USING ANALYTICAL METHODOLOGIES TO ASSESS THE
ORGANOLEPTIC CHARACTER OF
CITRUS ESSENTIAL OIL

A Dissertation
Submitted to
the Temple University Graduate Board

In Partial Fulfillment
of the Requirements for the Degree
DOCTOR OF PHILOSOPHY

---

by
Jessica Lynn Kovach
May 2019

Examining Committee Members:

Dr. Susan A. Jansen Varnum, Advisory Chair, Department of Chemistry
Dr. Michael J. Zdilla, TU Department of Chemistry
Dr. Stephanie L. Wunder, TU Department of Chemistry
Dr. Rachel B. Spigler, External Member, TU Department of Biology

©
Copyright
2019

by

Jessica L. Kovach

All Rights Reserved

ABSTRACT

Essential oils are natural products used to flavor food and beverages. With the increase in nutrition conscious consumers, manufacturers of food additives and food products are faced with the challenge of making healthy alternatives. In particular, food products going to market with label claims stating reductions in sugar and salt, organic certified, organic compliant, and all natural; moreover, the ingredients used in flavors must meet these label claims as well. More often than not, the challenge in using ingredients that follow these requirements is the pricing, the sourcing and the variability among those sources. Variability is common in the ingredients coming from nature such as fruits and plants because the area of cultivation can vary by the soil at the sight of planting and/or the climate in the region. Pricing is also problematic in naturally grown ingredients because it is a matter of supply and demand. Stock could be depleted from natural disasters, disease carrying pest(s), pests that consume the crop, and/or other causes for scarce supply of crop(s).

Essential oils are natural byproducts of fruit, peels, and leaves from plants that contribute to flavor formulae for a large variety of food products. Because the essential oils are a crop-based commodity, every variety has inherent differences based on the growing conditions and their ripening stages [1]. Nevertheless, each type of oil has marker chemicals that make up much of its composition; these marker chemicals have the tendency to degrade over time based on their interaction with light, oxygen exposure, and temperature. For companies that manufacture flavorings, understanding the variability among sources of essential oils as well as the possible degradants of essential oils is

valuable information to obtain because it is possible the variants and degradants will negatively impact the flavor profile. Flavor is without question the most important attribute of the food we consume and by default stability of said flavor(s) need to be understood [30].

The content in this dissertation involves the stability analysis of a common essential oil, Oil Mandarin Italian Select, from *Citrus Reticulata* Blanco. It has known off notes that form from unknown causes. Most common is the plastic note that has formed in carbonated products like soda. Studying this particular essential oil in various conditions is intended to shed light on what those degradants are and under which conditions they form to give mandarin oil an off-note when applied to high acid and carbonated beverage applications. Once the note is reproduced, a correlation between analytical data and sensory interpretation of the oil will be developed.

Mandarin essential oil being in the *Citrus* genus is traditionally analyzed via gas chromatography (GC) because of the high quantities of volatile constituents that give an oil high aroma activity. The volatile fraction of mandarin oil to be studied includes stability of methyl-N-methylanthranilate (MNMA), a major component giving mandarin its distinct grapey character, as well as gamma terpinene, thymol, sinensal, alpha pinene, beta pinene, myrcene, para cymene, alpha terpineol, and beta caryophyllene. Each of these ten compounds contributes to the unique flavor profile of mandarin oils when compared to orange and tangerine essential oils [1]. It was the common knowledge that para cymene can be perceived as rancid in aroma and the many interconversions the terpenes make that cause para cymene formation in *Citrus* oils, which made monitoring

the changes of this chemical in the three stability environments crucial. Attention is being paid to para-cymene, as a specific marker of degradation in *Citrus*.

The data obtained from the applied stability studies were challenging to understand as the marker chemicals are volatile and sensitive to chemical change. In this work the chemical changes and trends were analyzed under various storage conditions. Significant statistical analyses were employed to help define criteria of usability. The analyses were required because of natural variants and apparent inconsistencies of the data. Dixon Q Test and the Z Test were applied to determine outliers. Additionally, the Bland Altman method was applied to compare storage conditions and to determine if this statistical approach could be used to define significant changes in the marker chemical stability. The Bland Altman plots suggest that each component met the statistical limits of agreement, meaning the samplings were not significantly changing, statistically speaking. A final approach to assess the analytical data of the mandarin oil for significant change was the mass balance of each marker chemical from week 0 to week 24. Instrumental fluctuations have an acceptable range of +/- 20% in the industry; hence, a significant change criterion for a chemical in the mass balance must be one that exceeded +/- 20%. Unlike classical statistic methods, the mass balance was indicative that significant change had occurred to the compounds in the three studies. Upon sensory analysis of the oil samples, display of plastic note, oxidation, and overall loss of characteristic mandarin notes, the mass balance was found to correlate best to the significant change detected by sensory evaluation of the oil samplings. Due to the inadequate number of validated methods on *Citrus* essential oil research and the absence

of large groupings of terpenes validated in a unified methodology, reconciliation of mass balance is an underutilized method of assessment in the literature.

As a final assessment of the GC method validated, a product containing the selected mandarin oil was analyzed to evaluate the ability of the method to separate the oil components within a significantly more complicated matrix than the initial samples. The method was successful though not all marker chemicals were detected due to their low formulation concentration being below the level of detection of the method. This should not be seen as a failure of the method. For the major components of the essential oil studied, the method was quantitatively successful, meeting industry requirements.

To my parents, Frank and Diane Kovach

without your unwavering support, encouragement, and unconditional love

this process would not have been possible

ACKNOWLEDGMENTS

As I sit here and reflect on this journey, I am more than grateful for all of the support I had received over the years which made this possible. There is no other way to begin this thesis than to thank all of the individuals who encouraged, counseled, guided, and loved me through the end of this journey. I am sure just as much as this was a challenge for me, I became a challenge to all of you from time to time. For that, I want to thank all of you for enduring this long and bumpy ride with me. It would not have been possible any other way.

To my advisor Dr. Susan Jansen-Varnum, thank you! From the beginning of my first semester she was able to see potential in me, my goals, and the possibility of an executable plan to go beyond the master's program and join the chemistry department as a PhD student all while maintaining full time employment. This I know is a rare find in an advisor and for that I am forever grateful. Dr. Jansen-Varnum's flexibility and desire to work with students based on their individual interests while meeting program requirements was exactly what I needed; without her out of the box thinking, who knows how this journey through graduate school would have unfolded.

In addition to formulating the option to go about this untraditionally, I sincerely appreciate the efforts that went in to accomplishing the plan. Dr. Jansen-Varnum kindly allowed for countless virtual lunch dates with me over the years. Being able to collaborate on my original research proposal, my research, manuscript preparation, and of course, this defense, during my designated lunch time made the world of difference given my commitment to full time work hours. Not only did it prevent rushed evening meetings

on campus, but the conference calls with a Google share (thank goodness for that feature as well!) kept the research progressing when I was unable to see the forest from the trees.

I could go on and on in thanking Dr. Jansen-Varnum for all of her efforts in getting me to this final semester, and not one paragraph could truly express my deepest gratitude. I feel very fortunate to have been able to learn from, work for, and collaborate with such a dedicated and distinguished professor and because of that I look forward to having Dr. Jansen-Varnum in my personal network for life outside graduate school.

I would like to acknowledge my committee comprised of Dr. Michael Zdilla and Dr. Stephanie Wunder for their flexibility and understanding of the obstacles completing my PhD from afar conjured up over the last few years. I sincerely appreciate their availability for summer presentations, even when Skype was required, their input and advice that added to my growth as a writer and adapting to meet the deadlines I created for this defense. I would also like to thank Dr. Rachel Spigler for kindly agreeing to be an external committee member for my dissertation defense.

To my parents, Diane and Frank Kovach, this would have been IMPOSSIBLE without unconditional love and support you both gave. From counseling me through the idea of graduate school, the decision to go further than a masters, and your efforts to keep me sane when I became my worst enemy, thank you! I would also like to express my deepest appreciation that in all the pressures that came with choosing this path, you always reminded me that your expectations should never be a factor in my stress. I am

fortunate for your positive influence. The days of you listening to my griping are almost through, but for all of the times you allowed me the outlet to express my thoughts and concerns, know it was therapeutic regardless of whether we found a solution.

I cannot possibly find all the words to express how I feel about the support you bolstered me with, but know, this was only possible because of each of you. As much as I did the project, I was only capable because of the insurmountable amount of effort you both put into being parents. You instilled the drive, the determination, and some hardcore resilience mechanisms that were necessary to get me to this point. I want your efforts to be recognized in this moment just as much as the work displayed herein. I love you both more than anything in this world. From the bottom of my heart, thank you!

To my fiancé and best friend, Thomas M. Shiels, thank you for being my calm within this storm. Your patience, your flexibility, your help, and your support made this journey bearable. You were the closest thing I had to daily collaboration during this process and while I am glad to not rehash my daily findings anymore, I am appreciative for all the times you listened, provided new perspectives, as well as assistance. When I needed to study through the entire weekend for an exam, a cume, or a presentation, you posted up with me at the local B&N, made sure my coffee was filled, and made certain dinner was planned for. When I needed someone to troubleshoot the instrument or edit/critique my writing, you did that too. Ultimately, when I needed help, whether the ask was big or small, tedious or interesting, you provided as much assistance as you could and I am grateful. And, when it came time for me to inevitably wallow, you

always had words of encouragement. I sincerely appreciate the efforts you put in to make my life a little easier, to build my confidence, and to keep pushing me along. You are a paramount reason I was able to get through these last five years amongst the obstacles, and I want to thank you from the bottom of my heart. I love you!

I would like to express my appreciations to Dr. Michael Puppolo. Mike was another Ph.D student in Dr. Jansen-Varnum's lab that I sadly had not met until the end of his time at Temple University. Mike was always enthusiastic and had willingness to support my research any way he could. Mike became my virtual mentor! He provided valuable suggestions, guidance, advice, and valuable edits to both my ORP and my dissertation research; his advice and collaborations were extremely helpful throughout this process. I am deeply thankful for Mike's willingness to add perspective to my research and to work through questions that cropped up over the years.

I would also like to recognize Dr. Mohit Patel and Mrs. Tiffany Elbardarssi. Mohit was working on his Ph.D at the time I had entered the group and we spent many of hours troubleshooting both the HPLC and the QTOF LCMS. Mohit was always accessible and always had ideas or advice to share. He was also the organizer for my teaching assignment through Dr. Jansen-Varnum's summer science programs. Mohit is a very valuable group member and I sincerely appreciate the offerings he shared. Tiffany often worked behind the scenes for myself and for the remaining group members by the time I had entered the group. Tiffany was always on top of organizing materials for the research, planning my involvement in the summer camps, and also provided a platform to

discuss my research ideas when the topic was still underdeveloped. Tiffany ensures everything runs smoothly, which is something everyone around her benefits from including me. Thank you, Tiffany.

I would like to extend a thank you to my company and colleagues for providing me an environment to work that allowed for flexibility to accomplish my graduate degree. Whether it was Monday morning courses, cumulative exams, or afternoon courses I was always allowed to schedule my workload in accordance. Colleagues past and present provided platforms for discussions on my approaches and the resulting data that made interpretation and application of the research worthwhile. A special thank you to my boss, Dave, in particular for his willingness to evaluate my analytical data against his wealth of knowledge on flavors, for providing sensory analysis, and for being a mentor in the field. To my direct coworkers, and friends, Bruce, Shannon, and Carly for providing refuge from the stress, while we spend more time together than at home. The comic relief, the friendship, the support, encouragement and the ears to listen made coming to work a pleasurable experience during such a high stress time.

Lastly, to my undergraduate lab partner, Zac Gotlib, thank you for continuing to be a resource from the other side of the country especially as it pertained to editing my documents. As the memes say “only ‘lab partners’ have no ‘end’ in them”. Thanks for everything you taught me since the first day at Kutztown. Life would have been far less interesting without your humor and shenanigans!

TABLE OF CONTENTS

ABSTRACT .... **III**

ACKNOWLEDGMENTS .... **VIII**

LIST OF TABLES .... **XIX**

LIST OF FIGURES .... **XXIII**

CHAPTER 1 .... **1**

CITRUS ESSENTIAL OIL **1**

1.1 Introduction 1

1.2 Extraction methods 4

1.2.1 Supercritical Fluid Extraction (SFE) 8

1.2.2 Microwave Accelerated Distillation (MAD) 8

1.3 Huanglongbing (HLB) Disease and Impact on Flavor Quality 11

1.4 Evolution of Mandarin and its Essential Oil 14

1.5 Citrus Reticulata Blanco 17

1.6 Alpha and Beta Pinene 20

1.7 Myrcene 21

1.8 Para Cymene 23

1.9 Gamma Terpinene 24

1.10 Alpha Terpineol 25

1.11 Thymol 26

1.12 Methyl-N-Methylanthranilate (MNMA) 26

1.13 Beta Caryophyllene 27
1.14 Sinensal 28
1.15 Research Objectives 31
CHAPTER 2 32
LITERATURE REVIEW OF PROPERTIES AND ANALYTICAL TECHNIQUES TO CHARACTERIZE CITRUS OILS 32
2.1 Introduction 32
2.2 Citrus Essential Oil 34
2.3 Mandarin Specific Essential Oil Research 37
2.4 Non-Validated Methods 39
2.5 Validated Methods 43
2.6 Conclusions 44
CHAPTER 3 46
EXPERIMENTAL APPROACH: GAS CHROMATOGRAPHY 46
3.1 Introduction to Chromatography 46
3.2 Gas Chromatography Principles 48
3.3 Gas Chromatography Design 57
3.4 Internal Standard method 62
3.5 External Standard Method 64
CHAPTER 4 66
DATA ANALYSIS AND STATISTICAL METHODS 66

4.1 Introduction 66

4.2 Data analysis techniques in method validation 66

4.2.1 Weighted or Unweighted Regression: Homoscedasticity vs Heteroscedasticity 66

4.2.2. F -Test for extended linear range 67

4.2.3 Weighted Linear Regression (WLR) 68

4.3 Data analysis techniques in Stability Evaluation 70

4.3.1 Use of internal standardization 70

4.3.2 Use of External Standard 71

4.3.3 Molecular Ions and Base Peaks 72

4.3.4 Dixon Q Test 72

4.3.5 Z Test 73

4.3.6 Reconciling Mass Balance 74

4.3.7 Bland Altman Method 75

CHAPTER 5 77

METHOD DEVELOPMENT AND VALIDATION 77

5.1 Introduction 77

5.2 Experimental 79

5.2.1 Reagents and Chemicals 79

5.2.2 Method Development 80

5.2.3 Instrumentation and Experimental Methods 81

5.2.4 Sample Preparation 82

5.2.5 Method Validation 83
5.2.5.1 Specificity 83
5.2.5.2. Range 84
5.2.5.2.1 General Working Range 84
5.2.5.2.2 Extended Linear Behavior of Range 84
5.2.5.3 Linearity 84
5.2.5.4. Accuracy 85
5.2.5.5 Precision 85
5.2.5.6 Sensitivity 86
5.2.5.6.1 Limit of Detection (LOD) 86
5.2.5.6.2 Limit of Quantitation (LOQ) 86
5.2.5.7 System Suitability Testing 86
5.3 Validation Results and Discussion 87
5.3.1 Specificity 87
5.3.2 Range 92
5.3.2.1 Homoscedasticity vs. Heterscedasticity 96
5.3.2.2 Weighted Linear Regression (WLR) 98
5.3.3 Linearity 103
5.3.4 Accuracy 105
5.3.5 Precision 108
5.3.6 Sensitivity 110
5.3.7 System Suitability Testing 111

5.4 Conclusion 111

CHAPTER 6 113

STABILITY ANALYSIS 113

6.1 Introduction 113

6.2 Goal 114

6.3 Experimentation with Mandarin Oil 114

6.3.1 Reagents and chemicals 114

6.3.2 Effect of storage at ambient conditions 114

6.3.3 Effect of storage at elevated temperatures 115

6.3.4 Effect of oil in high acid tasting solution (HATS) 115

6.3.5 Sensory Evaluation 116

6.4 Results and Discussion 117

6.4.1 Use of Internal Standard Method 117

6.4.2 Use of External Standard Method 118

6.4.3 Molecular Ions and Base Peaks 118

6.4.4 Dixon Q Test 119

6.4.5 Z Test 150

6.4.6 Reconciling Mass Balance 166

6.4.7 Bland Altman Method 169

6.4.8 Forced Degradation Study 185

6.4.9 Sensory Evaluation 188

6.5 Conclusion 193

CHAPTER 7 .... **195**

APPLICATION OF *CITRUS RETICULATA* BLANCO **195**

7.1 Introduction 195

7.2 Experimental Method 196

7.2.1 Reagents 196

7.2.2 Instrumentation 196

7.2.3 Sample Preparation 197

7.2.4 Forced Degradation 197

7.3 Results and Discussion 197

7.3.1. Expired versus Fresh Sample Mass Balance 197

7.3.2 Mass Balance Following Forced Degradation 203

7.4 Conclusion 209

CHAPTER 8 .... **210**

CONCLUDING REMARKS **210**

8.1 Summary and Concluding Remarks 210

8.2 Future Studies 214

REFERENCES CITED **215**

LIST OF TABLES

Table 1-1: Organoleptic descriptors provided by Good Scents [50] 19

Table 5-1: Base peak ions used for additional peak identification 88

Table 5-2: Coefficient of determination (R^2) from 100 to 10,000 ppm........................... 94

Table 5-3: Correlation coefficient (r) of extended linear range 95

Table 5-4: Experimental F-value versus tabulated F-values for linear ranges 97

Table 5-5: Original versus weighted linear regression 99

Table 5-6: Percent bias original linear regression (OLR) 100

Table 5-7: Percent bias weighted linear regression (WLR)... 101

Table 5-8: Average magnitude of % bias [A] 102

Table 5-9: Coefficient of determination for 70%-130% concentration 104

Table 5-10: Coefficient of determination for LOQ-130% concentration 105

Table 5-11: Accuracy and Repeatability method validation results 107

Table 5-12: Precision method validation results... 109

Table 5-13: Respective LOQ and LOD concentrations with their S/N ratios 110

Table 6-1: Base peak ions for ion extraction from total ion chromatograph 119

Table 6-2: Dixon Q Test performed on alpha pinene in ambient oil study 121

Table 6-3: Dixon Q Test alpha pinene in hot box oil study... 122

Table 6-4: Dixon Q test alpha pinene in HAT solution study 123

Table 6-5: Dixon Q test beta pinene in ambient oil study 124

Table 6-6: Dixon Q Test beta pinene in hot box oil study... 125

Table 6-7: Dixon Q Test beta pinene in HAT solution study 126

Table 6-8: Dixon Q Test Myrcene in ambient oil study ... 127

Table 6-9: Dixon Q Test Myrcene in hotbox oil study ... 128

Table 6-10: Dixon Q Test Myrcene in HAT solution study ... 129

Table 6-11: Dixon Q Test para cymene in ambient oil study ... 130

Table 6-12: Dixon Q Test para cymene in hot box oil study ... 131

Table 6-13: Dixon Q Test para cymene in HAT solution study ... 132

Table 6-14: Dixon Q Test gamma terpinene in ambient oil study ... 133

Table 6-15: Dixon Q Test gamma terpinene in hotbox oil study ... 134

Table 6-16: Dixon Q Test gamma terpinene in HAT solution study ... 135

Table 6-17: Dixon Q Test alpha terpineol in ambient oil study ... 136

Table 6-18: Dixon Q Test alpha terpineol in hotbox oil study ... 137

Table 6-19: Dixon Q Test alpha terpineol in HAT solution study ... 138

Table 6-20: Dixon Q Test thymol in ambient oil study ... 139

Table 6-21: Dixon Q Test thymol in hotbox oil study ... 140

Table 6-22: Dixon Q Test thymol in HAT solution study ... 141

Table 6-23: Dixon Q Test MNMA in ambient oil study ... 142

Table 6-24: Dixon Q Test MNMA in hotbox oil study ... 143

Table 6-25: Dixon Q Test MNMA in HAT solution study ... 144

Table 6-26: Dixon Q Test beta caryophyllene in ambient oil study ... 145

Table 6-27: Dixon Q Test beta caryophyllene in hotbox oil study ... 146

Table 6-28: Dixon Q Test beta caryophyllene in HAT solution study ... 147

Table 6-29: Dixon Q Test sinensal in ambient oil study ... 148

Table 6-30: Dixon Q Test sinensal in hotbox oil study 149

Table 6-31: Dixon Q Test sinensal in HAT solution study 150

Table 6-32: Alpha Pinene Z test results in ambient oil study 151

Table 6-33: Alpha Pinene Z test results in hotbox oil study... 152

Table 6-34: Alpha pinene z-test in HAT solution study 152

Table 6-35: Beta Pinene z-test ambient oil study 153

Table 6-36: Beta Pinene z-test hot box oil study 153

Table 6-37: Beta Pinene Z test results in HAT solution study 154

Table 6-38: Myrcene Z test results in ambient oil study 154

Table 6-39: Myrcene Z test results in hotbox oil study 155

Table 6-40: Myrcene Z test results in HAT solution study 155

Table 6-41: Para Cymene Z test results in ambient study 156

Table 6-42: Para Cymene Z test results in hot box study 156

Table 6-43: Para Cymene Z test results in HAT solution study 157

Table 6-44: Gamma Terpinene Z test results in ambient study 157

Table 6-45: Gamma Terpinene Z test results in hotbox study....................................... 158

Table 6-46: Gamma Terpinene Z test results in HAT solution study............................ 158

Table 6-47: Alpha Terpineol Z test results in ambient oil study 159

Table 6-48: Alpha Terpineol Z test results in hotbox oil study 159

Table 6-49: Alpha Terpineol Z test results in HAT solution study 160

Table 6-50: Thymol Z test results in ambient oil study 160

Table 6-51: Thymol Z test results in hotbox oil study... 161

Table 6-52: Thymol Z test results in HAT solution study 161

Table 6-53: MNMA Z test results in ambient oil study 162

Table 6-54: MNMA Z test results in hotbox oil study.. 162

Table 6-55: MNMA Z test results in HAT solution study 163

Table 6-56: Beta Caryophyllene Z test results in ambient oil study 163

Table 6-57: Beta Caryophyllene Z test results in hotbox oil study................................ 164

Table 6-58: Beta Caryophyllene Z test results in HAT solution study 164

Table 6-59: Sinensal Z test results in ambient oil study 165

Table 6-60: Sinensal Z test results in hotbox oil study 165

Table 6-61: Sinensal Z test results in HAT solution study 166

Table 6-62: Reconciling Mass Balance of each stability study 168

Table 6-63: Sensory evaluation of mandarin oil by flavorist trained in Citrus 191

Table 6-64: Sensory evaluation of mandarin oil by flavorist trained in savory............. 192

Table 7-1: FID results for expired product 200

Table 7-2: FID results for freshly manufactured product 201

Table 7-3: Mass balance of fresh product versus expired product 202

Table 7-4: FID results of fresh product after five days at 40 °C.................................... 205

Table 7-5: FID results for fresh product after ten days at 40 °C.................................... 206

Table 7-6: Mass balance of fresh to five day hotbox product.. 207

Table 7-7: Mass balance of fresh to ten day hotbox product 208

LIST OF FIGURES

Figure 1-1: Isoprene unit, the basis of a terpene molecule 2

Figure 1-2: Basic structure of a monoterpene demonstrated by Myrcene 3

Figure 1-3: Basic structure of a sesquiterpene demonstrated by Farnesene 3

Figure 1-4: Cross section illustration of *Citrus* fruit anatomy adapted from Liu et al.[24].... 5

Figure 1-5: Microwave-accelerated distillation (MAD) extraction method modeled from Ferhat et al.[13] 10

Figure 1-6: Hydrodistillation (HD) extraction method modeled from Ferhat et al.[13]....... 11

Figure 1-7: Left to right, alpha pinene and beta pinene structure 21

Figure 1-8:Myrcene structure in nonlinear form 22

Figure 1-9: Para cymene structure 23

Figure 1-10: Gamma Terpinene structure 24

Figure 1-11: Alpha terpineol structure 25

Figure 1-12: Thymol structure 26

Figure 1-13: MNMA structure 27

Figure 1-14: Beta caryophyllene structure 28

Figure 1-15: Sinensal structure 29

Figure 1-16: Summary of terpene interconversions 30

Figure 3-1: Resolution calculation 50

Figure 3-2: Resolution calculation 50

Figure 3-3: Calculation for retention factor 50

Figure 3-4: Calculation for selectivity factor 51

Figure 3-5: Calculation for number of theoretical plates 51

Figure 3-6: Plate height calculation 53

Figure 3-7: Plate height calculation 53

Figure 3-8: Visual illustration of multiple flow paths in a packed column 54

Figure 3-9: Visual illustration of longitudinal diffusion 55

Figure 3-10: Mass transfer calculation 56

Figure 3-11: Cross section of the open tubular column varieties [6] 59

3-12: Internal standard calculations 63

Figure 4-1: Slope formula for weighted regression 69

Figure 4-2: Intercept formula for weighted regression 70

Figure 4-3: Correlation coefficient formula for weighted regression 70

Figure 4-4: Percent relative error formula for weighted regression 70

Figure 4-5: Assay formula 71

Figure 4-6: Q experimental formula for Dixon Q Test... 73

Figure 4-7: Z value formula used in Z Test 74

Figure 5-1**:** Representative TIC of mandarin essential oil from 0:00 - 68:00 minute 88

Figure 5-2: Base peak ion119 m/z for para cymene from 29:00 – 31.50 minutes............ 89

Figure 5-3**:** Representative TIC of a blank solution from 00:00- 68:00 minutes 89

Figure 5-4: Representative TIC of mandarin essential oil from 0:00 - 14:00 minutes..... 90

Figure 5-5: Representative TIC of mandarin essential oil from 14:00- 29:00 minutes.... 90

Figure 5-6: Representative TIC of mandarin essential oil from 28:50- 40:00 minutes.... 91

Figure 5-7: Representative TIC of mandarin essential oil from 40:00- 68:00 minutes.... 91

Figure 6-1: Bland Altman plot for alpha pinene under ambient stability conditions 170

Figure 6-2: Bland Altman plot for alpha pinene under hotbox stability conditions 170

Figure 6-3: Bland Altman plot for alpha pinene under HATS stability conditions........ 171

Figure 6-4: Bland Altman plot for beta pinene under ambient stability conditions 171

Figure 6-5: Bland Altman plot for beta pinene under hotbox stability conditions 172

Figure 6-6: Bland Altman plot for beta pinene under HATS stability conditions.......... 172

Figure 6-7: Bland Altman plot for myrcene under ambient stability conditions 173

Figure 6-8: Bland Altman plot for myrcene under hotbox stability conditions............. 173

Figure 6-9: Bland Altman plot for myrcene under HATS stability conditions 174

Figure 6-10: Bland Altman plot for para cymene under ambient stability conditions ... 174

Figure 6-11: Bland Altman plot for para cymene under hotbox stability conditions 175

Figure 6-12: Bland Altman plot for para cymene under HATS stability conditions...... 175

Figure 6-13: Bland Altman plot for gamma terpinene for ambient stability conditions 176

Figure 6-14: Bland Altman plot for gamma terpinene for hotbox stability conditions .. 176

Figure 6-15: Bland Altman plot for gamma terpinene for HATS stability conditions... 177

Figure 6-16: Bland Altman plot for alpha terpineol under ambient stability conditions 177

Figure 6-17: Bland Altman plot for alpha terpineol under hotbox stability conditions.. 178

Figure 6-18: Bland Altman plot for alpha terpineol under HATS stability conditions .. 178

Figure 6-19: Bland Altman plot for thymol under ambient stability conditions 179

Figure 6-20: Bland Altman plot for thymol under hotbox stability conditions 179

Figure 6-21: Bland Altman plot for thymol under HATS stability conditions.............. 180

Figure 6-22: Bland Altman plot for MNMA under ambients stability conditions 180

Figure 6-23: Bland Altman plot for MNMA under hotbox stability conditions 181

Figure 6-24: Bland Altman plot for MNMA under HATS stability conditions 181

Figure 6-25: Bland Altman plot for beta caryophyllene in ambient stability conditions 182

Figure 6-26: Bland Altman plot for beta caryophyllene in hotbox stability conditions . 182

Figure 6-27: Bland Altman plot for beta caryophyllene in HATS stability conditions .. 183

Figure 6-28: Bland Altman plot for sinensal under ambients stability conditions 183

Figure 6-29: Bland Altman plot for sinensal under hotbox stability conditions............. 184

Figure 6-30: Bland Altman plot for sinensal under HATS stability conditions 184

Figure 6-31: Reported reaction of gamma terpinene oxidation to para cymene [58] 187

Figure 6-32: Reported reaction of limonene to para cymene [61] 188

CHAPTER 1

CITRUS ESSENTIAL OIL

1.1 Introduction

Essential oils are a byproduct of raw plant materials which have been processed and isolated by physical processes [13]. The name essential oil comes from "essential" meaning a distinct aroma profile attributed to the natural, plant starting material [16, 19]. The term "oil" is used for the hydrophobic nature of essential oils, not because they provide nutritional value as would an oil containing acyl-glycerols [16]. Citrus essential oils find applications in many food commodities, but most abundant use of citrus oils can be found in beverage applications, specifically carbonated soft drinks [17].

Citrus essential oils are increasingly used in flavor production for their ability to be a natural flavor ingredient in an industry striving to make clean, all natural labels. Flavors made to taste like orange, lemon, lime, grapefruit, tangerine, mandarin have the challenge of tasting authentic without the addition of *Citrus* essential oils during the formulation process. Despite using natural ingredients over synthetic materials, *Citrus* flavors end up tasting more like a candy or commercialized flavor that results in artificial perceptions. In other words, *Citrus* flavors need the enhancement natural oils provides in order for the flavor to be perceived as true to fruit and therefore fully appreciated by the consumer. *Citrus* flavors are not like other fruit flavors which can be formulated from a blend of a dozen or more natural, flavor grade chemicals and taste identical to the desired

profile. The unique nature in the flavor profiles of *Citrus* is likely attributed to its complex cultivation.

Citrus essential oils are a complex mixture of volatile components, largely monoterpene hydrocarbons and their oxygenated derivatives, known as terpenoids [19, 62]. Hydrocarbons with the empirical formula $C_{10}H_{16}$ were the first chemical class defined as terpenes [14].

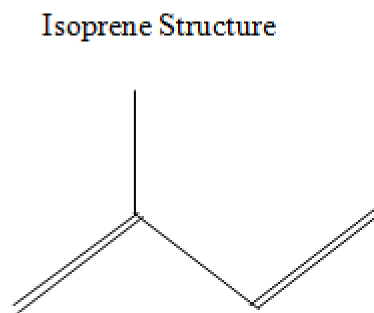


Figure 1-1: Isoprene unit, the basis of a terpene molecule

Terpenoid compounds related to these hydrocarbons are represented by the empirical formula $C_{10}H_{16}O$ or $C_{10}H_{18}O$ [14]. The class, terpenes, evolved to be a broader category than originally designated. Molecules whose empirical formulas as simple as C_5H_8, are known as isoprenes, while molecules with ten carbon hydrocarbons are all

defined as terpenes [14]. Monoterpenes are the C_{10} terpene class containing two isoprene units and sesquiterpenes follow with C_{15} which is three isoprene units in one molecular structure [14].

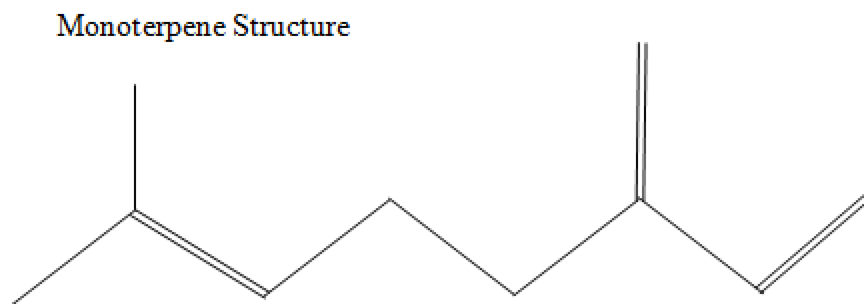


Figure 1-2: Basic structure of a monoterpene demonstrated by Myrcene

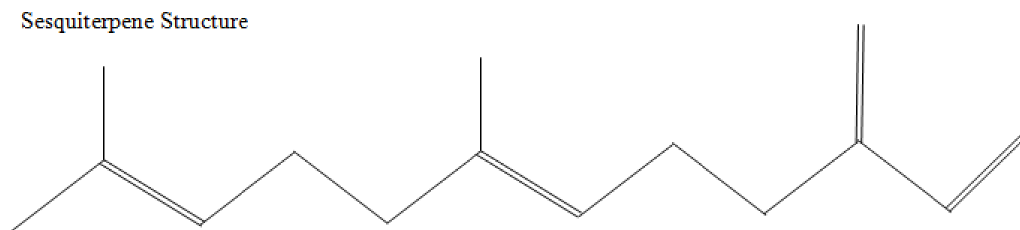


Figure 1-3: Basic structure of a sesquiterpene demonstrated by Farnesene

Due to the highly unsaturated nature of monoterpenes, these oils are inherently sensitive to changes in light, heat, oxidation or oxidative degradation, and hydration [13]. They can even react with other related compounds, increasing the complexity of the chemistry. Using natural oils containing an abundance of monoterpenes as flavor boosters, increases the risk of flavor instability, making new challenges for flavor manufacturers to combat to assure that their finished flavors will be shelf stable and marketable. Removing the terpene fraction increases storage time of a *Citrus* essential oil by preventing oxidation of the terpenes that usually result in off notes [28]. Terpene hydrocarbons, as most of the citrus molecules are classified, are diverse in aroma, ranging from cedar-like to medicinal in aroma with very piney and citrusy nuances for the mid-chain terpene hydrocarbons [21]. Terpene alcohols are classified as having floral character though diversity of the molecules gives an aromatic range of cedar to minty nuances [21]. As for the aromatic esters, the classifying aroma is well described as fermented for most compounds in this family [21].

Herein, chapter one introduces the many factors affecting the distribution of components as well as the changes in organoleptic properties in mandarin and other *Citrus* essential oils.

1.2 Extraction methods

Essential oil isolation has been restricted to only a physical process by the International Organization of Standardization; those physical means are defined as distillation, expression, or dry distillation [13]. Distillation uses steam, steam and water,

or just water [13]. Expression, also known as cold pressing (CP) in *Citrus*, is the most common way of isolating the volatile compounds from the *Citrus* peels, but requires centrifugation in order to fully separate the oil from the emulsion that occurs from the process [13, 16, 18]. The aromatic oils are held in small oils sacs/glands in the sub epidermal layer called the flavedo [24]. For a cross sectional views of a Citrus fruit see Figure 1-4 below.

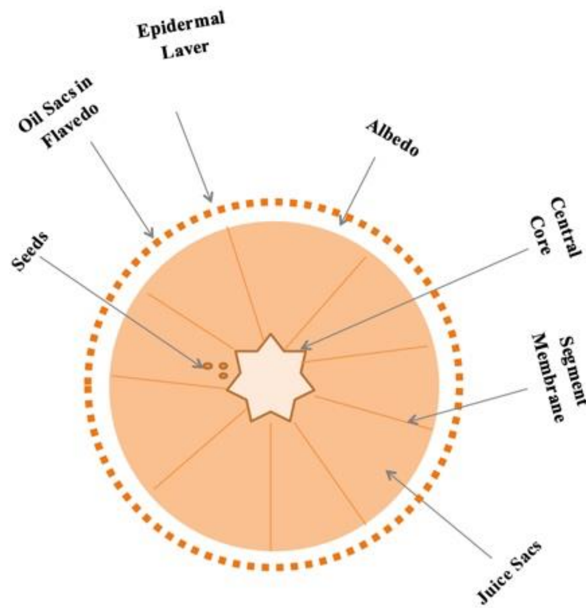


Figure 1-4: Cross section illustration of *Citrus* fruit anatomy adapted from Liu et al.[24]

Cold pressing is a temperature independent technique involving the abrasion of the citrus peel so that the cuticles and glands of the fruit expel the oils from the inner sacs [18, 21]. There is the option to cold press the entire fruit or the de-pulped peel of the fruit; the whole fruit saves time because the process is not interrupted, but the quality of oil from de-pulped peels is higher [62]. Cold pressing is desirable in citrus fruits compared to other fruits because of the thermally unstable aldehydes that are present in *Citrus* [18].

Distillation is usually used for economic reasons in countries abroad; however, cold pressing is more popular because it produces a larger yield [13, 16]. During the distillation process, the *Citrus* peels are steamed, boiled, or dry distilled; steam distillation being the most common [18, 21]. Distillation is based on two major factors, the immiscibility of the essential oil with water, and at boiling temperature the vapor pressure of water and the oil equaling ambient pressure [13]. When these conditions occur during distillation, compounds within the essential oil, which typically boil between 200 ℃ and 300 ℃, begin evaporation at a temperature closer to that of water, 100 ℃ [13, 21]. The evaporated oil becomes a steam capable of traveling through the tubing of the apparatus which is being cooled by an external source [13]. The essential oil vapors in that steam condense in a separate vessel named the "Florentine Flask" [14, 18]. Unfortunately water still persists from condensed steam in the Florentine flask, but the weight of the essential oil is much less than that of water, so water can be easily removed [14,21]. Typically the *Citrus* peel yields 0.05-5.0 % w/w essential oil; however, a select few factors tend to dictate how much yield is obtained. Some of these factors include the

length of time of the distillation, the temperature of the distillation, pressure of the distillation, as well as the origin of the fruit being distilled [13].

As science advances with time, advantages and disadvantages are discussed for most techniques used by the scientific community. Extraction of essential oils is no different. Both cold pressing and distillation have some disadvantages. For distillation, the prolonged use of high temperature is a concern because it causes modifications to the oil constituents and in some cases loss of compounds altogether due to thermal instability and volatility [15, 18]. In addition, a large handful of flavor constituents are not steam volatile, resulting in insufficient quantities in the final oil distilled [21]. With distillation, there is also the potential for oxidation and hydrolysis because of the chemical changes induced from the prolonged use of elevated temperatures [21].

Disadvantages of the cold pressing technique stem from its use of air and water agitation [15]. Agitation has been discovered as a catalyst for distortion of a given essential oil flavor profile which is problematic from an economic standpoint of manufacturers, and also problematic for companies buying the essential oil for application in flavors and fragrances. Specifically, water agitation can cause two common problems that affect the oil flavor profile. First, is a reduction of polar analytes in the oil because they are left behind in the water-based emulsion [15]. Second, is a reduction in citral and terpene alcohols, both of which contribute immensely to the flavor characteristics for *Citrus* and their oils [15, 21]. Without the polar constituents, citral, and terpene alcohols, flavor profiles of the expected *Citrus* variety begin to fall flat in organoleptic perception. Organoleptic is defined as the sensory perception one obtains through taste and smell. The use of agitation with air promotes hydrolysis, oxidation, and

resinification all of which negatively impact flavor profiles of essential oils from what is expected based on the starting material [15].

With these negative qualities of two historically common isolation techniques impacting the quality of the essential oils from a sensory perspective, new techniques have been developed to circumvent the problems associated with the traditional processes. Techniques such as supercritical fluid extraction (SFE) and microwave-accelerated distillation (MAD) are the two most commonly employed [13,16].

1.2.1 Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction uses solid starting material that interacts with supercritical fluids, usually CO_2, to remove polar and nonpolar molecules from the starting material at low temperatures [18]. Low temperatures are a great advantage over the high temperatures associated with distillation because fewer artifacts are formed and thermally sensitive molecules remain intact during the extraction process [18]. Use of CO_2 is also advantageous because it is nontoxic, has no taste or odor, and is tunable to different pressures and temperatures to achieve high selectivity during extraction [18].

1.2.2 Microwave Accelerated Distillation (MAD)

Microwave accelerated distillation uses inherent water content within a product with microwave energy to induce distillation without using steam, water, or solvents [13]. The MAD technique works by subjecting the natural product to a microwave reactor where the energy applied to the product would heat the inherent water content until the

glands burst to release the essential oil; the temperature of the extraction is 100 ℃ because of the boiling point of water [63]. Once released, the essential oil vapors travel to a condenser outside of the reactor allowing the oil to condense separately while the water vapor condenses back for reflux into the extraction to maintain an equal amount of humidity in the continuous process [13]. Advantages of MAD for extracting essential oils from *Citrus* peels include decreased extraction times, low energy consumption, low cost in use, negligible solvent or water use, and an overall environmentally friendly technique [13, 63]. Because of the desirable advantages of MAD over HD and CP, scarcely any artifacts are created thereby staying true to the authentic aroma and flavor profiles of natural *Citrus* products [13, 63].

The processing technique of the mandarin oil studied here was not disclosed by the manufacturer but it is suspected that the oil was distilled based on the knowledge of the vendor's facilities and product lines. Understanding the techniques common to oil extraction suggests that mandarin oil may have a different distribution of components and possible degradants if one of these other techniques were used; therefore presenting differently both chemically and organoleptically. Future studies using the methodologies developed here could be applied to compare the distribution of products in the same product extracted by different techniques.

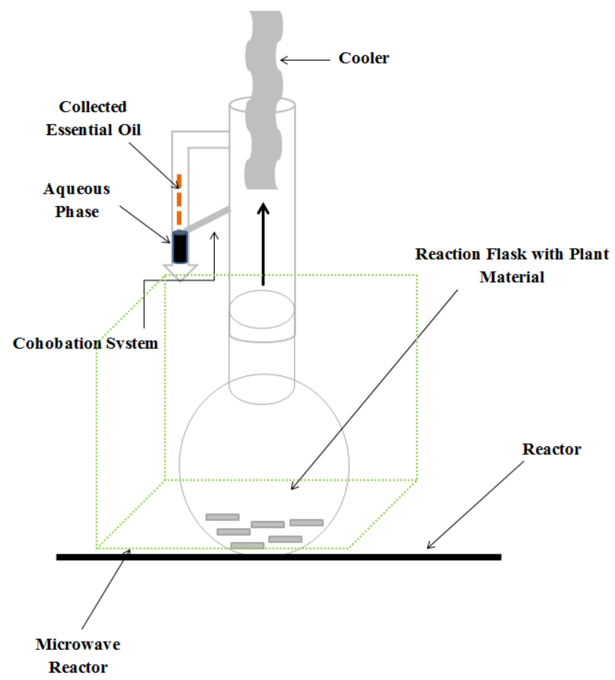


Figure 1-5: Microwave-accelerated distillation (MAD) extraction method modeled from Ferhat et al.[13]

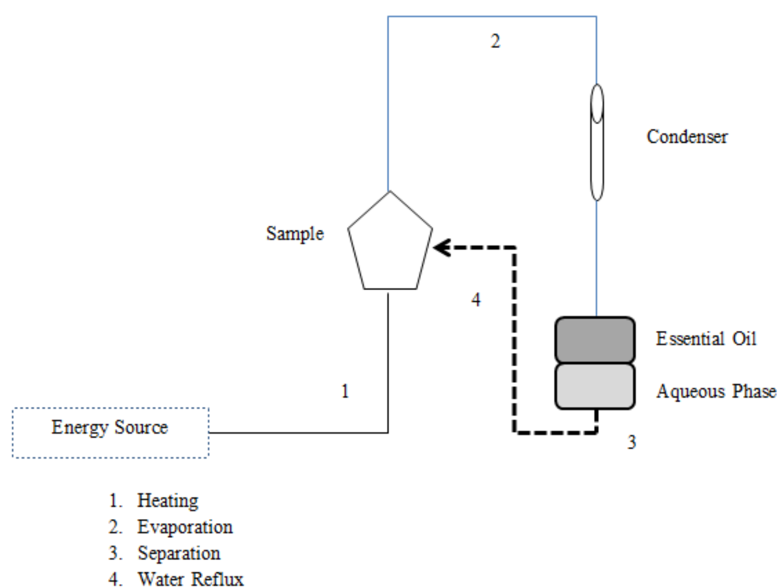


Figure 1-6: Hydrodistillation (HD) extraction method modeled from Ferhat et al.[13]

1.3 Huanglongbing (HLB) Disease and Impact on Flavor Quality

Citrus essential oils obtained from fruits are in high demand due to their versatility in potential uses. In order to keep up with the demands, enormous quantities of the fruits are necessary to produce enough essential oil from the peels. Unfortunately, in the last century, a disease targeting *Citrus* trees has been making it difficult for suppliers to keep pace with the growing market for *Citrus* essential oils as well as the other demanding market(s) for which *Citrus* fruits are used. Huanglongbing (HLB), also

referred to as citrus greening, is the disease spreading across counties, states and countries responsible for the decline in quality and quantity of *Citrus* trees [9]. HLB was first documented in China in 1919, and has been seen in parts of Florida since the mid-2000s [11, 22]. It eventually made a rapid spread to all counties in Florida that are known for their commercial cultivation of *Citrus* [10]. In the citrus season spanning 2007-2008, HLB had a negative economic impact totaling $8.9 million dollars [9]. The impact comes from the loss of trees, the pest management costs for the groves, and an overall decline in production which affected profit margins [11]. There is not a region on the globe that has successfully been able to manage HLB to avoid its spread. Because of this, research is still necessary to find a rapid diagnosis [11].

Citrus greening is considered a bacterial disease, caused by liberibacters. Liberibacters acts as a blocker for phloem, which is the vascular system of the citrus trees [10, 11, 22]. By blocking the trees' vascular system, trees struggle to take in nutrients leading to disease onset [10]. HLB is spread by a leaf-feeding insect that is very miniscule; an Asian Citrus Psylliad (ACP) [10, 11, 22]. Typical symptoms of HLB include large dropping of fruit prior to harvest, undersized/misshapen fruits at harvest, stunted tree growth, and distressed leaves [9, 10, 11]. Usually the fruits that do make it to harvest also contain a bitter fruit juice that is typically rendered a loss because its quality is too poor to warrant economic value [10]. Specifically, the juice of the citrus fruit from diseased trees presents with a decrease in the quantity of aroma active compounds as well as a decrease in the potency of the aroma actives that are able to express [9].

Over the years the owners of citrus groves have tried to find solutions to combat HLB, some had more rigorous evaluation of the citrus trees while others tried to rehab the

trees as to not eradicate them entirely [10, 11]. There have been no prevention strategies for HLB for *Citrus* groves making it a very serious disease for the genus [22]. It is not always easy to identify disease onset because "symptoms" of HLB may not present until two or more years after infection nor is it common for all symptoms to pronounce simultaneously to make it obvious [10, 22]. This makes it quite challenging to manage because not only has the symptomatic tree suffered but other trees within the grove and surrounding groves could be suffering from the "Bad- Neighbor effect" [10]. "Bad neighbor effect" is when a disease spreads because it was not identified in a localized region and has been able to spread to its surroundings [10]. As an example, in China it was found to take less than five years before an entire grove was fully infected by HLB due to the "Bad Neighbor Effect" [22].

There are two main programs in the literature for trying to circumvent HLB. The more aggressive option that was alluded to previously is named the Bové program. In the Bové program, the trees within a grove are evaluated intensely for signs exhibiting HLB; if signs appear, the tree is immediately eliminated from the grove [10, 22]. Pest management solutions are also applied to the groves as a preventative maintenance [11, 22]. Not only does this plan become difficult for trees with delayed symptoms, it drives up the cost of grove maintenance [10]. The second program is called Enhanced Foliar Nutrition in which the citrus trees get fed through their leaves so that the nutrition can still be absorbed without going through the vascular system [10]. This second option does not eradicate trees based on their presentation of symptoms, and instead works around them [10, 11]. It allows the groves to maintain their citrus trees so yield increases, but the cost of grove maintenance, like pest management, are significantly increased from

these approaches [10, 11]. Most industries are using the latter method since it allows for increased volume of production and masks the disease enough, thus avoiding eradication of the trees altogether [10].

While not all *Citrus* fruits make it through the evaluation process to production, most fruits harvested from enhanced foliar nutrition processes are salvageable for use in products such as Tropicana OJ, Simply Pure OJ, and other market products consumers have learned to love. Unfortunately, the fruit juices are no longer a standalone ingredient in these manufactured products because the need for natural or natural and artificial (N&A) flavors are required to bring the *Citrus* flavor back into an acceptable range for delicious consumption. It is here that flavor companies step in to mitigate the complications from HLB that cause low fruit yield possessing poor quality. Flavor companies are able to use the peel oil, and/or other food grade flavor molecules to enhance the sweetness, the fruity notes, the tart notes, or blocking of bitter and potential metallic notes. It is this reason a fuller understanding the aroma profiles of *Citrus* essential oils/fruits/ juices is so important for flavor companies. Allowing flavor companies to have this expertise, allows them to make flavors that are more true to the actual fruits' characteristics and provides solutions for mitigating the off-notes that are produced from groves affected by HLB.

1.4 Evolution of Mandarin and its Essential Oil

Mandarin essential oil comes from the peels of the mandarin fruit, which are classified as a type of orange, that contain more variation between cultivars than other

varieties of oranges [8, 27]. Loose-skinned oranges have had convoluted botanical nomenclature for centuries that led to four distinct groupings published in Ernest Guenther's third volume of The Essential Oils; The King, Satsuma, Mandarin, and Tangerines [17]. It was among these four groups that mandarin stood out to be the most evolved variety originating from China and later cultivated in Mediterranean countries [17]. Reeve described the spread of mandarin as going from China to Japan, India, Arabia, North Africa, and then eventually Europe where traders carried the mandarins westward to America in the 19th century [27]. Webber was more specific in describing the first mandarin tree as planted in England in 1805 and from there the Chinese mandarin spread to Malta, Sicily, and then additional areas within Italy [23]. It took until the years 1840-1850 for the China Mandarin to be introduced to America, but when it was, it was introduced by the Italian Consul in New Orleans, LA and from there extended its presence to Florida and California [17]. From understanding the different countries the mandarin has been cultivated, it has been concluded that the mandarin is capable of growing under conditions ranging from desert-like conditions to subtropics [28]. The tradeoff becomes the several dozen varieties that are produced from morphological characteristics [28].

It was ideated that the tangerine variety of loose skinned oranges was an underdeveloped mandarin, a horticultural strain if you will [17]. To complicate things further, both strains, mandarin and tangerine, were given the same botanical variety *Citrus nobilis* var. *Deliciosa* which changed to *Citrus Reticulata* Blanco [17]. Reeve commented during his article in *Perfumer & Flavorist*, how terminology between tangerines and mandarins really varies by geographic location [27]. Botanists disagree

with using tangerine and mandarin interchangeably because the essential oils each have difference in odor, flavor, chemical composition, and physicochemical properties [17]. John Wright explains that tangerine is much closer to that of the sweet orange oil than it is to mandarin oil [21]. The differences in mandarin and tangerine develop due to their soil conditions, climate, and cultural differences that influence chemical composition and physicochemical properties, not the essential oil isolation process [17]. It can be noticed that oils produced in Brazil and Florida have similarities in their aroma due to the long collaboration and sharing of plant material between the parties; Brazil had also collaborated with Italy to import plant material with the Sicilian strain so it could self-produce that specific variety of mandarin oil [17]. Having these cross country collaborations has allowed the mandarin oil varieties to grow as similarly as possible despite the geographical differences of each region. Collaborations like this are what reduces the crops' differences so that the varieties do not begin to take on new characteristics that create confusion as the tangerine once did. In 2011 it was published that 460 tons of mandarin oil was produced across the globe mainly from Brazil and Italy [21]. Mandarin oils are often used to make mango, peach and apricot flavors as a natural flavor modifier [21]. The official FEMA GRAS number for this mandarin oil is 2657; this is the indicator that the substance is Generally Regarded as Safe (GRAS) by the Flavor and Extract Manufacturers Association of the U.S. (FEMA) in edible applications [21]. To be identified as FEMA GRAS, a compound must be qualified by the Food and Drug Administration through scientific testing of the material to evaluate its safety for consumption. A private party with the authority to conduct scientific research on a material can also evaluate its safety for use in food products.

Blanco in the name *Citrus Reticulata* Blanco came from the white fibers networked throughout the fruit peel and juice filled flesh of the fruit; it was published in the Philippines in 1837 [17]. Mandarin essential oils can be classified into three different color varieties: red, yellow, and green; horticulturists lend the name tangerine to the deep red variety of mandarin essential oils [8, 17]. Green oils usually mean the fruit was immature when it was pulled from the tree; green oils are best used for fragrance applications over flavor applications [28]. Yellow essential oils are indicative of a more mature fruit than green oils and have applications in both fragrances and flavors [28]. Red oils are solely used in flavor applications and are indicative of a fully matured fruit [28]. Prices also vary based on the different varieties of the oils [1].

1.5 Citrus Reticulata Blanco

This research involves the analysis of *Citrus Reticulata Blanco* mandarin oil of an Italian variety, but other common mandarin oils of the Mediterranean are known as *Citrus deliciosa Tenore* [8]. In 1963 Kovats and Kuglar published methyl-N-methylanthranilate and thymol as being the main constituents in Sicilian mandarin oils, since then both chemicals have been the focus of most mandarin oil research published in literature [8].

The general composition of mandarin is 98% terpenes and up to 1.5% being oxygenated components to which are responsible for the mandarin aroma, and the rest of the constituents are flavonoids, waxes, and sesquiterpenes [28]. The descriptors of mandarin include terms such as citrus, mandarin, Concord grape, lavender, peely, thyme,

and orange, respectively [21]. The constituent composition of mandarin oil can be further decomposed to 72% limonene, 18% gamma terpinene, 0.8% methyl-N-methylanthranilate, 0.5% linalool, 0.2% decanal, 0.1% thymol, and 0.05% alpha sinensal [21]. Later, Wilson and Zoccali added beta pinene to the list of important chemicals to the mandarin oil aroma profile [1,8]. The added chemical classes of marker chemicals of mandarin oil fall into aromatic esters and monoterpene alcohols [1]. Below in Table 1 there is a summary of the organoleptic descriptors of the ten mandarin marker chemicals chosen for this study.

Due to these chemicals being markers of mandarin, a study was designed to better understand the stability and chemistry occurring within the essential oil. Specifically, a root to the commonly perceived plastic off-note in mandarin flavored products needs discovery. Upon understanding the chemical(s) responsible for plastic off notes, manufacturers of mandarin flavors have the ability to create flavor solutions or optimized processing procedures to reduce the likelihood of, prevent completely, or mask the off notes of concern.

Table 1-1: Organoleptic descriptors provided by Good Scents [50]

Compound	Primary Odor Descriptor	Primary Flavor Descriptor	Additional Descriptors
Alpha Pinene	Herbal	Woody	sweet, pine, woody, earthy, terpenic, herbal, camphoreous
Beta Pinene	Herbal	Pine	dry, resinous, hay, green, cooling, terpenic, spicy, peppery, eucalyptus, fresh
Myrcene	Spicy	Woody	peppery, terpenic, plastic, herbal, rose, celery, carrot, citrus, leafy, minty, tropical, vegetable, mango
Para Cymene	Terpenic	Terpenic	Fresh, citrusy, woody, spicy, lemon, cumin, cilantro, rancid, bell pepper, origanum
Gamma Terpinene	Terpenic	Terpenic	oily, woody, lemon, lime, herbal, tropical, sweet, citrus, fruity
Alpha Terpineol	Terpenic	Citrus	pine, lilac, floral, woody, lemon, lime, cooling, resinous, soapy
Thymol	Herbal	Phenolic	thyme, medicinal, camphoreous, spicy, chemical, woody
Methyl-N-Methylanthranilate	Floral	Fruity	mandarin, fruity, sweet, phenolic, winey, grape, fresh, floral, woody
Beta Caryophyllene	Spicy	Spicy	sweet, woody, clove, dry, peppery, powdery, nut skin
Sinensal	Citrus	n/a	citrus, orange, mandarin

1.6 Alpha and Beta Pinene

Alpha pinene and its isomer beta pinene are two of the most abundant bicyclic monoterpenes discovered [69]. Alpha pinene is found in larger quantities than beta pinene with ratios of 50-70% and 15 to 30% in essential oils, respectively [70]. The pinenes are precursor chemicals to other aroma compounds used in flavor creation such as limonene, para cymene, and alpha terpineol [70]. The pinenes while being abundant in essential oils are also challenging molecules to work with because they are unstable, very volatile, and have low solubility in water without the use of a co-solvent [70]. Alpha pinene is soluble in ethanol and oils and has aroma characterized as pine, citrus, spicy, woody, and turpentine [70]. Alpha pinene has been traced to positive biological activities; antimicrobial, anticancer, therapeutics, antitumor, antibacterial, and more when it is one of the main constituents in essential oils [70]. Alpha pinene has even been traced to have repellant properties [70]. Beta pinene differs slightly from alpha pinene in that it's insoluble in ethanol and water but remains soluble in oils [70].

Its aroma characteristics include cooling, woody, piney, and turpentine with nuances of mint, eucalyptus, and camphor properties at 10% solutions but at taste, its fresh, pine, woody, with nuances with spice, mint, and camphor [70]. Beta pinene is a precursor chemical to citral, citronellol, geraniol, citronellal, linalool, ionones, and menthol [70].

Because it is found in lower quantities in essential oils compared to alpha pinene, it is less studied for its biological effects and metabolic pathways as compared to alpha

pinene [70]. Because of the importance alpha pinene is showing for biological advances, and the abundance both alpha and beta pinene are found in essential oils, years of work have gone into understanding the transformations these molecules can undergo to be produced in large volumes as well as be precursors for other flavor and fragrance related compounds [69,70]. A large majority of this research is based on the biotransformation, using microbial agents [69, 70].

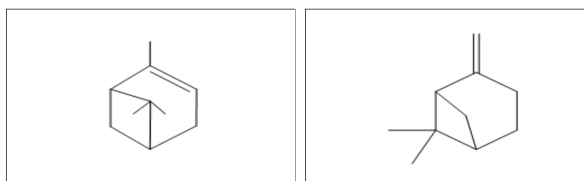


Figure 1-7: Left to right, alpha pinene and beta pinene structure

1.7 Myrcene

Myrcene is an acyclic terpene hydrocarbon with discovery most commonly associated with cannabis research, but also has published claims for its antibiotic, sedative, anti-inflammatory, and anti-mutagenic properties [86]. Myrcene was established as food safe in 1965 by the FDA and the Flavor Extract Manufacturing

Association (FEMA) [87]. Infrequently is myrcene extracted directly from plants or other natural sources, instead it is more economical to synthesize it from the thermal isomerization of beta pinene between 450 and 600 C for only a few seconds [87]. Myrcene is traditionally derived from 2,6-dimethyloctane and like most other terpenes, occurs naturally as the beta isomer form [88]. Myrcene aroma analysis is best at 5% in solution of alcohol or oil due to its insolubility in water at ambient temperatures [87]. Aroma descriptors used for myrcene include vegetative, green, sweet, woody, fruity, and herbaceous, with hints of anise and vanilla [87].

Myrcene was been deemed a spontaneous polymerizer at room temperature making it difficult for storing the pure material [88]. The polymerization of myrcene causing instability is noticeable in the industrial setting, particularly in flavor creation. When used as an individual ingredient in a flavor, a production lot of myrcene that is more than a few weeks old has the tendency to precipitate out of solution. When the production lot of myrcene is fresh, the same effect is not observed. Myrcene is a key marker in mandarin but its instability as a molecule makes analysis challenging.

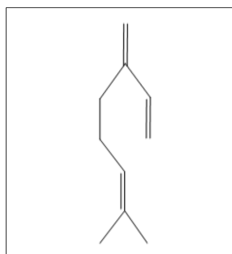


Figure 1-8:Myrcene structure in nonlinear form

1.8 Para Cymene

Para cymene is an aromatic hydrocarbon possessing a methyl and isopropyl group on its benzene ring and is associated with monoterpenes [60]. Para cymene is a commonly used intermediate for fragrances, herbicides, antioxidants, and pharmaceuticals [61].Its medicinal use is often to reduce coughing and phlegm, but requires encapsulation due to its short half-life [60]. Because para cymene is easily converted from limonene in high yields due to the similarity in their carbon structures, the process of doing so is very desirable for its green chemistry [61]. Research has also found it to be obtainable from gamma terpinene. By-products of the conversion include alpha terpinene, gamma terpinene, terpinolene, p-mentha-3ene, and p menthane; however the terpinenes should only be intermediates that get fully converted to para cymene [61]. Para cymene has several documented biological activities from the antibacterial, antioxidant, anti-inflammatory, antimicrobial, and anticancer properties the molecule possess [60].

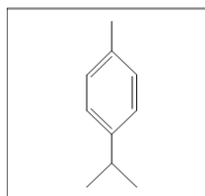


Figure 1-9: Para cymene structure

1.9 Gamma Terpinene

Gamma terpinene is a C10 monoterpene that can be found in nature from emissions of vegetation [92]. Gamma terpinene is present in many essential oils not only *Citrus* essential oils, for example it is a major constituent of thyme oil at 28% behind para cymene and thymol [93]. In 1977 Poulose found evidence of a biosynthetic pathway between gamma terpinene, para cymene, and thymol by which gamma terpinene can produce para cymene which in turn produces thymol [93].

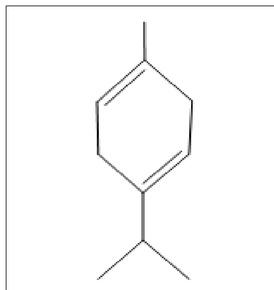


Figure 1-10: Gamma Terpinene structure

1.10 Alpha Terpineol

Alpha terpineol is a monoterpene alcohol derived from limonene, alpha pinene and beta pinene through acid catalyzed and bioconversion mechanisms [96]. It is naturally occurring in pine oil as well as petit grain oil from the bitter orange tree, with additional isomers being beta, gamma, and 4-terpineol [96]. Typically alpha terpineol is used for fragrance, flavor, and cosmetics because of its fresh, lilac aroma; however, its bioactivity makes it popular for use in pharmaceuticals [96]. The bioactivities of this molecule, much like the other terpenes, include antioxidant, anticancer, antibronchitis, antiulcer, antinociceptive, and antihypertensive activities [96].

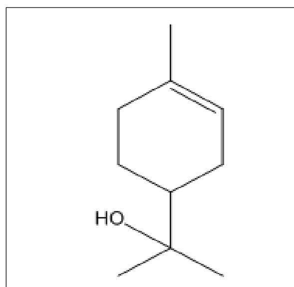


Figure 1-11: Alpha terpineol structure

1.11 Thymol

Thymol at no surprise is the primary aromatic monoterpene found in thyme oil [93]. It has been reportedly produced from para cymene since at least 1923 [93]. Thymol also has presence in tangerine oil varieties as well as Sicilian mandarin oils [8]. The quantities of thymol reported in oils range from 0.04-0.2% with a flavor threshold in water to be 1.7 ppm [8].

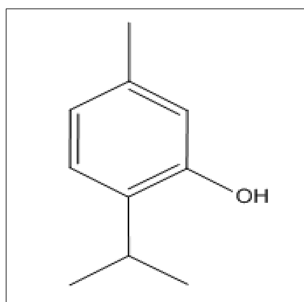


Figure 1-12: Thymol structure

1.12 Methyl-N-Methylanthranilate (MNMA)

MNMA has been designated a key compound in mandarin oils when combined with thymol, without the thymol synergies, the oil is characteristic of tangerine oil [8].

Infrequently is MNMA reported in mandarin oil literature because despite being an important aroma compound, the literature tends to focus on the key terpenes in mandarin only [84]. Nevertheless, it is an authenticity marker for mandarin in other parts of the world [84]. Recently, other MNMA research has identified it as the source of the tingling sensation associated with mandarins due to its analgesic properties for pain [84].

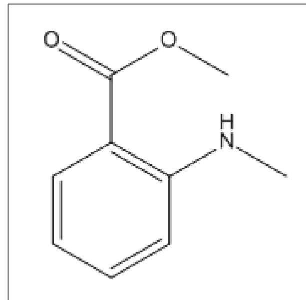


Figure 1-13: MNMA structure

1.13 Beta Caryophyllene

Beta caryophyllene is a sesquiterpene possessing a spicy character most commonly tasted in black pepper [95]. It was the first “dietary cannabinoid” while also being a GRAS material for use in food products [95]. Beta caryophyllene while present

in mandarin and black pepper also contributes greatly to cannabis, hops, cloves, rosemary, and more [95].

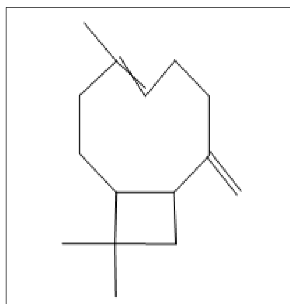


Figure 1-14: Beta caryophyllene structure

1.14 Sinensal

Sinensal is a sesquiterpene aldehyde with isomers alpha and beta, both of which are well known aroma compounds of the Chinese orange [88]. The alpha and beta forms of sinensal are present in a 2:1 ratio with alpha sinensal being the dominant isomer; nevertheless, both isomers possess the same aroma [97]. Aroma descriptors include citrus, floral, fatty, and green nuances [97]. Literature shows that sinensal is able to be synthesized from myrcene [88].

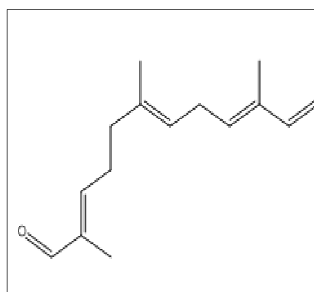


Figure 1-15: Sinensal structure

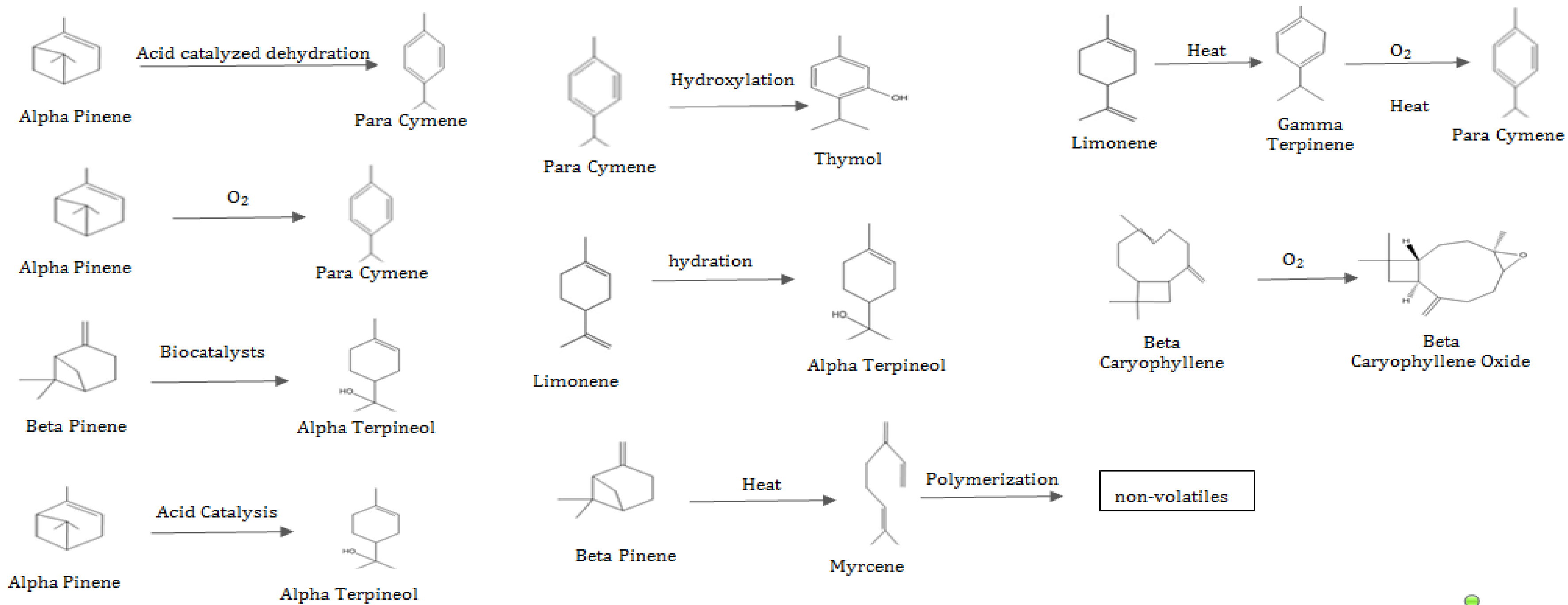


Figure 1-16: Summary of terpene interconversions

1.15 Research Objectives

The aims of this research were three-fold. First, the creation of a unified method for ten marker chemicals of *Citrus Reticulata* Blanco meeting the ICH Q2(R1) validation requirements for an analytical method [33], then, to determine if chemical changes can be identified by chromatographic analytical methods, with these changes altering the organoleptic profile of the essential oil, next to determine if there is a statistical correlation between the composition and organoleptic properties of the oil leading to a criterion for oil selection, and lastly if the statistical methods are insufficient, to develop a model that can correlate analytical data to sensory analyses. It is hypothesized that statistically significant differences in the distribution of organoleptic markers can be used to discriminate *Citrus* oil quality. These differences will correlate with olfactory perception, thus offering quantitative measures to support the descriptive qualification of the oil provided by the sensory analysis.

CHAPTER 2

LITERATURE REVIEW OF PROPERTIES AND ANALYTICAL TECHNIQUES TO CHARACTERIZE CITRUS OILS

2.1 Introduction

In recent years, global trends favoring natural products in flavors, fragrances, pharmaceuticals, and aromatherapy has driven an increase in the essential oils marketplace. Specifically, *Citrus*, is key class of essential oils that has a plethora of avenues for chemical research to be conducted due to the diversity of the class. *Citrus* includes orange, grapefruit, lemon, lime, mandarin, and tangerine essential oils plus the varieties of each subclass. Despite the increased interest in essential oils and the shifts in the focus of research, general research dates back to the 1950's and earlier. *Citrus* essential oil research has expanded into Huanglongbing (HLB) the disease of *Citrus* greening, biological, toxicological, and health benefits, health risks, use of multi-dimensional analysis techniques, and oil authenticity.

Mandarin is an example of a *Citrus* essential oil that has gained attention due to its specific flavor profile. Research on Mandarin essential oils primarily focuses on the impact of removing the oils coloration as well as the differentiation between sweet orange fruit/juice/oil compared to mandarin fruit/juice/oil. Typical analyses are performed using gas chromatography-olfactometry (GC-O), GCxGC, high performance liquid chromatography (HPLC), and other multidimensional techniques.

Current chemical research on monoterpenes, sesquiterpenes, thymol, methyl-N-methylanthranilate, and sinensal involve topics that diverge over different fields of study, such as healthcare and pharmaceuticals. Even within the broader study of essential oils, the applications and end use products extended much outside the realm of *Citrus* related and *Citrus* flavored food products. Specifically, research on terpenes lends itself to cannabis and hops related products likely due to popularity in craft beers, microbrews, and the legalization of medicinal marijuana throughout the U.S. The application of terpenes in nutraceuticals has demonstrated many health benefits relating to antibacterial, antifungal, anticancer, and antitumor properties. The utilization of terpenes in nutraceuticals also has its health risks such as skin sensitivity in UV light, dermal rashes, dermal irritation, not to mention the physiological effects that remain unknown. Thymol a primary aroma compound in mandarin that is applicable to savory food solutions based on its presence in dry spices and essential oils, for example thyme and oregano oils. Lastly, sinensal is a constituent in *Citrus* oils that acts as a differentiator among varieties.

The use of essential oils and terpene constituents is continuously expanding due to an increasingly trendy market. Moreover, the following work presents literature reviews and relevant articles on specific categories that explore the application of said essential oils, stability, and common constituents in *Citrus Reticulata* Blanco.

2.2 Citrus Essential Oil

HuanglongBing has been a dominant topic in published research on *Citrus* for quite some time, however, an influx of ideas have emerged concentrating on the benefits of the biological effects.

Toxicological effects were reported for d-limonene, a naturally occurring monoterpenes associated with *Citrus* and other flavors and fragrances. Research suggests that humans receive approximately 96% of their exposure to limonene from its use as a food additive and while its GRAS (Generally Regarded as Safe)-approved, there are reports that show limonene's hepatotoxicity and neurotoxicity are attributed to its easy absorption through the gastrointestinal tract for both animals and humans. Most research has been inconclusive making the assessment of limonene's effect difficult to accomplish, but warnings have been published indicating that too much exposure has the potential to be toxic. Ravichandran explains that future research on the topic should include limonene, its metabolites as well as the toxicity of the metabolites when ozone treatment is applied to food processing and products where limonene is present [71].

Research on limonene's biological effects was compiled by Viera et al.. Limonene was proven to possess anti-inflammatory activity along with antioxidant, anticancer, antinociceptive, antidiabetic, treatment of metabolic syndrome, gastroprotective and ulcer healing activities. The availability of this naturally produced chemical and its high concentration in essential oils make the chemical attractive to use as a natural therapeutic. Future research aims at preclinical and clinical trials of limonene

on the aforementioned conditions as well as investigations of limonene's effectiveness to treat colitis, asthma, even airway inflammation.

It should be noted that aside from well-known fragrance and food related applications of C*itrus* essential oil, *Citrus* as a genus, has reported medicinal benefits. Dosoky reported the biological activity for eleven *Citrus* oil variegates in July 2018 [73]. Specifically, references were made to the antibacterial and antimicrobial activity the oils possess. Dosoky et al. also identified the biological activity of key essential oil components: limonene, gamma terpinene, linalool, linalyl acetate, alpha terpineol, geranyl acetate, terpinolene, and beta pinene. While this genus of essential oils are generally regarded as safe (GRAS) further revelations have been made of the photo toxicity effects from expressed essential oils on humans that distilled essential oils do not possess; the difference is in the furocoumarin content in expressed essential oils [73]. Overall, it is these new reports on *Citrus* that are making it popular and versatile for applications extending outside the flavor and fragrance industries.

On a separate note, *Citrus* oil research has also been extended to multidimensional techniques with the purpose of finding applicability and usability of the methods to understand different views on the volatile compositions of essential oils. Techniques included GC x GC, GC x GC-FID, HPLC-GC, and GC x GC MS. GC x GC was shown to be applicable for fingerprinting the oils for the purpose of identifying oil origin, treatments of the oils, and the properties of oils that could affect the sensorial perceptions. The use of mass spectral detection in the techniques was pertinent for contaminants and adulteration research. GC x GC was also deemed suitable for co-

elution of enantiomers. After learning of the applicability of GC x GC analyses, the author concluded that a gradual increase in use of these techniques will become prominent in future literature [76].

Essential oils are often in high demand and costly to produce due to the effects of HLB and other poorly managed environmental conditions affecting cultivation. For these reasons that adulteration of essential oils is on the rise. Unfortunately, with the growing awareness revolving around adulteration of essential oils, trade deals worldwide are negatively impacted due to the slow process in analysis and verification of oil authenticity in the trade process [77].

Common adulteration methods include the use of synthetic materials, the use of volatile constituents from different natural sources, the addition of non-volatiles, and the use of vegetable oils. These methods are applied to decrease cost and maintain a consistent density of material. The modifications typically account for 8% of the composition for the oil so historically common analytical methods have difficulty detecting the alterations in composition [77].

In order for an oil to be considered authentic, it needs to be free of extra components, foreign bodies, and the raw materials must be pure. Common instrumental techniques used to assess authenticity include gas and liquid chromatography, electronic nose, mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and UV-Vis spectroscopy. In this application, these tools are used to determine whether essential oils conform to the upper and lower composition ranges defined in regulatory standards for the marker chemicals of a given essential oil. Furthermore, chiral

chromatography methods have contributed greatly to adulteration research of essential oils due to their ability to quantitate enantiomers [77]. The ever growing challenge is for analytical chemists to keep up with the subtle yet fraudulent ways essential oils are being produced by developing appropriate methods for quantitative analysis.

Unlike adulteration, the decoloration process of *Citrus* essential oils is known to improve upon the overall product quality. The process of removing color has the added ability to remove terpene hydrocarbons improving upon the deterioration of *Citrus* essential oils. Improvements can be seen in the extension of oil shelf life and increasing the oxygenated components to intensify the oil's aroma. This was confirmed by analyzing citral, limonene, and linalool in a sample of orange essential oil before and after decolorization. The compounds were quantified using an external standard method by GC-MS and an HP-5 column. The study demonstrated that decolorization provided a decrease in limonene content (94% to 22.1%) and increases in linalool and citral content (1.4% to 3.1%), while maintaining pleasant sensorial experiences in both aroma and taste [78].

2.3 Mandarin Specific Essential Oil Research

The flavor difference between orange and mandarin has been a convoluted topic in *Citrus* research until Feng et al. performed a comparative analysis. Feng et al. found sensory analysis to be key in differentiating orange flavor from mandarin flavor with the assistance of trained panelists. Aroma extraction dilution analysis (AEDA) was employed to obtain odor activity values (OAVs) for the volatile constituents of orange

and mandarin. Chemicals with odor activity values greater than 1 were identified as potent chemicals heavily influencing the aroma profile of the mandarin or orange. Key orange aroma components identified were ethyl butanoate, ethyl-2-methylbutanoate, octanal, decanal, and acetaldehyde. Key mandarin aroma components identified were linalool, octanal, alpha pinene, limonene, and (E,E)-2,4-decadienal. Chiral analysis for limonene and linalool was completed to further obtain the enantiomer composition. Limonene was detected in its (R)- enantiomer form in all samples analyzed which coincides with the (R)-enantiomers orange sensory characteristics and previous studies on the *Citrus* fruits. Linalool on the other hand was detected in its (S)-enantiomer form however the (S)-enantiomer does possess citrus characteristics lending itself well to the application [31].

Further research was performed on the aroma active components of mandarin by Huang et al. Characterization of these aroma active components in mandarin by GC-MS and GCO concluded that they are found to be tolerant of HLB. Huang et al. aimed to identify chemicals in the oils that were responsible for the aroma of the fruit hybrid [9]. Techniques such as solvent assisted flavor extraction (SAFE), GC-MS/O, and AEDA enabled Huang et al. to compile flavor dilution factors ranging from 2-256 for the aroma compounds. A flavor dilution value greater than 128 was considered to have significant importance to the oil's aroma. A flavor dilution value greater than 16 was assigned to the second grouping of molecules of significant interest. Group one consisted of alpha pinene, limonene, beta myrcene, linalool, and beta caryophyllene, all of which are crucial to the mandarin aroma based on aroma extraction dilution analysis. Group two molecules

included gamma terpinene, beta pinene, terpinolene, alpha terpineol, p-dimethylstyrene, linalool oxide, decanal, perilla aldehyde, and beta cubebene, which are also noted to be influential aroma compounds for mandarin. These chemicals when classified organoleptically are floral, lemony, peely, green, mint, sweet all of which are comparable to organoleptic descriptors of the natural mandarin peel oil itself [9].

2.4 Non-Validated Methods

Zoccali et al. (2017) evaluated the volatile oxygenated profile of mandarin essential oil with a non-validated methodology. This work consists of two chromatographic techniques using C7-C30 n-alkanes for retention index calculations without internal or external standardization or other calibration means. Mandarin essential oils were prepared by a 1:2 dilution in hexane. Initially, normal phase HPLC (NP-HPLC) was performed to collect concentrated fractions of the oil to obtain the volatile oxygenated fraction separate. Detection was made with photodiode array (PDA) scanning from 190-390 nm. Collected fractions were reduced under nitrogen to 100 ul and injected into a GC x GC-qMS system configured with a SLB-5ms column, an uncoated column for making the double loop, and a supelcowax-10 column. A mass spectral scan from 40-360 m/z was performed in electron ionization mode; and identification was supported by the FFNSC 3.0 mass spectral library [1]. Previous work by Zoccali et al. (2014) used a similar methodology with the addition of ‘The atlas of spectral data of sesquiterpene hydrocarbons’ [80].

In 2017 Xiao et al. analyzed a variety of mandarin fruits by both GC-MS and GC-O with HP-INNOWax and DB-5 columns. Similar to Zoccali et al., Xiao et al. evaluations used C7-C30 alkane mix for calculating retention indices as well as authentic standards, NIST08, and Wiley7n spectral libraries for the purpose of volatile constituent identification. In terms of methods for analyte quantitation, each chemical of interest was applied to a match matrix of fruit juice, and then set at eight concentration levels to build a calibration curve. The calibration curves were made plotting the peak area ratio of analyte to internal standards against the concentration ratio of analyte to internal standard. GC-O online analysis was performed, by four trained individuals, using the same polarity columns that as GC-MS testing, but an FID was implemented as the secondary detector to the Olfactory Detection Port (ODP). Prior to analysis samples were extracted using a DVB/CAR/PDMS SPME fiber in order to extract the constituents from the complex juice matrix for both standards and unknowns. It was concluded that 22 odor active chemicals were pertinent in creating mandarin aroma in juice. Key constituents included nonanal, hexanal, linalool, (R)-limonene, beta-ionone, decanal, gamma terpinene, and methyl butyrate. Limonene and gamma terpinene were noted as crucial compounds to the woody character mandarin delivers from its juice [79].

Huang et al. implemented an innovative modification of the instrumentation set-up by using a GC-MS with an ODP detector and a1:1 effluent split to characterize major aroma compounds found in the oil of a mandarin hybrid found to be tolerant of the disease HLB. Similar to previous studies, alkane mixtures were used to assess mass spectral data to identify aroma chemicals. All samples were vacuum distilled with the

SAFE method and SPME, with DVB/CAR/PDMS fiber, was used as a backup method of extraction in the event suspected volatiles were lost in SAFE. Huang et al. used a free fatty acid phase (FFAP) column to improve quantitation of the desired compounds. Aroma extraction dilution analysis was performed with the ODP to determine the threshold of detection and describe the character of the aroma at the different concentration ranges. Quantification of the aroma compounds was performed with an internal standard that was added at the beginning of the sample preparation to ensure the percentage of each compound could be calculated based on the peak area in the detection readout [9].

A literature search of validated methods on thymol, methyl-N-methylanthranilate (MNMA), and sinensal presented results that were inconclusive. For MNMA, minimal research has been conducted, but one article of interest is the determination of bitterness in *Citrus Reticulata* Blanco leaves. The work investigated how bitterness was attributed to MNMA by analysis of NMR and 1D and 2D MS [84].

Thymol research in the last five years revolves around its use as a nutraceutical and its associated health effects, but the methodologies performed were non-validated in nature.

Sinensal's most competing research from 2015 involves the analysis of two *Citrus* cultivars extracted with supercritical CO2 and analysis using GC-FID/MS. This method was again a profiling project, not one that contained methods validation. The presence of sinensal in the extracts was a point of differentiation between cultivars [85]. In a second article involving green mandarins Silvestre et al. investigated the use of vacuum

fractional distillation with analysis GC-MS using the same HP-INNOWax column as previously mentioned. No method validation was pursued but the presence of sinensal, thymol, MNMA, and all of the terpenes monitored in this *Citrus Reticulata* Blanco project were detected. The vacuum distillation process did not produce a detectable thermal degradation of the compounds, which is important to note as these compounds are known to be thermally unstable and need to be monitored during any essential oil processing [86].

The work presented thus far demonstrates a common theme to published research. These research topics primarily focus on profiling raw fruits, their juices, and the extracted essential oils to uncover unique differences in a sample's chemical composition. While commonalities may exist in the presented research such as the use of calibration curves of standard mixtures and the application of internal standards for normalization of chromatographic response; there is also a uniqueness that distinguishes and defines the present work, such as the use of a differing polarity GC column for a single assay utilizing both qualitative and quantitative detection techniques which has been scarce in literature to date. Furthermore, the primary gap in the preexisting research is the lack of validation methodologies establishing the suitability, repeatability, accuracy, precision, and sensitivity of an analytical technique and method for analysis of different compounds in complex matrices.

2.5 Validated Methods

Previously validated methods associated with *Citrus* essential oil, such as the work by Tranchida et al. (2013) differ from non-validated work in that analysis involved detection with a more advanced technique, triple quadrupole mass spectrometry, rather a simple gas chromatography methodology. The molecules of interest in the mandarin oil studied were specific to three preservatives, *O*-phenylphenol, butylated hydroxytoluene, and butylated hydroxy-anisole [81]. The validated work included an evaluation of accuracy, repeatability, linearity, sensitivity, etc. on the studied materials to ensure the method is suitable for its intended use.

More recently, Paolini et al. validated a fast GC-tandem mass spectrometry method for a variety of volatile organic compounds (VOCs), amongst which were terpenes, for products in the oenological class. The 15-minute method aimed to understand the difference in flavor profiles of internationally traded wines and spirits. Three products used to test the method were: balsamic vinegar, wine, spirits, and grape must. It should be noted that of the eleven terpenes validated, alpha terpineol was the only relatable compound to the presented research on *Citrus Reticulata* Blanco [82].

The work presented by Paolini et al. demonstrated the utilization of a validated method for accurate analysis of essential oils [82]. However, a validated work with an increased number of monoterpenes of interest was performed by Jemmali et al.. Jemmali et al. used GC-MS to screen columns to determine which possessed the best resolution. A HP-5ms column with 5% diphenyl and 95% dimethyl polysiloxane was selected for validation because of its ability to resolve the most amount of compounds. During the

screening study, Jemmali et al. noted that the HP-1ms demonstrated approximately 33% more resolved compounds than the RTX-1ms column. This is relevant because the Agilent equivalent to the RTX-1ms was used in the mandarin oil method development research completed in this dissertation. The Agilent equivalent of the RTX-1ms illustrated no challenges resolving terpenes in the work presented throughout this dissertation; however, it should be noted that there was a difference in the matrices for Jemmali et al. (resinous plant material) and this dissertation (essential oils). Jemmali et al. evaluated common terpenes of interest such as alpha pinene, beta pinene, and beta caryophyllene. The separation was accomplished on a HP-5ms in 22 minutes with derivatized samples, a temperature gradient, and a pressure-temperature vaporization (PTV) injector [83]. The only commonality to the mandarin oil research was the use of a temperature gradient; the PTV inlet was foregone and the essential oil was only extracted when subject to high acid tasting solution containing a high concentration of sugar.

2.6 Conclusions

In conclusion, it is evident from the literature review how unique and specific methodologies are for applications of *Citrus* and more specifically, mandarin essential oil. The review demonstrated that the analyte matrix was a critical factor in the success of a method and that some methodologies required extraction by a means of derivatization, SPME extractions, or supercritical fluid extractions to be successful. The analytical instrumentation utilized in the reviewed studies often differed from that described in the remainder of this dissertation. Most work involved the use of GC-MS

with olfactory detection, two dimensional GC, GV x GC, GC-FID/Olfactory. It should be noted that a list of HPLC methods of both normal and reversed phase were not reviewed as their application was not related to the work in this dissertation. Most importantly, this dissertation describes the application of a method validated to ICH standards listed in Q2(R1) for 10 compounds important to mandarin that is being presented for the first time. Due to gaps in the presented literature search such as the lack of validated methods for thymol, MNMA, and sinensal or the abundance of general screening methods as well as the lacking of methods to quantitate multiple terpenes in a unified method, it is clearly warranted that the efforts to expand this area of research is justified.

CHAPTER 3

EXPERIMENTAL APPROACH: GAS CHROMATOGRAPHY

3.1 Introduction to Chromatography

Chromatography is an analytical technique involving the separation of components from a mixture. The result of chromatography is the identification of each component of a given mixture as well as the ability to form qualitative or quantitative assumptions based on the separation results. An example of a quantitative analysis would be the determination of a specific quantity of a given analyte in the solution mixture in the form of parts per million or other concentration units. The principle of chromatography is that solute molecules in a mixture are introduced to a stationary phase where they temporarily bind until the mobile phase moves the molecules to a detector. The molecules are binding to said stationary phase based on their affinity to the material, their affinity for the mobile phase, and volatility, in the specific case of gas chromatography. It is those molecules with low affinitiy or high volatility that are poorly retained by the stationary phase and therefore elute to the dector sooner; compounds who have a high affinity for the stationary phase need more time to elute to the detector [6]. Stationary phase refers to a solid phase or a solid support with liquid absorbed on its surface, mobile phase is a gas or liquid component [44]. If the mobile phase is a gas, the chromatography is termed gas chromatography (GC). In order for GC to be a successful separation technique, the analytes should be volatile in nature so that the molecules can easily reach the gas state. In mixtures that are very volatile, the option to inject the

headspace (HS) gases above a sample also exists, this technique is denoted HS-GC. The analytes should be in the liquid state, with a matrix that does not contain water, examples would be ethanol or methanol. Ideally, the solvent should be equally as volatile as the sample, and have little affinity for the stationary phase so that it is one of the first components to elute the column and reach the detector. If the mobile phase is liquid, the separation technique is termed liquid chromatography (LC). Liquid chromatography is used for the separation of mixtures with semi-volatiles, non-volatiles, and thermally unstable molecules. In addition to these two techniques, there are nearly a dozen of other forms of chromatography; examples include thin layer chromatography (TLC), size exclusion chromatography (SEC), ion exchange chromatography, supercritical fluid chromatography (SFC), and column chromatography [44].

The approach used on Oil Mandarin Italian Select was gas chromatography, with mass spectral detection as well as flame ionization detection (GC-MS/FID). The volatility of the compounds of interest complemented the instrumental attributes, the molecules were easily resolved with minor method adjustments, and there was minimal difficulty in interpreting mass spectra for peak identifications. Gas chromatography was not the initial separation technique attempted. First, a reversed phase high performance liquid chromatography method with UV detection (RP-HPLC-UV) was developed to quantify terpenes in the essential oil for the purpose of converting that method to a compatible ultra-high performance liquid chromatography method with quadrupole time of flight mass spectral detection (UHPLC-QTOF-MS) under reversed phase conditions. There was significant challenge in developing a method that would have improved

figures of merit over the methods in literature. Run time and resolution were not improved with the liquid chromatography methods. Limonene being the primary "solvent" was present in very high concentration while other components were represented at less than 1% of the limonene concentration creating complications with the detection. The compounds of interest have nearly the same chromaphores and retention characteristics, so spectral detection that was analyte specific could not benefit the method. The terpenes having the same molecular formulas, molecular weights, and mass spectral ions also made mass specific detection complicated. Furthermore, the difficulty in developing a method different would require new technologies that would only be feasible if the methodology offered significant improvement over standard methods. The primary analysis technique for *Citrus* matrices in the flavor and fragrance industry is gas chromatography.

It is forecasted that SFC will become the preferred separation technique for complex *Citrus* products when it rises to an industry standard analysis technique. SFC was contemplated for this study, but obtaining access to the instrumentation was not feasible for the timeline and is currently not well established in the industry.

3.2 Gas Chromatography Principles

Gas Chromatography uses a column designed one of several ways with the stationary phase selected based on the polarity of analytes being separated. The mobile phase, or carrier gas, in gas chromatography is always inert as to not react with the sample or the stationary phase. Most common mobile phases are helium and nitrogen,

but on occasion hydrogen is used [6]. The mobile phase is passed through the column at high pressures where the vaporized sample then meets the gas stream so it can be carried through the chromatographic system [44]. As a mixture travels the length of the column the solutes will interact with the stationary phase differently causing a separation of the mixture to occur [6]. Separation is defined by the distribution coefficient (K_c), a ratio of the concentration of solute molecules in the stationary phase to the concentration of solute in the mobile phase [6, 51]. If K_c is large than there is a large retention of solute molecules on the stationary phase and when K_c is small, temperature is likely high and the retention is therefore low because most solutes are traveling through the mobile phase straight to the detector. The distribution coefficient being high is visually apparent by wider peak shape and when it is low the peaks are thin and narrow [51].

The coefficient of determination sets up for resolution. Resolution is a measurement of how well two solutes separate in a chromatographic column [6]. Chromatographic peaks should be thin and Gaussian; the wider a peak, the more likely the peak is to interfere with a surrounding solute peak [6, 52]. Resolution is dictated by three key chromatographic parameters 1) efficiency 2) selectivity and 3) retention [52]. The resolution equation encompassing those parameters has been written as seen in equation 3-1 and 3-2 [51].

$$\text{Rs} = \frac{\sqrt{N}}{4}\left(\frac{k}{k+1}\right)\left(\frac{\alpha - 1}{\alpha}\right)$$

Figure 3-1: Resolution calculation

$$\text{Rs} = \frac{\Delta t_R}{w_{av}} = \frac{0.589\Delta t_R}{w_{1/2av}}[6]$$

Figure 3-2: Resolution calculation

The N term is representative of the column efficiency, based on column dimensions plus the carrier gas, α is representative of the selectivity of the given stationary phase and temperature, and k is the retention factor dictated by the given stationary phase, temperature, and the column dimensions [51, 52]. To break this fundamental equation into its key parameters there are three separate equations to note: retention factor, k, selectivity, α, and efficiency via number of theoretical plates, N [6, 51, 52]:

$$k = \frac{t_R - t_0}{t_0}$$

Figure 3-3: Calculation for retention factor

$$\alpha = \frac{k_2}{k_1}$$

Figure 3-4: Calculation for selectivity factor

$$N = 16(\frac{t_R}{w})^2$$

Figure 3-5: Calculation for number of theoretical plates

Retention factor, k, is a measurement of retention, in unit time, of a solute on a column. This is calculated using the ratio of retention time of the desired solute to the retention time of a non-retained solute, like a solvent [53]. The longer a component is retained on the column, the more column volumes of mobile phase that are needed to push the component to elution. If a particular compound spent four times more time in the stationary phase as it did in the mobile phase, $5t_m$ to elute, the retention factor would be $(5t_m-t_m)/t_m = 4$; if the compound is not retained on the column, it spends all of its time in the mobile phase and therefore has a retention factor of zero [6]. It is ideal to have a retention factor in the 5-7 range, but between 3 and 10 is sufficient as long as analysis time is not being wasted to achieve these numbers [52, 53]. To separate two or more

compounds from each other, their retention factors should be different enough from each other which are where the selectivity factor comes into play [52].

The selectivity factor is a measure of how far apart the retention factor of two peaks are, which would describe how well the peaks could be distinguished by the system. To obtain the separation factor it is typical to perform a ratio of the retention factor, k, of each adjacent peak [53]. Alpha should be greater than one for a separation to take place, which means equations in Figure 3-4 above, k_2 should be larger than k_1; otherwise, less than or equal to one indicates co-elution [6, 52, 53]. Agilent Technologies and authors of quantitative chemistry textbooks, like Daniel Harris, publish the criteria that resolution equal to 1.5 can be deemed baseline resolved and resolution of 2 is considered great success during method development [6, 51, 52].

Efficiency of a chromatographic separation is evaluated by the number of theoretical plates, N; a measure of peak width for an analyte during separation [55]. This parameter is best when it is large as it indicates the column is efficient for the analyte, the peak is sharp and narrow, the detection was sufficient, and there is enough peak capacity to resolve complex samples [52]. Efficiency is a result of the compound and its retention, the column dimensions, the carrier gas chosen, and the flow rate of the carrier gas [55]. Plate number is limited by a few experimental conditions, specifically analysis time and analysis pressure [52]. The plate height, H, is also a measure of efficiency that finds its place in various forms of the theoretical plates formula.

$$H = \frac{L}{N}$$

Figure 3-6: Plate height calculation

The L term is length of column and the N term is the number of theoretical plates [55]. The smaller the plate height, the narrower the solute bandwidth, resulting in more plates within the column length [6, 55]. Efficient columns contain more plates than inefficient columns [6].

The van deemter equation relates the plate height back to the flow rate, u_x, and the selected column using the equation below: [6]

$$H \cong A + \frac{B}{u_x} + Cu_x$$

Figure 3-7: Plate height calculation

The A, B, and C terms are constants that are determined for a particular column and its stationary phase; meaning if you change the stationary phase or the column, each of these constants change [6]. The A term represents the multiple flow paths solutes can travel through a stationary phase, mainly just for packed GC columns and LC columns. Flow path is entirely dependent on the particle shape and diameter; however, in cases where the packed columns are used, the relationship is directly proportional to the plate height [55]. An approximation of what multiple flow paths in a packed column look like is depicted below [6, 56].

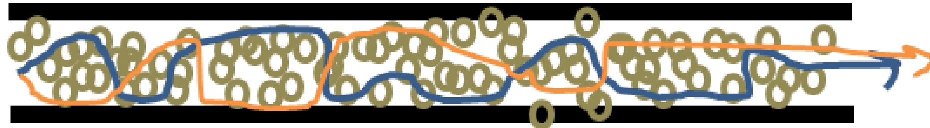


Figure 3-8: Visual illustration of multiple flow paths in a packed column

The B term, which is inversely related to the flowrate, represents longitudinal diffusion of the analyte band; this occurs from high concentration to low concentration

across the column. Longitudinal diffusion is less noticeable when the flowrate is high because the analyte spends less time in the column allowing less time for the band to broaden as much [6]. A depiction of longitudinal diffusion is depicted below [6].

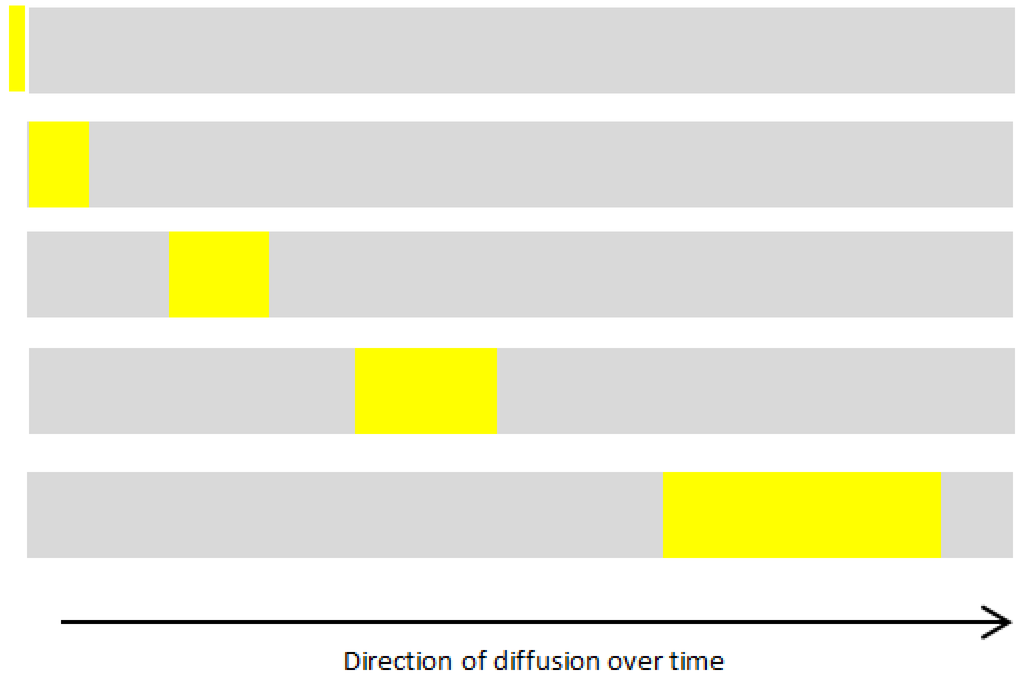


Figure 3-9: Visual illustration of longitudinal diffusion

The last term, Cu_x, is mass transfer, a proportional component to plate height and flow rate, u_x [55]. It is capturing the time required for the solute molecules to reach equilibrium between the mobile phase (C_m) and stationary phase (C_s). The fundamental equation exists as so [6]:

$$Cu_x = (C_s + C_m)u_x$$

Figure 3-10: Mass transfer calculation

Of the three important resolution parameters, Agilent Technologies has published in their supporting documentation that selectivity had the most impact on separation; however, because it is too difficult to predict thereby slowing method development processes, the number of theoretical plates should be a primary focus to chromatographers [52].

3.3 Gas Chromatography Design

The essential components of a gas chromatograph include the inlet where the sample is fed from its vial through a septum via a syringe, the capillary column housed inside the GC oven which is programmed to desired temperature(s), the gas lines that feed the carrier gas into the column as well as feeds gas to the detector(s), the detector(s) of choice is next in the design, and lastly the computer for data handling.

A GC inlet can be one of six varieties if you use an Agilent instrument as was used for this research. Options include a programmable temperature vaporization (PTV) inlet, a split-splitless inlet, a cool-on-column inlet, or MMI, purged packed, and volatiles inlets [51]. The instrument used for this research was setup to have PTV in the front inlet and split/splitless in the back inlet; the split splitless was the inlet used; however, an explanation of both is described. A PTV inlet is programmed to carry out various temperature settings when a sample is introduced before said sample is pushed through to the capillary column within. This inlet type can be run with a split ratio or splitless. An example of a PTV inlet is a thermal desorption unit (TDU).

A split/splitless inlet allows an analyst to select the amount of sample that enters the capillary column, a split ratio; however the temperature at the inlet is isocratic unlike the PTV inlet. Splitless mode means the full sample size injected is dumped onto the column. When a sample is very concentrated, this is not the best approach because eventually when the analytes reach the detector, the response is overloaded and resolution is compromised. The best option is to choose a split ratio in these instances so that partial sample volume is injected onto the capillary column as to not overload the response. The

remaining analyte that is unwanted gets purged out a separate valve called the split vent before the sample is pushed into the column. If a sample is very dilute, then a split ratio is not always the best choice and splitless mode should be considered for an optimal choice. Ultimately, the split ratio whether it's 100% or not dictates the efficiency of the sample injected which is seen visually by the sensitivities and peak shapes in the chromatogram [51]. In either inlet example, liners are placed inside the injector that come packed, unpacked, baffled, as well as straight that each come with their own advantages for sample introduction into the inlet. The goal of the research is what determines the best liner to implement on. The overarching function of the liners is to aid in vaporization properties of the sample injected into the hot inlet [51].

The capillary column has one primary function which is to carry the solutes down the column to the detection device. Columns can be packed or open tubular though open tubular is more commonly used. Open tubular columns are made of fused silica and polyimide to help them withstand temperatures up to 350℃ and moisture from the surrounding environment. Packed columns are better suited for larger sample capacity handling; however they are not as great at providing high resolution, sensitivity, and short analysis times [6]. Columns come in a variety of open tubular options, packed options, as well as sizes because the stationary phases are vast.

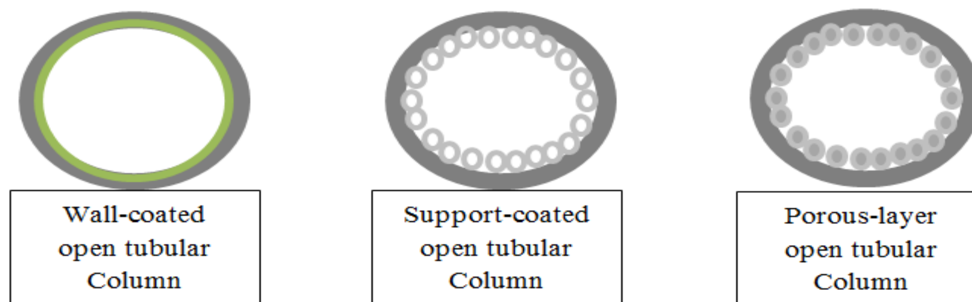


Figure 3-11: Cross section of the open tubular column varieties [6]

To decide between the plethora of options, manufacturers will often provide a guide indicating which column is better suited for each analysis type. In general, polarities of columns can range from very nonpolar such as a DB-1 column, to mid polar such as an RTX-1701 column, to polar such as a wax column. Many variations of GC columns exist between polar and nonpolar, and some variations are even dictated by the type of detector. The column is compatible with PTV and split/splitless inlets because it is connected from inside the GC oven to the bottom of the inlets and to the detectors. The capillary column has programmable parameters for pressure, flow rate, and its mode of use, all of which affect retention time and efficiency of the chromatographic separation [51]. The modes for the column are constant flow or constant pressure; the user can decide which parameter is of use to their research. The column temperature is controlled through the oven parameters.

Detection options vary for gas chromatography. There is the option to use mass spectrometry detection (GCMS), flame ionization detection (GC-FID), sulfur detection (GC-S), nitrogen-phosphorus detection (GC-NPD), and even olfactometry (GC-O). Each detection option has its benefits, so it's usually advantageous to have multiple options at one's disposal when evaluating complex and/or unknown samples. The most common is flame ionisation detection (FID), but also mass spectrometry (MS) is very useful. In this work, the gas chromatograph was setup to capture both FID and MS data with the use of a capillary flow technology (CFT) switch. A traditional GC is equipped with an FID because it a very simply designed detector that spans several orders of magnitude in its ability to produce a linear response [45]. Flame ionization detection is often times described as a carbon-selective detection method because of its ability to have a molar response equal to the carbon atom number for hydrocarbon molecules, and a relative response as long as several carbons are in the molecular structure [46]. Flames are an environment where very rapid reactions take place and in doing so, very excited particles are formed in the burning process; the flame is lit using a glow plug that serves as an igniter [45, 46]. The resulting flame is a make up of hydrogen and air with makeup gas coming from the carrier/mobile phase gas in the capillary column [46]. The molecules are exposed at the FID jet where an excess of air is flowing so the organic molecules can combust in the flame, the combustion process is referred to as natural ionization [45, 46]. Above the flame is a collector electrode that collects the charged particles that are created by applying a voltage of 200- 300 volts between the tip of the jet and the collector electrode; either of these two parts are grounded during the process. There are two types

of charged particles collected based on the detector design, either electrons or cations. To create the readout for data analysis, the current, usually very low in amperage, is converted to voltage and from there amplified by an electrometer. There is very minimal dead volume in FIDs so between the minimal injection volume and the rapid chemical signal acquisition, the band broadening effects seen in the chromatography due to the detector are negligible [46].

Mass spectrometry (MS) is a favorable detection technique used in chromatography because of its ability to identify the mixture components responsible for each chromatographic peak created in the readout. Mass spectrometry is used to determine the molecular mass associated with each peak seen in a chromatogram and because of this ability, in some industries like Forensics, GCMS has become a gold standard analytical technique.

Unlike other analytical spectroscopic techniques, MS does not use electromagnetic radiation, but instead it commonly uses electron impact to vaporize samples to generate radicals and cations [48]. Electron-impact mass spectrometer (EI-MS) is the more popular instrument used, and was used in this research. When a sample is vaporized in a vacuum, they travel to the ion source where a high energy electron beam strong enough to break chemical bonds bombards the molecules, ejecting an electron from the molecule and forming the molecular ion [48, 49]. The molecular ion is equivalent to the mass of the molecule [47, 48]. It is possible to have both positively and negatively charged molecules form; once in the form of a radical, cation, or anion, the molecule decomposes under fragmentation reactions to form fragment ions [48, 49].

Only fragment ions are detectable by the MS, meanwhile radicals go undetected by the MS as do neutral molecules [48]. The mass spectrometer sorts fragment ions at the mass analyzer according to mass to charge ratio, denoted m/z, which is usually equivalent to the mass because z is equivalent to unit charge [47].

In this research a quadrupole was the mass analyzer used because these instruments couple well with GC separations across the molecular weight ranges of components separated. Quadrupoles operate with four rods working in parallel that separate ions according to their trajectories occurring from the electric fields put off by the rods [49]. The graphical output becomes a histogram named the mass spectrum, that plots the relative abundance on the y-axis and the mass-to-charge ratio or the ionic mass plotted on the x-axis [47].

In an ordinary mass spectrum the molecular ion is the highest m/z plotted, unless isotopes are present, and the peak with the highest abundance in the mass spectrum is considered the base peak, which is commonly assigned a relative abundance of 100% so the other peaks can be scaled accordingly [47, 48]. Using an MS enables analysts to obtain molecular mass information, structural information, and identification information as well as the ability to quantify analytes from a complex mixture separated by the capillary column [49].

3.4 Internal Standard method

An internal standard is a known concentration of an added component to a sample being analyzed, but this component chosen as an internal standard is not a compound that

is likely to be found in the sample. The internal standard should be chemically inert with respect to interactions with other analytes, it should have a unique retention time, and the chosen compound also needs to be similar enough in chromatographic response to the expected compounds of interest [49]. The primary reason for using an internal standard is to eliminate the differences in detection from one sample to the next due to instrumental fluctuations beyond the analysts' control. Mass spectral detection and flame ionization detection data are based on absolute intensity measurements determined by counting the ions created in the flame or MS source. These ions when "collected" create a voltage signal. Low concentration and/or low sample volume with instrumental fluctuations in intensity measurements from voltage changes cause poor quality and reproducibility in detection. In other methodologies, such as HPLC, a reference wavelength is used and the signal from the analyte is ratioed against this reference value. The internal standard supplies an analogous reference value. Internal standards can be utilized in different ways. They can be used to find the response factor to an analyte of interest that is then used to determine concentration of analyte, Figure 3-12, or they can be used to normalize the injected data by converting each response to a ratio of the internal standard peak area with the analyte peak area for all injections [6].

$$\frac{Area\ of\ Analyte\ Peak}{Concentration\ of\ analyte} = F\left(\frac{area\ of\ internal\ standard\ peak}{concentration\ of\ internal\ standard}\right)$$

3-12: Internal standard calculations

In industrial settings it is common to find instruments being utilized for long term research as well as short term projects at once. As a result, the instruments experience preventative maintenance measures that impact the sensitivity and response of the detectors. In addition, there are every day changes that suggest column maintenance such as inlet allocations that require moving of the column daily, weekly, or monthly. Consumables are often forgotten about, but too have an impact on instrumental response and reproducibility if not maintained according to specificiation. For example, GC inlet liners build up residue from repeated injections that if remain unchanged slowly reduces the amount of analyte that makes it to the column. These common examples of maintenance attempt to demonstrate how an internal standard can account for routine changes to an instrument on top of the uncontrollable fluctuations in intensity based measurements that impact the quality of the data generated for research. The ultimate goal in any study, short term and long term, is to produce the most quantitative data possible, and internal standards aid in this effort.

3.5 External Standard Method

An external standard is used much like an internal standard in that it’s a compound of known concentration used to make reference quantification more accurate; however, it is injected into the instrument either immediately before or after the sample is analyzed. The external standard does not take into account the losses that occur during sample preparation [90]. Being that the detector response is capable of fluctuations one

run to the next, the external standard method cannot fully account for the changes beyond the analyst's control especially if the internal standard is not also added to the solution.

CHAPTER 4

DATA ANALYSIS AND STATISTICAL METHODS

4.1 Introduction

During the experimental design and execution of methods for analyzing and understanding the changes in marker chemicals of mandarin essential oil and its evolving process over the course of its recommended shelf life, a number of data analysis techniques were needed. These techniques are commonly a mix of analytical and statistical methods for assessing data output. Such methods are needed in the method validation phase of a project and find plentiful use through application and interpretation of stability studies. These methods are for the purpose of presenting the data in a form suitable for interpretation, particularly when evaluating stability data for indication of a significant chemical change.

4.2 Data analysis techniques in method validation

4.2.1 Weighted or Unweighted Regression: Homoscedasticity vs Heteroscedasticity

In the cases of analyte concentrations ranging by more than two orders of magnitude, the homoscedascity needs to be evaluated [36, 37]. Homoscedascity is evaluated by examining the variance over the range of analysis [36]. Variance is simply the square of the standard deviation and therefore represents the dispersion in the data.

Data are homoscedastic if the variance is not significantly different over the range of analysis. It is relatively common to see the accuracy values differ as concentration declines or as detector response changes and this comprises the reliability of the method. When the variance is not statistically equivalent throughout the range, the data are heteroscedastic [38, 37, 39]. This does not mean the data fails the typical regression assessment; however, it is common to see a high correlation coefficient, r, while experiencing greater error or bias in measurements associated with increasing "x-values" [36, 37]. The correlation coefficient, r, is only reporting the linear association of two types of data, it does not describe the agreement between the data; this is a common misconception [34]. By definition, the coefficient of determination, r^2, simply accounts for the variance in dependent variable predicted from the independent variable. The ideal approach is to find a linear fit that reduces the influence of larger concentrations that give measurements with larger variance than those with more consistent variance.

4.2.2. F -Test for extended linear range

Testing of homoscedasticity versus heteroscedasticity to evaluate the variance as a function of increasing concentration requires plotting of the residuals versus concentration and application of the F-Test to the lowest and highest concentration in the working range [36]. Residuals, R are computed from the difference in the observed signal (S_{exp}) and the signal calculated (S_{calc}) using the original linear regression (OLR) equation: $R = S_{exp} - S_{calc}$ [36, 38]. The preferred outcome of the residual plots is to observe data points that are randomly scattered about the concentration axis [38]. The data points for

each of the mandarin components studied had some scatter to them, but were not convincingly random.

For the data to be considered homoscedastic, mathematically not just visually, the tabulated F value must be greater than the experimentally calculated F value ($F_{tab} > F_{exp}$) and heteroscedastic when the data gives a larger experimental F than the tabulated value. Tabulated F- values are found in a distribution tables under the 99% confidence interval with n-1 degrees of freedom [38]. The experimental F value is determined from the ratio between the variance squared of the highest (s_2^2) and lowest (s_1^2) concentration level within the working linear range [36, 38].

4.2.3 Weighted Linear Regression (WLR)

When the data are heteroscedastic, weighted linear regression is analytically appropriate. Weighted linear regression reveals the random error associated with the original linear fit for a given scenario [37]. Weighting your linear regression model allows for a balance of error distributed across the investigated calibration range(s) [38]. Common weighted factors include $1/x$, $1/y$, $1/x^2$, $1/y^2$, $1/x^{1/2}$,$1/y^{1/2}$ [36, 38]. If using the inverse of y for weighting regression, consideration is being placed on the response variable and when using the inverse of x, weight is being placed on the concentration [36]. The best weighting factor is decided based upon which provided the smallest intercept, the best correlation coefficient compared to the 1x model, and which provided the best sum of percent relative error (%RE). Percent relative error is computed from the comparison of the predicted concentration, C_f, from the best weighted regression and the

nominal concentration, C_n, see equation in Figure 4-4 [18, 19, 20, 21]. Additional assessment would be a plot of %RE versus concentration to illustrate the scatter around the concentration axis; the best weighting factor would produce a band of scattered data points around the x-axis [18].

The weighted slope, y-intercept, and correlation coefficient can be calculated using equations in Figure 4-1, 4-2, and -43 below [36, 37, 38, 39]. The terms are as followed: w_i is the weighting factor, x_i and y_i are the original independent and dependent variables, n is the number of observations, $\overline{X_w}$ and $\overline{Y_w}$ are the average weighted independent and dependent variables, a_w is the slope of the weighted regression, b_w is the intercept of the weighted regression, and b is the original linear regression intercept. In cases like this one where the data appeared linear, but was in fact heteroscedastic, the weighted regression became the new calibration model for quantifying unknown samples containing the analytes of interest [38]. As one last assessment, the bias was evaluated for the simple least squares linear regression model as compared to the nominal concentration values as well as for the weighted least squares regression model. The average magnitude of the of the percent bias over each extended linear range was evaluated for the simple least squares linear regression model.

$$Slope, a_w = \frac{\sum_i w_i x_i y_i - n\overline{X_w}\,\overline{Y_w}}{\sum_i w_i x_i^2 - n\bar{X}_w^2}$$

Figure 4-1: Slope formula for weighted regression

$$Intercept, b_w = \bar{Y}_w - b\overline{X_w}$$

Figure 4-2: Intercept formula for weighted regression

$$correlation\ coefficient, r = \frac{\sum w_i * \sum w_i x_i y_i - \sum w_i x_i * \sum w_i y_i}{\sqrt{\sum w_i * \sum w_i x_i y_i - (\sum w_i x_i)^2} * \sqrt{\sum w_i * \sum w_i y_i^2 - (\sum w_i y)^2}}$$

Figure 4-3: Correlation coefficient formula for weighted regression

$$\%RE = \frac{C_f - C_n}{C_n} * 100$$

Figure 4-4: Percent relative error formula for weighted regression

4.3 Data analysis techniques in Stability Evaluation

4.3.1 Use of internal standardization

The use of the internal standard to normalize instrumental response between injections was first and foremost. Using the internal standard of toluene allowed the analyst to monitor the individual components of interest against the toluene week after

week. With toluene being a constant standard, any fluctuations that could occur from voltage fluctuation impacting intensity measurements and/or maintenance of the hardware, both the toluene and the analytes of interest are affected the same; therefore, data is best represented as a ratio of the peak areas opposed to taking analyte peak area at face value.

4.3.2 Use of External Standard

In addition to spiking each oil sample with a known concentration of internal standard to normalize the chromatography, a multiple standard with the components of interest was also injected prior to any mandarin oil injections. In doing so, the external standard was normalizing the data to account for instrumental fluctuations in analyte response between sample injections and further allowing quantitation in parts per million instead of an area ratio. The goal was to find clarity in the stability results that were becoming challenging to interpret by the internal standard method alone.

$$\text{Assay Concentration} = \frac{\text{Compound area ratio in oil}}{\text{Compound area ratio in standard}} * \text{ concentration of multiple standard}$$

Figure 4-5: Assay formula

4.3.3 Molecular Ions and Base Peaks

Assessment of stability data across 24 weeks of samplings can be performed with use of the mass spectra ions. The first process is to extract the molecular ion of each characterizing molecule and manually integrate its peak area in the total ion chromatogram. The same process is completed for the internal standard chromatographic peak. A ratio of the molecular ion responses is computed with toluene, much like in the internal standard method, to confirm there remains a form of normalization being used between each sampling. Once all ratios are obtained, the data is observed for outliers. The same process as above is then used for the base peaks of each molecule.

4.3.4 Dixon Q Test

Dixon Q test is performed to statistically assess for the presence of outliers in the samplings collected from the stability studies; this tool has been around since 1991. To properly apply the Q test, the data should be organized in ascending order for the average and standard deviation to be computed before any potential outlier data is removed. The apparent outlier is tested by comparing the gap between the values to the range of values. This comparison gives Q_{EXP} compared with Q_{CRIT} at the 95% confidence interval. In this case of seven values, the CI is 0.568 for the data in ascending order $X_1 < X_2 <....X_6 < X_7$ [57].

$$QEXP = \frac{X_7 - X_6}{X_7 - X_1}$$

Figure 4-6: Q experimental formula for Dixon Q Test

In the cases where the experimental Q value is greater than the critical Q value, the largest value taken as X_2 is considered an outlier and should be removed from the data set before proceeding to the z-test. A new average and standard deviation is then calculated any time a data set had an outlier removed so the new average can be carried into the z-test calculations.

4.3.5 Z Test

The purpose of the z test is to determine in units of standard deviation, the distance each data point is removed or separated from the sample average. The preferred result is to not have any data points in the set further removed than two standard deviations from the mean. In the event another data point is outside two standard deviations limits this point is not withing 95% confidence interval and requires investigation. The data point is not an outlier, but it is significantly different than the sample average.

$$Z\ value = \frac{X - \bar{X}}{\sigma}$$

Figure 4-7: Z value formula used in Z Test

4.3.6 Reconciling Mass Balance

Reconciliation of mass is based on the law of Conservation of Mass by which matter cannot be created or destroyed but rather transformed. A simple assessment of mass describes chemical changes in a solution over the length of a given study. Typically reconciliation of mass balance is applied in pharmaceutical work to understand the degradation components of a drug product when it's forcibly degraded. The concept is that when you begin a study with 100% of an ingredient and end with less, the mass is conserved in the system in the form of something else; moreover, when those two ingredients are summed, the final balance equals that from the beginning. For this research mass balance reconciliation was used to assess the change of a sample over the length of an experiment when analyzed by gas chromatography. Gas chromatography methods typically have acceptance criteria +/- 20% for a given measurement due to the high volatility of the analytes and the fluctuations in intensity measurements. When the mass change exceeds this limit, a noteworthy change is occurring causing general failure

of the the sample assay. In this work, the properties of the mixture may vary greatly when mass balance fails.

4.3.7 Bland Altman Method

Comparisons of methods, or sample treatment conditions are assessed in different manners. Frequently, the data obtained from two methods or treatments are simply analyzed by correlation studies. The reference treatment is the independent, x, variable, while the modified treatment or method is the dependent variable, y. Correlation coefficients, r, are not the most reliable means of analyzing data belonging to two separate methods under comparison because r does not report agreement between methods, but instead only the strength of relationship between the variables [66]. The linear strength of relationship between methods is signified by the closeness of the r-value to +1.0 or -1.0 [35]. To continue, scatter plots without a regression line are minimally useful in comparing methods and their agreement of measures because when data is plotted, the points cluster around what would be a regression line, making interpretation of the measurement differences a challenge [65]. Bland and Altman worked, prior to their 1986 publication in *The Lancet*, to develop a simple and quantitative model for comparing methods or sample treatments based on the average and standard deviation of data sets [35, 64]. They proposed a better model based on the average of the methods plotted against the difference in the methods [65]. Trends in the data, outliers, systematic differences in the methods explored, and the magnitude of difference between the methods can be more easily interpreted with this approach [34,

65]. Their expectation was that 95% of the difference data would fall within two standard deviations (+/-2s) of the mean; the bounds were termed the limits of agreement [35, 64]. The differences typically follow a normal distribution because most of the variation is removed from the model leaving just the measurement error; this is why it is expected for 95% of the data to fall within two standard deviation limits. Limits are calculated from the bias - 1.96s, s being the standard deviation, and bias + 1.96s where the bias is the average of the differences from the two methods [66].

CHAPTER 5

METHOD DEVELOPMENT AND VALIDATION

5.1 Introduction

It is common practice to use gas chromatography (GC) methods for the analysis of *Citrus* essential oils due to the high volatility of the *Citrus* oil components that make up the distinct flavor profiles. It is also common to add an Olfactory Detection Port (ODP) to a GC for olfactory determination of the eluting compounds and their individual aromas, this method is often referred to as GC-O. An ODP is a detection unit mounted on the outside of the instrument that allows a person to observe the odor of the chromatographic peaks being separated. The analyte flow is often split so that part of the analyte is sent to an electronic detector like a flame ionization detector (FID) and the remaining part is sent to the ODP. The analyte exits the ODP through a nose cone where a person is awaiting to smell the aromas of each chromatographic peak in real time. The operator then has the ability to digitally record notes about the aromas as well as an intensity measurement for later reference to the analysis. Based on the aroma of the eluting analytes, trained professionals are able to identify the likely chemical responsible. To be able to use an ODP for successful analysis of a chromatographic separation, a flavor chemist is usually the desired professional for evaluation, otherwise someone with a skilled sense of smell is necessary. It is beneficial to use an ODP in the flavor and fragrance industries since the formulas developed are based on the observations of the senses. The benefit of also using an analytical detector such as an FID or a mass

spectrometer (MS), is that you collect identifiable and quantitative data. The MS allows for peak identification as an analyte elutes the column, while the FID produces data much better to quantify concentrations of resulting analytes. While plenty of methods in the literature exist for *Citrus* essential oils using GCMS and GC-O, the search for a validated method for Oil Mandarin Italian Select and its marker chemicals was unsuccessful.

Method validation qualifies an analytical method by performing a series of tests that are indicative of the method's accuracy, precision, stability, sensitivity, suitability, linearity, and repeatability. The goal is to provide solid evidence that a methodology is effective for the purpose it is intended for [94]. Examples of common strategies to method development and validation include creating a method entirely from scratch and using the trial and error approach, developing based off previously published methods in literature, or validating a method already deemed suitable by a company's standard operating procedures [94]. Required tests are directed at a particular feature of the testing media so the methodology can be fine-tuned to deliver appropriate results and prove its suitable for its intended use. It is most commonly performed in pharmaceutical environments to ensure a drug product meets the expected standards for potency, stability, quality, and safety. The validity of a method means the analytical result of testing is reliable, consistent, and trustable. In other words, without a method validation it is difficult to assess reliability of the delivered results coming from the analytical methodology, whether those results appear good or bad. The Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH) have outlined methods, approaches, definitions of each required element, and suggested data

presentation to clearly demonstrate a method is validated for its intended use. All regulatory laboratories rely on validation methodologies to support analytical measurements.

This chromatographic method developed in this work with dual detection techniques was developed for a neat *Citrus* oil of the mandarin variety for a full quantitative profile of the oil as received and an assessment of chemical degradation that are necessary for the flavor industry as they discriminate sample characteristics for product formulation or destruction.

5.2 Experimental

5.2.1 Reagents and Chemicals

Oil Mandarin Italian Select was obtained from Citrus and Allied (C&A) along with alpha terpineol. Beta pinene and gamma terpinene were from Berje, while beta caryophyllene was purchased from Natural Advantage, alpha pinene was obtained from Vigon, methyl-N-methylanthranilate was from Advanced Biotech, and thymol and para-cymene were from Treatt USA. Toluene, the internal standard, sinensal, and myrcene were obtained from Sigma Aldrich. Ethanol was obtained from Brenntag. No futher purification of the compounds was required.

5.2.2 Method Development

The strategy used in developing the final method and subsequent executed in the validation was derived from the various methods published in the literature of *Citrus.* Resolution of our more complex *Citrus* components required further method development with iterative adjustments to meet resolution requirements. Specifically, multiple temperature gradients were screened to evaluate those providing an adequate separation while meeting reasonable runtime requirements. Method A used oven parameters from 40 °C to 280 °C at a rate of 5 °C /min with a 2 minute final hold time. Method A was a total of 50 minutes. Method B ramped up from 40 °C to 100 °C at 5 °C /min, then continued at 2 °C /min until 150 °C before increasing the rate back to 5 °C /min until 280 °C were reached; no hold time was used at 280 °C. Method B extended the overall run to 63 minutes. Method C ramped up 40 °C to 150 °C at 2 °C /min, before increasing the rate to 10 °C /min until 280 °C were reached; no hold time was used at 280 °C. Method C extended the overall run to 68 minutes.

Competing effects of two critical pairs, para cymene-limonene and beta pinene-myrcene, were assessed in each method to help select a method that would meet validation requirements. With the challenge in the separation, mass selective detection was used to aid the analysis. Peak purity and resolution, for para cymene-limonene and beta pinene-myrcene, were identified by mass selective detection; resolution of 1.65 and 2.13 was established when using method C, respectively. Method C meets the analytical standard for quantification and was used in the full validation work.

5.2.3 Instrumentation and Experimental Methods

Oil Mandarin Italian Select, herein referred to as mandarin oil, along with the individual standards were analyzed using gas chromatography-mass spectrometry and flame ionization detection (GCMS-FID) due to the volatility of the materials. Toluene was chosen as an internal standard because its elution time was early enough not to affect the detection of compounds of interest in the neat essential oil being studied. Electron impact (EI) mass spectrometric data was collected using an Agilent 5975C inert XL, triple axis mass spectrometer (MS) interfaced with an Agilent GC System 7890A equipped with flame ionization detection (FID) that used a capillary flow technology (CFT) splitter through the Aux EPC 3. Flow was split with a 4:1 ratio to the FID and MS, respectively, with 3.8 psi constant pressure through the Aux EPC.

The samples were injected into the Agilent split/splitless inlet using a Gerstel multipurpose sampler (MPS-2 XL) oriented with a 10 uL syringe. Injection volume of each sample was 1 uL. For successful separation, an Agilent CP8928 VF-1ms capillary column with dimensions of 50 m x 0.32 mm i.d, 0.40 uL film was used. Injection temperature was 250 °C with temperature programming beginning at 40 °C with no hold time and a rate of 5 °C/min until a final temperature of 280 °C was reached. The oven was held at 280 °C for 2 minutes giving a 68-minute runtime for each separation. Helium was the carrier gas while helium, hydrogen, and air were supplied for the FID. The inlet was programmed to have a 25:1 analyte split with a split flow of 25 mL/min. The FID had a temperature of 300 °C, while the mass spectrometer source had a temperature of

230 °C and the quadrupole was 150 °C. The mass spectrometer scanned masses low to high ranging from 20 - 400 m/z.

Libraries including the Wiley10, NIST14, and FFNSC3 spectral databases were used to identify and confirm the peaks in the TIC, while peak area from the FID trace was used in reporting the amount of analyte detected. Using FID for quantitation is beneficial because it is a universal and highly sensitive detector. Mass spectrometry data is useful for identifying molecules based on their fragmentation patterns.

5.2.4 Sample Preparation

Initially, working standards were prepared for each mandarin oil component from the dilution of the pure material from the manufacturers. A 10% solution in ethanol was made and from there the following concentrations were prepared: 100, 500, 1000, 5000, 7000, 10,000 (1%), 20,000 (2%), and 50,000 (5%) mg/L. Once the overall range of linearity was assessed, a 100% working standard solution was designated as in pharmaceutical practices; this concentration was 5000 ppm with the exception for sinensal, which was designated a 1% working standard concentration based on its specific linear range. Once more solutions were prepared ranging from the LOQ to 130% of the working standard concentration that were used in the linearity and accuracy portions of the method validation. In every sample injected, there was always a constant volume of toluene added as use as an internal standard, that volume was 100 ul to 1 mL of standard solution.

5.2.5 Method Validation

In this chapter, a single method using GCMS/FID for the detection of alpha and beta pinene, myrcene, para cymene, gamma terpinene, alpha terpineol, thymol, beta caryophyllene, methyl-N-methylanthranilate, and sinensal was developed and validated according to the ICH Q2 (R1) guidelines allowing the method to be validated domestically and internationally [33]. This was pursued as such because *Citrus* is in applications that span the globe.

The acceptance criteria is not defined in the ICH guidelines on method validation, moreover, validation expectations were defined by the analyst to include:

- Resolution of critical pairs 1.50 or greater
- A working and linear range for each compound with coefficient of determination or correlation coefficient of 0.9900 and greater
- Relative standard deviation of 10.0% or less for the suitability, accuracy, stability and precision testing of each compound

5.2.5.1 Specificity

The specificity of the analytes of interest was confirmed three ways. Analyte base peaks and analyte parent ions were monitored in scan mode in the chromatographic region of their elution time, while also using positive identification with samples of known concentration of analyte, table 1. In addition, use of the Wiley, NIST, and FFNSC3 libraries aided in peak identification by indicating the quality of each

component identification appointed when compared to standard mass spectra. The criterion was a quality of 85% or greater.

5.2.5.2. Range

5.2.5.2.1 General Working Range

The working range is determined with a full linearity and illustrates to what upper and lower concentration range, the method exhibits accurate, precise, and linear results. To establish linearity and range for the 10 citrus compounds of interest, calibration curves with six data points ranging from 100 ppm to 10,000 ppm were plotted based on FID response and regression lines were determined.

5.2.5.2.2 Extended Linear Behavior of Range

The extended linear behavior of each analyte was studied beyond 10,000 ppm. Concentrations of 20,000, 50,000, and 100,000 ppm were chosen as these equate to 2%, 5%, and 10% solutions of essential oil. The use rates of essential oils in flavor formulae vary making it important to monitor their chromatographic response at high concentrations that resemble use rate in flavor creation.

5.2.5.3 Linearity

Linearity under a defined concentration range for a given analyte, must possess the ability to obtain said analyte's concentration when detected in an unknown solution.

Unlike active pharmaceutical ingredients which have a target delivery concentration in each dose, essential oils have a varying use-rate in flavor formulae and also in their end use applications. To solve this difference, the range had to be investigated for each material as written about in section 4.2.6. Within that generic linear range, a 100% concentration was designated at the median concentration. All compounds with the exception of sinensal used 5000 ppm as the 100% solution; sinensal used 10,000 ppm as the 100% solution due to its loss in sensitivity at concentrations near the LOQ. Choosing a 100% concentration was important in order to follow ICH method validation criteria for linearity which is defined as 70% to 130% and the LOQ to 130%.

5.2.5.4. Accuracy

Accuracy was assessed doing triplicate injections of the LOQ, the 80% solution, the 100% solution, and the 120% solution for each analyte, Table 11. A recovery calculation for each analyte being validated was performed referencing the injections made in section 3.2.5 of the 100% solution and %RSD is calculated off the recovery assay for the triplicate injection of each 100% solution. The purpose was to compare experimental concentrations against theoretical concentrations for measure of closeness.

5.2.5.5 Precision

Precision was demonstrated by making six injections of the 100% solution for each analyte being validated then calculating the percent relative standard deviation (%RSD) of those injections.

5.2.5.6 Sensitivity

5.2.5.6.1 Limit of Detection (LOD)

In order to get a target S/N ratio of 3, the concentration of the compound's solution was adjusted based on a dilution factor targeting the signal-to-noise ratio. Each calculated LOD concentration was prepared and injected in triplicate following a sequence of blank injections. The average and standard deviation were calculated to decipher the degree of uncertainty in the measurements at the LOD due to instrument fluctuations.

5.2.5.6.2 Limit of Quantitation (LOQ)

In order to get a target S/N ratio of 10, the concentration of the compound's solution was adjusted based on a dilution factor targeting the signal to noise ratio. The LOQ concentration was then prepped and ran in triplicate. The average S/N ratio was calculated as was the standard deviation of the triplicate runs to decipher the degree of uncertainty in the measurements at the LOQ due to instrument fluctuations.

5.2.5.7 System Suitability Testing

System suitability is designed to show the instrumental electronics, the samples, and the parameters of analysis are functional for the intended purpose from one day to the next. Replicates of the 100% working standard solution were prepared and ran five times

if the %RSD was under 2% and ran six times if the %RSD was greater than 2%; this was repeated for a total of three days.

5.3 Validation Results and Discussion

5.3.1 Specificity

Alpha pinene, beta pinene, para cymene, gamma terpinene, methyl-N-methylanthranilate, and beta caryophyllene had quality of hits equal to or greater than 95%. Myrcene, thymol, and sinensal had quality of hits greater than or equal to 90%, and alpha terpineol was the sole compound at 86% quality of the match. Figure 5-1 illustrates the GC separation of the mandarin oil, with critical pair para cymene and limonene annotated. Selected ion monitoring of para cymene base peak, 119 m/z, was used to extract para cymene from the limonene peak, Figure 5-2. The chromatographic peak of para cymene lacks Gaussian shape despite being representative of a single extracted ion. As stated in section 3.1, limonene and para cymene were a critical pair that had resolution of 1.65, indicating there was baseline resolution present despite the visual assessment. Limonene was disregarded because it had such large concentration since it acts as a solvent in essential oils and it was not a component being quantified in this research. It should be noted that in the blank matrix containing ethanol and toluene at the same concentration as in the oil samples, there are no interfering components as demonstrated in Figure 5-3. Figures 5-4 through 5-7 illustrate the method separation for the ten compounds of interest.

Table 5-1: Base peak ions used for additional peak identification

Compound	Base Peak Ions (m/z)	Retention Time Scanned
alpha pinene	93	24.00 -25.00 min
beta pinene	93	27.00-28.00 min
myrcene	41	27.00-28.00 min
para cymene	119	29.00-31.00 min
gamma terpinene	93	32.50 - 34.00 min
alpha terpineol	59	42.00-43.00 min
thymol	135	48.50-49.50 min
methyl-N-methylanthranilate	165	55.50-56.50 min
beta caryophyllene	93	57.00-58.00 min
sinensal	93	64.00-65.00 min
toluene	91	13.00-14.00 min

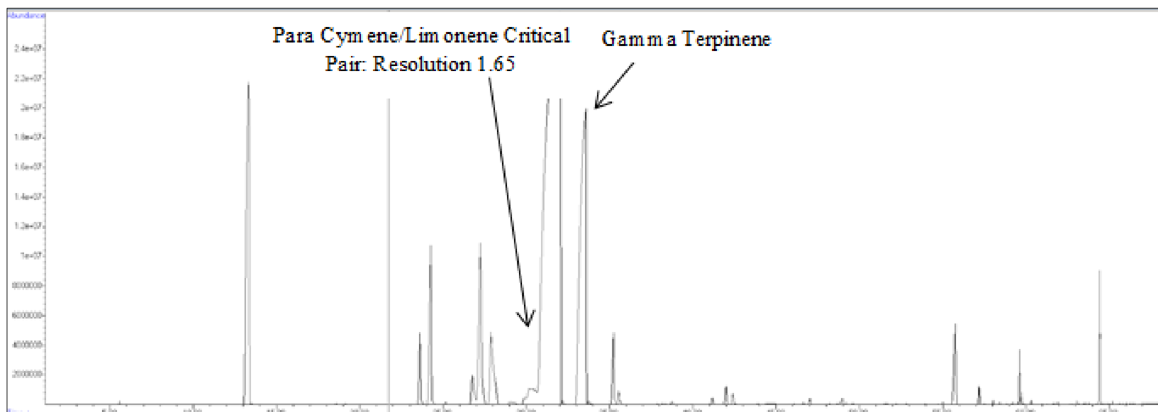


Figure 5-1: Representative TIC of mandarin essential oil from 0:00 - 68:00 minute

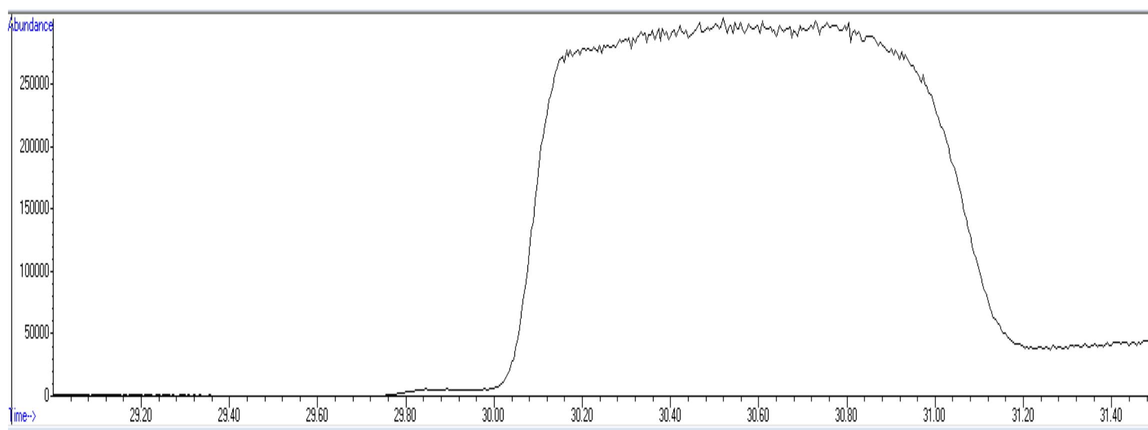


Figure 5-2: Base peak ion119 m/z for para cymene from 29:00 – 31.50 minutes

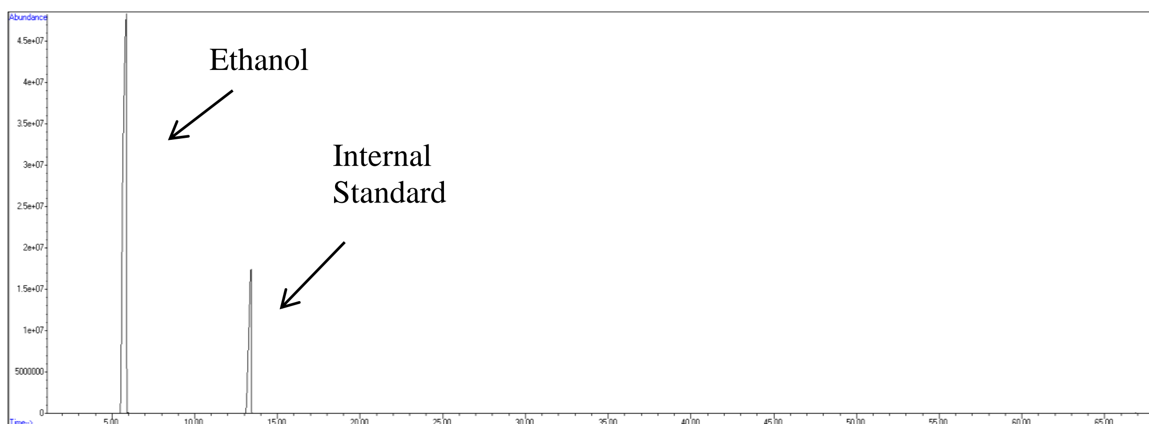


Figure 5-3**:** Representative TIC of a blank solution from 00:00- 68:00 minutes

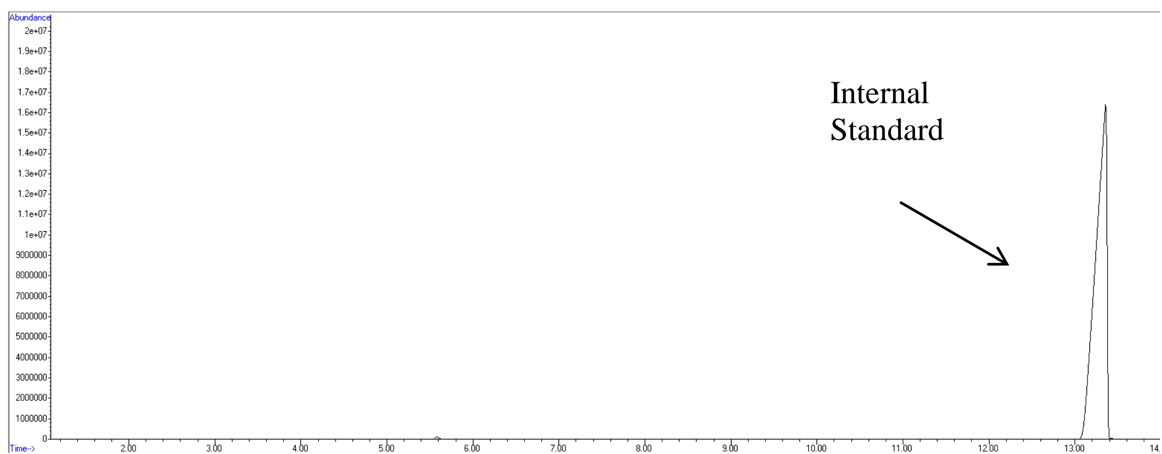


Figure 5-4: Representative TIC of mandarin essential oil from 0:00 - 14:00 minutes

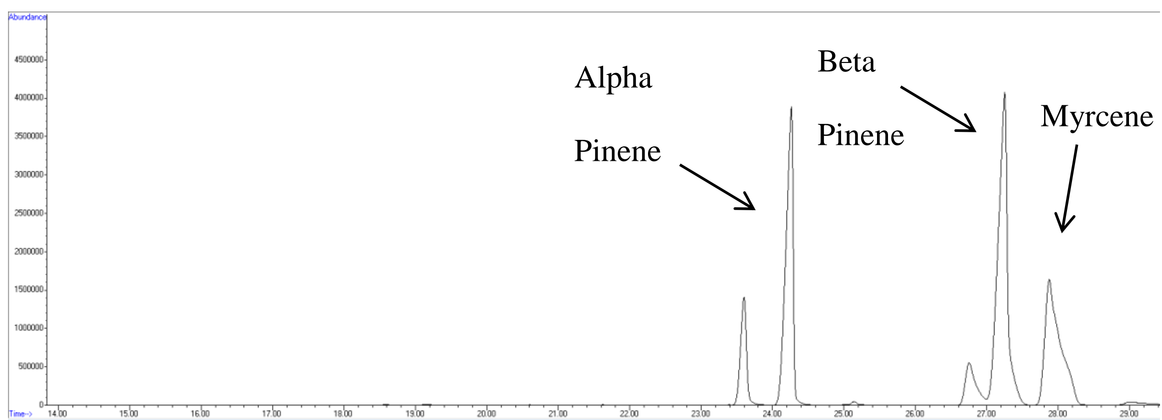


Figure 5-5: Representative TIC of mandarin essential oil from 14:00- 29:00 minutes

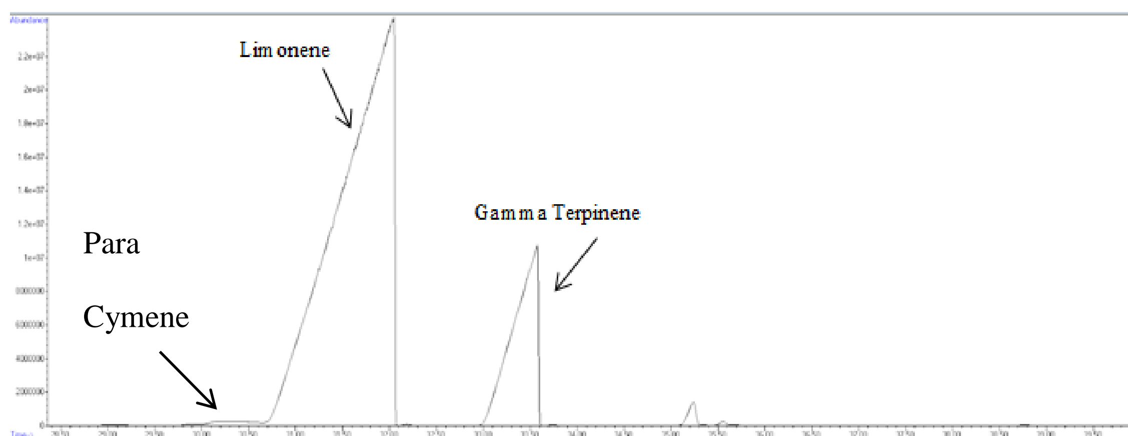


Figure 5-6: Representative TIC of mandarin essential oil from 28:50- 40:00 minutes

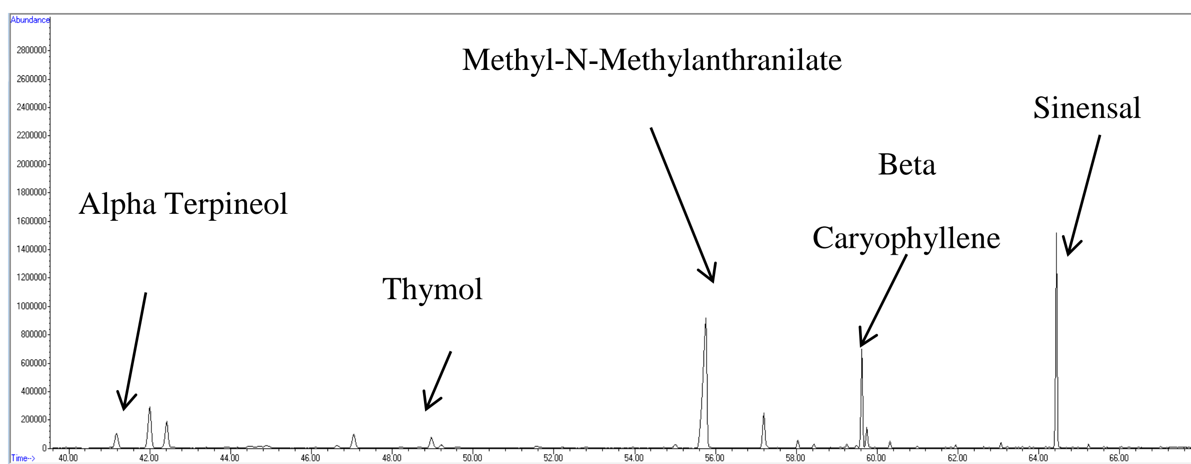


Figure 5-7: Representative TIC of mandarin essential oil from 40:00- 68:00 minutes

5.3.2 Range

With the exception of methyl-N-methylanthranilate and sinensal the Excel linear regression output for concentrations ranging from 100 ppm to 10,000 ppm provided correlation coefficients, r, for beta pinene, myrcene, para cymene, and gamma terpinene that were 0.9900 and greater. The correlation coefficients for alpha pinene, alpha terpineol, and beta caryophyllene were greater than 0.9990.

Alpha pinene, beta pinene, para cymene, alpha terpineol, methyl-N-methylanthranilate, sinensal, and beta caryophyllene each showed a better correlation coefficient as the concentration range extended from 20,000, 50,000, up to 100,000 ppm. Myrcene and gamma terpinene saw an increase in r from 20,000 ppm to 50,000 ppm, but then gamma terpinene's correlation coefficient plateaued near 100,000 ppm, while myrcene continued with a minor increase. Thymol's correlation coefficient experienced minimal change from one range to the next as all r-values were 0.99 and greater. The purpose of extending the linear ranges towards the high end of essential oil concentration was to observe where the drop in linearity occurred based on the correlation coefficient. The concluding ranges with best correlation included, alpha pinene, beta pinene, para cymene, beta caryophyllene all of which had the best linear results from 100 ppm to 10,000 ppm and 100 ppm to 100,000 ppm. Alpha terpineol, methyl-N-methylanthranilate, and sinensal had great correlation from 100 ppm to 50,000 ppm as well as at 100 ppm to 100,000 ppm. Thymol showed minor positive change in correlation at each increasing concentration range from 100 ppm to 10,000 ppm, 20,000 ppm, 50,000 ppm, and 100,000 ppm. Myrcene had the greatest correlation when the range extended from 100 ppm to

10,000 ppm despite myrcene's peak symmetry, clear detection by MS and FID, Gaussian peak shape at all concentration levels, and reproducibility. Once this initial screen was completed, linearity was demonstrated at ICH recommended concentrations.

Table 5-2: Coefficient of determination (R^2) from 100 to 10,000 ppm

Compound	A	B	C	D
Alpha Pinene	0.9993	y = 1.21E-05x +3.55E-05	0.9993	y = 2E-05x +3E-05
Beta Pinene	0.9969	y = 1.29E-05x -1.38E-03	0.9968	y = 1E-05x -0.0014
Myrcene	0.9972	y = 4.88E-06x -5.13E-04	0.9972	y = 5E-06x -0.0005
Para Cymene	0.9940	y = 1.33E-05x -1.60E-03	0.9940	y = 1E-05x -0.0016
Gamma Terpinene	0.9906	y =127E-05x -1.26E-03	0.9906	y =1E-05x -000013
Alpha Terpineol	0.9986	y =1.35E-05x -7.35E-04	0.9986	y =1E-05x -0.0007
Thymol	0.9829	y =8.98E-06 x+3.60E-03	0.9829	y =9E-06x +0.0036
Methyl-N-Methylanthranilate	0.9519	y =7.80E-06x -6.14E-04	0.9519	y =8E-06x -0.0006
Beta Caryophyllene	0.9995	y =1.69E-05x -4.49E-04	0.9995	y =2E-05x -0.0004
Sinensal[E]	0.9527	y =4.84E-07x -4.88E-05	0.9530	y =5E-07x -5E-05

*A = R^2 determined by method of least squares for area ratio versus concentration
*B = Linear regression equation determined by method of least squares for area ratio versus concentration
*C = R^2 determined for area ratio versus concentration
*D = Linear regression equation for area ratio versus concentration
*E= the LOQ for sinensal is 3100 ppm, therefore linear range was not 100- 10,000 ppm, but instead 3100 - 20,000 ppm.

Table 5-3: Correlation coefficient (r) of extended linear range

Investigation of Extended Linear Range			
Compound	A	B	C
Alpha Pinene	0.9767	0.9941	0.9971
Beta Pinene	0.9668	0.9934	0.9960
Myrcene	0.8545	0.9612	0.9683
Para Cymene	0.9962	0.9955	0.9979
Gamma Terpinene	0.9667	0.9929	0.9902
Alpha Terpineol	0.9433	0.9911	0.9916
Thymol	0.9974	0.9995	0.9985
Methyl-N-Methylanthranilate	0.9828	0.9920	0.9922
Beta Caryophyllene	0.9115	0.9747	0.9941
Sinensal[D]	0.9762	0.9968	0.9911

*A = r determined by linear regression performed in Excel for area ratio at 100 - 20,000 ppm
*B = r determined by linear regression performed in Excel for area ratio at 100 - 50,000 ppm
*C = r determined by linear regression performed in Excel for area ratio at 100 - 100,000 ppm
*D = r for ranges starting at 3100 ppm due to sinensal's limit of quantification

5.3.2.1 Homoscedasticity vs. Heterscedasticity

In this specific method, the linear ranges screened, spanned three orders of magnitude for all components validated, apart from sinensal that spanned two orders of magnitude because its LOQ was 3100 ppm. Specific ranges were 100-100,000 ppm, 100-50,000 ppm, 100- 20,000 ppm and 3100-100,000 ppm, 3100-50,000 ppm, and 3100-20,000 ppm, respectively. Due to the large linear range investigated, there was reason to question if variance in the analytical results would lead to a statistically significant bias in the end result that would affect quantitation of the mandarin characterizing compounds.

5.3.2.1.1 F-Test for extended linear range

As presented in table 5-4, within the three concentration ranges investigated, some compounds produced F-Tests that passed for homoscedasticity, but just as many compounds under the investigated linear ranges failed the F-Test for homoscedasticity. To proceed, the compounds classified as heteroscedastic required a weighted linear regression to account for the variance observed at the lower concentrations and upper concentrations in the range. These samples were statistically differing in their variance (Mansilha et al., 2010).

Table 5-4: Experimental F-value versus tabulated F-values for linear ranges

	100 - 100,000 ppm linear range				100- 50,000 ppm linear range				100- 20,000 ppm linear range			
Compound	n	dof	Ftab	Fexp	n	dof	Ftab	Fexp	n	dof	Ftab	Fexp
Alpha Pinene	9	8	6.03	327, 357.93	8	7	6.99	72809.14	7	6	8.47	29431.48
Beta Pinene	9	8	6.03	8,455.51	8	7	6.99	2567.29	7	6	8.47	834.88
Myrcene	9	8	6.03	22.09	8	7	6.99	22.08	7	6	8.47	8.63
Para Cymene	9	8	6.03	298.89	8	7	6.99	11.99	7	6	8.47	25.55
Gamma terpinene	9	8	6.03	309.49	8	7	6.99	9.77	7	6	8.47	1.04
Alpha Terpineol	9	8	6.03	0.00002	8	7	6.99	0.0072	7	6	8.47	0.0092
Thymol	9	8	6.03	6.91	8	7	6.99	30.98	7	6	8.47	6.05
Methyl-N-Methylanthranilate	9	8	6.03	8.66	8	7	6.99	49.01	7	6	8.47	7.348
Beta Caryophyllene	9	8	6.03	0.24	8	7	6.99	4.53	7	6	8.47	1.105
Sinensal	8	7	6.99	248.17	7	6	8.47	329.32	6	5	10.97	178.52

5.3.2.2 Weighted Linear Regression (WLR)

It is typical to use empirical determinations for the best weighting factor, though most common factors include 1/x, 1/y, $1/x^2$, $1/y^2$, $1/x^{1/2}$,$1/y^{1/2}$; in this case most compounds were best weighted with $1/x^2$ [36, 38]. By using the inverse of concentration, the regression model was being weighted higher by the left side of the calibration curve where concentration was closer to the LOQ and less error was perceived [36]. This softens the influence the higher concentrations, which carry a multi-magnitude response difference, have on the linear fit since these data points tend to carry the most variance [38]. The best weighting factor was decided based on which provided the smallest intercept, the best correlation coefficient compared to the 1x model, and which provided the best sum of percent relative error (%RE). Percent relative error was computed from the comparison of the predicted concentration from the best weighted regression and the nominal concentration using equation 4-4 [18, 19, 20, 21].

The weighted slope, y-intercept, and correlation coefficient were calculated using equations 4-1, 4-2, and 4-3 [36, 37, 38, 39]. In cases like this one where the data appeared linear, but was in fact heteroscedastic, the weighted regression became the new calibration model for quantifying unknown samples containing the analytes of interest [38]. The bias and average magnitude of the percent bias over each extended linear range was evaluated for the simple least squares linear regression model. The bias ranged from 8-25% with the exception of myrcene, which functioned better at lower concentrations to begin with. As you see in table 5-7, great improvement was achieved using weighted linear regression models over the original linear modeling. Doing so highlighted the improvement the weighted regression model had on accuracy.

Table 5-5: Original versus weighted linear regression

Compound	W_i = 1x Regression	W_i = 1x R-value	Weighted Regression ($w_i = 1/x^2$)	$W_i = 1/x^2$ R-value	∑%RE
Alpha Pinene	y = 1.00E-05x + 0.0002	0.9971	y = 1.104E -05x - 1.992E-07	0.9928	83.90
Beta Pinene	y = 1.00E-05x - 0.0011	0.9960	y = 1.147E-05x +1.508E-06	0.9907	98.19
Myrcene	y = 2.00E-06 x + 0.0109	0.9612	y = 3.928E-06x - 1.843E-05	0.9340	239.42
Para Cymene	y = 1.00E-05x + 0.0053	0.9979	y = 1.211E-05x - 8.279E-06	0.9949	71.52
Gamma Terpinene	y = 1.00E-05x - 0.0117	0.9902	y = 1.164E-05x +1.495E-05	0.9900	99.25
Alpha Terpineol	y = 1.00E-05x +0.0004	0.9916	y = 1.183E-05x -6.587E-07	0.9791	161.18
Thymol	y = 8.00E-06x +0.0105	0.9985	y = 1.094E-05x - 1.491E-05	0.9352	211.32
Methyl-N-Methylanthranilate	y = 8.00E-06x -0.0141	0.9922	y = 7.475E-06x -5.408E-06	0.9877	117.70
Beta Caryophyllene	y = 9.00E-06x +0.0232	0.9941	y = 1.435E-05x -3.451E-05	0.9628	228.94
Sinensal	y = 4.00E-07x +0.001	0.9911	y = 5.697E-07 -1.139E-07	0.9372	144.37

Data collected using the area ratio

Table 5-6: Percent bias original linear regression (OLR)

Concentration (mg/L)	Alpha Pinene	Beta Pinene	Myrcene	Para Cymene	Gamma Terpinene	Alpha Terpineol	Thymol	MNMA	Beta Caryophyllene	Sinensal
100	-8.01	-74.62	-5319.89	-523.39	1019.43	7.53	-1158.11	1769.41	-2440.69	N/A
500	20.69	6.44	-961.06	-77.67	225.55	28.56	-243.78	354.42	-423.73	N/A
1,000	8.06	8.87	-426.15	-29.65	123.52	16.12	28.84	177.66	-179.34	N/A
3,100	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-41.67
5,000	22.13	19.78	18.86	7.34	45.07	33.59	-4.38	40.99	37.51	4.51
7,000	23.05	19.79	52.39	16.37	26.74	28.63	-9.07	-2.42	46.29	26.86
10,000	18.61	30.96	94.01	31.24	42.33	36.59	5.50	23.72	63.90	22.10
15,000	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-13.35
20,000	-10.31	-12.45	-37.61	13.32	-6.14	-20.56	15.39	-19.12	-12.96	86.30
50,000	-6.49	-8.73	-22.63	-3.48	-7.52	-21.67	13.92	-19.75	-10.49	46.89
100,000	6.88	7.92	N/A	9.11	22.44	1.61	3.29	4.15	1.19	-20.35

[A] % bias = $(C_{exp} - C_{nom}) / C_{nom} \times 100$

Table 5-7: Percent bias weighted linear regression (WLR)

Concentration (mg/L)	Alpha Pinene	Beta Pinene	Myrcene	Para Cymene	Gamma Terpinene	Alpha Terpineol	Thymol	MNMA	Beta Caryophyllene	Sinensal
100	-4.02	8.42	34.27	6.96	8.77	-14.10	16.20	5.89	35.07	NA
500	11.85	10.10	24.34	9.93	8.51	-33.74	-12.91	6.99	18.22	NA
1,000	-0.85	3.58	17.67	3.82	6.35	-32.70	90.34	7.68	10.87	NA
3,100	NA	NA	NA	NA	NA	NA	NA	NA	NA	-2.41
5,000	-8.81	6.17	21.41	-2.23	7.48	15.76	-10.85	12.98	18.37	8.50
7,000	11.64	5.69	22.60	2.61	-3.37	9.18	-18.72	-22.65	14.70	14.16
10,000	7.57	15.06	32.26	12.92	13.68	13.57	-13.23	13.44	18.86	3.29
15,000	NA	NA	NA	NA	NA	NA	NA	NA	NA	-27.45
20,000	-18.66	-23.16	57.66	-4.15	-24.41	1.55	-10.81	-20.99	-37.31	39.60
50,000	-15.26	-20.22	55.19	-19.44	-22.58	15.49	-14.77	-17.14	-40.63	6.65
100,000	-3.17	-5.80	35.37	-9.46	4.10	25.07	-23.50	9.95	-34.92	-42.32

[A] % bias = $(C_{exp} - C_{nom}) / C_{nom} \times 100$

Table 5-8: Average magnitude of % bias [A]

Alpha Pinene		Beta Pinene		Myrcene		Para Cymene		Gamma Terpinene		Alpha Terpineol		Thymol		MNMA		Beta Caryophyllene		Sinensal	
OLR	WLR	OLR	WLR	OLR	WLR	OLR	WLR	OLR	WLR	OLR	WLR	OLR	WLR	OLR	WLR	OLR	WLR	OLR	WLR
13.80	9.09	14.37	11.22	141.85	33.31	23.52	8.07	62.41	11.31	21.65	17.91	40.52	14.97	80.28	13.98	96.93	24.24	25.10	18.05

[A] Excludes outliers observed in Table: 5-6 and Table: 5-7

5.3.3 Linearity

The coefficient of determination for each linearity range for each analyte were tabulated in Tables 5-8 and Table 5-9, respectively. Given the reproducibility, resolution, peak symmetry, and investigated concentration ranges for these components, there is minimal concern of their linear fit. It should be noted that myrcene is a compound that spontaneously polymerizes rendering it unstable; in the industry this compound is usable for only a few weeks before the sample is discarded for formulation and a fresh production sample is requested. In addition, myrcene is rarely added to flavor formulation directly due to its instability as a raw ingredient; therefore, it is commonly found in a flavor analysis by association with an essential oil or the formation from other terpene compounds for which it can interconvert.

Table 5-9: Coefficient of determination for 70%-130% concentration

Compound	R^2
Alpha Pinene	0.9930
Beta Pinene	0.9708
Myrcene	0.7271
Para Cymene	0.9274
Gamma Terpinene	0.9990
Alpha Terpineol	0.8753
Thymol	0.9706
Methyl-N-Methylanthranilate	0.8953
Beta Caryophyllene	0.9693
Sinensal	0.6678

Table 5-10: Coefficient of determination for LOQ-130% concentration

Compound	R^2
Alpha Pinene	0.9981
Beta Pinene	0.9980
Myrcene	0.9657
Para Cymene	0.9826
Gamma Terpinene	0.9993
Alpha Terpineol	0.9958
Thymol	0.9938
Methyl-N-Methylanthranilate	0.8890
Beta Caryophyllene	0.9975
Sinensal	0.8769

5.3.4 Accuracy

Concentrations recovered were within ± 20% of the target concentration except at the LOQ for each validated compound; however, the repeatability of the measurements at each concentration level were within a couple percent of one another. At the LOQ, the recovered concentration was superseding the target concentration most likely due to the variability that occurs near the compounds' threshold of accurate quantitation. Myrcene was only accurate and repeatable at the 100% concentration level, the LOQ was much to

high as was every other analyte, however the 80 and 120% concentrations were recovering 40% less than expected.

Table 5-11: Accuracy and Repeatability method validation results

	1		2		3		4		5		6		7		8		9		10	
Solution	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
LOQ	0.0016	214	0.0016	224	0.0001	213	0.0009	137	0.0015	124	0.0014	197	0.0014	141	0.0018	270	0.0019	161	0.0017	139
LOQ	0.0015	204	0.0015	212	0.0009	208	0.0010	151	0.0015	128	0.0015	200	0.0015	158	0.0018	270	0.0021	177	0.0018	141
LOQ	0.0016	209	0.0017	232	0.0010	218	0.0010	149	0.0015	126	0.0015	200	0.0016	167	0.0018	270	0.0025	211	0.0018	148
80%	0.0362	98	0.0387	105	0.0115	64	0.0420	108	0.0390	100	0.0358	104	03690	96	0.0379	96	0.0376	88	0.0036	112
80%	0.0362	98	0.0384	104	0.0116	65	0.0414	106	0.0391	101	0.0361	105	0.0361	94	0.0386	97	0.0378	89	0.0035	109
80%	0.0361	98	0.0382	103	0.0116	64	0.0420	108	0.0391	101	0.0379	110	0.0372	97	0.0380	96	0.0372	87	0.0036	114
100%	0.0454	98	0.0432	94	0.0233	103	0.0478	98	0.0469	97	0.0486	113	0.0469	98	0.0510	103	0.0411	77	0.0038	96
100%	0.0453	98	0.0427	93	0.0232	103	0.0489	100	0.0480	99	0.0480	112	0.0476	99	0.0468	94	0.0429	80	0.0038	94
100%	0.0473	102	0.0417	90	0.0234	104	0.0485	99	0.0492	101	0.0475	111	0.0479	100	0.0516	104	0.0446	84	0.0039	98
%RSD		2.53		1.73		0.37		1.12		2.34		1.21		1.18		5.24		4.15		2.06
120%	0.0554	100	0.0552	100	0.0176	65	0.0670	115	0.0543	93	0.0605	117	0.0559	97	0.0577	97	0.0569	89	0.0052	107
120%	0.0557	101	0.0545	98	0.0173	64	0.0675	115	0.0547	94	0.0567	110	0.0565	98	0.0593	100	0.0549	86	0.0051	106
120%	0.0558	101	0.0542	98	0.0178	66	0.0681	117	0.054	93	0.0544	105	0.0564	98	0.0597	100	0.0575	90	0.0052	107

*A = Area ratio to the internal standard

*B = Percent recovery

*1-10 = Alpha pinene, beta pinene, myrcene, para cymene, gamma terpinene, alpha terpineol, thymol, beta caryophyllene, methyl-N-methylanthranilate, and sinensal, respectively.

5.3.5 Precision

Data for the precision of the area ratio and the retention time of each analyte being validated is compiled into Table 5-11. Alpha and beta pinene, and myrcene had %RSD below 1%, while para cymene, gamma terpinene, alpha terpineol, thymol, and methyl-N-methylanthranilate had precision below 3%. Beta Caryophyllene fell below 4% but sinensal was on the brink of 11% RSD. With the exception of sinensal, all components meet the established criterion for this method.

Table 5-12: Precision method validation results

	1		2		3		4		5		6		7		8		9		10	
	Ratio	RT	Ratio	RT	Ratio	RT	Ratio	RT	Ratio	RT	Ratio	RT	Ratio	RT	Ratio	RT	Ratio	RT	Ratio	RT
Inj 1	0.0460	24.173	0.0460	27.132	0.0227	27.792	0.0480	30.243	0.0469	32.870	0.0435	42.182	0.0487	49.378	0.0510	57.250	0.0532	55.835	0.0032	64.466
Inj 2	0.0460	24.175	0.0460	27.132	0.0225	27.802	0.0490	30.256	0.0480	32.860	0.0422	42.174	0.0495	49.386	0.0468	57.240	0.0525	55.837	0.0038	64.468
Inj 3	0.0460	24.170	0.0460	27.132	0.0225	27.790	0.0480	30.257	0.0492	32.800	0.0441	42.167	0.0475	49.383	0.0516	57.240	0.0545	55.847	0.0041	64.471
Inj 4	0.0460	24.174	0.0460	27.143	0.0223	27.794	0.0500	30.258	0.0484	32.870	0.0420	42.165	0.0469	49.383	0.0510	57.230	0.0532	55.846	0.0042	64.470
Inj 5	0.0460	24.176	0.0460	27.133	0.0226	27.788	0.0480	30.250	0.0495	32.850	0.0445	42.178	0.0476	49.383	0.0482	57.230	0.0525	55.849	0.0045	64.470
Inj 6	0.0470	24.173	0.0470	27.140	0.0225	27.785	0.0510	30.265	0.0495	32.850	0.0414	42.164	0.0479	49.372	0.0489	57.230	0.0536	55.849	0.0042	64.471
%RSD	0.8840	0.0090	0.8800	0.0200	0.6376	0.0212	2.3920	0.0250	2.0820	0.0300	2.9990	0.0177	1.9920	0.0102	3.8300	0.0143	1.4760	0.0110	10.740	0.0030

*1-10 = Alpha pinene, beta pinene, myrcene, para cymene, gamma terpinene, alpha terpineol, thymol, beta caryophyllene, methyl-N-methylanthranilate, and sinensal, respectively.

5.3.6 Sensitivity

Table 5-13: Respective LOQ and LOD concentrations with their S/N ratios

Compound	LOQ (ppm)	S/N ± uncertainty*	LOD (ppm)	S/N ± uncertainty*
Alpha Pinene	77	10.694 ± 1.3280	22.63	6.7545 ± 3.9833
Beta Pinene	75	12.116 ± 1.4160	22.63	7.1336 ± 3.4074
Myrcene	158	11.3426 ± 0.0387	47.70	3.9839 ±1.6217
Para Cymene	66	9.0225 ± 3.3880	20.04	4.6999 ± 2.2044
Gamma Terpinene	117	11.9190 ± 1.7841	35.45	3.3 ± 0.000
Alpha Terpineol	88	6.611 ± 1.5790	23.69	5.7228 ± 2.9573
Thymol	108	4.8485 ± 4.2532	31.84	3.0947±0.4217
Methyl-N-Methylanthranilate	141	9.2121 ± 0.3285	42.41	3.4830 ± 0.3382
Beta Caryophyllene	65.86	10.0235 ± 0.7179	20.16	3.3432 ± 0.3787
Sinensal	3100	9.2192 ± 5.4436	1061	4.9464 ± 0.2211

*Uncertainty was determined through standard deviation of the triplicate injections

5.3.7 System Suitability Testing

The lowest %RSD for the suitability runs was 0.5555 for day two of alpha pinene validation with the highest %RSD being 5.613 from day one of sinensal validation. For all other compounds the %RSD are captured within that range and thereby meet the criterion.

5.4 Conclusion

The validation component of this research had two major challenges to overcome. First there was the challenge of creating resolution of multiple components that were in a natural mixture with dozens of inherent constituents. Second, understanding the variance of the method validated when it was to cover a concentration range spanning several orders of magnitude.

The GCMS/FID method for simultaneous detection and quantitation proposed was successfully developed and validated for ten mandarin characterizing components. All ten compounds were detected in the essential oil matrix in less than 70 minutes while meeting the guidelines for resolution. Specificity was confirmed by the use of mass selective detection, standard solution measurements, base peak ion monitoring in scan mode, and use of third party libraries. This seemingly exhaustive confirmation is necessary because of the complexity of the compounds. Selected ion extraction from the total ion chromatogram was also used for verification of peak purity. Through the analysis of variance, heteroscedasticity, and weighted linear regression, the accuracy of

the method over the large range (three or five) orders of magnitude was improved for each concentration range. This is reflected in the %bias.

CHAPTER 6

STABILITY ANALYSIS

6.1 Introduction

The physicochemical properties of essential oils and the potential alterations that affect the quality of oil are published in few places. This would be less problematic if degradation did not negatively affect the quality of flavor and fragrances, but unfortunately, that is not the case. When oils degrade, the components that form are usually detectable due to their odor threshold. Degradation needs to be monitored in essential oils used for flavorings because odor threshold renders the oil undesirable for product formulation. These affects are observed because odor threshold depends on molecules volatility. Moreover, even the analytes with low concentrations derived from oxidation or degradation reactions have the ability to contribute significantly to the overall aromatic profile of the essential oil [5].

The majority of components within essential oils fall among the same chemical class, making it is easy for those components to convert into one another through oxidation, isomerization, dehydration, or biotransformation reactions. The aforementioned reactions are usually triggered enzymatically or chemically [5]. Typical catalysts for these reactions include heat, light, exposure to oxygen, biological organisms, acid, and bases. Due to the interconversion of terpenes in essential oils, it is difficult to unambiguously identify degradation mechanisms that may alter the flavor and aroma profiles.

6.2 Goal

The goal of the stability study was to detect any change occurring in the oil that could give rise to new component(s) or show a reduction in any component(s) for the purpose of understanding what reaction(s) could be responsible for the putrid odor and taste in mandarin essential oils. The most intrusive off note of concern is the development of a plastic note reminiscent of a pool toy. Certainly not a "mouthwatering" aroma. Once the responsible compound(s) is/are identified, flavor houses are capable of developing a flavor system to mask and/or inhibit the off-note formation.

6.3 Experimentation with Mandarin Oil

6.3.1 Reagents and chemicals

Oil Mandarin Italian Select was obtained from Citrus and Allied (C&A), toluene, the internal standard, was obtained from Sigma Aldrich, and ethanol was obtained from Brenntag.

6.3.2 Effect of storage at ambient conditions

The mandarin essential oil samples were prepared neat with 2000 uL quantities in half- ounce amber bottles. Each bottle was sealed with a screw top cap and tape, before being stored at ambient temperature, 23 °C ± 5 °C. A total of six bottles were prepared for each oil so that every month a sample was being analyzed, one bottle could be pulled

from storage. Half of the sample of neat oil, 1000 uL, plus toluene was used for injection immediately onto the GCMS/FID and the other 1000 uL of sample was stored in a freezer at -10 ℃. The samples were frozen to stop progression of any reactions at each monthly stage in order to have a sample in reserve in the event additional aliquots of oil were needed for analysis. The study spanned 24 weeks with analysis conducted every fourth week.

6.3.3 Effect of storage at elevated temperatures

Each mandarin oil was prepared in the same fashion as explained in section 6.3.2, the difference being that instead of the samples being stored in ambient conditions, the samples were stored at 38 ℃ in a hotbox. Samples were again analyzed every fourth week for a total of 24 weeks; 1000 uL plus toluene was used for the immediate analysis, while 1000 uL was stored in a freezer at -10 ℃ to stop any reactions from progressing. The goal was to detect any oxidation or degradation reactions that were occurring in the oil to understand what components form and/or degrade to give the putrid odor and taste flavor houses are trying to prevent/mask.

6.3.4 Effect of oil in high acid tasting solution (HATS)

High acid tasting solution reconstituted with a 1:5 dilution of HATS to non-carbonated water was used to demonstrate mandarin oil in a specific application and observe how it reacts over time. The chosen survey study models a soda beverage,

without the carbonation, because soda products are known to display a bitter, plastic taste when mandarin oil is used in the formulation. The matrix is acidic. The study was performed at ambient conditions to mimic the environment of grocery store shelves and soda manufacturer warehouses. The objective was twofold, understand how the variable of introducing an acidic environment impacts oxidation or degradation reactions of the mandarin oil over a 24-week time period as well as understand if the products of the reactions are capable of organoleptically embodying the putrid, plastic off-note needing to be masked by additional flavorings.

6.3.5 Sensory Evaluation

To conclude the stability studies of mandarin oil exposed to ambient, hotbox, and HATS environments for 24 weeks, sensory evaluations of the neat oil samples were performed by trained flavor chemists (flavorists) certified by the Society of Flavor Chemists. Samples at time zero, 12 weeks, and 24 weeks were evaluated for presence of plastic off notes, oxidative off notes, and then any additional comments on the oil aroma profile that were noteworthy. Each stability set was evaluated sequentially starting with ambient time 0 and ending with HATS week 24.

It was advantageous to use at minimum two flavorists with differing disciplines for the sensory evaluation. Doing so provides unique assessment of different olfactory characteristics of a sample. While each flavorist has undergone the same training and testing to be certified a flavorist by the Society of Flavor Chemists, their careers diverge into different food categories. Each food category, sweet goods, beverages, savory,

vanilla, oral care, and snack foods inherently have flavor trends; therefore, a flavorist becomes well-versed to some flavor materials more than others. This leads to acquired sensitivities of olfactory perceptions that may cause one flavorist to distinguish an aroma that the other did not.

6.4 Results and Discussion

Upon subjecting the neat material or material in HATS to stability indicating conditions and applying the established analytical method, a variety of mathematical approaches were investigated to best understand the resulting data. Normalization of internal and external standards was used along with reconciliation of mass balance, Z test, Dixon Q test, and the Bland-Altman method in the data evaluation. Forced degradation was also applied to one component of interest.

6.4.1 Use of Internal Standard Method

Toluene was used as the internal standard for this series of experiments. The peak area ratios against that of toluene at first showed no real trend in any of the three 24-week stability studies. There were instances where the data suggested major degradation was occurring and other times the data looked as though the marker chemical(s) were increasing significantly. Comparing the area by ratio to the internal standard was not sufficient to resolve this apparent irregularity initially, though ultimately this method was selected.

6.4.2 Use of External Standard Method

Due to the lack of clarity in using the internal standard method alone, implementation of external standardization was then performed. This method provided few enlightening differences in the data trends for the analyst to get an understanding of what chemistry was occurring. Adding an internal standard to the external standard solution was also performed, but unfortunately, normalizing with both methods did not make the trends in the data any clearer, it did confirm the instrument was functioning well. Based on this assessment, the external standard method was deemed unnecessary.

6.4.3 Molecular Ions and Base Peaks

To further gain insight to the data obtained from the stability studies, deeper extraction of the main ions for each mandarin compounds of interest was performed. Area counts were obtained and comparisons to the main ions of toluene were made using ratios. When using the molecular ions, in a few of the characterizing mandarin components, more than one sampling looked suspiciously different than the others for extracted ion area ratio. When the ratios were evaluated based off using the base peak ions, ≤1 outliers in the data was observed. Because the base peaks are the more stable ion in a mass spectrum for a given molecule and presented less outlier data, extracted area ratio from base peak ions was used in additional statistical analysis. Outliers were confirmed statistically using Dixon Q Test and eliminated in the subsequent statistical analyses.

Table 6-1: Base peak ions for ion extraction from total ion chromatograph

Compound	Base Peak Ions (m/z)	Retention Time Scanned
Alpha Pinene	93	24.00 -25.00 min
Beta Pinene	93	27.00-28.00 min
Myrcene	41	27.00-28.00 min
Para Cymene	119	29.00-31.00 min
Gamma Terpinene	93	32.50 - 34.00 min
Alpha Terpineol	59	42.00-43.00 min
Thymol	135	48.50-49.50 min
Methyl-N-Methylanthranilate	165	55.50-56.50 min
Beta Caryophyllene	93	57.00-58.00 min
Sinensal	93	64.00-65.00 min
Toluene	91	13.00-14.00 min

6.4.4 Dixon Q Test

In using the Dixon Q test to confirm chemical outliers, alpha and beta pinene, gamma terpinene and alpha terpineol each had an outlier in the ambient study at week 20 and the hotbox studies at week 16, meanwhile there were no data samplings from the high acid tasting solution study that presented as outliers. Para cymene only showed an outlier data sampling in the ambient stability study at week 20. MNMA presented outliers

at week 16 for both the ambient and hotbox studies whereas beta caryophyllene and thymol each only had an outlier at week 16 in the hotbox study. Lastly, myrcene and sinensal had no outliers.

The reactivity of the compounds and their isomerization make it difficult to understand the origin of the outliers. It should be noted that the week 20 sample of the ambient study and the week 16 sample of the hotbox study were analyzed on the same days; the scheduled analyses were just offset by four weeks.

Table 6-2: Dixon Q Test performed on alpha pinene in ambient oil study

Dixon Q Test computed in Excel	
Alpha Pinene	Base Peak Ratio
Week 12	0.1475
Week 0	0.2071
Week 16	0.2096
Week 24	0.2210
Week 4	0.2229
Week 8	0.2240
Week 20 (outlier)	0.4539
Average	0.2409
STDEV	0.0977
Q (exp)	0.7503
Q (crit) @ 95% CI	0.5860
New Average	0.2054
New Stdev	0.0292

*Confidence Interval (CI)

Table 6-3: Dixon Q Test alpha pinene in hot box oil study

Dixon Q Test computed in Excel	
Alpha Pinene	Base Peak Ratio
Week 24	0.2033
Week 8	0.2071
Week 4	0.2141
Week 20	0.2183
Week 12	0.2183
Week 0	0.2229
Week 16 (outlier)	0.4078
Average (1)	0.2417
STDEV (1)	0.0736
Q (exp)	0.9041
Q (crit) @ 95% C.I	0.5860
New Average	0.2140
New StDev	0.0075

*Confidence Interval (CI)

Table 6-4: Dixon Q test alpha pinene in HAT solution study

Dixon Q Test computed in Excel	
Alpha Pinene	Base Peak Ratio
Week 20	0.1366
Week 4	0.1378
Week 16	0.1396
Week 8	0.1432
Week 0	0.1568
Week 12	0.2651
Week 24	0.3706
Average (1)	0.1928
STDEV (1)	0.0909
Q (exp)	0.4508
Q (crit) @ 95% C.I	0.5860
New Average	0.1928
New StDev	0.0909

*Confidence Interval (CI)

Table 6-5: Dixon Q test beta pinene in ambient oil study

Dixon Q Test computed in Excel	
Beta Pinene	Base Peak Ratio
Week 12	0.2095
Week 16	0.2844
Week 0	0.2894
Week 24	0.2919
Week 4	0.3100
Week 8	0.3184
Week 20 (outlier)	0.6395
Average (1)	0.3347
STDEV (1)	0.1203
Q (exp)	0.7467
Q (crit) @ 95% C.I	0.5860
New Average	0.2839
New StDev	0.0387

*Confidence Interval (CI)

Table 6-6: Dixon Q Test beta pinene in hot box oil study

Dixon Q Test computed in Excel	
Beta Pinene	Base Peak Ratio
Week 24	0.2792
Week 4	0.2909
Week 20	0.2930
Week 12	0.2969
Week 8	0.2985
Week 0	0.3100
week 16 (outlier)	0.5750
Average (1)	0.3348
STDEV (1)	0.1063
Q (exp)	0.8957
Q (crit) @ 95% C.I	0.5860
New Average	0.2947
New StDev	0.0101

*Confidence Interval (CI)

Table 6-7: Dixon Q Test beta pinene in HAT solution study

Dixon Q Test computed in Excel	
Beta Pinene	Base Peak Ratio
Week 4	0.1858
Week 20	0.1875
Week 16	0.1916
Week 8	0.1956
Week 0	0.2158
Week 12	0.3666
Week 24	0.5198
Average (1)	0.2661
STDEV (1)	0.1292
Q (exp)	0.4585
Q (crit) @ 95% CI	0.5860
New Average	0.2661
New StDev	0.1292

*Confidence Interval (CI)

Table 6-8: Dixon Q Test Myrcene in ambient oil study

Dixon Q Test computed in Excel	
Myrcene	Base Peak Ratio
Week 12	0.1431
Week 24	0.1641
Week 0	0.1669
Week 20	0.1741
Week 8	0.1764
Week 4	0.1800
Week 16	0.1816
Average (1)	0.1695
STDEV (1)	0.0133
Q (exp)	0.0416
Q (crit) @ 95% C.I	0.5860
New Average	0.1695
New StDev	0.0133

*Confidence Interval (CI)

Table 6-9: Dixon Q Test Myrcene in hotbox oil study

Dixon Q Test computed in Excel	
Myrcene	Base Peak Ratio
Week 16	0.1540
Week 24	0.1600
Week 20	0.1620
Week 8	0.1660
Week 4	0.1730
Week 0	0.1800
Week 12	0.1840
Average (1)	0.1684
STDEV (1)	0.0110
Q (exp)	0.1333
Q (crit) @ 95% C.I	0.5860
New Average	0.1658
New StDev	0.0110

*Confidence Interval (CI)

Table 6-10: Dixon Q Test Myrcene in HAT solution study

Dixon Q Test computed in Excel	
Myrcene	Base Peak Ratio
Week 20	0.0944
Week 16	0.0988
Week 4	0.1031
Week 12	0.1117
Week 24	0.1152
Week 8	0.1237
Week 0	0.1310
Average (1)	0.1111
STDEV (1)	0.0133
Q (exp)	0.1995
Q (crit) @ 95% C.I	0.5860
New Average	0.1111
New StDev	0.0133

*Confidence Interval (CI)

Table 6-11: Dixon Q Test para cymene in ambient oil study

Dixon Q Test computed in Excel	
Para Cymene	Base Peak Ratio
Week 4	0.1588
Week 0	0.1644
Week 8	0.2504
Week 12	0.3290
Week 16	0.3344
Week 24	0.3354
Week 20 (outlier)	0.6512
Original Average	0.3177
Original STDEV	0.1659
Q (exp)	0.6413
Q (crit) @ 95% C.I	0.5860
New Average	0.2621
New StDev	0.0842

*Confidence Interval (CI)

Table 6-12: Dixon Q Test para cymene in hot box oil study

Dixon Q Test computed in Excel	
Para Cymene	Base Peak Ratio
Week 12	0.0290
Week 0	0.1588
Week 4	0.3740
Week 20	0.4287
Week 8	0.4542
Week 24	0.5417
Week 16	1.1146
Average (1)	0.4430
STDEV (1)	0.3459
Q (exp)	0.5277
Q (crit) @ 95% confi	0.5860
New Average	0.4430
New StDev	0.3459

*Confidence Interval (CI)

Table 6-13: Dixon Q Test para cymene in HAT solution study

Dixon Q Test computed in Excel	
Para Cymene	Base Peak Ratio
Week 4	0.0007
Week 0	0.0008
Week 8	0.0109
Week 20	0.0115
Week 16	0.7562
Week 12	1.3497
Week 24	2.2495
Average (1)	0.6256
STDEV (1)	0.8864
Q (exp)	0.4001
Q (crit) @ 95% C.I	0.5860
New Average	0.6256
New StDev	0.8864

*Confidence Interval (CI)

Table 6-14: Dixon Q Test gamma terpinene in ambient oil study

Dixon Q Test computed in Excel	
Gamma Terpinene	Base Peak Ratio
Week 12	0.7570
Week 8	0.9733
Week 0	0.9746
Week 24	0.9917
Week 4	1.0120
Week 16	1.0409
Week 20 (outlier)	1.6552
Average (1)	1.0578
STDEV (1)	0.2794
Q (exp)	0.6839
Q (crit) @ 95% C.I	0.5860
New Average	0.9582
New StDev	0.1018

*Confidence Interval (CI)

Table 6-15: Dixon Q Test gamma terpinene in hotbox oil study

Dixon Q Test computed in Excel	
Gamma Terpinene	Base Peak Ratio
Week 24	0.9148
Week 4	0.9344
Week 8	0.9748
Week 20	0.9873
Week 0	1.0120
Week 12	1.0311
Week 16 (outlier)	1.4988
Average (1)	1.0504
STDEV (1)	0.2018
Q (exp)	0.8009
Q (crit) @ 95% C.I	0.586
New Average	0.9757
New StDev	0.0446

*Confidence Interval (CI)

Table 6-16: Dixon Q Test gamma terpinene in HAT solution study

Dixon Q Test computed in Excel	
Gamma Terpinene	Base Peak Ratio
Week 16	0.4856
Week 20	0.5011
Week 4	0.7062
Week 8	0.7137
Week 0	0.7841
Week 12	1.0406
Week 24	1.4377
Average (1)	0.8098
STDEV (1)	0.3338
Q (exp)	0.4171
Q (crit) @ 95% C.I	0.5860
New Average	0.8098
New StDev	0.3338

*Confidence Interval (CI)

Table 6-17: Dixon Q Test alpha terpineol in ambient oil study

Dixon Q Test computed in Excel	
Alpha Terpineol	Base Peak Ratio
Week 8	0.0056
Week 12	0.0068
Week 0	0.0094
Week 4	0.0104
Week 24	0.0115
Week 16	0.0122
Week 20 (outlier)	0.0239
Average (1)	0.0114
STDEV (1)	0.0060
Q (exp)	0.6393
Q (crit) @ 95% C.I	0.5860
New Average	0.0093
New StDev	0.0026

*Confidence Interval (CI)

Table 6-18: Dixon Q Test alpha terpineol in hotbox oil study

Dixon Q Test computed in Excel	
Alpha Terpineol	Base Peak Ratio
Week 24	0.0081
Week 0	0.0104
Week 4	0.0112
Week 8	0.0132
Week 20	0.0143
Week 12	0.0143
Week 16 (outlier)	0.0271
Average (1)	0.0141
STDEV (1)	0.0062
Q (exp)	0.6719
Q (crit) @ 95% C.I	0.5860
New Average	0.0119
New StDev	0.0025

*Confidence Interval (CI)

Table 6-19: Dixon Q Test alpha terpineol in HAT solution study

Dixon Q Test computed in Excel	
Alpha Terpineol	Base Peak Ratio
Week 16	0.0069
Week 20	0.0072
Week 4	0.0073
Week 8	0.0076
Week 0	0.0081
Week 12	0.0135
Week 24	0.0207
Average (1)	0.0102
STDEV (1)	0.0052
Q (exp)	0.5213
Q (crit) @ 95% C.I	0.5860
New Average	0.0102
New StDev	0.0052

*Confidence Interval (CI)

Table 6-20: Dixon Q Test thymol in ambient oil study

Dixon Q Test computed in Excel	
Thymol	Base Peak Ratio
Week 12	0.0041
Week 24	0.0056
Week 16	0.0077
Week 0	0.0078
Week 4	0.0085
Week 8	0.0086
Week 20	0.0114
Average (1)	0.0077
STDEV (1)	0.0023
Q (exp)	0.3880
Q (crit) @ 95% C.I	0.5860
New Average	0.0077
New StDev	0.0023

*Confidence Interval (CI)

Table 6-21: Dixon Q Test thymol in hotbox oil study

Dixon Q Test computed in Excel	
Thymol	Base Peak Ratio
Week 20	0.0064
Week 24	0.0073
Week 8	0.0084
Week 12	0.0085
Week 0	0.0085
Week 4	0.0085
Week 16 (outlier)	0.0135
Average (1)	0.0087
STDEV (1)	0.0023
Q (exp)	0.7011
Q (crit) @ 95% C.I	0.5860
New Average	0.0080
New StDev	0.0009

*Confidence Interval (CI)

Table 6-22: Dixon Q Test thymol in HAT solution study

Dixon Q Test computed in Excel	
Thymol	Base Peak Ratio
Week 16	0.0040
Week 20	0.0042
Week 4	0.0048
Week 8	0.0052
Week 0	0.0063
Week 12	0.0103
Week 24	0.0157
Average (1)	0.0072
STDEV (1)	0.0043
Q (exp)	0.4643
Q (crit) @ 95% C.I.	0.5860
New Average	0.0072
New StDev	0.0043

*Confidence Interval (CI)

Table 6-23: Dixon Q Test MNMA in ambient oil study

Dixon Q Test computed in Excel	
MNMA	Base Peak Ratio
Week 24	0.0388
Week 12	0.0419
Week 8	0.0554
Week 0	0.0581
Week 4	0.0621
Week 20	0.0901
Week 16 (outlier)	0.1946
Average (1)	0.0773
STDEV (1)	0.0544
Q (exp)	0.6710
Q (crit) @ 95% C.I	0.5860
New Average	0.0577
New StDev	0.0183

*Confidence Interval (CI)

Table 6-24: Dixon Q Test MNMA in hotbox oil study

Dixon Q Test computed in Excel	
MNMA	Base Peak Ratio
Week 20	0.0454
Week 24	0.0536
Week 12	0.0586
Week 4	0.0620
Week 0	0.0621
Week 8	0.0626
Week 16 (outlier)	0.0948
Average (1)	0.0627
STDEV (1)	0.0154
Q (exp)	0.6522
Q (crit) @ 95% C.I	0.5860
New Average	0.0574
New StDev	0.0068

*Confidence Interval (CI)

Table 6-25: Dixon Q Test MNMA in HAT solution study

Dixon Q Test computed in Excel	
MNMA	Base Peak Ratio
Week 20	0.0287
Week 16	0.0288
Week 4	0.0352
Week 8	0.0370
Week 0	0.0475
Week 12	0.0753
Week 24	0.1141
Average (1)	0.0524
STDEV (1)	0.0317
Q (exp)	0.4547
Q (crit) @ 95% C.I	0.5860
New Average	0.0523
New StDev	0.0317

*Confidence Interval (CI)

Table 6-26: Dixon Q Test beta caryophyllene in ambient oil study

Dixon Q Test computed in Excel	
Beta Caryophyllene	Base Peak Ratio
Week 12	0.0016
Week 24	0.0026
Week 0	0.0033
Week 8	0.0035
Week 16	0.0036
Week 4	0.0036
Week 20	0.0053
Average (1)	0.0033
STDEV (1)	0.0011
Q (exp)	0.4416
Q (crit) @ 95% C.I	0.5860
New Average	0.0033
New StDev	0.0011

*Confidence Interval (CI)

Table 6-27: Dixon Q Test beta caryophyllene in hotbox oil study

Dixon Q Test computed in Excel	
Beta Caryophyllene	Base Peak Ratio
Week 20	0.0027
Week 24	0.0028
Week 4	0.0034
Week 8	0.0035
Week 12	0.0035
Week 0	0.0036
Week 16 (outlier)	0.0050
Average (1)	0.0035
STDEV (1)	0.0007
Q (exp)	0.5963
Q (crit) @ 95% C.I	0.5860
New Average	0.0033
New StDev	0.0004

*Confidence Interval (CI)

Table 6-28: Dixon Q Test beta caryophyllene in HAT solution study

Dixon Q Test computed in Excel	
Beta Caryophyllene	Base Peak Ratio
Week 16	0.0012
Week 20	0.0012
Week 8	0.0022
Week 4	0.0022
Week 0	0.0028
Week 12	0.0031
Week 24	0.0039
Average (1)	0.0024
STDEV (1)	0.0010
Q (exp)	0.3061
Q (crit) @ 95% C.I	0.5860
New Average	0.0024
New StDev	0.0010

*Confidence Interval (CI)

Table 6-29: Dixon Q Test sinensal in ambient oil study

Dixon Q Test computed in Excel	
Sinensal	Base Peak Ratio
Week 12	0.0067
Week 24	0.0180
Week 0	0.0261
Week 16	0.0261
Week 8	0.0270
Week 4	0.0288
Week 20	0.0347
Average (1)	0.0239
STDEV (1)	0.0090
Q (exp)	0.4033
Q (crit) @ 95% C.I	0.5860
New Average	0.0239
New StDev	0.0090

*Confidence Interval (CI)

Table 6-30: Dixon Q Test sinensal in hotbox oil study

Dixon Q Test computed in Excel	
Sinensal	Base Peak Ratio
Week 20	0.0208
Week 24	0.0212
Week 8	0.0282
Week 0	0.0288
Week 12	0.0288
Week 4	0.0313
Week 16	0.0351
Average (1)	0.0278
STDEV (1)	0.0051
Q (exp)	0.2671
Q (crit) @ 95% C.I	0.5860
New Average	0.0278
New StDev	0.0051

*Confidence Interval (CI)

Table 6-31: Dixon Q Test sinensal in HAT solution study

Dixon Q Test computed in Excel	
Sinensal	Base Peak Ratio
Week 16	0.0079
Week 20	0.0080
Week 8	0.0170
Week 4	0.0176
Week 12	0.0194
Week 0	0.0218
Week 24	0.0278
Average (1)	0.0171
STDEV (1)	0.0072
Q (exp)	0.3022
Q (crit) @ 95% C.I	0.5860
New Average	0.0171
New StDev	0.0072

*Confidence Interval (CI)

6.4.5 Z Test

The Z test was used to understand, how well the individual components of the samples individually behaved, statistically speaking, under the various treatment conditions. The Z-test reports the “distance” a sample measurement deviates from the mean in units of the standard deviation. Beta pinene, alpha terpineol, thymol and MNMA under HAT solution conditions showed no outlier behavior based on the Dixon Q Test; however, individual sample data showed that week 24 for each compound

exceeded the Z critical value of 1.960. When the Z-test fails, the sample is said to be statistically different and sometimes termed an outlier. Alpha pinene, para cymene and gamma terpinene each came close to exceeding the Z critical value for week 24 of exposure to HAT solution; however, they just barely passed with Z values of 1.83-1.95. Myrcene, beta caryophyllene, and sinensal showed no significant data by the Z test for either stability analysis conducted. It is of no surprise that at the end of the HAT solution stability test, most of the mandarin marker chemicals failed or near failed the Z-test. Half a shelf life exposed to acidic conditions is an extreme circumstance for the essential oil to maintain its integrity and by seeing each compound begin to behave erratically is an indicator that the stability has been lost. It is a surprise that an additional statistical technique failed to demonstrate the change in oil quality over the course of the three separate stability studies.

Table 6-32: Alpha Pinene Z test results in ambient oil study

Alpha Pinene	Base Peak Ratio	z-values	|z- values|	Compared to z crit = 1.960
Week 12	0.1475	-1.9799	1.9799	greater than
Week 0	0.2071	0.0599	0.0599	less than
Week 16	0.2096	0.1455	0.1455	less than
Week 24	0.2210	0.5356	0.5356	less than
Week 4	0.2229	0.6007	0.6007	less than
Week 8	0.2240	0.6383	0.6383	less than
Average	0.2054			
STDEV	0.0292			

Table 6-33: Alpha Pinene Z test results in hotbox oil study

Alpha Pinene	Base Peak Ratio	z-values	|z- values|	Compared to z crit = 1.960
Week 24	0.2033	-1.4350	1.4350	less than
Week 8	0.2071	-0.9197	0.9197	less than
Week 4	0.2141	0.0069	0.0069	less than
Week 20	0.2183	0.5715	0.5715	less than
Week 12	0.2183	0.5794	0.5794	less than
Week 0	0.2229	1.1969	1.1969	less than
Average	0.2140			
STDEV	0.0075			

Table 6-34: Alpha pinene Z-test in HAT solution study

Alpha Pinene	Base Peak Ratio	z-values	|z- values|	Compared to z crit = 1.960
Week 20	0.1366	-0.6182	0.6182	less than
Week 4	0.1378	-0.6054	0.6054	less than
Week 16	0.1396	-0.5855	0.5855	less than
Week 8	0.1432	-0.5453	0.5453	less than
Week 0	0.1568	-0.3958	0.3958	less than
Week 12	0.2651	0.7950	0.7950	less than
Week 24	0.3706	1.9551	1.9551	less than
Average	0.1928			
STDEV	0.0909			

Table 6-35: Beta Pinene Z-test ambient oil study

Beta Pinene	Base Peak Ratio	z-values	|z- values|	Compared to z crit = 1.960
Week 12	0.2095	0.7378	0.7378	less than
Week 16	0.2844	1.0016	1.0016	less than
Week 0	0.2894	1.0193	1.0193	less than
Week 24	0.2919	1.0281	1.0281	less than
Week 4	0.3100	1.0918	1.0918	less than
Week 8	0.3184	1.1214	1.1214	less than
Average	0.2839			
STDEV	0.0387			

Table 6-36: Beta Pinene Z-test hot box oil study

Beta Pinene	Base Peak Ratio	z-values	|z- values|	Compared to z crit = 1.960
Week 24	0.2792	-1.5396	1.5396	less than
Week 4	0.2909	-0.3751	0.3751	less than
Week 20	0.2930	-0.1751	0.1751	less than
Week 12	0.2969	0.2111	0.2111	less than
Week 8	0.2985	0.3697	0.3697	less than
Week 0	0.3100	1.5090	1.5090	less than
Average	0.2947			
STDEV	0.0101			

Table 6-37: Beta Pinene Z test results in HAT solution study

Beta Pinene	Base Peak Ratio	z-values	|z- values|	Compared to z crit = 1.960
Week 4	0.1859	-0.6216	0.6216	less than
Week 20	0.1875	-0.6086	0.6086	less than
Week 16:	0.1916	-0.5770	0.5770	less than
Week 8:	0.1956	-0.5455	0.5455	less than
Week 0:	0.2158	-0.3891	0.3891	less than
Week 12:	0.3666	0.7782	0.7782	less than
Week 24:	0.5198	1.9636	1.9636	less than
Average	0.2661			
STDEV	0.1292			

Table 6-38: Myrcene Z test results in ambient oil study

Myrcene	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 12	0.1431	-1.9846	1.9846	greater than
Week 24	0.1641	-0.4034	0.4034	less than
Week 0	0.1669	-0.1925	0.1925	less than
Week 20	0.1741	0.3496	0.3496	less than
Week 8	0.1764	0.5228	0.5228	less than
Week 4	0.1800	0.7938	0.7938	less than
Week 16	0.1816	0.9143	0.9143	less than
Average (1)	0.1695			
STDEV (1)	0.0133			

Table 6-39: Myrcene Z test results in hotbox oil study

Myrcene	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 16	0.1540	-1.3138	1.3138	less than
Week 24	0.1600	-0.7674	0.7674	less than
Week 20	0.1620	-0.5853	0.5853	less than
Week 8	0.1660	-0.2211	0.2211	less than
Week 4	0.1730	0.4162	0.4162	less than
Week 0	0.1800	1.0536	1.0536	less than
Week 12	0.1840	1.4178	1.4178	less than
Average (1)	0.1684			
STDEV (1)	0.0110			

Table 6-40: Myrcene Z test results in HAT solution study

Myrcene	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 20	0.0944	-1.2550	1.2550	less than
Week 16	0.0988	-0.9249	0.9249	less than
Week 4	0.1031	-0.6023	0.6023	less than
Week 12	0.1117	0.0429	0.0429	less than
Week 24	0.1152	0.3054	0.3054	less than
Week 8	0.1237	0.9431	0.9431	less than
Week 0	0.1310	1.4907	1.4907	less than
Average (1)	0.1111			
STDEV (1)	0.0133			

Table 6-41: Para Cymene Z test results in ambient study

Para Cymene	Base Peak Ratio	z-values	|z- values|	Compared to z crit = 1.960
Week 4	0.1588	-1.2268	1.2268	less than
Week 0	0.1644	-1.1603	1.1603	less than
Week 8	0.2504	-0.1386	0.1386	less than
Week 12	0.329	0.7951	0.7951	less than
Week 16	0.3344	0.8593	0.8593	less than
Week 24	0.3354	0.8712	0.8712	less than
Average	0.2621			
STDEV	0.0842			

Table 6-42: Para Cymene Z test results in hot box study

Para Cymene	Base Peak Ratio	z-values	|z- values|	Compared to z crit = 1.960
Week 12	0.0290	-1.1968	1.1968	less than
Week 0	0.1588	-0.8215	0.8215	less than
Week 4	0.3740	-0.1995	0.1995	less than
Week 20	0.4287	-0.0414	0.0414	less than
Week 8	0.4542	0.0325	0.0325	less than
Week 24	0.5417	0.2854	0.2854	less than
Week 16	1.1146	1.9413	1.9413	less than
Average	0.4430			
STDEV	0.3459			

Table 6-43: Para Cymene Z test results in HAT solution study

Para Cymene	Base Peak Ratio	z-values	|z- values|	Compared to z crit = 1.960
Week 4	0.0007	-0.7050	0.7050	less than
Week 0	0.0008	-0.7049	0.7049	less than
Week 8	0.0109	-0.6935	0.6935	less than
Week 20	0.0115	-0.6928	0.6928	less than
Week 16	0.7562	0.1474	0.1474	less than
Week 12	1.3497	0.8169	0.8169	less than
Week 24	2.2495	1.8320	1.8320	less than
Average	0.6256			
STDEV	0.8864			

Table 6-44: Gamma Terpinene Z test results in ambient study

Gamma Terpinene	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 12	0.7570	-1.9766	1.9766	greater than
Week 8	0.9733	0.1475	0.1475	less than
Week 0	0.9746	0.1611	0.1611	less than
Week 24	0.9917	0.3286	0.3286	less than
Week 4	1.0120	0.5277	0.5277	less than
Week 16	1.0409	0.8117	0.8117	less than
Average (1)	0.9582			
STDEV (1)	0.1018			

Table 6-45: Gamma Terpinene Z test results in hotbox study

Gamma Terpinene	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 24	0.9148	-1.3662	1.3662	less than
Week 4	0.9344	-0.9276	0.9276	less than
Week 8	0.9748	-0.0209	0.0209	less than
Week 20	0.9873	0.2588	0.2588	less than
Week 0	1.0120	0.8135	0.8135	less than
Week 12	1.0311	1.2425	1.2425	less than
Average (1)	0.9757			
STDEV (1)	0.0446			

Table 6-46: Gamma Terpinene Z test results in HAT solution study

Gamma Terpinene	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 16	0.4856	-0.9714	0.9714	less than
Week 20	0.5011	-0.9250	0.9250	less than
Week 4	0.7062	-0.3106	0.3106	less than
Week 8	0.7137	-0.2880	0.2880	less than
Week 0	0.7841	-0.0770	0.0770	less than
Week 12	1.0406	0.6912	0.6912	less than
Week 24	1.4377	1.8809	1.8809	less than
Average (1)	0.8098			
STDEV (1)	0.3338			

Table 6-47: Alpha Terpineol Z test results in ambient oil study

Alpha Terpineol	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 8	0.0056	-1.4168	1.4168	less than
Week 12	0.0068	-0.9594	0.9594	less than
Week 0	0.0094	0.0318	0.0318	less than
Week 4	0.0104	0.4130	0.4130	less than
Week 24	0.0115	0.8323	0.8323	less than
Week 16	0.0122	1.0991	1.0991	less than
Average (1)	0.0093			
STDEV (1)	0.0026			

Table 6-48: Alpha Terpineol Z test results in hotbox oil study

Alpha Terpineol	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 24	0.00811	-1.5416	1.5416	less than
Week 0	0.01040	-0.6160	0.6160	less than
Week 4	0.0112	-0.3048	0.3048	less than
Week 8	0.0132	0.5266	0.5266	less than
Week 20	0.01430	0.9602	0.9602	less than
Week 12	0.0143	0.9757	0.9757	less than
Average (1)	0.0119			
STDEV (1)	0.0025			

Table 6-49: Alpha Terpineol Z test results in HAT solution study

Alpha Terpineol	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 16	0.0070	-0.6279	0.6279	less than
Week 20	0.0072	-0.5761	0.5761	less than
Week 4	0.0073	-0.5571	0.5571	less than
Week 8	0.0076	-0.5006	0.5006	less than
Week 0	0.0081	-0.4144	0.4144	less than
Week 12	0.0135	0.6451	0.6451	less than
Week 24	0.0207	2.0310	2.0310	greater than
Average (1)	0.0102			
STDEV (1)	0.0052			

Table 6-50: Thymol Z test results in ambient oil study

Thymol	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 12	0.0041	-1.5236	1.5236	less than
Week 24	0.0056	-0.8884	0.8884	less than
Week 16	0.0077	-0.0079	0.0079	less than
Week 0	0.0078	0.0517	0.0517	less than
Week 4	0.0085	0.3588	0.3588	less than
Week 8	0.0086	0.3968	0.3968	less than
Week 20	0.0114	1.6126	1.6126	less than
Average (1)	0.0077			
STDEV (1)	0.0023			

Table 6-51: Thymol Z test results in hotbox oil study

Thymol	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 20	0.0064	-1.7298	1.7298	less than
Week 24	0.0073	-0.7093	0.7093	less than
Week 8	0.0084	0.5050	0.5050	less than
Week 12	0.0085	0.6052	0.6052	less than
Week 0	0.0085	0.6471	0.6471	less than
Week 4	0.0085	0.6817	0.6817	less than
New Average	0.0080			
New StDev	0.0009			

Table 6-52: Thymol Z test results in HAT solution study

Thymol	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 16	0.0040	-0.7393	0.7393	less than
Week 20	0.0042	-0.7026	0.7026	less than
Week 4	0.0048	-0.5577	0.5577	less than
Week 8	0.0052	-0.4756	0.4756	less than
Week 0	0.0063	-0.2045	0.2045	less than
Week 12	0.0103	0.7113	0.7113	less than
Week 24	0.0157	1.9685	1.9685	greater than
Average (1)	0.0072			
STDEV (1)	0.0043			

Table 6-53: MNMA Z test results in ambient oil study

MNMA	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 24	0.0388	-1.0319	1.0319	less than
Week 12	0.0419	-0.8650	0.8650	less than
Week 8	0.0554	-0.1268	0.1268	less than
Week 0	0.0581	0.0217	0.0217	less than
Week 4	0.0621	0.2374	0.2374	less than
Week 20	0.0901	1.7646	1.7646	less than
Average (1)	0.0577			
STDEV (1)	0.0183			

Table 6-54: MNMA Z test results in hotbox oil study

MNMA	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 20	0.0454	-1.7664	1.7664	less than
Week 24	0.0536	-0.5599	0.5599	less than
Week 12	0.0586	0.1854	0.1854	less than
Week 4	0.0620	0.6811	0.6811	less than
Week 0	0.0621	0.6955	0.6955	less than
Week 8	0.0626	0.7644	0.7644	less than
Average (1)	0.0574			
STDEV (1)	0.0069			

Table 6-55: MNMA Z test results in HAT solution study

MNMA	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 20	0.0287	-0.7478	0.7478	less than
Week 16	0.0288	-0.7458	0.7458	less than
Week 4	0.0352	-0.5429	0.5429	less than
Week 8	0.0370	-0.4856	0.4856	less than
Week 0	0.0475	-0.1530	0.1530	less than
Week 12	0.0753	0.7240	0.7240	less than
Week 24	0.1141	1.9510	1.9510	less than
Average (1)	0.0524			
STDEV (1)	0.0317			

Table 6-56: Beta Caryophyllene Z test results in ambient oil study

Beta Caryophyllene	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 12	0.0016	-1.5778	1.5778	less than
Week 24	0.0026	-0.6924	0.6924	less than
Week 0	0.0033	-0.0342	0.0342	less than
Week 8	0.0035	0.1373	0.1373	less than
Week 16	0.0036	0.2088	0.2088	less than
Week 4	0.0036	0.2546	0.2546	less than
Week 20	0.0053	1.7037	1.7037	less than
Average (1)	0.0033			
STDEV (1)	0.0011			

Table 6-57: Beta Caryophyllene Z test results in hotbox oil study

Beta Caryophyllene	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 20	0.0027	-1.3087	1.3087	less than
Week 24	0.0028	-1.2332	1.2332	less than
Week 4	0.0034	0.3383	0.3383	less than
Week 8	0.0035	0.6550	0.6550	less than
Week 12	0.0035	0.6651	0.6651	less than
Week 0	0.0036	0.8835	0.8835	less than
Average (1)	0.0033			
STDEV (1)	0.0004			

Table 6-58: Beta Caryophyllene Z test results in HAT solution study

Beta Caryophyllene	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 16	0.0012	-1.1812	1.1812	less than
Week 20	0.0012	-1.1741	1.1741	less than
Week 8	0.0022	-0.1749	0.1749	less than
Week 4	0.0022	-0.1673	0.1673	less than
Week 0	0.0028	0.3917	0.3917	less than
Week 12	0.0031	0.7311	0.7311	less than
Week 24	0.0039	1.5747	1.5747	less than
Average (1)	0.0024			
STDEV (1)	0.0010			

Table 6-59: Sinensal Z test results in ambient oil study

Sinensal	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 12	0.0067	-1.9034	1.9034	less than
Week 24	0.0180	-0.6539	0.6539	less than
Week 0	0.0261	0.2392	0.2392	less than
Week 16	0.0261	0.2452	0.2452	less than
Week 8	0.0270	0.3397	0.3397	less than
Week 4	0.0288	0.5389	0.5389	less than
Week 20	0.0347	1.1943	1.1943	less than
Average (1)	0.0239			
STDEV (1)	0.0090			

Table 6-60: Sinensal Z test results in hotbox oil study

Sinensal	Base Peak Ratio	z-values	|Z- values|	compared to Z crit = 1.960
Week 20	0.0208	-1.3588	1.3588	less than
Week 24	0.0214	-1.2443	1.2443	less than
Week 8	0.0282	0.0826	0.0826	less than
Week 0	0.0288	0.1978	0.1978	less than
Week 12	0.0288	0.2065	0.2065	less than
Week 4	0.0313	0.6856	0.6856	less than
Week 16	0.0351	1.4306	1.4306	less than
Average (1)	0.0278			
STDEV (1)	0.0051			

Table 6-61: Sinensal Z test results in HAT solution study

Sinensal	Base Peak Ratio	z-values	|Z- values|	Compared to Z crit = 1.960
Week 16	0.0079	-1.2831	1.2831	less than
Week 20	0.0080	-1.2561	1.2561	less than
Week 8	0.0170	-0.0138	0.0138	less than
Week 4	0.0176	0.0785	0.0785	less than
Week 12	0.0194	0.3240	0.3240	less than
Week 0	0.0218	0.6555	0.6555	less than
Week 24	0.0278	1.4950	1.4950	less than
Average (1)	0.0171			
STDEV (1)	0.0072			

6.4.6 Reconciling Mass Balance

To evaluate the ambient, thermal and acid catalyzed stability of mandarin essential oil a reconciliation of mass balance was evaluated. Mass balance calculations were performed to assess week zero and week 24 for each compound under each of the three stability-indicating environments. The intent of this study was to explain the changes in marker chemicals to infer if any chemical(s) could be responsible off notes in the mandarin oil. The goal was to demonstrate the analytical ability to detect degradation of the marker aroma chemicals during each of the stability indicating environments.

An area ratio was computed for all ten compounds of interest, using the method of internal standard; converting to a percentage demonstrated component changes at a larger magnitude' thereby making it easier to interpret significance of each change. The

criterion for rejection of a molecule's stability was a change outside +/- 20%. Compounds deviating beyond +/- 20% are considered suspect.

In the mass balance for all three studies para cymene experienced change that pushed it far from the +/- 20% acceptability. Thymol also experienced chemical change exceeding the threshold of acceptance. Beta caryophyllene only fell below the acceptable criterion in the ambient study but experienced less than 1% change in the hotbox and HAT solution studies. Alpha terpineol increased out of the acceptable boundaries in both the hotbox and HAT solution studies, and lastly, MNMA exceed the criterion for the hotbox study by 4%. Most intriguing was the change in sinensal that only occurred in the high acid tasting solution stability study at week 24 in which it decreased to 27%.

Table 6-62: Reconciling Mass Balance of each stability study

	Ambient Conditions			Hot Box Conditions			High Acid Tasting Solution		
Compound	Ratio as %	± 20%		Ratio as %	± 20%		Ratio as %	± 20%	
Alpha Pinene	103.0242	120-80	pass	93.1034	120-80	pass	114.3878	120-80	pass
Beta Pinene	101.9689	120-80	pass	96.7972	120-80	pass	118.6505	120-80	pass
Myrcene	98.3278	120-80	pass	88.8889	120-80	pass	111.7362	120-80	pass
Para Cymene	187.0696	120-80	fail	324.5614	120-80	fail	396.7543	120-80	fail
Gamma Terpinene	92.0381	120-80	pass	86.0351	120-80	pass	80.6201	120-80	pass
Alpha Terpineol	99.5442	120-80	pass	133.3333	120-80	fail	136.4865	120-80	fail
Thymol	136.0082	120-80	fail	150.0000	120-80	fail	130.4348	120-80	fail
MNMA	103.4532	120-80	pass	124.4898	120-80	fail	112.3494	120-80	pass
Beta Caryophyllene	51.5072	120-80	fail	99.9463	120-80	pass	100.1081	120-80	pass
Sinensal	90.3304	120-80	pass	107.1429	120-80	pass	27.8788	120-80	fail

6.4.7 Bland Altman Method

The Bland-Altman method was used to assess each mandarin component over 24 weeks, under each of the three studies. In this instance, one data set was the area ratio data observed from each study individually, over the 24 weeks. The second data set was the system suitability area ratio data from the three day validation period, averaged. The system suitability data was each mandarin component under unstressed conditions. The stressed conditions data obtained was compared to the unstressed data obtained. The Bland-Altman method was applied to demonstrate whether the stressed chemical data fell within the 95% confidence limit of data typical of an unstressed chemical.

In Summary, under three conditions for 24 total weeks, each component showed an increasing linear trend however the data points fell within the 95% confidence interval, meeting the criteria required for equivalence. Simply put, this means that stressed conditions are still comparable to the unstressed conditions for each of the ten components over 24 weeks. However, this increasingly linear trend as the mean difference gets larger indicates a proportional bias in the data [34]. It is concluded that a possible source of this bias is due to stressed conditions applied for an extended period of time while comparing the data in Bland-Altman Analysis to fresh, unstressed sample data. See Figures 6-1 through 6-30 for all Bland Altman plot under the three storage studies.

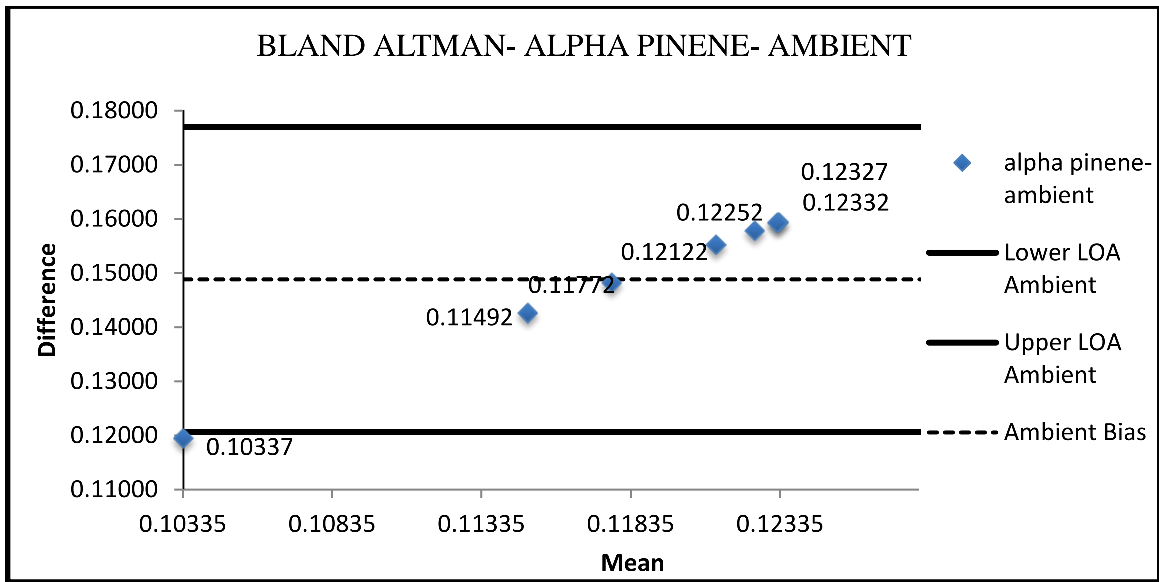


Figure 6-1: Bland Altman plot for alpha pinene under ambient stability conditions

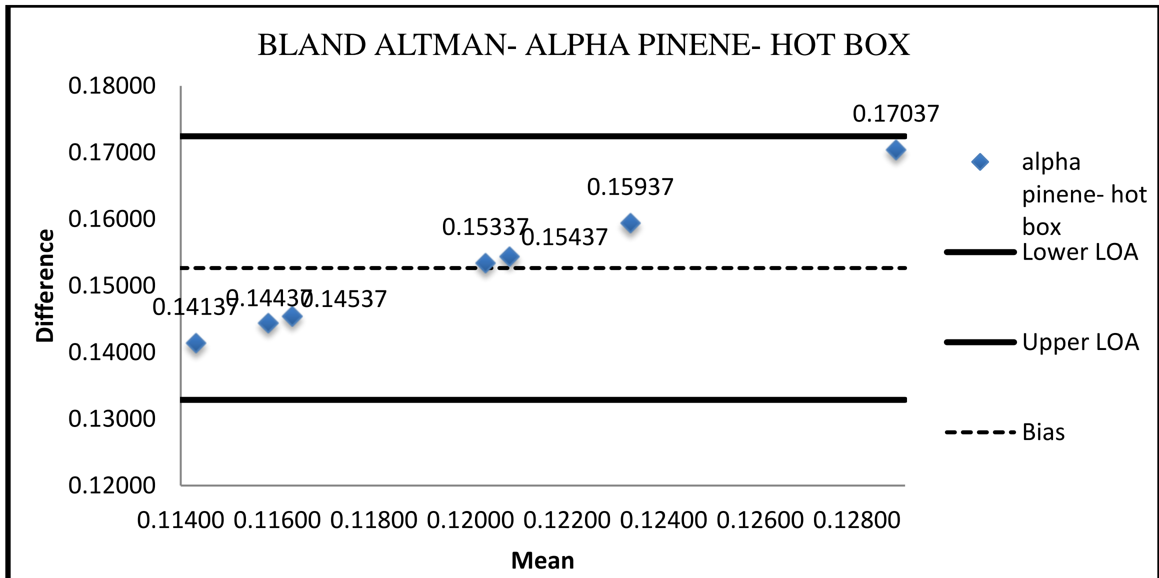


Figure 6-2: Bland Altman plot for alpha pinene under hotbox stability conditions

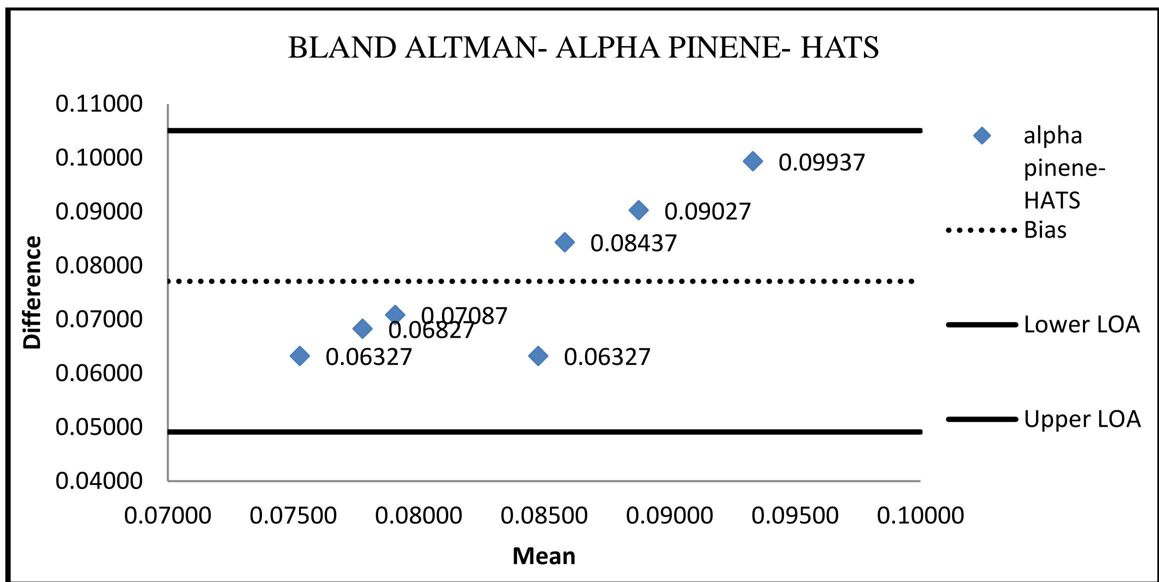


Figure 6-3: Bland Altman plot for alpha pinene under HATS stability conditions

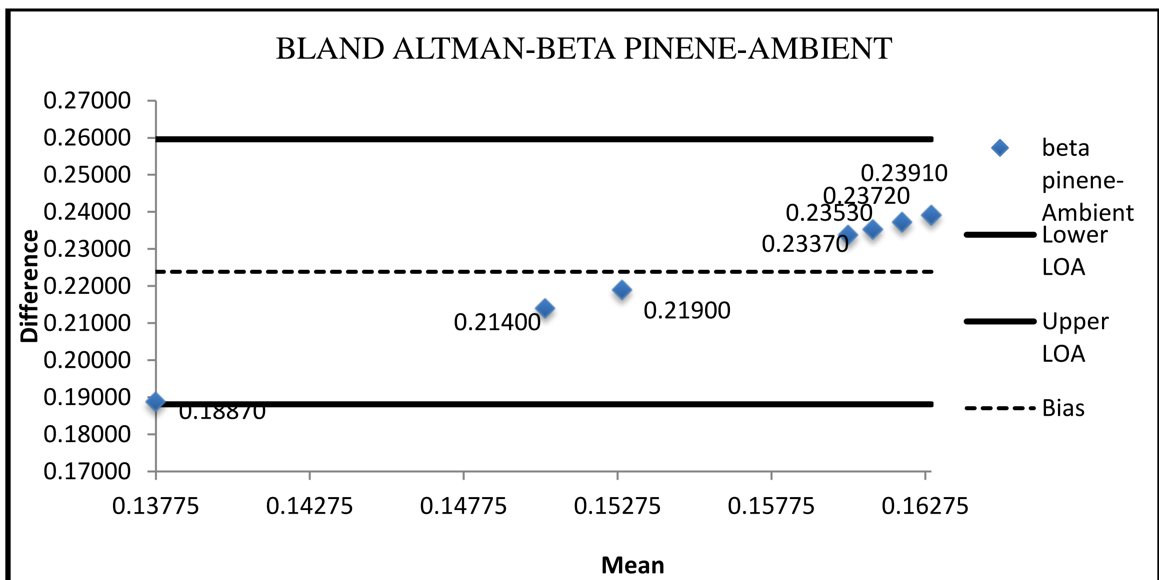


Figure 6-4: Bland Altman plot for beta pinene under ambient stability conditions

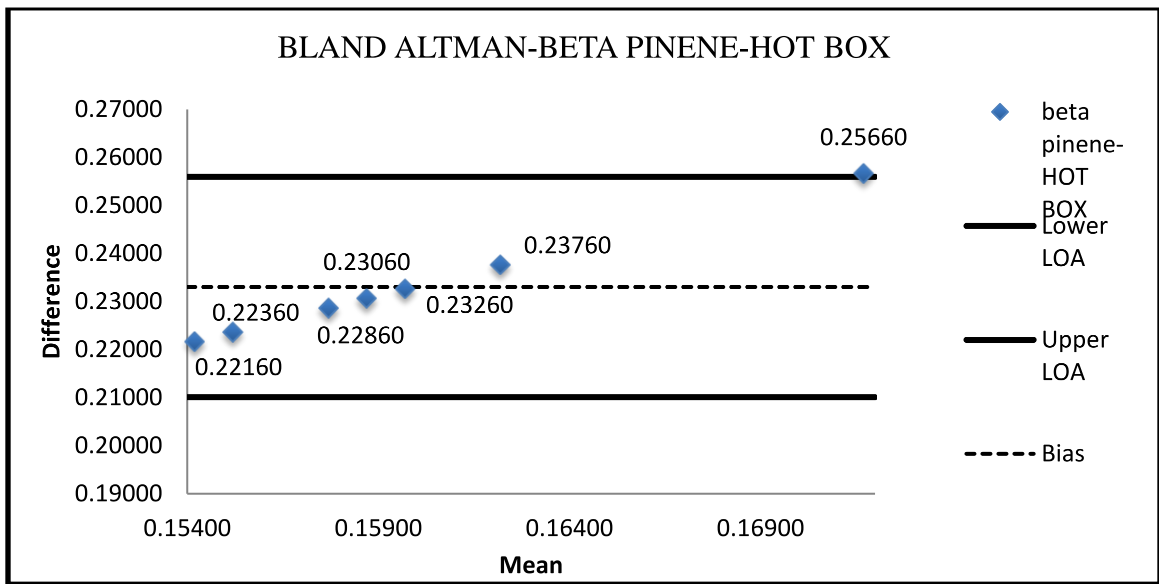


Figure 6-5: Bland Altman plot for beta pinene under hotbox stability conditions

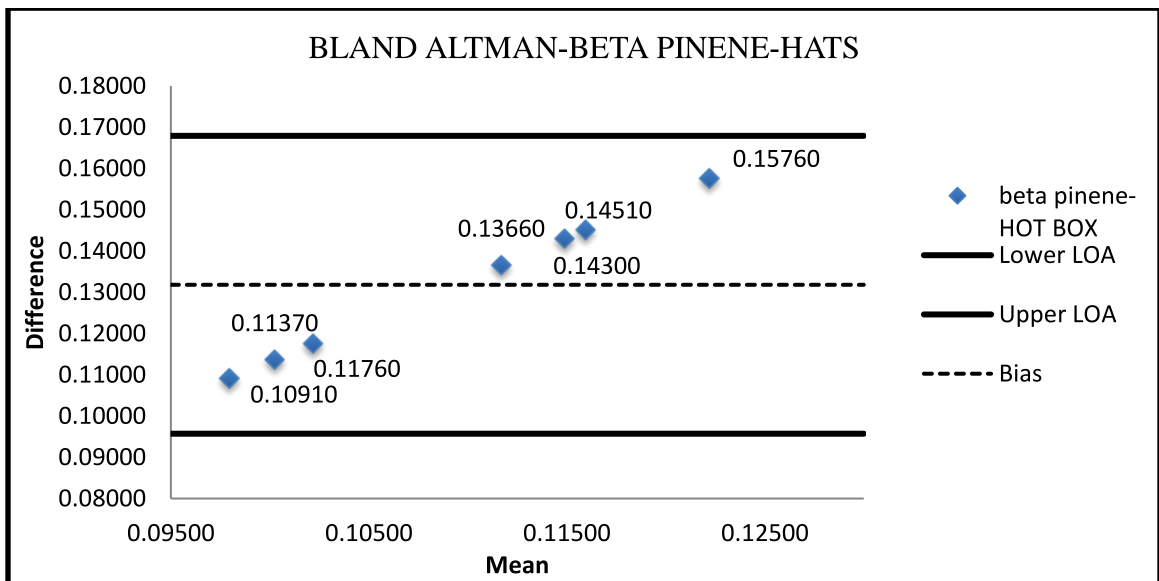


Figure 6-6: Bland Altman plot for beta pinene under HATS stability conditions

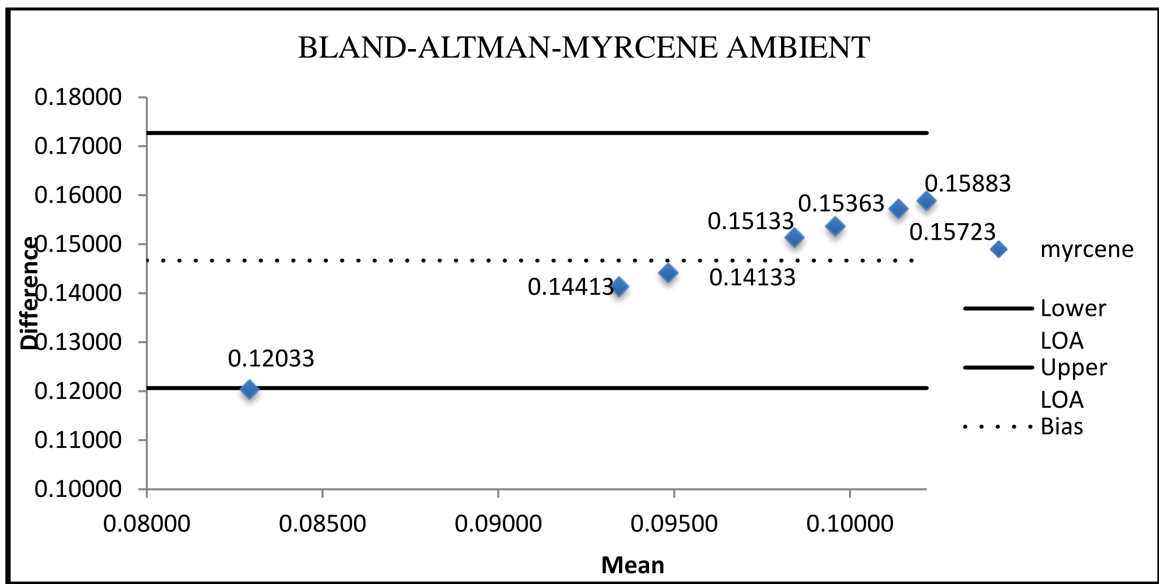


Figure 6-7: Bland Altman plot for myrcene under ambient stability conditions

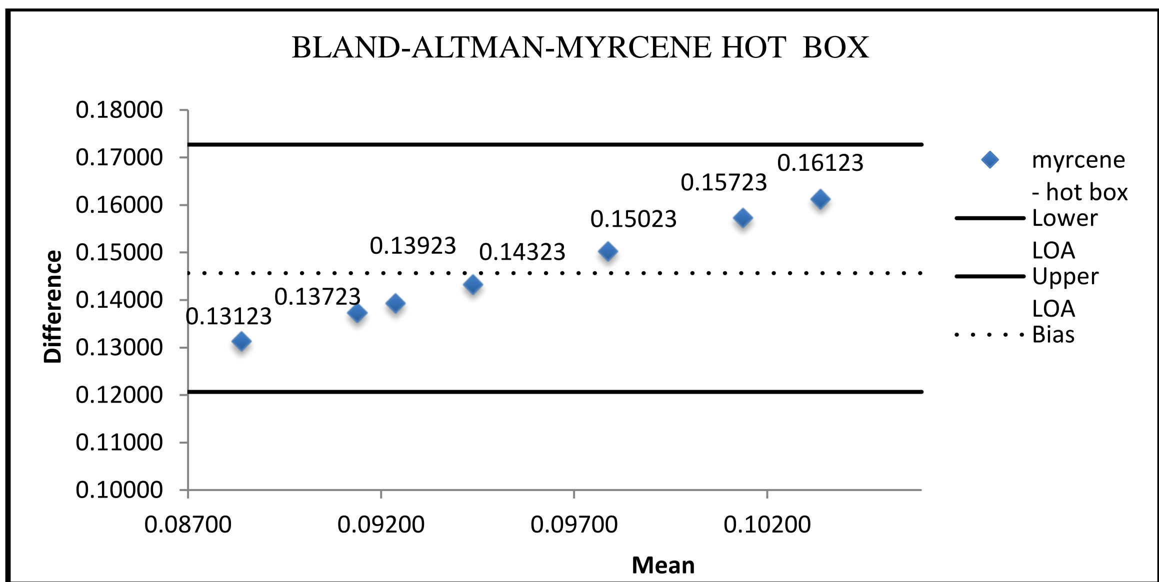


Figure 6-8: Bland Altman plot for myrcene under hotbox stability conditions

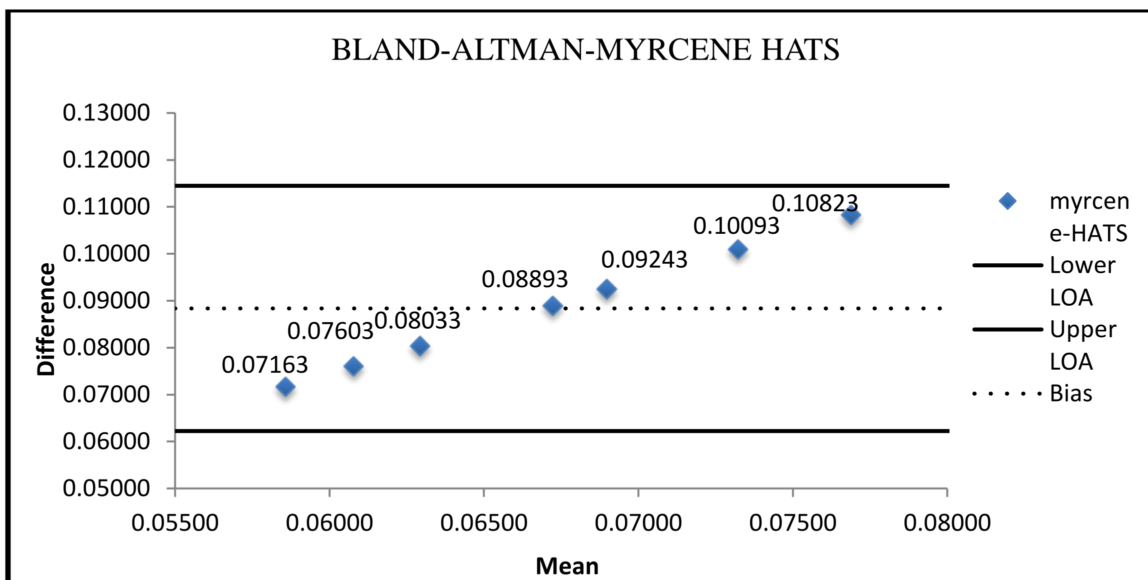


Figure 6-9: Bland Altman plot for myrcene under HATS stability conditions

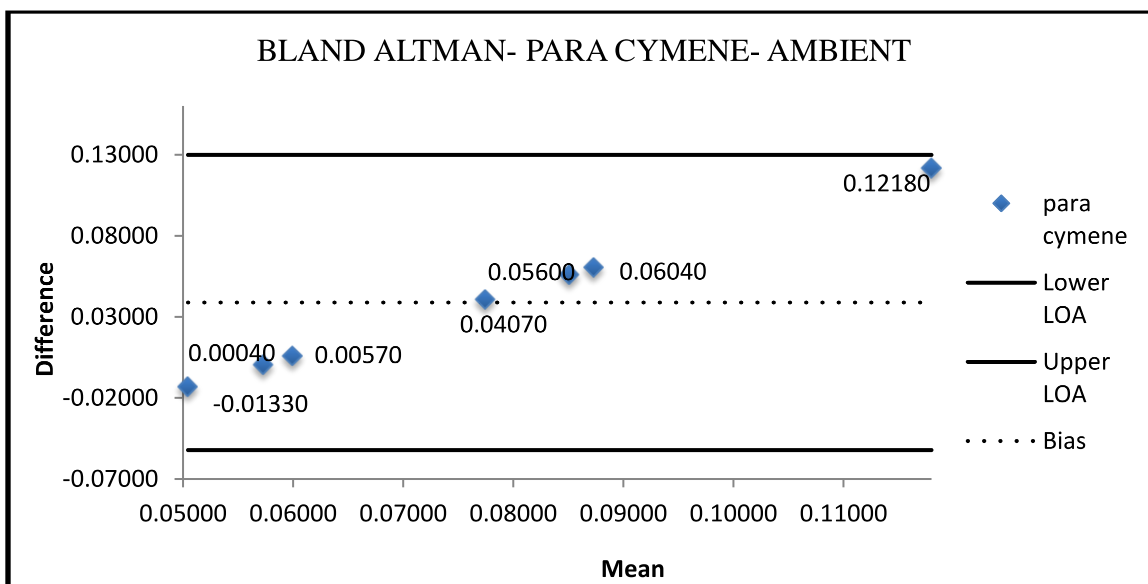


Figure 6-10: Bland Altman plot for para cymene under ambient stability conditions

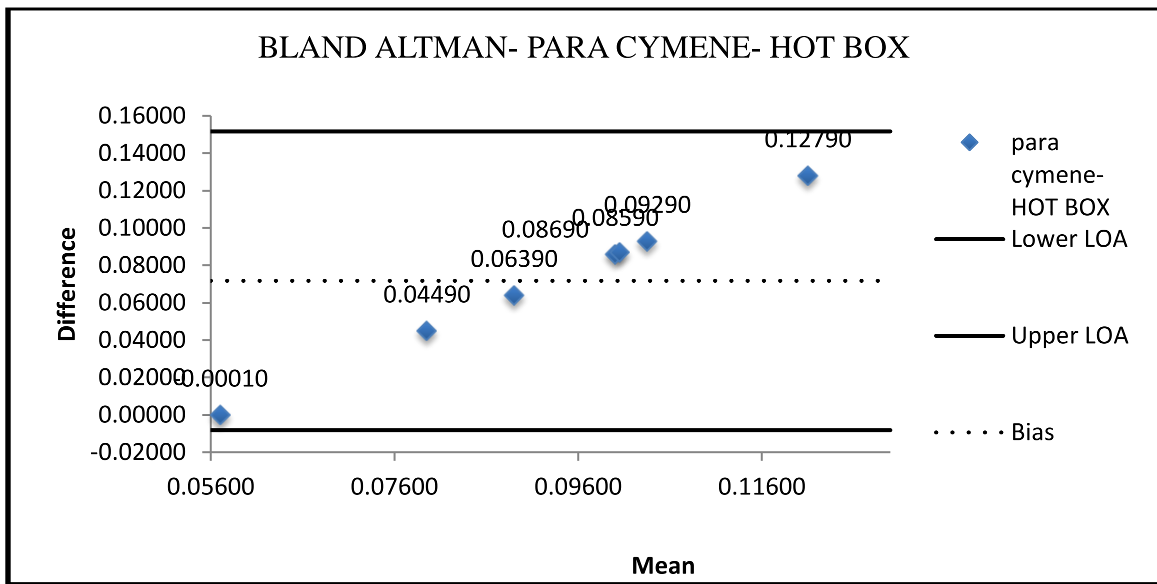


Figure 6-11: Bland Altman plot for para cymene under hotbox stability conditions

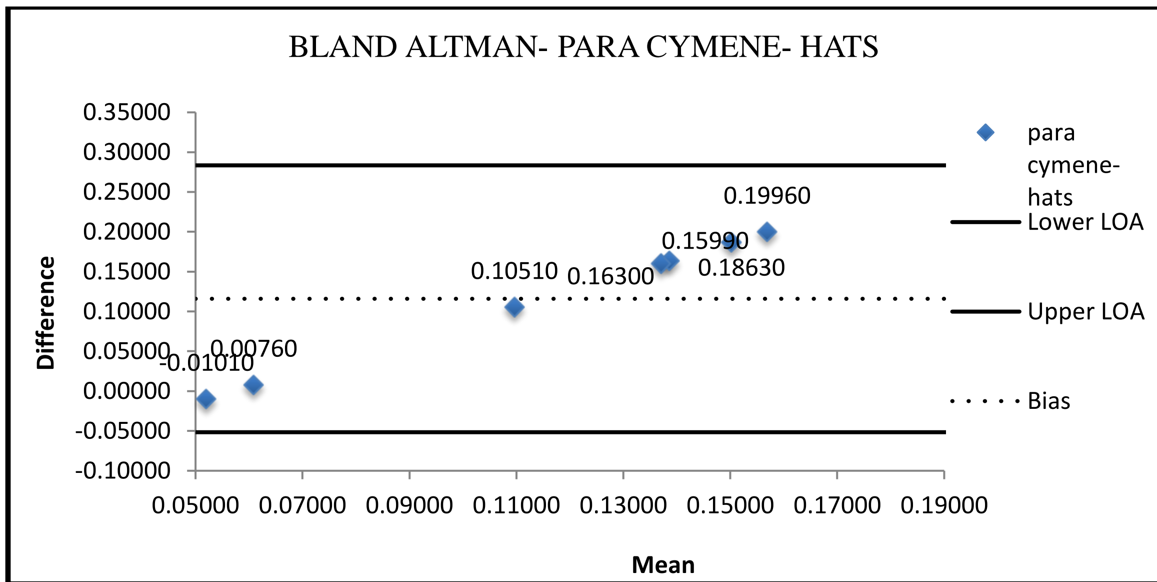


Figure 6-12: Bland Altman plot for para cymene under HATS stability conditions

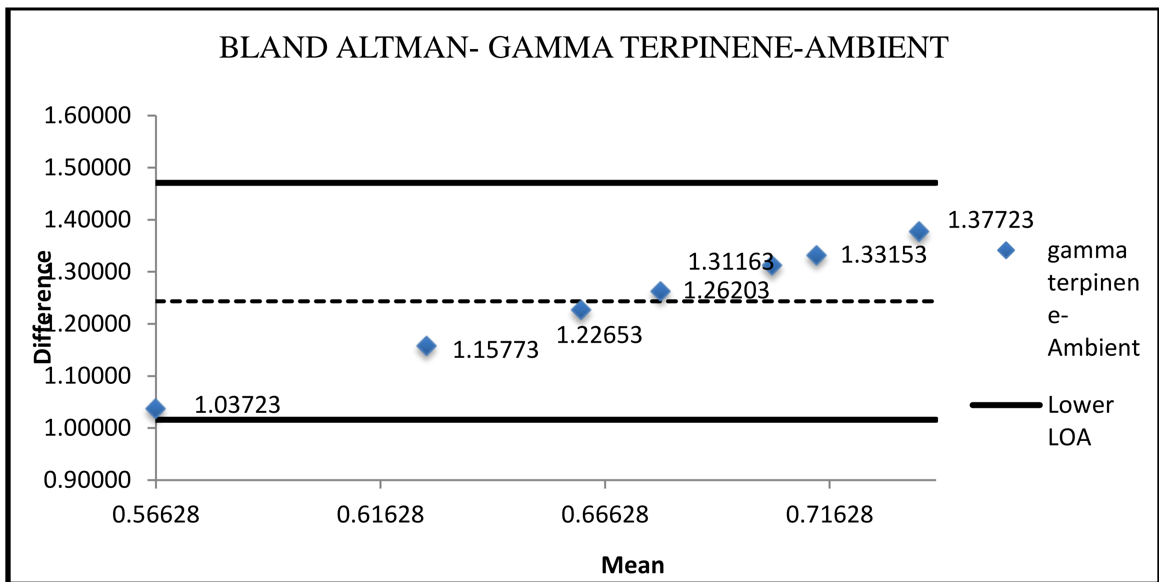


Figure 6-13: Bland Altman plot for gamma terpinene for ambient stability conditions

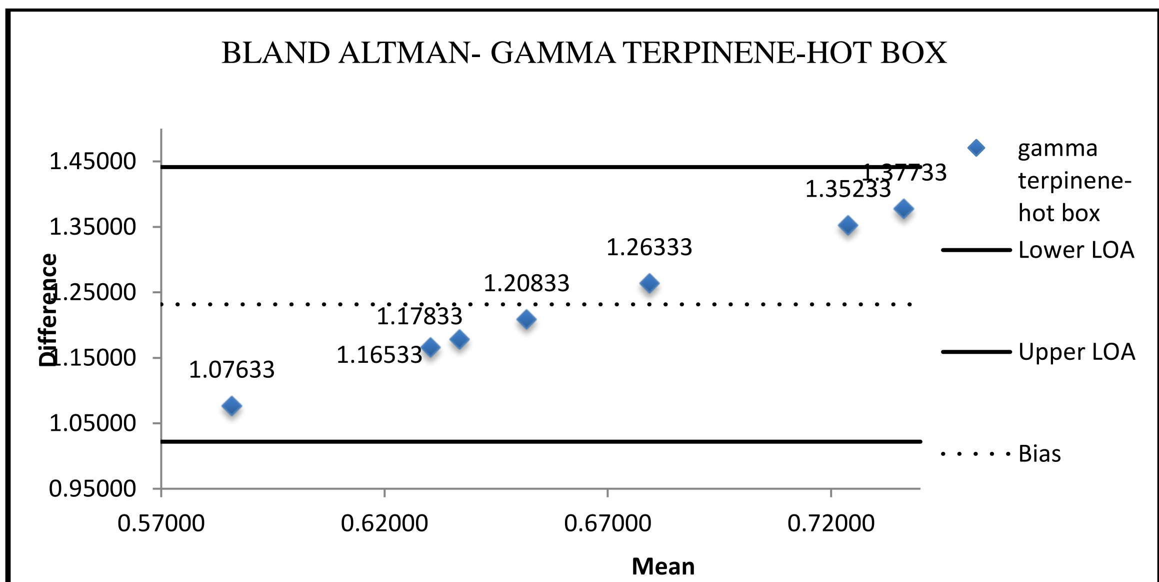


Figure 6-14: Bland Altman plot for gamma terpinene for hotbox stability conditions

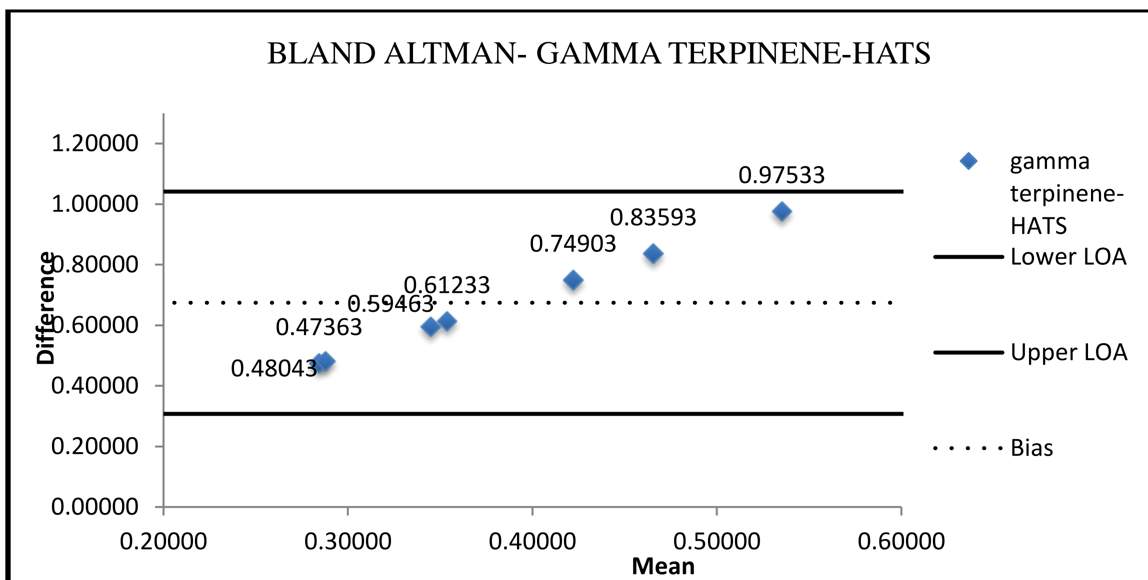


Figure 6-15: Bland Altman plot for gamma terpinene for HATS stability conditions

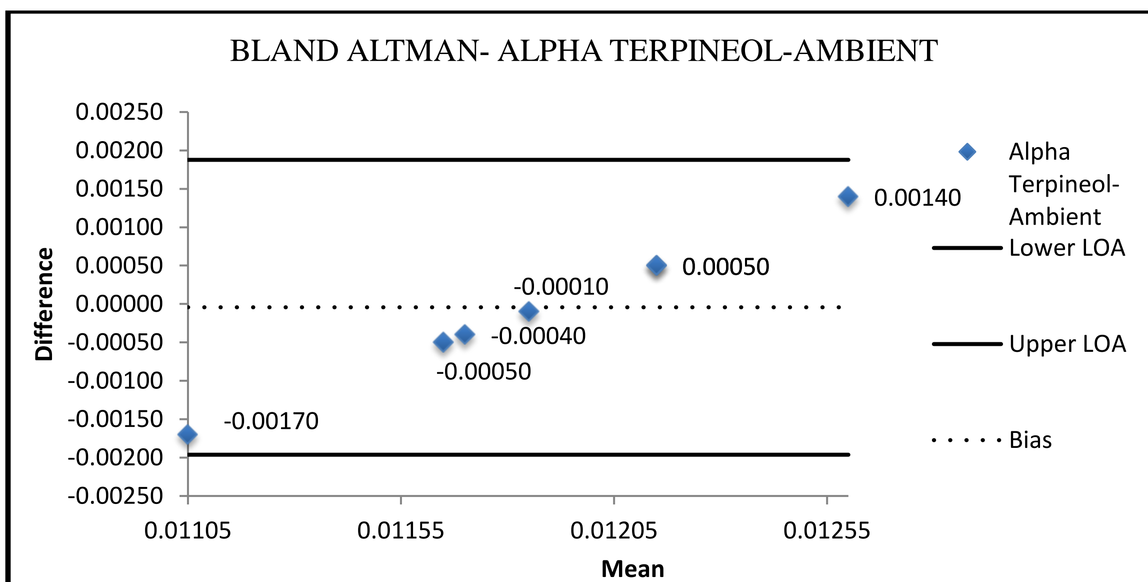


Figure 6-16: Bland Altman plot for alpha terpineol under ambient stability conditions

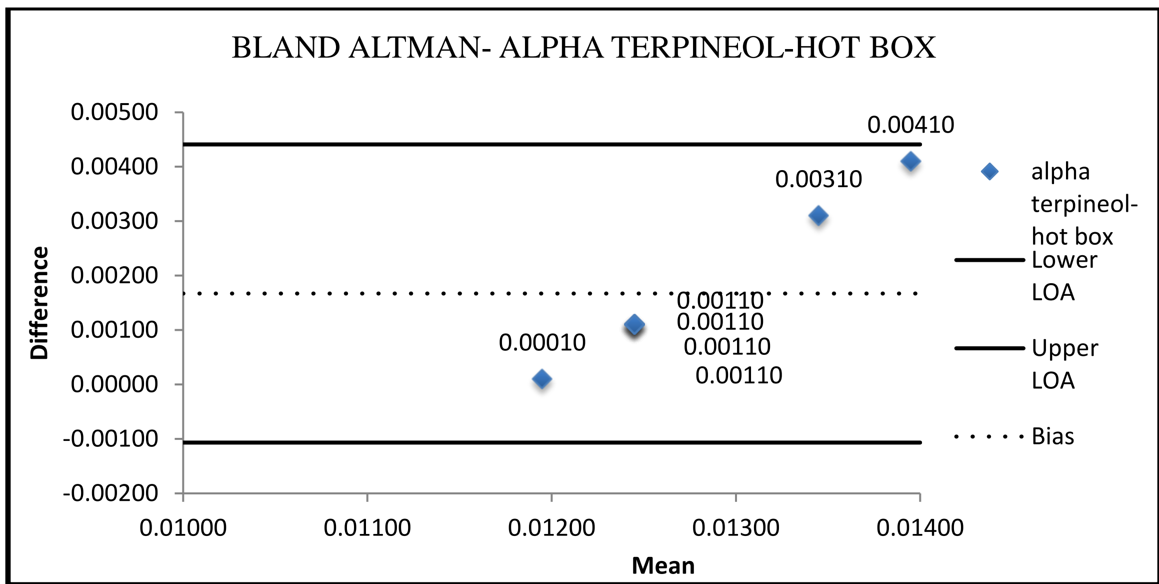


Figure 6-17: Bland Altman plot for alpha terpineol under hotbox stability conditions

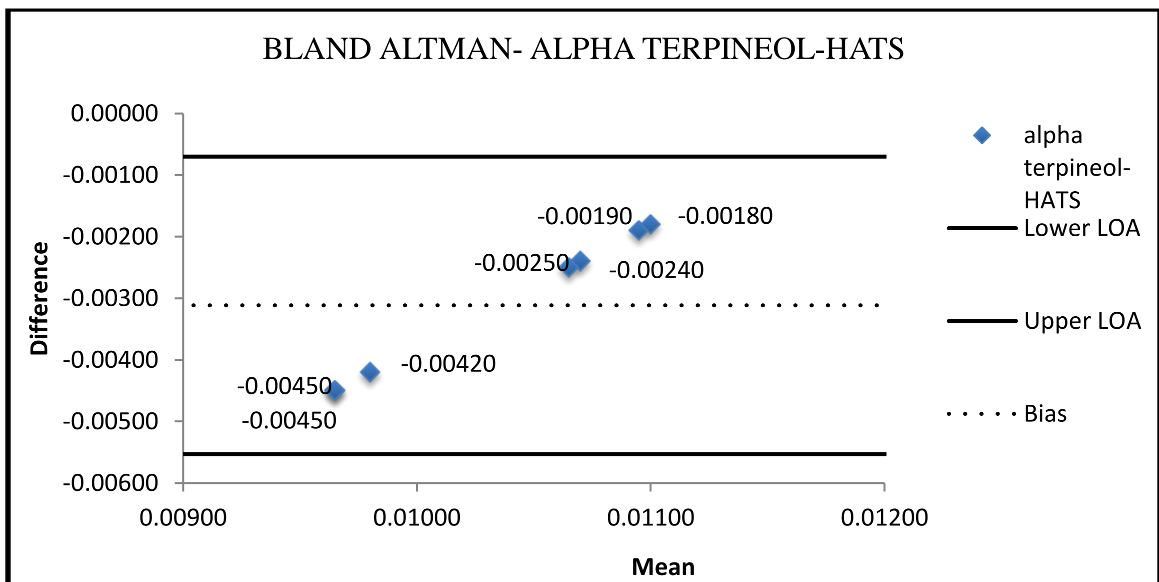


Figure 6-18: Bland Altman plot for alpha terpineol under HATS stability conditions

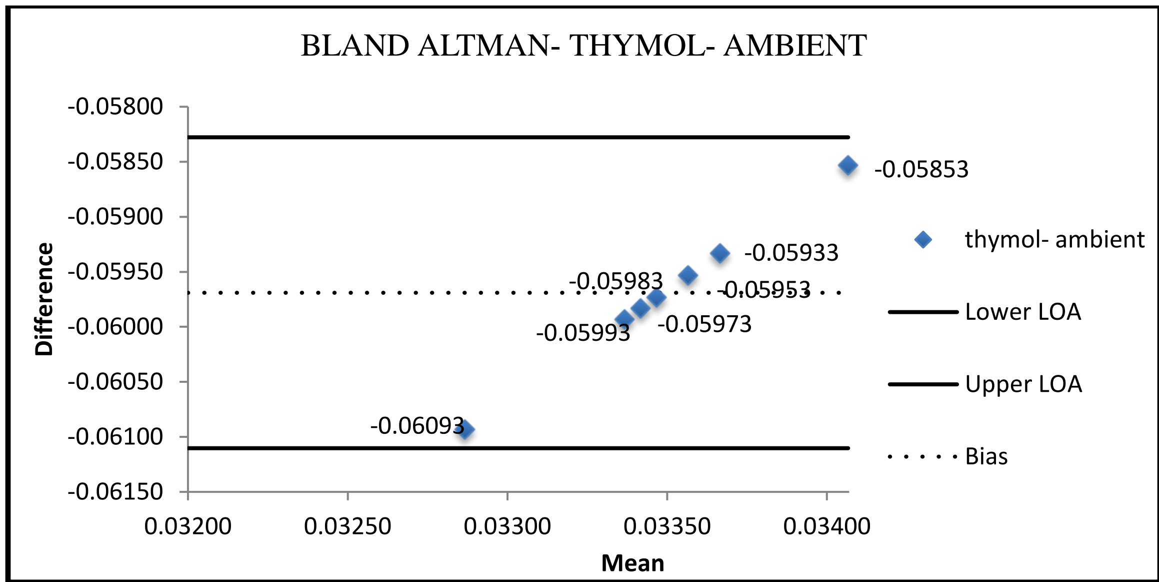


Figure 6-19: Bland Altman plot for thymol under ambient stability conditions

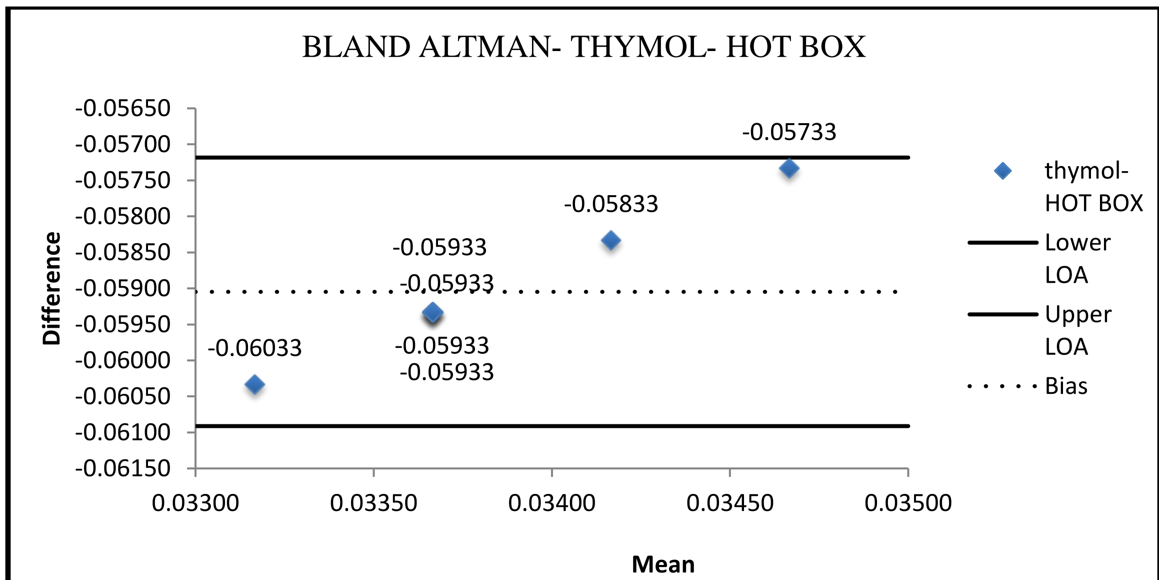


Figure 6-20: Bland Altman plot for thymol under hotbox stability conditions

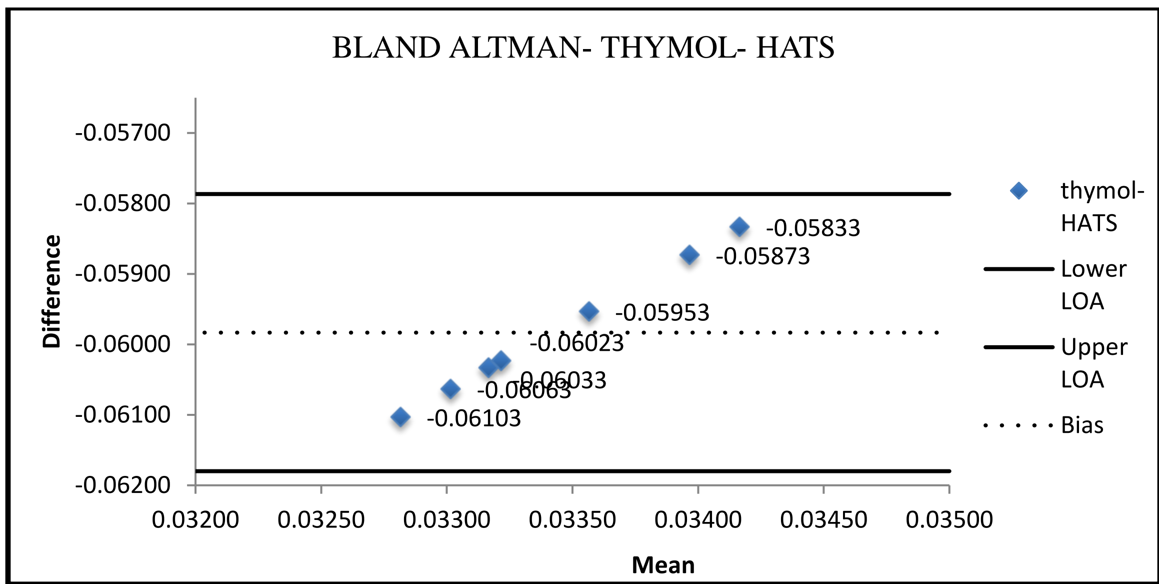


Figure 6-21: Bland Altman plot for thymol under HATS stability conditions

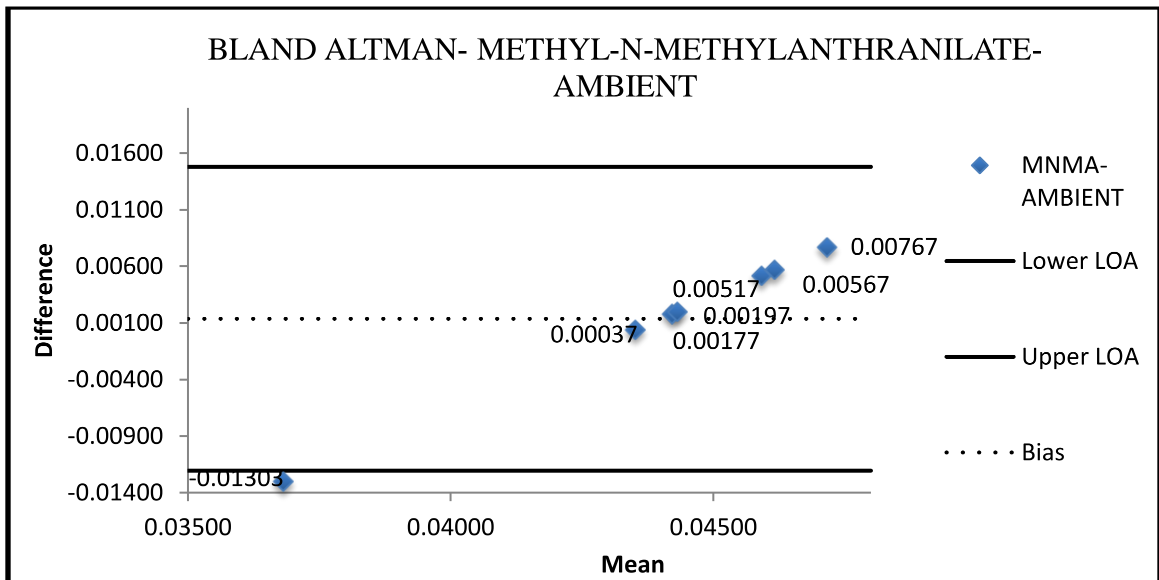


Figure 6-22: Bland Altman plot for MNMA under ambients stability conditions

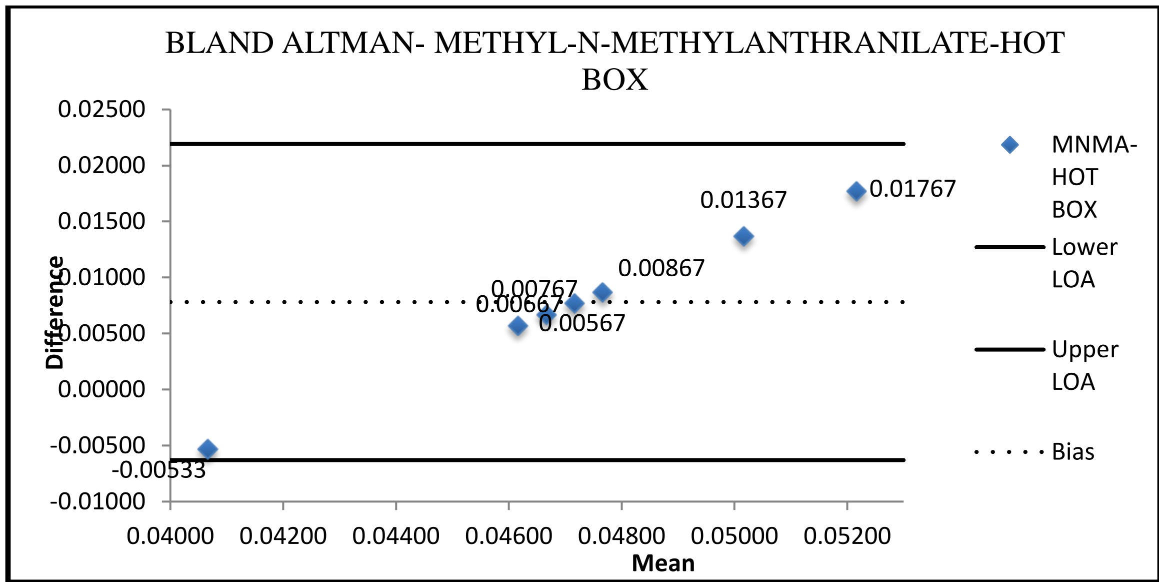


Figure 6-23: Bland Altman plot for MNMA under hotbox stability conditions

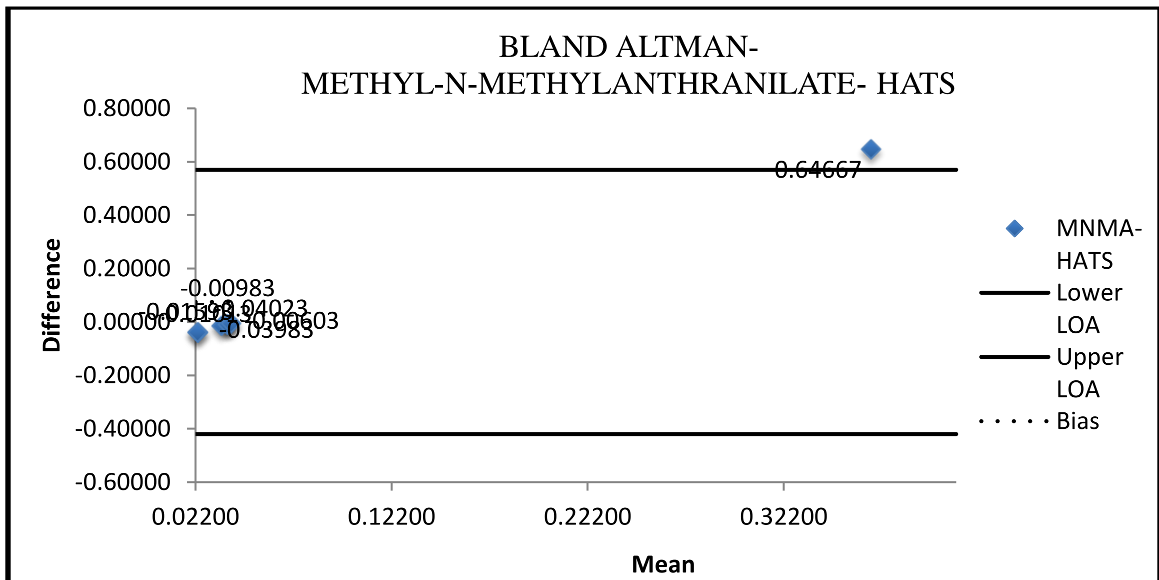


Figure 6-24: Bland Altman plot for MNMA under HATS stability conditions

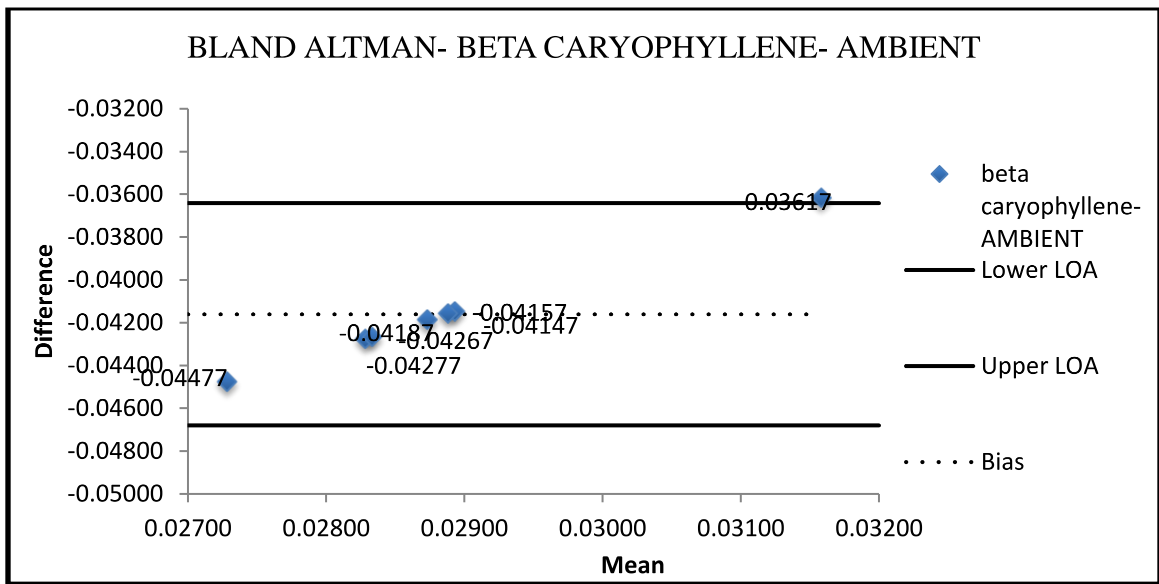


Figure 6-25: Bland Altman plot for beta caryophyllene in ambient stability conditions

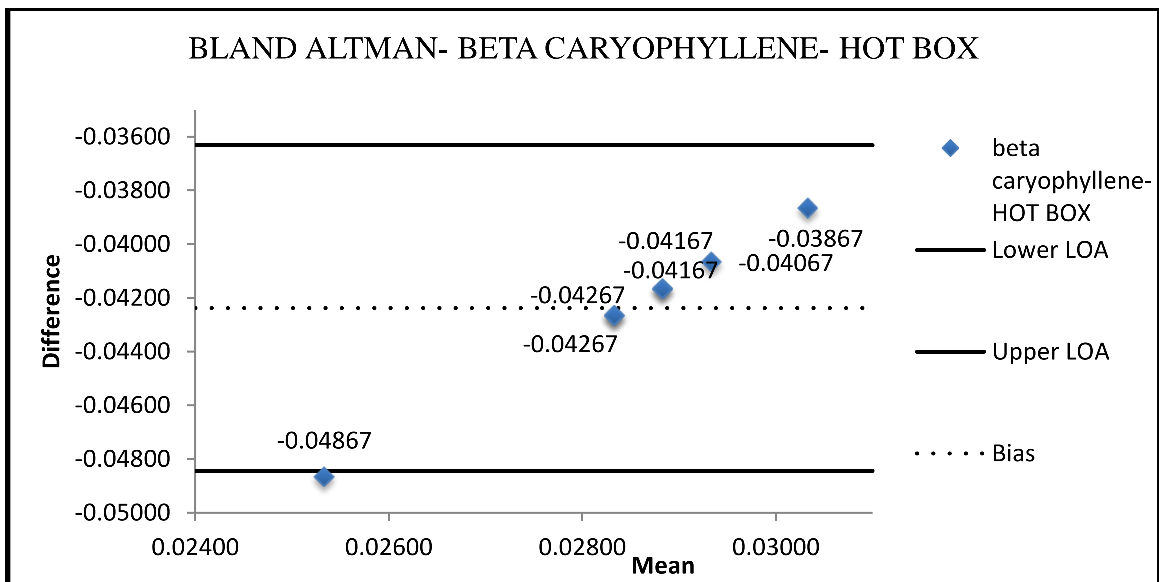


Figure 6-26: Bland Altman plot for beta caryophyllene in hotbox stability conditions

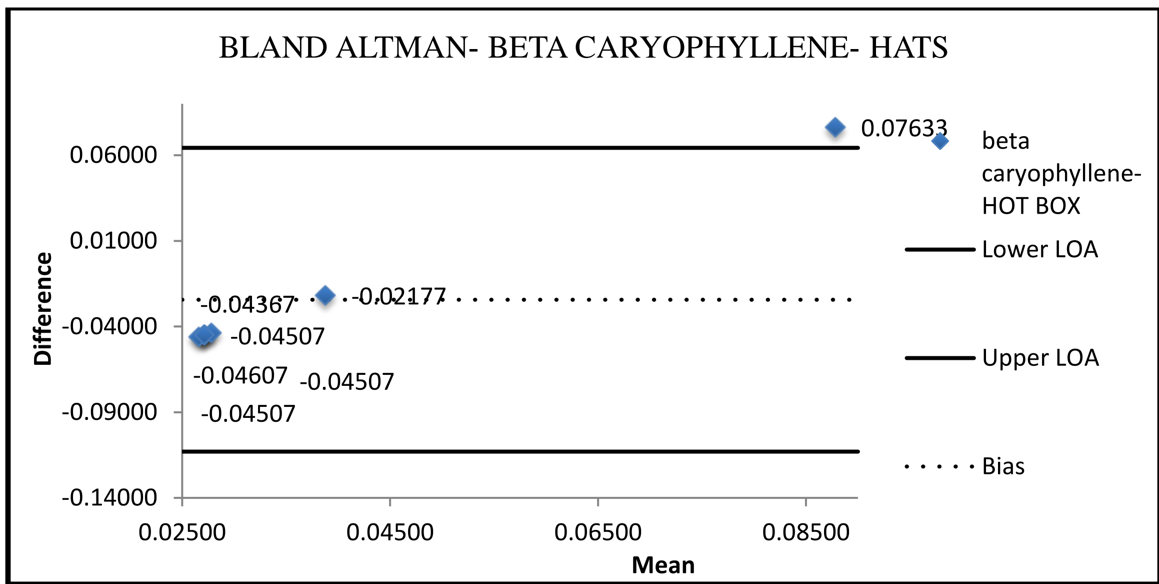


Figure 6-27: Bland Altman plot for beta caryophyllene in HATS stability conditions

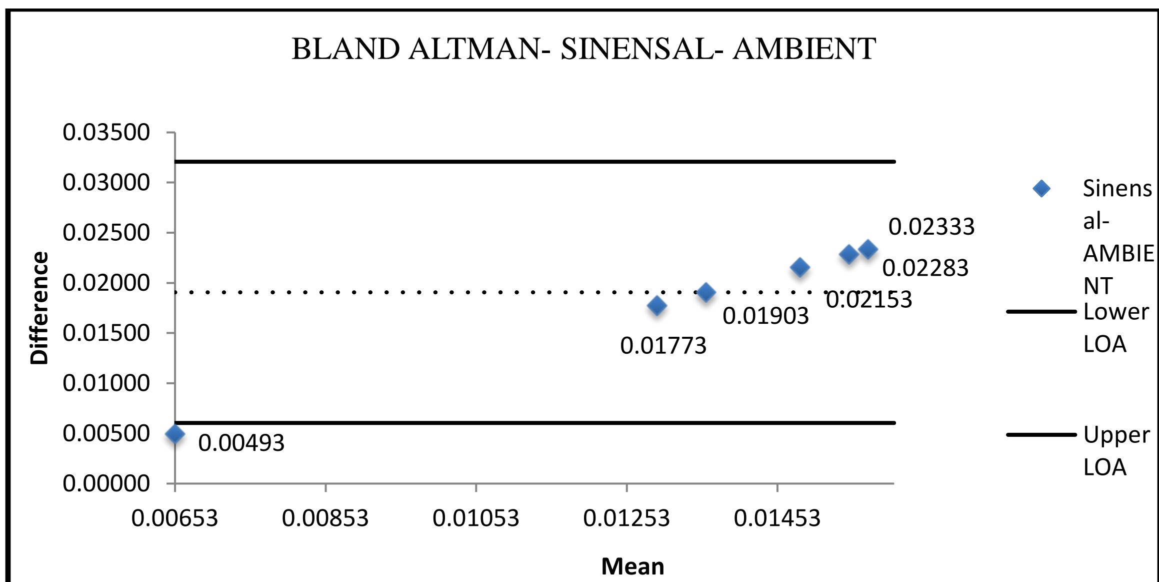


Figure 6-28: Bland Altman plot for sinensal under ambients stability conditions

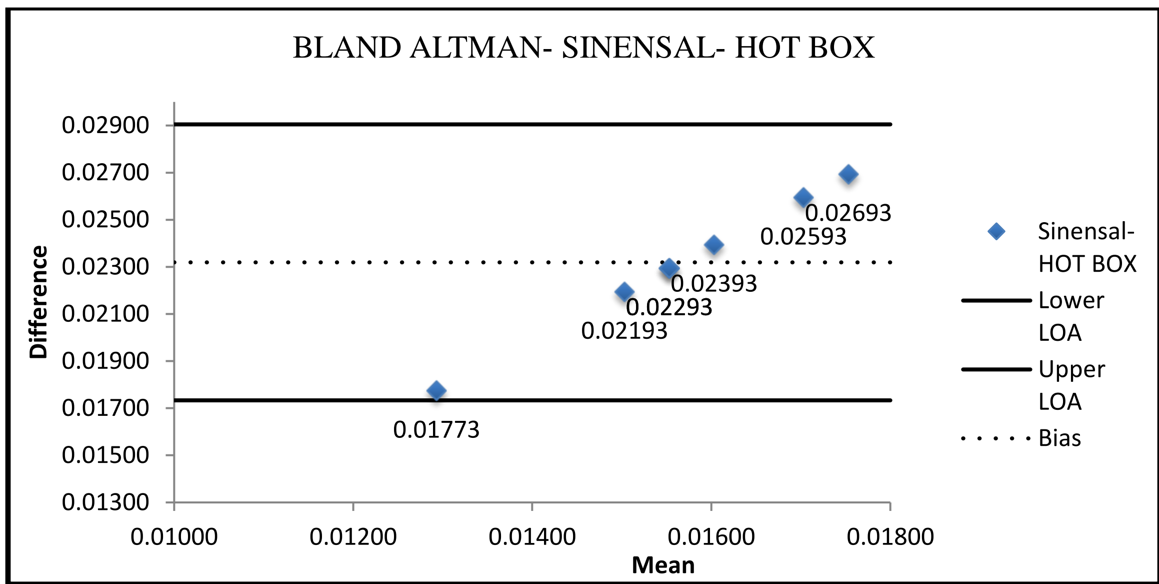


Figure 6-29: Bland Altman plot for sinensal under hotbox stability conditions

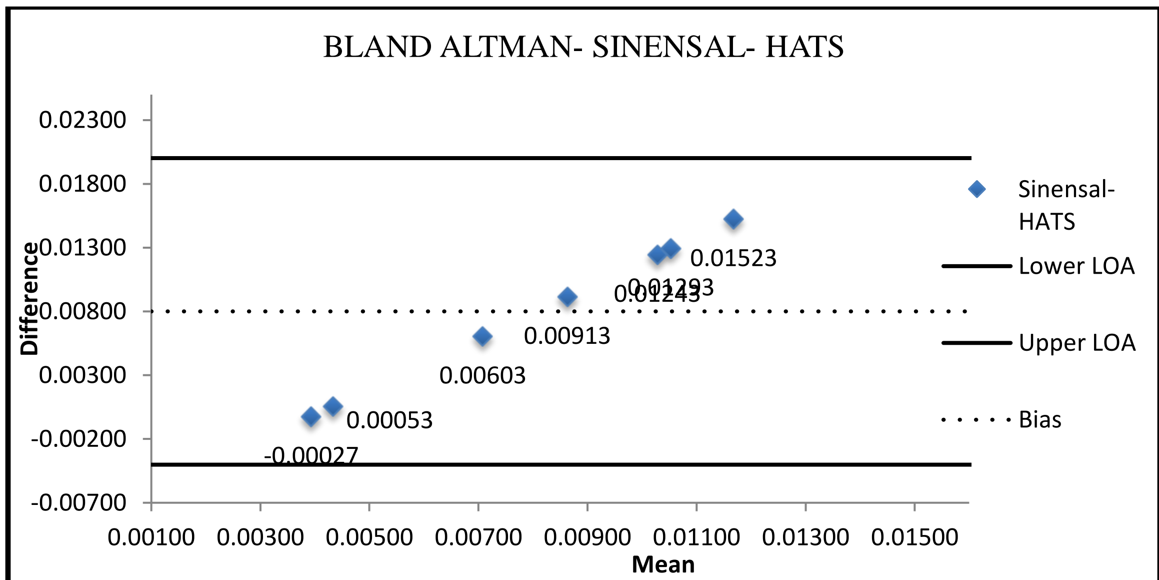


Figure 6-30: Bland Altman plot for sinensal under HATS stability conditions

6.4.8 Forced Degradation Study

In reconciling the mass balance of the mandarin oil components in each 24 week stability study, para cymene was a component that consistently fell out of the acceptable range of +/- 20% change. It is commonly known that para cymene has organoleptic properties described as pungent, bitter, and rancid [60]; these same organoleptic descriptors are also used, by flavorists and product developers, to describe mandarin oil as it ages with the addition of plasticky. It is for these reasons this specific component was analyzed further.

To investigate this change, para cymene was subjected to an accelerated degradation study in which aliquots of sample were jarred in amber bottles at 40°C for five and ten days then analyzed according to the validated GCMS/FID method. Overall the accelerated study showed a loss in para cymene as compared to the time zero sample. The hot box samples were then analyzed by a different individual on a different instrument with an OV-1 and a FFAP carbowax column installed. The results were analyzed in terms of area percent, and once again, the area percent of para cymene decreased under forced degradation conditions.

Interestingly, in this short study para cymene was found to degrade when it was subjected to ambient temperature and 40°C for ten days. With this validated method specifically, para cymene's area ratio to toluene went from 2.8919 at time zero down to 2.7965 after ten days in ambient conditions and down to 2.4664 after ten days at 40°C.

When the other method was applied for changes in area percent, para cymene at time zero was 98.073% of the total area and at day ten of 40℃, the area percent was 97.869%.

Contradictory, in each environment used in the stability study of the mandarin oil there was a rise in para cymene. The likely cause for the rise in para cymene in the oil itself is due to limonene and gamma terpinene degradation. What has been known is limonene and gamma terpinene were very large contributors to the oil and each have properties that cause it to degrade to para cymene [58, 60]. In comparison of the response factors of para cymene and limonene, limonene has a larger response factor than para cymene, 0.60 and 0.01 in the oil, respectively. This means that even the slightest degradation of limonene to para cymene is enough to cause noticeable change in the mandarin oil flavor profile. The reason for this being that small changes in para cymene representation increases the pungency of the oil and unfortunately stands out organoleptically to consumers and flavor chemists.

Asikainen reported in 2013 that gamma terpinene when exposed to molecular oxygen yielded para cymene when catalyzed with benzoquinone; however, if no catalyst was involved the autoxidation occurred over the course of a few weeks [58]. Our study times exceeded this, suggesting that this reaction involving gamma terpinene is highly likely; Figure 6-31 below [58]. Nguyen reported in 2012 the use of heat and a Vietnamese catalyst to generate para cymene from limonene [61]. The results shows that different levels of acidified Vietnamese montmorillonite and heat increased the para cymene yield from limonene; reaction in Figure 6-32 below [61]. Our study seems consistent with that of Nguyen. In the mandarin oil, over 90% limonene in composition

was subjected to the high acid tasting solution as one stability indicating procedure and a heated environment for the other. These conditions are similar to those in the controlled studies of Nguyen and Asikainen.

In summary, it is highly plausible that the increase in para cymene from limonene and gamma terpinene degradation reactions from exposure to heat or acid is the cause of the off noting in mandarin essential oil.

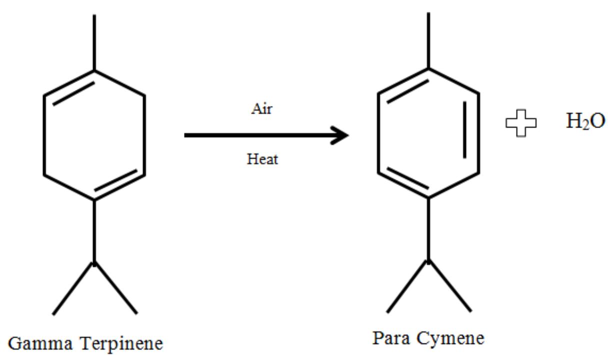
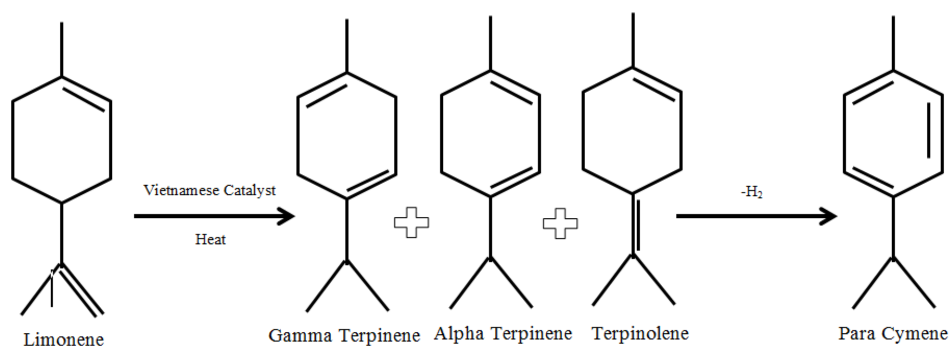


Figure 6-31: Reported reaction of gamma terpinene oxidation to para cymene [58]



*Excludes use of heavy solvents

Figure 6-32: Reported reaction of limonene to para cymene [61]

6.4.9 Sensory Evaluation

The flavorists who performed the sensory evaluations of the neat oils have experience ranging ten to 40 years in the industry. While the one flavorist is trained in differentiating among *Citrus* varieties, the other is trained in savory and seasoning solutions that have a different class of chemicals; for example spicy, vegetative, and meaty aroma chemicals much like those found on your dinner plate.

Each flavorist was given a fresh sampling of the mandarin oil as a reference; using reference samples is a common practice in the workplace, as it gives a benchmark for comparison against treated samples. Flavorists were given a blotter containing an oil sample from week 0, 12, and 24 of the three stability studies. When an unstressed sample

of mandarin oil, from week 0 for ambient and hot box studies, was beyond its shelf life but had been stored chilled in an amber bottle, both flavorists felt the sample was equivalent to a newly manufactured lot of mandarin based on its organoleptic character. The descriptors were that of a typical mandarin, citrus, peely, aldehydic, grape-like, and fishy. From the flavorist trained in *Citrus,* fishy notes were noticed immediately and dropped off by the end of the sequence in which HATS samples were evaluated. For the other flavorist, the fishy notes were only apparent when the sample was aged at 24 weeks in ambient conditions. Fishy notes are associated with amine compounds which were not a subject of interest but in future work should be added to the validated methodology.

The flavorist trained in *Citrus* noticed oxidation in all samples except the week 0 samples for all three studies where the savory flavorist picked up the oxidation at week 24 of the hot box study and weeks 12 and 24 of the HATs study. Oxidation was described as a reduction in the citrusy, juicy, fruity, character and a rise in the more potent terpenes that carry less desirable aromas, para cymene for example. Week 24 of the hot box and the later weeks of the HATs samples presented the para cymene pungent, terpene character for the tenured flavorist. In addition to oxidation, the other key change in the oil aroma was the presence of a plastic note, often associated with aged mandarin. Alas, the flavorist with roots in savory and 40 years in the business was able to smell that off note in the HATs week 24 sampling.

Interestingly, the savory flavorist had noticed a spike in spicy terpene notes in the early stages of the HATs study which was found to taper off by the end of the HATs study; the *Citrus* flavorist did not find this note in their evaluation. When asked if that

spicy terpene note was characteristic of a cinnamon, nutmeg spice or herbal, thyme spice the answer was “thyme”. This is important behavior to note because thymol, being the major thyme leaf oil monoterpene, is also an aroma active chemical in mandarin essential oils that separates it from sweet orange oils. This flavorist also called out the presence of caryophyllene notes in the time zero HATs sample which was a pleasant surprise since beta caryophyllene was another aroma active chemical in mandarin that was important to the unique flavor profile. Further descriptions of the evaluations from both flavorists are summarized in tables 6-63 and 6-64.

Table 6-63: Sensory evaluation of mandarin oil by flavorist trained in Citrus

Sample Name	Plastic Note	Oxidized Note	Overall Description	Chemical Indicators
Ambient/ Hot Box Week 0	No	No	fishy, grapey, citrus, peel, aldehydic end	MNMA, Amines
Ambient Week 12	No	Yes	less fresh, fishiness is stronger "old fish", powdered orange note	Amines
Ambient Week 24	No	Yes	rotting fish, low to no citrus character	Diaminohexane
Hot Box Week 12	No	Yes	powdery, very fishy, citrus peel is still apparent, powdery grape, less oxidized than Ambient 12	MNMA, Terpenes
Hot Box Week 24	No	Yes	powdery, less fish character, strong citrus/grape - not too different than week 12	MNMA, Terpenes
HATS Time zero	Yes	No	vegetative, celery character with underlying citrus	
HATS Week 12	Yes	Yes	oxidized orange not mandarin, powdery, aspirin, low vegetative character of time 0	
HATS Week 24	Yes	Yes	very mild citrus/powder. Plastic character is mild too, almost like "burnt plastic" but not overpowering b/c overall aroma is faded	Para Cymene

Table 6-64: Sensory evaluation of mandarin oil by flavorist trained in savory

Sample Name	Plastic Note	Oxidized Note	Overall Description	Chemical Indicators
Ambient/ Hot Box Week 0	No	No	Typical mandarin, balanced, nothing out of the ordinary	
Ambient Week 12	No	No	Authentic anthranilate note in mandarin has dissipated	MNMA
Ambient Week 24	No	No	Strong fishy character	Amines
Hot Box Week 12	No	No	Negligible change from Time 0	
Hot Box Week 24	No	Yes	Reduction of sweet/fruity/juicy notes; high terpene note indicative of para cymene; orange oil character	Para cymene
HATS Time zero	Yes	No	Spicy; caryophyllene notes; most drastic change thus far	Thymol, Beta Caryophllene
HATS Week 12	Yes	Yes	Slight oxidation; spicy terpene note fading; higher resemblance of orange than mandarin	Thymol
HATS Week 24	Yes	Yes	Terpeney but not spicy terpenes, no resemblance to mandarin at all; loss of fruity/juicy/sweet notes ; plastic note very high	Para Cymene, Sinensal

6.5 Conclusion

In summary, the simplified conclusion is that the analytical data for the three stability studies confirmed the oil was found to be statistically stable under the given conditions for 24 weeks presented by each compound data samplings remaining bound by the limits of agreement in the Bland Altman method. The sensory evaluation revealed the oil was experiencing significant change from its freshest form indicating it is not stable after 24 weeks. Unfortunately, this is the gap in the flavor industry currently; as humans our sense of smell and taste are far more sensitive to flavor and their changes than an analytical method is to date. As a result, flavor chemists mistrust analytical data and use the evaluations by their senses to understand when a problem with a material such as an essential oil is significant enough to reject for use. It is this challenge that analytical chemists in the flavor and fragrance industry face in the coming years of analytical method development.

The most popular tool on the rise in flavor and fragrance analysis is olfactory chromatographic methods because it is the best form of instrumentation to blend the sensorial perceptions with analytical chemistry, to date. Despite its popularity in literature for flavors and fragrance applications, it is not an industry standard approach to analysis due to its requirement of specialized evaluators. Future approaches to the current technologies include the need for increased sensitivity to the degree of change in a compound's behavior starting with lower levels of detection and quantitation. Finding an instrument that can detect 5-10x lower than the LOD and LOQ presented in this research

is probably a valuable starting point since applications are delivering flavors at sub 10 ppm levels.

Until the industry standard shifts towards olfactory methods, this research presented a tool that could better correlate standard analytical approaches with sensory data that can relieves decision making pressures from flavor chemists; this tool being the reconciliation of mass balance. In the presented stability data the mass balance reconciliation, Table 6-62, showed some disagreement with Bland Altman method, particularly for para cymene and thymol for each study, overall correlating well with the sensory results in Table 6-64. Para cymene increased over the course of each study, from 187 % increase to nearly 400 % increase, likely from the limonene and gamma terpinene degradation reactions. Thymol presented increases of 130-150 % amongst the three studies likely from the over production of para cymene. Based on this positive correlation demonstrated, the application of the mass balance for reconciliation has been identified to pass, flag for suspicion, or completely fail a raw material. Criterion includes passing materials within +/- 20% while flagging, at minimum, materials whose mass balance indicates change above or below the established criterion.

CHAPTER 7

APPLICATION OF *CITRUS RETICULATA* BLANCO

7.1 Introduction

Previously, *Citrus Reticulata* Blanco was applied to stability conditions for up to 24 total weeks, which is half of the essential oils recommended shelf life. It was learned that according to the Bland Altman analysis, the ten components studied did not have a significant change in their stability to warrant concern of the oil's flavor profile, analytically. This was purely a study on the analytical chemistry technique's ability to detect changes. It should be noted that despite the data points for each compound falling within the limits of agreement, there was an increasing trend in the Bland Altman plots of each molecule that hinted a negative change was still occurring. The mass balance reconciliation concurred that a change was occurring by presenting some changes which fell outside the reasonable boundaries for change, +/- 20%. The organoleptic assessment correlated closer to the mass balance reconciliation data than it did to the Bland Altman analysis in regard to the changes in the oil's flavor profile.

In this chapter the application of *Citrus Reticulata* Blanco to a commercial product was investigated to understand the changes *Citrus Reticulata* Blanco could undergo when applied to a manufactured product. The purpose was to demonstrate the validated method on a finished application containing the mandarin oil of interest. Herein, *Citrus Reticulata* Blanco in the commercial product will be referred to as "the product" or "the sample", interchangeably. The stability and sensorial appeal of a

flavored market product is the business objective for flavor companies. In this chapter we analyze a commercial sample that is recently produced, and therefore is at the beginning of its recommended shelf life, against a sample passed its recommended shelf life. Further, a forced degradation, as applied to para cymene, was conducted for five and ten days at 40 °C to observe the changes in the mandarin oil components when applied to a matrix containing other Generally Regarded As Safe (GRAS) chemicals.

7.2 Experimental Method

7.2.1 Reagents

Six milliliters of product containing Oil Mandarin Italian Select was obtained and toluene, the internal standard, was obtained from Sigma Aldrich.

7.2.2 Instrumentation

The GCMS/FID methodology validated in chapter 4 was applied to the sample in undiluted form. Toluene was once again used as the internal standard as was used in every sample applied to the GCMS/FID method. Libraries including Wiley10, NIST14, and FFNSC3 spectral databases were used to identify and confirm the peaks in the total ion chromatogram (TIC). Mass spectrometry data was useful for identifying molecules based on their fragmentation patterns. Base peak ion areas for each of the ten validated molecules were once again extracted and converted to a ratio against the area of the base peak ion in the internal standard.

7.2.3 Sample Preparation

A 1 mL aliquot of the sample was treated with 100 uL of toluene and otherwise injected as is. No extraction was necessary as all ingredients in the sample were GC friendly and non-interfering.

7.2.4 Forced Degradation

A half ounce amber bottle was filled with 5 mL of sample then placed in a hot box for five days before a 1 mL aliquot was prepared along with toluene to a GC vial for injection. The bottle was restored under the same conditions until day 10 when again another 1 mL aliquot was prepared with toluene for injection.

7.3 Results and Discussion

7.3.1. Expired versus Fresh Sample Mass Balance

In the analysis of the expired product, four key mandarin chemicals were not detected, gamma terpinene, thymol, methyl-N-methylanthranilate, and sinensal. Table 7-1 summarizes the compounds detected; an area of zero and an area ratio also of zero indicated these components were not detected. In the fresh product, two key marker compounds were also undetected; thymol and methyl-N-methylanthranilate, indicated by an area and area ratio of zero while gamma terpinene and sinensal were present. Table 7-2 summarizes the detected compounds. It is plausible the chemicals are not detected in the fresh product because their final concentration in the product is well below the LOD.

This is not uncommon in the flavor industry because minimal quantities of flavor chemicals are needed to provide a powerful tasting market product.

To evaluate the results of the ambient stability of product at the beginning and end of the shelf life, a reconciliation of mass balance was evaluated to assess the changes of a sample that may be attributed to thermal stability. The goal was to analytically demonstrate the discrepancies in fresh versus aged flavored products when stored in ambient conditions for the products entire shelf life. An area ratio was computed for all ten compounds of interest, using the method of internal standard; this ratio was then converted to a percentage. Using a percentage versus a ratio made assessing the component changes more straightforward. The criterion for rejection of a molecule's stability was a change outside +/- 20%. Compounds deviating beyond +/- 20% presented noteworthy behavior during the stability study. A boundary of +/- 20% change was set as the accuracy of the validated method was evaluated at +/- 20% of the working standard concentration. If boundaries were any larger the divide in analytical and sensory data would increase. Analytical data has been trailing the sensitivity of the human senses for decades, by increasing the range to +/- 30% would indicate a sample or a compound is passable far longer than it should be.

The mass balance of fresh sample to the expired sample presented alpha pinene and myrcene as the only components to maintain a level of stability that fell within +/- 20%; meanwhile every other mandarin compound that could be detected fell outside the acceptable window of change. Alpha pinene experienced a loss of 1% and myrcene experienced a loss of 12%. Table 7-3 summarizes these results. In further comparison of

the mass balances from the expired to fresh lots of product, they were not equivalent as one would expect based on the conservation of mass. The fresh sample had a total mass balance ratio of 3.60 and the expired sample was 2.74. Total sum of areas for all integrated peaks was taken as a ratio with the internal standard peak area in both cases. Total integration was chosen to account for all peaks in the chromatography that could have increased or decreased in addition to the ten chemicals of interest in this study. The expectation was to see equivalent ratios indicating the mass balance overall was equivalent from fresh to expired product, while only the levels of individual mandarin components were seeing degradation. Instead, the lower mass balance in the expired product indicates the degradation has decreased compound areas lower than the level of detection, or that the degradants formed are low/non-volatile in composition.

Table 7-1: FID results for expired product

Expired Sample	Toluene Base Peak Ion Area	Compound Base Peak Ion Area	Base peak Area Ratio
Alpha Pinene	1,046,255,550	10,256,384	0.0098
Beta Pinene	1,046,255,550	4,712,249	0.0045
Myrcene	1,046,255,550	14,437,678	0.0138
Para Cymene	1,046,255,550	657,264	0.0006
Gamma Terpinene	1,046,255,550	0	0.0000
Alpha Terpineol	1,046,255,550	809,679	0.0008
Thymol	1,046,255,550	0	0.0000
MNMA	1,046,255,550	0	0.0000
Beta Caryophyllene	1,046,255,550	237,201	0.0002
Sinensal	1,046,255,550	0	0.0000

Table 7-2: FID results for freshly manufactured product

Fresh Sample	Toluene Base Peak Ion Area	Compound Base Peak Ion Area	Base peak Area Ratio
Alpha Pinene	1,188,885,739	11,771,746	0.0099
Beta Pinene	1,188,885,739	7,296,638	0.0061
Myrcene	1,188,885,739	18,636,054	0.0157
Para Cymene	1,188,885,739	1,071,337	0.0009
Gamma Terpinene	1,188,885,739	1,562,962	0.0013
Alpha Terpineol	1,188,885,739	1,425,908	0.0012
Thymol	1,188,885,739	0	0.0000
MNMA	1,188,885,739	0	0.0000
Beta Caryophyllene	1,188,885,739	364,828	0.0003
Sinensal	1,188,885,739	585,729	0.0005

Table 7-3: Mass balance of fresh product versus expired product

Fresh vs. Expired Samples	Fresh Base Peak Area Ratio	Expired Base Peak Area Ratio	Ratio (%)	± 20%	
Alpha Pinene	0.0099	0.0098	99.0047	80-120	pass
Beta Pinene	0.0061	0.0045	73.3851	80-120	fail
Myrcene	0.0157	0.0138	88.0330	80-120	pass
Para Cymene	0.0009	0.0006	69.7134	80-120	fail
Gamma Terpinene	0.0013	0.0000	0.0000	80-120	fail
Alpha Terpineol	0.0012	0.0008	64.5244	80-120	fail
Thymol	0.0000	0.0000	0.0000	80-120	N/A
MNMA	0.0000	0.0000	0.0000	80-120	N/A
Beta Caryophyllene	0.0003	0.0002	73.8806	80-120	fail
Sinensal	0.0005	0.0000	0.0000	80-120	fail

7.3.2 Mass Balance Following Forced Degradation

In the sample stressed for five days at 40 ºC thymol and methyl-N-methylanthranilate went undetected as they were undetected in the fresh sample was well. While this appears inconsequential, it is confirmation that degradation of the other components in the product did not form thymol or methyl-N-methylanthranilate when exposed to elevated temperatures. Sinensal is the only compound that looked to have experience a decrease outside of the accepted criterion after undergoing stressed conditions. It is possible that the decreased resulted in an increase of the beta sinensal isomer that was not monitored by the method. Table 7-4 further summarizes the analysis.

As it pertains to the sample stressed for ten days at 40 ºC, gamma terpinene, thymol, methyl-N-methylanthranilate, and sinensal were undetected. The complete loss in those four compounds after ten days of exposure to a stressed environment resembled the analysis of the expired product. Para cymene was significantly higher in the ten-day sample than what was present in the expired product. This is an indication that the freshly made product stressed for ten days at 40 ºC is a harsher environment than 365 days or more at ambient temperatures. Table 7-5 further summarizes the analysis.

To further evaluate the thermal stability of the fresh lot of product a reconciliation of mass balance was evaluated. Mass balance calculations were performed to assess used to a fresh versus stressed sample for five and ten days in a hot box at 40 ºC. The intention of this study was to explain the discrepancies that arise in flavored products and their ingredients by analytically demonstrating the degradation of the ten marker

chemicals of mandarin when exposed to elevated temperatures for an extended period of time. The same procedure as used in 6.3.1 was followed using the same criterion for rejection of a molecule's stability. Again, compounds deviating beyond +/- 20% presented noteworthy behavior during the thermal stability study.

In the mass balance of the fresh sample to the sample stressed under elevated heat for five days, except for sinensal, all detectable compounds experienced minimal changes and therefore remained in the acceptable boundary of +/- 20%. The compounds were reconciled at 84-94% of their mass balance; moreover, a loss was seen but not significant enough for concern. Table 7-6 summarizes these results.

In the mass balance of the fresh sample compared to the sample that underwent ten days in the hot box, every detectable compound had significant enough change to be pushed outside the acceptable boundary of +/- 20%. Para cymene experienced a drastic change of 1050%, while myrcene degraded to only 4.5% left in the product.
Table 7-7 summarizes these results.

Table 7-4: FID results of fresh product after five days at 40 °C

Fresh Day 5 @ 40 °C	Toluene Base Peak Ion Area	Compound Base Peak Ion Area	Base peak Area Ratio
Alpha Pinene	1,342,609,068	11,143,916	0.0083
Beta Pinene	1,342,609,068	6,984,777	0.0052
Myrcene	1,342,609,068	17,884,342	0.0133
Para cymene	1,342,609,068	1,142,772	0.0009
Gamma Terpinene	1,342,609,068	1,598,406	0.0012
Alpha Terpineol	1,342,609,068	1,447,982	0.0011
Thymol	1,342,609,068	0	0.0000
MNMA	1,342,609,068	0	0.0000
Beta Caryophyllene	1,342,609,068	346,623	0.0003
Sinensal	1,342,609,068	471,904	0.0004

Table 7-5: FID results for fresh product after ten days at 40 °C

Fresh Day 10 @ 40 °C	Toluene Base Peak Ion Area	Compound Base Peak Ion Area	Base peak Area Ratio
Alpha Pinene	1,022,575,016	6,283,373	0.0061
Beta Pinene	1,022,575,016	3,945,373	0.0039
Myrcene	1,022,575,016	715,105	0.0007
Para Cymene	1,022,575,016	9,678,734	0.0095
Gamma Terpinene	1,022,575,016	0	0.0000
Alpha Terpineol	1,022,575,016	700,418	0.0007
Thymol	1,022,575,016	0	0.0000
MNMA	1,022,575,016	0	0.0000
Beta Caryophyllene	1,022,575,016	200,559	0.0002
Sinensal	1,022,575,016	0	0.0000

Table 7-6: Mass balance of fresh to five days hotbox product

Fresh vs. Stressed Samples	Fresh Base Peak Area Ratio	Day 5 Base Peak Area Ratio	Ratio (%)	± 20%	
Alpha Pinene	0.0099	0.0083	83.8277	80-120	pass
Beta Pinene	0.0061	0.0052	84.7657	80-120	pass
Myrcene	0.0157	0.0133	84.9786	80-120	pass
Para Cymene	0.0009	0.0009	94.4548	80-120	pass
Gamma Terpinene	0.0013	0.0012	90.5585	80-120	pass
Alpha Terpineol	0.0012	0.0011	89.9212	80-120	pass
Thymol	0.0000	0.0000	0.0000	80-120	N/A
MNMA	0.0000	0.0000	0.0000	80-120	N/A
Beta Caryophyllene	0.0003	0.0003	84.1317	80-120	pass
Sinensal	0.0005	0.0004	71.3424	80-120	fail

Table 7-7: Mass balance of fresh to ten-day hotbox product

Fresh vs. Stressed Samples	Fresh Base Peak Area Ratio	Day 10 Base Peak Area Ratio	Ratio (%)	± 20%	
Alpha Pinene	0.0099	0.0061	62.0579	80-120	fail
Beta Pinene	0.0061	0.0039	62.8652	80-120	fail
Myrcene	0.0157	0.0007	4.4613	80-120	fail
Para Cymene	0.0009	0.0095	1050.3581	80-120	fail
Gamma Terpinene	0.0013	0.0000	0.0000	80-120	fail
Alpha Terpineol	0.0012	0.0007	57.1098	80-120	fail
Thymol	0.0000	0.0000	0.0000	80-120	N/A
MNMA	0.0000	0.0000	0.0000	80-120	N/A
Beta Caryophyllene	0.0003	0.0002	63.9144	80-120	fail
Sinensal	0.0005	0.0000	0.0000	80-120	fail

7.4 Conclusion

At elevated temperatures the stability of each component is minimal and as such degradation is expected. In fact, it was demonstrated that thermal degradation over a few days can produce degradation much like a product that has expired. In further comparison of the mass balances from the expired to fresh lots of product, they were not equivalent as one would expect based on the conservation of mass. The findings suggest that there was degradation of compounds beyond the limit of detection and/or chemical changes occurred to materials that produced low/non-volatile degradants. Due to the complexity of the thermal degradents, the individual components could not be identified at this point in time due to the complexity of peaks in the chromatogram. Future research could point to this particular subject.

CHAPTER 8

CONCLUDING REMARKS

8.1 Summary and Concluding Remarks

Prior to the start of this research it was established mandarin oil can portray an off note described as a plastic pool toy. Common market products that develop this off note when mandarin oil is used in formulation are sodas and carbonated beverages. The cause of this plastic off note is infrequently mentioned in the current literature, it is for that reason a stability study on a mandarin essential oil sourced from *Citrus Reticulata* Blanco was performed. The goal of the stability study was to understand the effects temperature evokes on the mandarin oil when exposed for 24 weeks. To avoid the effects light could have on the study, all samples were kept in amber bottles. The temperatures chosen include ambient room temperature, which could not be controlled by the analyst and 40 °C in a hot box which was controlled. The hot box temperature was chosen based on what was slightly elevated to the room and was also a likely temperature scenario for a product in a warehouse, tracker trailer, or store shelf. Further, the oil was also exposed to high acid tasting solution (HATS) made of the combination of citric acid, sodium benzoate, water, and sugar for mocking the mandarin oil in a soda beverage. The HATS study was also performed at ambient temperature.

Upon successfully validating a GCMS/FID method against the ICH guidelines for accurately analyzing each sample from the shelf life study, the data had to be analyzed by a few different techniques to get a logical understanding of the chemical changes that occurred. The statistical approaches were employed to find an acceptable reporting style

of changes in marker chemicals of *Citrus* essential oil to find an appropriate reporting approach for analytical data that was conventional and easily understood by flavorists and other scientists in the industry. The evaluation of the stability study data were challenging due to the volatility of the molecules in the essential oil and the constant interconversions of the molecules amongst one another. The statistical results remained ambiguous in that it could not discriminate a change in the markers of the oil thereby failing the first hypothesis.

Trouble in the data analysis techniques explored resulted from the analytical expectation for which data of each marker chemical falling within two standard deviations of the mean met the 95% confidence interval and thus had insignificant changes during the stability studies. This is an analytical standard not a criterion established in this research. Despite being a standard expectation, the confidence of the statistical analysis methods was low because the sensitivity of the method revealed inconsistencies in the chromatographic peaks that when plotted for visual analysis were unpredictable. This discrepancy led to further researching for an appropriate method of capturing the chemical changes, including the involvement of sensory evaluation to understand if an aroma change was occurring or not.

The addition of evaluating the mandarin samples conducted by flavor chemists with strong sensory skill was to identify changes in the oil's aroma that were not being seen as statistically significant. Most important were plastic notes, notes indicative of oxidation, and overall changes to the characteristic mandarin aroma. The flavor chemists evaluated time zero, week 12, and week 24 samples from all three studies. Fish notes were detected as well as increases in pungent terpene notes, loss of juicy/fruity/sweet

notes, loss of grape notes, and sudden spike then decrease in the spicy character. The fishy nuances are coming from the amine compound class and were not a part of this research; however, future work could encompass these compounds for the characterization and further stability analysis of mandarin essential oils. This project was initially focused on the investigation of plastic notes not fish notes. With the addition of a sensory analysis on the samples it was again apparent the statistical methods were not sufficient to discriminate desirable samples from those needing further consideration.

Following the sensory and analytical evaluations of the 24-week stability study data, the best correlation between the chemical data and the sensory was that provided by the mass balance approach with a change criterion beginning at +/- 20%. The mass balance was computed from the differences observed between week 0 and week 24, Table 6-62. The data correlated quite well to the oil aroma evaluation, particularly by the flavor chemist holding 40 years of experience in the business, Table 6-64. The mass balance approach was not amongst the statistical treatments of the stability data originally investigated because for flavor and fragrance molecules, the smallest of changes in mass can have a drastic impact on the odor activity, while large changes in mass may appear as sensorially insignificant. Nevertheless, in comparing the mass balance with the sensory results, this study produced a new approach to assaying essential oils of the *Citrus* genus that can be executed as a screen or qualifying assay for usage. In the industry as it stands, the typical screen is the presence of impurities that remove a raw ingredient from its acceptable specification, but a correlation to the sensory effects of these out of specification materials is not established.

Using the mass balance approach with an acceptance criterion of +/- 20% allows for passing, flagging, or failing of a material. The +/- 20% criterion can be used to pass a material if the marker chemicals have not changed beyond this threshold without consulting a flavor chemist or conducting further testing. If a material has marker chemicals falling outside the threshold, the sample is deemed suspicious and should undergo further testing before it is applied to its intended formulation. This could be accomplished prior to use in flavor formulae as a preventative measure opposed to after. Currently, problematic raw ingredients such as essential oils are not identified until the formulation process is already under contract with a customer. In the cases where the material changes more than +/- 50% the sample would need to be rejected and a fresh lot should be qualified for the intended formulation. Having such a screening procedure like this in place in a production facility begins to bridge the gap between analytical and sensory assay. It relieves flavor chemists from troubleshooting all material issues and thereby allows analytical chemists to provide trustworthy results regarding flavor formulations in the industry that are currently not established due to the ambiguity of the common statistical approaches. To date, the lack of validated methods on these compounds prevents the quantitative discrimination by mass balance to be an accurate approach for qualifying flavor materials such as essential oils for use in flavor formulae. Due to this absence in the literature, this approach is not utilized on essential oils or flavor molecules in discerning quantitative stability changes; moreover, a justified foundation for its use in the flavor and fragrance industry has now been provided.

8.2 Future Studies

As the *Citrus* industry continues to boom, the methods studied within can be assessed against the other varieties or to earlier stages in the stability analysis. The approaches could also be applied to the amine compounds in the mandarin oil to correlate the sensory findings to the analytical results. Studies of new analytical methodologies could be applied to *Citrus* to better correlate data to sensory characteristics for the whole genus. A starting point could be the introduction of supercritical fluid chromatography for building up its use in the industry since volatile molecules are heavily used in flavors and fragrances. In addition, studies of the different processing methods of essential oils could be applied to understand the distribution of products in the same material extracted by different techniques. It would be interesting to correlate sensory analyses to the oils of different extraction methods to understand the distribution of aroma character and activity. Ultimately, with the findings here that mass balance correlated best to sensory data, this approach could be applied to a variety of flavor ingredients or flavor systems to understand its usability for different market products, different flavor ingredients, and different troubleshooting exercises that are encountered in the laboratories of manufacturing companies in this targeted industry.

REFERENCES CITED

1. Zoccali, M., Tranchida, P. Q., Bonaccorsi, I. L., Dugo, P., Mondello, L., & Dugo, G. (2016, 10). Detailed Profiling of the Volatile Oxygenated Fraction of Mandarin Essential Oils by Using the Off-Line Combination of High-Performance Liquid Chromatography and Comprehensive Two-Dimensional Gas Chromatography-Mass Spectrometry. *Food Analytical Methods, 10*(4), 1106-1116. doi:10.1007/s12161-016-0668-2
2. Turek, C., & Stintzing, F. C. (2013, 01). Stability of Essential Oils: A Review. *Comprehensive Reviews in Food Science and Food Safety, 12*(1), 40-53. doi:10.1111/1541-4337.12006
3. Turek, C., & Stintzing, F. C. (2012, 04). Impact of different storage conditions on the quality of selected essential oils. *Food Research International, 46*(1), 341-353. doi:10.1016/j.foodres.2011.12.028
4. Turek, C., & Stintzing, F. C. (2011, 04). Application of high-performance liquid chromatography diode array detection and mass spectrometry to the analysis of characteristic compounds in various essential oils. *Analytical and Bioanalytical Chemistry, 400*(9), 3109-3123. doi:10.1007/s00216-011-4976-5
5. Turek, C., & Stintzing, F. C. (2011, 11). Evaluation of Selected Quality Parameters to Monitor Essential Oil Alteration during Storage. *Journal of Food Science, 76*(9). doi:10.1111/j.1750-3841.2011.02416.x
6. Harris, D. C. (2010). *Quantitative chemical analysis*. Freeman.
7. Magni, F. (2004, 05). Book Reviews: HPLC of Peptides and Proteins: Methods and Protocols. By Marie-Isabel Aguilar (Editor). Proteomics, 4(5), 1517-1517. doi:10.1002/pmic.200490024

8. Wilson, C. W., & Shaw, P. E. (1981, 05). Importance of thymol, methyl N-methylanthranilate, and monoterpene hydrocarbons to the aroma and flavor of mandarin cold-pressed oils. *Journal of Agricultural and Food Chemistry, 29*(3), 494-496. doi:10.1021/jf00105a014

9. Huang, M., Valim, M. F., Feng, S., Reuss, L., Yao, L., Gmitter, F., & Wang, Y. (2017, 03). Characterization of the Major Aroma-Active Compounds in Peel Oil of an HLB-Tolerant Mandarin Hybrid Using Aroma Extraction Dilution Analysis and Gas Chromatography-Mass Spectrometry/Olfactometry. Chemosensory Perception. doi:10.1007/s12078-017-9221-y

10. Spreen, T. H., & Zansler, M. L. (2016, 12). Economic Analysis of Incentives to Plant Citrus Trees in Florida. HortTechnology, 26(6), 720-726. doi:10.21273/horttech03424-16

11. Halbert, S. E., & Manjunath, K. L. (2004, 09). Asian Citrus Psyllids (Sternorrhyncha: Psyllidae) And Greening Disease Of Citrus: A Literature Review And Assessment Of Risk In Florida. *Florida Entomologist, 87*(3), 330-353. doi:10.1653/0015-4040(2004)087

12. Zaid, A. N., Abualhasan, M. N., Jaradat, N., Marar, M., Mansoor, K., & Qa'Dan, F. (2016, 06). Development and validation of a new analytical HPLC method for the estimation of carvone in suppositories. *Journal of Pharmaceutical Investigation, 46*(6), 557-563. doi:10.1007/s40005-016-0264-x

13.Ferhat, M. A., Meklati, B. Y., & Chemat, F. (2007). Comparison of different isolation methods of essential oil from Citrus fruits: Cold pressing, hydrodistillation and microwave 'dry' distillation. Flavour and Fragrance Journal,22(6), 494-504. doi:10.1002/ffj.1829

14. *The essential oils: Volume one: History, origin in plants, production, analysis*. (1948). D. Van Nostrand Company.

15. Castro, M. L., Jiménez-Carmona, M., & Fernández-Pérez, V. (1999, 11). Towards more rational techniques for the isolation of valuable essential oils from plants. *TrAC Trends in Analytical Chemistry, 18*(11), 708-716. doi:10.1016/s0165-9936(99)00177-6

16. Hao Fan Dissertation from; Rutgers in Jan 2014

17. *The essential oils: Volume three: Individual Essential Oils of the Plant Families Rutaceae and Labiatae*. (1964). D. Van Nostrand Company.

18. Stratakos, A. C., & Koidis, A. (2015). Chapter 4 - Methods for Extracting Essential Oils. In *Essential Oils in Food Preservation, Flavor and Safety* (pp. 31–38). https://doi.org/http://dx.doi.org/10.1016/B978-0-12-416641-7.00095-X

19. Ameh, S. J., & Obodozie-Ofoegbu, O. (2016). Chapter 11 - essential oils as flavors in carbonated cola and citrus soft drinks. In V. R. Preedy (Ed.), *Essential oils in food preservation, flavor and safety* (pp. 111-121). San Diego: Academic Press.

20. Dugo, P., Bonaccorsi, I., Ragonese, C., Russo, M., Donato, P., Santi, L., & Mondello, L. (2010, 11). Analytical characterization of mandarin (Citrus deliciosa Ten.) essential oil. *Flavour and Fragrance Journal, 26*(1), 34-46. doi:10.1002/ffj.2014

21. Wright, John. *Flavor Creation*. Allured Pub. Corp., 2011.

22. Bové, J.M. (2006) Huanglongbing: A Destructive, Newly-Emerging, Century-Old Disease of Citrus[1]. *Journal of Plant Pathology,* 88(1), 7-37. doi:10.4454/jpp.v88i1.828

23. *The Citrus industry*. (1967). University of California.

24. Liu, Y., Heying, E., & Tanumihardjo, S. A. (2012, 10). History, Global Distribution, and Nutritional Importance of Citrus Fruits. *Comprehensive Reviews in Food Science and Food Safety, 11*(6), 530-545. doi:10.1111/j.1541-4337.2012.00201

25. Woods, L., & Doull, J. (1991, 08). GRAS evaluation of flavoring substances by the Expert Panel of FEMA. Regulatory Toxicology and Pharmacology, 14(1), 48-58. doi:10.1016/0273-2300(91)90051-v

26. Hall, R.H., & Oser, B.L. (1960). Recent progress in the consideration of flavoring ingredients under the Food Additives Amendment III. GRAS Substances. *Journal of the Institute of Food Technology,* 19(2), 151-197.

27. Reeve, D., & Arthur, D., (2002). Riding the citrus trails: when is a mandarin a tangerine? *Perfumer & Flavorist,* 27, 20-22 NO DOI

28.Danielski, L. (2008).Deterpenation of mandarin (Citrus reticulata) peel by means of countercurrent multistage extraction adsorption/desorption with supercritical CO.2 *Journal of Supercritical Fluids DOI: 10.1016/j.supflu.2007.09.007*

29. Elmaci, Y., & Altug, T. (2005, 04). Flavor Characterization Of Three Mandarin Cultivars (Satsuma, Bodrum, Clementine) By Using Gc/ms And Flavor Profile Analysis Techniques. *Journal of Food Quality, 28*(2), 163-170. doi:10.1111/j.1745-4557.2005.00009.x

30. Weerawatanakorn, M., Wu, J., Pan, M., & Ho, C. (2015, 06). Reactivity and stability of selected flavor compounds. *Journal of Food and Drug Analysis, 23*(2), 176-190. doi:10.1016/j.jfda.2015.02.001

31. Feng, S., Suh, J. H., Gmitter, F. G., & Wang, Y. (2017, 12). Differentiation between Flavors of Sweet Orange (Citrus sinensis) and Mandarin (Citrus reticulata). *Journal of Agricultural and Food Chemistry,66*(1), 203-211. doi:10.1021/acs.jafc.7b04968

32. Njoroge, S. M., Mungai, H. N., Koaze, H., Phi, N. T., & Sawamura, M. (2006, 11). Volatile Constituents of Mandarin (Citrus reticulata Blanco) Peel Oil from Burundi. *Journal of Essential Oil Research, 18*(6), 659-662. doi:10.1080/10412905.2006.9699197

33. ICH. (2005). *Guideline i. Validation of analytical procedures: Text and methodology q2(r1).*

34. Giavarina, D. (2015). Understanding Bland Altman analysis. *Biochemia Medica, 25*(2), 141-151. doi:10.11613/bm.2015.015

35. Myles, P., & Cui, J. (2007, 09). I. Using the Bland–Altman method to measure agreement with repeated measures. *British Journal of Anaesthesia, 99*(3), 309-311. doi:10.1093/bja/aem214

36. Almeida, A., Castel-Branco, M., & Falcão, A. (2002, 07). Linear regression for calibration lines revisited: Weighting schemes for bioanalytical methods. *Journal of Chromatography B, 774*(2), 215-222. doi:10.1016/s1570-0232(02)00244-1

37. Mansilha, C., Melo, A., Rebelo, H., Ferreira, I., Pinho, O., Domingues, V., . . . Gameiro, P. (2010, 10). Quantification of endocrine disruptors and pesticides in water by gas chromatography–tandem mass spectrometry. Method validation using weighted linear regression schemes. *Journal of Chromatography A, 1217*(43), 6681-6691. doi:10.1016/j.chroma.2010.05.005

38. Silva, C. P., Emídio, E. S., & Marchi, M. R. (2015, 01). Method validation using weighted linear regression models for quantification of UV filters in water samples. *Talanta,131*, 221-227. doi:10.1016/j.talanta.2014.07.041

39. *Statistics and Chemometrics for Analytical Chemistry*. (2010). Pearson Education.

40. National Academies of Sciences, Engineering, and Medicine. 2018. *A Review of the Citrus Greening Research and Development Efforts Supported by the Citrus Research and Development Foundation: Fighting a Ravaging Disease*. Washington, DC: The National Academies Press. doi: https://doi.org/10.17226/25026.

41. Shaw, P. E. (1979, 03). Review of quantitative analyses of citrus essential oils. *Journal of Agricultural and Food Chemistry, 27*(2), 246-257. doi:10.1021/jf60222a032

42. Marlet, C., & Lognay, G. (2010, 09). Development and validation by accuracy profile of a method for the analysis of monoterpenes in indoor air by active sampling and thermal desorption-gas chromatography-mass spectrometry. *Talanta, 82*(4), 1230-1239. doi:10.1016/j.talanta.2010.06.044

43. Mirhosseini, H., Salmah, Y., Nazimah, S., & Tan, C. (2007, 01). Solid-phase microextraction for headspace analysis of key volatile compounds in orange beverage emulsion. *Food Chemistry, 105*(4), 1659-1670. doi:10.1016/j.foodchem.2007.04.039

44. Coskun, O. (2016). Separation Techniques: CHROMATOGRAPHY. *Northern Clinics of Istanbul*. doi:10.14744/nci.2016.32757

45. Boček, P., & Janák, J. (1971, 12). Flame ionisation detection. *Chromatographic Reviews, 15*(2-3), 111-150. doi:10.1016/0009-5907(71)80016-9

46. Poole, C. F. (2015, 11). Ionization-based detectors for gas chromatography. *Journal of Chromatography A, 1421*, 137-153. doi:10.1016/j.chroma.2015.02.061

47. Hoffmann, E. D., & Stroobant, V. (2012). *Mass spectrometry: Principles and applications*. John Wiley & Sons.

48. Loudon, G. M. (2009). *Organic chemistry*. Roberts and Co. (pages 558-560)

49. Christina Helen Nilsen Chromatography of metabolites in plasma and urine following oral administration of anthocyanin rich capsules University of Stavanger Spring 2010 https://brage.bibsys.no/xmlui/bitstream/handle/11250/182430/Nilsen%2C%20Christina%20Helen.pdf?sequence=1

50. Flavor, Fragrance, Food and Cosmetics Ingredients information. (n.d.). Retrieved from http://www.thegoodscentscompany.com

51. Jones, S. (2, 2009) *Understanding Gas Chromatography* [PDF Format]. Agilent Technologies E-Seminar Retrieved from: https://www.agilent.com/cs/library/eseminars/public/Understanding%20Gas%20Chromatography-%20What%20is%20really%20going%20on%20inside%20the%20box.pdf

52. Champion, B. (12, 2009) *HPLC Separation Fundamentals* [PDF format]. Agilent Technologies E-Seminar Retrieved from: https://www.agilent.com/cs/library/slidepresentation/Public/HPLC%20Separation.pdf

53. IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997)doi:10.1351/goldbook.r05317**54.**

54. Srivastava, M. (2011). High-performance thin-layer chromatography (HPTCL). Springer.

55. Giddings, J. C. (1991). Unified separation science. New York: Wiley.

56. Dean, R. B., & Dixon, W. J. (1951, 04). Simplified Statistics for Small Numbers of Observations. *Analytical Chemistry, 23*(4), 636-638. doi:10.1021/ac60052a025

57. Rorabacher, D. B. (1991, 01). Statistical treatment for rejection of deviant values: Critical values of Dixon's "Q" parameter and related subrange ratios at the 95% confidence level. *Analytical Chemistry, 63*(2), 139-146. doi:10.1021/ac00002a010

58. Asikainen, M., Jauhiainen, O., Aaltonen, O., & Harlin, A. (2013). Continuous catalyst-free aromatization of γ-terpinene using air as an oxidant. *Green Chemistry*, *15*(11), 3230. doi:10.1039/c3gc41224e

59. Gu, H., Liu, G., Wang, J., Aubry, A., & Arnold, M. E. (2014, 09). Selecting the Correct Weighting Factors for Linear and Quadratic Calibration Curves with Least-Squares Regression Algorithm in Bioanalytical LC-MS/MS Assays and Impacts of Using Incorrect Weighting Factors on Curve Stability, Data Quality, and Assay Performance. *Analytical Chemistry,86*(18), 8959-8966. doi:10.1021/ac5018265

60. Marchese, A., Arciola, C., Barbieri, R., Silva, A., Nabavi, S., Sokeng, A. T., . . . Nabavi, S. (2017, 08). Update on Monoterpenes as Antimicrobial Agents: A Particular Focus on p-Cymene. *Materials,10*(8), 947. doi:10.3390/ma10080947

61. Nguyen, T-T. T., Duus, F., & Le, T. N. (2013). Solvent Free Preparation of p-Cymene from Limonene Using Vietnamese Montmorillonite. *Journal of Engineering Technology and Education*, *9*(5), 94-99.

62. Dugo, G., & Di_Giacomo, A. (2002). *Citrus: The genus citrus*. Taylor & Francis.

63. Mohamadi, M., Shamspur, T., & Mostafavi, A. (2013, 02). Comparison of microwave-assisted distillation and conventional hydrodistillation in the essential oil extraction of flowers Rosa damascena Mill. *Journal of Essential Oil Research, 25*(1), 55-61. doi:10.1080/10412905.2012.751555

64. Altman, D. G., & Bland, J. M. (2017, 06). Assessing Agreement between Methods of Measurement. *Clinical Chemistry, 63*(10), 1653-1654. doi:10.1373/clinchem.2016.268870

65. Altman, D. G., & Bland, J. M. (1983, 09). Measurement in Medicine: The Analysis of Method Comparison Studies. *The Statistician, 32*(3), 307. doi:10.2307/2987937

66. Bland, J. M., & Altman, D. G. (2010, 08). Statistical methods for assessing agreement between two methods of clinical measurement. *International Journal of Nursing Studies, 47*(8), 931-936. doi:10.1016/j.ijnurstu.2009.10.001

67. Westgard, J. O. (2008, 03). Use and Interpretation of Common Statistical Tests in Method Comparison Studies. *Clinical Chemistry, 54*(3), 612-612. doi:10.1373/clinchem.2007.094060

68. Dewitte, K., Fierens, C., Stockl, D., & Thienpont, L. M. (2002). Application of the bland-altman plot for interpretation of method-comparison studies: A critical investigation of its practice. [Letter]. *Clinical Chemistry, 48*(5), 799-801.

69. Marmulla, R., & Harder, J. (2014, 07). Microbial monoterpene transformations-a review. *Frontiers in Microbiology, 5*. doi:10.3389/fmicb.2014.00346

70. Vespermann, K. A., Paulino, B. N., Barcelos, M. C., Pessôa, M. G., Pastore, G. M., & Molina, G. (2017, 01). Biotransformation of α- and β-pinene into flavor compounds. *Applied Microbiology and Biotechnology, 101*(5), 1805-1817. doi:10.1007/s00253-016-8066-7

71. Ravichandran, C., Badgujar, P. C., Gundev, P., & Upadhyay, A. (2018, 10). Review of toxicological assessment of d-limonene, a food and cosmetics additive. Food and Chemical Toxicology, 120, 668-680. doi:10.1016/j.fct.2018.07.052

72. Sharifi-Rad, M., Varoni, E. M., Iriti, M., Martorell, M., Setzer, W. N., Contreras, M. D., . . . Sharifi-Rad, J. (2018, 05). Carvacrol and human health: A comprehensive review. Phytotherapy Research, 32(9), 1675-1687. doi:10.1002/ptr.6103

73. Dosoky, N., & Setzer, W. (2018, 07). Biological Activities and Safety of Citrus spp. Essential Oils. International Journal of Molecular Sciences, 19(7), 1966. doi:10.3390/ijms19071966

74. Chhikara, N., Kour, R., Jaglan, S., Gupta, P., Gat, Y., & Panghal, A. (2018). Citrus medica: Nutritional, phytochemical composition and health benefits – a review. Food & Function, 9(4), 1978-1992. doi:10.1039/c7fo02035j

75. Vieira, A., Beserra, F., Souza, M., Totti, B., & Rozza, A. (2018, 03). Limonene: Aroma of innovation in health and disease. Chemico-Biological Interactions, 283, 97-106. doi:10.1016/j.cbi.2018.02.007

76. Tranchida, P. Q., Franchina, F. A., & Mondello, L. (2017, 05). Analysis of essential oils through comprehensive two-dimensional gas chromatography: General utility. Flavour and Fragrance Journal, 32(4), 218-227. doi:10.1002/ffj.3383

77. Do, T. K., Hadji-Minaglou, F., Antoniotti, S., & Fernandez, X. (2015, 03). Authenticity of essential oils. TrAC Trends in Analytical Chemistry, 66, 146-157. doi:10.1016/j.trac.2014.10.007

78. Ren, J., Zhang, Y., Fan, G., Wang, M., Zhang, L., Yang, Z., & Pan, S. (2018, 03). Study on the optimization of the decolorization of orange essential oil. Food Science and Biotechnology, 27(4), 929-938. doi:10.1007/s10068-018-0354-9

79. Xiao, Z., Wu, Q., Niu, Y., Wu, M., Zhu, J., Zhou, X., . . . Kong, J. (2017, 09). Characterization of the Key Aroma Compounds in Five Varieties of Mandarins by Gas

Chromatography–Olfactometry, Odor Activity Values, Aroma Recombination, and Omission Analysis. Journal of Agricultural and Food Chemistry, 65(38), 8392-8401. doi:10.1021/acs.jafc.7b02703

80. Zoccali, M., Bonaccorsi, I. L., Tranchida, P. Q., Dugo, P., Mondello, L., & Dugo, G. (2015, 07). Analysis of the sesquiterpene fraction of citrus essential oils by using the off-line combination of high performance liquid chromatography and gas chromatography-based methods: A comparative study. Flavour and Fragrance Journal, 30(6), 411-422. doi:10.1002/ffj.3262

81. Tranchida, P. Q., Zoccali, M., Franchina, F. A., Bonaccorsi, I., Dugo, P., & Mondello, L. (2013, 01). Fast gas chromatography combined with a high-speed triple quadrupole mass spectrometer for the analysis of unknown and target citrus essential oil volatiles. Journal of Separation Science, 36(3), 511-516. doi:10.1002/jssc.201200742

82. Paolini, M., Tonidandel, L., Moser, S., & Larcher, R. (2018, 08). Development of a fast gas chromatography-tandem mass spectrometry method for volatile aromatic compound analysis in oenological products. Journal of Mass Spectrometry, 53(9), 801-810. doi:10.1002/jms.4259

83. Jemmali, Z., Chartier, A., & Elfakir, C. (2016, 04). Development of a gas chromatography–mass spectrometry method to monitor in a single run, mono- to triterpenoid compounds distribution in resinous plant materials. Journal of Chromatography A, 1443, 241-253. doi:10.1016/j.chroma.2016.03.031

84. Correa, E., Quiñones, W., & Echeverri, F. (2015, 10). Methyl-N-methylanthranilate, a pungent compound from *Citrus Reticulata* Blanco leaves. Pharmaceutical Biology, 54(4), 569-571. doi:10.3109/13880209.2015.1044618

85. Jerkovic, I., Jerković, J., Drulžić, Z., Marijanović, M., Gugić, S., Jokić, & Roje. (n.d.). GC-FID/MS Profiling of Supercritical CO2 Extracts of Peels from Citrus aurantium, C. sinensis cv. Washington navel, C. sinensis cv. Tarocco and C. sinensis cv. Doppio Sanguigno from Dubrovnik Area (Croatia). *NATURAL PRODUCT COMMUNICATIONS, 10*(7), 1315-1318.

86. Silvestre, W., Agostini, F., Muniz, L., & Pauletti, G. (2016, 06). Fractionating of green mandarin (Citrus deliciosa Tenore) essential oil by vacuum fractional distillation. Journal of Food Engineering, 178, 90-94. doi:10.1016/j.jfoodeng.2016.01.011

87. Umnahanant, P., Zafar, A., Kankala, V., & Chickos, J. (2019, 04). Vapor pressure and vaporization enthalpy studies of ( )-longifolene, (−)-isolongifolene and β-myrcene by correlation gas chromatography. The Journal of Chemical Thermodynamics, 131, 583-591. doi:10.1016/j.jct.2018.12.013

88. Behr, A., & Johnen, L. (2009, 12). Myrcene as a Natural Base Chemical in Sustainable Chemistry: A Critical Review. ChemSusChem,2(12), 1072-1095. doi:10.1002/cssc.200900186

89. Krings, U., & Berger, R. (n.d.). Terpene bioconversion--how does its future look? *NATURAL PRODUCT COMMUNICATIONS, 5*(9), 1507-1522.

90. Oliveira, E. C., Muller, E. I., Abad, F., Dallarosa, J., & Adriano, C. (2010). Internal standard versus external standard calibration: An uncertainty case study of a liquid chromatography analysis. *Química Nova, 33*(4), 984-987. doi:10.1590/s0100-40422010000400041

91. Diana F.J. (2009) Method Validation and Transfer. In: Huynh-Ba K. (eds) Handbook of Stability Testing in Pharmaceutical Development. Springer, New York, NY

92. Aschmann, S. M., Arey, J., & Atkinson, R. (2011, 08). Formation of p-cymene from OH γ-terpinene: H-atom abstraction from the cyclohexadiene ring structure. Atmospheric Environment, 45(26), 4408-4411. doi:10.1016/j.atmosenv.2011.05.04

93. Poulose, A., & Croteau, R. (1978, 04). Biosynthesis of aromatic monoterpenes. Archives of Biochemistry and Biophysics,187(2), 307-314. doi:10.1016/0003-9861(78)90039-5

94. Peris-Vicente, J., Esteve-Romero, J., & Carda-Broch, S. (2015, 11). Validation of Analytical Methods Based on Chromatographic Techniques: An Overview. Analytical Separation Science,1757-1808. doi:10.1002/9783527678129.assep0

95. Hartsel, J. A., Eades, J., Hickory, B., & Makriyannis, A. (2016). Cannabis sativa and Hemp. Nutraceuticals, 735-754. doi:10.1016/b978-0-12-802147-7.00053-x

96. Khaleel, C., Tabanca, N., & Buchbauer, G. (2018, 04). α-Terpineol, a natural monoterpene: A review of its biological properties. Open Chemistry, 16(1), 349-361. doi:10.1515/chem-2018-0040

97. Lan-Phi, N. T. (2010, 10). Gas Chromatography-Olfactometry and Aromaactive Components in Citrus Essential Oils. Citrus Essential Oils, 201-227. doi:10.1002/9780470613160.ch5

98. Gratien, A., Johnson, S. N., Ezell, M. J., Dawson, M. L., Bennett, R., & Finlayson-Pitts, B. J. (2011, 04). Surprising Formation ofp-Cymene in the Oxidation of α-Pinene in Air by the Atmospheric Oxidants OH, O3, and NO3. *Environmental Science & Technology, 45*(7), 2755-2760. doi:10.1021/es103632b

99. Utami, Herti & Budiman, Arief & Sutijan, Sutijan & Roto, Roto & Sediawan, Wahyudi. (2010). Synthesis of -Terpineol from Turpentine by Hydration in a Batch Reactor.