DEVELOPMENT AND VALIDATION OF AN IMPROVED METHOD FOR DETERMINATION OF CHLOROPROPANOLS IN PAPERBOARD FOOD PACKAGING BY GC-MS

By

WAN-YUN LIU

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ABSTRACT OF THE THESIS

Development and Validation of an Improved Method for Determination of Chloropropanols in Paperboard Food Packaging by GC-MS

By WAN-YUN LIU

Thesis Director:

Thomas G. Hartman

Paper used for food packaging is often manufactured with wet strength resin additives to impart moisture resistance. Wet strength resins formulated with epichlorohydrin form undesirable chloropropanol type by-products including 3-chloro-1,2-propanediol (3-MCPD) and 1,3-dichloro-2-propanol(1,3-DCP). Chloropropanols are suspect carcinogens. These compounds were recently added to the California Proposition 65 list (known or suspect human carcinogens) which mandates labeling of products sold in the state of California, creating a dilemma for food packaging processors. Food packaging manufacturers are concerned with migration of chloropropanols from packaging into foods or beverages. Therefore, analytical methods for determination of chloropropanols in paperboard food packaging are of great importance. However, previously described methods are not developed for paperboard food packaging samples, or not for EU standard aqueous extraction study of paperboard samples.

The objective of this research is to develop an improved analytical method for determination of 3-MCPD and 1,3-DCP in paper type food packaging. The refined method uses aqueous extraction, matrix-spiking of a deuterated surrogate internal standard (3-MCPD-d_5), cleanup using Extrelut solid phase extraction, derivatization using silylation reagent, and GC-MS analyses of the chloropropanols as their corresponding trimethyl silyl ethers. The new method is applicable to paper type food packaging sample for EU standard aqueous extraction study and aqueous food stimulant migration test. Also, the method uses 10 times less sample size, solvents and reagents than previously described methods, reducing the cost and time for analysis. The derivatization procedure was also improved. The overall validation data suggest the method is precise and rugged. The limit of detection of aqueous extract is 0.010 ppm (w/w) for both 3-MCPD and 1,3-DCP. Analytical system precision is 3.36%RSD for 3-MCPD and 7.65%RSD for 1,3-DCP.

The new method has been applied to the analysis of over 100 commercial paperboard packaging samples. The data is being used to guide development of next generation wet strength resins with reduced chloropropanols content, and also used for risk assessments to calculate VSD (virtual safe dose).

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LISTS OF ABBREVIATIONS

1,3-DCP	1,3-Dichloro-2-propanol
3-MBPD	3-Monobromo-1,2-propanediol
3-MCPD	3-Monochloro-1,2-propanediol
4-MI	4-Methylimidazole
BHT	2,6-bis(1,1-dimethylethyl)-4-methylphenol
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CEN	European Committee for Standardization
ECD	Electron capture detector
FDA	Food and Drug Administration
GC-FID	Gas chromatography - flame ionization detector
GC-MS	Gas chromatography - mass spectrometry
HFB	Heptafluorobutyryl
HFBA	Heptafluorobutyric acid anhydride
HFBI	Heptafluorobutyrylimidazole
HPLC	High-performance liquid chromatography
HVP	Hydrolyzed vegetable protein
LOD	Limit of detection
LOQ	Limit of quantification
MF	Melamine formaldehyde
NSRL	No significant risk levels
OEHHA	Office of Environmental Health Hazard Assessment
PAE	Polyamidoamine-epichlorohydrin
PBA	Phenylboronic acid
QM	Quantity in material
SIM	Selected ion monitoring
SML	Specific migration limit
TDI	Tolerable daily intake
TMCS	Trimethylchlorosilane
TMS	Trimethyl silyl
TOCl	Total organic chloride
UF	Urea formaldehyde
VSD	Virtual safe dose

1. INTRODUCTION

Paper-based packages and containers are part of our everyday lives. Paperboards and cartons are largely used as food packaging material for milk, beverages, and disposable cups. Despite the well-acceptance of paper type food packaging to the consumers, studies have shown that the migration of substances from this "traditional" food packaging can be a source of food contamination (Castle *et al*. 1997; Arvanitoyannis and Bosnea, 2004). In order to protect consumers from the migration of harmful substances, the contaminants inside the food packaging should be monitored and regulated.

3-chloro-1,2-propanediol (3-MCPD) and 1,3-Dichloro-2-propanol (1,3-DCP) are known contaminants in paperboard packaging (Devore *et al*. 1991; Boden *et al*. 1997) They are formed as by-products of wet strength additive, polyamidoamine-epichlorohydrin (PAE) resin. The PAE resin is the predominant additive used in wet strength paper to impart moisture resistance. This polymer is typically manufactured with epichlorodrin, which is the precursor of 3-MCPD and 1,3-DCP in paperboards.

3-MCPD and 1,3-DCP are part of chloropropanols. Chloropropanols are known as food processing contaminants in acid-hydrolyzed vegetable protein (HVP) since 1978 (Velisek *et al*.). Subsequent studies revealed the presence of chloropropanols in soy sauce, and in a wide range of processed foods and food ingredients. Because of health concerns with these contaminants, there have been numerous studies on the occurrence and analytical methods of chloropropanols in food products (Brereton *et al*. 2001; Hamlet *et al*.

2002; Baer *et al*. 2010). However, the presence and determination methods of chloropropanols in paperboard food packaging have been discussed much less.

Studies have shown that 3-MCPD and 1,3-DCP are suspect carcinogens (Lynch *et al.* 1998; Cho *et al.* 2008). The European Community set a tolerable daily intake (TDI) of 2 μg/kg bodyweight for 3-MCPD (Commission Regulation No.1881/2006). In 2010, the California Office of Environmental Health Hazard Assessment (OEHHA) reviewed and concluded the evidence of carcinogenicity of 3-MCPD and 1,3-DCP. Based on the reports of OEHHA, 3-MCPD and 1,3-DCP were added to the California Proposition 65 list as "known to the state of California to cause cancer". Products containing chemicals in the list are required by law to be labeled accordingly in the state of California. In order to follow this regulation, the food packaging industry needs an analytical method for determination of chloropropanols migration from food packaging into foodstuff or beverages. For instances, paper packaging manufacturers would like to insure that any migration of chloropropanols is below the virtual safe dose (VSD).

Previously published analytical methods mainly focused on the chloropropanol content (especially 3-MCPD) in HVP, soy sauce, and other food stuff. Brereton *et al*. (2001) published a collaborative study on determination of 3-MCPD in malt extract, soup powder, bread crumbs, salami sausage, cheese alternative and HVP. This method is recommended by the European Standard (EN 14573). It has also been accepted by AOAC Internal as an official method for analytical determination of 3-MCPD. Nevertheless, this method is not considered to be perfect. Retho and Blanchard (2005)

and Cao *et al*. (2009) critically evaluated the limitations of HFBI derivatization process used by Brereton method. The use of a large volume of expensive solvent, diethyl ether (at least 250 ml of diethyl ether used for each sample) is also an issue of this method. Moreover, this method is not designed for paperboard samples.

Publications about the analysis of chloropropanols in paperboard samples are limited. The study conducted by Boden *et al*. (1997) is one of them. Boden *et al.* developed an analytical method to test the amount of 3-MCPD and 1,3-DCP in paperboard samples by GC-MS. However, the method did not apply to migration study of paperboard packaging. Furthermore, the sample preparation process did not follow the European standard aqueous extraction study for paperboards (CEN, 1993). The limit of detection of this method was 0.04 mg/kg, which is not optimal for the requirement of regulations.

In order to evaluate the safety of a packaging sample, two different types of tests were given by Arvanitoyannis and Bosnea (2004). The first one is used for quantification of contaminants present in the packaging material (quantity in material, known as QM). The other one is specific migration test, which determines the quantity of contaminants that could possibly migrate from packaging into the foodstuff (specific migration limit, known as SML). Both tests are essential for analysis of packaging samples.

Because of the consumer health concerns, the mandate of California proposition 65, and the lack of appropriate analytical method for paperboard samples, an improved analytical method for determination of 3-MCPD and 1,3-DCP in paper type food packaging is of great importance. In this study, such analytical method was developed and

validated. The European Standard cold water extraction and migration test were used to determine the QM and SML, respectively. The overall analytical procedure was adapted from Brereton method, with changes in sample preparation procedure, derivatization reagent, and sample size. The method was conducted mainly by GC-MS. Nevertheless, a GC-FID condition for this method was also established for analysis of chloropropanols. While GC-MS provides more sensitivity and selectivity for analytes, GC-FID is more practical for food packaging manufacturers to perform as a part of their routine quality control.

2. LITERATURE REVIEW

2.1. Additives in Paper Industry and Wet- Strength Resin

2.1.1. Additives in Paper Industry

The overall paper manufacturing process involves debarking and chipping, pulping, bleaching and washing, adding additives, paper finishing and drying. The structure of paper is based on a layered fibrous network with cellulose and hemicellulose as the main chemical components. Chemical additives are also an important part of paper. They are used in paper industry to modify the properties of paper (categorized as functional chemicals) or to improve the production process (categorized as processing aids). Without them, paper would be an inferior material with low mechanical strength and brownish color. Figure 1 shows the percentages of market share of different chemical additives (Ginebreda *et al.* 2011). Wet-strength resin holds 7% market share and is one of the major additives used in paper industry. The main additives in paper industry and their functions are listed below (Smith 1995; Ginebreda *et al.* 2011):

- Retention aids: Used to increase the retention of added substance in the cellulose fibers. They act with other additives to improve the overall performance of paper.
- Sizing agents: Used to make the fiber network in paper more hydrophobic and resistant to the penetration of water or other fluids.
- Wet-strength agents: Used to impart wet strength and obtain better mechanical properties in wet conditions.

- Dry-strength agents: Used to obtain better mechanical strength in dry conditions.
- Coating agents: Used to improve the appearance, smoothness, brightness, and printability of paper.
- Other functional chemicals: including dyes and pigments, optical brightening agents, grease resistant agents, and flame retarders. Their purposes are self-explanatory.
- Process chemicals (Processing aids): including pitch dispersants, defoamers, biosides, cleaners, and deinking agents. They are used to enable better runnability and higher productivity throughout the manufacturing process.

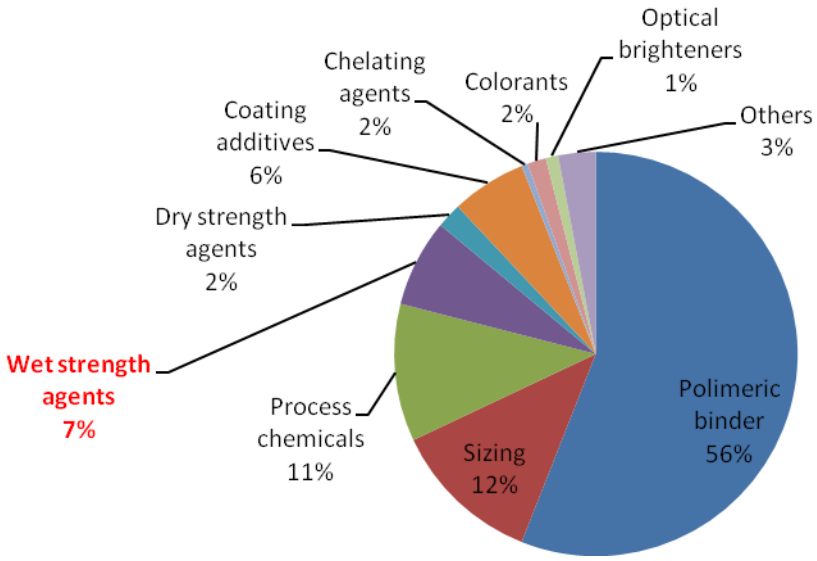


Figure 1: Market shares of different synthetic chemical additives related to the global consumption [Adapted from Ginebreda *et al*, 2011]

2.1.2. Wet-Strength Resins

Paper without wet-strength additives is weak in wet condition. The cellulose fibers would swell in water and the fiber-fiber interaction would be damaged. This is because of the

natural hydrogen bonding in the fiber network. In order to overcome this problem, wet-strength resins are added to the pulp slurry during paper production. The additives adhere to the pulp and form a network to protect or reinforce the fiber network. Protection and reinforcement are the two proposed mechanisms to explain the development of wet-strength (Espy 1995). Protection mechanism assumes that the additive forms an insoluble network by cross-linking with itself, and the network inhibits fiber separation; reinforcement mechanism suggests that the additive forms linkages with cellulose or hemicellulose by covalent bond, and these linkages reinforce the strength of fiber network since they are not broken by water. The main attributes of wet-strength resins are:

- Water soluble or water dispersible, thus allowing better distribution.
- Cationic, thus facilitating interactions with anion pulp fibers.
- Polymeric, thus forming strong networks.
- Reactive, thus promoting the formation of linkages with cellulose or with themselves (Espy 1995).

There are many different wet-strength resins. Urea formaldehyde resin (UF), melamine formaldehyde resin (MF), and polyamidoamine-epichlorohydrin resin (PAE) are commonly used ones (Ginebreda *et al.* 2011). The repeat units of these polymers are shown in Figure 2. It was suggested that UF resins only form cross-links with themselves, while MF resins also cross-link with cellulose. Therefore, MF resins may provide better

wet-strength performance. However, UF resin is often preferred to the more expansive MF resin with regard to cost. A mixture of UF and MF is also used for cost saving.

PAE resins were developed as the alternative of these formaldehyde-containing resins. Formaldehyde is a highly toxic compound. For the reasons of health, safety, and environmental concerns, the use of formaldehyde-containing resins has been avoided, and the use of PAE resins has been largely increased. PAE resins were also considered to provide superior performance in the form of greater wet tensile strength (Devore *et al.* 1991). PAE resins are now the predominant wet-strength additive.

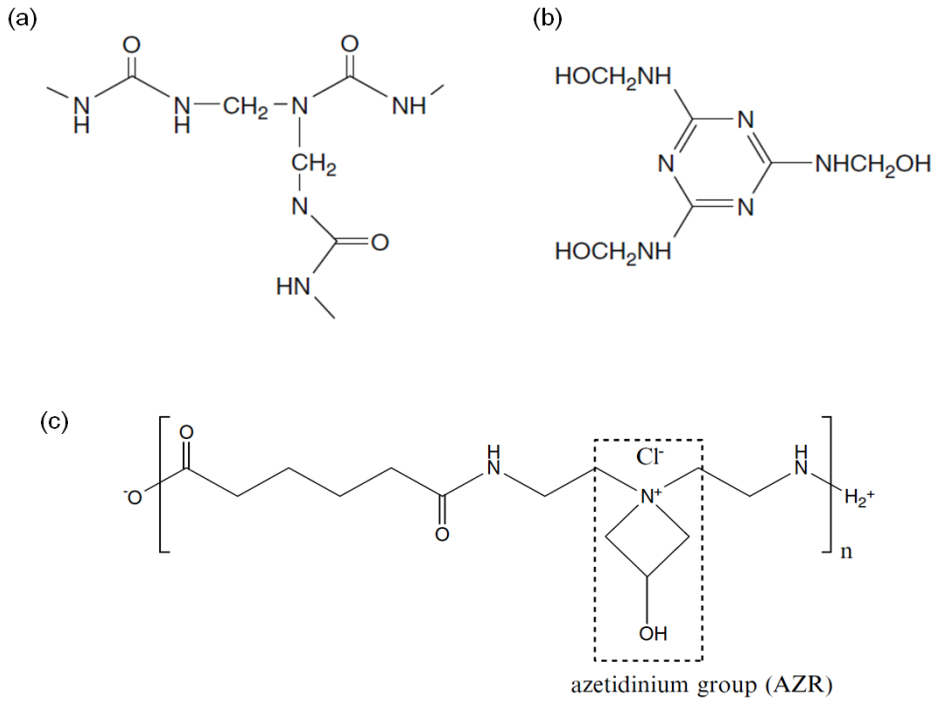


Figure 2: Repeat unit of wet-strength resins (a) urea formaldehyde resin, (b) melamine formaldehyde resin, (c) polyamidoamine-epichlorohydrin resin [Adapted from Ginebreda *et al.* 2011]

2.1.3. Formation of PAE Resins

The production of PAE resin is performed by a two step process. First, the reaction of adipic acid and diethylenetriamine gives a polyamidoamine oligomer. Then, the addition of epichlorohydrin in a water solution produces the final polymer structure of PAE resins. The repeat unit is shown in Figure 2(c). Figure 3 illustrates the formation of PAE resin.

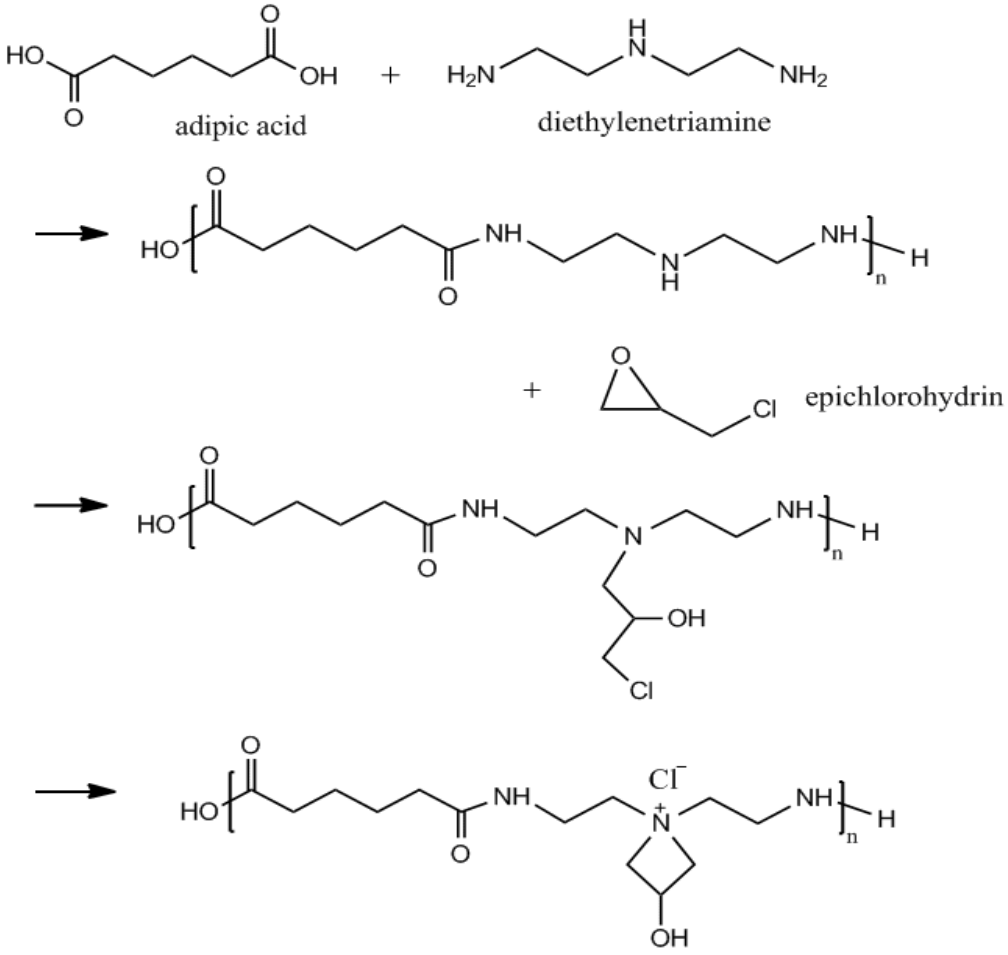


Figure 3: Formation of PAE resins

The azetidinium group is the most reactive part in PAE resins. It can further crosslink with other secondary amine groups in the PAE resins or interact with the

carboxylate groups of the paper fiber network. Fisher (1997) indicated that the wet-strength performance of PAE resins is related to the azetidinium equivalent weight. Higher cationic charge density from azetidinium groups in PAE resin produces better wet tensile strength. Further research by Obokata and Isogai (2007) concluded that the ester bond formation between azetidinium groups of PAE and carboxyl groups of cellulose fibers are the major cause of wet-strength development of PAE resins.

2.1.4. Formation of Chloropropanols in PAE Resins

During the second step of the production of PAE resins (addition of epichlorohydrin), 3-MCPD and 1,3-DCP are formed as by-products. 1,3-DCP is the product of the reaction between epoxide group of epichlorohydrin and chlorine ion; 3-MCPD is formed by the hydrolysis of epichlorohydrin in water (Boden *et al.* 1997). Figure 4 shows the formation of 3-MCPD and 1,3-DCP. It is noticed that there is an equilibrium between epichlorohydrin and 1,3-DCP in aqueous condition. The subsequent formation of 3-MCPD from the equilibrium may be an explanation for the higher levels of 3-MCPD than 1,3-DCP in paperboards.

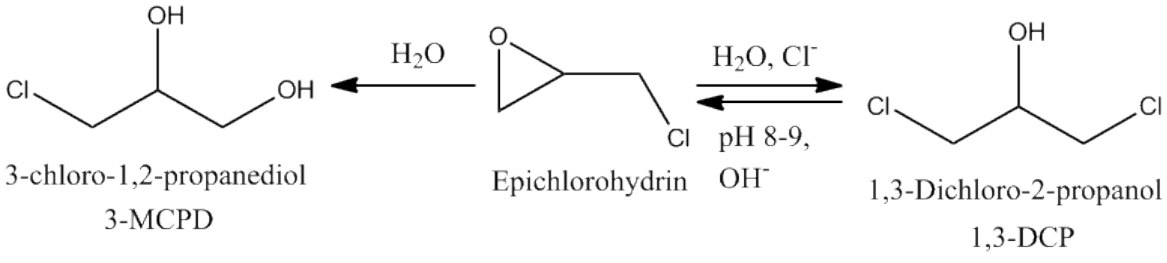


Figure 4: Formation of chloropropanols from epichlorohydrin [adapted from Hamlet *et al.* 2002]

2.2. Chloropropanols in Foods

The collective term “chloropropanols” is used to describe a group of food contaminants which consist of three carbon alcohols or diols and one or two chlorine atoms. The structures of six chloropropanols found in foodstuff are shown is Figure 5. It was found that the two chiral chloropropanols, 3-MCPD and 2,3-DCP, are present in foods as racemic mixture of their (*R*)- and (*S*)-enantionmers (Velisek *et al.* 2002). Figure 6 shows the monochloropropanediol isomers and their relationship with their precursor, glycerol. One positional isomer (2-MCPD) and the two enantionmers of 3-MCPD are formed from the substitution of hydroxyl groups by chlorine ion at different positions on the glycerol backbone.

There have been numerous studies on chloropropanols in foodstuff. In order to better understand chloropropanols, it is important to review their occurrences in foods.

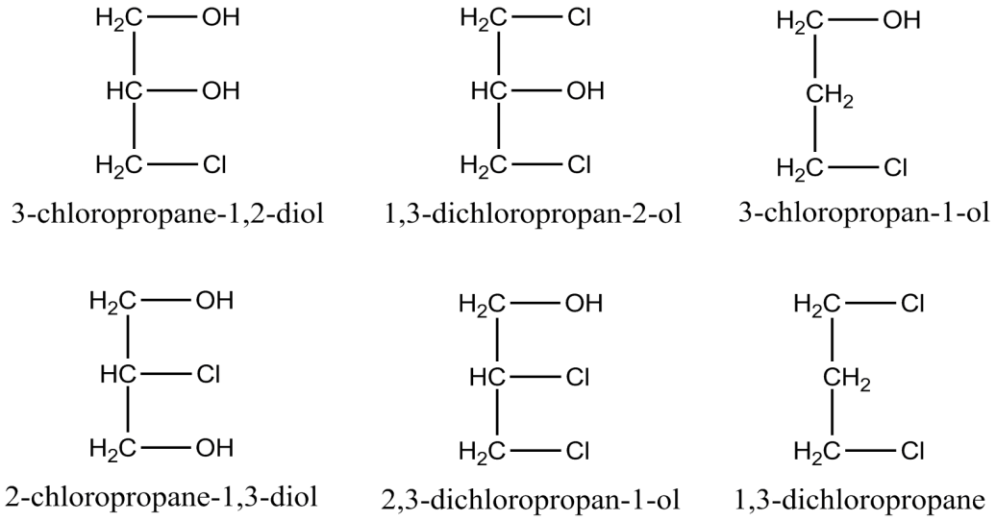


Figure 5: Chloropropanols found in Foods

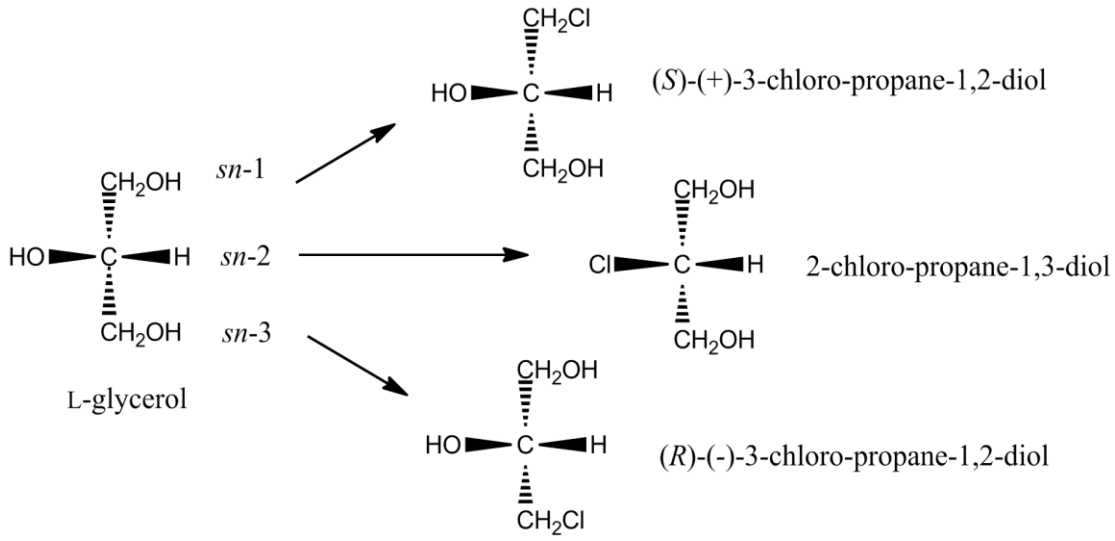


Figure 6: Monochloropropanediol isomers and their relationship to L-glycerol. [adapted from Hamlet *et al.* 2002]

2.2.1. Occurrences of Chloropropanols in Foods

Chloropropanols were first found in acid-hydrolyzed vegetable protein (HVP) by Velisek *et al.* in 1978. It was shown that the hydrochloric acid used in the production of HVP could react with residual glycerol and lipids in the proteinaceous material to yield a range of chloropropanols (Figure 5). The major chloropropanols found in HVP was 3-MCPD, with a smaller amount of 2-MCPD, 1,3-DCP, and 2,3-DCP. In the 1990s, high concentrations of 3-MCPD have been reported in some samples of soy sauce (Meierhans *et al*. 1998). This contamination could be a result from the addition of HVP in the soy sauce product, or from the use of acid hydrolysis of the soybeans to prepare the soy sauce (Crews *et al.* 2002). Traditionally, soy sauce is manufactured by enzyme fermentation of soybeans and roasted wheat with *Aspergillus oryzae* or *Aspergillus sojae* (Velisek 2009). Soy sauce produced by traditional fermentation should be free from 3-MCPD. However,

the natural fermentation process is long and time-consuming. Acid hydrolysis of soy beans can accelerate the production of soy sauce. A blend of naturally fermented and acid hydrolysis soy sauce is also used in retail soy sauce products. HVP is often added to soy sauce to improve the savory taste. A survey of chloropropanols in soy sauces and related products available in the USA was conducted by Nyman *et al*. (2003). The highest levels found were 876 ppm of 3-MCPD and 9.8 ppm of 1,3-DCP, which are significantly higher than the levels of chloropropanols in HVP or other foods. It was suggested that the direct acid treatment of the soy ingredients and high levels of residual lipids in the soy protein ingredients are the reasons for the high levels of chloropropanols.

In the recent decade, studies have shown that chloropropanols can be found in a wide range of foods other than HVP, soy sauce, and their related products. Generally, foods with high salt content (soups and sauces), high fat content (meats and dairy products), and heat processing (cereal and bakery products) have a higher possibility to contain chloropropanols (Hamlet *et al* 2002). The levels of chloropropanols in these foods are usually below 1 ppm (w/w) or even at ppb range, much lower than those reported in some soy sauces. Nevertheless, the risk of high intake of chloropropanols result from the quantity eaten rather than from high levels of chloropropanols in the food products because chloropropanols occur in a wide range of foods.

It was also suggested that the occurrence of 3-MCPD in the salami products may originated from the sausage casings treated with wet-strength resins (Hamlet *et al* 2002). Other food contact materials such as tea bags, coffee filters, and the absorbents packaged

with meats may also be the sources of chloropropanols since their production process all involves wet-strength resin.

Among all chloropropanols found in foods, 3-MCPD and 1,3-DCP are the most important in terms of their abundance and toxicity. Studies and regulations are mainly focus on these two compounds.

2.2.2. Mechanism of Formation of Chloropropanols in Foods

The formation of chloropropanols in foods is generally from three pathways: acid hydrolysis, heat processing, and from chloroesters (Baer *et al.* 2010).

- Acid hydrolysis:

  Acid hydrolysis is the reason that chloropropanols found in HVP. The reaction of hydrochloric acid with the residual oil in the vegetable protein forms chloropropanols. The precursors in the residual oil are triacylglycerols, phospholipids, and glycerols (Collier *et al.* 1991). The reaction involves neucleophilic substitution of an acyl group in the oil precursor by the chlorine anion. The position of the neucleophilic substitution is activated by its neighboring ester groups. Prolonged heating and acidic condition are necessary for the reaction. In Collier's study, triacylglycerols were found to be the main oil processor which yields more chloropropanols than glycerols and phospholipids. The mechanism of formation of chloropropandiols from triacylglycerols is shown in Figure 7.

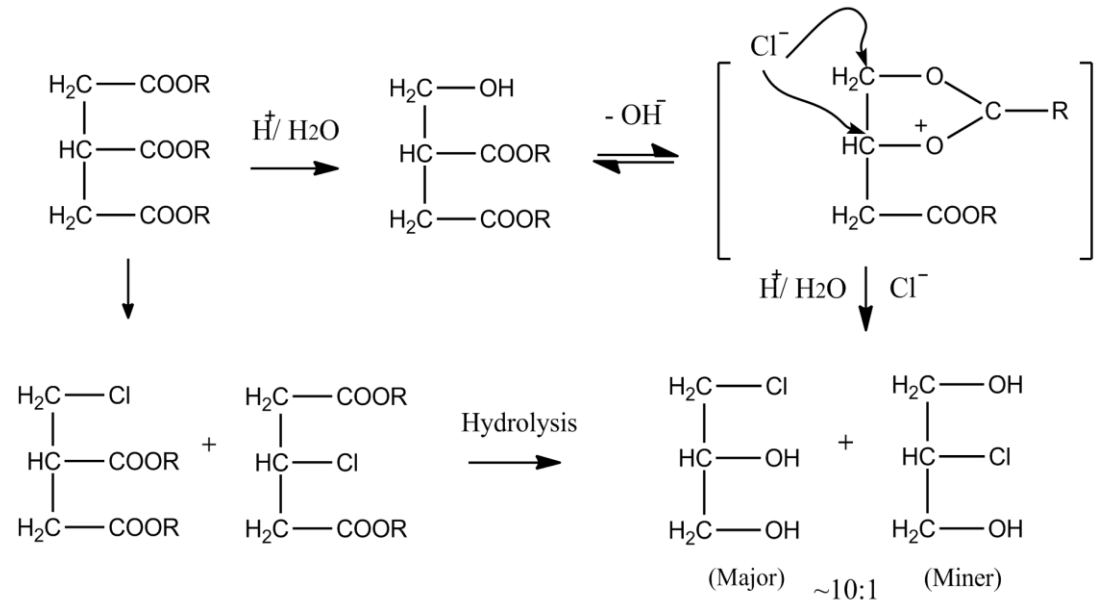


Figure 7: Formation of chloropropandiols from triacylglycerols. [adapted from Collier *et al.* 1991]

- Heat Processing:

  Chloropropanols are also formed in foods without HVP. Lipids and sodium chloride are the suggested precursors. The water content, sodium chloride content, temperature, pH value, and lipid precursors (glycerols, phospholipid, or triacylglycerols) are the factors influencing the formation of chloropropanols. Higher sodium chloride content generates higher levels of chloropropanols. Increased temperature above 160℃ also promotes the formation of chloropropanols. (Baer *et al.* 2010)

  Chloropropanols are formed in bakery products during heat processing. Free glycerol was shown to be the major precursor of 3-MCPD in leavened dough (Hamlet *et al*.

2004). The mechanism is shown in Figure 8. The reaction intermediate, glycidol, has a chemical structure similar to epichlorohydrin.

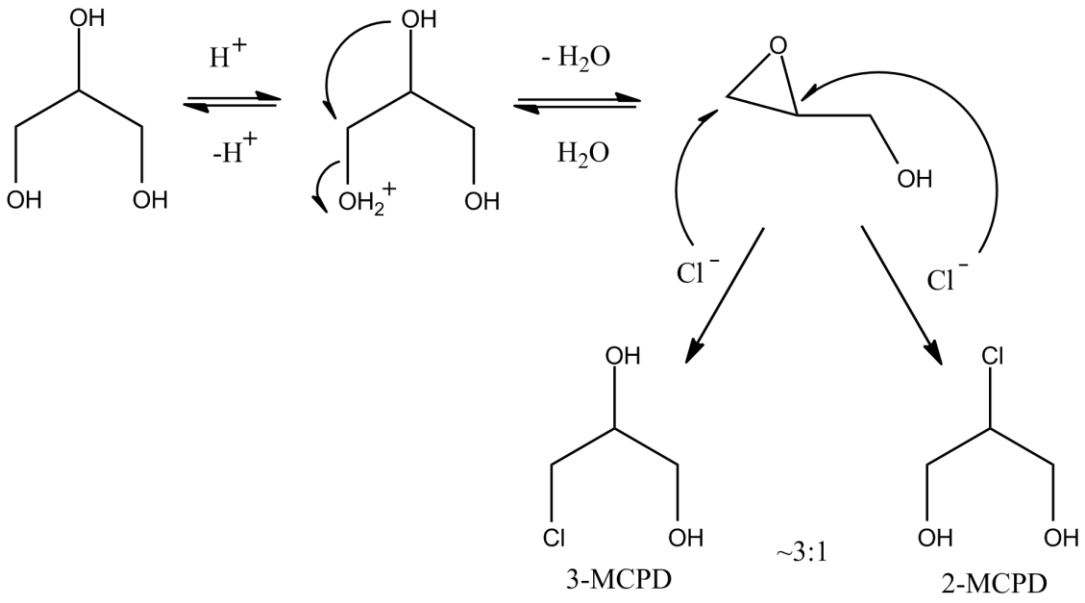


Figure 8: Formation of MCPD from glycerol in breads [Adapted from Hamlet *et al.* 2004]

- Chloroesters

  Although fatty esters of 3-MCPD and 1,3-DCP (mono- and di-esters) can form in HVP, they have drawn less attention since the majority of them were removed during the filtration of the HVP (Hamlet and Sadd 2009). However, the presence of 3-MCPD-esters at a higher concentration than the level of 3-MCPD in some edible oils has been reported (Zelinkova *et al.* 2007). The possible toxicity of chloroesters have not been studied, but it is concerned that 3-MCPD may be released from its chloroesters. It has been suggested that lipase can hydrolysis 3-MCPD esters in vitro

(Hamlet and Sadd 2009). Therefore, the residue lipase activities of certain food ingredients should be controlled. Chloroesters are currently the new interests in this area.

2.3. Methods for Mitigation

2.3.1. In Foods

Since HVP is a widely used ingredient in savory products such as prepared sauces, soups, and gravy mixes, measures were taken to minimize the concentration of chloropropanols in HVP. Three main approaches are adopted by the manufacturers (Velisek 2009):

- Control of the acid hydrolysis process and subsequent neutralization: the temperature and heating time of acid hydrolysis are modified to reduce the formation of chloropropanols. Following neutralization of the hydrolysate also reduces the levels of chloropropanols in the final product.
- Alkaline treatment: this approach is used to remove the formed chloropropanols in the hydrolysate by alkali, since chloropropanols are unstable under alkaline condition.
- Counter current liquid-liquid extraction: this approach is used to remove the chloropropanols in the final product.

A number of different manufacturing processes are also used to reduce to levels of chloropropanols in soy sauce. The amount of residual lipids in the soy protein ingredients is needed to be minimized.

2.3.2. In Paperboards

Devore *et al.* (1991) has published a method for reduction of total organic chloride (TOCl, including chloropropanols) in PAE resins. The method is based on a modified production process and a reduced level of epichlorohydrin in PAE resin. Another approach is using micro-organisms or enzymes for dehalogenation (Riehle 2003, 2005).

Kymene® is the most widely used commercial product of the PAE resins. A second generation of Kymene® products using a different manufacturing process has been developed to reduce the levels of chloropropanols. Subsequent research on post-treatment of Kymene® further reduced the levels of chloropropanols. The post-treatment using a microbial dehalogenation process produces a third generation of Kymene® resin with levels of chloropropanols lower than 10 ppm. (Riehle 2005)

Besides the reduction of chloropropanols in paperboards, the prevention of migration was another approach to enhance product safety. Pace and Hartman (2010) have demonstrated that polyethylene extrusion-coated film can act as a functional barrier to the migration of 3-MCPD.

2.4. Health Risks of Chloropropanols

2.4.1. 3-MCPD

Studies have revealed that 3-MCPD affects male fertility, decrease body weight, and cause renal tumor in rats (Lynch *et al*. 1998; Cho *et al*. 2008). It has been demonstrated

that the genotoxicity of 3-MCPD *in vitro* was not expressed *in vivo* (El Ramy *et al* 2007, Jeong *et al* 2010). Therefore, 3-MCPD has been classified as non-genotoxic carcinogen. In 2010, the California Office of Environmental Health Hazard Assessment (OEHHA) reviewed and concluded the evidence of carcinogenicity of 3-MCPD. The evidences include treatment-related kidney tumors in rats, positives findings in a variety of *in vitro* genotoxicity tests, metabolism of 3-MCPD to glycidol (a genotoxic carcinogen), and the structure-activities considerations with other carcinogens.

2.4.2. 1,3-DCP

1,3-DCP is also carcinogenic in rats. Unlike 3-MCPD, 1,3-DCP is considered to be a genotoxic carcinogen (JECFA 2006). 1,3-DCP has also been suggested to cause necrosis in liver (L'Huillier *et al*. 2002). California OEHHA concluded the evidence of carcinogenicity of 1,3-DCP, including treatment-related kidney, liver, tongue, thyroid tumors in rats, positives findings in a variety of *in vitro* genotoxicity tests, metabolism of 1,3-DCP to multiple genotoxic compounds, and the structure-activities considerations with other carcinogens.

2.5. Regulatory Information

Considering the lack of genotoxicity of 3-MCPD *in vivo*, the tolerable daily intake (TDI) of 2 μg/kg bodyweight for 3-MCPD was established by the European Commission (SCF 2001). Tolerable intake was not set for 1,3-DCP because of its genotoxicity. The

maximum reportable limit of 20 μg/kg of 3-MCPD in soy sauce and HVP was established by the European Community (EC No.1881/2006). The Food and Drug Administration (FDA) sets a limit of 1 mg/kg of 3-MCPD and 0.05mg/kg of 1,3-DCP in HVP used in foods (dry basis) (Joint FAO/WHO Food Standard Programme, 2001). Table 1 summarized the maximum limits of chloropropanols in foodstuffs adopted by different countries. Most regulatory controls are adopted for HVPs and soy sauces.

Table 1: International maximum limits and specifications for chloropropanols [Adapted from Hamlet and Sadd, 2009]

Country/Region	3-MCPD mg/kg	1,3-DCP mg/kg	Scope
Australia/ New Zealand	0.2	0.005	Soy/oyster sauce
Canada	1		Soy/oyster sauce
China	1		HVP
European Community	0.02		HVP and soy sauce
Korea	0.3		Soy sauce containing HVP
	1		HVP
Malaysia	0.02		Liquid foods with HVP
	1		HVP
Switzerland	0.2	0.05	Savory Sauce
Thailand	1		Hydrolyzed soybean protein
United States	1	0.05	HVP

Germany has set regulations for paper and board in contact with foods. The levels of 1,3-DCP need to be non-detectable in the aqueous extract prepared by EN 645 method (CEN 1993) with a limit of detection of 2μg/l. The levels of 3-MCPD in the aqueous extract must not exceed the limit of 12μg/l in any case. These limitation are about 50

μg/kg of 1,3-DCP and 300 μg/kg of 3-MCPD considering the weight of paperboard subject to extraction.

Based on the reports of OEHHA, 3-MCPD and 1,3-DCP were added to the California Proposition 65 list as "known to the state of California to cause cancer" in October 2010. The California Proposition 65 is also known as the "Safe Drinking Water and Toxic Enforcement Act of 1986". It requires the State of California to publish a list of chemicals known to cause cancer, or birth defects, or other reproductive harm at least annually. A clear warning (Figure 9) is required by law to be put on the products containing the chemicals on the list. 3-MCPD and 1,3-DCP are currently on the list of first priority for No Significant Risk Levels (NSRL) development. NSRL is used to establish a safe harbor level for Proposition 65. As a consequence of this regulation, methods for analysis of chloropropanols are of great importance.

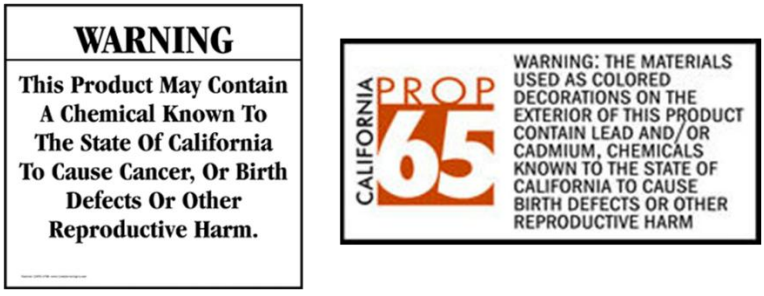


Figure 9: Warning labels for California Proposition 65.

2.6. Analytical Methods for Determination of Chloropropanols

The analysis of chloropropanols at trace concentrations in foods could be challenging. There is no suitable chromophore in their structures, so approaches based on

high-performance liquid chromatography (HPLC) is not applicable. The low volatility of chloropropanols (especially chloropropanediols) also makes direct analysis by GC-MS difficult. Moreover, the low molecular weight of chloropropanols causes problems in distinguishing the target ions from the background noise. These limitations have been overcome by derivatization methods. A derivatization method is required to produce more volatile analytes and prevent the undesired interaction of chloropropanols with other components in GC systems. Derivatization also gives more reliable characteristic ions due to increased molecular weights. Most analytical methods published in these decades apply GC-MS analysis of volatile and stable derivatives of chloropropanols.

Compare to 3-MCPD, 1,3-DCP is more volatile and less polar. This makes it possible to analyze in GC-MS without derivatization. Schuhmacher *et al.* (2005) reported a GC-MS methods for determination of 1,3-DCP in water without derivatization. Liquid-liquid extraction of 1,3-DCP by ethyl acetate was used in this method. Crews *et al.* (2002) also developed a headspace method to quantify the volatile 1,3-DCP by GC-MS. However, methods for simultaneous determination of 3-MCPD and 1,3-DCP are still preferred.

There are three most commonly used derivatization methods (Wenzl *et al.* 2007): heptafluorobutyryl (HFB) ester derivatives, boronic acid derivatives, and dioxolane derivatives.

2.6.1. Heptafluorobutyryl (HFB) Ester Derivative

Van Bergen *et al.* (1992) developed an analytical method for determination of chloropropanols in protein hydrolysates. The method used solid phase extraction to clean up water extract by Extrelut column and heptafluorobutyrylimidazole (HFBI) for derivatization. Hamlet (1998) also reported a method using the same derivatization reagent. Based on these two studies, Brereton *et al.* (2001) developed a refined method for determination of 3-MCPD as its HFBI derivative by GC-MS, tested the method in a wide range of foods, and validated the method by interlaboratory study. This method is recommended by the European Standard, and it is also the official method of AOAC International.

Subsequent studies have been conducted to optimize Brereton method. The method was originally designed for 3-MCPD. Nyman *et al.* (2003) modified the solid phase extraction stage of the method and applied it to analysis of 1,3-DCP in soy sauce. Simultaneously determination of 3-MCPD and 1,3-DCP by similar procedure was done by Chung *et al.* (2002), Xu *et al.* (2006), and Abu-El-Haj *et al.* (2007). Their methods were all based on Brereton method but with different changes in solid phase extraction stage (different elution solvent and different absorbent). Moreover, they used heptafluorobutyric acid anhydride (HFBA) instead of HFBI for derivatization. HFBA is a cheaper choice and it also creates the HFB ester derivatives. The formation of 3-MCPD HFB ester derivatives is shown in Figure 10.

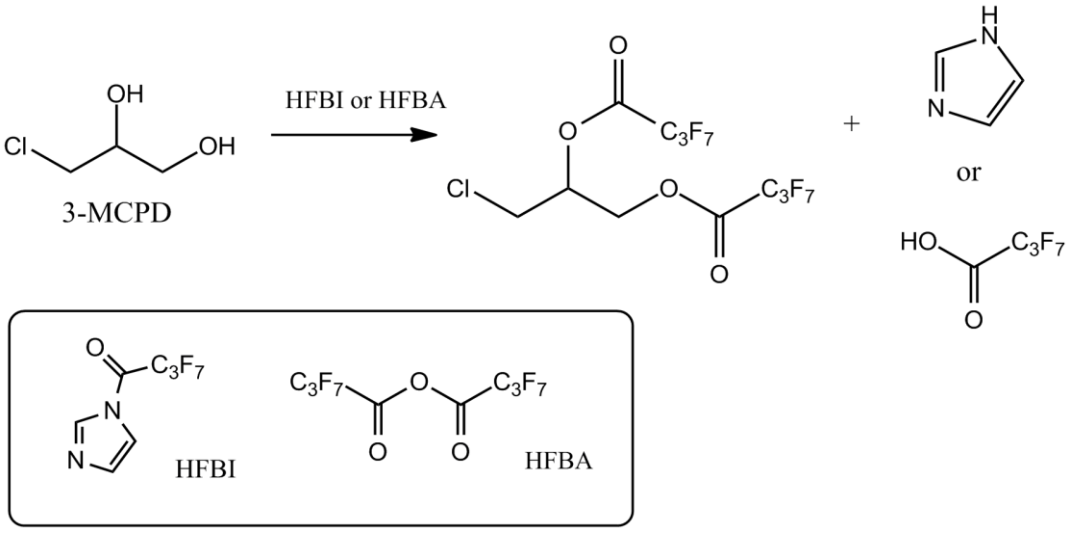


Figure 10: Derivatization reactions of 3-MCPD with HFBI or HFBA [Adapted from Hamlet and Sadd 2009].

Although HFBI/HFBA is the most commonly used derivatization reagent for determination of choropropanols, Retho and Blanchard (2005) and Cao *et al*. (2009) have critically evaluated the limitations of the HFBI or HFBA derivatization process. First, the reagent can react with all neucleophilic compounds present in the extract, resulting noisy background and low selectivity. Second, the abundances of the characteristic ions are low. Moreover, the reagent is very moisture sensitive, creating difficulties to perform the derivatization. The stabilities of the HFBI derivatives were also questioned by Gonzalez *et al.* (2011). They have compared the stabilities of HFBI derivatives with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatives of 3-MCPD and 1,3-DCP. Their results suggested that BSTFA derivatives demonstrated a greater stability over time than HFBI derivatives.

2.6.2. Boronic Acid Derivatives

Boronic acids react with 1,2- and 1,3- diols to form cylic dioxaborolane/dioxaborinane derivatives. Phenylboronic acid (PBA) is the commonly used reagent to give boronic derivatives. This method has been used to determine the 3-MCPD and 3-MCPD esters in different foods (Divinova *et al.* 2004, Kusters *et al.* 2010). The derivatization reaction of 3-MCPD with PBA is shown in Figure 11.

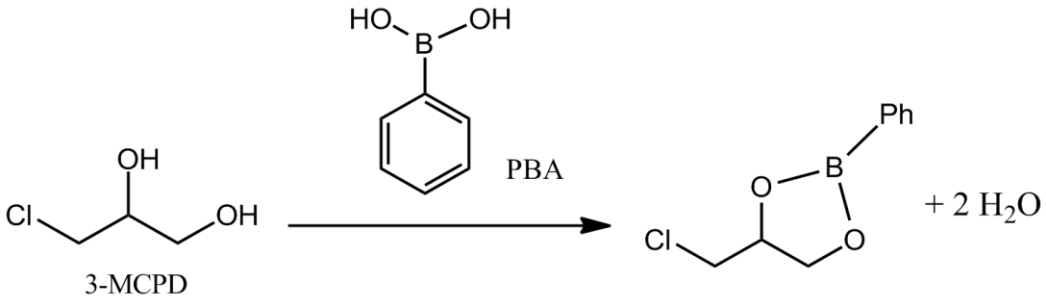


Figure 11: Derivatization reaction of 3-MCPD with PBA [Adapted from Hamlet and Sadd 2009].

2.6.3. Dioxolane Derivatives

Meierhans *et al.* (1998) reported a method using the reaction of diols with ketones to form cycic acetals in the presence of toluene-4-sulfonic acid (TsOH). This method has been modified (Dayrit and Ninonuevo 2004) and applied to analysis of chloropropanols in a wide range of foods (Retho and Blanchard 2005). The derivatization reaction of 3-MCPD with ketones is shown in Figure 12.

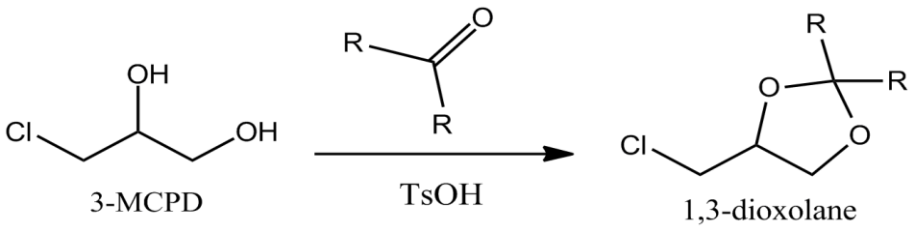


Figure 12: Derivatization reaction of 3-MCPD with ketones [Adapted from Hamlet and Sadd 2009].

Boronic acid derivatives and dioxolane derivatives can only used to analyze the levels of monochloropropandiols. 1,3-DCP cannot derivatized by these two methods.

2.6.4. Determination of Chloropropanols in Paperboards

Pesselman and Feit (1998) have addressed the safety issue of epichlorohydrin used for coating materials, adhesives, and resin stabilizers. They developed a method for analysis of 3-MCPD in aqueous solution by GC with electron capture detector (GC-ECD). A derivatization method using butaneboronic acid was used. 3-MCPD was analyzed as its boronic acids derivative.

Boden *et al.* (1997) developed an analytical method to test the amount of 3-MCPD and 1,3-DCP in paperboard samples by GC-MS. This method was designed to test the total quantity of chloropropanols in the packaging material. Migration test was not conducted in this study. Acetonitrile was used as the extraction solvent and BSTFA was used as the derivatization reagent. The limit of detection was 0.04 mg/kg. This is a relatively high limit comparing to other GC-MS methods discussed above.

3. OBJECTIVES

3.1. Objective

The overall objective is to develop an improved analytical method for simultaneous determination of 3-MCPD and 1,3-DCP in paper type food packaging by GC-MS. This refined method is specifically developed for paperboard samples, and is applicable for various sample preparation methods of paperboards, including European Standard cold water extract study (EN 645:1993) and migration test.

Besides the application to paperboard sample, the method should also meet the following expectations:

- Using a better derivatization method.
- Lowering the solvent use, cost and time for analysis.
- Quantifying 3-MCPD and 1,3-DCP at the μg kg^{-1} level to meet the requirement of the US and Europe regulation.

3.2. Specific Tasks

- Development of an refined procedure for determination of 3-MCPD and 1,3-DCP in paperboard food packaging by GC-MS based on the Brereton method (AOAC international method) The major adjustments are a different derivatization method (using silylation reagent BSTFA), different sample preparation steps for paper type samples, and a scaled-down sample size.

- Validation of the refined method.
- European Standard cold water extraction study of paperboard samples.
- Migration test of paperboard samples using the single-side extraction cell.
- Unskived edge extraction to analysis the migration of chloropropanols from unskived (uncoated) edges of paper type food packaging samples.
- Comparison of the extracted chloropropanol contents of EU standard cold water extract and water extract of homogenized paper sample.
- Performing the method on a gas chromatography coupled with flame ionization detector (GC-FID) for analysis of chloropropanols.

4. EXPERIMENTAL DESIGN

4.1. Experimental Design Overview

As mentioned on the previous section, this refined analytical method for determination of chloropropanols is based on the AOAC method, but with some adjustments. After the overall analytical procedure and GC-MS conditions were established, validation study of this method was performed. Finally, a GC-FID condition for conducting this analytical method was also developed.

Figure 13 shows the flow chart of the overall analytical procedure. The overall process can be divided into four parts:

- Aqueous extraction: Chloropropanols were extracted by distilled water. According to the different purposes of different tests, there were four different methods for preparing water extract: European standard cold water extraction, migration cell extraction, unskived edge extraction, cold water extraction of homogenized sample.
- Solid-phase extraction by Extrelut® NT column: Chloropropanols were partitioned into diethyl ether using a glass column packed with Extrelut® NT.
- Derivatization: Chloropropanols were derivatized by silylation reagent, Sylon BFT (BSTFA+TMCS, 99:1).
- GC-MS analysis: Chloropropanols were analyzed by GC-MS as their corresponding trimethyl silyl esters.

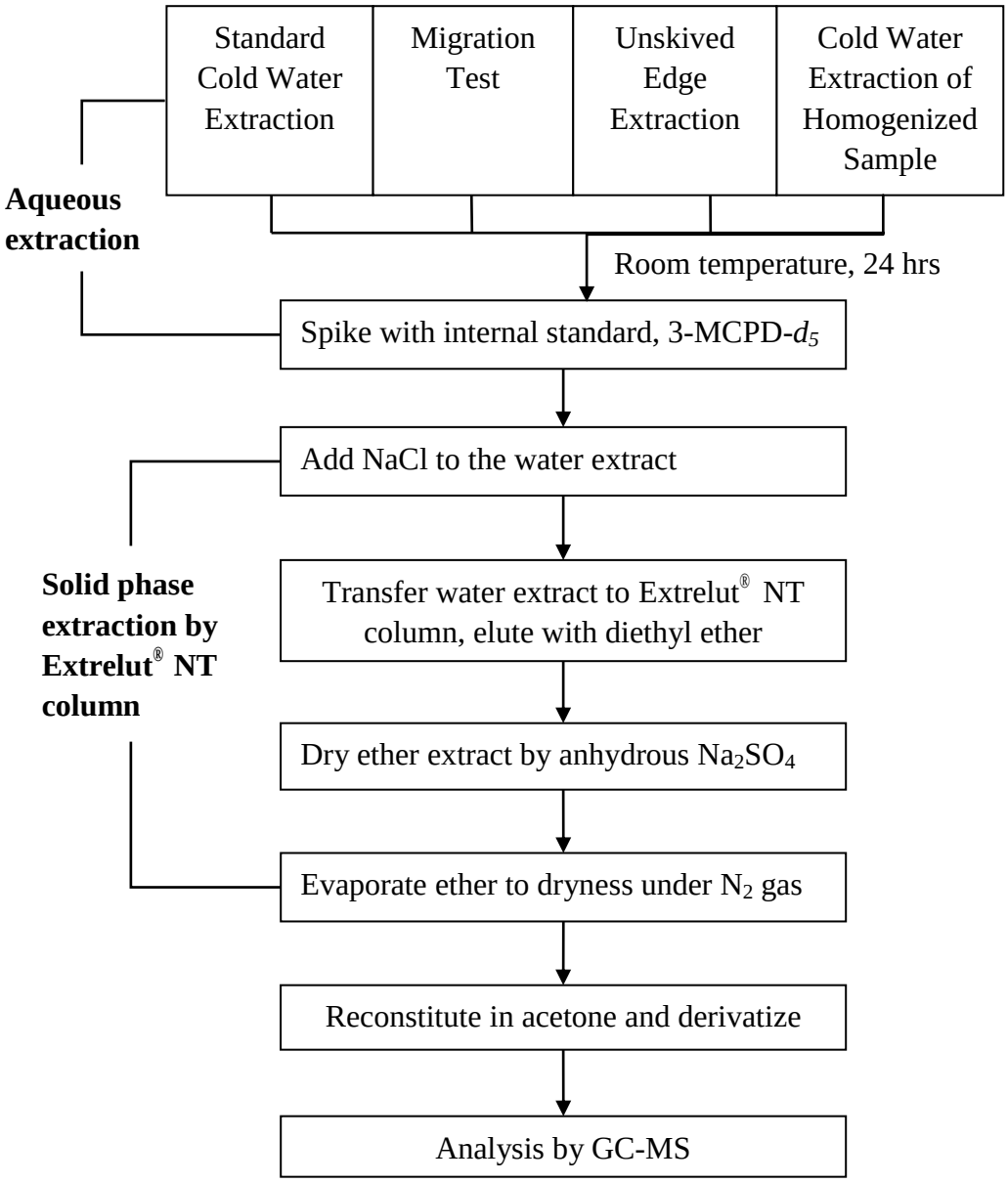


Figure 13: Flow chart of overall analytical procedure

4.2. Reagents and Materials

Chemicals and Reagents were supplied as follows: (±)3-Chloro-1,2-propanediol (3-MCPD), 98% (99.6% in this specific lot), from Sigma-Aldrich, Milwaukee, WI.; 1,3-dichloro-2-propanol (1,3-DCP), 98% (99.3% in this specific lot), from Sigma-Aldrich, Milwaukee, WI, USA; 3-chloro-1,2-propanediol-1,1,2,3,3,-d_5 (3-MCPD-d_5), used as surrogate internal standard, 98 atom % D, 97% chemical purity (98.1 atom % D and 99% chemical purity in this specific lot), from Sigma-Aldrich, Milwaukee, WI, USA; sodium chloride and anhydrous sodium sulfate were purchased from Fisher Scientific, Fair Lawn, NJ, USA; derivatization reagent kit, Sylon BFT (BSTFA + TMCS 99:1, 0.1ml ampoules) was provided by Supelco Analytical, Bellefonte, PA, USA; Extrelut® NT20 sorbent (LC and GC grade) was purchased from EM Science, Cherry Hill, NJ, USA.

Acetone (HPLC grade, submicron filtered) and ethyl ether (ACS grade, BHT stabilized) were purchased from Fisher Scientific, Fair Lawn, NJ, USA. Ethyl ether was redistilled before use on rotary evaporator to get rid of BHT. Distilled water was prepared in house by a Waters Milli-Q Nanopure™ system.

4.3. Stock Standard Solutions

Separate stock standard solutions of 3-MCPD and 1,3-DCP were prepared in acetone at concentration of 10 mg/ml. From these solutions, a series of standard solutions of 3-MCPD and 1,3-DCP at 1, 0.1, 0.01, 0.001 mg/ml were prepared by dilution with

acetone, respectively. Stock solution of internal standard, 3-MCPD-d_5, were prepare in acetone at concentration of 1 mg/ml.

4.4. Calibration Standards

For preparation of calibration standards, appropriate amount of 3-MCPD and 1,3-DCP stock solutions, 10 µl of 3-MCPD-d_5 stock solution (1 mg/ml), and 20 µl of Sylon BFT were mixed in acetone. The amount of acetone added was calculated so that the final volume of each calibration standard was 100 µl.

Table 2: Volume of each chloropropanols standard added to the calibration standards and their final concentrations (for GC-FID calibration curve)

Final conc. of calibration standards in µg per 100 µl		Volume (µl) of 3-MCPD 1mg/ml Stock solution	Volume (µl) of 1,3-DCP 1 mg/ml stock solution	Volume (µl) of 3-MCPD 0.1 mg/ml stock solution	Volume (µl) of 1,3-DCP 0.1mg/ml stock solution
3-MCPD and 1,3-DCP final conc.	I.S. final conc.				
10	10	10	10	0	0
5	10	5	5	0	0
1	10	0	0	10	10
0.5	10	0	0	5	5
0.1	10	0	0	1	1

Table 2 shows the volume of each 3-MCPD and 1,3-DCP stock solutions added to the calibration standards and their final concentrations. This table was used to build a calibration curve for GC-FID system. For GC-MS, stock solutions of 3-MCPD and 1,3-DCP at lower concentrations (0.01, 0.001 mg/ml) were also used to get calibration standards at lower concentrations (3-MCPD and 1,3-DCP at 0.05, 0.01, 0.005, 0.001 µg

per 100µl). The calibration standards were prepared in Reacti-Vials and followed by the derivatization process. The result calibration curves and calculations are discussed in the results and discussions section.

4.5. Preparation of Water Extract

In most of the published analytical methods, chloropropanols were extracted with a saturated sodium chloride solution. However, Retho and Blanchard (2005) pointed out that the saturated sodium chloride in water does not promote the extraction of chloropropanols in the aqueous phase. Moreover, the European standard EN 645:1993 (CEN, 1993) describes an official method for preparation of a cold water extract from paper or paperboard sample. This method is used for "investigations of certain extractives in paper or board intended to come into contact with foodstuffs" (CEN, 1993). Thus, pure water extraction is preferred in this study. Distilled water (Milli-Q) was used to prepare water extract. Four different methods for preparation of water extract were used. Figure 14 shows the pictures of the preparation of water extract.

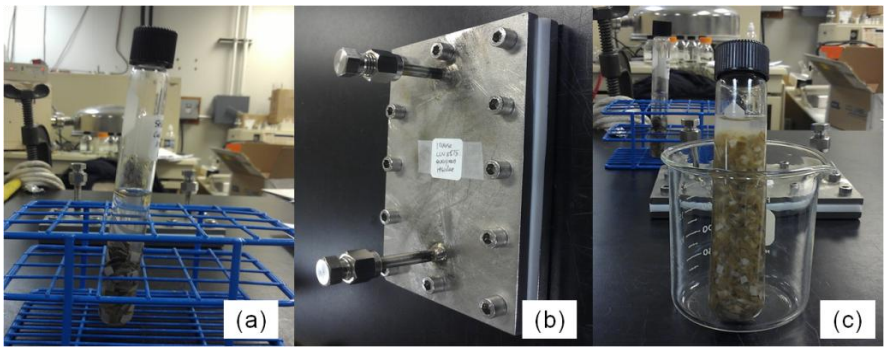


Figure 14: Sample preparation methods: (a) cold water extraction, (b) migration test, (c) water extraction of homogenized sample

4.5.1. EU Standard Cold Water Extraction

Cold water extract of each paperboard sample was prepared in accordance with the European standard method (CEN, 1993) except a scaled-down sample size. A paper or paperboard sample was cut into small pieces with a scissor. Each piece was smaller than 1 cm^2. The scissor was cleaned in between samples by wiping with methanol. Then weigh about 1 g of test pieces to an accuracy of 0.001g. Paperboard pieces were transfer into a 20 ml size test tube with Teflon-lined screw cap closures. 10 ml of distilled water was added to the test tubes. The test sample was incubated 24 hours at room temperature with periodically agitations.

After 24 hour incubation, the sample was spiked with 10 μl of 3-MCPD-d_5 stock solution (1 mg/ml). This is about 10 ppm (w/w) of internal standard.

4.5.2. Migration Test

Although the European Standard recommends a cold water extraction method for analysis of paperboard food packaging, this method dose not simulate the real conditions of the use of the paperboard in typical beverage carton applications. In a real scenario, the cartons come in contact with beverages, and chloropropanols may migrate from the packaging to the beverage. In order to study the migration of chloropropanols, migration tests using single-side extraction cell were conducted.

Figure 15 shows the single side extraction cell, which was designed by Dr. Thomas G. Hartman (the thesis director) and produced by Scientific Instrument Services, Inc.,

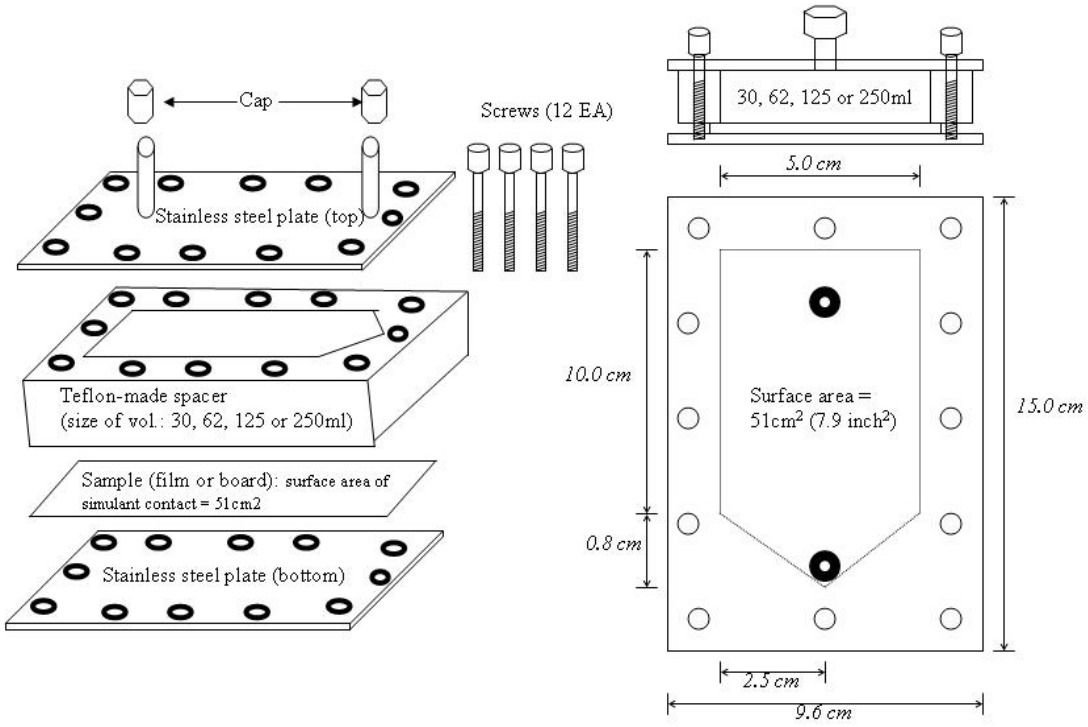


Figure 15: Single-side extraction cell for migration test [Adapted from Pace and Hartman, 2010]

Ringoes, NJ, USA. The extraction cell assembly includes two stainless steel plates with a Teflon spacer between the plates. A board or film sample is placed between the Teflon spacer and the bottom plate. The spacer holds the solvent used for extraction. The surface area of solvent contact side is 51 cm^2. The top plate contains two tube ports for filling and emptying the solvent. This device can extract migratable contaminants from one single side of packaging material.

In this study, a 30 ml size Teflon spacer was used, 30 ml of distilled water was filled into the extraction cell, and the beverage contact side of a carton sample was extracted. After 24 hour incubation, 10 ml of water extract was transferred to a 16 ml size sample vial and spiked with 10 µl of 3-MCPD-d_5 stock solution.

The migration test was performed either with a slit in the paperboard sample or without a slit. The slit in a sample simulates the unskived (with no coating) edge of the 5th panel in a beverage carton. In a polyethylene extrusion-coated packaging, the 5th panel is attached to the 1st panel to form a carton. The edge of the 5th panel may be completely coated with polyethylene (skived), or may not be coated (unskived). Figure 16 shows the 5th panel of a carton and a illustration of a slit in a sample. The slit was cut by a clean blade before extraction. The length of slit in a sample is determined by the following equation:

$$\frac{\text{length of slit}}{\text{extracted area of sample (51 cm}^2\text{)}} = \frac{\text{length of unskived edge}}{\text{beverage contact surface area}}$$

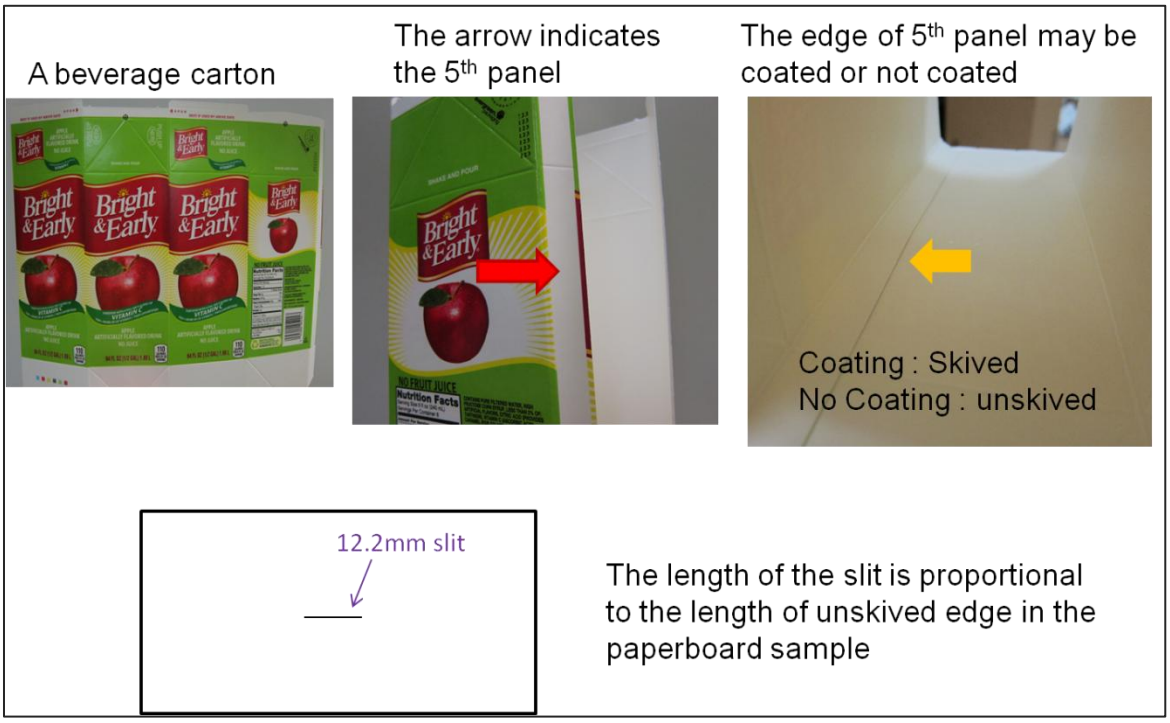


Figure 16: The 5th panel of a carton and the illustration of a slit in a sample.

Migration tests with a slit in the paperboard sample were used to determine the migration of chloropropanols from unskived edge of the 5th panel in a carton.

4.5.3. Unskived Edge Extraction

Unskived edge extraction is another procedure to analysis the migration of chloropropanols from unskived edge. A 2.5 × 2.5 cm^2 paperboard was immersed into 30ml of distilled water in a 50 ml size test tube. The edges of the sample should be completely under the water level. After 24 hour incubation, 10 ml of water extract was transferred to a 16 ml size sample vial and spiked with 10 μl of 3-MCPD-d_5 stock solution. The total length of extracted edge was 10 cm. This procedure dose not simulate the edge of the 5th panel in a carton, but analyze the chloropropanol migration from a 10 cm unskived edge.

4.5.4. Water Extraction of Homogenized Paperboard Sample

It was questioned that the European Standard cold water extraction method can get the maximum extractable chloropropanols from paperboard samples. In order to investigate this problem, a paperboard sample was homogenized to achieve the maximum surface area for extraction, and then the normal procedure of cold water extraction was conducted.

Paperboard sample was homogenized by a blender with a stainless steel mini container (from Waring commercial, Torrington, CT, USA). 4 g of sample (cut into small

pieces) and 35 ml of distilled water was added to the blender for homogenization. After homogenization, the sample was transferred to a 50 ml size test tube. 5 ml of distilled water was used to rinse the container, and the water was also transferred to the test tube to make the total water volume of 40 ml. After 24 hours incubation, the sample was spiked with 40 μl of 3-MCPD-d_5 stock solution. (10 ppm, w/w)

4.6. Solid-phase Extraction by Extrelut® NT20 column

Every water extract obtained from the procedures described in section 4.5 was subjected to solid-phase extraction by Extrelut® NT20 column. Extrelut® NT is a diatomateous earth sorbent. Figure 17 shows the working principle of Extrelut® NT column. The main purpose of this step was to transfer extracted chloropropanols from aqueous phase to organic phase, and remove the water from the extract. The sorbent kept water in its structure and the water acted as the stationary phase during elution. In this study, the sorbent used was Extrelut® NT20, which can take up to 20 ml of aqueous sample if one pack of sorbent was used. This step also filtered out remaining small fibers from paper samples (especially homogenized samples), and cleaned up the unwanted hydrophilic substances in the water extract.

The Extrelut® NT20 column was prepared by packing 1.9 g of Extrelut® NT20 sorbent into a 10 ml size glass pipet between two plugs of glass wool (the prepacked columns are also available from the vendor). 1.5 g of NaCl was added to 5 ml of water extract to enhance the effectiveness of solid-phase extraction. 2.3 ml of water extract was

applied to the Extrelut® NT20 column. 2 ml represents 1/5 of sample (extra volume due to NaCl). After 15~20 minutes equilibrium, the sample was eluted with redistilled diethyl ether. 20 ml of ether eluate was collected, and dried with anhydrous sodium sulfate. Finally, the ether was evaporated to dryness under nitrogen gas and the extract was reconstituted with acetone in a Reacti-Vial. Then the extract was ready for derivatization.

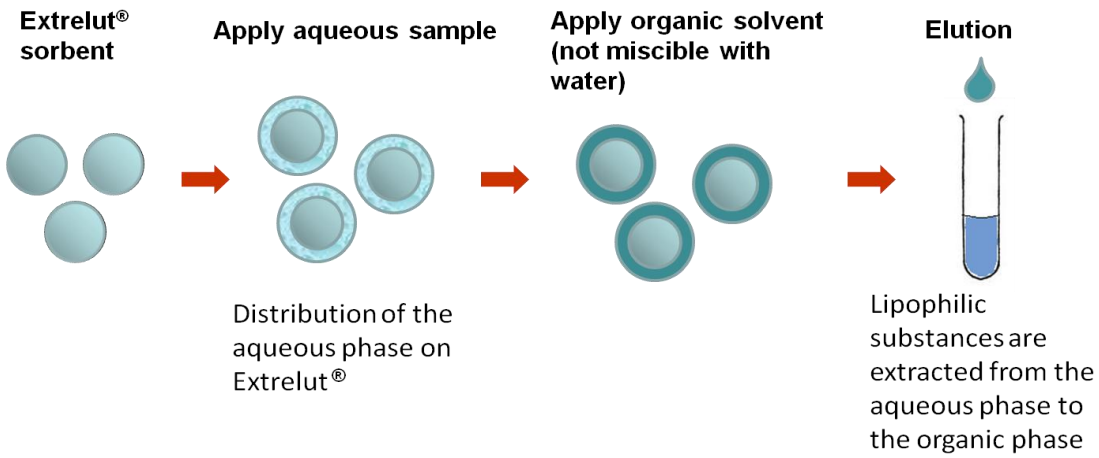


Figure 17: The principle of Extrelut® NT column

4.7. Derivatization Procedure

Besides the limitations mentioned earlier in the literature review section, there are also other problems of HFBI derivatization procedure. The procedure uses isooctane as solvent, and there is a solubility problem of chloropropanols in isooctane. The derivatization procedure is difficult and time-consuming. Moreover, the moisture sensitive reagent is not available in a small ampule packaging, so it readily reacts with the moisture in air and loses its reactivity.

In order to avoid the inconveniences and drawbacks of HFBI, a silylation reagent, Sylon BFT, was used for derivatization. This reagent contains the combination of BSTFA [N,O-bis(trimethylsiyl)trifluoroacetamide] and TMCS (trimethylchlorosilane), 99:1. The mechanism of silylation is shown on Figure 18. Trimethyl silyl derivatives are formed. The procedure is very simple: mix the reagent with the extract and heat at 80 ℃ for an hour.

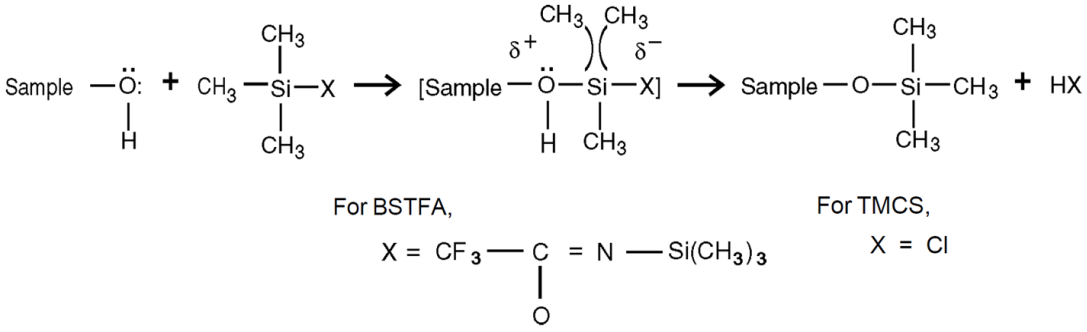


Figure 18: The mechanism of silylation [Adapted from Suplelco.]

4.8. Gas Chromatography - Mass Spectrometry Analysis Conditions

GC-MS analyses were carried out on a Varian 3400 GC interfaced with a Finnigan TSQ-7000 triple stage quadrupole tandem mass spectrometer. The GC was equipped with a capillary column ZB-5NS (from Phenomonex) and a split/splitless injector. This column is 30 meter × 0.32 mm I.D. × 0.25 μm film thickness. The injection volume was 1.0 μl in a splitless mode. The injector temperature was set at 260 ℃. The carrier gas was helium at 10 psi. The column was temperature programmed from 50 ℃ (hold for 3 minutes) to 150 ℃ at a rate of 10 ℃ per minute, then from 150 ℃ to 280 ℃ at a rate of 20 ℃ per

minute with a 10-minute hold at 280 ℃. The temperature of GC-MS transfer line was set at 280 ℃. The mass spectrometer was operated in electron ionization mode (70 eV). The ion source was at 250 ℃. The scan range of MS was 35-550 *m/z*. Xcaliber data system was used for operating and data analysis.

The scan rate of MS was set at 0.3 sec, which is faster than the common setting of 1 sec. An increased scan rate results in better separation of peaks (especially the peaks of 3-MCPD and 3-MCPD-d_5), but it also decrease the intensity of signals. The setting was a balance between these two effects.

4.9. Gas Chromatography - Flame Ionization Analysis Conditions

GC-FID analyses were carried out on a Varian 3400 GC equipped with a flame ionization detector and a SPB^{TM}-1 capillary column (from Supelco). This column is 60 meter × 0.25 mm I.D. × 1 μm film thickness, with longer length and thicker film than the column used for GC-MS analysis. The reason for this choice was to obtain better resolution of chloropropanol peaks, especially for the peaks of 3-MCPD and 3-MCPD-d_5. The injection volume was 1.0 μl. The injector temperature was set at 280 ℃. The carrier gas is helium at 19.0 psi, with a linear carrier velocity of 32 cm/sec at 100℃. The detector temperature was set at 320℃, using air/hydrogen at a rate of 400/40 ml per minute, with helium as the make-up gas. The column was temperature programmed from 50 ℃ (hold for 3 minutes) to 160 ℃ at a rate of 10 ℃ per minute, then hold at 160 ℃ for 3 minute,

then from 160 ℃ to 280 ℃ at a rate of 10 ℃ per minute with a 10-minute hold at 280 ℃. PeakSimple software was used for data analysis.

4.10. Method Validation

Validation of an analytical method is necessary to ensure that the method is qualified and fitted for its intended use. González and Herrador (2007) described a holistic approach of validation. They divided the process into four parts:

- Applicability, fitness for purpose, and acceptability limits: the basic information about the analytical method, including the identity of analyte, the concentration range covered, the material used and the sample matrix, and the corresponding protocol (analytical procedure).
- Selectivity and specificity: the ability of the method to measure the analyte in presence of all the potential sample components and interferences.
- Calibration study: including the linearity, dynamic range, LOD, and LOQ
- Accuracy study: including accuracy, precision, and robustness.

The first two parts can be derived from the description of the procedure in previous sections, and the mass spectra and other chromatogram data presented in the next section. The calibration data will also be discussed in the next section. Other validation studies include analytical system precision, analytical method precision, and between batch precision and accuracy are described as follows:

- Analytical system precision: accessed by running 6 analyses of 3-MCPD at 0.131μg/100 μl and 1,3-DCP at 0.122 μg/100μl (also containing internal standard 3-MCPD-d_5 at 11.17μg/100μl)
- Analytical method precision: accessed by running 6 analyses of a cold water extract of a paperboard sample. The sample was previously analyzed and confirmed to contain both 3-MCPD and 1,3-DCP.
- Between batch precision and accuracy: accessed by analyzing the standard solution of 3-MCPD at 1.007μg/100 μl and 1,3-DCP at 1.017μg/100 μl (also containing internal standard 3-MCPD-d_5 at 10.145μg/100μl) daily in the period from 4/27 to 5/2/11.

5. RESULTS & DISCUSSIONS

5.1. Gas Chromatography-Mass Spectrometry: data analysis

5.1.1. Selected ion chromatogram and mass spectra

Chloropropanols were analyzed by GC-MS as their corresponding trimethyl silyl esters. Figure 19 shows the selected ion chromatogram of a solution containing TMS derivatives of 12.15 µg of 1,3-DCP, 13.13µg of 3-MCPD, and 11.17 µg of 3-MCPD-d_5 in 100 µl of acetone. Their characteristic ions are shown in Table 3. The retention times were 9.042 (scan number 351) for 3-MCPD-d_5-di-TMS, 9.085 (scan number 356) for 3-MCPD-di-TMS, and 7.069 (scan number 119) for 1,3-DCP-TMS.

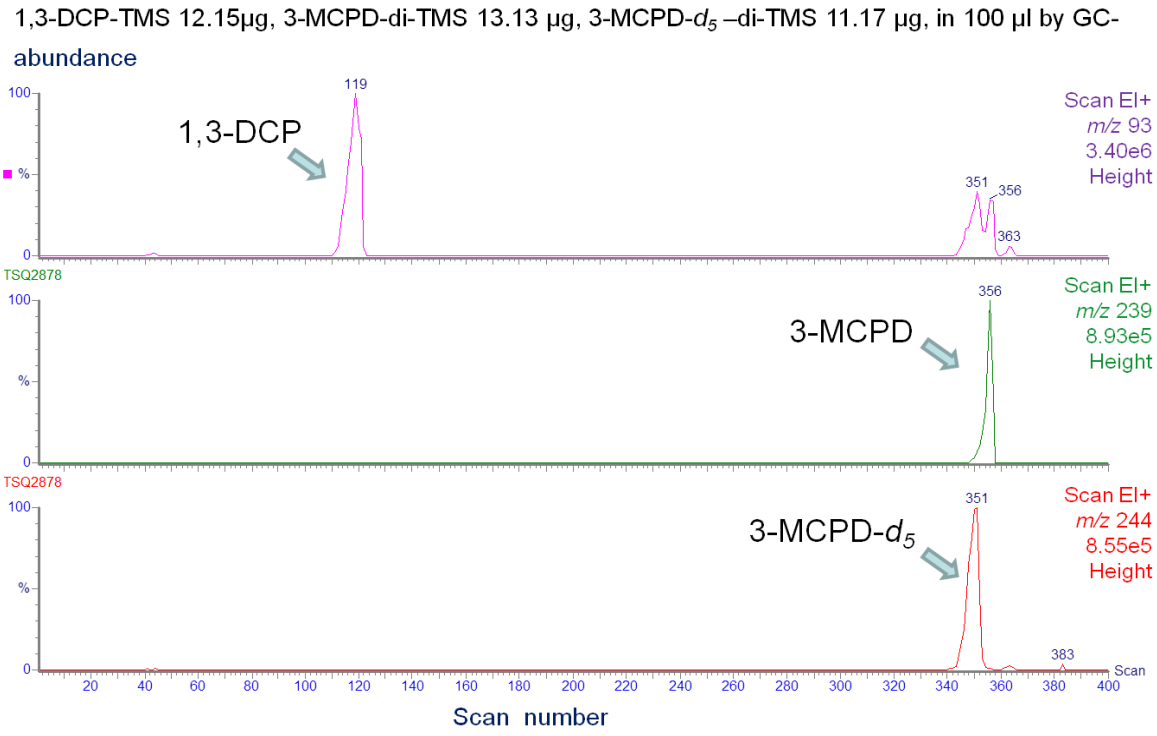


Figure 19: Selected ion chromatogram of chloropropanols

Table 3: Characteristic ion and quantifier ion for the chloropropanols

	3-MCPD-d_5-di-TMS	3-MCPD-di-TMS	1,3-DCP-TMS
Characteristic ion (*m/z*)	244, 119, 104	239, 116, 101	93
Quantifier ion (*m/z*)	244	239	93

Figure 20, Figure 21, and Figure 22 present the mass spectra of the TMS derivatives of 3-MCPD-d_5, 3-MCPD, and 1,3-DCP from the same solution. Their chemical structures are also shown in these figures. The derivatization reagent reacts with two hydroxyl groups in the 3-MCPD-d_5 and 3-MCPD, forming their di-TMS derivatives with molecular weights of 259 for 3-MCPD-d_5-di-TMS and 254 for 3-MCPD-di-TMS. The electron ionization voltage breaks the intact molecular ion, resulting in their characteristic ions. The ions of *m/z* 244 and 239 are result from the loss of a methyl group from 3-MCPD-d_5-di-TMS or 3-MCPD-di-TMS. The loss of a CD_2O-TMS or a CH_2O-TMS from the molecular ions of 3-MCPD-d_5-di-TMS or 3-MCPD-di-TMS forms the ions of *m/z* 119 and 116. Then, the further loss of a methyl group forms the *m/z* 104 and 101. The characteristic ion of 1,3-DCP-TMS is selected from the diagnostic fragmentation pattern of *m/z* 93 and 95, which exhibit a 2-unit difference in mass and a abundance ratio of 3:1. The pattern indicates the presence of chlorine and its stable isotope, $^{35}Cl/^{37}Cl$. The major characteristic ions used in this study, *m/z* 244, 239, 93, were also selected as the qualifier ions in other published literatures that utilized TMS derivatives for analysis of chloropropanols by GC-MS (Cao *et al*. 2009; Gonzalez *et al*. 2011; Racamonde *et al*. 2011) It was noticed that a ion of *m/z* 73 was appeared in the mass spectra of all three

chloropropanols. This ion is the trimethylsilyl fragment from the derivatives of chloropropanols.

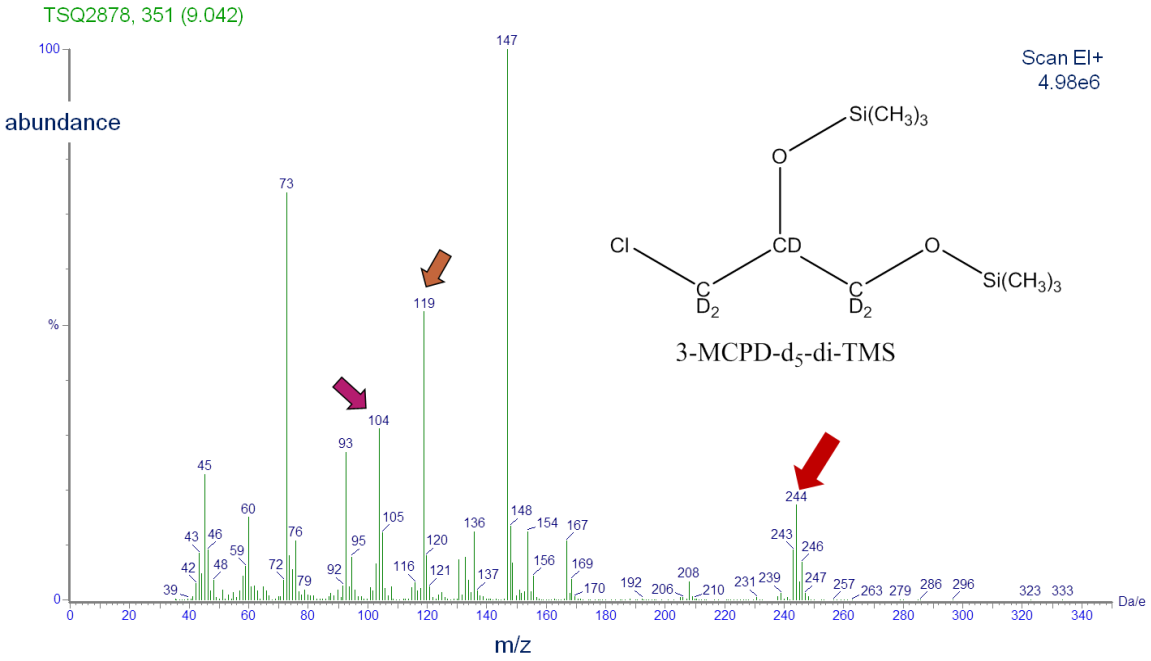


Figure 20: Mass spectrum of 3-MCPD-d_5-di-TMS.

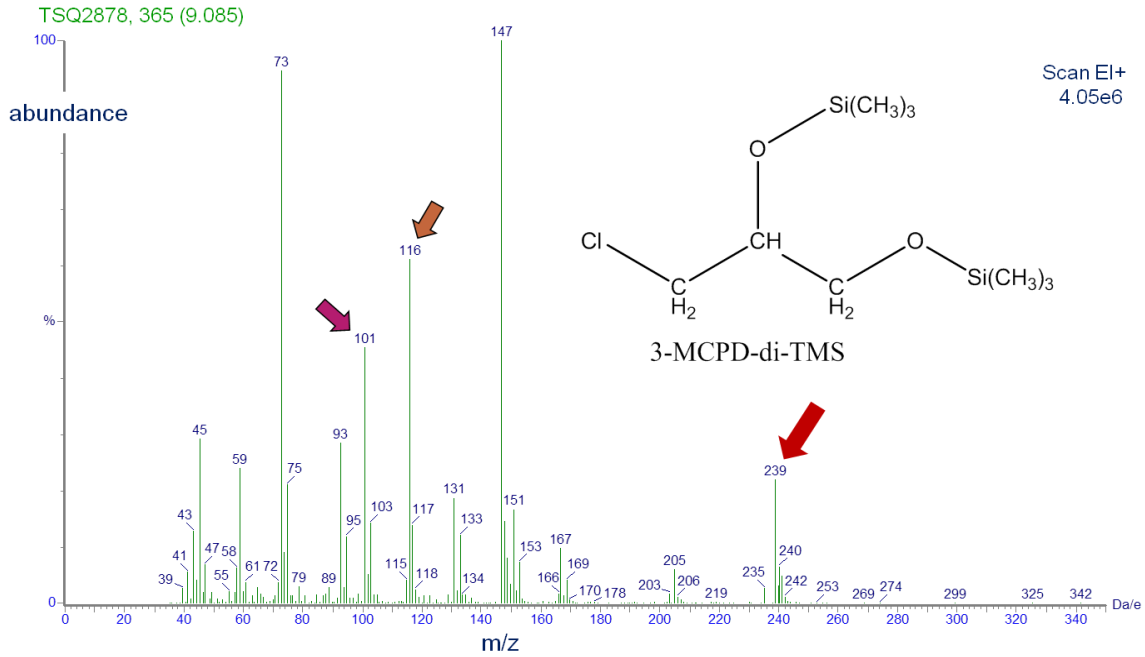


Figure 21: Mass spectrum of 3-MCPD-di-TMS

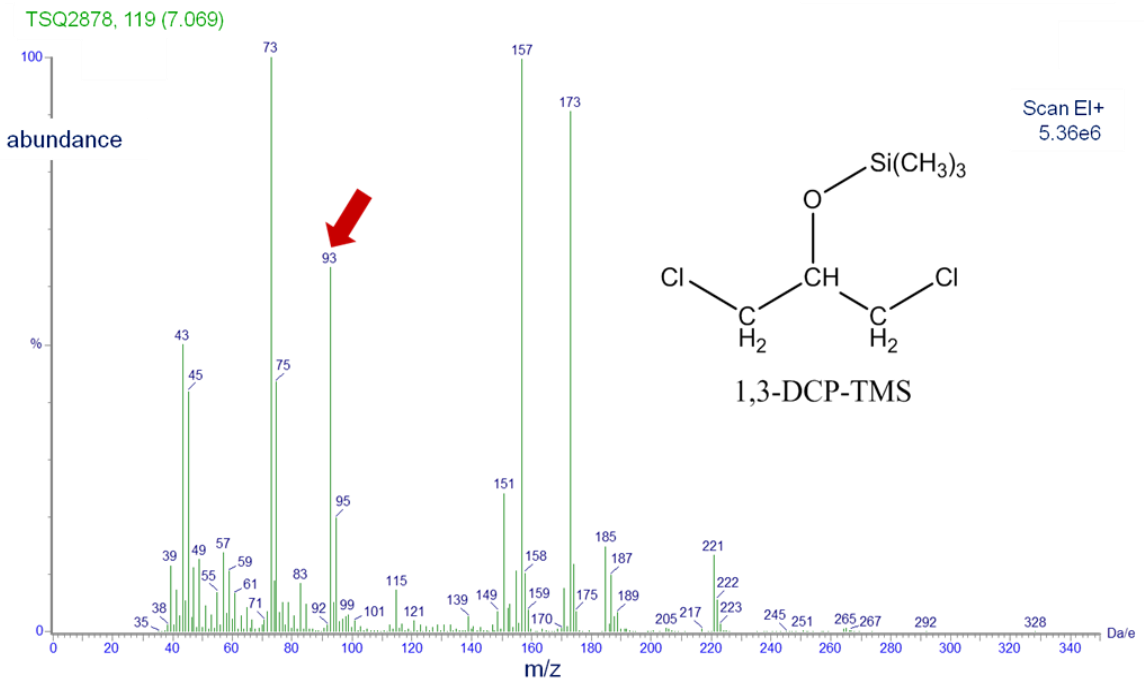


Figure 22: Mass spectrum of 1,3-DCP-TMS

The reason for choosing 3-MCPD-d_5 as internal standard is that this deuterated surrogate molecule has a structure similar to 3-MCPD, and it acts likes 3-MCPD, the major target for analyses. Comparing the fragmentation patterns in Figure 20 and Figure 21, a lot of similarities can be found.

Figure 23 presents another selected ion chromatogram of a real paperboard sample. The sample was prepared by cold water extraction method. This chromatogram shows that with all the possible interferences in the sample solution, a clear chromatogram can still be obtained.

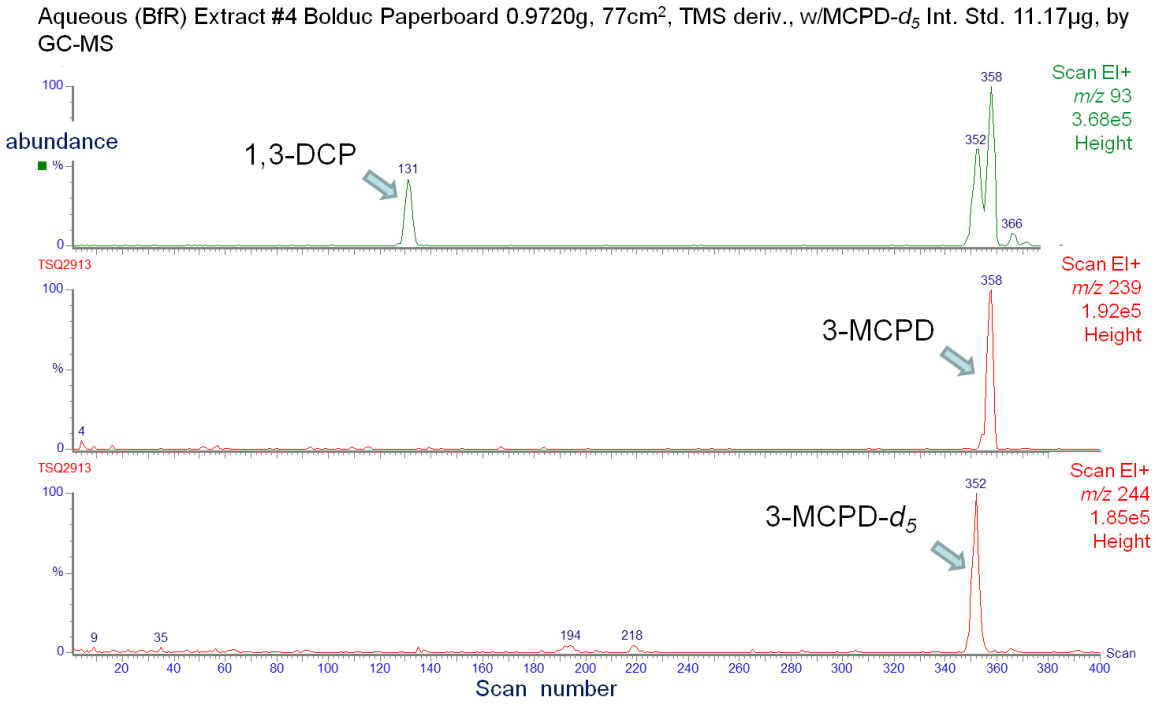


Figure 23: Selected ion chromatogram of a paperboard sample

5.1.2. GC-MS Calibration Curves

Table 4 and Table 5 present the results of calibrations of 3-MCPD and 1,3-DCP by GC-MS, respectively. The calibration curves were obtained by plotting the peak area ratio of 3-MCPD/I.S. or 1,3-DCP/I.S. versus the concentrations of standard solutions of 3-MCPD or 1,3-DCP. Liner regressions were used. The resulting calibration curves are shown in Figure 24.

For 3-MCPD, a seven point calibration was performed (Table 4). The dynamic range of calibration is approximately 0.01~10 µg/100µl. (0.013~13.13µg/100µl). The 3-MCPD calibration is linear in this dynamic range (R-squared > 0.99). Higher injected concentrations of 3-MCPD tend to cause a non-linear calibration.

For 1,3-DCP, a nine point calibration was performed (Table 5). The dynamic range of calibration is approximately 0.01~100 μg/100μl. (0.012~121.5 μg/100μl). The 1,3-DCP calibration is linear in this dynamic range (R-squared > 0.999).

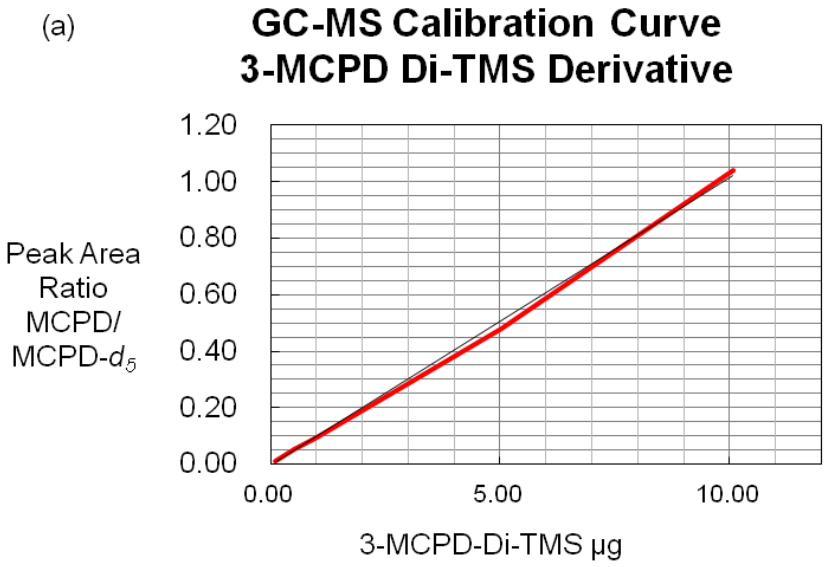
Table 4: 3-MCPD Calibration data (GC-MS)

3-MCPD Conc. in ug per 100ul	**3-MCPD-d_5 Int. Std. Conc. in ug per 100ul**	**Peak Area Ratio m/z 239/244**	**3-MCPD Regression Output:**	
			Constant	0
13.130	11.17	0.5986	Std Err of Y Est	0.008713
6.565	11.17	0.3062	R Squared	0.998565
1.313	11.17	0.0406	No. of Observations	7
0.657	11.17	0.0240	Degrees of Freedom	6
0.131	11.17	0.0055		
0.066	11.17	0.0027	X Coefficient(s)	0.045664
0.013	11.17	0.0014	Std Err of Coef.	0.000591

Table 5: 1,3-DCP Calibration data (GC-MS)

1,3-DCP Conc. in ug per 100ul	**3-MCPD-d_5 Int. Std. Conc. in ug per 100ul**	**Peak Area Ratio**	**1,3-DCP Regression Output:**	
121.500	11.17	60.3737	Constant	0
60.750	11.17	31.4645	Std Err of Y Est	0.465766
12.150	11.17	6.3668	R Squared	0.999510
6.075	11.17	2.5066	No. of Observations	9
1.215	11.17	0.3938	Degrees of Freedom	8
0.608	11.17	0.1762		
0.122	11.17	0.0769	X Coefficient(s)	0.501097
0.061	11.17	0.0288	Std Err of Coef.	0.003412
0.012	11.17	0.0137		

(a)



(b)

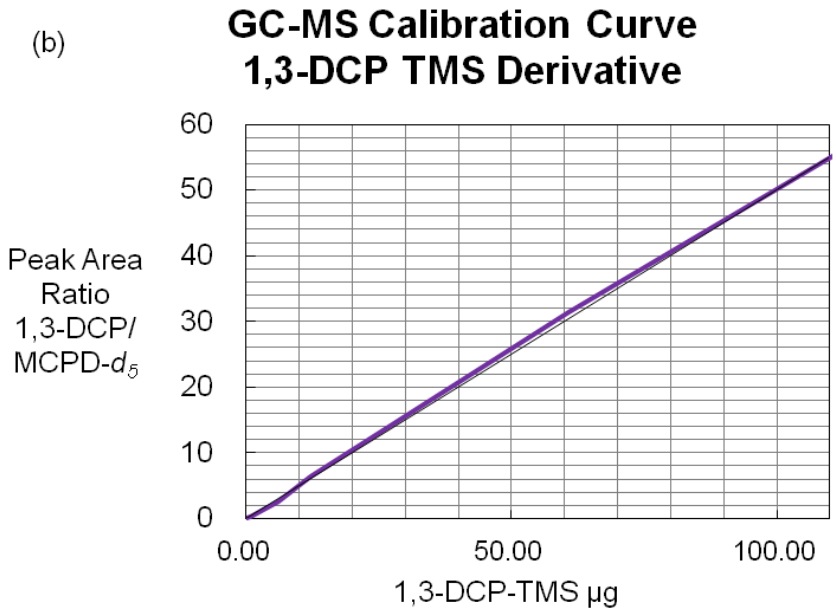


Figure 24: (a) 3-MCPD GC-MS calibration curve; (b) 1,3-DCP GC-MS calibration curve.

5.1.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection was determined with a signal-to-noise ratio of 3, where the noise was selected from peaks adjacent to the peak of the TMS derivatives of chloropropanols. Figure 25 shows the signal-to-noise ratio for determination of LOD. The LOD is approximately 10 µg/kg for both 3-MCPD and 1,3-DCP, considering the extracted sample weight and its equivalence to the standard extraction method.

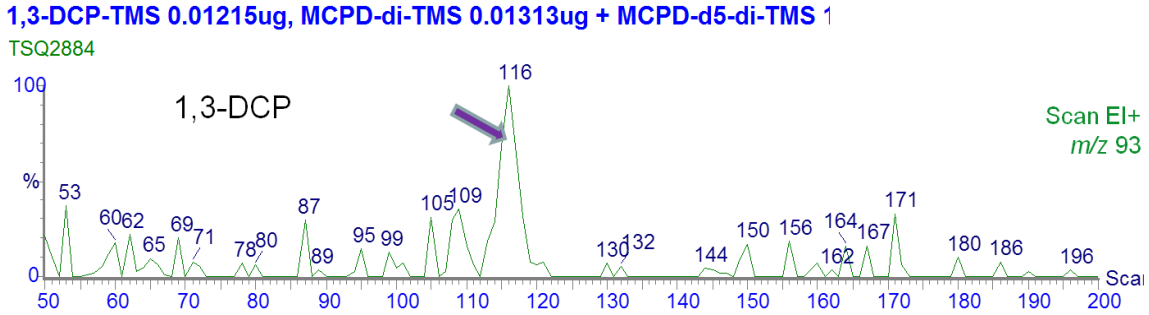


Figure 25: The signal-to-noise ratio for determination of LOD.

The limit of quantification was determined with a signal-to-noise ratio of 10, where the noise was selected from peaks adjacent to the peak of the TMS derivatives of chloropropanols. Figure 26 shows the signal-to-noise ratio for determination of LOQ. The LOQ is approximately 50 µg/kg for both 3-MCPD and 1,3-DCP.

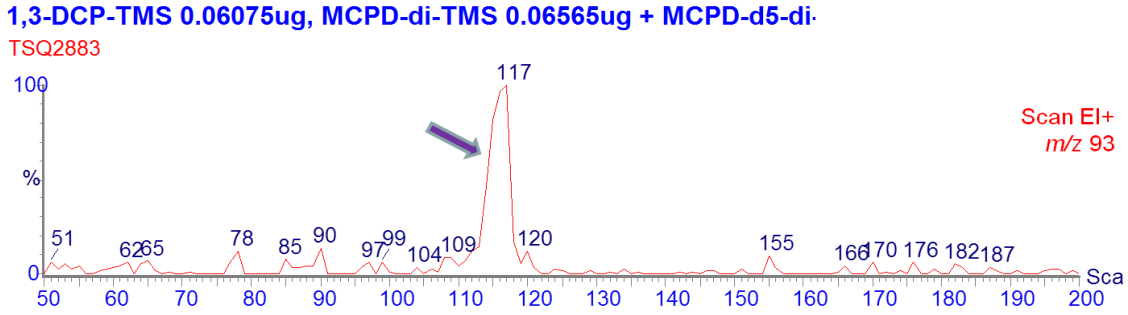


Figure 26: The signal-to-noise ratio for determination of LOQ.

5.1.4. GC-MS in Selected Ion Monitoring (SIM) mode

In selected ion monitoring mode, *m/z* 244, 239, 93 were monitored for the analysis of 3-MCPD-d_5, 3-MCPD, and 1,3-DCP. Since only these three ions of interest were being monitored, matrix interferences were lower. As a result, better selectivity and sensitivity were obtained. Figure 27 shows the selected ion chromatogram of chloropropanols by GC-MS in SIM mode. The tested solution contains TMS derivatives of 0.001 µg of 1,3-DCP, 0.001µg of 3-MCPD, and 10.425 µg of 3-MCPD-d5 in 100 µl of acetone. The concentration is ten times lower than the lowest concentration analyzed in full scan mode. This chromatogram suggests that in SIM mode, a solution with ten times lower concentrations of 3-MCPD and 1,3-DCP than in full scan mode can be analyzed.

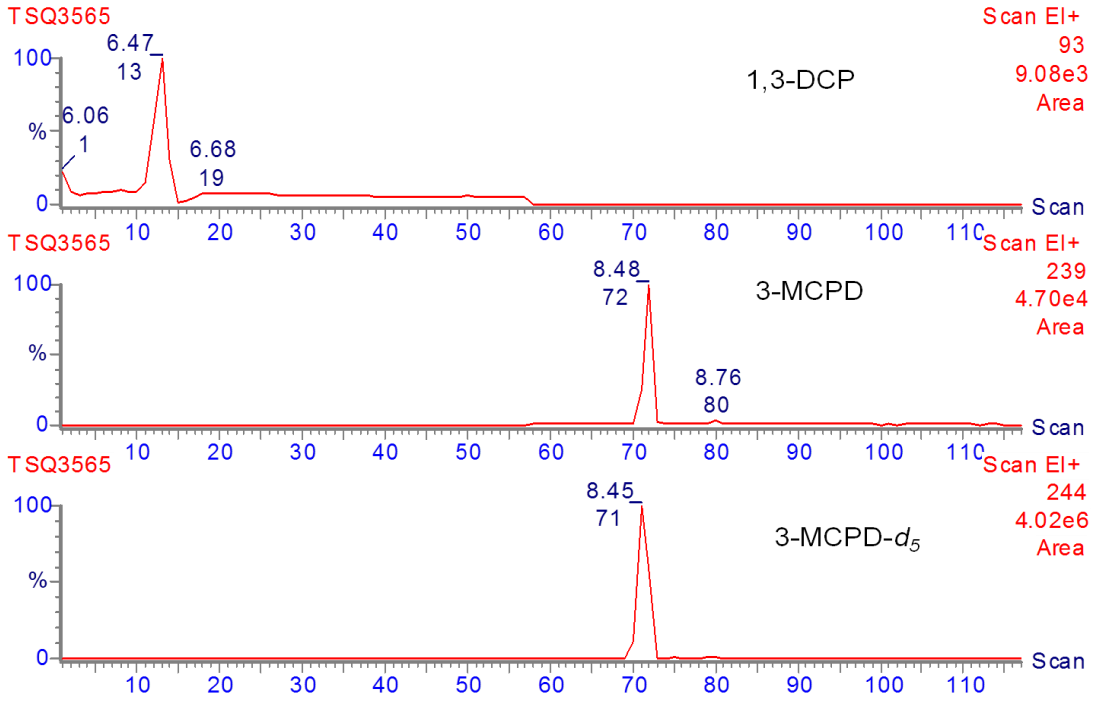


Figure 27: selected ion chromatogram of chloropropanols by GC-MS in SIM mode.

5.2. GC-FID: data analysis

5.2.1. GC-FID chromatogram

After the analytical procedure using GC-MS was established, a GC-FID method was also developed. Figure 28 shows the GC-FID chromatogram of a solution containing the TMS derivatives of 1.017 μg of 1,3-DCP, 1.007 μg of 3-MCPD, and 10.425 μg of 3-MCPD-d_5 in 100 μl of acetone. The peak identification was performed by injections of a series of standard solutions with different concentrations. The changes in peak area with different standard concentrations provided the information for peak identification. The retention times were 20.21 for 1,3-DCP-TMS, 23.16 for 3-MCPD-d_5-di-TMS, and 23.23 for 3-MCPD-di-TMS. It was noticed that the peaks of 3-MCPD-d_5-di-TMS and 3-MCPD-di-TMS were overlapped. A different column (SPB^{TM}-1 capillary column, with long length and thicker film than the column used in GC-MS method) and different temperature programs were used to get optimized separation. Different internal standards such as 3-monobromo-1,2-propanediol (3-MBPD) or a mixture of benzene-d_6, toluene-d_8 and naphthalene-d_8 were also considered to avoid the overlap between internal standard and analyte. However, the pretrial injections suggested that 3-MBPD-di-TMS was unstable in the conditions used in GC-FID methods. Moreover, 3-MCPD-d_5 was still more preferable for its similar chemical properties with 3-MCPD. The similarity between internal standard and analytes was essential in this method because the matrix spike of internal standard in the sample preparation process was the basis of quantification. An

internal standard with different chemical properties may act differently from analytes in the extraction, elution, or derivatization process, and affect the accuracy of quantification. Therefore, 3-MCPD-d_5 was still used as the internal standard in GC-FID method. The chromatogram in Figure 28 presents the result.

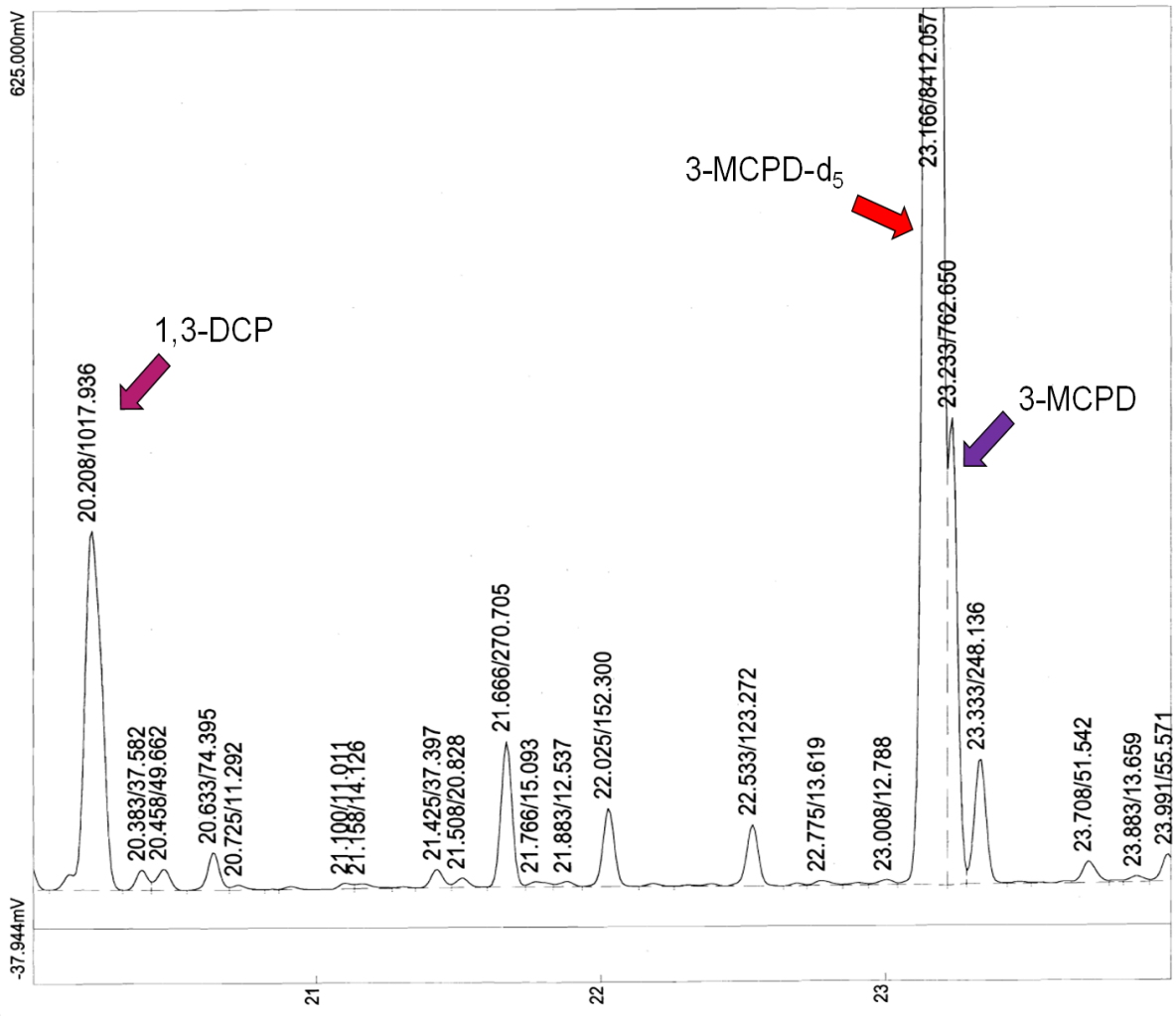


Figure 28: GC-FID chromatogram of chloropropanols.

5.2.2. GC-FID calibration curve

Despite the overlap between the peaks of 3-MCPD-d_5-di-TMS and 3-MCPD-di-TMS, linear calibration curves of 3-MCPD and 1,3-DCP was still obtained to validate the GC-FID method, and for quantification. The resulting calibration curves are shown in Figure 29. A five point calibration was performed for both 3-MCPD (Table 6) and 1,3-DCP (Table 7). The dynamic range of calibrations are approximately 0.1~10 μg/100μl. (0.1007~10.07 μg/100μl of 3-MCPD, and 0.1017~10.165 μg/100μl of 1,3-DCP). The calibrations of 3-MCPD and 1,3-DCP are linear in this dynamic range (R-squared > 0.99).

From the GC-FID chromatogram of a method blank injection, an interference peak was found at the same retention time of 1,3-DCP (co-elution). As a result, background subtraction was conducted in every calculation related to 1,3-DCP.

Table 6: 3-MCPD calibration data (FID)

3-MCPD Conc. ug/100ul	**3-MCPD-d_5 Conc. ug/100ul**	**Peak Area Ratio 3-MCPD/I.S.**	**3-MCPD Regression Output:**	
			Constant	-0.0078
10.0700	10.425	1.0400	Std Err of Y Est	0.0209
5.0350	10.425	0.4782	R Squared	0.9983
1.0070	10.425	0.0930	No. of Observations	5
0.5035	10.425	0.0533	Degrees of Freedom	3
0.1007	10.425	0.0111		
			X Coefficient(s)	0.1026
			Std Err of Coef.	0.0025

Table 7: 1,3-DCP calibration data (FID)

1,3-DCP Conc. ug/ 100ul	3-MCPD-d_5 Conc. ug/ 100ul	Peak Area Ratio 1,3-DCP/I.S.	Corrected Peak Area Ratio	1,3-DCP Regression Output:	
				Constant	0.0409
10.1650	10.425	0.7247	0.6708	Std Err of Y Est	0.0075
5.0825	10.425	0.3701	0.3162	R Squared	0.9995
1.0165	10.425	0.1078	0.0539	No. of Observations	5
0.5083	10.425	0.0745	0.0206	Degrees of Freedom	3
0.1017	10.425	0.0540	0.0001		
				X Coefficient(s)	0.0668
				Std Err of Coef.	0.0009

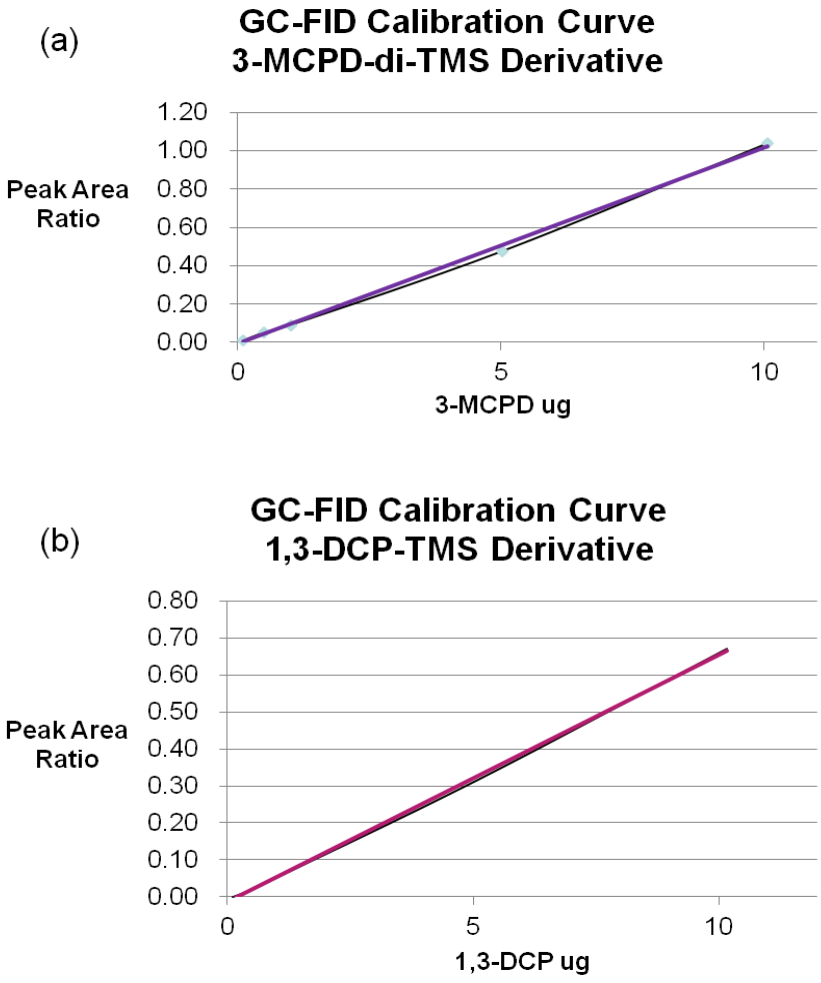


Figure 29: (a) 3-MCPD GC-FID calibration curve; (b) 1,3-DCP GC-FID calibration curve.

5.2.3. GC-FID limitations

The advantage of GC-FID over GC-MS is that GC-FID is more practical for manufacturers to utilize for routine quality control. A GC-MS is more expansive and requires specialized expertise to run and maintain. Therefore, the conversion from GC-MS method to GC-FID method is important. However, several limitations of GC-FID were appeared in this study to prevent the use of GC-FID to analyze chloropropanols. First, the sensitivity was worse than GC-MS. The detection limit of GC-FID method was ten times higher than GC-MS method, and not ideal for regulatory requirements (usually in μg/kg range). Second, there was overlap between the peaks of 3-MCPD-d_5-di-TMS and 3-MCPD-di-TMS. Bad resolution causes problems in the integration of peak areas and the quantification. Third, there were many background interferences. The co-elution problem of 1,3-DCP became more serious when aqueous extract of paperboard sample was analyzed. As a result, the data from GC-FID method were not really reliable. Some paperboard samples were analyzed by GC-FID, and the resulting levels of chloropropanols were significantly higher than the results obtained by GC-MS. In conclusion, GC-FID is not recommended in this study.

5.3. Analytical System Precision

Analytical system precision was accessed by running 6 analyses of a solution containing 3-MCPD at 0.131μg/100 μl, 1,3-DCP at 0.122 μg/100μl, and internal standard 3-MCPD-d_5 at 11.17μg/100μl. The data of the system precision measurements are given

on the Table 8 and Table 9. The System precision of 3-MCPD expressed as RSD% is 3.36% (n=7). The mean backfit to calibration is 0.119±0.004 μg/100μl. The System precision of 1,3-DCP expressed as RSD% is 7.65% (n=7). The mean backfit to calibration is 0.151±0.0115 μg/100μl.

Table 8: Analytical system precision:3-MCPD

3-MCPD Conc. ug/100μL	3-MCPD-d_5 Conc. ug/100μL	Peak Area Ratio 3-MCPD/I.S.	Backfit 3-MCPD Conc. ug/100uL	Mean (n=7) 3-MCPD Conc. ug/100uL	S.D. (n=7) 3-MCPD Conc. ug/100uL	Analytical System Precision RSD %
0.131	11.17	0.0055	0.1212	**0.1190**	**0.0040**	**3.36**
0.131	11.17	0.0052	0.1147			
0.131	11.17	0.0056	0.1227			
0.131	11.17	0.0052	0.1132			
0.131	11.17	0.0053	0.1170			
0.131	11.17	0.0056	0.1231			
0.131	11.17	0.0055	0.1213			

Table 9: Analytical system precision: 1,3-DCP

1,3-DCP Conc. ug/100ul	3-MCPD-d_5 Conc. ug/100ul	Peak Area Ratio 1,3-DCP/I.S.	Backfit 1,3-DCP Conc. ug/100ul	Mean (n=7) 1,3-DCP Conc. ug/100ul	S.D. (n=7) 1,3-DCP Conc. ug/100ul	Analytical System Precision RSD %
0.122	11.17	0.0769	0.1535	**0.1510**	**0.0115**	**7.65**
0.122	11.17	0.0804	0.1604			
0.122	11.17	0.0758	0.1512			
0.122	11.17	0.0753	0.1502			
0.122	11.17	0.0765	0.1526			
0.122	11.17	0.0812	0.1620			
0.122	11.17	0.0636	0.1269			

5.4. Analytical Method Precision

Analytical method precision was accessed by running 6 analyses of a cold water extract of a paperboard sample. The sample was previously analyzed and confirmed to contain both 3-MCPD and 1,3-DCP. The analytical method precision data are given at Table 10 and Table 11. The mean concentration of 3-MCPD was 18.47±2.98 ppm (16.13 %RSD); The mean concentration of 1,3-DCP was 1.34±0.12 ppm (9.35 % RSD).

Table 10: Analytical Method Precision: 3-MCPD

Sample name	3-MCPD-d_5 Int. Std. Conc. ug/100ul	Peak Area Ratio 3-MCPD/I.S.	3-MCPD Conc. ppm w/w	Mean (n=6) 3-MCPD Conc. ppm w/w	S.D. (n=6) 3-MCPD Conc. ppm w/w	Analytical Method Precision RSD %
#6	11.17	0.8255	18.08	**18.47**	**2.98**	**16.13**
#6	11.17	0.6637	14.54			
#6	11.17	1.0859	23.78			
#6	11.17	0.8380	18.36			
#6	11.17	0.8114	17.77			
#6	11.17	0.8337	18.26			

Table 11: Analytical Method Precision: 1,3-DCP

Sample name	3-MCPD-d_5 Int. Std. Conc. ug/100ul	Peak Area Ratio 1,3-DCP/I.S.	1,3-DCP Conc. ppm w/w	Mean (n=6) 1,3-DCP Conc. ppm w/w	S.D. (n=6) 1,3-DCP Conc. ppm w/w	Analytical Method Precision RSD %
#6	11.17	0.6897	1.38	**1.34**	**0.13**	**9.35**
#6	11.17	0.5751	1.15			
#6	11.17	0.7092	1.42			
#6	11.17	0.7229	1.44			
#6	11.17	0.6085	1.21			
#6	11.17	0.7162	1.43			

5.5. Between Batch Precision and Accuracy

Between batch precision and accuracy was monitored in a six-day period. A solution containing 1.007µg of 3-MCPD, 1.017µg of 1,3-DCP, and 10.425µg of 3-MCPD-d_5 at 100µl of acetone was analyzed daily. A total of 17 measurements were performed. The data are presented in Table 12 and Table 13. The mean backfit of 3-MCPD was 0.86±0.05 µg/100µl (6.35 %RSD). The mean % deviation from nominal calibration was 15% for 3-MCPD. The mean backfit of 1,3-DCP was 1.02±0.03 µg/100µl (3.01 %RSD). The mean % deviation from nominal calibration was 4% for 1,3-DCP.

Table 12: Between Batch Precision: 3-MCPD

				Backfit		Mean (n=2~6)		
Analysis Date	3-MCPD-d_5 Conc. ug/100uL	3-MCPD Conc. ug/100uL	Peak Area Ratio 3-MCPD/I.S.	3-MCPD Conc. ug/100uL	%Deviation Nominal Calibration	3-MCPD Conc. ug/100uL	S.D. (n=2~6)	%RSD
4-27-11	10.425	1.007	0.0845	0.8992	11%	0.8552	0.0346	4.04%
4-27-11	10.425	1.007	0.0779	0.8352	17%			
4-27-11	10.425	1.007	0.0815	0.8703	14%			
4-27-11	10.425	1.007	0.0765	0.8215	18%			
4-27-11	10.425	1.007	0.0762	0.8189	19%			
4-27-11	10.425	1.007	0.0831	0.8861	12%			
4-28-11	10.425	1.007	0.0875	0.9287	8%	0.8678	0.0537	6.18%
4-28-11	10.425	1.007	0.0792	0.8475	16%			
4-28-11	10.425	1.007	0.0771	0.8273	18%			
4-29-11	10.425	1.007	0.0772	0.8282	18%	0.8526	0.0345	4.05%
4-29-11	10.425	1.007	0.0822	0.8771	13%			
4-30-11	10.425	1.007	0.0836	0.8907	12%	0.8391	0.0728	8.68%
4-30-11	10.425	1.007	0.0730	0.7877	22%			

5-1-11	10.425	1.007	0.0892	0.9456	6%	0.9143	0.0442	4.84%
5-1-11	10.425	1.007	0.0828	0.8831	12%			
5-2-11	10.425	1.007	0.0841	0.8952	11%	0.8329	0.0881	10.58%
5-2-11	10.425	1.007	0.0713	0.7706	23%			
Mean				**0.8596**	**15%**	**0.8604**	**0.0547**	**6.35%**

Table 13: Between Batch Precision: 1,3-DCP

Analysis Date	3-MCPD-d_5 Conc. ug/100uL	1,3-DCP Conc. ug/100uL	Corrected Peak Area Ratio 1,3-DCP/I.S.	Backfit 1,3-DCP Conc. ug/100uL	%Deviation Nominal Calibration	Mean (n=2~6) 1,3-DCP Conc. ug/100uL	S.D. (n=2~6)	%RSD
4-27-11	10.425	1.0165	0.06872	1.2249	20%	1.1616	0.0452	3.89%
4-27-11	10.425	1.0165	0.06800	1.2140	19%			
4-27-11	10.425	1.0165	0.06305	1.1399	12%			
4-27-11	10.425	1.0165	0.06266	1.1341	12%			
4-27-11	10.425	1.0165	0.06211	1.1259	11%			
4-27-11	10.425	1.0165	0.06243	1.1307	11%			
4-28-11	10.425	1.0165	0.05710	1.0508	3%	1.0298	0.0218	2.11%
4-28-11	10.425	1.0165	0.05578	1.0311	1%			
4-28-11	10.425	1.0165	0.05419	1.0074	1%			
4-29-11	10.425	1.0165	0.05246	0.9815	3%	0.9981	0.0236	2.37%
4-29-11	10.425	1.0165	0.05469	1.0149	0%			
4-30-11	10.425	1.0165	0.05494	1.0186	0%	0.9937	0.0352	3.54%
4-30-11	10.425	1.0165	0.05162	0.9689	5%			
5-1-11	10.425	1.0165	0.05378	1.0012	2%	0.9855	0.0222	2.26%
5-1-11	10.425	1.0165	0.05168	0.9698	5%			
5-2-11	10.425	1.0165	0.05132	0.9644	5%	0.9389	0.0360	3.84%
5-2-11	10.425	1.0165	0.04792	0.9134	10%			
Mean				**1.0524**	**4%**	**1.0179**	**0.0307**	**3.01%**

5.6. Solution Stability

The data obtained from between batch precision study can be also used to exam the solution stability. The solution was stored at room temperature in a Reacti-Vial with a Teflon-lined, screw cap closure. During this six-day period, no statistical difference was observed in the concentrations of both 3-MCPD and 1,3-DCP. The data suggest that freshly prepared solutions of 3-MCPD and 1,3-DCP TMS derivatives, stored at room temperature in Reacti-Vial with a Teflon-lined cap, are stable for six days.

The study conducted by Gonzalez *et al.* (2011) also suggested that the TMS derivatives of 3-MCPD and 1,3-DCP were stable during a 30-day period.

5.7. Water Extraction of Homogenized Sample

In order to determine the effect of homogenization, the same paperboard sample was treated with both EU standard cold water extraction and homogenization. The data are provided in Table 14 and Figure 30. The mean concentration of 3-MCPD in normal cold water extract was 5.56±1.05 ppm; the mean concentration of 3-MCPD in water extract of homogenized sample was 5.01±0.69 ppm. 1,3-DCP was below the detection limit (10 ppb). A Student's t test was performed to verify the influence of homogenization. The p value is above 0.05, indicating that there is no difference between these two data sets. The result suggests that these two sample preparation method can extract similar amount of chloropropanols. An increased surface area did not extract more chloropropanols form paperboard sample.

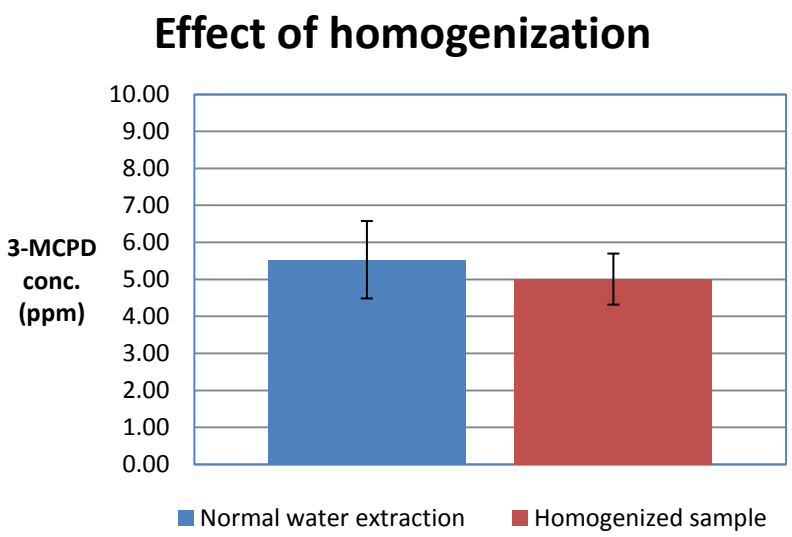


Figure 30: Comparison of the results from cold water extract and water extract of homogenized sample.

Table 14: Comparison of regular cold water extraction and water extraction of homogenized sample

Cold Water Extraction (No homogenization)						
I.S. Conc. ug/100ul	**3-MCPD Peak Area**	**3-MCPD-d_5 Peak Area**	**Peak Area Ratio**	**3-MCPD conc. in ppm (w/w)**	**Mean (n=4) 3-MCPD conc.**	**S.D. (n=4) 3-MCPD conc.**
10.425	906953	3000844	0.3022	6.62	5.56	1.05
10.425	971093	3450395	0.2814	6.16		
10.425	970670	4099711	0.2367	5.18		
10.425	734123	3773936	0.1945	4.26		
Cold Water Extraction of Homogenized Sample						
I.S. Conc. ug/100ul	**3-MCPD Peak Area**	**3-MCPD-d_5 Peak Area**	**Peak Area Ratio**	**3-MCPD conc. in ppm (w/w)**	**Mean (n=4) 3-MCPD conc.**	**S.D. (n=4) 3-MCPD conc.**
10.425	610371	3171611	0.1924	4.21	5.01	0.69
10.425	695739	3227427	0.2156	4.72		
10.425	798882	3306073	0.2416	5.29		
10.425	851536	3212717	0.2651	5.80		
t-test						
0.435975745 >0.05						

5.8. Analysis of Paperboard Packaging Samples

Over 100 commercial paperboard packaging samples were analyzed by this refined method. In this section, several results are presented as examples.

5.8.1. EU Standard Cold Water Extraction of Samples from project # 5575

In this project, five paperboard samples were analyzed by GC-MS using EU Standard cold water extraction procedure. The data are provided in Table 15 and Table 16. The levels of 3-MCPD range from 3.62 ppm to 23.82 ppm. The levels of 1,3-DCP range from 0.47 ppm to 3.44 ppm. For the same paperboard sample, the level of 3-MCPD are generally higher than the levels of 1,3-DCP.

Table 15: The levels of 3-MCPD in paperboard samples from project #5575, tested by EU standard cold water extraction.

Sample no.	Sample Wt. in Grams	3-MCPD-d_5 Int. Std. Conc. ug/ 100ul	3-MCPD m/z 239 Peak Area	3-MCPD-d5 m/z 244 Peak Area	Peak Area Ratio 3-MCPD/I.S.	3-MCPD Conc. ppm w/w	Mean (n=2) 3-MCPD Conc. ppm w/w
Sample #1	0.9496	11.17	723	4601	0.1571	3.62	**3.62**
Sample #1	0.9496	11.17	792	5058	0.1566	3.61	
Sample #2	0.9882	11.17	415	2424	0.1712	3.79	**3.69**
Sample #2	0.9882	11.17	412	2546	0.1618	3.59	
Sample #3	1.0204	11.17	3649	2992	1.2196	26.17	**23.82**
Sample #3	1.0204	11.17	4011	4009	1.0005	21.47	
Sample #4	0.9720	11.17	4686	4842	0.9678	21.80	**20.30**
Sample #4	0.9720	11.17	5302	6355	0.8343	18.80	
Sample #5	0.9909	11.17	3538	5289	0.6689	14.78	**13.14**
Sample #5	0.9909	11.17	4053	7796	0.5199	11.49	

Table 16: The levels of 1,3-DCP in paperboard samples from project #5575, tested by EU standard cold water extraction.

Sample no.	Sample Wt. in Grams	3-MCPD-d_5 Int. Std. Conc. ug/ 100ul	1,3-DCP m/z 93+95 Peak Area	3-MCPD-d5 m/z 244 Peak Area	Peak Area Ratio 1,3-DCP/I.S.	1,3-DCP Conc. ppm w/w	Mean (n=2) 1,3-DCP Conc. ppm w/w
Sample #1	0.9496	11.17	966	4601	0.2100	0.44	**0.47**
Sample #1	0.9496	11.17	1186	5058	0.2345	0.49	
Sample #2	0.9882	11.17	755	2424	0.3115	0.63	**0.58**
Sample #2	0.9882	11.17	666	2546	0.2616	0.53	
Sample #3	1.0204	11.17	5619	2992	1.8780	3.67	**3.44**
Sample #3	1.0204	11.17	6575	4009	1.6401	3.21	
Sample #4	0.972	11.17	6079	4842	1.2555	2.58	**2.40**
Sample #4	0.972	11.17	6862	6355	1.0798	2.22	
Sample #5	0.9909	11.17	5283	5289	0.9989	2.01	**1.72**
Sample #5	0.9909	11.17	5552	7796	0.7122	1.43	

5.8.2. EU Standard Cold Water Extraction of Samples from project # 5750

In this project, eight paperboard samples were analyzed by GC-MS using EU Standard cold water extraction procedure. These paperboard samples were manufactured with second generation Kymene® wet-strength resin, which contains reduced amount of chloropropanols. The resulting 3-MCPD concentrations are provided at Table 17, and the levels of 3-MCPD are between none detected to 0.53ppm (with a detection limit of 0.01 ppm). 1,3-DCP was none detected in all of these paperboard samples (with a detection limit of 0.01 ppm). Comparing to the data of previous samples (project#5575), the concentrations of chloropropanols in these paperboards (project #5750) are significantly lower.

Table 17: The levels of 3-MCPD in paperboard samples from project #5750, tested by EU standard cold water extraction.

Sample no.	Sample Wt. in Grams	3-MCPD-d_5 Int. Std. Conc. ug/ 100ul	3-MCPD m/z 239 Peak Area	3-MCPD-d5 m/z 244 Peak Area	Peak Area Ratio 3-MCPD/I.S.	3-MCPD Conc. ppm w/w	Mean (n=2) 3-MCPD Conc. ppm w/w
Sample #1	1	10.425		2046447		n.d.	**n.d.**
Sample #1	1	10.425		2946937		n.d.	
Sample #2	1	10.425	32944	1213169	0.0272	0.27	**0.31**
Sample #2	1	10.425	50505	1441359	0.0350	0.35	
Sample #3	0.9962	10.425	268984	5182865	0.0519	0.51	**0.53**
Sample #3	0.9962	10.425	295213	5438131	0.0543	0.54	
Sample #4	0.9965	10.425	15418	862021	0.0179	0.18	**0.18**
Sample #4	0.9965	10.425	15382	796939	0.0193	0.19	
Sample #5	0.9967	10.425	39925	3552816	0.0112	0.11	**0.11**
Sample #5	0.9967	10.425	17414	1540360	0.0113	0.11	
Sample #6	1.0015	10.425	59619	5179800	0.0115	0.11	**0.10**
Sample #6	1.0015	10.425	44336	5402407	0.0082	0.08	
Sample #7	1.0006	10.425	14752	3602386	0.0041	0.04	**0.04**
Sample #7	1.0006	10.425	12474	2782857	0.0045	0.04	
Sample #8	1.0012	10.425	23504	4093715	0.0057	0.06	**0.06**
Sample #8	1.0012	10.425	17865	3069723	0.0058	0.06	

5.8.3. EU Standard Cold Water Extraction, Migration Test, and Unskived Edge Extraction of Samples from project # 5705

Four samples were analyzed using EU Standard cold water extraction, migration test, and unskived edge extraction. Table 18 provides the data of cold water extraction. Table 19 provides the data from unskived edge extraction. The results suggest that the migration of chloropropanols from the unskived edges in paperboard sample can be a source of chloropropanol contamination. Table 20 presents the data of migration test. The 1,3-DCP was none detected in this test. The paperboard samples with a slit result in higher

3-MCPD levels, indicating 3-MCPD migrated from the slit. The slit simulates the unskived edge of 5th panel in a carton. Therefore, the 5th panel of the beverage carton should be coated to prevent the migration of chloropropanols.

Table 18: The levels of chloropropanols in paperboard samples from project #5705, tested by EU standard cold water extraction.

Cold water extraction 1g of sample		
Sample no.	**Mean (n=3) 3-MCPD Conc. ppm (w/w)**	**Mean (n=3) 1,3-DCP Conc. ppm (w/w)**
Sample #1	3.14	0.13
Sample #2	5.15	0.10
Sample #3	4.14	0.12
Sample #4	4.08	0.12

Table 19: The data of unskived edge extraction of paperboard samples from project #5705

Unskived edge extraction 12.5 cm^2 of extracted surface (both side), 10 cm of edge		
Sample no.	**Mean (n=3) 3-MCPD Conc. ppm (w/w)**	**Mean (n=3) 1,3-DCP Conc. ppm (w/w)**
Sample #1	2.08	0.53
Sample #2	2.49	0.40
Sample #3	2.87	0.68
Sample #4	2.82	0.51

Table 20: The data of migration test of paperboard samples from project #5705

Migration test 51 cm^2 of extracted surface			
Sample no. and description	**Sample Wt. in Grams**	**Sample in cm^2**	**Mean (n=2) 3-MCPD Conc. ppm w/w**
Sample #1, Matte-Side, No Slit	1.5437	51	**0.03**
Sample #1, Matte-Side, w/ 12.2 mm Slit	1.5437	51	**0.10**
Sample #2, Matte-Side, No Slit	1.4083	51	**0.06**
Sample #2, Matte-Side, w/ 12.2 mm Slit	1.4083	51	**0.12**
Sample #3, Matte-Side, No Slit	2.0744	51	**0.03**
Sample #3, Matte-Side, w/ 12.2 mm Slit	2.0744	51	**0.07**
Sample #4, Matte-Side, No Slit	1.4648	51	**0.05**
Sample #4, Matte-Side, w/ 12.2 mm Slit	1.4648	51	**0.30**

5.8.4. EU Standard Cold Water Extraction, Unskived Edge extraction, and Migration Test of Samples from project # 5722

Three samples were analyzed by EU standard cold extraction, unskived edge extraction, and migration test. The results are concluded in Table 21, Table 22, and Table 23. From the results of cold water extraction, the levels of 3-MCPD in paperboard samples range from 0.41 ppm to 0.85 ppm; the levels of 1,3-DCP in paperboard samples range from 0.04 to 0.06 ppm. The results of unskived edge extraction and migration test suggest the migration of chloropropanols from both unskived edge and paperboard surface into water.

Table 21: The levels of chloropropanols in paperboard samples from project #5722, tested by EU standard cold water extraction.

Cold water extraction **1g of sample**		
Sample no.	**Mean (n=2) 3-MCPD Conc. ppm (w/w)**	**Mean (n=2) 1,3-DCP Conc. ppm (w/w)**
Sample #1	0.85	0.11
Sample #2	0.42	0.04
Sample #3	0.41	0.04

Table 22: The data of unskived edge extraction of paperboard samples from project #5722

Unskived edge extraction **12.5 cm2 of sample, 10 cm of Edge**		
Sample no.	**Mean (n=3) 3-MCPD Conc. ppm (w/w)**	**Mean (n=3) 1,3-DCP Conc. ppm (w/w)**
Sample #1	0.07	0.008
Sample #2	0.08	0.010
Sample #3	0.08	0.013

Table 23: The data of migration test of paperboard samples from project #5722

Migration test **51 cm2 of extracted surface**		
Sample no.	**Mean (n=3) 3-MCPD Conc. ppm (w/w)**	**Mean (n=3) 1,3-DCP Conc. ppm (w/w)**
Sample #1	0.04	n.d.
Sample #2	0.02	n.d.
Sample #3	0.08	n.d.

5.8.5. EU Standard Cold Water Extraction of Pulp Samples

Besides paperboard samples, the method were also used to test recycled pulp samples. Since recycled pulp is manufactured from recycled paper or paperboard, it is necessary to exam its safety for use. For example, its volatile and semi-volatile profile, and optical brightener additive content are needed to be analyzed. Chloropropanols could be one of the contaminants present in the recycled pulp. Two recycled pulp samples were tested by EU standard cold water extraction. The sample preparation method was the same as paperboard samples: 1g of pulp samples were cut into small pieces and extracted with 10 ml water for 24 hours. The results are shown in Table 24. Both 3-MCPD and 1,3-DCP were none detected in these two pulp samples (with a detection limit of 0.01 ppm).

Table 24: The levels of chloropropanols in pulp samples, tested by EU standard cold water extraction

Cold water extraction 1g of sample		
Sample no.	**Mean (n=2) 3-MCPD Conc. ppm (w/w)**	**Mean (n=2) 1,3-DCP Conc. ppm (w/w)**
Sample #1	n.d.	n.d.
Sample #2	n.d.	n.d.

In summary of the analysis of paperboard samples, the quantity in material (QM) of chloropropanols in paperboard samples was determined by EU standard cold water

extraction. The levels of 3-MCPD are generally at 0.1~100 ppm, while the levels of 1,3-DCP are usually below 1 ppm or even none detected (with a detection limit of 0.01 ppm). Paperboard manufactured with second generation Kymene® wet-strength resin contain lower amount of 3-MCPD (below 1ppm), and no 1,3-DCP was detected. The migration test and unskived edge extraction were used to determine the migration of chloropropanols from the food contact surface or from the unskived edge. The resulting level of chloropropanols is lower than the quantity in material. However, migration is the major source of chloropropanol contamination from paperboard sample. The data also suggest that the unskived edge of 5^{th} panel in a beverage carton can be a source of chloropropanol contamination.

6. CONCLUSION

In conclusion, an improved analytical method for determination of chloropropanols in paperboard packaging was developed and validated. This new method fulfills the expectations described in the objective section. First, it was specifically designed for paperboard sample. Second, it utilizes a better derivatization method than the one used in AOAC method. Third, the method uses 10 times less sample size, solvents, and reagents than previously described method, lowing the cost and time for analysis. Last but not least, the method is capable of quantifying 3-MCPD and 1,3-DCP at the μg/kg range to meet the requirement of the US and Europe regulation.

The overall validation data suggest the method is precise and rugged. The LOD of aqueous extract is 0.01 mg/kg for both 3-MCPD and 1,3-DCP. When the SIM mode of GC-MS is used, the detection limit can be ten times lower. Linear calibration (R squared > 0.99) can be obtained in the dynamic range of 3-MCPD or 1,3-DCP. The analytical system precision, method precision, and between batch precision were below 10% RSD with an exception of 16% RSD of method precision for 3-MCPD. A precision data below 10% RSD can be considered as a great precision. However, 16% RSD is still a good precision for analysis of a real sample.

Various sample preparation methods for paperboard packaging samples were used in this study. EU standard cold water extraction was used to determine the total quantity in material (QM). Migration test and unskived edge extraction were used to determine the

migration of chloropropanols from paperboard surface or unskived edge. The data from analysis of commercial paperboard samples show the levels of 3-MCPD in paperboard samples are generally 0.1~100 ppm, and the levels of 1,3-DCP are usually below 1 ppm or even none detected. The paperboards manufactured with second generation Kymene® wet-strength resin contain lower amount of chloropropanols. The migration of chloropropanols from packaging to aqueous solution is a source of contamination, especially the migration of chloropropanols from the unskived edge.

The result of water extraction of homogenized sample suggests that EU Standard cold water extraction can provide the extraction efficiency similar to the homogenized sample. Therefore, EU standard cold water extraction method is ideal for determination the levels of chloropropanols in paperboard sample.

GC-FID conditions and calibrations for this method were also established. However, the low sensitivity, bad resolution, and co-elution problem prevent the use of GC-FID method to analyze chloropropanols. GC-MS method is still preferable for analysis of chloropropanols.

All in all, this study reports a refined method to analyze chloropropanols in paperboard samples. The method has been applied to the analysis of over 100 commercial paperboard packaging samples. The data is being used to guide the development of next generation wet strength resins with reduced chloropropanols content, and also to ensure the levels of chloropropanols in products are below the regulatory limit. The method is also useful for risk assessments to calculate VSD (virtual safe dose) of 3-MCPD and

1,3-DCP. VSD is determined for carcinogens which are not assumed to have a threshold, and is corresponding to a one-in-a-million risk of cancer, assuming a lifetime exposure at the level. If the level of a carcinogen in the products is below the VSD, there is no need to put the warning label of California Proposition 65 in the products.

California Proposition 65 creates big challenges among the food industry. For example, in January 2012, the state of California added the compound 4-methylimidazole, also known as 4-MI or 4-MEI, to its list of known carcinogens. 4-MI forms the caramel coloring in soda. In order to avoid the cancer warning label in their cans and bottles, Pepsi and Coca-Cola have asked their ingredient suppliers to change their manufacturing process to produce caramel colorings with lower level of 4-MI. Chloropropanols in paper type food packaging is at similar position as 4-MI. This study provides a method to determine the levels of chloropropanols in paper type food packaging, and further guide the food packaging manufacturers to produce paperboards with lower levels of chloropropanols. Consequently, the manufacturers can produce the products with the levels of chloropropanols below the VSD, and avoid the cancer warning label in their products.

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