DEVELOPMENT AND VALIDATION OF A PURGE & TRAP- THERMAL DESORPTION- GAS CHROMATOGRAPHY- MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF PROPYLENE CHLOROHYDRINS (PCH) in HYDROXYPROPYL STARCH AND/OR PROPYLENE OXIDE FUMIGATED FOOD PRODUCTS

by

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ABSTRACT OF THE THESIS

DEVELOPMENT AND VALIDATION OF A PURGE & TRAP- THERMAL DESORPTION- GAS CHROMATOGRAPHY- MASS SPECTROMETRY (P&T-TD-GC-MS) METHOD FOR THE DETERMINATION OF PROPYLENE CHLOROHYDRINS (PCH) IN HYDROXYPROPYL STARCH AND PROPYLENE OXIDE- FUMIGATED FOOD PRODUCTS

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Propylene oxide has been widely used in food industry to modify food functionality and to sterilize food products. Examples include hydroxypropylation of starch and cellulose to modify cold water solubility and sterilization of nutmeat like almonds. In this process, some undesirable side chemicals such as PCH are produced. PCH are mutagens and potential carcinogens. The level of PCH permissible in modified starch is regulated internationally. Food Chemical Codex, Joint FAO/WHO Expert Committee on Food Additives (JECFA) and European Commission on food additives have set the limit of PCH in hydroxypropyl starch, which is less than 1mg/kg. However, because few toxicology guideline studies are available on PCH, the hazardous effects have not been fully understood and the allowable residue content of PCH in many food

products has not been specified by regulations.

Recent independent studies conducted in the US and Europe have suggested that the certified international method for PCH quantification in modified starch may underestimate the amount of residual PCH. The objective of this research is to develop an alternate method for PCH quantification and to independently evaluate the results of a newly proposed solvent extraction GC-MS analytical method (AVEBE unpublished study).

The P&T-TD-GC-MS method includes four steps: solvent extraction with water or MEOH, Purge & Trap concentration, thermal desorption and GC-MS analysis. It incorporates 3-chloro-1-propanol (a PCH structural isomer) as a matrix-spiked internal standard. The method is approximately 100 times more sensitive than the proposed European solvent extraction-GC-MS method, allowing for full-scan mass confirmation in contrast to the European method that uses selected ion monitoring. The P&T-TD-GC-MS method also eliminated the requirement of a highly specific GC injector (Programmed Thermal Volatilizer or PTV) which is mandated by the European method and not commonly available to most labs.

With this P&T-TD-GC-MS method, the LOD of both PCH1 and PCH2 is 0.025mg/kg and their LOQ is 0.1 mg/kg. A series of method validation tests, including precision, system suitability and recovery were conducted to determine the accuracy and sensitivity of the P&T-TD-GC-MS method in this study.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLISTRATIONS

List of Abbreviations

CA	California
CFR	Code of Federal Regulation
CNS	Central Nervous System
DS	Degree of Substitution
DP	Degree of Polymerization
EPA	Environmental Protection Agency
EU	European
FDA	Food and Drug Administration
GLPs	Good Laboratory Practices
I.S.	Internal Standard
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	Limit of Detection
LOQ	Limit of Quantification
MA	Massachusetts
MEOH	Methanol
MN	Minnesota
NJDHSS	New Jersey Department of Health and Senior Service
NTP	National Toxicology Program
OSHA	Occupational Safety and Health Administration
PA	Pennsylvania
PBH	Propylene Bromohydrins
PCH	Propylene Chlorohydrins
PCH1	1-chloro-2-propanol
PCH2	2-chloro-1-propanol
PCH3	3-chloro-1-propanol
P&T-TD-GC-MS	Purge& Trap- Thermal Desorption- Gas Chromatography-Mass Spectrometry
PPO	Propylene oxide
PTV	Programed Thermal Volatilizer
RSD	Relative Standard Deviation
SARA	Superfund Amendments and Reauthorization Act
SE	Standard Error
SD	Standard Deviation
USDOD	United States Department of Defense
USDOE	United States Department of Energy
VOCs	Volatile Organic Compounds

1. Introduction

Hydroxypropyl starch is produced by modifying native starch with propylene oxide in an aqueous environment. Some desirable functionalities are achieved with this modification, including decreased starch gelatinization temperature, increased starch granule swelling and dispersion rate, increased clarity and cohesiveness of dispersions, increased starch paste low-temperature stability and increased solubility and flexibility of starch films (Rutenberg and Solarek 1984). Because of these properties, hydroxypropyl starch has been applied in formulating many food products, like frozen pudding, frozen pie filling and surimi food. However, in starch hydroxypropylation process, propylene chlorohydrins are formed as undesirable by-products. PCH pose a potential negative health effect when consumed, and their content in modified starch has been put under strict regulation worldwide.

Fumigation is an important way to sterilize food products. Propylene Oxide is a main fumigant used for bacteria, mold, yeast and insect reduction in nutmeats (except peanuts) and cocoa powder authorized by FDA/EPA (Griffith and Warren 2001; Isikber et al. 2006). When fumigating with propylene oxide, PCH tend to form by the reaction among propylene oxide, inorganic chloride and moisture in the food products.

PCH exist in two isomers, 1-chloro-2-propanol and 2-chloro-1-propanol, which are formed as by-products in starch hydroxypropylation or propylene oxide- fumigation process. PCH are known as mutagens and potential carcinogens (NJDHSS 2004). Currently, few guideline studies are available on PCH, which include acute, sub chronic,

developmental, reproduction or chronic toxicity studies (EPA 2006). A search in the open literature provides some information concerning short term inhalation, digestion, eye and skin exposure hazards caused by PCH exposure. Based on the in depth review conducted by a subcommittee of the National Academy of Science and a committee on nutrition from American Academy of Pediatrics, it is inadvisable to use hydroxypropyl starches in foods intended for infants and young children (National Academy of Science 1978; Chairman 1971).

The current certified international method for PCH detection was developed by JECFA. This method, however, may underestimate PCH content in hydoxypropyl starch according to recent unpublished AVEBE's study. Currently, a European proposed solvent extraction GC-MS method, which aims at achieving a higher accuracy and sensitivity are under development and evaluation.

Because of a lack of appropriate analytical method for PCH quantification and to independently evaluate the proposed European methanol extraction GC-MS method, a P&T-TD-GC-MS method for PCH quantification in hydroxypropyl starch has been developed and validated in this study. In this study, the calibration curves for PCH were built; precision and recovery were tested; limit of detection (LOD), limit of quantification (LOQ) and system suitability (chromatographic resolution, peak tailing) were determined to make sure results obtained by the P&T-TD-GC-MS method are close to the true values in future routine tests.

2. Literature Review

2.1 Hydroxypropyl Starch Application

Hydroxypropyl starch is modified by the etherification of native starch with propylene oxide. Under normal conditions at ambient temperature, waxy starch pastes show little tendency to gel or retrograde. However, under refrigerated prolonged storage condition, syneresis tends to occur in waxy paste, thus the quality and shelf life of food products which are formulated with native starch will be reduced (Kruger et al. 1967). In order to solve this problem, hydroxypropyl groups are introduced into native starch by modifying the starch with PPO. The introduction of hydroxypropyl groups can disrupt inter- and intra- molecular hydrogen bonds, so that starch granular structure is weakened and the motional freedom of starch chains in amorphous regions is increased (Kavlani Neelam et al. 2012). Because of the modification, hydroxypropyl starch exhibit many desirable functional properties, like freeze-thaw stability, cold water solubility, and increased thickening property, which allows it to be widely used in many food applications.

Because of the improved freeze-thaw stability, hydroxypropyl starches have been applied in making surimi food, microwave reconstitution and frozen pie filling (Hunt et al. 2009; Glyn O. Phillps 2000). Also, hydroxypropyl tapioca starch has been successfully applied in making frozen pudding (D'Ercole 1972).

Because of the increased thickening properties, hydroxypropyl starch has been used in conjunction with other thickeners in food formulation to achieve desirable

thickening performance, for example with carrageenan in milk system and with xanthan gum in salad dressings (Rutenberg and Solarek 1984). Hydroxypropyl starches with a degree of substitution (DS) of 0.05-0.10 have been applied in gravies, sauces, fruit pie fillings and puddings as thickening agent (Hjermstad 1984).

In addition, hydrolysates of hydroxypropyl starches containing more than 15 weight percentage of polymers of 2-6 DP (degree of polymerization), prepared by enzymatic or acid hydrolysis, are suitable as a low-calorie sweetener component in bakery products (Quarles and Alexander 1992).

Also, hydroxypropyl starch with a natural amylose content of more than 50 weight percent can be used to produce an edible, water-soluble, protective film coating for foods (Mitan 1969). And hydroxypropyl starch has also been applied in making biodegradable plastic materials.

Because the hydroxypropyl starch has a confirmed function in preventing diabetes, inhibiting blood sugar elevation and preventing other diseases that are caused by elevated blood sugar, it has been expected to be used in formulating food products medicine and pet food (Shimotoyodome et al. 2004).

2.2 The Formation of Propylene Chlorohydrins in Food Products

2.2.1 Starch Hydroxypropylation Process

In industrial practice, hydroxypropyl starch is usually produced by esterifying native starch with PPO in aqueous slurries of starch granules under alkaline condition with temperatures up to 50℃. Because PPO-air mixture is explosive, during the reaction

a blanket of nitrogen is recommended in a closed pressure vessel. The ideal pH for the hydroxypropylation reaction is 11.5, and sodium hydroxide is commonly used to provide the desirable pH.

Sodium sulfate is added to starch suspension prior to the PH adjustment to repress starch granules from swelling and to protect their integrity (Tsuzuki 1968). Along with the increase of hydroxypropylation degree, more sodium sulfate will be required, since highly hydroxypropylated starch will swell and become difficult to purify when salt is washed out. After a desired degree of hydroxypropylation is reached, the starch slurry is neutralized and dried. Figure 1 shows the mechanism of starch hydroxypropylation.

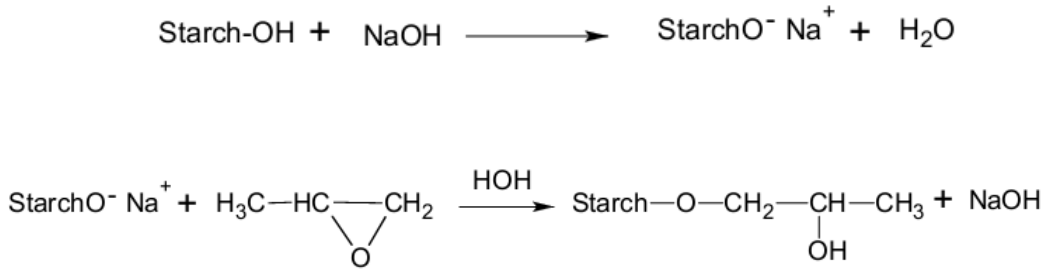

Figure 1: Mechanism of Starch Hydroxypropylation

The highly strained three-membered epoxide ring of propylene oxide is responsible for its reactive nature. The reaction kinetics (SN_2 type) is second order and dependent on concentration of both reactants.

Propylene oxide substitution occurs primarily at the HO-2-Hydroxyl group in the starch anhydroglucose unit (Leegwater et al. 1973; Chlebicki and J. 1975). The above phenomenon is possibly because of the high relative acidity of the HO-2 group due to its proximity to the anomeric center or the reactivity of HO-6 may be more dependent on

alkali concentration (Wurzburg et al. 1986). In addition, the NMR data indicate that the hydroxypropyl groups are distributed with a ratio of 7:2:1 on the 2-O, 3-O, and 6-O positions (Xu and Seib 1997).

A particularly desirable level of hydroxypropyl substitution ranges from 10 to 20%. Usually the DS should not exceed 40%. Since the etherification reaction efficiency may vary, depending on particular reaction conditions, it is necessary to employ an excess amount (5% - 40%) of PPO to achieve the desired level of substitution (Kesler et al. 1970). The excess PPO can react with inorganic chloride in starch suspension and produce PCH as undesirable byproducts.

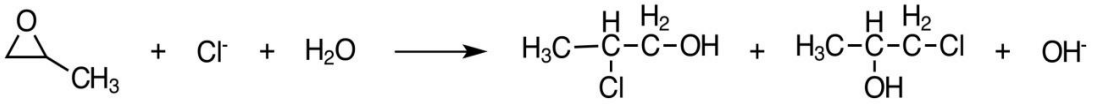

Figure 2: Mechanism of PCH Production in the Process of Starch Hydroxypropylation

2.2.2 Food Fumigation

Fumigation is an important way to sterilize food products. In this process, gaseous pesticides are usually used as fumigants to control insects, mites, nematodes, wireworms, rodents and to a lesser extent bacteria, molds and yeasts in stored food products. Since 1958, PPO is a major fumigant authorized by FDA/EPA for controlling bacteria, mold, yeast and insect disinfestations in nutmeats (except peanuts) and cocoa powder (Griffith and Warren 2001; Isikber et al. 2006). One advantage of using PPO for sterilization is that it gives no after flavor on treated food materials. (Wesley et al. 1965). It has also

been used to sterilize spices, canned soup ingredients and dehydrated mashed potato granules (Bruch and Koesterer 1961; Steele and Hadziyev 1976; Griffith and Warren 2001).

PPO used for food fumigation is regulated by CFR 40 part 185.15. It establishes a residue PPO tolerance of 300ppm for nutmeats, cocoa powder and spices. PCH are formed during PPO sterilization process, even in nominally dry materials. During fumigation with PPO, the hydrogen from moisture and the trace amount of chorine that naturally occurred in the materials is sufficient for PCH formation (Wesley et al. 1965).

PCH is considered to be the residue of concern for dietary risk assessment and tolerance reassessment purposes because residues persist at high levels and are likely to be present in treated commodities at the time of consumption. The residual PCH amount in canned soup has been reported to be 6.8 ppm when the ingredients were sterilized with gaseous PPO (Wesley et al. 1965). The formation of chlorohydrins has been demonstrated in flour and pepper after fumigating with ethylene and propylene oxide (Ragelis et al. 1966). GC, infrared and NMR were used in this study to confirm the presence and identity of ECH and PCH. PCH1 was identified in PPO fumigated wheat flour at the concentration of 10 ppm, and PCH2 at about 2 ppm. In the following study, PCH1 was found in six PPO treated food products, including walnut meat, tapioca starch, flour, cocoa, glazed cherries, glazed citron, and the level was between 4-47 ppm (Ragelis et al. 1968). The “cold sterilization” process with PPO for dehydrated potato granules would result in 12 ppm PCH with 94% being PCH1 residue (Steele and Hadziyev 1976).

An estimation of PCH1 intake from food has been determined (Rosenkranz et al. 1975; Carr and Rosenkranz 1978). These studies reported a consumption of 1lb food product fumigated with PPO would result in an intake of as much as 21mg PCH1.

PBH (propylene bromohydrins) are also formed in PPO sterilization process. However, PBH residues are minimal relative to PCH and PPO residues in food commodities at the time of consumption; therefore, PBH is not regard as a residue of concern by EPA.

2.3 Health Risks of PCH

The toxicity database for PCH is inadequate. There are few guideline studies, including acute, subchronic, developmental, reproduction or chronic toxicity studies regarding PCH (EPA 2006). Although a developmental toxicity study and a few sub-chronic studies conducted on rats can be found in open literature, these studies lack sufficient study details or have deficiencies by EPA evaluation: poor stability of the test compound, studies conducted before Good laboratory Practices (GLPs) were established, thus they provide little useful information regarding the toxicity of PCH. However, despite the scanty nature of the available literature on PCH toxicity, the following section will present toxicity information on PCH compounds that are available from previous literatures.

2.3.1 Acute Toxicity

Some acute toxicity studies were conducted on animal subjects and summarized in Table 1 (Yang 1987).

Table 1: Acute Toxicity of PCH

Subject		PCH1	PCH2
rats	Single-dose oral, LD_{50}	0.1-0.3 g/kg	0.24 g/kg
	Inhalation with saturated vapor. Max exposure with no death	-	15min
guinea pig	Single-dose oral, LD_{50}	-	0.72 g/kg
rabbit	Single skin application, LD_{50}	-	[1]1
	Primary skin irritation	~ 0.5g/kg	0.53g/kg
	Corneal injury	Yes, marked	[2]8

[1]Grade of 1-10; 1= no reaction from undiluted material; 10= most toxic (Smyth et al. 1962)
[2]Scale of 1-10; 1= no observed reaction; 10= most severe (Smyth et al. 1962)

A search in the open literature provides some information concerning short term inhalation, digestive, eye and skin exposure hazards of PCH in human. Short term inhalation or ingestion of PCH1 can lead to vomiting, digestive disorders, headache, symptoms of drunkenness, kidney and liver damage. Short term as well as long term skin or eye exposure toy PCH1 can cause irritation. The ingestion of PCH2 can cause gastrointestinal irritation with nausea, vomiting and diarrhea, and ingestion of large amount may cause CNS depression. Eye exposure to PCH2 may cause irritation, chemical conjunctivitis and corneal damage. Skin exposure may cause irritation, dermatitis and cyanosis of the extremities.

2.3.2 Chronic and Subchronic Toxicity

Two unpublished studies concerning the chronic and sub-chronic toxicity were presented in a report by FAO in 1974 (Yang 1987). The two studies were, respectively, conducted in a 25 weeks and 22 weeks period with rats (strains unspecified) as subjects.

A deficiency of the studies was caused by the high volatility of PCH compounds. During the feeding process, there were significant loss of PCH by stirring and the feed being left for a prolonged time in open air. Thus, the data from these studies were of little guidance in understanding the chronic and subchronic toxicity of PCH.

2.3.3 Mutagenicity

PCH1 had mutagenic effect on TA 1535 and TA 100 strains of *Salmonella typhimurium* (Carr and Rosenkranz 1978; Pfeiffer and Dunkelberg 1980). It also showed genotoxicity in *E. coli* Pol A1- test (Hyman et al. 1980).

A mixture of PCH isomers (PCH1: PCH2 = 75: 25) was used to test mutagenicity. The mixture showed mutagenicity to Salmoella strain TA 1530 but not to strain TA 1538 (Rosenkranz et al. 1975). In the study conducted by Pfeiffer and Dunkelberg, a mixture of unknown composition of PCH isomers was used on Salmonella and was found to have a mutagenicity effect to strain TA 1535 and TA 100, but not to strain TA 98 and TA 1537 (Pfeiffer and Dunkelberg 1980). A dose dependent mutagenic effect of PCH mixture (PCH1: PCH2 = 72: 25) to Salmonella strain TA 1535 and strain TA 100 was further confirmed (Biles and Piper 1983). Because the mutagenicity effect of pure PCH2 was not available, it remains undetermined if the mutagenic effect of PCH mixture was caused solely by PCH1 or by both PCH1 and PCH2.

2.3.4 Carcinogenicity

The tumorigenicity effect of PCH1 on strain A mice was evaluated (Theiss et al. 1979) through a 24 week period of pulmonary adenoma induction with PCH1 but none

was found.

A direct exposure to PCH is suspected to be carcinogenic to human. Previous retrospective cohort studies have drawn different conclusions on the relationship between pancreatic, lymphopoietic cancer incidences with working in chlorohydrins production environment. In one study (Benson and Teta 1993), a significant trend was observed for lymphopoietic, haematopoietic cancer and its subcategory leukaemia. However, another study (Olsen et al. 1997) showed that workers who had direct exposure to ECH and PCH had not experienced a significant increased risk for pancreatic, lymphopoietic or haematopoietic cancer, however a prolonged 10 years of observation would be necessary to confirm the risk.

2.4 Regulatory Information

PCH are not listed under the Superfund Amendments and Reauthorization Act of 1986 Section 302/304, Section 313 (SARA), which is also known as Emergency Planning and Community Right-to-Know Act (EPCRA). PCH are not regulated as radionuclides reportable under Comprehensive Environmental Response, Compensation and Liberty Act of 1980 (CERCLA, 40 CFR 302.4), or have export requirement under Toxic Substances Control Act 12(b) (TSCA), or require an Occupational Safety and Health Administration (OSHA) process safety plan. However, PCH1 is listed on the EPA Toxic Substances Control Act (TSCA) inventory.

When it comes to state regulations, PCH1 appears on the hazardous substances lists of MA, NJ, PA, but are not regulated by MN or CA. The New Jersey Department of

Health and Senior Service (NJDHSS) listed both of PCH1 and PCH2 as mutagens and potential carcinogens. A limit of 1 ppm is recommended for PCH airborne exposure (NJDHSS 2004). PCH are not regulated under California Proposition 65.

A search of regulatory information of PCH in countries other than the US provides the following information. PCH is classified as class B3 (combustible) and D2-B (toxic) with Canadian WHMIS (Workplace Hazardous Materials Information System) classification. PCH1 is regulated by European Commission regulations as flammable, has danger of very serious irreversible effects, has possibility of forming flammable/explosive vapor-air mixture.

PCH content is regulated in modified starch and food fumigated with PPO. Residual PCH in modified starch is regulated in the Code of Federal Regulations with a upper limit of 5ppm as shown below in Table 2 (Code of Federal Regulations, Title 21 2013).

Table 2: Regulations for PCH Content in Food Starch — Modified in the United States

a): Food starch may be esterified by the treatment with one of the following	
Use	**Limitations**
Epichlorohydrin, not to exceed 0.1 percent, and propylene oxide, not to exceed 10 percent, added in combination or in any sequence	Residual propylene chlorohydrin not more than 5 parts per million in food starch-modified.
Epichlorohydrin, not to exceed 0.1 percent, followed by propylene oxide, not to exceed 25 percent	Do.
Propylene oxide, not to exceed 25 percent	Do.
b): Food starch may be esterified and etherified by treatment of the following	

Use	Limitations
Phosphorus oxychloride, not to exceed 0.1 percent, and propylene oxide, not to exceed 10 percent	Residual propylene chlorohydrin not more than 5 parts per million in food starch-modified.

c): Food starch may be modified by treatment of the following

Use	Limitations
Chlorine, as sodium hypochlorite, not to exceed 0.055 pound of chlorine per pound of dry starch; 0.45 percent of active oxygen obtained from hydrogen peroxide; and propylene oxide, not to exceed 25 percent	Residual propylene chlorohydrin not more than 5 parts per million in food starch-modified.

Food Chemical Codex, JECFA and European Commission on Food Additives have set the limit of PCH in hydroxypropyl starch and hydroxypropyl distarch phosphate, which is less than 1mg/kg (Food Chemical Codex 1996; Food Additives in Europe 2000; Status of Safety Assessment of Food Additives Presently Permitted in Europe 2002). The EEC Scientific Committee on Food additives is pushing the limit of PCH in hydroxypropyl distarch phosphate to 0.1 ppm (Otto.B.Wurzburg 1995). As one of the major manufacturers and exporters of modified tapioca starch, Thailand also has regulations concerning PCH content (<1ppm) in chemically modified starches approved for food applications (Breuninger et al. 2009). Table 3 shows the PCH tolerances in PPO treated food commodities (EPA 2011).

Table 3: Tolerance for Propylene Chlorohydrins in Food Fumigated with PPO

Tolerances Established Under 40 CFR 180.491	
Commodity	**Tolerance(ppm)**
Basil, dried leaves	6000
Cacao bean, dried bean	20.0
Cacao bean, cocoa powder	20.0
Figs	3.0

Garlic, dried	6000
Grape, raisin	4.0
Herbs/spices, group 19, dried, except basil	1500
Nut, pine	10.0
Nut, tree, group 14	10.0
Nutmeats, processed, except peanuts	10.0
Onion, dried	6000
Pistachio	10.0
Plum, prune, dried	2.0

Besides the above regulations concerning modified starch and PPO treated food commodities, hydroxypropyl starch is regulated when applied to infant food. On the basis of an in-depth review conducted by a subcommittee of the National Academy of Science and a committee on nutrition from American Academy of Pediatrics, modified food starches used in infant foods were generally approved with an exception of hydroxypropyl starch or hydroxypropyl distarch phosphate (Review of Safety and Suitability of Modified Food Starches in Infant Food 1978; Chairman 1971). In addition, Commission of the European Communities Scientific Committee for Food advises that propylene oxide-modified starches should not be used in foods for infant and young children (Otto.B.Wurzburg 1995).

2.5 Analytical Method

The analysis of PCH at trace concentrations in hydroxypropyl starch can be challenging. PCH are polar compounds with a low molecular weight of 94, which could contribute to its early elution in the chromatogram and cause trouble in distinguishing the target ions from background noise. PCH has a hydroxyl group, which tends to interact with the starch matrix through hydrogen bonding, posing a challenge for a quantitative

extraction.

There are few published methods on instrumental detection and quantification of PCH. Some methods shown below were previously used or are currently under development for PCH detection and quantification in solutions or food matrixes.

PCH was initially analyzed with a chemical method (Cannon 1950). PCH were hydrolyzed into propylene glycol with sodium bicarbonate. After neutralization, propylene glycol is oxidized with periodic acid to give formaldehyde and acetaldehyde. The wave height difference created by applying different voltage to aldehyde solutions enabled the quantification of PCH. Figure 3 shows the related chemical equations. The study was not conducted in a food matrix and was only applicable to the analyses of chlorohydrins in aqueous solutions or water soluble solvents. Additionally, the sensitivity of the method was unknown. So it has little significance pertaining to our study.

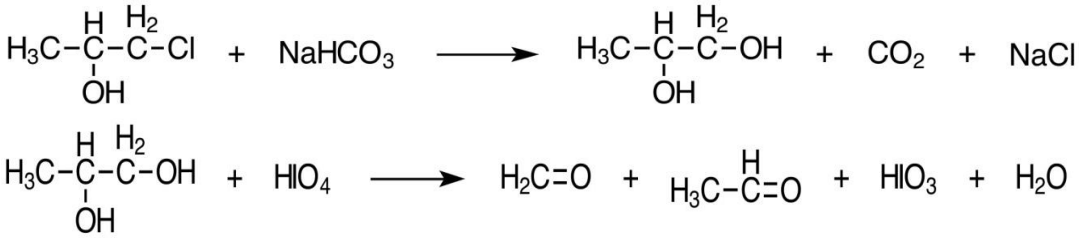

Figure 3: Chemical Equations on PCH Quantification Adopted from Cannon (1950)

A method for PCH quantification in hydroxypropyl starch was developed by JECFA, which has been recognized as the certified international method. In the method description, 50 g of hydroxypropyl starch sample is first hydrolyzed with sulfuric acid. After neutralization with sodium hydroxide, the acidified starch suspension is extracted

with diethyl ether and analyze by direct injection- GC-FID (Modified Starch 2001). This method has its limitation as it may underestimate the amount PCH in hydroxypropyl starch stated in a recent unpublished study conducted by AVEBE. Currently some other detection methods for PCH quantification in hydroxypropyl starch are under development by the starch industry to achieve better accuracy and sensitivity.

One analytical method (Ingredion, unpublished study) currently under development uses pentane sonication extraction-GC, equipped with a halogen specific detector, to quantify residual PCH in hydroxypropyl starch.

Another method, which was mentioned previously as the European proposed solvent extraction-GC-MS method, uses methanol (w/ 4% water) for starch extraction (AVEBE unpublished study). After stirring overnight, the supernatant is analyzed by direct injection- GC-MS. The method requires a programmed temperature vaporization (PTV) injector, which is not a common injector for most GC systems unless optionally equipped.

Other possible method may include chemical derivatization- GC- ECD (electron capture detector). PCH derivatized with a chosen derivatization agent (e.g. TMS) to form a silyl or tosyl derivatives can increase the molecular weight and its sensitivity to electron capture detector, also prevent the undesirable interactions between hydroxyl groups and other components in GC system. This technique has been used in previous study (Ragelis et al. 1968). However, a quantitative conversion to either silyl or tosyl derivatives failed because of PCH volatility, sensitivity to PH changes and slow reactivity of the compound

The derivatization agent and the reaction conditions were not provided.

Methods (chemical or GC procedures) has been applied in chlorohydrins (ECH & PCH) quantification in fumigated food products (Wesley et al. 1965). In the chemical procedures, the water slurry of a food sample was steam distilled. Under alkaline condition, the distillate was heated on steam to hydrolyze the chlorohydrin. Then acidified the solution with nitric acid and the chloride was determined by the Volhard method.

For samples which contain less than 10 ppm chlorohydrins, GC equipped with an FID detector was used to quantify chlorohydrin's concentration. However, in this study conducted by F. Wesley et al. (1965), no presence evidence of PCH was presented using GC-FID.

Anhydrous ether was used to extract PCH from PPO treated wheat flour samples, and the extracts were analyzed by GC-FID (Ragelis et al. 1966). This method was able to quantify PCH1 in flour sample at a level of 10 ppm, and detect PCH2's presence (around 2ppm).

In order to apply the method to other food categories covered under current regulations besides PPO treated flour, in a subsequent study (Ragelis et al. 1968), the extracts from three isolative methods, including ether extraction, sweep co-distillation and steam distillation, respectively, were analyzed with GC-FID in the efforts to quantifying PCH1 in PPO treated cocoa, nut meats, tapioca starch, flour, glazed cherries and glazed citron.

2.6 The Purge & Trap Sample Preparation Method

Purge and Trap has been used to extract VOCs from a solid or liquid matrix for introduction into a GC- MS for separation and identification. The VOCs are concentrated onto a desorption tube, followed by thermal desorption into a GC. Sample matrices can range from soil, plastics, foods, flavor, fragrance, emulsions and water. Tenax-TA is used as adsorbent in the trap in this study. Tenax-TA is the most widely used as an adsorbent compared with other porous polymer resins or various forms of activated graphitized carbons (Hartman et al. 1993). Figure 4 shows the internal design of an adsorption tube which is consisted of the packed glass lined tube (GLT) used in a Short Path Thermal Desorption Unit designed by Hartman et al.

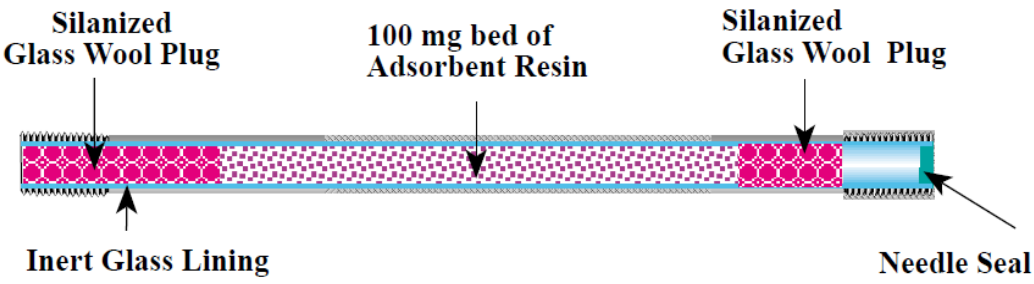

Figure 4: Cross Section of Packed GLT Tube (Figure Courtesy of Scientific Instrument Service, Inc., Ringoes, NJ.)

Purge & Trap system can be applied with sample in either liquid or solid form. In this study, a salt matrix is used to hold 200ul liquid extract in the Purge and Trap vessel which is designed for the isolation and concentration of volatile and semi-volatile compounds from solid sample matrices into a desorption tube (Figure 4). Figure 5 shows the design of a Purge and Trap vessel for solid sample matrices.

The thermal desorber is interfaced to a GC through its injection port. The purge gas is supplied directly to the desorption tube and is flow regulated. The thermal desorption device primarily performs the desorption function to let the trapped samples go into the GC for separation and analysis. Figure 6 shows theory of short path thermal desorption system operation.

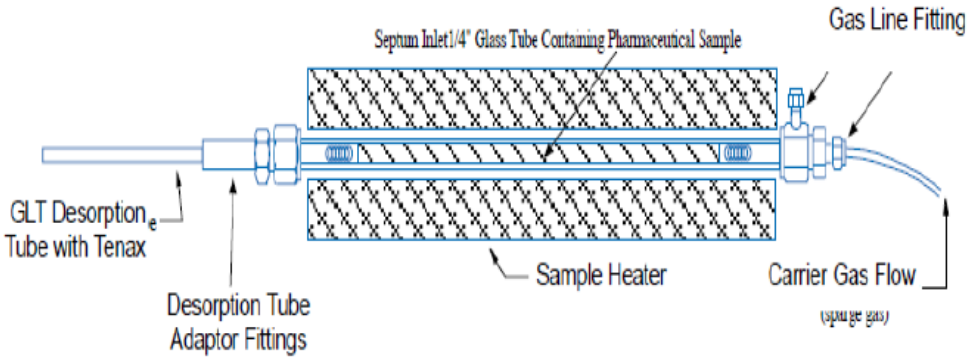

Figure 5: Purge and Trap Vessel Useful for the Isolation and Concentration of Volatile and Semi-volatile Flavor Components from Solid Sample Matrices. (Figure Courtesy of Scientific Instrument Service, Inc., Ringoes, NJ.)

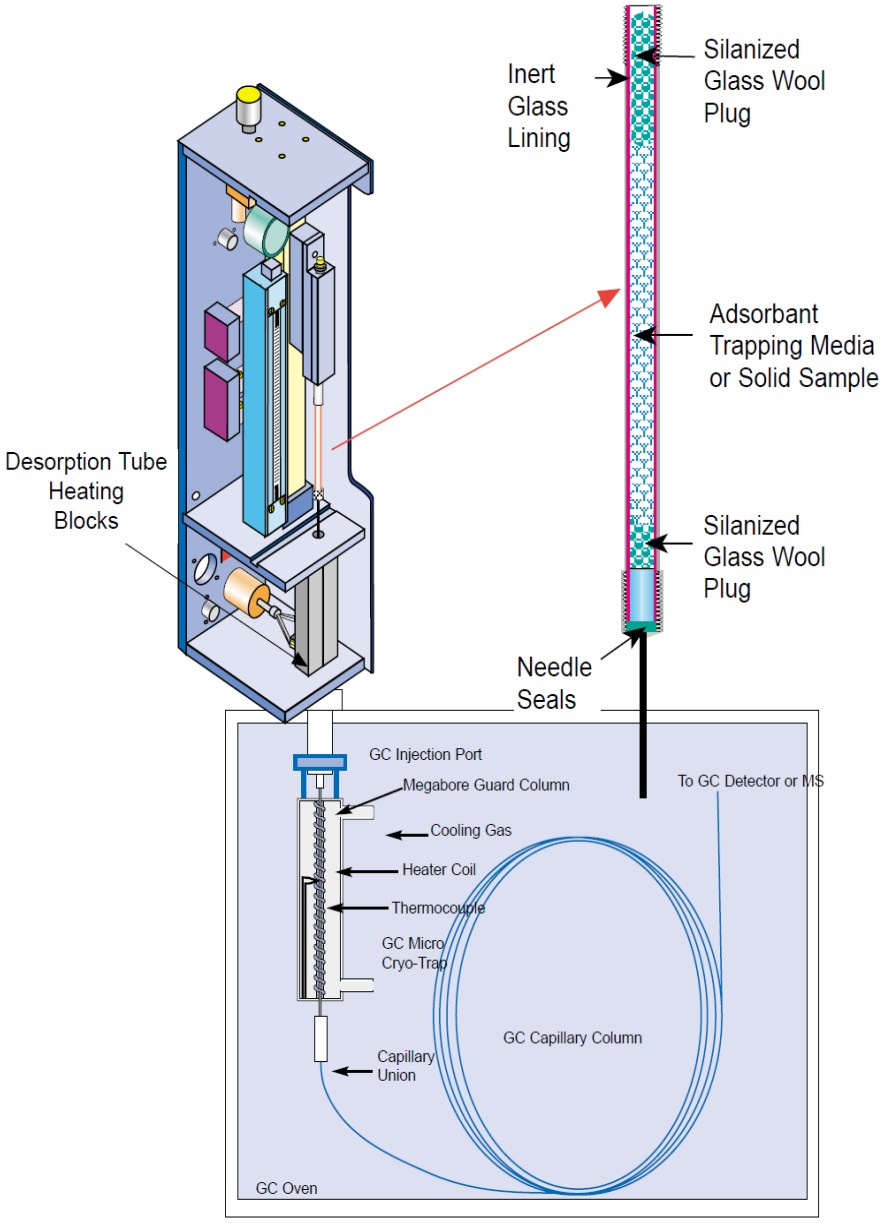

Figure 6: Short Path Thermal Analysis, Theory of Operation (Figure Courtesy of Scientific Instrument Service, Inc., Ringoes, NJ.)

3. Objectives

3.1 Objective

The overall objective of this study is to develop a P&T- TD- GC- MS method for the accurate and convenient quantification of PCH in hydroxypropyl starch.

Solvent with good extraction efficiency was compared and chosen (water or MEOH) in this study. After method development and validation, a ring test which involved 3 other independent labs was conducted to compare the P&T-TD-GC-MS method with the newly developed European solvent extraction GC-MS method. Also to broaden the method's application, test conditions and analysis parameters are modified to adopt the method to the quantification of PCH in fumigated almond sample.

The method should also meet the following requirements:

- Lowering the solvent use, cost and time for analysis
- Be able to quantify PCH at the mg/kg level to meet the residue requirement in US and Europe regulations.

3.2 Specific Tasks

- Develop a P&T-TD-GC-MS method for accurate quantification of PCH in hydroxyporpyl starch.
- Compare the extraction efficiency of water and MEOH.
- Conduct validation tests on the developed method.
- Compare the results of using different Purge & Trap absorption salt matrix (sodium chloride or anhydrous sodium sulfate) on PCH content.

- Compare this method with the European proposed MEOH extraction GC-MS method.
- Adapt the method to apply it to fumigated almond analysis.

4. Hypothesis

Water or methanol extraction of hydroxypropyl starch is expected to disrupt hydrogen bonding affinity between PCH and the starch matrix, resulting in quantitative extraction. The resulting extracts will contain dilute concentration of PCH. Attempts to concentrate the extracts via evaporation of solvent will result in evaporative loss of PCH. It is hypothesized that purge and trap concentration of PCH using Tenax adsorbent with subsequent analysis by thermal desorption-GC-MS will afford a sensitive, accurate and reproducible analytical method for determination of trace level PCH concentration in foods.

5. Experimental Design

5.1 Experimental Design Overview

In this study, the overall analytical procedures including sample preparation and instrumental analysis conditions were established, and validation tests were conducted.

Figure 7 shows the setup of overall analytical procedures. The analytical process includes 3 parts:

- Aqueous extraction

  Sample starch was extracted with 10ml MEOH or distilled water in 20ml test tube with Teflon lined closure. The extraction was spiked with 1ppm 3-chloro-1-propanol as the internal standard prior to the overnight extraction at 40℃ with agitation (adapted to room temperature in ring test section) overnight.

  Water and methanol were compared concerning PCH extraction efficiency in hydroxypropyl starch matrix. Both water and methanol have affinity toward polar compounds through intermolecular forces including hydrogen bonding, dipole-dipole and van der Waal's forces. Through the formation of hydrogen bonding between water or MEOH and PCH, the hydrogen bonding between PCH and the starch matrix will be disrupted, thus, promoting a better extraction of PCH from the hydroxypropyl starch matrix.

  In some PCH quantification methods, ether was used in PCH extraction. This is due to the fact that PCH have better solubility in ether compared with water or alcohol. However, using ether tends to extract copious amounts of background

materials along with chlorohydrins. Also diethyl ether has a characteristic fragment ion with m/z=45, which may lead to an overestimation of PCH1 quantification, which has the same characteristic fragment ion, when the selected ion m/z=45 is used for quantification.

In the aqueous extraction, 3-chloro-1-propanol was used as the internal standard. Internal standards normalize GC-MS data and compensate for potential drift in running and gain setting over time. Calibrations made with internal standard methodology are typically stable for extended periods. In previous studies (Ingredion and AVEBE unpublished studies), chlorobenzene was used as internal standard. By comparing the performance of chlorobenzene or 3-chloro-1-propanol as the internal standard in this study, chlorobenzene was ruled out due to poor chromatographic peak shape because of its non-polarity when analyzed with a polar Carbowax column. In contrast, 3-chloro-1-propanol was selected as an ideal internal standard. The reasons are mentioned in Section 6.1.

- Purge and Trap concentration

200ul extract was introduced by a syringe to the Purge & Trap absorption salt matrix in a glass tube (Figure 19). The glass tube was then placed in the Purge & Trap system. The PCH and I.S. in the extract was allowed to concentrate into an adsorption trap. The adsorption tube would then be thermally desorbed at 250℃ for 5 min.

Purge and Trap concentration of PCH could eliminate the evaporative loss of

PCH via the procedure of solvent evaporation and facilitate an accurate analysis of PCH.

For samples extracted with water, a dry purge step was required. This involved flushing the desorption tube with N_2 for a set time period prior to TD- GC-MS. Dry purge effectively remove moisture from the desorption tube and prevent the formation of ice during cryo- focusing on the GC column at -20℃.

- GC-MS analysis

In choosing an appropriate GC column for PCH separation, 3 kinds of column (Carbowax, DB-1, Equality^{TM}-1701) of different polarity, were compared, and Equality^{TM}-1701 was chosen because of its intermediate polarity which is suitable for alcohol separation. In addition, the column has a low temperature limit of minus 20 degree Celsius, which enables cryo-focusing of the thermally desorbed compounds and allows for a better separation of the mixture on the GC column.

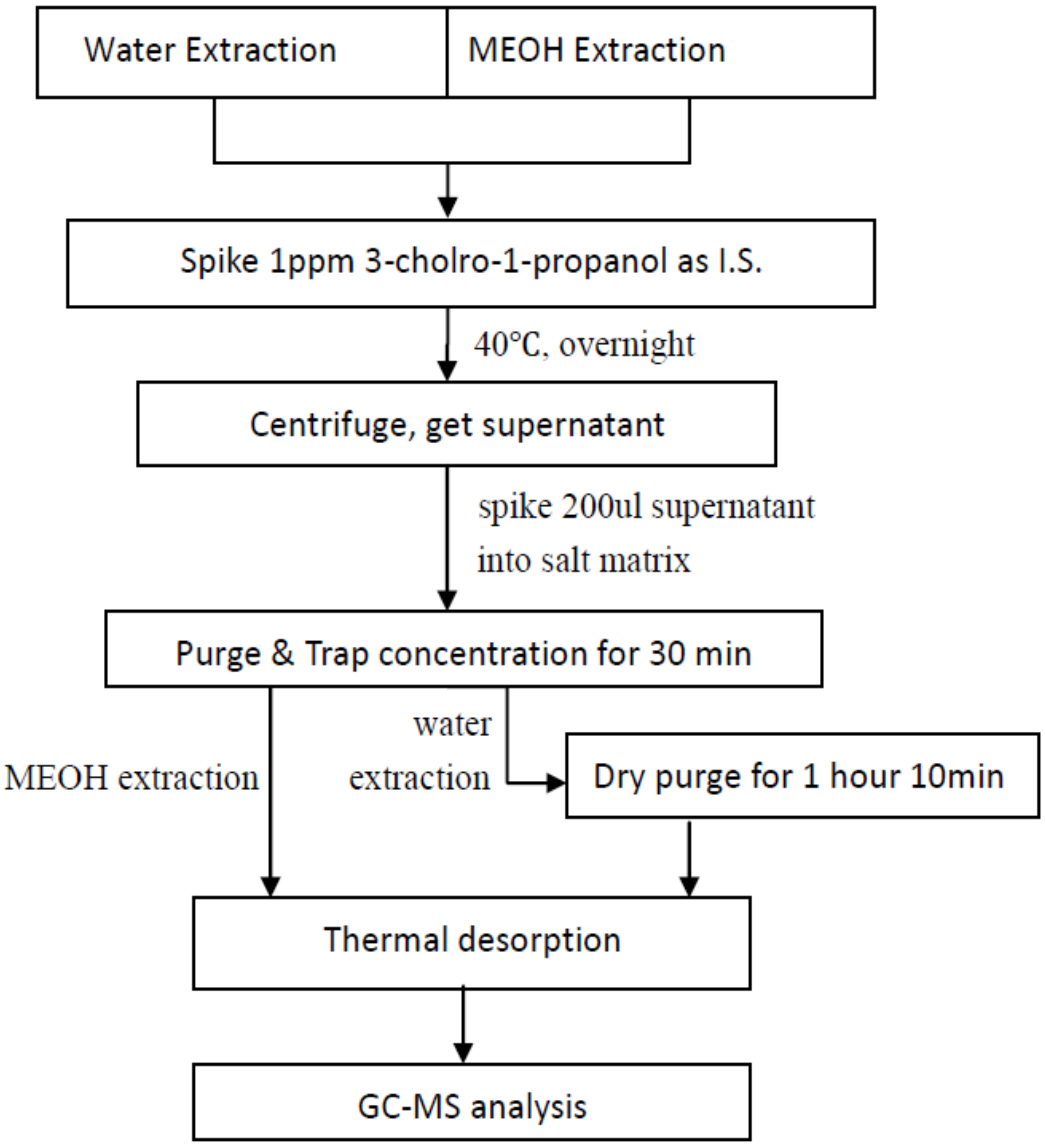

Figure 7: Flow Chart of the Analytical Procedures

5.2 Reagents and Materials

Chemicals and reagents were obtained as follows: 1-chloro-2-propanol (PCH1), purity 75.9%, with 23.4% 2-chloro-1-propanol (PCH2), from Sigma Aldrich, Saint Louis, MO; 2-chloro-1-propanol (PCH2), purity 96.6%, from Sigma Aldrich, Steinheim, Germany; 3-chloro-1-propanol (PCH3), used as internal standard, purity 99.4%, from Sigma Aldrich, Saint Louis, MO; sodium chloride and anhydrous sodium sulfate were purchased from Fisher Scientific, Fair Lawn, NJ.

Methanol (HPLC grade) was purchased from Fisher Scientific, Fair Lawn, NJ. Distilled water was prepared in house by Waters Milli-Q Nanopure^{TM} system.

5.3 Stock Standard Solutions

Stock Solution A (PCH3 in MEOH, 40ug/ml)

Weigh 10.06 mg PCH3 (corrected for purity 99.4%) in a 16ml glass vial with Teflon lined closure, then pipette 10ml MEOH into the glass vial and mix well. Store the 1mg/ml internal standard solution at room temperature. Solution stability is 6 days based the standard stability test. Pipette 8 ml of 1mg/ml internal standard solution into 200ml volumetric flask; fill the volumetric flask up to the 200ml volumetric line with MEOH to make the final concentration of PCH3 at 40ug/ml.

Stock Solution B (PCH1, 10mg/ml)

Weigh 131.75mg (corrected for purity 75.9%) PCH1 in a 16ml glass vial, then pipette 10ml MEOH into the vial and mix well. Store the solution at room temperature. Solution stability is 6 days based on the standard stability test.

Stock Solution C (PCH2, 10mg/ml)

Weigh 103.09mg (correct for purity 97%) PCH2 in a 16ml glass vial, then pipette 10ml MEOH into the vial and mix well. Store the solution at room temperature. Solution stability is 5 days based on the standard stability test.

Stock Solution D (PCH1, 1mg/ml)

Weigh 13.18mg (correct for purity 75.9%) PCH1 in a 16ml glass vial with Teflon closure, then pipette 10ml MEOH into the glass vial and mix well. Store the solution at room temperature. Solution stability is 6 days.

Stock Solution E (PCH2, 1mg/ml)

Weigh 10.35mg (correct for purity 96.6%) PCH2 in a 16ml glass vial, then pipette 10ml MEOH into the glass vial and mix well. Store the solution at room temperature. Solution stability is 5 days.

Stock Solution F (PCH1, 0.1mg/ml)

Pipette 1 ml stock solution B into 16ml glass vial, then pipette 9ml MEOH solvent into the glass vial and mix well. Store the solution at room temperature.

Stock Solution G (PCH2, 0.1mg/ml)

Pipette 1 ml stock solution C into 16ml glass vial, then pipette 9ml MEOH solvent into the glass vial and mix well. Store the solution at room temperature.

5.4 Preparation of PCH1 Calibration Standards

Prepare calibration solutions freshly on the morning of the day of analysis. Transfer 10ml 40ug/ml internal standard solution (A) into each 16ml glass vials. Then,

respectively transfer 400ul, 200ul, 100ul, 40ul, 20ul, 10ul, 4ul, 10ug/ul PCH1 solution (B) into the glass vials.

Table 4: PCH1 Calibration Curve Standards Preparation

PCH1 stock solution (mg/ml)	# of ul of PCH1 in 16ml glass vial	PCH1 concentration in ug/ml
B	400	400
B	200	200
B	100	100
B	40	40
B	20	20
B	10	10
B	4	4

5.5 Preparation of PCH2 External Calibration Curve Standards

Prepare calibration solutions freshly on the morning of the day of analysis. Transfer 10ml 40ng/ul internal standard solution (A) into each 16ml glass vials. Then, respectively transfer 400ul, 200ul, 100ul, 40ul, 20ul, 10ul, 4ul, 10ug/ul PCH2 solution (C) into the glass vials.

Table 5: PCH2 Calibration Curve Standards Preparation

PCH2 stock solution (mg/ml)	# of ul of PCH2 in 16ml glass vial	PCH2 concentration in ug/ml
C	400	400
C	200	200
C	100	100
C	40	40
C	20	20
C	10	10
C	4	4

5.6 Preparation of PCH in Matrix Calibration Curve Standards

Prepare calibration solutions freshly on the morning of the day of analysis. Weigh 2.1459g blank starch sample (moisture content=6.8%) into 20ml test tube and extract the sample with 10ml MEOH or water. Spike 50ul 40ug/ml I.S. into each test tube. Then, respectively transfer 2ul, 10ul (0.1ug/ul PCH1 solution); 2ul, 10ul, 20ul (1ug/ul PCH1 solution) into each test tube to make the final PCH1 concentration of 0.1 to 10 ppm, and 1.38ul, 6.92ul (0.1ug/ul PCH2 solution); 1.38ul, 6.92ul, 13.83ul (1ug/ul PCH2 solution) into 20ml test tube to make the final PCH2 concentration of 0.1 to 10 ppm.

Table 6: In Matrix Calibration Curve Standards Preparation

stock solution	# of ul of PCH1	stock solution	# of ul of PCH2	PCH1 conc. in ppm	PCH2 conc. in ppm
F	2	G	1.38	0.1	0.1
F	10	G	6.92	0.5	0.5
D	2	E	1.38	1	1
D	10	E	6.92	5	5
D	20	E	13.83	10	10

5.7 Starch Sample Extraction Preparation Procedure

Accurately weigh 2.0000 g starch (on dry weight basis, corrected for moisture content) into 20 ml test tube with Teflon lined closure. Label the test tube with sample ID information and preparation date. Pipette 10ml MEOH or water with 10.0ml pipette. Then spike 2.0ul of 1mg/ml PCH3 with 10.0ul syringe. This will deliver 2.0ug of internal standard to the extraction system. And the final concentration of PCH3 in each test tube will be 0.04ug/ml in MEOH or Water or 1 parts per million (ppm w/w) relative to the

starch sample based on a nominal 2.0 g weight on dry bases. Vortex test tube and extract overnight at 40 degree Celsius (at room temperature in the ring test).

5.8 Purge and Trap and Thermal Desorption Condition

The P&T-TD equipment used in this study was developed jointly by the Center for Advanced food Technology (CAFT), Rutgers University and Scientific Instrument Services (SIS) (Hartman et al. 1991). The PCH compounds are purged out from the absorption salt matrix and concentrated into the Tenax-TA adsorbent trap in the GLT tube in this process. Nitrogen of 99.999% purity is used as purge gas. This process allows the inert gas stream to strip the analytes from the salt matrix and concentrate them on an adsorbent trap. Purge & Trap is conducted for 30min at 100℃ with nitrogen gas flow rate of 50ml/min.

For samples prepared by water extraction, dry purge (purge gas: nitrogen of 99.999% purity; flow rate: 50ml/min) was for 1 hour and 10 minutes at room temperature to remove moisture in the adsorbent trap.

After Purge & Trap, the adsorption trap was connected to the short path thermal desorption unit. Prior to injected into the GC, the desorption tube was flushed with Helium for 10 seconds. Following 30 seconds of injection that allowed the pressure in the GC system to equilibrate, the desorption tube was heated to 250℃ and held for 5min of desorption (see Figure 6).

5.9 Gas Chromatography- Mass Spectrometry Analysis Conditions

GC-MS analysis was performed on a Varian 3400 GC interfaced with a Finnigan

MAT 8230 magnetic mass spectrometer. The GC is equipped with a capillary column Equity™ -1701 (60m ×0.32mm i.d. ×1.0um film thickness; Supelco, Bellefonte, PA). Helium was used as a carrier gas at 20psi. And the split ratio was at 10:1. The column was temperature programmed from -20℃ to 260℃ at a rate of 15℃ per minute. The temperature of GC-MS transfer line was set at 260℃. The mass spectrometer was operated in electron ionization mode (70eV). The ion source was at 250℃. The mass scan range was 35-150 (or 35-100 m/z in the ring test). The Finnigan MAT SS300 data system was used for operating. Data collection and data analysis was conducted using the Micromass Masslynx program.

5.10 Method Validation

Method validation is important to ensure that the results obtained from every test in a routine analysis is very close to the unknown true value (Ph. Hubert et al. 2003). A holistic approach of studying method validation has divided the method validation process into four parts shown below (Gustavo González and Ángeles Herrador 2007):

- Applicability, fitness for purpose, and acceptability limits: including introductive information about identity of analyte, the concentration range covered, the material used as test matrix, the corresponding protocol and the intended application (Thompson et al. 2002).

- Selectivity and specificity: the ability to measure the analytes in the presence of potential sample components accurately.

- Calibration study: including the analyses of linearity, dynamic range, LOD

and LOQ.

- Accuracy study: including bias/trueness, precision and robustness.

Accuracy can be assessed by the analyses of 2 parameters: bias/trueness and precision. Bias/ Trueness can be determined by conducting 2 procedures, including using an internal inspection material with an assigned value and conducting recovery test. In this study, PCH3 at the concentration of 1ppm (nominal to 2g of starch on dry weight basis) was used as the internal standard. The recovery tests were conducted with both water and MEOH extracts and are presented in the Section 6.9.

The first two parts has been described in previous sections and a sample chromatogram and mass spectra of the target compounds is given in the next section. Calibration data and other validation tests are presented in the Section 6.2.

6. Results & Discussion

6.1 Selected Ion Chromatogram and Mass Spectra

PCH were analyzed by GC-MS. Figure 8 shows the selected ion chromatogram of the standard with 10 ppm PCH1, 3 ppm PCH2 in 10 ml 40ng/ul PCH3 in methanol. The retention time is 10.41 min for PCH1, 10.84 min for PCH2 and 12.55 min for PCH3. Figure 9, 10, 11 respectively presents the mass spectra of PCH1, PCH2 and PCH3, from the same standard solution. The characteristic ions of each compound are shown in Table 7.

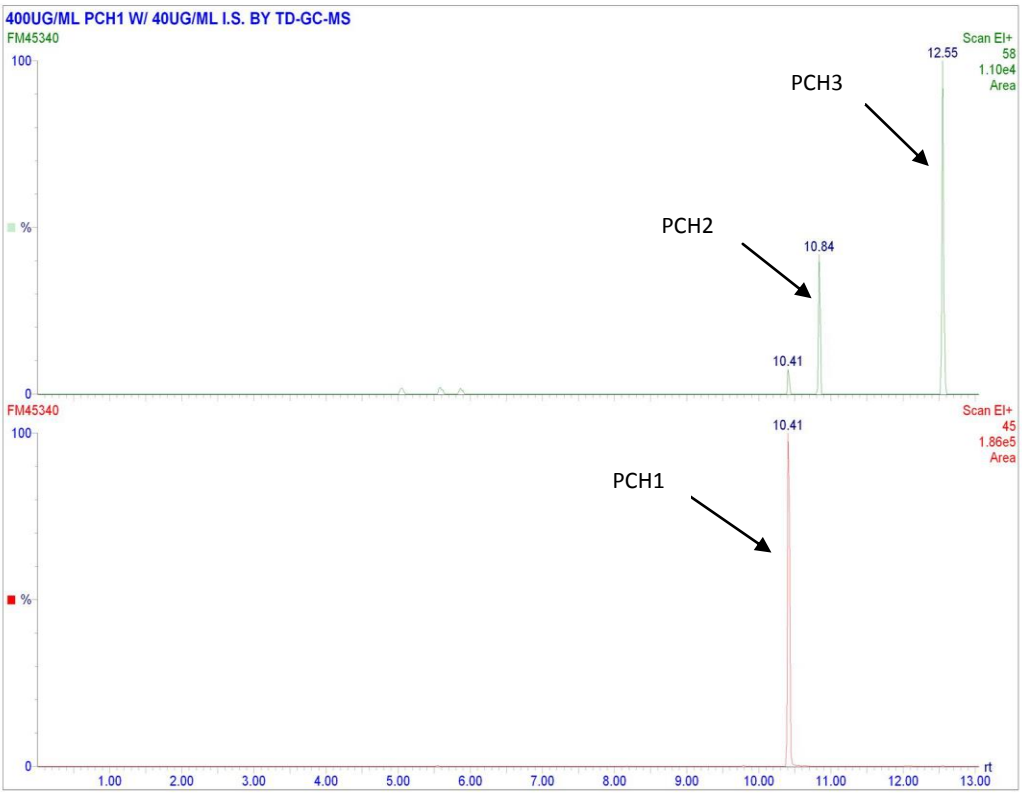

Figure 8: Selected Ion Chromatogram for PCH

Table 7: Characteristic Ion and Quantifier Ion for PCH

	PCH1	PCH2	PCH3
Characteristic ion (m/z)	45,79, 81	58, 63, 65	58,76,78
Quantifier ion (m/z)	45	58	58

PCH have a molecular weight of 94. In an ionization source, when a compound is impacted by electrons, it tends to fragment into charged ions of different masses. Take PCH1 for example, it shows peaks at m/z= 79, corresponding to loss of a CH_3- group. In Figure 9, in addition to the peak at m/z= 79, there is a small peak at m/z = 81 because of the presence of ^{37}Cl isotope in the molecule with a rough ratio of 3:1 (^{35}Cl: ^{37}Cl). In Figure 10 and 11, m/z = 58 corresponding to loss of a Cl^-. And m/z = 76 and 78 corresponding to a loss of a H_2O- group; m/z = 62 corresponding to a loss of CH_3- and a -OH group; m/z =63 corresponding to a loss of -CH_2OH group. Based on different constitutional structure of PCH compounds, the mass spectrum will show different pattern as can be seen in Figures 9, 10 and 11.

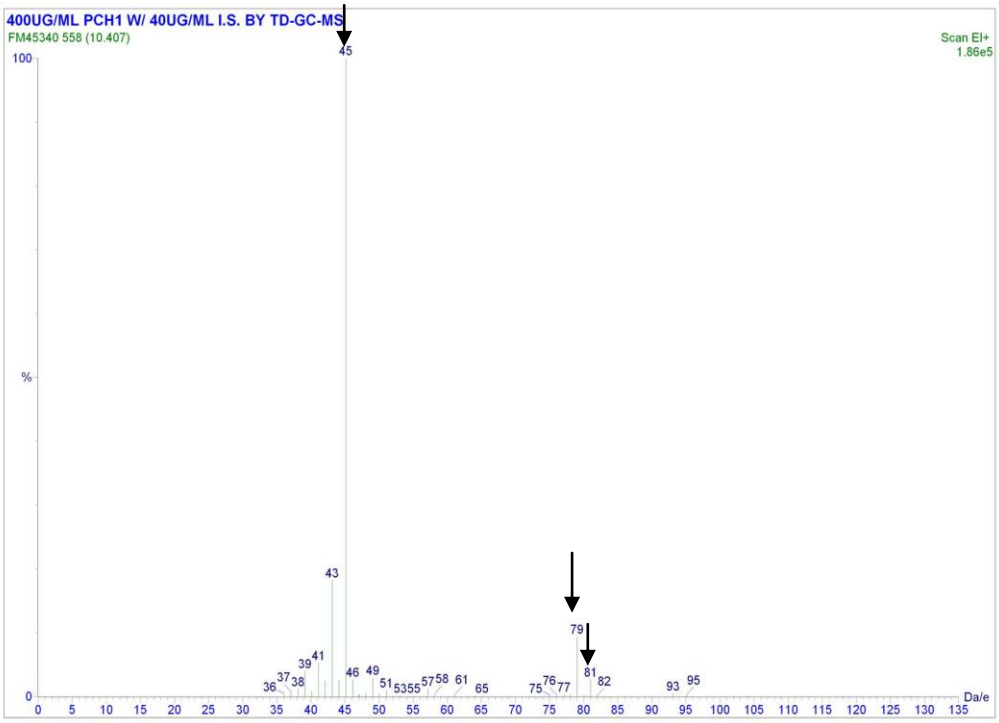

Figure 9: Mass Spectrum of PCH1

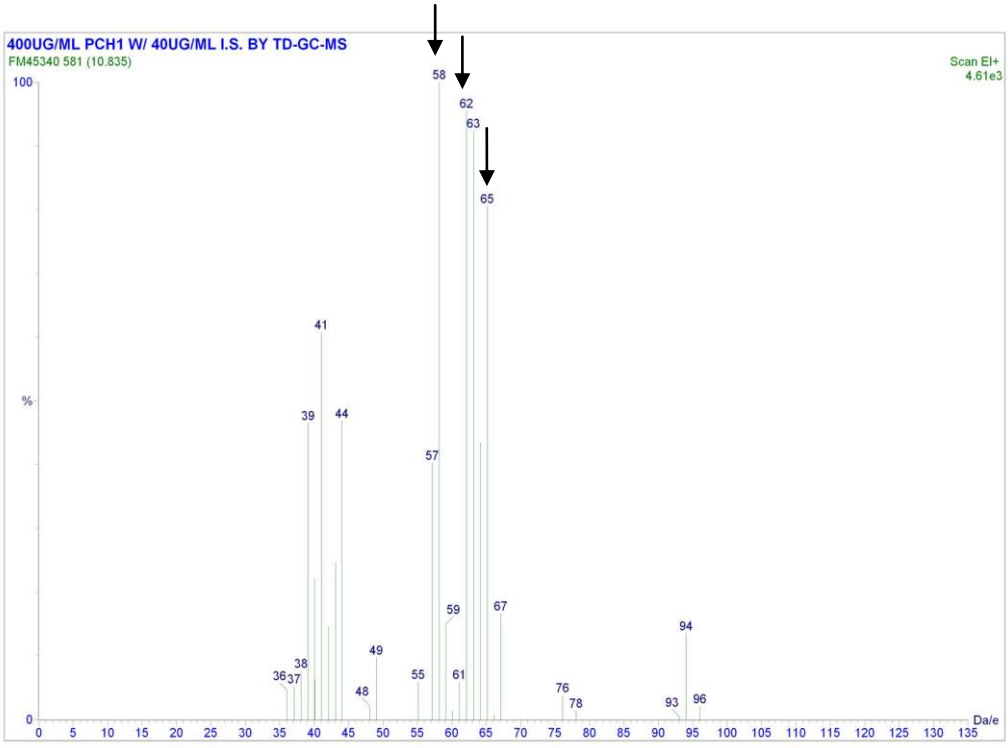

Figure 10: Mass Spectrum of PCH2

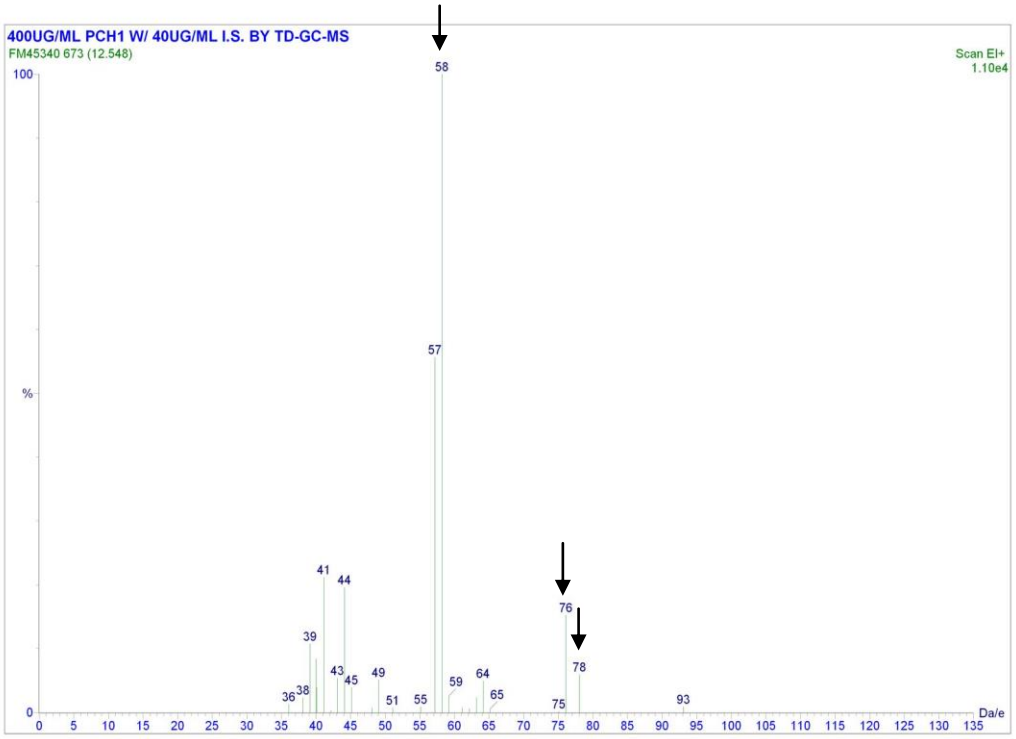

Figure 11: Mass Spectrum of 3-Chloro-1-propanol

The reasons of choosing PCH3 as the internal standard are as follows:

- PCH3 is a constitutional isomer of PCH1 & PCH2 and is a chloropropanol, so it will undergo similar hydrogen bonding interactions in a starch matrix. Comparing the fragmentation patterns of PCH1 and PCH2 with PCH3, a lot of similarities can be found.
- PCH3 has good peak shape on column Equality^{TM} -1701.
- PCH3 is available as a high purity standard from Sigma-Aldrich and well separated from PCH1 and PCH2 on the GC column.

6.2 GC-MS Calibration Curves

The calibration curve data of PCH1 and PCH2 were, respectively, presented in

Tables 8 and 9 (calculated based on total ion current, TIC). The calibration curves were obtained by plotting the peak area ratio of PCH1/I.S. or PCH2/I.S. as dependent variables versus the concentrations of standard solutions of PCH1 or PCH2 as independent variables. Linear regressions were applied. The calibration curves were shown in Figure 12 and 13.

For PCH1, a seven-point calibration was performed as shown in Table 8. The dynamic range of the calibration is from 0.1ppm to 10ppm (nominal to 2 g of starch sample on a dry weight basis). The calibration is linear in the dynamic range with a R-square value >0.99.

For PCH2, a seven-point calibration was performed as shown in Table 9. The dynamic range of the calibration is from 0.1ppm to 10ppm (nominal to 2g of starch sample on a dry weight basis). The calibration is linear in the dynamic range with a R-square value >0.99.

Table 8: P&T-TD-GC-MS Assay for PCH1 Calibration Curve
PCH1 Calibration Curve –by MEOH Extraction with GC-MS Analysis (TIC):

PCH1 Concentration ug/ml	*PCH1 concentration in ppm	peak area ratio PCH1/IS	Average of peak area ratio	PCH1 Regression Output	
400	10	18.30548	17.87171	Constant	-0.3421
		17.43794		Est of Std Err	0.43454
200	5	7.916458	8.383595	R Squared	0.9963
		8.850732		No. of Observation	7
100	2.5	3.350902	3.400858	Degree of Freedom	5
		3.450814			
40	1	1.450074	1.292607	X Coefficient	1.7919
		1.135139			
20	0.5	0.801563	0.808974		
		0.816385			
10	0.25	0.356257	0.359616		
		0.362975			
4	0.1	0.188093	0.161887		
		0.135682			

*based on 2g of starch on a dry weight basis

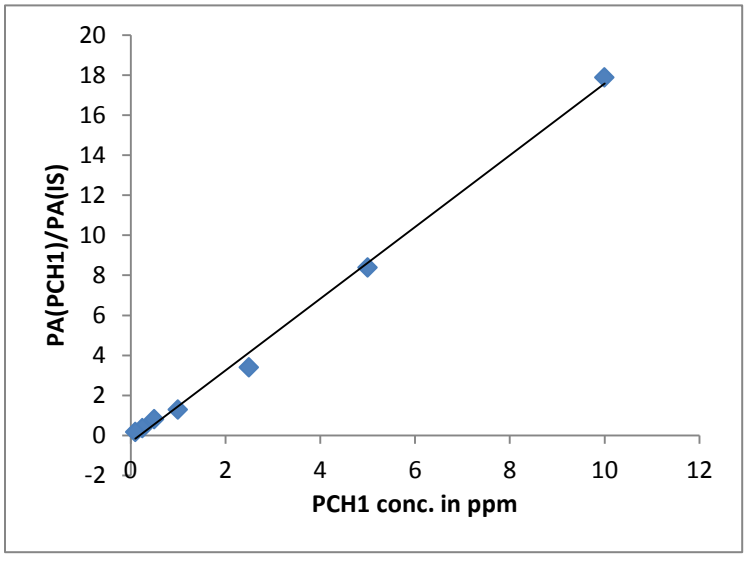

Figure 12: PCH1 Calibration Curve (TIC)

Table 9: P&T-TD-GC-MS Assay for PCH2 Calibration Curve
PCH2 Calibration Curve –by MEOH Extraction with GC-MS Analysis (TIC):

PCH2 concentration ug/ml	*PCH2 concentration in ppm	peak area ratio PCH2/IS	Average of peak area ratio	PCH2 Regression output	
400	10	4.192214	4.307205	Constant	-0.0062
		4.273646		Est of Std Err	0.09195
		4.455756		R Squared	0.997
200	5	2.287617	2.321119	No. of Observation	7
		2.354621		Degree of Freedom	5
100	2.5	1.306949	1.164524		
		1.022098		X Coefficient	0.4386
40	1	0.333423	0.322013		
		0.310604			
20	0.5	0.179889	0.178182		
		0.176474			
10	0.25	0.114814	0.10823		
		0.101646			
4	0.1	0.045163	0.04314		
		0.041117			

*based on 2 g of starch on a dry weight basis

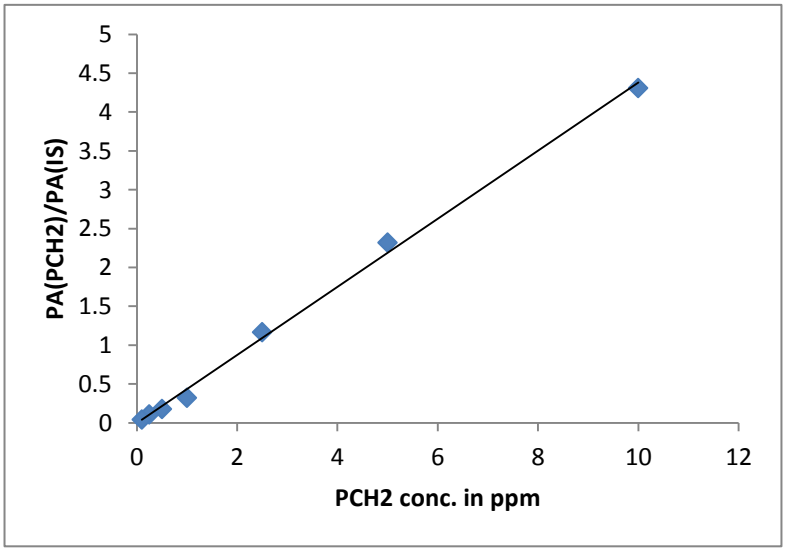

Figure 13: PCH2 Calibration Curve (TIC)

6.3 LOD and LOQ

LOD was determined with a signal-to-noise ratio of 3, where the noise was selected from peaks that are adjacent to the PCH1 or PCH2 peak in a chromatogram. Figure 14 shows the signal-to-noise ratio for the determination of the LOD for PCH1. The LOD is approximately 0.025 mg/kg for PCH1. Figure 15 shows the signal-to-noise ratio for the determination of the LOD for PCH2. The LOD is approximately 0.025 mg/kg for PCH2.

The LOQ was determined with a signal-to-noise ratio of 10, where the noise was selected from peaks adjacent to the PCH1 or PCH2 peak in a chromatogram, Figure 16 shows the signal to noise ratio for the determination of LOQ for PCH1. The LOQ is approximately 0.1 mg/kg for PCH1. Figure 17 shows the signal-to-noise ratio for the determination of the LOQ for PCH2. The LOQ is approximately 0.1 mg/kg for PCH2.

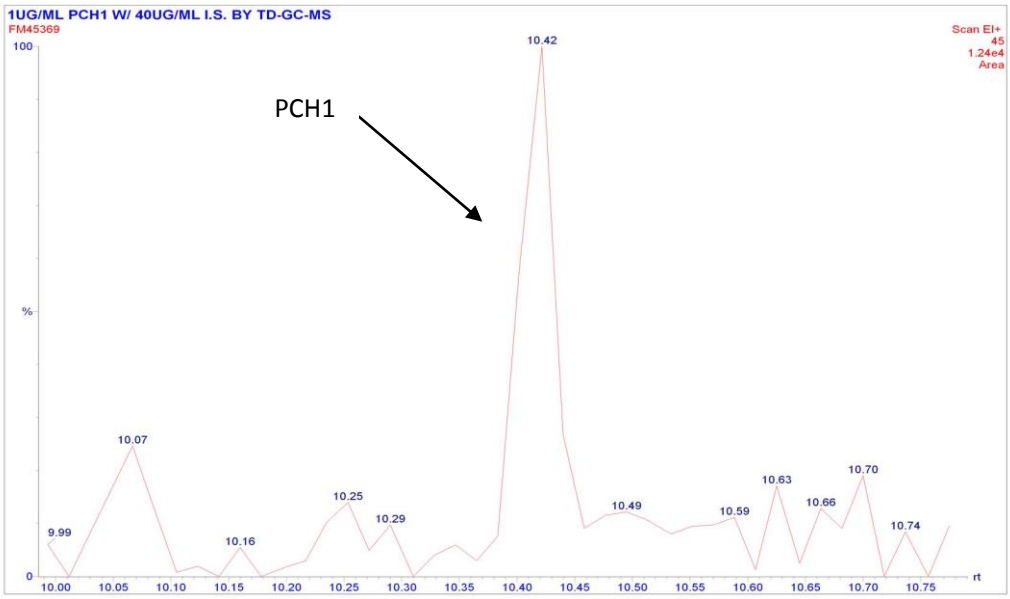

Figure 14: The Signal-to-noise Ratio for Determination of LOD of PCH1

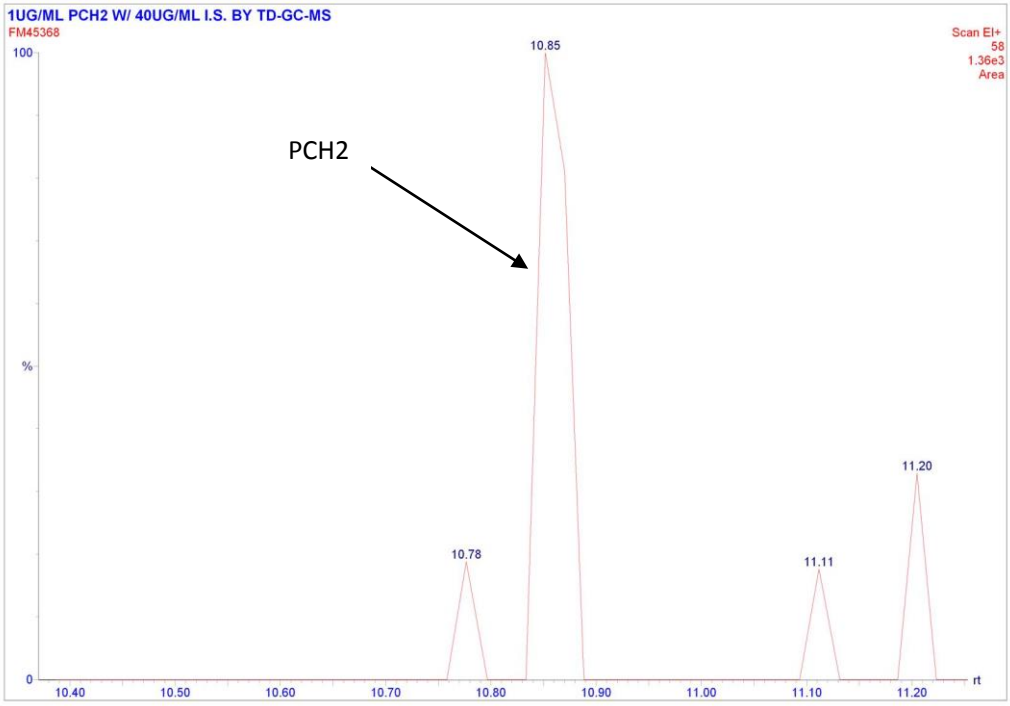

Figure 15: The Signal-to-noise Ratio for Determination of LOD of PCH2

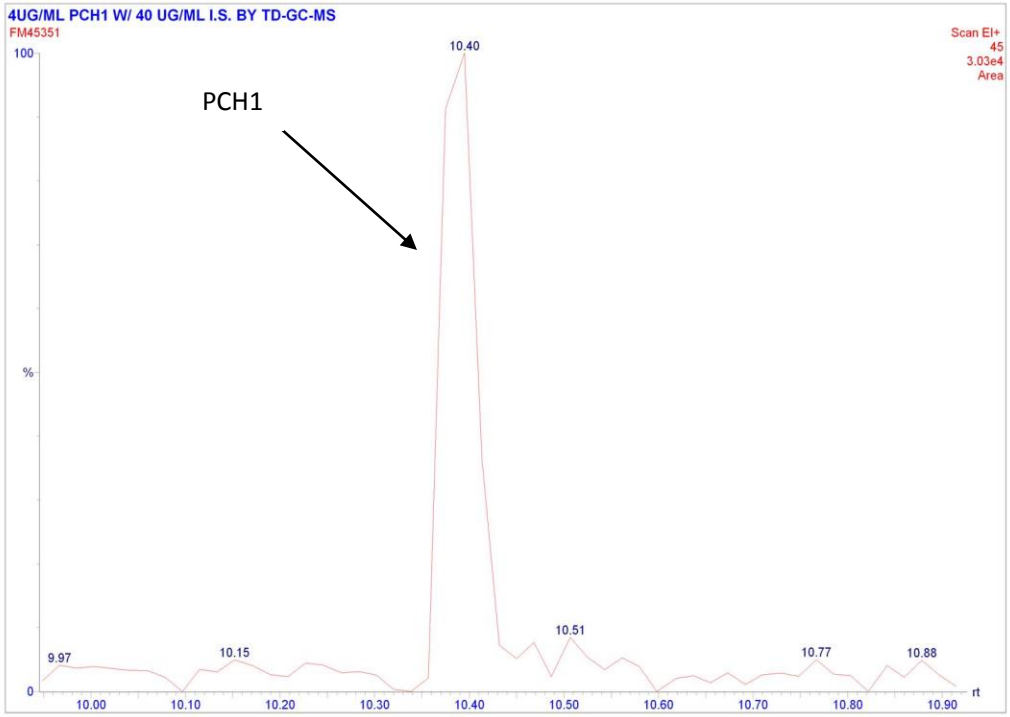

Figure 16: The Signal-to-noise Ratio for the Determination of LOQ of PCH1

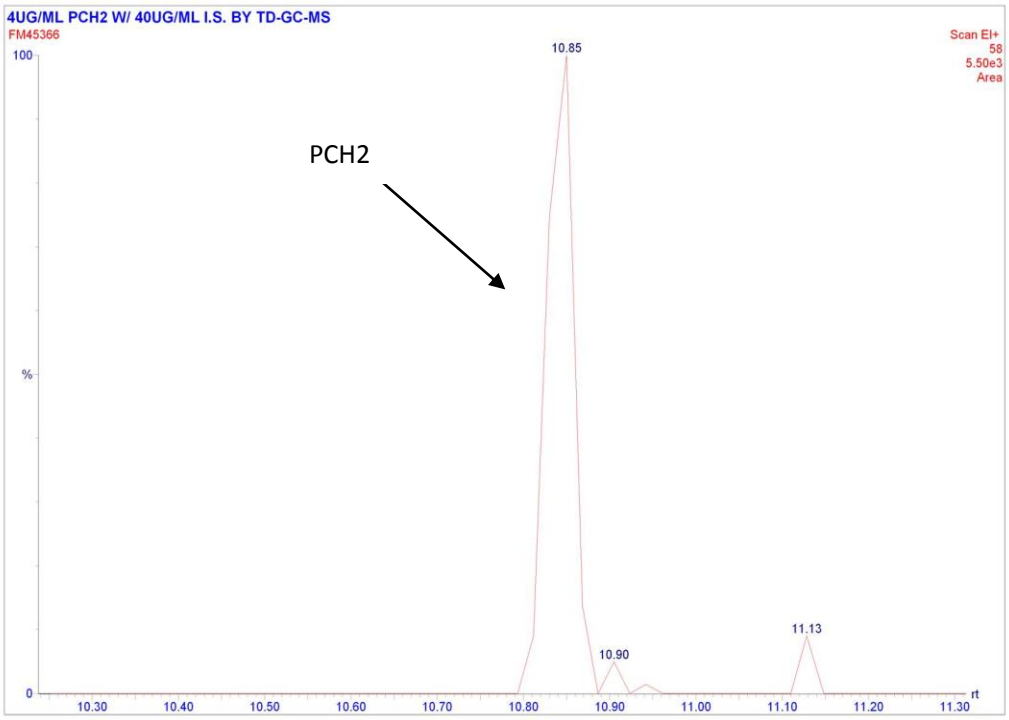

Figure 17: The Signal-to-noise Ratio for the Determination of LOQ of PCH2

6.4 Analytical System Precision

Analytical system precision was accessed by running six analyses of a chosen standard solution with the PCH concentration of 0.4mg/ml (PCH1: 75.9%, PCH2: 23%), the PCH3 concentration of 0.4mg/ml in MEOH. The system precision data are given in Table 10 and 11. The system precision of PCH1 expressed as RSD% is 4.73 (n=6). The mean backfit to calibration is 1.49±0.07 ppm. The system precision of PCH2 expressed as RSD% is 5.34 (n=6). The mean backfit to calibration is 0.13±0.007 ppm.

Table 10: P&T-TD-GC-MS Assay for PCH1 System Precision Data

GC-MS analysis file	PCH1 peak area	I.S. peak area	peak ratio PCH1/I.S.	Mean (n=6)	S.D. (n=6)	RSD%
FM44710	8646	5548	1.56	1.49	0.07	4.73
FM44711	8394	5231	1.60			
FM44712	9708	6835	1.42			
FM44713	10215	7024	1.45			
FM44714	9752	6685	1.46			
FM44715	9492	6442	1.47			

Table 11: P&T-TD-GC-MS Assay for PCH2 System Precision Data

GC-MS analysis file	PCH2 peak area	I.S. peak area	peak ratio PCH2/I.S.	Mean (n=6)	S.D. (n=6)	RSD%
FM44710	774	5548	0.14	0.13	0.007	5.34
FM44711	700	5231	0.13			
FM44712	854	6835	0.12			
FM44713	945	7024	0.13			
FM44714	828	6685	0.12			
FM44715	791	6442	0.12			

6.5 Analytical Method Precision

Analytical method precision was accessed by running six analyses of an exemplar hydroxypropyl starch sample (designated as NS1) extraction (with water or MEOH). Starch sample NS1 was known to be modified with PPO and contain PCH. Table 12 shows the analytical method precision data. With MEOH extraction, the mean concentration of PCH1 was 1.29±0.07 ppm (5.64 %RSD); the mean concentration of PCH2 was 0.32±0.03 ppm (8.02 %RSD). With water extraction, the mean concentration of PCH1 was 0.91±0.032 ppm (3.52 %RSD); the mean concentration of PCH2 was 0.31±0.045 ppm (14.58 %RSD).

Table 12: Method Precision
Sample NS1 with MEOH or Water Extraction Analysis Data by P&T-TD-GC-MS (TIC)

	PCH1		PCH2	
	MEOH extract	Water extract	MEOH extract	Water extract
Mean(n=6)	1.29	0.91	0.32	0.31
S.D.(n=6)	0.07	0.032	0.03	0.045
RSD%	5.64	3.52	8.02	14.58

A general criterion for good precision is that RSD% should below 10%. As we can see from the system precision, RSD% for both PCH1 (4.73%) and PCH2 (5.34%) are below the 10% limit. For method precision, the RSD% for PCH2 with water extraction is a bit above 10% with a value of 14.58%. However, considering that the method precision test was conducted in a real starch matrix, a bit above the 10% still indicate an acceptable precision of applying water extraction-P&T-TD-GC-MS method in PCH2 quantification.

6.6 Between Batch Precision

Between batch precision was monitored in a six-day period. A standard solution with PCH at 0.4mg/ml (PCH1: 75.9%, PCH2: 23%) and internal standard at 0.4mg/ml. A total of 20 measurements were performed. The data are presented in Table 13 and 14. As we can see from the between batch precision data, the analytical system showed good consistency during sample testing. The mean backfit for PCH1 was 1.5±0.08 (5.42 %RSD). The mean backfit for PCH2 was 0.104±0.011 (10.58 %RSD). As it can be noticed from Table 14, on day 5, the content of PCH2 in the standard solution has decreased.

Table 13: Example PCH1 between Batch Accuracy & Precision Data

Analysis Date	PCH1 peak area	I.S. peak area	peak ratio PCH1/I.S.	Mean (n=2-7)	S.D.	%RSD
Day 0	9851	7137	1.38	1.41	0.041	2.90
	9559	6858	1.39			
	9108	6239	1.46			
	9263	6266	1.48			
	8532	6102	1.40			
	7973	5823	1.37			
	8107	5768	1.41			
Day 1	10252	6580	1.56	1.53	0.023	1.52
	9734	6421	1.52			
	9577	6301	1.52			
Day 2	7836	5003	1.57	1.55	0.027	1.75
	7852	5139	1.53			
Day 3	8968	5950	1.51	1.52	0.023	1.52
	8805	5677	1.55			
	8862	5845	1.52			
Day 4	7987	5282	1.56	1.53	0.023	1.52
	9029	6222	1.52			
	8213	5725	1.52			
Day 5	7087	4501	1.57	1.64	0.092	5.62
	5861	3443	1.70			
Mean				1.50	0.08	5.42

Table 14: Example PCH2 between Batch Accuracy & Precision Data

Analysis Date	PCH2 peak area	I.S. peak area	peak ratio PCH2/I.S.	Mean (n=2-7)	S.D.	%RSD
Day 0	715	7137	0.10	0.11	0.0076	6.98
	707	6858	0.10			
	705	6239	0.11			
	724	6266	0.12			
	701	6102	0.11			
	573	5823	0.10			
	659	5768	0.11			

Day 1	718	6580	0.11	0.11	0.0028	2.57
	696	6421	0.11			
	716	6301	0.11			
Day 2	565	5003	0.11	0.11	0.0011	0.92
	588	5139	0.11			
Day 3	671	5950	0.11	0.11	0.0028	2.55
	610	5677	0.11			
	653	5845	0.11			
Day 4	530	5282	0.10	0.10	0.0070	7.09
	653	6222	0.10			
	522	5725	0.09			
Day 5	365	4501	0.081	0.077	0.0056	7.35
	251	3443	0.073			
Mean				0.104	0.011	10.58

6.7 Solution Stability

The data obtained from between batch precision can be used to study standard solution stability. The standard was stored at room temperature in glass vial with Teflon lined closure. During a 6-day period, no statistical difference was observed in the studied concentration for PCH1. However, a decrease of the content of PCH2 standard solutions was observed on day 5. The data suggest that freshly prepared PCH1 standard solutions, stored at room temperature in glass vial with a Teflon-lined closure are stable for 6 days, and freshly prepared PCH2 standard solutions are stable for 5 days.

6.8 System Suitability

Well-separated peaks are very important to the accuracy of quantification. Resolution is a parameter to assess how well two close peaks are separated from each

other. The data for resolution is presented in the spreadsheet Table 15 and 16. The resolution is calculated as (RT I.S. - RT PCH)/0.5(Peak Width of I. S. + Peak Width of PCH). All RT and Peak Width values are time in minutes. Resolution values of PCH1 ranged from 4.54-6.27. Resolution values of PCH2 ranged from 20.00- 25.86. Resolution values obtained are all above the minimum value of 2 which is required in a system suitability assay (Validation of Chromatographic Methods 1994).

Table 15: PCH1 Chromatographic Resolution Data

	GC RT in minutes		**Peak width at base in minutes**		
GC-MS file	PCH1	PCH2.	PCH1	PCH2	*Resolution
FM44771	10.57	11.01	0.10	0.094	4.54
FM44772	10.59	11.01	0.076	0.085	5.22
FM44773	10.56	10.98	0.077	0.058	6.22
FM44774	10.59	11.01	0.075	0.073	5.68
FM44775	10.61	11.05	0.077	0.089	5.30
FM44776	10.57	11.01	0.094	0.077	5.15
FM44777	10.63	11.05	0.076	0.072	5.68
FM44783	10.77	11.2	0.075	0.090	5.21
FM44784	10.76	11.2	0.075	0.075	5.87
FM44785	10.73	11.18	0.076	0.073	6.04
FM44798	10.63	11.05	0.076	0.058	6.27
FM44799	10.61	11.06	0.077	0.071	6.08
FM44808	10.63	11.07	0.076	0.080	5.64
FM44809	10.66	11.1	0.076	0.075	5.83
FM44810	10.64	11.08	0.077	0.075	5.79
FM44815	10.66	11.08	0.077	0.076	5.49
FM44816	10.63	11.07	0.077	0.078	5.68
FM44817	10.64	11.08	0.077	0.077	5.71

*Resolution= (RT Int. Std. Peak- RT PCH1 Peak)/0.5(Peak width of Int. Std. peak+ Peak width of PCH1 Peak)

Minimum Value of Resolution for Assay >2

Table 16: PCH2 Chromatographic Resolution Data

	GC RT in minutes		**Peak width at base in minutes**		
GC-MS file	PCH2	I.S.	PCH2	I.S.	*Resolution
FM44771	11.01	12.72	0.094	0.077	20.00
FM44772	11.01	12.74	0.085	0.077	21.36
FM44773	10.98	12.71	0.058	0.098	22.18
FM44774	11.01	12.72	0.073	0.076	22.95
FM44775	11.05	12.76	0.089	0.077	20.60
FM44776	11.01	12.71	0.077	0.075	22.37
FM44777	11.05	12.76	0.072	0.076	23.11
FM44783	11.20	12.92	0.090	0.075	20.85
FM44784	11.20	12.91	0.075	0.076	22.65
FM44785	11.18	12.90	0.073	0.076	23.09
FM44798	11.05	12.77	0.058	0.075	25.86
FM44799	11.06	12.78	0.071	0.076	23.40
FM44808	11.07	12.78	0.080	0.076	21.92
FM44809	11.10	12.81	0.075	0.078	22.35
FM44810	11.08	12.79	0.075	0.077	22.50
FM44815	11.08	12.81	0.076	0.077	22.61
FM44816	11.07	12.78	0.078	0.077	22.06
FM44817	11.08	12.79	0.077	0.076	22.35

*Resolution= (RT Int. Std. Peak- RT PCH2 Peak)/0.5(Peak width of Int. Std. peak+ Peak width of PCH2 Peak)

Minimum Value of Resolution for Assay >2

Peak tailing will negatively affect the accuracy of quantitation. Usually, a tailing factor below 2 is an important parameter in the determination of system suitability (Validation of Chromatographic Methods 1994). Tailing factor (T) = $W_{0.05}/2f$. A sample

chromatographic peak is shown in figure 18. The data for assessing tailing factor were presented in Table 17. And from the calculated results, all tailing factors of PCH1 and PCH2 are below 2.

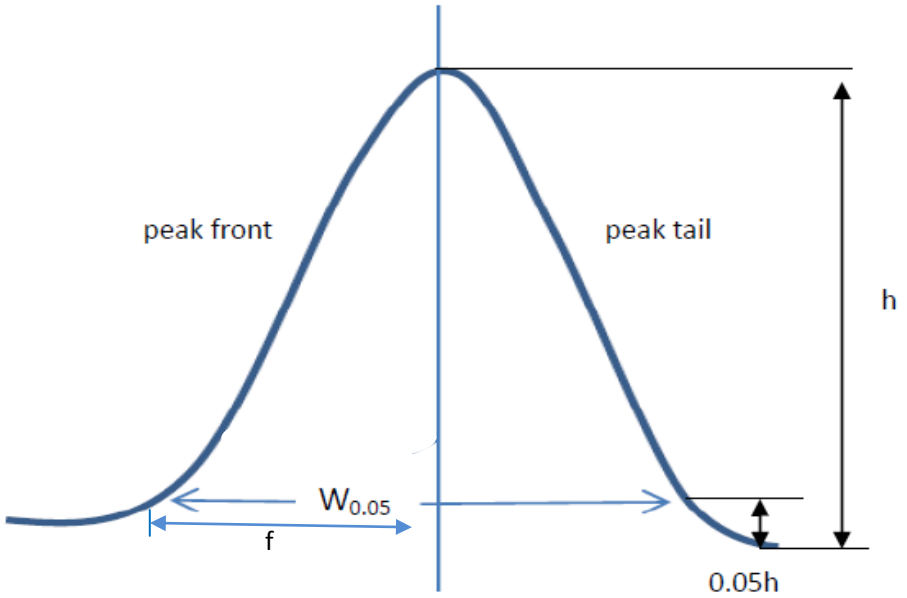

Figure 18: A Sample Chromatographic Peak ($W_{0.05}$= Peak Width at 0.05 Peak Height, f= Distance between Peak Maximum and Peak Front)

Table 17: Tailing Factor for System Suitability Assessment

	PCH1			PCH2		
GC-MS file	W	f	Tailing factor(T)	W	f	Tailing factor(T)
FM44771	0.056	0.02	1.40	0.082	0.037	1.11
FM44772	0.065	0.033	0.98	0.065	0.018	1.80
FM44773	0.065	0.035	0.93	0.12	0.045	1.33
FM44774	0.067	0.037	0.90	0.073	0.036	1.01
FM44775	0.063	0.026	1.21	0.072	0.038	0.95
FM44776	0.065	0.027	1.20	0.068	0.034	1.00
FM44777	0.063	0.035	0.90	0.065	0.028	1.16
FM44783	0.067	0.036	0.93	0.080	0.031	1.29
FM44784	0.069	0.034	1.01	0.074	0.054	0.68

FM44785	0.068	0.033	1.03	0.072	0.037	0.97
FM44798	0.069	0.038	0.91	0.058	0.020	1.45
FM44799	0.060	0.023	1.30	0.066	0.032	1.03
FM44808	0.066	0.033	1.00	0.077	0.037	1.04
FM44809	0.063	0.024	1.31	0.071	0.037	1.00
FM44810	0.058	0.017	1.70	0.070	0.035	1.00
FM44815	0.065	0.021	1.55	0.057	0.020	1.42
FM44816	0.066	0.036	0.92	0.065	0.037	0.88
FM44817	0.070	0.033	1.06	0.068	0.037	0.92

6.9 Spiking & Recovery

Recovery test was performed in six replicates with starch sample EK9005 extracts (with MEOH or water), and spiked with 10 ppm of PCH1 and 10 ppm of PCH2. The hydroxypropyl starch sample EK9005 had been washed enough times to eliminate the presence of PCH. Sample EK9005 was analyzed un-spiked and found to be free of PCH compounds (or below detection limit). The absorption salt used in recovery study is sodium chloride. The data of PCH recovery is presented in Table 18.

Table 18: Recovery Data
EK9005 Spiked with 10ppm PCH1 and 10ppm PCH2 by MEOH or Water Extraction

	MEOH Extraction		Water extraction	
	TIC	Selected ion	TIC	Selected ion
PCH1	99.4%	99.95%	94.1%	127.7%
PCH2	98.7%	67.4%	81.7%	80.4%

The recovery test was conducted to assess the bias of P&T-TD-GC-MS method for PCH quantification in hydroxypropyl starch matrix. It serves to monitor the P&T-TD-GC-MS method operation procedures including, solvent extraction, evaporation

loss, Purge& Trap loss.

Recovery data in Table 18 shows a decent recovery of spiked PCH in blank starch extracted with methanol. Recovery from the water extraction of the PCH spiked blank starch was not as good as it is anticipated that recovery would be higher by substituting sodium sulfate as the adsorption matrix instead of sodium chloride when water extraction is used. Possible explanations are mentioned in Section 6.11.

6.10 Analysis of Starch Samples

In this test, three hydroxypropylated starch samples, including NS1, NS14, EK9005 (EK9005 that had been washed many times to eliminate PCH content was used as a blank), were analyzed using P&T-TD-GC-MS with MEOH or water extraction. NS14 was highly hydroxypropylated and formed a gel with water. So no water extract was obtained. The results shown in Table 19 were calculated based on both total ion current and selected ion current. The absorption matrix was sodium chloride. The levels of PCH obtained ranged from 3.06 ppm to below the limit of detection.

Table 19: PCH Conc. (ppm) in Sample NS1, NS14, EK9005 with Water or MEOH Extraction

	NS1(n=6)				NS14(n=4)		EK9005(n=4)			
	MEOH		Water		MEOH		MEOH		Water	
	TIC	SI*	TIC	SI*	TIC	SI*	TIC	SI*	TIC	SI*
PCH1	1.29	0.79	0.90	0.83	2.75	1.79	-	-	-	-
PCH2	0.32	0.52	0.31	0.55	0.31	0.34	-	-	-	-
Total	1.61	1.32	1.21	1.38	3.06	2.13	-	-	-	-

*SI: Calculation was based on selected ion current

6.11 The Effect of Different P&T Absorption Salt Matrix on PCH Concentration

Sodium chloride and sodium sulfate were compared to evaluate their effects on PCH content results. Approximately 1.20g salt was used to absorb 200ul sample MEOH or water extract. The absorption matrix was placed between glass wool. The distance of the glass wool to the head of glass tube is 14cm to ensure an evenly heated environment of 100℃. The experiment was performed in four replicates with MEOH or water extracts of hydroxypropyl starch sample NS1.

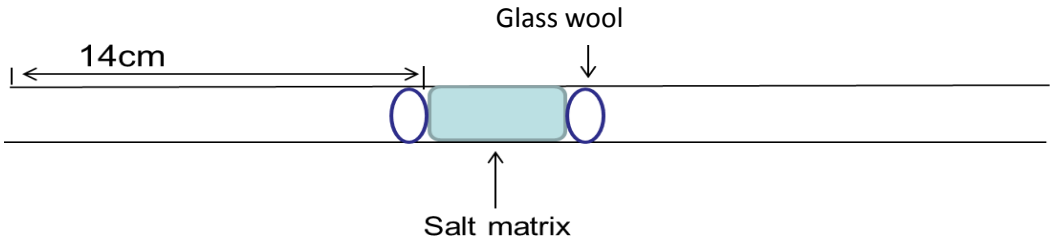

Figure 19: Absorption Salt Matrix in P&T Vessel Designed for Solid Samples

The reasons that a salt is used to hold the liquid extract is, first, salt will increase the boiling point of water above 100℃, thus, reducing the emanating moisture. Secondly, because of the salting out effect, PCH are more likely to partition into the gas phase and be purged and trapped into the Tenax-TA adsorbent.

Table 20: PCH Content Comparison in Two Salt Matrixes (Sample NS1)

	MEOH ext.				**Water ext.**			
	NaCl		**Na_2SO_4**		**NaCl**		**Na_2SO_4**	
	TIC	*SI	TIC	SI	TIC	SI	TIC	SI
Mean (n=4)	1.54	1.38	1.52	1.37	1.23	1.32	1.59	1.60
S.D.	0.103	0.13	0.034	0.11	0.061	0.12	0.018	0.058
RSD%	6.66	9.19	2.24	7.99	4.96	8.98	1.10	3.58

*SI: selected ion

Since sodium chloride contains chlorine in the molecule, it is possible that during the concentration process, any residual PPO in the starch sample may react with the chloride ion in water to form PCH. To verify this possibility, sodium sulfate and sodium chloride as salt matrixes were used side by side to compare the levels of PCH obtained.

From the data listed in Table 20, there's no significant difference in PCH content with MEOH extraction, using sodium chloride or sodium sulfate. However, the data with water extraction told a different story. It appeared that water extraction in sodium sulfate matrix was much higher than that in sodium chloride matrix. Previous sample analyses and method precision validation were conducted in sodium chloride matrix, and PCH content from sample with MEOH extraction was higher than that with water extraction. This fact may not be solely explained by different solvent extraction efficiencies as previously proposed based on this test, but also because of the different interactions between salt and water. When water extract was spiked into the sodium chloride matrix, sodium chloride will partially ionize and form sodium cation and chloride anion, which will change water hydrogen bonding network and pose a negative effect on PCH successfully elution from the salt matrix. When water extraction was spiked into anhydrous sodium sulfate matrix, sodium sulfate would combine with at most ten water molecules and form an aquo-complex. This tends to reduce the interaction between water molecules and PCH through hydrogen bonding and was in favor of PCH eluting from sodium sulfate matrix.

Two tailed t-test was applied to examine if there was significant difference by

using the two salt absorption matrixes. When the samples were extracted with MEOH, and the data calculated based on total ion content, $|t|=0.39 < t_{0.05}(6) = 2.447$. So there is no significant difference of using sodium chloride or sodium sulfate when sample were extracted by MEOH. When extract (with MEOH or water) was spiked in sodium chloride matrix and data calculated based on TIC, $|t|= 5.18 > t_{0.05}(6) = 2.447$. So there is a significant difference of using MEOH or Water extract in sodium chloride matrix. When sample extracted with water, and data calculated based on TIC, $|t|= 11.27 > t_{0.05}(6) = 2.447$. So there is a significant difference of using sodium chloride or sodium sulfate when sample extracted with water. Based on the data shown in the Table 20 and conclusion from t tests, sodium sulfate is adopted as the salt matrix in the following ring test.

7. Proposed Changes in the Ring Test Study

Although the P&T-TD-GC-MS method shows acceptable recovery, system and method precision, the accuracy of this method has yet to be determined. In order to assess the accuracy of the P&T-TD-GC-MS method and the European proposed solvent extraction GC-MS method, a ring test was conducted among 4 labs (Rutgers, AVEBE Netherlands, AVEBE Sweden, and ISSI NJ).

In the ring test, each lab received the same sets of starch samples. And the chemicals used to build calibration curves, including PCH, PCH2 and PCH3, all have the same batch numbers.

For the ring test, the following modifications were made to increase the accuracy of the P&T-TD-GC-MS method:

- Mass spectrometer scan range is reduced to 35-100 from 35-150 to allow more scans concentrating on the range of 35-94.
- Starch samples are extracted at room temperature, which is used as other labs extraction condition, thus, making the final PCH content more comparable.
- Build calibration curves with and without starch matrix.
- Glass tubes with diameter of 1/4 inch (OD) rather than 1/2 inch (OD) are used in methanol extraction to reduce dead volume. It is not applicable to water extracts because more moisture will be trapped into the desorption tube, when it’s changing to glass tubes with 1/4 inch (OD).

8. Ring Test

8.1 Calibration Curves with Blank Hydroxypropyl Starch as Spike Matrix

The calibration curve data of PCH1 and PCH2 were respectively presented in Table 21 and 22 (calculated based on total ion current, TIC). The calibration curves were obtained by plotting the peak area ratio of PCH1/I.S. or PCH2/I.S. as dependent variables versus the concentrations of standard solutions of PCH1 or PCH2 as independent variables. Linear regressions were applied. The calibration curves were shown in Figure 20 and Figure 21.

For PCH1, a five-point calibration was performed as shown in Table 21. The dynamic range of the calibration is from 0.1ppm to 10ppm (nominal to 2 g of starch sample on dry weight basis). The calibration is linear in this dynamic range with R-square >0.99.

For PCH2, a five-point calibration was performed as shown in Table 22. The dynamic range of the calibration is from 0.1ppm to 10ppm (nominal to 2g of starch sample on dry weight basis). The calibration is linear in this dynamic range with R-square >0.99.

Table 21: P&T-TD-GC-MS Assay for PCH1 Calibration Curve –by MEOH Extraction with GC-MS Analysis (TIC)

*PCH1 concentration in ppm	peak area ratio PCH1/IS	Average of peak area ratio	PCH1 Regression Output	
10ppm	18.74892	20.2095	Constant	1.2203
	20.89072		Est of Std Err	0.1669
	20.98884		R Squared	0.9997
5ppm	10.76064	10.67757	No. of Observations	5
	10.58146		Degrees of Freedom	3
	10.6906			
1ppm	3.616585	3.329339	X Coefficient	1.899
	3.624429			
	2.747003			
0.5ppm	1.908135	2.192858		
	1.920511			
	2.749928			
0.1ppm	1.30183	1.216441		
	1.084593			
	1.262899			

*based on 2g of starch on dry weight basis

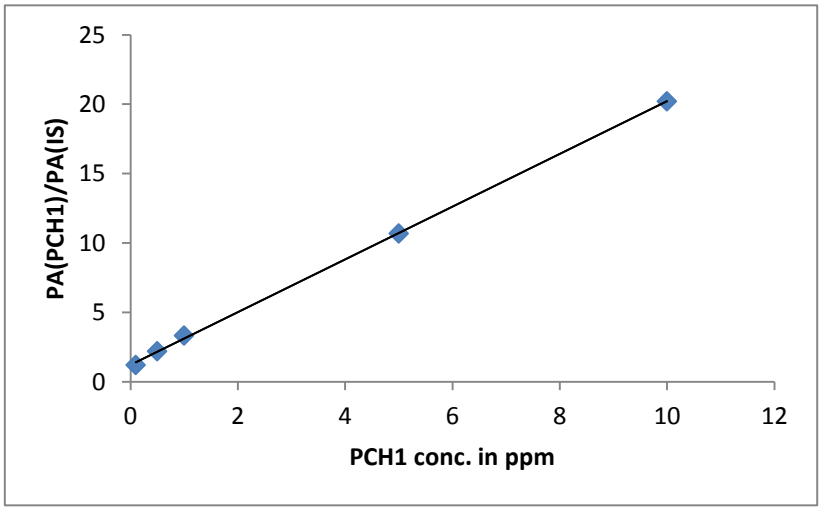

Figure 20: PCH1 Calibration Curve by MEOH Extraction (TIC)

Table 22: P&T-TD-GC-MS Assay for PCH2 Calibration Curve –by MEOH Extraction with GC-MS Analysis (TIC)

*PCH2 concentration in ppm	peak area ratio PCH2/IS	Average of peak area ratio	PCH2 Regression Output	
10ppm	2.736011	4.969395	Constant	0.0033
	4.561626		Est of Std Err	0.06959
	7.610548		R Squared	0.9992
5ppm	2.711702	2.594849	No. of Observations	5
	2.502809		Degrees of Freedom	3
	2.570035			
1ppm	0.420976	0.436698	X Coefficient	0.5005
	0.519406			
	0.369712			
0.5ppm	0.318159	0.276815		
	0.277502			
	0.234785			
0.1ppm	0.036276	0.046973		
	0.054253			
	0.05039			

*based on 2g of starch on dry weigh basis

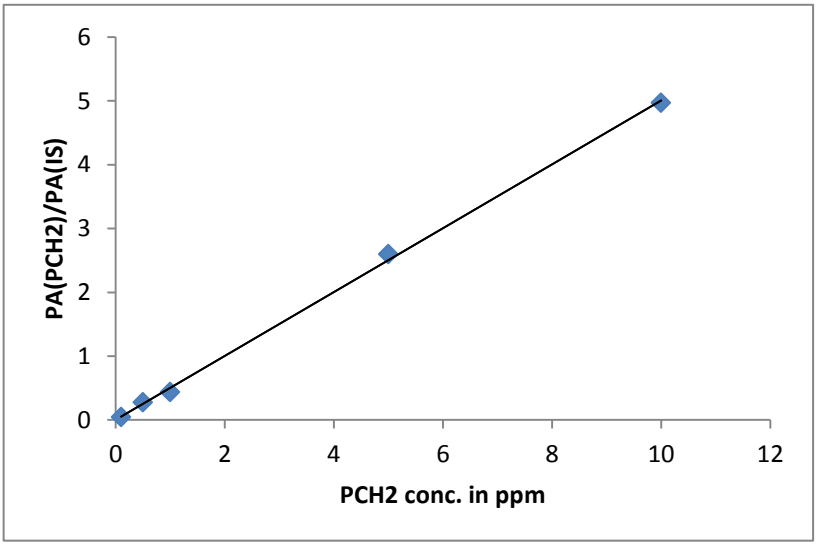

Figure 21: PCH2 Calibration Curve by MEOH Extraction (TIC)

The calibrations were also built with water extraction. And the data were calculated based on both total ion content and selected ion content. All in matrix linear regression curves equations were presented in Table 23.

Table 23: Linear Calibration Equations (in Blank Starch Matrix)

	PCH1		PCH2	
	TIC	Selected ion	TIC	Selected ion
MEOH	y=1.899x+1.22	y=2.98x-0.065	y=0.5x+0.0033	y=0.169x-0.0353
	R^2=0.9997	R^2=0.9988	R^2=0.9992	R^2=0.9977
Water	y=2.8833x+0.21	y=5.29x+0.7085	y=0.6419x+0.188	y=0.2754x+0.0489
	R^2=0.998	R^2=0.9876	R^2=0.9819	R^2=0.997

8.2 Starch Samples Analyzed with Methanol Extraction, P&T-TD-GC-MS Method

A total of 12 granular and pregelatinized starch samples coded in duplicate were analyzed with MEOH extraction, P&T-TD-GC-MS in mass spectrometry lab, Rutgers University. The same set of samples were independently analyzed by AVEBE Netherlands, AVEBE Sweden and ISSI lab in NJ, using the newly proposed European methanol extraction GC-MS method.

A separate set that does not include pregelatinized samples were analyzed with water extraction, P&T-TD-GC-MS method. Table 24 shows PCH concentration in all the ring test samples based on the P&T-TD-GC-MS method (calculated based on in starch matrix calibration as previously shown in Table 23).

Table 24: Sum of Conc. of PCH1 and PCH2 in ppm

PCH mean conc. in ppm (n=2)				
Sample	**MEOH extract**		**Water extract**	
	TIC	**Selected ion**	**TIC**	**Selected ion**
A	0.00	0.07	0.02	0.01
A	0.09	0.20	0.00	0.00
B	0.75	0.50	0.60	0.59
B	0.75	1.14	0.56	0.45
C	1.28	1.48	0.94	0.89
C	1.39	1.47	0.91	0.95
D	1.76	1.94	1.07	1.86
D	2.60	2.31	1.36	1.27
E	2.50	2.57	2.93	3.16
E	1.83	1.55	1.64	1.14
F	0.59	0.64	0.20	0.18
F	0.20	0.17	0.25	0.30
G	0.46	0.56	0.23	0.20
G	0.57	0.64	0.21	0.14
H	0.31	0.33	0.10	0.035
H	0.13	0.29	0.10	0.038
I	0.26	0.49	0.11	0.10
I	0.20	0.59	0.07	0.06
J	0.35	0.56	0.07	0.06
J	0.40	0.26	0.25	0.20
K	0.55	0.15		
K	0.59	0.27		
L	0.32	0.40		
L	0.04	0.21		

* Sample A-D: pre-spiked samples

Sample E-L: samples were pre-tested with the European proposed MEOH extraction- GC- MS method by AVEBE Netherlands

8.3 Calibration Curves Built without Blank Hydroxypropyl Starch Matrix

Besides in matrix calibration, calibration without blank starch matrix were built based on P&T –TD –GC –MS was also built to analyze ring test samples.

The calibration curve data of PCH1 and PCH2 were respectively presented in

Table 25 and 26 (calculated based on total ion current, TIC). The calibration curves were obtained by plotting the peak area ratio of PCH1/I.S. or PCH2/I.S. as independent variables versus the concentrations of standard solutions of PCH1 or PCH2 as dependent variables. Linear regressions were applied. The calibration curves were shown in Figure 22 and Figure 23.

For PCH1, a five-point calibration was performed as shown in Table 25. The dynamic range of the calibration is from 0.1ppm to 10ppm (nominal to 2 g of starch sample on dry weight basis). The calibration is linear in this dynamic range with R-square of 0.951. The F value calculated for this calibration model is $14.51 > F_{0.05}(1, 3) = 10.13$. Thus the calibration model is considered suitable.

For PCH2, a five-point calibration was performed as shown in Table 26. The dynamic range of the calibration is from 0.1ppm to 10ppm (nominal to 2g of starch sample on dry weight basis). The calibration is linear in this dynamic range with R-square >0.99.

Table 25: P&T-TD-GC-MS Assay for PCH1 Calibration Curve –by MEOH Extraction w/ No Starch with GC-MS Analysis (TIC)

*PCH1 concentration in ppm	peak area ratio PCH1/IS	Average of peak area ratio	PCH1 Regression Output	
10ppm	31.35178	31.56637	Constant	3.6832
	31.78095		Est of Std Err	0.3017
5ppm	16.68307	17.66797	R Squared	0.9995
	18.65287		No. of Observations	5
1ppm	6.362683	6.442819	Degrees of Freedom	3
	6.522954			
0.5ppm	5.701373	5.460716	X Coefficient	2.791

	5.22006		
0.1ppm	3.955357	3.609341	
	3.263326		

*based on 2g of starch on dry weight basis

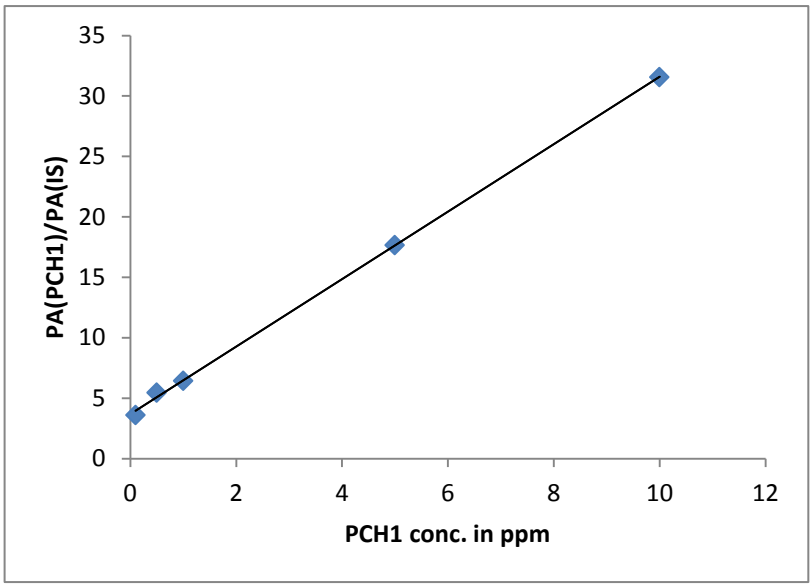

Figure 22: PCH1 Calibration Curve by MEOH Extraction (TIC)

Table 26: P&T-TD-GC-MS Assay for PCH2 Calibration Curve –by MEOH Extraction w/ No Matrix Starch with GC-MS Analysis (TIC)

*PCH2 concentration in ppm	peak area ratio PCH2/IS	Average of peak area ratio	PCH1 Regression Output	
10ppm	8.909091	10.26883	Constant	-0.1005
	11.62857		Std Err of Est	0.4344
5ppm	4.326772	4.290972	R Squared	0.9922
	4.255172		No. of Observations	5
1ppm	0.888889	0.953426	Degrees of Freedom	3
	1.017964			
0.5ppm	0.649886	0.643057	X Coefficient	1.0066
	0.636228			
0.1ppm	0.049603	0.050542		
	0.051481			

*based on 2g of starch on dry weight basis

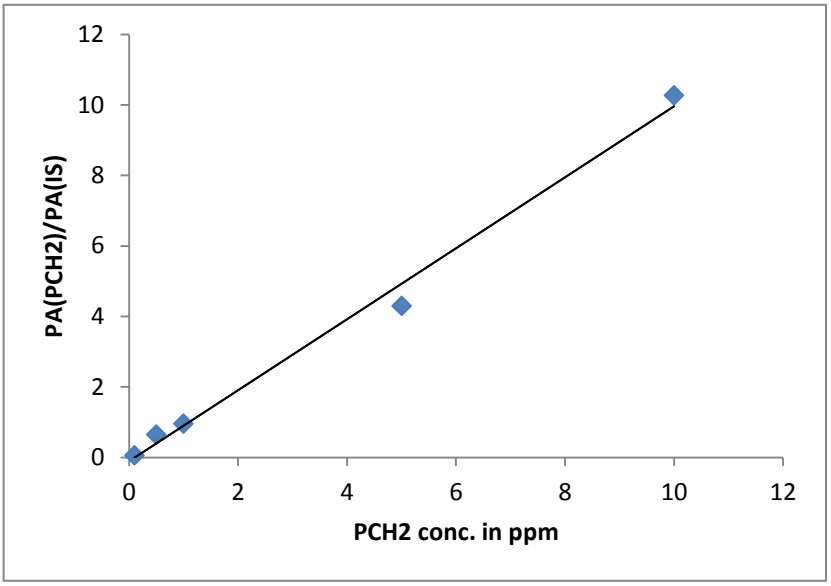

Figure 23: PCH2 Calibration Curve by MEOH Extraction (TIC)

The calibrations were also built with water extraction. And the data were calculated based on both total ion content and selected ion content. All linear regression (without starch matrix) equations were shown in Table 27.

Table 27: Linear Calibration Curve Equations (without Blank Starch Matrix)

Cal. Equation	PCH1		PCH2	
	TIC	Selected ion	TIC	Selected ion
MEOH	y=2.791x+3.68	y=3.417x+0.457	y=1.0066x-0.1	y=0.528x-0.0877
	R^2=0.9995	R^2=0.9982	R^2=0.9922	R^2=0.9629
Water	y=6.1334x-1.272	y=9.28x-1.82	y=1.829x-0.206	y=0.7362x+0.1
	R^2=0.9983	R^2=0.995	R^2=0.9847	R^2=0.9782

8.4 Starch Samples Analyzed with Methanol Extraction, P&T-TD-GC-MS Method

Table 28 shows PCH concentration in all the ring test samples based on the

P&T-TD-GC-MS method (calculated based on the calibration built up without blank starch matrix as previously shown).

Table 28: Sum of Conc. of PCH1 and PCH2 in ppm

PCH mean conc. in ppm (n=2)				
Sample	**MEOH extract**		**Water extract**	
	TIC	**Selected ion**	**TIC**	**Selected ion**
A	0.26	0.04	0.38	0.22
A	0.41	0.43	0.21	0.21
B	0.79	0.57	0.65	0.52
B	0.78	0.96	0.63	0.47
C	1.07	1.25	0.81	0.67
C	1.14	1.23	0.78	0.71
D	1.34	1.45	0.99	1.19
D	1.82	1.85	0.86	0.85
E	1.75	1.57	1.61	1.69
E	1.37	1.15	1.06	0.77
F	0.36	0.57	0.46	0.30
F	0.47	0.40	0.49	0.35
G	0.62	0.49	0.47	0.31
G	0.68	0.56	0.47	0.30
H	0.54	0.54	0.42	0.23
H	0.44	0.40	0.42	0.23
I	0.16	0.43	0.42	0.26
I	0.48	0.52	0.40	0.24
J	0.56	0.51	0.40	0.24
J	0.59	0.48	0.49	0.31
K	0.67	0.11		
K	0.36	0.21		
L	0.20	0.39		
L	0.39	0.16		

* Sample A-D: pre-spiked starch samples

Sample E-L: commercial starch samples were pre-tested with the European proposed MEOH extraction- GC- MS method by AVEBE Netherlands

8.6 Starch Samples Analyzed with European Proposed Solvent Extraction GC-MS Method

A total of 12 granular and pre-gelatinized starch samples were blind coded in duplicated and analyzed using European methanol extraction-GC-MS method, respectively, by AVEBE Netherlands and ISSI in New Jersey. The data were presented in Table 29.

These 12 samples were pretested by AVEBE with the European methanol extraction- GC- MS method and the contents of PCH in these samples were listed in the first column (A-D were pre-spiked and the levels of PCH in E-L were previously determined by AVEBE).

By comparing the data of pretested samples and the data from ring test as shown in Table 29, the European methanol extraction method did not show good reproducibility.

Table 29: Data from European Solvent Extraction GC-MS Method

		PCH mean conc. in ppm				
		AVEBE Netherlands		**AVEBE Sweden**		**ISSI NJ**
Sample	*	*In-matrix*	*Without matrix*	*In-matrix*	*Without matrix*	*In-matrix*
A	0	0.3	0.2	0.22	0.17	0.00
A	0	0.5	0.35	0.21	0.16	0.00
B	0.5	0.75	0.6	0.26	0.21	0.34
B	0.5	0.7	0.6	0.28	0.24	0.32
C	1	1.1	0.95	0.32	0.26	0.70
C	1	1.1	0.95	0.34	0.3	0.57
D	1.5	1.8	1.6	0.38	0.31	0.95
D	1.5	1.65	1.45	0.35	0.27	1.18
E	2.71	1.89	1.79	1.32	1.31	2.01
E	2.71	1.26	1.18	1.57	1.43	1.99
F	0.61	0.32	0.29	0.4	0.38	0.24
F	0.61	0.38	0.34	0.44	0.42	0.27
G	0.89	0.74	0.69	0.8	0.86	0.29
G	0.89	0.82	0.77	0.72	0.76	0.42
H	0.15	0.08	0.06	0.23	0.17	0.00

H	0.15	0.1	0.07	0.23	0.18	0.09
I	0.06	0.1	0.08	0.27	0.22	0.11
I	0.06	0.1	0.08	0.26	0.21	0.09
J	0.21	0.09	0.07	0.24	0.19	0.00
J	0.21	0.1	0.08	0.25	0.2	0.00
K	1.13	1.51	1.43	1.53	1.63	1.08
K	1.13	1.35	1.28	1.18	1.24	1.48
L	0.25	0.26	0.23	0.48	0.42	0.19
L	0.25	0.22	0.19	0.46	0.44	0.19

* Sample A-D: pre-spiked starch samples

Sample E-L: commercial starch samples were pre-tested with the European proposed MEOH extraction- GC- MS method by AVEBE Netherlands

Pre-spiked samples E-L were used for recovery calculation. The recovery data are shown in Table 30, 31.

Table 30: Recovery of EU Method Based on Pre-spiked Samples

	AVEBE Netherlands		AVEBE Sweden		ISSI
	Without matrix	In-matrix	Without matrix	In-matrix	In-matrix
0.5ppm	63%	68%	12%	11%	66%
1ppm	66%	71%	11.5%	11.5%	64%
1.5ppm	82%	88%	8.3%	10%	72%

Table 31: Recovery of P&T-TD-GC-MS Method Based on Pre-spiked Samples

	MEOH extraction				Water extaction			
	In-matrix		Without matrix		In-matrix		Without matrix	
	TIC	SIM	TIC	SIM	TIC	SIM	TIC	SIM
0.5ppm	141%	137%	90%	106%	114%	103%	69%	56%
1ppm	129%	134%	77%	100.5%	91.5%	91.5%	50%	47.5%
1.5ppm	142.3%	132.7%	83%	94.3%	80.3%	104%	42%	53.7%

As it can be concluded from the recovery data, values obtained from the European proposed methanol extraction GC-MS method shows an underestimation compared to the pre-tested values. Using the methanol extraction-P&T-TD-GC-MS method (with in-matrix calibration) shows a highly overestimation compared to the pre-tested values

from the European proposed methanol extraction-GC-MS method. A possible explanation could be a co-extraction of compounds that have a similar retention time in the applied GC system. Possible way to modify this situation would be to use m/z=79 instead of m/z=45 for PCH1 quantification, m/z=65 instead of m/z=58 for PCH2 quantification. With the water extraction- P&T-TD-GC-MS method, based on in-matrix calibration, a decent recovery of PCH compounds was achieved compared to the pretested values with the European proposed methanol extraction-GC-MS method. Both in-matrix calibration and calibration without starch matrix were utilized to calculate the commercial starch samples that were provided. The results showed a pronounced difference, as it evident in Table 31. The in-matrix extraction procedure was conducted in a similar extraction environment as was done for the starch samples and was more representative of the real sample analysis.

Figure 24 compared the results obtained from the commercial starch samples (E-L) using EU Proposed MEOH extraction-GC-MS method with the pretested values provided. As can be seen, the shape of fold lines from all 3 parties that employed the EU proposed MEOH extraction-GC-MS method based on the pre-tested values. It shows that different calibration method (in-matrix or without matrix) would not lead to a pronounced difference in the results obtained. Similar plots of the results based on the P&T-TD-GC-MS using methanol and water are given in Figure 25 and 26, respectively. The matching of fold lines in both figures was poor. This means that irrespective of whether methanol or water was used for extraction, whether the calculation of the PCH

values based on the in-matrix or without matrix equations, or whether the areas were determined by total ion current or the selected ion current, consistent results by the P&T-TD-GC-MS method cannot be obtained.

It was agreed that Rutgers's results have strengthened the confidence that PCH levels are as found by the EU method. In order to further assess the accuracy of European Proposed MEOH extraction-GC-MS method, several adjustments have been proposed, such as modifying the calibration range, and conducting method validation tests.

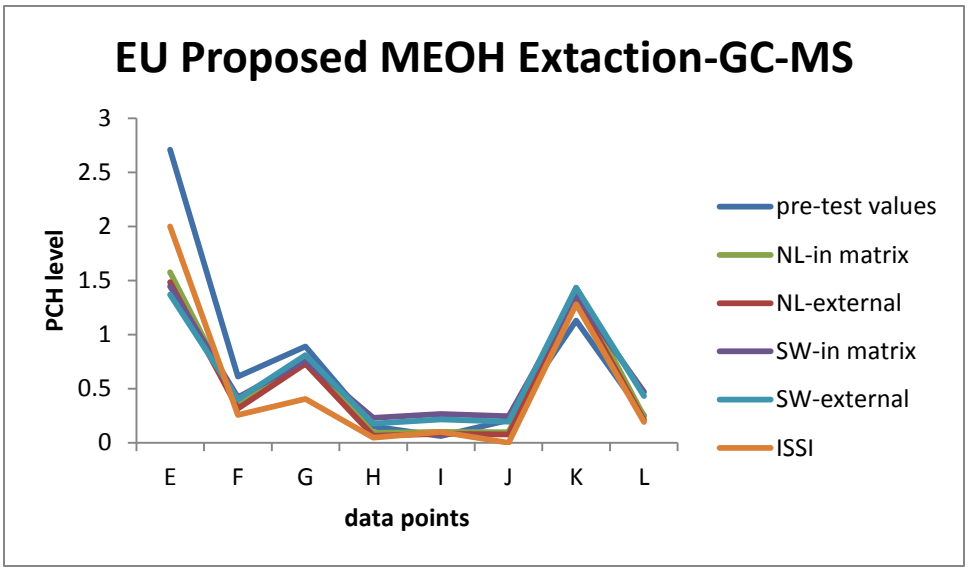

Figure 24: Comparison of Commercial Samples with EU Proposed MEOH Extraction-GC-MS Method

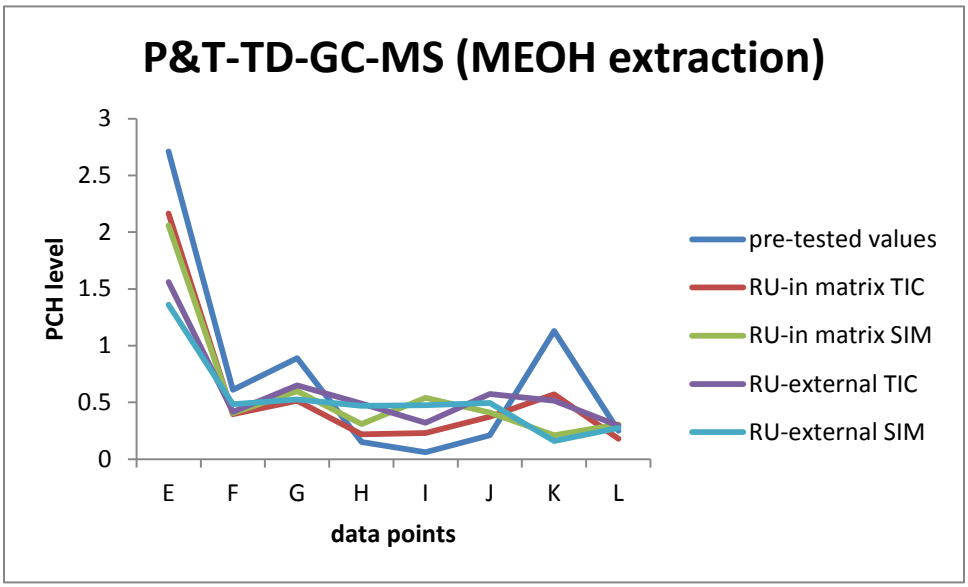

Figure 25: Comparison of Commercial Samples with MEOH Extraction-P&T-TD-GC-MS Method

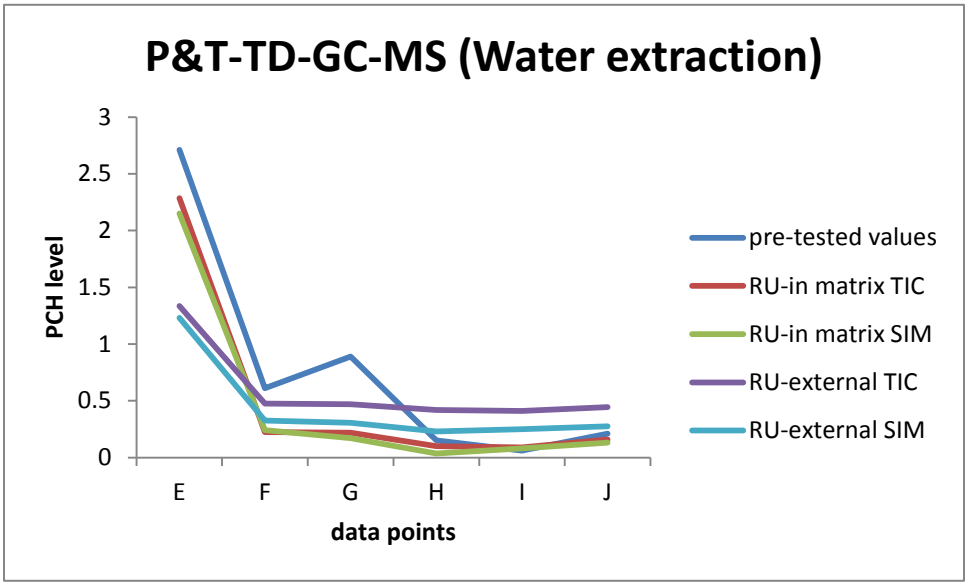

Figure 26: Comparison of Commercial Samples with Water Extraction-P&T-TD-GC-MS Method

9. P&T-TD-GC-MS Method Adoption in Quantifying PCH in Fumigated Almond

Sliced almond samples, 12-1, 12-3, HJ1 (Campos Brothers Farms, Caruther, CA) were used to quantify PCH content. In the analyses, 10g of each sample was and then rapidly sealed into a purge and trap apparatus. The sample as purged at 100℃ with nitrogen at a flow rate of 50ml/min for 30min into a desorption tube containing Tenax, previously, spiked with internal standards (10.0ug of benzene-d6, toluene-d8 and naphthalene-d8) for quantification purpose. The charged adsorbent trap was then connected to short path thermal desorption system and thermally desorbed (at 250℃ for 5min) into the GC-MS.

The results indicated that samples 12-3 and HJ1 had been treated with PPO as PCH and PBH were found. PBH are generally not considered as residues by the EPA, because PBH are present at very low concentrations in commodities at the time of consumption. Table 31 shows PCH concentrations obtained. Figure 24 and 25 show the chromatograms of the samples 12-3 and HJ1, respectively. In order to apply this P&T-TD-GC-MS method in quantifying PCH in fumigated almonds sample, method validation tests as described for the hydroxypropyl starch samples are needed.

Table 32: PCH Concentration (ppm) in Sliced Almond Sample 12-3 and HJ1

	PCH1 Conc. (ppm)	PCH2 Conc.(ppm)
12-3	0.799	0.035
HJ1	0.056	--

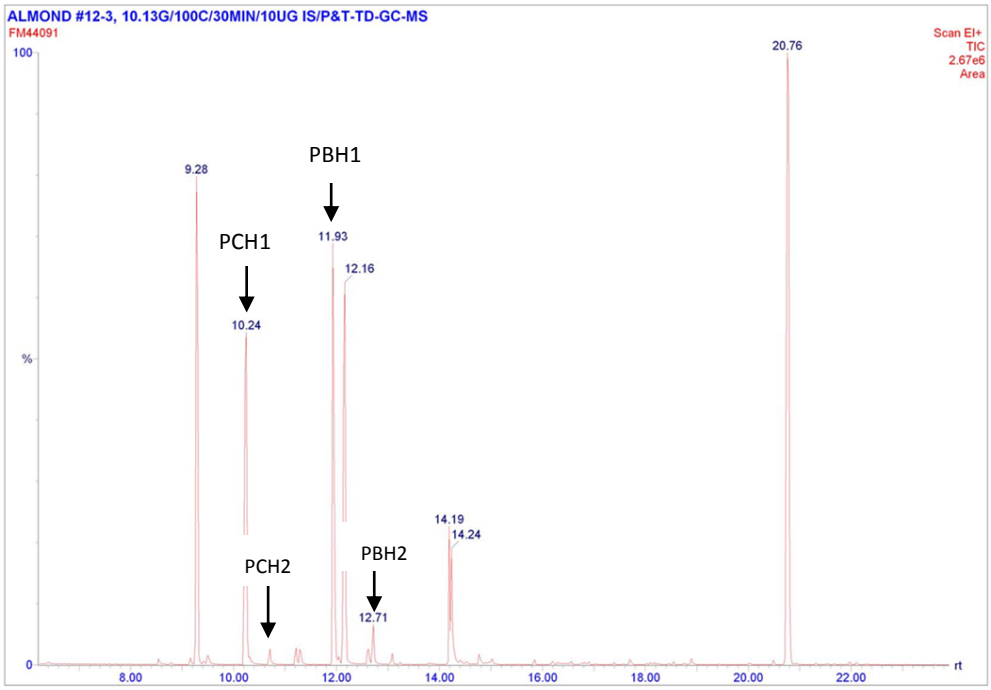

Figure 27: GC Chromatogram of Almond Sample 12-3 (*I.S.1: d-6 benzene; I.S.2: d-8 toluene; I.S.3: d-8 naphthalene)

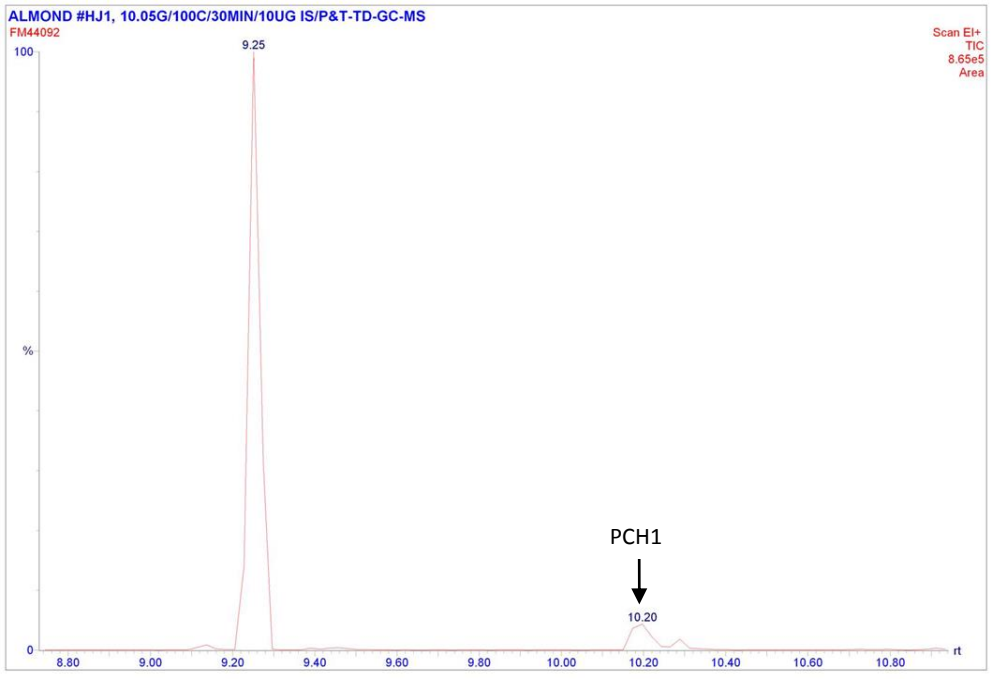

Figure 28: GC Chromatogram of Almond Sample HJ1 (*I.S.1: d-6 benzene)

10. Conclusion

The P&T-TD-GC-MS method for PCH quantification in hydroxypropyl starch and PPO fumigated almond has been developed. And the method has been validated on its application in PCH quantification from the hydroxypropyl starch matrix.

The method meets the expectations described in the objective section. Firstly, the method use reduced amount of solvent and starch sample compared with the European proposed MEOH extraction- GC- MS method and the certified international method by JECFA; consequently lowing the cost for PCH analysis in hydroxypropyl starch. This P&T-TD-GC-MS method uses 4 times reduced starch sample size and solvent volume compared with the European proposed MEOH extraction- GC- MS method, and 25 times reduced starch sample size compared to the certified international method for PCH quantification. 3-chloro-1-propanol was analyzed to replace chlorobenzene as a more suitable internal standard in this study. In addition to quantify PCH in hydroxypropyl starch matrix, by modification, the method is able to be applied in quantifying PCH in PPO- fumigated almond sample. Moreover, the method is able to quantify PCH at trace level and successfully met the detection limit requirements of US and Europe regulations.

The overall validation data suggest the method is precise and rugged. Linear calibration curves (R^2> 0.99) can be achieved in a dynamic range of PCH from 0.1 to 10 ppm (nominal to 2 g of starch on dry bases). This method has an LOD of 0.025 mg/kg for both PCH1 and PCH2, which meets the detection limit regulated by the US and Europe regulations. The analytical system precision, method precision, and between

batch precision were below 10 % RSD with an exception of 14.6% RSD for PCH2 in method precision test. An RSD value below 10% means good precision. However, considering that method precision test conducted in real starch sample matrix, 14.6 %RSD is acceptable.

In the method development process, two salts (sodium chloride and sodium sulfate) were compared as the P&T absorption salt matrix with their effects on PCH content. No significant difference of PCH content observed between using NaCl and Na_2SO_4 with MEOH extraction. With water extraction, using NaCl as salt matrix result in a lower PCH content value compared to using Na_2SO_4 or compared to using MEOH extraction. Based on this test, sodium sulfate was adopted as P&T absorption salt matrix in the ring test.

In the ring test, it was agreed that Rutgers results have strengthened the confidence that PCH levels are as found by the EU method. In order to further assess the accuracy of European Proposed MEOH extraction-GC-MS method, several adjustments have been proposed, including modifying the calibration range, conducting method validation tests etc.

The introduce level of the P&T-TD-GC-MS method is 200ul, which is considered to be 100 times more concentrated compare to the EU proposed MEOH extraction-GC-MS method, in which the spiked amount is 2ul. Thus, the P&T-TD-GC-MS method is approximately 100 times more sensitive than the European proposed MEOH extraction-GC-MS method, allowing for full-scan mass confirmation in

contrast to the European method that uses selected ion monitoring. And the method also eliminates the usage of a PTV injector which is mandatory in the proposed European solvent extraction-GC-MS method.

In all, the study establishes an alternative method to quantify PCH in hydorxyproyl starch and has been modified and applied in quantifying PCH in PPO-fumigated almond. The method is expected to be used in future routine analyses of PCH in hydroxypropyl starch and provide valuable guidance to the modified starch manufacturers on producing hydroxypropyl starch and hydroxypropyl distarch phosphate in keeping PCH residues within the acceptable regulated levels.

Reference

Benson, L. and Teta, M.J. (1993) *Mortality due to Pancreatic and Lymphopoietic Cancers in Chlorohydrin Production Workers*. British Journal of Industrial Medicine **50**:7.

Biles, R.W. and Piper, C.E. (1983) *Mutagenicity Of Chloropropanol In A Genetic Screening Battery.* Fundamental and Applied Toxicology **3**:7.

Breuninger, W. F., et al. (2009) *Tapioca/ Cassava Starch: Production and Use.* In: BeMiller J, Whistler R (eds) Starch: Chemistry and Technology.

Bruch, C.W. and Koesterer, M. G. (1961) *The Microbicidal Activity of Gaseous Propylene Oxide and Its Application to Powdered or Flaked Foods*. Journal of Food Science **26** (4):8.

Cannon, W. A. (1950) *Simultaneous Determination of Ethylene and Propylene Chlorohydrins*. Analytical Chemistry **22** (7):2.

Carr, H.S. and Rosenkranz, H.S. (1978) *Interference of Derivatives of the Flame Retardant Tris(2,3-dibromopropyl) Phosphate: Halogenated Propanols.* Mutation Research **57**:4.

Chairman, L.J.(1971) *Modified Food Starches for Use in Infant Foods*. Nutrition Reviews **29**(3):5.

Chlebicki, J. (1975) *Kinetics and Mechanism of the Propylene Oxide Reaction with Alcohols in the Presence of Base Catalysts*. Rocz Chem:**49.**

Code of Federal Regulations, Title 21 (2013). vol 3.

D'Ercole, A.D. (1972) *Process of Preparing Frozen Pudding Composition.*US Patent 3,669,687.

Environmental Protection Agency (2011) *Propylene Oxide; Proposed Tolerance Actions.*

Environmental Protection Agence (2006) *Reregistration Eligibility Decision for Propylene Oxide* .

Food Additives in Europe 2000: Status of Safety Assessments of Food Additives Presently Permitted in EU (2002).

Food Chemicals Codex (1996). National Academy of Sciences, Washington DC.

Glyn, O. and Phillps, P.A.W. (2000) *Handbook of Hydrocolloids.*

Griffith, T. and Warren, M. (2001) *Propylene Oxide, a Registered Fumigant, a Proven Insecticde.* Paper Presented at the Proc. Int. Conf. Controlled Atmosphere and Fumigation in Stored Products, Fresno, CA.

Gustavo González, A. and Ángeles Herrador, M. (2007) *A Practical Guide to Analytical Method Validatio*n, *Including Measurement Uncertainty and Accuracy Profiles.* TrAC Trends in Analytical Chemistry **26** (3):227-238. doi:10.1016/j.trac.2007.01.009.

Hartman, T.G., et al. (1993) *Flavor Characterization Using Adsorbent Trapping- Thermal Desorption or Direct Thermal Desorption- Gas Chromatography- Mass Spectrometry.* In: Ho C-T, Manley CH (eds) Flavor Measurement Marcel Dekker Inc.

Hartman, T.G., et al.(1991) *Short Path Thermal Desorption: Food Science Applications*. Food Technology **45** (7):2.

Hjermstad (1984) *Production and uses of hydroxypropyl starch*. In: BeMiller J, Whistler R (eds) Starch Chemistry and Technology. 2nd edition Academic Press Inc., Sao Paulo, p 10.

Hunt, A., et al. (2009) *Roles of Starch in Surimi Seafood: A Review*. Food Reviews International **25** (4):299-312. doi:10.1080/87559120903155834.

Hyman, J., et al. (1980) *The E.coli Pol A1- Assay: A Quantitative Procedure for Diffusible and Non-diffusible Chemicals.* Mutation Research **74**:5.

Isikber, A.A., et al.(2006) *Propylene Oxide as a Potential Quarantine Fumigant for Insect Disinfestation of Nuts*. Paper Presented at the 9th International Working Conference on Stored Product Protection, Sao Paulo, Brazil.

Kavlani Neelam, et al. (2012) *Various Techniques for the Modification of Starch and the Applications of Its Derivatives International Research*. Journal of Pharmacy **3** (5):7.

Kesler, C.C. and Carey, P. (1970) *Sugar Products Prepared from Hydroxypropylated Starch.* US Patent.

Kruger, L.H. and Rutenberg, M.W. (1967) *Production and Uses of Starch Acetates.* In Starch: Chemistry and Technology **2**.

Leegwater, D.C., et al. (1973) *Analysisi of an Acid Hydrolysate of a Low-substitited Hydroxypropyl Starch*. Die Stärke:**25**.

Mitan F.J. (1969) *Process of Coating Food*. US Patent 3,427,951.

Modified Starch. In: 57th Meeting of JECFA, 2001. p 19.

National Academy of Science (1978) *Review of Safety and Suitability of Modified Food Starches in Infant Food.* Under FDA contract 223-76-2061.

New Jersey Department of Health and Senior Service (2004) *1-Chloro-2-Propanol Hazardous Substance Fact Sheet.*

Olsen, G.W., et al.(1997) *Mortality from Pancreatic and Lymphopoietic Cancer among Workers in Ethylene and Propylene Chlorohydrin Production.* Occupational and Environmental Medicine **54** (8):592-598.

Otto.B.Wurzburg (1995) *Modified food starches*. In: Food Additive Toxicology.

Pfeiffer, E.H. and Dunkelberg, H. (1980) *Mutagenicity of Ethylene Oxide and Propylene Oxide and of the Glycol and Halohydrins Formed Fromed Them during the Fumigation of Food Stuff.* Food and Cosmetics Toxicology **18**:4.

Ph. Hubert, et al.(2003) *Validation of Quantitative Analytical Procedure.* Harmonization of Approaches STP Pharma Pratiques **13** (3):38.

Quarles, J.M. and Alexander, R.J. (1992) *Hydroxypropyl Starch Hydrolyzate Product*. United States Patent.

Ragelis, E.P., et al. (1966) *Note on Determination of Chlorohydrins in Food Fumigated with Ethylene Oxide and Propylene Oxide.* Journal of the Association of Official Analytical Chemists **49** (5):3.

Ragelis, E.P., et al. (1968) *Isolation and Determination of Chlorohydrins in Food Fumigated with Ethylene Oxide or with Propylene Oxide*. Journal of the Association of Official Analytical Chemists **51** (3):7.

Rosenkranz, H.S., et al.(1975) *Chloropropanol, a Mutagenic Residue Resulting from Propylene Oxide Sterilization*. Mutation Research **30**:2.

Rutenberg, Solarek (1984) *Starch Derivatives: Productions and Uses.* In: Whistler R, BeMillar J (eds) Starch Chemistry and Techonology Academic Press Inc., Orlando, p 55.

Shimotoyodome, A., et al.(2004) *Hydroxypropylated Starch for Preventing/treating Diabetes*. Europe Patent 2308320A2.

Smyth, H.F., et al.(1962) *Range Finding Toxicity Data: List VI.* American Industrial Hygiene Association Journal:**13**.

Steele, L. and Hadziyev, D. (1976) *Sterilization of Dehydrated Potato Granules with Propylene Oxide.* European Food Research and Technology **162**:13.

Theiss, J.C., et al. (1979) *Introduction of Pulmonary Adenomas in Strain A Mice by Substituted Organohalides*. Cancer Research **39**:5.

Thompson, M., et al.(2002) *Harmonized Guidelines for Single-laboratory Validation of Methods of Analysis*. Pure Appl Chem **74**:21.

Tsuzuki T (1968) *Hydroxypropyl Starch Ether*. US Patent 3,378,546.

Wesley, F., et al.(1965) *The Formation of Persistent Toxic Chlorohydrins in Foodstuffs by Fumigation with Ethylene Oxide and Propylene Oxide*. Journal of Food Science **30** (6):6.

Wurzburg O.B., et al. (1986) *Modified Starches: Properties and Uses.*

Xu, A. and Seib, P.A. (1997) *Determination of the Level and Position of Substitution in Hydroxypropylated Starch by High-Resolution H-NMR Spectroscopy ofAlpha-limit Dextrins.* Journal of Cereal Science **25**:10.

Yang, R.S.H. (1987) *Propylene Chlorohydrins: Toxicology, Metabolism, and Environmental Fate.* Reviews of Environmental Contamination and Toxicology **99** (13):47