted cereals				
Barley[f]	3	1314	557-1896	[54]
Barley	8	646	44-1386	[66]
Wheat	6	438	89-1020	[66]
Rye	6	245	25-684	[66]
Wheat flour	1	-	<5	[35, 36]

[a] maize, rapeseed, soybean, sunflower; [b] data not available; [c] mixed sunflower, rape seed, coconut, corn and palm oils; [d] 3-CPD bound in monoesters [e] milk fat, lard, tallow; [f] laboratory trial samples roasted at 220, 230 and 240°C for 35 min

Although there is some evidence that the chemical refining process may yield lesser amounts of 3-CPD esters compared to the physical method [70], it seems that CPD-ester formation in refined oils

may be influenced more by the crude oil characteristics. Refined palm and olive oils appear to generate more 3-CPD esters compared to seed oils such as maize, rapeseed, soybean and sunflower [31, 71]. Crude palm oils derived from pulps differ markedly from seed oils in their composition and can contain relatively high levels of potential 3-CPD-ester precursors such as chloride ions, and DAG [31, 71]. In a direct analysis of refined oils using LC/MS, collision *et al* demonstrated evidence of a correlation between DAG and 3-CPD-esters indicating that the former lipids may be significant precursors.

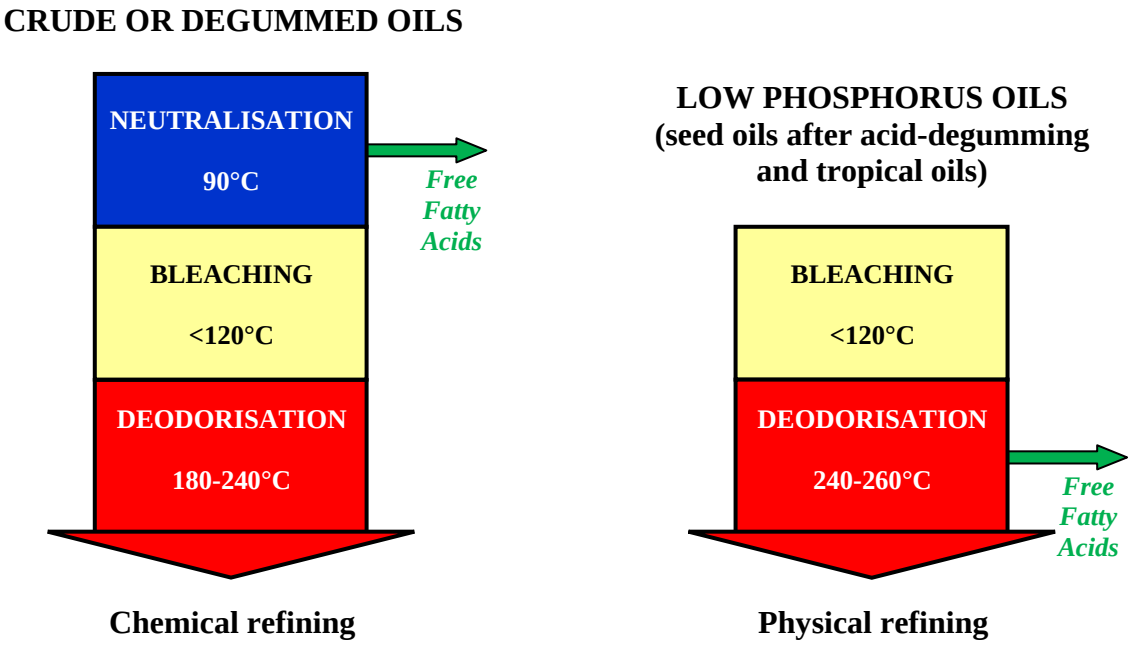


Figure 6. Overview of the main processes of edible oil refining

Irrespective of the refining process used (physical / chemical), the majority of 3-CPD esters appear to be formed during the deodorization step [70, 71], a high temperature process (e.g. 260°C for 20 min) in which steam is injected into the oil to remove undesirable steam volatile components. It seems that amounts of CPD-esters are formed rapidly during the deodorisation stage and are more temperature dependent rather than process time dependent [70, 71]. However in laboratory trials, extended heating of oils at high temperatures led to a decrease in 3-CPD-esters indicating that formation may limited by precursor availability and/or decomposition reactions [31, 72].

The contribution to 3-CPD-ester formation from the bleaching process is less clear. It is known that bleaching earths can be activated with acid treatments to enhance their efficiency and that they could therefore be a potential source of chloride ions (e.g. from hydrochloric acid). While some studies have reported that the bleaching process can contribute 20-30% [70] to the overall formation of 3-CPD-

esters during refining, other workers reported a decrease during the bleaching of a pre-refined palm oil [71].

7.1.2 Thermally treated cereals

Several laboratories have reported the formation of 3-CPD-esters in roasted cereals such as barley (and malt), rye and wheat [33, 54, 66, 67]. The generation of 3-CPD-esters was correlated to time and temperature with the highest amounts being formed at the higher temperatures (see Figure 7). Presumably the esters are formed from naturally occurring lipids and chloride ions present in the cereals by mechanisms analogous to that in the refined oils.

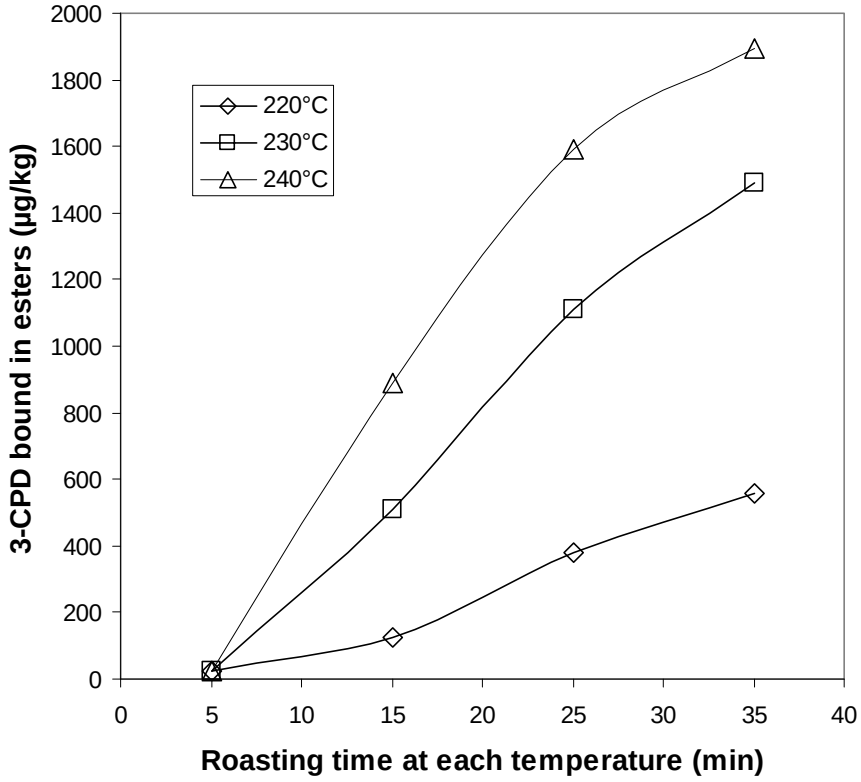


Figure 7. Formation of 3-CPD bound in esters during the roasting of barley over 5 – 30 min at temperatures of 220, 230 and 240°C (data extracted from [54])

Roasted cereals and malts are used extensively in the brewing and baking industries and are also important constituents of coffee surrogates and cereal-based beverages. Although amounts of 3-CPD-esters are relative high in these ingredients, they typically constitute a small proportion of consumed products, e.g. beer, or in the case of cereal-based beverages, are extracted with water and filtered before consumption. In the case of the latter products, 3-CPD-esters have similar solubility properties to lipids and are poorly extracted into water. Hence the overall contribution to 3-CPD exposure from the esters in these ingredients is likely to be very minor.

7.2 Retail foods

Published data on the occurrence of 3-CPD-esters in retails foodstuffs is summarised in Table 7. Not surprisingly, products using or containing significant amounts of refined oils in manufacture, e.g. potato crisps, French fries and infant formulae, have been found to contain some of the highest amounts of 3-CPD-esters. In addition to contributions from ingredient fats and oils, intrinsic thermal formation may also be significant in products and contain the precursors of 3-CPD-esters and attain sufficiently high temperatures during their manufacture. Hence products with relatively high surface area to volume ratios such as nuts, biscuits and crackers may fall into this category.

There is much less knowledge of CPD-esters in non-thermal processed food products. Robert *et al*. observed the formation of 3-CPD in savoury model systems comprising vegetable oil, salt and lipase model systems. The authors proposed that 3-CPD-esters might be formed as intermediates in the generation of 3-CPD in these systems by a lipase-catalysed transesterification of TAG. However it is equally likely that 3-CPD may have arisen from CPD-esters now known to be present in the oils as a consequence of the refining process, and that this mechanism may also explain the spurious occurrence of the free chloropropanol in e.g. cured fish, dairy products and salami [73].

Table 7. Concentrations of 3-CPD-esters measured in retail foods (expressed as total 3-CPD bound in esters on commodity weight basis)

Foods	No samples	mean ($\mu g\ kg^{-1}$)	range ($\mu g\ kg^{-1}$)	reference
Biscuits (excluding infant biscuits)	6	521	249-696	[55]
Bouillon cube	5	502	380-670	[68]
Bread				
assorted, UK	5	27[a]	<11-42	[55]
wheat, UK	1	-	6.7	[35, 36]
crumb	1	-	4.9	
crust	1	-	547	
toast	7	86[a]	60-160	
wheat-rye, CZ	20	-[b]	5.7 – 84.9	[74]
Breakfast cereals	5	12	11-12	[55]
Chicken, coated	2	342	260-423	[55]
Coffee	15	140[a]	<100-390	[75]
Coffee creamer	15	385	130-730	[68]
Coffee surrogates	5	721	145-1184	[33]
Cream (aerosol)	10	359	50-730	[68]
Cracker	1	-	140	[67]
Crispbread	1	-	420	[67]
Doughnut	1	-	1210	[67]
Gingerbread	1	-	359	[55]
Infant and Baby food				
Formula, starter	6	380	286-588	[34]
Formula, follow-up	5	253[a]	<75-464	[34]
Milk, growing-up	3	144	62-291	[34]
Jarred foods	5	-	<11	[55]
Infant biscuits	2	208	110-306	[55]
Processed cereals	5	96[a]	<11-230	[55]
Nuts, roasted	3	457	433-500	[31]
Pickled herring	1	-	280	[67]
Potato products				
Crisps	20	198	48-1186	[55]
Crisps	16	-[b]	229-1008	[76]
Crisps	30	-[b]	97.6-606.3	[69]
French fries	16	-[b]	100-258	[76]
pre-frying	16	-[b]	27-64	[76]
French fries	1	-	6100	[67]
French fries	20	105	35-397	[55]
Home cooked French fries / potato products	17	74	14-225	[55]
Mashed	3	-[b]	37.5-275	[69]
Raw	1	-	2.2	[69]
Salami	1	-	1760	[31]
Vegetable crisps	1	-	157	[55]
Vegetable soup	1	-	<11	[55]

[a] derived value from reported data (one half of the reported limit of detection value used to calculate the mean); [b] data not available

8. Formation routes

The most prevalent chloropropanol esters widespread in thermally processed foods are 3-CPD esters, but 2-CPD, 1,3-DCP and 2,3-DCP esters might also occur at lower or insignificant concentrations. Chloropropanol esters can be subsequently hydrolysed to free chloropropanols that may be otherwise formed directly from glycerol and allyl alcohol or may come from other sources [73]. Due to the fragmentary data available, the major pathways of chloropropanol esters formation in foods are still unclear as all details are not known and suggested mechanisms need to be verified.

The most important precursors of chloropropanol esters identified in foods are fats derived from glycerol (glycerolipids such as TAG, partial acylglycerols, i.e. DAG, MAG and glycerophospholipids – GPL) and chloride ions, either naturally present or added as sodium chloride. The main physical factors influencing the formation of chloropropanol esters are water activity, temperature and time.

8.1 Formation of precursors

Glycerolipids are involved in a variety of biological functions. All eukaryotic organisms and even a few prokaryotes have the ability to synthesise TAG as a storage form of energy (e.g. in plant seeds and animal adipose tissue) and generate the metabolic energy. Glycerophospholipids are used for the maintenance of barrier properties in cell membranes and signal transduction across membranes. The glycerolipid biosynthesis and catabolism has been studied intensively.

In plant seeds, TAG degradation occurs in the seed oil body (and other plant tissues) where TAG is deposited and stored. The free fatty acid liberated by the action of lipases is transported to glyoxysomes where they are converted to acetyl-CoA via the β-oxidation pathway. The acetyl-CoA is eventually converted to carbohydrates via glyoxylate cycle. It is generally thought that the hydrolytic attack of TAG primarily occurs at the *sn*-1 or *sn*-3 positions by TAG lipases (EC 3.1.1.3) but not at the *sn*-2 position, and s*n*-2 MAG are naturally racemised to *sn*-1 MAG to be completely hydrolysed by MAG lipases (EC 3.1.1.23) to glycerol (see Figure 8).

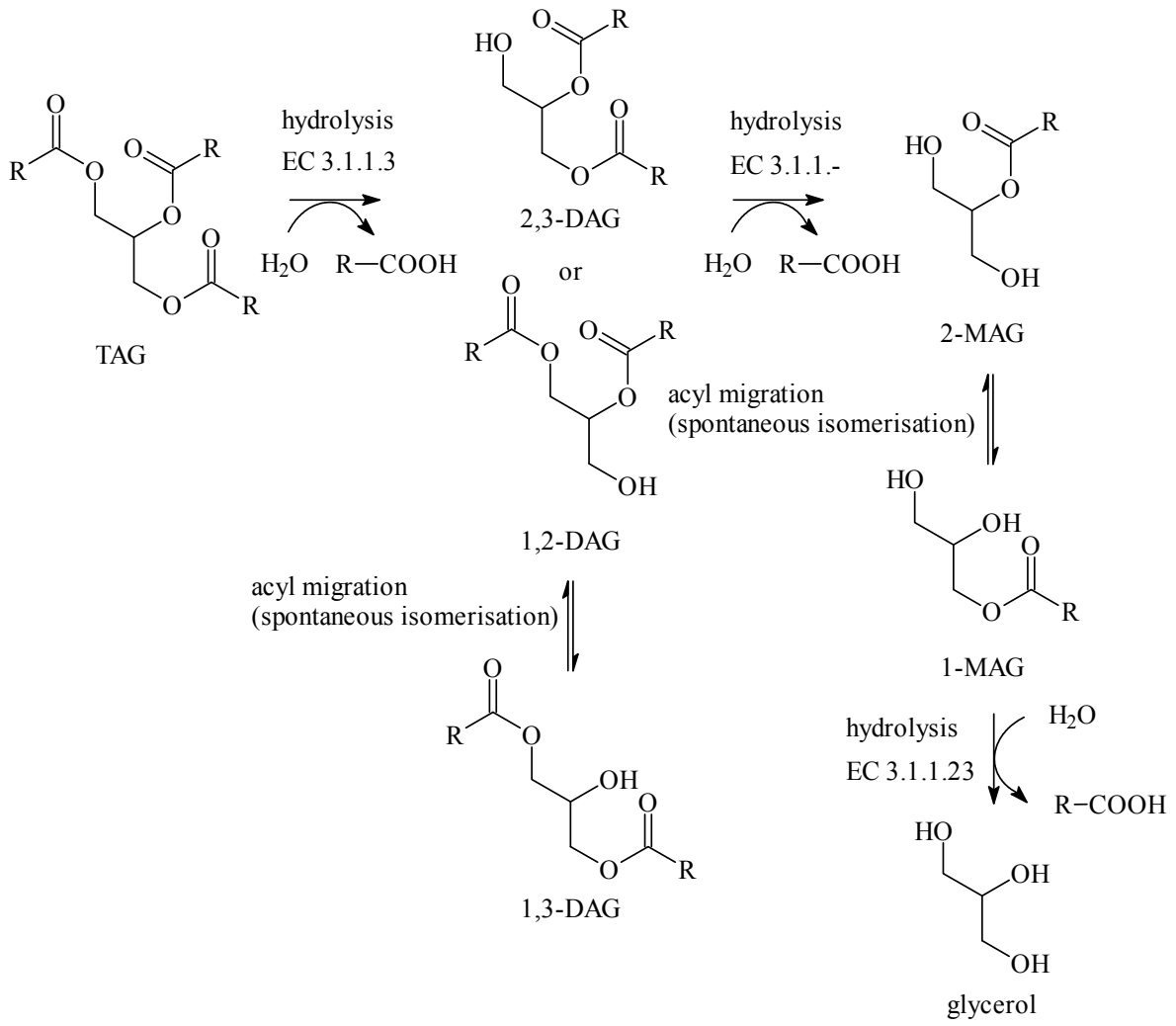


Figure 8. Degradation of plant TAG

DAG levels are especially high in oils produced from fruit pups such as olive and palm oils. For example, the DAG level in virgin olive oils generally ranges from 1 to 3% depending on the ripeness and variety of olive fruit [77]. Freshly made virgin olive oils from healthy olive fruits contain almost solely 1,2-DAG but those coming from poor-quality fruits show a significant increase of 1,3-DAG content. During storage of virgin olive oils, the amount of 1,2-DAG decreases, whereas that of 1,3-DAG and the total DAG amounts increase. Isomerisation of DAG also proceeds during the refining process. Alkaline neutralisation of the oil results in a decrease of total DAG content and an increase of 1,3-DAG content (see Table 8). Deodorisation of the oil results in a slight increase of total DAG and 1,3-DAG contents [78]. The MAG amount in olive oils is much lower than that of DAG. In olive oils produced using various olive varieties and containing 0.73-1.52% 1,2-DAG and 0.40-2.48% 1,3-DAG, the 1-MAG concentrations ranged from 0.05% to 0.11% [79].

Table 8. DAG composition of different olive oils (adapted from reference [78])

Cultivar	Alteration	Total DAG (%)	1,2-DAG (%)	1,3-DAG (%)
Pictual	none	1.21	1,09	0,06
Pictual	piled for 14 days	6.52	4.88	1.48
Pictual	infected by fungi	1.72	0.07	0.99
Pictual	piled for 14 days, neutralised	6.22	4.04	1.96

Similarly to virgin olive oils, the freshly extracted palm oils have a low DAG content (2.3%-4%). However, the conditions prevailing in the industry, such as harvesting conditions, transportation of fruits to factories etc., result in commercial oils having DAG contents of 4.0-7.8% [80, 81]. DAG content also increases during storage under different conditions at elevated temperatures. The 1,2-isomers predominate over the 1,3-isomers in freshly extracted oils, in distinct contrast to commercial oils (Table 9). The amount of MAG in palm oils from industrial production is lower than that of DAG. In palm oils with the DAG contents of 5.3%, 7.6% and 7.6%, the corresponding MAG concentrations were 0.44%, 0.44% and 0.51%, respectively [81].

Table 9. Average DAG contents in palm oil products (adapted from reference [81])

Product	Total DAG (%)	1,2-DAG (%)	1,3-DAG (%)
RBD palm oil	6.0	1.8	4.2
Palm olein	6.1	1.8	4.3
Palm stearin	4.4	1.2	3.1

In most other fats and oils, the concentration of DAG ranges from 0.8 to 5.8% and MAG are present in much smaller quantities than DAG (less than 0.2%). For example: the total DAG content in sunflower oil, soybean oil, coconut oil, rapeseed oil, palm kernel oil, and corn oil was 2.2, 2.3, 2.6, 2.8, 3.9 and 4.1%, respectively [82].

In enzymatically active materials, such as some cereal products, partial acylglycerols and glycerol form by lipase catalysed TAG hydrolysis. The wheat kernel contains much less DAG (0.04-0.2%) than oils [83] although this and amounts of glycerol increased markedly in wholemeal flours after milling [84]. The content of glycerol in yeasted bread dough was 0.2-0.4% after 90 min proof [84, 85].

The hydrolysis of dietary fats by lipases proceeds analogously. The human fat-digestive enzymes include TAG- and phospholipases. TAG lipase catalyzes the hydrolysis of free TAG to fatty acid, MAG- and DAG. The human lipases include the pre-duodenal lingual and gastric lipases (EC 3.1.1.3), extra-duodenal pancreatic and hepatic lipases, endothelial lipase (EC 3.1.1.3), and lipoprotein lipase (EC 3.1.1.34) which hydrolyses TAG in chylomicra (see footnote 1 on page 6). Hydrolysis is regiospecific and results in the release of free fatty acid from the 1(3)-positions of TAG and formation

of 2-MAG. Isomerisation of 2-MAG to 1(3)-MAG occurs to some extent, and these can be degraded completely to glycerol and free fatty acids.

During thermal processes (e.g. frying) at approximately 190°C, fats thermally and oxidatively decompose to a great number of various products. Water and steam hydrolyze TAG and GPL, producing DAG, MAG, free fatty acid, glycerol and other products. Other non-volatile polar products include partial acylglycerols containing chain scission products, TAG oligomers and oxidized TAG. Glycerol partially decomposes, partially evaporates, since it volatilises above 150°C, and the reaction equilibrium is thus shifted in favour of the hydrolysis products. The extent of hydrolysis is a function of various factors, such as fat temperature, interface area between fat and the aqueous phase, amount of water and steam (water hydrolyses lipids more quickly than steam) [86].

The phenomenon of acyl migration in acylglycerols and sugars has been studied since the dawn of stereochemical research [87]. Spontaneous isomerisation of 1,2(2,3)-DAG to 1,3-DAG (Figure 9) [38] and of 2-MAG to 1(3)-MAG (Figure 10) proceeds through a five-member intramolecular ring intermediate (cyclic ketal, substituted 1,3-dioxolane) initiated by a nucleophilic attack of a primary hydroxyl oxygen on the secondary acyl carbonyl group. Subsequent ring-opening leads to the formation of the more thermodynamically stable isomers of DAG and MAG (1,3-DAG and 1- or 3-MAG). The isomerisation proceeds in both solvents and liquid state. As might be expected, acyl migration does not occur in TAG [88].

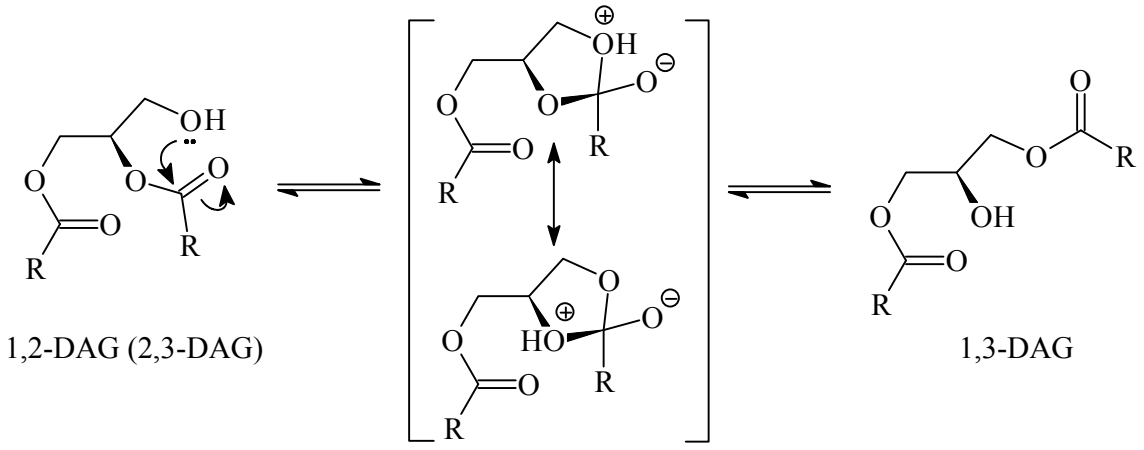


Figure 9. Mechanism of acyl migration in DAG

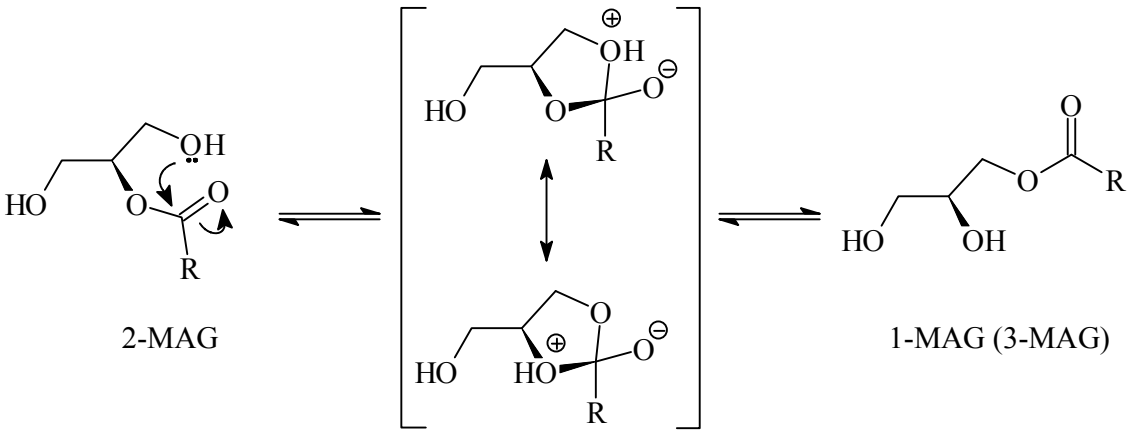


Figure 10. Mechanism of acyl migration in MAG

The acyl migration reaction (S_N2 nucleophilic substitution) rates are subject to both acid and base catalysis which facilitates the initial nucleophilic attack by the primary hydroxyl group. The isomerisation is also greatly promoted by the increasing of temperature. Water activity rather than water content is another key factor influencing acyl migration rate, which decreases with the increase of water activity [38]. Another factor that influences the equilibrium is the length and degree of unsaturation of the acyl group. 1,2-DAG (2,3-DAG) / 1,3-DAG ratio comprises 30-40 / 60-70 [89] of an equilibrated mixture and long-chain DAG typically display a ratio 1,2-DAG (2,3-DAG) / 1,3-DAG of 33 / 67 at equilibrium [90]. The typical ratio for MAG ranges from 20 / 80 [90] to 10 / 90 [88], while for long chain MAG, commonly occurring in foods, the typical ratio of 2-MAG / 1(3-MAG) is 10 / 90 [91, 92].

8.2 Formation of chloropropanol esters

8.2.1 Formation of chloropropanol esters from TAG and partial acylglycerols

The 3-CPD esters are formed during thermal processing of foods at high temperatures and in relatively high amounts during the refining of edible oils, mainly in the deodorisation step. A simplified reaction scheme leading to the formation of 3-CPD esters and free 3-CPD from TAG is given in Figure 11 (isomerisation reactions of MAG and DAG and reactions leading to 2-CPD are not given).

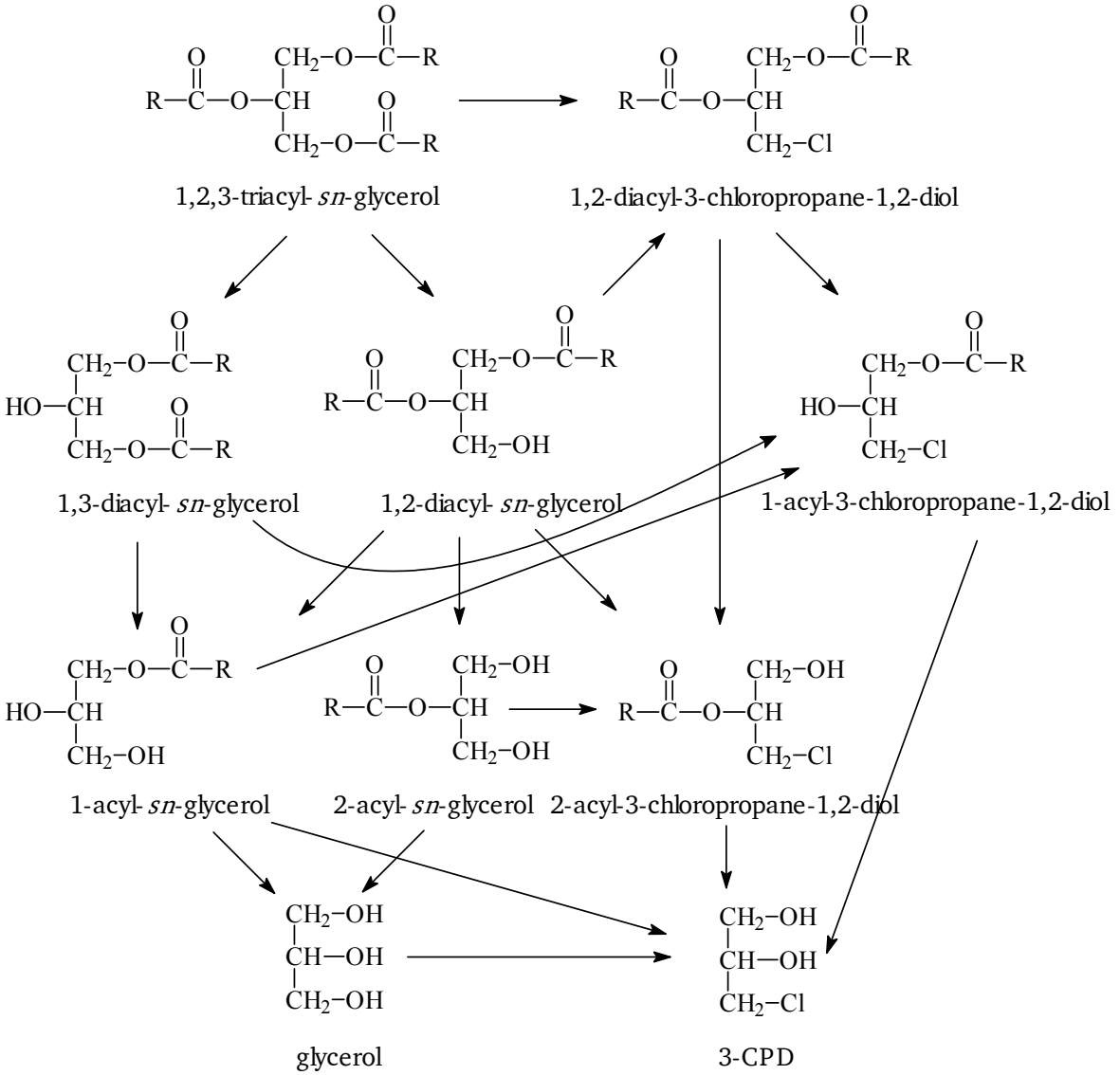


Figure 11. Formation of 3-CPD from TAG (adapted from reference [17])

The mechanism for the formation of 3-CPD esters from TAG in HVP was first proposed by Collier *et al*. [93]. Principally, TAG may undergo two types of reactions (Figure 12). The first reaction starts with acid hydrolysis to DAG and the vicinal DAG ester group(s) provide an anchimeric assistance resulting in the formation of an acylated cyclic acyloxonium ion intermediate (4-hydroxymethylester of 2-alkyl-1,3-dioxolane) under acidic conditions. More detailed reaction mechanism is given in Figure 13. The acyloxonium ion ring structure may then open by a nucleophilic reagent chloride ion yielding 3-CPD diester. The second pathway that might take place in the frame of this complex reaction is a direct substitution of the acyl group (acidolysis by HCl) by chloride anion in the TAG, which gives the 3-CPD diester as well. Analogously, the direct substitution of the hydroxyl group in partial acylglycerols by chloride anion might also take place. Hydrolysis of the 3-CPD diester provides

free 3-CPD (probably via 3-CPD monoesters). The mixed glycerol esters involving two or three different acyl residues yield mixed 3-CPD diesters.

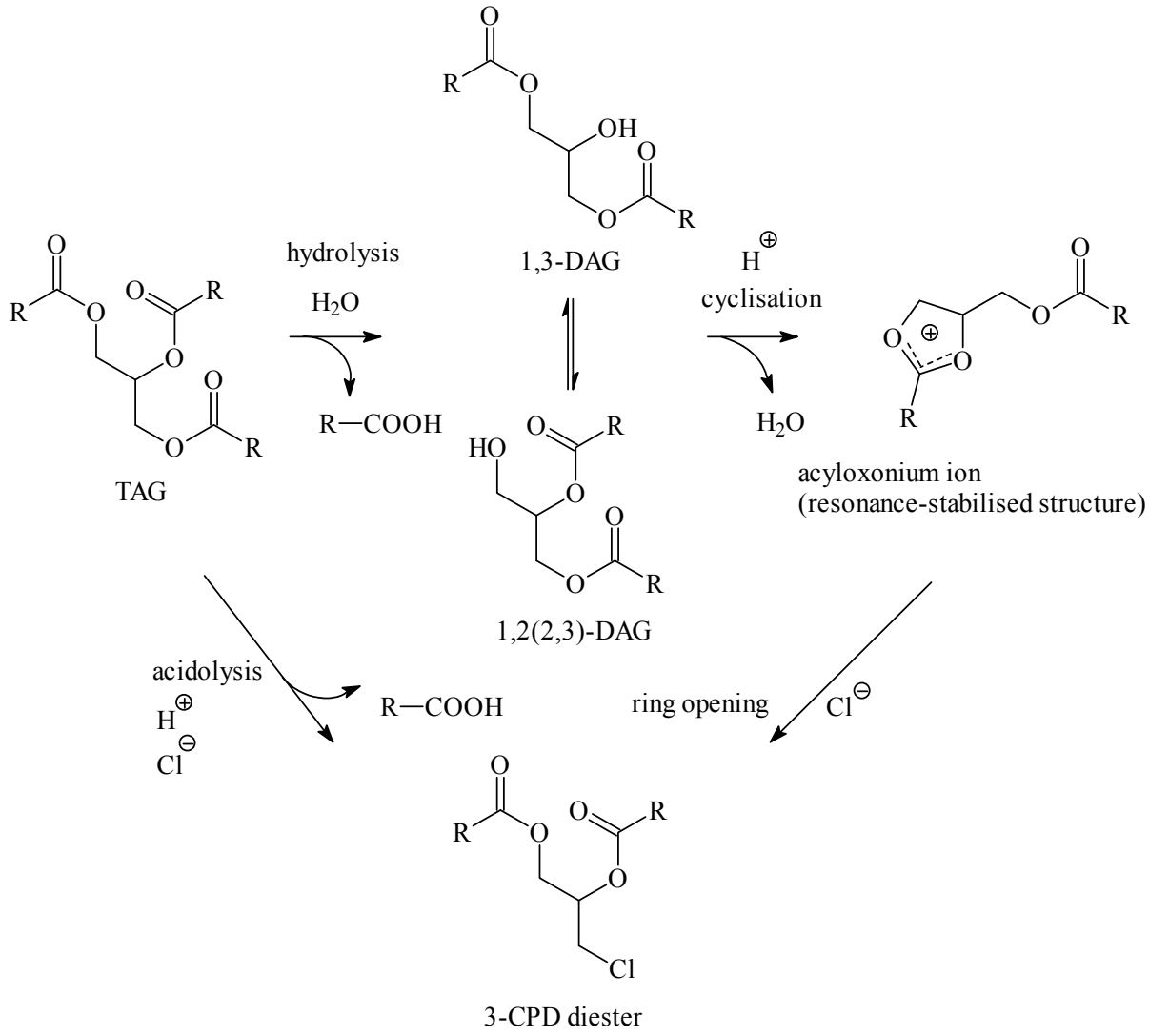


Figure 12. Formation of 3-CPD diesters from TAG *via* DAG (adapted from references [93] and [94])

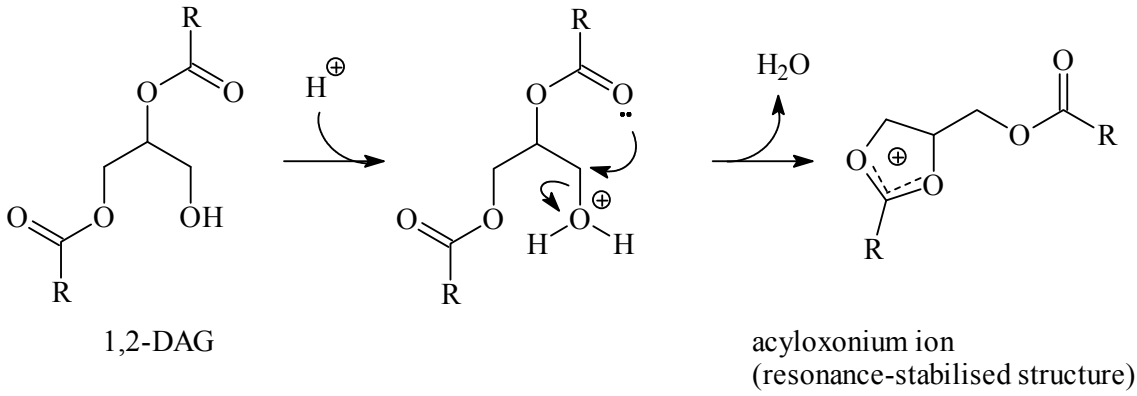


Figure 13. Formation of acyloxonium ion from 1,2-DAG

Analogously to DAG, cyclic acyloxonium ion intermediates with free (unesterified) hydroxymethyl group (4-hydroxymethyl-2-alkyl-1,3-dioxolanes) might form by cyclisation of either 1-MAG or 2-MAG (Figure 14), which is subsequently opened by chloride anion to yield 3-CPD monoester. 3-CPD diester can be hydrolyzed to 3-CPD monoesters and these to free 3-CPD.

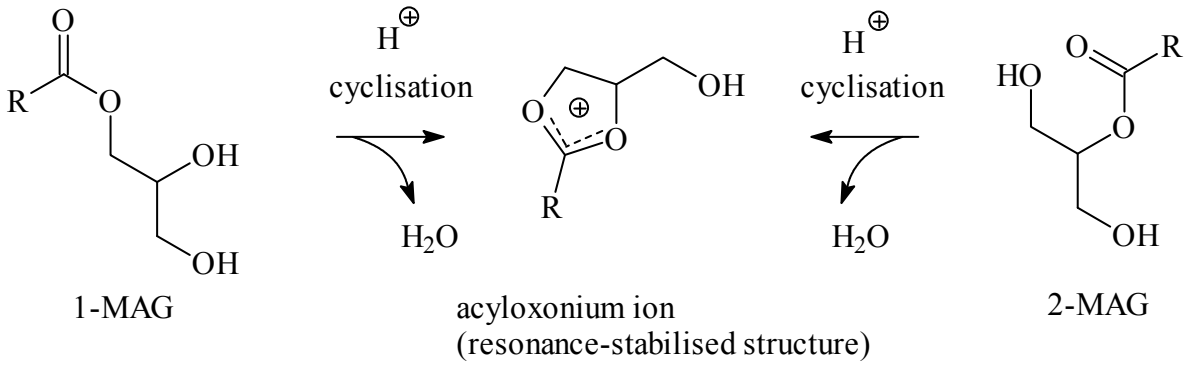
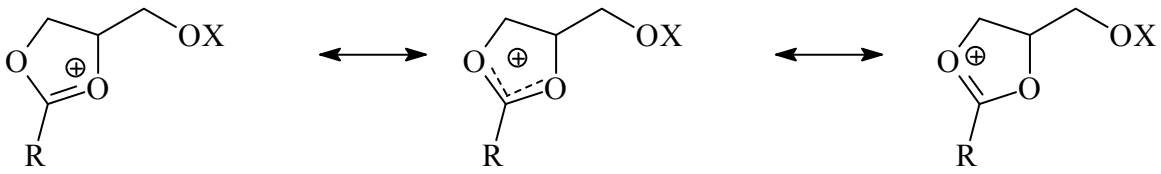


Figure 14. Formation of acyloxonium ions from MAG (adapted from reference [95])

In the presence of Lewis acids or under H^+ catalysis, cyclic acyloxonium ions are expected to form from TAG and DAG also during the refining of edible oils. Studies using tripalmitin and dipalmitin have indicated the formation of acyloxonium ions in both acylglycerols and their subsequent chlorination to form 3-CPD diesters and monoesters when heated at 90°C in the presence of $ZnCl_2$ [94]. The main factors for the formation of 3-CPD esters are the presence of chloride ions, suitable precursors, as well as temperature and time. In particular, the increasing amounts of MAG and DAG in the oil during the refining show a linear correlation with the 3-CPD esters. The ionic nature of chloride precludes its easy access into the hydrophobic environment of the oil. These studies have also indicated that covalently bound chlorine in organic compounds such as in sucralose is also able to efficiently chlorinate glycerol [96], supporting the hypothesis of an oil-soluble chlorinating agent, specifically during palm oil refining, that can be formed through the reaction of carotenoid radical cations with halogens to form complexes. Such complexes may bring chloride ions into the proximity of the lipids to effect chlorination through reaction with acyloxonium ions.

The acyloxonium ion derived from DAG (see Figure 13) exists as a resonance-stabilised molecule, which lowers the potential energy of the substance. The charge delocalised ions can be represented by two contributing structures (resonance structures or canonical forms), acyloxonium ion 1 and 2 (Figure 15). The resonance structures derived from MAG (see Figure 14) are represented by acyloxonium ion 3 and 4, respectively (Figure 15).



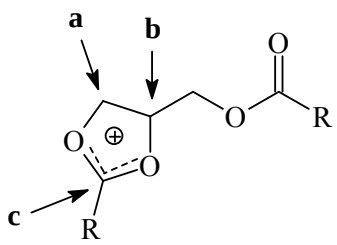
acyloxonium ion 1, X = acyl
acyloxonium ion 3, X = H

resonance-stabilised structure

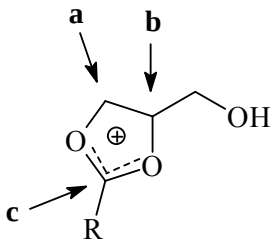
acyloxonium ion 2, X = acyl
acyloxonium ion 4, X = H

Figure 15. Resonance structures of acyloxonium ions 1-4

Acyloxonium ions undergo reaction at three different sites. The attack of a nucleophile (chloride, hydroxyl group) to the acyloxonium ion at C-4 and C-5 results in ring opening (Figure 16) under the formation of CPD esters. The most prevalent isomer among the chloropropanols is 3-CPD, but 2-CPD might also occur. The nucleophilic attack of chloride on C-5 (pathway **a**) is easier due to electron rich and less sterically hindered carbon, therefore, 3-CPD diester is the major reaction product formed from the acyloxonium ion derived from DAG. The acyloxonium ion derived from MAG yields 3-CPD 2-ester as the major product and the nucleophilic attack of chloride on C-4 (pathway **b**) yields either 2-CPD diester or 2-CPD monoester. It can be supposed that the 3-CPD 2-ester is in equilibrium with 3-CPD 1-ester. The mechanism of 3-CPD monoesters isomerisation is analogous to isomerisation of MAG (see Figure 10).



acyloxonium ion derived from DAG
(resonance-stabilised structure)



acyloxonium ion derived from MAG
(resonance-stabilised structure)

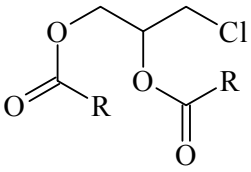
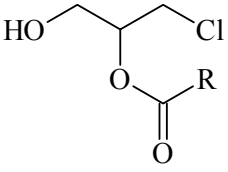
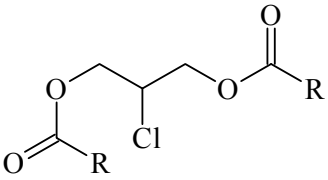
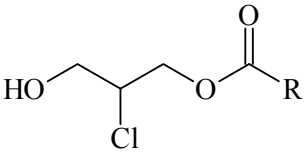
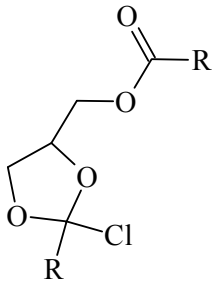
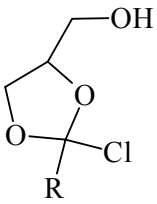
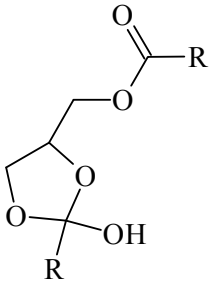
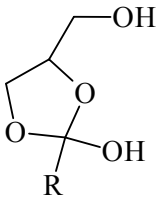
Pathway	Nucleophile	Product	Product
a	$Cl^{\ominus}$		
		3-CPD diester	3-CPD 2-ester
b	$Cl^{\ominus}$		
		2-CPD diester	2-CPD monoester
c	$Cl^{\ominus}$		
		ortho ester-type product	ortho ester-type product
c	$HO^{\ominus}$		
		orthoacid (acetal)	orthoacid (acetal)

Figure 16. Reactions of acyloxonium ions 1-4

The mechanism recently proposed for the formation of 3-CPD monoesters [94] includes the formation of the acyloxonium ion 2 (see Figure 15) that forms directly from TAG in acidic media. Whether this

reaction really proceeds, is not yet known but the same acyloxonium ion can form by cyclisation of DAG as it was shown above (see Figure 13). Relatively high concentrations of both DAG and 3-CPD diesters in refined palm oil support the formation of acyloxonium ion 2 from DAG. This mechanism further requires the formation of acyloxonium ion 6 from 3-CPD diester under acidic conditions. The same chlorine-containing acyloxonium ion (acyloxonium ion 6) may form by cyclisation of a 3-CPD monoester (Figure 17). Ring opening of the corresponding resonance structures (Figure 18) by hydroxyl ions (water) yields 3-CPD 1-ester and 3-CPD 2-ester. Ring opening of the acyloxonium ion resonance structures by chloride yields esters of DCPs. A list of potential products formed from acyloxonium ions 5 and 6 are summarized in Figure 19.

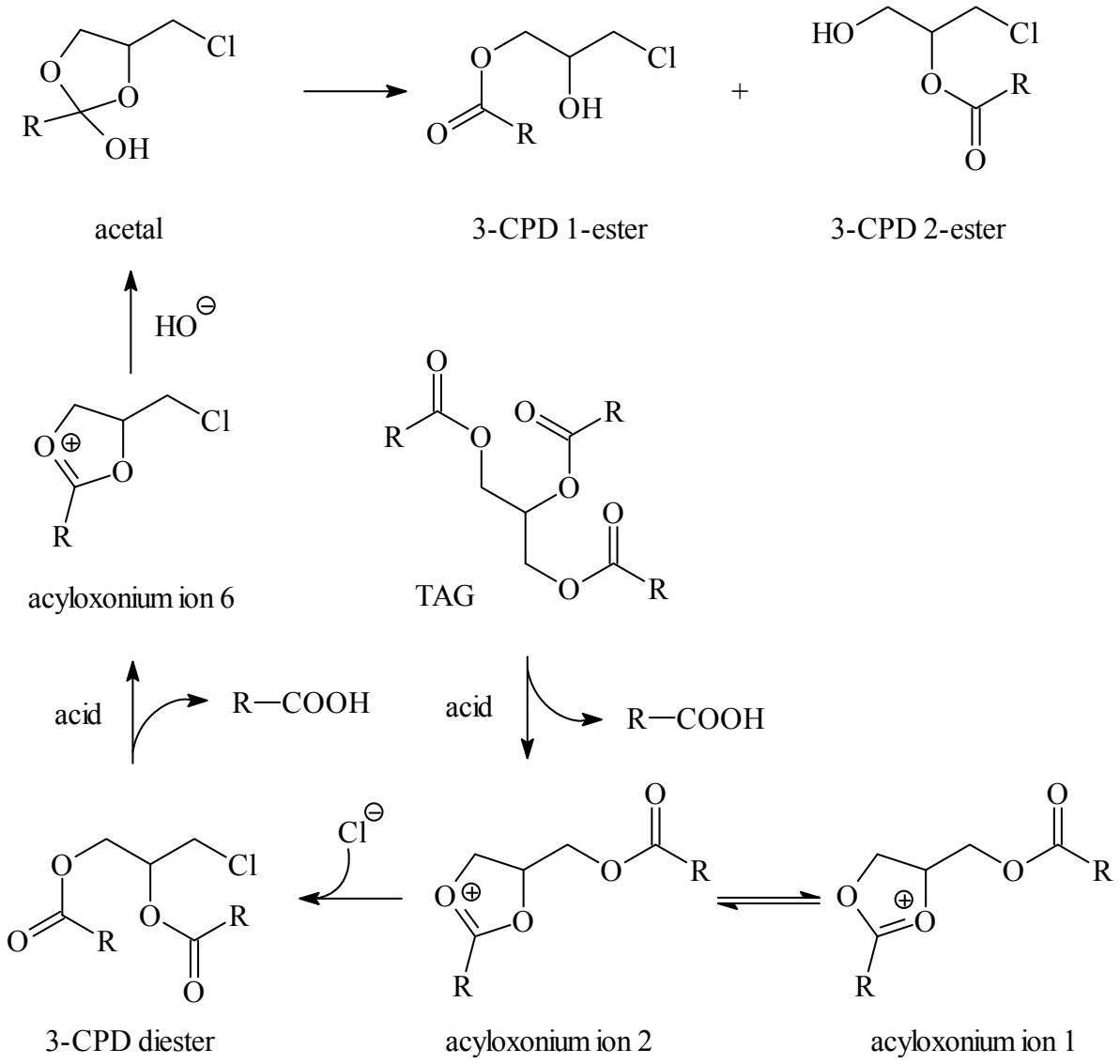


Figure 17. Mechanism proposed for the formation of 3-CPD di- and monoesters (adapted from reference [94])

Analogously to the glycosylation reaction of sugars[2], quite different products might be expected by the nucleophilic attack of either chloride or hydroxyl ions (water) on C-2 (pathway **c**) [98]. The attack of chloride yields unstable ortho-ester type product (according to the nomenclature used in sugar chemistry). In the case of acyloxonium ion derived from DAG, this product is readily hydrolysed to a mixture of DAG isomers and HCl. Analogous reaction of acyloxonium ion derived from MAG leads to a mixture of MAG isomers and HCl. The attack of hydroxyl ion (water) on C-2 of acyloxonium ion derived from DAG (MAG) yields an orthoacid (acetal), which has the same structure as the intermediate in DAG (MAG) (see Figure 8 and Figure 9) isomerisation. This intermediate decomposes to a mixture of DAG (MAG) isomers (see Figure 12. Formation of 3-CPD diesters from TAG *via* DAG (adapted from references [93] and [94])

and Figure 14).

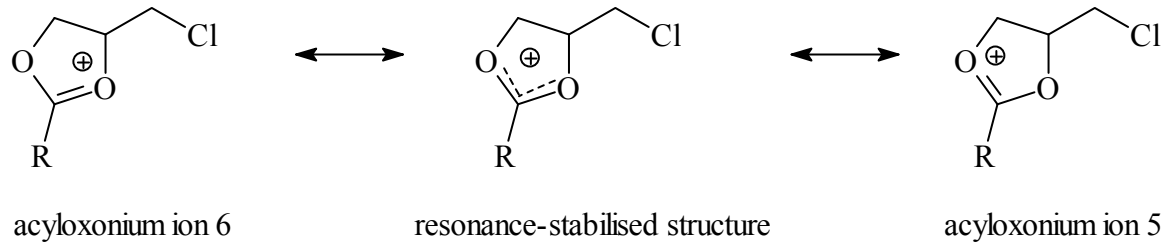
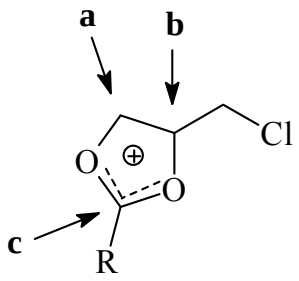


Figure 18. Resonance structures of acyloxonium ions 5 and 6

[2] The formation of a 1,2-*trans* glycosidic bond in sugars is usually achieving using neighbouring group participation by an acyl group on O-2 of the donor. The initially formed oxocarbenium ion is in equilibrium with the more stable, charge delocalised acyloxonium ion formed by participation of the acyl group and the semiacetal hydroxyl. Nucleophilic ring opening of the acyloxonium ion (only possible from the top face of the ring) gives stereoselectively 1,2-*trans* glycoside, whereas attack on the acyl carbon results in orthoester formation. Under the usually acidic glycosylation conditions, the orthoester rearranges to the more stable glycoside *via* the acyloxonium ion. A solvent of low polarity favours the acyloxonium ion. A more polar solvent or a nucleophile of low reactivity favours attack on the oxocarbenium ion causing the formation of both 1,2-*trans* and 1,2-*cis* glycosides [97].



acyloxonium ion derived from 3-CPD diesters
(resonance-stabilised structure)

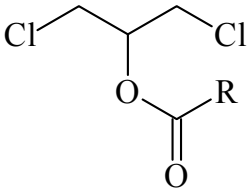
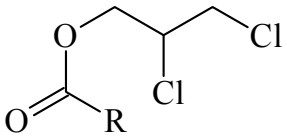
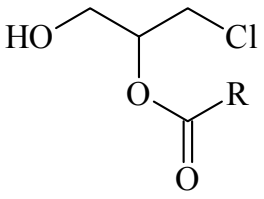
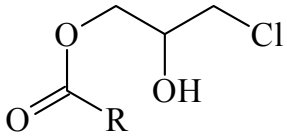
Pathway	Nucleophile	Product
a	$Cl^{\ominus}$	 1,3-DCP ester
b	$Cl^{\ominus}$	 2,3-DCP ester
c	$HO^{\ominus}$	 3-CPD 2-ester and  3-CPD 1-ester

Figure 19. Reactions of acyloxonium ions 5 and 6

8.2.2 Formation of chloropropanol esters from other precursors

Studies on HVP have indicated [93] that CPD esters might also form from glycerol in the presence of acetic acid (Figure 20) under acidic conditions but the potential of higher fatty acids has not been studied. CPD esters can yield free CPD by hydrolysis. The ability of sodium chloride to chlorinate glycerol is greatly enhanced in the presence of amino acids and phosphate-containing compounds such as deoxyguanosine monophosphate. In addition, amino acid hydrochloride salts have greater ability to chlorinate glycerol than a mixture of sodium chloride and amino acids [94].

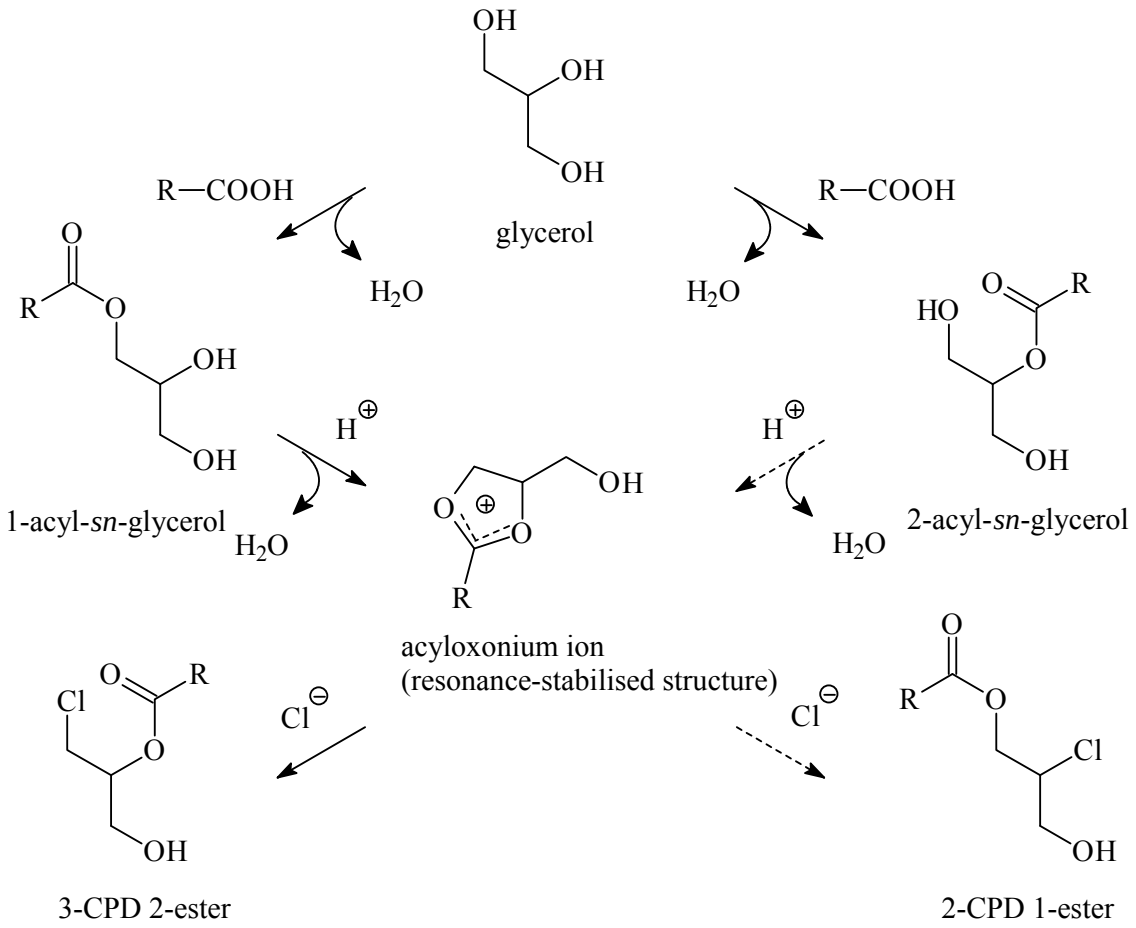


Figure 20. Formation of CPD esters from glycerol in the presence of organic acids (adapted from references [17, 93])

During the production of HVP, phospholipids are apparently hydrolyzed to totally deacylated derivatives, e.g. 3-*sn*-phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphatidylcholine) yields *sn*-glycero-3-phosphocholine (Figure 21). The 1,2-diacyl-*sn*-glycero-3-phosphatidylcholine can react with chloride ions yielding e.g. the corresponding 1-chloroderivative, which is hydrolyzed to 3-CPD. The substituted phosphate group in the deacylated derivatives can be replaced by chloride ions, which leads to CPDs. Similarly to glycerol, phospholipids show little regioselectivity due to facile intramolecular isomerisation of *sn*-glycero-3-phosphocholine to *sn*-glycero-2-phosphocholine [93]. However, this isomerisation can occur via a 5-membered cyclic phosphate ion species [95] and may be significant in directing chloride ion nucleophiles in lysophospholipids such as lysophosphatidylcholine. It is possible that cyclic acyloxonium ions may also form in this case [95].

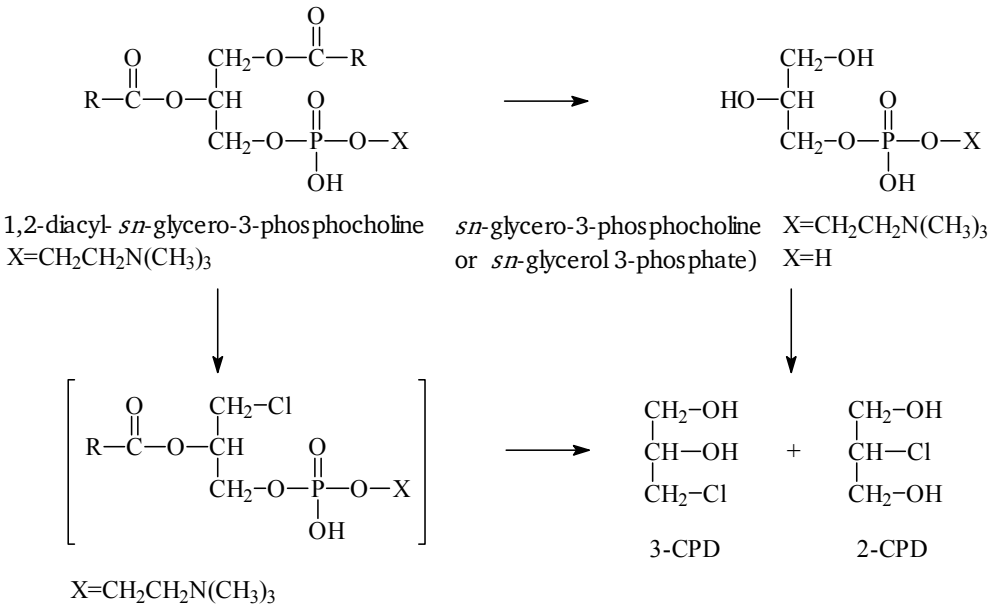


Figure 21. Formation of chloropropanediols from glycerophospholipids (adapted from reference [17])

Sphingophospholipids are a class of sphingolipids that are derived from *N*-glycerol analogues called sphingosines (2-amino-1,3-dihydroxyalkanes). They are long-chain bases substituted on the amino group by a fatty acid derived acyl group. In mammals, the long-chain base moiety is (4*E*)-sfing-4-enine, i.e. (2*S*,3*R*,4*E*)-2-aminooctadec-4-ene-1,3-diol, known as sphingosine. The acyl residue is derived from oleic acid. *N*-acylated sphingosine is known as ceramide. In contrast, the sphingoid base composition of plants is more variable, being composed of up to eight different C18-sphingoid bases derived from (2*S*,3*R*)-sphinganine [99]. Similarly to the isomerisation of partial acylglycerols, the acyl migration between adjacent amino and hydroxyl groups in ceramides also involves the formation of a five-membered ring (derived from oxazolidine in this case) under acidic conditions (Figure 22) [100] but the structure analogous to the acyloxonium ion has not been described. In case, such structure exists, its reaction with chloride would yield chlorine-containing analogues of ceramides.

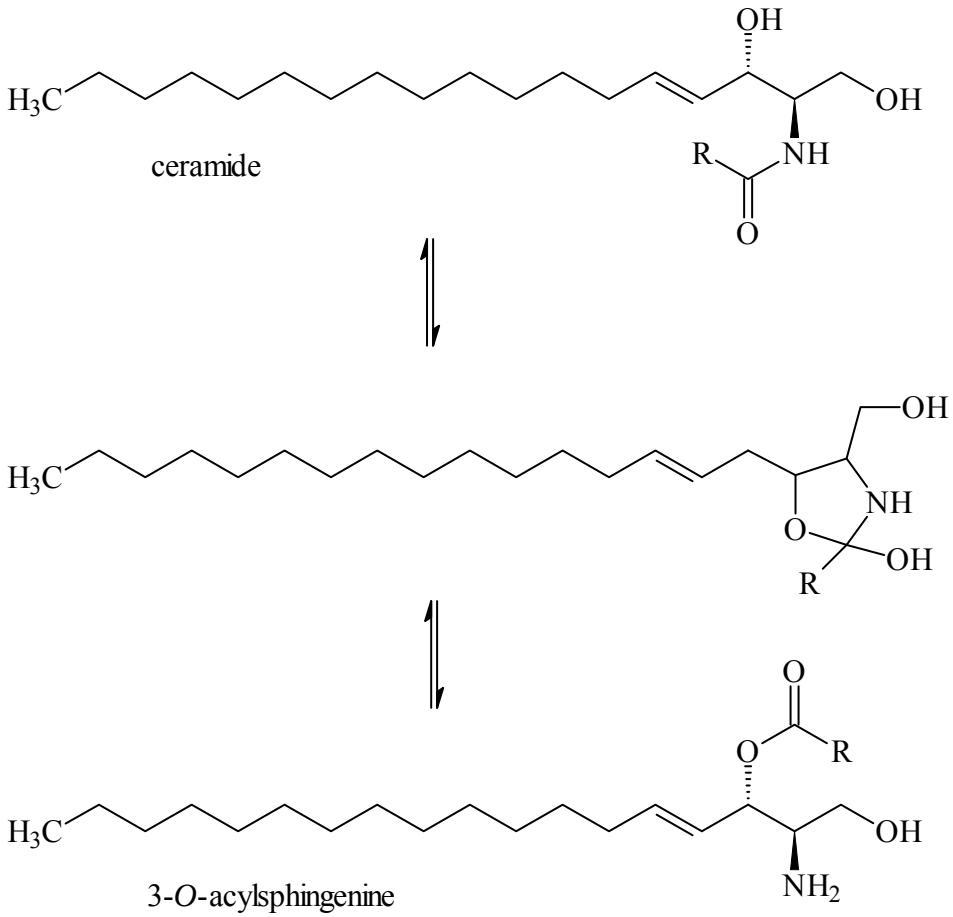


Figure 22. Isomerisation of ceramide (adapted from reference [100])

8.3 Formation of glycidol esters

Fatty acid esters of glycidol have been recently found in refined palm in relatively high quantities and in foods commonly formulated using palm oil (see also Section 11.1). They can potentially be found in all processed foods containing CPD esters and unesterified CPD. Preliminary findings indicate that glycidol esters occurring in refined oils may correlate with the DAG content [41]. It is probable that glycidol esters may act as intermediates in the formation of 3-CPD (ring opening by chloride and fatty acid hydrolysis) but this hypothesis was not thoroughly verified yet. Whether free glycidol occurs in these products is not known. By analogy, the basic reactions of glycidol esters, such as their formation and decomposition, are supposed to be analogous to the reactions of free glycidol.

The combination of a hydroxy group and chlorine atom in neighbouring carbon atoms is responsible for the most common reaction of vicinal chlorohydrins which is dehydrochlorination to form substituted oxiranes (epoxides) [101]. It was found that chloropropanediols (such as 3-CPD) has considerable stability and decompose slowly in slightly acidic pH of commercial HVP (at pH about 5.5) stored at room temperature [102]. Dehydrochlorination of chloropropanediols can be also expected to proceed in processed foods and refined vegetable oils. Generally, in slightly acidic and

neutral media the dehydrochlorination of vicinal chlorohydrins involves the elimination of the chloride anion resulting in the formation of an intermediate carbocation. The hydroxyl group of the chlorohydrin acts as a nucleophilic reagent giving rise to a protonated epoxide (conjugated acid) from which the epoxide arises by elimination of a proton (Figure 23) [4].

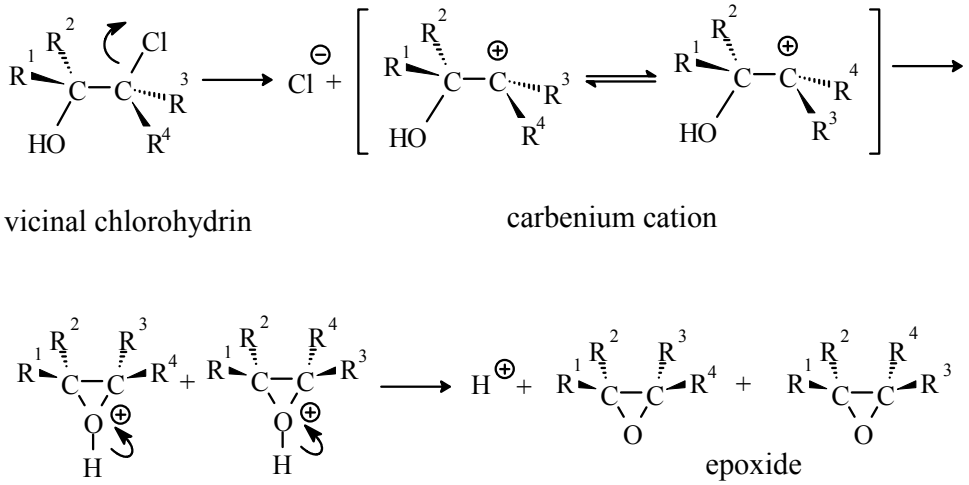


Figure 23. Mechanism of dehydrochlorination of vicinal chlorohydrins in neutral and acidic media (adapted from reference [4])

In alkaline media the decomposition of chloropropanediols is very rapid. The reaction with hydroxyl anions brings the alcohol function of the chlorohydrin to equilibrium with its corresponding alkoxide (alcoholate). The alkoxide oxygen attacks the carbon that bears the leaving chloride atom to give the epoxide. This step determines the reaction rate of dehydrochlorination. As in other nucleophilic substitution reactions, the nucleophile approaches the carbon from the side opposite the bond to the leaving chloride so that the intramolecular S_N reaction takes place with conversion of configuration at the carbon that bears the chloride leaving group. The reaction mechanism is outlined in Figure 24 [4].

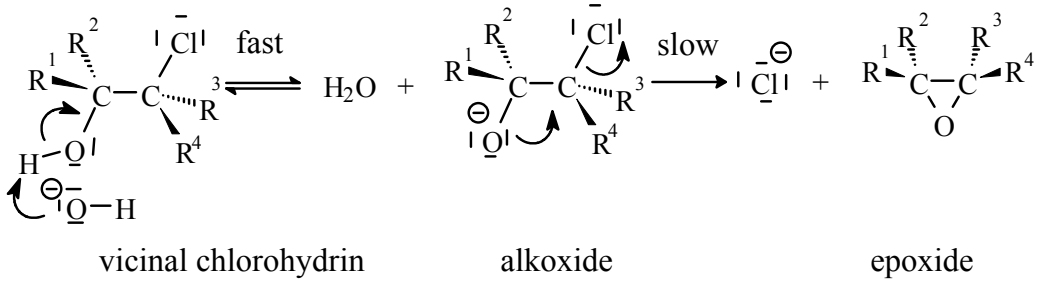


Figure 24. Mechanism of dehydrochlorination of vicinal chlorohydrins in alkaline medium (adapted from reference [4])

Analogously to other vicinal chlorohydrins, (2*R*)-3-chloropropane-1,2-diol ($R^1 = H$, $R^2 = CH_2OH$, $R^3 = R^4 = H$) gives (2*R*)-hydroxymethyloxirane (also known as oxiranemethanol, oxiranylmethanol or glycidol) by a base-promoted ring closure while (2*S*)-3-chloropropane-1,2-diol ($R^1 = CH_2OH$, $R^2 = R^3 = R^4 = H$) yields enantiomeric (2*S*)-hydroxymethyloxirane (Figure 25) and a racemic mixture of both isomers is thus obtained from the treatment of HVP with alkali.

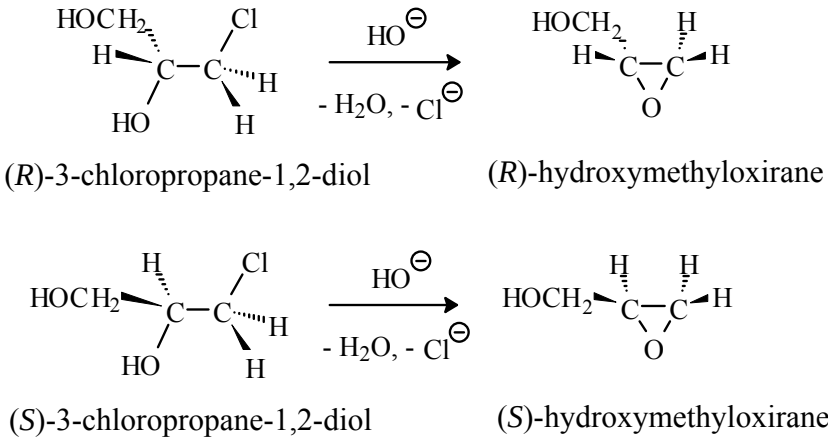


Figure 25. Dehydrochlorination of 3-CPD to glycidol (adapted from reference [4])

Glycidols are not stable under alkaline conditions but the rate of 3-CPD decomposition is higher than the rate of their decomposition. As a result, glycidols accumulate in HVP treated with alkali [103]. The concentration of glycidol depends on the temperature and pH of the HVP hydrolysate.

Accordingly, symmetric cyclisation of the prochiral 2-CPD (elimination of proton occurs from either C1 or C3 hydroxyl, followed by ring closure) always leads to a racemic mixture of both optically active glycidols.

Epoxides are very reactive compounds in which the epoxide ring can be opened by a variety of nucleophiles (water, alcohols, thiols, amines, acids etc.). Nucleophilic ring opening of epoxides has many of the features of an S_N2 reaction (inversion of configuration is observed at the carbon at which substitution occurs) (Figure 26). Asymmetrical epoxides are attacked at the less substituted, less sterically hindered carbon of the ring from the side opposite to the carbon-oxygen bond [101]. Accordingly, the reaction of 3(2)-CPD derived glycidols with water (hydroxyl ion) yields glycerol.

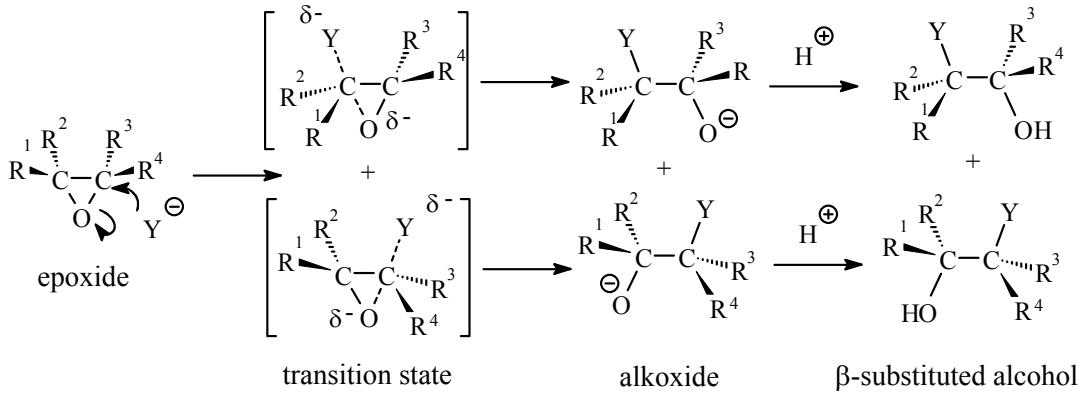


Figure 26. Nucleophilic ring opening of epoxides (adapted from reference [4])

Extending the above glycidol reactions to those of glycidol esters, it can be supposed that the major precursors of glycidol esters are 1-CPD esters that have the necessary combination of a hydroxyl group and chlorine atom in neighbouring carbon atoms. Opening of the epoxide ring by chlorine anion would yield 3-CPD 1-esters or 2-CPD 1-esters, opening of the ring under acidic conditions would yield 1-MAG.

8.4 Summary of major formation routes

The simplified reaction mechanisms for the major pathways possibly involved in the formation of chloropropanol esters from TAG, based on the reactions described above, are outlined in Figure 27. These reactions can explain the formation of all acylated CPD and DCP compounds. Hydrolysis of TAG yields a mixture of 1,2-diacyl- and 1,3-DAG (1,2-DAG isomerises to 1,3-DAG), which is further hydrolysed to a mixture of 1-MAG and 2-MAG (1-MAG isomerises to 2-MAG). The partial acylglycerols are activated through the reaction with H^+ (H_3O^+) and protonation of oxygen in the carbonyl group owes the necessary draft of electrons under the formation of the cyclic acyloxonium ions that exists as a resonance hybride of two structures. The acyloxonium ion derived from DAG is attacked by chloride ion yielding CPD diesters while the attack of chloride ion to the acyloxonium ion derived from MAG yields CPD monoesters. The CPD monoesters having vicinal chlorohydrine structure (3-CPD 1-ester, 3-CPD 2-ester, 2-CPD monoester) eliminate hydrochloric acid to form glycidol esters. Formation of glycidol esters is discussed in section 11.1.5 “Formation of glycidol esters”. Acyloxonium ions formed from 3-CPD monoesters are attacked by chloride ions yielding 1,3-DCP and 2,3-DCP esters. As a result, in thermally processed foods, the major chloropropanol esters will then be 3-CPD diesters and 2-CPD diesters that will occur in lower amounts, similarly to CPD monoesters, DCP esters will occur in very low quantities.

Hydrolysis of MAG to glycerol, hydrolysis of CPD esters to CPDs, hydrolysis of DCP esters to DCPs and reactions of glycerol with chlorides are not shown as well as ring opening reaction of acyloxonium

ions by water which is a weaker nucleophile than chloride ion. Direct substitution of acyl groups (acidolysis by HCl) and direct substitution of the hydroxyl group in partial acylglycerols by chloride anion were not considered.

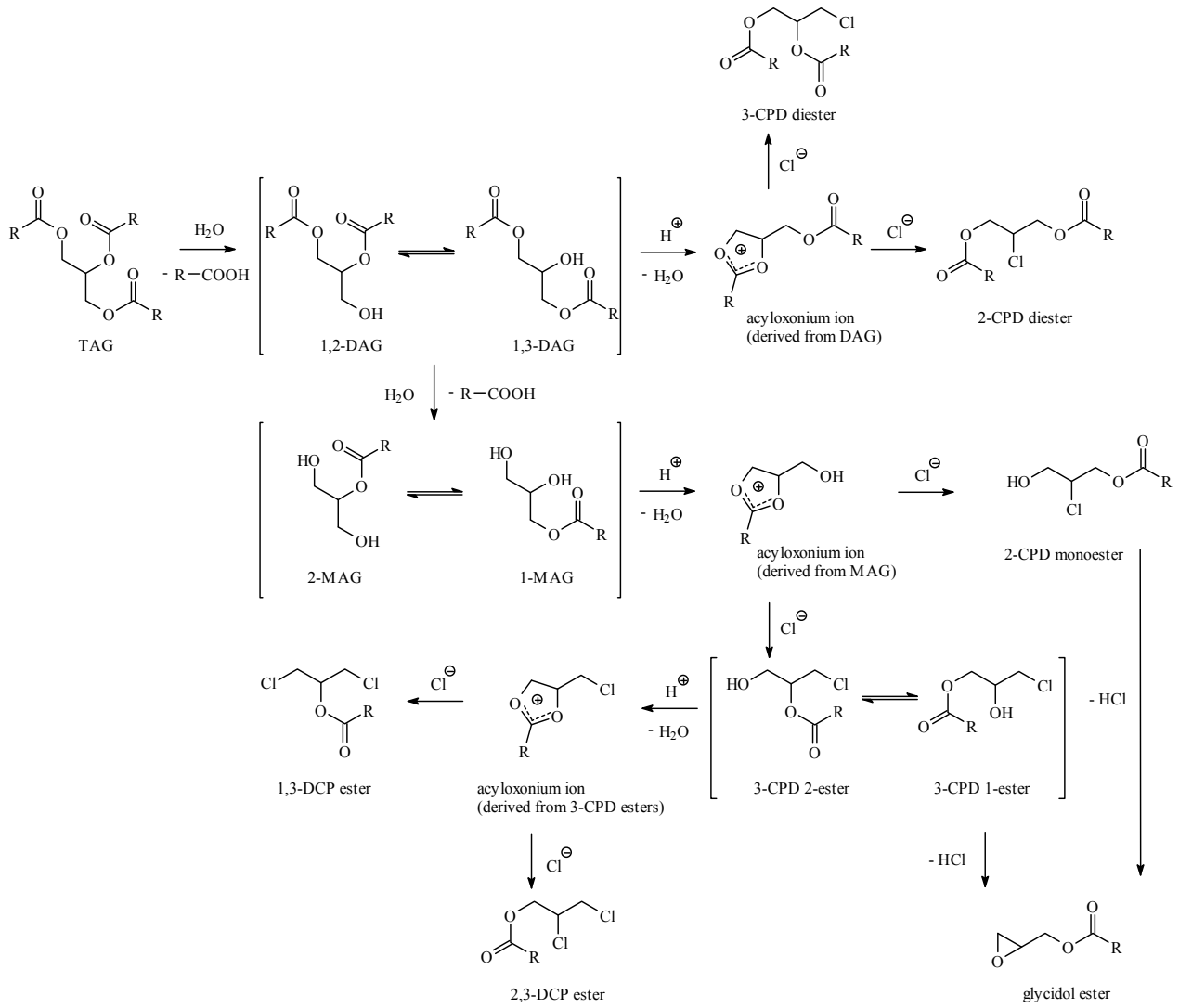


Figure 27. Proposed mechanisms of CPD esters, DCP esters and glycidol esters formation

In tissue fluids, such as human breast milk and cow´s milk, CPD-esters probably form by quite different reaction mechanisms. This pathway is suggested to start from 2-MAG released by the action of lipases on the dietary TAG. This 2-MAG is taken up by enterocytes, reesterified by acyltransferases and then incorporated into tissue lipids by the mechanisms discussed in the section 5 “Toxicological aspects".

A question remains if there are other “bound” forms of CPD (DCP) in addition to esters such as chlorine-containing ceramide analogues, 1,3-dioxolans (e.g. from food associated carbonyls), reaction products with thiol groups of proteins etc. [104, 105].

9. Mitigation

The mitigation of CPD and CPD-esters in foodstuffs in general can be achieved by two independent ways:

- reduction of CPD and CPD-esters formation during the food processing
    - reduction of precursor contents in raw materials
    - change of physical and chemical process parameters
        - temperature
        - processing time
        - water activity
        - pH
        - presence of other reactants
- degradation of induced CPD and CPD-esters
    - physical methods
    - chemical methods

From the perspective of manufacturers, the content of CPD and CPD-esters in the final products can be reduced by more appropriate selection of materials. Perhaps the most important from this perspective seems to be the choice of fats, oils or fatty ingredients, especially their origin (e.g. palm, soybean, rapeseed, etc) and type (e.g. virgin, refined) of oil.

9.1 Reduction of CPD and CPD-esters formation during food processing

TAG, the major compounds of food lipids, are not direct precursors of CPD and CPD-esters. These are partial esters, i.e. mono- and DAG. They occur naturally in small amounts in crude animal fats and vegetable oils. However, partial hydrolysis of TAG during oil extraction increases the concentrations of mono- and DAG. Most fats and oils obtained from low quality sources that have abnormally high free fatty acid concentrations will also have correspondingly high mono- or DAG concentrations. This is caused by the cellular decompartmentalisation of lipases that can interact with water to hydrolyze free fatty acid from glycerol or sterols [106]. Heat and pressure also accelerate fatty acid hydrolysis. Fatty acid hydrolysis and thus mono- or DAG formation are especially prevalent in olives [107], palm fruits and safflower seeds [108]. The level of DAG in virgin olive oils ranges from 1–3% depending on the ripeness and variety of olive fruit. The levels of DAG in fresh and commercial palm oils range from 1.7–3.8%, and 4.0–7.5%, respectively. In most other fats and oils the concentration of DAG ranges from 0.8–5.8% and MAG are present in much smaller quantities than DAG (less than 0.2%) [77].

A decrease in formation of CPD and CPD-esters can therefore be achieved by a lower concentration of partial esters of glycerol in the raw materials or by reducing their production by chemical or enzymatic hydrolysis of TAG in the technological process. A promising possibility in the refining process of vegetable oils could be the reduction of DAG in the raw material before processing. It could be

achieved by breeding of new species of oleipherous plants with lower lipase activities or by enzymatic esterification of DAG to TAG. The requirement of a lower content of precursors was first applied in the production of protein hydrolysates. Reduction of residual lipids, which are exposed to action of hydrochloric acid during the process and form partial esters of glycerol, led to lower production of CPD.

The second principle precursors are chloride ions. Their natural content in food materials is sufficient to generate amounts of CPD and CPD-esters in the mg/kg ranges, e.g. in the refining of vegetable oils. Washing of raw oils before processing could reduce the content of chlorides [82, 109] and hence CPD-ester formation. Furthermore, chemicals and materials used in the refining of oils (e.g. water, acids, basic solutions, auxiliary means, bleaching earths) can be significant sources of chloride ions and it may be equally important to control their use [94]. A promising way for reducing the formation of CPD-esters during deodorisation of oils may be temperature control, because this parameter had a very strong influence on formation of CPD-esters whereas the impact of process time seemed to be less critical [71]. These technological possibilities are the subject of the current investigation efforts.

Amounts of generated CPD and CPD-esters may also be influenced by other substances which are able to prevent the formation or degrade them. The most active compounds decomposing 3-CPD were sodium bicarbonate, followed by the sodium carbonate, cysteine and glutathione [110]. These substances occur naturally in foods or may be added to foods commonly as additives (e.g. as baking enhancers). Addition of glutathione reduced the amount of generated 3-CPD by about 80%, cysteine by 42%, sodium carbonate by 14%, and sodium bicarbonate by 8% compared with a model that did not contain such substances.

By studying the influence of reaction conditions in model systems it was found that decreasing amounts of water in the reaction mixture gave a decrease of 3-CPD [111, 112] due to lower formation of partial esters of glycerol. Other research studied formation of 3 CPD-PP in a model mixture of tripalmitin and sodium chloride at 170° C for 16 hours [113]. In this study two systems were analyzed, one without addition of water and one with the addition of 30% (w/w) water. It was evident that in the presence of water, tripalmitin was hydrolyzed to dipalmitin. The consequence of this phenomenon was the rapid formation of 3-CPD-PP. The calculated rate constant for the models without the addition of water ($k_{1,2\,(0\,\%)}$) and with 30% (w/w) water ($k_{1,2\,(30\,\%)}$) were $(5{,}41 \pm 0{,}32) \cdot 10^{-5}\ s^{-1}$ and $(1{,}18 \pm 0{,}09) \cdot 10^{-4}\ s^{-1}$ respectively.

9.2 Degradation of induced CPD and CPD-esters

It was found that CPD have considerable stability in slightly acidic and neutral pH, but decomposes very rapidly in alkaline media *via* glycidol to glycerol [61, 62, 102]. Hence alkalisation is a method that is used commercially to reduce the level of CPD in protein hydrolysates. Volatile dichloropropanols have been formerly eliminated from HVPs by stripping the raw hydrolysate with

overheated vapour at a reduced pressure or by evaporation [17], but chloropropanediols and their esters can not be removed by the stripping process as they do not distil with water vapour.

In addition to optimisation of the refining process, it should also be possible to remove CPD-esters from the product by absorption of the esters at solid surfaces or by decomposition. All these possibilities will have to be assessed in the near future [109].

The effect of various factors on degradation of 3-chloropropane-1,2-diol esters was studied in model systems closely resembling processed foods [72, 113]. The decomposition rate of 3-CPD-PP was measured in model systems at 100-230°C for 16 hours. The amount of 3-CPD-PP rapidly decreased with increasing temperature (see Figure 28). For example, the concentration of 3-CPD-PP decreased after 0.5 hour at 100°C to 72% (w/w) of the original amount and at 200°C after 16 hours this ester was not detected.

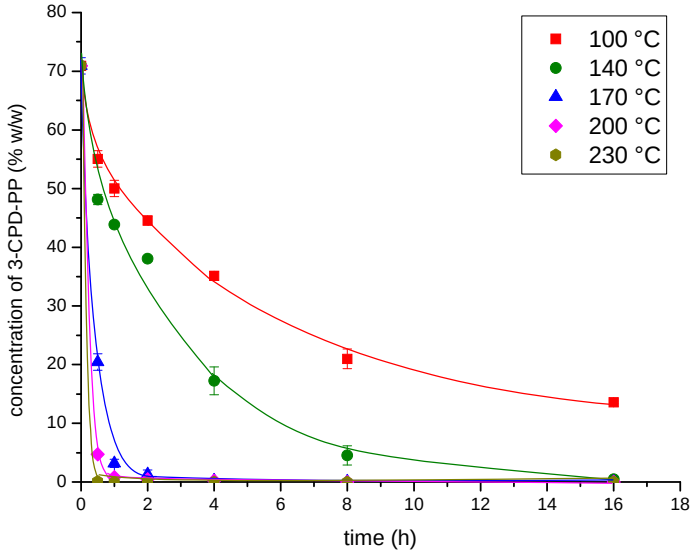


Figure 28. Degradation of 3-CPD-PP in model systems

Degradation of 3-CPD-PP followed first order kinetics according to equation 1 and the rate constants given in Table 10.

$$c = c_0 . e^{-k_{2,3}\tau} \qquad \text{Equation 1}$$

Where: C and C_0 are the concentrations at time t and $t=0$ respectively; and $k_{2,3}$ is the first order rate constant.

Table 10. Rate constants of 3-CPD-PP degradation

T (°C)	Rate constant $k_{2,3}$ (s^{-1})
100	$(4.94 \pm 0.88) .10^{-5}$
140	$(1.05 \pm 0.13) .10^{-4}$
170	$(8.24 \pm 0.16) .10^{-4}$
200	$(1.35 \pm 0.22) .10^{-3}$
230	$(3.72 \pm 0.09) .10^{-3}$

The kinetic parameters of the reaction were calculated from equation 2.

$$k_{2,3} = A_{2,3} \cdot e^{-\frac{E_{a(2,3)}}{R.T}} \qquad \text{Equation 2}$$

Where: E_a is the activation energy (44.97 $kJ.mol^{-1}$); A is the pre-exponential factor (= 122.3 s^{-1}); R is the molar gas constant; and T is the temperature.

However, strategies to reduce CPD and CPD-ester levels have not yet been fully explored and may not be possible for all foodstuffs. These strategies need to consider whether interventions to reduce the risk of CPD and CPD-esters might increase the risk of other process contaminants, such as furan or acrylamide [114]. Optimisation of the refining process is also a challenge because it is a balancing act between the necessary purification steps of the oil and the potential formation of other process-derived contaminants [109].

10. Future prospects and conclusions

Following the recent reports of CPD-esters in edible oils it is evident that these process contaminants are now widely distributed in the food chain and that amounts of the bound form of CPD are much higher than the free diol.

Although toxicological and biokinetic studies of 3-CPD-esters are in their infancy the immediate concern has been the potential release of carcinogenic 3-CPD in the GI tract by the action of intestinal lipases. However preliminary studies appear to suggest that a complete hydrolysis of all CPD-esters is unlikely due to the specificity of intestinal lipases. These lipases preferentially hydrolyse fatty acids on the *sn*-1 and *sn*-3 positions of the glycerol backbone and hence it is the mono-esters of 3-CPD are likely to determine the actual amounts of 3-CPD released directly into the GI tract. The fate of the remaining 3-CPD-*sn*-2-monoesters is not known although it is proposed that they may be metabolised by the same mechanisms as ingested TAG. Since these mono- and diesters of CPD are likely to have

different metabolic pathways it will be important to determine relative amounts in foods. The fate of ingested glycidol esters, also present in edible oils, and their potential conversion to CPD-esters by hydrochloric acid in the stomach may also contribute to dietary loads.

It seems that methods of analysis used to measure total 3-CPD bound in its fatty acid esters, particularly those using alkalic transesterification to release 3-CPD, may not be sufficiently robust and that efforts should be directed towards developing procedures for direct determination, which show much promise.

The composition of the unrefined oils appears to dictate the overall amounts of CPD-esters formed during refining with highest amounts being formed in oils from fruit pulps such as palm. These fruit pulps can have relatively high levels of partial acylglycerol precursors (see below) due to inherent lipase activity in the pulp. As might be expected, formation is temperature sensitive (Arrhenius) and during refining of the oil highest amounts of CPD-esters are formed in the final high temperature deodorisation process. However, the contribution to this formation from other steps in the process is not clear.

From our learning of 3-CPD mechanisms during the manufacture of acid-HVP it would appear that the immediate precursors of CPD-esters in foods are likely to be the mono- and diacylglyerols including possibly the phosphatidylglycerols. These partial acylglycerols have the ability to form “activated” cyclic acyloxonium ion intermediates under thermal processes which can efficiently direct the chloride ion substituent. However, evidence for the direct formation of acyloxonium intermediates from TAG under hydrophobic conditions has recently been presented. The implications of the latter findings for edible oil refining need careful consideration. Furthermore, the hydrophilic nature of chloride ions precludes their availability in oil such matrices and the role of covalently bound and oil soluble forms of chloride precursors also needs to be investigated.

Several potential mitigation measures for CPD-esters in foodstuffs have already been identified. However, it is important that nutritionists, food chemists and toxicologists jointly consider the wider risks and benefits of these measures. This will ensure that interventions to reduce these contaminants do not have a negative impact on health and nutrition or increase the risk of producing other undesirable compounds.

10.1 Knowledge gaps

Toxicokinetics

- Biokinetic studies should be undertaken to determine:
    - the extent to which CPD-esters are hydrolysed in the intestinal enterocytes
    - fate of glycidol esters in the gut - do they form CPD-esters in the stomach by the action of hydrochloric acid?

Occurrence / intake

- Mono- and diesters of CPD are likely to have different metabolic pathways *in vivo*, consequently the relative amounts of mono- and diesters should be determined in foods
- Are non-thermally processed foods, e.g. fermented products a significant source of CPD-esters?

Formation routes in foods

- Is glycidol a precursor of CPD-esters e.g. in refined oils?
- During the refining of oils deodorization seems to be a critical step, but it is not clear how other refining steps contribute to the formation
- What are the oil soluble sources of chloride ions in foods?
- Are there other "bound" forms of CPD in foods?

11. Appendices

11.1 The occurrence of fatty acid esters of glycidol in foodstuffs

Glossary and acronyms

BfR Bundesinstitut für Risikobewertung (The Federal Institute of Food Safety)

CVUA Chemisches und Veterinäruntersuchungsamt

DG SANCO Directorate General for Health and Consumer Affairs

DGF Deutsche Gesellschaft fur Fettwissenschaft (German Society for Fat Science)

EC European Commission

EFSA European Food Safety Authority

FDA Food and Drug Administration

FSA UK Food Standards Agency

IARC International Agency for Research on Cancer

JECFA FAO/WHO Expert Group on Food Additives

LOAEL Lowest observed adverse effect level

MOE Margin of exposure

TDI Tolerable Daily Intake

11.1.1 Background

Glycidol esters (GE) are the esterified form of glycidol (3-hydroxy-1,2-epoxypropane), a well known industrial chemical and human carcinogen [106].

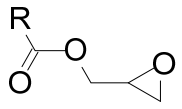


Figure 29. Structure of a fatty acid ester of glycidol (R=alkyl)

Glycidol esters are believed to be formed in refined edible fats and oils and hence products using these ingredients (e.g. infant formulae) may be at risk of contamination. In keeping with the 3-CPD-esters, the main risk here is the potential release of toxic glycidol in the GI tract. In a recent risk assessment, the BfR [116] has concluded that infants who are fed exclusively industrially prepared infant milk formula could take in harmful levels of glycidol. The BfR has called for the urgent development of robust analytical methods and biokinetic studies to support reliable risk assessments.

11.1.2 Health effects

Toxicological investigations of glycidol-fatty acid esters are not yet available. Free glycidol has an oral LD(50) of 850 mg/kg in rats and 450 mg/kg in mice following administration of an 18 percent solution. Symptoms of acute exposure affect the nervous, pulmonary and hepatic systems [117] [118].

Glycidol is classified by IARC as probably carcinogenic to humans (Group 2A) [115] and has been placed in category 2 by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) MAK Commission [119].

Glycidol induces a wide spectrum of genotoxic effects *in-vitro* (gene mutations, chromosomal aberrations, sister chromatid exchanges, unscheduled DNA synthesis) in various mammalian cells [106, 119]. It can alkylate DNA directly, and forms DNA adducts *in vitro* when incubated with DNA [120].

11.1.2.1 Fate on ingestion

Hydrolysis of 3-CPD esters in the mammalian gut by pancreatic lipases has been demonstrated *in vitro* and it is therefore reasonable to assume that glycidol might be released from its esters in the same manner [121]. Furthermore, epoxide ring opening followed by nucleophilic addition (e.g. by chloride ions) occurs readily under acid conditions i.e. similar to those in the human the stomach. Hence under gastrointestinal tract conditions, glycidol-esters and glycidol may be transformed into MAG and glycerol or their respective 3-CPD-esters and 3-CPD [119]. Until the metabolic fate of GE is known, current risk assessments must consider potential exposure to both glycidol and 3-CPD.

11.1.2.2 Exposure

A "margin of exposure" (MoE) determination for genotoxic carcinogens has been proposed by the European Food Safety Authority [122]. This tries to relate the carcinogenic effect to dose in animals and predicts safe exposure levels for humans. A reference value for the effect dose from the animal experiments is defined. T25 is the chronic dose in mg/kg body weight per day within the life span at which causes a specific tissue cancer in 25% of the test animals.

In a preliminary estimate of exposure to glycidol, the BfR assumed a glycidol content in refined edible fat of 1 mg/kg with an average daily fat intake of 20 g and a maximum of 80 g. This results in a hypothetical glycidol exposure of 20 or 80 micrograms per day.

11.1.3 Analysis

Analytical methods need to be able to detect a minimum of about 0.05 mg/kg glycidol as ester to monitor the level of 0.067 mg/kg that would produce the prescribed MoE value of at least 10,000, calculated as glycidol content of oils used for infant formula milk.

The BfR has declared an urgent need for research to develop and validate a suitable analytical method for the quantitative determination of glycidol and its fatty acid esters in refined edible fats and oils.

The analytical method commonly used for 3-CPD determination for GC-MS involves derivatisation with phenylboronic acid in NaCl solution. As part of that procedure glycidol is quantitatively transformed into 3-CPD. The method therefore detects the sum of 3-CPD esters and GE.

In the method published by the German Society for Fat Science [123] both 3-CPD esters and GE are determined. At first the total content of ester-bound 3-CPD and glycidol ester is measured by methanolysis and reaction with phenylboronic acid. Then a second sample is treated with a mixture of propanol and sulphuric acid which opens the epoxide ring of the glycidyl ester and the resulting glycidol is quantitatively removed. From these experiments the total level of GE can be calculated by difference.

It has been argued however that there is no evidence that glycidol is the only substance which contributes to the difference in results before and after glycidol removal, and that direct LCMS analysis of CPD mono-esters and di-esters and glycidyl esters gives different but truer results as there is no chemical modification [41].

Intact GE can be measured by gas chromatography (GC) or liquid chromatography (LC) with mass spectrometric detection (GCMS, LCMS).

11.1.3.1 GCMS

Synthetic GE have been determined by gas chromatography (GC) on a 50% phenyl methyl silicone phase (OV-17) after derivatisation of the free –OH group to its trimethylsilyl ether [124]. This method did not have the sensitivity or resolution to quantify trace levels of GE in edible oils containing TAG and DAG.

Current GC methods use a high temperature non-polar column 100% dimethylpolysiloxane phase capillary column such as DB-1HT with an oven temperature programmed to reach 340°C.

11.1.3.2 LCMS

The most well-characterised analytical methods produced so far are those of Masukawa *et al*. at the Kao Corporation Research Laboratories [125], the Archer Daniels Midland Company, ADM [41, 126] and Weißhaar and Perz [127]. Masukawa *et al*. and others tested three procedures with the final one using a two-stage solid phase extraction procedure with end determination by LCMS.

The first procedure simply dispersed the oil sample in a 1:1 methanol:propanol mixture prior to LCMS. The peak shapes were broad and frequently split at the top, and the baselines were noisy compared with those of standards. These problems increased on repeated sample injections. Subsequent procedures almost eliminated these problems by applying solid phase extraction (SPE) clean-up.

Isolation of glycidyl esters from acylglycerols (TAG, DAG and MAG) is most conveniently based on SPE. The glycidyl esters have no free hydroxyl group whereas DAG and MAG have two and one respectively and will be retained more strongly on polar SPE phases such as silica. TAG similarly have no free hydroxyl group but have three alkyl long chains in contrast to the single one in glycidyl

esters and can be resolved using a reversed-phase mode SPE. In practice Masukawa *et al*. and others used a column of each type sequentially.

In the second Masukawa procedure the oil sample was applied to a normal- phase SPE cartridge first and reversed-phase SPE cartridge second. In the third procedure the reversed-phase SPE cartridge preceded the normal- phase one. This method gave better recoveries and was validated further. In brief the oil was dispersed in acetonitrile and applied to the reversed-phase SPE cartridge The cartridge was washed twice with acetonitrile and the extracts combined and evaporated. The residue was dissolved in chloroform and applied to a normal-phase SPE cartridge. Glycidyl esters were eluted with chloroform for determination by LCMS.

The LCMS procedure of Masukawa and others used a 2.1 mm I.D. x 100 mm, 1.7 mm C18 column with a small particle size. The mobile phases were A (acetonitrile/methanol/water 17:17:6) and B (2-propanol). The gradient programme was A 98% (B 2%) at the start, a linear gradient of A 98% to 85% (B 2% to 15%) between 0.0 and 15.0 min, isocratic elution with A 5% (B 95%) from 15.1 to 25.0 min, and finally isocratic elution with A 98% (B 2%) from 25.1 to 40.0 min The flow rate was 0.2 mL/min and the column temperature 40°C.

Glycidyl linolenate was the earliest eluting ester and DAG and TAG were eluted after the glycidyl esters. The retention time of glycidyl linolenate had to be more than 5 minutes to avoid ion suppression or enhancement in the MS. The use of the mobile phase specified led to a retention time of about 6 mins for the linolenyl ester of glycidol.

The MS method was Atmospheric Pressure Chemical Ionization (APCI) in positive ion mode. The negative ion mode gave no molecular ions or adducts. The APCI mode was 10 times more sensitive than electrospray (ESI-MS). Selected ion monitoring (SIM) was used to measure the protonated molecular ions $[M + H]^+$ of five glycidyl esters (palmitate m/z 313, stearate m/z 341, oleate m/z 339, linoleate m/z 337 and linolenate m/z 335. The esters were quantified using calibration standards between 0.05 and 5 mg/mL.

No useful product ions were observed in the daughter ion spectra when collision induced-dissociations were tested using tandem MS.

Method performance. The limit of detection (LOD) for glycidyl ester standards based on a signal to noise ratio of 3:1 ranged from 0.0014 to 0.0037 mg/mL and the limit of quantification (LOQ) ranged from 0.0045 to 0.012 mg/mL.

The relative standard deviation (RSD%) for retention times and area measurements, for six consecutive injections of glycidyl ester standards were in the range of 0.18-0.23% and 4.3-7.0%. All calibration line correlation of determination (R2) were greater than 0.9989 in the range between 0.05 and 5 mg/mL.

The Masukawa method was validated by application to three commercial edible oils, one of which was high in DAG.

In the procedures from ADM the sample was simply dissolved in mobile phase and injected into the HPLC Column (3 µm C18 ,100Å 50 x 3.0 mm). The mobile phase was a gradient of methanol:acetonitrile 90:10 vs methylene chloride:methanol:acetonitrile 80:10:10, both containing 0.026 mM sodium acetate to induce ionisation of the esters. A Time of Flight MS instrument was used.

The ADM method could detect both mono- and di-esters of CPD with a limit of quantitation for total CPD esters of about 500 µg/kg . The limit of quantitation for total glycidyl esters was about 100 µg/kg, equivalent to about 20 µg/kg glycidol.

Weishaar and Perz developed a method for the direct detection of glycidyl esters and separated glycidyl esters from TAG using size exclusion chromatography (SEC) as their attempts to use the dual SPE column method of were unsucessful. The SEC method separated GC from TAG completely in about 30 minutes [127]. Applications have so far been limited on account of the unknown purity of the standards used.

11.1.3.3 Enantioselective analysis

Glycidol enatiomers might have different biological effects and so their individual determination can be of interest. Separation of free glycidol enatiomers can be achieved by reaction of the epoxy function with 1-naphthyl isocyanate to give the 1-naphthyl carbamate and resolution using a C18 based chiral LC column [128]. The derivatisation also adds a UV-absorbing chromophore to the glycidol enabling detection by UV. Similar approaches might be applicable to esterified glycidol.

11.1.3.4 Availability and synthesis of standards

Glycidol stearic acid ester is readily available commercially. Glycidol esters can be synthesised by reaction of the relevant fatty acid chloride (prepared from fatty acid and thionyl chloride) with glycidol in diethyl ether in the presence of pyridine. The esters can be purified by silica-gel column chromatography to remove residual fatty acids and MAG by-products [125]. They can also be prepared by transesterification of the fatty acid methyl esters with glycidol using sodium methoxide as catalyst or via conversion of epichlorohydrin with the salts of the corre¬sponding fatty acids. Weishaar and Perz obtained poor yields from the transesterification method and adapted the epichlorohydrin procedure [127].

11.1.4 Occurrence

Glycidol-fatty acid esters can potentially be found in all foods that contain refined vegetable fats and oils. The highest levels of glycidol-fatty acid esters have been measured in refined palm oil, and also therefore in foods commonly formulated with palm oil including infant formulas and infant follow-on formulas. It is likely that foods have a high percentage of palm oil also have the highest levels of glycidol-fatty acid esters.

Accurate information on the levels of glycidol-fatty acid esters can only be made by use of reliable analytical methods. Glycidol is classified internationally as a probable human carcinogen. The few data available so far have been acquired from qualitative, non-validated methods

CVUA Stuttgart analysed four palm oil based vegetable fats for glycidol-fatty acid esters. The palm fats were studied because in a BfR collaborative study of 3-CPD fatty acid esters large method-dependent differences were observed in the 3-CPD levels measured [129-132]. The presence of glycidol-fatty acid esters in these refined oils was put forward as a possible reason for these differences, where the glycidol could be cleaved and then chlorinated during the 3-CPD ester analytical process. Whether glycidol in free form occurs in the edible fat, is not yet known. The CVUA study found no glycidol-fatty acid esters in unrefined oils (virgin olive oil, crude palm oil).

In a study based on direct determination by LCMS [41] found that GE were present in significant quantities in some commercial vegetable oils and their level seemed to be correlated to the diglyceride content. Several oils analysed by direct LCMS contained GE but no 3-CPD esters, suggesting that GE are more widely distributed in vegetable oils than 3-CPD esters.

Weishaar and Perz [127] applied the indirect SEC method to the analysis of GE in refined palm and other oils. They demonstrated that GE were absent from virgin olive oil and unrefined palm oil, and that the concentration of GE to be significantly higher in refined palm oils. This indicates that GE are formed during the deodorization step of refining. The deodorization conditions affected the levels of GE much more than the 3-CPD esters.

11.1.5 Formation

Glycidol-fatty acid esters are formed during refining of vegetable fats and oils. Refining is used to remove undesirable compounds such as free fatty acid and carbonyl compounds that cause unpleasant odour and taste. Refining comprises principally bleaching by heating in the presence of a acid-washed earth, and deodorizing. It seems that mono- and DAG may be the precursors of GE formed by an intermolecular mechanism under the conditions of high temperature refining. Preliminary findings indicate that GE measured in these refined oils may correlate the with DAG content [41]. It is probable that GE are also intermediates in the formation of 3-CPD-esters but under conditions of limited chloride ion availability, e.g. during the deodorisation step of oil refining, the reaction does not proceed further [121]. However, in a recent study, palm oil fortified with glycidol stearate and subjected a range of bleaching procedures using hydrochloric acid treated adsorbents did lead to the formation of 3-CPD diesters, but with no loss of glycidol stearate, indicating that CPD-esters may be formed by a different route [41].

11.1.6 Future work

ILSI has recommend future research into GE [121], in particular;

An agreed-upon method is needed to quantify GE.

Reports of the presence of GE in refined palm oil need to be substantiated.

Their formation mechanisms need to be elucidated.

The toxicology of GE needs to be addressed.

The fate of GE after consumption needs to be studied.

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