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Identification of Potential Volatile Contributors to Bitterness in Chocolate

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ABSTRACT

Chocolate is a highly appreciated food around the world whose unique flavor is due to a complex mix of both volatile and non-volatile compounds. Bitterness is a basic taste that is extremely important to chocolate flavor and, although has been shown to be affected by other modalities, has been thought to have been primarily caused by non-volatile compounds in chocolate. Odor induced taste effects occur when odor-active volatile organic compounds in foods change the perception of specific tastes, and while odor induced taste effects have been well studied for tastes such as saltiness and sweetness, just a few studies report odor induced effects on bitterness. Therefore, the goals of this study are to 1.) investigate if and how chocolate odor affects overall perceived bitterness and 2.) present a list of potential chemical compounds that may be causing odor induced effects on overall perceived bitterness. This is the first time any volatile contributors to bitterness have been explored in chocolate.

In addition, volatile sulfur compounds, particularly dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) which are produced during the roasting process, have been noted for their importance to chocolate flavor. Despite this, the effect of roasting on these compounds has never been thoroughly studied, and so a third goal of this research was to investigate how roasting of cocoa affects volatile sulfur compound composition in chocolate.

Using chocolate samples made from cacao from three different origins and roasted according to an experimental design (McClure et al., 2021) we found evidence for odor induced effects on perceived bitterness in chocolate through human sensory analysis. To identify potential volatiles causing this effect, chocolate samples were evaluated using gas chromatography-mass spectrometry (GC-MS) and raw data was analyzed using PARADISe to identify and semi-quantify compounds. Two primary lists of compounds were identified via correlation and partial least squares regression analysis, with 2,3-Butanedione and Ethyl Butanoate being the two compounds with significant correlation values to overall bitterness, VIP scores of over 1.5, and selectivity ratios of over 10. Volatile sulfur compounds were

evaluated in the samples using comprehensive gas chromatography with mass spectrometry and sulfur selective detection (GCxGC-MS/SCD). Semi-quantified volatiles were analyzed by ANOVA and multivariate statistical methods to determine the effect of roasting and origin on sulfur compounds. Both the concentration and diversity of sulfur compounds increased significantly ($p<0.05$) by all three experimental factors, with roasting temperature by far exceeding the effects of roasting time and cacao origin. These findings warrant future investigations into the effect of sulfur compounds on the sensory perception of chocolate in the future.

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Chapter 1

Introduction

Due to its unique taste, texture, and aroma, chocolate is one of the most popular and widely enjoyed foods. Chocolate is a usually sweetened, solid paste made from cacao with a distinct melt-in-mouth texture that is attributed to the unique fatty acid composition in cacao beans (Aprotosoaie et al. 2016). Also known as cocoa after it is processed, cacao is made from the processed seeds (cacao beans) of a small, tropical evergreen tree in the Malvaceae family known as *Theobroma cacao* (Aprotosoaie et al. 2016). Cacao is one of the most important global commodities, with more than four million tons of cocoa beans being consumed annually as of 2018 in a market that has an ever-increasing demand in more and more difficult circumstances to supply those demands (World Cocoa Foundatation 2014; Barchart 2019). Cacao is grown mostly by small-scale farmers living within 15-20° North and South of the equator, with production having increased 3.9% over 2017 (Barchart 2019), although recently after record levels of production, cacao production has seen a decline due to increasingly difficult growing conditions (ICOO 2022).

The fruits of the *T. cacao* tree contain seeds, called cocoa beans, which, after being harvested, undergo a 5-7-day fermentation process in large heaps either on banana leaves or in wooden boxes. After fermentation the beans are dried, sold and transported to chocolate manufacturers where they are cleaned, deshelled, roasted, ground into a cocoa liquor, pressed, conched and potentially mixed with other ingredients, such as sugar, flavorings, and stabilizers, and tempered into chocolate products (Beckett et al. 2017).

Chocolate's unique flavor is due to a complex mix of both volatile (aromatic) and non-volatile compounds held within a matrix of fat (Afoakwa et al. 2008). Volatile flavor chemicals, or the aroma fraction, is one of the main contributors to flavor in chocolate and from a chemical standpoint extremely

complex. Thus far, over 200 volatile compounds have been identified in roasted chocolate (Afoakwa et al. 2008; Tran et al. 2015; Ziegleder 2017; Magagna et al. 2017). Besides the aroma (=smell) of chocolate, other constituents in chocolate such as non-volatile polyphenols, methylxanthines, and non-volatile acids contribute basic taste and mouthfeel qualities of bitterness, astringency, and sourness, and the ~ 50% fat (= cocoa butter) is important for the melting and flavor release of chocolate. The resulting, multi-sensorial flavor perception is thus a complex interaction of aroma volatiles and non-volatile constituents.

The aroma of chocolate is a result of both the genetic variety of cacao and its lengthy and labor-intensive processing (Afoakwa et al. 2008; Fowler and Coutel 2017). Of particular importance to developing chocolate aroma is the roasting step, in which numerous Maillard reactions and Strecker degradation reactions convert flavor precursors produced by fermentation and drying into main classes of key flavor-active components, such as pyrazines, aldehydes and other hetero atom-containing aromatic compounds (Afoakwa et al. 2008; Ziegleder 2017). A recent report by McClure et al. (2022) studied the effect of roasting temperature and time on chocolate flavor and acceptability and found that in general, roasting decreased bitterness and astringency and increase liking.

Although bitterness is an important flavor component of chocolate (Gaudette and Pickering 2013; Ziegleder 2017), chocolate is a notable outlier among bitter foods as bitterness is generally disliked by humans in most foods (Drewnowski and Gomez-Carneros 2000; Fischer et al. 2005). This is hypothesized to be the result of evolution to detect bitter-tasting toxins (Keast et al. 2003). Bitterness in chocolate is thought to be primarily caused by non-volatile methylxanthines (i.e., caffeine and theobromine) and flavonoids (i.e., epicatechin, catechin, and epicatechin oligomers), with some contribution to bitterness caused by a variety of 2,5-diketopiperazines (DKPs) and other minor compounds (Stark et al. 2006; Afoakwa et al. 2008; Ziegleder 2017). Even with a growing list of new compounds identified to contribute to bitterness in chocolate, perceived bitterness in chocolate is still not fully understood (Stark et al. 2006; Afoakwa et al. 2008; Ziegleder 2017).

Previous studies investigating bitterness in chocolate have used nose clips to exclude any potential cross-modal interferences from volatiles on bitterness perception and focus solely on which non-volatile chemicals are bitter-tasting compounds (Stark et al. 2006). This of course makes sense as the compounds that cause bitter taste perception are sensed on the tongue by bitter taste receptors (Lawless and Heymann 2010; Gaudette and Pickering 2013). However, there are other signals to the brain from other sensory modalities that also affect bitterness perception, including other tastes, aromas, and somatosensory, aural, and visual inputs (Lawless and Heymann 2010). Therefore, in order to determine what compounds in chocolate are causing bitterness these other modalities would have to be controlled for (Stark et al. 2006).

Such an approach, however, ignores the contribution of these other modalities on overall bitterness perception. This study therefore sought to 1.) investigate if and how chocolate odor affects overall perceived bitterness and 2.) present a list of potential aroma compounds that may be affecting overall perceived bitterness.

Odor induced taste effects

Odor induced taste enhancement (Djordjevic et al. 2004; Frøst et al. 2021) as well as suppression of taste (Lawless 1979; Green et al. 2010) have both been well documented in literature. The most well studied odor induced taste effects has been focusing on sweetness (Djordjevic et al. 2004; Boakes and Hemberger 2012) and saltiness (Djordjevic et al. 2004; Lawless and Heymann 2010). Such studies have focused on these cross-modal interactions as potential strategies to reduce sugar and salt while still maintaining flavor in food (Lawrence et al. 2011; Boakes and Hemberger 2012; Wang et al. 2019), with a recently published study examining potential odor contributors to the enhancement of umami in seaweed extracts (dashi) (Frøst et al. 2021). Besides enhancement of taste, odor induced textural enhancement has also been studied for creaminess, and fattiness in order to reduce fat in foods while still maintaining taste

(Frøst and Janhøj 2007; Syarifuddin et al. 2016). While odor and other modalities have been known to affect bitterness perception (Djordjevic et al. 2004; Lawless and Heymann 2010) any investigation into specific bitterness enhancing odors has been surprisingly absent in literature. Blocking olfactory input with e.g., a nose-clip has been a common method to study sensory interaction between smell and taste (Ovejero-López et al. 2005; Labbe et al. 2006; Frøst et al. 2021). Retronasal olfactory perception is very easily blocked with a simple nose clip, thus effectively blocking any odor perception which allows us to study the effect of aroma volatiles on bitterness perception in chocolate.

Identifying specific compounds that are able to trigger odor-taste cross-modal interactions can be difficult due to the complex nature of food aroma composition, especially in chocolate. A report by (Frøst et al. 2021) was able to successfully identify potential compounds causing odor induced enhancement of umami in seaweed extracts (dashi): Sensory participants were asked to evaluate various dashi samples with and without olfactory input by using a nose-clip and rate the perceived intensities of relevant sensory attributes, such as umami and salty taste. In parallel, volatile chemical analysis for each of the different seaweed extracts was conducted, and a partial least squares regressions (PLSR) model was then used to identify which aroma compounds would best predict the difference in perceived umami with (i.e., blocked olfactory sensation) and without the nose clips; i.e., which compounds were likely involved in the increase in perceived umami taste intensity. Here, the same approach was used to identify which if any compounds may be causing odor induced enhancement of chocolate bitterness. The resulting list contains potential compounds which could then be further investigated in future validation studies.

Sulfur Compounds

A second aim of this project was to characterize the volatile sulfur aroma composition of chocolate as a function of cacao origin and roasting temperature and time, using advanced sulfur selective detection instrumentation (comprehensive gas chromatography paired with sulfur chemiluminescence and

flame ionization detectors) available at the Institute of Analytical Chemistry and Food Chemistry (ACFC) at the Technical University of Graz (TUG). Volatile sulfur compounds, especially dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS), have been noted for their importance to chocolate flavor (Afoakwa et al. 2008; Liu et al. 2015a), although remain understudied. Although sulfur compounds have been found in much higher concentrations in milk chocolate, various sulfur compounds have been reported in 100% chocolate, so called cocoa liquor. These sulfur compounds are most likely resulting from sulfur-containing amino acids present in the cocoa bean (Liu et al. 2015a). Sulfur-containing aroma compounds including dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) have been reported to form via Strecker degradation of the amino acid methionine, indicating that most sulfur aroma compounds in 100% chocolate are formed during roasting (Bedrosian et al. 1959). Despite the importance of these compounds stressed in literature, the effect of roasting on volatile sulfur compounds has never been thoroughly studied (Afoakwa et al. 2008; Liu et al. 2015a). One reason for that could be due to the analytical challenge of measuring volatile sulfur compounds, resulting from their high reactivity and low concentration levels. The second aim of this study was therefore to determine the effects of roasting temperature and time and cacao origin on the formation of volatile sulfur compounds in 100% chocolate.

Chapter 2

Hypothesis

Hypothesis 1.

Perceived bitterness in 100% chocolate samples will differ depending on whether olfactory input is blocked or not with nose clips, indicating an olfactory component to bitterness perception in chocolate via cross-modal interaction effects.

Hypothesis 2.

Potential volatile compounds affecting bitterness perception in 100% chocolate will be detectable by measuring volatile aroma composition by gas chromatography-mass spectrometry and gas chromatography-sulfur selective detection and can be compared to bitterness differences perceived by humans.

Hypothesis 3.

Roasting temperature and time and cacao origin will affect the concentration of dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) in 100% chocolate.

Chapter 3

Materials and Methods

The same samples as previously reported (McClure et al., 2021) have been used for all experiments.

Chocolate Samples and Chemicals

All chocolate samples were sourced from three different origins – Madagascar, Peru, and Ghana, as described in detail in McClure et al. (2020; 2021). Raw cacao from these origins was roasted according to an experimental design (Table 1: Randomized modified I-optimal experimental design for roasting. Samples from Ghana were evaluated with and without a nose clip. , covering a roasting temperature range from 24^{O}C to 171^{O}C and a roasting time range from 0 to 80 min, as detailed in McClure et al. (2020; 2021). The resulting design space included a total of 9 treatments (8 unique temperature-time combinations plus 1 duplicated center point) for each origin, resulting in a total of 27 chocolate samples.

Roasted samples were turned into 100% chocolate as described in McClure et al. (2020; 2021). Chocolate samples were evaluated by human sensory evaluation (see McClure et al. 2021 and *p.7*) and volatile analysis by gas chromatography with various detectors (see *Sensory Evaluation*). All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) and Fluka Chemie GmbH (Buchs, Switzerland), and were of analytical grade or higher in purity.

Sensory evaluation

All 27 chocolate liquor samples (i.e., all treatments across all 3 origins of cacao) were assessed as described in detail by McClure et al. (2021).

A subset of the samples (i.e., all samples from Ghana) were in addition evaluated by a second group of consumers (n= 108; 18-64 years of age), recruited in the same way as for the first sensory evaluation from the Penn State SEC database. The same familiarization with the prototypical flavors and the generalized labelled magnitude scale (gLMS) as described in McClure et al. (2021) was used, and similarly, samples were presented in a counter-balanced manner according to a modified Williams Latin Square design.

During the evaluation however, participants were asked to wear a nose clip to prevent retronasal olfactory perception. While wearing their nose clip, participants were asked to evaluate five samples on three consecutive days for overall liking on a 9-point hedonic scale, followed by rating the perceived intensities for the attributes astringent, sour, bitter, sweet, and cocoa/dark chocolate. During the mandatory 2-min break in between samples, participants were asked to cleanse their palate with room temperature reverse osmosis water. Finally, data on chocolate preference, chocolate consumption frequency, gender, age, and ethnicity were collected after the last sample assessment on day three.

Table 1: Randomized modified I-optimal experimental design for roasting. Samples from Ghana were evaluated with and without a nose clip.

Roast Order and Sample Code	Time (min)	Temperature (°C)	Origin
1	40	114	Peru
2	40	114	Ghana
3	54	151	Madagascar
4	80	84	Peru
5	0	24	Peru
6	20	171	Ghana
7	80	84	Ghana
8	20	171	Madagascar
9	54	151	Peru
10	55	64	Peru
11	40	114	Peru
12	0	24	Ghana
13	40	114	Ghana
14	40	114	Madagascar
15	11	105	Peru
16	55	64	Madagascar
17	11	105	Madagascar
18	54	151	Ghana
19	11	105	Ghana
20	40	114	Madagascar
21	80	135	Peru
22	80	135	Ghana
23	0	24	Madagascar
24	80	84	Madagascar
25	55	64	Ghana
26	80	135	Madagascar
27	20	171	Peru

Volatile Analysis

Approximately 250 g of each chocolate sample were double wrapped in heavy duty aluminum foil, placed in labelled plastic bags and transported by air in insulated tote bags from State College, Pa, USA to Graz, Austria, for volatile analysis. Upon arrival, samples were removed from the bags and aluminum foils, broken up into coarse pieces, and stored in 40 mL glass vials at 4^{O}C in the dark.

Volatile profiling by gas chromatography – mass spectrometry (GC-MS)

Optimization of headspace solid phase microextraction (HS-SPME) parameters

A center point sample was chosen at random to be representative of all chocolate samples (14: Madagascar, 40 min, 114^{O}C) to test the effects of different enrichment programs (40^{O}C at 20 min, 40^{O}C at 40 min, 60^{O}C at 20 min, 80^{O}C at 20 min) on quantity and quality of resulting chromatograms. Cocoa liquor samples (100.0 $\pm$ 0.5 mg) were prepared in triplicate by weighing samples into 20 mL headspace (HS) vials (Shimadzu Europa GmbH) along with a glass-coated magnetic stir bar a glass, capped with a polytetrafluoroethylene (PTFE)-lined silicone septum magnetic crimp cap and measured by gas chromatography-flame ionization detection (GC-FID) (see p.13). The optimal parameters were determined to be extraction at 60^{O}C for 20 min as GC-FID chromatograms showed number of different compounds and peak areas to stabilize at 60^{O}C with no noticeable difference between 60-80^{O}C, and because 60^{O}C for 20 min was the most time efficient compared to longer extraction times.

Optimization of sample weight for HS-SPME-GC-MS analysis

A center point sample was chosen at random (11: Peru, 40 min, 114^{O}C) to test the effects of different sample weights (50 mg, 100 mg, 250 mg, 500 mg) on GC chromatograms. The cocoa liquor samples were prepared for each weight in duplicate ($\pm$ 1.5 mg) by weighing samples into 20 mL HS vials (Shimadzu Europa GmbH) along with a glass-coated magnetic stir bar a glass and capping with a polytetrafluoroethylene (PTFE)-lined silicone septum magnetic crimp cap and measurement by GC-FID (see section *p.13*). Data showed no discernable difference between the different sample weights, therefore, a sample weight of 100 mg was chosen to conserve sample and as it was much easier to accurately weigh than 50 mg.

Optimization of Internal Standard (IS) for HS-SPME-GC-MS analysis

Two internal standards (IS) were tested (Napthelene-D8, Toluene-D8) using the center point cocoa liquor sample (1: Peru, 40 min, 114^{O}C) by spiking duplicate cocoa liquor samples (100.0 $\pm$ 0.5 mg), weighed into 20 mL HS vials (Shimadzu Europa GmbH) along with a glass-coated magnetic stir bar a glass with 100 ng of Napthelene-D8 (10 mg/L in methanol) and Toluene-D8 (10 mg/L in methanol). Samples were then enclosed with a polytetrafluoroethylene (PTFE)-lined silicone septum magnetic crimp cap and analyzed by gas chromatography-mass spectrometry (GC-MS) (see 13) . Both IS were found to function as suitable IS as both did not appear naturally in the cocoa liquor samples and were easily identifiable, therefore, both IS were used in subsequent analysis.

The effect of Alkalinization on GC-MS analysis of chocolate

Cocoa liquor is ~50% fat, and as such contains also detectable levels of free fatty acids (FFA). FFAs are particularly difficult to analyze by GC because of their high polarity which can lead to "tailing" peaks and subsequent co-elution with other compounds. Therefore, the effects of neutralizing FFAs with an alkaline solution on chocolate aroma composition was studied.

A center point cocoa liquor sample (1: Peru, 40 min, 114^{O}C) was chosen to test the effects of adding alkaline solutions (100 μL 0.5 *N* KOH, 100 μL 1.0 *N* KOH, 100 μL 2.0 *N* KOH, 1 mL 0.5 *N* KOH) to chocolate samples on neutralizing FFA. Cocoa liquor samples (100 $\pm$ 0.5 mg) were prepared and analyzed for each alkaline solution in duplicate as described above.

The two strongest alkali solution, 100 μL 2.0 *N* KOH and 1 mL 0.5 *N* KOH, completely neutralized all FFAs. Although all FFAs were neutralized revealing previously co-eluted and thus, obscured compounds, adding a strong alkali also affected the overall volatile profile of the chocolate samples by also changing relative concentrations of other compounds considerably, thus, making the alkalized data quantitatively unrepresentative of the chocolate samples. Some examples of volatiles that

changed considerably between non-alkalized and alkalized chocolate samples are shown in Table 2, demonstrating the wide-ranging effects of alkalization on chocolate volatiles, as previously reported in Ziegleder (2017). While alkalization is useful for qualitatively identifying compounds that FFAs might otherwise obscure, alkalization also changes the volatile profile to a degree that it is not recommended for quantitatively analyzing chocolate.

Table 2: Examples of chemicals that change concentration by being alkalized. Shown are peak areas for the same chocolate sample analyzed with ("alkalized") and without ("non-alkalized") the addition of 100 μL 2.0 N KOH alkali solution as well as the peak area ratio of the two treatments.

Compound	Alkalized	Non-Alkalized	Alkalized: Non-Alkalized ratio
2,3-Butanedione	2.72E+07	3.70E+06	7.35
Butanal, 3-methyl-	1.92E+06	2.98E+07	15.5
Acetoin	1.99E+06	2.16E+07	0.09
2,3-Butanediol	5.21E+07	7.69E+07	0.68
Oxime-, methoxy-phenyl-_	5.25E+07	1.44E+07	3.65
Butanoic acid, 4-hydroxy-	1.69E+06	3.70E+06	0.46
Pyrazine, 2,3-dimethyl-	8.70E+06	4.28E+06	2.03
1-Methoxy-2-propyl acetate	3.89E+07	2.57E+07	1.52
Benzaldehyde	4.80E+07	2.32E+07	2.07
Pyrazine, trimethyl-	4.09E+07	2.27E+07	1.80
Tricyclo[2.2.2.0(1,4)]octane	7.90E+06	2.72E+06	2.90
Ethanone, 1-(1H-pyrrol-2-yl)-	8.86E+06	8.91E+06	0.99
Acetophenone	7.56E+06	3.94E+06	1.92
Pyrazine, tetramethyl-	2.11E+08	1.50E+08	1.40
Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate	1.02E+07	3.38E+06	3.03
Linalool	4.13E+07	2.64E+07	1.56
Phenylethyl Alcohol	3.29E+07	2.03E+07	1.63
Pyrazine, 3,5-diethyl-2-methyl-	8.40E+06	5.28E+06	1.59
Octanoic acid, ethyl ester	4.21E+06	3.47E+06	1.21
Benzeneacetic acid, ethyl ester	9.09E+06	8.80E+06	1.03
Acetic acid, 2-phenylethyl ester	2.21E+07	1.99E+07	1.11

Volatile profiling by gas chromatography – mass spectrometry (GC-MS)

For quantitative analysis of cocoa volatiles, cocoa liquor samples (100.0 ± 0.5 mg) were weighed into 20 mL HS vials (Shimadzu Europa GmbH) together with a glass-coated magnetic stir bar, spiked with 100 ng of Napthelene-D8 and 100 ng of Toluene-D8, and enclosed with a polytetrafluoroethylene (PTFE)-lined silicone septum magnetic crimp cap (Shimadzu Europa GmbH). Volatiles were extracted onto a 2 cm SPME (Supelco, Bellefonte, PA, USA) fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS) for 20 min at 60^{O}C after which the fiber was thermally desorbed in a SPME inlet liner (Supelco) held at 270°C in splitless mode (purge valve on at 1 min). Sampling was performed automatically using a CTC PAL 1 with a Chromtech Single magnetic mixer (CTC Analytics, Switzerland). For the separation of the volatiles, a Shimadzu QP-2020 GC MS system (Shimadzu Europa GmbH) with a single quadrupole mass-selective detector was used in scan mode from 35.00 to 400.00 m/z and a scan rate of 1250 scans/sec, with the detector multiplier voltage set 50 V below tuning voltage to prevent detector saturation. A 30 m Restek Rxi5MS (0.25 mm inner diameter and 1 µm film thickness, Restek, Bellefonte, PA, USA) with the following temperature program was used: 35.0°C held for 3 min, ramp of 4°C/min to 180°C over a period of 36.25min, and a final ramp of 20°C/min to 280 over 5min with a final hold of 2 min. Ultrapure helium as a carrier gas was used in constant flow mode with a total flow rate of 54.3 mL/min, and a column flow of 0.95 mL/min. The MS interface was held at 280°C, and the ion source temperature was set to 200°C. All samples were analysed in triplicate.

Volatile profiling by comprehensive gas chromatography – mass spectrometry (GCxGC-MS)

Due to the problems with identifying VOCs in chocolate as previously discussed (e.g., complexity, overlapping peaks, high levels of FFAs, tailing compounds), chocolate samples were also analyzed GCxGC-MS (Appendix B). Comprehensive gas chromatography (GCxGC) facilitates the

separation of complex mixtures through the combination of orthogonal GC columns (e.g., different polarities) (Tranchida et al. 2016; Siegmund et al. 2018) The basic concept is to subject chromatographic bands, potentially containing overlapping and co-eluting compounds, to an additional orthogonal separation mechanism in a continuous and sequential manner, to achieve well-separated compounds that can then be identified by a detector (e.g., mass spectrometry MS) (Tranchida et al. 2016; Siegmund et al. 2018).

For GCxGC-MS analysis, cocoa liquor samples (100.0 ± 0.5 mg) were prepared as described in *volatile profiling by gas chromatography – mass spectrometry (GC-MS)* (p.22). Comprehensive GC×GC-MS analysis was performed on a Shimadzu GC-2010 Plus with cryogenic cooling with liquid nitrogen, coupled with a fast-scanning quadrupole mass selective detector (Shimadzu Europa GmbH). The same extraction conditions as described in *volatile profiling by gas chromatography – mass spectrometry (GC-MS)* (p.22) were used, and extracted volatiles were thermally desorbed in a SPME inlet liner held at 270°C in splitless mode (purge valve on at 1 min) using an OPTIC IV injector with Evolution Workstation OPTIV 4 (version 4.04) (GL Science BV, The Netherlands). Sampling was performed automatically using a Shimadzu AOC 5000 autosampler. The thermal modulator ZOEX ZX-1 (Zoex Corp., Houston, TX USA) was used to modulate between the two complementary GC columns, coupling in the first dimension a nonpolar 30 m RXi1HT column (0.25 mm inner diameter and 0.25 μm film thickness; Restek, Bellefonte, PA, USA) with a midpolarity 2 m Restek Rtx200 (0.15 mm inner diameter x 0.15 μm film thickness; Bellefonte, PA, USA) column in the second dimension. The following temperature program was used: 35.0 °C (2 min) with a ramp of 4^{O}C/min to 220°C over 46.25 min, and a final ramp of 20^{O}C/min to 280^{O}C over 3min with a final hold time of 3min with a helium carrier gas in constant flow mode (30.0 cm/s at 85.0 kPa) and a modulation frequency of 4 s with a hot jet temperature of 300°C and a hot jet pulse of 350 ms. Mass spectra were acquired in scan mode (35−300 amu with 50 scans per sec, EI 70 eV). The interface temperature was set to 270°C, and the ion source temperature was 200°C.

Profiling of volatile sulfur compounds with GCxGC-SCD/FID

For quantitative analysis of volatile sulfur compounds in chocolate comprehensive chromatography was coupled with sulfur chemiluminescence and flame ionization detection (GCxGC-SCD/FID). Cocoa liquor samples were prepared as described on *p.13*, but with the modification of having no internal standards. Not having an internal standard is a weakness of this experiment and it is suggested that future research study potential internal standards for use in SCD. Comprehensive GC×GC-SCD/FID analysis was performed on Shimadzu GC-2010 Plus with cryogenic cooling option with liquid nitrogen cooling (Shimadzu Europa GmbH) coupled with SCD and FID (Shimadzu Europa GmbH). Extracted volatiles (see *p.13*) were thermally desorbed in a SPME inlet liner held at 270°C in splitless mode (purge valve on at 1 min),using a CTC PAL 1 autosampler with a Chromtech Single magnetic mixer (CTC Analytics, Switzerland). The first dimension used a nonpolar 20 m SLB-5MS column (0.18 mm inner diameter and 0.18 μm film thickness; Supelco, Bellefonte, PA, USA) and the second dimension column was a midpolarity 5 m SLB 35MS column (0.32 mm inner diameter and 0.25 μm film thickness, Supelco) with the following temperature program: 25.0°C (1 min) with a ramp of 5°C/min to 190°C (33 min) with a constant helium carrier gas flow (linear velocity of 14.7 cm/s at 102.0kPa). A modulation frequency of 6 s and a loop fill time of 400 ms was carried out by a Trajan flow modulator. SCD data was acquired with a sampling rate of 8 ms, a delay time of 0.0 min and no detector subtraction, with the detector interface temperature set to 200°C and the SCD furnace temperature held at 850°C. The detector used a H_2 flow of 80.0 mL/min, a N_2 flow of 40.0 mL/min, an O_2 flow of 10.0 mL/min, and an O_3 flow of 25.0 mL/min. FID data was acquired with a sampling rate of 8 ms, a delay time of 0.0 min and no detector subtraction, with the interface temperature set to 300°C. The helium makeup gas flow was 24.0 mL/min and the air and hydrogen flows were 200 mL/min and 32 mL/min, respectively. All samples were analysed in triplicate.

To facilitate identification of common sulfur volatile compounds, a standard mix of sulfur compounds containing dimethyl sulfide (DMS; ; $0.1\mu g/\mu L$) dimethyl disulfide (DMDS; ; $0.1\mu g/\mu L$), and

dimethyl trisulfide (DMTS; $0.1 \mu g/\mu L$) was analyzed the same way, and only DMDS and DMTS were successfully identified in the cocoa samples by comparing the retention indexes (RI) for DMDS and DMTS in the standard mix to the RIs in the chocolate samples (DMDS: 745 ± 10 RI ; DMTS: 976 ± 10 RI).

Data analysis

Volatile data analysis by PARADISe

Due to the high prevalence of co-eluding compounds, GC-MS data from all chocolate samples from the Ghana origin were analyzed by the program PARADISe, an automated PARAFAC2 based Deconvolution and Identification system which is able to analyze and separate overlapping signals and lower the signal-to-noise ratios in raw GC-MS data, and identify and semi-quantify compounds by reporting peak areas of deconvoluted compounds(Johnsen et al. 2017). Identified and semi-quantified compounds were expressed in internal standard equivalent (ISE) concentrations using the IS Napthene-D8. Only samples from the Ghana origin were analyzed in this way as those were the only ones tested for volatile contributors to bitterness.

ChromSquare

All comprehensive data was analyzed by the GCxGC analytical software program ChromSquare (v 2.4, Shimadzu Europa GmbH) to identify and semi-quantify all volatiles in the 27 chocolate samples.

Statistical Analysis

Analysis of Variance (ANOVA) as well as two multivariate analysis, principal component analysis (PCA) and partial least squares regression (PLSR), were performed to investigate the relationships between the volatile compounds and difference in bitterness intensities of the chocolate samples. All data analysis was done using RStudio (Redmond, WA) v. 1.2.1334, build 1379 (f1ac3452) running R (Vienna, Austria) version 3.6.0 Patched (2019-06-04 r76666) with the additional packages Tidyverse (v1.3.1: Wickham, 2021), readxl (v1.3.1: Hadley et al, 2019). The package Hmisc (v4.6.0: Harrell Jr, 2021) was used to create the correlation matrix and the package mdatools (v0.12.0: Kucheryavskiy, 2021) was used to create the PLSR models.

Correlation Analysis

A correlation analysis between all identified volatiles and the difference in bitterness ratings (ΔBitt) was conducted across all samples from the Ghana origin. Only compounds that showed a absolute correlation coefficient R^2 of greater than 0.5 and were significant at α=0.0001 were included to selectively narrow down the list of potential volatiles that could be causing odor induced enhancement of bitterness in chocolate.

Partial Least Squares Regression Analysis (PLSR)

PLSR was used to create a model to predict the difference in bitterness (ΔBitt) from volatiles, similar to the approach taken by Frost et al. (Frøst et al. 2021), to identify which compounds might be causing odor induced enhancement of bitterness. The PLSR model was created using the chemical data from the first two Ghana replicates (e.g., 1 and 2) for every sample (i.e., test set) and verified using

chemical data from the last Ghana replicates (e.g., 3) from every sample (i.e., validation set). Compounds with variable importance in the projection (VIP) scores of greater than 1.5 and/ or compounds with selectivity ratios of over 10 were considered the best candidates for bitterness enhancing volatiles, in order to select only the best candidates and in accordance with prior literature (Chong and Jun 2005).

Analysis of Variance (ANOVA)

A fixed effects ANOVA model was run on the samples evaluated with GCxGC-SDC to test if dimethyl disulfide and dimethyl trisulfide differed significantly between samples.

Chapter 4

Results

Perception of bitterness intensity was significantly affected by roasting conditions as previously described by McClure et al. (2022), with roasting temperature having a larger effect on bitter intensity than roasting time. In general, roasting at higher temperatures (135ºC and 151ºC) led to lowest average bitterness ratings of 25 on a gLMS scale compared to chocolate samples that were unroasted or roasted at lower temperatures, with mean bitterness ratings of over 30 on the gLMS scale.

When comparing mean bitterness ratings of all 7 chocolates from Ghana, i.e., those samples that were evaluated with and without noseclip (Table 3), i.e., without retronasal olfaction and with, an interesting pattern emerges: The average bitterness intensity was lower for every sample when participants had their olfactory perception blocked by wearing a nose clip. The degree of bitterness reduction experienced by the chocolate consumers ranged from 2.9 to 8.9 units on the gLMS scale, equivalent to a 11 to 27% reduction. This measurable difference, which is similar to the maximum difference in bitterness between the highly roasted and unroasted chocolate, is surprising though as bitter taste perception is not elicited by aroma compounds, and the nose clip only prevented retronasal olfaction, not taste perception. This finding provides evidence for the presence of odor-induced enhancement of bitter taste perception in chocolate.

Table 3 also summarizes the differences in bitterness ratings (ΔBitt) for each sample which is also an indirect measure of the degree to which odor may affect overall bitterness, with larger differences indicating greater contributions of odor to total perceived bitterness.

The largest ΔBitt is seen for the samples roasted for 80 min at 84ºC (a difference of 8.9 points), 55 min at 64ºC (a difference of 8.50 points), and 40 min at 114ºC (a difference of 7.76 points), the latter is also one of the duplicated center points. Interestingly, the other center point saw the lowest ΔBitt ratings (a difference of 2.91 points). Similar differences in perception of the

two center points were also noted by McClure et al. (2022) for other sensory attributes, such as astringency and sourness. This could be explained either by human variability and/or variability in the roasting process for the center point. According to McClure et al. (2021), this variability did not lead to detectable differences in major non-volatile compounds, including methylxanthines, flavonoids, and 2,5-diketopiperazines.

Table 3: Average Bitterness Ratings with and without olfactory input and Bitterness Difference (ΔBitt) for Chocolate Samples from Ghana roasted at different time and temperatures.

Treatment		Average Bitterness Rating		
Time (min)	**Temperature (°C)**	**+ Olfactory**	**- Olfactory**	**ΔBitt**
40	114	31.1	23.5	7.67
20	171	29.7	24.2	5.50
80	84	32.7	23.8	8.91
0	24	29.7	22.1	7.58
40	114	26.2	23.3	2.91
54	151	25.5	22.0	3.46
11	105	31.9	25.5	6.39
80	135	25.2	21.4	3.82
55	64	32.3	23.8	8.50

Based on these findings, volatile profiling was conducted next to identify potential volatile compounds that could cause odor induced enhancement of bitterness in chocolate. Due to the magnitude of previously reported volatiles in roasted cocoa and chocolate (Afoakwa et al. 2008; Tran et al. 2015; Ziegleder 2017; Magagna et al. 2017) an untargeted, exploratory approach to volatile analysis was taken.

Correlation Analysis

A total of 198 volatiles were identified with the PARADISe software, although due to the possibility of misidentification of compounds using identification software identified compounds were also encoded (i.e., C001 - C198).

Volatile organic compounds (VOCs) that correlate the most strongly with the change in bitterness are thought to be potential causes of odor induced enhancement of bitterness, so Table 4 summarizes those volatiles that significantly correlate with ΔBitt at α=0.001 and and show an absolute correlation coefficient of greater than 0.5, thus, could be potential bitterness enhancers. Volatiles showing either significant positive or negative correlation coefficients were included as odor-induced enhancement of bitterness could result from either the presence or absence of a compound. This approach however does not account for the fact that odor-induced enhancement of bitterness is most likely the result of a mixture of different VOCs whose combined are responsible for bitterness enhancement. Besides a simple correlation analysis to identify potential compounds smaller was paired with a partial least squares regression (PLSR) model to evaluate which VOCs best predict ΔBitt ratings.

Partial Least Squares Regression (PLSR) analysis

A PLSR model was created using the chemical data from the first two replicates for every sample and externally validated using the third analytical replicate data from every sample. The resulting root mean squared error (RMSE) of prediction plot in **Error! Reference source not found.** suggests a first minimum for a one-component PLCR model. Using this one-dimensional model, only 25.2% of the variation for the predictor X matrix (VOC data) is explained, however, the same model is capturing 88.6% of the variation of the response Y matrix (ΔBitt). Correlation coefficients are 0.543 for the calibration data, 0.349 for the cross-validation (CV), and 0.390 for the external validation data. With this PLSR model, calibration data grouped fairly well with the validation data, indicating the validity and stability of the model (Figure 2).

Table 4: Chemicals that significantly correlate to difference in bitterness

Chemical Code	Chemical	correlation coeff	p value
DMDS	Dimethyl disulfide	-0.61879	0.00058
C005	Acetic acid, methyl ester	6.50E-01	2.42E-04
C008	2,3-Butanedione	6.67E-01	1.46E-04
C014	2-Pentanone	6.92E-01	6.27E-05
C018	Acetoin	6.63E-01	1.64E-04
C019	1H-2,8a-Methanocyclopenta[a]cyclopropa[e]cyclodecen-11-one, 1a,2,5,5a,6,9,10,10a-octahydro-5,5a,6-trihydroxy-1,4-bis(hydroxymethyl)-1,7,9-trimethyl-, [1S-(1�,1a�,2�,5�,5a�,6�,8a�,9�,10a�)]-	6.43E-01	3.00E-04
C021	1-Butanol, 3-methyl-	7.29E-01	1.59E-05
C022	Hexadecane, 1,1-bis(dodecyloxy)-	7.42E-01	9.35E-06
C023	Propanoic acid, 2-hydroxy-, methyl ester, (�)-	6.26E-01	4.76E-04
C027	Propanoic acid, 2-methyl-	6.61E-01	1.74E-04
C030	Butanoic acid	7.20E-01	2.29E-05
C031	2,3-Butanediol, [S-(R*,R*)]-	6.13E-01	6.70E-04
C033	1H-1,2,3,4-Tetrazole, 5-hydrazino-	-6.32E-01	4.09E-04
C034	2-Hexadecanol	7.20E-01	2.29E-05
C036	trans-2-Methyl-4-hexen-3-ol	6.30E-01	4.31E-04
C037	Furfural	-6.15E-01	6.39E-04
C041	Butanoic acid, 2-methyl-	6.65E-01	1.55E-04
C048	2-Heptanone	6.52E-01	2.29E-04
C052	1-(Methoxymethoxy)-3-methyl-3-hydroxybutane	6.30E-01	4.31E-04
C060	3-Pentanol, 2,4-dimethyl-	6.45E-01	2.84E-04
C067	2(3H)-Furanone, dihydro-5-methyl-	6.63E-01	1.64E-04
C068	Butanoic acid, ethyl ester	6.96E-01	5.52E-05
C070	Glycerin	6.56E-01	2.05E-04
C073	Chloro(2-methyloxiran-2-yl)acetic acid, t-butyl ester	-6.34E-01	3.89E-04
C103	2-Pyrrolidinone	-6.54E-01	2.17E-04
C137	Benzamidomethyl benzoate	-6.43E-01	3.00E-04
C141	Tricyclo[4.3.1.1(3,8)]undecan-1-amine	-6.79E-01	9.79E-05
C162	Stearic acid, 2-hydroxy-1-methylpropyl ester	7.04E-01	4.25E-05
C185	2,4,6-Trimethylmandelic acid	-6.69E-01	1.36E-04
C190	4(1H)-Pyrimidinone, 6-hydroxy-	-6.11E-01	7.03E-04

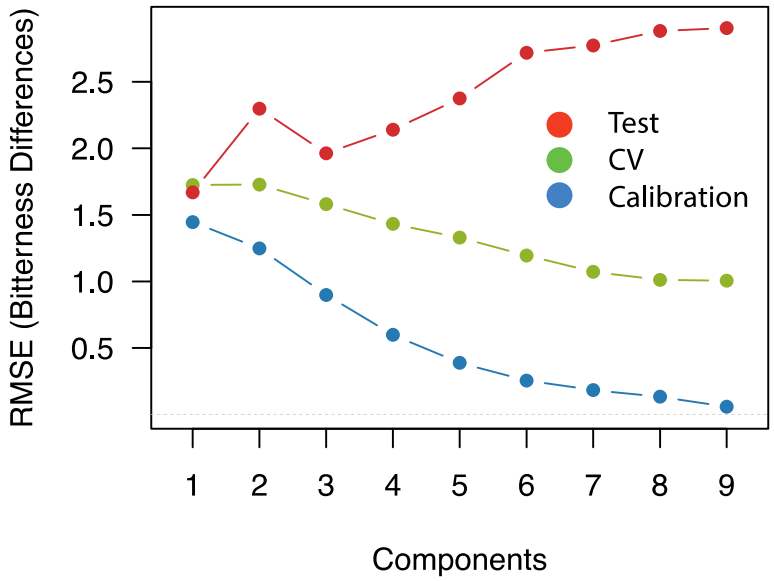


Figure 1: PLSR root mean squared error (RSME) plot shown with the calibration data (in blue), the cross-validation (CV) data (in green) and the external validation data (in red).

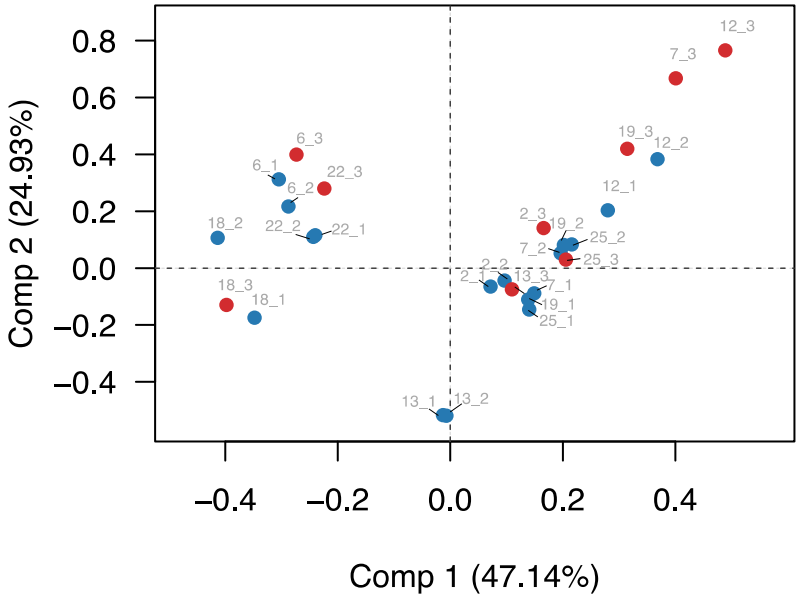


Figure 2: PLSR scores plot showing the calibration samples (in blue) and the external validation samples (in red). Individual samples are labelled by their sample number (see Table 1) and their analytical replicate number (_1, _2, and _3).

Figure 3 shows the PLSR loadings plot for the predictor variables (VOCs) shown in blue and the response variable (ΔBitt) shown in red. Points further from the origin are more important in the model and VOCs shown closer to the red ΔBitt point are stronger, positively correlated predictors of ΔBitt. Points opposite from the red ΔBitt are negatively correlated whereas VOCs that are orthogonal show no correlation to changes in bitterness. Due to the sheer number of compounds, variables of importance in projection (VIP) scores and selectivity ratios were calculated for important compounds.

Figure 4 shows a plot of the VIP scores and selectivity ratios from the PLSR model, whereas **Error! Reference source not found.** lists all compounds that were most closely associated with ΔBitt based of their VIP scores being above 1.5 and/or their selectivity ratios being above 10. The VIP score and selectivity ratio cut-offs were chosen to be rather restrictive to reduce the list of potential compounds to be considered. Volatiles C008 (2,3-Butanedione) and C068 (Butanoic acid, ethyl ester) were the only compounds that showed (1) significant correlation values (Table 4), (2) VIP scores above 1.5 (**Error! Reference source not found.**), and (3) selectivity ratios of greater than 10 (**Error! Reference source not found.**). Especially C068 (Butanoic acid, ethyl ester) stands out with its exceptionally high selectivity ratio of 44.5.

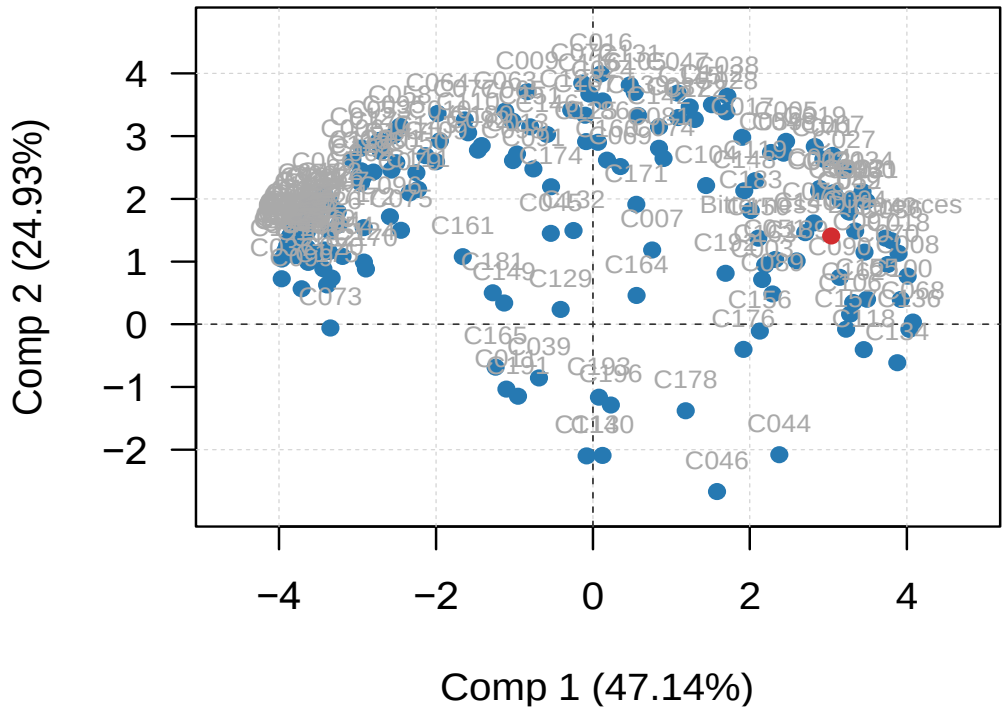


Figure 3: Loadings Plot of Volatile Organic Compounds (blue) and Bitterness Differences (red)

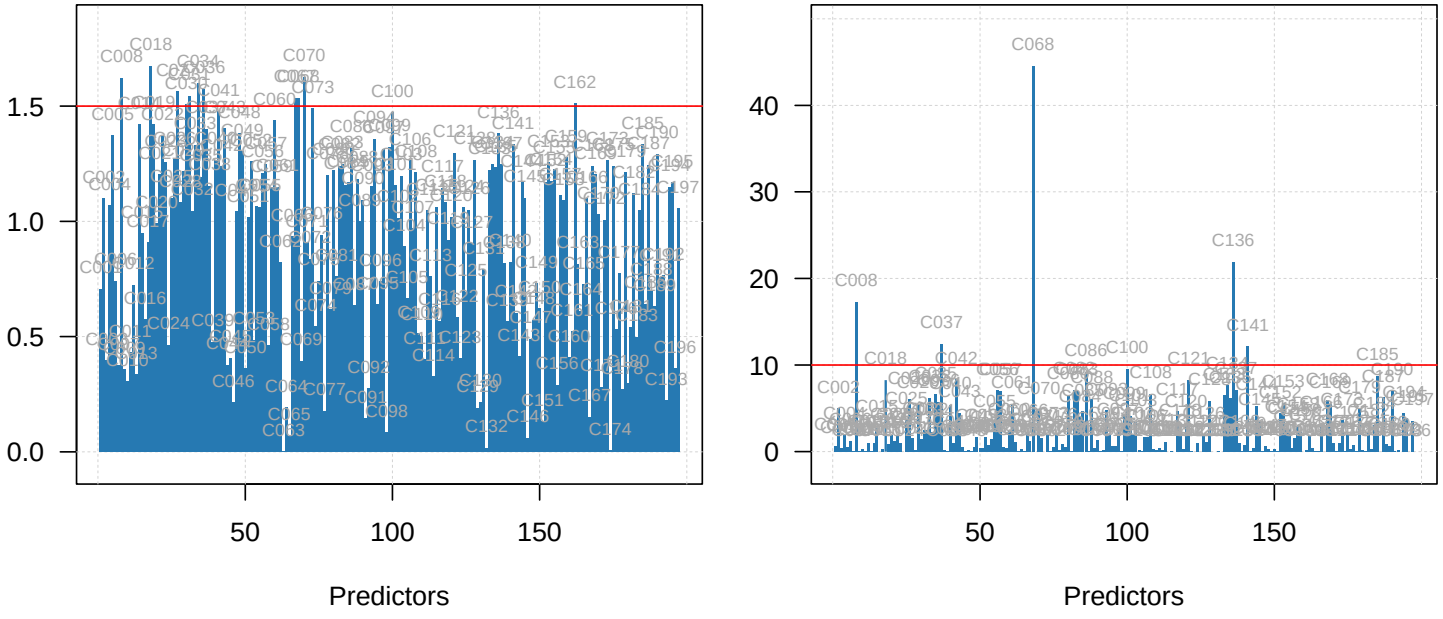


Figure 4: VIP Scores (left) and Selectivity Ratios (right) from PLSR model with the respective cut-off values indicated as red lines

Table 5: Significant chemicals as determined by VIP scores (VIP > 1.5) and Selectivity ratio (SR > 10)

Code	Chem	VIP	SR
C008	2,3-Butanedione	1.62	17.3
C018	Acetoin	1.67	-
C027	Propanoic acid, 2-methyl-	1.56	-
C030	Butanoic acid	1.51	-
C031	2,3-Butanediol, [S-(R*,R*)]-	1.54	-
C034	2-Hexadecanol	1.60	-
C036	trans-2-Methyl-4-hexen-3-ol	1.57	-
C037	Furfural	-	12.4
C067	2(3H)-Furanone, dihydro-5-methyl-	1.53	-
C068	Butanoic acid, ethyl ester	1.53	44.5
C070	Glycerin	1.63	-
C136	Dispiro[2.1.2.1]octane, 1,1,2,2,6,6,7,7-octamethyl-	-	21.9
C141	Tricyclo[4.3.1.1(3,8)]undecan-1-amine	-	12.2
C162	Stearic acid, 2-hydroxy-1-methylpropyl ester	1.51	-

Volatile sulfur compound analysis by GCxGC-SCD/FID

All samples were analyzed from volatile sulfur compounds with GCxGC-SCD/FID. While only two compounds - dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) - were semi-quantified, many more unidentified sulfur compounds coming from the chocolate appeared in the SCD chromatogram.

The sulfur compounds dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) differed significantly between the sample treatments (origin x roasting) as evaluated by ANOVA: DMDS: $F(26,54)=15.97$, $p < 0.001$; DMTS: $F(26,54)= 391.67$, $p < 0.001$. As seen in Figure 5 and Figure 6, the concentration of DMDS and DMTS are mostly affected by roasting temperature, as the most dramatic

increases in these compounds concur with increases in roasting temperatures (e.g., in the samples from Ghana, DMTS concentration rises over 270-fold from unroasted cacao to cacao roasted at the highest temperature of 171ºC). Both figures also demonstrate that not only roasting temperature affects DMDS and DMTS concentration in chocolate as DMDS and DMTS concentrations also differ between genetic origins (e.g., at 171ºC concentration of DMTS in the samples from Ghana is nearly 1.42 times that of the concentration of DMTS in samples from Peru (Figure 6)). Similarly, concentration of DMDS and DMTS also differs due to roasting time (e.g., even though Madagascar samples are roasted up to 171ºC, peak concentration of DMDS and DMTS is seen when the samples were roasted to 151ºC for a longer time as seen in Figure 5 and Figure 6). The concentration of DMDS and DMTS also vary for the replicated center point (114ºC for 40min), indicating that similarly to the human assessors, the two replicated center points show differences in volatile sulfur compounds.

Regarding the precision of the measurements, in the majority of cases the standard error of the mean remained relatively low, ranging from 0.01 to 1.46 relative concentration units. Samples with notably high standard errors included the center point sample 20 from Madagascar roasted at 114ºC for 40 min with a SE=1.04 for DMDS and the unroasted sample 5 from Peru left at 24ºC for 0 min with a SE=1.46 for DMDS and SE=1.28 for DMTS.

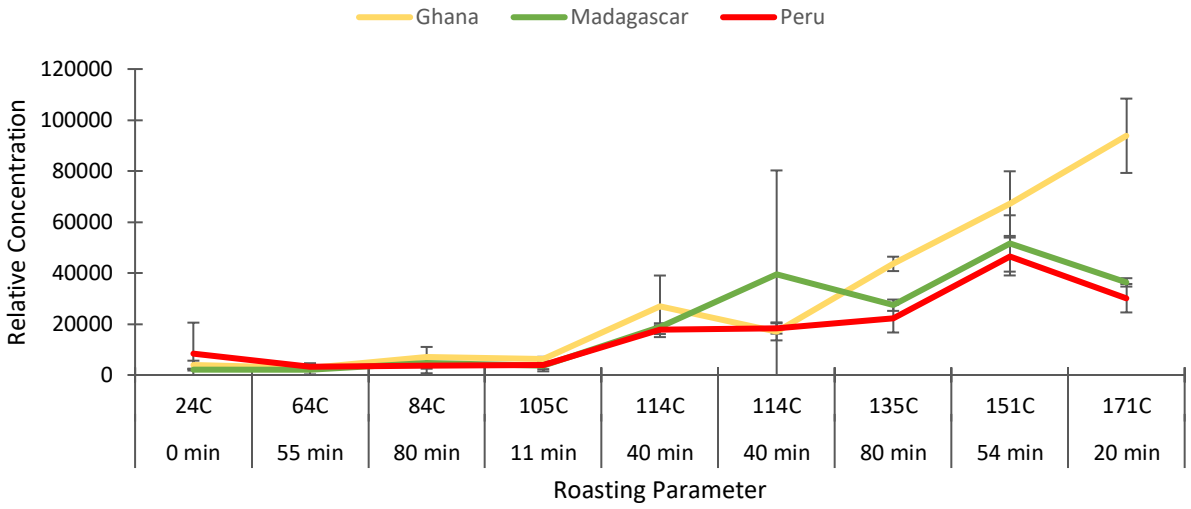


Figure 5: Dimethyl Disulfide Relative Concentration at all Roasting Parameters for all Origins. Error bars indicate standard deviation

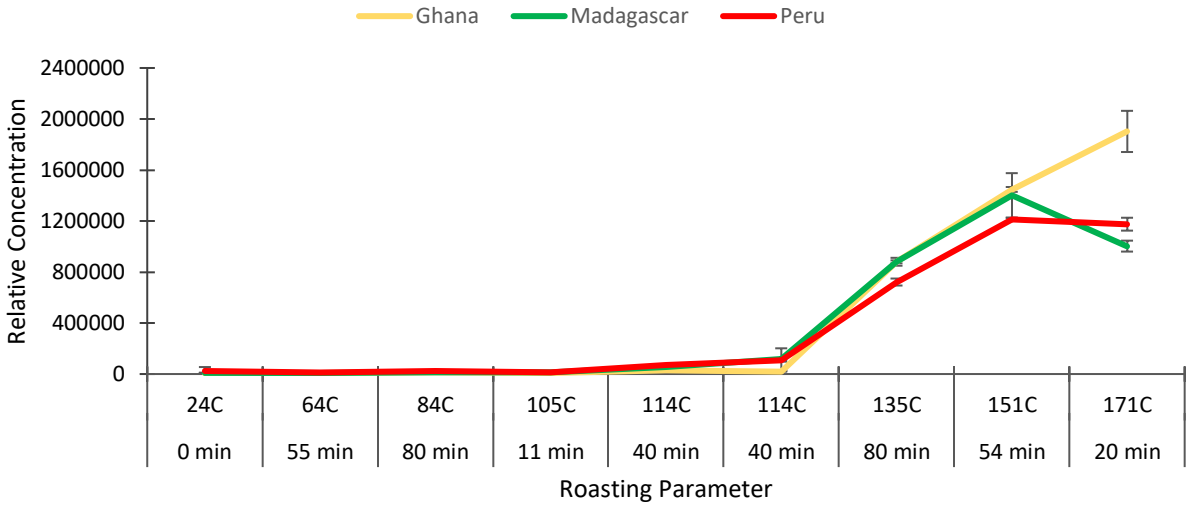


Figure 6: Dimethyl Trisulfide Relative Concentration at all Roasting Parameters for all Origins. Error bars indicate standard deviation.

While no in-depth analysis was conducted, the shear variety of volatile sulfur compounds that appear in roasted cocoa was a surprising finding. Besides the 2 identified and semi-quantified DMDS and DMTS at least a dozen of unknown volatile sulfur compounds also appears in the SCD chromatograms and warrant further research (**Error! Reference source not found.**). For example, three different sulfur compounds appear at roughly the same retention time of 20 min in the first dimension. These compounds

were only separated from each other in the second dimension, demonstrating the separation power of comprehensive GCxGC-SCD. This also indicates that the already scarce literature on volatile sulfur compounds in roasted cocoa is most likely underreporting how many different sulfur compounds are present in roasted cocoa, due to the use of non-comprehensive chromatography.

Additionally, this complexity in the number of sulfur species seems to also be affected by roasting parameters as seen in the example Figure 8, which depicts example GCxGC-SCD chromatograms of two chocolate samples from the same origin, with one being unroasted and the other being roasted for the highest time temp combination. As can be seen in Figure 8, more blobs appear in the chocolate sample roasted at the higher temperature than the sample roasted at the lower temperature, indicating at roasting has some effect on the number of sulfur species formed during roasting in addition to concentration of volatile sulfur compounds formed during roasting. This is the first time this phenomenon has been reported in chocolate and should be further investigated to evaluate exactly how roasting affects the formation of sulfur compounds in chocolate.

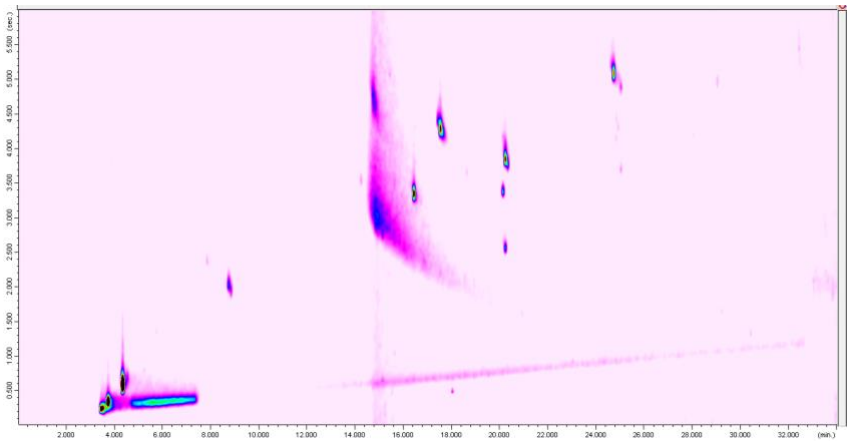


Figure 7: Example GCxGC-SCD chromatogram of a roasted chocolate sample (Ghana, 112ºC for 40 min).

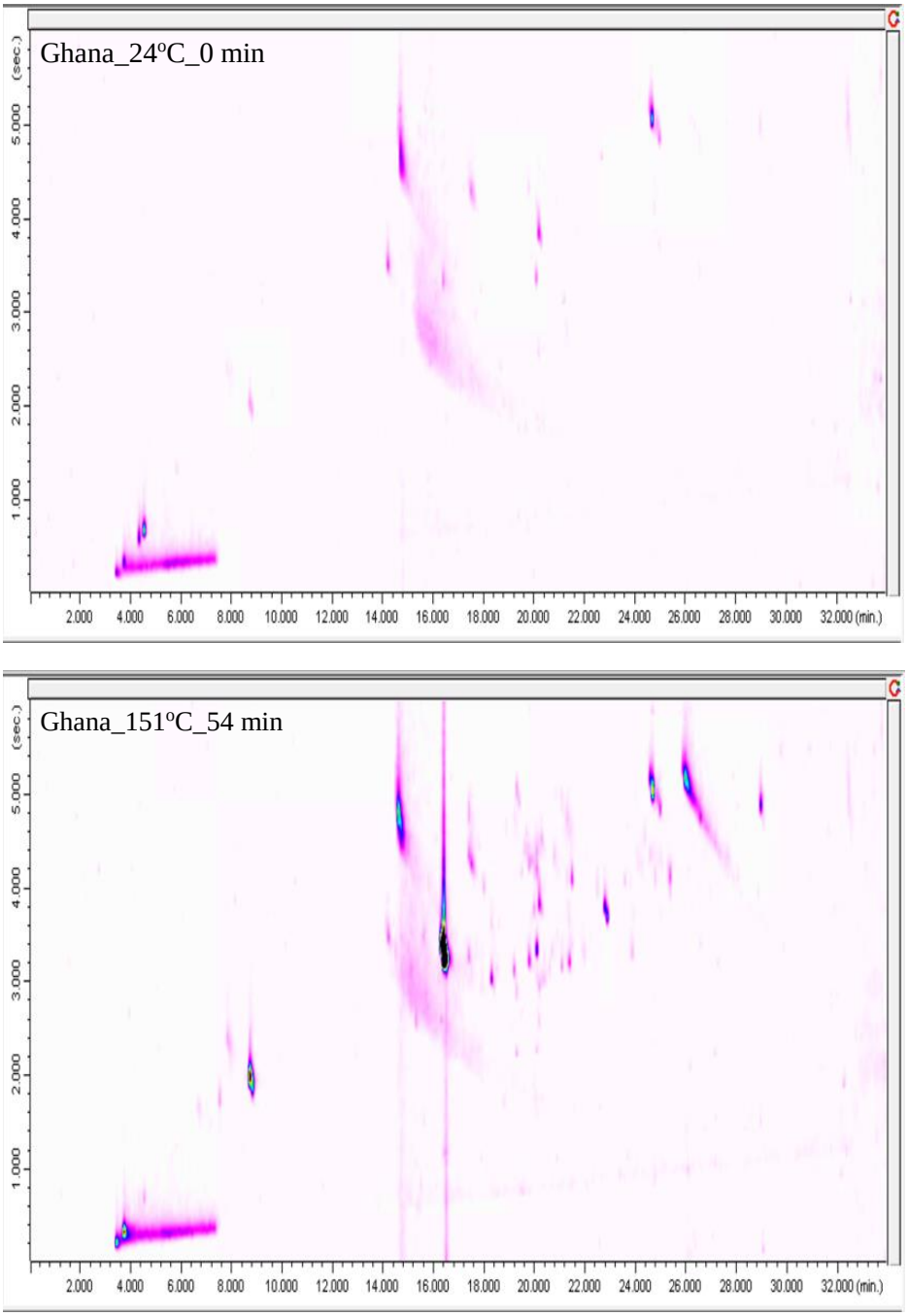


Figure 8: Example GCxGC-SCD chromatograms of unroasted chocolate samples (24ºC for 0 min, sample 12) (above) and highly roasted chocolate samples (151ºC for 54 min, sample 18) (below) from Ghana

Chapter 5

Conclusion and Discussion

This research project addressed several goals. The first goal was to investigate how odor affects perceived bitterness in 100% chocolate. Chocolate when evaluated by consumers without a nose clip was found to be more bitter than chocolate evaluated with a nose clip (e.g., without odor perception) in all roasting treatments, indicating the presence of aroma volatiles that induced a cross-modal bitterness enhancement in chocolate (Table 3). This is an especially interesting discovery as bitterness in chocolate is thought to be primarily caused by non-volatile compounds such as methylxanthines (i.e., caffeine and theobromine) flavonoids (i.e., epicatechin, catechin, and epicatechin oligomers), some 2,5-diketopiperazines (DKPs), and other minor non-volatile compounds (Stark et al. 2006; Afoakwa et al. 2008; Ziegleder 2017), but volatile aroma contributions to bitterness have thus far not been studied. Bitterness perception has been previously shown to be affected by other modalities, such as sweet taste or salty taste (Djordjevic et al. 2004; Lawless and Heymann 2010). Cross-modal interactions, such as the one shown here, has been reported only once before, where the addition of a cocoa flavor to a cocoa beverage enhanced the bitterness in cocoa beverages using a similar nose-clip method (Labbe et al. 2006).

The second goal of this research project was to identify potential volatiles that could be inducing the observed odor induced enhancement of bitterness. Volatiles of the chocolate samples were profiled using GC-MS, resulting data was then processed to identify and semi-quantify compounds and analyzed statistically via correlation analysis and partial least squares-regression (PLS-R), similar to the approach reported by Frøst et al. (2021). Two primary lists of compounds were identified by correlation analysis (Table 4) and PLS-R (Error! Reference source not found.), with 2,3-Butanedione and Ethyl Butanoate being the two compounds with a high chance of affecting overall bitterness perception. A third

compound, Dimethyl disulfide (DMDS), whose importance to chocolate flavor and olfaction has been suggested previously (Afoakwa et al. 2008), was also identified as a potential contributor to bitterness due to its significant correlation to overall bitterness (ΔBitt) (Table 4), and a VIP score in the PLS-R model of over 1. Dimethyl trisulfide (DMTS), which also has been noted for its importance to flavor and olfactory sensation in chocolate but whose specific effect has not been investigated (Liu et al. 2015), saw similar but slight weaker results leading to its exclusion from previous lists.

Of note is that in a mixture as complex as chocolate the observed odor induced enhancement of bitterness could be a result of the presence or absence of any one or mix of compounds identified here. Most research into odor induced taste effects has been focusing on compounds that increase sweetness perception (Djordjevic et al. 2004; Boakes and Hemberger 2012) and saltiness perception (Djordjevic et al. 2004; Lawrence et al. 2011), as well as umami taste perception as of recent (Frøst et al. 2021); little work has investigated volatile compounds that are able to increase bitterness perception. The chocolate aroma composition is extraordinarily complex (Afoakwa et al. 2008; Tran et al. 2015; Ziegleder 2017; Magagna et al. 2017) so future validation studies are needed to pinpoint the exact volatiles able of inducing bitterness in chocolate, starting with the identified volatiles suggested here.

The final goal of this research was to investigate how roasting of cocoa affects volatile sulfur compound composition in chocolate, specifically, in regard to dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) which have been noted for their importance to flavor in chocolate, but never thoroughly investigated (Afoakwa et al. 2008; Liu et al. 2015a). It was hypothesized that since DMDS and DMTS result from the Strecker degradation of methionine and other sulfur containing amino acids that both would increase with increasing roasting temperature. Using advanced sulfur selective detection instrumentation (GCxGC-SCD/FID), it was found that DMDS and DMTS both increased as a result of increasing roasting temperature, thus confirming our hypothesis (Figure 5, Figure 6).

Most surprisingly in the analysis of volatile sulfur compounds was the presence of dozens of other unidentified sulfur compounds appearing after roasting in the GCxGC-SCD chromatogram (Figure

7**,** Figure 8). The amount of sulfur species in these chromatograms also seemed to be influenced by roasting (Figure 8). Future work should try to identify these compounds and examine how they may affect flavor in chocolate.

Limitations of this project primarily involve the inability to identify every detected volatile compound. Reliance on deconvolution software such as PARADISe and spectral matching with entries in the NIST database, even with their strengths, leave the possibility that compounds could have been unknowingly misidentified. Identification of volatile sulfur compounds besides those that could be identified by matching with commercially available sulfur compounds was also limited by the low concentrations of sulfur compounds present in the samples that make the use of mass spectrometry less successful. Although less likely, there is the chance of VOCs present in the environment that may have been mistaken to be present in the chocolate itself due to the use of the high sensitivity of the analytical instrumentation used here. This project also analyzed solely unsweetened chocolate, so findings here may not be representative of commercial chocolate that contain other ingredients besides cocoa (e.g., sucrose, vanilla, lecithin, etc.), however, since all chocolate contains cocoa, our findings should be applicable to the cocoa portion of other chocolate.

Among the strengths of this study is the combination of instrumental analysis and human sensory perception that made use of a large number of actual chocolate consumers of all genders, ages, and ethnicities to evaluate these samples instead of relying on a trained panel, making our sensory results more strongly representative of the general population. The use of the same chocolate samples as described in McClure et al. (2021) that were created based on a comprehensive experimental design for roasting, including a raw unroasted chocolate, also allowed for a comprehensive study of how roasting affects volatile composition for all reasonably conceivable roasting treatments, making our results widely applicable. The use of extremely sensitive analytical equipment such as comprehensive gas chromatography with both non-specific mass spectrometry and sulfur-selective detection and powerful

analytical deconvolution software such as PARADISe aided in the identification and semi-quantitation of volatile compounds as accurate and precise as they can currently be.

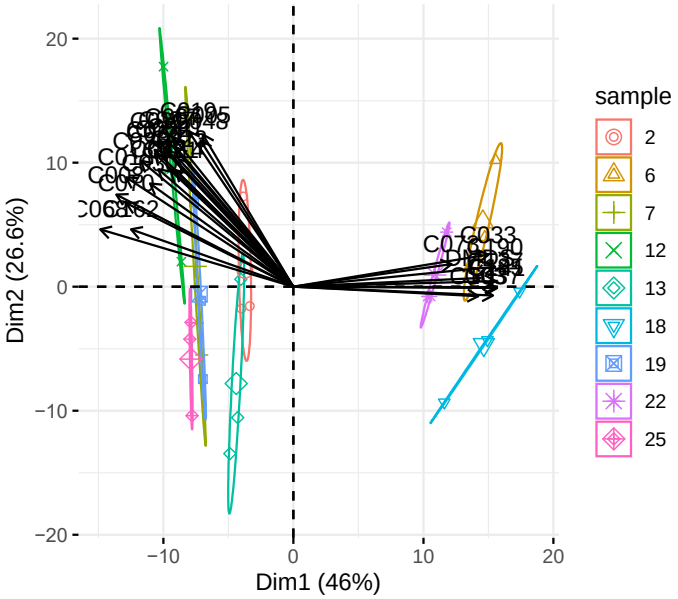
Funding

This material is based upon work supported by the Austrian Marshal Plan Foundation and the Penn State Schreyer Honors College. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author and do not necessarily reflect the views of the Austrian Marshal Plan Foundation or the Penn State Schreyer Honors College. Sensory research was conducted at the Penn State Food Science Sensory Evaluation Center by Drs. Alan McClure and Helene Hopfer. Analytical work was conducted at the Institute of Analytical Chemistry and Food Chemistry at the Technical University of Graz by Aaron Wiedemer and Dr. Erich Leitner.

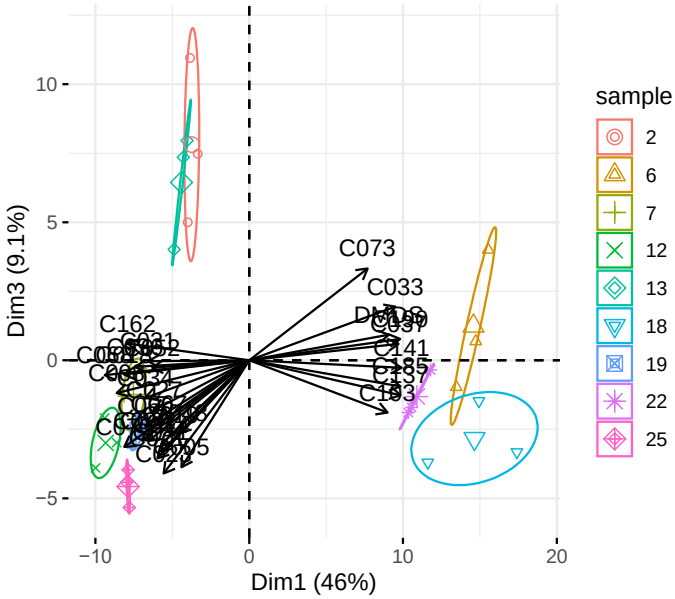
Appendix A

PCAs for Ghana Samples grouped by sample with compounds with high correlation to difference in bitterness ratings.

B.) PCs 1 & 2



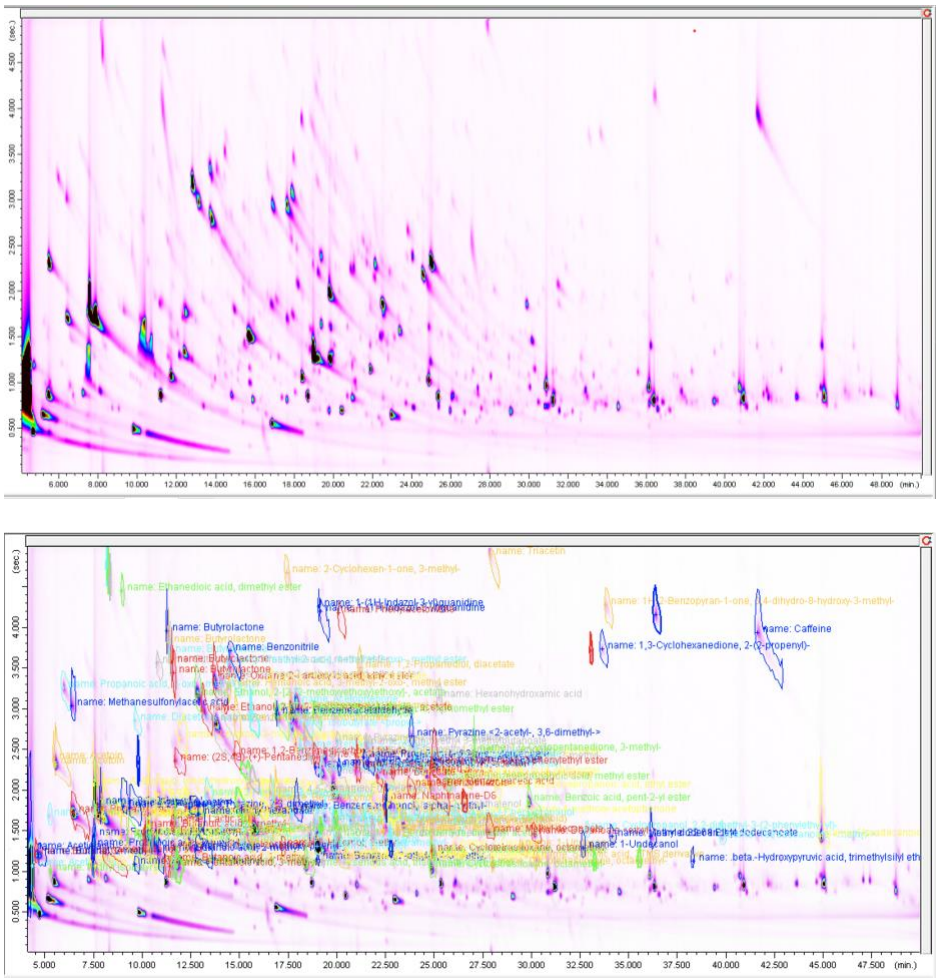
A.) PCs 1 & 3



Appendix B

GCxGC-MS Results

200 VOCs were identified by comprehensive gas chromatography – mass spectrometry. Appendix B shows a screenshot of the comprehensive chromatogram of chocolate. As is seen, there are a myriad of compounds that are only visible due to the second dimension of separation, indicating how complex chocolate aroma is and why it is so important to use GCxGC-MS.



Appendix B: GCxGC-MS TIC (sample 2)

Appendix C

R Script

```
#'  Undergraduate Thesis
#'  Aaron Wiedemer
#'  3/27/2022
#   ++++++++++++++++++++

#### Data Set Up ####
library(readxl)
library(tidyverse)
?tidyverse
?readxl

# Data

GC.Gh<-read_excel("/Users/aaron/OneDrive - The Pennsylvania State
University/Thesis/Data/Ghana GC PCA/20220201_Ghana data for R-Aaron's MacBook Pro.xlsx",
sheet = 4,col_names = TRUE) # data with sulfur

# makes samples, replicates, time and temperature data factor data
GC.Gh$sample<-as.factor(GC.Gh$sample)
GC.Gh$replicate<-as.factor(GC.Gh$replicate)

# Check
str(GC.Gh)
summary(GC.Gh)
dim_desc(GC.Gh)

# ++++++++++++++++++
#### Correlation ####
# ++++++++++++++++++
library("Hmisc")

# creates a correlation matrix for delta bitterness, finding the correlation and p-values between
each variable
diff.cor<-rcorr(x = as.matrix(GC.Gh[, -c(1:2,5:6)]),
```

```
            type='spearman')

# ++++++++++++++++++++++++++++
# flattenCorrMatrix
# ++++++++++++++++++++++++++++
# cormat : matrix of the correlation coefficients
# pmat : matrix of the correlation p-values
flattenCorrMatrix <- function(cormat, pmat) {
  ut <- upper.tri(cormat)
  data.frame(
    row = rownames(cormat)[row(cormat)[ut]],
    column = rownames(cormat)[col(cormat)[ut]],
    cor  =(cormat)[ut],
    p = pmat[ut]
  )
}

# organizes correlation matrix into a row, column, and their correlation and p-value
diff.cor.flat<-flattenCorrMatrix(diff.cor$r, diff.cor$P)

# shows correlations for just bitterness differences
diff.cor.sub<-diff.cor.flat[which(diff.cor.flat$row=="Bitterness Differences"),]

# a subset of the bitterness rating correlations where the absolute value of the correlation is
greater then 0.5 and the p-value is below 0.001.  This low p-value was used to mitigate type I
error.
(diff.cor.maj<-subset(diff.cor.sub,abs(diff.cor.sub$cor)>0.5&diff.cor.sub$p<0.001))

summary(str(diff.cor.maj))
levels(diff.cor.maj$row)

# ++++++++++++++++++++++++
## Code identifier ##
# ++++++++++++++++++++++++

# load chemical names
chem.names<-as.data.frame(read_excel("/Users/aaron/OneDrive - The Pennsylvania State
University/Thesis/Data/Ghana GC PCA/20220201_Ghana data for R-Aaron's MacBook
Pro.xlsx",sheet = 6)) # list of chemical names and their respective codes

chem.names$Code<-as.factor(chem.names$Code)
chem.names$Chem<-as.factor(chem.names$Chem) #changes data to factor data
```

```
# check
head(chem.names)
str(chem.names)

# makes row in diff.cor into factor data
diff.cor.maj$row<-as.factor(diff.cor.maj$row)
diff.cor.maj$column<-as.factor(diff.cor.maj$column)


(for (i in diff.cor.maj$column) {print(rownames(chem.names[which(chem.names$Code==i),]))

})


(chem.names1<-
chem.names[c(1,7,10,16,20,21,23,24,25,29,32,33,35,36,38,39,43,50,54,62,69,70,72,75,105,139
,143,164,187,192),]) #Important chemicals used in PCA



chemicals<-cbind(chem.names1,diff.cor.maj)
summary(chemicals)
head(chemicals)
chemicals<-chemicals[,-c(3,4)]

# bitterness rating correlations where the absolute value of the correlation is greater then 0.5
and the p-value is below 0.001.

# list of important chemical codes, names, and correlations to delta bitter.  Only chemicals
which were significantly correlated at a=0.001 and had either a correlation above 0.5 or below -
0.5 where chosen as potentially important

print(chemicals)

# export data to excel

library("writexl")
write_xlsx(chemicals,"/Users/aaron/OneDrive - The Pennsylvania State
University/Thesis/Data//pca.chem.corr.2.xlsx")

# ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
#### PLS ####
# ++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++++
```

```
## Packages ##
library(mdatools)

#check version
?mdatools
# [Package mdatools version 0.12.0 Index]

## Data ##
idx <- seq(3,27,3) #index to select third replicate
# predictor variable
Xc <- GC.Gh[-idx,8:206]
# response matrix
yc <- GC.Gh[-idx,7]
# verification predictor variable
Xt <- GC.Gh[idx,8:206]
# verification response matrix
yt <- GC.Gh[idx,7]


# ++++++++++++++++++++++++++
## PLS calibration model ##
# ++++++++++++++++++++++++++

GC.Gh.pls <- pls(Xc,yc,9,scale = TRUE,center = TRUE, info="bitterness prediction model", cv=1,
ncomp.selcrit = "min",x.test = Xt,y.test = yt) # centered and mean scaled
summary(GC.Gh.pls)
# 88.70477% of Y is accounted for

# RSME plots
par(mfrow = c(1,1))
plotRMSE(GC.Gh.pls,show.legend = T)

GC.Gh.pls$ncomp.selected  # suggested number of componenets = 1

# From the RSME plot and the suggested number of components form the pls algorythm, the
first minimum for RSME is 1 so we will use the first component in the pls model

# regression coeffs
GC.Gh.pls$coeffs$values
summary(GC.Gh.pls$coeffs)

par(mfrow = c(1,1))
plotRegcoeffs(GC.Gh.pls,type = "h", show.labels = TRUE)
```

```
## PLS prediction ##
res<-predict(GC.Gh.pls,Xt,yt)
res$y.pred # predicted bitterness differences
res$y.ref # reference distances

# ++++++++++++++++++++
## PLS Validation ##
# ++++++++++++++++++++

plotXScores(GC.Gh.pls,show.labels=T,show.legend =T) # xvariance explained
plotXYScores(GC.Gh.pls,show.labels=T,show.legend=F)
# Groupings seem good


## Loadings Plot ##
plotXYLoadings(GC.Gh.pls,show.labels=T,show.legend=F) # cmpds near this will be correlated to
Bittrness differences
# Complex, therfore will try to identify important compouds with VIP scores and Selectivity
Ratios (RV)

# +++++++++++++++++++++++
### Variable Selection ###
# +++++++++++++++++++++++

# ++++++++++++++++++++++
## Code identifier ##
# ++++++++++++++++++++++

# load chemical names
chem.names<-read_excel("/Users/aaron/OneDrive - The Pennsylvania State
University/Thesis/Data/Ghana GC PCA/20220201_Ghana data for R.xlsx",sheet = 4) # list of
chemical names and their respective codes

chem.names$Code<-as.factor(chem.names$Code);chem.names$Chem<-
as.factor(chem.names$Chem) #changes data to factor data

# check
head(chem.names)
str(chem.names)
```

```
## VIPS ##

par(mfrow = c(1,1))
par(mar = c(5,2.5,1,1))
plotVIPScores(GC.Gh.pls, type = "h", show.labels = TRUE,ncomp=1,show.legend=F, main = '') #
VIP Scores Plot
abline(h=1.5, col = 'red', lty = 1)

vip<-vipscores(GC.Gh.pls) # list of VIPSs
# DMDS and DMTS have VIPs > 1
vip.1<-as.data.frame(vip[which(vip>1.5),])# chem codes and their VIP scores
colnames(vip.1)<-"VIP"
vip.1$Code<-row.names(vip.1)
print(vip.1)
vip.2<-(chem.names[which(vip>1.5)-2,])# chemicals w/ VIP scores >1.5
vip.2<-as.data.frame(vip.2)
print(vip.2)

vip.c<-merge(vip.2,vip.1,by="Code")
print(vip.c) # list of VIP scores, chemicals, and chemical ID codes


## Selectivity Ratio ##

plotSelectivityRatio(GC.Gh.pls, type = "h", show.labels = TRUE, show.legend=F,main = '') #
Selectivity ratio
# C068 is ethyl bytyrate
abline(h=10, col = 'red', lty = 1)

sr<-selratio(GC.Gh.pls) # list of selectivity ratios
summary(sr)

sr.1<-as.data.frame(sr[which(sr>10),])# chem codes and their SR
colnames(sr.1)<-"SR"
sr.1$Code<-row.names(sr.1)
print(sr.1)
sr.2<-(chem.names[which(sr>10),])# chemicals w/ SR > 10
sr.2<-as.data.frame(sr.2)
print(sr.2)

sr.c<-merge(sr.2,sr.1,by="Code")
print(sr.c) # list of SR, chemicals, and chemical ID codes
```

```
# ++++++++++++++++++++++
#### Sulfur Report ####
# ++++++++++++++++++++++

#### Data Set Up ####
library(readxl)
library(tidyverse)

s3<-read_excel("/Users/aaron/OneDrive - The Pennsylvania State
University/Thesis/Data/SCD/SCD Report.xlsx", sheet = 8,col_names = TRUE) # Final in SCD
report

dim(s3)


s3$Time<-as.factor(s3$Time)
s3$Temp<-as.factor(s3$Temp)
s3$Origin<-as.factor(s3$Origin)
s3$Sample<-as.factor(s3$Sample)
s3$Origin<-as.factor(s3$Origin)
s3$Replicate<-as.factor(s3$Replicate)

# Check
str(s3)
summary(s3)
dim_desc(s3)

# ++++++++++++
#### ANOVA ####
# ++++++++++++

s.mat<-as.matrix(s3[,6:7])

# Fixed ANOVA for chemicals as effected by time and temperature
s.lm<-lm(s.mat~(Sample),data=s3)
summary(s.lm)
s.aov <- aov(s.lm)
s.aov.sum<-summary(s.aov)
print(s.aov.sum)

# ++++++++++++++++++
#### Appendix ####
# ++++++++++++++++++
```

```
# ++++++++++++++++++
#### PCA ####
# ++++++++++++++++++

library(FactoMineR)
library(factoextra)

## Data set up ##
GC.Gh2<-(GC.Gh[,-c(1,2)])

str(GC.Gh2)
summary(GC.Gh2)
dim_desc(GC.Gh2)

# ++++++++++++++++++
#### PCA model ####
# ++++++++++++++++++

GC.Gh.PCA<-PCA(GC.Gh2,graph = FALSE,ncp = 3) #makes a PCA with 3 dimensions

get_eigenvalue(GC.Gh.PCA) # eigenvalues for PCA. 81.75% of the cumulative variability is
explained by 3 dimensions, hence why I chose it
fviz_eig(GC.Gh.PCA) # eigenvalue scree plot

## Dimension Description ##

GC.Gh.PCA$var$contrib

GC.Gh.dim<-dimdesc(GC.Gh.PCA,axes = c(1:3),proba = 0.05)

GC.Gh.dim$Dim.1 # mostly characterized by C141, C082, C133 and negatively correlated with
C136, C068, C134, and C008.  Also somewhat correlated with DMDS and DMTS
GC.Gh.dim$Dim.2 # mostly characterized by C016, C009, C112, and negatively correlated with
C132, C178, C124
GC.Gh.dim$Dim.3 # mostly characterized by C114, C130, C196 and negatively correlated with
C142, C092, C045
# Within each dimension, this function shows every component that significantly correlates
with each dimension.

# +++++++++++++++
#### Biplot ####
# +++++++++++++++
```

```
var<-as.matrix(diff.cor.maj$column)

# PCA biplot with three individual values clusters as determined by the means algorithm.
Selected variables are determined by the diff.cor.maj
par(mfrow = c(1,2))

fviz_pca_biplot(GC.Gh.PCA,
        axes = c(1,2),
        select.var= list(name=var),
        geom.ind = "point",
        col.ind = GC.Gh$sample,
        addEllipses = TRUE,
        ellipse.type = "confidence",
        col.var = "black",legend.title = "sample")

fviz_pca_biplot(GC.Gh.PCA,
        axes = c(1,3),
        select.var= list(name=var),
        geom.ind = "point",
        col.ind = GC.Gh$sample,
        addEllipses = TRUE,
        ellipse.type = "confidence",
        col.var = "black",legend.title = "sample")
# PCA biplot with three individual values clusters as determined by the means algorithm.
Selected variables are determined by the diff.cor.maj

# this graph is messy, identify potential compounds by identifying their correlations
```

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Academic Vita

Aaron Wiedemer

Education

Aug 2018 – May 2022 **Bachelor of Science in Food Science, Minor in Jazz Performance**
The Pennsylvania State University, Schreyer Honors College, University Park, PA

- Honors Thesis: Identification of Potential Volatile Contributors to Bitterness in Chocolate
- Dean's list: all semesters
- Department Head's list: all semesters

Study Abroad:

- Technical University of Graz, *Graz, Austria (Fall 2021)*
- AgroParis Tech, *Paris, France (Summer 2019)*
- *Guilin, Xi'an, Shanghai, and Beijing China (Summer 2016)*

Research Experience

2021 – Present **Identification of Potential Volatile Contributors to Bitterness in Chocolate**
P.I. Erich Leitner, PhD
Technical University of Graz, Institute of Analytical Chemistry and Food Chemistry &
P.I. Helene Hopfer, PhD
The Pennsylvania State University, College of Agricultural Sciences, Department of Food Science

Currently researching potential volatile compounds that cause bitter and astringent cross-modal interactions in chocolate aroma processed under different roasting conditions for my honors thesis. Designed and ran experiments using analytical methods such as GCxGC-MS, GC-MS, GC-SCD, and GC-FID. Analyzed large sets of analytical chemistry data using GC-MS data analysis software such as Shimadzu LabSolutions, XC-MS, and metaboanalyst. Recipient of the Austrian Marshal Plan Scholarship (€5,000)

Summer 2021 **Research and Development Internship**
Shawn Yoon, PhD
Ingredion Inc., Global Research and Development Team, Applied Biochemistry and Mechanisms Group, Bridgewater, New Jersey

Conducted research for a specific application for Ingredion Inc. Gained experience in food biotechnology, fermentation science, and starch chemistry. Designed and ran over 50 experiments using TLC, SDS-PAGE, and spectrophotometry.

Summer 2019 **The Effect of Environment and Genotype on Plant Metabolites in Cultivars of Theobroma Cacao Essential to Flavor; Total Fat content and Volatile Profile**
P.I. Helene Hopfer, PhD
The Pennsylvania State University, College of Agricultural Sciences, Department of Food Science

Discovered how genetic and harvest related differences in *Theobroma cacao* affect total fat content and volatile flavor compound composition in both roasted and raw chocolate. Designed and ran experiments using analytical methods such HS-SPME-GC-MS and TD-NMR. Analyzed large sets of analytical chemistry data using PARADISe GC-MS data analysis software and Minitab . Recipient of a Penn State College of Agricultural Sciences Student Research Grant ($3,175).

Grants and Awards

2022 Gamma Sigma Delta Poster Competition, 3rd place ($100)

2021 Austrian Marshal Plan Scholarship *(€5,000)*

2021 Rumbaugh Agricultural Leadership Award *($1,000)*

2021	Knighltly Scholarship in Food Science *($1,462)*
2019 & 2020	Star Kay White Scholarship in Food Science *($1,184)*
2019 & 2020	Horace T. Woodward Scholarship in the College of Agriculture *($2,000)*
2019	Penn State College of Agricultural Sciences Student Research Grant *($3,175)*
2018	Frank S. and Nina Cobb Scholarship *($1,200)*
2018	Young Scholarship in Agricultural Sciences *($510)*

Teaching Experience

Spring 2021 & Spring 2022	Undergraduate Teaching Assistant Helene Hopfer, PhD *Food Science 410 – Chemical Methods of Food Analysis. Penn State University* Assisted in teaching, holding office hours, and grading for a 400-level Major course
Summer 2018	Private Music Instructor *Saxophone* Taught middle school students music theory and saxophone performance
Summer 2018	Middle School Music Education Intern Joseph Bassin, PhD *Chatham Middle School, New Jersey* Assisted in teaching and leading 6th and 7th grade band classes

Presentations

March 2022	**Boy, that *Doesn't* Stink! The Effect of Roasting and Cacao Origin on Sulfur Compounds in Chocolate** *Penn State Gamma Sigma Delta Poster Conference*
May 2021	**What is Food Chemistry?** Yelena Naumova, PhD *Chatham High School, AP Chemistry, Chatham, NJ*

Work Experience

Summer 2018	Grill Chef *Chipotle, New Providence, New Jersey, Summer 2018* Made and served food with a team to >200 persons a day

Certifications

2021	Lean Six Sigma White Belt Certification *Ingredion Inc., Bridgewater, New Jersey*

Student Leadership and Involvement

2020 - Present	Penn State Representative *IFT College Bowl* Competed in IFT's annual Intercollegiate Food Science Trivia Competition
2019 - Present	Thespian *Penn State Thespians* Organized large crowds and aided productions
2019 - Present	Social Captain *Penn State LGBTQ+ Roundtable* Organized events to support the LGBTQ+ on campus
2019	Team Leader *National Dairy Council Product Development Competition* Led a team of student product developers to create a high protein matcha drink

2018 - Present	President Elect, Vice President, Treasurer, Fundraising Chair *Food Science Club at Penn State* Led Penn State's official Food Science club and helped raise > $7,000 annually
2018	Penn State Football Crowd Usher *Penn State Football* Helped manage crowds of over 120,000 people at the biggest college football event in the nation

Community Service

2021	COVID-19 Vaccine Distributor *Centre Volunteers in Medicine, COVID-19 Vaccine Clinic* Vaccinated 3,000 high risk individuals
2019	Building recycler *ReFarm sustainable food project* Recycled dilapidated buildings for construction materials
2018 - Present	Volunteer *Penn State Boulevard, Community Service and THON Organization* Recipient of "Best Canvasser" Award, helped raise >$20,000 for children with pediatric cancer annually
2015 - 2018	Team Leader *RISE - Home construction and community development in Northern Appalachia* Worked with a team to make homes warmer, dryer, and safer in Northern Appalachia

Professional Memberships

2020 - Present	Professional Manufacturing Confectioners Association (PMCA)
2020 - Present	Gamma Sigma Delta Honors Society (GSD)
2019 - Present	International Food Technologists (IFT)
2019 - Present	Penn State Cacao Research Network (CCRN)

Computer Skills

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PARADISe GC-MS Data Software XCMS GC-MS Data Software

Metaboanalyst metabolomics software

XCMS chromatography mass spectrometry software

Microsoft Office (Outlook, Word, Excell, etc.)

Schimatzu postrun analysis

ChromSquare comprehensive gas chromatography software

MuseScore

Media development (podcasts, videos, etc.)

Google apps