IDENTIFICATION OF VOLATILE COMPOUNDS CONTRIBUTING TO PENNYCRESS AROMA

A THESIS
SUBMITTED TO THE FACULTY OF UNIVERSITY OF MINNESOTA
BY

PEISHAN LUO

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DR. GARY A. REINECCIUS, ADVISOR

JANUARY, 2020
ACKNOWLEDGEMENTS

There are many people to whom I wish to express my sincere gratitude and appreciation during this work. Life is not easy, but it becomes easier when getting help and encouragement from others.

Foremost, I would like to express my deepest appreciation to my advisor, Dr. Gary A. Reineccius. I am very grateful for the privilege to have Gary as my advisor, not only in my research and study, but also in my life and career. His support, encouragement, wisdom, kindness and generosity have always influenced me positively, helping me release my fear of the unknown.

My appreciation also extends to my laboratory colleagues in the flavor lab. Thanks to Vaidhyanathan Anantharamkrishnan, who provided me with every bit of guidance, assistance and support that I needed in my work. Thanks to Yara Benavides Paz, who served as my sensory panelist and provided me with useful advice in my research. Thanks to Katie Enzenauer and Bruna Barbon Paulo for their friendship and encouragement throughout my time in the lab as well as in my daily life. I also need to thank Jean-Paul Schirle-Keller, who helped me solve any analytical problems that I faced, making the experiments going well.

I would also like to thank my defense committee members Dr. George A. Annor and Dr. Leonard F. Marquart for their value time and helpful guidance. Besides, I have to thank my former advisor Dr. Daniel J. O'Sullivan for helping me join the Food Science M.S. program here.
Special thanks to Dr. David Marks and Ratan Chopra (Department of Plant and Microbial Biology), who shared their abundant knowledge and expertise on pennycress, and also provided pennycress sample for this research. My special thanks also expressed to Julie Kirihara (Center for Mass Spectrometry and Proteomics) for her patience, kindness and help in the mass spectrometry center.

Most importantly, I owe this accomplishment to the endless love from my parents and my elder brother. I cannot image how can I reach this point without their support.
DEDICATION

This thesis is dedicated to my parents for always supporting me and encouraging me to do what I want to do. They gave me the courage to overcome the fear of “from having everything to having nothing” in a new environment. Thanks for them giving me the wings to fly further and higher.

I also dedicated this thesis to my elder brother who always tries his best to protect me like a shield whenever problems try to reach me. Without my brother, I won’t be the person I am today.
ABSTRACT

Pennycress (*Thlaspi arvense* L.) is an extremely cold-tolerant oilseed in the mustard family. Pennycress has an unpleasant mustard-like aroma, which limits its application in the food industry. However, no publications have documented the determination of volatile compounds in pennycress.

To identify volatile compounds that contribute to pennycress aroma, wild-type pennycress seeds were evaluated using solvent assisted flavor evaporation (SAFE), combined with gas chromatography-olfactometry (GC-O) and aroma extraction dilution analysis (AEDA). In this research, twenty-nine aroma-active compounds were perceived by three panelists. With the aid of gas chromatography–mass spectrometry (GC-MS), retention indices, aroma descriptors and standard chemicals verification, ten volatile compounds were identified: 2,5/-2,6-dimethyl-3-methoxypyrazine (grassy, FD 4,000), allyl isothiocyanate (onion-like, FD 50), hexanal (green, FD 10), (E)-2-octenal (earthy, FD 100), acetic acid (sour, FD 100), (E)-2-nonenal (woody, FD 50), 1-octanol (grassy, FD 10), 1-nonanol (green, FD 50), (R)-2-methylbutanoic acid/3-methylbutanoic acid (cheesy, FD 100) and phenethyl alcohol (rose-like, FD 50).

Beyond the compounds absolutely identified, a number of additional volatile compounds were tentatively identified. By matching odor descriptions, mass spectra and retention index, 7 volatile compounds were tentatively identified: 1-pentanol, octanal, (E)-2-penten-1-ol, (E)-2-heptenal, (Z)-3-hexen-1-ol, (E, E)-3,5-octadien-2-one and pentanoic acid. All these tentatively identified compounds have been found in Brassicaceae family.
This research provided a preliminary analysis of pennycress aroma profile. The identified odor-active compounds might attract general attention to compounds and metabolic pathways that haven’t been greatly noticed in previous pennycress studies. So, the results in this research propose a direction for further pennycress analysis, from the prospect of aroma profile and olfactory perception, which provides more clues for breeding programs or relative research groups to pointedly design processes, minimizing the undesirable aroma.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... I
DEDICATION ................................................................................................................................. III
ABSTRACT ........................................................................................................................................ IV
TABLE OF CONTENTS ................................................................................................................... VI
LIST OF TABLES ............................................................................................................................. VIII
LIST OF FIGURES ........................................................................................................................ IX
LIST OF ABBREVIATIONS ........................................................................................................... XI

## CHAPTER 1. INTRODUCTION .................................................................................................. 1
  1.1 BACKGROUND ......................................................................................................................... 1
    1.1.1 Food Flavor ......................................................................................................................... 1
    1.1.2 Flavor Analysis ................................................................................................................... 2
    1.1.3 Pennycress .......................................................................................................................... 3
  1.2 RESEARCH OBJECTIVE .......................................................................................................... 4

## CHAPTER 2. LITERATURE REVIEW ....................................................................................... 5
  2.1 PENNYCRESS ......................................................................................................................... 5
    2.1.1 Overview of Pennycress ...................................................................................................... 5
    2.1.2 Current Application of Pennycress ..................................................................................... 6
    2.1.3 Commercialization Problems of Pennycress ..................................................................... 8
    2.1.4 Sources of Pennycress Flavor ............................................................................................ 9
    2.1.5 Palatability of Pennycress ................................................................................................ 14
    2.1.6 Volatile Compounds in Brassicaceae Family ................................................................... 15
  2.2 VOLATILES ANALYSIS ......................................................................................................... 19
    2.2.1 Volatile Compounds .......................................................................................................... 19
      2.2.1.1 Source of Volatile Compounds in Foods .................................................................... 20
      2.2.1.2 Volatile Compounds and Olfactometry Perception ..................................................... 21
    2.2.2 Gas Chromatography ....................................................................................................... 23
      2.2.2.1 Gas Chromatography Detectors .............................................................................. 24
      2.2.2.2 Gas Chromatography-Mass Spectrometry (GC-MS) ................................................ 25
    2.2.3 Gas Chromatography-Olfactometry (GC-O) .................................................................. 27
  2.3 VOLATILES EXTRACTION .................................................................................................... 31
    2.3.1 Solvent Extraction ............................................................................................................. 32
    2.3.2 Solvent Assisted Flavor Evaporation (SAFE) .................................................................. 32
    2.3.3 Stir Bar Sorptive Extraction (SBSE) .................................................................................. 35

## CHAPTER 3. MATERIALS AND METHODS ............................................................................. 37
  3.1 OVERVIEW OF EXPERIMENTAL FLOW .......................................................................... 37
  3.2 MATERIALS ............................................................................................................................ 37
3.2.1 Pennycress ................................................................. 37
3.2.2 Chemicals ............................................................... 38
3.3 METHODS ................................................................. 38
3.3.1 Isolation and Extraction of Volatile Compounds ................. 38
3.3.1.1 Solvent Assisted Flavor Evaporation (SAFE) ............... 39
3.3.1.2 Stir Bar Sorptive Extraction (SBSE) ......................... 41
3.3.2 Aroma Extract Dilution Analysis (AEDA) ....................... 41
3.3.3 Separation and Detection of Volatile Compounds ............... 42
3.3.3.1 Gas Chromatography-Flame Ionization Detection-Olfactometry (GC-FID-O) ........................................ 42
3.3.3.2 Gas Chromatography-Mass Spectrometry-Olfactometry (GC-MS-O) .... 43
3.3.3.3 Gas Chromatography-Mass Spectrometry (GC-MS) .......... 43
3.3.3.4 Gas Chromatography-Flame Ionization Detector (GC-FID) ..... 44
3.3.4 Identification of Volatile Compounds .......................... 44

CHAPTER 4. RESULTS AND DISCUSSION ................................ 46
4.1 GAS CHROMATOGRAPHY-OLFACTOMETRY ANALYSIS .......... 46
4.1.1 Identification of Volatile Compounds in Pennycress .......... 48
4.1.2 Analysis of Odor-Active Volatile Compounds .................. 56
4.1.2.1 Allyl isothiocyanate (AITC) ....................................... 57
4.1.2.2 2,5-/ 2,6-Dimethyl-3-methoxypyrazine ..................... 64
4.1.2.3 Fatty Acid-Derived Volatiles ................................. 71
4.1.2.4 Acids ................................................................. 76
4.1.2.5 Pleasant Odorant .................................................... 83
4.2 FUTURE WORK ........................................................... 84

CHAPTER 5. CONCLUSION ....................................................... 87

BIBLIOGRAPHY .................................................................. 89

APPENDICES .................................................................... 104

APPENDIX 1. OTHER VOLATILE COMPOUNDS TENTATIVELY IDENTIFIED IN PENNYCRESS VOLATILES EXTRACT THROUGH MASS SPECTRUM AND RETENTION INDEX .......................... 104
APPENDIX 2. GAS CHROMATOGRAM OF SAFE VOLATILES EXTRACT (WITH C6-C25 ALKANES ADDED) FROM PENNYCRESS OIL (TOP) AND PENNYCRESS SEEDS (BOTTOM) BY GC-MS ON DB-WAX COLUMN .................................................... 107
LIST OF TABLES

Table 1. Sinigrin hydrolysis products and their occurrence in plants......................... 19
Table 2. Volatile compounds identified in pennycress volatiles extract. ....................... 53
Table 3. Tentatively identified volatile compounds in pennycress volatiles extract. ....... 55
LIST OF FIGURES

Figure 1. The general structure of glucosinolates.............................................................. 10
Figure 2. The pathway of glucosinolates enzymatic degradation products..................... 11
Figure 3. The pathway for the biosynthesis of glucosinolates........................................... 12
Figure 4. The structure of sinigrin. ...................................................................................... 13
Figure 5. Structures of the main glucosinolates identified in Brassicaceae....................... 16
Figure 6. Formation of volatile compounds from the hydrolysis of sinigrin in cruciferous plants................................................................. 18
Figure 7. Simplified diagram of gas chromatography - mass spectrometry (GC-MS). .... 27
Figure 8. Gas chromatography-olfactometry (GC-O). ......................................................... 29
Figure 9. Solvent assisted flavor evaporation (SAFE) apparatus........................................ 33
Figure 10. Simplified diagram of (A) stir bar sorptive extraction (SBSE) and (B) solid phase micro extraction (SPME). ................................................................. 36
Figure 11. Experimental work flow .................................................................................... 37
Figure 12. Illustration of solvent assisted flavor evaporation (SAFE) method. ............... 40
Figure 13. Diluted sample for aroma extract dilution analysis (AEDA). ............................ 42
Figure 14. Gas chromatogram of pennycress volatiles extract from SAFE by GC-FID-O on a DB-WAX column. ................................................................. 47
Figure 15. Gas chromatogram of pennycress volatiles extract from SAFE by GC-FID on a HP-5MS column................................................................. 47
Figure 16. Combined aromagram results of pennycress volatiles extract on DB-wax column for three panelists................................................................. 48
Figure 17. Total ion chromatogram of pennycress volatiles extract from SAFE by GC-MS on Stabilwax column................................................................. 49
Figure 18. Total ion chromatograph of pennycress volatiles extract from SAFE by GC-MS-O on HP-5MS column................................................................. 50
Figure 19. Gas chromatogram (top) and aromagram by three panelists (bottom) of pennycress volatiles extract on DB-WAX column................................................................. 51
Figure 20. Structures of 10 identified odorants in wild-type pennycress aroma extract. 57
Figure 21. GC-MS mass spectrum of allyl isothiocyanate (AITC) (no.7).......................... 58
Figure 22. HPLC analysis of desulfated glucosinolates from wild-type (top) and the Nutty mutant (bottom) pennycress ................................................................. 61
Figure 23. Terminal biosynthetic pathway of sinigrin........................................................ 62
Figure 24. GC-MS mass spectrum of 2,5-dimethyl-3-methoxypyrazine (no.10)............ 65
Figure 25. GC-MS mass spectrum of 2,6-dimethyl-3-methoxypyrazine (no.10)............ 65
Figure 26. The comparison of mass spectrum between no.10 odorant (middle), 2,5-diemethyl-3-methoxy pyrazine (upper) and 2,6-diemethyl-3-methoxy pyrazine (bottom). 67
Figure 27. GC-MS mass spectrum of hexanal (no.1)......................................................... 72
Figure 28. GC-MS mass spectrum of (E)-2-octenal (no.9).............................................. 73
Figure 29. GC-MS mass spectrum of (E)-2-nonenal (no.14)........................................... 73
Figure 30. GC-MS mass spectrum of 1-octanol (no.16)................................................. 75
Figure 31. GC-MS mass spectrum of 1-nonanal (no.18).................................................. 75
Figure 32. GC-MS mass spectrum of 3-methylbutanoic acid (no. 19)............................ 77
Figure 33. GC-MS mass spectrum of 2-methylbutanoic acid (no. 19).......................... 77
Figure 34. The two enantiomers of 2-methylbutanoic acid..................................... 79
Figure 35. GC-MS mass spectrum of acetic acid (no. 11)....................................... 81
Figure 36. GC-MS mass spectrum of phenethyl alcohol (no. 25)............................ 83
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAB</td>
<td>Acetic acid bacteria</td>
</tr>
<tr>
<td>AC</td>
<td>Allyl cyanide</td>
</tr>
<tr>
<td>AEDA</td>
<td>Aroma extract dilution analysis</td>
</tr>
<tr>
<td>AITC</td>
<td>Allyl isothiocyanate</td>
</tr>
<tr>
<td>ASTM</td>
<td>American Society for Testing and Materials</td>
</tr>
<tr>
<td>ATC</td>
<td>Allyl thiocyanate</td>
</tr>
<tr>
<td>CETP</td>
<td>1-Cyano-2,3-epithiopropane</td>
</tr>
<tr>
<td>CHARM</td>
<td>Combined hedonic aroma response measurement</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron capture detector</td>
</tr>
<tr>
<td>EI</td>
<td>Electron impact</td>
</tr>
<tr>
<td>FD</td>
<td>Flavor dilution</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detector</td>
</tr>
<tr>
<td>FPD</td>
<td>Flame photometric detector</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC-FID</td>
<td>Gas chromatography-flame ionization detector</td>
</tr>
<tr>
<td>GC-FID-O</td>
<td>Gas chromatography-flame ionization detector-olfactometry</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GC-MS-O</td>
<td>Gas chromatography-mass spectrometry-olfactometry</td>
</tr>
<tr>
<td>GC-O</td>
<td>Gas chromatography-olfactometry</td>
</tr>
<tr>
<td>HPO</td>
<td>Hydroperoxide</td>
</tr>
<tr>
<td>HPOL</td>
<td>Hydroperoxide lyase</td>
</tr>
<tr>
<td>HS</td>
<td>Headspace</td>
</tr>
<tr>
<td>KI</td>
<td>Kovats index</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipoxygenases</td>
</tr>
<tr>
<td>LRIs</td>
<td>Linear retention indices</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>OAVs</td>
<td>Odor activity values</td>
</tr>
<tr>
<td>P&amp;T</td>
<td>Purge and trap</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PID</td>
<td>Photo ionization detector</td>
</tr>
<tr>
<td>RI</td>
<td>Retention index</td>
</tr>
<tr>
<td>RT</td>
<td>Retention time</td>
</tr>
<tr>
<td>SAFE</td>
<td>Solvent assisted flavor evaporation</td>
</tr>
<tr>
<td>SBSE</td>
<td>Stir bar soprtive extraction</td>
</tr>
<tr>
<td>SDE</td>
<td>Simultaneous distillation extraction</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid phase micro extraction</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TCD</td>
<td>Thermal conductivity detector</td>
</tr>
<tr>
<td>TD</td>
<td>Thermal desorption</td>
</tr>
<tr>
<td>TGSC</td>
<td>The good scents company</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion chromatogram</td>
</tr>
<tr>
<td>USDA</td>
<td>U.S. Department of Agriculture</td>
</tr>
<tr>
<td>VOCs</td>
<td>Volatile organic compounds</td>
</tr>
</tbody>
</table>
CHAPTER 1. INTRODUCTION

1.1 BACKGROUND

In today’s food market, the hottest trend is very clear: plant-based food products. According to the newest data released by the Plant Based Foods Association and The Good Food Institute on July 2019 (Simon, 2019), the total U.S. plant-based food market reached a value of $4.5 billion. Plant-based food market includes diverse plant-based alternatives, like plant-based milk, meat, cheese, condiments, dressings categories. Comparatively, the growth of the plant-based food market is tremendous: that five times faster than total U.S. retail food sales in the past year. As the interest in plant-based food is increasing, more and more novel plant materials are being investigated for introducing as food ingredients. While the introduction of new plant materials enriches the variety of plant-based food products, it might also limit consumer acceptance of these new products due to flavor issues. Pennycress has great potential for use in food product development, because of its high content of protein and fat as well as its low cost of planting (Selling et al., 2013). However, pennycress has an undesirable flavor (Vaughn, Isbell, Weisleder, & Berhow, 2005), and thus its application in food products will be largely limited if the flavor problem cannot be solved. To address the flavor problem of pennycress, the analysis of pennycress flavor is necessary.

1.1.1 Food Flavor

Food flavor is a complex sensation impacted by various sensory stimuli, in which taste and smell play major roles (Laing & Jinks, 1996). Taste is perceived on the tongue and mouth, related to non-volatile components in the food (Williams, 1974), whereas
smell is derived from volatiles, which are sensed by olfactory receptors located in the olfactory region in our nose. Both volatile and non-volatile compounds have a significant influence on the flavor profile (Diez-Simon, Mumm, & Hall, 2019).

The aroma of a food may arise from a complex combination of volatile compounds, or it might result from relatively few character-impact odorants (R. Marsili & McGorrin, 2011). Among these diverse aroma compounds, their structure and therefore, volatility vary widely (Miettinen, 2004). Each volatile compound has the potential to make its own impact on the overall aroma, i.e., it might contribute to the characteristic odor, add a particular nuance to a flavor or it might make no contribution at all to aroma. So, in order to determine the influence of volatile compounds on overall aroma, flavor analysis is necessary to be introduced.

1.1.2 Flavor Analysis

Flavor analysis is generally divided into two areas: sensory analysis and instrumental analysis (Chambers IV & Koppel, 2013). Sensory analysis directly reflects human perception of flavor, while instrumental analysis precisely indicates qualitative and quantitative evaluation of flavor.

In sensory analysis, one of the most comprehensive methods is descriptive analysis, which is considered to be a preferred technique for food aroma evaluation, due to its high reliability and consistency. As human olfaction is able to detect and discriminate between a large amount of odorants (Hatt, 2004), sensory descriptive analysis conducted by trained sensory panels can provide a detailed profile of scent attributes of a food. For a practical application, sensory analysis is usually coupled with
instrumental measurement (Chambers IV & Koppel, 2013), linking olfactory perception by humans to volatile compounds identification via instruments.

Instrumental methods perform flavor analysis based on the isolation, identification and quantification of each volatile compound in a matrix. The most common instrumental method is gas chromatography (GC), which can be coupled with mass spectrometry (MS). Gas chromatography-mass spectrometry (GC-MS) is a powerful tool for the separation and identification of aroma components. When equipped with a olfactometric port, GC-MS can be developed into GC-MS-olfactometry (GC-MS-O), which enables aroma compounds to be detected and identified by GC-MS, and also sensed by human nose (Chambers IV & Koppel, 2013). The combination of sensory and instrumental analysis not only increases the sensitivity of volatiles detection, but also contributes to compound confirmation. Those tentatively identified compounds through GC-MS can be further verified via the comparison of human response between the target compound in a sample and the standard compound. Sensory and instrumental analysis play significant roles in identifying odor-active compounds in a sample. However, not all these volatiles contribute to the overall aroma, so the introduction of screening methods are necessary, through which, the sensory significant odor-active compounds are selected for additional sensory studies (Delahunty, Eyres, & Dufour, 2006).

1.1.3 Pennycress

Field Pennycress (*Thlaspi arvense* L.) is an oilseed belonging to the mustard family. Since pennycress is high in protein (25-27%) and fat/oil (20-36%) (Chopra, Johnson, et al., 2019; Thomas, Hampton, Dorn, David Marks, & Carter, 2017), it has a
high potential to become an ideal ingredient in food products. However, as it is a member of the mustard family, it has a common mustard-like aroma, which is a deterrent to be accepted by consumers. Hence, the unpleasant aroma of pennycress will largely limit its introduction and application in the food industry. Nowadays, however, more and more research groups and companies have noticed the growth potential of pennycress, and various research has been done on pennycress’s breeding, protein isolation and oil analysis. However, flavor-related research on pennycress is extremely limited.

1.2 RESEARCH OBJECTIVE

The objective of this research was to identify volatile compounds which contribute to the pennycress aroma thus providing valuable information for breeding programs or relative research groups more possible to design processes to minimize the undesirable aroma.
CHAPTER 2. LITERATURE REVIEW

2.1 PENNYCRESS

2.1.1 Overview of Pennycress

Pennycress (*Thlaspi arvense* L.) is an oilseed crop belonging to Brassicaceae (mustard family) (Dorn, Johnson, Daniels, Wyse, & Marks, 2018). Similar to other Brassicaceae species, pennycress exhibits both winter and spring type annual growth habits, and its ability to survive the winter distinguishes itself from many other Brassicaceae species. Pennycress originated in Eurasia, and is a widespread crop throughout temperate North America (Mitich, 1996; Vaughn et al., 2005). The common name of pennycress is field pennycress, and it is also known as stinkweed, frenchweed or fanweed (Best & Mcintyre, 1975).

Pennycress is currently being developed as a “new” crop aimed at helping solve global food issues. As global population keeps growing, the demand for food continues to rise. The increasing food demand will require farmers worldwide to improve crop production, not only by increasing the amount or area of agricultural lands, but also improving land use efficiency to attain higher crop yields (Jordan et al., 2016; Tilman, Balzer, Hill, & Befort, 2011). Nevertheless, the use efficiency of agricultural land is greatly impacted by extreme weather (e.g., floods, severe storm, summer drought and cold snap). According to the U.S. Department of Agriculture (USDA), it is estimated that 90% of crop loss is the result of extreme weather (Foerster, 2019). Particularly, extreme cold is an annual concern for farmers in Midwestern United States, where winters are long and hard. In the U.S. Midwest, corn and soybean are usually the primary crops,
however, the extremely low winter temperatures make it impossible for corn and soybean overwinter. Since few traditional crops can survive winters in the Midwest, the agricultural landscape typically remains barren over winter. For farmers, the fallow soil in winter is a great waste of land resource as well as potential income (Dorn, Fankhauser, Wyse, & Marks, 2013), because they cannot get any benefits from their agricultural land at that time. In addition, as the soil is not covered with any crops, it is subject to water and wind erosion. Thus, nutrients (e.g., nitrate) will be lost from the soil. This creates additional problems because excess NO$_3$ will go into the waterways, which is harmful to water quality. Therefore, the planting of cold-tolerant crops during winter would play an important role in protecting the landscape as well as water resources (Moser, Shah, Winkler-Moser, Vaughn, & Evangelista, 2009).

Pennycress is a crop that will survive winter in the United States. Pennycress has a high tolerance of low temperature, as low as -30 °C (Sedbrook, Phippen, & Marks, 2014), which makes it an ideal winter annual crop. As pennycress has a short life cycle (planted in fall and harvested in early summer), it can serve as a double or relay crop in the traditional soybean and corn rotation. Since pennycress can survive when traditional crops cannot, pennycress planting will not require any additional land or replace traditional crops. In addition, there is little need of more agricultural inputs (e.g., fertilizers, pesticides) or water for pennycress cultivation (Claver, Rey, López, Picorel, & Alfonso, 2017).

**2.1.2 Current Application of Pennycress**
Pennycress is an ideal oilseed crop, with 20-36% oil content (Thomas et al., 2017). The fatty acid profile of pennycress oil was investigated by Moser et al. (2009) and they reported that the oil from dried wild pennycress seeds contains 55.6% monounsaturated fatty acids, 38% polyunsaturated fatty acids and 4.6% saturated fatty acids. Among the fatty acids, erucic acid (32.8%) is the most abundant fatty acid (Moser et al., 2009). Although pennycress seed is high in oil, its oil hasn’t been accepted as an edible oil for human consumption because of its high level of erucic acid (Ioana, Socaci, & Socaciu, 2012). It has been reported that diets with high amounts of erucic acid are harmful to the heart (Altendorf, 2017). Although pennycress oil is not edible thus far, it can be used as high-quality feedstocks for biodiesel production. Pennycress oil meets the biodiesel requirement established by the United States American Society for Testing and Materials (ASTM), which shows pennycress oil is an accepted biofuel. Pennycress oil’s high erucic acid content also makes it perform better at lower temperatures, which is better than biodiesel made from soybean oil. In addition, when pennycress is part of a double cropping system with soybeans, the total oilseed yield was found to be 18-28% greater than the yield from soybeans alone, despite soybean yield being decreased by 18-20% due to competition for resources in double cropping system (Altendorf, 2017). The economic return from growing pennycress has the potential to be higher due to the high yield of pennycress, with traditional farming yielding 700 to 900 lbs/acre, and up to 1,500 to 2,000 lbs/acre in research trials (Kleba & Ismail, 2018). It is relevant that more research is being done to increase the yield of pennycress.
The remaining material “press cake” after oil extraction potentially becomes a by-product for protein production. Pennycress is high in protein. The study conducted by Hojilla-Evangelista et al. (2015) showed that pennycress seeds have 20% crude protein content and pennycress press cake has 26%. Gordon & Mark (2015) compared two traditional methods for protein isolation, acid precipitation and saline extraction, to isolate protein from pennycress press cake and seed meal, and they found that some functional properties of protein (e.g., solubility, emulsifying capacity, foaming capacity, heat coagulability) are influenced by the extraction method used. Saline extraction produced protein with lower purity and higher solubility, while acid-precipitated press cake protein had higher stability to heat denaturation, and greater foam capacity. The pennycress press cake protein from both methods exhibited promising emulsifying and foaming properties, which are desired to be used as pressurized foams, whipped products and emulsions (Gordon & Mark, 2015). Pennycress protein shows potential in industrial applications, however, currently it is still not a commercially edible protein for human consumption in the U.S. (Dorn, Fankhauser, Wyse, & Marks, 2015). Nevertheless, as a result of increasing research efforts on pennycress protein isolation and digestibility (Kleba & Ismail, 2018), future application and utilization of pennycress protein will be promising.

2.1.3 Commercialization Problems of Pennycress

As already noted, while pennycress is an environmentally friendly crop which is high in both oil and protein, the current application of pennycress is limited. The commercialization of pennycress is a complex problem related to different factors, which
have been discussed by Jordan et al. (2016). First, the cultivation of pennycress is likely to have an unexpected impact on agricultural landscape. As pennycress is not a native plant to North America, the cultivation of “invasive” pennycress is possible to make an unexpected change in the original composition of landscape, such as soil nutrients, pest dynamics and pollinators. Second, pennycress will not be successful without the utilization of new plant breeding technologies, which might lead to a public concern for genetically modified crops. New planting/breeding technologies used in pennycress cultivation include genome editing methods aimed to induce precisely-targeted mutations. While new breeding technology helps to improve pennycress properties, it also brings about another problem due to the “artificial change”. Third, production of pennycress products possibly brings a concern for sustainability. Biofuel is currently the main product for pennycress, however, it is still unknown whether it will have an effect on soil, water, biodiversity, landscape resources and so on. The sustainable commercialization of pennycress and its products are associated with its demand on market. Most of farmers are more willing to plant traditional crops, instead of an unfamiliar crop with limited demand, which might be risky for them.

Despite the commercialization of pennycress being challenging, it still has a promising potential for industrial applications. By contrast, its application on food products for human consumption is more challenging, due to its off flavors and toxicity (Sedbrook et al., 2014).

2.1.4 Sources of Pennycress Flavor
The lack of palatability is one of the biggest problems for pennycress to become a food ingredient. Generally, plants from the Brassicaceae family have a strong pungent odor typically described as “mustard-like”; pennycress has this characteristic flavor. According to the description from Lisen (Sundgren, 2015), the flavor of pennycress is like “a mix of pepper, mustard, and onion with a slight bitterness”. Based on research conducted on other species in Brassicaceae family, it is generally accepted that pennycress’s unpleasant flavor is caused by its high glucosinolate content. Glucosinolates are an important group of phytochemicals abundant in Brassicaceae (Holst & Williamson, 2004), they are β-thioglucoside-N-hydroxysulfates with a sulphur-linked β-D-glucopyranose moiety and an amino acid-derived side (Figure 1) (Bell, Oloyede, Lignou, Wagstaff, & Methven, 2018; Holst & Williamson, 2004). Glucosinolates comprise a large family of more than 100 molecules with common core structure and different side chains (Fahey, Zalcmann, & Talalay, 2001; Halkier & Gershenzon, 2006). The differences in the chemical nature of side chains lead to the differences in hydrolysis products of glycosylates (Fenwick & Heaney, 1983), which are catalyzed by the enzyme myrosinase (β-thioglucosidase glucohydrolase, EC 3.2.3.1) (Al-Gendy, El-gindi, Hafez, & Ateya, 2010).

![Figure 1. The general structure of glucosinolates.](image)
Myrosinase is an enzyme related to plant defense in the Brassicaceae. In the presence of water, myrosinase causes the cleavage of the thio-glucose bond, releasing a molecule of D-glucose and thiohydroximate-O-sulfonate (Figure 2), which is an unstable intermediate that will be spontaneously rearranged into different compounds (Al-Gendy et al., 2010), including epithionitriles, thiocyanates, nitriles, isothiocyanates and oxazolidinethiones (Figure 2) (Al-Gendy et al., 2010). The structures of these compounds are dependent on the side chain of glucosinolates, the existence of different medium, and the hydrolysis conditions (Fenwick & Heaney, 1983).

![Glucosinolate degradation pathway](Figure 2)

Glucosinolates in plants are thought to impart resistant to herbivores and microbes (Altendorf, 2017). The precursors of glucosinolates are considered to be protein and nonprotein amino acids (Haughn, Davin, Giblin, & Underhill, 1991). The biosynthesis of glucosinolates from amino acids requires at least five steps (Figure 3): amino acids, N-

Glucosinolates are common in Brassicaceae family and they can be found in different parts of the plant with the seeds usually containing the highest amounts. The concentration of glucosinolate is not only dependent on the parts/organs of plants and the planting stages of pennycress, but also related to the environmental conditions where pennycress is being grown.

Sinigrin, the common name of allyl glucosinolate (Figure 4), is the main glucosinolate in cruciferous vegetables (Altendorf, 2017). When plant tissues are crushed, sinigrin will be hydrolyzed by the enzyme myrosinase into smaller compounds. Shofran et al. (1998) indicated that the general hydrolysis products of sinigrin are allyl isothiocyanate (AITC), allyl cyanide (AC), 1-cyano-2,3,epithiopropane (CETP) and allyl...
thiocyanate (ATC). Since these degradation products are volatile compounds, they are responsible for the characteristic odor of the mustard family. However, physical damage is just one of the factors leading to the development of the characteristic off-flavor. Dai & Lim (2014) found that the release of AITC from sinigrin requires water, so dry milling would fail to activate the liberation of AITC from mustard seeds. Also, an adequate heat treatment before milling will deactivate myrosinase, which prevents the formation of degradation compounds during crushing (Sedbrook et al., 2014).

![Figure 4. The structure of sinigrin.](image)

Even though the production of all off-flavored degradation products could theoretically be prevented by avoiding wedding the seeds or conducting a preboiling treatment before milling, the production of degradation compounds is still hard to totally avoid. For example, plants may be physically damaged during delivery from field to processing plant under an open environment where moisture is hard to control. Therefore, protecting pennycress from being exposed to damage or moisture is not a practical way to prevent the formation of off-flavor. By contrast, reducing glucosinolate content in
pennycress is a better approach to avoiding any risk of producing any flavored compounds, and further guarantee the palatability of pennycress.

As noted earlier, glucosinolates are also considered to be toxic on consumption. Pennycress has a name of “horse killer” due to its high content of glucosinolates which is harmful to horses. Besides horses, other livestock (e.g., cattle, pig) will also get sick or even die when consuming a large amount of seeds or leaves from pennycress. As Best & McIntyre (1975) mentioned, livestock may develop “chronic enteritis, hemorrhagic diarrhea, colic, abortion, nephritis and hematuria, apathy and paralysis of heart and respiration” by over-consumption of pennycress (Best & McIntyre, 1975). Moreover, pennycress will also taint the flavor of milk and flesh from the cattle that are fed pennycress. Once livestock have consumed the seeds or leaves of pennycress, the unpleasant flavor or odor from pennycress will remain in flesh and even milk up to 8 hours after eaten (Bond, Davies, & Turner, 2007; United States Department of Agriculture & Animal and Plant Health Inspection Service, 2015). Additionally, the glucosinolates would remain in the seed meal or press cake after finishing oil extraction.

Due to the glucosinolates inherent to pennycress and the challenges they present, the most promising solution is considered to be breeding research focused on reducing glucosinolate content. Chopra et al. (2019) has proposed that marker-assisted breeding strategies could be successful in producing a pennycress devoid of glucosinolates.

2.1.5 Palatability of Pennycress

Although wild-type pennycress is high in glucosinolates (toxic to livestock), it has been reported that it can be consumed by human beings. According to Bond et al. (2007),
field pennycress is cultivated as a food plant in some European countries. The young leaves of pennycress are traditionally eaten raw or cooked, even though it has a bitter taste and mustard-like odor. Pennycress has been included in the diet in different ways. It can be mixed with salads and other foods in a small amount, or it can be cooked in a soup, and even treated as a pot herb. Seeds can be also added to salad, or ground as a mustard substitute. In addition to being consumed as food, pennycress seeds are also used in Tibetan medicine. According to Barker (2003), pennycress seed has a function of diuretic and rheumatism relief. In Mongolia, it serves as a prescription medicine, used for healing lung and kidney diseases.

Although pennycress has the history of serving as food or medicine in some countries, currently, the issues of whether pennycress should be considered as a “noxious weeds” is still not confirmed, since the requirements to be noxious weeds are different among different places. Sedbrook et al. (2014) reported that “pennycress is not on the USA or Canadian federal lists of noxious weeds”. Besides, according to the State Noxious-Weed Seed Requirements revised on November, 2019 (Agricultural Marketing Service, 2019), Minnesota law categorized *Thlaspi arvense* (pennycress) as a “restricted” noxious-weed seed instead of “prohibited”, which means that pennycress is allowed to be commercial under restricted labeled and below certain amounts.

2.1.6 Volatile Compounds in Brassicaceae Family

The aroma of pennycress comes from volatile organic compounds (VOCs), but current research on flavor analysis of pennycress is scarce. No publications have reported the comprehensive determination of volatile components in pennycress. As pennycress is
a member of Brassicaceae family, it obtains genetic similarity with its Brassicaceae relatives, including *Armoracia lapathifolia* (horseradish), *Brassica nigra* (black mustard), and *Brassica oleracea* (cauliflower, broccoli, cabbage, Brussels sprout) (Rask et al., 2000). Since the volatile profile of pennycress is still unknown, reviewing those researches on other members of Brassicaceae species will be helpful in understanding the volatile components of pennycress.

Volatiles analysis has been conducted on various Brassicaceae species. Blažević et al. (2010) studied volatile compounds of *Aurinia sinuate* (Brassicaceae) and found that the primary detected VOCs were degradation products from three glucosinolates, including glucoberteroin, glucobrassicanapin and glucoalyssin (Figure 5) (Al-Gendy et al., 2010).

![Figure 5. Structures of the main glucosinolates identified in Brassicaceae. (Source: Al-Gendy et al., 2010)](source.png)

The main degradation compounds were: 6-(methylthio)hexanenitrile, 6-(methylsulfinyl)hexanenitrile, 5-(methylsulfinyl)pentyl isothiocyanate, 5-
(methylthio)pentyl isothiocyanate, 4-pentenyl isothiocyanate, 5-hexenenitrile and 5,6-epithiohexanenitrile. Verzera et al. (2010) conducted research on volatile compounds of *Isatis tinctoria* L. (Brassicaceae), in which, thirty components were identified, comprising 99.6% of total volatile compounds. The most abundant classes of components were isothiocyanates and thiocyanates, including methyl thiocyanate, 3-butenyl isothiocyanate, cyclopentyl isothiocyanate. A study on the volatile profile of *Arabidopsis thaliana* (Brassicaceae) was conducted by Rohloff & Bones (2005), and they detected 102 volatile compounds, including 18 isothiocyanates and 9 nitriles. In addition, the volatile isothiocyanates in seeds of fourteen different Brassica were studied by Jensen et al. (1953), and they found that allyl isothiocyanates were identified from 10 Brassica species. Also, it was common to see two or three different isothiocyanates appeared in one species.

Studies on volatile analysis of different Brassicaceae species indicated that isothiocyanates were the major glucosinolate degradation products in the Brassicaceae family. It was also found that some plants had a limited number of isothiocyanates while some plants contained more. This result conforms to the number of glucosinolates identified in the Brassicaceae plants. Most plants usually contain six or less glucosinolates, while some plants contain up to 23 glycosylates (Fahey et al., 2001).

As Vaughn et al. (2005) found that sinigrin was the sole glucosinolate in pennycress, the volatile compounds contributing to pennycress aroma would most likely be the hydrolytic degradation products of sinigrin including allyl isothiocyanate (AITC), allyl thiocyanate (ATC), 1-cyano-2,3-epithiopropane (CETP), allyl cyanide (AC) (Figure
6) (Chin, Zeng, & Lindsay, 1996; Dai & Lim, 2014). Table 1 shows the aroma description of these four hydrolysis products and their common plant sources (Shofran et al., 2006).

![Chemical Structure](image)

Figure 6. Formation of volatile compounds from the hydrolysis of sinigrin in cruciferous plants.
(Source: Al-Gendy et al., 2010)
Each of these volatile compounds (Figure 6) could contribute to a characteristic odor in a plant. Since the volatile profile in a plant is typically quite complex, it is generally difficult to determine what particular component makes the greatest contribution to perceived aroma. However, since the volatile compounds in pennycress are considered to come from the hydrolysis of sinigrin, knowledge of sinigrin degradation products can be helpful in understanding/determining the volatile compounds that are most to be problematic in pennycress.

**2.2 VOLATILES ANALYSIS**

**2.2.1 Volatile Compounds**

Aroma compounds are required to be volatile molecules which have a high vapor pressure at room temperature. They are perceived only when they are released from a food matrix and reach the olfactory area, evoking a smell consciousness. The transmission of a volatile compound in a food to the vapor phase (air) is greatly

---

**Table 1. Sinigrin hydrolysis products and their occurrence in plants.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plant sources</th>
<th>Aroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>AITC</td>
<td>mustard, horseradish, cabbage, cauliflower, Brussels sprouts, turnips, kale, collards</td>
<td>mustard, horseradish</td>
</tr>
<tr>
<td>ATC</td>
<td>stinkweed, horseradish</td>
<td>garlic</td>
</tr>
<tr>
<td>CETP</td>
<td>cabbage, Brussels sprouts, crambe seed</td>
<td>sulfurous</td>
</tr>
<tr>
<td>AC</td>
<td>cabbage, cauliflower, Brussels sprouts</td>
<td>mustard, horseradish</td>
</tr>
</tbody>
</table>

(Source: Shofran et al., 2006)
dependent on the interaction of a volatile with non-volatiles in a food, e.g., lipids, protein and carbohydrates (Guichard, 2015). Aroma perception can be affected in aroma compound moving into the lipid phase (reducing its vapor pressure), by reacting with a protein, or being trapped by a carbohydrate (e.g., starch) (Fisk, Boyer, & Linforth, 2012; Herrera-Jimnéz, Escalona-Buenda, Ponce-Alquicira, Verde-Calvo, & Guerrero-Lagarreta, 2007).

2.2.1.1 Source of Volatile Compounds in Foods

Commonly, the aroma of a food is the result of volatile compounds inherent to the food itself, or it can be formed during food processing, preparation and storage (Cui et al., 2017). In storage, any change of storage conditions (e.g., temperature, oxygen content, light exposure) would lead to an aroma difference (Averbeck & Schieberle, 2011). In processing, volatiles are likely to be generated by fermentation, enzymatic reaction, lipid peroxidation and thermal degradation (e.g., Maillard reaction, caramelization) (Tylewicz, Inchingolo, & Rodriguez-Estrada, 2017).

While the aroma of meat is primarily formed through thermal reaction of non-volatiles (Arshad et al., 2018; Resconi, Escudero, & Campo, 2013), plant aroma is mainly derived from four classes of volatiles: terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives and amino acid derivatives. The creation of plant volatiles results from biosynthetic pathways, related to the coordination of carbon, nitrogen and sulfur metabolism as well as energy (Dudareva, Klempien, Muhlemann, & Kaplan, 2013; Dudareva, Negre, Nagegowda, & Orlova, 2006). Plant volatiles can be released from most parts of their anatomy (Borges, 2015), among which, they are constitutively emitted
from flowers, leaves, roots (Kigathi, Weisser, Reichelt, Gershenzon, & Unsicker, 2019). Although seeds are usually not the volatile-producing part for most species, the seeds of Brassicaceae and some other families do produce volatile compounds, which are degraded from non-volatile glucosinolates when the tissue is disrupted (Xue et al., 2013).

Generally, plants emit volatile compounds with various biological or ecological properties (Liu et al., 2018). The emission of plant volatiles enables themselves to interact with their surrounding environment. For example, in response to herbivore attack, plants will release volatiles to attract enemies of herbivore (Clavijo McCormick, Unsicker, & Gershenzon, 2012), so plant damage can lead to an increasing emission of volatile compounds. The release of volatile compounds from plants are complex, i.e., some volatiles are produced upon cell disruption, and some emitted due to enzyme reaction. Thus far, more than 11 thousand volatiles have been identified in foods. However, among this vast number of volatile compounds, only a limited number of them have important impact on an overall aroma (Tylewicz et al., 2017).

### 2.2.1.2 Volatile Compounds and Olfactometry Perception

A large number of volatile chemicals exist in foods at a wide range of concentrations. These compounds also vary greatly in their sensory thresholds and thus their concentration in a food can be a poor indication of the contribution to flavor. Odor threshold represents the lowest concentration of an aroma compound that 50% of the people evaluating a compound can perceive it. The volatile compounds with low odor thresholds are typically most likely to have a significant influence on overall aroma even if present at low concentrations (Cliff, Stanich, Trujillo, Toivonen, & Forney, 2011). So,
to some extent, odor threshold can be considered as one of the indicators of the importance of odorants. Odor threshold values are impacted by diverse factors, it varies when compounds are dissolved in different mediums (Lam & Proctor, 2003), since the tasting medium influences the volatility of the compound (Buttery, Guadagni, & Ling, 1973). Besides the tasting medium, odor threshold value is also influenced by molecular structure or functional group in a compound. Takeoka et al. (1995, 1996) evaluated the interaction between the change of compound structure and its odor threshold value in water, in which, they found that the change of odor threshold was not consistent when adding a methyl group to different compounds. The addition of methyl group increased the odor threshold for pentyl acetate, hexyl acetate, however, for ethyl acetate and propyl acetate, the odor threshold was decreased. It indicated that the odor threshold of a molecule is not reliably predicted based on the structure difference or similarity. Besides that, human perception on a odorant is also impacted by the interaction between olfactory receptor and some functional groups. As Genva et al. (2019) reported, human olfaction is more sensitive to some functional groups, such as oxines (-NOH), thiols (-SH), and nitro groups (-NO₂), which are correspondingly responsible for green camphoraceous, sulfurous and sweet ethereal character. This implied that the perceived odor of a volatile compound can be largely influenced by functional groups. However, some volatile compounds with same functional group have analogous aroma, while some have dissimilar odor. So even functional groups do associate with odor character, they are still not the indications of the odor of a compound (Genva et al., 2019). Therefore, the characteristics of odorants should be determined on the olfactory analysis of the
compound in a specific condition, instead of relying on the prediction based on molecular structure.

The aroma of pennycress is most likely the result of a combination of various volatile compounds, with each compound contributing to a specific odor. To understand the importance of individual volatiles to the overall pennycress aroma, those non-volatile compounds (e.g., lipids, protein, fiber, starch) and water (probably derived from plants, solvent, air) are required to be isolated from volatile compounds, since, with minor exceptions, there are less than 1% volatile compounds in a food matrix (Elmore, 2015). Therefore, the isolation of non-volatile components and water from the food would maximize the concentration of volatile compounds for instrumental detection, and make guest mammographic (e.g., non-volatile components, water) analysis impossible. Besides, in a complex volatiles extract, each volatile compound needs to be separated from a pennycress aroma matrix for single compound analysis.

2.2.2 Gas Chromatography

Gas chromatography (GC) is a commonly used technique for volatile separation in a mixture. The first modern GC was introduced by James and Martin in 1952, for the separation of volatile fatty acids (Bartle & Myers, 2002; Kolomnikov, Efremov, Tikhomirova, Sorokina, & Zolotov, 2018). GC experienced a rapid development in the first two decades and is a mature technique today (Ettre, 2002). The application of GC coupled with mass spectrometry led to a tremendous increase in the number of identified aroma compounds in foods, i.e., from 500 in 1963 to over 11 thousand today (Reineccius, 2006; Yahia, 2017).
Gas chromatography separates volatile compounds depending on the interaction between mobile phase and stationary phase in the column. The mobile phase is called a carrier gas, which is an inert gas (i.e., helium, nitrogen, or hydrogen), carrying the gaseous sample through the column, while the stationary phase is a thin film of some high boiling polymer (most commonly based on the poly siloxane structure). Due to different interactions with the stationary phase, each component of a sample mixture hopefully will be eluted in the column at a different time. The interaction strength can be influenced by the GC operating conditions, and the polarity of the stationary phase. Since polar compounds interact more strongly with polar stationary phases, polar columns work better for the separation of polar compounds, and non-polar columns are better for separation of non-polar compounds separation. After being separated, each compound of the sample exits the column and goes into a detector (Rausch, 2009).

2.2.2.1 Gas Chromatography Detectors

The GC detector is located at the end of the column and is used for monitoring the eluting analytes, converting their interaction into an electronic signal (Jackson, Walton, & Campbell, 1997). There are various detectors available for GC, and each one has its unique selectivity, sensitivity and thus a different application. The detectors can be generally classified into two types, universal and selective detectors. As for universal detectors, flame ionization detector (FID) and thermal conductivity detector (TCD) are the most common detectors: they are both sensitive to a wide range of analytes. While TCD is not sensitive to trace analytes (Uyanik, 1998), FID is able to detect compounds with concentrations as low as one nanogram (Mansfield, 2008). FID can response to most
molecules ionized in a hydrogen/air flame, leading to a wide range in linear response as well as a high sensitivity (Holm, 1999). With a 100 times lower detection limit than TCD, FID has an ideal sensitivity for volatiles detection and has become the most widely used GC detector. With the use of n-alkanes and standard compounds, FID can tentatively identify unknown compounds by comparing Kovats Index (KI) between the number calculated on the column and the one listed on the literature (Kováts, 1958).

A universal detector shows a response for almost all types of analytes, whereas a selective detector only works for a specific type of analyte. The common selective detectors are electron capture detector (ECD), photo ionization detector (PID), and flame photometric detector (FPD). Although selective detectors have certain detection limit, they have advantages for better gas chromatographic performance on target analytes. However, in the modern application of GC, it is very common that a mass spectrometer is connected to the GC, which is available for compound identification of peaks from the mass spectrum.

2.2.2.2 Gas Chromatography-Mass Spectrometry (GC-MS)

Mass spectrometry is an analytical method for measuring mass-to-charge ratio (m/z) of ions by a mass spectrometer, which operates under vacuum. A mass spectrometer (Figure 7) is composed of three components: an ion source, a mass analyzer, and a mass detector (Rausch, 2009), which correspondingly contributes to sample ionization, ion separation and ion detection, thus producing a mass spectrum (Siuzdak, 2004). Based on the unique mass spectrum and elution time of each compound, mass spectrometry is able to determine the identity of most detected analytes. As mass
spectrometry is an effective technique for compound identification, it has been used with GC for identifying unknown volatiles from a complex mixture, which is called gas chromatography-mass spectrometry (GC-MS) (Figure 7). Different compounds are separated in the GC column and then enter into a mass spectrometer. The successful detection of ions in GC-MS requires a high vacuum, maintained via a vacuum pump connected to mass spectrometer (Sellier & Guiochon, 1970).

GC-MS can detect and identify volatile compounds at concentrations as low as $10^{-5}$ g/L (Friedrich & Acree, 2000). However, compound identification via mass spectrometry is a challenge. Compounds with similar characteristics usually have very close retention times as well as similar mass spectra, so may result in “a same mass trace in the same retention window” (Lisec, Schauer, Kopka, Willmitzer, & Fernie, 2006). To avoid the mistakes caused by instrumental errors (the instrument making an incorrect assigned identity), it is very necessary to conduct manual inspection of spectra. By conducting a manual inspection of ion fractions, comparing individual mass traces from an unknown sample to the one from library, it is possible to identify unknown compounds. The combination of GC and MS leads to an efficient tool for mixtures analysis, which decreases the time necessary for peak identification and comparison as well as the time involved in separation, detection and identification.
2.2.3 Gas Chromatography-Olfactometry (GC-O)

The development of GC has made a significant contribution to the research on odors as it has allowed the separation, qualification and quantification of volatile compounds comprising an odor (Delahunty et al., 2006). The odor from a food product usually is composed of numerous volatiles, however, not all volatile compounds are aroma-active (Cheng et al., 2015; Wu et al., 2018). Only when volatile compounds are available to be perceived by olfactory receptors, they can be considered as aroma-active compounds. Actually, in foods, only a small amount of volatiles compounds, around 5%, are aroma-active compounds (Weiss & Christlbauer, 2019). In a food product, the availability and intensity of volatile compounds perceived by the human nose can be influenced by the concentration and odor threshold of individual compounds.
Although the application of GC-MS is able to detect and identify volatile compounds in a mixture, however, to determine the contribution of individual volatiles to the odor quality of a food product, knowing whether the compound is present or absent in a mixture is not enough (Delahunty et al., 2006). It is important to understand how a volatile compound is perceived in the nose. The combination of gas chromatography with olfactometry has allowed volatiles separated by gas chromatographic instruments and detected through olfactometry to be assessed for contribution to the overall perceived odor.

GC-olfactometry (GC-O) (Figure 8) is a technique combining instrumental separation with sensory detection for aroma-active volatiles selection, which allows for an efficient comparison of volatile compounds contributing to perceived odors (Mansfield, 2008). Volatile compounds are separated by the GC column and split into two portions, one portion goes to physical detectors, usually flame ionization detector (FID) or mass spectrometer, where a signal is recorded and converted into a chromatogram. Another portion of volatiles goes to a sniffing port, where humans use their nose to detect and distinguish each aroma eluting from the GC column. The person sniffing the GC effluent will, then make a descriptive record of each smell at its respective elution time (R. T. Marsili, 2006). The application of GC-MS-O effectively eliminates the need for separating GC-MS and GC-O runs, which reduces the risk of inappropriate match between compounds and aromas (Mansfield, 2008). It is commonly believed, for the majority of odor molecules, human nose has a higher sensitivity than any physical detectors. As reported, the human nose is able to smell odorants at
concentrations as low as $10^{-12}$ g/L ($10^{-19}$ ppm), and so thus, no physical instruments has been developed to achieve the same sensitivity as the human nose (Mansfield, 2008). Therefore, the compounds with low odor threshold as well as low concentration are probably often detected by human sniffing but poorly detected by the physical detector as the concentration is frequently below its detection limit. So, the introduction of GC-O has greatly increased the sensitivity of aroma compound detection.

Figure 8. Gas chromatography-olfactometry (GC-O).

GC-O can locate aroma-active compounds in a volatile mixture, however, not all these aroma-active volatiles have important impact on the overall odor (Grosch, 1994). The impact of above compounds on aroma perception is related to the compound’s concentration and odor threshold. Odor activity values (OAVs) were introduced as a rough method to measure the olfactory importance of a compound to a mixture (Mansfield, 2008). OAVs are defined as “the concentration of an odorant divided by its
odor threshold” (Mayol & Acree, 2001). This approach assesses odorant importance in terms of the “ratio of the concentration of an odorant to its threshold concentration” (Audouin, Bonnet, Vickers, & Reineccius, 2001). Since OAV concept was proposed in 1957 (Patton & Josephson, 1957), it has been used with GC-O as a screening tool to differentiate volatile compounds. Two other major screening methods are: combined hedonic aroma response measurement (CHARM) analysis and aroma extract dilution analysis (AEDA), which have been introduced for determining key aroma compounds in food. Both methods are dilution methods, in which, an aroma extract is diluted and analyzed by GC-O. The highest dilution that an aroma is able to be perceived by human nose is defined as dilution value. CHARM analysis measures Charm value, the dilution value throughout the entire time a compound elutes, and AEDA measures flavor dilution (FD) factor, which means the maximum dilution value detected (Grosch, 1994). Both Charm analysis and AEDA propose that the bigger the dilution has, the higher potential that a compound is a significant contributor to the overall aroma (Delahunty et al., 2006). However, both methods are just screening procedures, which are rough tools to select compounds that are most likely to make a significant contribution to the overall aroma. The actual contribution of a volatile compound to overall aroma needs to be determined by further sensory studies. Although GC-O techniques have limitations, they are still considered as the best screening methods for current flavor analysis (Audouin et al., 2001).
2.3 VOLATILES EXTRACTION

As Mansfield (2008) reported, odor-active compounds have been shown to influence the overall aroma of a food when they are at concentrations as low as $10^{-11}$ ppt. GC-MS can detect and identify compounds at a concentration above $10^{-5}$ ppt. Volatiles can be detected and analyzed only when their concentrations reach the detection limit of instruments or human senses. Thus, the task of isolating and concentrating volatiles from a food is critical. There are diverse methods available for volatiles extraction, such as headspace techniques (e.g., purge and trap (P&T)), sorptive extraction (e.g., stir bar sorptive extraction (SBSE)), solid phase micro extraction (SPME), distillation methods (e.g., simultaneous distillation extraction (SDE)) and so on. Among different methods, there is no doubt that the selection of extraction method has a significant influence on further analysis of those extracted volatile compounds. Unfortunately, when choosing an extraction technique, there is no perfect option since each method has its specific advantages and weaknesses. Different methods will have different sensitivity, selectivity and reproducibility, depending upon the sample type and the ultimate goal of the study (High, Bremer, Kebede, & Eyres, 2019). To decide on an extraction method, one needs to take various factors into account, including the type of sample and the objective of the research. Within a variety of volatiles extraction techniques, one of the best choices for pennycress seeds is very likely to be solvent assisted flavor evaporation (SAFE), which is excellent in producing a “clean” volatiles extract, where fat/oil can be largely removed. SAFE extraction usually follows solvent extraction, which provides an initial compound extract.
2.3.1 Solvent Extraction

Solvent extraction is a simple and efficient technique for isolating flavor essence from food products (Wong & Parks, 1968). In conducting a solvent extraction, an appropriate organic solvent is mixed with the target food sample. Through contact with the solvent, volatile constituents will be tend to be extracted into the solvent (Birch, 2000). Various solvents can be used for solvent extraction, and diethyl ether is one of the most common organic solvents. Diethyl ether is a somewhat polar aprotic solvent, in which, polar compounds are readily dissolved, and non-polar substances are also soluble (Pulster, Bourgeois, & Harbison, 2015). Diethyl ether can be a good organic solvent for extracting organic aldehydes, alcohols, esters and ketones (Tomaszewski, 2016). As it is simple and efficient, solvent extraction has been extensively used in essential oil extractions, particularly for botanical material. However, it is not an ideal method as the solvent will also dissolve unwanted products (Houck & Siegel, 2010). It can even develop emulsions in aqueous system and extract lipids in fat-containing samples (Wong & Parks, 1968). Nevertheless, for further volatiles analysis, non-volatile compounds have to be separated from volatiles extract.

2.3.2 Solvent Assisted Flavor Evaporation (SAFE)

Solvent Assisted Flavor Evaporation (SAFE) is one of the best methods to separate volatile compounds from non-volatile compounds, while minimizing artifact formation. SAFE is a versatile technique to obtain the careful and direct isolation of volatile compounds from a complex food matrix, and it was originally designed for analysis of volatiles in dairy products, which contained fats (Engel, Bahr, & Schieberle,
The SAFE apparatus (Figure 9) is connected to a high vacuum pump, providing a reduced pressure in the system. The reduced pressure allows volatiles to be extracted at a low temperature, which prevents or reduces the formation of thermally induced artifacts.

![Diagram of SAFE apparatus](source)

**Figure 9.** Solvent assisted flavor evaporation (SAFE) apparatus. (Source: Engel et al., 1999)

As Engel et al. (1999) described, the SAFE apparatus comprises of a dropping funnel, a cooling trap and a central head bearing two “legs”, which separately fix an evaporating flask and collecting flask with ground joints. The head, “legs” and evaporating flask are thermostatically heated via outlets and water bath, whereas cooling trap and collecting flask are cooled with liquid nitrogen. High vacuum is introduced into SAFE apparatus via the outlet connected to cooling trap, while the stopcock of the
dropping funnel is closed. As for the high vacuum system, it can be obtained from a roughing pump and an oil diffusion pump. A roughing pump is responsible for decreasing the pressure inside diffusion chamber to lower or equal to $10^{-2}$ mbar. It is the low pressure that enables diffusion pump to attain a high vacuum ($10^{-3}$ to $10^{-9}$ mbar) since a diffusion pump will fail in activating or exhausting against atmospheric pressures (Hoffman, 1979). With sample dropped into the evaporating flask from the dropping funnel, the distillation procedure starts. Once the sample is vaporized, the volatile compounds, solvent and water enter the distillation head, and condense in the collecting flask which is cooled with liquid nitrogen. By contrast, non-volatile compounds remain in the evaporating flask, which are depleted of volatiles.

The application of SAFE provides a high recovery of volatiles and allows non-volatiles to be carefully separated from volatiles, which enables SAFE to be suitable for high-fat (50% fat) matrices (Hausch, Lorjaroenphon, & Cadwallader, 2015), aqueous foods (e.g., milk and beer) and aqueous food suspensions (e.g., fruit pulps). SAFE has become a preferred technique for a “clean” and high-recovery volatiles extract (W. Zhu & Cadwallader, 2019). According to Elmore (2015), the aroma extract from SAFE can be considered as the volatiles that mostly represent the aroma in the food itself. Besides its high efficiency in volatiles extraction, the stability of compact distillation unit in SAFE system also enables it to have the advantage of saving time as well as costs (Zhou, Liu, Liu, & Song, 2019). While volatile extracts obtained by SAFE are highly “clean”, they are not dry, so the volatile extract must be dried over anhydrous sodium/magnesium
sulfate for water removal (Tomaszewski, 2016). After dried and further concentrated, SAFE volatiles extract can be directly used for gas chromatographic analysis.

2.3.3 Stir Bar Sorptive Extraction (SBSE)

Although SAFE can provide a highly pure extract of volatile compounds, it requires a relatively bigger amount of sample, and it is time-consuming. In the case of sample amount limited, sorptive extraction would be a preferred extraction method, as it only requires a small amount of sample, and it is simple as well.

One of the efficient sorptive methods is stir bar sorptive extraction (SBSE). SBSE method was first introduced in 1999 as a technique for volatile analytes enrichment. In SBSE, a magnetic stir bar coated with polydimethylsiloxane (PDMS) phase, commercially known as “Twister”, is used to stir into the aqueous sample. Through extraction, volatile and semi-volatile analytes are sorbed into the PDMS phase. Afterwards, followed by thermal desorption (TD) and gas chromatographic analysis, volatile compounds are desorbed from the “Twister” and transferred into a GC system (Baltussen, Sandra, David, & Cramers, 1999; Horák, Kellner, Čulík, Jurková, & Čejka, 2007).

The principle of SBSE is very similar with another sorptive extraction method — solid phase micro extraction (SPME). In SBSE, PDMS stir bar is placed in aqueous sample (liquid phase), whereas in SPME, the PDMS fiber is exposed to the headspace (HS) of sample (gas phase) (Figure 10). These two extraction methods both use PDMS phase for sampling, however, the volume of PDMS layer in SBSE is 50 to 250 times bigger than SPME (Jakubowska, Henkelmann, Schramm, & Namiesnik, 2009). As a
result, SBSE provides a higher recovery and sensitivity than SPME (Castro & Ross, 2015).

Figure 10. Simplified diagram of (A) stir bar sorptive extraction (SBSE) and (B) solid phase micro extraction (SPME).
(Source: Gama, Melchert, Paixão, & Rocha, 2019).
3.1 OVERVIEW OF EXPERIMENTAL FLOW

Volatile Compounds were isolated from pennycress seeds by solvent extraction and solvent assisted flavor evaporation (SAFE). The volatiles were analyzed with aroma extract dilution analysis (AEDA) involving gas chromatography-olfactometry (GC-O). The volatiles were also analyzed via gas chromatography-mass spectrometry (GC-MS) to aid in compound identification. The experimental work flow is shown in Figure 11 below.

![Experimental work flow](image)

Figure 11. Experimental work flow.

3.2 MATERIALS

3.2.1 Pennycress

Wild-type pennycress seeds MN106 and four types of mutant pennycress seeds Nutty (aop2-1), A7-95 (tt4-1), A7-191 (tt8-2), A7-63 were used in this research. They were all planted in St. Paul, MN, harvested in June 2018, and stored in laboratory at room temperature (US20190225977, 2019; US20190082718, 2019).

All pennycress seeds were provided by the research group led by Dr. David Marks (the University of Minnesota Pennycress breeding program).
3.2.2 Chemicals

Diethyl ether (stabilized with BHT) was obtained from Acros Organics (Morris Plains, NJ, USA), and BHT was removed from diethyl ether through fractional distillation. Anhydrous magnesium sulfate was obtained from Sigma-Aldrich (St. Louis, MO, USA). Boileezers were obtained from Fisher Scientific Co. (Pittsburgh, PA, USA). A mixture of n-alkanes standards C₆ to C₂₅ (2ml/L each, in pentane) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Standard compounds 2,5-dimethyl-3-methoxypyrazine was from Ambeed, Inc. (Arlington Hts, IL, USA), and allyl isothiocyanate, hexanol, (E)-2-octenal, acetic acid, (E)-2-nonenal, 1-octanol, 1-nonanol, isovaleric acid, phenethyl alcohol were all obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.3 METHODS

3.3.1 Isolation and Extraction of Volatile Compounds

1000g of wild-type pennycress seeds were ground into flour using a coffee grinder (Epica Products, Brick, NJ, USA). Aliquots of 300g, 300g, 200g, and 200g of pennycress flour were separately weighed into 1000ml flasks, with 380ml, 380ml, 270ml, 270ml of distilled diethyl ether added correspondingly. A magnetic stir bar (3 inch × 1/2 inch) was added to each flask and then the flasks were placed on a magnetic stir plate, stirring for 90 min. The solvent extract was filtered using a 24.0 cm Whatman No.1 filter paper (Whatman, Hillsboro, OR, USA), collecting filtrate and solid residue separately. The solid residue was re-extracted with distilled diethyl ether again (for 90 min), and
filtered again. The extraction process repeated a third time. The collected filtrates were pooled to total 1450ml. The pooled filtrate was transferred into a round bottle flask, boilerezers added, and then connected to a fractional distillation apparatus. Through distillation, 650ml of solvent extract was the final volume used for solvent assisted flavor evaporation (SAFE).

3.3.1.1 Solvent Assisted Flavor Evaporation (SAFE)

Extract was added to the dropping funnel of SAFE apparatus “BAENG” (Glasbläserei Bahr, Manching, Germany) (Figure 12), which was heated to 45°C by a heating bath circulator (EX100, Neslab Instruments Inc., Newington, NH, USA). The receiving flask was immerged with liquid nitrogen, while the vacuum safety cooling trap of the instrument was filled with liquid nitrogen. The SAFE apparatus was connected to a high vacuum system, which contained an oil diffusion pump (Diffstak, Edwards, UK) and a roughing pump (General Electric Company, New York, USA), maintaining a final vacuum to equal to or lower than $10^{-4}$ Torr. When opening the valve, extract from dropping funnel started going into the SAFE extraction system, and the volatile extraction procedure began. Non-volatile compounds dropped into the round bottle flask immersed in 45°C warm water, whereas volatile compounds were evaporated, and then condensed in the receiving flask. During SAFE extraction, non-volatiles (190ml) and volatiles (430ml) were totally collected. After SFAE extraction finished, the volatile extract was thawed at room temperature and dried over anhydrous magnesium sulfate, stirred for 15 min and filtered. Finally, the volatile extract was concentrated under a
gentle stream of nitrogen to approximately 0.4ml and transferred to a 2ml vial for storage until instrumental analysis.

The non-volatiles from SAFE extraction was collected and it was used as the sample for “volatiles analysis of pennycress oil” since it was mainly fat. Pennycress non-volatiles extract (called as pennycress oil) was extracted with distilled diethyl ether (via solvent extraction method) for 90 min, then further extracted via SAFE (procedures were the same as mentioned above). After SAFE extraction, volatiles extract was collected as “pennycress oil volatiles extract”.

Figure 12. Illustration of solvent assisted flavor evaporation (SAFE) method. (Source: Engel et al., 1999)
3.3.1.2 Stir Bar Sorptive Extraction (SBSE)

5 g of pennycress seeds were weighed, ground into flour and mixed with 20ml of distilled water in a 50ml flask. A PDMS-coated stir bar “Twister” (10 mm × 0.5 mm, Gerstel, Mülheim an der Ruhr, Germany) was then immersed into the above solvent mixture. The flask was sealed and located on a stir plate for extraction overnight. After overnight extraction, the stir bar was removed from solvent mixture, and rinsed with distilled water, then wiped with delicate task wiper (Kimberly-Clark Worldwide, Inc., Roswell, GA). Afterwards, the stir bar was ready for GC analysis.

3.3.2 Aroma Extract Dilution Analysis (AEDA)

Volatile extract was diluted with distilled diethyl ether to obtain different concentrations: 1/10, 1/50, 1/250, 1/1000, 1/3000, 1/4000, 1/5000 of the stock volatiles extract (Figure 13). Each dilution was analyzed by three panelists (two females and one male) in GC-FID-O, from non-diluted to highest diluted sample. To avoid olfactory fatigue of panelists, the sniffing procedure of each sample was divided into 2 GC time sections, 2-25 min and 24-47 min. In each sniffing test, each panelist smelled one GC time section (2-25min or 24-47min) of each sample. During the sniffing test, panelists were asked to describe the odor of perceived odorants, and the corresponding eluting times were recorded together via a voice recorder. Each perceived aroma was assigned a flavor dilution (FD) factor to the highest dilution that the aroma was still perceived by panelists.
3.3.3 Separation and Detection of Volatile Compounds

3.3.3.1 Gas Chromatography-Flame Ionization Detection-Olfactometry (GC-FID-O)

A HP 5890 Series II Gas Chromatograph (Hewlett-Packard, Alto Palo, CA, USA) was employed for GC-FID-O analysis. A 30 m length × 0.25 mm I.D. × 0.25 µm film thickness DB-WAX column (J&W Scientific Inc., Folsom, CA, USA) was used for chromatographic separation. 2µl of sample was manually injected via a 10 µl syringe (model 80377, Hamilton Co., Reno, NV, USA) and injection port temperature was 220°C. The carrier gas was hydrogen, with a constant pressure of 70.3 kPa and 10:1 split ratio, and the flow was 1.7ml/min. The temperature program of GC oven was as follows: the initial temperature was 40°C, with 0 min hold time, increased to 80°C with a 3°C /min rate, then increased to 220°C with a 5°C /min rate and held for 5 min. The total run time of the chromatographic analysis was 47 min. The effluent was split into two sections at the end of GC column by a splitter. One portion was directed into flame ionization detector (FID), held at 250°C, and the other portion was introduced into a sniffing port, where the temperature was controlled by a variable autotransformer (type 3PN1010,
Staco Energy Products Co., Dayton, OH, USA). A gentle stream of humidified air was also introduced to the sniffing port, providing a humid sniffing environment for panelists.

### 3.3.3.2 Gas Chromatography-Mass Spectrometry-Olfactometry (GC-MS-O)

The GC-MS-O analysis was performed using an Agilent 6890 Gas Chromatograph (Agilent Technologies, Inc., Palo Alto, CA, USA), a Hewlett Packard 5973 Mass Selective Detector (Hewlett-Packard, Alto Palo, CA, USA), and a sniffing port. A 30 m length × 0.25 mm I.D. × 0.25 µm film thickness HP-5MS column (Agilent Technologies, Santa Clara, CA, USA) was used for chromatographic separation. 2µl of sample was manually injected via a 10 µl syringe, and injection port temperature was 220°C. Carrier gas was helium, with a constant pressure of 94.3 kPa and 10:1 split ratio, and the flow was 1.7ml/min. The temperature program was the same that described above. An MSD Chemstation was used to control the instrument and analyze data. The Mass spectrometer transfer line was 230°C, and MS parameters were as follows: operation was in electron impact (EI) ionization mode at 69.9 eV, scanning a range of 29 to 350 m/z, with 4.37 scans/sec. In the sniffing port, the temperature was maintained at 225°C by a variable autotransformer (POWERSTAT®, The Superior Electric Co., Bristol, CT, USA), and a gentle stream of humidified air was introduced into sniffing port as well. Compounds were tentatively identified by comparison of scanned mass spectrum with MS fragmentation patterns in NIST11.L Mass Spectral Library.

### 3.3.3.3 Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS was performed using an Agilent Technologies 6890N Network GC System (Agilent Technologies, Inc., Palo Alto, CA, USA) and an Agilent Technologies
5973 inert Mass Selective Detector. A Stabilwax ®-DA column (30 m length × 0.25 mm I.D. × 0.25 µm film thickness, Restek, Bellefonte, PA, USA) was used for chromatographic separation. 2µl of sample was manually injected via a 10µl syringe, and injection temperature was 220°C. Carrier gas was hydrogen, with a constant pressure of 30.0 kPa and 10:1 split ratio, and the flow was 1.7ml/min. The temperature program was the same that described above. An MSD Chemstation was used for instrument control and data analysis. The mass spectrometer trans line temperature was 230°C, the MS was operated in electron impact (EI) ionization mode at 70eV with a mass range of 29-350 m/z. Compounds were tentatively identified by comparison of scanned mass spectrum with MS fragmentation patterns in pal600k.L Mass Spectral Library.

3.3.3.4 Gas Chromatography-Flame Ionization Detector (GC-FID)

The GC-FID was performed using a Hewlett Packard HP 5890 Series II GC system (Hewlett-Packard, Alto Palo, CA, USA), with a HP-5MS column (30 m length × 0.25 mm I.D. × 0.25 µm film thickness, Agilent Technologies, Santa Clara, CA, USA). Injection port temperature was 220°C, and the detector temperature was 250 °C. 2µl of sample was manually injected via a 10 µl syringe. Carrier gas was hydrogen, with a constant pressure of 70.3 kPa and 10:1 split ratio, and the flow was 1.7ml/min. The temperature program was the same that described above.

3.3.4 Identification of Volatile Compounds

Compounds were tentatively identified by comparison of scanned mass spectrum with MS fragmentation patterns in pal600k.L or NIST11.L Mass Spectral Library. To further verify identification, Linear Retention Indices (LRIs) were calculated and
compared with published Retention Index (RI) value listed on literatures. 2µl mixture of 
C₆ - C₂₅ alkanes was injected with 2µl volatiles extract via a 10µl syringe to obtain the 
retention time (RT) of each compound in sample and each alkane, for attaining the 
calculation of RI. The formula for RI calculation was as follow (Kováts, 1958):

\[ I = 100 \times \left[ n + \left( t_u - t_n \right) / \left( t_N - t_n \right) \right], \]

\( I \): retention index (RI); 
\( n \): the number of carbon atoms in the alkane with the lower retention time; 
\( N \): the number of carbon atoms in the alkane with the higher retention time; 
\( t_u \): the retention time of unknown compound; 
\( t_N \): the retention time of N-alkane; 
\( t_n \): the retention time of n-alkane.

To further confirm the identification of assumed compound, a standard compound 
was injected and detected in GC-FID-O. Once the retention time and odor of the standard 
compound matched the target peak in sample, the standard compound was injected with 
the volatiles extract sample in GC-FID-O for monitoring the standard compound in 
volutiles extract. Co-chromatography of the standard with the tentatively identified 
compound was considered to be an identification.
CHAPTER 4. RESULTS AND DISCUSSION

4.1 GAS CHROMATOGRAPHY-OLFACTOMETRY ANALYSIS

Pennycress volatiles extract were analyzed using GC-FID-O on DB-WAX column and GC-FID on HP-5MS column, and their corresponding gas chromatograms are shown in Figure 14 and 15 separately. From these two gas chromatograms, the obvious difference on peak separation implied that pennycress volatiles extract had a better separation on DB-WAX column, a polar column. So, in this research, a polar column (DB-WAX or Stabilwax column) was used in GC-O analysis, combined with AEDA, for the screening of aroma-active compounds.

The GC-O analysis was conducted by three panelists (1 male and 2 females). With the summary of sniffing results from all panelists, a total of 29 odor-active compounds were found, in which, each odorant had a FD factor not less than 10, and all odorants were able to be perceived by at least two panelists at a very close retention time (less than 0.3 min of time difference).
Figure 14. Gas chromatogram of pennycress volatiles extract from SAFE by GC-FID-O on a DB-WAX column.

Figure 15. Gas chromatogram of pennycress volatiles extract from SAFE by GC-FID on a HP-5MS column.
4.1.1 Identification of Volatile Compounds in Pennycress

By performing different dilutions in AEDA, possible key aroma-active compounds were determined as shown in the aromagram of pennycress volatiles extract (Figure 16). This plot displays three panelists’ olfactory perception of pennycress volatiles as fractionated portions at specific retention indices (Kamath, Asha, Ravi, Narasimhan, & Rajalakshmi, 2001). Retention indices (RI) were calculated with the aid of external n-alkanes, corresponding to retention times.

Figure 16. Combined aromagram results of pennycress volatiles extract on DB-wax column for three panelists.

* The height of the lines corresponds to highest flavor dilution value reached by panelist 1 (◆), panelist 2 (■) and panelist 3 (▲). In peaks 6, 8, 19 results for the panelist 1 and 2 were identical, so only results of panelist 2 were clearly visible. In peaks 12, 15, 16, 23 results for the panelist 1 and 3 were identical, so only results of panelist 1 were clearly visible. In peaks 1, 5, 10, 22, 24, 25, 26, 29 results for the panelist 2 and 3 were identical, so only results of panelist 2 were clearly visible.
For compound identification, the mass spectra were obtained via GC-MS on a Stabilwax column, and its total ion chromatogram (TIC) is shown in Figure 17. In addition, to reconfirm that Stabilwax column, a polar column is more suitable than non-polar column, for the separation of volatile compounds in pennycress extract, a HP-5MS column was used in the GC-MS-O to analyze pennycress extract sample (Figure 18). The comparison of TICs acquired from Stabilwax and HP-5MS column indicated again that a polar column provided a better separation and resolution for volatile compounds in pennycress extract, verifying that a polar column is preferred in this research.

![Figure 17](image1.png)

Figure 17. Total ion chromatogram of pennycress volatiles extract from SAFE by GC-MS on Stabilwax column.
Figure 18. Total ion chromatograph of pennycress volatiles extract from SAFE by GC-MS-O on HP-5MS column.

The aroma-active compounds were located in the gas chromatographic profile by matching retention times of descriptive odors in an aromagram with retention times of FID-signal by GC-FID-O on DB-WAX column. A match of gas chromatogram and aromagram is shown in Figure 19 below.
Figure 19. Gas chromatogram (top) and aromagram by three panelists (bottom) of pennycress volatiles extract on DB-WAX column.
Among 29 aroma-active compounds detected by GC-O on DB-WAX column, 10 volatile compounds (Table 2) were identified based on mass spectra, retention indices, aroma descriptors and GC-O analysis of standard chemicals. In 10 of these identified odorants, 8 were fully identified and verified by GC-O analysis of standard compounds, whereas two compound identities were uncertain between two isomers due to the lack of a standard compound of one isomer and the difficulty of isomer separation.
Table 2. Volatile compounds identified in pennycress volatiles extract.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Compounds</th>
<th>Aroma Descriptors</th>
<th>FD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RI&lt;sup&gt;c&lt;/sup&gt;</th>
<th>ID&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexanal</td>
<td>green</td>
<td>10</td>
<td>1088</td>
<td>RI&lt;sup&gt;e&lt;/sup&gt;, MS&lt;sup&gt;f&lt;/sup&gt;, AD&lt;sup&gt;g&lt;/sup&gt;, Std&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>Allyl isothiocyanate</td>
<td>onion-like, grassy</td>
<td>50</td>
<td>1366</td>
<td>RI, MS, AD, Std</td>
</tr>
<tr>
<td>9</td>
<td>(E)-2-Octenal</td>
<td>earthy, vegetable-like</td>
<td>100</td>
<td>1439</td>
<td>RI, MS, AD, Std</td>
</tr>
<tr>
<td>10</td>
<td>2,5-Dimethyl-3-methoxypyrazine</td>
<td>grassy, nutty</td>
<td>4000</td>
<td>1445</td>
<td>RI, MS, AD, Std</td>
</tr>
<tr>
<td>11</td>
<td>2,6-Dimethyl-3-methoxypyrazine</td>
<td></td>
<td></td>
<td></td>
<td>N/A&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>Acetic acid</td>
<td>sour, acid</td>
<td>100</td>
<td>1463</td>
<td>RI, MS, AD, Std</td>
</tr>
<tr>
<td>16</td>
<td>(E)-2-Nonenal</td>
<td>woody, vegetable-like</td>
<td>50</td>
<td>1546</td>
<td>RI, MS, AD, Std</td>
</tr>
<tr>
<td>18</td>
<td>1-Octanol</td>
<td>citrus, fruity, grassy</td>
<td>10</td>
<td>1579</td>
<td>RI, MS, AD, Std</td>
</tr>
<tr>
<td>19</td>
<td>1-Nonanol</td>
<td>green, woody</td>
<td>50</td>
<td>1682</td>
<td>RI, MS, AD, Std</td>
</tr>
<tr>
<td>19</td>
<td>(R)-2-Methylbutanoic acid</td>
<td>cheesy</td>
<td>100</td>
<td>1687</td>
<td>N/A</td>
</tr>
<tr>
<td>19</td>
<td>3-Methylbutanoic acid</td>
<td></td>
<td></td>
<td></td>
<td>RI, MS, AD, Std</td>
</tr>
<tr>
<td>25</td>
<td>Phenethyl alcohol</td>
<td>rose, coconut</td>
<td>50</td>
<td>1946</td>
<td>RI, MS, AD, Std</td>
</tr>
</tbody>
</table>

<sup>a</sup> Flavor Dilution Factor; <sup>b</sup> Retention Index of unknown compounds calculated on a DB-WAX column; <sup>c</sup> Retention Index of standard chemicals calculated on DB-WAX column; <sup>d</sup> Identification Basis; <sup>e</sup> Retention Index calculated was agreed with retention index of standard chemicals; <sup>f</sup> Mass Spectrum detected was agreed with pal600k mass spectral database; <sup>g</sup> Aroma descriptors were agreed with literature; <sup>h</sup> Standard compounds injected had similar retention time and odor with unknown compounds; <sup>i</sup> Not available.
Besides the 10 (above) volatile compounds identified with the aid of standard chemicals verification via GC-O, additional volatile compounds were tentatively identified through GC-O and GC-MS data. While GC-O provides the odor character of unknown odorants, GC-MS can produce mass spectra and retention index of those unknown compounds, so, by comparing odor descriptions, mass spectra and retention index with library/reference, the identity of unknown volatiles in pennycress can be tentatively determined. A total of 7 tentatively identified compounds is shown in Table 3. Although the identities of these 7 compounds are just tentatively determined, they all have been previously reported in diverse Brassica species.

In addition, 60 volatile compounds (present in Appendix 1) were tentatively identified on the basis of mass spectrum and retention index. Even though compounds identification solely via GC-MS does not provide any indication of the contribution of individual volatile compounds to the overall pennycress aroma, it did create a more complete profile of volatile compounds in pennycress. Since this is the first work on aroma analysis of pennycress, making a more comprehensive database of volatile compounds in pennycress can provide more information for further studies.
Table 3. Tentatively identified volatile compounds in pennycress volatiles extract.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>GC-O RT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Aroma descriptors&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tentative compound identification</th>
<th>RI Cal (GC-O)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>RI Cal (GC-MS)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Ref&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Previously reported&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>12.709</td>
<td>grassy, fruity</td>
<td>1-Pentanol</td>
<td>1266</td>
<td>1253</td>
<td>1256</td>
<td>[1]</td>
</tr>
<tr>
<td>4</td>
<td>13.680</td>
<td>green</td>
<td>Octanal</td>
<td>1291</td>
<td>1289</td>
<td>1286</td>
<td>[1]</td>
</tr>
<tr>
<td>5</td>
<td>14.580</td>
<td>metallic, mushroom-like</td>
<td>(E)-2-Penten-1-ol</td>
<td>1315</td>
<td>1316</td>
<td>1335</td>
<td>[2]</td>
</tr>
<tr>
<td>6</td>
<td>14.680</td>
<td>earthy, nutty</td>
<td>(E)-2-Heptenal</td>
<td>1317</td>
<td>1321</td>
<td>1320</td>
<td>[3]</td>
</tr>
<tr>
<td>8</td>
<td>17.450</td>
<td>green, metallic</td>
<td>(Z)-3-Hexen-1-ol</td>
<td>1389</td>
<td>1386</td>
<td>1338</td>
<td>[2]</td>
</tr>
<tr>
<td>13</td>
<td>22.196</td>
<td>green, cucumber-like</td>
<td>(E, E)-3,5-Octadien-2-one</td>
<td>1535</td>
<td>1519</td>
<td>1575</td>
<td>[4]</td>
</tr>
<tr>
<td>20</td>
<td>27.406</td>
<td>oily, fatty</td>
<td>Pentanoic acid</td>
<td>1730</td>
<td>1739</td>
<td>1721</td>
<td>[5]</td>
</tr>
</tbody>
</table>

<sup>a</sup>Approximate retention time of odor perceived by panelists via GC-O; <sup>b</sup>Aroma described by panelists via sniffing test in GC-O; <sup>c</sup>Retention Index of unknown compounds calculated based on RT from GC-O on a DB-WAX column; <sup>d</sup>Retention Index of unknown compounds calculated based on RT from GC-MS on a DB-WAX column; <sup>e</sup>Retention Index from references (polar columns); <sup>f</sup>Literature references that have reported the tentatively identified compound.

References:
[1] Zhao, Tang, & Ding (2007): potherb mustard (Brassica juncea, Coss.);
[2] Ikeura, Kobayashi, & Hayata (2012): cabbage (Brassica oleracea var. capitata L.);
[3] Krumbein, Kläring, Schonhof, & Schreiner (2010): broccoli (Brassica oleracea var. italica);
4.1.2 Analysis of Odor-Active Volatile Compounds

Among 10 identified odor-active volatile compounds (Table 2), the aromagram (Figure 16) indicated that 2,5-/2,6-dimethyl-3-methoxypyrazine (no.10) had the highest FD factor of 4,000, which was 40 times of the maximum FD factor of any other odor-active compounds. Allyl isothiocyanate (AITC) (no.7), commonly considered as a key volatile compound in pennycress, had a FD factor of 50. Other identified volatiles are: “green” hexanal (no.1, FD 10), “earthy” (E)-2-octenal (no.9, FD 100), “sour” acetic acid (no.11, FD 100), “woody” (E)-2-nonenal (no. 14, FD 50), “fruity, grassy” 1-octanol (no.16, FD 10), “green” 1-nonanol (no.18, FD 50), “cheesy” (R)-2-methylbutanoic acid /3-methylbutanoic acid (no.19, FD 100) and “rose-like” phenethyl alcohol (no. 25, FD 50). The structures of identified compounds are shown in Figure 20 below.
Figure 20. Structures of 10 identified odorants in wild-type pennycress aroma extract.

4.1.2.1 Allyl isothiocyanate (AITC)

AITC is considered as one of the most important volatiles in pennycress. It is a hydrolysis product of a glucosinolate (sinigrin), which is abundant in wild-type pennycress seeds with a content higher than 100 μmol/g of seeds (Chopra, Johnson, et al., 2019). AITC (no.7) (Figure 21) was perceived by all three panelists in pennycress volatiles extract being described as “onion-like”, “grassy”, matching the descriptive odor “mustard-like”, “onion-like”, “horseradish-like” in previous researches (Giarratana et al., 2015; Kroener & Buettner, 2018; McGorrin, 2011; Muscolino et al., 2016). An AITC
standard solution had the same sensory character (“onion-like”) on GC-O sniffing as odorant no.10 in pennycress volatiles extract.

![GC-MS mass spectrum of allyl isothiocyanate (AITC) (no.7).](image)

Figure 21. GC-MS mass spectrum of allyl isothiocyanate (AITC) (no.7).

Based on pennycress having a strong mustard character, AITC can be considered as a character-impact volatile in pennycress. AITC is an organosulfur phytochemical with high volatility, and commonly occurs in the family Brassicaceae (Kim et al., 2015). AITC is derived from sinigrin (allyl-glucosinolate), which is the only glucosinolate present in pennycress (Hojilla-Evangelista et al., 2015). When pennycress seeds were ground, cells were disrupted and cell-bond myrosinase would interact with sinigrin. With water or moisture contacted, the hydrolysis of sinigrin happened, resulting in the production of AITC (Dai & Lim, 2014).

As AITC is a hydrolysis product of sinigrin, it does not exist in pennycress seed itself – there must be an enzymatic hydrolysis of the sinigrin to produce free AITC. Since the generation of AITC needs adequate moisture for enzymatic activity to occur, for detecting AITC in pennycress volatiles extract, participation of water/moisture in
volatiles extraction process or in sample preparation procedure is required. Thus, it is interesting that AITC was detected when the solvent (diethyl ether) used for volatiles extraction contained no moisture and water was not added to the pennycress seed flour at any steps in flavor analysis. Actually, the pennycress seed used in this research had a moisture content of 9%. Hence, even if the flavor isolation process didn’t contain any added-water to promote/allow AITC formation, a certain amount of AITC was present in the pennycress seeds. By contrast, it is reported that excess water is possible to restrict AITC evolution through binding sites or diluting substrate or/and enzyme system (Hensley, 2005), which are also vital for AITC production. Hensley (2005) added different amount of deionized water to two jars of pennycress seed meals and compared AITC values, from which, it was found that no statistical difference was perceived between the water added and “dry” samples. Hence, while admitting water was needed for AITC generation, Hensley (2005) claimed that relative humidity didn’t impact the concentration of AITC in the pennycress seed meals. Thus, though no water was deliberately added in sample treatment or volatile extraction process, AITC was still found in the volatiles extract. According to GC-O analysis, extracted AITC exceeded the detection limit for both instrumental detector and human olfaction. So, even though AITC might be not completely hydrolyzed from pennycress seeds, the concentration of the extracted and detected AITC were enough for its identification in this research.

Due to the undesirable “onion-like” aroma, the presence of AITC in a food product will impact consumer’s sensory perception thus influence their acceptance. With a high content of protein, pennycress is highly expected to be an alternative protein
source. Nevertheless, AITC might interact with amino acids and proteins in pennycress seeds (Kawakishi & Kaneko, 1987; Murthy & Rao, 1986), causing modified proteins. The functionality of whey protein isolate was reported to be modified after reaction with AITC (Keppler et al., 2017), while Kawakishi & Kaneko (1987) observed a decrease in the digestibility of protein-AITC adducts. Due to the reaction between AITC and protein, the existence of AITC in pennycress still makes an impact on the odor of pennycress protein.

Although pennycress protein has a promising potential as a food ingredient, today it is considered as a by-product from pennycress oil press cake, which remains after pennycress oil processing (Hojilla-Evangelista et al., 2015). At present, pennycress oil is the primary application of pennycress production, however, pennycress oil so far is just suitable for biofuel instead of cooking oil due to its unacceptably high content of erucic acid (Dorn et al., 2015). This high level of erucic acid is considered to be harmful to human health. Despite that, McGinn et al. (2019) claimed that pennycress oil had a promising potential to be developed as edible cooking oil, and they are working on that via genetic modification. However, even though health issues of pennycress oil may be solved, its unpleasant flavor problem might still remain.

To understand aroma profile of pennycress oil, we used SAFE to extract volatile compounds from pennycress oil, followed by GC-MS analysis with n-alkanes added. The GC-MS profile (with C_6 to C_{25} of n-alkanes added) of volatiles extract from pennycress oil is similar to the volatiles extract from ground pennycress seeds (shown in Appendix 2). Besides, AITC was detected in aroma extract of pennycress oil, which demonstrated
that AITC was likely to have an influence on the aroma of pennycress oil as well as the seeds.

Since the presence of glucosinolate has restricted the application of pennycress, substantial research is under way to remove glucosinolates from pennycress via breeding programs. The research group led by David Marks (University of Minnesota) has succeeded in breeding mutant pennycress (named as “Nutty”) with low glucosinolate levels (less than 20 μmol/g of seeds), a significant decrease compared with wild-type pennycress (over 100 μmol/g of seeds) (Chopra, Johnson, et al., 2019). According to Chopra et al. (2019), besides a reduction of sinigrin detected in Nutty mutant, an obvious accumulation of 3-methylsulfinylpropyl and 3-methylthiopropyl glucosinolates was also found (Figure 22), and they are precursors of sinigrin.

![HPLC analysis of desulfated glucosinolates from wild-type (top) and the Nutty mutant (bottom) pennycress.](source)

(Source: Chopra, Johnson, et al., 2019)
The Nutty mutant was associated with the mutation in a gene (named Ta-aop2-1), which encodes an enzyme with Arabidopsis AOP2, a glucosinolate biosynthetic gene that catalyzes the conversion of 3-methylsulfinylpropyl glucosinolate to sinigrin (Figure 23). Hence, the mutant gene limited the synthesis of the glucosinolate sinigrin in Nutty mutant pennycress, regulating the generation of volatile AITC. Yet at the same time, the accumulation of 3-methylsulfinylpropyl glucosinolate (glucoiberin) and 3-methylthiopropyl glucosinolate (glucoibervirin) in Nutty mutant type would bring about their corresponding hydrolysis products 3-methylsulfinylpropyl isothiocyanate and 3-methylthiopropyl isothiocyanate. According to Velisek (2014), 3-methylsulfinylpropyl isothiocyanate contributes to broccoli odor while 3-methylthiopropyl isothiocyanate has cauliflower and horseradish odor note. Therefore, in mutant type Nutty, though the emission of sinigrin-derived volatiles has been limited, the release of glucoiberin-derived and glucoibervirin-derived volatiles are accelerated. But the reason for the overall decrease in glucosinolates in Nutty mutant still remains unclear, which needs further work for a complete understanding.

\[
\begin{array}{c}
3\text{-methylthiopropyl} \\
\downarrow \\
3\text{-methylsulfinylpropyl} \\
\downarrow \\
AOP2 \\
\downarrow \\
sinigrin
\end{array}
\]

Figure 23. Terminal biosynthetic pathway of sinigrin.
(Source: Chopra, Johnson, et al., 2019)
According to Chopra et al. (2019), Nutty mutant seed was reported to “have a pleasant and nutty flavor”, and the garlicky odor of pennycress leaves was considered to be absent as well. To basically learn about the change of aroma profile of pennycress with different glucosinolate concentrations, we performed aroma analysis using stir bar sorptive extraction (SBSE) on wild-type and Nutty mutant pennycress seeds. SBSE is simple and efficient for volatiles extraction, more importantly, it is suitable for small amounts of sample, which satisfied the limited amount (5 gram) of mutant type provided for this research.

Through SBSE, volatile compounds were absorbed in PDMS-coated magnetic stir bar and further analyzed by GC-O, from which, “onion-like” AITC was perceived by panelists in wild-type pennycress seeds. By contrast, AITC was not detected in the Nutty mutant pennycress seeds, via both FID detector and sniffing port, which implied that the concentration of AITC released from Nutty mutant pennycress seeds was below detection limit of both human olfactory and instrument. Although SBSE is less sensitive than SAFE, which we used for volatiles extraction from a large amount of seeds, the apparent difference in AITC detected from two different types indicated that SBSE was sufficient for AITC comparison.

In addition to AITC detection, we also compared overall aroma of the water extract of wild-type and mutant type ground pennycress seeds. As for the mutant type, the water extract of its ground seeds exhibited an unpleasant overall aroma compared with pennycress whole seeds. While claimed that Nutty mutant seeds had a pleasant flavor, in our judgement, the water extract of its ground seeds presented a strong “onion-like”
overall aroma. In terms of water extract of different pennycress types, the “onion-like” odor of Nutty mutant was weaker than the wild-type. That could be because less AITC was released, while more 3-methylsulfinylpropyl and 3-methylthiopropyl isothiocyanates were emitted from Nutty mutant. Overall, the odor of Nutty mutant could not be considered “pleasant” or acceptable, which contradicted to the conclusion of Chopra et al. (2019). However, since seed disruption and solvent extraction could enhance the production and release of volatile compounds from pennycress, it would be reasonable that whole seeds of mutant pennycress and the aroma extract of its ground seeds had different overall aroma profiles. However, when introducing pennycress seeds into food product development, it is inevitable that processing may lead to enhancement of some unpleasant volatiles. Hence, the problem of unpleasant odor hasn’t been solved in low-glucosinolate mutant pennycress.

In conclusion, odor analysis of Nutty mutant type demonstrated that glucosinolate is not the only precursor that causes unpleasant volatiles, and AITC is not the only volatile compound that contributes to pennycress’s unaccepted aroma. So, in addition to the “well-known” AITC in pennycress, other volatiles must also be considered.

4.1.2.2 2,5-/2,6-Dimethyl-3-methoxypyrazine

In terms of flavor dilution (FD) value, no.10 odorant was a unique odorant in all extracted pennycress volatiles. No.10 odorant obtained a FD factor of 4,000; much higher than any other odorants. Odorant no.10 was tentatively identified via mass spectra to be 2,5-dimethyl-3-methoxypyrazine (Figure 24) or 2,6-dimethyl-3-methoxypyrazine (Figure 25).
To further clarify the identity of no.10 odorant, a pure standard was required. Unfortunately, very little information about 2,6-dimethyl-3-methoxypyrazine is in the literature. Also, a pure standard of 2,6-dimethyl-3-methoxypyrazine was not available for purchase. So, only the 2,5-dimethyl-3-methoxypyrazine standard was used for compound identity verification, from which, we found that 2,5-dimethyl-3-methoxypyrazine
standard (RT 19.365 min) had an almost same retention time as no.10 odorant (RT 19.351 min), and its perceived “grassy” odor is also similar with no.10 odorant.

With dilution analysis on a 2,5-dimethyl-3-methoxypyrazine solution (stock solution: 1µl/1ml in distilled diethyl ether), we found that its 4,000 times diluted solution could be still perceived through a sniffing port in GC-O, exhibiting an extremely low odor threshold. Even though the concentration of our stock solution of 2,5-dimethyl-3-methoxypyrazine standard was probably higher than no.10 odorant in pennycress volatiles extract, its capability of being detected after diluting 4,000 times could be enough to imply its extremely low odor threshold, which was consistent with no.10 odorant.

For lack of 2,6-dimethyl-3-methoxypyrazine standard compound, it was difficult to confirm whether 2,6-dimethyl-3-methoxypyrazine would have the same performance as no.10 odorant. Nevertheless, as 2,5-dimethyl-3-methoxypyrazine and 2,6-dimethyl-3-methoxypyrazine are structural isomers, it is highly likely that they have similar odor characteristics. Comparing the mass spectrum of no.10 odorant (Figure 26) with two assumed compounds, the mass spectrum of 2,6-dimethyl-3-methoxypyrazine was shown to be closer than 2,5-dimethyl-3-methoxypyrazine. Thus, we are unable to exclude the possibility of 2,6-dimethyl-3-methoxypyrazine existing in pennycress, even though 2,5-dimethyl-3-methoxypyrazine (RT 19.365 min, “grassy” odor) had almost matched the characteristics of no.10 odorant (RT 19.351 min, “grassy” odor), with similar retention
time and odor descriptors. Accordingly, we cannot rule out that no.10 odorant is the mixture of 2,5-dimethyl-3-methoxypyrazine and 2,6-dimethyl-3-methoxypyrazine.

Figure 26. The comparison of mass spectrum between no.10 odorant (middle), 2,5-diemethyl-3-methoxy pyrazine (upper) and 2,6-diemethyl-3-methoxy pyrazine (bottom).
Unlike AITC, which has been considered to play an important role in pennycress aroma, the detection of 2,5-dimethyl-3-methoxypyrazine or 2,6-dimethyl-3-methoxypyrazine in pennycress seeds was unexpected. Both 2,5-dimethyl-3-methoxypyrazine and 2,6-dimethyl-3-methoxypyrazine have never been reported to be found in mustard family, thus their pathway in pennycress remains unknown and brings interest as well.

Both 2,5-dimethyl-3-methoxypyrazine and 2,6-dimethyl-3-methoxypyrazine are methoxypyrazines, which are claimed to be “very powerful” heterocyclic aromatic compounds with low olfactory thresholds (i.e., as low as 1 nanogram per liter) (Rauhut & Kiene, 2019). Methoxypyrazines have been reported to possess “green, vegetal, herbaceous” aroma notes and have impacted odor characters of wines, green peas, bell peppers, potatoes, beets, asparagus, et al. (Di Gaspero & Foria, 2015; Reynolds, 2010; Taylor, McDougall, & Stewart, 2007).

2,5-dimethyl-3-methoxypyrazine has been reported to have an extremely low odor threshold of 56 ng/L in air by Czerny & Grosch (2000) and 100 ng/L in water by Mihara et al. (1991). 2,5-dimethyl-3-methoxypyrazine has been found in pepper (black and white) and wine (as a flavor defect). According to different literature references, its sensory character has been described differently by various researchers where sample, medium and concentration were changed. Cai et al. (2007) described the odor of 2,5-dimethyl-3-methoxypyrazine as “moldy” and “earthy” from Harmonia axyridis beetles, and Jagella & Grosch (1999) claimed that it smelled “earthy” and “cocoa-like” in black and white pepper, while Botezatu & Pickering (2012) suggested that it brought
“earthy/musty” and “green/vegetal” aromas to wine. 2,5-dimethyl-3-methoxypyrazine is considered as a problem for wine aroma, and it has been found in ladybug-tainted (by either *Coccinella septempunctata* or *Harmonia axyridis* beetles) wine (Botezatu & Pickering, 2012). Cai et al. (2007) stated that 2,5-dimethyl-3-methoxypyrazine was produced by *H. axyridis* through detecting and identifying headspace volatiles released by live *H. axyridis*. With nesting and crushing, ladybug beetles will transfer 2,5-dimethyl-3-methoxypyrazine into grapes thus taint the aroma of wine. While ladybug tainting a field of pennycress seems problematic, the extremely low sensory threshold might make this possible.

2,6-dimethyl-3-methoxypyrazine was stated to have an aroma like “musty”, “foul drains”, or “sour dishcloths” (Yasuhara, Yamanaka, & Ogawa, 1986), similar with the odor character of no.10 odorant (“grassy”). 2,6-dimethyl-3-methoxypyrazine contamination of plants or foodstuffs has been reported to be due to an aerobic bacterium (Mottram, Patterson, & Warrilow, 1984). Schulz et al. (2004) identified both 2,6-dimethyl-3-methoxypyrazine and 2,5-dimethyl-3-methoxypyrazine in the myxobacterium *Chondromyces crocatus* through headspace analysis. Thus, it is possible that either 2,6-dimethyl-3-methoxypyrazine or 2,5-diemethyl isomer detected is derived from microorganisms, which probably exist in soil, as soil has been regarded to be one of primary habitats of bacteria (Effmert, Kalderás, Warnke, & Piechulla, 2012). Bacteria can occur either on the surface of soil or in the soil core, and it can also associate with belowground parts of plants. As Junker & Tholl (2013) suggested, plant scent may be modified by bacteria through direct and indirect mechanisms. Bacteria can stimulate
volatiles emitted from plants due to its resistance to the attacking pathogen and avirulent
pathogen strains, and bacteria can also add new aroma compounds to plants through
catabolism, where plant volatiles are served as carbon source for bacterial. In the
meantime, plant volatiles used as carbon source would be reduced. Besides, bacteria also
have the ability to release volatile compounds with their own metabolism, impacting a
plant’s scent (Helletsgruber, Dötterl, Ruprecht, & Junker, 2017). Above all, bacteria can
have a large effect on the odor profile of plants. Accordingly, the detection of 2,6-
dimethyl-3-methoxypyrazine might be the consequence of microbial interaction.

If either beetles or bacteria is the only way of producing 2,5-dimethyl-3-
methoxypyrazine/ 2,6-dimethyl-3-methoxypyrazine, they can be both considered as
external volatiles detected in pennycress. So, the occurrence of 2,5-/2,6-dimethyl-3-
methoxypyrazine can be regarded to result from contamination from external
environment. We conducted SBSE and GC-O analysis on five types of pennycress seeds,
including wild-type and four different mutant types, in which, no.10 odorant (“grassy”
odor) was perceived by panelists in all analyzed samples. That implied that no.10 odorant
was present in all these pennycress samples, demonstrating that the presence of 2,5-/2,6-
dimethyl-3-methoxypyrazine was not an incidence in specific pennycress sample. It is
possible to be emitted from all pennycress planted in the same cultivation conditions. To
understand the source of 2,5-/2,6-dimethyl-3-methoxypyrazine in pennycress, additional
investigations on environment would be needed, which could further enable to
understand the contribution of 2,5-/2,6-dimethyl-3-methoxypyrazine to pennycress
aroma.
Although the 2,5-/2,6-dimethyl-3-methoxypyrazine detected in pennycress is probably the result of “contamination”, which is possible to control or avoid, as 2,5-/2,6-dimethyl-3-methoxypyrazine has an extremely low odor threshold and thus even a low chance of 2,5-/2,6-dimethyl-3-methoxypyrazine generation could strongly influence pennycress aroma. Consequently, it is very important to conduct further analysis on 2,5-/2,6-dimethyl-3-methoxypyrazine in pennycress to determine its source.

4.1.2.3 Fatty Acid-Derived Volatiles

Among 10 odorants identified in this research, 5 were fatty acid-derived volatiles, including three fatty aldehydes: hexanal (no.1, FD 10), (E)-2-octenal (no.9, FD 100), (E)-2-nonenal (no.14, FD 50), and two fatty alcohols: 1-octanol (no.16, FD 10) and 1-nonanol (no. 18, FD 50).

Fatty acid profile of wild-type pennycress seed oils was investigated by Chopra et al. (2019), and they concluded that the primary fatty acids of MN106 were erucic (C22:1, 35%), linoleic (C18:2, 18%), oleic (C18:1, 13%), linolenic (C18:3, 10%) and eicosenoic (C20:1, 10%) acid. In these fatty acids, erucic acid is normally considered as one of the biggest problems in wild-type pennycress since high contents of erucic acid is harmful for human consumption, which would limit edible applications of pennycress products. Currently, many researches are searching for ways to decrease the erucic acid in pennycress. Besides, an increase of oleic acid has also drawn attention since oleic acid obtains a high oxidative stability as well as a low saturated fat content, which plays a role in storage stability and positive health effect. On the contrary, to oleic acid, linolenic and
linoleic acids have low oxidative stability, causing themselves to be subject to oxidization, which further produces undesirable flavors in food.

Hexanal (Figure 27) is a saturated fatty aldehyde with a “green” odor. It has been identified in diverse Brassica species, such as broccoli (Brassica oleracea var. italica Plenck), cabbage (Brassica oleracea subsp. capitata L.), and brussels sprouts (Reddy & Guerrero, 2000; Rinaldi et al., 2013; Ulrich, Krumbein, Schonhof, & Hoberg, 1998).

![Figure 27. GC-MS mass spectrum of hexanal (no.1).](image)

Same as hexanal, (E)-2-octenal (Figure 28) and (E)-2-nonenal (Figure 29) are also fatty-acid-derived aldehydes, but they are unsaturated aldehydes. They both have been identified in mustard species Brassica oleracea L. var. costata D (De Pinho et al., 2009), and they have been also found in other plants, like Jalapeño pepper (Capsicum annuum L.), peach (Prunus persica L.), and in fungi Truffles (Tuber spp.) (Azcarate & Barringer, 2010; Feng et al., 2019; J. C. Zhu & Xiao, 2019). Besides, the odor of (E)-2-octenal and (E)-2-nonenal are similar, also having been described as “green” character.
These three fatty-acid-derived aldehydes hexanal, (E)-2-octenal, (E)-2-nonenal all possess a “green” odor note, and they all can be derived from linoleic acid through the action of lipoxygenases (LOX) and hydroperoxide lyase (HPOL) enzymes (Matsui, Takaki, Shimada, & Hajika, 2011). LOX enzymes catalyze the oxidation of linoleic acid, leading to the generation of hydroperoxide (HPO), and HPOL enzymes subsequently...
cleave these HPOs to form aldehydes. According to Azcarate (2010), hexanal is generated from the 13-HPO of linoleic acid, while (E)-2-nonenal is formed from its 9-HPO. (E)-2-octenal is produced from the 10-HPO of linoleic acid, which results from 9-HPO rearrangement. Due to the oxidatively instability of linoleic acid when interacting with enzyme LOXs and molecular oxygen, the existence of linoleic acid in pennycress would lead to the generation of its derived-aldehydes, impacting pennycress aroma profile. Especially, when pennycress seeds are ground during processing, tissue disrupted, the formation of these volatile aldehydes will be enhanced thus their concentration will be increased, due to an increased interaction with enzymes and oxygen (Azcarate, 2010). Currently, research is underway to reduce the amount of linoleic acid in pennycress (Sedbrook et al., 2014), assuming this will provide a longer shelf-life oil. However, there is limited literature relating pennycress flavor or aroma problems to the presence of linolenic acid.

Different from previous three fatty-acid-derived aldehydes, 1-octanol (Figure 30) and 1-nonanol (Figure 31) are both fatty alcohols. 1-Octanol has been identified in various species in Brassicaceae family, such as Brassica fruticulosa Cyr., Brassica Incana Ten. and Brassica juncea Coss (Tripodi, Verzera, Dima, Conduarso, & Ragusa, 2012; Zhao, Tang, & Ding, 2007). 1-Nonanol has been found in cabbage (Brassica oleracea L.) and pac choi (Brassica rapa var. Mei Qing Choi), which are also classified in the family of Brassicaceae (Rajkumar et al., 2017; Talavera-Bianchi, Adhikari, Chambers IV, Carey, & Chambers, 2010).
Based on the good scents company (TGSC) website, the odor of 1-octenal is described as “waxy, green, orange, aldehydic, rose, mushroom”, and 1-nonanol is defined to be “fresh, clean, fatty, floral, rose, orange, dusty, wet, oily”, which are in a broad range of odor descriptors, some can be considered as “pleasant”, while some are usually used for describing “unpleasant” aromas. Corresponding odorants of 1-octenal and 1-nonanal
are no.16 and no.18; they were described as “citrus, fruity, grassy” and “green, woody” respectively, by panelists in this research. These panelists also performed GC-O sniffing analysis on standard chemicals of 1-octenal and 1-nonanal, in which, they described 1-octenal to be “fresh, minty, flower-like”, while describing 1-nonanal as “fresh, green”. These odor descriptors were similar to those obtained from odorants no.16 and no.18 in pennycress volatiles extract. Thus, even though aroma descriptors of 1-octenal / 1-nonanal showed a large difference among different studies, the identity of no.16 and no.18 were still able to be verified via matching GC-O result of their standard chemicals.

In general, 1-octenal and 1-nonanol are largely responsible for vegetable-like odor notes.

As a fatty alcohol, the formation of 1-octanol comes from a fatty acid: octanoic acid, which is also called caprylic acid. According to Alhotan et al. (2017), caprylic acid is one of fatty acids in pennycress, which supports the presence of 1-octanol in pennycress. As for 1-nonanol, it is derived from nonanoic acid, also named as pelargonic acid. Based on “fatty acids compositions of pennycress” summarized by Alhotan et al. (2017), pelargonic acid was not present in pennycress. However, pelargonic acid can be produced through the oxidation cleavage of oleic acid, one of major fatty acids in pennycress. In conclusion, these five volatile compounds are all derived from fatty acids in pennycress, thus, it is hypothesized that fatty acids have great impact on pennycress aroma.

4.1.2.4 Acids

In addition to those fatty-acid-derived volatiles, acids can be also important for an aroma profile. No.19 odorant, with a FD value of 100, was perceived by panelists to be a
“cheesy” or even “smelly socks-like” aroma. Based on mass spectrum, no.19 odorant was initially identified as 3-methylbutanoic acid (Figure 32) or/and 2-methylbutanoic acid (Figure 33).

Figure 32. GC-MS mass spectrum of 3-methylbutanoic acid (no. 19).

Figure 33. GC-MS mass spectrum of 2-methylbutanoic acid (no. 19).

3-Methylbutanoic acid, commonly known as isovaleric acid, is a short-chain carboxylic acid with a “cheesy”, “rancid”, “sweaty” odor, corresponding to no.19.
odorant. As a natural fatty acid, it has been found in various foodstuffs, like cocoa beans, cheese and lamb (Attaie & Richter, 1996; Frauendorfer & Schieberle, 2008; Sutherland & Ames, 1996). While a cheesy odor is not similar to “mustard-like” odor in Brassicaceae family, 3-methylbutanoic acid has been identified in diverse mustard plants, such as white mustard seeds (Sinapis alba L.), rapeseeds (Brassica napus L.) and Asian skunk cabbage (Symplocarpus renifolius) (Oguri, Sakamaki, Sakamoto, & Kubota, 2019; Ortner, Granvogl, & Schieberle, 2016).

As for 2-methylbutanoic acid, it exhibits two enantiomeric forms, (S)- and (R)-2-methylbutanoic acid (Figure 34), which are a pair of chiral molecules that are non-superimposable on mirror images with each another (Tucker, 2000). Usually, enantiomers show similar chemical and physical characteristics, and they possess identical mass spectra as well as gas chromatographic retention time, which leads to the difficulty of distinguishing from each other (Tester & Karkalas, 2003; Wang et al., 2014). However, the enantiomers of some volatile compounds can have an obvious difference in their odors, due to their different interaction with other chiral molecules, resulting in a different reaction with olfactory receptors (Bentley, 2006; Pickenhagen, 1989). It is reported that (S)-2-methylbutanoic acid has a pleasant fruity aroma, while (R)-2-methylbutanoic acid has a cheesy and sweaty odor, which is usually considered as an undesirable odor. (S)-2-methylbutanoic acid has been identified in fermented pear mash (Zierer, Schieberle, & Granvogl, 2016), and (R)-2-methylbutanoic has been found in cocoa beans. Due to the difficulty of distinguishing (S)- and (R)-2-methylbutanoic acid from each other, there is limited literature recording their common sources. But (R/S)-2-
methylbutanoic acid has been generally reported to occur in *Brassica* species: white mustard seeds (*Sinapis alba* L.) and rapeseeds (*Brassica napus* L.), where 3-methylbutanoic acid has been found as well (Ortner et al., 2016).

![Methylbutanoic Acid Structures]

**Figure 34.** The two enantiomers of 2-methylbutanoic acid.

To verify the identity of no.19 odorant between 2-methylbutanoic acid and 3-methylbutanoic acid, we conducted a separate GC-O analysis on pure reference compounds (S)-2-methylbutanoic acid and 3-methylbutanoic acid, whereas (R)-2-methylbutanoic acid was not available for evaluation. From GC-O analysis, (S)-2-methylbutanoic acid and 3-methylbutanoic acid had similar retention times, and sniffing detection showed that (S)-2-methylbutanoic had a slightly fruity aroma, while 3-methylbutanoic acid had a cheesy odor. In addition, to further understand the gas chromatographic separation when both volatiles occur in the same solution, a 1:1 standard mixture of (S)-2-methylbutanoic acid and 3-methylbutanoic acid was analyzed by GC-O. The gas chromatogram showed that (S)-2-methylbutanoic acid and 3-methylbutanoic acid eluted in the same peak, and sniffing result indicated that the cheesy odor was detected obviously whereas a fruity aroma was not clear. Although the analysis of (R)-2-methylbutanoic acid was not possible in this research, its elution time should be
the same as (S)-2-methylbutanoic acid as they are enantiomers. From the above, 2-methylbutanoic acid and 3-methylbutanoic acid were verified to have same gas chromatographic performance. Since no.19 odorant was identified as both 2-methylbutanoic acid and 3-methylbutanoic acid through mass spectrometry, it is possible that these two isomers both exist in pennycress volatiles and they elute at an almost same retention time, very close to no.19 odorant. So, 3-methylbutanoic acid and (R/S)-2-methylbutanoic acid are both likely to occur in pennycress. Nevertheless, in terms of olfactometry perception of panelists in this research, the cheesy odor note of 3-methylbutanoic acid was much more noticeable than the fruity odor of (S)-2-methylbutanoic acid, when they were at the same concentration. Hence, if 3-methylbutanoic acid and (S)-2-methylbutanoic acid are both existing in pennycress volatiles, the odor of (S)-2-methylbutanoic acid would be easily “hidden” by 3-methylbutanoic acid. Besides, no.19 odorant was described as “cheesy”, which is not the characteristic aroma of (S)-2-methylbutanoic acid. Hence, even though (S)-2-methylbutanoic acid probably occurs in pennycress volatiles, it did not contribute to no.19 odor, instead, the “cheesy” aroma might be the result of only 3-methylbutanoic acid or (R)-2-methylbutanoic acid, or the mixture of both.

According to Frauendorfer & Schieberle (2008), 3-methylbutanoic acid and 2-methylbutanoic acid were both identified in cocoa powder, and they had an odor threshold of 22 μg/kg (0.02 ppm) and 203 μg/kg (0.203 ppm) respectively, which can be considered as a low odor threshold (≤ 1ppm). In addition, based on feedback from panelists, this “cheesy”, “sweaty” odor was perceived from GC-O analysis. Therefore,
the presence of 3-methylbutanoic acid or/and (R)-2-methylbutanoic acid is thought to contribute an undesirable odor to pennycress.

Acetic acid (no.11) was also identified as a key volatile in pennycress. No.11 odorant emitted a “sour” and “vinegar-like” aroma, with a FD factor of 100. Acetic acid (Figure 35), systematically named ethanoic acid, is a monocarboxylic volatile acid possessing a strong aroma of vinegar, corresponding to odor descriptor of no.11 odorant.

![Figure 35. GC-MS mass spectrum of acetic acid (no. 11).](image)

Acetic acid has been documented to be a main volatile component in vinegar, juice, wine and alcoholic beverage. It is produced by acetic acid bacteria (AAB), a group of gram-negative or gram-variable aerobic bacteria assigned in the family Acetobacteraceae. AAB have been isolated from fruits and flowers, which is acidic and has a rich content of carbohydrate. AAB also contribute to the flavor of fermented beverages and foods, where sugars and ethanol are oxidized into acetic acid (Lynch, Zannini, Wilkinson, Daenen, & Arendt, 2019; Sengun & Karabiyikli, 2011). Acetic acid has been reported to be found in Brassica and Arabidopsis, both belong to Brassicaceae.
family, which supports the possibility of acetic acid being present in pennycress. Guarino et al. (2017) studied the emission of volatile organic compounds (VOCs) in *Brassica oleracea* var botrytis under infested and healthy condition. They identified acetic acid and also concluded that pest infestation greatly increased the release of acetic acid, which could be the result of plant metabolism change and/or the increasing occurrence of microorganisms due to pest infestation.

*Arabidopsis* is considered as a model for pennycress gene characterization due to their similar gene duplication (Chopra et al., 2018). Rasheed et al. (2018) investigated acetic acid pathway genes in *Arabidopsis*, which contribute to acetic acid fermentation pathway, where pyruvate is converted to acetaldehyde and further oxidized to acetic acid. In addition, pyruvate was found in pennycress’s glycolysis pathway according to the metabolic map of pennycress embryos from Tsogtbaatar et al. (2015). Acetic acid has an odor threshold ranging from 0.59 to 40 ppm (van Thriel et al., 2006). When it is in a high concentration, it will contribute to a “strong sour” odor note, which is usually considered as undesirable. In the sniffing process of pennycress volatiles, acetic acid was clearly recognized by panelists due to its unique odor. While a “mustard-like” aroma predominated, a sour odor was also perceived as a part of the overall aroma of pennycress volatiles water extract. However, this sour odor brought an impression of unwanted spoilage of pennycress. With an acidic odor, the presence of acetic acid is possible to have an undesirable influence on pennycress aroma.
4.1.2.5 Pleasant Odorant

Obviously, the overall aroma of pennycress is unpleasant, however, that does not mean only unpleasant odorants are present in pennycress. No. 25 odorant, with a FD value of 50, was perceived and described as “rose” and “coconut”, which was considered as pleasant to panelists (at analyzed concentration). This volatile was identified to be phenethyl alcohol (Figure 36), with the help of GC-O analysis on phenethyl alcohol standard chemical.

Figure 36. GC-MS mass spectrum of phenethyl alcohol (no. 25).

Phenethyl alcohol, also named benzeneethanol, is a phenol-derived higher alcohol (Li, Sun, Li, Liu, & Huang, 2014; Petropulos et al., 2014). With a rose-like odor note, it is regarded as the primary component in rose oil acquired from the rose blossom. In addition, phenethyl alcohol has been identified in blackberry, contributing to a “floral, perfume, peach” odor note, and isolated in nonacidic soy sauce, with a “floral/sweet” odor description as well as traditional soypaste with “sweet floral” aroma (Klesk & Qian, 2003; Lee, Seo, & Kim, 2006; Zhang, Li, Lo, & Guo, 2010). Phenethyl alcohol was also
found in diverse *Brassica* species. Tollsten & Bergström (1988) demonstrated that phenethyl alcohol served as a “flower-fragrance” component in *B. nigra*, *B. napus*, *B. campestris* and *Brassica juncea*. Pollner & Schieberle (2016) also reported that phenethyl alcohol brought a “honey-like” odor to rapeseed oil. Phenethyl alcohol has a low odor threshold, which was reported as 0.221 ppm in rapeseed oil, 0.044 ppm in liquid paraffin, and 0.039 ppm in propylene glycol (Pollner & Schieberle, 2016; Tsukatani, Miwa, Furukawa, & Costanzo, 2003). Generally, phenethyl alcohol serves as fragrance or food flavorings due to its pleasant floral odor. In contrast to those undesirable odorants identified earlier, phenethyl alcohol has a positive impact on pennycress aroma. That said, the desired sensory properties of a food ingredient is generally blandness - no odor character which then can be flavored as desired.

**4.2 FUTURE WORK**

As this research is the first study on aroma analysis of pennycress, it represents preliminary identification of volatile compounds that primarily contribute to pennycress aroma. Further investigation will be needed for a more precise and complete aroma profile as well as a view into the taste of pennycress. Certainly, more concentrated volatiles extract would be helpful in identifying extremely low odor threshold compounds present at very low concentrations. Some compounds could be smelled but not detected by instrumental analysis. Increasing concentration of pennycress volatiles is also practical for perceiving more odorants, which will result in a more complete aroma profile of pennycress.
Based on GC-O analysis of identified volatiles, the perceived aroma of odorants in pennycress volatiles extract and their corresponding standard chemicals were very similar. However, since odors can be different when compounds are at different concentrations, it would influence the accuracy when comparing odors between unknown compounds and standard chemicals. So, adjusting the concentration of standard chemical to be almost the same as its corresponding odorant might assist in increasing the odor similarity between identified compounds and detected odorants. Further, comparing overall aroma of pennycress aroma extract with a standard mixture of identified compounds at approximate concentrations could be worthwhile to determine how closely a mixture of identified volatiles mirrors pennycress aroma. In addition to aroma, taste is also an important factor that impacts the flavor profile of pennycress thus influences human perception. Therefore, conducting taste test on pennycress samples will contribute to an understanding of human taste perception on pennycress, which provides a more comprehensive evaluation of flavor profile. When pennycress are introduced into food factory as food ingredients, a complete flavor profile might serve as an useful reference for consumer acceptance.

Additional work aimed at spiking volatile profiles with individual pure compounds will be helpful to understand the contribution of compounds to pennycress aroma. To perform aroma analysis (through GC-O analysis and overall aroma perception) on mutant pennycress sample (provided by pennycress breeding program), where specific volatile compounds or its precursor are decreased, would be valuable to conclude whether identified volatiles are key active-odor compounds to pennycress aroma.
2,5/2,6-dimethyl-2-methoxypyrazine was unexpectedly found in pennycress from this research. Due to their extremely low odor threshold, it is worthwhile to further analyze the importance of 2,5/2,6-dimethyl-3-methoxypyrazine to pennycress aroma. In addition to an investigation of soil and storage condition, the evaluation of 2,5/2,6-dimethyl-3-methoxypyrazine in no-glucosinolate mutant pennycress will be an useful way to investigate its contribution to pennycress aroma. Furthermore, a closer cooperation with breeding groups can enhance the genomics studies in pennycress breeding programs. Monitoring the flavor (smell and taste) profile of mutant pennycress samples will provide the most relevant information to pointedly design processes for minimizing unpleasant odor.
CHAPTER 5. CONCLUSION

Solvent assisted flavor evaporation (SAFE) isolation of volatiles from wild-type pennycress seeds, combined with gas chromatography-olfactometry (GC-O) and aroma extraction dilution analysis (AEDA) of extracted volatiles, perceived twenty-nine aroma-active compounds, among which, ten odorants were identified with the aid of gas chromatography–mass spectrometry (GC-MS), retention indices, aroma descriptors and standard chemicals verification.

The odorant with highest flavor dilution (FD) value was identified to be 2,5/-2,6-dimethyl-3-methoxypyrazine, which to the best of our knowledge hasn’t been reported in any pennycress research, and it was first identified in Brassicaceae plants. Among 10 identified volatiles, half of these compounds were fatty-acid-derived compounds. Three “green” fatty aldehydes: hexanal, (E)-2-octenal and (E)-2-nonenal, which were most likely formed from linoleic acid degradation, having been rarely considered as a significant concern in published pennycress work. An “onion-like” volatile compound AITC was identified and concluded to have a FD value of 50 in wild-type pennycress volatiles. AITC has been widely considered as the most important aroma compound in pennycress, however, in this research, it was shown to be not the only reason for unpleasant odor of pennycress.

In addition, a more complete profile of volatile compounds in pennycress was achieved by conducting compounds identification via GC-MS. By matching odor descriptions, mass spectra and retention index, seven volatile compounds were tentatively identified.
The identification of aroma-active compounds in wild-type pennycress seeds provides the initial analysis of pennycress aroma, which might bring attention to compounds and metabolic pathways that have been overlooked in previous pennycress research. Besides, a relatively comprehensive database of pennycress aroma profile can give an overview on pennycress aroma and provide more information as well as a direction for further studies.


Averbeck, M., & Schieberle, P. (2011). Influence of different storage conditions on changes in the key aroma compounds of orange juice reconstituted from concentrate. European Food Research and Technology, 232(1), 129–142. https://doi.org/10.1007/s00217-010-1366-8


Advances, 7(2), 45442–45451. https://doi.org/10.1039/c7ra09355a


Herrera-Jimnéz, M., Escalona-Buenda, H., Ponce-Alquicira, E., Verde-Calvo, R., &


McGinn, M., Phippen, W. B., Chopra, R., Bansal, S., Jarvis, B. A., Phippen, M. E., …


Petropulos, V. I., Bogeva, E., Stafilov, T., Steføva, M., Siegmund, B., Pabi, N., &


Wu, Y., Zhang, W., Duan, S., Song, S., Xu, W., Zhang, C., … Wang, S. (2018). In-depth


APPENDICES

Appendix 1. Other volatile compounds tentatively identified in pennycress volatiles extract through mass spectrum and retention index.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$RI_{cal}^a$</th>
<th>$RI_{ref}^b$</th>
<th>ID$^c$</th>
<th>Ref$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alcohols</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Butanol</td>
<td>1025</td>
<td>1035</td>
<td>MS$^e$, RI$^f$</td>
<td>[1]</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>1038</td>
<td>1052</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>2-Pentanol</td>
<td>1122</td>
<td>1142</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>1144</td>
<td>1152</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>1-Penten-3-ol</td>
<td>1161</td>
<td>1176</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>3-Penten-2-ol</td>
<td>1172</td>
<td>1182</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>1210</td>
<td>1195</td>
<td>MS, RI</td>
<td>[5]</td>
</tr>
<tr>
<td>3-Penten-1-ol, (E)-</td>
<td>1282</td>
<td>1255</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>1360</td>
<td>1354</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>2-Hexen-1-ol, (E)-</td>
<td>1410</td>
<td>1410</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>1-Heptanol</td>
<td>1460</td>
<td>1460</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>6-Hepten-1-ol, 2-methyl-</td>
<td>1469</td>
<td>1480</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td>2,3-Butanediol</td>
<td>1579</td>
<td>1567</td>
<td>MS, RI</td>
<td>[6]</td>
</tr>
<tr>
<td>1,2-Butanediol</td>
<td>1690</td>
<td>n/a$^g$</td>
<td>MS</td>
<td>n/a</td>
</tr>
<tr>
<td>2,4-Heptadien-1-ol</td>
<td>1695</td>
<td>1696</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td>2-Nonen-1-ol</td>
<td>1716</td>
<td>n/a</td>
<td>MS</td>
<td>n/a</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>1874</td>
<td>1885</td>
<td>MS, RI</td>
<td>[6]</td>
</tr>
<tr>
<td>Dihydro-beta-ionol</td>
<td>1962</td>
<td>1966</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td><strong>Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Propenoic acid, 2-methyl-</td>
<td>1774</td>
<td>n/a</td>
<td>MS, RI</td>
<td>n/a</td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>1847</td>
<td>1847</td>
<td>MS, RI</td>
<td>[2]</td>
</tr>
<tr>
<td>Heptanoic acid</td>
<td>1954</td>
<td>1965</td>
<td>MS, RI</td>
<td>(3)</td>
</tr>
<tr>
<td>3-Hexenoic acid, (E)-</td>
<td>1959</td>
<td>1957</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>2061</td>
<td>2065</td>
<td>MS, RI</td>
<td>[2]</td>
</tr>
<tr>
<td>Nonanoic acid</td>
<td>2167</td>
<td>2159</td>
<td>MS, RI</td>
<td>[3]</td>
</tr>
<tr>
<td>2-Octenoic acid</td>
<td>2184</td>
<td>2182</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td>Decanoic acid</td>
<td>2272</td>
<td>2298</td>
<td>MS, RI</td>
<td>[4]</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>2430</td>
<td>2425</td>
<td>MS, RI</td>
<td>[6]</td>
</tr>
<tr>
<td>Dodecanoic acid</td>
<td>2484</td>
<td>2503</td>
<td>MS, RI</td>
<td>[4]</td>
</tr>
<tr>
<td>Benzeneacetic acid</td>
<td>&gt;2500$^h$</td>
<td>2550</td>
<td>MS</td>
<td>[10]</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>&gt;2500</td>
<td>2713</td>
<td>MS</td>
<td>[4]</td>
</tr>
<tr>
<td>Pentadecanoic acid</td>
<td>&gt;2500</td>
<td>2822</td>
<td>MS</td>
<td>[4]</td>
</tr>
<tr>
<td>Compounds</td>
<td>RI&lt;sub&gt;cal&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>RI&lt;sub&gt;ref&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ID&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ref&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>-------------------------------------------------------------</td>
<td>------------------------------</td>
<td>-----------------------------</td>
<td>----------------</td>
<td>---------------</td>
</tr>
<tr>
<td>9-Octadecenoic acid, (Z)-</td>
<td>&gt;2500</td>
<td>3157</td>
<td>MS</td>
<td>[4]</td>
</tr>
<tr>
<td>Benzoic acid, 4-methoxy-</td>
<td>&gt;2500</td>
<td>n/a</td>
<td>MS</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Ketones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3-Pentanedione</td>
<td>1065</td>
<td>1071</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>3-Hydroxy-2-pentanone</td>
<td>1337</td>
<td>1349</td>
<td>MS, RI</td>
<td>[6]</td>
</tr>
<tr>
<td>1-Hydroxy-2-butanone</td>
<td>1370</td>
<td>1380</td>
<td>MS, RI</td>
<td>[6]</td>
</tr>
<tr>
<td>3-Octen-2-one</td>
<td>1405</td>
<td>1408</td>
<td>MS, RI</td>
<td>[8]</td>
</tr>
<tr>
<td>5-Ethylcyclopent-2-en-1-one</td>
<td>1418</td>
<td>1426</td>
<td>MS, RI</td>
<td>[8]</td>
</tr>
<tr>
<td>2-Cyclohexen-1-one, 3,5,5-trimethyl-</td>
<td>1577</td>
<td>1576</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td>Pantolactone</td>
<td>2028</td>
<td>2029</td>
<td>MS, RI</td>
<td>[9]</td>
</tr>
<tr>
<td>2-Pentadecanone, 6,10,14-trimethyl-</td>
<td>2123</td>
<td>2122</td>
<td>MS, RI</td>
<td>[8]</td>
</tr>
<tr>
<td>1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-</td>
<td>2265</td>
<td>2260</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td>2,5-Pyrrolidenedione, 3-ethyl-4-methyl-</td>
<td>2359</td>
<td>n/a</td>
<td>MS</td>
<td>n/a</td>
</tr>
<tr>
<td>2,5-Pyrrolidinedione</td>
<td>2466</td>
<td>2458</td>
<td>MS, RI</td>
<td>[9]</td>
</tr>
<tr>
<td><strong>Aldehydes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Pentenal, (E)-</td>
<td>1130</td>
<td>1135</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>Heptanal</td>
<td>1206</td>
<td>1186</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>2-Hexenal, (E)-</td>
<td>1217</td>
<td>1225</td>
<td>MS, RI</td>
<td>[1]</td>
</tr>
<tr>
<td>2,4-Heptadienal, (E,E)-</td>
<td>1488</td>
<td>1496</td>
<td>MS, RI</td>
<td>[8]</td>
</tr>
<tr>
<td>Pentanal</td>
<td>&lt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>984</td>
<td>MS</td>
<td>[1]</td>
</tr>
<tr>
<td><strong>Esters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Caproic acid vinyl ester</td>
<td>1660</td>
<td>n/a</td>
<td>MS</td>
<td>n/a</td>
</tr>
<tr>
<td>Pentanedioic acid, dimethyl ester</td>
<td>1703</td>
<td>1699</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td>gamma-Heptalactone</td>
<td>1792</td>
<td>1796</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td><strong>Terpenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Limonene</td>
<td>1195</td>
<td>1186</td>
<td>MS, RI</td>
<td>[3]</td>
</tr>
<tr>
<td>(+)-Longicyclene</td>
<td>1476</td>
<td>1489</td>
<td>MS, RI</td>
<td>[7]</td>
</tr>
<tr>
<td>beta-Bisabolene</td>
<td>1720</td>
<td>1720</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td>2,4-Hexadiene, 3,4-dimethyl-, (E,Z)-</td>
<td>2277</td>
<td>n/a</td>
<td>MS</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Furanic compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furan, 2-pentyl-</td>
<td>1232</td>
<td>1232</td>
<td>MS, RI</td>
<td>[8]</td>
</tr>
<tr>
<td>5-Ethyl-2(5H)-furanone</td>
<td>1751</td>
<td>1755</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
</tbody>
</table>
Continued.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>RI\textsuperscript{a}</th>
<th>RI\textsuperscript{b}</th>
<th>ID\textsuperscript{c}</th>
<th>Ref\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>2(3H)-Furanone, dihydro-5-pentyl- (gamma-Nonalactone)</td>
<td>2021</td>
<td>2020</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
<tr>
<td>2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-</td>
<td>2325</td>
<td>2325</td>
<td>MS, RI</td>
<td>[10]</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Retention Index of unknown compounds calculated on a DB-WAX column; \textsuperscript{b} Retention Index from references (polar columns); \textsuperscript{c} Identification Basis; \textsuperscript{d} Reference sources of listed RI\textsubscript{ref}; \textsuperscript{e} Mass Spectrum detected was agreed with pal600k mass spectral database; \textsuperscript{f} Retention Index calculated was agreed with references; \textsuperscript{g} Not available; \textsuperscript{h} RI\textsubscript{cal} is not precise (e.g., >2500; <1000) since alkane C26 was not available and RT for alkane C9 was not determined.

Appendix 2. Gas chromatogram of SAFE volatiles extract (with C₆ to C₂₅ alkanes added) from pennycress oil (top) and pennycress seeds (bottom) by GC-MS on DB-WAX column.