

The Pennsylvania State University  
The Graduate School  
Department of Food Science

**CHARACTERIZATION OF PERSEORANJIN, A NATURAL ORANGE PIGMENT  
FOUND IN HASS AVOCADO (*PERSEA AMERICANA*) SEED,  
AND ITS USES AS A NATURAL FOOD COLORANT**

A Thesis in  
Food Science  
by  
Rachel M. Shegog

©2015 Rachel M. Shegog

Submitted in Partial Fulfillment  
of the Requirements  
for the Degree of  
Master of Science

December 2015

The thesis of Rachel M. Shegog was reviewed and approved\* by the following:

Joshua D. Lambert  
Associate Professor of Food Science  
Thesis Adviser

Ryan J. Elias  
Associate Professor of Food Science

Gregory R. Ziegler  
Professor of Food Science

Emmanouil Chatzakis  
Director of The Pennsylvania State University NMR Facility

Robert F. Roberts  
Professor of Food Science  
Head of the Food Science Department

\*Signatures are on file in the Graduate School

## ABSTRACT

The global natural colors market is estimated to reach US \$ 2.3 billion by 2020. This is due in part to increasing consumer desire for natural food ingredients and the desire of food companies to produce “clean-label” products. We examined an extract of Hass avocado (*Persea americana*) seeds crushed in water as a potential source of natural orange colorants. The extract color is pH dependent, and can produce a range of colors from pale yellow near pH 2.5 to deep red and brown colors near pH 10. Treating the extract under alkaline conditions to pH 12.32 shifts the extract’s color range towards the red end of the visible light spectrum. After treatment with alkali, the extract can produce rich orange colors even at low pH values near 2.5, while the native extract produces only pale yellow in this range.

A polyphenol oxidase (PPO) catalyzed reaction produced the most abundant pigment in this extract, 2-(4-hydroxy-8-(2-((5-hydroxy-2-oxo-2,6,7,7a-tetrahydrobenzofuran-6-yl)oxy)ethyl)-5-oxo-6-((3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-yl)methoxy)-5H-benzo[7]annulen-3-yl)acetic acid, or perseoranjin, which is a novel glycosylated benzotropolone compound with carboxylic acid and fused-ring butenolide containing side chains. Though the color is stable at room temperature, liquid chromatography-mass spectrometry (LC-MS) indicates that the individual compounds are not stable, forming dimers and other compounds in aqueous solution. The most abundant colored fraction showed Perseoranjin to have a parent ion  $[M+H]^+$  with  $m/z$  603.1675 in positive mode. Based on the presence of an abundant  $m/z$  441 fragment ( $\Delta m/z$  162), we hypothesized that the compound was a glycosylated compound. The same extract also contained other  $[M+H]^+$  ions including an unrelated  $m/z$  603.1687 compound, a  $m/z$  917.2639 compound with a  $m/z$  603 moiety, and finally an  $m/z$  1205 dimer produced from the combination of two  $m/z$  603 compounds.

Using LC-MS-based metabolomics and principal component analysis (PCA) of the colored and PPO-inhibited-uncolored extracts, we found approximately forty-nine masses unique to the colored or uncolored extract as well as some known compounds present in both. Abscisic acid and perseitol, the 7-member sugar alcohol, were present in both extracts while epicatechin, catechin, procyanidin B2 and salidroside were found only in the uncolored extract. Here, we report the results of our on-going studies to chemically characterize this new orange pigment. We also provide some preliminary results on its colorant properties in various food products. These results will support a new, value-added use for avocado seeds which are frequently viewed as a low-value waste product.

## TABLE OF CONTENTS

LIST OF FIGURES.....	vii
LIST OF TABLES .....	x
ACKNOWLEDGEMENTS .....	xi
Chapter 1: Literature Review .....	1
1.1 Color in Foods.....	1
1.1.1 Importance of Color in Foods.....	1
1.1.2 Artificial Food Colorants .....	4
1.1.3 Natural Food Colorants.....	7
1.2 Role of Polyphenol Oxidase in Pigment Formation .....	9
1.3 Benzotropolone Compounds.....	12
1.4 Avocado ( <i>Persea americana</i> ) Seed as a Source of Bioactive Phytochemicals.....	15
1.4.1 Ethnobotanical and Historical use of Avocado Seed.....	16
1.4.2 Composition and Chemical Components of Avocado Seeds.....	17
1.4.3 Biological Activity.....	20
1.4.4 Safety of Avocado Seed Extract .....	31
1.5 Hypothesis and Objectives .....	31
Chapter 2: Materials and Methods .....	33
2.1 General .....	33
2.1.1 Preparation of a Semi-pure CASE.....	33
2.2 Color and Stability of Semi-pure CASE in some Commercial and Model Food Products .....	34
2.2.1 Color studies .....	34
2.2.2 Stability Studies in Model Sugar Drinks .....	34
2.3 Effect of pH on the color of CASE .....	36
2.4 Preparation of Colored and Uncolored Avocado Seed Extracts for Metabolomics.....	36
2.5 Structure Elucidation of the Colored Compound.....	37
2.5.1 HPLC Purification .....	37
2.5.2 High Resolution MS/MS Analysis.....	38
2.5.3 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR)...	39

2.5.4	High Resolution NMR Analysis .....	39
Chapter 3: Results .....		41
3.1	Colorant Properties of Semi-pure CASE in a panel of Commercial Food Products .....	41
3.2	Stability of Semi-pure CASE in a Model Sugar Drink .....	45
3.3	Effect of pH on Color of Semi-pure CASE.....	48
3.4	Principal Component Analysis (PCA) of Colored and Uncolored Avocado Seed Extracts.....	52
3.5	Structure Elucidation.....	59
Chapter 4: Discussion.....		73
Chapter 5: Conclusions and Future Work.....		76
REFERENCES.....		78
APPENDIX: SUPPLEMENTAL DATA.....		84

## LIST OF FIGURES

<b>Figure 1:</b> Some common yellow-red-orange natural colorants.....	8
<b>Figure 2:</b> Some pigments produced via polyphenol oxidase activity. ....	11
<b>Figure 3:</b> Some potentially bioactive compounds found in avocado seeds. ....	19
<b>Figure 4:</b> From left, photos show the inside, front, and top of a light box.....	36
<b>Figure 5:</b> Semi-pure CASE in white grapefruit juice, apple juice, and Sprite®. Concentration of semi-pure CASE used is shown above each sample in the units of mg/mL.....	41
<b>Figure 6:</b> $\Delta E$ values of semi-pure CASE in Sprite®, apple juice, and white grapefruit juice.	42
<b>Figure 7:</b> Semi-pure CASE in white cake. Photos show the tops (A) and middles (B) of the cupcakes. Concentration of CASE is shown in mg/mL. Baked samples were prepared in duplicate. ....	42
<b>Figure 8:</b> $\Delta E$ values of semi-pure CASE in cupcake tops and middles. ....	43
<b>Figure 9:</b> Semi-pure CASE was added to a white no-color-added cheese powder (blank). Warm milk was then added to the resulting samples in order to prepare a cheese sauce. Dry cheese powders are shown on the left (A), while the prepared cheese sauce is shown on the right (B). ....	44
<b>Figure 10:</b> $\Delta E$ values of CASE in a white no-color-added Kraft® cheese powder (left) and in prepared cheese sauce (right). $\Delta E$ of regular Kraft® cheese powder and prepared sauce were also calculated and appear as red lines on the graphs.....	45
<b>Figure 11:</b> Change in $\Delta E$ of light treated samples at 26 °C (A), and samples kept in the dark at 4 °C (B), 23 °C (C), and 40 °C (D). ....	46
<b>Figure 12:</b> CASE in model sugar drink samples on day 36 of the stability study. ....	47
<b>Figure 13:</b> Change in 445 nm absorbance of light treated samples at 26°C (A) and samples kept in the dark at 4°C (B), 23°C (C), and 40°C (D). ....	48
<b>Figure 14:</b> Pretreatment (A), base treated (B), and pH readjusted (C) semi-pure CASE samples compared to a control sample.....	49

<b>Figure 15:</b> Full LC profile of the semi-pure CASE in water control sample (top) and pH adjusted semi-pure CASE in water (bottom). The peak of interest, perseoranjin, appears at 15 min on both chromatograms. Line colors are pink, 280 nm; blue, 320 nm; green, 445 nm.....	50
<b>Figure 16:</b> LC profile and areas of maximum absorbance for perseoranjin peak in semi-pure CASE in water (top) and pH adjusted semi-pure CASE in water. Line colors are pink, 280 nm; blue, 320 nm; green, 445 nm. ....	51
<b>Figure 17:</b> PCA clustering scores for colored (solid line, C) and uncolored (dashed line, U) samples analyzed in positive mode. ....	52
<b>Figure 18:</b> PCA of colored and uncolored extracts in positive mode. ....	53
<b>Figure 19:</b> PCA of colored (solid line, C) and uncolored (dashed line, U) extracts in negative mode. ....	54
<b>Figure 20:</b> PCA of colored and uncolored extracts in negative mode. ....	55
<b>Figure 21:</b> Semi-pure, post-amberlite CASE. Samples were analyzed at 280 nm (top, black) and at 445 nm (bottom, red). ....	60
<b>Figure 22:</b> Post-C18 rough “F12” samples were analyzed at 280 nm (bottom, black) and at 445 nm (top, red). ....	60
<b>Figure 23:</b> UV-vis trace of persinoranjin shows maxima in the 400-480 nm range. ....	61
<b>Figure 24:</b> MS/MS analysis indicated an $[M+H]^+$ 603.1675 parent peak. ....	62
<b>Figure 25:</b> Analysis of perseoranjin included a $[M+H]^+$ 917.2639 peak (A), the compound of interest, $[M+H]^+$ 603.1675 peak (B), another $[M+H]^+$ 603.1687 peak (C), and $[M+H]^+$ 1205 dimer produced from the combination of two $[M+H]^+$ 603 compounds (D). ....	62
<b>Figure 26:</b> ATR-FTIR analysis of perseoranjin, the most prominent colored compound. ....	63
<b>Figure 27:</b> Structure of 2-(4-hydroxy-8-(2-((5-hydroxy-2-oxo-2,6,7,7a-tetrahydrobenzofuran-6-yl)oxy)ethyl)-5-oxo-6-((3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-yl)methoxy)-5H-benzo[7]annulen-3-yl)acetic acid, with the common name perseoranjin. ....	64



<b>Figure 28:</b> $^1\text{H}$ NMR of “F12” in $(\text{CD}_3)_2\text{SO}$ .....	67
<b>Figure 29:</b> $^{13}\text{C}$ NMR of perseoranjin in $(\text{CD}_3)_2\text{SO}$ .....	68
<b>Figure 30:</b> DEPT-edited HSQC of perseoranjin in $(\text{CD}_3)_2\text{SO}$ . ....	69
<b>Figure 31:</b> HMBC NMR of perseoranjin in $(\text{CD}_3)_2\text{SO}$ . Arrows on the structure indicate correlations. ....	70
<b>Figure 32:</b> COSY analysis of perseoranjin in $(\text{CD}_3)_2\text{SO}$ . ....	71
<b>Figure 33:</b> TOCSY analysis of perseoranjin in $(\text{CD}_3)_2\text{SO}$ .....	72
<b>Figure 34:</b> Some potential precursors for enzymatic synthesis of perseoranjin.....	75
<b>Figure 35:</b> $^1\text{H}$ NMR of “F12” in $\text{D}_2\text{O}$ . ....	86
<b>Figure 36:</b> $^{13}\text{C}$ of NMR perseoranjin in $\text{D}_2\text{O}$ . ....	87
<b>Figure 37:</b> DEPT-edited HSQC perseoranjin of in $\text{D}_2\text{O}$ . ....	88
<b>Figure 38:</b> HMBC of perseoranjin in $\text{D}_2\text{O}$ . ....	89
<b>Figure 39:</b> COSY analysis of perseoranjin in $\text{D}_2\text{O}$ . ....	90
<b>Figure 40:</b> TOCSY analysis of perseoranjin in $\text{D}_2\text{O}$ .....	91
<b>Figure 41:</b> NOESY analysis of perseoranjin in $\text{D}_2\text{O}$ .....	92
<b>Figure 42:</b> Effect of semi-pure CASE on viability of LNCaP cells. ....	93

## LIST OF TABLES

<b>Table 1:</b> Color additives subject to certification and permanently listed (unless otherwise indicated) for use in food. ....	5
<b>Table 2:</b> Structures and physical properties of some important benzotropolones.....	14
<b>Table 3:</b> Summary of current findings regarding the effects of avocado seed extracts. ....	21
<b>Table 4:</b> Sampling time points (days) on which each treatment group was sampled.....	35
<b>Table 5:</b> UV-Vis spectrometer data of the control and pH adjusted samples at 445 nm and 480 nm. ....	49
<b>Table 6:</b> Compounds found in colored and uncolored avocado seed extracts via principal component analysis. ....	56
<b>Table 7:</b> NMR assignments for perseoranjin in (CD <sub>3</sub> ) <sub>2</sub> SO. Asterisks indicate C-H connections confirmed by DEPT-edited-HSQC experiments.....	65

## ACKNOWLEDGEMENTS

I am deeply grateful to my adviser, Dr. Joshua Lambert, for giving me the opportunity to work in his laboratory and study for my Master of Science degree. His advisement and encouragement has been invaluable. I would also like to thank the members of my thesis committee, including Dr. Gregory Ziegler, Dr. Ryan Elias, and Dr. Emmanuel Hatzakis. Their guidance and suggestions for my experiment planning and product development has been invaluable. I would like to express my gratitude for Dr. Carlos Pacheco for his unending assistance and support throughout the course of my NMR experimentation. For his assistance with my metabolomics experiments I would like to thank Dr. Phillip Smith and the metabolomics research laboratory of Dr. Andrew Patterson .

Lastly I would like to thank the students and staff of the Food Science Department for their support and encouragement. In particular I would like to thank the undergraduate students Juan Mogoginta and Joshua Wilkins for their assistance with purifying extract, and I would like to thank my lab mates for their friendship and support during my studies.

This research was funded in part by a RAIN grant from the Pennsylvania State University College of Agriculture.

## **Chapter 1: Literature Review**

### **1.1 Color in Foods**

The global natural and synthetic food color market is estimated to reach US \$ 2.3 billion by 2019, with North America dominating the market, followed closely by Europe.<sup>1</sup> This figure reflects the ubiquitous application of added food colorants throughout the world. Though the use of added food colorants has continued to grow, consumers have become increasingly concerned by the perceived negative health risks which may be associated with artificial food colors. This change in consumer desire can be seen by the change in the global food colors market, as natural food colors have begun to dominate the market, increasing from 54.9% in 2014 to a predicted 60% by 2020, with particular interest in those compounds responsible for yellow, orange, red, and pink colors.<sup>2</sup> Though it seems consumers are beginning to show preference for natural food colors over those not found in nature, it cannot be overstated that being of natural origin (i.e. being produced by a living organism) does not signify that the consumption of such compounds is safe. In this review of food colors, the history and significance will first be reviewed, followed by a discussion of both artificial and natural colorants through the current day.

#### **1.1.1 Importance of Color in Foods**

The sense of sight has long played a critical role in both food choice and safety. Primates possess trichromatic vision, allowing them the ability to see a much larger range of colors than other mammals.<sup>3</sup> The development of trichromatic vision in primates is believed to evolutionarily coincide with their loss of many of their olfactory receptor genes, which could indicate the primate's heightened reliance on visual and auditory cues as opposed to their mammalian counterparts who rely heavily on the sense of smell.<sup>4</sup> One way in which those cues

have aided primates is the ability to visually observe color trends of colored plants and animals. Though vibrantly colored organisms appear beautiful, their conspicuous appearance can often indicate toxicity, unpalatability, or ability to escape predation.<sup>5</sup> This phenomenon is called aposematism or warning coloration. Several examples of aposematism can be seen in the red berry-like fruit of the yew (*Taxus spp.*, *Taxaceae*),<sup>6</sup> the green-yellow fluorescence of *Cortinarius orellanus* mushrooms,<sup>7</sup> and the great variety of colors found in poison dart frogs (Dendrobatidae), to name a few.<sup>8</sup> Some organisms such as day moths and butterflies (Lepidoptera) have evolved the ability to sequester toxic compounds from plants and store these compounds near the surface of their wings. There, those vibrantly colored compounds are visible to others and serve as a warning to potential predators.<sup>9</sup> Vibrant coloration is not exclusively linked to toxicity. Some edible organisms produce a variety of colors for reasons unrelated to aposematism, while some toxic organisms remain entirely neutral in color.

In humans, however, food color is irrefutably linked to their perception of food safety and flavor.<sup>10</sup> Johnson et al.<sup>11</sup> asked panelists to rate strawberry flavored drinks which had been sweetened with sucrose. The results showed a linear relationship between redness and perceived sweetness. Consumers rated the drinks with the most added red color as sweeter, more pleasant, and as having the most acceptable color. Another study by Johnson et al.<sup>12</sup> found that when accompanied by an increase in red color, the amount of sucrose in cherry flavored drinks could be reduced with no change in the taster's perception of sweetness. The researchers hypothesized that this reaction is related to the taster's association of the cherry-red color with optimal ripeness and sweetness in the fruit. The color-flavor relationship extends to more than just sweetness, or even flavor. A study by Pangborn and Hansen<sup>13</sup> found that in uncolored peach nectar samples, panelists were able to perceive changes in sweetness and sourness of samples,

but in colored samples no trend in those taste perceptions were found. Maga<sup>14</sup> found that green color increased panelists sensitivity to changes in sweetness, while yellow decreased sensitivity. Green and yellow both decreased panelists sensitivity to sourness, while red decreased sensitivity to bitterness. Color can have an effect on sensory properties other than taste. In a study by Rohm et al.<sup>15</sup> panelists were asked to rate the spreadability of two different butters samples, one of which was significantly more yellow than the other. When panelists rated the butters in a dark room under red light used to mask sample color, their ratings agreed with the spreadability ratings determined via cone penetration experiments. However, when panelists rated the same butters in plain daylight, they rated the more yellow butter as having higher spreadability.

Clearly color has a direct connection with human's sensory perception of foods. This can be both a help and a hindrance when it comes to marketing new food products. While vibrant novel colors catch the attention of consumers, it tends to only be helpful in the case of nondescript flavors, such as new flavors of soft drinks like Mountain Dew®.<sup>10</sup> However, when it comes to foods in which a color-flavor connection has already been established, novel colors are generally not received well by consumers. The production of Crystal Pepsi® in the 1990's is a classic example.<sup>16</sup> Marketed as a clear, cola flavored soft drink, Crystal Pepsi® was initially popular with consumers. It wasn't long after its release that consumers stopped buying Crystal Pepsi and returned to the more tradition, caramel colored sodas. These modern examples point towards the importance of food coloring, but adding coloring to food and pharmaceuticals is by new means a modern idea. Archaeologists believe that some of the earliest uses of food colorings took place around 1500 BCE, but literary writings such as Homer's Iliad and Pliny the Elder discuss the use of saffron in coloring foods, and the use of additives to color wines as early as 500 BCE. Ancient Egyptian writings tell of the use of added colorants in drugs.<sup>17</sup> Food colors are

still of great value in the pharmaceutical and organic food markets where added coloring is needed to control for the natural variation in color between batches of product. Color can be particularly important in pharmaceuticals when consumers associated certain colors with specific diseases and their treatments.<sup>18</sup> As added colors play such an important role in food and drugs, it is important to distinguish the differences between the meanings of artificial, natural, and synthetic colorants.

### **1.1.2 Artificial Food Colorants**

Artificial colorants are those pigments which have been fully discovered and synthesized in the laboratory, and are not of natural origin. In the past, known toxic substances such as copper sulfate, were used to color foods. In 1938, the Food & Drug and Cosmetic act (FD&C) produced a list of colorants allowed for use in foods, and required a higher purity standard for those compounds. Since then, artificial colorants have continued to gain popularity due to their increased stability under a variety of heat, light, time, and other storage conditions. Although there are standards as to what colors are allowable in food, the data on the long term effects of these compounds is limited, as is knowledge as to what dose is actually consumed by individuals on a regular basis. Still, the use of artificial colorants in the United States has increased 5 fold from 5 mg/capita/day in 1950 to 68 mg/capita/day in 2012.<sup>19</sup> Currently, the Food and Drug Administration's (FDA) website lists thirteen certified FD&C colors and lakes permanently listed for use in food (Table 1).<sup>20</sup> A lake is made by combining a dye with salts to prepare an insoluble material which can be dispersed into a food product. Lakes are particularly useful due to their ability to be dispersed in oils.

Although these artificial colorants are allowable for use in the United States, many of them have already been banned or required to carry a warning label in other countries due to

concern about their negative health risks. One of the foremost concerns is that of behavioral changes in children. Because of the many factors which influence a child's behavior, showing

FD&C Color	Common Name	Compound Type	Allowable Uses
FD&C Blue #1	Brilliant Blue	Triphenylmethane	Food, drugs, and cosmetics including cosmetics for eye area.
FD&C Blue #1 Aluminum Lake			Drugs and cosmetics for eye area.
FD&C Blue #2	Indigotine	Sulfonated indigo	Food and ingested drugs; sutures.
FD&C Blue #2 Aluminum Lake on alumina			Bone cement.
FD&C Green #3	Fast Green	Triphenylmethane	Food, drugs, and cosmetics, not for use in eye area.
FD&C Red #3	Erythrosine	Xanthene	Food and ingested drugs. May not be used in cosmetics, external drugs, and lakes.
FD&C Red #40 and its Aluminum Lake	Allura Red	Azo	Food, drugs, cosmetics, including drugs and cosmetics for eye area.
FD&C Yellow #5	Tartrazine	Azo	Food drugs, and cosmetics including drugs and cosmetics for eye area,
FD&C Yellow #5 Aluminum Lake			Drugs and cosmetics for eye area,
FD&C Yellow #6	Sunset Yellow	Azo	Food, drugs, and cosmetics; not for use in eye area,
Other FD&C Lakes			Provisionally listed. May be prepared from any of the above FD&C colors except FD&C Red #3.
Citrus Red #2	Citrus Red	Azo	Skins of mature oranges at $\leq 2$ ppm by wt. of whole fruit.
Orange B	Orange B	Pyrazolone	Surfaces and casings of frankfurters or sausages $\leq 150$ ppm by wt. of finished product.

**Table 1:** Color additives subject to certification and permanently listed (unless otherwise indicated) for use in food.

a correlation between artificial colorant intake and behavior change is particularly difficult. Two of the most influential studies citing this effect were conducted by Stevenson et al.<sup>21,22</sup> indicated



that artificial colors, sodium benzoate, or both increased hyperactivity in children aged 3, 8 and 9 years. Unfortunately it is difficult to determine what specifically was responsible for this result, as the foods and beverages consumed by the children contained the preservative sodium benzoate as well as a mixture of the artificial colors sunset yellow, carmoisine, tartrazine, and ponceau 4R.

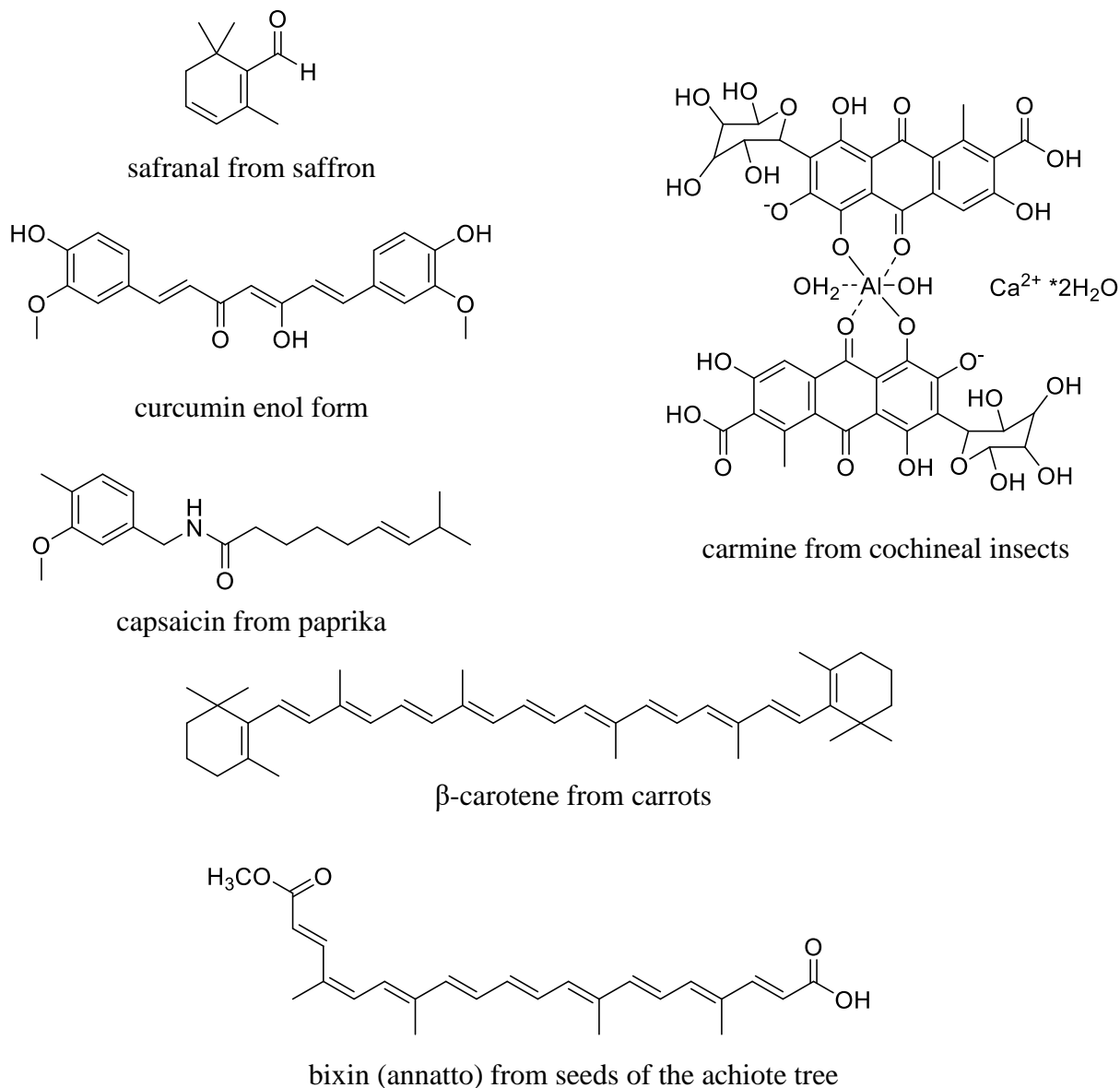
Allergic reactions specifically to FD&C yellow #5, or tartrazine, are another example of inconclusive data. Allergic or adverse reactions, such as urticaria (hives), eczema, angioedema, asthma and hyperactive behavior have been frequently reported. People with asthma or aspirin sensitivity are believed to be at a higher risk for adverse reactions to tartrazine and other azo type dyes, and adverse reactions are most often reported within these populations. This is of particular concern as many corticosteroids and antihistamines contain these colorants.<sup>23</sup> However, well controlled reviews and randomized, controlled trials indicate no apparent increase in risk to those with asthma or aspirin sensitivity.<sup>24</sup>

A multigenerational study of rats by Erickson et al.<sup>25</sup> attempted to determine whether there could be a synergistic interaction between the behavior changes caused by consumption of artificial colors (FD&C Red 40, FD&C Yellow 5, FD&C Yellow 6, and FD&C Blue 1) in the drinking water of juvenile and adolescent rats and behavior changes caused by multigenerational prenatal stress. The artificial colors induced the greatest effect during the time of exposure, while multigenerational stress produced lifelong behavior changes. The study showed few synergistic effects between the two causes of behavior changes. Further studies are needed to determine the effects of artificial food dyes in humans, particularly children. Parents should be particularly aware of the possible risk to their child and ensure that their children are not consuming more than the ADI of these artificial food colors.

### 1.1.3 Natural Food Colorants

A natural colorant can be defined as any pigment which is produced by any organism such as a plant, animal, fungi, or microorganism.<sup>26</sup> In its use in a food, a natural colorant can either be extracted from its natural source, such as in the case of safranal from saffron, or after discovery can be synthesized in a laboratory for use, as is commonly done with  $\beta$ -carotene found in carrots. Some common yellow-red-orange natural colors are shown below (Figure 1). The general perception of consumers is that natural food colorants are innately safer than their artificial counterparts. It is true that many natural colorants offer a variety of health benefits due to their antioxidant properties. However, the dose of any compound to be consumed must always be taken into consideration. As an example,  $\beta$ -carotene is found in many fruits and vegetables and is converted to vitamin A within the body. When consumed in foods, it is difficult to overdose on  $\beta$ -carotene as the body will only convert as much as needed to vitamin A.<sup>27</sup> However, when taken in high doses  $\beta$ -carotene can increase the risk of lung cancer and heart disease in people who are heavy smokers or drinkers.<sup>28</sup> When natural products are isolated and used as food additives, consumers may be at risk of consuming unsafe doses.

Carmine has gained much attention in recent years when consumers discovered that products containing this cochineal insect derived food colorant were being labeled as vegetarian. Carmine has been known to cause asthma and respiratory distress in people who commonly work with it, such as butchers or factory workers.<sup>29,30</sup> Another common natural food colorant is annatto, found in the seeds of the achiote tree. In a study by Hagiwara et al.<sup>31</sup> groups of 10 female and 10 male Sprague-Dawley rats were fed annatto extract in their diet at levels of 0, 0.1, 0.3, and 0.9% for thirteen weeks. No negative effects were observed in terms of body weight, food and water consumption, ophthalmology or hematology data. Blood chemistry results



**Figure 1:** Some common yellow-red-orange natural colorants.

revealed that both males and females in the 0.3 and 0.9% groups experienced changes including increased alkaline phosphatase, phospholipid, total protein, albumin, and albumin globulin ratio, as well as marked increases in absolute and relative liver weight. Further study showed hepatocyte hypertrophy due to abundant mitochondria. The No Observed Adverse Effect Level (NOAEL) was determined to be a dietary level of 0.1% (69 mg/kg bw/day for males, 76 mg/kg

bw/day for females). Because high-dose consumption of many natural colorants has not been studied, caution must be used when replacing artificial colorants with natural colorants. More studies are needed to determine the safety of natural colorants, particularly in the high-risk population such as children, the elderly, and those with certain illnesses.

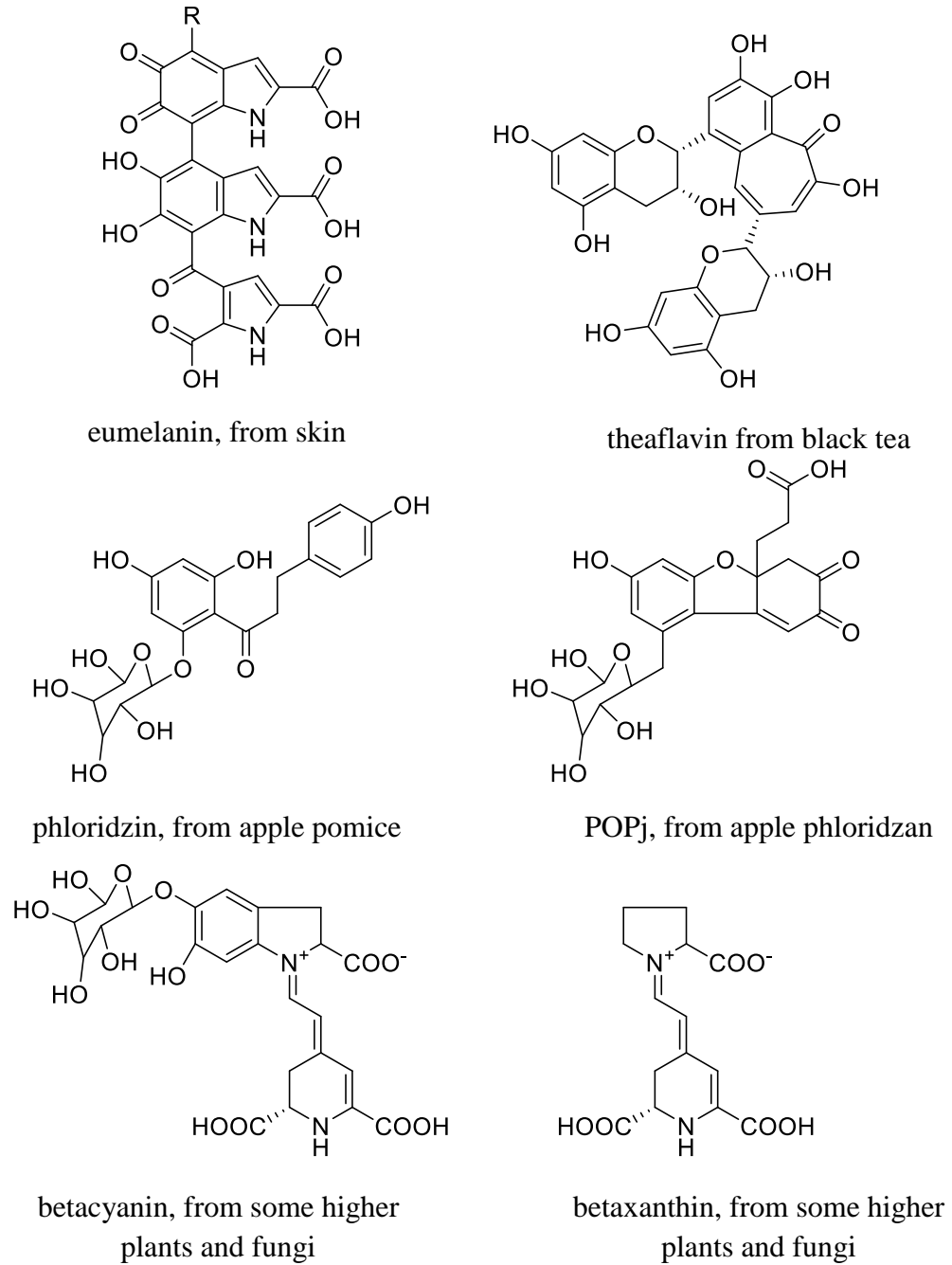
The time and money needed to take a natural colorant from its discovery in nature to it being FDA approved for use in foods can be a hindrance to those interested in adding new natural colorants to the market. Of course, as with any food additive, the main concern is for the safety of consumers. Once an interested party has a colorant they would like to have approved by the FDA, they can petition the FDA to review their product for use in foods. According to the FDA website, in addition to safety and toxicological data a petition must include information about the compound including identity of the proposed color additive, physical, chemical, and biological properties, chemical specifications, manufacturing process description, stability data, intended uses and restrictions, labeling information, analytical methods for enforcing chemical specifications, analytical methods for determination of the color additive in products, identification and determination of any substance formed in or on products because of the use of the color additive, proposed exemption from batch certification, an environmental assessment or claim for categorical exclusion, and an estimate of probably exposure.<sup>32</sup> Preparing data for a new colorant petition to the FDA could take years and become an expensive process. For this reason addition of new natural colorants to the market may be a slow process.

## **1.2 Role of Polyphenol Oxidase in Pigment Formation**

Polyphenol oxidases (PPO) are enzymes (EC 1.14.18.1) found almost universally in all varieties of organisms including bacteria, insects, crustaceans, mammals, fungi, and plants.<sup>33</sup>

They are divided into the two subclasses of tyrosinases and laccases.<sup>34</sup> PPO contributes to the production of the brown pigment melanin in mammals and is responsible for the browning which occurs in plants when the flesh of a fruit or vegetable is sliced or bruised in the presence of oxygen. PPO has a dinuclear copper active site which exerts these effects through the ability to bind an external diphenol molecule and oxidize it to an O-quinone which is released with a water molecule.<sup>35</sup> These reactive O-quinones are then converted to red, brown, and black pigments via non-enzymatic pathways.<sup>36</sup> These pigments are usually viewed as undesirable as they contribute to major economic losses as fruits and vegetables undergo browning during aging or damage after harvest. There are some situations in which PPO contributes to the production of the desirable, characteristic pigments attributed to some foods. Several pigments are pictured in Figure 2. In the case of black teas, PPO is responsible for production of red-brown pigments formed during fermentation called theaflavins and thearubigins.<sup>37</sup> PPO is also responsible for the formation of the yellow-orange color which occurs in apple cider.<sup>38</sup>

Due to the increasing interest in natural colorants, there is now much focus turning to the production of these colorants. A review article by Ridgway et al.<sup>39</sup> discussed the use of PPO for the production of “designer antioxidant and colourant flavonoids.” A paper by Guyot et al.<sup>40</sup>



**Figure 2:** Some pigments produced via polyphenol oxidase activity.

did just that by using mushroom PPO to convert phloridzin, a common compound found in apple pomice, to a yellow pigment (POPj) which they recommend could be used as a potential natural replacement for tartrazin. Another group of PPO produced pigments are the red and yellow

betalains. Betalains are produced only in higher fungi, and in higher plants of the order Caryophyllales which includes members such as cacti, carnations, amaranths, and beets. Interestingly, betalains seem to replace anthocyanins in these plants, and the two compounds are not produced simultaneously in the same plants. In 2013, Nakatsuka et al.<sup>41</sup> were able to overcome this barrier through the combined expressions of a tyrosinase gene from shiitake mushroom and a DOPA 4,5-dioxygenase gene from the four-o'clock plant, resulting in betalain production in cultured cells of tobacco BY2 and *Arabidopsis* T87. These examples demonstrate just a few of the many ways PPO can be used in the production of natural pigments, *in vitro*, *in vivo*, and as well as through the use of natural products in the laboratory.

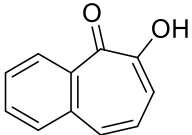
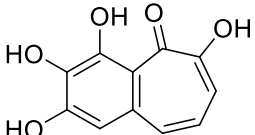
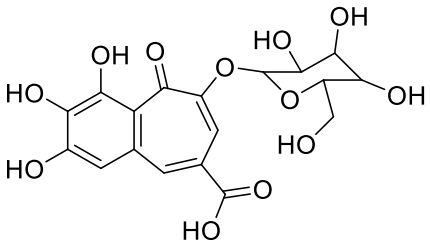
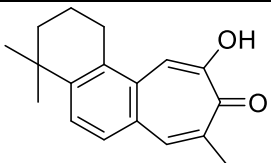
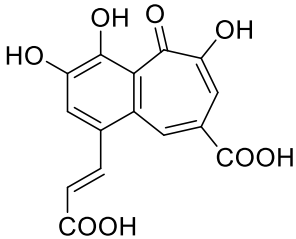
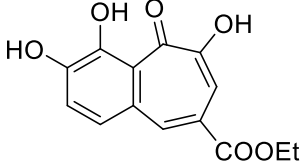
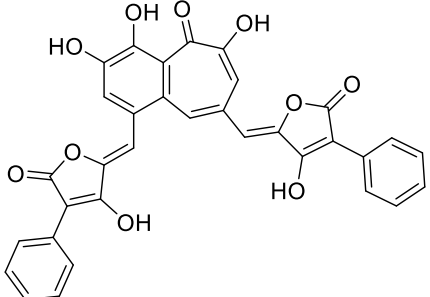
### 1.3 Benzotropolone Compounds

One particular class of pigmented compounds is benzotropolones. Benzotropolones are characterized by a seven-membered tropolone ring attached to a six-membered aromatic ring (Table 2) and have been found throughout nature in mushrooms, black teas, Chinese sage, and *Mesotaenium berggrenii*, an extremophyte living on glaciers.<sup>42-45</sup> Benzotropolones are generally yellow, orange, red, or brown in color, although one instance of a “dark solid with green metallic luster” was observed in the case of aurantricholine. Upon addition of base, aurantricholine changed irreversibly to green-black, while upon addition of acid it produced yellow compounds of undetermined structure.<sup>46</sup> Benzotropolone-glycosides tend to have low solubility in organic solvents and may only be easily dissolved in water, making structure elucidation complex. Another common property of some is that they may be unstable, even at low temperatures or upon standing in organic solvents. Benzotropolones have been reported to have health beneficial properties due to their antioxidant nature. For example, theaflavins (Figure 2), and their polymerized form, thearubigins, have been reported to aid in weight loss and metabolic

syndrome due their ability to decrease appetite, reduce adipose tissue, increase metabolism and energy levels and protect and enhance lean body mass.<sup>47,48</sup> Theaflavins have also been shown to be useful in the treatment of alcoholic liver diseases.<sup>49</sup> As the desire for natural alternatives to artificial colorants continues to grow, more research will be needed on the potential positive and negative health effects of these and other benzotropolones.



**Table 2:** Structures and physical properties of some important benzotropolones.

Structure	Name	Origin	Properties	Ref.
	Benzotropolone moiety	-	-	-
	Purpurogallin	Tinder fungus ( <i>Fomes fomentarius</i> ), cabbage	Red crystalline solid; soluble in DMSO, not in water.	50-52
	purpurogallin carboxylic acid-6-O-beta-D-glucopyranoside	<i>Mesotaenium berggrenii</i> (Zygnematophyceae, Chlorophyta), an extremophyte living on glaciers	Brown vacuolar solid; hardly soluble in organics.	45
	Salvione	Chinese sage ( <i>Salvia miltiorrhiza</i> )	Cytotoxicity in vero cells.	43
	Crociodin	Bolete fungus ( <i>Leccinum crociodium</i> )	Orange-red solid.	44
	Goupiolone A	Kabukalli tree ( <i>Goupia grabra</i> )	Genotoxic, orange solid.	53
	Aurantricholine	Golden orange tricholoma mushroom ( <i>Tricholoma aurantium</i> )	Fluorescent, orange-red solid; unstable, decomposes even on standing in organics.	46

#### 1.4 Avocado (*Persea americana*) Seed as a Source of Bioactive Phytochemicals<sup>A</sup>

The avocado (*Persea americana* Mill. Lauraceae) is a large drupe and has the highest oil content of all fruits, with the possible exception of the olive. The avocado's status as a food varies with the region where it is consumed and the degree of familiarity with which it is regarded by the local populace. The fruit is a traditional dietary staple in Guatemala and nearby countries. It is also an ingredient of widely consumed, elaborately prepared traditional foods in Mexico such as guacamole, which is composed of pureed avocado pulp and other vegetables.<sup>54</sup> The archaeological record reveals them to be one of the oldest food plants of Mexico (8000 BCE).<sup>55</sup> Avocados are endemic to the tropical regions of the New World but are now cultivated all throughout the world. The avocado is a member of the commercially important plant family, Lauraceae, and has eight well-defined geographical eco- types, three of which (so called horticultural races) comprise the commercial avocado crop. These horticultural races are the Mexican (*P. americana* var. *drymifolia*), the West Indian (*P. americana* var. *americana*) and the Guatemalan (*P. nubigena* var. *nubigena* and *P. nubigena* var. *guatemalensis*)<sup>55</sup>. Current commercial varieties of avocado are hybrids of these races. For example the Hass variety belongs to the Guatemalan-Mexican hybrid group.<sup>56</sup> Other common varieties in commerce include the Bacon, Feurte, Gwen, Lamb Hass, Pinkerton, Reed and Zutano.<sup>57</sup> The Hass variety is commonly grown in Mexico and California and is one of the major varieties in commerce, particularly in North America.<sup>58</sup> The fruits of the Hass variety are pear to ovoid shaped and the skin is tough, leathery and dark brown or black when ripe. It also contains a smaller seed than most other common varieties.<sup>59</sup>

---

<sup>A</sup> Section 1.4 has been published as a review: Dabas, D.; Shegog, R.M.; Lambert, J.D.; Ziegler, G.R. Avocado (*Persea americana*) Seed as a source of Bioactive Phytochemicals. *Curr. Pharm. Des.* **2013**, *19*(34), 6133-40.

Total U.S. avocado production during the 2007/08 season was 170,000 tons: total world production in 2007/08 was 1.4 million tons with the largest producer being Mexico.<sup>54</sup> The European Union and North America are the largest importers of avocados in the world.<sup>56</sup> To date, most chemical and bioactivity studies have focused on the pulp (mesocarp) of the avocado. Avocado pulp contains up to 30% oil which is rich in monounsaturated fatty acids. These fatty acids have been proposed to have beneficial effects with respect to cardiovascular disease risk factors.<sup>60,61</sup> The pulp also contains several bioactive phytochemicals including carotenoids (lutein, zeaxanthin, carotene, cryptoxanthin), B vitamins, vitamins C and E, D-mannoheptulose,  $\beta$ -sitosterol, and persenone A and B. These constituents have demonstrated antifungal, antitumor and antioxidant activities.<sup>62</sup> Avocado pulp has been found to have *in vitro* growth inhibitory effects against multiple cancer cell lines. Lutein, tocopherols, and other carotenoids are proposed as the active compounds.<sup>63</sup> Avocado pulp is also well known for its beneficial skin properties. The unsaponifiable fraction of avocado pulp in combination with soybean oil unsaponifiable is used for treatment of osteoarthritis, and new research shows its anti-carcinogenic and anti-inflammatory effects.<sup>57,62,64-66</sup> Available studies will be reviewed in order to provide insight into potential directions for future studies.

#### **1.4.1 Ethnobotanical and Historical use of Avocado Seed**

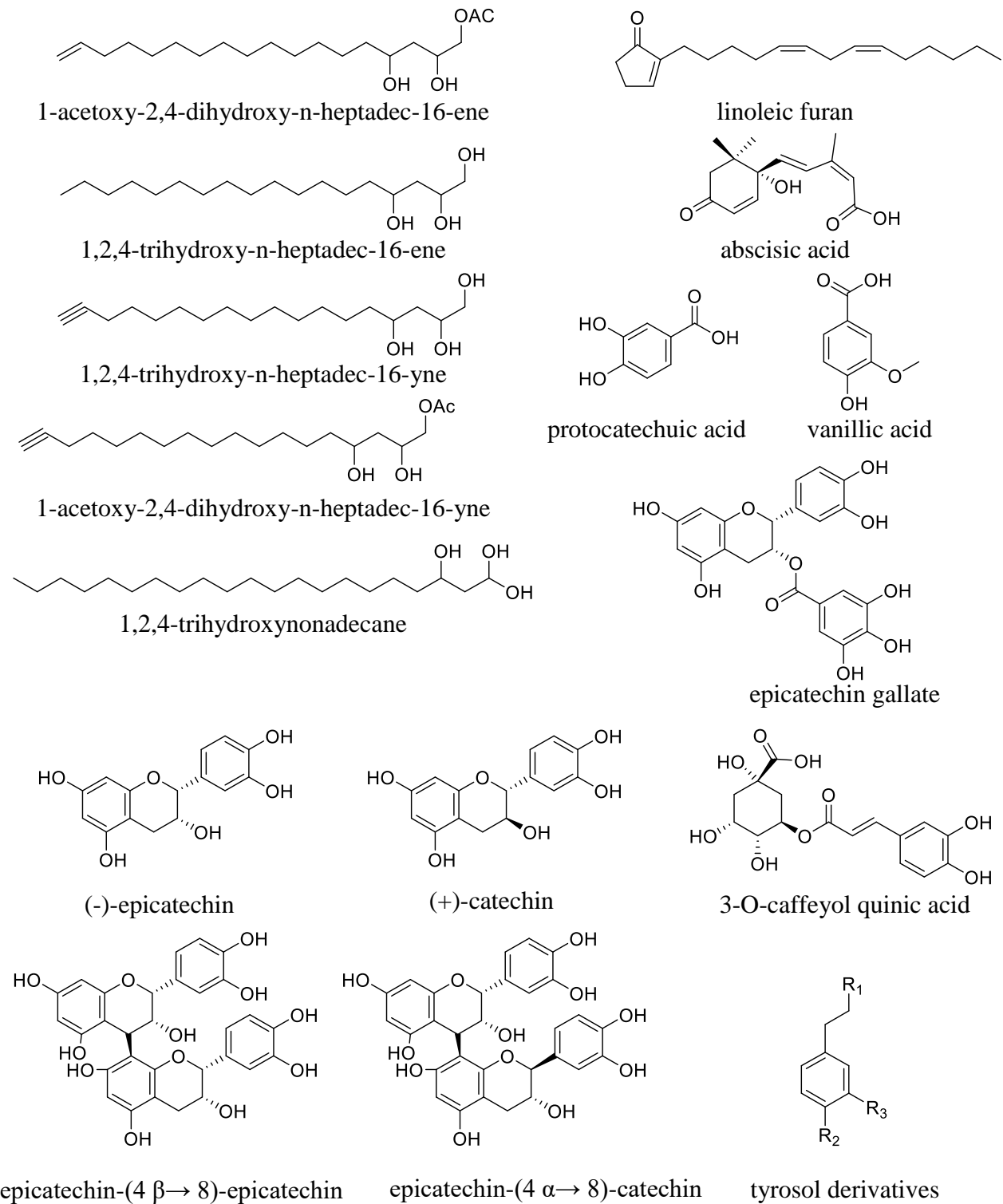
Ethnopharmacological studies of the Aztec and Mayan cultures showed the use of decoctions of avocado seeds for the treatment of mycotic and parasitic infections.<sup>67</sup> Seed preparations have been reported for use in cases of diabetes, gastrointestinal irregularity, and for its anti-anemic and diuretic effects.<sup>68</sup> A piece of the seed or the decoction put into a tooth cavity is reported to relieve toothache. A powder prepared from avocado seed is believed to

cure dandruff and the seed oil is sometimes applied to skin eruptions.<sup>59</sup> Topical application of avocado seed paste has been used for treatment of arthritis. In Nigeria, it is mixed with soups and puddings because it is considered to be effective for management of hypertension.<sup>69</sup> The seed was also used to treat snakebite. Crushed seed has been used as a contraceptive and as an abortive agent.<sup>68</sup> In African tradition, the ground seeds were consumed to treat whitlows and dysentery.<sup>70</sup> Historically, colored exudate from avocado seeds was used as indelible ink by the *Conquistadors* in the 1500s.<sup>59</sup>

#### **1.4.2 Composition and Chemical Components of Avocado Seeds**

The avocado seed represents up to 16% of the total weight of the fruit, has a rich phytochemical profile and a long history of ethnobotanical use.<sup>71,72</sup> The proximate composition of the seeds of two varieties of avocado, Hass and Feurte, was determined as follows: moisture content, 54.1% and 50.2%; ash content, 1.2% and 1.3%; protein, 2.4% and 2.5%; sugars, 3.5% and 2.2%; starch, 27.5% and 29.6 %; and fat 0.8 % and 1.0%, respectively.<sup>73</sup> Seeds of both varieties had lower fat content than the corresponding pulp. The lipid and fatty acid composition of the pulp and seed of the Feurte, Bacon, and Hass varieties were investigated and the total lipid content of the seeds has been determined as 1.1, 1.6 and 1.1%, respectively. The majority of lipids in the pulp were neutral lipids (95 – 99%) with a smaller amount of glycolipids (2.5 – 3.2%) and phospholipids (0.7 – 2.1%). By contrast in the seed, the percentage of neutral lipids was (77 – 80%) and the percentage of the other two fractions – glycolipids (12 – 13%) and phospholipids (7.4 – 10.9%) was higher. The neutral lipids were largely monoacylglycerols, diacylglycerols, free sterols and free fatty acids with a lesser amount of triacylglycerols in the neutral lipids of seeds than pulp.<sup>74</sup> Phytochemical studies on avocado seeds have identified various classes of natural products including

phytosterols, triterpenes, fatty acids, furanoic acids, abscisic acid, proanthocyanidins (PAC)s and other polyphenols (Figure 3).<sup>57,73</sup> The levels of these compounds in the seeds vary with the variety of avocado, conditions of growth and stage of maturity. Measured levels are also influenced by the method of extraction employed in the experiment. Soong and Barlow<sup>75</sup> determined the phenolic compound levels in the seed and pulp to be 88.2 and 1.3 mg/g gallic acid equivalents (GAE) respectively. Wang<sup>76</sup> determined the phenolics in the seed of Hass variety to be 51.6 mg/g GAE. By comparison, the phenolic compound content for the pulp was 4.9 mg/g GAE. The content of PACs in the Hass variety was found to be 47.7 mg/g. Phenolic content was found to be 137 g/mg tannic acid in seed.<sup>77</sup> Total phenolic content of ethyl acetate, acetone and methanolic extracts of seed were evaluated with the acetone extract containing the highest concentration of phenolic compounds (6082 mg GAE/g) followed by methanol and ethyl acetate extracts.<sup>78</sup> Catechin, epicatechin, and A and B type PACs (degree of polymerization = 2 – 6) and leucoanthocyanidins (3, 4-flavandiols) were identified as the major phenolic compounds in avocado seeds.<sup>75,79,80</sup> Epicatechin-(4 $\alpha$ →8)-catechin, epicatechin-(4 $\beta$ →8)-epicatechin, and the A-type trimers, A<sub>2</sub>-(+)-catechin and A<sub>2</sub>-(-)-epicatechin have been reported in seed (Figure 3).<sup>71</sup> 3-O-caffeyol quinic acid (57.5  $\mu$ g/g DW), 3-O-coumaroyl quinic acid (13.6  $\mu$ g/g DW), procyanidin trimer A (170  $\mu$ g/g DW), catechin/epicatechin-gallate (152  $\mu$ g/g DW) were isolated from the methanol extracts of Hass avocado seeds (Figure 3).<sup>81</sup> The Pahua-Ramos group reported that protocatechuic acid, kaempferide and vanillic acid are also present in the avocado seed (Figure 3).<sup>82</sup>



**Figure 3:** Some potentially bioactive compounds found in avocado seeds.

### **1.4.3 Biological Activity**

Despite its long history or ethnobotanical uses, the avocado seed is largely considered a waste product and is an underutilized resource. Consumption of the seed has not been widely reported in modern times nor have safe intake levels been determined. Modern scientific research into the potential bioactivities of avocado seeds remains in the early stages. Existing studies show a variety of health beneficial effects. Consumption of the seed showed anti-hypertensive, anti-diabetic, and hypo-cholesterolemic effects while extracts of seed, skin, and flesh showed anti-cancer and anti-inflammatory effects when consumed. As the seed also contains a variety polyunsaturated fatty acids which have been shown to have beneficial effects when used topically on the skin and hair. Finally, the seed has been shown to have antimicrobial and insecticidal effects and is currently used in a Ugandan anti-malarial drink called Artavol which reduced malarial related fevers and hospital visits in 80% of subjects over an 8 month period.<sup>83</sup> Current knowledge on the biological activities of avocado seed extracts is summarized in Table 3.

<b>Effect</b>	<b>Model</b>	<b>Extract/dose</b>	<b>Main Effect</b>	<b>Reference</b>
<b>Anticancer</b>	MDA-MB-231 breast cancer cells	Methanolic extract at concentrations greater than 100 µg/mL	Induction of apoptosis measured by increased caspase-3, caspase-7 and poly (ADP ribose) polymerase (PARP) cleavage.	Lee et al. 2008 <sup>77</sup>
<b>Anti-inflammatory</b>	Secretory PLA <sub>2</sub>	Lipidic polyols	Inhibit activity of PLA <sub>2</sub> .	Etozioni 2003 <sup>84</sup>
	Keratinocytes <i>in vitro</i>	PFA isolated from pulp and seed	Decreased generation of pro-inflammatory mediators IL-6, PGE <sub>2</sub> , and cyclobutane pyrimidine dimers after exposure to UVB radiation.	Rosenblat et al. 2010 <sup>85</sup>
<b>Antidiabetic</b>	Alloxan-induced diabetic rats	450 and 900 mg/kg bw seed extract treatment for 14 d	Reduced blood glucose levels by 47-55%. Histological study suggested restorative effect on pancreatic islets.	Edem 2009 <sup>86</sup>
	Alloxan-induced diabetic rats, non-diabetic rats	300 and 600 mg/kg bw aqueous seed extract for 21 d	Reduced plasma glucose concentrations by 73% and 78% respectively in diabetic rats. Plasma glucose concentrations of non-diabetic rats were reduced by 35-39%.	Edem, Ekanem, and Ebong 2009 <sup>87</sup>
<b>Antihypertensive</b>	NaCl induced hypertensive rats	200-700 mg/kg bw aqueous seed extract for 4 wk	Reduced blood pressure at all doses; reduced plasma TG, TC, and LDL at doses at 500 mg/kg bw and above.	Imafidon and Amaechina 2010 <sup>88</sup>
	NaCl hypertensive rats	200-700 mg/kg bw aqueous seed extract for 5 wk	Dose-dependently reduced blood pressure, cholesterol, glucose, urea and sodium levels.	Kate and Lucky 2009 <sup>89</sup>

**Table 3:** Summary of current findings regarding the effects of avocado seed extracts.



<b>Antihypertensive cont.</b>	Acetylcholine-induced hypertensive rats	260 mg/kg aqueous seed extract for 10 d	39.3-43.5% reduction in mean arterial pressure compared to baseline; heart rate reduced by 9.4-19.8%	Anaka, Ozolua, and Okpo 2009 <sup>90</sup>
<b>Hypo-cholesterolemic</b>	Mice	125, 250, and 500 mg ASF/kg bw dried avocado seed "flour" for 6 d	Significant reduction in TC and LDL-C.	Pahua-Ramos et al. 2012 <sup>82</sup>
	Cholesterol induced hypercholesterolemic mice	50-300 mg/kg bw methanolic seed extract for 10 d	Dose-dependent reduction in TC, TG, LDL-C, and VLDL-C	Asaolu 2010 <sup>91</sup>
	Rabbits	100 and 200 mg/kg bw aqueous seed extract for 2 mos	Dose-dependent reduction in TC, TG, LDL, and total lipids in the plasma	Nwaoguikpe and Braide 2011 <sup>92</sup>
<b>Dermatological uses</b>	Keratinocytes in vitro	Up to 5 µg/mL PFA derived from hexane seed extract	Increased cell viability; reduction in number of sunburned cells after irradiation; enhanced DNA repair	Rosenblat et al. 2010 <sup>85</sup>
	Keratinocytes in vitro	10 µg/mL water-ethyl acetate seed extract and HSCCC fraction of the seed	Proliferation and cell viability	Ramos-Jerz, Villanueva, and Deters 2007 <sup>71</sup>
	Human subjects	5-10% unsaponifiable fraction of avocado seed lipids applied to skin for 2-6 wk	Reduction of stretch marks, keratosis, redness, hypopigmentation, hyperpigmentation and ridging; increased skin moisture retention and skin elasticity; increased skin thickness.	Moy 1999 <sup>93</sup>

**Table 3 continued.**

<b>Dermatological uses continued</b>	In vitro rat skins and tibias of 17 day old chick embryos	Unsaponifiable component of avocado seed oil	Inhibited action of lysyl oxidase, which can lead to wrinkles and lack of skin elasticity	Werman, Mokady, and Neeman 1990 <sup>94</sup>
	Hairless mice	3% natural or synthetic isolated linoleic furan from seed extract	Enhanced metabolic activity in dermal and epidermal tissues leading to increased elasticity, protein content, DNA content, skin thickness, fibroblast population, collagen synthesis, and glucose metabolism.	Counts and Hube 1996 <sup>95</sup>
	Human subjects	Avocado seed soaked in mineral oil for 4 wk then applied to the scalp	Prevents frying and falling of hair	Ruiseco 1989 <sup>96</sup>
<b>Antimicrobial</b>	Select gram positive and gram negative bacteria in vitro	125-250 µg/mL ethanolic seed extract	Antimicrobial effects shown against Salmonella enteritidis, Citrobacter freundii, Pseudomonas aeruginosa, and Enterobacter aerogenes.	Raymond, Chia and Dykes 2010 <sup>97</sup>
	Candida spp, Cryptococcus neoformans, and Malassezia pachydermatis in vitro	Methanolic seed extract at 0.125-0.625 mg/mL, 0.08-0.156 mg/mL, and 0.312-0.625 mg/mL, respectively	Fungicidal effects	Leite et al. 2009 <sup>98</sup>

**Table 3 continued.**

<b>Effect</b>	<b>Model</b>	<b>Extract/dose</b>	<b>Main Effect</b>	<b>Reference</b>
<b>Antimicrobial continued</b>	<i>S. aureus</i> and <i>B. subtilis in vitro</i>	Crude terpenoid and alkaloid fractions from solvent seed extraction	Growth inhibition	Nagaraj et al. 2010 <sup>99</sup>
<b>Insecticidal</b>	<i>Aede aegypti</i>	16.7 mg/mL hexane seed extract; 8.9 mg/mL methanol extract	LC <sub>50</sub> against <i>Aedes aegypti</i> larvae (yellow fever vector)	Leite et al. 2009 <sup>98</sup>
	<i>Trypanosoma cruzi in vitro</i>	>500 µg/mL methanolic extract	Moderate activity against mobilization of epimastigotes and trypomastigotes of <i>Trypanosoma cruzi</i>	Abe et al. 2005 <sup>100</sup>
<b>Toxicity</b>	Rats	2-10 g/kg aqueous extract administered as single dose	Showed no signs of toxicity in two wk following gavage	Ozolua et al. 2009 <sup>90</sup>
	Rats	2.5 g/kg bw for 28 d	No gross or hematological changes observed	Michaelakis et al. 2009 <sup>101</sup>
	Mice	1767 mg/kg bw	Determined to be LD <sub>50</sub>	Ramos et al. 2012 <sup>82</sup>

**Table 3 continued.**

#### **1.4.3.1 Antioxidant Activity of Avocado Seeds**

Avocado seeds have been found to have higher antioxidant activity than the pulp and this activity has been attributed to the high content of the phenolic compounds. The antioxidant capacity of avocado seeds has been studied mainly using electron transfer-based assays. Soong and Barlow found that an ethanol:water (1:1, v/v) extract of the avocado seed contained 1160 µmol/g and 1484 µmol/g ascorbic acid equivalent antioxidant capacity

(AEAC) scavenging assay and the ferric reducing antioxidant power assay, respectively. These values were 55 and 155 times greater than that of the pulp, respectively.

The antioxidant activity of the ethyl acetate, acetone and methanol extracts of the avocado seed were compared using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay: the acetone extract had the highest antioxidant activity (158.3 mmol Trolox equivalent (TE)/g fresh weight).<sup>78</sup> The radical scavenging capacity of 20 µg/mL of avocado seed methanolic extract using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was comparable to 15 µM Trolox. Nagaraj prepared alkaloid and terpenoid rich extracts of avocado seeds and determined radical scavenging activity using the DPPH assay.<sup>99</sup> The terpenoid extract had higher antioxidant activity (4.9 µg/mL AEAC) than the alkaloid fraction (3.4 µg/mL AEAC). The antioxidant activity of the seed extract has also been evaluated using the oxygen radical absorbance capacity assay, a hydrogen atom transfer-based reaction. The seed extract was found to contain 428.8 TE/g, whereas the pulp contained only 11.6 TE/g.<sup>76</sup> Total antioxidant levels were higher in the peel and seed tissues than in the pulp. The antioxidant activity of seeds was due to the presence of ascorbic acid and phenolic compounds, whereas D-mannoheptulose was found to be responsible for the antioxidant activity of the pulp.<sup>102</sup>

#### **1.4.3.2 Anticancer Effects**

To date, only one paper has reported the anticancer effects of extracts of the avocado seed and peel. Treatment of MDA-MB-231 human breast cancer cells with the methanolic extract of avocado seed (100 µg/mL) led to induction of apoptosis as measured by increased caspase-3, caspase-7 and poly (ADP ribose) polymerase cleavage. DNA laddering was also observed. The peel extract showed similar, but much more potent effects. This observed

differences in anticancer effects correlated with higher total phenolic compound levels and higher total flavonoid levels in the peel than in the seed<sup>77</sup>. Further studies are required to determine the spectrum of anticancer activity of the avocado seed methanolic extract, the active compounds, and whether anticancer activity is observed *in vivo*.

#### **1.4.3.3 Anti-inflammatory Effects**

Arachidonic acid is the precursor to a large family of compounds called eicosanoids which include the cyclo-oxygenase derived prostaglandins and lipoxygenase-derived leukotrienes. Eicosanoids play important physiologic and pathologic functions including mediation of inflammation. The enzyme Phospholipase A2 (PLA<sub>2</sub>) releases arachidonic acid from membrane phospholipids from the *sn*-2 position and thus plays an important role in inflammation.<sup>103</sup> Lipidic polyols purified from avocado seeds were found to inhibit activity of secretory PLA<sub>2</sub>. The active compounds were identified as 1-acetoxy-2,4-dihydroxy-n-heptadec-16-ene (olefin A), 1,2,4-trihydroxy-n-heptadec-16-ene (olefin B), 1-acetoxy-2,4-dihydroxy-n-heptadec-16-yne (acetylene A) and 1,2,4-trihydroxy-n-heptadec-16-yne (acetylene B) (Figure 3). Compared to the control, almost complete inhibition was observed after treatment with 12.2 mM of olefin A, 7.5 mM of acetylene A, 0.17 mM olefin B and 0.08 mM of acetylene B<sup>84</sup>. Although these findings are interesting, they must be interpreted with care since the effective concentrations are quite high. Treatment of keratinocytes (1 µg/mL for 60 min) with polyhydroxylated fatty alcohols (PFA) isolated from seed and pulp prior to exposure to ultra violet (UV) B radiation resulted in decreased generation of pro-inflammatory mediators including interleukin (IL)-6 and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), as well as reduced formation of cyclobutane pyrimidine dimers. In human skin explants, treatment with

PFA significantly reduced UV-induced cellular damage. The major constituents were identified as olefin A and acetylene B (Figure 3).<sup>85</sup>

#### **1.4.3.4 Antidiabetic Effects**

Avocado seed extracts have been shown in laboratory models to reduce blood glucose levels and ameliorate diabetes. Treatment of alloxan-induced diabetic rats with an ethanolic seed extract (450 or 900 mg/kg bw) for 14 d reduced blood glucose levels by 47 – 55%. Histological examination of the pancreas of treated rats suggested that the extract protected pancreatic islet cells.<sup>86</sup> Edem *et al.*<sup>87</sup> found that treatment with an aqueous seed extract (300 or 600 mg/kg bw) for 21 d reduced plasma glucose concentrations by 73 and 78% respectively in alloxan-induced diabetic rats. The glucose levels of non-diabetic rats were also reduced by 35 – 39% suggesting that the effects are not specific to the disease model. The authors hypothesized that the hypoglycemic effects are due to the presence of insulin-mimetic substances in the extract which stimulates production of insulin by cells or enhances glucose utilization. This mechanism remains to be further studied.

#### **1.4.3.5 Antihypertensive Effects**

Avocado seed extract is used traditionally in Nigeria for the treatment of hypertension.<sup>88</sup> An aqueous seed extract from Fuerte avocados (200 - 700 mg/kg bw) significantly reduced the blood pressure in NaCl-induced hypertensive rats after 4 wk of treatment. A reduction in plasma triglycerides (TG), total serum cholesterol (TC) and serum low density lipoprotein-associated cholesterol (LDL-C) levels was also observed at doses of 500 mg/kg bw and higher.<sup>88</sup> In a similar study 200 – 700 mg/ kg bw aqueous avocado seed extract dose-dependently reduced blood pressure, cholesterol, glucose, urea and sodium levels in hypertensive rats after 5 wk.<sup>89</sup> Acute treatment of acetylcholine-induced hypertensive rats

with aqueous seed extract (260 mg/kg bw) for 10 d led to a reduction in mean arterial pressure (39 – 44% reduction) and heart rate (9.4-19.8% reduction).<sup>90</sup>

#### **1.4.3.6 Cholesterol-lowering Effects**

Recent research has suggested that avocado seeds can reduce cholesterol in laboratory animal models. Avocado seed flour (125, 250 and 500 mg/kg bw) significantly reduced TC and LDL-C in mice after 6 d.<sup>82</sup> In another study, a dose-dependent reduction in TC, TG, LDL-C and very low density lipoprotein-associated cholesterol in hypercholesterolemic rats after treatment with a methanolic extract of avocado seed (50 – 300 mg/kg bw) for 10 d.<sup>91</sup> Nwaoguikpe and Braide<sup>92</sup> observed similar effects in rabbits after treatment with an aqueous seed extract (100 and 200 mg/kg bw) for 2 mos.

#### **1.4.3.7 Dermatological Effects**

Components of avocado seeds may promote the growth of keratinocytes. Pretreatment of keratinocytes with avocado seed-derived PFAs improved cell viability following UVB irradiation compared to vehicle-treated controls.<sup>85</sup> The treatment also reduced the number of sunburned cells in human skin explants, enhanced DNA repair, and reduced the secretion of pro-inflammatory mediators IL-6 and PGE<sub>2</sub>. Proliferation and cell viability of keratinocytes were also enhanced by treatment with 10 µg/mL of a water- ethyl acetate extract of the avocado seed.<sup>104</sup> Avocado seed unsaponifiable fatty acids (UFA) have been patented for use in a cosmetic base to improve skin quality in human subjects based of several different studies. In the first study, after a six week topical treatment, 28 of 40 volunteers showed at least mild or moderate improvement in stretch marks and keratoses. In a second study, treatment for 4 wk with UFA improved stretch marks compared to controls. A third study observed that redness, hypopigmentation, hyperpigmentation and ridging were reduced

significantly as a result of treatment. A fourth study observed a 33% increase in skin moisture retention and an 8% increase in skin elasticity occurred compared to the control after 2 wk of UFA application. Addition of metal chelators improved the UFA effects on the damaged skin.<sup>93</sup> The unsaponifiable component of avocado seed oil was shown to inhibit the action of lysyl oxidase. This enzyme initiates crosslinks in collagen and the overabundance of these cross-links results in wrinkles and a lack of skin elasticity.<sup>94</sup> Hairless mice treated for 14 d with 3% natural or synthetic isolated linoleic acid-acid conjugated furan enhanced metabolic activity in the skin, caused an increase in elasticity, protein content, DNA content and an increased epidermal thickness in the treatment group. Compounds with a similar structure, having at least 9 carbons units attached to number 2 position of furan ring were also considered effective. The compounds have been the subject of two patents.<sup>95,96</sup>

#### **1.4.3.8 Antimicrobial Activity**

The antimicrobial activity of an ethanolic avocado seed extract was tested in select gram positive and gram negative bacteria. The extract was found to be effective against *Salmonella enteritidis*, *Citrobacter freundii*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes* (minimum inhibitory concentration (MIC) = 125 to 250 µg/mL).<sup>97</sup> The antimicrobial activity of crude terpenoid fractions and crude alkaloid fractions obtained using solvent extraction was assessed and both the fractions were found to inhibit the growth of *Staphylococcus aureus* and *Bacillus subtilis*.<sup>99</sup> Fungicidal effects of a methanolic avocado seed extract were observed against *Candida* spp, *Cryptococcus neoformans* and *Malassezia pachydermatis* with MIC = 0.125 to 0.625 mg/mL, 0.08 to 0.156 mg/mL and 0.312 to 0.625 mg/mL.<sup>98</sup> These results are interesting, but it is unclear how these extracts will perform in



*vivo*. It is also unclear what phytochemicals account for the anti-microbial and anti-fungal activity. Further studies are needed in these areas.

#### **1.4.3.9 Insecticidal Effect**

There is a pressing need for safe and effective new insecticides to deal with the spread of insects representing agricultural pests or disease vectors.<sup>101</sup> Leite *et al.*<sup>98</sup> tested the larvicidal activity and *in vitro* insecticidal potential of hexane and methanol extracts of avocado seeds and found that the median inhibitory concentration against *Aedes aegypti* larvae (Yellow Fever Vector) was 16.7 mg/mL and 8.9 mg/mL for hexane and methanol extracts, respectively. Phytochemical analysis revealed the presence of 1,2,4-trihydroxydecane and  $\beta$ -sitosterol in the hexane fraction and flavonoids, tannins, alkaloids and triterpenes in the methanol extract: though the presence of these compounds was not directly related to the insecticidal effects. A methanol extract of the avocado seed showed moderate activity against the mobilization of epimastigotes and trypomastigotes of *Trypanosoma cruzi in vitro* (MIC > 500  $\mu$ g/mL). A series of 1,2,4-trihydroxy heptadecane derivatives and 1,2,4-trihydroxy nonadecane derivatives were isolated as active compounds (Figure 3).<sup>100</sup>

#### **1.4.3.10 Colorant Effects**

Historical reports indicate that the Spanish *Conquistadors* used a colored exudate from avocado seeds as an indelible ink.<sup>59</sup> Studies in our laboratory have reported the development of a stable orange pigment, perseoranjin, when avocado seeds are crushed in the presence of oxygen.<sup>105</sup> This color development was dependent on the action of the enzyme polyphenol oxidase, indicating that the resulting pigment is a polyphenolic compound. The pigment was found to be a glycosylated benzotropolone with carboxylic acid and fused-ring butenolide moieties.

#### 1.4.4 Safety of Avocado Seed Extract

Crucial to its use in various applications including as a natural colorant and as a potential medicinal agent is the safety of an avocado seed extract. Contrary to popularly held opinions, the fact that avocado seed extracts are derived from natural sources implies nothing about their inherent safety. To date, there have been a limited number of studies on this topic. Oral administration of a single bolus dose of an aqueous extract of avocado seed (2 – 10 g/kg bw) was found to result in no significant toxic effect in rats within two subsequent weeks of observation. Sub-chronic administration of the same extract (2.5 g/kg bw) for 28 d also caused no-significant gross toxicological or hematological changes.<sup>69,101</sup> In another study, the median lethal dose of dried avocado seed in mice was determined as 1767 mg/kg bw when avocado seed flour was delivered via oral gavage.<sup>82</sup> These doses are quite high and suggest the overall safety of the aqueous seed extract. These data, however, remain in the preliminary stage and further studies are needed to more carefully determine the maximum tolerated dose of this extract. Additional studies are needed to determine the safe dose range for avocado seed extracts prepared with other solvents, since these are likely to enrich different compounds.

#### 1.5 Hypothesis and Objectives

An orange colored avocado seed extract has been previously reported by Dabas et al.<sup>105</sup> Consumer concern about the perceived health risks of artificial colors has driven companies to seek natural alternatives in order to produce “clean label” products. **I hypothesize that a polyphenol oxidase catalyzed reaction is producing a benzotropolone pigment in avocado seed extract. I hypothesize that this pigment will be shelf, light, and heat stable.**

To test these hypotheses, I propose the following objectives:

- A. To explore the stability and colorant properties of a semi-purified colored avocado seed extract.
- B. To use metabolomics to explore phytochemical differences between colored and uncolored avocado seed extracts.
- C. To identify the most prominent compound responsible for the yellow-orange color in avocado seed extract.

Removal of artificial colorants from food products is increasing. In many countries including most of Europe, popular artificial colorants such as red #3, yellow #5, and yellow #6 have already been banned from use in food products or are required to carry a warning label when used. The United States is following suit as many major companies are removing artificial colorants from their product lines, for example Kellogg's® cereals and Kraft's® macaroni and cheese. The search for natural red and yellow colors is particularly urgent, as many current options are expensive, contain allergens, or are animal derived. These natural colors also have poor shelf and heat stability. Avocado seed extract represents a novel source of yellow-orange-red natural colors, which are stable under a variety of conditions. The use of colored avocado seed extract will be particularly appealing for products with long shelf-lives such as beverages and candies, as well as products which are baked or undergo pasteurization. Using avocado seed extract as a natural colorant also provides a new value-added use for avocado seeds which are typically viewed as a low-value waste product.

## Chapter 2: Materials and Methods

### 2.1 General

Hass avocados, Great Value Pure Cane Sugar, Sprite®, Great Value 100% Apple Juice, Ocean Spray 100% White Grapefruit Juice, Pillsbury Traditional Vanilla Cake Mix, and other baking food materials were purchased from local grocery stores. Chemicals used were reagent grade and were used as supplied except where noted. Lyophilization was performed using a Virtis Genesis 25 XL Pilot Lyophilizer (Warminster, PA). Organic solvents were removed using a rotary evaporator (Heidolph; Germany).  $L^*a^*b^*$  values were determined using a Minolta CR-200 Chroma Meter (Japan). All liquid chromatography (LC) samples were filtered using 25 mm syringe filter with 0.45  $\mu\text{m}$  cellulose acetate membrane (VWR; Radnor, PA) prior to injection. All other reagents were of the highest quality available.

#### 2.1.1 Preparation of a Semi-pure CASE

After removal from the avocados, seeds were cleaned, peeled, and chopped by hand into small pieces. The pieces were then were blended with deionized, distilled water in a laboratory blender (Waring; Wilmington, NC) for 60 s. The resulting seed/water mixture was placed in the refrigerator at 4 °C for 24 h. After 24 h, the supernatant was gravity filtered through blotting paper (grade 703, VWR). The filtered supernatant was frozen in plastic trays and lyophilized to produce a dried, raw extract (~3.73% yield). The dried raw extract was further purified by flash chromatography using a nitrogen pressurized (< 2ppm moisture, Penn State General Stores) glass column packed with amberlite XAD7-HP (Sigma; Cleveland, OH). The column was eluted with water to removed sugars from the extract, and a colored fraction eluted using methanol containing 0.1% (v/v) acetic acid. The organic solvent was removed using a rotary evaporator,

and the remaining water frozen and lyophilized to produce a semi-pure colored extract (~29% yield). This semi-pure CASE was used in all color and stability studies.

## **2.2 Color and Stability of Semi-pure CASE in some Commercial and Model Food Products**

### **2.2.1 Color studies**

Semi-pure CASE was added to Sprite® (pH 3.29), apple juice (pH 3.71), white grapefruit juice (pH 3.25), and white cupcake mix at final concentrations of 0, 0.25, 0.75, 1, 3, 5, 8, and 10 mg/mL. For the cupcakes, L\*a\*b\* values were determined after baking and were measured on both the middles and tops of the cakes. For each food product, L\*a\*b\* values were found and  $\Delta E$  values calculated using the uncolored food product as the control and using the equation

$$\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2}$$

where  $L_0$ ,  $a_0$ , and  $b_0$  are the respective values of the uncolored food sample.

To prepare cheese sauces, regular orange and color-additive free white Kraft® cheese powders were provided by Wincrest Bulk Foods (Munnsville, NY). Orange cheese powders were prepared using 10, 100, and 200 mg CASE/g prepared dry cheese powder. L\*a\*b\* values were determined for each dry cheese powder as well as prepared cheese sauce (1 g cheese powder combined with 2 mL warm, skim milk). L\*a\*b\* values were used to calculate  $\Delta E$  for each sample, with white cheese powder used as the control.

### **2.2.2 Stability Studies in Model Sugar Drinks**

Samples were prepared by adding semi-pure CASE to a model sugar drink solution ( 2.6 M sodium citrate buffer containing 500 g/L sucrose). The solutions were adjusted to either pH 2.5 or pH 5.85. Semi-pure CASE was added to final concentrations of 0, 1, and 5 mg/mL. The samples were prepared in duplicate and placed into screw-cap GC vials. After being sealed into

the GC vials, samples were bubbled with nitrogen to remove oxygen from the sample and the headspace. The samples were then divided into four treatment groups and sampled as outlined below in Table 4.

Dark, 40°C	Dark, 23°C	Dark, 4°C	Light, 26°C
1	1	-	1
2	2	-	2
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-
8	8	8	8
-	15	15	15
-	21	21	21
-	29	29	29
-	37	37	37
-	43	43	43
-	50	50	-
-	56	56	-

**Table 4:** Sampling time points (days) on which each treatment group was sampled.

Sample testing at each time point included pH determination, L\*a\*b\* values, and UV-Vis spectroscopy. At each time point, a 3 mL aliquot was removed from each sample using a gas-tight syringe while bubbling nitrogen through the sample in order to retain an oxygen free environment. Light treated samples were placed into a prepared “light box” which was made using two 15 in x 15 in x 15 in Styrofoam boxes with 2” thick walls. 1 in x 2 in holes were cut into opposite sides of each box and a 60 Hz GE Slim Line Fluorescent Fixture, having dimensions of 14 in x 1 7/8 in x 1 in was placed into the box by placing either end into the pre-cut holes. Three 2.5 in x 3 in holes were cut into the front of the light boxes, and a 6.5 in x 2.5 in hole was cut into the top of the boxes to allow for air flow and decrease the chance of temperature fluctuations caused by opening the boxes to remove samples. Figure 4 shows the

inside, front, and top of a light box. The light intensity was tested in both boxes, at each sample position using a Sper Scientific Light Meter (Scottsdale, AZ) and was found to be 4400 lux.



**Figure 4:** From left, photos show the inside, front, and top of a light box.

### **2.3 Effect of pH on the color of CASE**

Two identical samples of semi-pure colored extract were prepared by dissolving 0.05 g of the extract in 10 mL distilled, deionized water. Samples were stirred with stir plates. The native pH of the treatment and control samples was 3.32 and 3.42, respectively. The treatment sample was treated with 10 M NaOH until the sample reached pH 12.32. The treatment sample was then treated with 6 M HCl until pH 1.59 was reached. In all cases an equivalent volume of water was added to the control sample in order to maintain similar concentrations. The final pH of the control sample was 3.50, and the treatment sample was finally adjusted to pH 3.57.

### **2.4 Preparation of Colored and Uncolored Avocado Seed Extracts for Metabolomics**

Five biological replicates were prepared of both colored and uncolored extracts. Each replicate contained approximate 10 g portions from two avocado seeds, totaling 20 g of seed per replicate. Colored replicates were prepared by blending ~20 g of seeds into 400 mL of deionized, distilled water. For uncolored replicates, ~20 g of seeds was blended into 400 mL of deionized distilled water containing tropolone (5.0 mg, 0.041 mmol).

MS/MS analysis was completed at the Penn State Metabolomics Core facility with the help of Dr. Phillip Smith, in the laboratory of Dr. Andrew Patterson. Samples (5  $\mu$ L) were separated by reverse phase HPLC using a Prominence 20 UFLCXR system (Shimadzu, Columbia MD) with a Waters (Milford, MA) BEH C18 column (100mm x 2.1mm 1.7  $\mu$ m particle size) maintained at 55°C and a 20 minute aqueous acetonitrile gradient, at a flow rate of 250  $\mu$ L/min. Solvent A was HPLC grade water with 0.1% formic acid and Solvent B was HPLC grade acetonitrile with 0.1% formic acid. The initial condition were 97% A and 3 % B, increasing to 45% B at 10 min, 75% B at 12 min where it was held at 75% B until 17.5 min before returning to the initial conditions. The eluate was delivered into a 5600 (QTOF) Triple time of flight (TOF) using a Duospray™ ion source (all AB Sciex, Framingham, MA). The capillary voltage was set at 5.5 kV in positive ion mode and 4.5 kV in negative ion mode, with a declustering potential of 80V. The mass spectrometer was operated in IDA (Information Dependent Acquisition) mode with a 100 ms survey scan from 100 to 1200  $m/z$ , and up to 20 MS/MS product ion scans (100 ms) per duty cycle using a collision energy of 50V with a 20V spread. Principal component analysis was processed using square root mean square analysis. Known compounds were identified using the Scripps METLIN metabolomics database.

## **2.5 Structure Elucidation of the Colored Compound**

### **2.5.1 HPLC Purification**

The semi-pure, post-amberlite CASE was further purified using an Agilent PrepStar system with 440-LC fraction collector (Santa Clara, CA). The extract was dissolved in deionized, distilled water to a final concentration of 20 mg/mL and filtered. Samples (10 mL) were injected onto a Viva C18 250 mm x10 mm x 5  $\mu$ m column (Restek, Bellefonte, PA). Samples were separated using a gradient of deionized water containing 0.1% acetic acid and acetonitrile. The



percentage of acetonitrile increased with time as follows; 0 min, 5% ; 0-40 min, 5-30%; 40-45 min, 30-95%; 45-48 min, 95%; 48-49 min, 95-5%; 49-51 min, 5% at a flow rate of 4 mL/min. Fractions were collected at 30 s intervals (2 mL each) from 19.5 min to 26 min. The peak of interest eluted in fraction 12 (f12) at approximately 22 min. The 12<sup>th</sup> fractions from all following injections were combined and lyophilized to produce rough F12.

Once dried, the rough F12 samples were diluted with deionized water and 10 mL samples were injected onto an Ultra Aromax 250 mm x 10 mm x 5 µm column (Restek). Samples were resolved using a gradient method of deionized water containing 0.1% acetic acid, and methanol. The percentage of methanol was increased as a function of time as follows: 0 min, 48%; 0-13.5 min, 48-65%; 13.5-14.5 min, 65%; 14.5-15 min, 65-48%; 15-17 min, 48%, at a flow rate of 4 mL/min. Fractions were collected at 24 sec intervals (1.6 mL each from 8.9 min to 14.5 min. The peak of interest eluted as the later of 2 overlapping peaks at approximately 10.6 min to produce “pure F12.”

“Pure F12” fractions were combined and lyophilized. Injections (20 µL) were made onto an ultra Aromax 150 mm x 4.6 mm x 5 µm column (Restek; Bellefonte, PA). A gradient method was used with solvent being filtered DDI water with 0.1% acetic acid and solvent B being methanol. The method was as follows: 0 min, 45%; 0-25, 45-62%; 25-28 min, 62%; 28-29 min, 62-45%; 29-31 min, 45%, at a flow rate of 4 mL/min. If additional purification was necessary, the fractions were again fractionated using the PrepStar system.

### **2.5.2 High Resolution MS/MS Analysis**

MS/MS analysis was completed at the Penn State Metabolomics Core facility with the help of Dr. Phillip Smith, in the laboratory of Dr. Andrew Patterson. Samples (5 µL) were

separated by reverse phase HPLC using a Prominence 20 UFLCXR system (Shimadzu, Columbia MD) with a Waters (Milford, MA) BEH C18 column (100mm x 2.1mm 1.7  $\mu$ m particle size) maintained at 55°C and a 20 minute aqueous acetonitrile gradient, at a flow rate of 250  $\mu$ L/min. Solvent A was HPLC grade water with 0.1% formic acid and Solvent B was HPLC grade acetonitrile with 0.1% formic acid. The initial conditions were 97% A and 3 % B, increasing to 45% B at 10 min, 75% B at 12 min where it was held at 75% B until 17.5 min before returning to the initial conditions. The eluate was delivered into a 5600 (QTOF) TripleTOF using a Duospray™ ion source (all AB Sciex, Framingham, MA). The capillary voltage was set at 5.5 kV in positive ion mode and 4.5 kV in negative ion mode, with a declustering potential of 80V. The mass spectrometer was operated in IDA (Information Dependent Acquisition) mode with a 100 ms survey scan from 100 to 1200  $m/z$ , and up to 20 MS/MS product ion scans (100 ms) per duty cycle using a collision energy of 50V with a 20V spread. Principal component analysis was processed using square root mean square analysis.

### **2.5.3 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR)**

ATR analysis was conducted at the Materials Characterization Lab in the Penn State Materials Research Institute with the help of Maxwell Weatherington in the laboratory of Dr. Joshua Stapleton. ATR experiments were conducted with a neat sample using a Bruker Vertex 70v (Billerica, MA) scanning the mid-IR range.

### **2.5.4 High Resolution NMR Analysis**

Nuclear Magnetic Resonance (NMR) studies were conducted at the Penn State NMR Core Facility with the help of Dr. Emmanuel Hatzakis and Dr. Carlos Pacheco using approximately 1 mg of perseoranjin. Experiments used a Bruker Avance III HD NMR operating at 500.20 MHz, equipped with a 5 mm CPPBBO-BB-1H/19F/ D Z gradient probe. Standard

Bruker pulse sequences were used. 1D  $^1\text{H}$  and  $^{13}\text{C}$  experiments, as well as 2D Correlation Spectroscopy (COSY), Total Correlation Spectroscopy (TOCSY), Heteronuclear Multiple Bond Correlation (HMBC), and Distortionless Enhancement by Polarization Transfer (DEPT) edited Homonuclear Single bond Quantum Correlation (HSQC) experiments were conducted on two separate samples, one in  $(\text{CD}_3)_2\text{SO}$  and one in  $\text{D}_2\text{O}$  (Supplemental Data). Additionally a 2D Nuclear Overhauser Effect Spectroscopy (NOESY) and selective TOCSY and Rotating Frame NOESY (ROESY) experiments were conducted on the  $\text{D}_2\text{O}$  sample. Data was processed using MestReNova software. Details of each experiment are listed below.

1D  $^1\text{H}$ : 500.20 Hz; acquisition time, 2.3396; spectral width, 7002.8; spectral size, 65536; acquired size, 16384; relaxation delay, 1.0000; pulse width, 10.1300.

1D  $^{13}\text{C}$ : 125.79 Hz; acquisition time, 1.1010; spectral width, 29761.9; spectral size 262144; acquired size, 32768; relaxation delay, 8.0000; pulse width, 10.0000.

COSY: (500.20, 500.20) Hz; acquisition time, 0.1532; spectral width (6684.5, 6684.5); spectral size (1024, 1024); acquired size (1024, 128), relaxation delay, 2.0000; pulse width, 10.1300.

TOCSY: (500.20, 500.20) Hz; acquisition time, 0.2048; spectral width (5000.0, 5000.0); spectral size (1024, 1024); acquired size (1024, 316); relaxation delay, 1.5000; pulse width, 9.7500.

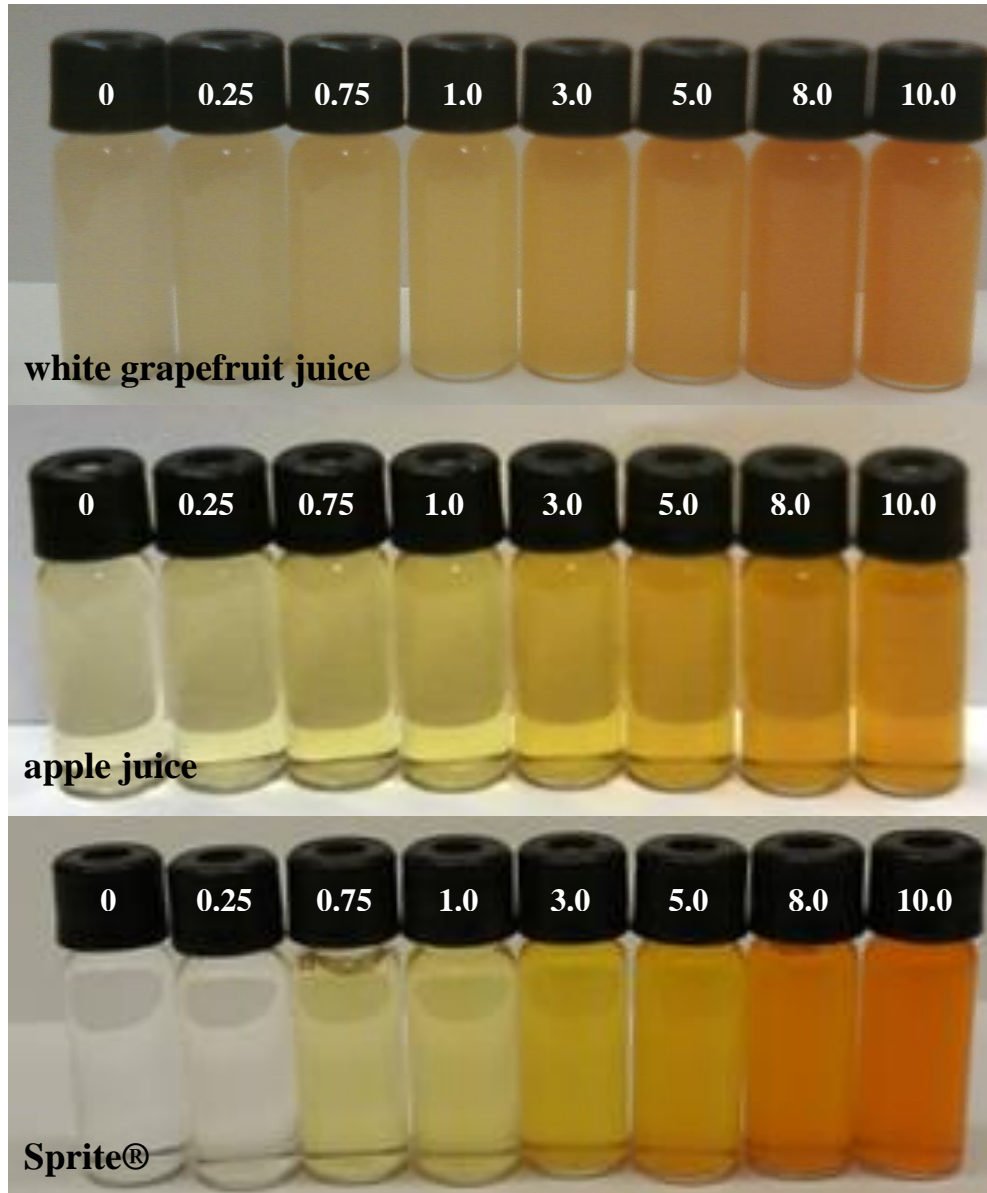
HSQC-DEPT: (500.20, 125.78) Hz; acquisition time, 0.0786; spectral width (6510.4, 22624.4); spectral size (2048, 2048); acquired size (512, 150); relaxation delay, 1.5000; pulse width, 9.7500.

HMBC: (500.20, 125.79) Hz; acquisition time, 0.1365; spectral width (7500.0, 28901.7); spectral size (1024, 1024); acquired size (1024, 256); relaxation delay, 1.5000; pulse width, 9.7500.

## Chapter 3: Results

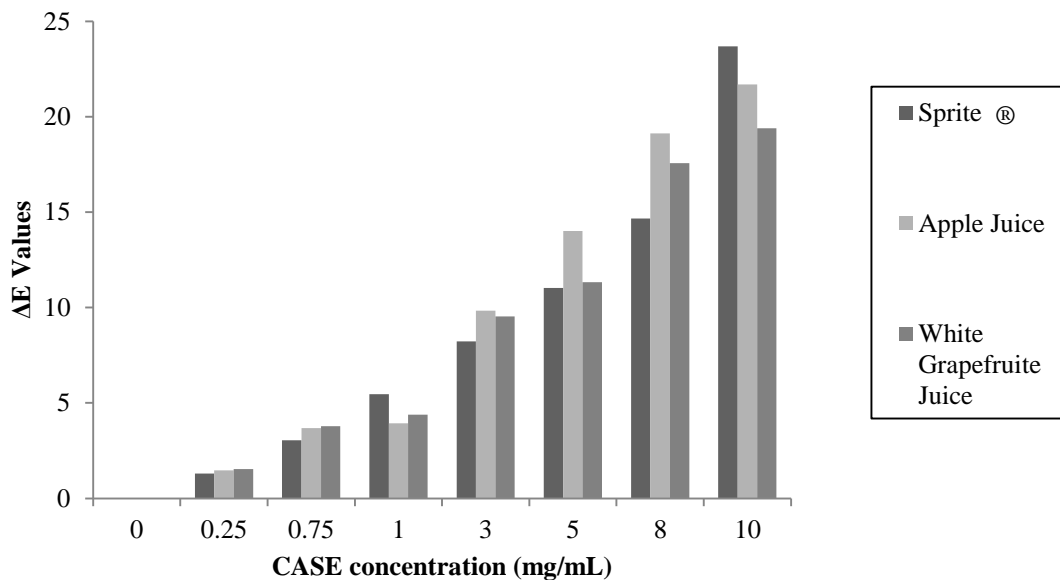
### 3.1 Colorant Properties of Semi-pure CASE in a panel of Commercial Food Products

Semi-pure CASE was added to three commercial beverages to assess the behavior of the pigment in a variety of matrices. The results for white grapefruit juice, apple juice, and Sprite® are shown below in Figure 5.



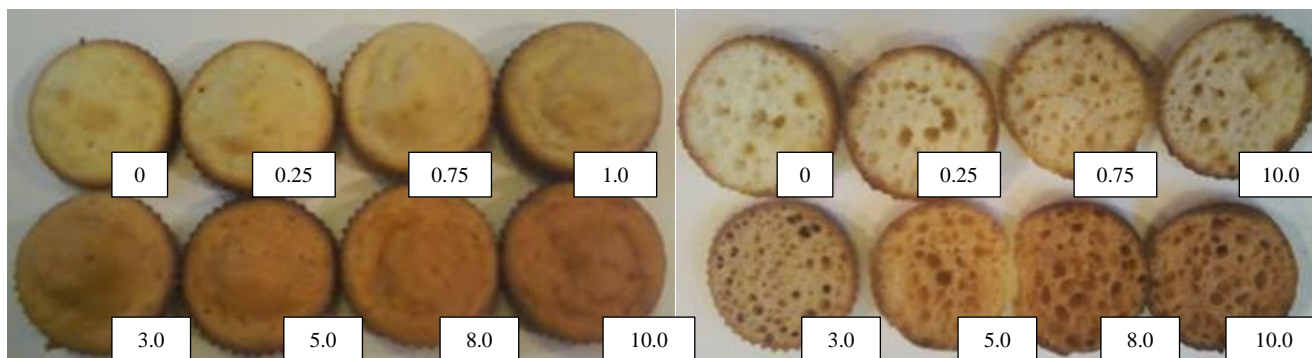
**Figure 5:** Semi-pure CASE in white grapefruit juice, apple juice, and Sprite®. Concentration of semi-pure CASE used is shown above each sample in the units of mg/mL.

Visually, the color of the extract appeared to be the most vibrant in the Sprite®. Figure 6 shows the calculated  $\Delta E$  values for the samples.



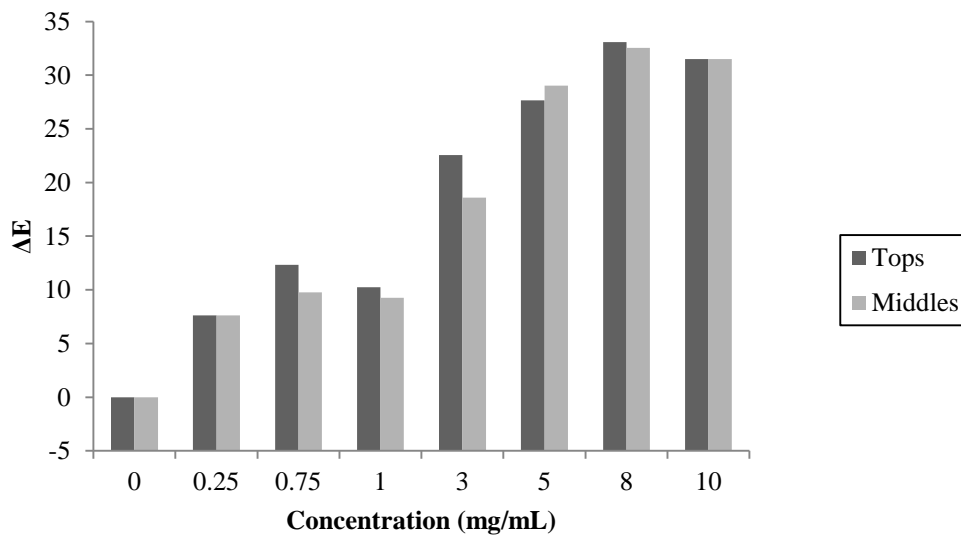
**Figure 6:**  $\Delta E$  values of semi-pure CASE in Sprite®, apple juice, and white grapefruit juice.

When used in baking, the semi-pure CASE retained its colorant properties well, although it should be noted that higher concentrations of the extract produced a smaller loaf volume, denser crumb, and appeared to lead to decreased browning on the crust of the cupcakes (Figure 7).



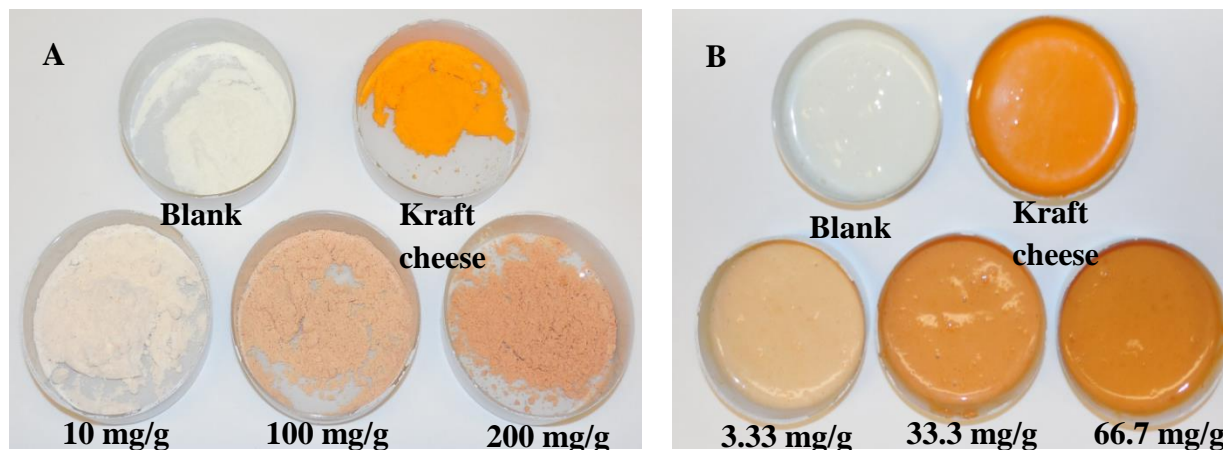
**Figure 7:** Semi-pure CASE in white cake. Photos show the tops (A) and middles (B) of the cupcakes. Concentration of CASE is shown in mg/mL. Baked samples were prepared in duplicate.

The reason for these effects is likely due to the high polyphenol content of the semi-pure CASE. The Maillard reaction is responsible for the desirable browning and crust formation on baked or fried products. The reaction occurs when a carbonyl group from a sugar reacts with an amine group from an amino acid producing glycosimines which undergo further reactions to produce the desirable brown crust and aromas of baked goods. Because semi-pure CASE contains a high concentration of polyphenols, the carbonyl groups from these compounds may react with amino acids, producing a different product.<sup>106</sup> This is one of the primary limitations of using semi-pure CASE as opposed to a more pure colorant. In terms of  $\Delta E$ , the addition of semi-pure CASE showed a steady climb in values in both the tops and middles of the cupcakes, but appeared to reach an asymptote near 8 mg/mL (Figure 8).



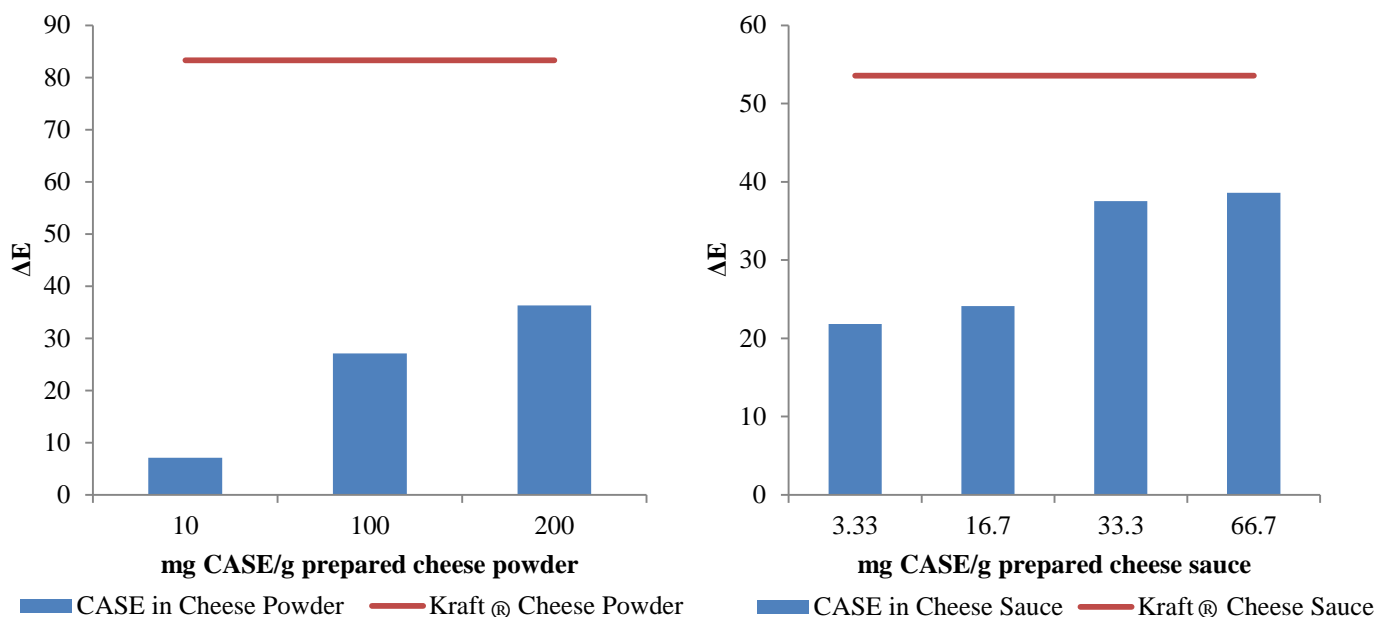
**Figure 8:**  $\Delta E$  values of semi-pure CASE in cupcake tops and middles.

Semi-pure CASE was also added to white cheese powder (blank) and compared to regular orange Kraft® cheese powder (Figure 9, A). To create a cheese sauce, warm milk (2 mL/g) was added to the cheese powders (Figure 9, B). The CASE produced a strong orange



**Figure 9:** Semi-pure CASE was added to a white no-color-added cheese powder (blank). Warm milk was then added to the resulting samples in order to prepare a cheese sauce. Dry cheese powders are shown on the left (A), while the prepared cheese sauce is shown on the right (B).

color, but was not as bold or bright as the regular Kraft® cheese powder. The  $\Delta E$  values appear to reach a maximum around 100 mg/g dry sample or 33.3 mg/g prepared sample (Figure 10). A similar result was observed in the white cake mix around 8 mg/mL semi-pure CASE.

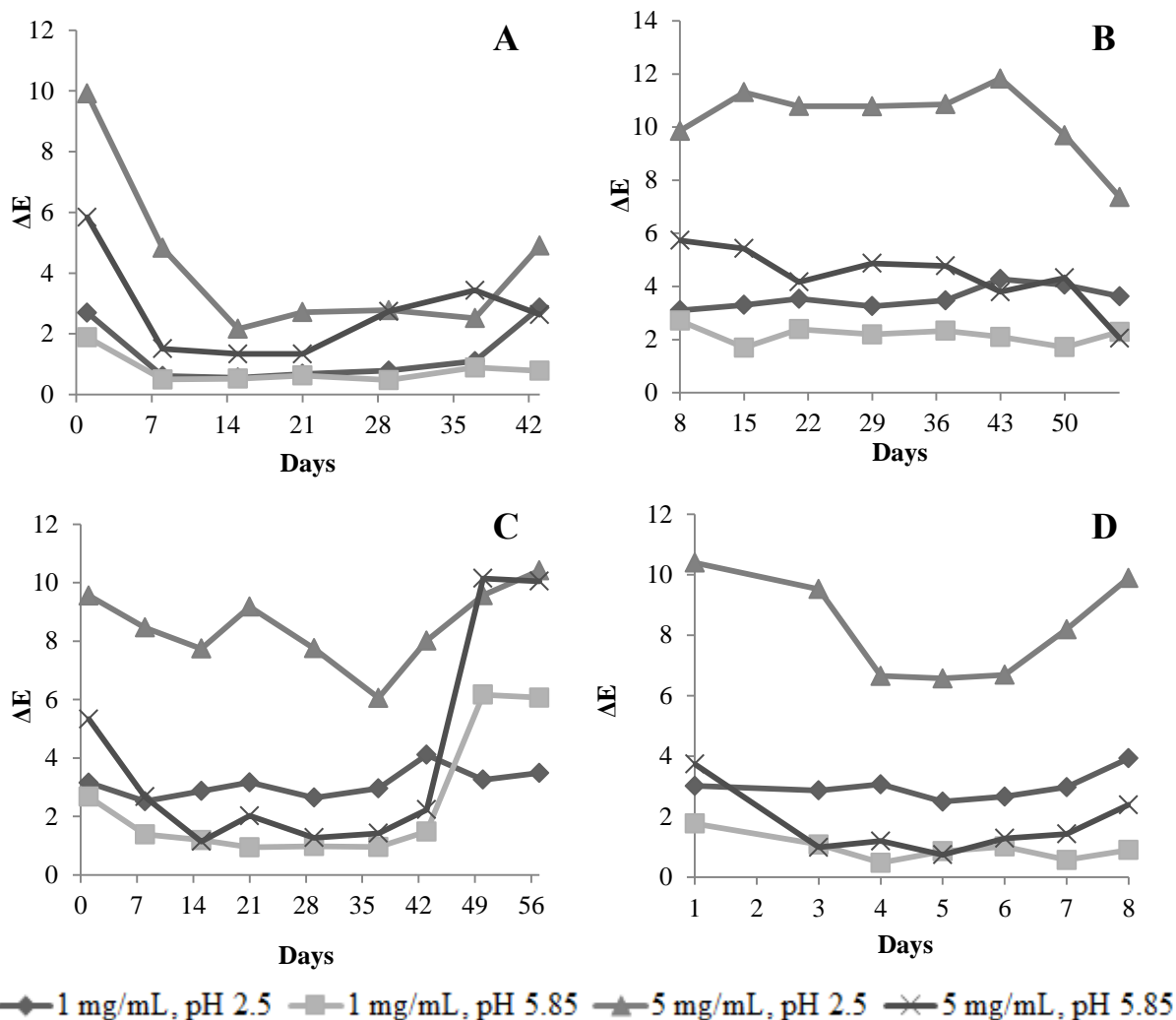


**Figure 10:**  $\Delta E$  values of CASE in a white no-color-added Kraft® cheese powder (left) and in prepared cheese sauce (right).  $\Delta E$  of regular Kraft® cheese powder and prepared sauce were also calculated and appear as red lines on the graphs.

### 3.2 Stability of Semi-pure CASE in a Model Sugar Drink

Semi-pure CASE samples were prepared in a model sugar drink with concentrations of 1 mg/mL or 5 mg/mL, and at pH 2.5 and pH 5.85. Treatment groups consisted of three dark groups at 4 °C, 23 °C, and 40 °C, and one treatment group of light treated samples at 26 °C. Samples were prepared in duplicate, and  $L^*a^*b^*$  measurements of each sample were performed twice.  $\Delta E$  values for the samples can be seen in Figure 11. In general, the greater the  $\Delta E$  value, the more likely it is that the corresponding color change is perceptible to the human eye. It is widely accepted that a change of  $\Delta E \leq 2.5$  is insignificant, or likely to be imperceptible to the human eye.<sup>107</sup> After deoxygenation by bubbling nitrogen through samples, a change in pH in the order of -0.35 pH units was observed for all samples.





**Figure 11:** Change in  $\Delta E$  of light treated samples at 26 °C (A), and samples kept in the dark at 4 °C (B), 23 °C (C), and 40 °C (D).

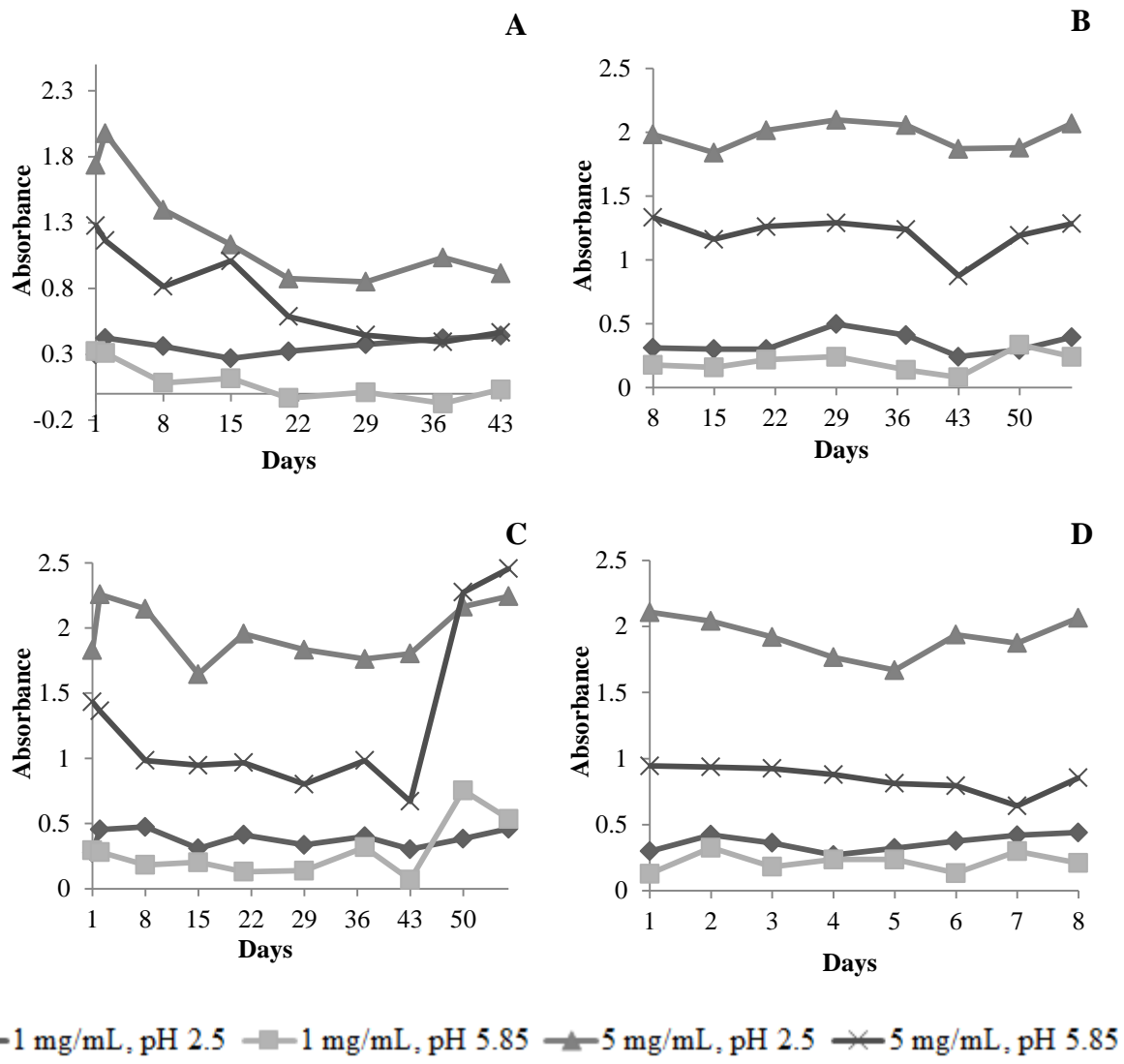
The pH of all samples then remained constant throughout the remainder of the experiment. Some variation in  $\Delta E$  is expected due to the nature of the experiment. Apart from some minor fluctuations, samples retained their bright colors even after 50 d. Photos of the samples on day 36 are shown below in Figure 12. Samples were measured on the spectrometer during the experiment to determine any change in sample absorbance at 445 nm, the wavelength corresponding to the most prominent colored compound (Figure 13). Though the light treated samples showed a minor decrease in  $\Delta E$  values over time, they showed no significant change in

absorbance at 445 nm. Both the 1 mg/mL at pH 5.85 and the 5 mg/mL at pH 2.5 samples experienced increases in  $\Delta E$  and absorbance at 445 in the darkened, room temperature samples during the final three weeks. This may have been due to the formation of a precipitate, as no such trend is observed in any of the other samples.

Concentration	1 mg/mL						5 mg/mL					
	pH 2.5			pH 5.85			pH 2.5			pH 5.85		
Light	+	-	-	+	-	-	+	-	-	+	-	-
Temp (°C)	26	23	4	26	23	4	26	23	4	26	23	4

**Figure 12:** CASE in model sugar drink samples on day 36 of the stability study.

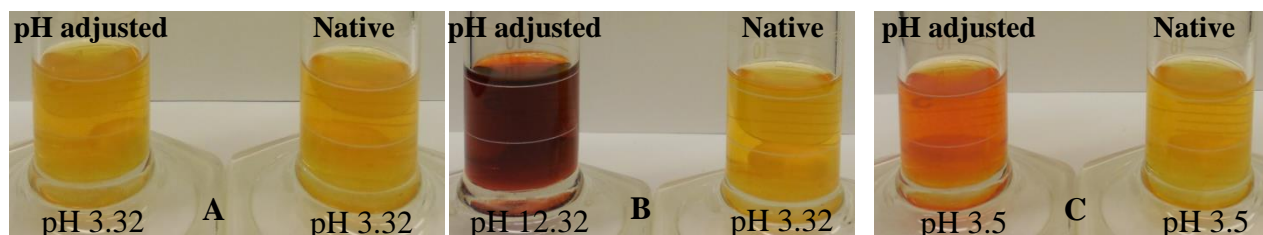


**Figure 13:** Change in 445 nm absorbance of light treated samples at 26°C (A) and samples kept in the dark at 4°C (B), 23°C (C), and 40°C (D).

### 3.3 Effect of pH on Color of Semi-pure CASE

The effect of pH on raw CASE samples has been previously reported.<sup>108</sup> Here, the effect of pH in semi-pure CASE is reported. Semi-pure CASE in water at a final concentration of 5 mg/mL has a pH of 3.32 and a yellow color (Figure 14). Adjusting the pH to neutral levels again produced a deeper orange color, and increasing the pH to 10-12 created a dark red, and finally

brownish red color. Upon returning the sample to its native pH range, and even lower to pH 1.59 produced a sample in which color was still pH dependent, but the color range had been shifted to a more orange-red range.

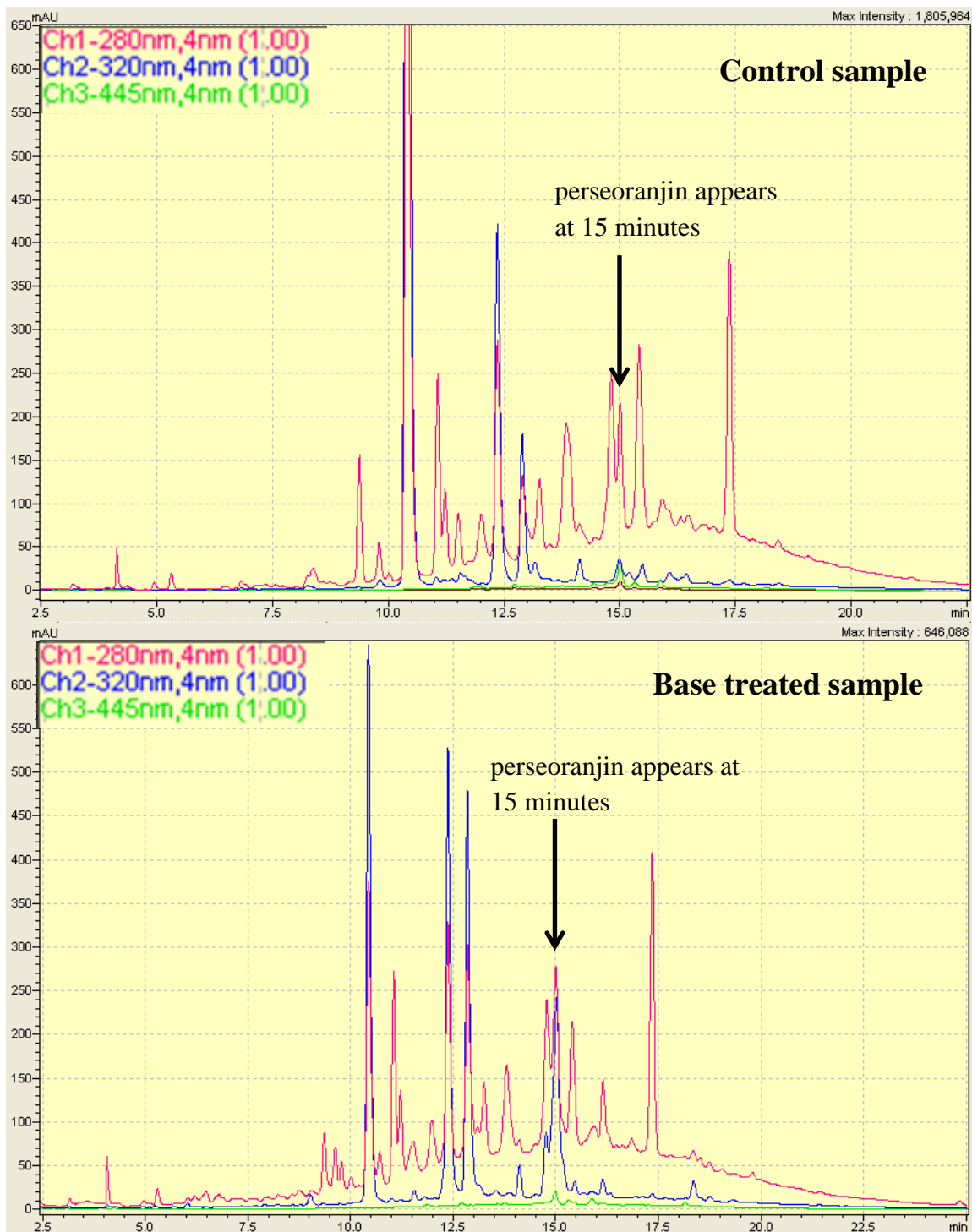


**Figure 14:** Pretreatment (A), base treated (B), and pH readjusted (C) semi-pure CASE samples compared to a control sample.

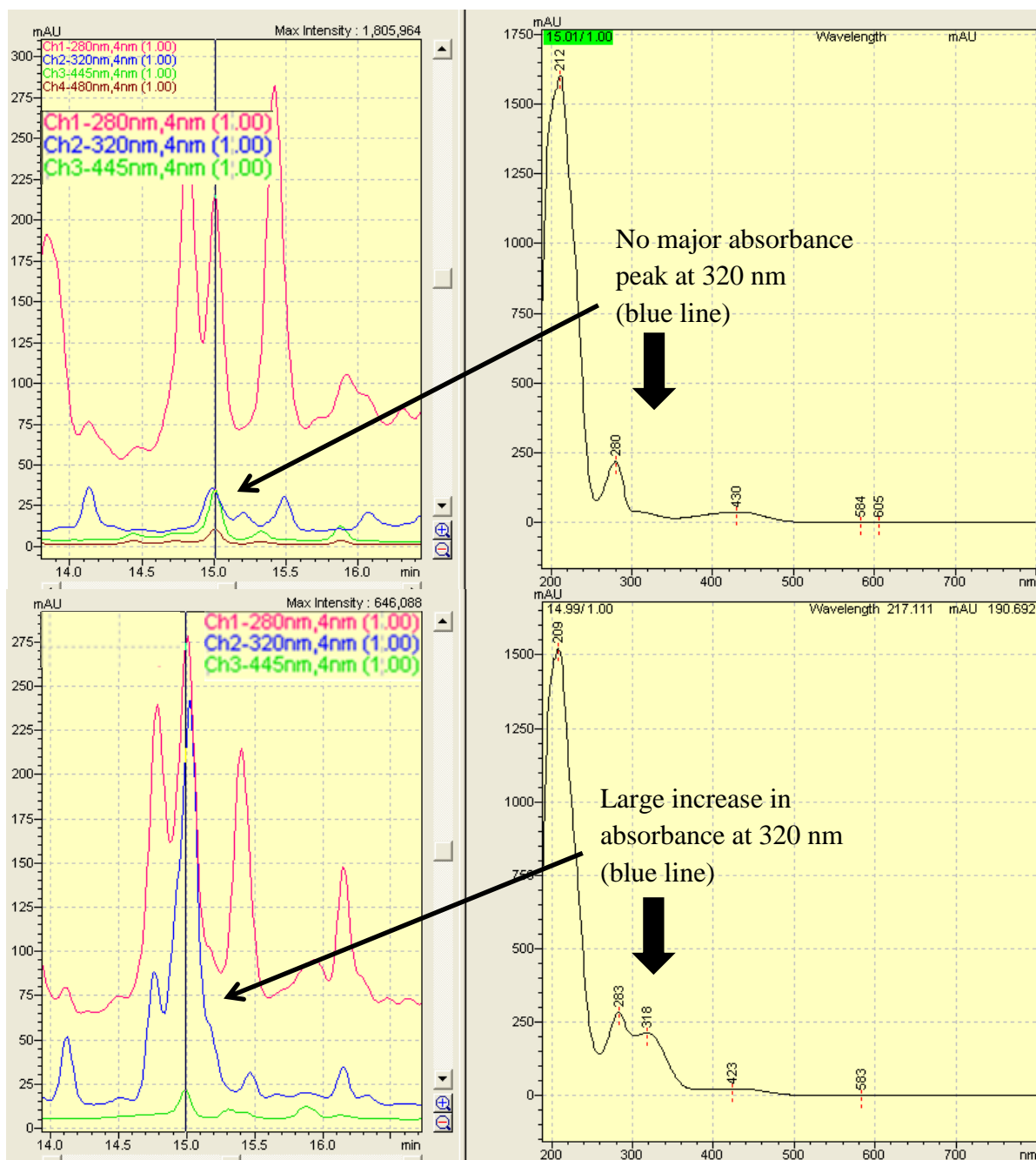
Spectrometer data showed an increase in sample absorbance at both 445 nm and 480 nm (Table 5). Liquid chromatography profiles showed that the most prominent colored peak, perseoranjin, was still present after treatment with base. The 280 nm profile remained as well, though decreased, while a substantial increase was seen in the 320 nm profile (Figure 15 and 16). This red-shift in color and increase in the 320 nm peak indicates that there may be some extended conjugation forming in compounds where conjugation may have previously been limited.

	pH adjusted	Native	pH adjusted	Native
pH	ABS (445 nm)		ABS (480 nm)	
3.32	0.462		0.273	
12.32	3.203	-	3.540	-
3.5	0.526	0.340	0.305	0.186

**Table 5:** UV-Vis spectrometer data of the control and pH adjusted samples at 445 nm and 480 nm.



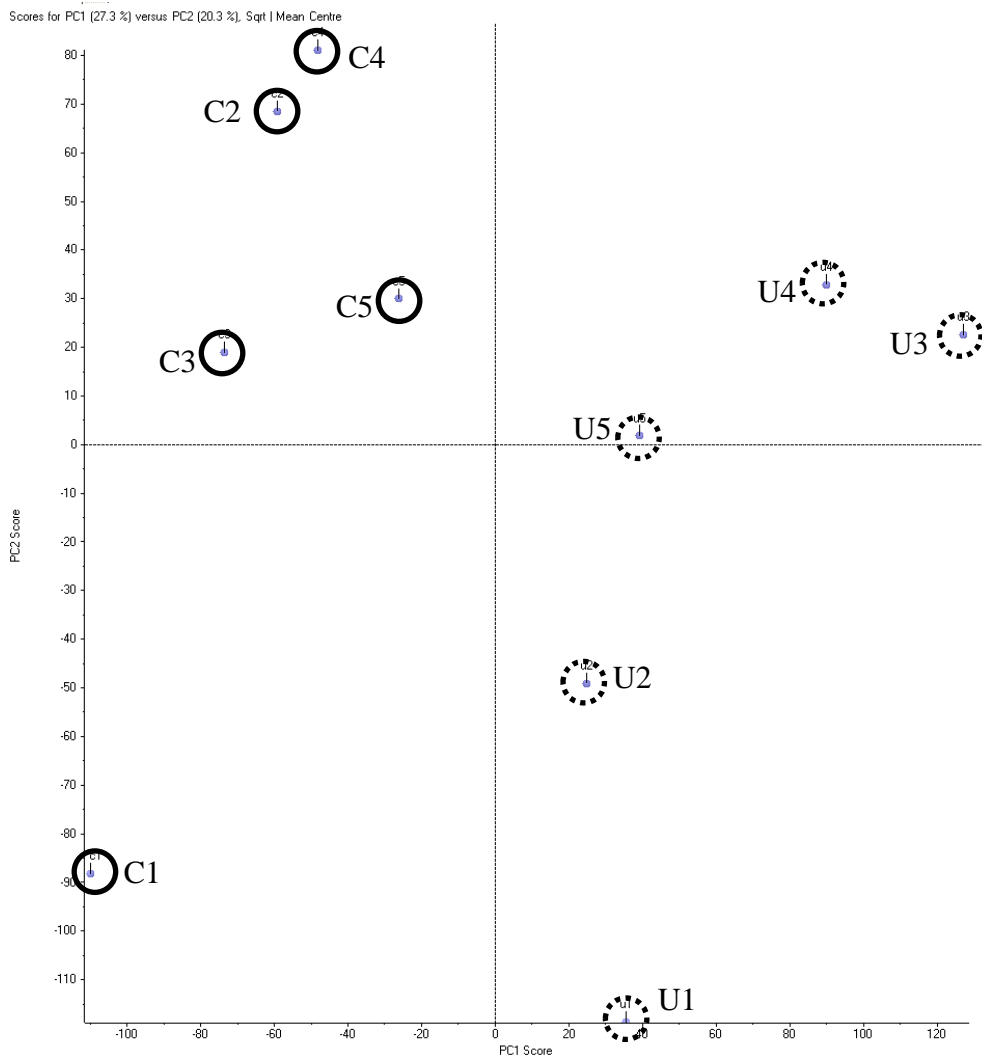
**Figure 15:** Full LC profile of the semi-pure CASE in water control sample (top) and pH adjusted semi-pure CASE in water (bottom). The peak of interest, perseoranjin, appears at 15 min on both chromatograms. Line colors are pink, 280 nm; blue, 320 nm; green, 445 nm.



**Figure 16:** LC profile and areas of maximum absorbance for perseoranjin peak in semi-pure CASE in water (top) and pH adjusted semi-pure CASE in water. Line colors are pink, 280 nm; blue, 320 nm; green, 445 nm.

### 3.4 Principal Component Analysis (PCA) of Colored and Uncolored Avocado Seed Extracts

An uncolored avocado seed extract was prepared by inhibiting the action of PPO with tropolone. By comparing biological replicates of colored and uncolored extracts, it was possible to determine masses unique to each sample. Figure 17 shows the clustering of masses in samples analyzed in positive mode. Variation between samples is observed due to difficulty in totally inhibiting PPO action, and because natural samples tend to vary greatly, even within crops. That variation can be observed in these data by the divergence between clustering of



**Figure 17:** PCA clustering scores for colored (solid line, C) and uncolored (dashed line, U) samples analyzed in positive mode.

replicates, as seen in Figure 17. Masses near the upper left tended to be present at higher concentrations in the colored samples, while samples towards the lower right tended to be present at higher concentrations in the uncolored samples. Figure 18 shows each unique mass found in the samples in positive mode. The clustering of samples analyzed in negative mode is

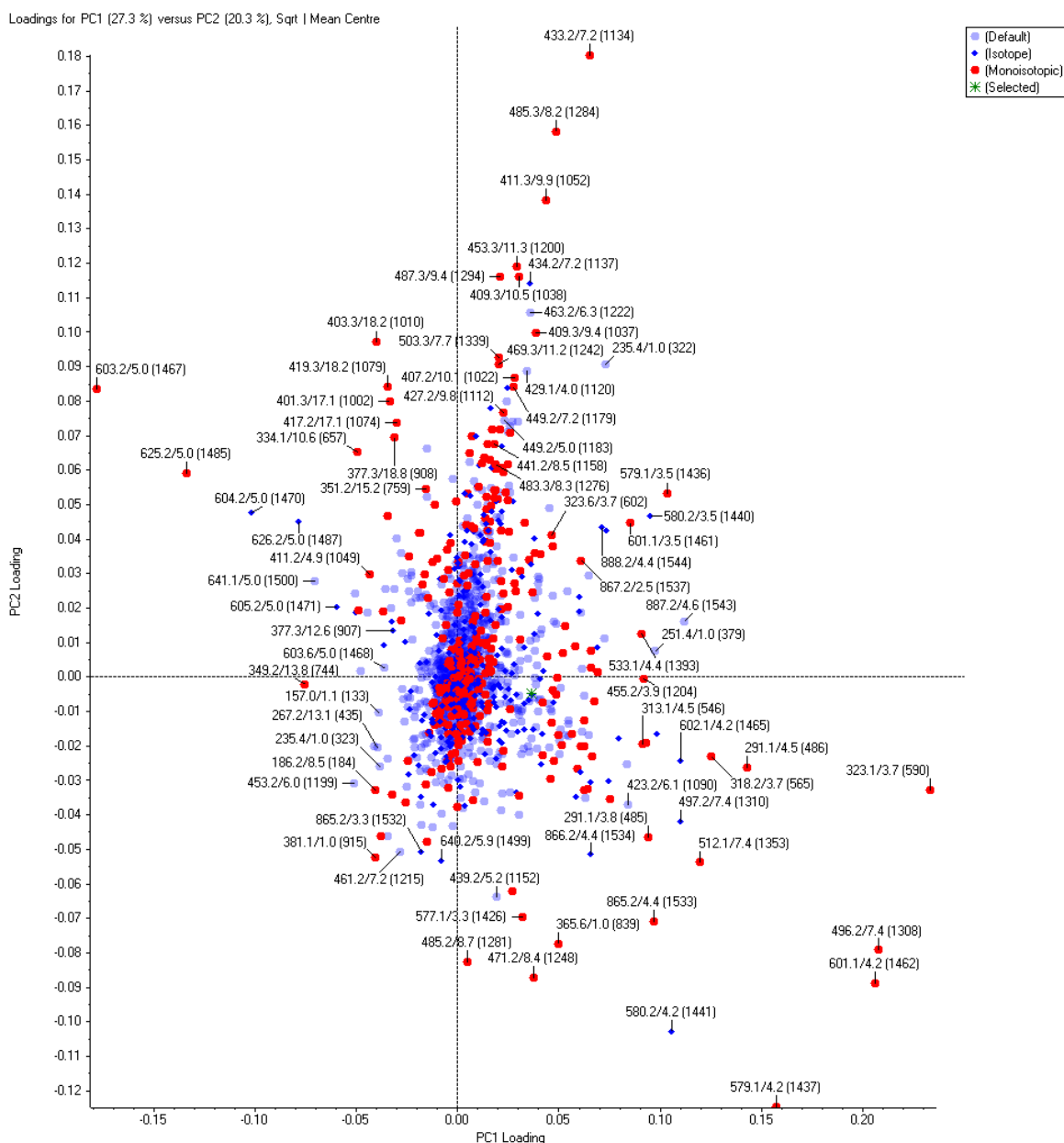
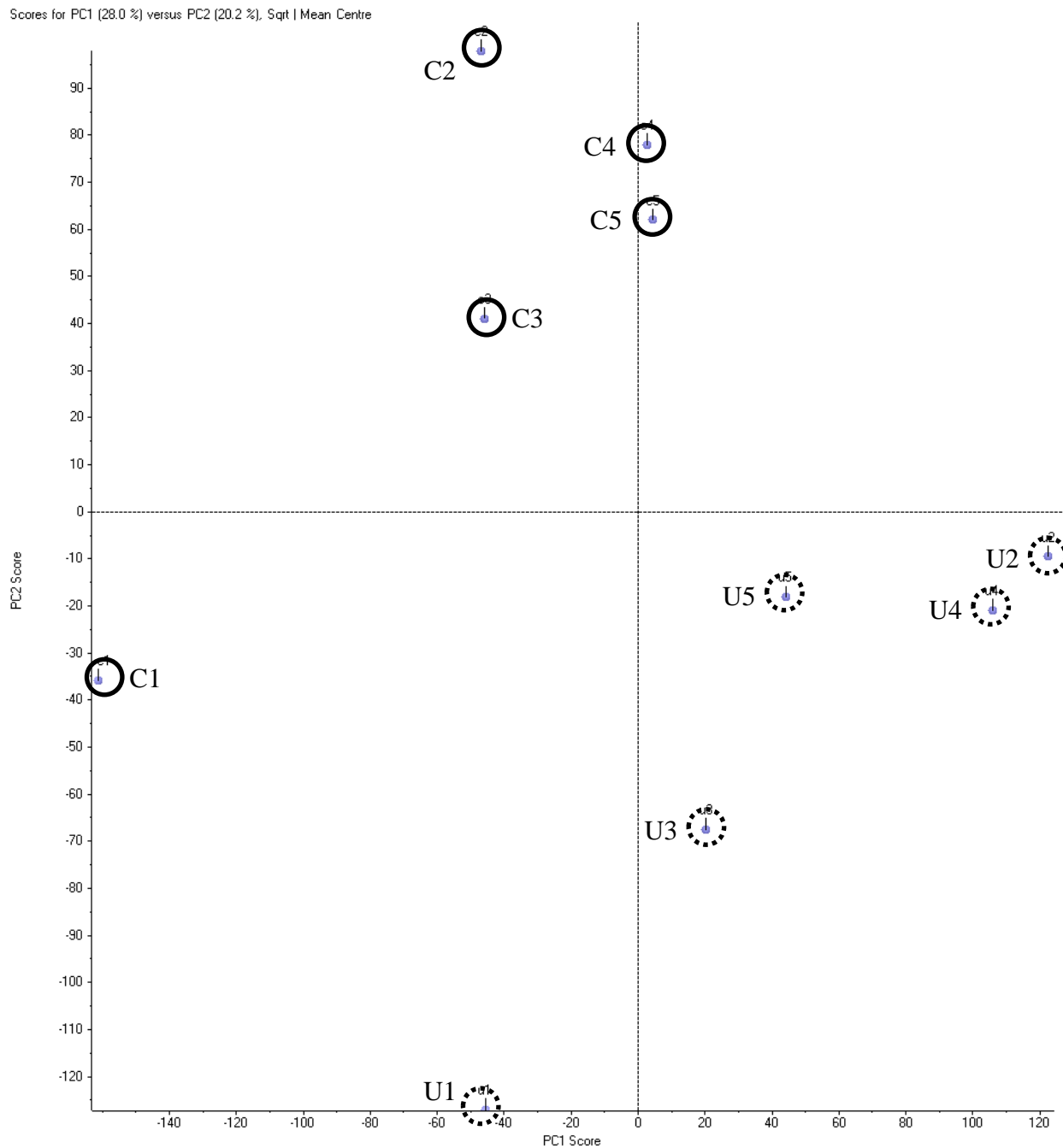


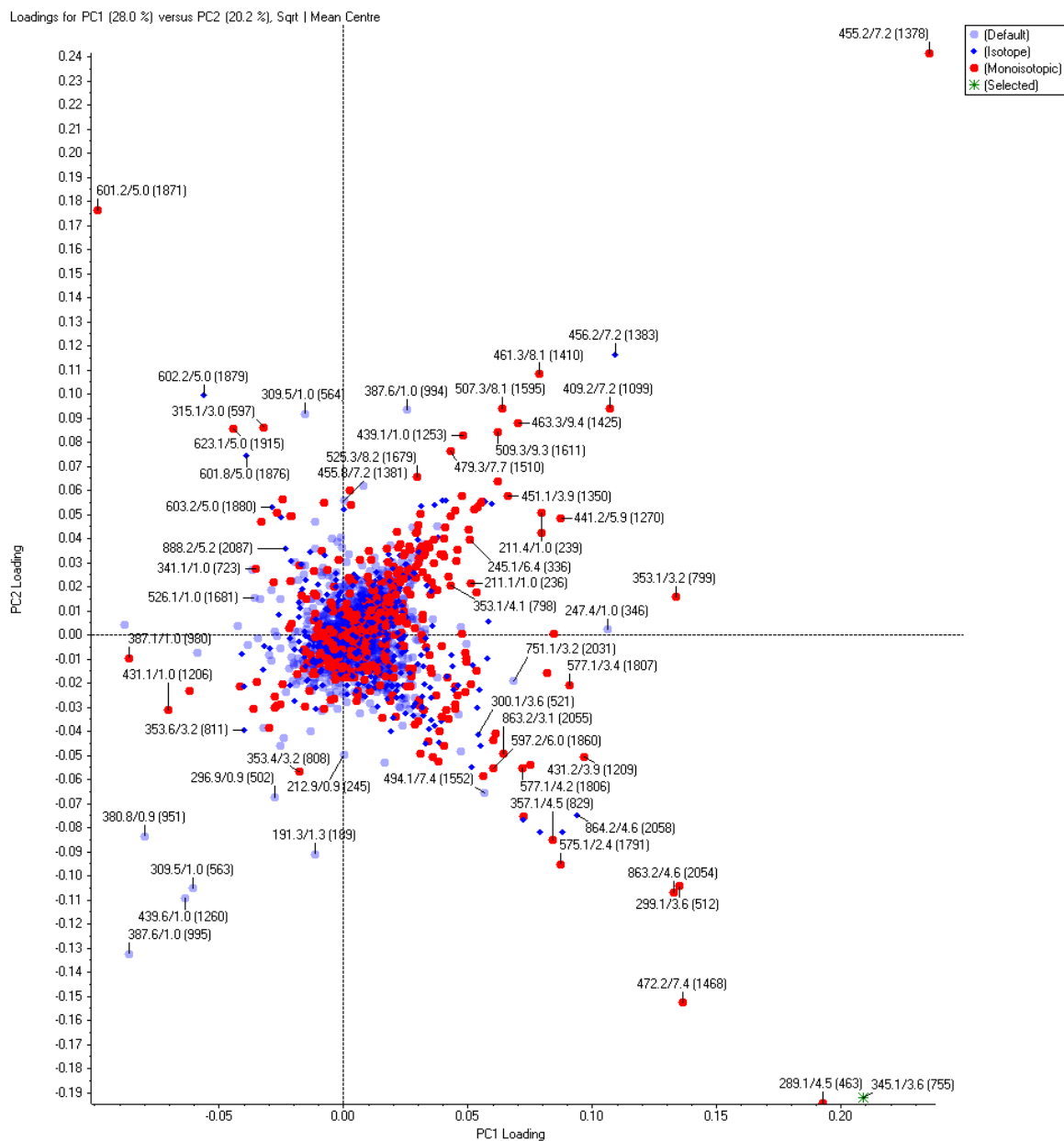
Figure 18: PCA of colored and uncolored extracts in positive mode.



shown in Figure 19, while the total principal component analysis is shown in Figure 20. Again masses near the upper left tended to be present at higher concentrations in the colored samples, while samples towards the lower right tended to be present at higher concentrations in the



**Figure 19:** PCA of colored (solid line, C) and uncolored (dashed line, U) extracts in negative mode.



**Figure 20:** PCA of colored and uncolored extracts in negative mode.

uncolored samples. Principal component analysis showed approximately forty-nine masses unique to the colored or uncolored extract. Abscisic acid, and perseitol, the 7-member sugar alcohol, were present in both extracts while epicatechin, catechin, procyanidin B2 and salidroside were found only in the uncolored extract. Table 6 shows a list of masses found in one or both of the extracts.

**Table 6:** Compounds found in colored and uncolored avocado seed extracts via principal component analysis.

Extract	Compound	Retention time (min)	Mode	Molecular Ion	Fragments
both	perseitol	0.96	negative	211.082	193.0171, 149.0457, 131.0353, 119.0347, 113.0243, 101.025, 89.0255, 85.0309, 71.0163, 59.0173, 57.038, 55.0227
both	abscisic acid	4.05	positive	265.1413	247.1325, 135.134, 229.1225, 219.1386, 217.1219, 211.1116, 203.1054, 196.0858, 187.1131, 175.0743, 161.0945, 147.0797, 135.0791, 128.0619, 115.0552, 95.0498, 91.0547
uncolored	epicatechin/ catechin	4.51	negative	289.073	271.0623, 247.0636, 245.0829, 227.0725, 221.0833, 205.0518, 203.0726, 187.0408, 161.0616, 159.0459, 151.0404, 137.0252, 125.0247, 123.0456, 109.0303, 97.0303, 95.051
uncolored	catechin/ epicatechin	3.76	negative	289.0726	245.0828, 123.0459, 109.031
uncolored	Procyanidin B2	4.17	positive	579.1484	439.1004, 427.1019, 411.1085, 409.0885, 303.0826, 301.0698, 291.0857, 289.0682, 287.0547, 259.0612, 247.0601, 229.0499, 215.0698, 205.0465, 201.0542, 191.0333, 187.0373, 179.0321, 177.0547, 175.0398, 167.0334, 165.0542, 163.0382, 159.0445, 149.0222, 147.0443, 139.0381, 135.0439, 127.039, 123.0435, 109.0304, 68.9977
uncolored	salidroside	3.67	positive	323.1096	None
uncolored	no ID	3.64	negative	299.1134	179.0547, 137.061, 119.0494, 101.0245, 89.025, 71.0155
uncolored	no ID	3.64	negative	345.1193	299.1134, 179.0561, 161.0457, 137.0613, 119.0439, 113.0249, 89.0255, 71.0157, 59.0168
uncolored	no ID	2.45	negative	575.127	557.1182, 531.1353, 513.41, 487.143, 449.0897, 423.0757, 407.0825, 363.0927, 351.0499, 327.0516, 325.0733, 309.0438, 307.0617, 287.0576, 243.0306, 241.0524, 217.0513, 175.041, 167.0355, 125.0245

**Table 6 continued.**

uncolored	no ID	3.86	negative	431.1571	299.1094, 191.0582, 149.047, 119.05, 99.0113, 89.0259, 71.0144, 59.0145
uncolored	no ID	4.52	negative	357.0588	311.0537, 289.0721, 245.0821, 203.0711, 137.0245, 109.0302
uncolored	no ID	5.8	negative	437.0509	419.0418, 391.049, 285.3968, 285.0378, 284.0348, 283.0264, 227.0345, 171.0448, 151.0035, 123.0059
uncolored	no ID	4.16	negative	577.1356	451.1055, 425.0901, 407.0788, 339.0898, 289.0725, 287.0565, 245.0819, 203.0691, 137.0238, 125.0244
uncolored	no ID	3.43	negative	577.1423	559.1265, 457.1053, 425.0921, 407.0798, 339.0899, 289.0736, 245.0829, 161.0252, 125.0248
uncolored	no ID	2.42	negative	863.1943	711.1417, 693.1323, 649.1332, 575.1234, 513.123, 449.0925, 407.0818, 297.0422, 287.0565, 243.0302, 167.0353
uncolored	no ID	6.04	negative	597.1882	477.1443, 357.1041, 345.1067, 339.0859, 315.0899, 233.0458, 209.0467, 191.0366, 167.0354, 125.0244
uncolored	no ID	7.37	negative	540.149	494.1429, 472.1618, 472.1854, 350.0873, 321.0949, 254.043, 232.0646, 212.0338, 172.0403, 144.0457, 132.0454
uncolored	no ID	5.8	negative	575.1223	539.101, 449.0882, 423.0769, 407.0779, 327.0521, 289.0725, 287.0548, 285.0419, 177.0193, 175.0397, 163.0038, 125.0247
uncolored	no ID	4.17	positive	601.1302	449.0829, 431.716, 311.0526
uncolored	no ID	7.4	positive	496.157	none
uncolored	no ID	4.53	positive	291.0866	207.0651, 165.0548, 161.0593
uncolored	no ID	3.67	positive	318.1545	265.1079, 247.0967, 229.0857, 147.0437, 139.0387, 123.0439, 115.0543, 111.0441, 91.0552, 77.0399, 65.0406, 55.0207
uncolored	no ID	7.4	positive	512.1319	none

**Table 6 continued.**

uncolored	no ID	4.42	positive	865.1955	713.1505, 695.1389, 575.1172, 205.0844, 187.0751, 163.0598, 145.0497, 127.0387, 121.0653, 85.0299, 77.0401, 69.0351, 57.036, 53.0416
uncolored	no ID	3.8	positive	291.0859	207.0643, 179.0682, 165.0539
uncolored	no ID	3.67	positive	470.1613	399.0965, 339.0746, 320.1014, 161.0598, 147.0436, 139.0388, 123.0439, 119.0485, 115.0544, 111.0438, 91.0554, 77.0391
uncolored	no ID	4.53	positive	313.0674	279.0533
uncolored	no ID	4.64	positive	575.1019	539.098, 529.134, 279.0533, 261.0269, 251.0664, 219.0314, 201.0065, 177.0222, 170.406, 158.9965, 140.9861, 121.0652, 98.9752, 77.0406
uncolored	no ID	1.04	positive	365.6434	203.052, 185.0414
uncolored	no ID	8.35	positive	471.2209	335.095
uncolored	no ID	3.33	positive	577.1332	541.1306, 451.0998, 449.0806
uncolored	no ID	3.66	positive	385.081	339.3446
uncolored	no ID	4.56	positive	330.0386	311.4504, 279.0465, 237.0408, 201.0073, 175.005, 163.006, 126.969, 110.9749, 98.9766, 68.9664
uncolored	no ID	4.03	positive	617.6813	311.0522, 287.0526, 191.0045, 173.019, 160.9945, 140.0411, 139.0389
uncolored	no ID	4.53	positive	329.041	190.9962, 172.9939, 160.988
uncolored	no ID	7.4	positive	336.107	192.0642, 174.0522, 146.0596, 132.9961
uncolored	no ID	5.27	positive	383.1665	221.1129, 128.049
uncolored	no ID	3.9	positive	471.1259	None
uncolored	no ID	4.8	positive	577.1338	559.1175, 451.0739, 435.0754, 409.0917, 301.0684, 289.0726, 275.0703, 271.0583, 245.0411, 163.0373, 123.0434
colored	no ID	4.99	negative	603.1596	449.1087, 439.1136, 421.0948, 299.0563, 271.0261, 259.0621, 175.04
colored	no ID	4.99	negative	623.1428	471.0916, 449.1119, 381.0565, 293.0443, 269.0619, 269.0464, 227.0335
colored	no ID	3.34	negative	447.1531	315.108, 191.0565, 174.9567, 135.0455, 89.0257

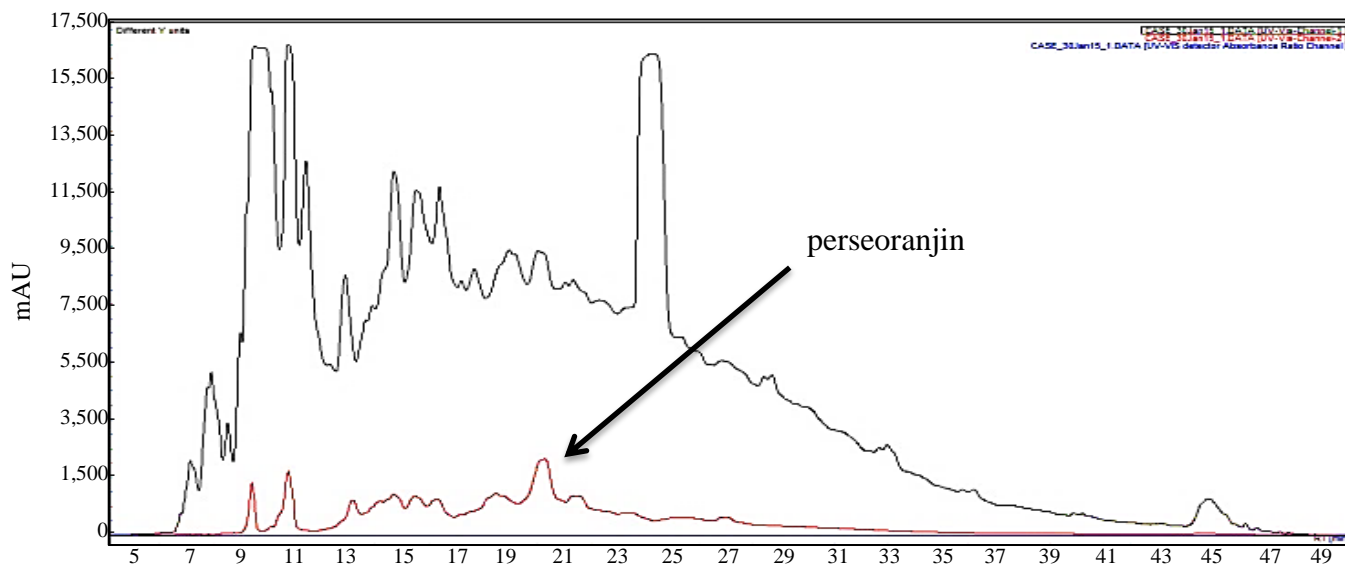
**Table 6 continued.**

colored	no ID	4.96	negative	733.2036	581.1564, 571.1712, 439.1058, 421.0892, 259.0599
colored	no ID	5.18	negative	887.2102	725.1714, 449.1034, 394.0628
colored	no ID	4.99	negative	691.1336	645.1358, 623.1358, 623.1447, 539.0832, 471.0935, 449.1107, 381.0565, 309.0367, 293.4312, 269.0458, 225.0515
colored	no ID	4.99	negative	601.4094	none
colored	no ID	10.59	positive	334.1114	306.1059, 230.0734, 229.0682
colored	no ID	5.01	positive	625.6052	473.1048, 311.0514, 203.0624, 127.0308, 126.0243, 105.0458, 77.0403, 58.9978, 51.0265
<b>*colored</b>	<b>no ID</b>	<b>5.01</b>	<b>positive</b>	<b>603.169</b>	451.1201, 441.1167, 395.1102, 289.0697, 271.0589, 243.0636, 215.0697, 147.0432

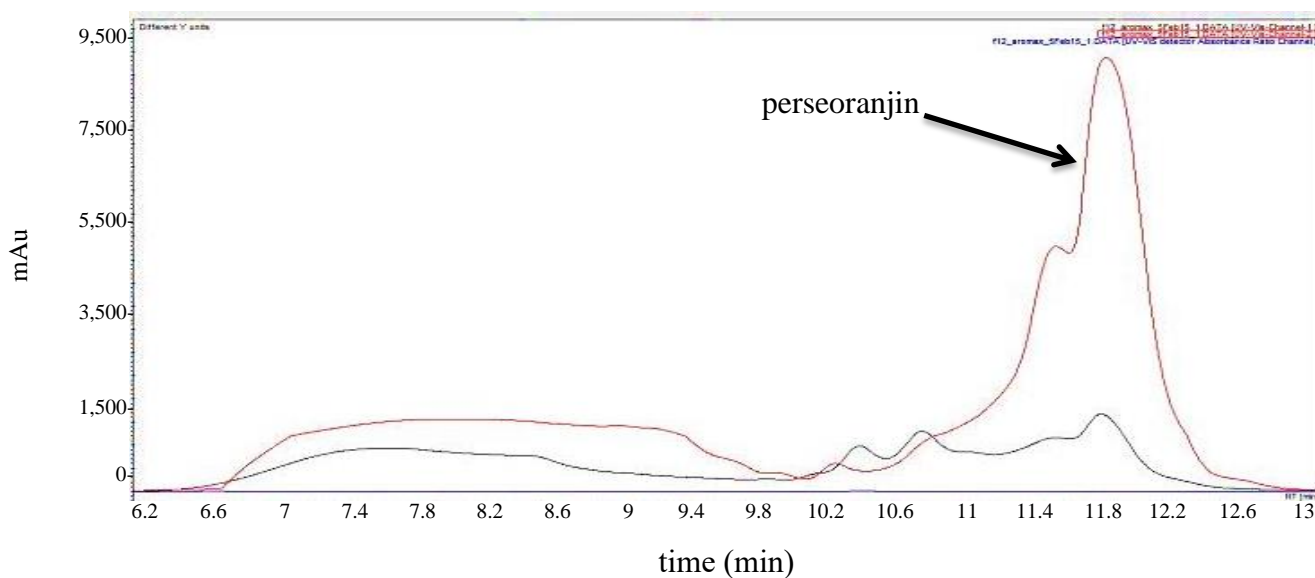
\* [M+H]<sup>+</sup> 603.169 was explored further in these studies

### 3.5 Structure Elucidation

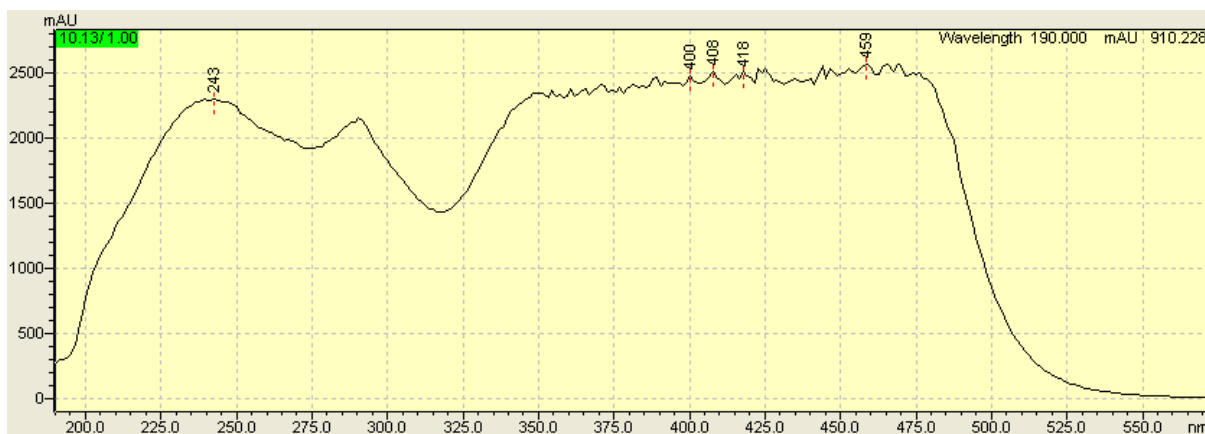
Purification of the extract consisted of multiple chromatographic steps. As is common with natural products, changes in the chromatogram were encountered between seed batches. The initial purification step was filtration and purification with amberlite, leading to a redder extract (~29% yield). The semi-pure, post amberlite extract was then purified using a preparatory C18 HPLC column (Figure 21). A single fraction from that analysis, “F12”, was further purified using a Restek ultra aromax preparatory HPLC column (Figure 22). Perseoranjin eluted as the second of two overlapping compounds. A UV-vis trace of perseoranjin is shown in Figure 23.



**Figure 21:** Semi-pure, post-amberlite CASE. Samples were analyzed at 280 nm (top, black) and at 445 nm (bottom, red).



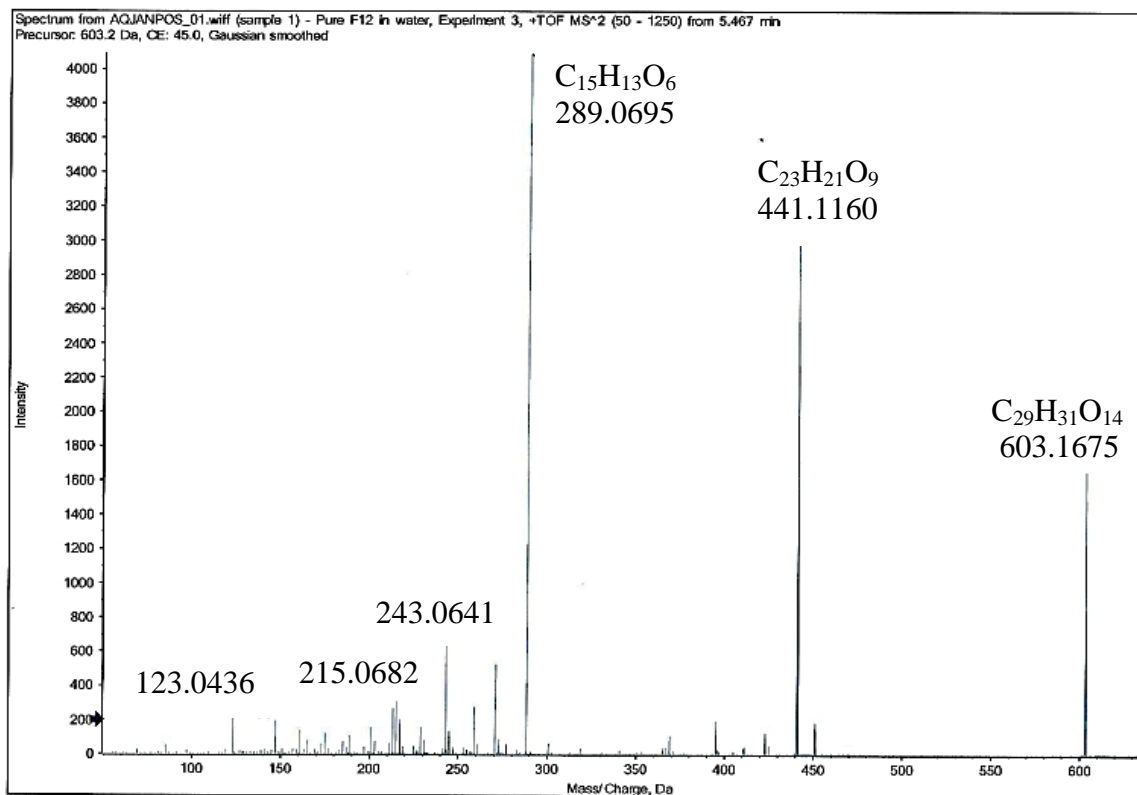
**Figure 22:** Post-C18 rough “F12” samples were analyzed at 280 nm (bottom, black) and at 445 nm (top, red).



**Figure 23:** UV-vis trace of persinoranjin shows maxima in the 400-480 nm range.

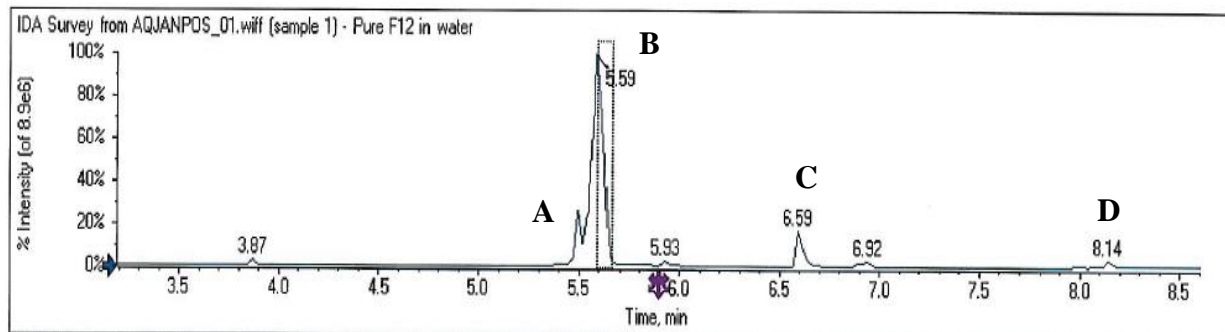
Persinoranjin, collected from the ultra aromax column, was analyzed via high resolution MS/MS. Total concentration in seed extract was not calculated due to the limited quantity, but it is believed to be in the low PPB range. The HRMS calculated molecular formula for  $[M+H]^+$  603.1675 was  $C_{29}H_{31}O_{14}$ . An abundant  $m/z$  441.1160 fragment ( $\Delta m/z$  162), indicated the presence of a monosaccharide (Figure 24). After passing through 3-6 purification steps, the extract still retained some impurities including compounds of  $[M+H]^+$  603.1687,  $[M+H]^+$  917.2639 which contained an  $m/z$  603 fragment, and  $[M+H]^+$  1205 which was a dimer produced from the combination of two  $[M+H]^+$  603 compounds (Figure 25).





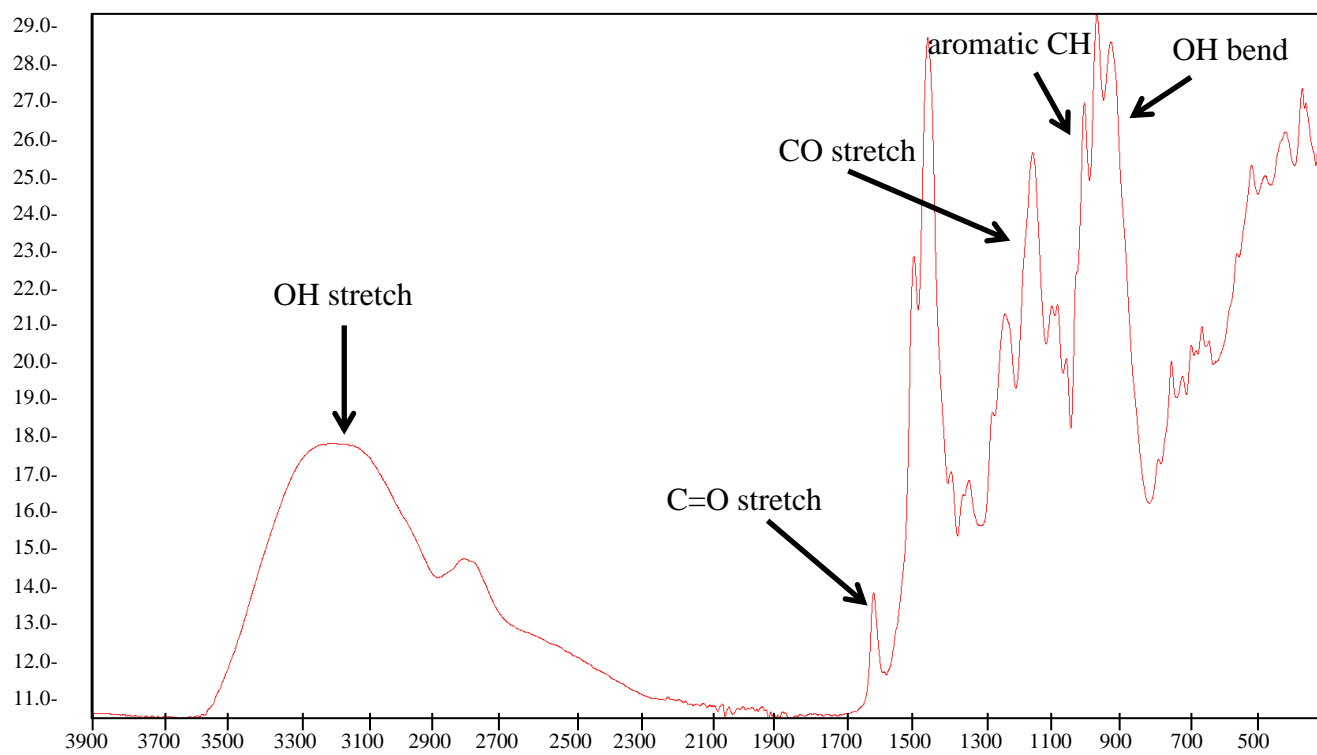
**Figure 24:** MS/MS analysis indicated an  $[M+H]^+$  603.1675 parent peak.

Full MS data for the purified compound “F12” are listed below. The spectrum is shown above in Figure 24. MS  $m/z$  (relative intensity): 603.1675 (42.1%), 441.1160 (74.4%), 289.0695 (100%), 243.0641 (15.2%), 215.0682 (7.3%), 123.0436 (4.9%).



**Figure 25:** Analysis of perseoranjin included a  $[M+H]^+$  917.2639 peak (A), the compound of interest,  $[M+H]^+$  603.1675 peak (B), another  $[M+H]^+$  603.1687 peak (C), and  $[M+H]^+$  1205 dimer produced from the combination of two  $[M+H]^+$  603 compounds (D).

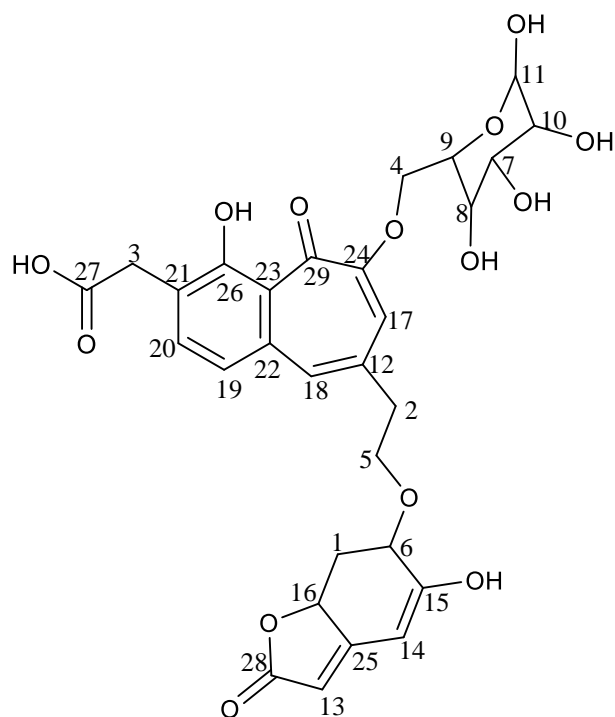
Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) analysis showed a broad OH band at  $3300\text{ cm}^{-1}$  and a peak at  $1740\text{ cm}^{-1}$  indicating the presence of carboxylic acid and/or ketone. Several important peaks have been assigned in Figure 26. The full ATR data for perseoranjin is as follows. IR ( $\text{cm}^{-1}$ ): 3300, 2900, 1740, 1640, 1580, 1475, 1350, 1300, 1200, 1120, 1100, 1030, 850, 800, 650, 550, 500.



**Figure 26:** ATR-FTIR analysis of perseoranjin, the most prominent colored compound.

Based on the above data, as well as high resolution NMR analysis, the compound was found to be 2-(4-hydroxy-8-(2-((5-hydroxy-2-oxo-2,6,7,7a-tetrahydrobenzofuran-6-yl)oxy)ethyl)-5-oxo-6-((3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-yl)methoxy)-5H-benzo[7]annulen-3-yl)acetic acid, or perseoranjin, a glycosylated benzotropolone with multiple side chains, having a

molecular formula of  $C_{29}H_{30}O_{14}$  (Figure 27). A summary of the NMR data for perseoranjin can be seen in Table 7. Spectra from samples in  $(CD_3)_2SO$  can be seen in Figures 28-33.



**Figure 27:** Structure of 2-(4-hydroxy-8-(2-((5-hydroxy-2-oxo-2,6,7,7a-tetrahydrobenzofuran-6-yl)oxy)ethyl)-5-oxo-6-((3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-yl)methoxy)-5H-benzo[7]annulen-3-yl)acetic acid, with the common name perseoranjin.

NMR Correlations of perseoranjin in (CD <sub>3</sub> ) <sub>2</sub> SO				
Position	C ppm	H ppm	HMBC	COSY
1*	28.9362	2.777, 2.647		6, 16
2*	43.8557	2.099, 1.943		5
3*	50.2423	3.523, 3.338		
4*	61.442	3.639, 3.400	29	
5*	64.1187	3.782, 3.364		2
6*	64.3734	4.1		1
7*	70.3375	3	10	
8*	73.8364	2.86	10	
9	76.8902	4.47		4
10*	77.2339	3.05	7, 8	
11*	79.9933	5.09		
12	88.9941		2, 17	
13*	90.704	6.14	14, 25	
14	102.7507		13	
15	103.2829		1	
16*	103.331	4.01		1
17*	113.1622	6.49		
18*	115.1565	6.94		
19*	115.3991	6.75		
20*	118.3498	6.73	18	
21	130.1123		19, 20	
22	145.2667	8.91	19, 20	
23	145.3811	8.91	18	
24	155.383			
25	165.3164		13	
26	166.5427		3	
27	172.4969			
28	178.3405			
29	192.7777		4	

**Table 7:** NMR assignments for perseoranjin in (CD<sub>3</sub>)<sub>2</sub>SO. Asterisks indicate C-H connections confirmed by DEPT-edited-HSQC experiments.

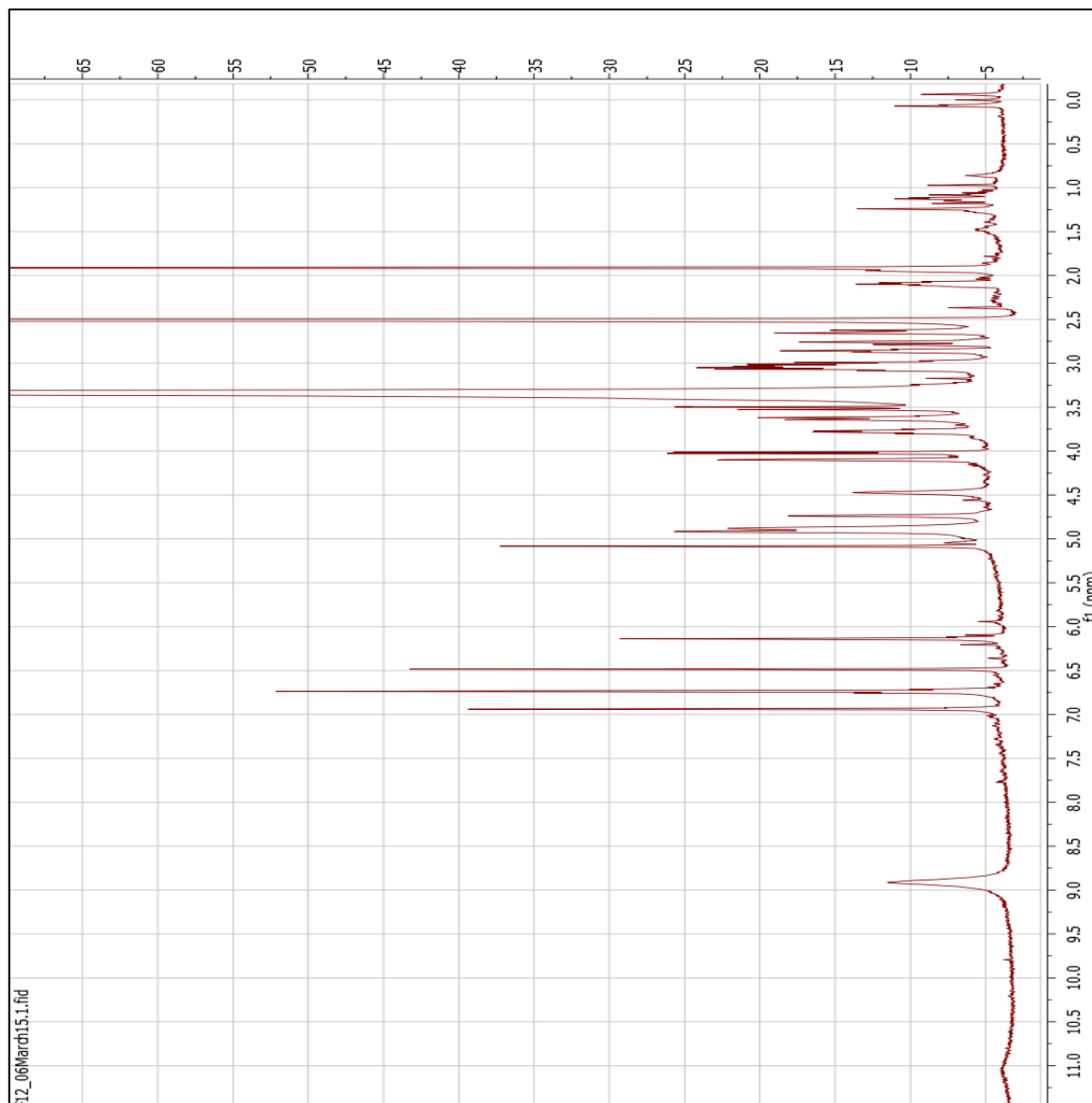
The full data for perseoranjin in  $(\text{CD}_3)_2\text{SO}$  are listed below. Additional experiments were conducted on perseoranjin dissolved in  $\text{D}_2\text{O}$ , however it was primarily the  $(\text{CD}_3)_2\text{SO}$  data that were used in assigning positions in the structure. Data of perseoranjin in  $\text{D}_2\text{O}$  can be found in the supplemental information, Figures 35-41.

Figure 28:  $^1\text{H}$  NMR (500 MHz,  $(\text{CD}_3)_2\text{SO}$ )  $\delta$  8.91 (s, 2H), 6.94 (s, 1H), 6.74 (dd, 2H), 6.48 (s, 1H), 6.14 (s, 1H), 5.08 (s, 1H), 4.90 (d,  $J = 15.5$  Hz, 1H), 4.14 – 4.06 (m, 2H), 4.02 (d,  $J = 7.7$  Hz, 1H), 3.78 (td,  $J = 9.3, 6.1$  Hz, 1H), 3.63 (d,  $J = 11.7$  Hz, 1H), 3.51 (d,  $J = 14.6$  Hz, 1H), 3.33 (m, 3H), 3.03 (dp,  $J = 25.5, 9.3, 8.9$  Hz, 1H), 2.86 (t,  $J = 8.3$  Hz, 1H), 2.77 (dd,  $J = 16.6, 4.0$  Hz, 1H), 2.64 (d,  $J = 16.0$  Hz, 1H), 2.10 (ddd,  $J = 14.5, 8.6, 5.7$  Hz, 1H), 1.91 (m, 1H).

Figure 29:  $^{13}\text{C}$  NMR (126 MHz,  $(\text{CD}_3)_2\text{SO}$ )  $\delta$  192.76 (C=O, C29), 178.33 (C=O, C28), 172.48 (C=O, C27), 166.53 (C26), 165.30 (C25), 155.37 (C24), 145.37 (C23), 145.25 (C22), 130.10 (C21), 118.34 (CH, C20), 115.39 (CH, C19), 115.15 (CH, C18), 113.16 (CH, C17), 103.32 (CH, C16), 103.28 (C15), 102.74 (CH, C14), 90.70 (CH, C13), 88.99 (C12), 79.99 (CH, C11), 77.23 (CH, C10), 76.89 (CH, C9), 73.83 (CH, C8), 70.33 (CH, C7), 64.37 (CH, C6), 64.11 ( $\text{CH}_2$ , C5), 61.44 ( $\text{CH}_2$ , C4), 50.24 ( $\text{CH}_2$ , C3), 43.85 ( $\text{CH}_2$ , C2), 28.94 ( $\text{CH}_2$ , C1).

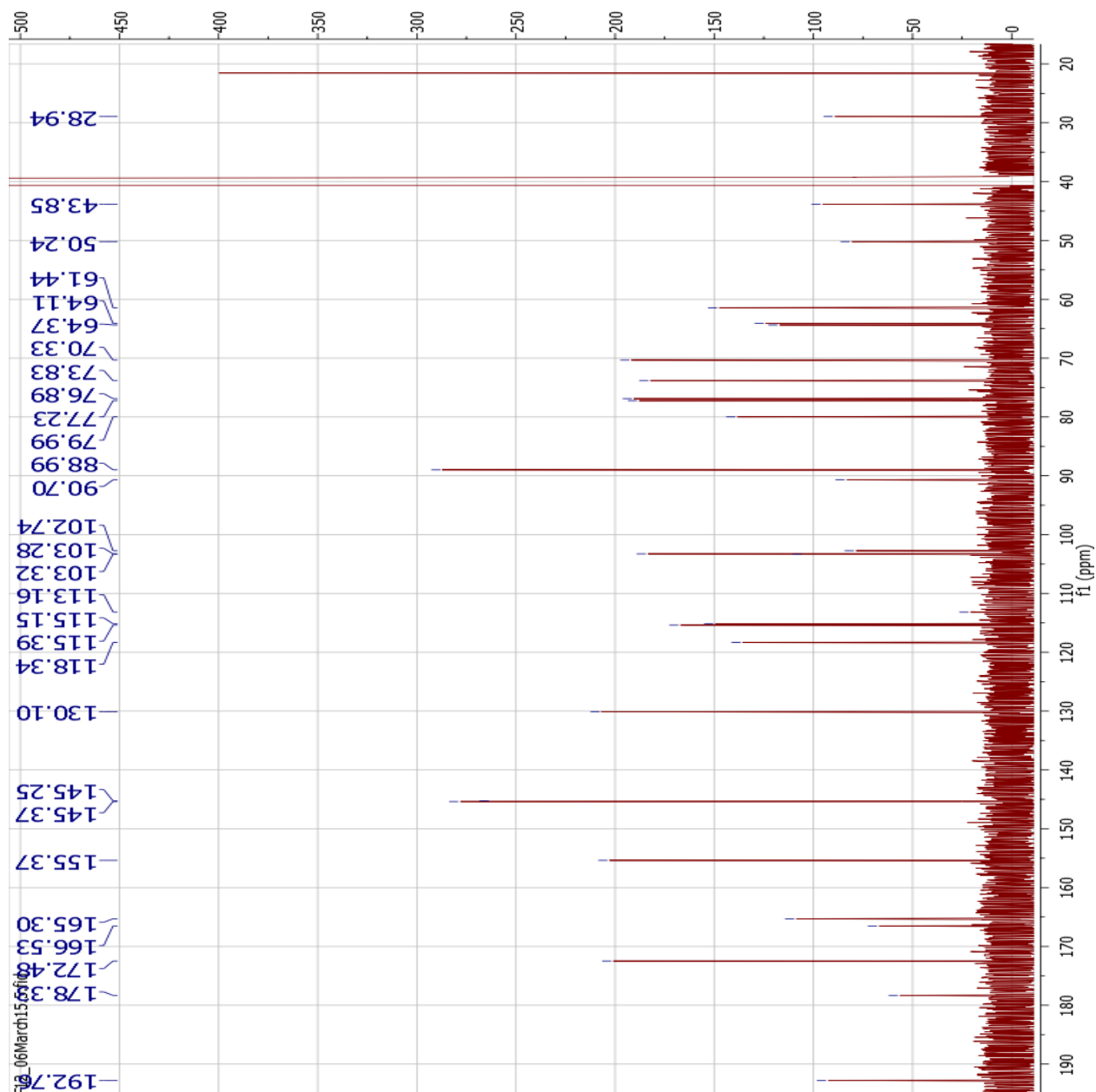
Purified perseoranjin samples contained some structurally similar contaminants, and some peaks may be seen in the NMR data due to these contaminants. The proton spectrum (Figure 26) showed many multiplets in the 5-2 ppm region, indicative of sugar protons and OH groups. There are five  $\text{CH}_2$  groups, however, for those groups on carbons 3, 4, and 5, one proton signal from each  $\text{CH}_2$  group is hidden by a large water peak at approximately 3 ppm, making them difficult to identify. The spectrum also showed a very broad, low intensity peak near 11 ppm. This peak is likely to be due to the interaction between the OH on C26 and the C=O at

C29. These HO – O=C correlations have been observed in similar compounds, often appearing very downfield around 10-13 ppm. It was necessary to use  $(\text{CD}_3)_2\text{SO}$  and  $\text{D}_2\text{O}$  as solvents due to low solubility of perseoranjin in any organic solvent. Unfortunately these solvent cause some difficulties when trying to identify  $\text{CH}_2$  protons, and the many OH groups which possess protons which exchange rapidly in these solvents, leading to a decrease in the intensity of their signals.



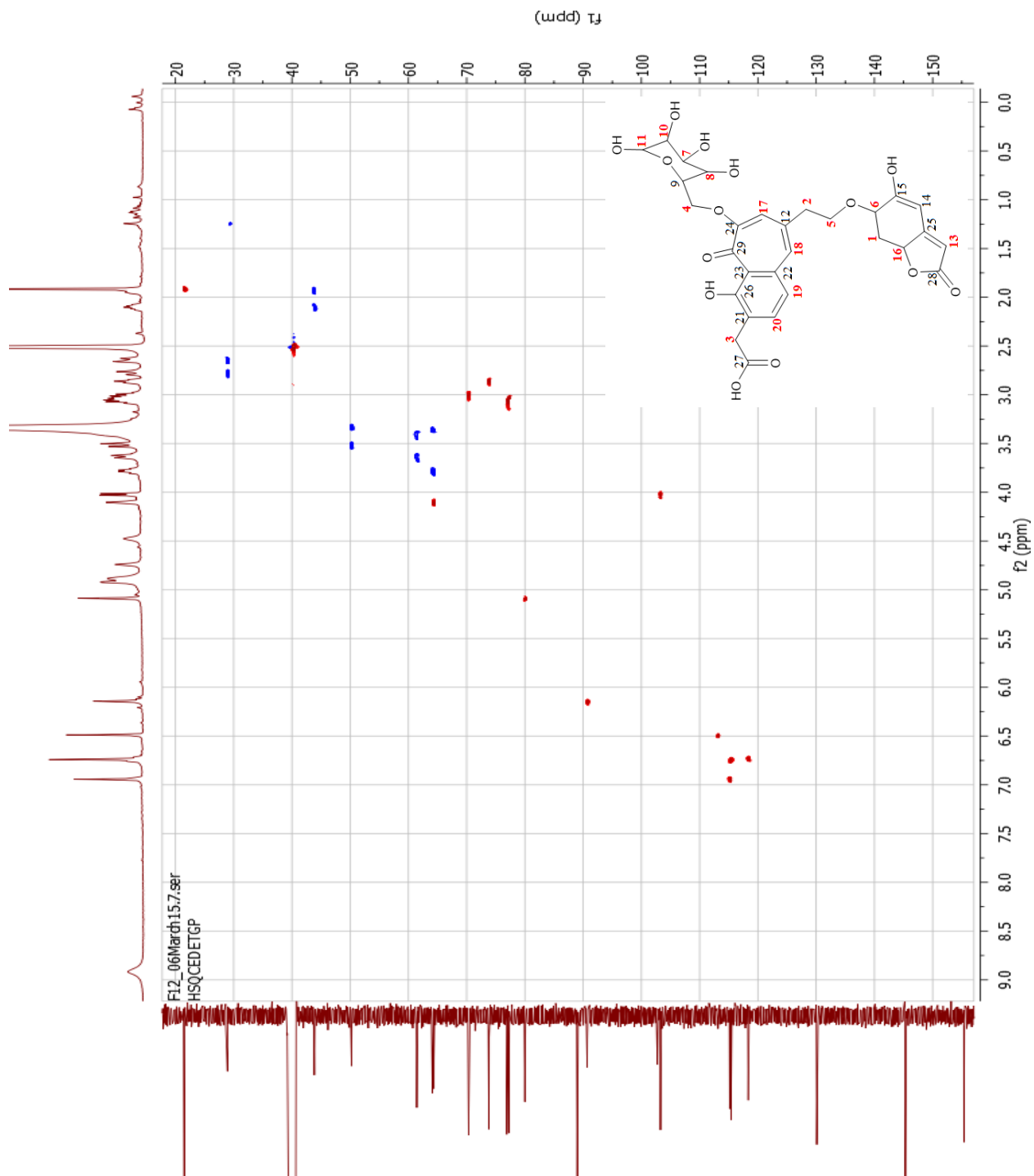
**Figure 28:**  $^1\text{H}$  NMR of "F12" in  $(\text{CD}_3)_2\text{SO}$ .

In the carbon spectrum, 30 peaks were found, 29 of which were assigned to perseoranjin. Assigned peaks are marked below. A carbon at 113.16 ppm had an extremely low intensity, however, it had correlations in both the DEPT-edited-HSQC as well as in the HMBC, indicating that it was a true peak, the carbon of which belonged to perseoranjin.



**Figure 29:**  $^{13}\text{C}$  NMR of perseoranjin in  $(\text{CD}_3)_2\text{SO}$ .

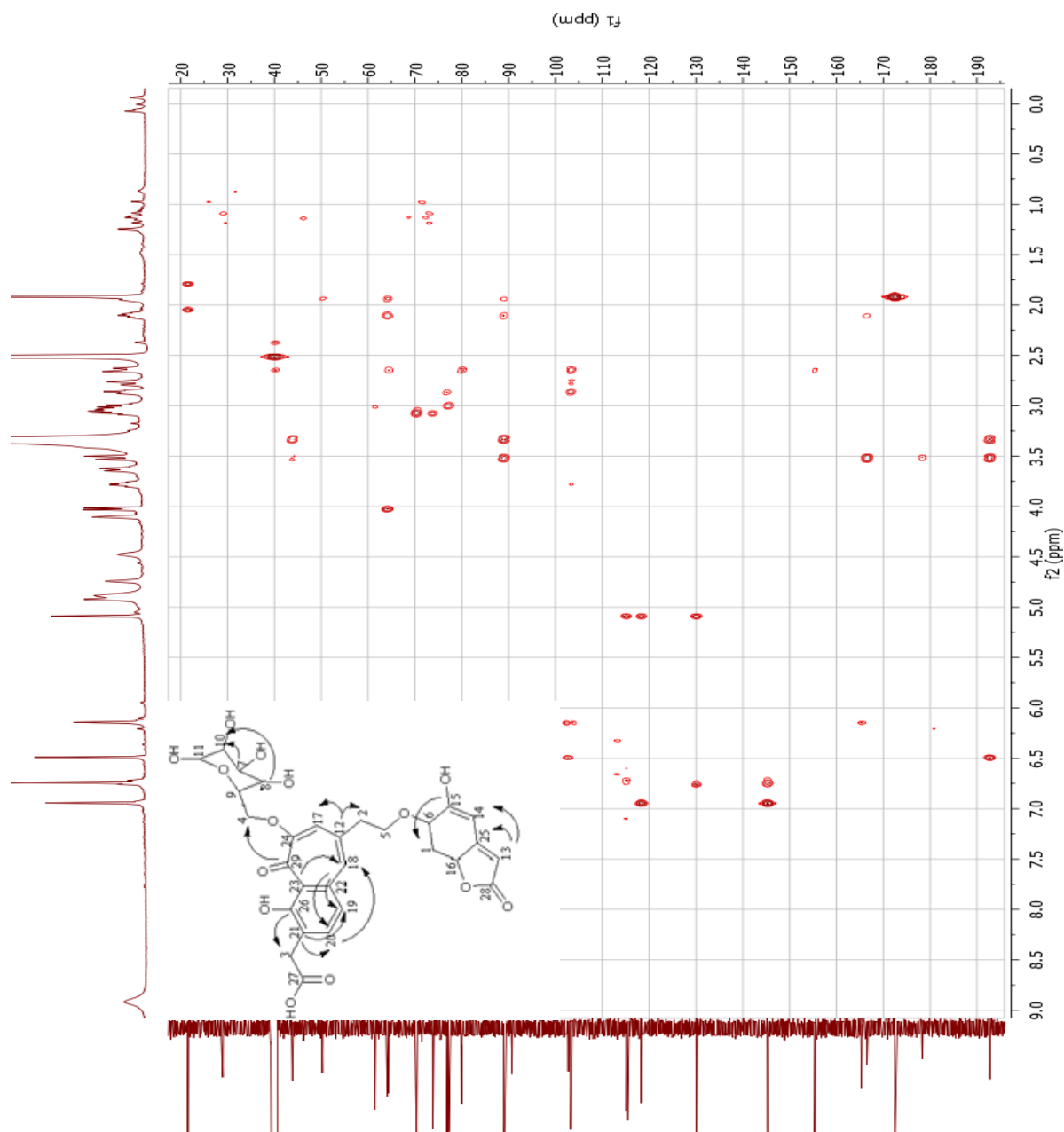
DEPT-edited-HSQC confirmed sixteen CH connections, including the presence of five CH<sub>2</sub> groups, indicated by red numbers in Figure 28.



**Figure 30:** DEPT-edited HSQC of perseoranjin in (CD<sub>3</sub>)<sub>2</sub>SO.

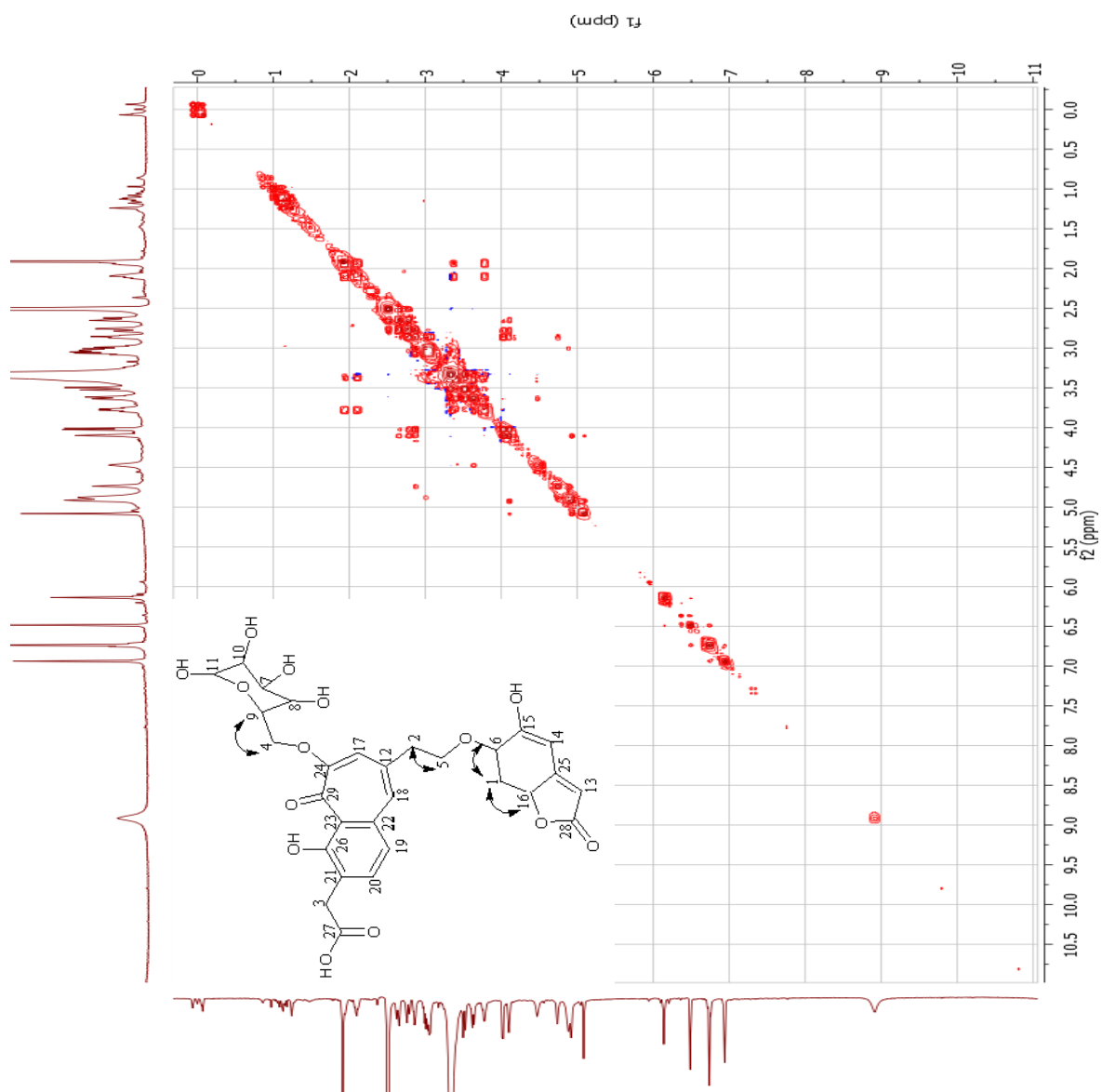


HMBC spectrum correlations, indicated by arrows, are shown below in Figure 29. A correlation between carbon 29 and 4 indicated the presence of the monosaccharide on the tropolone ring, while a correlation between carbon 26 and 3 indicated the presence of another, isolated CH<sub>2</sub> on the aromatic ring.



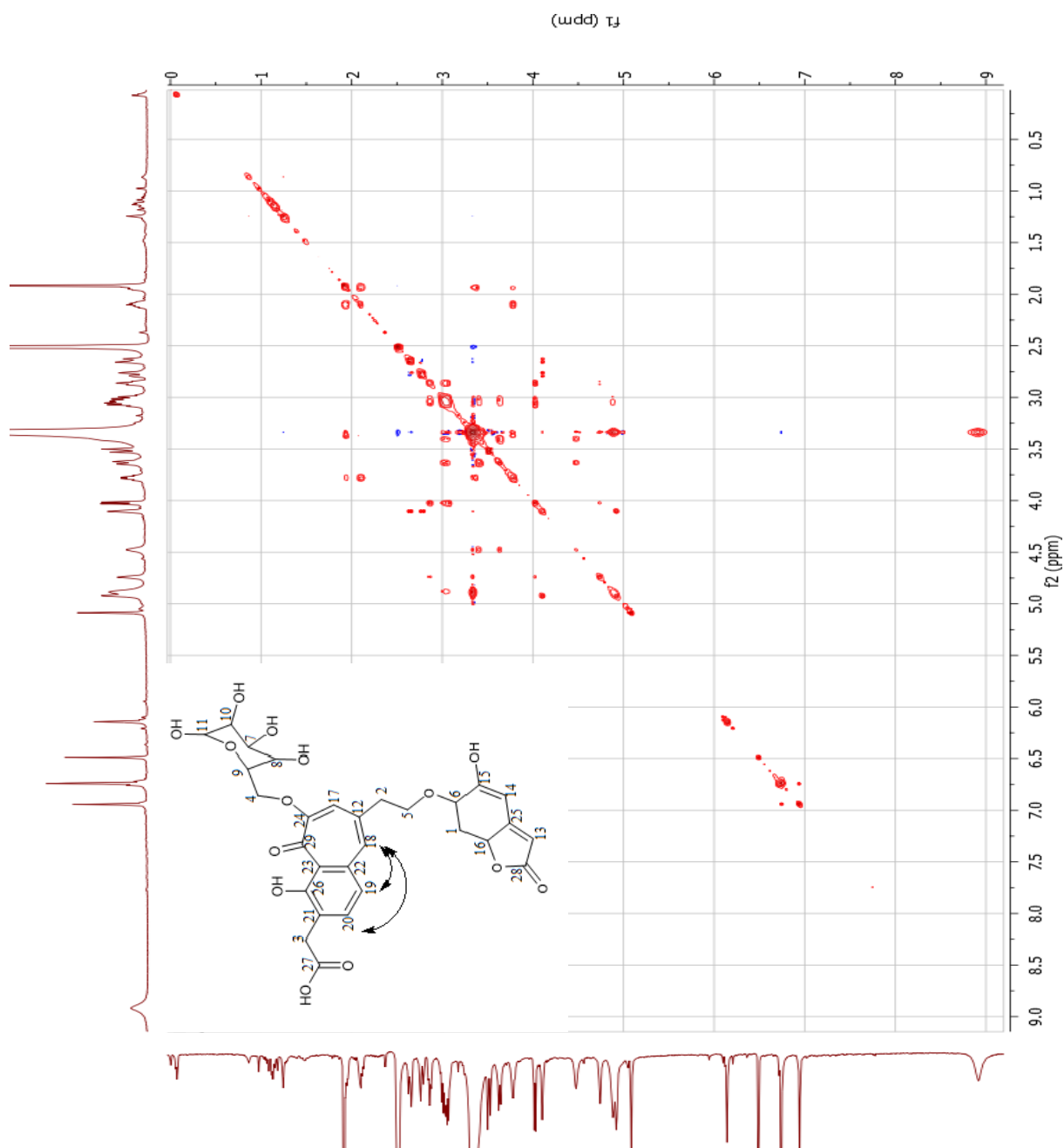
**Figure 31:** HMBC NMR of perseoranjin in (CD<sub>3</sub>)<sub>2</sub>SO. Arrows on the structure indicate correlations.

COSY correlations (Figure 30) were crucial in determining the presence of two adjacent CH<sub>2</sub> groups on carbons 2 and 5. It also indicated the presence of a separate, non-aromatic ring spin-system around carbons 6, 1, and 16. The lack of a COSY correlation to carbon 3 indicated its isolation from adjacent protons, while a single COSY correlation between carbon 4 and 9 indicated that it was the CH<sub>2</sub> of the monosaccharide.



**Figure 32:** COSY analysis of perseoranjin in (CD<sub>3</sub>)<sub>2</sub>SO.

TOCSY correlations indicated connections between protons of the monosaccharide. A correlation between carbon 18, 19, and 20 indicated their close proximity on the benzotropolone moiety. A correlation between 18 and 19 was also seen in the NOESY spectrum of a prepared D<sub>2</sub>O sample (Supplemental Data, Figure 41).



**Figure 33:** TOCSY analysis of perseoranjin in (CD<sub>3</sub>)<sub>2</sub>SO.

## Chapter 4: Discussion

In the stability study, semi-pure CASE proved to be a stable colorant over a variety of light and temperature conditions. As it is water soluble, semi-pure CASE lends itself particularly well to beverage and candy uses, but would also do well as a component of a flavor or sauce mix. Color stability under heat conditions is a common concern when working with natural colorants as many can lose their color during baking or pasteurization. Semi-pure CASE was shown to be stable during both storage at 40° C and baking. However, special care must be taken when considering the final texture and mouthfeel of semi-pure CASE colored food products, as high concentrations may lead to a denser crumb texture, or an antioxidant related decrease in Maillard browning.<sup>108</sup> For other food products such as frostings and fillings, it may be possible to combine semi-pure CASE with alumina or some other material to create a lake. The use of semi-pure CASE in food products is possible, however it is necessary to add a concentration 10-100 times more semi-pure CASE than the corresponding amount of artificial colorant needed to achieve a similar final color. This is due to the low concentration of colored compounds within the extract. Perseoranjin is believed to be particularly potent, as it produces a vibrant color in the seed extract despite its presence in the low PPB range. It will be important to assure the safety of semi-pure CASE consumption before production of foods with added semi-pure CASE, especially those with relatively large amounts of semi-pure CASE.

Perseoranjin will be able to produce a wide range of colors from pale yellow, to orange, to red, to a deep red-brown color. This wide range of colors is achievable by placing the compound under alkali conditions before readjusting to the desired pH. This means that even in low pH foods CASE can provide a range of yellow and orange colors, which is ideal for its use

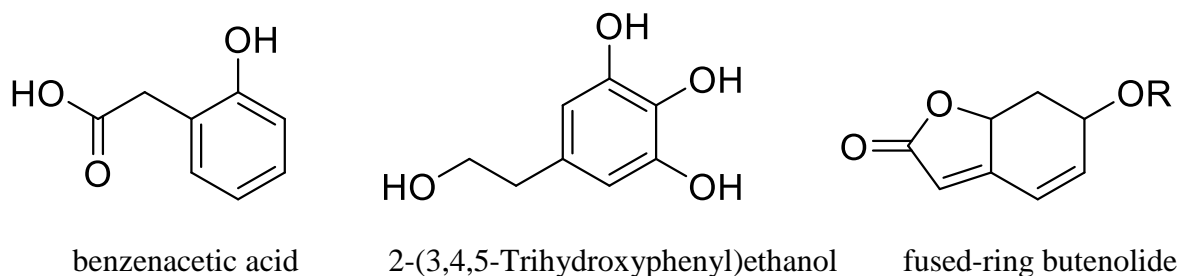
in beverages juices and sodas, as well as the many unique fall/Halloween treats such as orange colored milk.

Comparison of colored and uncolored seed extracts showed a number of known compounds as well as some of undetermined structure. Some of these compounds may act as precursors for perseoranjin, however, due to the low amount needed for complete formation of perseoranjin, it is unlikely that a change in the overall concentration of those precursors would be observed. Preparation of an uncolored extract proved to be particularly difficult, as even immediate addition of the seeds to a tropolone solution led to a slightly yellow extract. Once a seed is cut or damaged in anyway, it immediately begins forming the orange compounds. For that reason we were unable to produce a completely colorless extract.

The inhibition of color formation caused by the addition of tropolone implied the likelihood of a benzotropolone moiety in the colored compound. High resolution mass spectrometry, as well as NMR analysis, confirmed the presence of a monosaccharide which is believed to be the cause of the compound's low solubility in organic solvents. ATR confirmed the presence of C=O bonds, and specifically the presence of a carboxylic acid. The presence of an aromatic ring-CH<sub>2</sub>-carboxylic acid system was confirmed through a combination of NMR experiments.

Perseoranjin was indeed found to be a novel glycosylated benzotropolone compound. COSY and TOCSY experiments confirmed the presence of another spin system, removed from the benzotropolone moiety which was found to be a ring-fused butenolide moiety similar to that found in plants of the Ranunculaceae family such as buttercups and crow's foot.<sup>109</sup> An initial synthesis attempt could make use of tyrosinase from mushrooms or horseradish peroxidase to

provide the enzymatic formation of the 7-membered ring. Compounds such as benzenacetic acid and 2-(3,4,5-Trihydroxyphenyl)ethanol (Figure 34) could combine via enzymatic synthesis to form the benzotropolone moiety including the  $\text{CH}_2\text{-COOH}$  on the aromatic ring, as well as



**Figure 34:** Some potential precursors for enzymatic synthesis of perseoranjin.

OH groups which could easily be involved in the addition of multiple side chains including a monosaccharide and a fused-ring butenolide moiety.

Esters can hydrolyze via a variety of mechanisms, particularly in the case of lactones. In the presence of a strong base lactones can hydrolyze to form their parent compound, a bifunctional straight chain compound.<sup>110</sup> The color dependence of perseoranjin on pH may be due to the deprotonation of various OH and  $\text{CH}_2$  groups, and the opening of the butenolide rings at high pH. Ring opening and the deprotonation of OH and  $\text{CH}_2$  groups could lead to an increase in the number of double bonds in the compound, causing an increase in conjugation which is observed as a red-shift in the color spectrum.

## Chapter 5: Conclusions and Future Work

A colored avocado seed extract was shown to have stable color under a variety of heat, light, and shelf conditions and was able to produce a variety of yellow, orange, and red colors. Purification of perseoranjin from avocado seeds proved to be a very time and resource intensive process with many steps. In the future, synthetic production of perseoranjin is likely to provide a more efficient method of acquiring the compound than the extensive time and materials needed to purify the compound directly from seeds.

While a semi-pure extract may be useful in some applications, the high concentration needed may prove to be a hindrance for its use in foods. This issue was directly observed when semi-pure CASE was used in baking, as the high polyphenol content lead to a smaller loaf volume, denser crumb, and decrease in crust browning. In the future, a synthetic route for the production of perseoranjin will make it possible to expand its uses as a synthesized natural colorant. In addition, some other studies will need to be conducted in order to determine the safety of consumption of the semi-pure extract and perseoranjin, and to help determine an allowable daily intake levels (ADI) for consumers.

Perseoranjin is not the only compound responsible for the color in avocado seed extract, so another area of study will be to further explore CASE and determine the structures of other colored compounds, particularly the other 603, 917, and 1205 peaks seen in the mass spectrum of the fraction containing perseoranjin. The whole extract presents as a dark or reddish orange color, while perseoranjin is a more yellow orange. Further analysis of the whole extract could potentially determine the source of the redder color, which is of particular interest to the natural color market.<sup>2</sup> One useful area of study would be to work on fractionating CASE to obtain a mixture of 5-10 colored compounds. This purification could remove up to 90% of the

compounds present in semi-pure CASE while vastly increasing the color activity. This extract could be prepared as outlined in this thesis by using preparatory HPLC, or other chromatographic methods may be used.

In previous work, CASE was shown to have some beneficial anti-cancer, anti-inflammatory, and anti-oxidant properties when tested *in vitro* in human cancer cell lines.<sup>108</sup> Those effects were not replicated using the semi-pure CASE in the present study. This indicates that the colored compounds are likely not responsible for these health beneficial effects. It is more likely that other polyphenol compounds in the extract are responsible for the effects observed in cell line studies. Further analysis of whole extracts and extracts with differing purity levels may be useful for aiding in the determination of which compounds are responsible for those effects. This process, called high-throughput screening, is widely used by pharmaceutical companies and research hospitals to quickly determine the activity of a variety of compounds, extracts, or fractions. In such instances assays are conducted via robotics, and statistical modeling programs are used to predict which substances may have beneficial health properties. These methods are useful when working with a large number of compounds or fractions, however, a small-scale process could be completed by using metabolomics analysis of prepared fractions. Cancer cells could be treated with various fractions of semi-pure CASE to determine each fraction's effect on cell viability. If a fraction produces interesting results, it could be further explored using metabolomics. Scaling-up the purification of perseoranjin, conducting safety studies of extracts, and determining the source of CASE's health beneficial effects should be the primary goals when considering the future of this work.



## REFERENCES

- (1) MarketsandMarkets. Natural & synthetic food color market worth \$2.3 billion by 2019 <http://www.marketsandmarkets.com> (accessed Oct 8, 2015).
- (2) Future Market Insights. Natural Food Colours Market : Global Industry Analysis and Opportunity Assessment 2014 - 2020 <http://www.futuremarketinsights.com> (accessed Oct 8, 2015).
- (3) Dulai, K. S.; von Dornum, M.; Mollon, J. D.; Hunt, D. M. *Genome Res.* **1999**, *9*, 629–638.
- (4) Gilad, Y.; Wiebe, V.; Przeworski, M.; Lancet, D.; Pääbo, S. *PLoS Biol.* **2004**, *2* (1), 120–125.
- (5) Servedio, M. R. *Evolution* **2000**, *54* (3), 751–763.
- (6) Wilson, C. R.; Sauer, J. M.; Hooser, S. B. *Toxicol.* **2001**, *39*, 175–185.
- (7) Keller-Dilitz, H.; Moser, M.; Ammirati, J. F. *Mycol. Soc. Am.* **1985**, *77* (5), 667–673.
- (8) Summers, K.; Clough, M. E. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98* (11), 6227–6232.
- (9) Nishida, R. *Annu. Rev. Entomol.* **2002**, *47*, 57–92.
- (10) Garber Jr, L. L.; Hyatt, E. M.; Starr, R. G. *J. Mark. Theory Pract.* **2000**, *8* (4), 59–72.
- (11) Johnson, J. L.; Dzendolet, E.; Clydesdale, F. M. *J Food Prot.* **1983**, *46* (1), 21–28.
- (12) Johnson, J.; Clydesdale, F. M. *J. Food Sci.* **1982**, *47* (3), 747–752.
- (13) Pangborn, R. M.; Hansen, B. **1963**, *76* (2), 315–317.
- (14) Maga, J. A. *Chem. Senses Flavor* **1974**, *1*, 115–119.
- (15) Rohm, H.; Strobl, M.; Jaros, D. *Z Leb. Unters Forsch A* **1997**, *205* (2), 108–110.
- (16) Peracchio, L. a.; Meyers-Levy, J. *J. Consum. Res.* **1994**, *21* (1), 190.
- (17) Burrows, A. *Compr. Rev. Food Sci. Food Saf.* **2009**, *8*, 394–408.
- (18) Swartz, C. J.; Cooper, J. *J. Pharmaceutical Sci.* **1962**, *51* (2), 89–99.
- (19) Stevens, L. J.; Burgess, J. R.; Stochelski, M. a; Kuczek, T. *Clin. Pediatr. (Phila).* **2014**, *53* (2), 133–140.
- (20) FDA. Color Additive Status List <http://www.fda.gov> (accessed Oct 8, 2015).
- (21) Bateman, B.; Warner, J. O.; Hutchinson, E.; Dean, T.; Rowlandson, P.; Gant, C.; Grundy, J.; Fitzgerald, C.; Stevenson, J. *Arch. Dis. Child.* **2004**, *89* (6), 506–511.
- (22) Mccann, D.; Barrett, A.; Cooper, A.; Crumpler, D.; Dalen, L.; Grimshaw, K.; Kitchin, E.; Lok, K.; Porteous, L.; Prince, E.; Sonuga-Barke, E.; Warner, J. O.; Stevenson, J. *Lancet* **2007**, *370*, 1560–1567.
- (23) Drugs.com. FD&C Yellow No. 5 <http://www.drugs.com/inactive/fd-c-yellow-no-5-250.html> (accessed Oct 1, 2015).
- (24) Ardern, K. D. *Cochrane database Syst. Rev.* **2001**, No. 4, CD000460.

- (25) Erickson, Z. T.; Falkenberg, E. a; Metz, G. a S. *PLoS One* **2014**, 9 (6), e92132.
- (26) Luning, P.; Van der Spiegel, M.; Marcelis, W. J. In *Food Colorants: Chemical and Functional Properties*; Socaciu, C., Ed.; CRC Press, 2007; p 557.
- (27) However, A. Beta - carotene <http://umm.edu> (accessed Oct 8, 2015).
- (28) Tanvetyanon, T.; Bepler, G. *Cancer* **2008**, 113 (1), 150–157.
- (29) Tabar-Purroy, A. I.; Alvarez-Puebla, M. J.; Acero-Sainz, S.; García-Figueroa, B. E.; Echechipía-Madoz, S.; Olaguibel-Rivera, J. M.; Quirce-Gancedo, S. *J. Allergy Clin. Immunol.* **2003**, 111 (2), 415–419.
- (30) Anibarro, B.; Seoane, J.; Vila, C.; Mugica, V.; Lombardero, M. *Int J Occup Med Env. Heal.* **2003**, 16 (2), 133–137.
- (31) Hagiwara, A.; Imai, N.; Ichihara, T.; Sano, M.; Tamano, S.; Aoki, H.; Yasuhara, K.; Koda, T.; Nakamura, M.; Shirai, T. *Food Chem. Toxicol.* **2003**, 41 (8), 1157–1164.
- (32) FDA. Color Additives: FDA’s Regulatory Process and Historical Prospectives <http://www.fda.gov/ForIndustry/ColorAdditives/RegulatoryProcessHistoricalPerspectives/> (accessed Oct 20, 2015).
- (33) Mayer, A. M. *Phytochemistry* **2006**, 67 (21), 2318–2331.
- (34) Mustafa, R.; Muniglia, L.; Rovel, B.; Girardin, M. *Food Res. Int.* **2005**, 38, 995–1000.
- (35) EC 1.14.18.1 <http://enzyme.expasy.org/EC/1.14.18.1> (accessed Oct 10, 2015).
- (36) Doğan, S.; Turan, P.; Doğan, M. *Process Biochem.* **2006**, 41 (12), 2379–2385.
- (37) Sang, S.; Tian, S.; Stark, R. E.; Yang, C. S.; Ho, C. T. *Bioorganic Med. Chem.* **2004**, 12 (11), 3009–3017.
- (38) Phillips, M. *The Apple Grower: A Guide for the Organic Orchardist*, illustrate.; Chelsea Green Publishing, 2005.
- (39) Ridgway, T.; Tucker, G.; Wiseman, H. *Biotechnol. Genet. Eng. Rev.* **1997**, 14, 165.
- (40) Guyot, S.; Serrand, S.; Le Quéré, J. M.; Sanoner, P.; Renard, C. M. G. C. *Innov. Food Sci. Emerg. Technol.* **2007**, 8 (3), 443–450.
- (41) Nakatsuka, T.; Yamada, E.; Takahashi, H.; Imamura, T.; Suzuki, M.; Ozeki, Y.; Tsujimura, I.; Saito, M.; Sakamoto, Y.; Sasaki, N.; Nishihara, M. *Sci. Rep.* **2013**, 3.
- (42) Menet, M.; Sang, S.; Yang, C. S.; Ho, C.-T.; Rosen, R. T. *J. Agric. Food Chem.* **2004**, 52, 2455–2461.
- (43) Ginda, H.; Kusumi, T.; Ishitsuka, M. O.; Kakisawa, H.; Weijie, Z.; Jun, C.; Tian, G. Y. *Tetrahedron* **1988**, 29 (36), 4603–4606.
- (44) Kerschensteiner, L.; Löbermann, F.; Steglich, W.; Trauner, D. *Tetrahedron* **2011**, 67 (8), 1536–1539.
- (45) Remias, D.; Schwaiger, S.; Aigner, S.; Leya, T.; Stuppner, H.; Lütz, C. *FEMS Microbiol. Ecol.* **2012**, 79 (3), 638–648.

- (46) Klostermeyer, D.; Knops, L.; Sindlinger, T.; Polborn, K.; Steglich, W. *European J. Org. Chem.* **2000**, *13* (4), 603–609.
- (47) Kandaswami, C.; Stohs, S.; Coyle, C. Composition and method for promoting weight loss. US 7989009 B2, 2006.
- (48) Cornelius, D. W.; Haynes, G. L. Metabolic enhancing properties of theaflavins and thearubigins. US 20090098224 A1, 2007.
- (49) Li, S.; REN, X.; Liu, J. Method of using theaflavin. US 20150094364 A1, 2014.
- (50) Barltrop, J. A.; Nicholson, J. S. *J. Chem. Soc.* **1948**, No. 116, 116–120.
- (51) Houghton, J. D.; Hendry, G. A. F. *Natural Food Colorants*, 2, illustr.; Media, S. S. and B., Ed.; 2012.
- (52) Huang, Y. L.; Tsujita, T.; Tanaka, T.; Matsuo, Y.; Kouno, I.; Li, D. P.; Nonaka, G. I. *Phytochemistry* **2011**, *72* (16), 2006–2014.
- (53) Fukui, N.; Ohmori, K.; Suzuki, K. *Helv. Chim. Acta* **2012**, *95* (11), 2194–2217.
- (54) Knight, R. J.; Schaffer, B. A.; Wolstenholme, B. N.; Whiley, A. W. In *The avocado: botany, production, and uses.*; CAB International: Wallingford, UK, 2002; pp 1–12.
- (55) Heiser, C. B. *Annu. Rev. Ecol. Syst.* **1979**, *10* (1), 309–326.
- (56) Chanderbali, A. S.; Albert, V. a.; Ashworth, V. E. T. M.; Clegg, M. T.; Litz, R. E.; Soltis, D. E.; Soltis, P. S. *Bioessays* **2008**, *30* (4), 386–396.
- (57) Ding, H.; Chin, Y. W.; Kinghorn, a. D.; D'Ambrosio, S. M. *Semin. Cancer Biol.* **2007**, *17* (5), 386–394.
- (58) USDA. *Avocado Situation and Outlook for Selected Countries*; 2006.
- (59) Morton, J. F.; Dowling, C. F. *Fruits of Warm Climate*, illustrate.; Morton JF: Winterville, NC, 1987.
- (60) Colette, C.; Percheron, C.; Pares-Herbute, N.; Michel, F.; Pham, T.-C.; Brillant, L.; Descomps, B.; Monnier, L. *Int. J. Obes. Relat. Metab. Disord.* **2003**, *27* (6), 648–656.
- (61) Pieterse, Z.; Jerling, J. C.; Oosthuizen, W.; Kruger, H. S.; Hanekom, S. M.; Smuts, C. M.; Schutte, a. E. *Nutrition* **2005**, *21* (1), 67–75.
- (62) Lu, Q. Y.; Arteaga, J. R.; Zhang, Q.; Huerta, S.; Go, V. L. W.; Heber, D. *J. Nutr. Biochem.* **2005**, *16* (1), 23–30.
- (63) Lu, Q. Y.; Zhang, Y.; Wang, Y.; Wang, D.; Lee, R. P.; Gao, K.; Byrns, R.; Heber, D. *J. Agric. Food Chem.* **2009**, *57* (21), 10408–10413.
- (64) Altinel, L.; Saritas, Z. K.; Kose, K. C.; Pamuk, K.; Aksoy, Y.; Serteser, M. *Tohoku J. Exp. Med.* **2007**, *211* (2), 181–186.
- (65) Kawcak, C. E.; Frisbie, D. D.; McIlwraith, W.; Werpy, N. M.; Park, R. D. *Am. J. Vet. Res.* **2007**, *68* (6), 598–604.
- (66) Boileau, C.; Martel-Pelletier, J.; Caron, J.; Msika, P.; Guillou, G. B.; Baudouin, C.;

- Pelletier, J.-P. *Arthritis Res. Ther.* **2009**, *11* (2), R41.
- (67) Kunow, M. A. *Maya medicine : traditional healing in Yucatan.*, illustrate.; University of New Mexico Press: Albuquerque, 2003.
- (68) Duke, J. A.; Martinez, R. V. *Amazonian ethnobotanical dictionary*; CRC Press: Boca Raton, FL, 1994.
- (69) Ozolua, R.; Anaka, O.; Okpo, S.; Idogun, S. *African J. Tradit. Complement. Med.* **2009**, *6* (4), 573–578.
- (70) Hatfield, G. *Encyclopedia of folk medicine : old world and new world traditions*; ABC-CLIO: Santa Barbara, CA, 2004.
- (71) Ramos-Jerz, M. D. R. Phytochemical analysis of avocado seeds (*Persea americana* Mill., c.v. Hass), Technical University Carolo-Wilhelmina, 2007.
- (72) Olaeta, J. a; Schwartz, M.; Undurraga, P.; Contreras, S. In *Proceedings VI World Avocado Congress*; Chile, 2007; pp 1–8.
- (73) Weatherby, L. S.; Glenn, S. *Ind. Eng. Chem.* **1931**, 1421–1423.
- (74) Takenaga, F.; Matsuyama, K.; Abe, S.; Torii, Y.; Itoh, S. *J. Oleo Sci.* **2008**, *57* (11), 591–597.
- (75) Soong, Y. Y.; Barlow, P. J. *Food Chem.* **2004**, *88* (3), 411–417.
- (76) Wang, W.; Bostic, T. R.; Gu, L. *Food Chem.* **2010**, *122* (4), 1193–1198.
- (77) Lee, S. G.; Yu, M. H.; Lee, S. P.; Lee, I. S. *J. Korean Soc. Food Sci. Nutr.* **2008**, *37* (3), 269–275.
- (78) Villa-Rodríguez, J. a.; Molina-Corral, F. J.; Ayala-Zavala, J. F.; Olivas, G. I.; González-Aguilar, G. a. *Food Res. Int.* **2011**, *44* (5), 1231–1237.
- (79) Prabha, T.; Patwardhan, M. *J. Food Sci. Technol.* **1980**, *17*, 215–217.
- (80) Rodríguez-Carpena, J.-G.; Morcuende, D.; Andrade, M.-J.; Kylli, P.; Estévez, M. *J. Agric. Food Chem.* **2011**, *59* (10), 5625–5635.
- (81) Kosińska, A.; Karamać, M.; Estrella, I.; Hernández, T.; Bartolomé, B.; Dykes, G. a. *J. Agric. Food Chem.* **2012**, *60* (18), 4613–4619.
- (82) Pahua-Ramos, M. E.; Ortiz-Moreno, A.; Chamorro-Cevallos, G.; Hernández-Navarro, M. D.; Garduño-Siciliano, L.; Necoechea-Mondragón, H.; Hernández-Ortega, M. *Plant Foods Hum. Nutr.* **2012**, *67* (1), 10–16.
- (83) Correspondent, S. Ugandan scientists develop anti-malarial drink  
<http://www.theeastafrican.co.ke/news/Ugandan-scientists-develop-anti-malarial-drink/-/2558/2121176/-/12xj3g4/-/index.html> (accessed Oct 22, 2015).
- (84) Etozioni, S. Lipidic polyols from plants as a natural source for phospholipase A2 inhibitors, Technion Israel Institute of Technology, 2003.
- (85) Rosenblat, G.; Meretski, S.; Segal, J.; Tarshis, M.; Schroeder, A.; Zanin-Zhorov, A.; Lion, G.; Ingber, A.; Hochberg, M. *Arch. Dermatol. Res.* **2011**, *303* (4), 239–246.

- (86) Edem, D. *Eur. J Sci Res* **2009**, No. 33, 669–678.
- (87) Ebong, P.; Edem, D.; Ekanem, I. *Pak. J. Pharm. Sci.* **2013**, No. 22, 272–276.
- (88) Imafidon, K. E.; Amaechina, F. C. **2010**, 4 (2), 116–121.
- (89) Kate, I. E.; Lucky, O. O. *World J. Med. Sci.* **2009**, 4 (2), 143–146.
- (90) Anaka, O. N.; Ozolua, R. I.; Okpo, S. O. **2009**, 3 (10), 485–490.
- (91) Tola, B. *J. Med. Med. Sci.* **2010**, 1, 126–128.
- (92) Nwaoguikpe, R. N.; Braide, W. *African J. Pharm. Pharmacol. Res.* **2011**, 1 (2), 23–29.
- (93) Moy, L. S. *Cosmetic Formulation and Method for Amelioration of Skin Keratoses and Striae Distensae*. 5759555, 1998.
- (94) Werman, M.; Mokady, S.; Neeman, I. *J. Agric. Food Chem.* **1990**, 38, 2164–2168.
- (95) Counts, D.; Huber, R. *Lipidic Furans and Retinol Palmitate Compositions Useful for Skin Therapeutics*. US Patent 5514709, 1996.
- (96) Ruiseco, M. *Oil Based Scalp Treatment Composition*. US Patent 4849214, 1989.
- (97) Raymond Chia, T. W.; Dykes, G. a. *Pharm. Biol.* **2010**, 48 (7), 753–756.
- (98) Jaime, J.; Leite, G.; Helena, É.; Brito, S.; Cordeiro, R. A.; Sâmia, R.; Brilhante, N.; Júlio, J.; Sidrim, C.; Bertini, L. M.; Morais, S. M. De; Fábio, M.; Rocha, G. **2009**, 42 (2), 110–113.
- (99) Nagaraj, M.; Sandhya, V.; Supriya, G.; Manju, R.; Kumari, P.; Bole, S.; Lalitha, V.; Kiran, B. *World Appl. Sci. J.* **2010**, 9 (6), 695–698.
- (100) Abe, F.; Nagafuji, S.; Okawa, M.; Kinjo, J.; Akahane, H.; Ogura, T.; Martinez-Alfaro, M. A.; Reyes-Chilpa, R. *Biol. Pharm. Bull.* **2005**, 28 (7), 1314–1317.
- (101) Michaelakis, A.; Strongilos, A. T.; Bouzas, E. a.; Koliopoulos, G.; Couladouros, E. a. *Parasitol. Res.* **2009**, 104 (3), 657–662.
- (102) Tesfay, S. Z.; Bertling, I.; Bower, J. P. *South African J. Bot.* **2012**, 79, 159–165.
- (103) Sudhir, K. *J. Clin. Endocrinol. Metab.* **2005**, 90 (5), 3100–3105.
- (104) Ramos-Jerz, M.; Villanueva, S.; Deters, A. In *55th Internation Congress and Annual Meeting of the Society for Medicinal Plant Research*; Graz, Austria, 2007.
- (105) Dabas, D.; Elias, R. J.; Lambert, J. D.; Ziegler, G. R. *J. Food Sci.* **2011**, 76 (9), 1335–1341.
- (106) Moskowitz, M. R.; Bin, Q.; Elias, R. J.; Peterson, D. G. *J. Agric. Food Chem.* **2012**, 60 (45), 11245–11252.
- (107) Salameh, Z.; Tehini, G.; Ziadeh, N.; Ragab, H. A.; Berberli, A.; Aboushelib, M. N. *Int. J. Esthet. Dent.* **2014**, 9 (1), 1–9.
- (108) Dabas, D. *A colored avocado seed extract with antioxidant, anti-carcinogenic and anti-inflammatory effects*, The Pennsylvania State University, 2012.

- (109) Guerriero, A.; Pietra, F. *Phytochemistry* **1984**, 23, 2394–2396.
- (110) Gómez-Bombarelli, R.; Calle, E.; Casado, J. *J. Org. Chem.* **2013**, 78 (14), 6880–6889.

## APPENDIX: SUPPLEMENTAL DATA

### NMR Analysis of perseoranjin in D<sub>2</sub>O

Nuclear Magnetic Resonance (NMR) studies were conducted at the Penn State NMR Core Facility with the help of Dr. Emmanuel Hatzakis and Dr. Carlos Pacheco using approximately 1 mg of perseoranjin. Experiments used a Bruker Avance III HD NMR operating at 500.20 MHz, equipped with a 5 mm CPPBBO-BB-1H/19F/ D Z gradient probe. Standard Bruker pulse sequences were used. 1D <sup>1</sup>H and <sup>13</sup>C experiments, as well as 2D Correlation Spectroscopy (COSY), Total Correlation Spectroscopy (TOCSY), Heteronuclear Multiple Bond Correlation (HMBC), and Distortionless Enhancement by Polarization Transfer (DEPT) edited Homonuclear Single bond Quantum Correlation (HSQC) experiments were conducted on two separate samples, one in (CD<sub>3</sub>)<sub>2</sub>SO and one in D<sub>2</sub>O (supplemental information). Additionally a 2D Nuclear Overhauser Effect Spectroscopy (NOESY) and selective TOCSY and Rotating Frame NOESY (ROESY) experiments were conducted on the D<sub>2</sub>O sample. Data was processed using MestReNova software. Details of each experiment are listed below.

1D <sup>1</sup>H: 500.20 Hz; acquisition time, 2.3396; spectral width, 6009.6; spectral size, 65535; acquired size, 16384; relaxation delay, 1.0000; pulse width, 10.1300.

1D <sup>13</sup>C: 125.79 Hz; acquisition time, 1.1010; spectral width, 29761.8; spectral size, 65536; acquired size, 32768; relaxation delay, 2.0000; pulse width, 10.0000.

COSY: (500.20, 500.20) Hz; acquisition time, 0.1532; spectral width (6684.5, 6684.5); spectral size (1024, 1024); acquired size (1024, 256); relaxation delay, 2.0000; pulse width, 10.7500.

TOCSY: (500.20, 500.20) Hz; acquisition time, 0.2048; spectral width (5000.0, 5000.0); spectral size (1024, 1024); acquired size (1024, 256); relaxation delay, 1.5000; pulse width, 10.75000.

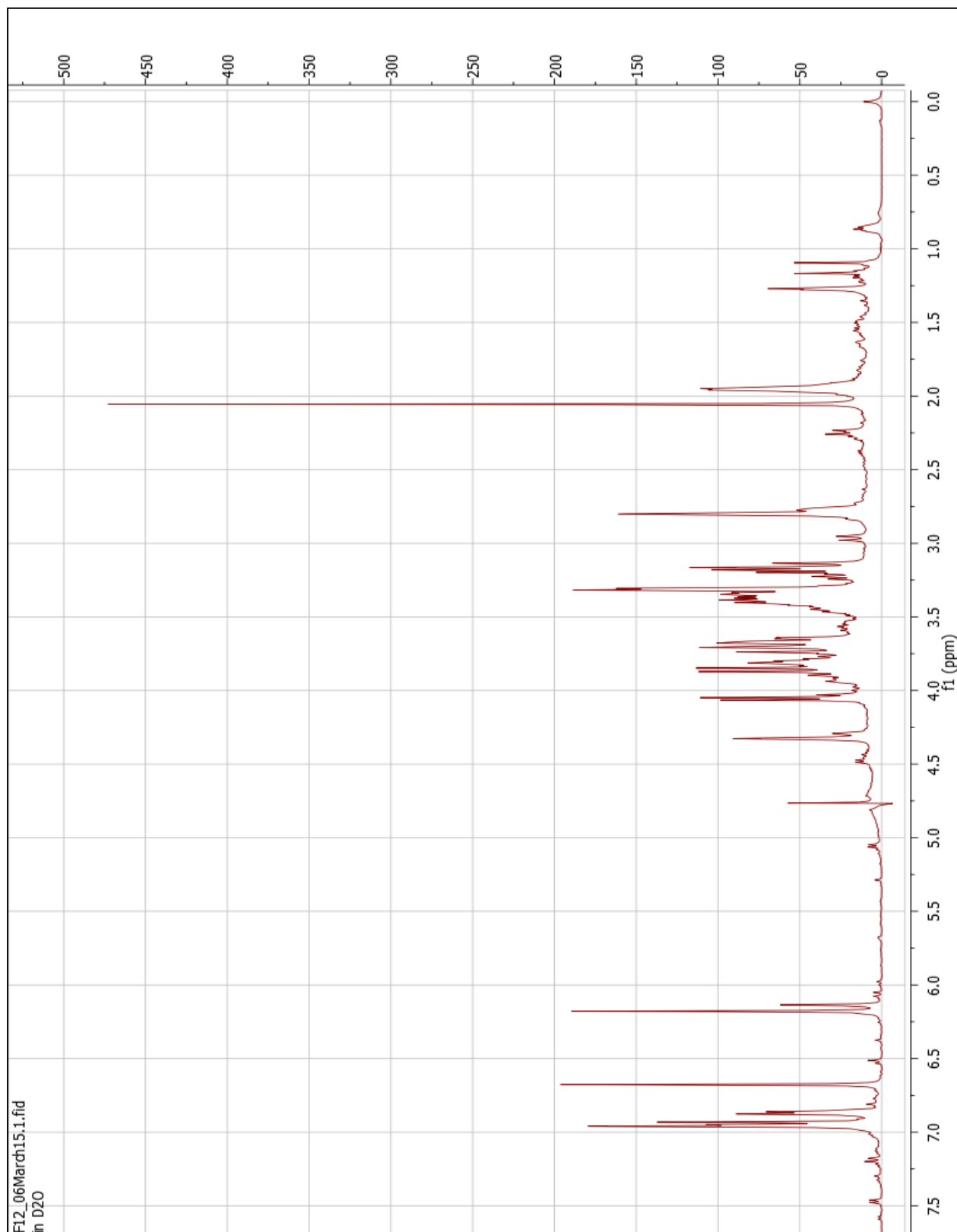
NOESY: (500.20, 500.20) Hz; acquisition time, 0.1862; spectral width (5498.5, 5500.6); spectral size (1024, 1024); acquired size (1024, 312); relaxation delay, 2.0000; pulse width, 10.7500.

HSQC-DEPT: (500.20, 125.78) Hz; acquisition time, 0.0786; spectral width (6510.4, 22624.4); spectral size (512, 512); acquired size (512, 256); relaxation delay, 1.5000; pulse width, 10.1300.

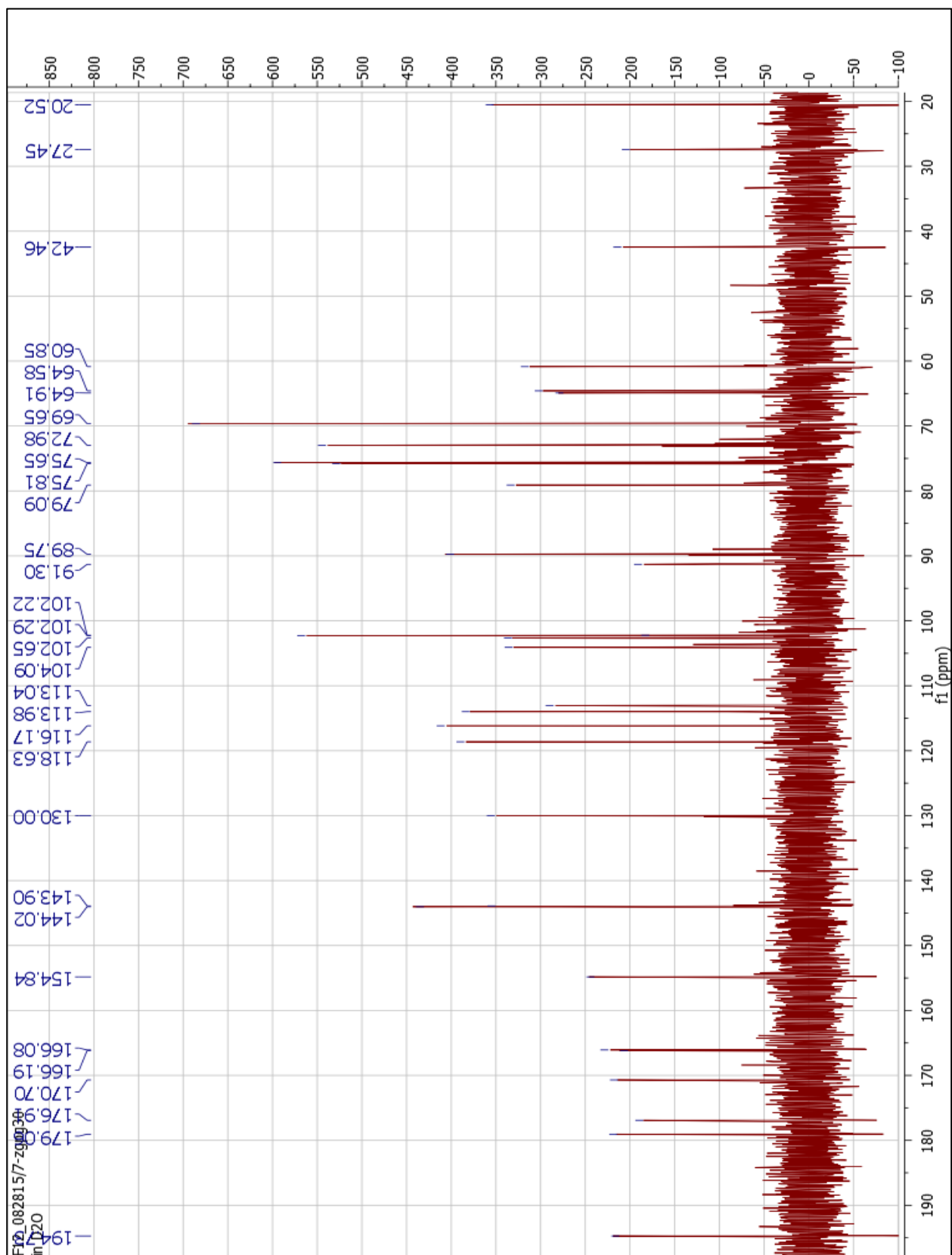
HMBC: (500.20, 125.79) Hz; acquisition time, 0.1365; spectral width (7500.0, 28901.7); spectral size (1024, 1024); acquired size (1024, 256); relaxation delay, 1.5000; pulse width, 9.7500.

NMR spectra of perseoranjin in D<sub>2</sub>O are shown in Figures 35-41.

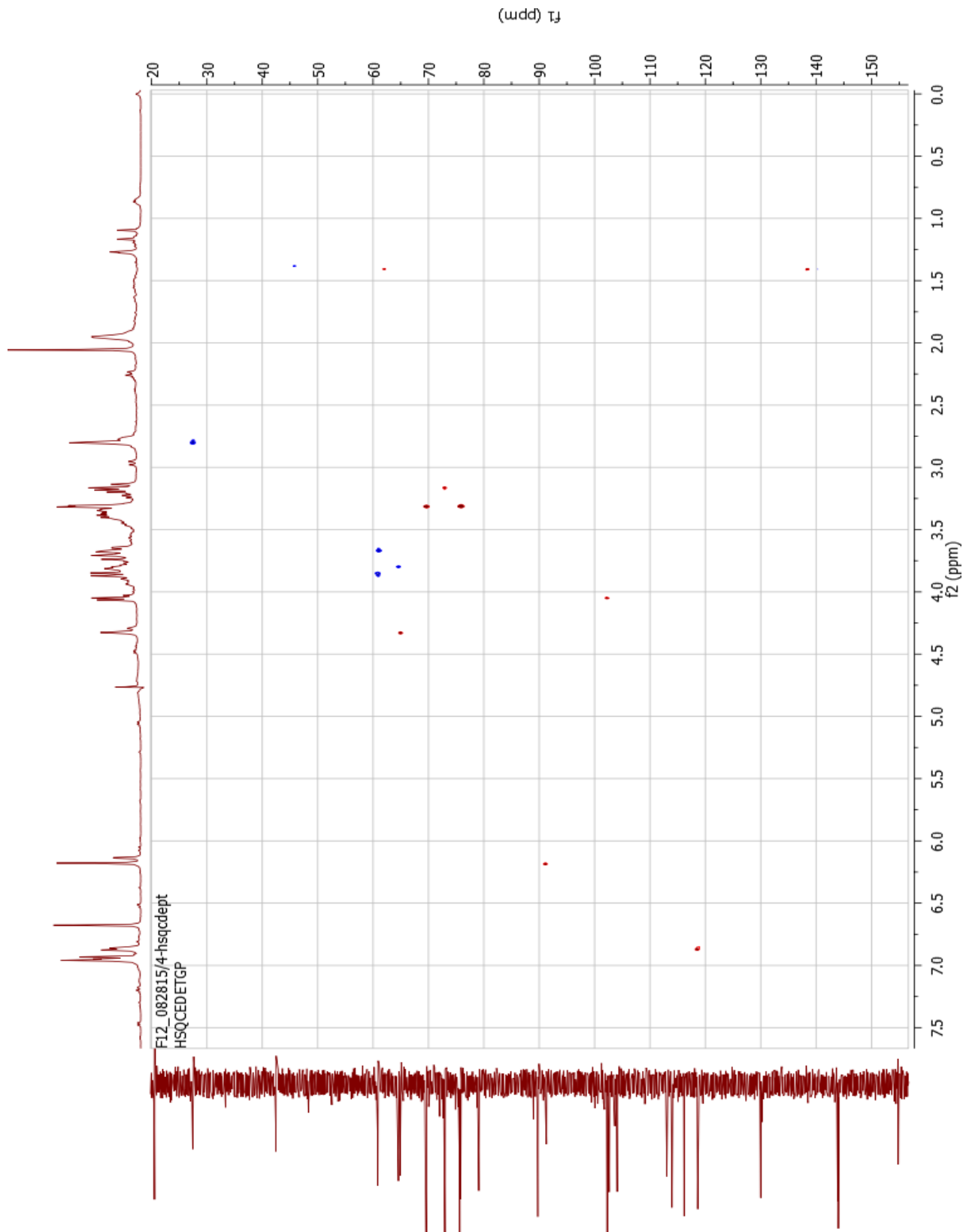




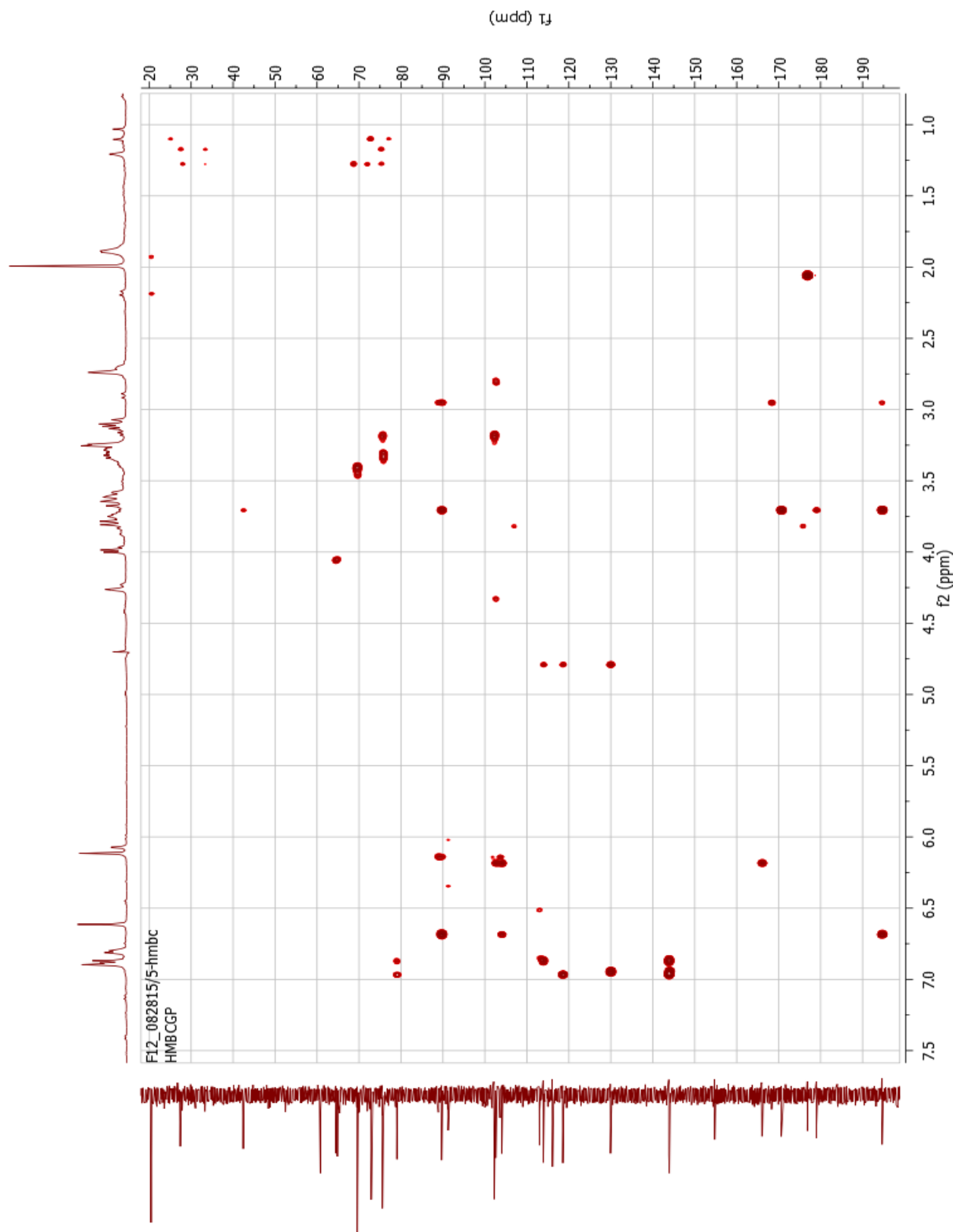
**Figure 35:**  $^1\text{H}$  NMR of "F12" in  $\text{D}_2\text{O}$ .



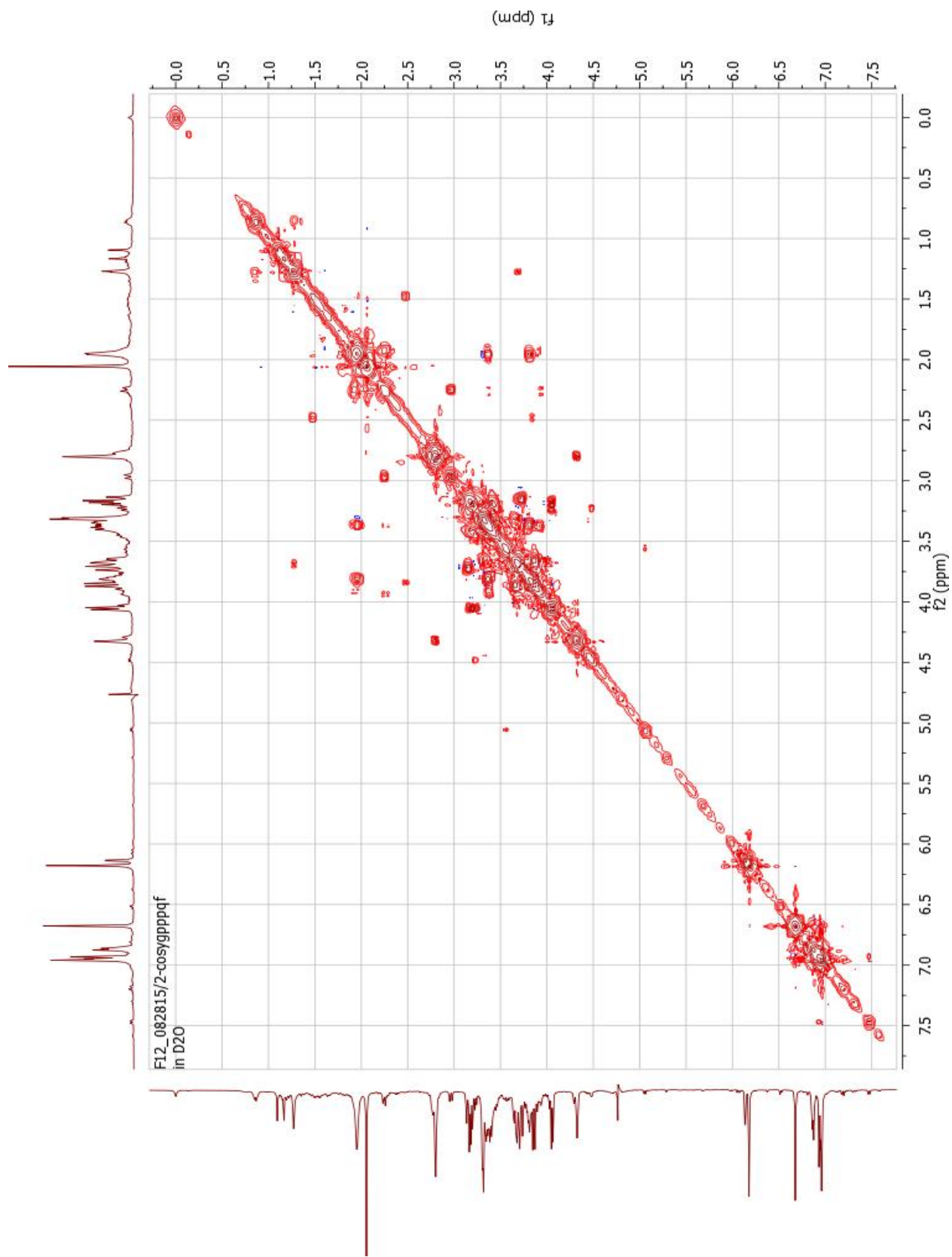
**Figure 36:**  $^{13}\text{C}$  of NMR perseoranjin in  $\text{D}_2\text{O}$ .



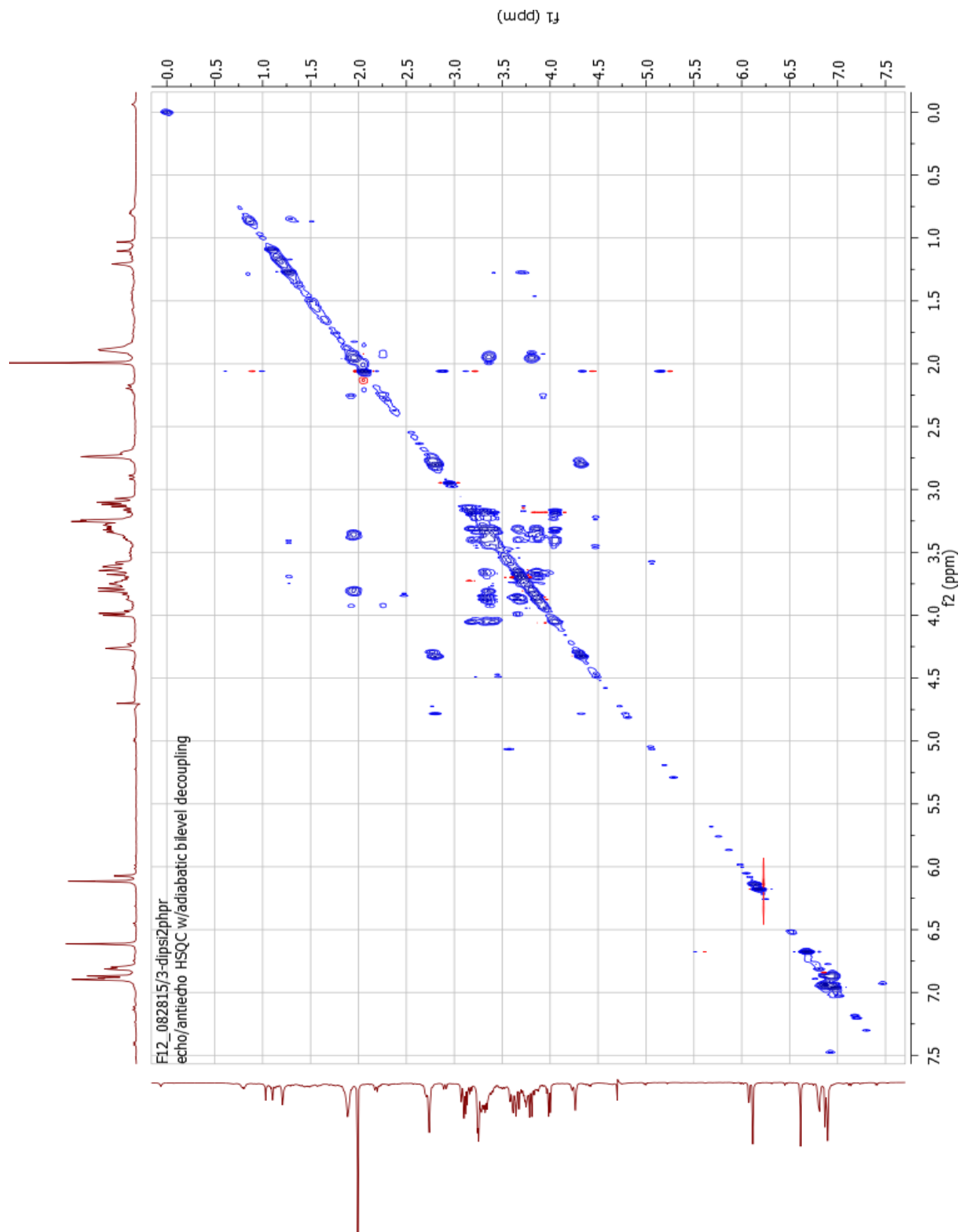
**Figure 37:** DEPT-edited HSQC perseoranjin of in D<sub>2</sub>O.



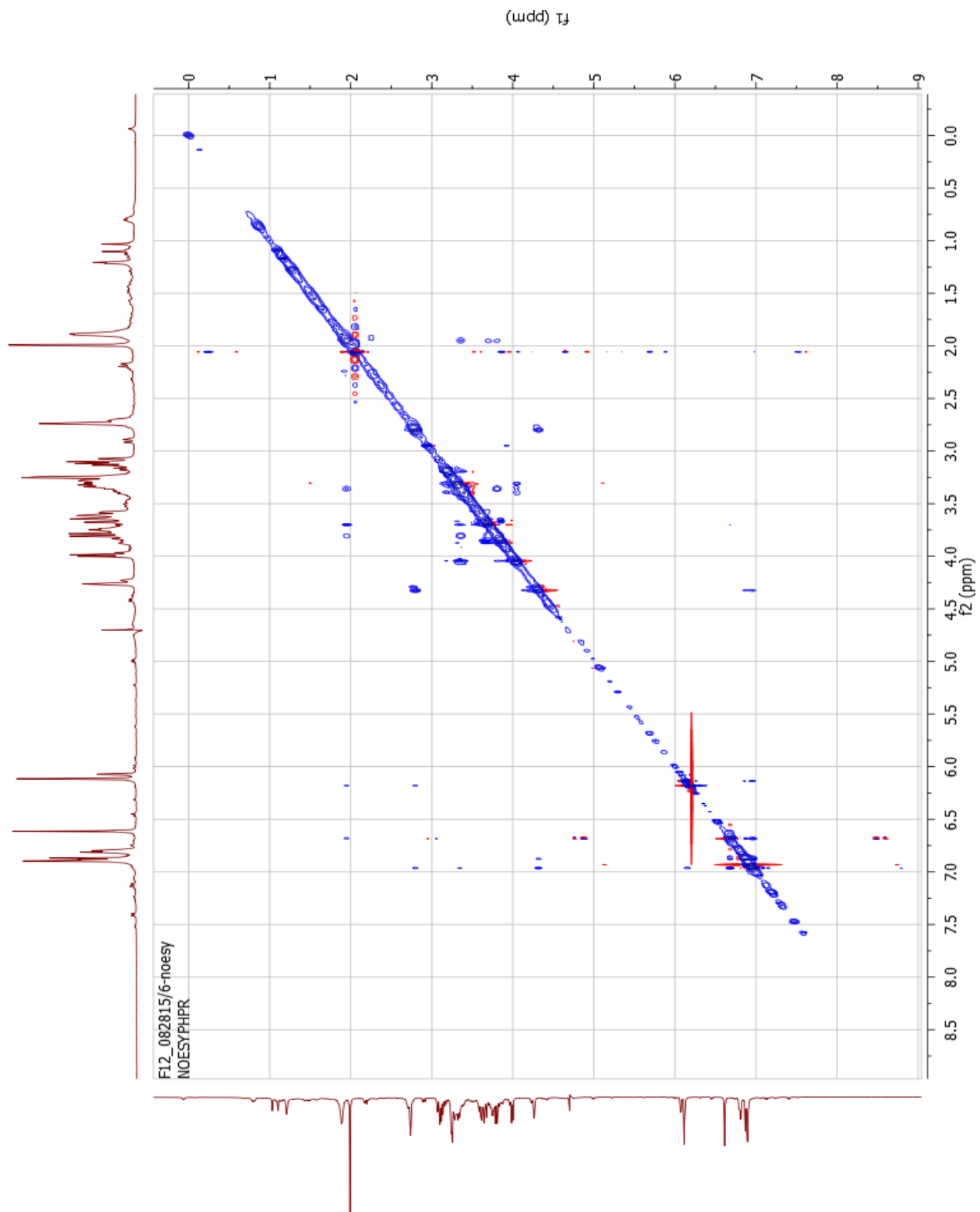
**Figure 38:** HMBC of perseoranjin in D<sub>2</sub>O.



**Figure 39:** COSY analysis of perseoranjin in D<sub>2</sub>O.



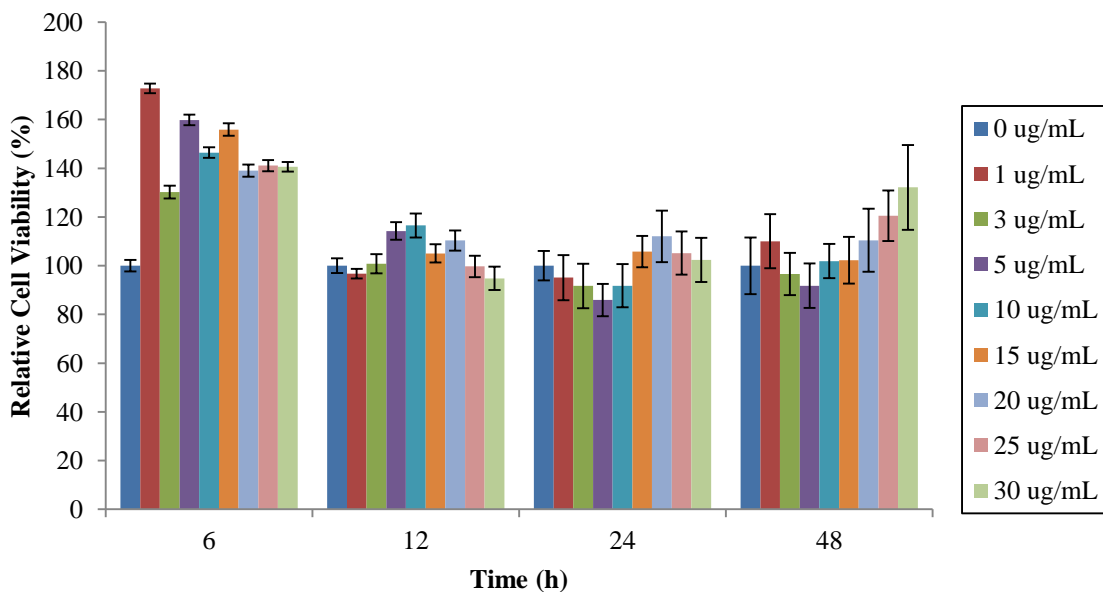
**Figure 40:** TOCSY analysis of perseoranjin in D<sub>2</sub>O.



**Figure 41:** NOESY analysis of perseoranjin in D<sub>2</sub>O.

## Effect of semi-pure CASE on Viability of Human Cancer Cells

Previous studies have shown the anti-cancer effects of some colored avocado seed extracts in LNCaP human prostate cancer cell lines.<sup>108</sup> Following the same protocol, the effect of semi-pure CASE on cell viability was determined using the MTT assay. The concentrations of semi-pure CASE used were 0, 1, 3, 5, 10, 15, 20, 25, and 30  $\mu\text{g/mL}$ . In brief, cells were seeded (104 cells/well) in 96 well plates and allowed to attach overnight. The cells were treated with CASE for 6, 12, 24, and 48 h. After CASE treatment, cells were combined with MTT and absorbance read at 540 nm. Figure 42 shows the results of this experiment. In this study, semi-pure case did not show any decrease in the viability of LNCaP cells over 48 hours. In some cases, a non-significant trend of increasing cell viability with increasing semi-pure CASE was observed, which could be due to the high polyphenolic content of the extract.



**Figure 42:** Effect of semi-pure CASE on viability of LNCaP cells.



## VITA

### RACHEL M. SHEGOG

#### EDUCATION

- M.S., Food Science. The Pennsylvania State university, University Park, PA (2012-2015)
- B.S., Chemistry. The Pennsylvania State University, University Park, PA. (2008-2012)

#### EXPERIENCE

- Undergraduate researcher, Laboratory of Food Science and Disease Prevention. Department of Food Science, The Pennsylvania State University, University Park, PA. (2011-2012)
- Undergraduate researcher, Inorganic chemistry laboratory, The Pennsylvania State University, Beaver Campus, Monaca, PA. (2009-2010)
- College tutor of math, chemistry, English. Center for Academic Achievement, The Pennsylvania State University, Beaver Campus, Monaca, PA. (2008-2010)

#### SELECT PUBLICATIONS AND PRESENTATIONS

- Ziegler, G.R.; Lambert, J.D.; Shegog, R.M.; Dabas, D.; Hatzakis, E. Compounds and Compositions and Methods for Coloring Edible Materials. USPTO Provisional Patent. Submitted November 4<sup>th</sup>, 2015.
- Shegog, R.M.; Elias, R.J.; Ziegler, G.R.; Lambert, J.D. Hass avocado (*Persea americana*) seed as a natural colorant. Proceedings of the Annual Meeting of the American Society of Pharmacognosy and International Conference on the Science of Botanicals, August, 2014. Published: *Planta. Med.* 80(10):797-98.
- Shegog, R.M.; Dabas, D.; Ziegler, G.R.; Lambert, J.D. (2013) Avocado (*Persea americana*) seed as a source of bioactive phytochemicals. *Curr. Pharm. Des.* 19(34):6133-40.
- Shegog, R.M.; Elias, R.J.; Ziegler, G.R.; Lambert, J.D. Characterization of an orange pigment found in Hass avocado seed (*Persea americana*). Presented at the 2015 Gamma Sigma Delta Research Expo, The Pennsylvania State University, University Park, PA, March, 2015.
- Shegog, R.M.; Elias, R.J.; Ziegler, G.R.; Lambert, J.D. Characterization of an orange pigment found in Hass avocado seed (*Persea americana*). Presented at the 2015 Graduate Research Symposium, The Pennsylvania State University, University Park, PA, March, 2015.

#### HONORS AND AWARDS

- Robert D. and Jeanne L. McCarthy Memorial Graduate Teaching Award and Graduate Scholarship (2014)
- Travel Award, Sahakian Family Endowment for Graduate Education in Agricultural Sciences (2014)
- First Place in oral presentations, Penn State Beaver Undergraduate Research Fair
- Presentation title "The Preparation of a Model Compound for an Iron (III) Phenyl POSS Ionomer" (2010)