ABSTRACT

FAN, XINYUE. Cucurbitacins in Bitter and Pickling Cucumber and Cucurbitacin C Reduction during Cucumber Fermentation and Acidification. (Under the direction of Dr. Suzanne Johanningsmeier and Dr. Jonathan Schultheis).

Cucurbitacins are a group of triterpene compounds found in nature, most commonly in cucurbitaceous plants. In cucumber plants, the occurrence of cucurbitacins could result in bitter cucumber, which can result in significant product loss. In recent years, many researchers studied cucurbitacins biosynthesis, occurrence and bioactivities because of potential medicinal and toxic characteristics. However, no research studied cucurbitacin stability during cucumber fermentation and acidification. The goal of this project was to develop sample preparation, quantitative and qualitative methods for cucurbitacin analysis in the pickling cucumber, and determine the stability of cucurbitacin C during cucumber fermentation and acidification.

In the first study, five cucurbitacins or isomers were tentatively identified in fresh pickling cucumber fruits, leaves or peels by liquid chromatography time-of-flight mass spectrometry. All samples were freeze-dried and extracted with methanol. Identification of cucurbitacins was performed by comparing the exact mass to the theoretical mass of expected adducts, both in the positive and negative ionization mode in combination with UV absorbance at 230 nm. Cucurbitacins B, C or Q, and C glycoside were detcted in cucumber leaves. Cucurbitacins C or Q, F or O, and P were found in pickling cucumber fruits purchased from a local market, and cucurbitacins F or O, and P were observed in cucumber peel of a separate lot of commercial pickling cucumbers. This method was sensitive enough to detect cucurbitacins in a typical pickling cucumber cultivar and fewer interfering ions were observed in negative ionization mode.

In the second study, bitter (Hanzil) and non-bitter (Vlaspik) pickling cucumber were grown under controlled greenhouse conditions with multiple harvests (n = 5) and/or greenhouse areas (n=3) as replications. Temperature ranged between 30 °C during the day to 18 °C at night. Cucumbers were cut longitudinally and each section allocated to “raw,” “fermented,” and/or “acidified” treatments. Acidified cucumber was preserved with 3% sodium chloride (NaCl), lactic acid (110 mM) and sodium benzoate (12 mM). Naturally fermented cucumber was brined (3% NaCl) and incubated at 28°C for 14 days. Samples were stored at -80°C, freeze-dried, and extracted with methanol for cucurbitacin analysis by liquid chromatography time-of-flight mass spectrometry. Cucurbitacins C, C-glycoside, F or O, and P were tentatively identified in cucumber fruits by accurate mass in the negative ionization mode. Cucurbitacin C was also confirmed and quantified with an authentic standard. Hanzil contained primarily cucurbitacin C (13.31 ppm -110.42 ppm), which was not detected in Vlaspik even under simulated drought. In Hanzil, cucurbitacin C concentration increased with cucumber size, ranging from 27.99 ± 7.67 ppm in size 2A to more than 74 ppm in size 4+ fruits. Structurally, the endocarp had the highest cucurbitacin C concentration (88.66 ± 44.33 ppm) followed by the mesocarp (30.61 ± 15.11 ppm) and the exocarp (2.58 ± 1.75 ppm), which likely explains the increase in overall cucurbitacin content with increasing cucumber size. Cucurbitacin C was 3-fold lower in acidified cucumber (15.65 ± 10.33 ppm) and 10-fold less in fermented cucumber (4.90 ± 3.94 ppm) than in fresh cucumber (47.90 ± 22.79 ppm).

This research could provide the pickle industry an analytical method for cucurbitacin analysis if they have bitter pickle products. Also, the stability of cucurbitacin C during cucumber fermentation and acidification was determined in this research, which serves as the basis to further study whether fermentation could be used as a de-bittering method for bitter pickling

cucumber. This research also determined which part is the best source of cucurbitacin C in cucumber fruits to further explore its bioactivities and toxicities.

Cucurbitacins in Bitter and Pickling Cucumber and Cucurbitacin C Reduction during Cucumber Fermentation and Acidification.

by
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APPROVED BY:

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DEDICATION

To my parents, Changbin Fan and Yajuan Yang, for their over twenty years unconditional support of me.

To my future myself, never give up. Persistence can make failures into miracles, which is one of the most important things that I have learned when I was 21 to 24 years old.

BIOGRAPHY

Xinyue Fan was born on May 9, 1996 to Changbin Fan and Yajuan Yang. She attended Beijing University of Agriculture in China in 2014 for her bachelor's degree. During her undergraduate studies, her research focused on the yeast varieties on the quality of wine from Huailai Wine Region under the supervision of Prof. Demei Li. She also contributed to the development of quinoa milk beverage and probiotic chocolate. Besides research, she was actively involved in the College of Food Science and Engineering at BUA. She served as the president of class of 2014 and vice president of the press center. She was awarded the Third-class Academic Scholarship three times and Great Northern Agriculture Scholarship twice during the time at BUA. In July 2018, she earned her Bachelor of Engineering degree as an outstanding graduate in Beijing due to her excellent undergraduate work. Then, she came to the USA to pursue a Master's degree at North Carolina State University. She is conducting her graduate research under the supervision of Dr. Suzanne Johanningsmeier in the USDA-ARS Food Science and Market Quality &Handling Research Unit in Raleigh.

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CHAPTER 1. Review of biosynthesis, bioactivities and occurrence of cucurbitacins in cucumber

1.1 Abstract

Cucurbitacins are a group of triterpene compounds found in nature, most commonly in cucurbitaceous plants. In cucumber plants, the occurrence of cucurbitacins could result in bitter cucumber, which causes consumers' complaints and significant product loss. With the rapid development of analytical methods and genetic technologies, many researchers studied the biosynthesis, occurrence and bioactivities of cucurbitacins in recent years. These have been studied because of their complex chemical structure and potential medicinal and toxic characteristics. However, due to the structural diversity and low concentrations in plants, it is hard to obtain authentic standard compounds to explore their characteristics, which presented difficulties in testing their toxicity and bitterness level. This article reviews current research on occurrence and biosynthesis of cucurbitacins in cucumber plants as well as cucurbitacin toxicity and bioactivities. Analytical chemistry methods in cucurbitacin analysis and bitter perceptions in food were also explored in this review. This review aims to provide information as the basis for future research on analyzing cucurbitacins in cucumber fruits and studying cucurbitacins stabilities during cucumber fermentation and acidification.

1.2 Occurrence of bitterness in cucumber

Pickling cucumber is used for making pickles in the United States. Some pickle producers reported that bitter cucumbers were occasionally found in their product, which caused some consumer complaints and loss of plenty of pickle products. According to Shang et al. (2014), a class of compounds called cucurbitacins is the main cause of bitterness in cucumbers. Cucurbitacins are triterpenoids that are widely distributed in cucumber, melon and pumpkin (Chen et al., 2005). Research by Hideki et al. (2007) reported that cucumbers contained only one form of cucurbitacins, cucurbitacin C. They isolated cucurbitacin C from cucumber plants to investigate the relationship between its content and bitterness of the plant parts. They determined that the stem end was richer in cucurbitacin C and was more bitter than other parts of the fruit. Their results showed that one cucumber fruit (about 100 g) contained less than 1 mg cucurbitacin C (10 ppm), while the threshold level for bitterness was less than 0.1 mg/L (0.1 ppm) of cucurbitacin C in aqueous solution. These authors concluded that cucurbitacin C concentration determines the bitterness of cucumber. Cucurbitacin C was first identified in a special variety, *Cucumis sativus var.* Hanzil (Enslin et al., 1960). Cucurbitacin C concentration was quantified in different parts of cucumber plants (Qing et al., 2014). Cucurbitacin C was not detected in non-bitter fruits, female flowers, roots and leafstalks. Cucurbitacin C was detected in leaves and the stem with the young leaves having the highest amount of cucurbitacin C (510 ppm). Cucurbitacins A, B, C, D, E and I were identified in cotyledons of different varieties of cucumber seedlings (Mukherjee et al., 2013). More than one cucurbitacin might be present in a cucumber plant. Beyond the presence of cucurbitacins on the plant vegetation, it is important to study the occurrence of cucurbitacins in pickling cucumber fruit to better understand their relationship to bitterness of pickle products.

The occurrence of bitter fruit is influenced by several factors, including low temperature, drought, genetics and fertilizer application. Research by Kano et al. (2003) determined that low temperature, a high nitrogen rate (two times the usual application) and a bitter genetic line caused bitterness in cucumbers. All three of these factors resulted in more total nitrogen, amino-acid nitrogen and protein in bitter cucumber than non-bitter cucumber. The bitterness of these cucumbers may relate to a compound called cucurbitacin C, which is synthesized from mevalonic acid via 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA). HMG-CoA reductase activity was higher in bitter than non-bitter cucumber. In a few words, the occurrence of bitterness in cucumber was associated with active protein synthesis which led to high activity of HMG-CoA reductase. HMG-CoA reductase can catalyze the synthesis of cucurbitacin C, which led to the occurrence of bitter cucumber (Figure 1.1) (Kano et al., 2003). Research by Shang et al. (2014) also found that fruits become bitter when grown at low temperature and was influenced at the genetic level. When cucumbers were grown under low temperature, a single-nucleotide polymorphism (SNP)-1601 was essential for regulating Bi gene (bitter gene) expression. A natural mutant corresponding to SNP-1601 showed that its fruits were nonbitter even under such low temperature conditions (18℃ day, 12℃ night). In 2018, hydrogen sulfide (H_2S) was determined as a key intermediary to induce cucurbitacin C formation in cucumber under low growing temperature (Liu et al., 2019). The low temperature increased the content and production rate of H_2S which helps cucumber plants resist stress. The H_2S upregulated the gene encoding (Bi gene) cucurbitacin C synthetase thus increasing cucurbitacin C generation.

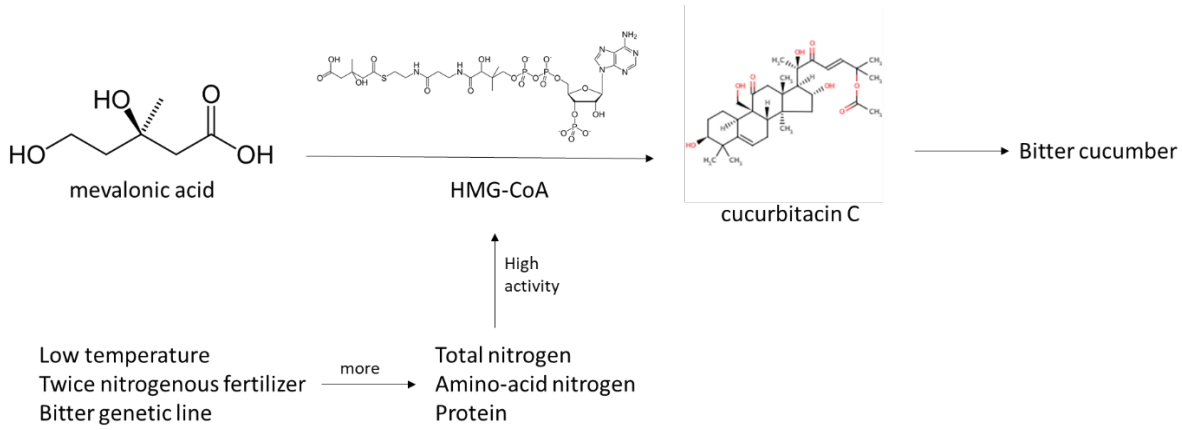


Figure 1.1. Factors effecting the occurrence of bitter cucumber and proposed mechanism.

1.3 Structure and chemical properties of cucurbitacins

Cucurbitacins are composed of a variety of triterpenoid structures. The basic structure of cucurbitacins is named 19-(10→9b)-abeo-10alanost-5-ene (Figure 1.2). In general, cucurbitacins are known for bitterness, toxicity and medical functions and can be isolated from plants and fruits in the Cucurbitaceae family. Interestingly, cucurbitacins were also recently isolated from mushroom and shell-free marine mollusks (Chen et al., 2005). In contrast to the typical bitterness, a kind of cucurbitacin called Siamenoside I was found to have sweet flavor which can be a potential sugar substitute for diabetics (Chen et al., 2005). This compound has four glucosides linked on side chains. Cucurbitacins are soluble in petroleum ether, chloroform, benzene, ethyl acetate, methanol and ethanol, but are insoluble in ether and only slightly soluble in water (Kaushik et al., 2015). The absorption maxima for ultraviolet light of cucurbitacins is between 228-234 nm due to the $\alpha, \beta -$ unsaturated ketones in side chain or in the cucurbitane skeleton (Kaushik et al., 2015).

Traditionally, cucurbitacins were divided into twelve categories, incorporating cucurbitacins A–T (Chen et al., 2005). Cucurbitacin C and Q, D and L, F and O, J and K are three groups of isomers, meaning these cucurbitacins have the same molecular formulas but

different structures. Besides the structural isomers, diversity in side chain groups is another consideration (Figure 1.3). For example, cucurbitacin A has the same structure as cucurbitacin B with an additional hydroxyl group on C-19. Cucurbitacin D has a hydroxyl group on C-25 instead of an acetyl group at the same position in cucurbitacin B. In addition, the position of double bonds play an important role in making different cucurbitacins. Cucurbitacins E, I, J, K, and L have a double bond between C-1 and C-2 while cucurbitacins A, B, C, and D have a single bond linking those two carbons (Gry et al., 2006). Additional research is needed to determine the influence of structure on toxicity and biological activities.

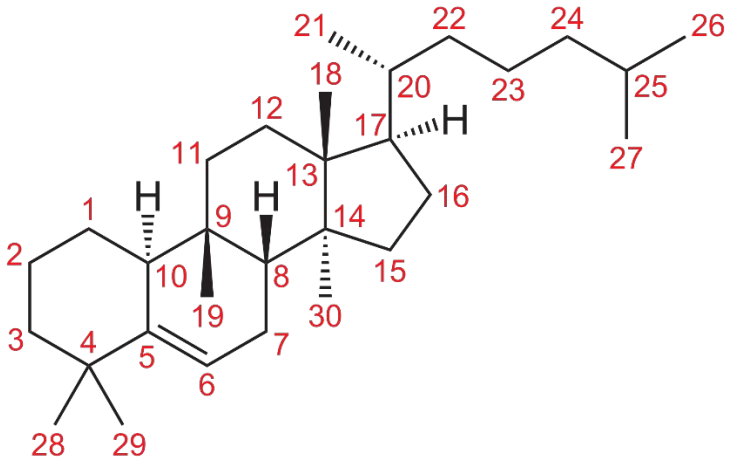
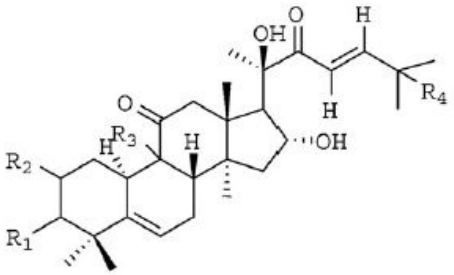
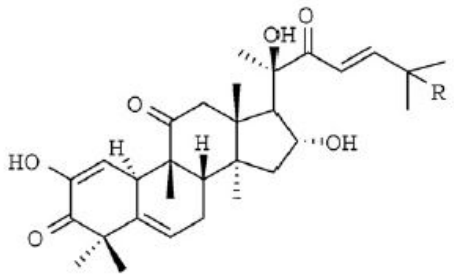


Figure 1.2. Basic structure of cucurbitacin.



(1)	Cucurbitacin A	R_1 = =O	R_2 = -OH	R_3 = $-CH_2OH$	R_4 = -OC OC H_3
(2)	Cucurbitacin B	R_1 = =O	R_2 = -OH	R_3 = -H	R_4 = -OC OC H_3
(3)	Cucurbitacin C	R_1 = -OH	R_2 = -H	R_3 = $-CH_2OH$	R_4 = -OC OC H_3
(4)	Cucurbitacin D	R_1 = =O	R_2 = -OH	R_3 = -H	R_4 = -OH



(5)	Cucurbitacin E	R = -OC OC H_3
(6)	Cucurbitacin I	R = -OH

Figure 1.3. Structure of cucurbitacins A, B, C, D, E and I (Mukherjee et al., 2013).

Table 1.1. Cucurbitacin formulas, molecular weights and masses.

Cucurbitacin	MW (g/mol)	Chemical formula	Monoisotopic mass	References
A	574.7022	$C_{32}H_{46}O_9$	574.3142	Kaushik et al. (2015)
B	558.7028	$C_{32}H_{46}O_8$	558.3193	Bajcisik et al. (2017)
C	560.7187	$C_{32}H_{48}O_8$	560.3349	Jevtic et al. (2017)
D	516.6662	$C_{30}H_{44}O_7$	516.3087	Kaushik et al. (2015)
E	556.6870	$C_{32}H_{44}O_8$	556.3036	Bajcisik et al. (2017)
F	518.6820	$C_{30}H_{46}O_7$	518.3244	Jevtic et al. (2017)
G	556.7285	$C_{30}H_{52}O_9$	556.3611	Kaushik et al. (2015)
H	534.6814	$C_{30}H_{46}O_8$	534.3193	Kaushik et al. (2015)
I	514.6503	$C_{30}H_{42}O_7$	514.2931	Bajcisik et al. (2017)
J	532.6656	$C_{30}H_{44}O_8$	532.3036	Kaushik et al. (2015)
K	532.6656	$C_{30}H_{44}O_8$	532.3036	Kaushik et al. (2015)
L	516.6662	$C_{30}H_{44}O_7$	516.3087	Kaushik et al. (2015)
O	518.6820	$C_{30}H_{46}O_7$	518.3244	Kaushik et al. (2015)
P	520.6979	$C_{30}H_{48}O_7$	520.3400	Kaushik et al. (2015)
Q	560.7187	$C_{32}H_{48}O_8$	560.3349	Kaushik et al. (2015)
S	498.6509	$C_{30}H_{42}O_6$	498.2981	Kaushik et al. (2015)
E glycoside	718.8276	$C_{38}H_{54}O_{13}$	718.3564	Bajcisik et al. (2017)
C glycoside	722.8593	$C_{38}H_{58}O_{13}$	722.3877	Zhong et al. (2017)
I 2-glucoside	676.7909	$C_{36}H_{52}O_{12}$	676.3459	Metlin

1.4 Biosynthesis and metabolism of cucurbitacin C in cucumber plants

In 2014, research by Shang et al. identified biosynthesis of cucurbitacin C in cucumber at the genetic level. There are nine cucumber genes in the biosynthesis pathway for cucurbitacin C and four catalytic steps. There are two genes, Bl (Csa5G1562200) and Bt (Csa5G157230), which control the cucurbitacin pathway in leaves and fruits, and one gene, Bi, that regulates cucurbitacin C biosynthesis in the entire plant. The Bl gene regulates cucurbitacin C biosynthesis by activating transcription of Bi in cucumber leaves (Figure 1.4), while the Bt gene regulates cucurbitacin C biosynthesis by activating transcription of Bi in cucumber fruit (Figure 1.4). Nine genes in the cucurbitacin C biosynthetic pathway were identified, including one oxidosqualene cyclase (OSC), seven cytochrome P-450 enzymes (P 450s) and one acyltransferase. The first step in cucurbitacin C biosynthesis is Bi encoding a cucurbitadienol synthase that catalyzes the cyclization of 2,3-oxidosqualene into the tetracyclic cucurbitane skeleton. The other three steps of cucurbitacin C biosynthesis related to three of these genes, Csa3G903540, Csa6G088160 and Csa6G088700. The products of Csa3G903540 and Csa6G088160 were named as 19-hydroxy cucurbitadienol and 19,25-dihydroxy cucurbitadienol, respectively. Finally, the Csa6G088700 gene encodes an acyltransferase (ACT) enzyme that can acetylate deacetyl-cucurbitacin C to yield cucurbitacin C (Figure 1.5) (Shang et al., 2014). A similar biosynthesis pathway was also found in melon (*Cucumis melo*) and watermelon (*Citrullus lanatus*) for cucurbitacins B and E, respectively (Zhou et al., 2016). The gene annotations and orientations within chromosomes 6 and 11 are exactly the same as those of Bi clusters. In addition, they found that both cucurbitacin B and cucurbitacin E had the same first biosynthetic steps as cucurbitacin C. Acyltransferases also acetylate the cucurbitacin precusors, cucurbitacin D and cucurbitacin I, in the production of cucurbitacin B and cucurbitacin E, respectively, which is similar to the final step of cucurbitacin

C biosynthesis from deacetyl cucurbitacin C in cucumber. The other cytochrome P450s were responsible for tailoring of the cucurbitacin core skeleton, resulting in the key structural variations among cucurbitacins C, B and E in cucumber, melon, and watermelon, respectively (Zhou et al., 2017).

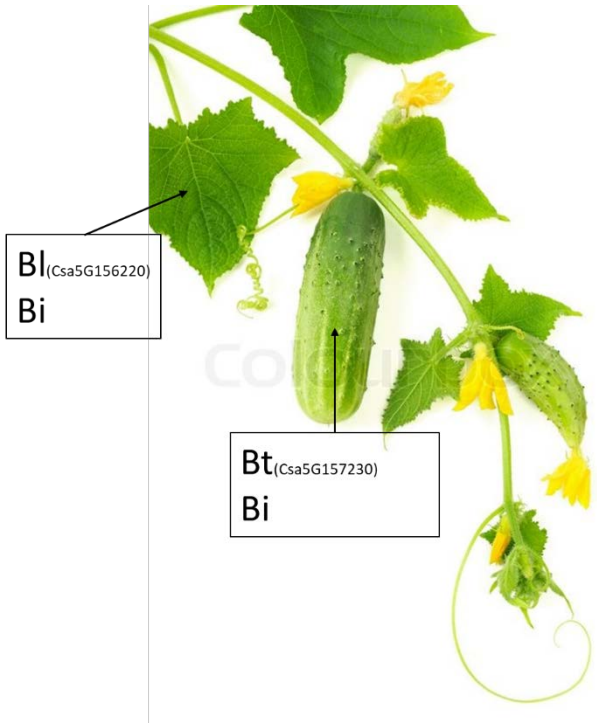


Figure 1.4. Two genetic factors controlling the pathway in bitter leaves and fruits of *Cucumis sativus.*

Figure 1.5. Biosynthetic pathway from deacetyl cucurbitacin C to cucurbitacin C (Shang et al., 2014).

Cucurbitacin C can be further metabolized in cucumber plants. It is glucosylated into cucurbitacin C 3-O-β-D-glucopyranoside through the action of uridine 5'-diphospho-glucuronosyltransferase (UDP-glucosyltransferase) UDT73AM3 (Zhong et al., 2017). This

mechanism was hypothesized as detoxification by glycosylation in cucumber plant and needs further investigation (Zhong et al., 2017). Cucurbitacin C content also significantly decreased as leaves turned yellow, with the production of 23,24-Dihydrocucurbitacin C as the next metabolite of cucurbitacin C (Qing et al., 2014).

1.5 Bioactivities of cucurbitacins

One function of cucurbitacins is to defend against pests and insects during cucumber plant growth (Davidovich-Rikanati et al., 2014). Cucurbitacins in cucumber plants showed cytotoxicity and anti-cancer activity. In addition, cucurbitacins are also used as a purgative, anti–inflammatory and anti-fertility agent (Mukherjee et al. 2013). Cucurbitacin B was identified to inhibit prostate cancer cell growth (Gao et al. 2014). Cucurbitacin B and E specifically have been linked to anti-artherosclerotic activity with inhibitory effects on lipid oxidation products such as malonaldehydes in animal studies(Kaushik et al., 2015). Cucurbitacin E was found to reduce adipocyte formation by inhibiting JAK-STAT5 transcription in fat tissue in mice. In addition, cucurbitacin E was found to enhance insulin signaling. Therefore, cucurbitacin E could be used as a new medicine for reducing visceral obesity and metabolic diseases at a low dosage (0.5 mg/kg in mice) (Murtaza et al., 2017). Cucurbitacin D and cucurbitacin I showed significant cytotoxicity against human cancer cell lines (Mukherjee et al., 2013; Blaskovich et al., 2003). It was also discovered that cucurbitacin C had high cytotoxicity in vitro to HepG2 ((human hepatoma, IC50 = 49.21 ± 1.10 uM) and A549 (human lung cancer, IC50 = 2.34 ± 0.51 uM), while cucurbitacin C 3-O-β-D-glucopyranoside had less toxicity on these cells than cucurbitacin C (IC50 > 100 uM) (Zhong et al., 2017). The bioactivities of cucurbitacins need further research to determine cucurbitacins effects on healthy cells and side effects on the body. Cucurbitacins

can also be a strong antifeedant for many insects (Davidovich-Rikanati et al., 2015), inspiring researchers to develop cucurbitacins as a pesticide or a potential insect growth regulator.

1.6 Toxicity of cucurbitacins

Cucurbitacins have been studied due to high toxicity, severe poisoning, and death in animals, mainly cattle and sheep, after consumption of fruits of *Cucumis* and *Cucurbita*. Wild species of watermelon have been used for pharmaceutical purposes, but because of high toxicity, such species have been restricted (Rymal et al., 1984). Cucurbitacin E has an LD50 in mice of 2.0 mg/kg; cucurbitacin A, 1.2 mg/kg in mice and 2.0 mg/kg in rats; cucurbitacin B, 1.1 mg/kg in mice; and cucurbitacin C 6.8 mg/kg in mice (Rymal et al., 1984). Recently, Sigma Aldrich reported the LD50 in the Safety Data Sheet. The LD 50 of cucurbitacin B was 14 mg/kg for mouse (Cucurbitacin B hydrate SDS, MilliporeSigma, US), cucurbitacin E was 340 mg/kg for rat (Cucurbitacin E SDS, MilliporeSigma, US), and cucurbitacin I was 5 mg/kg for mouse (Cucurbitacin I hydrate SDS, MilliporeSigma, US). Cucurbitacin E is responsible for toxicity in watermelon and squash (Rymal et al., 1984). The quantity of cucurbitacin E found in bitter yellow straightneck squash fruit averaged 3.10 mg/g of fresh fruit, while frozen zucchini contained 7.20 mg/g of flesh at the stem end with 2.7 mg/g in the central portion of the fruit. More than twenty human health poisoning cases were reported after consumption of three grams of bitter zucchini in Australia between 1981 and 1982 (Dolan et al., 2010, Rymal et al., 1984). Most patients reported digestive problems including nausea, vomiting and diarrhea after the ingestion of bitter tasting pumpkin soup or squash, and these two cases were linked to hair loss associated with cucurbitacin poisoning (Assouly, 2018). However, the levels were not measured in the foods that made people sick, so the amounts that are safe for consumption are still unknown.

The toxicity of cucurbitacins is also related to their hydrophobicity. Bartalis and Halaweish (2005) identified the relationship between hydrophobicity and basal cytotoxicity using HepG2 cells as a model system. Hydrophobicity and alkylation of the C1 hydroxyl were positively associated with cytotoxicity. The presence of an unsaturated bond between C1- C2 along with the acetylation of the C25 hydroxyl group increased both cytotoxicity and lipophilicity of cucurbitacins (Bartalis and Halaweish, 2005).

1.7 Analysis of cucurbitacins

Since plants only have a small quantity of cucurbitacins, isolation and purification or very sensitive analytical instruments are required for cucurbitacins analysis. Cucurbitacins are soluble in petroleum ether, chloroform, benzene, ethyl acetate, methanol and ethanol. Many research studies used petroleum ether, chloroform, ethyl acetate, methanol or ethanol to extract cucurbitancins from plants and used silica gel chromatography or high-performance liquid chromatography (HPLC) to do further purification. Cucurbitacin C has been isolated from cucumber fruits or leaves (Hideki et al., 2007), while cucurbitacins B, D, E and I have been isolated from *Cucumis sativus* (cucumber), fruits of *Aquilaria sinensis* (agarwood), *Cucurbita andreana, Cucurbita texana, Cucurbita okeechobeensis, Cucurbita pepo* (zucchini) and other plants (Mei et al. 2012, Halaweish and Tallamy 1993, Bajcsik et al. 2017).

Hideki et al. (2007) extracted cucurbitacin C from cucumber leaves for use as a standard. The process began with crushing young leaves of cucumber plants in petroleum ether at -20℃. Then, petroleum ether was discarded and the residue was obtained after vacuum filtration. The residue was extracted with methanol and the methanol extract was concentrated under reduced pressure. The concentrated methanol extract was dissolved in water, successively extracted with petroleum ether and dichloromethane, and the dichloromethane evaporated and suspended in a

small amount of methanol. Cucurbitacin C was further purified by HPLC with a C18 column using methanol: water (55:45). Halaweish et al. (1993) isolated cucurbitacin B, D, E and I from *Cucurbita andreana, C. texana* and *C. okeechobeensis*. Chloroform was used to extract cucurbitacins from plants and a solid phase extraction column packed with 0.2 mg of C18 reverse phase silica gel was used for further purification. For extraction conditions, the efficiency with which chloroform, methanol and chloroform-methanol (1:1) extracted cucurbitacins from plants was compared. Although methanol extraction efficiency was higher (93%) than chloroform (84.5%), samples extracted with methanol had a very sticky residue after evaporation, which made it difficult to dissolve for further analysis. For sample purification, a solid phase extraction column packed with 0.2g of C-18 reverse phase silica gel was used with methanol:water (80:20) as eluent (Halaweish et al., 1993). Therefore, methanol could be used as an extraction solvent for cucurbitacins and a C18 column could provide separation of cucurbitacins for analysis.

Liquid chromatography mass spectrometry (LC-MS) has been used to detect drugs, metabolites, and pesticides in water and food (Lactorte and Fernandez-Alba, 2005, Guale et al., 2013). LC-MS is more expensive but more sensitive and powerful than standard HPLC methods. Liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS) and liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) are used for determining known and unknown compounds with high resolution (Lactorte and Fernandez-Alba, 2005, Guale et al., 2013). Since there exist many different cucurbitacins and most do not have standards, these mass spectrometry techniques are employed in analyzing cucurbitacins in plant and drug materials. Most studies utilized water with 0.1% formic acid and acetonitrile with 0.1% formic acid as mobile phases as well as a C18 column for chromatographic separation

(Jevtic et al. 2017, Bajcsik et al., 2017; Xiao et al., 2018; Chawech et al., 2015; Shang et al., 2014). In addition, since the extraction procedure for cucurbitacins usually contains several steps including column separation and concentration and the resulting concentration is very low, known concentrations of internal standards, such as 4-hydroxybenzophenone, glycyrrhizic acid, or estrone, have been added at the beginning of extraction for quantitative analysis of cucurbitacins (Molavi et al., 2006; Hunsakunachai et al., 2019; Xiao et al., 2018). Cucurbitacins A, C, D, E, I, P, Q, and S and several cucurbitacin glycosides have been putatively identified by LC-QTOF-MS/MS. Most of them formed sodium adducts in positive ionization mode and formic acid adducts in negative ionization mode (UI Haq et al., 2019). Therefore, cucurbitacins could be separated by reversed column with acetonitrile or methanol as mobile phase. Cucurbitacins could be detected by LC-TOF-MS both in positive and negative ionization modes.

1.8 Drying cucumber

Given that cucurbitacins have some medicinal functions, some have tried to dry plant tissue to preserve the inherent cucurbitacins. Shadung et al. (2016) found that a suitable drying temperature for preserving cucurbitacin A and cucurbitacin B in fruit of wild cucumber and wild watermelon was 52℃. Higher temperatures resulted in more cucurbitacin loss during drying while lower temperature (30-40℃) resulted in cucurbitacin materials decaying by *Penicillium simplicissimum* (Shadung et al. 2016).

Freeze drying has been identified as a superior process to preserve food quality compared to conventional drying. There are 3 steps during the freeze drying process, including freezing, primary drying and secondary drying. Freezing converts most of the water into ice, leaving the solute in a glassy or crystalline phase. For freezing a single material, freezing temperature must be below a eutectic temperature where the maximal ice formation in products is. In some

products, this temperature has been measured by measurements of electrical resistance or differential scanning calorimetry, while in many others this temperature does not exist (Oetjen, 2004). For fruits and vegetables, the freezing temperature was usually in the range of -26 ℃ to -16 ℃ (Ghio et al., 2000). After products are frozen, primary drying is initated under vacuum while the temperature of shelves is increased. Primary drying, also called sublimation drying, causes ice to sublimate in the frozen products. The energy of increasing shelf temperature is consumed for sublimation, so when the product temperature is around the shelf temperature, primary drying ends and secondary drying starts. Secondary drying is also called desorption drying. During secondary drying, the water that cannot crystallize can be removed (Bhesh et al. 2013, Oetjen 2004). Temperature and the rate of temperature increase influence product quality in secondary drying (Bhesh et al. 2013; Oetjen 2004). Iwaniw and Mittal (1990) found that freeze drying strawberry had similar quality at low temperature and high rate of temperature increase as well as high temperature and low rate of temperature increase. In order to maximize preservation of chemicals in cucumber material, freeze drying would be a good approach to concentrate cucurbitacin in cucumber samples.

1.9 Bitter perception and bitterness in food

Bitterness in food has been long studied in a number of different fields such as food chemistry, pharmaceutical sciences and sensory analysis. Bitter taste has served as a warning and indicator of poisonous food in nature (Lee et al., 2015; Kurihara et al., 1994). However, culturally and traditionally humans also enjoy bitter foods such as coffee, tea, hops, squash, melon, chocolate, some alcoholic beverages and cucumber (Cavallo et al., 2019).

In humans, bitter taste is perceived by 25 members of the G protein-coupled receptors located on the tongue which are called T2Rs (Eskin and Aliani, 2017). Sensitivity of bitter

perception among humans varies greatly. This variability is based on genetic variations in oral sensation. A well-known study of sensitivities of bitter perception among humans involved the perception of phenylthiocarbamide (PTC) and 6-n-propyl-2-thiouracil (PROP). Those who were sensitive to PTC or PROP were categorized as supertasters (Eskin and Aliani, 2017). Delwiche et al. (2001) found that when people were sensitive to PROP they gave higher net bitterness ratings but did not differ from others in bitterness ranking (Delwiche et al., 2001, Eskin and Aliani, 2017). In addition, humans could distinguish temporal profiles of bitterness before and after swallowing (Higgins et al., 2021). Higgins et al. (2021) hypothesized that the differences in the temporal percepts might cause by the distribution of the bitter taste receptors throughout the oral cavity, chemical structural properties of the bitterants, or affinity to salivary proteins.

Phytochemicals are chemicals produced by plants to keep plants healthy. Phytochemicals including phenols, polyphenols, flavonoids, isoflavones, terpenes, and glucosinolates which are rejected by humans due to their bitter taste were determined to act as plant toxins (Drewnowski and Gomez-Carneros, 2000). The food industry is currently trying to reduce these compounds during selective breeding and food processing (Drewnowski and Gomez-Carneros, 2000). Some amino acids including isoleucine, leucine, tyrosine, tryptophan, and valine are also bitter-tasting compounds with thresholds ranging from 5 mmol/L to 21 mmol/L (Rotzoll et al., 2006). Significant research aims to analyze bitter compounds in food or medicine by sensory evaluation techniques, and electronic tongue combined with analytical chemistry methods and animal studies (Eskin and Aliani, 2017, Chen et al., 2012; Han et al., 2018; Kubec et al., 2018). Since bitter compounds are usually unpleasant, electronic tongue and animal studies were used to test bitter level of medicine to reduce human panelists in those type of studies. Since the toxicity of cucurbitacins is unknown, electronic tongue or animal studies would be better for testing the

bitter level of cucumbers. However, these methods are limited to a few bitter compounds. It needs further research to determine if these methods work for cucurbitacins.

1.10 Pickle processing

Fresh pickling cucumber can be preserved by fermentation or acidification. In 2020, 12,724 hundredweight (cwt) of cucumbers were produced in the US (USDA 2020 vegetables summary). Florida, Michigan and North Carolina are the main producing areas of cucumbers. Pickling cucumbers are usually grown in summer and it takes about 50 to 60 days for harvest. Cucumber fermentation is the traditional process to preserve cucumber. Cucumber is usually brined with 6% NaCl (Franco et al. 2016). The low salt fermentation technology was developed to reduce or eliminate the necessity for a desalting step (Fleming et al., 2002). Recently, BAG-IN-BOX technology was developed to prevent generating excess salt and organic waste. Whole cucumbers were washed, blanched, cooled, transferred to a tank and covered with brine which consisted of NaCl, acetic acid, $Ca(OH)_2$ and $CaCl_2$ (Fleming et al., 2002). Since the salt concentration could influence the microorganisms during cucumber fermentation (Perez Diaz et al., 2020) and there were no previous research focusing on bitter cucumber fermentation, it will be interesting to try low salt fermentation since a reduced salt fermentation enhanced microbial diversity (Perez Diaz et al., 2020), which will maximize the diversity of microorganisms that may help with cucurbitacin reduction. During natural cucumber fermentation, several microorganisms including aerobic bacteria, lactic acid bacteria (LAB), yeasts and molds are present. Raw cucumbers contain LAB as a minor part of their natural microbiota. LAB will outcompete the other natural microbiota due to the ability to survive in an extreme environment during fermentation. The dominant LAB in cucumber fermentation are from the *Lactobacillus, Pediococcus, Lactococcus and Leuconostoc* genera (Fleming et al., 1984; Perez Diaz et al.,

2020). *L. plantarum*, a facultative heterofermentater, produces lactic acid from glucose and fructose via the Embden-Meyerhoff-Parneas pathway and is the dominant LAB species in cucumber fermentation (Fleming et al., 1984; Perez Diaz et al., 2020). The metabolism of LAB is complicated. Many enzymes including glucosyltransferase are involved in phenolics metabolism, fatty acid metabolism, carbon metabolism and nitrogen metabolism, which convert flavonoid glycoside, phenolic acids, phenolic acid esters and tannins into other compounds (Filannino et al., 2018). Several LAB, including *L. plantarum* FMNP01 isolated from mango fruits and *Leuconostoc mesenteroides*, had glycosyltransferase genes (Li et al., 2014; Arguello-Morales et al., 2000; Funane et al., 2000). Since cucurbitacin C could be metabolized into cucurbitacin C glycoside in cucumber plant by a glycosyltransferase, LAB fermentation might decrease cucurbitacin content of bitter pickling cucumber. However, the relative bitterness of cucurbitacins and cucurbitacin glycosides is unknown.

During production of acidified cucumbers, which are also called fresh-pack pickles, acetic acid is directly added to preserve cucumber (Perez-Dıaz et al., 2014). Cucumbers are washed and transferred to jars directly and covered with brine. The cover brine usually contains NaCl, acetic acid, sodium benzoate, $CaCl_2$, and flavors as whole spices or emulsions. Products are typically pasteurized at 75℃ for 15 min for shelf stability (Fleming et al., 1984). $CaCl_2$ maintains the firmness of cucumber (Perez-Diaz et al., 2014). Acetic acid and sodium benzoate are effective for killing pathogens in less than 24 hours and assuring the microbial stability of cucumbers stored in bulk at 30℃ (Perez-Diaz and McFeeters, 2008). In this study, since the stability of cucurbitacins is unknown during cucumber storage in acidic brines, acidified cucumber could serve as a control group to identify if changes in cucurbitacin contents are due to fermentation.

1.11 Hypothesis

Cucurbitacin C can be metabolized into cucurbitacin C glycoside during cucumber fermentation since lactic acid bacteria commonly associated with cucumber fermentation contain glycosyltransferase genes.

1.12 Research Objectives

1). Develop sample preparation, quantitative and qualitative methods for cucurbitacin analysis by LC-TOF-MS.

2). Understand the occurrence of cucurbitacins in cucumber fruits and leaves

3). Determine the distribution of cucurbitacin C in exocarp, mesocarp, endocarp and different sizes of bitter cucumber

4). Determine the stability of cucurbitacin C during cucumber fermentation and acidification

1.13 Significance

This research could provide the pickle industry an analytical method for cucurbitacin analysis if they have bitter pickle products. Also, the stability of cucurbitacin C during cucumber fermentation and acidification will be determined in this research, which serves as the basis to further study whether fermentation could be used as a de-bittering method for bitter pickling cucumber. This research will also determine which part is the best source of cucurbitacin C in cucumber fruits to further explore its bioactivities and toxicity properties.

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Cucurbitacin E safety data sheet. Sigma Aldrich. Retrieved from https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=SML0577&brand=SIGMA&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fsearch%3Fterm%3Dcucurbitacin%26interface%3DAll%26N%3D0%26mode%3Dmatch%2520partialmax%26lang%3Den%26region%3DUS%26focus%3Dproduct.
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Cucurbitacin I hydrate safety data sheet. Sigma Aldrich. Retrieved from https://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=US&language=en&productNumber=C4493&brand=SIGMA&PageToGoToURL=https%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fsearch%3Fterm%3Dcucurbitacin%26interface%3DAll%26N%3D0%26mode%3Dmatch%2520partialmax%26lang%3Den%26region%3DUS%26focus%3Dproduct

Accessed 02/04/2021.

CHAPTER 2. Analysis of cucurbitacins in pickling cucumber by liquid chromatography time-of-flight mass spectrometry

2.1 Abstract

Cucurbitacins are triterpene compounds that can contribute to bitterness in cucumbers. Five cucurbitacins or isomers were tentatively identified in fresh pickling cucumber fruit, leaves or peels by liquid chromatography time-of-flight mass spectrometry. All samples were freeze-dried and extracted with methanol prior to analysis. Identification of cucurbitacins were performed by comparing the exact mass to the theoretical mass of expected adducts, both in positive and negative ionization mode in combination with UV absorbance at 230 nm. Cucurbitacins B, C or Q, and C glucoside were tentatively identified in cucumber leaves. Cucurbitacins C or Q, F or O, and P were tentatively identified in pickling cucumber fruits purchased from a local market, and cucurbitacins F or O, and P were tentatively identified in cucumber peel of a separate lot of commercial pickling cucumbers. Further study is needed to confirm identifications and identify which cucurbitacins are responsible for bitterness in pickling cucumber. This analytical method could be used for analyzing potential changes in cucurbitacins during food processing including fermentation as well as acidification, and a thermal treatment.

2.2 Introduction

Cucurbitacins are a group of triterpenes that contribute to bitterness in Cucurbitaceae, including squash, pumpkin, watermelon, melon, cucumber, and zucchini (Chen et al., 2005). Cucumbers were found to taste bitter when they were grown under stress conditions, including low temperature and excess nitrogenous fertilizer (Shang et al., 2014; Kano and Goto, 2003). Many cucumber producers also reported that drought could induce bitter cucumber (personal communication). In 2014, Shang et al. pointed out that the Bi gene regulated cucurbitacin C's occurrence in cucumber plant. When cucumbers were grown under low temperature, single-nucleotide polymorphism (SNP)-1601 was essential for regulating Bi expression in response to low temperature. The mutation corresponding to SNP-1601 showed that fruits were non-bitter even under low temperature conditions (18℃ day, 12℃ night) that would normally induce bitterness of cucumbers without the mutation (Shang et al., 2014). Cucurbitacins may have many medicinal properties including anti-inflammatory activity, anti-tumor activity, anti-atherosclerotic activity and anti-diabetic activity (Kaushik et al., 2015). Pharmaceutical industries are trying to study cucurbitacins bioactivities and develop new medicines using cucurbitacins in recent years (Kaushik et al., 2015). However, several human poisoning cases were reported where individuals presented digestive problems including nausea, vomiting and diarrhea after consuming 3 g of bitter zucchini containing cucurbitacins (Dolan et al., 2010; Rymal et al., 1984). In addition, another cucurbitacin poisoning case was reported that showed hair loss as the primary symptom (Assouly, 2018). Therefore, it is important to identify and isolate these compounds from plants and further study their toxicity and biological properties.

More than 20 types of cucurbitacins have been identified in different plants in recent years (Chen et al., 2005). Cucurbitacins were first observed in cucurbitaceae by Enslin et al. in

1954. They used paper chromatography for isolating cucurbitacins from different plants and predicted their formula and structures using ultra-violet (UV) absorption spectra. They showed formulae and properties for cucurbitacins A, B, C, D, E, F, G, I, J, and L. However, cucurbitacins B, C, D, F, and L formulas that were tentatively identified by them had two more hydrogens than in more recent findings (Enslin et al., 1954, 1957). Cucurbitacin I's formula was $C_{30}H_{44}O_8$ which had one more water molecule than today's formula (Table 2.1). Although identification procedures were very complicated due to the lack of modern sensitive analytical equipment at that time, the detailed structure of cucurbitacin C was further elucidated and it was discovered that this compound was found in *Cucumis sativus var*. Hanzil (Enslin et al., 1960). In their study, they did not find cucurbitacin C in other cultivars, which suggests that cucurbitacin C was absent or in very low concentrations in the other cultivars studied.

In recent years, cucurbitacins have been analyzed by high performance liquid chromatography (HPLC) and mass spectrometry (MS). In cucumber plants, cucurbitacin C was identified in bitter cucumber fruits, cucumber stems and cucumber leaves (Shang et al., 2014; Hideki et al., 2007). However, other cucurbitacins and cucurbitacin derivatives were also reported in cucumber plants including fruits, leaves, and stems (Rice et al., 1981). The pickling cucumber (*Cucumis sativus*) which is commonly used for fermented and acidified pickles in the United States frequently tastes slightly bitter (informal sensory test); however, no previous research identified cucurbitacin C in pickling cucumber.

Liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS) has been used for drug and metabolite research in recent years (Guale et al., 2013; Lacorte et al., 2006). LC-TOF-MS is a highly sensitive and powerful technique for determining known and unknown compounds from biological samples and drugs. Due to the variety of cucurbitacins and their

derivatives, and the lack of available standards for most of these compounds, LC-TOF-MS has been used for qualitative analysis of cucurbitacins in plants (Chawech et al., 2015; UI Haq et al., 2019). In previous research, cucurbitacins were not detected in non-bitter cucumber varieties by HPLC. In this study, cucurbitacins were hard to detect and identify in pickling cucumber by HPLC due to low concentration, matrix interferences and lack of authentic standards. Therefore, we optimized sample preparation and adapted a highly sensitive method for identifying cucurbitacins in pickling cucumber by LC-TOF-MS.

2.3 Materials and Methods

2.3.1 Chemicals

LC-MS grade acetonitrile, water and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Cucurbitacin E standard was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3.2 Sample preparation

2.3.2.1 Freeze drying

Pickling cucumbers purchased from a local market were cut crosswise into slices of 6 mm thick. Cucumber peels were collected from a separate lot of cucumbers from a commercial manufacturer, and cucumber leaves were obtained from an experimental field. All materials were stored at -80 ℃ for at least 12 hrs. Frozen samples were placed into a VirTis freeze dryer (Genesis XL, SP Scientific, NY, USA). A thermocouple was placed into the center of the tray on each shelf for product temperature monitoring. During primary drying, shelf temperature was set to -10℃. After product temperature reached around -10 ℃, shelf temperature was raised to 20 ℃ for secondary drying. After completion of freeze drying, samples were removed from the freeze dryer and pulverized with a Krups F203 grinder (Krups, Solingen, Germany). Freeze-

dried cucumber powder was stored in 50 ml culture tubes and kept desiccated at -80 ℃. Sample weights were measured before and after freeze drying for moisture determination.

2.3.2.2 Cucurbitacin extraction

Freeze dried cucumber powder (0.10 g or 0.20 g) was extracted with 10 ml methanol by sonication (FS60 sonicator, Fisher Scientific, Pittsburgh, PA,USA) for 15, 30, 45, 60, 75, 90, 105, 120 min to determine the optimal extraction time. The extract was centrifuged (Sorvall Legend XTR, Thermo Fisher Scientific, Waltham, MA, USA) at 7085 g for 10 min at room temperature. The supernatant was transferred to a new tube. This methanol extraction process was repeated a total of 3 times on the pellet. Supernatant was collected individually for analysis of extraction efficiency. The extraction efficiency was calculated by the following equation:

$$\text{Extraction Efficiency \%} = \frac{\text{Abs}}{\text{sum of all Abs}} \%$$

Supernatant from each of three extraction cycles was filtered through 0.22 μm polypropylene membranes by syringe filters and analyzed on a Cary 300 Bio UV-Visible spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA) for absorbances at 230 nm wavelength. Single cycle extract was filtered through 0.22 μm polypropylene membranes and concentrated 20 fold by evaporating to dryness under a flow of nitrogen and reconstituting in a small volume of methanol. Concentrated extract was analyzed by UPLC-ESI-DAD-TOF-MS in both positive and negative ion modes.

2.3.3 UPLC-ESI-TOF-MS analysis

2.3.3.1 Instrumental and method parameters

LC/MS analysis of the Cucurbitacin standards and sample extracts was performed using an Agilent 1260 Infinity II LC (Santa Clara, CA, USA) coupled to an Agilent 6230 Time-of-Flight (TOF)-MS system equipped with an Agilent Jet Stream dual electrospray ionization (ESI)

source. The HPLC system included a binary pump, Agilent 1260 Autosampler, a photodiode array detector and a Waters BEH RP-C18 column (1.7 μm, 100 × 2.1 mm). The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The UV wavelength was set at 230 nm. The flow rate was 0.25 mL/min and the injection volume was 5 μL. A linear gradient with the following concentration of mobile phase B (t min, B %) was used: (0, 10%), (5, 25%), (10, 100%), (15, 100%), (15.001, 10%). Post run time was set to 13 min to re-equilibrate the column for the next injection.

The ESI-TOF mass spectrometer was operated in both positive and negative ESI modes to maximize compound detection. Agilent MassHunter Workstation software was used to acquire data (version B.07.00). The capillary voltage was set to 3500V, gas temperature was 350°C, gas flow: 12 L/min. Also, reference ions including ammonium trifluoroacetate, purine and hexakis phosphazine were used for accurate mass measurement. Hexakis phosphazine generates m/z of 922.0098 for positive ion mode, and formic acid adduct with m/z of 1033.9881 for negative ion mode.

2.3.3.2 Limit of detection (LOD) and limit of quantification (LOQ) of cucurbitacin E (CuE)

In order to determine whether cucurbitacins ionized better in positive mode or negative mode, and which mode was more sensitive to detect cucurbitacins, CuE standard was used to test LOD and LOQ in both modes. CuE standard was purchased from Sigma-Aldrich (St. Louis, MO, USA) and a 1 mg/ml stock solution in methanol was made. The stock solution was stored at -20°C and used for making a 10 ppm CuE solution which was serially diluted to make solutions ranging from 5 ppm to 0.05 ppm. A five-point calibration curve was run in triplicate, both positive and negative ion modes. LOD and LOQ of CuE were calculated based on slope and

intercept of standard curves of CuE. The LOD and LOQ calculations were based on the following equations:

$$LOD = 3.3 \times S_y/S,$$

$$LOQ = 10 \times S_y/S$$

where S_y is the standard error of the intercept and S is the slope of the calibration curve.

2.3.4 Data analysis

MS total ion current (TIC) data were collected and UV data were collected at wavelength 230 nm. MassHunter Qualitative Analysis software was used to generate mass spectra for compounds of interest and to identify cucurbitacins based on exact mass and isotopic abundance information from the literature reference list in Table 2.1. Data for calibration curves were processed by MassHunter TOF Quantitative Analysis.

Table 2.1. Nineteen known cucurbitacin formulas and the exact masses of the parent compound and most prominent ESI adducts.

Cucurbitacin	MW (g/mol)	Chemical formula	Monoisotopic mass	$[M+Na]^+$	$[M+FA-H]^-$	References
A	574.7022	$C_{32}H_{46}O_9$	574.3142	597.3034	619.3124	Kaushik et al. (2015)
B	558.7028	$C_{32}H_{46}O_8$	558.3193	581.3085	603.3175	Bajcisik et al. (2017)
C	560.7187	$C_{32}H_{48}O_8$	560.3349	583.3241	605.3331	Jevtic et al. (2017)
D	516.6662	$C_{30}H_{44}O_7$	516.3087	539.2979	561.3069	Kaushik et al. (2015)
E	556.6870	$C_{32}H_{44}O_8$	556.3036	579.2928	601.3018	Bajcisik et al. (2017)
F	518.6820	$C_{30}H_{46}O_7$	518.3244	541.3136	563.3226	Jevtic et al. (2017)
G	556.7285	$C_{30}H_{52}O_9$	556.3611	579.3503	601.3593	Kaushik et al. (2015)
H	534.6814	$C_{30}H_{46}O_8$	534.3193	557.3085	579.3175	Kaushik et al. (2015)
I	514.6503	$C_{30}H_{42}O_7$	514.2931	537.2823	559.2913	Bajcisik et al. (2017)
J	532.6656	$C_{30}H_{44}O_8$	532.3036	555.2928	577.3018	Kaushik et al. (2015)
K	532.6656	$C_{30}H_{44}O_8$	532.3036	555.2928	577.3018	Kaushik et al. (2015)
L	516.6662	$C_{30}H_{44}O_7$	516.3087	539.2979	561.3069	Kaushik et al. (2015)
O	518.6820	$C_{30}H_{46}O_7$	518.3244	541.3136	563.3226	Kaushik et al. (2015)
P	520.6979	$C_{30}H_{48}O_7$	520.3400	543.3292	565.3382	Kaushik et al. (2015)
Q	560.7187	$C_{32}H_{48}O_8$	560.3349	583.3241	605.3331	Kaushik et al. (2015)
S	498.6509	$C_{30}H_{42}O_6$	498.2981	521.2874	543.2963	Kaushik et al. (2015)
E glycoside	718.8276	$C_{38}H_{54}O_{13}$	718.3564	741.3457	763.3546	Bajcisik et al. (2017)
C glycoside	722.8593	$C_{38}H_{58}O_{13}$	722.3877	745.3769	767.3859	Zhong et al. (2017)
I 2-glucoside	676.7909	$C_{36}H_{52}O_{12}$	676.3459	699.3351	721.3441	Metlin

2.4 Results and discussion

2.4.1 Optimized sample preparation

During sonication, the water bath in the sonicator became noticeably hot after 60 min, posing an explosion danger for the methanol extraction. Ice was added at the beginning of sonication for extraction times greater than 75 min. More cucurbitacins were extracted as time increased from 15 – 75 min (Figure 2.1). Cucurbitacin extraction did not show significant difference between 60 min and 75 min. When ice was added to the sonicator, cucurbitacin extraction decreased, indicating that temperature aids in extraction. In consideration of safety concerns and extraction efficiency, 60 min was chosen as the best time for cucurbitacin extraction.

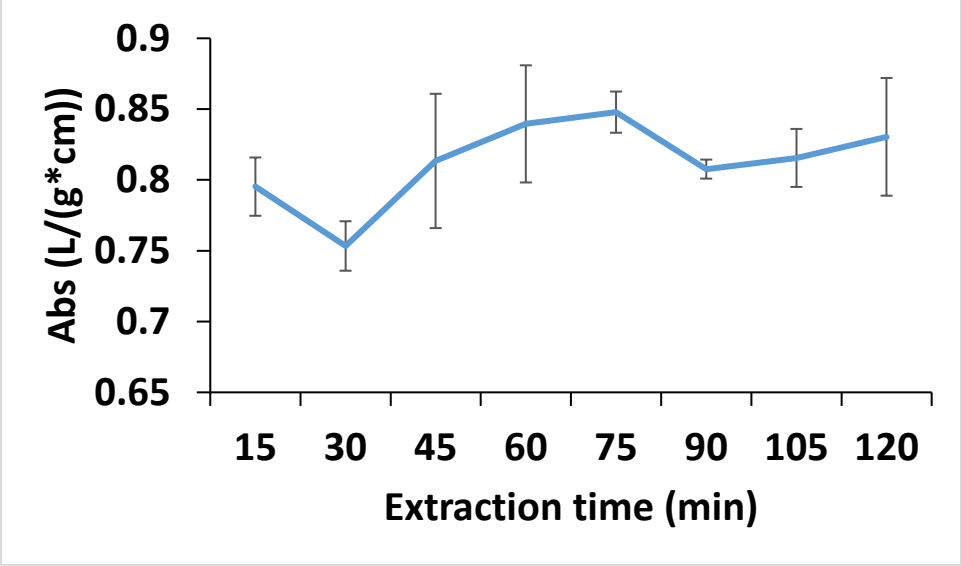

Figure 2.1. Extraction of cucurbitacins at different sonication times.

The extraction efficiency for 0.2 g cucumber powder with 10ml methanol (Table 2.2) and 0.1 g cucumber powder added with 10 ml methanol (Table 2.3) was compared to determine which extraction was more efficient.

The absorbance of the fourth extraction of 0.10 g cucumber powder was very close to zero while the absorbance of the fifth extraction of 0.20 g cucumber powder was much higher,

indicating that cucurbitacin or other compounds that absorb at 230 nm remained in the sample and was not fully extracted into the methanol. In addition, the single cycle extraction efficiency for 0.10 g cucumber powder was around 80% compared to 70% for 0.20 g cucumber. Given the greater extraction efficiency of 0.10 g cucumber powder, this amount was chosen for the final method.

Table 2.2. Extraction efficiency for 0.20 g cucumber powder in 10 ml methanol.

Replicate 1	Abs (230nm)	replicate 2	Abs (230nm)	Extraction efficiency ± SD %
Cycle 1	0.8	Cycle 1	0.7895	71 ± 2.9
Cycle 2	0.1792	Cycle 2	0.1936	88 ± 2.5
Cycle 3	0.0552	Cycle 3	0.0902	94 ± 0.51
Cycle 4	0.0352	Cycle 4	0.0416	98 ± 0.21
Cycle 5	0.0215	Cycle 5	0.0259	
Total	1.0911		1.1408	

Table 2.3. Extraction efficiency for 0.10 g cucumber powder in 10 ml methanol.

Replicate 1	Abs (230nm)	Replicate 2	Abs (230nm)	Extraction efficiency ± SD %
Cycle 1	0.4061	Cycle 1	0.4176	82± 4.7
Cycle 2	0.0584	Cycle 2	0.0695	94 ± 4.1
Cycle 3	0.0111	Cycle 3	0.0363	99 ± 0.92
Cycle 4	0.0021	Cycle 4	0.0093	
Total	0.4777		0.5327	

2.4.2 Ionization, LOD and LOQ of cucurbitacin E in UPLC-TOF-MS

In positive ion mode, the sodium ion adduct ($[M+Na]^+$, m/z 579.29209) of cucurbitacin E was the most abundant ion while the sodiated dimer adduct ($[2M+Na]^+$, m/z 1135.59460) was also present. In negative mode, the formic acid adduct ($[M+FA-H]^-$, m/z 601.30191) was generated during ionization (Figure 2.2). The retention time for CuE was 11.7 min. Comparing the ionization in positive and negative modes, the abundance in positive mode (2,053,390) was more than 2-fold higher than in negative mode (943,346), but there were more interfering ions.

The slope in positive mode was higher than in negative mode. This result indicated that the positive ion mode was more sensitive than negative mode.

The calibration curves for 0.05 ppm – 5 ppm CuE in positive and negative modes are shown in Figure 2.3. The S_y in positive mode and negative mode were 27,565 and 6,246, respectively. The LOD and LOQ in positive mode were 0.085 ppm and 0.26 ppm, respectively. The LOD and LOQ in negative mode were 0.06 ppm and 0.17 ppm, respectively. These LOD and LOQ were calculated back to the fresh cucumber sample (Table 2.4). The two steps of concentration, freeze drying and concentration 20-fold under nitrogen, were both accounted for in the calculation. From previous literature, the minimum lethal dose for rabbits by intravenous injection was 0.7 mg/kg body weight for cucurbitacin A and 0.5 mg/kg body weight for cucurbitacin B (Enslin, 1954). If cucurbitacins' concentration is higher than these lethal dosages, it should be detected by this method since the LOD (fresh weight basis) in this method was much lower than the lethal dose.

Therefore, even though the positive ionization mode produced a greater abundance of CuE ions and was slightly more sensitive, the LOD in negative mode was lower than in positive mode (Table 2.4) and R^2 was higher on negative mode, because there were less interfering ions in the negative mode. Given these observations, negative ionization mode will have better performance in quantitative analysis.

Table 2.4. LOD and LOQ for Cucurbitacin E extracted from cucumber and leaves samples (fresh weight basis).

	Positive mode		Negative mode	
	LOD (ppm)	LOQ (ppm)	LOD (ppm)	LOQ (ppm)
Cucumber fruit	0.026	0.077	0.017	0.052
Cucumber leaves	0.091	0.275	0.062	0.187
Cucumber peel	0.035	0.107	0.024	0.073

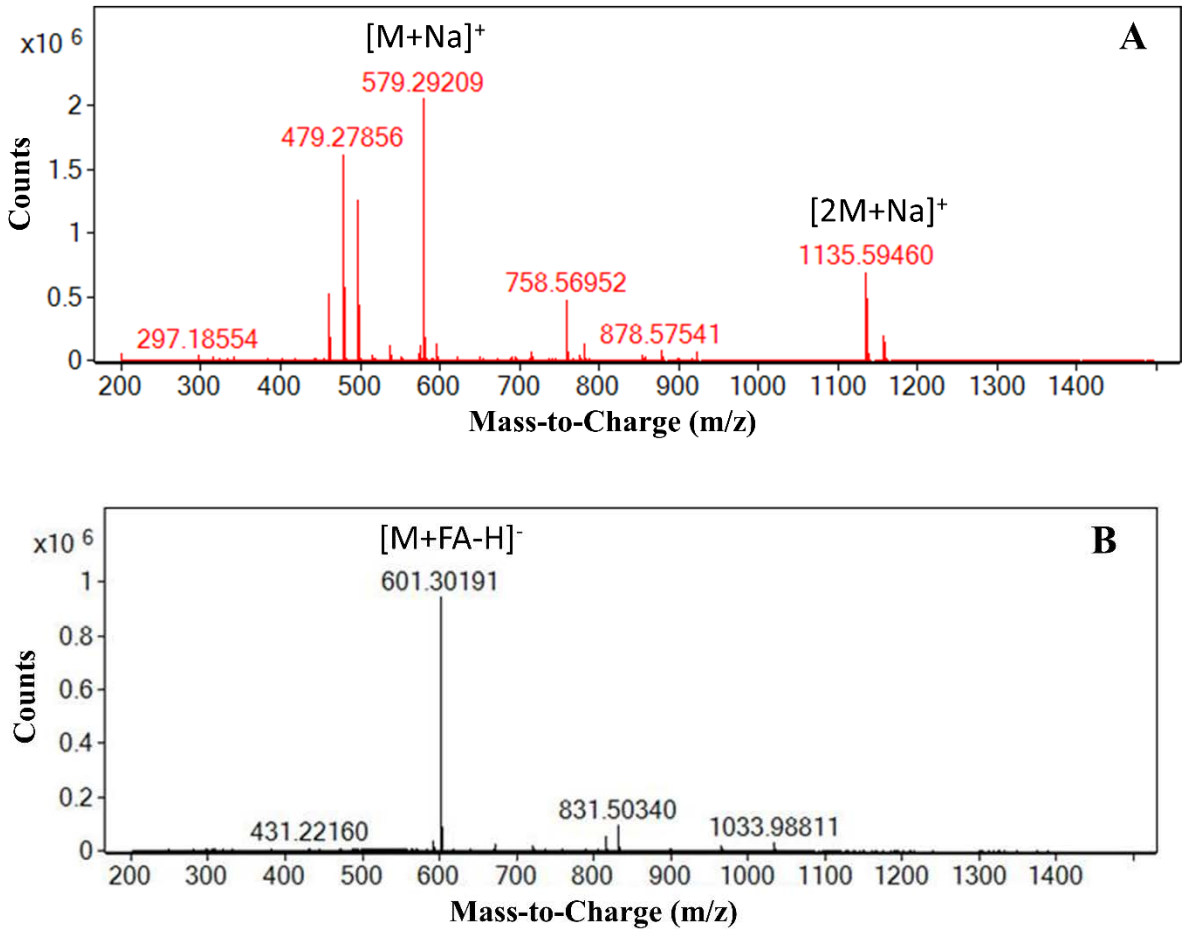

Figure 2.2. Mass spectrum of Cucurbitacin E in positive mode (A) and negative mode (B). In positive ion mode, the sodium ion adduct ([M+Na]+, m/z 579.29209) of cucurbitacin E was the most abundant ion while the sodiated dimer adduct ([2M+Na]+, m/z 1135.59460) was also present. In negative mode, the formic acid adduct ([M+FA-H]- , m/z 601.30191) was generated during ionization.

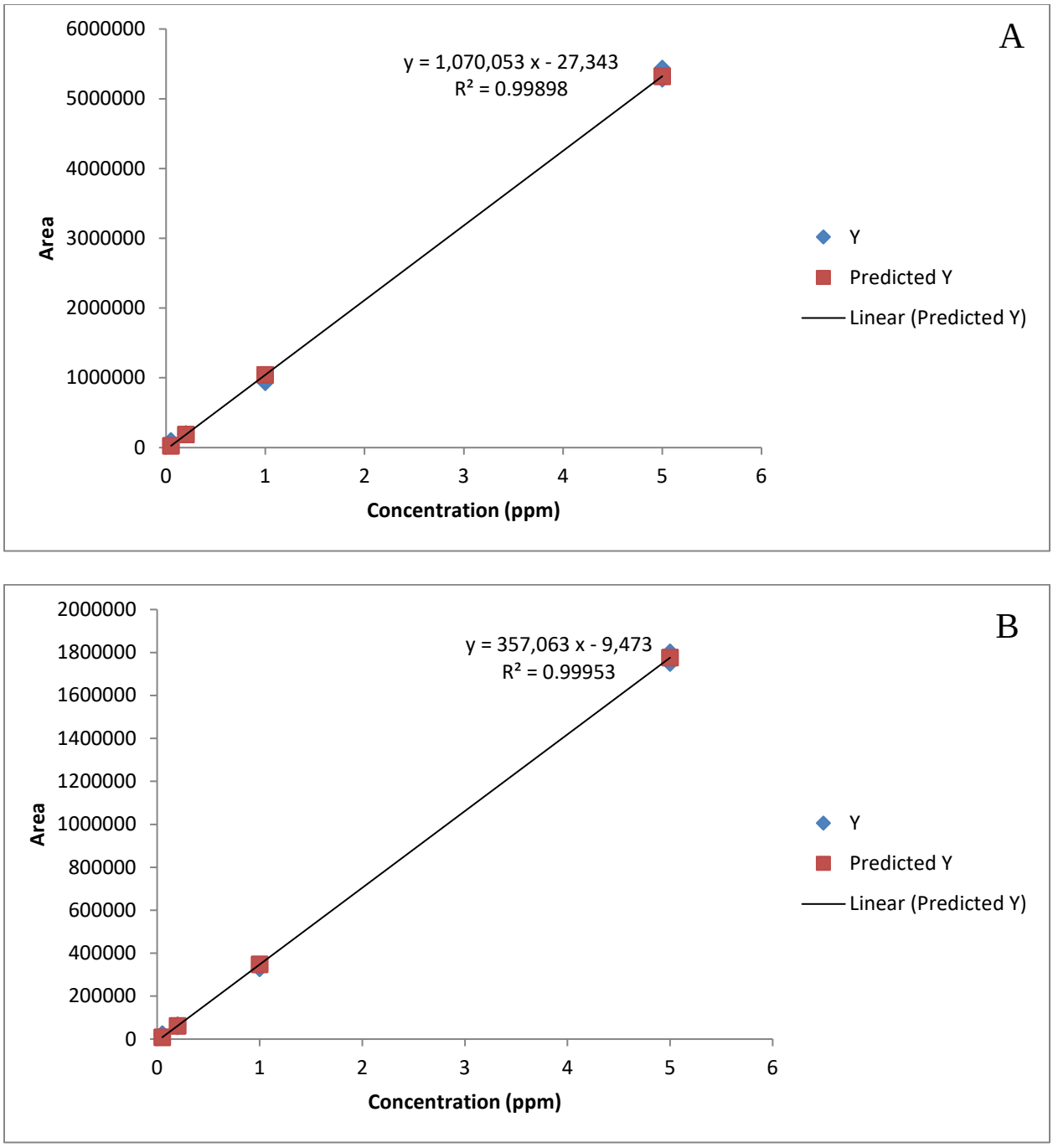

Figure 2.3. Calibration curves of Cucurbitacin E in positive (A) and negative (B) ionization modes for m/z 579.29284 and m/z 601.30182, repectively.

3.4.3 Identification of cucurbitacins in pickling cucumber

Five cucurbitacins were tentatively identified in cucumber fruit, leaves or peel based on the expected accurate mass of the adduct ion, retention time and isotopic abundance in positive and negative modes (Table 2.5). The retention time of the same compound should be the same both in positive and negative modes. CuE ionized in both positive and negative mode with $[M+Na]^+$ and $[M+FA-H]^-$ adducts at the same retention time. Assuming other cucurbitacins ionize similarly to CuE, they could be tentatively identified in samples by creating extracted ion chromatograms (EICs) of the corresponding adduct's expected accurate mass. The range of m/z for generating EICs was restricted to 10 ppm mass error. Cucurbitacins C and Q, and F and O are two groups of isomers, meaning they have the same molecular formulas but different structures. This method cannot distinguish between isomers based on mass alone, which was one of the limitations of this method.

Cucurbitacin B was tentatively identified in cucumber leaves only. The retention time for eluting cucurbitacin B was 11.25 min. The mass spectrum showed the $[M+Na]^+$ and $[M+FA-H]^-$ adducts (Figure 2.4). Cucurbitacin B is known as one of the compounds resulting in the bitterness in cucumber (Mukherjee et al., 2013). However, cucurbitacin B was not found in pickling cucumber fruit or peel tissues. Only one previous study isolated cucurbitacin B from cucumber leaves (Mashchenko et al., 1976). Since the samples that were used in this study did not have an obvious bitter taste, cucurbitacin B may have been below the detection limit. Cucurbitacin B might only be present in extremely bitter cucumber fruit rather than in most standard commercial cultivars fruits and peels.

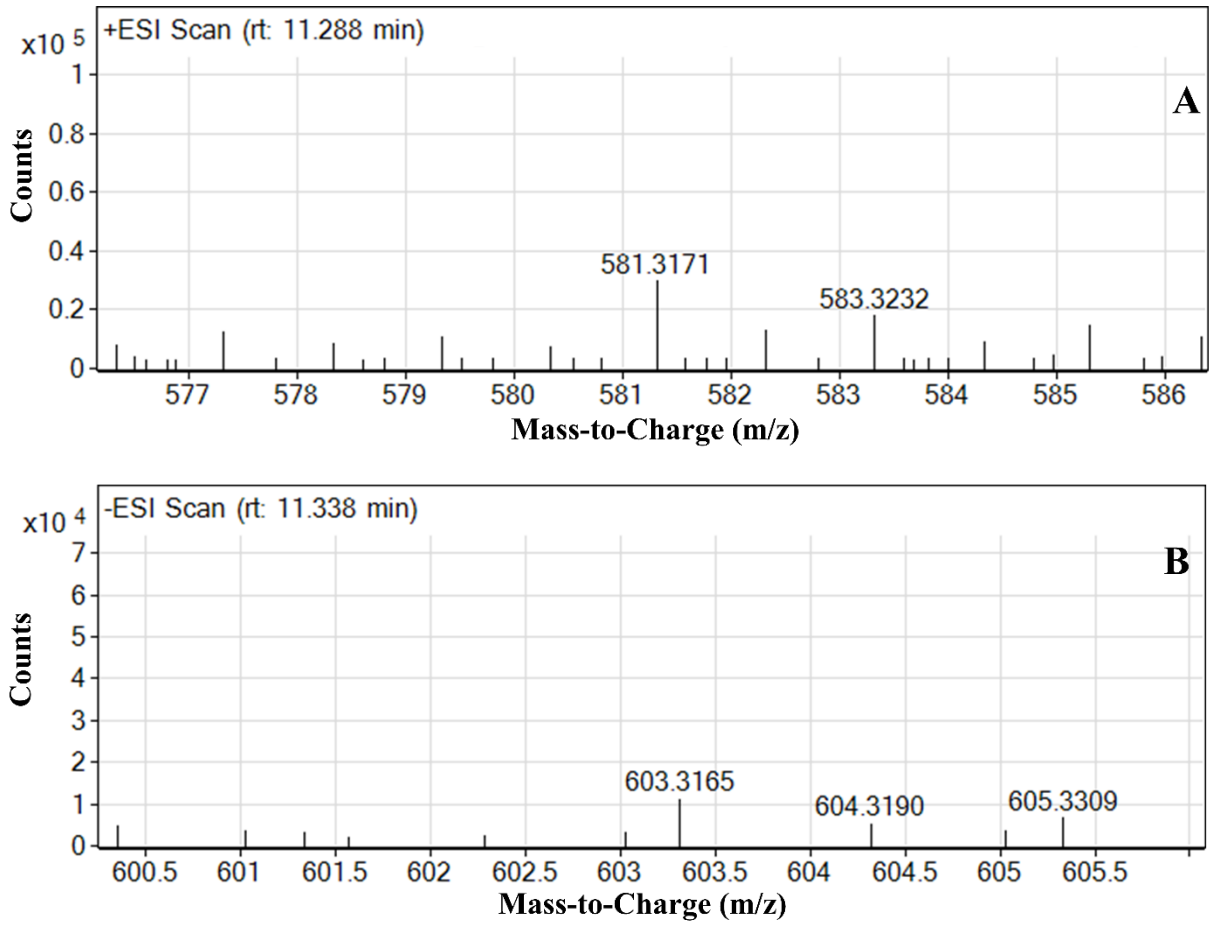

Figure 2.4. Mass spectrum of cucurbitacin B in cucumber leaves in positive (A) and negative (B) modes.

Cucurbitacin C or Q was detected in both cucumber leaves and fruit. The mass spectrum of this compound in cucumber leaves is shown in Figure 2.5. Cucurbitacin C has been considered the main bitter compound in cucumber (Shang et al., 2014; Hideki et al., 2007; Liu et al., 2019). In this study, this compound had the largest peak area compared to other tentatively identified cucurbitacins in cucumber leaves. In contrast, the abundance of this compound was quite low in pickling cucumber fruits. Hideki et al.' methods could not detect cucurbitacin C in cucumber fruit if its concentration was less than 0.1 mg/L (0.1 ppm) by the HPLC, but these fruits were reported to still taste bitter (Hideki et al., 2007). The LOD for our method was much lower than 0.1 mg/L for CuE; therefore, it should be sensitive enough to detect cucurbitacins in

cucumber that do not taste extremely bitter. Additionally, cucurbitacin C could be metabolized into cucurbitacin C glucoside by UDP-glucosyltransferase UGT73AM3 in cucumber plants (Zhong et al., 2017). This method also detected cucurbitacin C glucoside in cucumber leaves (Figure 2.6), which indicated that UDP-glucosyltransferase UGT73AM3 may be present in cucumber leaves. The activity of this enzyme in cucumber fruits would require further research. Hydrophobicity and alkylation of the C1 hydroxyl were positively associated with cytotoxicity. The presence of an unsaturated bond between C1- C2 along with the acetylation of the C25 hydroxyl group increased both cytotoxicity and lipophilicity of cucurbitacins (Bartalis and Halaweish, 2005). Therefore, since glucoside eluted prior to the parent cucurbitacins in the C18 column in our experiment, cucurbitacin C glucoside might have lower toxicity than cucurbitacin C. In addition, cucurbitacin C might have less toxicity than cucurbitacin E based on the retention time in our experiment. This result matched with the LD50 that was reported by Rymal et al. with cucurbitacin E 2.0 mg/g for mice and cucurbitacin C 6.8 mg/kg for mice (Rymal et al., 1984).

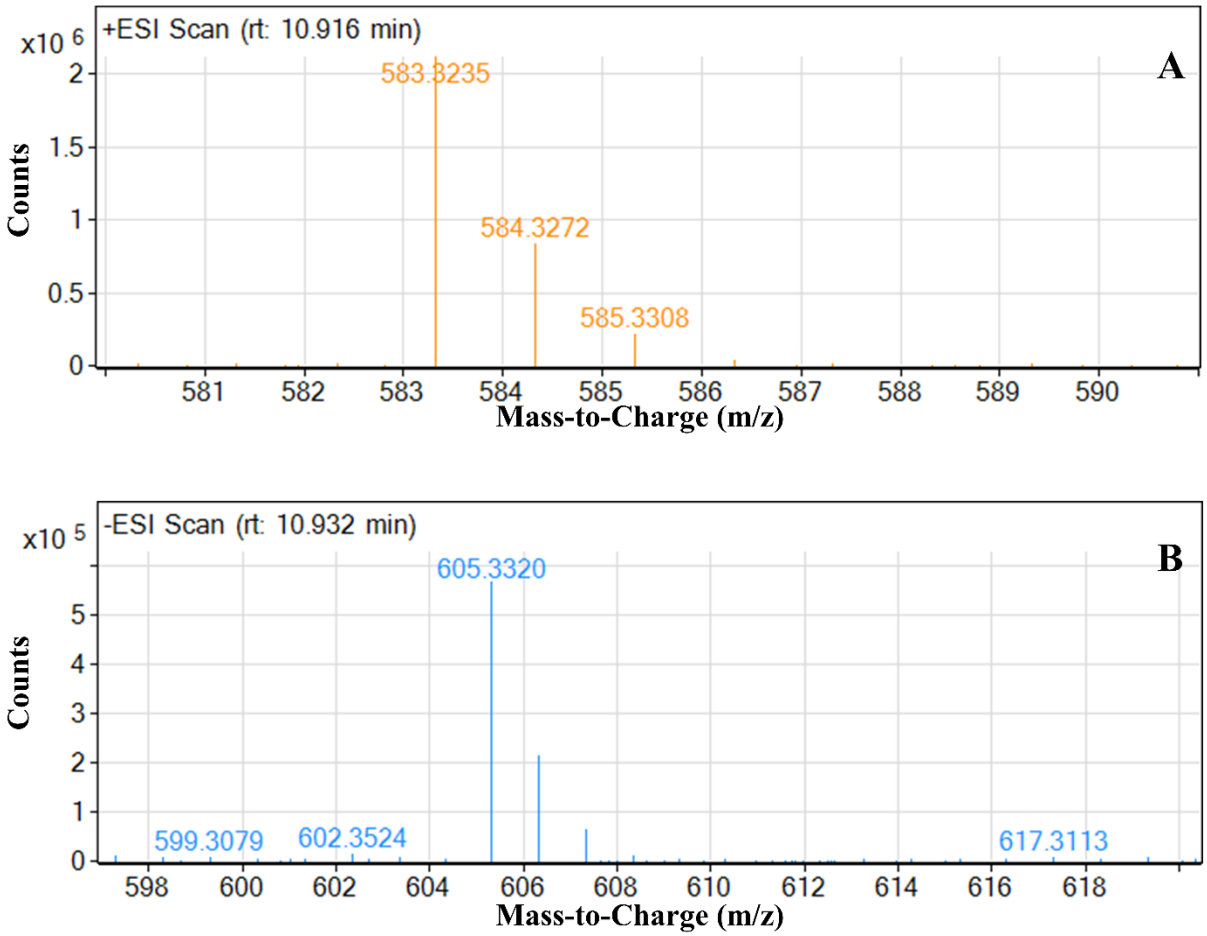

Figure 2.5. Mass spectrum of cucurbitacin C or Q in cucumber leaves in positive (A) and negative (B) modes.

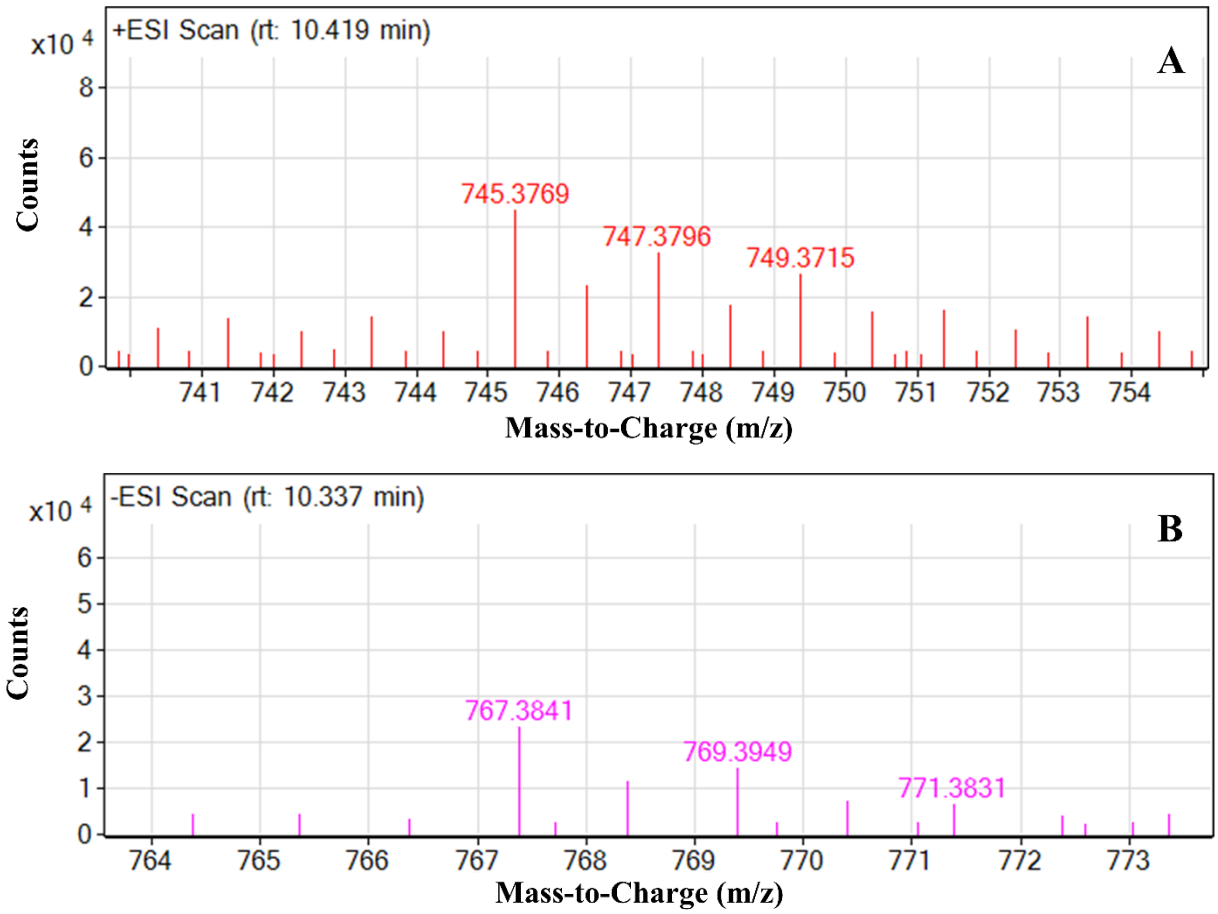

Figure 2.6. Mass spectrum for cucurbitacin C glucoside in cucumber leaves in positive (A) and negative (B) modes.

Cucurbitacin F or O, and cucurbitacin P were tentatively identified in both cucumber fruit and peel. Cucurbitacin F was found in cucumber leaves by Jevtić et al. (2017) in the negative ion mode. However, in this study, the mass spectrum peaks of cucurbitacins F or O, and P may have been an isotopic peak of other compounds that have less ~1 Da mass difference in both positive modes and negative modes, since they were not the most abundant peaks in the same compound group (Figure 2.7, Figure 2.8). Further research is required to confirm the presence of these cucurbitacins in cucumber. The five cucurbitacins tentatively identified in this study require structural confirmation by MS/MS or NMR to enhance their analysis (Kubec et al., 2018).

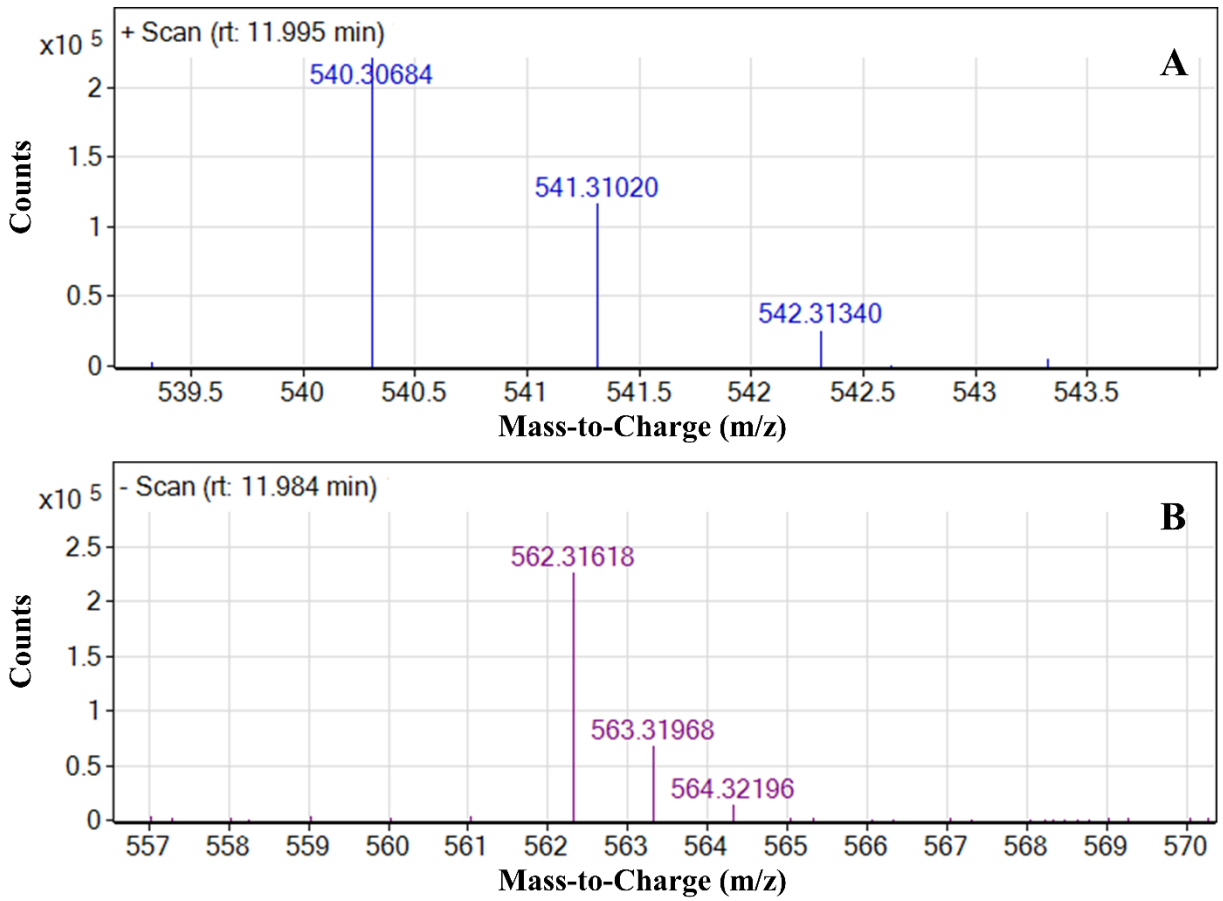

Figure 2.7. Mass spectrum for cucurbitacin F or O in cucumber fruit in positive (A) and negative (B) modes.

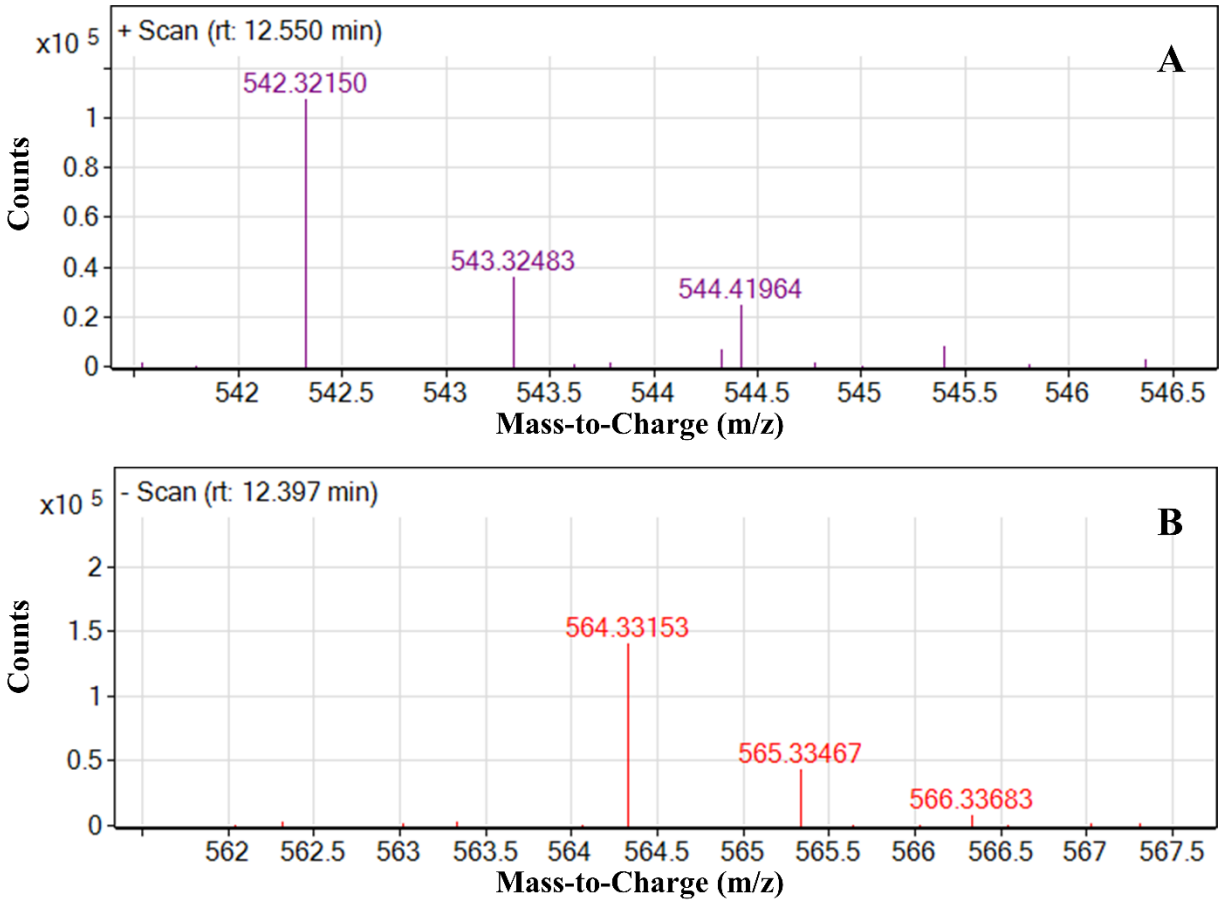

Figure 2.8. Mass spectrum for cucurbitacin P in cucumber fruit in positive (A) and negative (B) modes.

3.4.4 Cucurbitacins screening by infrared, matrix-assisted laser desorption electrospray ionization (IR-MALDESI) MS

The $[M+Na]^+$ adducts of cucurbitacins were searched in samples analyzed by IR-MALDESI-MS in positive ion modes with mass accuracy restricted to 5 ppm (See appendix). This experiment was performed with the method employed by Fideler et al. (2018) using data obtained from fresh cucumbers in those experiments. According to the IR-MALDESI-MS results, cucurbitacins A, J or K were tentatively identified in cucumber peel. However, these cucurbitacins were not identified in the samples analyzed by LC-TOF-MS. Since samples analyzed by IR-MALDESI did not have extraction procedures, these cucurbitacins might be lost

during freeze drying or sample preparation. More samples need to be analyzed by IR-MALDESI to further confirm if cucurbitacins A, J or K are present in cucumber fruits. These cucurbitacins also need to be analyzed by MS/MS to confirm identification.

Table 2.5. Cucurbitacins tentatively identified in pickling cucumber leaves, fruit peel, and fruit.

Name	Molecular Formula	RT (min)	Ion Type	Exact Mass	Peak Area	Materials
	$C_{32}H_{46}O_8$	11.255	$[M+Na]^+$	581.3085	275,187	cucumber leaves
Cucurbitacin B		11.255	$[M+FA-H]^-$	603.3175	128,270	cucumber leaves
	$C_{32}H_{48}O_8$	10.903	$[M+Na]^+$	583.3241	54,368	cucumber fruit
		10.900	$[M+FA-H]^-$	605.3331	16,330	cucumber fruit
		10.891	$[M+Na]^+$	583.3241	13,619,577	cucumber leaves
Cucurbitacin C or Q		10.899	$[M+FA-H]^-$	605.3331	4,388,408	cucumber leaves
	$C_{30}H_{46}O_7$	11.979	$[M+Na]^+$	541.3136	608,563	Cucumber fruit
		11.959	$[M+FA-H]^-$	563.3226	406,285	Cucumber fruit
		11.935	$[M+Na]^+$	541.3136	1,292,941	cucumber peel
Cucurbitacin F or O		11.939	$[M+FA-H]^-$	563.3226	682,156	cucumber peel
	$C_{30}H_{48}O_7$	12.359	$[M+Na]^+$	543.3292	498,850	cucumber fruit
		12.356	$[M+FA-H]^-$	565.3382	401,311	cucumber fruit
		12.340	$[M+Na]^+$	543.3292	609,277	cucumber peel
Cucurbitacin P		12.345	$[M+FA-H]^-$	565.3382	193,406	cucumber peel
	$C_{38}H_{58}O_{13}$	10.270	$[M+Na]^+$	745.3769	940,389	cucumber leaves
Cucurbitacin C glucoside		10.279	$[M+FA-H]^-$	767.38592	531,041	cucumber leaves

Mass accuracy for generating EICs was restricted to 10 ppm.

3.4.5 Unknown compound

Since pickling cucumber peel is normally perceived as more “bitter” than cucumber mesocarp and endocarp (informal sensory test), we also did compound screening based on the compound list generated by MassHunter software and found the compound that had higher concentration in cucumber peel than cucumber fruit. A compound with m/z 537.3034 ± 10 ppm in positive mode (Figure 2.9) and a compound with m/z 559.3103 ± 10 ppm in negative mode showed similar isotopic patterns at the same retention time. After accounting for the sodium or formic acid adducts’ mass, this compound’s predicted monoisotopic mass was estimated at 514.31. A compound called NSC375096 in the Metlin database with formula $C_{27}H_{46}O_9$ has this mass (Figure 2.10). The isotopic pattern of this compound is shown in Figure 2.11. This compound appears to be present at higher concentration in the peels.

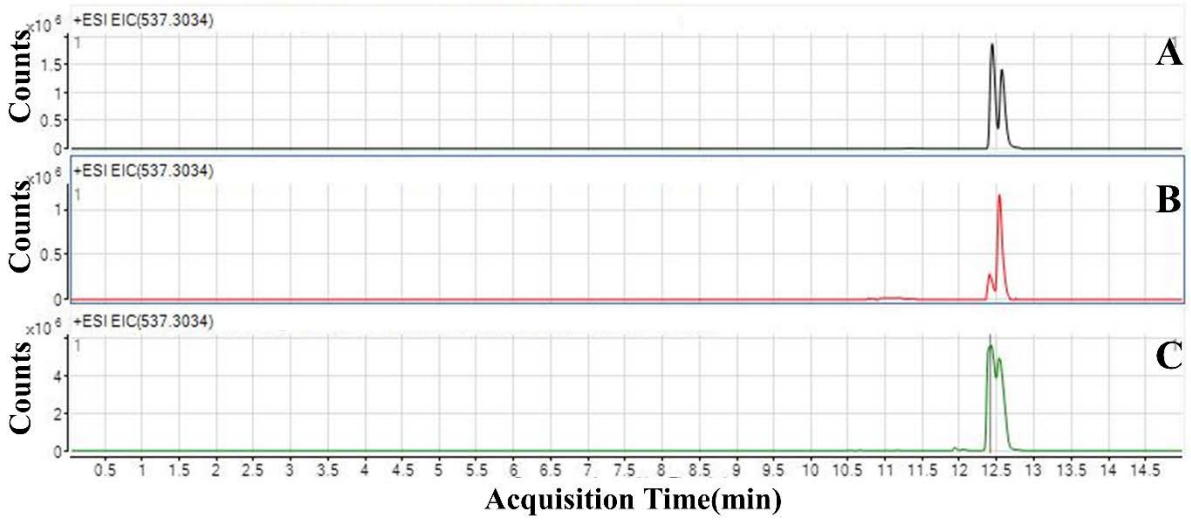

Figure 2.9. EICs of m/z 537.3034 in cucumber fruit (A), cucumber leaves (B) and cucumber peel (C) in positive ionization mode.

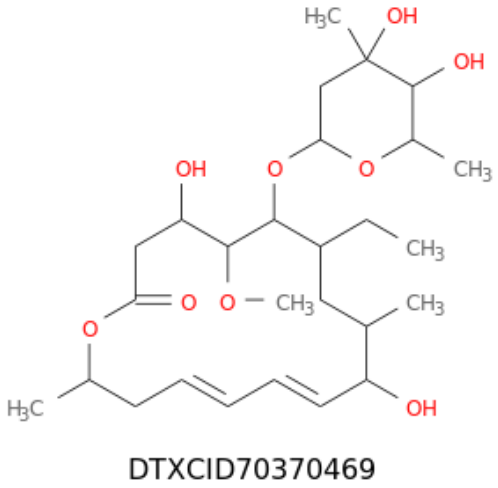

Figure 2.10. Structure of NSC3750.

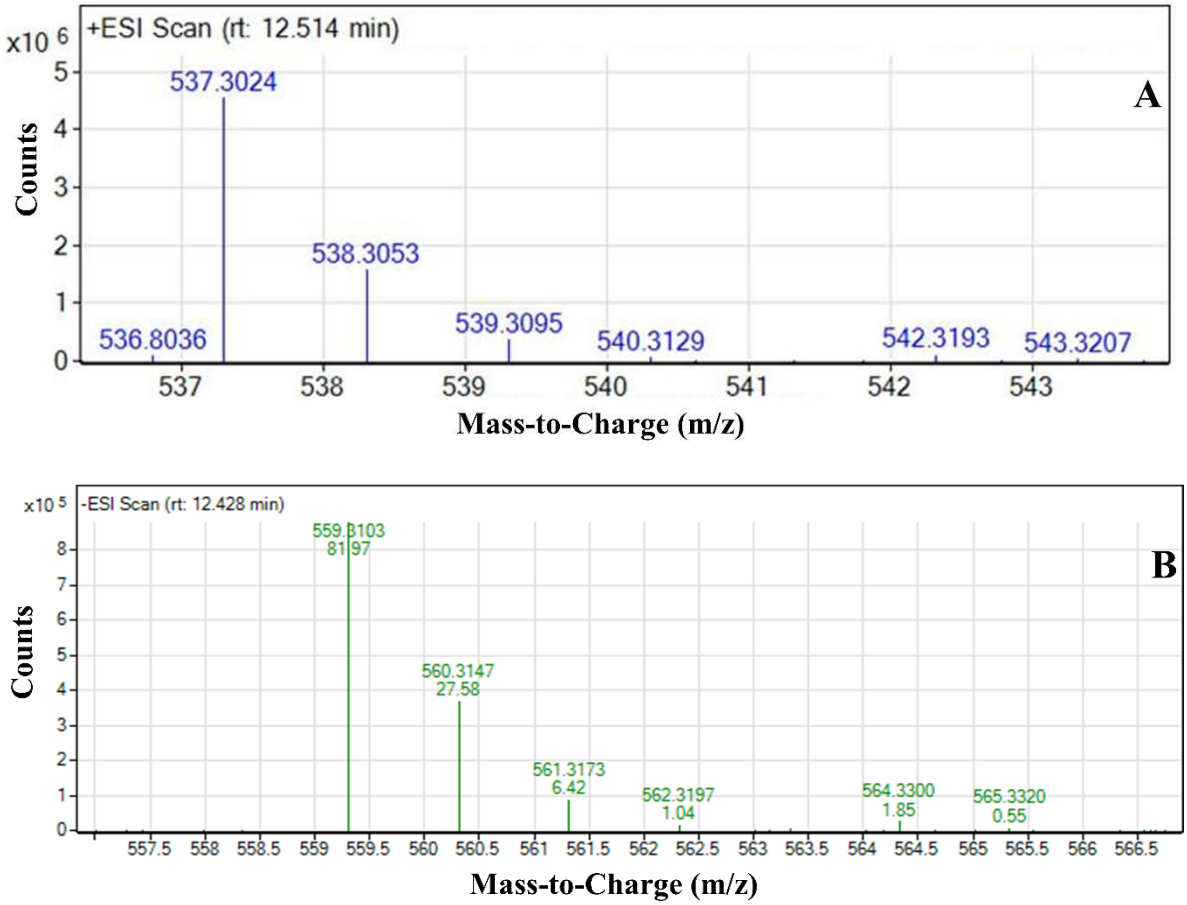

Figure 2.11. Mass spectrum of unknown compound in positive (A) and negative (B) modes.

2.5 Conclusion

Five cucurbitacins were tentatively identified in pickling cucumber fruits or leaves. CuE was not detected in picklng cucumber. Cucurbitacins B, C or Q and C glucoside were tentatively identified in pickling cucumber leaves;cucurbitacins C or Q, F or O and P were tentatively identified in pickling cucumber fruit; and cucurbitacins F or O and P were tentatively identified in pickling cucumber peels. This method was sensitive enough to detect cucurbitacins in a typical pickling cucumber cultivar and less interfering ions were observed in negative ionization mode. Future research could also investigate whether these five cucurbitacins are present in other types of cucumbers and which cucurbitacin is the major compound responsible for bitter taste. This method could be used as an analytical method for analyzing the changes in cucurbitacin contents in cucumber during food processing, including fermentation and thermal treatment.

2.6 Acknowledgement

I would like to thank Dr. Suzanne Johanningsmeier for guidance throughout this experiment, Rong Reynolds for assistance with UPLC-TOF analysis and reviewing this paper, Jennifer Fideler Moore for reviewing this paper, and Dr. Todd Wehner for providing cucumber leaves.

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CHAPTER 3. Occurrence of cucurbitacins in pickling and bitter cucumber and reduction by cucumber fermentation and acidification

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Target Journal: Food Chemistry

3.1 Abstract

Cucurbitacins are triterpene compounds that can contribute bitterness to cucurbits. Although bitterness is rare in pickling cucumber, it can result in significant product loss. In this study, high performance liquid chromatography-time-of-flight mass spectrometry (LC-TOF-MS) was employed to identify and quantify cucurbitacins in pickling cucumber. Bitter (*Hanzil*) and non-bitter (*Vlaspik*) pickling cucumber cultivars were grown under controlled greenhouse conditions with multiple harvests (n = 5) and/or greenhouse areas (n=3) as replications. Temperature ranged between 30 ℃ during the day to 18 ℃ at night. Cucumbers were cut longitudinally and each section allocated to “raw,” “fermented,” and/or “acidified” treatments. Acidified cucumber was preserved with 3% sodium chloride (NaCl), lactic acid (110 mM) and sodium benzoate (12 mM). Naturally fermented cucumber was brined (3% NaCl) and incubated at 28°C for 14 days. Samples were stored at -80°C, freeze-dried, and extracted with methanol for cucurbitacin analysis by LC-TOF-MS. Cucurbitacins C, C-glycoside, F or O, and P were tentatively identified in cucumber fruits by accurate mass in negative ionization mode. Cucurbitacin C (CuC) was confirmed and quantified with an authentic standard. *Hanzil* contained primarily CuC (13.31 ppm -110.42 ppm), which was not detected in *Vlaspik* even under simulated drought. In *Hanzil*, CuC concentration increased with cucumber size, ranging from 27.99 ± 7.67 ppm in size 2A to more than 74 ppm in size 4+ fruits. Endocarp had the highest CuC concentration (88.66 ± 44.33 ppm) followed by mesocarp (30.61 ± 15.11 ppm) and

exocarp (2.58 ± 1.75 ppm), which likely explains the increase in overall cucurbitacin content with increasing cucumber size since seed cavities become bigger with the growth of the cucumber. CuC was 3-fold lower in acidified cucumber (15.65 ± 10.33 ppm) and 10-fold less in fermented cucumber (4.90 ± 3.94 ppm) than in fresh cucumber (47.90 ± 22.79 ppm). CuC glycoside was higher only in acidified cucumber, indicating that the reduction in CuC content occurred by different mechanisms in the two pickling processes. This study serves as the basis for research into fermentation as a potential de-bittering method and identified bitter cucumber endocarp as a source of CuC for further exploration of its anti-cancer and anti-inflammatory effects.

Key words Cucurbitacin C, Bitter cucumber, Lactic acid fermentation, LC-TOF-MS, *Cucumis sativus*, Cucurbitacin C-glycoside

3.2 Introduction

Cucurbitacins are triterpenoids that are widely distributed in Cucurbitaceae, such as cucumber, melon and pumpkin. More than 20 kinds of cucurbitacins have been isolated from cucurbitaceous plants (Chen et al. 2005, Kaushik et al. 2015). In cucumber, cucurbitacin C (CuC) has been identified as the major cucurbitacin that makes cucumber taste bitter (Shang et al., 2014). Humans could taste bitterness in cucumber even when CuC concentration was less than 0.1 mg/L (Hideki et al., 2007). Because of this bitter taste, cucurbitacins serve as a strong antifeedant for many herbivores (Da & Jones, 1971). Furthermore, these bitter compounds can be toxic for humans. The median lethal dose (LD 50) of cucurbitacin B was 14 mg/kg for mouse (Cucurbitacin B hydrate safety data sheet (SDS), MilliporeSigma, US); cucurbitacin E was 340 mg/kg for rat (Cucurbitacin E SDS, MilliporeSigma, US); and cucurbitacin I was 5 mg/kg for mouse (Cucurbitacin I hydrate SDS, MilliporeSigma, US). Twenty- two cases of human poisoning after eating as little as three grams of bitter zucchini were reported in Australia from 1981 to 1982, and in Alabama and California in 1984 (Dolan et al., 2010). Most of these cases reported digestive problems, including nausea, vomiting and diarrhea after the ingestion of cucurbitacin-containing vegetables. Two additional cases reported hair loss associated with cucurbitacin poisoning (Assouly, 2018). However, cucurbitacins also showed several bioactivities which could be used as a potential pharmaceutical (Kaushik et al., 2005). Cucurbitacin B was identified to inhibit prostate cancer cell growth with 50% inhibitory concentration (IC 50) of 0.3 mM in an in vitro assay (Gao et al. 2014). Cucurbitacin D showed significant cytotoxicity against a cervical cancer cell line with IC 50 of 400 nM (Sikander et al., 2016). However, the effects of these cucurbitacins on healthy cells has not been studied. Research on the toxicity and bioactivities of CuC was limited due to its unique occurrence in

only cucumber plant. Therefore, it is important to understand the distribution of CuC in cucumber fruit to determine which part would be the best source for isolating CuC for further explore its toxicity and pharmaceutical effects.

Bitter cucumbers were produced if cucumbers were grown under stress or of a specific cucumber cultivar. Cucumber plants grown under low temperature (average minimum air temperature was under 12℃) or with twice the usual dose of nitrogen fertilizer produced more cucurbitacins, resulting in unusually bitter cucumbers (Kano and Goto, 2003). Many cucumber producers also reported that drought would induce bitter cucumber (Personal communication), and a passage written by Rosie Lerner also reported that drought, cold and poor nutrition could also result in bitter cucumber (Lerner, n.d.). In addition, CuC was first identified in a certain cultivar, *Cucumis sativus* var. *Hanzil* (Enslin et al., 1960). There are nine cucumber genes in the pathway for biosynthesis of CuC and four catalytic steps (Shang et al., 2014). In 2017, this group also found that cucumber plants metabolized this toxic bitter compound into cucurbitacin C 3-0-β-D-glucopyranoside. Even though watermelon that contained higher contents of cucurbitacin E glycoside were found to be bitter (Kim et al., 2018), glycoside has less cytotoxicity than the triterpenoid (Chang et al., 2018). During glucosylation, UDP-Glucosyltransferase UGT73AM3 served as the enzyme in cucumber plants (Zhong et al., 2017). Therefore, transferring cucurbitacin into cucurbitacin glycoside might be considered as a potential method to reduce the toxicity of bitter cucumber.

Recently, lactic acid fermentation was found to be a method to remove or change the undesired compounds that is toxic or contribute to off flavor (Filannino et al. 2018). In plant food, the metabolism of lactic acid bacteria (LAB) is complicated. Many enzymes including glucosyltransferase are involved in phenolics metabolism, fatty acid metabolism, carbon

metabolism and nitrogen metabolism, which converts flavonoid glycoside, phenolic acids, phenolic acid esters and tannins. With metabolism of lactic acid bacteria, toxic or undesirable plant food constituents are removed by hydrolysis, decarboxylation and enzymatic effects. (Filannino et al. 2018). For example, vicine and convicine (alkaloid glycoside) were hydrolyzed by *L. plantarum* in faba bean flour (Rizzello et al., 2016).

During natural cucumber fermentation, several microorganisms including aerobic bacteria, LAB, yeasts and molds are associated with cucumber. Raw cucumbers contain LAB as a minor part of their natural microbiota. LAB will outcompete the other natural microbiota due to LAB's ability to survive in extreme environment during fermentation (Franco et al., 2016). The dominant LAB in cucumber fermentation comprised *Lactobacillus, Pediococcus, Lactococcus* and *Leuconostoc* (Fleming et al., 1984, Perez Diaz et al., 2020). The homofermentative organism *L. plantarum* that produces lactic acid from glucose and fructose via the Embden-Meyerhoff-Parneas pathway is the dominant LAB species in cucumber fermentation (Fleming et al., 1984). In other lactic acid fermented foods, exopolysaccharides (EPS) produced by LAB are used to improve the texture (Galle and Arendt, 2014, Jolly and Stingele,2001). Interestingly, Laure Jolly and Francesca Stingele found that EPS clusters contain genes encoding glycosyltransferase from *Streptococcus thermophilus, Streptoccus macedonicus, Lactobacillus helveticus, Lactobacillus delbrueckii* subsp*. bulgaricus and Lactobacillus lactis strains* (Jolly & Stingele, 2001), but most of these are not closely related to cucumber fementation. Various lactobacilli strains possess glucosyltransferase genes (Kralj et al., 2003), including *Leuconostoc citreum* and *Lactobacillus reuteri, fermentum, sake,* and *parabuchneri* that are relevant to cucumber fermentation. The *L. plantarum* strain FMNP01 isolated from mango fruit had more than 200 unique genes, including glycosyltransferase (Li et al., 2014). In addition, many researchers found that *Leuconostoc*

mesenteroides had glycosyltransferase genes (Arguello-Morales et al., 2000; Funane et al., 2000), and *L. mesenteroides* is active at the beginning of cucumber fermentation (Franco et al., 2016; Fleming et al., 1984).

Cucurbitacins may also have antimicrobial activity. CuC at a concentration of 10 mg/mL inhibits growth of *Phytophtora cacforium* (fungi) (Miro, 1995). Cucurbitacin B inhibited growth of *Staphylococcus aureus* with the minimum inhibitory concentrations ranging from 0.12 to 0.44 mg/L (Hassan et al., 2017). Since cucurbitacins have antimicrobial activity, cucurbitacins may also have an influence on the microorganisms in cucumber fermentation.

We hypothesize that cucurbitacin C can be metabolized into cucurbitacin C glycoside during cucumber fermentation since lactic acid bacteria associated with cucumber fermentation may contain glycosyltransferase genes. The objectives of this study were to determine the occurrence and distribution of cucurbitacins in cucumber fruit and explore whether fermentation could be a potential processing method to reduce cucurbitacins in cucumber.

3.3 Materials and methods

3.3.1 Cucumber production

Three cultivars of cucumber were germinated on February 11, 2020: one flat of Sire, one flat of Hanzil, and two flats of *Vlaspik*. Hanzil seeds (accession # PI 173889) were obtained from the U.S. National Plant Germplasm (https://npgsweb.ars-grin.gov/gringlobal/search.aspx, USDA, ARS, Iowa State University) while other seeds were donated from Seminis owned by Bayer Crop Science (Missouri, US). A commercial mix of Vlaspik (88%) was blended with 12% Sire which serves as a pollinizer. Soil (SunGro germination mix, Sun Gro Horticulture, Massachusetts, US) was moistened with water until some excess moisture could be squeezed out by hand. Then, flats were filled with soil and dibbled a quarter inch for sowing each seed. Each

flat had 72 cells with 1 seed sown per cell. Flats were lightly watered, covered with plastic sheets to retain a high humidity, and placed into a 30℃ incubator for 46 hrs. Then, cucumber plants were transported to the greenhouse with temperature ranging between 30℃ during the day to 18℃ at night for enabling the plant to grow until its roots permeated the soilless media and the plant was easy to pull out from the cell with roots and soil intact.

Cucumbers were transplanted into pots (Classic 2100, 19.58 L, 14 in× 9 7/8 in, Nursery Supplies Inc.) on March 4, 2020. The pot was filled with soil (Pro-Line Growing Mix, Jolly Gardener, Poland, ME). Each cucumber plant was pulled out from its cell, and the root ball was placed about one cm below the surface of the soil in the pot. Each pot had four plants. Five pots of the same cultivar (Hanzil or Vlaspik) were grouped together, and each group was randomly assigned to different locations in the greenhouse to account for environmental variation (Figure 3.1). An additional five pots of Vlaspik were planted to test the effect of induced drought during fruit maturation. Otherwise, all production practices (fertilizer, irrigation, etc.) were optimized during the season for all other cucumbers. To induce water stress, three days prior to first harvest watering was stopped to the five pots of the Vlaspik drought plants. From that point on the plants in the drought treatment plots were only watered when wilting was visible. This management practice to induce drought was repeated for the remainder of the study. Sixteen pots of Sire pollinizer cultivar were strategically placed between and outside of each replication in order to promote fruit set and high-quality fruits. One box of bumble bees (Natupol Excel, Zuid-Holland, Netherlands) was placed in the greenhouse for pollination once female flowering began. Cucumber fruits were first harvested on April 10, 2020. Then, cucumber harvests continued over the next 5 weeks to collect a sufficient number of fruits from each replicate for the different experiments.

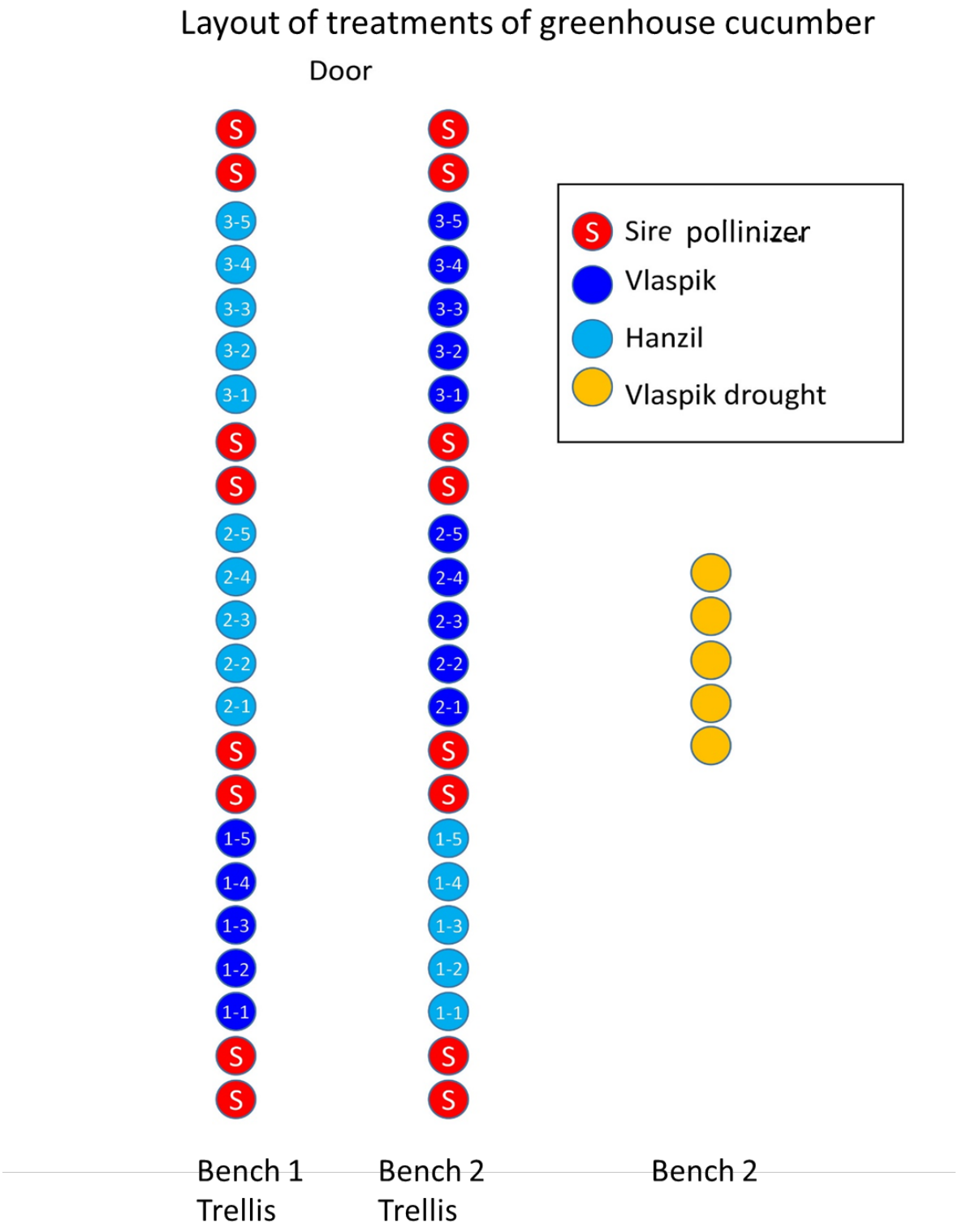

Figure 3.1. Greenhouse diagram.

3.3.2 Cucurbitacin C content during cucumber fermentation and acidification

3.3.2.1 Experimental design

There were three treatments: fresh, acidified and fermented cucumber for each of two cucumber cultivars (Hanzil and Vlaspik). Cucumbers were sorted by size and graded based on diameter (USDA grading standards for pickling cucumber: https://www.ams.usda.gov/grades-standards/pickling-cucumbers-grades-and-standards). Size 2 cucumbers from each greenhouse plot were pooled and cut into thirds longitudinally. Each third was allocated to "raw", "acidified", or "fermented" treatments. Cucumbers harvested on April 21, 2020 and on April 25, 2020 were used as blocks for this experiment. For each harvest, there were three replicates for Hanzil (one replicate per plot), three replicates for Vlaspik (one replicate per plot), and 1 replicate for Vlaspik-drought. Each replicate was composed of 19 to 29 or 13 to 16 cucumbers for Hanzil and Vlaspik, respectively.

3.3.2.2 Cucumber brining

Raw samples were vacuum sealed in polymer bags and stored at -80°C. Acidified and fermented treatments were packed by weight into jars using a 55% cucumber to 45% brine volumetric pack-out ratio. The acidified brine contained sufficient quantities of sodium chloride (NaCl), lactic acid and sodium benzoate to equilibrate to 3% (513 mM), 0.991% (110 mM), and 0.173% (12 mM), respectively. The fermented brine contained 3% NaCl after equilibration. Acidified and fermented cucumbers were incubated at 28°C for 14 days. Acidified and fermented cucumbers were moved to refrigerated storage until further sample processing. Acidified or fermented cucumbers were removed from the brine, drained for one minute, vacuum-sealed in polymeric bags, and stored at -80°C.

3.3.3 Cucurbitacin occurrence and distribution in Vlaspik and Hanzil

3.3.3.1 Cucurbitacin C distribution in different size fresh and fermented Hanzil cucumber

Size 2A, 2B, 3A, 3B, 4+ cucumber from three harvests were graded and cut in half longitudinally. Each half was allocated to the “raw” or “fermented” treatment. Cucumbers from all plots of the same harvest were pooled together to obtain enough cucumbers for each grading size. Three cucumber harvests on May 2, 2020, May 8, 2020 and May 13, 2020 were used to generate three replicates. For Hanzil, each replicate was composed of 11-17, 10-19, 8-28, 8-21 or 7-14 cucumbers for 2A, 2B, 3A, 3B and 4+, respectively. For Vlaspik, each replicate was composed of 10-12, 11-13, 9-18, 12-14 or 4-7 cucumbers for 2A. 2B, 3A, 3B and 4+, respectively. Raw samples were vacuum sealed in polymer bags and stored at -80 °C. Fermented treatments were packed by weight into jars using a 55 % cucumber to 45 % brine volumetric pack-out ratio and naturally fermented in 3% salt at 28°C for 14 days. Then, fermented cucumber was moved to refrigerated storage until further sample processing. Cucumbers were removed from the brine, drained for one minute, vacuum-sealed in polymeric bags, and stored at -80°C.

3.3.3.2 Cucumber sectioning

Freshly harvested cucumbers size 3A and 3B from each cultivar were sectioned into exocarp, mesocarp and endocarp to determine the distribution of CuC in cucumber fruits. All cucumbers were washed with tap water before sectioning. Exocarp was removed from cucumbers with a kitchen peeler. Mesocarp and endocarp were separated using a sharp chef’s knife. After sectioning, each section was weighed, vacuum-sealed in a polymeric bag, and stored at -80°C. Samples were temporarily placed in a -20°C freezer for short term storage of sections during the sectioning experiment. Cucumbers harvested on May 2nd, 2020 and May 8th, 2020

were used for this experiment to generate two replications for each size of each cultivar. Each replicate composed of 20-30 or 13-18 cucumbers for Hanzil and Vlaspik, respectively.

3.3.4 Sample analysis

3.3.4.1 Sample lyophilization

Frozen cucumbers were ground in a Robot Coupe fitted with an S blade ((RSI 2Y)1, Ridgeland, MS). One 50 ml tube of cucumber homogenate was collected from each sample and stored in a -80℃ freezer for quantification of sugars and organic acids. The remaining sample was weighed into containers and stored at -80℃ overnight in preparation for freeze drying. After samples were frozen, they were placed into a VirTis freeze dryer (Genesis, NY, USA). A thermocouple was placed into the center of each sample tray for product temperature monitoring. During primary drying, the shelf temperature was set to -10℃. Once the product temperature reached around -10℃, the shelf temperature was raised to 20℃ for secondary drying for 24 hrs. Freeze-dried samples were removed and pulverized with a grinder (WSG60 3 Cup Electric Power Wet/Dry Grinder - 120V, 750W, Waring, USA). Freeze-dried cucumber powder was stored in 50 ml culture tubes under desiccation at -80℃. Sample weights were measured before and after freeze drying for moisture determination.

3.3.4.2 Sample preparation for cucurbitacin analysis

Freeze dried cucumber powder (~0.10 g for fresh samples, ~0.20 g for pickle samples) was weighed by an analytical balance and sonicated with 10.0 ml methanol for 1 hr at 25℃ (Fisher Scientific, Pittsburgh, PA, USA). Then, each sample was centrifuged at 7085 g for 10 min. Supernatant was filtered with a 0.22 um syringe membrane and transferred into a new tube. Each pellet was recovered with 10.0 ml methanol and the extraction steps described above were repeated. Equal amounts of methanol extract from each extraction cycle were transferred to an

LC-MS vial and vortexed. The extraction efficiency for the two cycles combined was higher than 98% (Appendix B).

3.3.4.3 Analysis of cucurbitacin by LC-TOF-MS

LC-TOF-MS analysis of cucurbitacin standards and sample extracts was performed using an Agilent 1260 Infinity II liquid chromatography system (LC) (Santa Clara, CA, USA) coupled to an Agilent 6230 Time-of-Flight (TOF)-MS equipped with an Agilent Jet Stream dual electrospray ionization (ESI) source. The HPLC system included a binary pump, Agilent 1260 Autosampler, a photodiode array detector set at 230 nm, and was equipped with a Waters BEH RP-C18 column (1.7 μm, 100 × 2.1 mm). The mobile phase consisted of LC-MS grade water with 0.1% formic acid (solvent A) and LCMS grade acetonitrile with 0.1% formic acid (solvent B). The flow rate was 0.25 mL/min and the injection volume was 5 μL. A linear gradient with the following concentration of mobile phase B (t min, B %) was used: (0, 0%), (2, 0%), (2.01, 0%), (4, 10%), (14, 100%), (19, 100%), (19.01, 0%). Post run time was set to 13 min to re-equilibrate the column for the next injection. The first 4 min of eluent was diverted to waste for salt removal. The ESI-TOF mass spectrometer was operated in negative ESI mode. The capillary voltage was set to 3500V, gas temperature to 350℃, and gas flow to 12 L/min. Also, reference ions included ammonium trifluoroacetate, purine and hexakis phosphazine. The formic acid adduct of hexakis phosphazine with m/z of 1033.9881 was used for accurate mass adjustment. Agilent MassHunter Workstation software (version B.07.00) was used to acquire data.

Qualitative analysis of cucurbitacins was performed by creating extracted ion chromatograms (EICs) of the corresponding formic acid adduct's expected accurate mass (Table 2.1, Chapter 2). The range of m/z for generating EICs was restricted to 10 ppm mass error.

CuC standard compound was purchased from BOC Sciences Inc. (NY, USA) and dissolved in methanol to make a 200 ppm CuC stock solution. The stock solution was serially diluted in methanol to create a fresh standard curve ranging from 0.2 ppm to 5 ppm for each run of the instrument. The R^2 was 0.99, limit of detection was 0.094 ppm, limit of quantification was 0.28 ppm, and the relative standard deviation of each extraction was 4% (Appendix B).

3.3.5 Fermentation biochemistry

Quantification of glucose, fructose, ethanol, malic acid, succinic acid and lactic acid was performed using high performance liquid chromatography (HPLC) (Agilent 1260 Infinity, Agilent Technologies Inc., Santa Clara, CA, USA) according to Fideler et al. (2018). For fresh samples, samples (2.5-3g) were thawed and ground at 30 Hz for 4 min in a RetschMill (Retsch Gmbh, Haan, Germany) 5 ml capsule with three 9 mm beads to completely break cell walls to release sugars and acid for quantitative analysis. The resulting cucumber homogenate was centrifuged at 15115 g for 20 min at 25℃ (Eppendorf F35-6-30 centrifuge, Hamburg, Germany). The supernatant (400 ul) was diluted with 400 ul 0.03 N sulfuric acid mobile phase. Diluted samples were placed into a 4℃ refrigerator for 2 hrs to allow for protein precipitation. Then, samples were centrifuged at 7245 g for 10 min at 4℃. For acidified and fermented samples, brine samples were thawed and centrifuged at 7245 g for 10 min at 4℃. Supernatant was transferred to an HPLC vial and loaded into a refrigerated autosampler tray for analysis. The HPLC system was equipped with an Aminex HPX-87H resin column (300 × 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) maintained at 37℃ with a flow of 0.03 N H_2SO_4 mobile phase at 0.6 mL/min. Acids were detected by an Agilent 1260 Infinity diode array detector (Agilent Technologies Inc., Santa Clara, CA, USA) set at 210 nm, and sugars were detected by an Agilent Infinity refractive index detector (Agilent Technologies, Santa Clara, CA, USA) connected in

series. External standard calibration was performed using eight-point calibration curves (0.5–100 mM) for all analytes.

The pH was analyzed by Accumet AR 25 pH meter (Fisher Scientific, Pittsburgh, PA, USA) with a gel-filled plastic pH electrode that was calibrated in pH 2.00, pH 4.01, and pH 7.00 buffer in cucumber homogenate or brine at 25℃.

3.3.6 Statistical analysis

The statistical analysis was performed in JMP Pro 14 (SAS Institute Inc., Cary, NC, USA). CuC and CuC-glycoside concentrations or ion counts in fresh, acidified and fermented cucumber were log-transformed and were taken as response variables in additive factorial effects models of the form

$$Y_{hi} = \mu + \tau_i + \beta_h + \epsilon_{hi}$$

where i =1, 2, 3 and h =1, 2, 3 are indices and τ_i and β_h are factorial effects for treatments and blocks, respectively. ϵ_{hi} are assumed to be independent, normally distributed experimental error terms with constant unknown error variance.

CuC content in different size cucumbers were log-transformed and were taken in the analysis of covariance model of the form

$$Y_i = \beta_0 + \beta_1 X_{i1} + \beta_2 z_i + \beta_3 z_i X_{i1} + \epsilon_i$$

where i =1, 2, 3, 4, 5 and X_{i1} is an indicator variable for the fresh treatment. X_{i1} = 1 if observation i was fresh, X_{i1} = 0 if observation i was fermented. The average diameter of each grading size was used as a continuously valued covariate z_i in the model. The experimental error terms, ϵ_{hi} are assumed to be independent, normally distributed experimental error terms with constant unknown error variance.

CuC content in different parts of cucumber were log-transformed and were taken as response variables in additive factorial models of the form

$$Y_{ij} = u + \tau_i + \epsilon_{ij}$$

where $i = 1, 2, 3$ is index and τ_i is a factorial effect for treatments. ϵ_{ij} are assumed to be independent, normally distributed experimental error terms with constant unknown error variance.

3.4 Results and discussion

3.4.1 Qualitative analysis of cucurbitacins in Vlaspik and Hanzil

CuC was detected in Hanzil fruit and confirmed with the authentic standard. This compound is the dominant cucurbitacin in Hanzil fruit (Appendix Figure B9.), which matched the results by Enslin et al. (1960). CuC was also previously found in cucumber leaves and stems (Qing et al., 2014), *Shinsyo Hakuhi* cultivar (Horie et al., 2007) and wild cultivar fruits (Shang et al., 2014). However, the CuC concentration was below the detection limit in Vlaspik, even under the simulated drought growing conditions (data not shown). CuC glycoside, which could be metabolized from CuC by UDP-glucosyltransferase UGT73AM3 in cucumber plants (Zhong et al., 2017), was also tentatively identified in Hanzil fruit (Figure 3.2).

Cucurbitacins F or O, and P were tentatively identified both in Vlaspik and Hanzil. For cucurbitacin F or O (Figure 3.3), there were two closely-eluting peaks at ~15.0 min in both Hanzil and Vlaspik. Cucurbitacins F and O are a pair of stereoisomers which are not able to be distinguished based on mass only; thus, cucurbitacin F or O might present in cucumber fruit simultaneously. Cucurbitacin F was previously putatively identified in cucumber leaves by Jevtić et al. (2017). A derivative of cucurbitacin F called 25-acetylcucurbitacin F was isolated from *Sloanea zuliaensis* (Rodriguez et al., 2003) and demonstrated potent cytotoxic activity against

breast, lung, and central nervous system human cancer cell lines with the concentration required to inhibit 50% of cell growth of 0.110, 0.065 and 0.087 ug/mL, respectively (Rodriguez et al., 2003). However, the effects on healthy cells were not determined in this study. Cucurbitacin O was identified in *Cucumis prophetarum* (Afifi et al., 1999). In this study, Hanzil cucumber fruit had an additional peak at 12.270 min with m/z 563.32255, while this peak was absent in Vlaspik. Since CuC was only found in Hanzil and is acetylated from deacetyl CuC by acetyltransferase in cucumber plant (Shang et al., 2014). Deacetyl CuC has the same mass as cucurbitacin F or O. This additional peak might be the deacetyl CuC. Further research is required to identify the structure of this compound and determine which compounds contribute to the bitterness of cucumber. Cucurbitacin P (Figure 3.4) that was previously found in *Cucumis prophetarum* (Afifi et al.,1999) showed two closely eluting peaks ~15.5 min, which was first reported in cucumber fruit in this study. Additional research needs to identify the structure of this compound and determine if the split peak represents an unknown isomer of cucurbitacin P.

3.4.2 Fermentation biochemistry

The pH, organic acids, sugars and ethanol of fresh, acidified and fermented cucumber were analyzed to determine if cucumber fermentation progressed normally (Table 3.1). Since Hanzil fruit was firstly used in cucumber fermentation, it is also important to compare the composition of Hanzil and Vlaspik that might influence cucumber fermentation. Due to the equilibration of cucumber with brines, glucose, fructose, malic acid and succinic acid were much higher in fresh samples than in acidified samples. In addition, the two cucumber cultivars (Hanzil and Vlaspik) did not have a significant difference in those components except for malic acid and succinic acid. Fresh Hanzil had higher concentrations of malic acid and succinic acid than Vlaspik ($P < 0.05$). The concentration of malic acid was 24.14 ± 1.62 mM and 21.47 ± 2.36 mM

in Hanzil and Vlaspik, respectively. The concentration of succinic acid was 30.21 ± 3.50 mM and 22.75 ± 3.58 mM in Hanzil and Vlaspik, respectively. Even though succinic acid is a slightly bitter compound (Succinc acid, PubChem, 2021), the differences in succinic acid content between Hanzil and Vlaspik may not be detectable since no previous research reported that succinic acid would be a cause of bitter cucumber. Further research is required to determine the succinic acid concentration that could result in perceptible differences in bitter taste. For fermented cucumber, the ethanol concentration was higher than previously reported for cucumber fermentations (less than 10 mM) (Lu et al., 2002), indicating the potential presence of yeasts in samples (Walker and Walker, 2018). Sugar consumption by yeasts would explain why only a portion of the sugars were converted to lactic acid. Further research needs to determine microbial differences between greenhouse cucumber and field cucumber that might cause this problem. Besides, low salt fermentation without added acid might be another concern (Etchells et al., 1961; Guillou and Floros, 1993). In addition, the cucumber fermentations were not complete in this experiment as evidenced by the presence of residual glucose in fermented cucumber. The pH, organic acids, sugars and ethanol in different sizes of cucumbers are shown in Appendix B. The same trend was observed as described above in those samples.

3.4.3 Cucurbitacin C stability during cucumber fermentation and acidification

CuC concentration significantly decreased during cucumber fermentation and acidification (Figure 3.5) of bitter cucumber. CuC was 3 fold lower in acidified cucumber and 10 fold lower in fermented cucumber than in the fresh cucumber. Interestingly, CuC-glycoside significantly increased in acidified cucumber but decreased in fermented cucumber (Figure 3.6). The main structure of CuC and the glucosyltransferase might be stable in acid medium (Huang and Woodam, 1997), so it is possible that the cucumber glucosyltransferase continued to transfer

CuC into CuC glycoside during acidification. The reduction of CuC content in acidified cucumber might be due to CuC glycoside formation and partial dilution in brine since CuC was also detected in acidified cucumber brine (6.14 ± 3.96 ppm). The CuC concentration in acidified brine did not reach full equilibration. In fermented cucumber, some samples' CuC glycoside was too low to be detected and all samples showed that CuC glycoside was lower in fermented cucumber than fresh cucumber. Therefore, CuC might be metabolized into other molecules by cucumber fermentation microbiota. However, this hypothesis needs to be further studied to determine the dynamic change of CuC during cucumber fermentation and acidification.

3.4.4 Cucurbitacin C distribution in different sizes and parts of Hanzil cucumber

Cucumber size had a significant effect on CuC concentration ($p = 0.0027$). It was the first reported that CuC concentration increased with cucumber size, ranging from 27.99 ± 7.67 ppm in size 2A Hanzil cucumber to 73.94 ± 52.87 ppm in size 4+ Hanzil cucumber (Figure 3.7). Furthermore, endocarp had the highest CuC concentration (88.66 ± 44.33 ppm) followed by mesocarp (30.61 ± 15.11 ppm) and exocarp (2.68 ± 1.75 ppm) (Figure 3.8), which was different than cucurbitacin I distribution in wild Bottle gourd (Lagenaria siceraria (Molina) Standl.) with the highest cucurbitacin I concentration in the mesocarp (Attar and Ghane, 2018). Since the ratio of endocarp is higher in more mature cucumber fruit (Table 3.2), the CuC concentration in different parts of Hanzil is likely to explain the increase in overall CuC content with increasing cucumber size. Besides, CuC was stable and accumulated in cucumber fruit, whereas CuC concentration significantly decreased in all sizes of Hanzil after cucumber fermentation ($p < 0.0001$) (Figure 3.7).

3.5 Conclusion

Cucurbitacins F or O, and P were tentatively identified both in Hanzil (bitter) and Vlaspik (non-bittter) cucumber fruit types. In addition, CuC was identified as the dominant cucurbitacin in Hanzil, and was present in much higher concentrations than the other cucurbitacins. CuC concentration increased with size with the highest concentration found in the endocarp. Bitter cucumber endocarp was identified as a source of CuC for further exploration of its anti-cancer, anti-inflammatory and other biological effects.

The reduction of CuC concentration during acidification and fermentation was shown for the first time. Since CuC-glycoside only increased in acidified cucumber, mechanisms of the reduction in CuC content are different in the two pickling processes. This research also showed that CuC could move to the brine during acidification and fermentation. Therefore, CuC could be metabolized during cucumber fermentation. However, CuC was not metabolized into CuC glycoside as CuC glycoside decreased in this study. This finding serves as the basis for research into fermentation as a potential de-bittering method.

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Table 3.1. pH, organic acids, sugars and ethanol content of fresh, acidified and fermented cucumber.

	Fresh Hanzil	Fresh Vlaspik	Acidified Hanzil	Acidified Vlaspik	Fermented Hanzil	Fermented Vlaspik
pH	5.92 ± 0.11	5.93 ± 0.13	3.19 ± 0.07	3.19 ± 0.03	4.18 ± 0.28	4.67 ± 0.46*
Glucose (mM)	57.38 ± 4.56	50.07 ± 7.83	32.98 ± 0.68	30.77 ± 2.51	10.11 ± 9.20	3.76 ± 8.40
Fructose (mM)	68.43 ± 4.77	60.64 ± 8.39	39.23 ± 0.85	37.07 ± 2.80	19.87 ± 14.48*	8.13 ± 15.71
Ethanol (mM)	2.72 ± 0.78	1.89 ± 0.96	2.25 ± 0.45	1.51 ± 0.47	27.46 ± 16.36	34.42 ± 12.16
Malic acid (mM)	24.14 ± 1.62*	21.47 ± 2.36	15.24 ± 1.90*	13.38 ± 0.38	1.59 ± 0.67	1.02 ± 0.56
Succinic acid (mM)	30.21 ± 3.50*	22.75 ± 3.58	17.98 ± 4.67	15.26 ± 3.31	17.08 ± 4.18	16.69 ± 1.55
Lactic acid (mM)	0.78 ± 0.70	0.56 ± 0.57	98.95 ± 8.00	97.87 ± 4.83	32.86 ± 11.32*	15.05 ± 13.69

Values are the means of replicates ± standard deviation. Mean values with "*" represent statistically significant differences ($p < 0.05$) between the two varieties in each treatment using Student's T test.

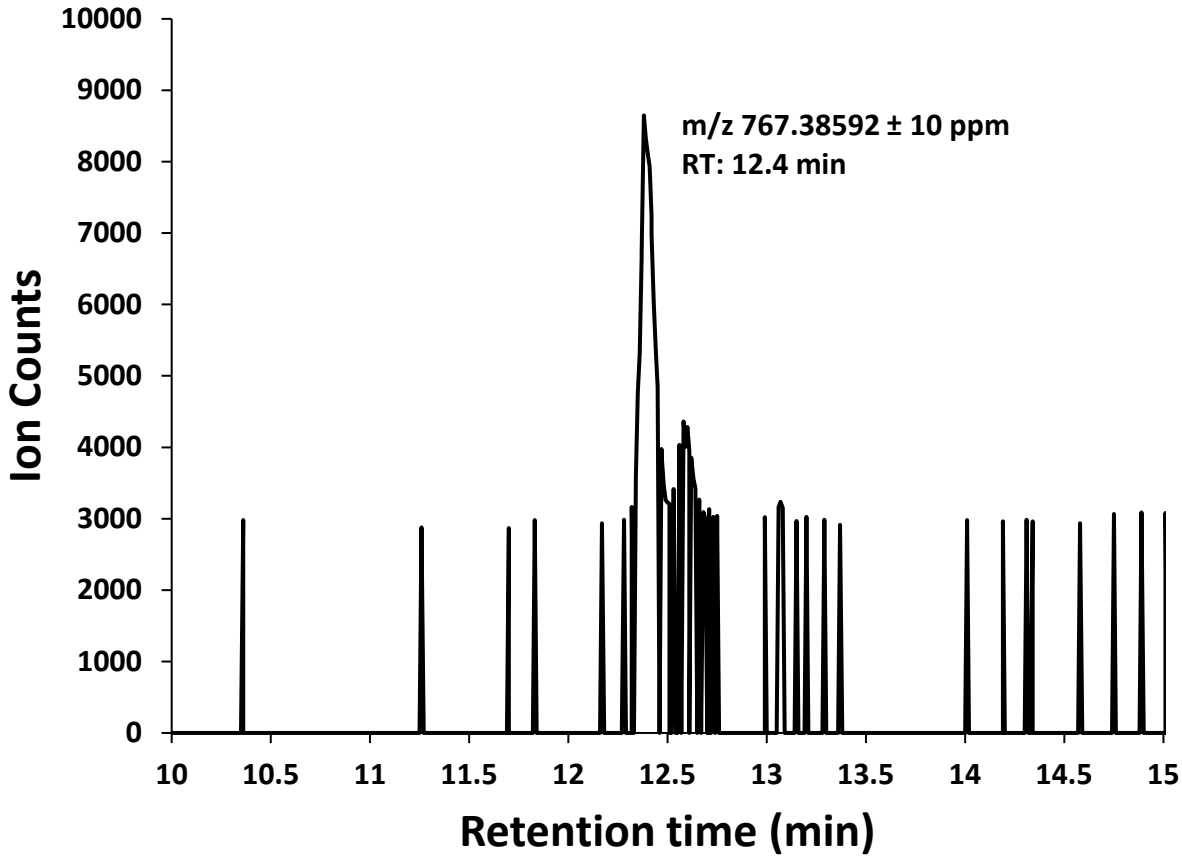


Figure 3.2. Cucurbitacin C glycoside tentatively identified in Hanzil cucumber.

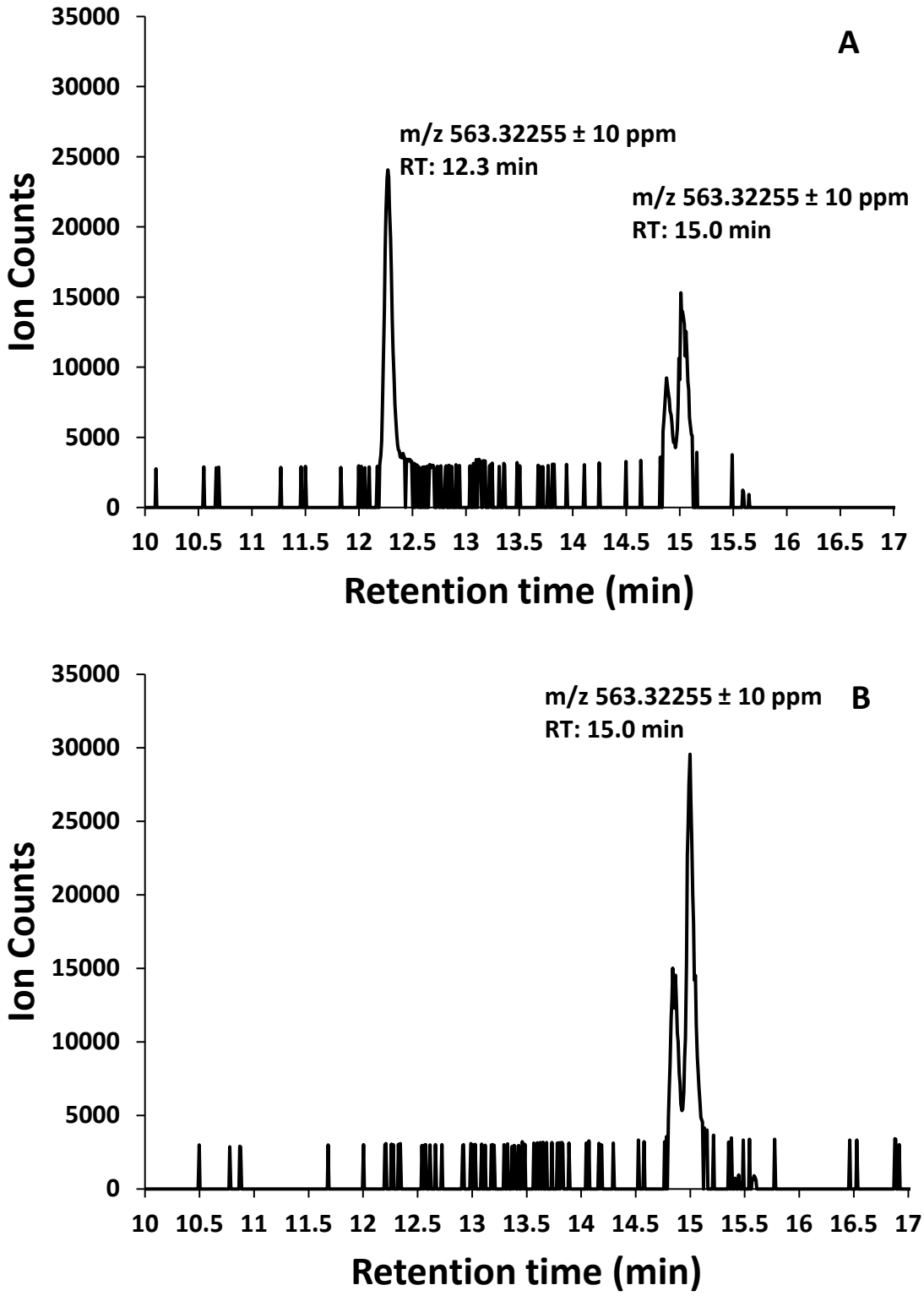


Figure 3.3. Cucurbitacin F or O tentatively identified in Hanzil (A) and Vlaspik (B) cucumber.

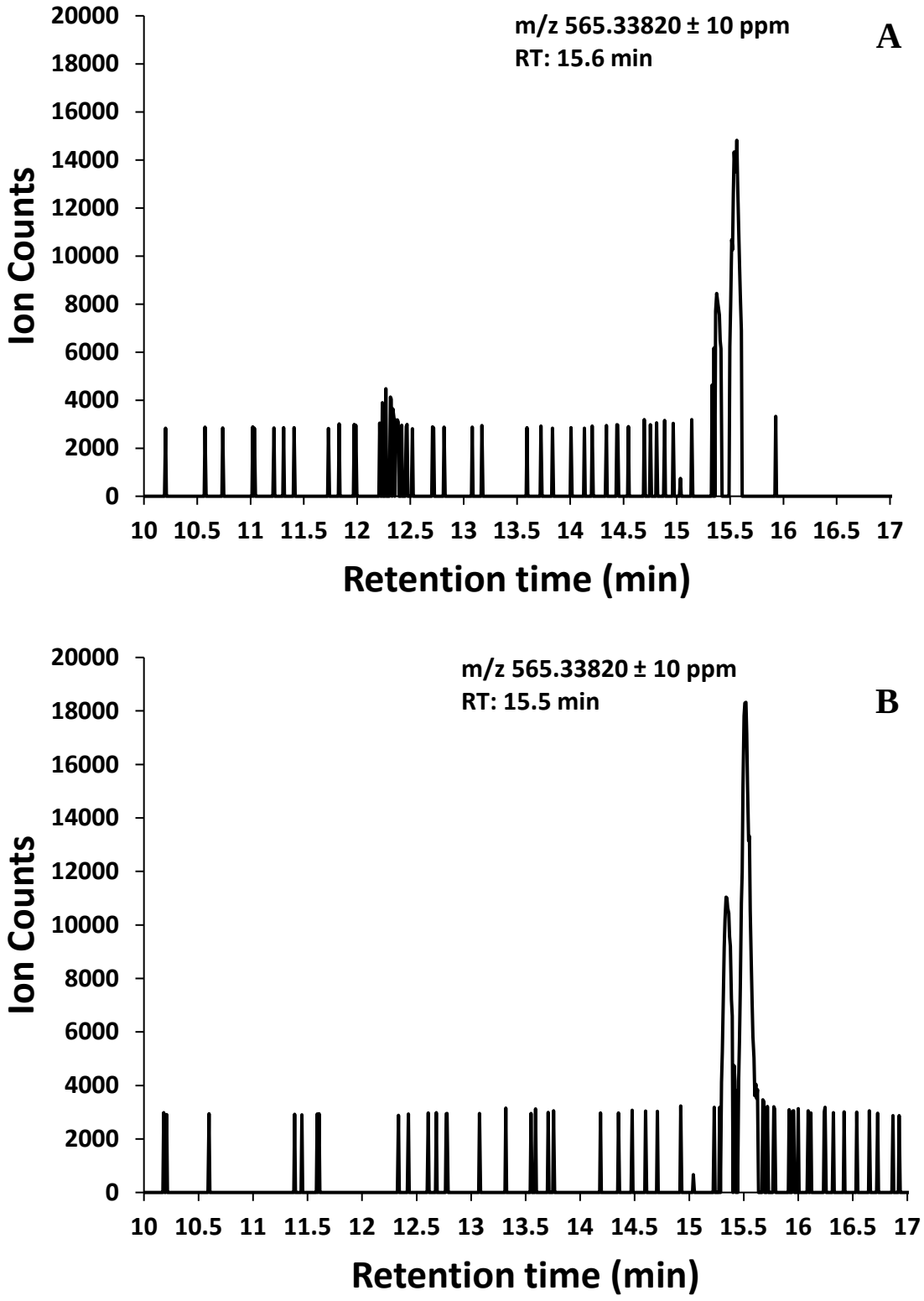


Figure 3.4. Cucurbitacin P was tentatively identified in Hanzil (A) and Vlaspik (B) cucumber.

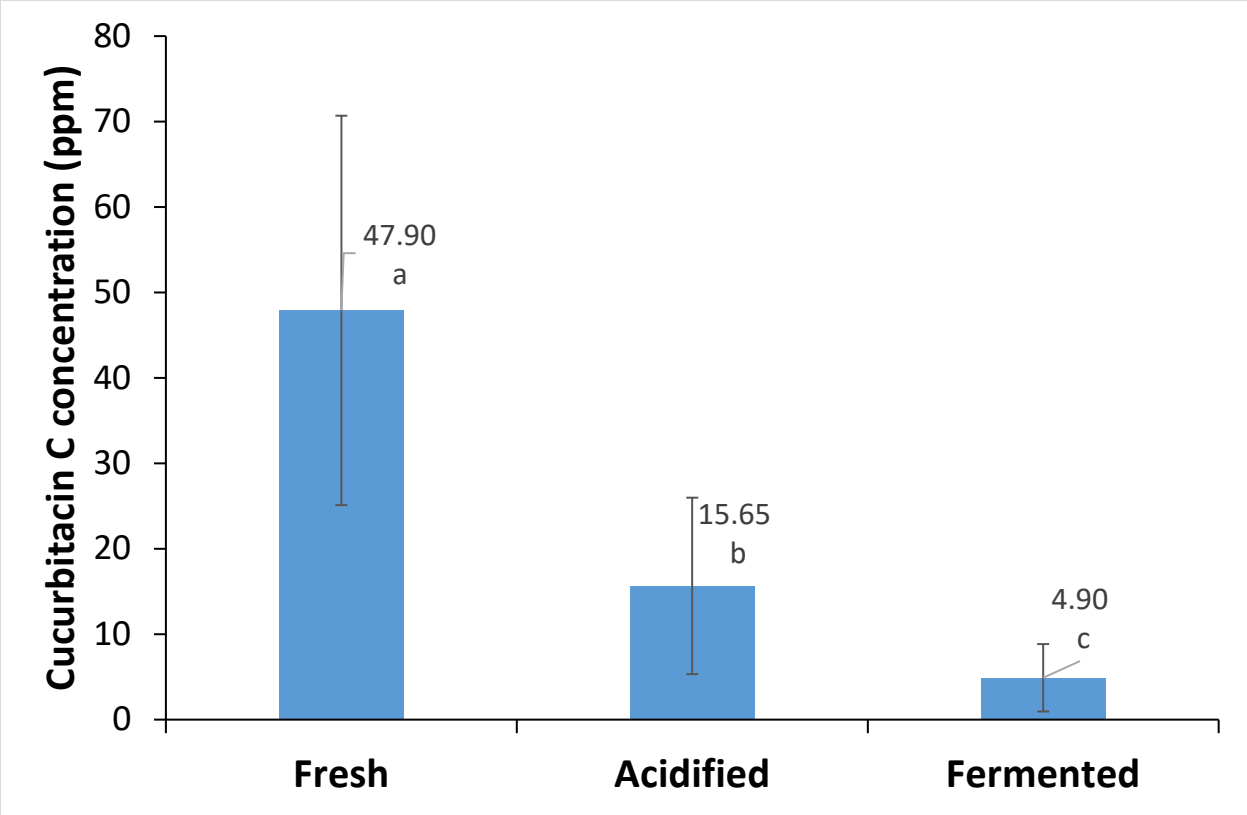


Figure 3.5. Cucurbitacin C content in fresh, acidified and fermented ‘Hanzil’ cucumber. Values are the means of replicates. Mean values with different lowercase letters showed statistically significant differences ($p < 0.05$) according to Tukey HSD test.

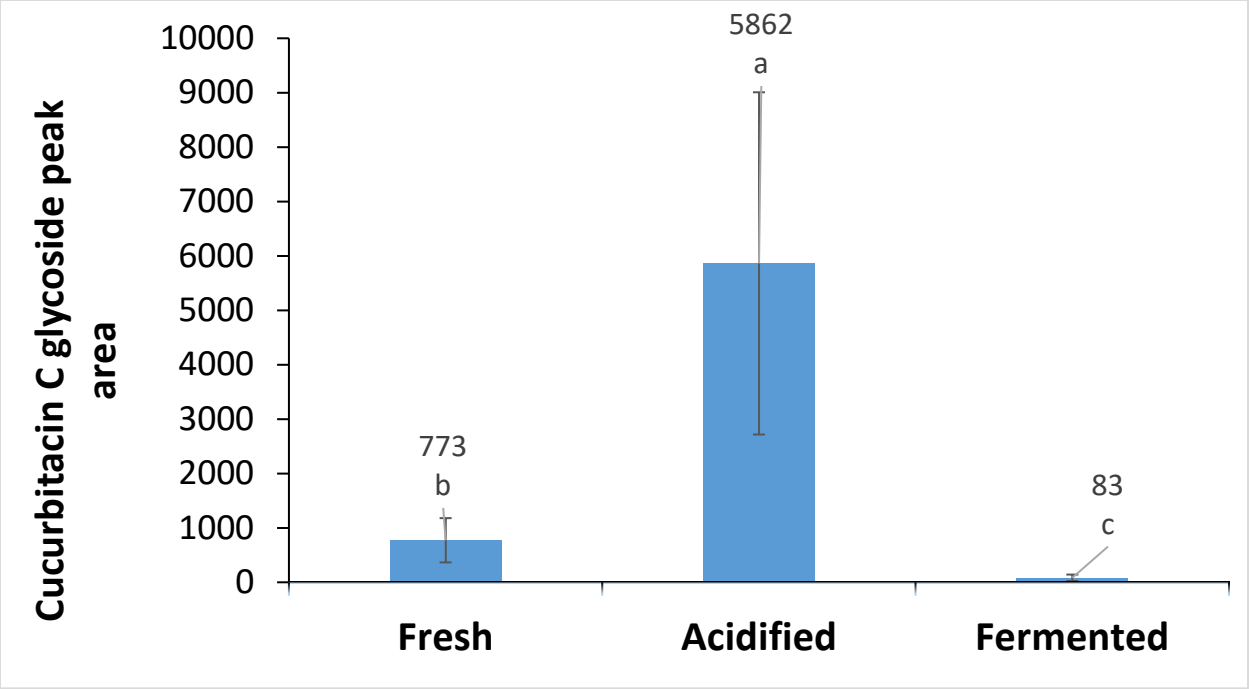


Figure 3.6. Cucurbitacin C glycoside content in fresh, acidified and fermented 'Hanzil' cucumber. Values are the means of replicates. Mean values with different lowercase letters showed statistically significant differences ($p < 0.05$) according to Tukey HSD test.

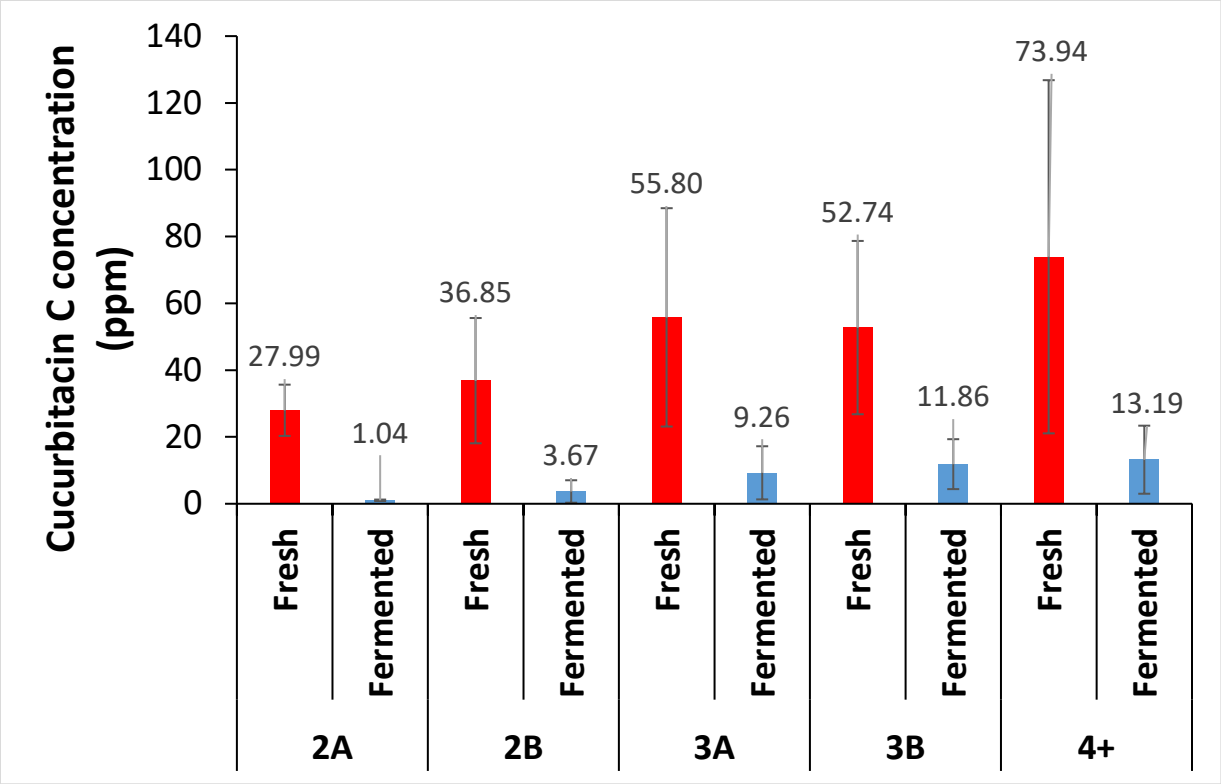


Figure 3.7. Cucurbitacin C concentration in different sizes fresh and fermented Hanzil cucumber.

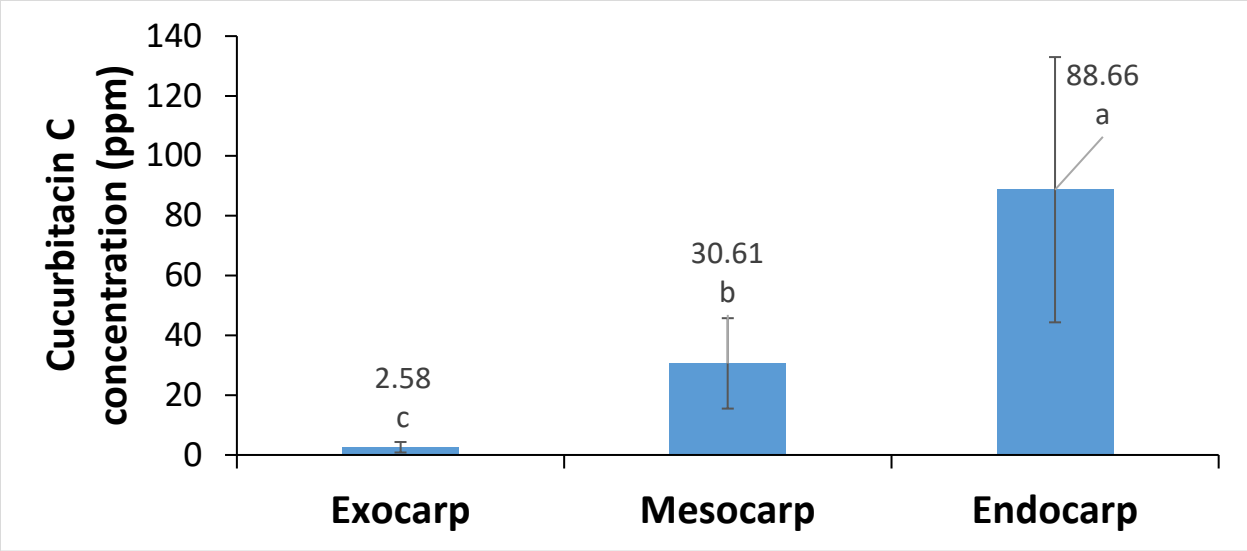


Figure 3.8. Cucurbitacin C content in exocarp, mesocarp and endocarp of 'Hanzil' cucumber. Values are the means of duplicate composite samples. Mean values with different lowercase letters showed statistically significant differences ($p < 0.05$) according to Tukey HSD test.

Table 3.2. Ratio of exocarp, mesocarp and endocarp.

Variety	Size	Exocarp %	Mesocarp %	Endocarp %
Hanzil	3A	17.45 ± 1.40	39.74 ± 2.02	42.80 ± 0.62
Hanzil	3B	15.09 ± 0.33	40.89 ± 1.13	44.02 ± 0.80
Vlaspik	3A	16.92 ± 0.00	57.31 ± 3.00	25.77 ± 2.99
Vlaspik	3B	14.51 ± 0.81	57.43 ± 2.00	28.06 ± 1.19

Values are the means of replicates ± standard deviation.

APPENDICES

Appendix A: Supplementary Material to Chapter 2

IR-MALDESI results of cucurbitacins A and J or K

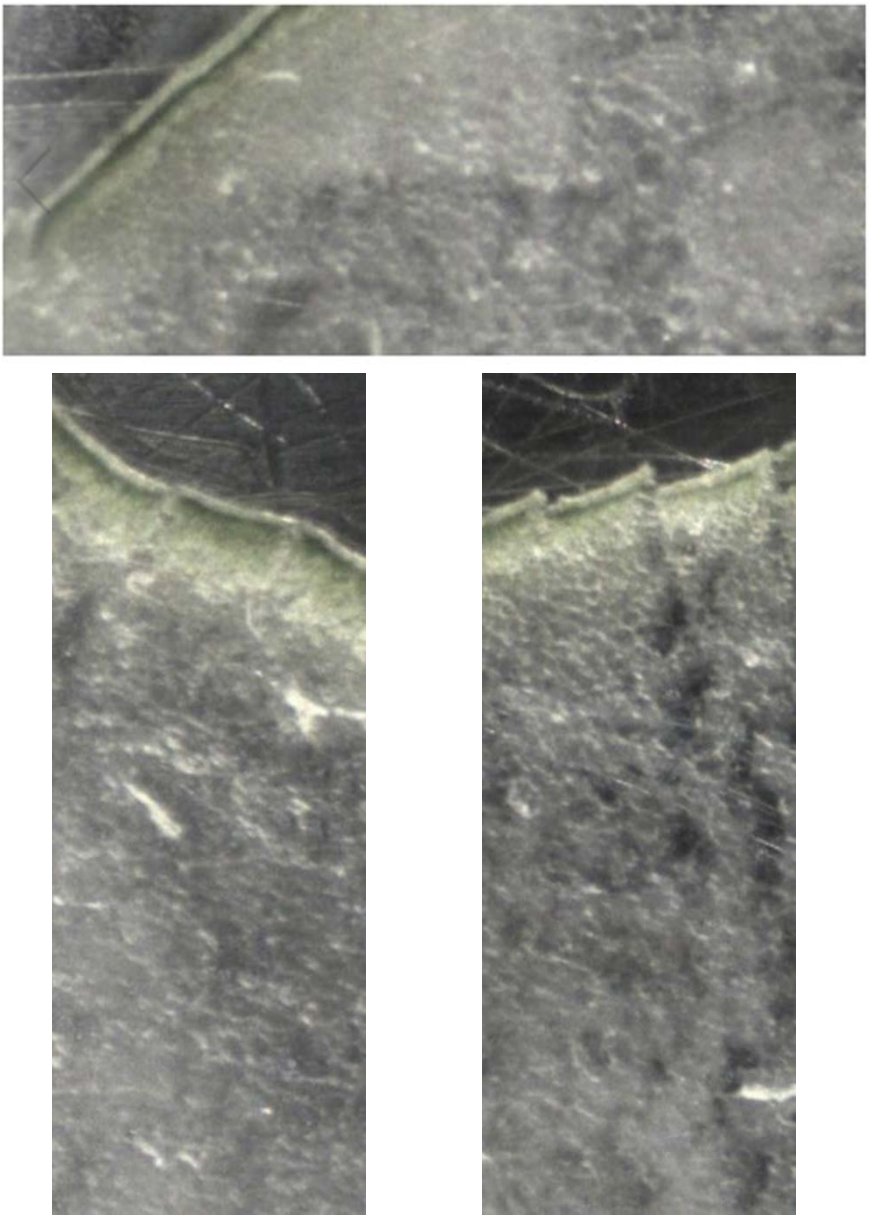


Figure A1. Image orientation of three fresh pickling cucumber pieces for three independent replicates.

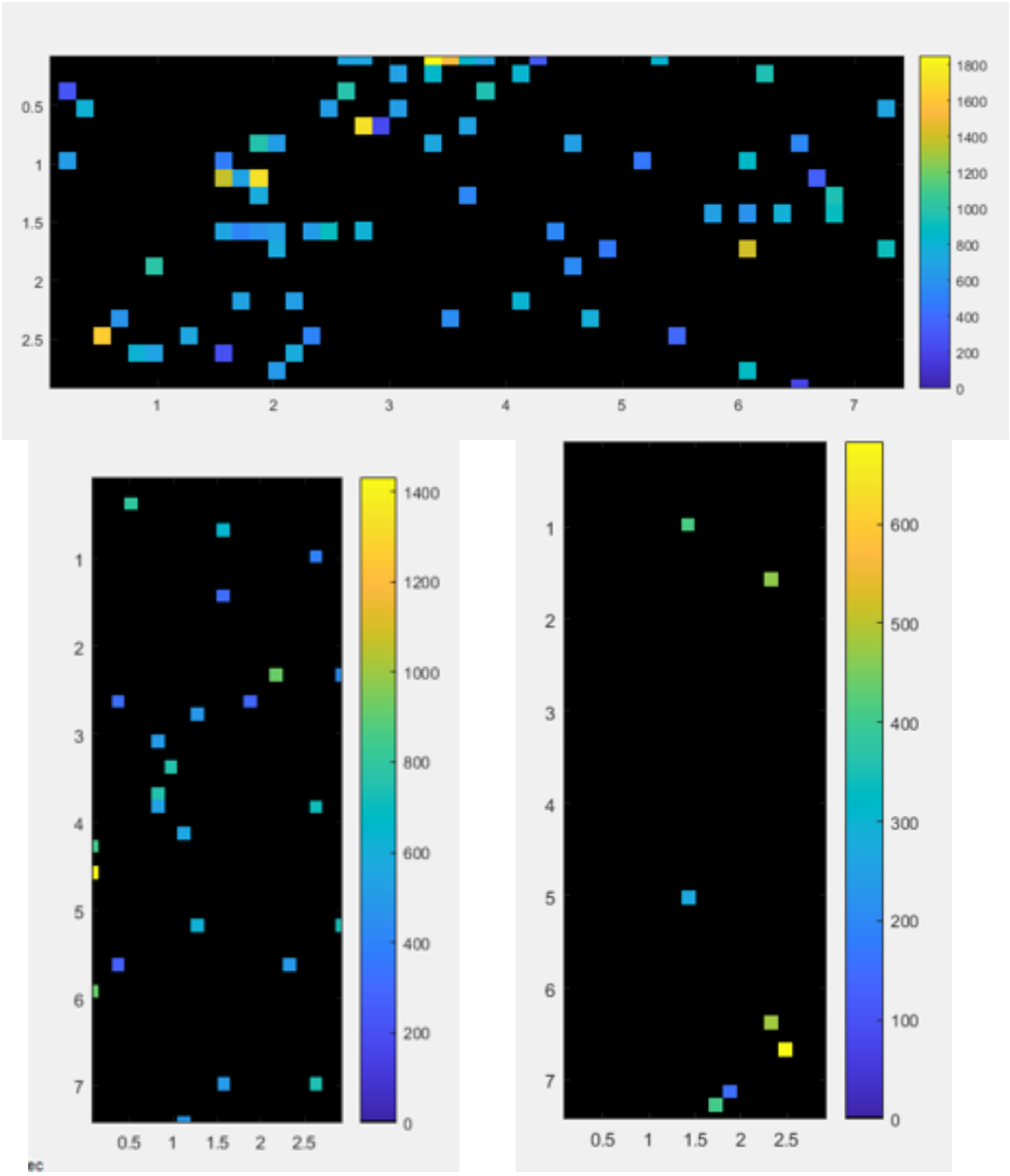


Figure A2. IR-MALDESI result of cucurbitacin A. The first cucumber showed a clear distinction between cucumber piece and background. Cucurbitacin A was tentatively identified in the first piece, but was not detected in the other two pieces.

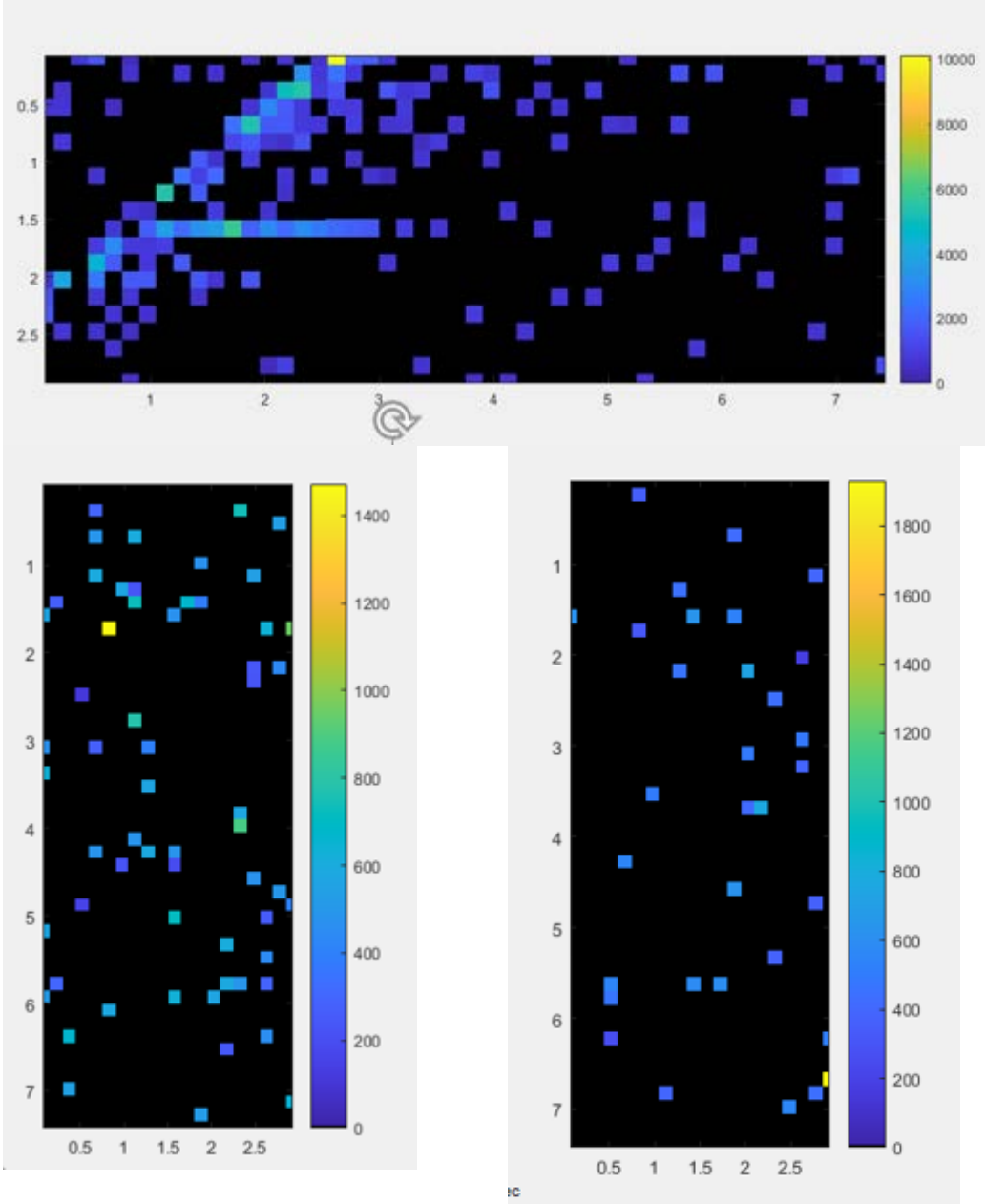


Figure A3. IR-MALDESI result of cucurbitacin J or K. The first cucumber showed a clear distinction between cucumber piece and background. Cucurbitacin J or K was tentatively identified in the first piece while the other two pieces did not have cucurbitacin J or K.

Appendix B: Supplementary Material to Chapter 3

Calibration curve and limit of detection (LOD) and limit of quantification (LOQ) of Cucurbitacin C

The calculations of LOD and LOQ were based on method and formula in Chapter 2. The standard error of intercept was 26379. The LOD was 0.9 ppm (mg/kg fresh weight) and LOQ was 2.8 ppm (mg/kg fresh weight).

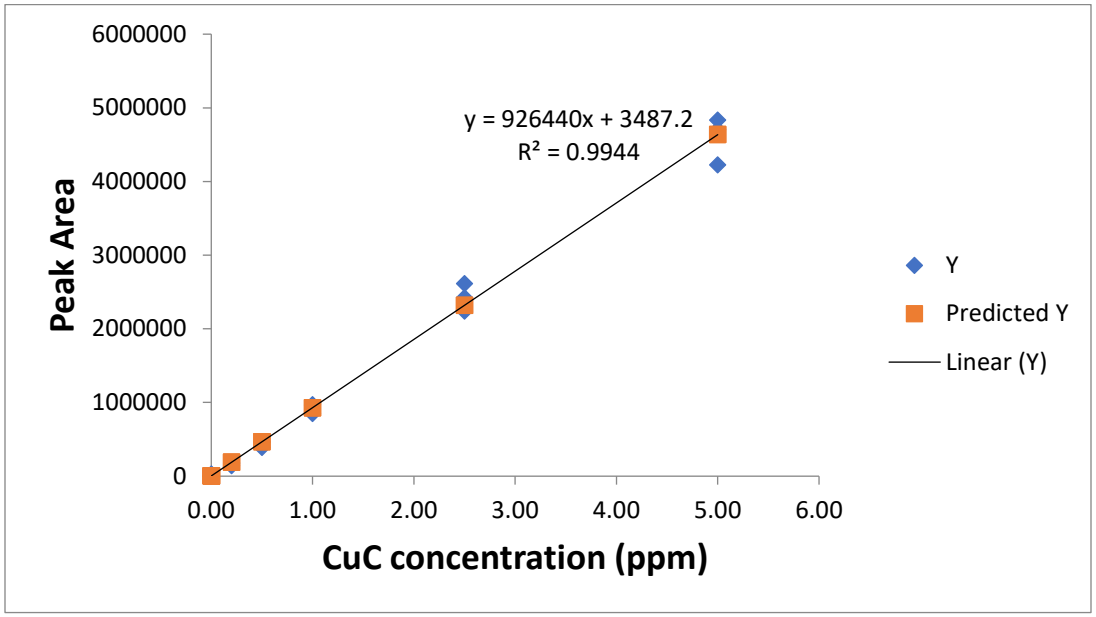


Figure B1. Calibration curve of cucurbitacin C using LC-TOF-MS. CuC standards with concentration ranging from 0.2 – 5 ppm were repeated and injected three times for generating calibration curve. The methanol blank was injected 10 times.

Extraction efficiency and reproducibility of Cucurbitacin C extraction

Method

Fresh Hanzil cucumber powder (0.10 g), acidified cucumber powder (0.20 g) or fermented cucumber (0.20 g) was extracted with 10 ml methanol and sonicated at 25℃ for 1 hr. Samples were then centrifuged at 7085 g for 10 min and supernatant was filtered with a 0.22 um syringe membrane filter into a new tube. Continue adding 10 ml methanol to pellet and repeat these steps 3 times. Another aliquot from the same freeze-dried sample was used to generate replicates.

Results

FFA 022 (Fresh Hanzil, size 2, plot 1, harvest date 4/25/2020) was used to determine the extraction efficiency for cucurbitacin C from fresh cucumber. The extraction efficiency of a single extraction cycle was around 92%. The extraction efficiency was increased to 98% with a second extraction. The % RSD of two cycles were 0.37% between two replicates.

FFA 029 (Acidified Hanzil, size 2, plot 2, harvest date 4/25/2020) was used to determine the extraction efficiency of acidified cucumber. The extraction efficiency of a single cycle was around 91%-92%. The extraction efficiency was increased to 98% if it was extracted twice. The RSD% of two cycles were 0.04% between two replicates.

FFA 030 (fermented Hanzil, size 2, plot 2, Harvest date 4/25/2020) was used to determine the extraction efficiency of fermented cucumber. The extraction efficiency of a single cycle was around 88%-89%. The extraction efficiency was increased to 96%-97% if it was extracted twice. The RSD% of two cycles were 0.81% between two replicates.

Table B1. Extraction efficiency and reproducibility of cucurbitacin C in fresh, acidified and fermented cucumber.

Sample type	Replicate 1	Peak Area 1 (EIC m/z 605.33312)	Extraction efficiency	Replicate 2	Peak Area 2 (EIC m/z 605.33312)	Extraction efficiency	Extraction efficiency ± SD %	RSD %
Fresh	Cycle 1	4,425,199	91.99%	Cycle 1	4,315,438	91.63%	91.81 ± 0.25	0.27
	Cycle 2	273,987	97.68%	Cycle 2	308,895	98.19%	97.93 ± 0.36	0.37
	Cycle 3	71,577	99.17%	Cycle 3	47,609	99.20%	99.18 ± 0.02	0.02
	Cycle 4	39,995		Cycle 4	37,719			
	Total	4,810,757		Total	4,709,661			
Acidified	Cycle 1	5,830,156	91.35%	Cycle 1	5,675,063	91.54%	91.44 ± 0.13	0.15
	Cycle 2	442,550	98.28%	Cycle 2	421,849	98.34%	98.31 ± 0.04	0.04
	Cycle 3	65,689	99.31%	Cycle 3	54,601	99.22%	99.27 ± 0.06	0.06
	Cycle 4	43,815		Cycle 4	48,145			
	Total	6,382,211		Total	6,199,658			
Fermented	Cycle 1	2,906,352	88.13%	Cycle 1	3,063,505	88.81%	88.47 ± 0.48	0.54
	Cycle 2	253,729	95.83%	Cycle 2	280,331	96.93%	96.38 ± 0.78	0.81
	Cycle 3	74,668	98.09%	Cycle 3	78,471	99.21%	98.65 ± 0.79	0.80
	Cycle 4	62,986		Cycle 4	27,339			
	Total	3,297,735		Total	3,449,646			

pH, organic acid, sugars and ethanol concentrations in different size cucumber before and after 14 days fermentation at 28℃

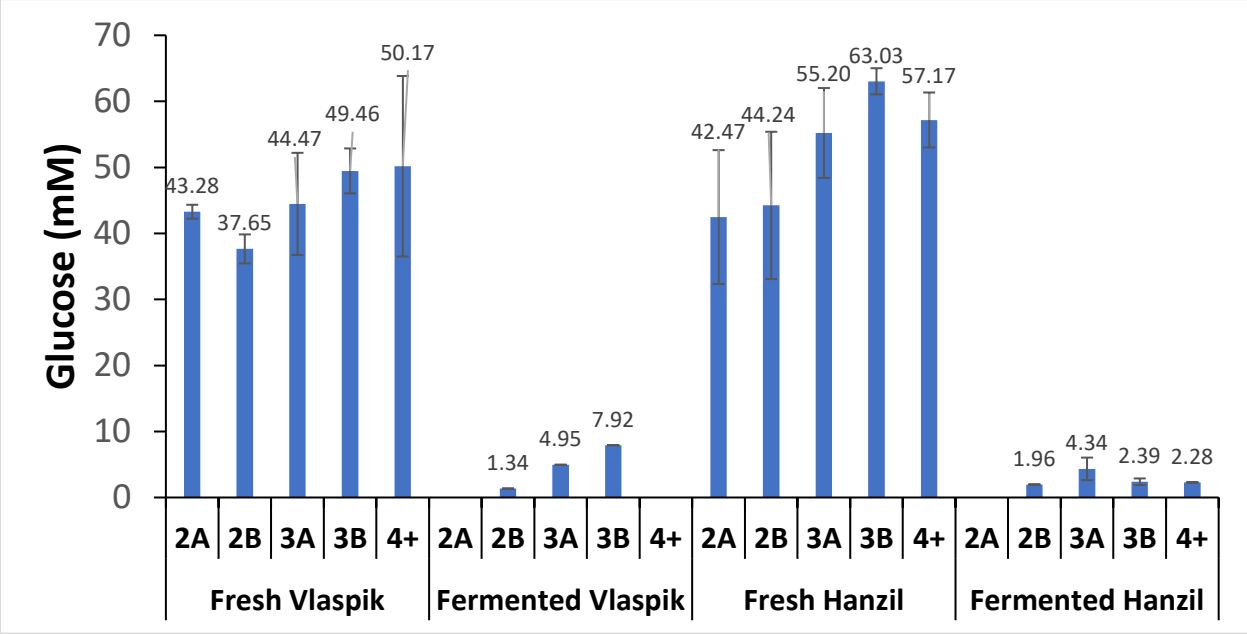


Figure B2. Glucose content of different sized fresh, and fermented Hanzil and Vlaspik cucumber. The residue glucose in size 3B fermented Vlaspik indicated incomplete fermentation.

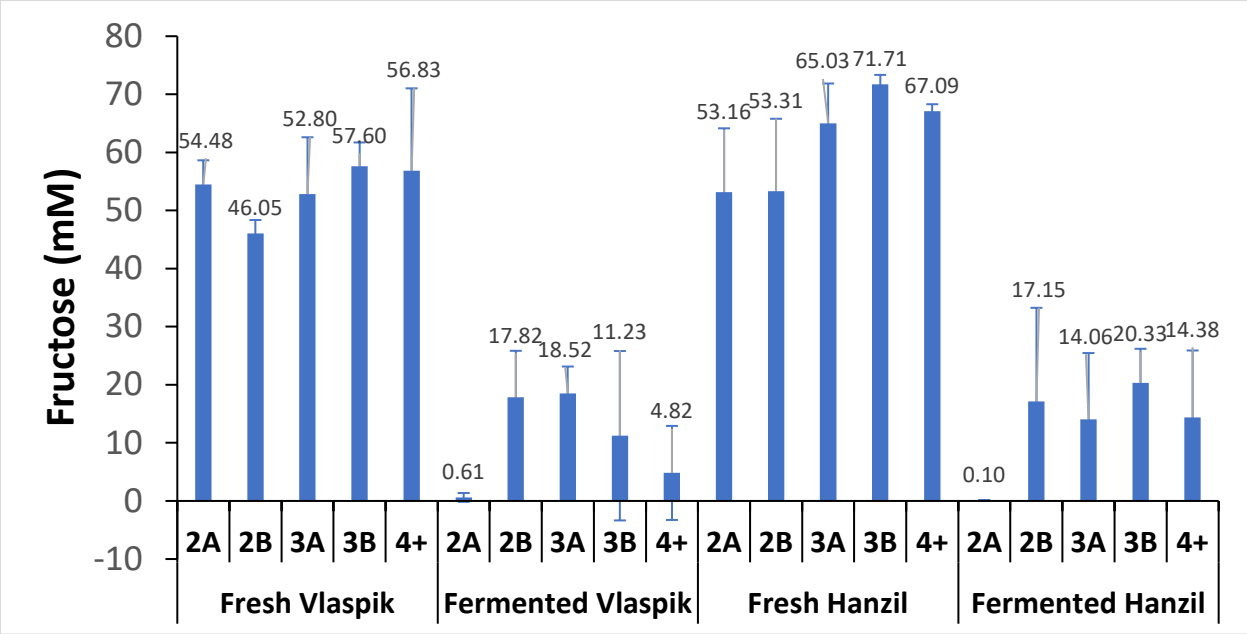


Figure B3. Fructose content of different sized fresh, and fermented Hanzil and Vlaspik cucumber.

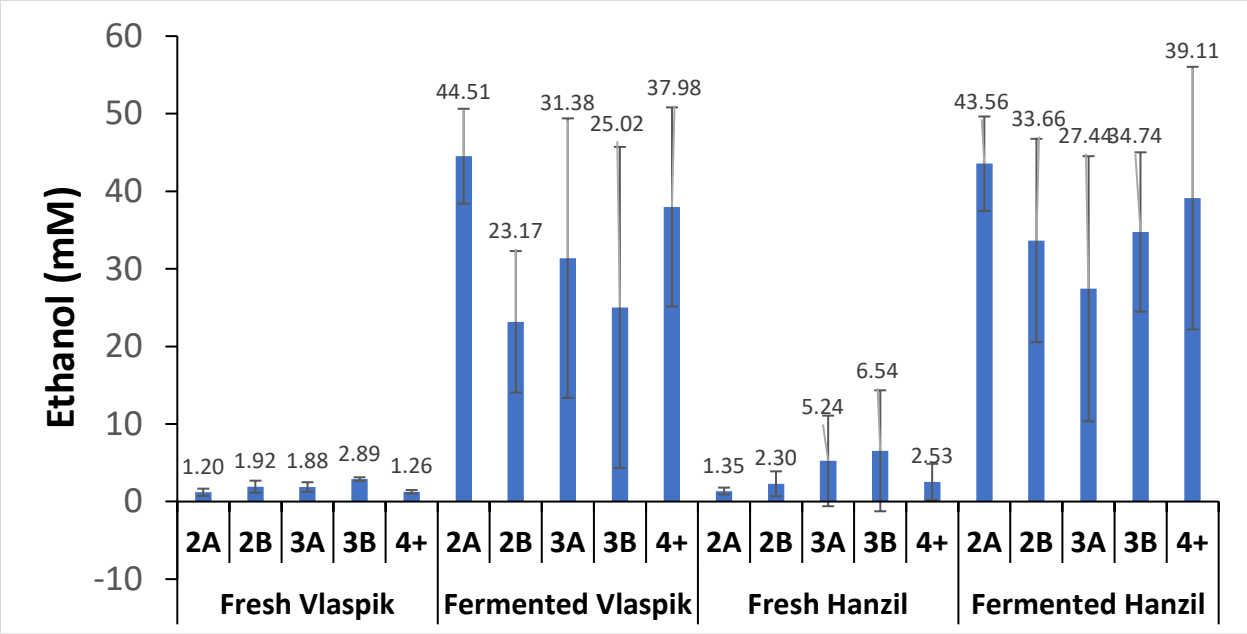


Figure B4. Ethanol content of different sized fresh, and fermented Hanzil and Vlaspik cucumber. The ethanol concentration was higher than normal cucumber fermentation, which indicated the presence of alcohol fermentation in this experiment.

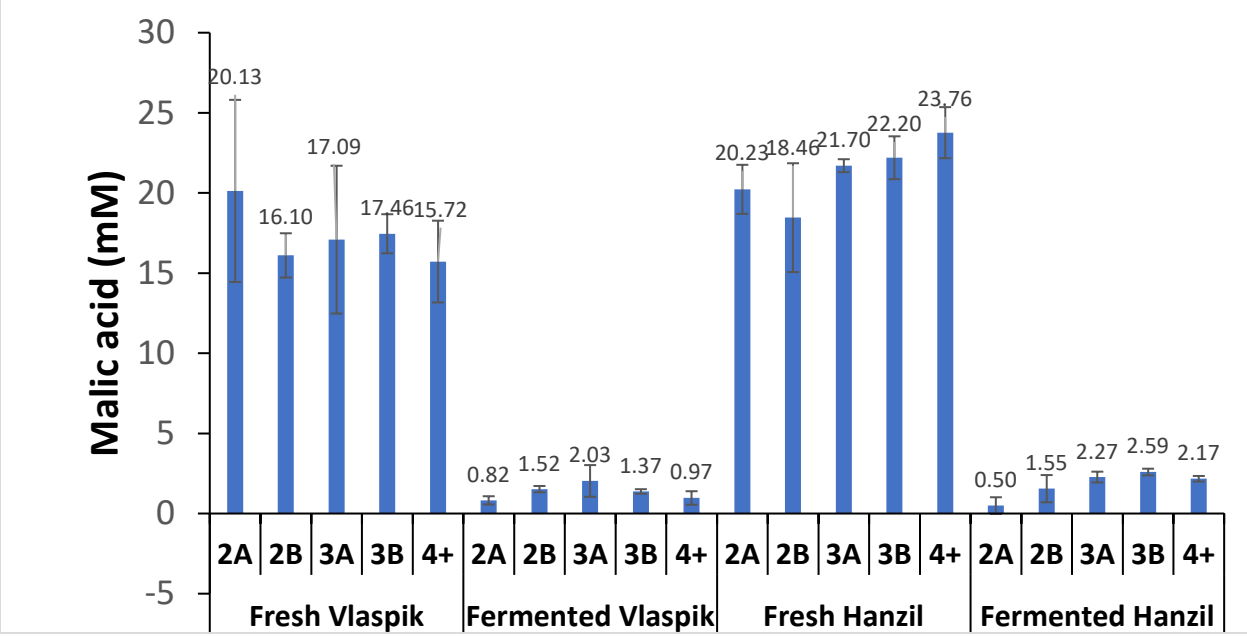


Figure B5. Malic acid content of different sized fresh, and fermented Hanzil and Vlaspik cucumber.

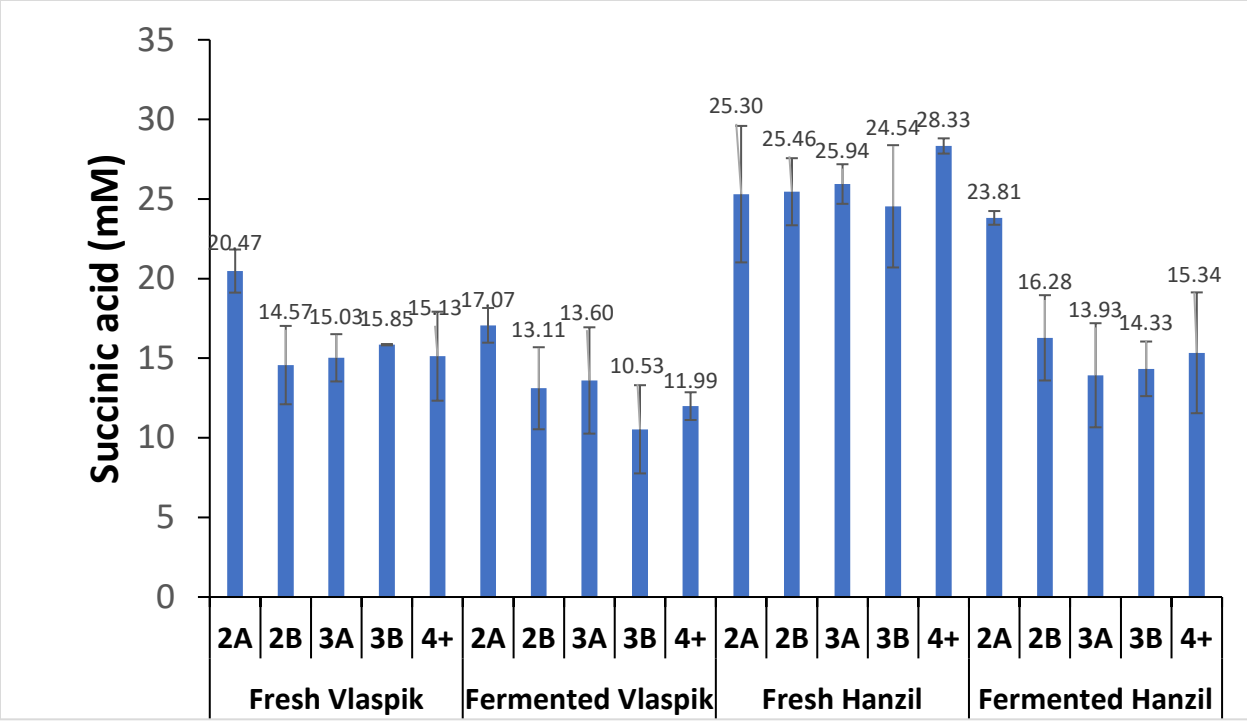


Figure B6. Succinic acid content of different sized fresh, and fermented Hanzil and Vlaspik cucumber. Fresh Hanzil had higher succinic acid than fresh Vlaspik.

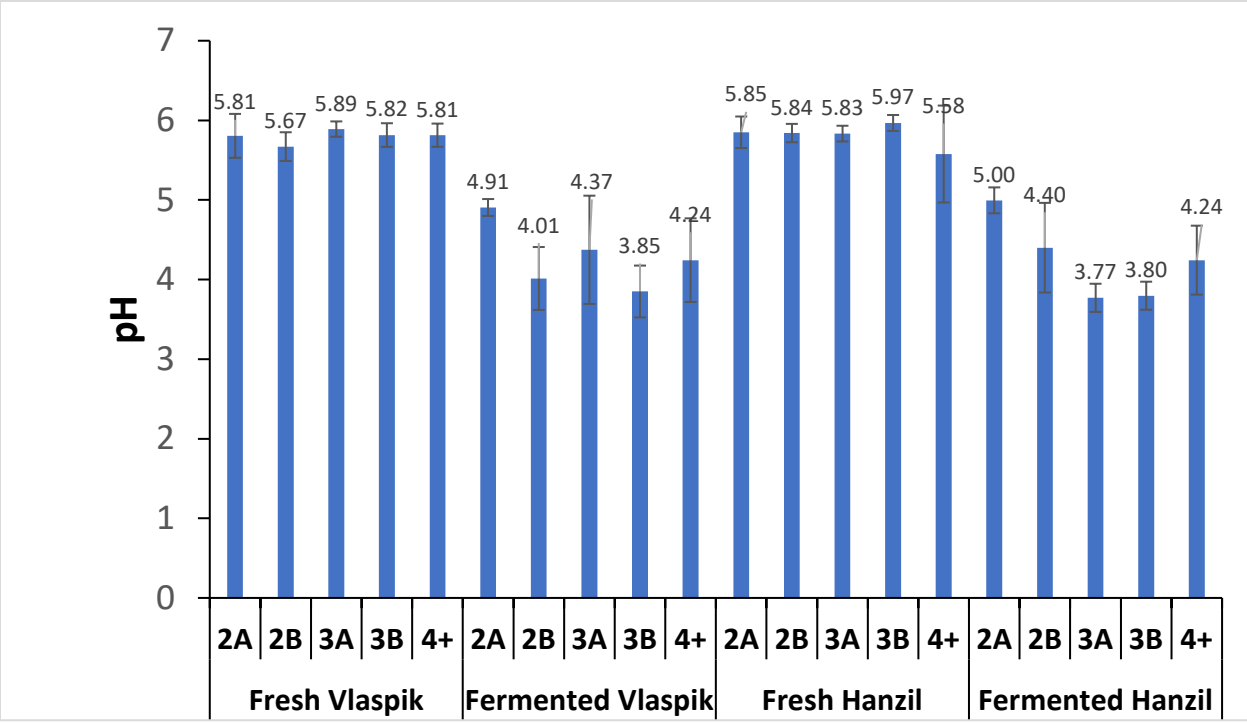


Figure B7. pH of different sized fresh, and fermented Hanzil and Vlaspik cucumber.

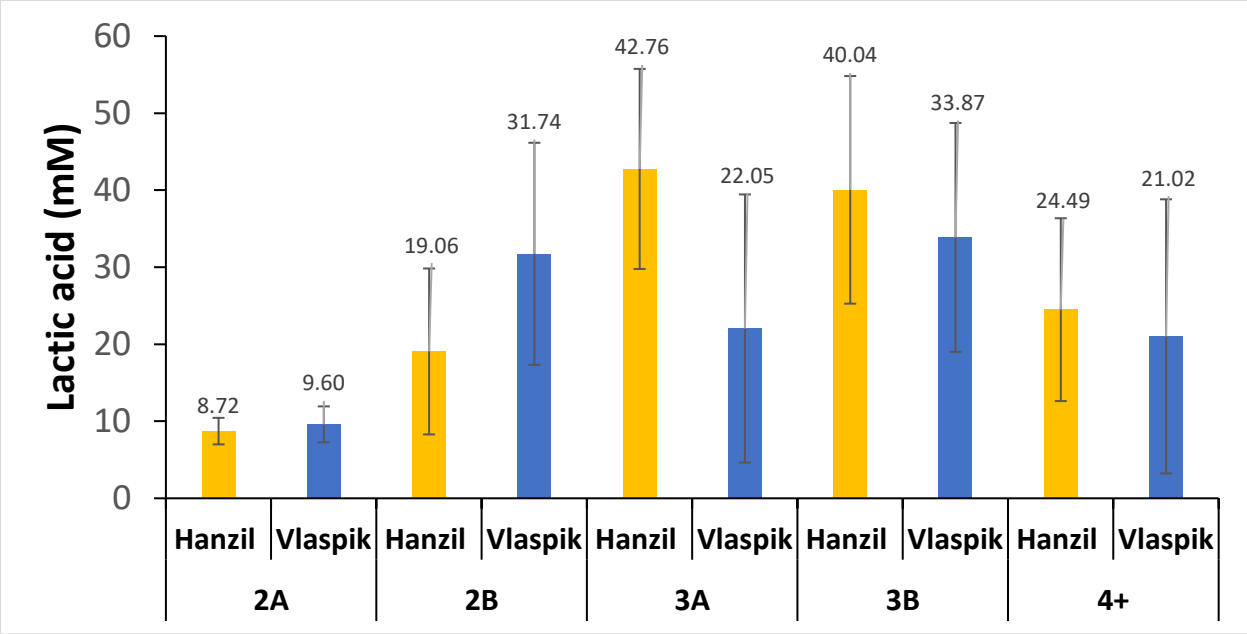


Figure B8. Lactic acid content of different sized Hanzil and Vlaspik cucumbers after fermentation for 14 days at 28 °C.

Semi-quantitative analysis of cucurbitacins F or O, P and C in Vlaspik and Hanzil.

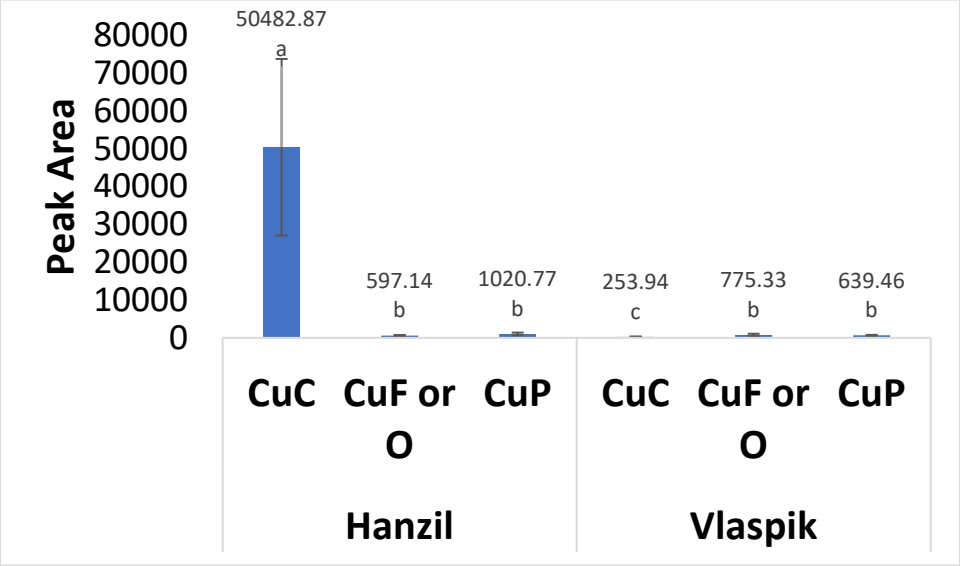


Figure B9. Semi-quantitative analysis of cucurbitacin C (CuC, m/z 605.33312), cucurbitacin F or O (CuF or O, m/z 563.32255), cucurbitacin P (CuP, m/z 565.33820) in Hanzil and Vlaspik cucumber. Mean values with different lowercase letters showed statistically significant differences ($p < 0.05$) according to Tukey HSD test.

Appendix C

Standard Operating Procedure (SOP) for freeze-drying cucumber for chemical analysis

I. Process Fundamentals

The drying process is very important for sample storage and analyzing very low concentration compounds in cucumber. Conventional drying usually requires high temperature for evaporating water, which might break down some compounds or lose some volatile compounds during the drying process. Freeze drying has been identified as a superior process to preserve food quality over conventional drying. There are 3 steps during the freeze-drying process, including freezing, primary drying and secondary drying. Water is converted into ice during freezing and sublimates during primary drying under vacuum at sub-zero temperatures. Secondary drying removes the remaining water that cannot crystallize by increasing the temperature while still under vacuum. Freeze drying fresh cucumber samples, frozen samples and pickles are described in this method.

II. Materials

- Liquid nitrogen
- Labels and Ziploc freezer bags
- Cucumber or pickle samples
- Desiccant (Anhydrous indicating drierite, 8 mesh size, ≥98% calcium sulfate, <2% cobalt chloride)
- Open containers (5*5*2 inch) with 100-200 g samples; 5*7*2 inch containers with maximum of 250 g cucumber homogenate; or trays for sliced cucumber (may vary)
- Glass Jars (A 48 oz pickle jar could fit 8 - 50 ml tubes)
- Vacuum sealer bags (Avid Armor 8’’*12’’)

III. Equipment

- Freeze dryer (VirTis, Genesis 25XL, capacity of the condenser: 25L)
- Mortar and pestle
- Sharp Chef's Knife
- Waring WSG60 3 Cup Electric Power Wet/Dry Grinder - 120V, 750W
- Robot Coupe with S blade (RSI 2Y1, Ridgeland, MS) S blade (part number: 27055 https://www.webstaurantstore.com/robot-coupe-27055-smooth-s-blade/64927055.html)
- -80℃ Freezer

IV. Safety Information & Hazard Warnings

- Liquid Nitrogen
    - Hazards
        - Extremely cold (−195.79 °C) The vaporization of liquid nitrogen can rapidly freeze skin tissue and eye fluid, resulting in cold burns, frostbite, and permanent eye damage even by brief exposure.
        - Liquid under pressure
    - Personal Protective Equipment (PPE)
        - Lab coat
        - Cryogenic Gloves
        - Safety glasses with side shields
        - Cryogenic Apron
    - Storage
        - Keep container tightly closed in a dry, cool, and well-ventilated place.
        - Protect from sunlight.

- Spill Measures
  - Prevent further leakage or spillage if safe to do so.
  - Clean contaminated objects and areas.
- First Aid Measures
  - If inhaled
    - If breathed in, move person into fresh air.
  - In case of skin contact
    - In case of contact with liquefied gas, thaw frosted parts with lukewarm water. If there is change in skin color or pain after contact with liquid nitrogen, call 911.
  - In case of eye contact
    - Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.
  - If swallowed
    - Rinse mouth with water.

- Vacuum pump oil and flushing liquid
  - Hazards
    - No Occupational Safety and Health Administration's (OSHA) hazards
  - Personal Protective Equipment (PPE)
    - Lab coat
    - Safety glasses with side shields

        - Gloves (specify the type)

- Storage
    - Keep container tightly closed in a dry, cool, and well-ventilated place.
    - Protect from sunlight.
- Spill Measures
    - Prevent further leakage or spillage if safe to do so.
    - Clean contaminated objects and areas with absorbent material (e.g. cloth, fleece).
- First Aid Measures
    - If inhaled
        - Move to fresh air
    - In case of skin contact
        - Wash contact areas with soap and water. Remove contaminated clothing.
    - In case of eye contact
        - Flush thoroughly with water. If irritation occurs, get medical attention.
    - If swallowed

- Do not induce vomiting. If vomiting occurs naturally, have victim lean forward to reduce risk of aspiration. Call a poison control center immediately.

- Vacuum pump
    - Hazards
        - Physical hazard: potential burn risk if touched after pump gets hot during operation
        - Fire hazards if pump malfunctions or overheats and ignites nearby flammable or combustible materials.
    - Personal Protective Equipment (PPE)
        - Lab coat
        - Safety glasses with side shields
        - Gloves
    - First Aid Measures
        - In case of skin contact, rinse affected area with cool water for five to ten minutes or until the pain subsides; apply moisturizing lotion; take pain medication if needed and follow up with a medical care provider if burn is severe.

- Robot Coupe
    - Hazards

        - Physical hazard: extremely sharp "S" blade presents risk of cuts
    - Personal Protective Equipment (PPE) & Preventive Practices
        - Lab coat
        - Safety glasses with side shields
        - Cut resistant gloves for handling the blade
        - Make sure blade is securely in place and lid is securely locked prior to turning on the Robot Coupe
    - Storage
        - Place the sharp "S" blade inside the Robot Coupe, keep the lid screwed on and store on a flat surface
    - First Aid Measures
        - In case of laceration when handling the sharp "S" blade, clean and dry the wounded area, and use a first aid kit to dress the wound. If serious incident occurs, seek professional medical care immediately.

V. Training Requirements

- Chemical Hygiene Plan
- Compressed Gas Regulator Safety Training
- Introduction to Cryogenic Safety
- Liquid Nitrogen Handling
- Freeze Dryer

- Robot Coupe

VI. Procedure

1. Cucumber Sampling

   a). For fresh cucumber or pickles, cut cucumber into 6 mm slices with a sharp knife. Place cucumber slices into a mortar and pour liquid nitrogen over the slices immediately for preventing enzymatic reactions. Use tongs to mix cucumbers to ensure every cucumber slice is in direct contact with liquid nitrogen. Remove cucumbers from mortar after liquid nitrogen evaporates, and put them into a Ziploc bag, press bag to remove excess air, seal, and store in -80 ℃ freezer immediately.

   b). For frozen cucumber, move cucumbers into ambient temperature and thaw for several minutes until they can be broken with a hammer (or other tools). Break whole cucumbers into several chunks and grind them in the Robot coupe for 20-30 seconds (may take up to 60 seconds if large pieces of material are still present after 30 seconds).

   c). For pickle samples that need to be sub-sampled, remove pickles from jars and place them into a drainer for 30 seconds. Place the pickles into vacuum sealer bag and vacuum seal. Store samples at -80 ℃ at least overnight or until ready for freeze-drying. Proceed starting with step b.

2. Weigh the container without lid and write down the weight on the container. Then, weigh samples with the container and record sample + container weight to five significant figures.
3. Lid the containers and store at-80 ℃ overnight.

   Check the oil level in the freeze-dryer vacuum pump from the sight glass. The level of the oil should be between the Min/Max marks. Manufacturers recommend changing the

oil after 2000 hrs of operation *or sooner* if the oil color has darkened or appears cloudy. *Note:* For cucumber samples (~95 % moisture content), it was necessary to change the oil after ~ 300-400 hrs of operation (3 or 4 cycles) when there was ~4 kg of cucumber samples in each batch. The freeze dryer oil change alarm is set to 480 hrs. Be sure to reset the alarm after each oil change to reset this clock. Detailed maintenance information is available at https://www.spscientific.com/basic-lyophilizer-maintenance/

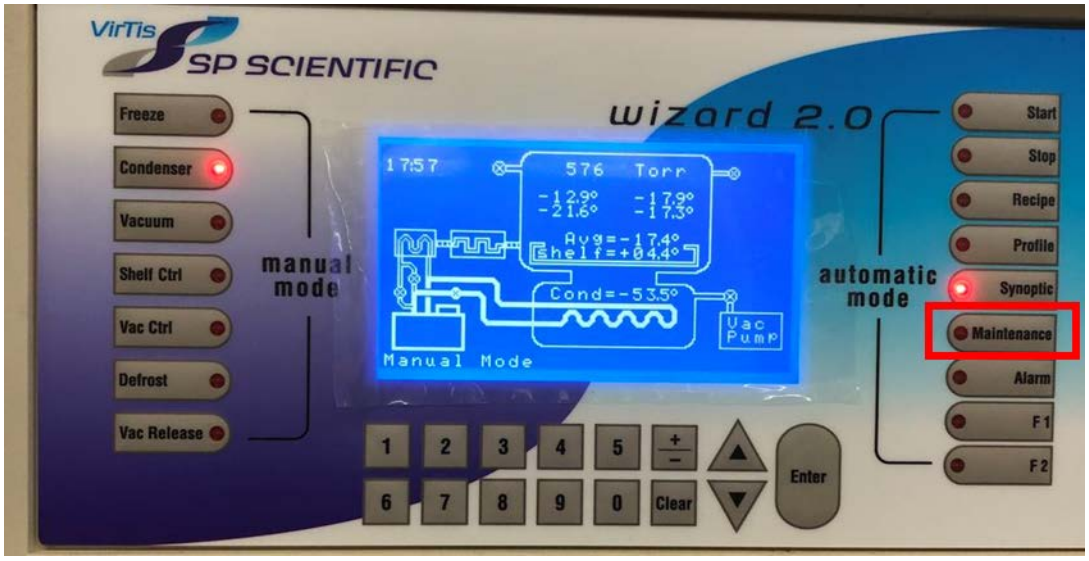
Newly changed oil (left). Oil that needs to be changed (right).

4. Remove samples from freezer and place the sample trays or containers on the freeze-dryer shelves. Insert four thermocouples into the center of samples for measuring product temperature. If sample containers vary in content, insert the thermocouple into those on each shelf with the highest sample mass. Before closing the freeze dryer door, clean the door and gasket with a clean, damp paper towel.

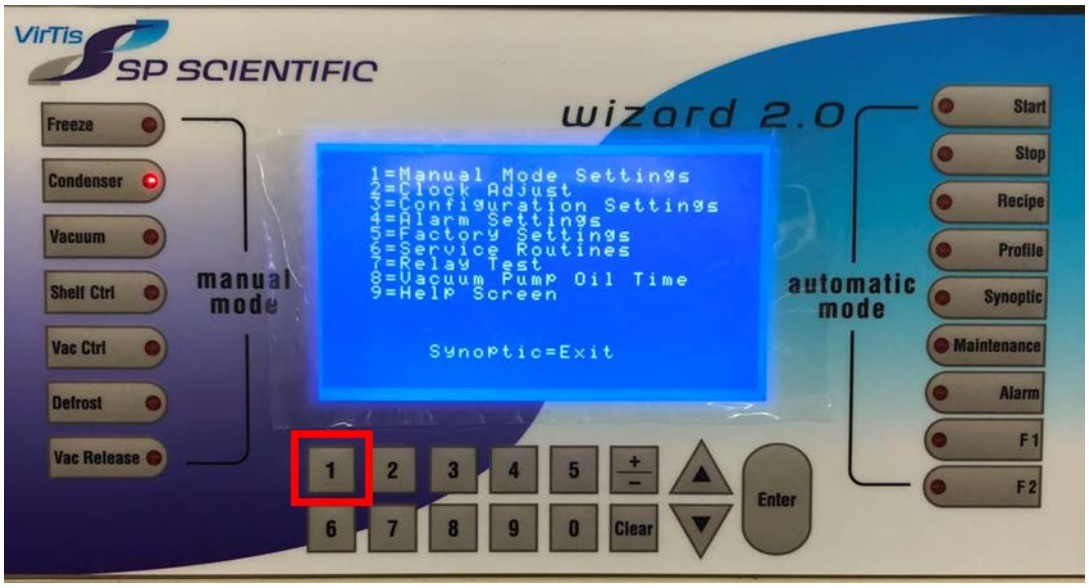
5. Press “freeze” button to turn the freezer on.
6. Turn off the freezer after the shelf temperature reaches -40 ℃. Then, press the “condenser” button.
7. After the condenser temperature is around -50 to -60 ℃, press “vacuum”. After vacuum reaches 200 mTorr, set vacuum control to 200 mTorr and shelf control to -10 ℃ to initiate primary drying. Wait for about 72 hours until the product temperature is -10℃ (If freeze drying frozen cucumber homogenate, it takes around 65 hours. *Tip*: the best indicator of complete primary drying is by monitoring the product temperature).

Setting shelf temperature instructions:

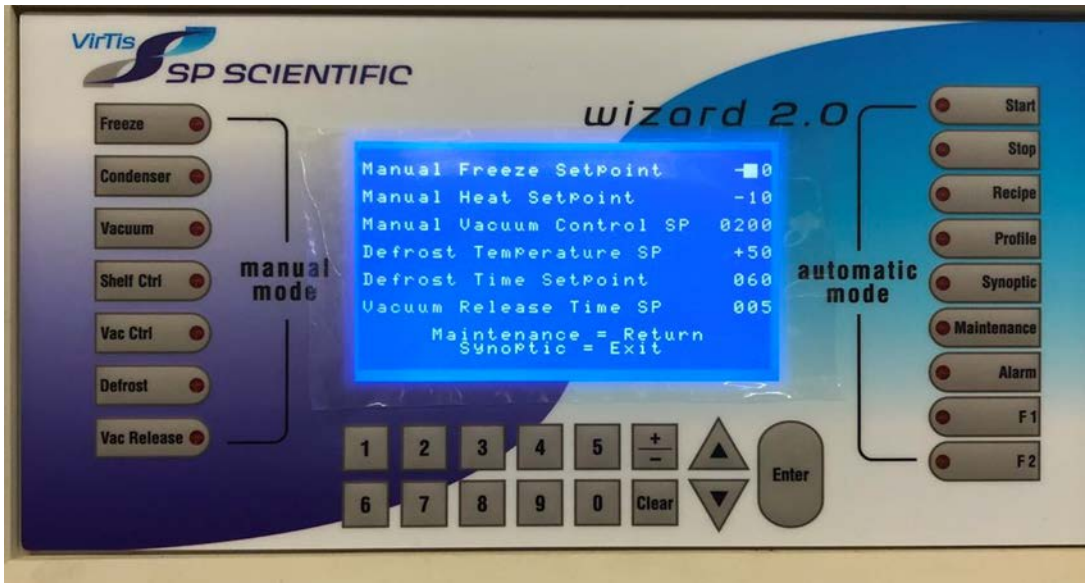
Step one: Press “Maintenance”



Step two: Press “1”



Step three: set shelf temperature of Manual Heat Setpoint. Then, press “Synoptic” to exit.



8. Set shelf temperature to 20℃ to initiate secondary drying and wait for 24 hours. Remove cucumber from freeze dryer and quickly close containers with matching lids for transport to the lab.

Condenser after freeze drying

9. Weigh dried cucumbers (record weight of dried cucumber +container) and calculate moisture content. Keep the containers tightly closed between measurements. If there are several containers, only remove 3 or 4 samples for grinding at one time to prevent re-absorption of moisture from the atmosphere. Keep the rest of the containers in the freeze dryer with the condenser on.
10. Grind freeze-dried cucumbers with a dry grinder mill for 20 seconds continuously (If needed, pulsing for several times).
11. Transfer the ground, freeze-dried cucumber powder into 50 ml centrifuge tubes, cap quickly, and place in a large glass jar containing desiccant. Store this glass jar in the -80°C freezer.
12. After freeze drying, press defrost to remove the ice in the condenser. Then, clean and dry the condenser. The condenser *must* be completely dry before starting the next run.

VII. Quality Assurance

For frozen samples, the sample temperature was measured as 0.4℃ after grinding with the robot coupe. The final freeze-dried product should not be spongy or sticky and be easily ground into a free-flowing light green powder. The moisture content for whole, fresh pickling cucumber should be around 95 %.

VIII. Storage Requirements

- Sample storage: Freeze-dried samples must be stored dessicated (to prevent re-absorption of moisture) in well-sealed containers at -80°C (or other temperature that is known to be suitable for the analytes of interest).

IX. Waste Disposal

The cucumber waste should be disposed of in the compost bin.

The used vacuum oil should be submitted as unwanted material to NCSU EHS for disposal.

X. References

1. Bhesh Bhandari, Nidhi Bansal, Min Zhang, and Pierre Schuck. 2013. Handbook of food powders: processes and properties. Oxford; Philadelphia: WP, Woodhead Publishing, 2013.
2. Iwaniw DC, Mittal GS. 1990. Process optimization of freeze-dried strawberries. Can.Agric.Eng. 32(2):323-8.
3. Oetjen G. 2004. Freeze-drying. Weinheim: Cambridge: Wiley-VCH, c2004.
4. https://www.spscientific.com/basic-lyophilizer-maintenance/

XI. Approval and Revision Information

Date of Approval:	September 15, 2020
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Support Scientist:	Xinyue Fan and Rong Reynolds
Department:	USDA - ARS SEA- Food Science Research Unit

SY Signature:  , Date: 9/15/2020

Revisions

Revision No.	Revision Date	Purpose of Revision